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Simian virus 40 DNA replication *in vitro*: purification and characterization of replication factors from mouse cells

Hiroyoshi Ariga

Institute of Medical Science, University of Tokyo, 4-6-1, Shirokane-dai, Minato-ku, Tokyo 108, Japan

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ABSTRACT

We have previously developed simian virus 40 (SV40) DNA replication system in vitro (Ariga and Sugano, J. Virol. 48, 481, 1983). This system is composed of human HeLa or mouse FM3A nuclear extract and cytoplasmic extract of SV40 infected CosI cells. Here FM3A nuclear extract was fractionated by DEAE Sephacel and single-stranded DNA cellulose chromatography into three components required for accurate in vitro SV40 DNA replication. One fraction (A fraction) contained DNA polymerase-primase, and the second component (B fraction) contained DNA topoisomerase. Third component was further purified to near homogeneity using DEAE-Sephacel, single-stranded DNA cellulose, and glycerol gradient centrifugation. The purified protein (named factor I) bound to the origin containing fragment of SV40 DNA. The factor I enhanced the initiation of SV40 DNA replication catalyzed by SV40 infected CosI cytoplasm alone. When all four fractions consisting of A, B fractions, factor I, and SV40 infected CosI cytoplasm were mixed together, the system was reconstituted, meaning that initiation and subsequent elongation were completed to generate the full sized daughter molecules.

INTRODUCTION

The genome of simian virus 40 (SV40) represents a simple, but appropriate, model for a single mammalian replicon. SV40 replicates in the nuclei of permissive cells such as monkey cells, as small circular chromosomes whose histone composition and nucleosome structure are indistinguishable from those of its host (1). The SV40 genome has been completely sequenced and contains 5243 base pairs (2, 3). With the exception of initiation, the viral DNA replication appears to be carried out by the host cells enzymes.

In order to understand the molecular mechanism of SV40 DNA replication, it is necessary to develop an in vitro soluble system which undergoes both the initiation, subsequent elongation

and termination of SV40 DNA synthesis. We have already developed such a system consisting of HeLa or FM3A nuclear extract and SV40 infected CosI cytoplasm, in which exogenously added SV40 DNA or cloned DNA containing SV40 origin can serve as a template in a quite similar manner to that in vivo (4, 5).

In this study I addressed the general question of what kinds of replication factors are needed to confer SV40 DNA replication. In particular, I attempted to purify a component that is responsible for the initiation step of DNA replication, using a complementation assay. I separated a FM3A nuclear extract into three fractions. Two of the fractions contained DNA polymerase, DNA topoisomerase mainly, whereas one fraction includes a factor that enhances the initiation event by the aid of T Ag. This factor was purified and characterized. This factor showed no enzymatic activity, but bound to the SV40 origin containing fragment.

MATERIAL AND METHODS

Conditions for in vitro reaction

FM3A nuclear extract and SV40-infected CosI cytoplasm were prepared as described previously (5, 6). The reaction mixture (100 ul) contained 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05 mM dATP, 0.05 mM dGTP, 0.05 mM dTTP, 1.5 uM α-^{32}P dCTP (410 Ci/mmole; 5,000 to 15,000 cpm/pmole), 3.75 mM ATP, 20 µl of FM3A nuclear extract (protein concentration 2.5 mg/ml) or 10 µl each of column fraction, 2 µl of Cos-I cytoplasm (protein concentration 20 mg/ml), and 0.4 µg of form I pSV0. The pSV0 is the DNA containing SV40 origin spanning 0.64-0.71 map unit of SV40 genome cloned in pBR322 (5). After incubation of the reaction mixture for 60 min at 37 °C and subsequent digestion with proteinase K (10 ug) and sodium dodecyl sulfate (0.2 %) for 20 min at 37 °C, the DNA was precipitated with ethanol. The DNA was dissolved in 50 µl of 40 mM Tris-1 mM EDTA-5 mM sodium acetate (pH 7.8) and electrophoresed on 1 % agarose gels in the same buffer. The gels were dried and autoradiographed on Kodak XRS X-ray film.

Filter binding assay for protein-DNA interaction

The pSV0 was digested with HindIII and PvuII and 5'-end of the resulting fragments were labeled with γ-^{32}P ATP following alkaline phosphatase treatment. Binding reaction (100 µl) were performed in 10 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 5 µg bovine serum albumin, 0.1 µg of the ^{32}P-labeled DNA fragment, and purified protein from FM3A cells. After incubation at 25 °C for 30 min, the reaction mixture was filtered through a nitrocellulose filter (Millipore HA 0.45 µm). Filters were washed two times with 1 ml of a buffer containing 10 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM DTT. DNA was extracted from filters overnight at 37 °C with 500 µl of 0.25 % sodium dodecyl sulfate and 40 µg of proteinase K. The extracted DNA was precipitated with ethanol, redissolved in buffer, and electrophoresed as described above.

Protein assays

Protein concentrations were measured by the procedure of Bradford with bovine serum albumin as the standard (7). The protein composition on indivisual and pooled column fractions was determined by electrophoresis on an 10 % sodium dodecyl sulfate-polyacrylamide gel (8).

RESULTS

SV40 DNA replication system consisted of the cytoplasmic extract from SV40-infected CosI cells and nuclear extract from human HeLa or mouse FM3A cells (4, 5). SV40-infected CosI cytoplasm was source of SV40 T antigen and permissive factor. Therefore, if nuclear extract from mouse FM3A is used for the fractionation analysis, other proteins necessary for SV40 DNA replication should be provided. The reaction performed with SV40-infected CosI cytoplasm alone catalyzed the initiation of SV40 DNA replication as shown later (Fig. 6). Therefore, when the proteins fractionated from the nuclear extract are added to the SV40-infected CosI cytoplasm, the proteins of interest will be the enhancing proteins in initiation or the accessory proteins in elongation of SV40 DNA replication. For that purpose, the complementation assay is used; the measurement of enhancing

activity after the addition of fractionated nuclear extract to the basal SV40-infected CosI cytoplasm.

Fractionation of the replication proteins from mouse cells.

The purification scheme is shown in Fig. 1. All the procedures were carried out at 0-4 °C. A crude nuclear extract (22 ml) (5) was prepared from 6 x 10^9 mouse FM3A cells and prepared by successive centrifugation at 100,000 x g for 30 min. The high speed supernatant (S-100) was dialyzed against buffer A (25 mM Tris-HCl, pH 7.5, 7 mM mercaptoethanol, 0.1 mM EDTA, and 10 % glycerol) and applied to a DEAE-Sephacel column (2.5 x 10 cm) equilibrated with buffer A. The column was washed with 60 ml of the buffer at a flow rate of 15 ml/hr and eluted with a 160 ml linear gradient of 0-0.5 M NaCl in buffer A. Fractions (4 ml each) were collected. The complementation assay was carried out using 10 µl each from fractions in reaction mixture (Fig. 2). Two active peaks (fractions 17 and 29) that enhance the incorporation of the open circular (form II) and linear (form III) DNA on neutral agarose gel, were obtained. The appearance of form II after the reaction was very reasonable. The reasons for presence of form III are following. The reaction performed by SV40 infected CosI cytoplasm gives the initiation and limited elongation of SV40 DNA replication. The initiation site performed in reaction of CosI cytoplasm was located in in vivo

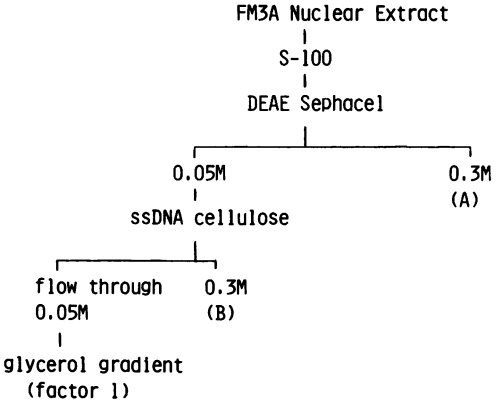

Fig. 1. Scheme for the fractionation of SV40 DNA replication factors.

ori region. The replicative intermediates performing the limited elongation are relatively rich in form III DNA (4). And other possibility is that product DNA would be of the structure sensitive to nuclease because the SV40-infected CosI cytoplasm is not sufficient for making complex necessary for complete DNA replication which can protect the nuclease attack. Other fractions such as fractions 41 and 45 were also active, but the intensity of the bands was weaker than that of the reaction with SV40-CosI cytoplasm alone. The fractions except for fractions 17, 29, 41, and 45 gave no bands, suggesting that fractions contained DNase or inhibitors such as proteinase. Both fractions

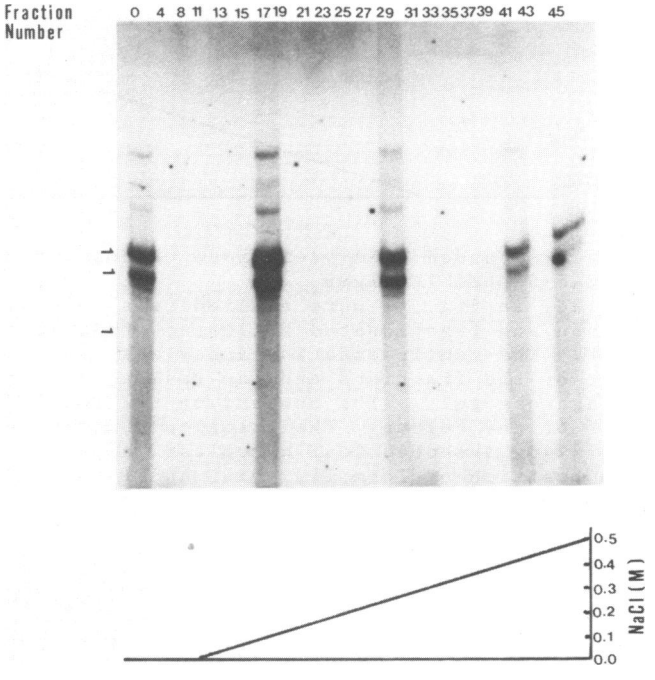

Fig. 2. DEAE-Sephacel chromatography of the FM3A S-100 fraction. The S-100 fraction was applied to DEAE-Sephacel column and fractionated by linear gradient at 0-0.5 M NaCl as described in the text. The complementation assay was carried out using 10 µl of aliquot of fractions and the radioactive bands were visualized on agarose gel. The numbers on the top indicate the fraction number of the column. The fraction 0 means the reaction catalyzed by SV40 infected CosI cytoplasm alone. The arrow shows the form II, form III and form I pSV0 DNA from the top, respectively.

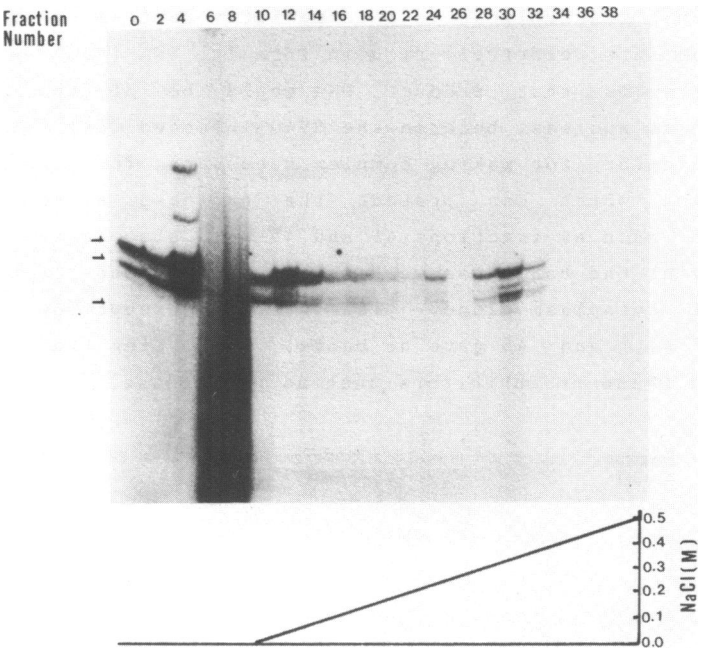

Fig. 3. Single-stranded DNA cellulose chromatography of the fractions in DEAE-Sephacel column.

The fractions in Fig. 2 were applied to single-stranded DNA cellulose column and fractionated by linear gradient at 0-0.5 M NaCl as in text. The complementation assay was carried out using 10 μl aliquot of the fractions and the radioactive bands were visualized on agarose gel. The numbers on the top indicate the fraction number of the column. The fraction 0 means the reaction performed by SV40 infected CosI cytoplasm alone. The arrow represents the position of form II, III, and I pSV0 DNA from the top, respectively.

were concentrated by 80 % ammonium sulfate and dialyzed against buffer A. Fraction eluted at 0.05 M NaCl contained the DNA binding activity as shown later. Therefore, the fractions were applied to a single-straned DNA (ssDNA) cellulose column. The column was washed with 10 ml of buffer A at a flow rate of 15 ml/hr and eluted with a 20 ml linear gradient of 0-0.5 M NaCl in buffer A. Fractions (0.5 ml each) were collected. The complementation assay was carried out as in DEAE Sephacel column (Fig. 3). The three active peaks were observed. The fractions eluted at 0.3 M NaCl contained DNA topoisomerase (fraction B) (data not shown). The fractions eluted at low NaCl concentration

Table 1. Summary of the purification of the factor I.

Step Fraction	Volume (ml)	Amount of protein (mg)	(%)	Total activity
1. S-100	52	50	100	5,200
2. DEAE-Sephacel	50	11	22	5,000
3. Single-stranded DNA cellulose	2	2	4	200
4. glycerol gradient	0.6	0.2	0.4	60

The activity used here is that one unit is five fold incorporation of form II DNA after the addition of 10 μl of sample to reaction mixture.

contained DNA binding activity. Fifty per cent of the binding activity applied to the column flowed through (fraction number 3-4) and the other 50 % of the activity eluted at 0.05 M NaCl (Fig. 3) Almost all the nuclease activities were separated from the binding activity in this step. The fractions containing DNA binding activity (flow through, number 3, and 4 fractions), were mixed together, and were further purified through 15-30 % glycerol gradient containing buffer A and 50 mM NaCl. After the complementation assay, active fractions corresponding to the position of marker bovine serum albumine were obtained and stored at -70 °C before use. The activity which enhances SV40 DNA replication was designated as factor I here (Table 1). The proteins present in the gradient fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by Coomassie brilliant blue staining (data not shown). Main band corresponding to 68,000 dalton in molecular weight and also several minor bands were detected. Considering the pattern of active peak in glycerol gradient centrifugation, 68 K polypeptide must be the factor I aimed at. However, the complete purification of factor I to a homogeneity is under way.

Characterization of factor I

Enzymatic activity of factor I (glycerol gradient fraction) was examined. Although enzymatic activities including DNA polymerase , DNA polymerase , DNA polymerase , RNA polymerase, topoisomerase, deoxyribonuclease, and ATPase were tested, no

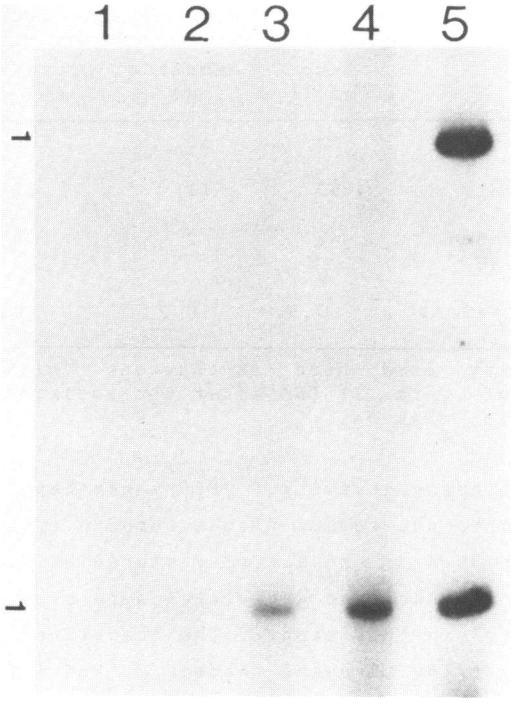

Fig. 4. DNA binding of factor I.
The pSV0 was digested with PvuII and HindIII. The resulting two fragments (pBR322 and SV40 sequences) were treated with alkaline phosphatase, and the 5'ends were labeled with polynucleotide kinase and γ-^{32}P ATP. The various amount of factor I (single-stranded DNA cellulose fraction) was incubated with ^{32}P-HindIII-PvuII fragment of pSV0. The reaction mixture was filtered through nitrocellulose filter. The ^{32}P-fragment trapped on filter was eluted and separated on agarose gel as described in Materials and Methods. The amount of factor I incubated was 0 µg (lane 1), 0.05 µg (lane 2), 0.1 µg (lane 3), and 0.2 µg (lane 4). The lane 5 indicates the input DNA fragments without reaction.

activities were detected. During course of purification of factor I, factor I could bind both single- and double-stranded DNA cellulose at low NaCl concentration. Therefore, binding activity to DNA was tested. The pSV0 that was used as a template in complementation assay, was digested with PvuII and HindIII to separate between SV40 origin containing fragment and vector pBR322 (5). The 5'-end of the resulting fragments were labeled with γ-^{32}P ATP by polynucleotide kinase and alkaline

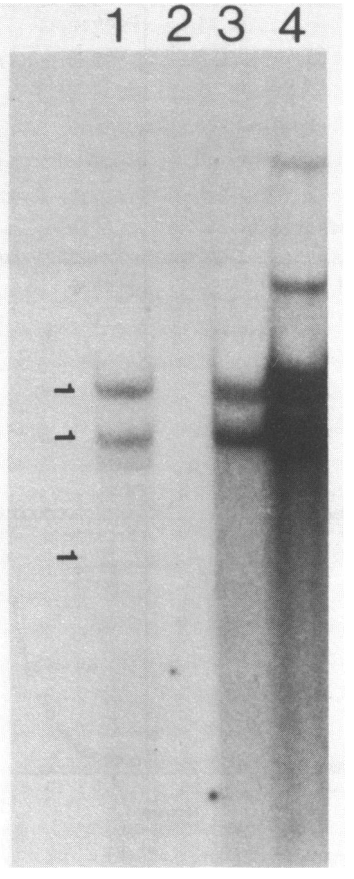

Fig. 5. Effect of factor I on SV40 DNA replication.
The DNA synthesis was carried out in a mixture containing SV40 infected CosI cytoplasm or factor I. The products were visualized after agarose gel electrophoresis as in Materials and Methods. Lane 1, reaction with SV40 infected CosI cytoplasm alone; lane 2, reaction with 0.2 µg factor I alone; lane 3, reaction with SV40 infected CosI cytoplasm plus 0.05 µg factor I; lane 4, reaction with SV40 infected CosI cytoplasm plus 0.2 µg factor I.

phosphatase. ^{32}P-fragments were mixed with factor I and filtered through nitrocellulose filter to retain only the protein-DNA complex. The DNA fragments trapped on nitrocellulose filter were eluted after the digestion with proteinase K and sodium dodecyl sulfate and separated on agarose gel (Fig. 4). It is quite clear that only the SV40 fragment could bind the factor I whereas

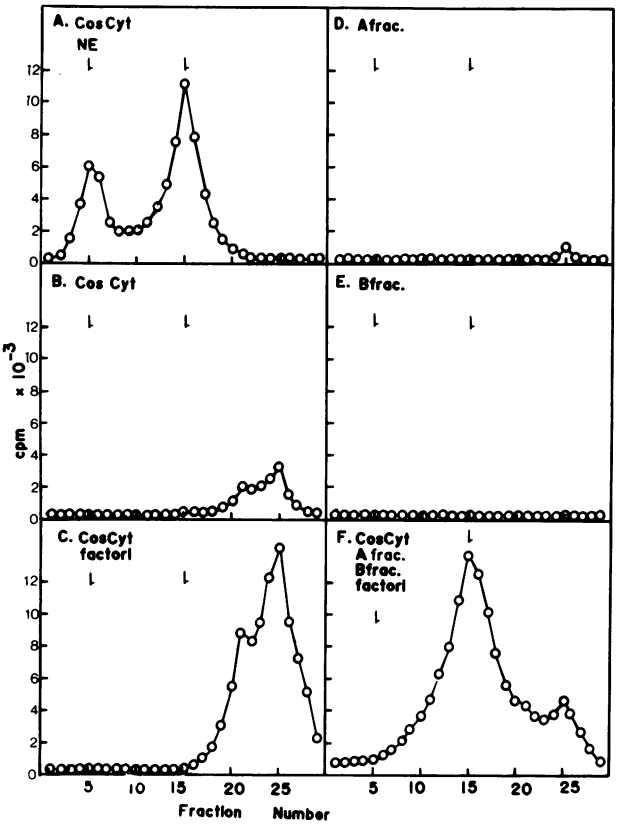

Fig. 6. Size determination of the product in reconstituted system.

The DNA synthesis reaction was carried out in various conbination of the fractions. After the reaction on pSVO for 60 min at 37 °C, DNA extracted with phenol, loaded onto 5 to 20 % alkaline sucrose gradient containing 0.1 M NaOH, 0.5 M NaCl, and 10 mM EDTA, and centrifuged in the 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 DNA from the bottom left, respectively. In this condition circular and linear DNA formed a single peak. (A) reaction with FM3A nuclear extract and SV40-CosI cytoplasm. (B) reaction with SV40-CosI cytoplasm alone. (C) reaction with SV40-CosI cytoplasm and 0.2 μg factor I. (D) reaction with A fraction alone. (E) reaction with B fraction alone. (F) reaction with SV40-CosI cytoplasm, A fraction, factor I, and B fraction.

pBR322 could not. When ^{32}P-labeled BstNI fragments of SV40 DNA were treated with factor I as the same manner with pSVO, several fragments bound factor I (data not shown). However, the

strongest binding fragment was G fragment that contains the origin and the promoter of SV40 genome. Furthermore, the same binding reaction was carried out under the condition of 0.5 M NaCl, only G fragment could bind to the factor I. These results suggest that factor I may recognize specific sequences present in the regulatory region of SV40 DNA such as origin, promoter, or enhancer.

Effect of factor I on SV40 DNA replication

As shown in Fig. 2 and 3, only form II and III were synthesized in the reaction containing only SV40 infected CosI cytoplasm that is the source of T Ag and predicted permissive factor. After the addition of factor I in the reaction mixture, the patterns on neutral agarose gel were not changed. However, approximately ten fold increase of DNA synthesis was observed (Fig. 5). The reaction containing factor I alone did not support any DNA synthesis, indicating that factor I contained neither DNA polymerase nor deoxyribonuclease. These phenomenon were much clearly understood in alkaline sucrose gradient centrifugation (Fig. 6). SV40 infected CosI cytoplasm gave rise to the DNA smaller than mature form of DNA with the peak at 4-5S corresponding to the Okazaki fragment. After the addition of factor I, overall patterns of DNA synthesis was not changed, but the total incorporation increased approximately ten fold (Fig. 7 B and C). These results suggest that factor I enhances the initiation event coupled with SV40 T Ag.

Reconstitution of SV40 DNA replication system

Three active fractions were obtained in DEAE-Sephacel and ssDNA cellulose chromatography using complementation assay. The A fraction contained DNA polymerase . When the A fraction was incubated on pSVO as a template, the small amount of small sized DNA was obtained in alkaline sucrose gradient (Fig. 6D). These were clearly derived from the repair-reaction because the density-transfer experiment using bromodeoxyuridine triphosphate in place of dTTP gave the unsubstituted DNA bands in neutral cesium chloride equilibrium centrifugation. This repair synthesis must be performed by the action of DNA polymerase coupled with deoxyribonuclease. When the B fraction was incubated on pSVO, DNA peak was under detection (Fig. 6E),

presumably because only DNA polymerase , even when DNA primase was included in B fraction, could not use closed circular double stranded DNA efficiently. When A fraction, B fraction, and SV40-CosI cytoplasm were mixed together, the pattern was the similar to that performed by CosI cytoplasm. When A fraction or B fraction was incubated with factor I, overall patterns of synthesized DNA were not changed as compared to those in A or B fraction alone (data not shown). When three fractions consisting of A, B and factor I were used in complementation assay containing SV40 infected CosI cytoplasm, however, plenty amount of the full sized pSVO was obserbed (Fig. 6F). This result indicates that at least two factors are needed to elongate the DNA strand.

DISCUSSION

This communication described reconstitution of SV40 DNA replication system with four partially purified proteins. One fraction is cytoplasmic extract from SV40 infected monkey CosI which is the source of viral T Ag and presumable permissive factor. This fraction is capable of initiating DNA synthesis. Indeed, DNA fragments synthesized in this fraction were located only at SV40 origin containing fragment of pSVO (data not shown). This fraction also contains the detectable amount of several enzymes including DNA polymerase and DNA primase. Therefore, it is likely that initiation of SV40 DNA replication occurs by RNA priming mechanism catalyzed by DNA primase followed by DNA polymerase . The T Ag present in this fraction does bind to the origin of SV40 DNA to recognize the initiation point. Therefore, it is conceivable that T Ag makes complex with DNA polymerase and primase to faciliate the initiation event.

The factor I purified from mouse FM3A cells here was the DNA binding protein which may recognize the regulatory region of SV40 genome. The efficiency to initiation of DNA replication catalyzed by SV40-CosI cytoplasm increases at least ten fold after the addition of factor I. Recently, sequence specific DNA binding proteins have been reported.; The DNA replication protein designated as nuclear factor I (NFl) was purified from HeLa cells (9). The NFl specifically binds to the sequencese within inverted terminal repeat of adenovirus DNA and is needed for

adenovirus DNA replication in vitro (9). These sequences are also present in many eukaryotic DNA (10). The similar proteins were purified from mouse and hamster cells (11), and chicken erythrocyte (12). There have also been reported several transcription factors; SPI which binds to the 21 base repeats of SV40 DNA (13), SLI to the rRNA promoter (14), TATA box binding protein (15), SV40 enhancer binding protein (1), and a Drosophila RNA polymerase II transcription factor (16). Especially, the protein of interest was NFI which specifically enhances the initiation of adenovirus DNA replication in vitro. It has been suggested that binding of NFI faciliates the interaction between the precursor terminal protein which is the initiator protein of adenovirus DNA replication and the conserved site and helps to unwind the DNA at the origin or change the double-stranded DNA structure. Therefore, it is conceivable that the factor I purified in this study recognizes some sequences and makes complex with other replication proteins such as SV40 T Ag or DNA polymerase-primase. The study to show the sequences recognized by factor I is under going.

SV40 DNA replication system was reconstituted by at least four fractions. A fraction (A fraction) was 0.3 M NaCl extract of DEAE-Sephacel column. This fraction contains DNA polymerase and DNA primase which catalyzes the oligoribonucleotide synthesis. It is possible that A fraction also posseses the DNA polymerase stimulatory protein, C1C2 (17). However, only fraction A alone without SV40 infected CosI cytoplasm could not initiate DNA replication over the detectable level. This is very acceptable because initiation is promoted by SV40 T Ag. The B fraction is 0.3 M NaCl extract of ssDNA cellulose column, which contains DNA topoisomerase. Only when four fractions were mixed together, the elongation of DNA synthesis of daughter molecule could be achieved. However, the denatured supercoiled DNA which should be observed after ligation and segregation of daughter molecules, could not be seen even after four fractions were mixed together. The reason for this is that factor(s) are missing in this reconstitution system. There is general idea that the plenty of late replicative intermediates composed mainly of the catener accumulate in SV40 DNA replication immediately before

segregation of the daughter strands and that protein factors are necessary to complete this step (18). In a present fractionation study I used a complementation assay in which the activity enhancing the initiation and the limited elongation performed by SV40 infected CosI cytoplasm, was measured. Therefore, this assay is apt to eliminate the factors necessary for the termination step of SV40 DNA replication. Other factors are clearly needed for the complete SV40 DNA replication in vitro. The A, B fractions and SV40 infected CosI cytoplasm still contain plenty of proteins. Therefore, further purification of proteins necessary for SV40 DNA replication should be done.

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