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c-*myc* protein can be substituted for SV40 T antigen in SV40 DNA replication

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ABSTRACT

Replicating activity of SV40 origin-containing plasmid was tested in human cells as well as in monkey CosI cells. All the plasmids possessing SV40 ori sequences could replicate, even in the absence of SV40 T antigen, in human HL-60 and Raji cells which are expressing c-myc gene at high level. The copy numbers of the replicated plasmids in these human cells were 1/100 as high as in monkey CosI cells which express SV40 T antigen constitutively. Exactly the same plasmids as the transfected original ones were recovered from the Hirt supernatant of the transfected HL-60 cells. Furthermore, replication of the SV40 ori-containing plasmids in HL-60 cells was inhibited by anti-c-myc antibody co-transfected into the cells. These results indicate that the c-myc protein can be substituted for SV40 T antigen in SV40 DNA replication.

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 or human cells, as small circular chromosomes whose histone composition and nucleosome structure are indistinguishable from those of its host (1). With the exception of initiation where the early gene product, T antigen, plays a crucial role, the viral DNA replication appears to be carried out by the host cells enzymes (1). The T antigen initiates viral DNA replication by binding the origin region (ori) of SV40 DNA.

Recently, we have found that the product of cellular proto-oncogene c-myc can promote cellular DNA replication by binding the putative replication ori of cellular DNA (2), which is quite reminiscent of relationship between SV40 T antigen and SV40 DNA replication. For the proof of this idea, we have cloned the c-myc protein binding sequences from human cells that, in a

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consequence, worked as an autonomous replicating plasmid after transfection into human and mouse cells (2).

In this paper, we addressed the question whether the DNA replication function of c-myc protein is indeed the similar to that of SV40 T antigen. The results showed that the c-myc product can be substituted for T antigen in terms of SV40 DNA replication.

MATERIALS AND METHODS

Plasmids

pSV0, pSVOD, pHT3, and pHT4 were pBR322 containing SV40 origin. The cloned regions of SV40 genome were shown in Fig. 1. pSV0 and pSVOD were reported previously (3). SV40-origin containing regions in pHT3 or pHT4 were derived from XS15 or XS16 (4), respectively. XS15 or XS16 were digested with XhoI and HindIII and the XhoI sites of SV40 origin-containing fragments were changed to AccI sites after treatment of Klenow fragment and addition of AccI linker. Then, the fragments were inserted into AccI and HindIII sites of pBR322 and these were named pHT3 or pHT4, respectively.

Cells

HL-60 (human promyelocytic leukemia cell line), U937 (human histiocytic lymphoma), Raji (human Burkitt lymphoma cell line), and monkey CosI cells (5) were used. The former three cells of human origin were obtained from ATCC via Japanese Cancer Research Resources Bank and cultured in RPMI1640 medium supplemented with 10 % fetal calf serum. CosI cells were from Y. Gluzman and cultured in Dulbecco's modified Eagle medium supplemented with 10 % fetal calf serum.

Replication of plasmid DNA in HL-60 cells

To check the replication of plasmid DNA, human HL-60 cells were transfected with plasmid DNA by a liposome-mediated gene transfer technique with nearly 100 % efficiency (3). This procedure gave the similar transfection efficiency to the cells including human HL-60, U937, Raji, mouse FM3A, NS-1, and rat 3Y1 cells. Liposome was constructed from phosphatidylserine by the published procedure (6). Seven μg of plasmid DNA was mixed with liposome composed of 1 μmole of phosphatidylserine, incubated at

37 ° C for 10 min. After addition of buffer containing 30 mM EDTA, 10 mM Tris-HCl (pH 7.5) and 0.145 M NaCl into DNA-liposome mixture, the mixture was incubated at 37 ° C for 40 min and centrifuged at 8,000 x g for 3 min. The pellet was incubated in the buffer containing 2 mM $MgCl_2$ and phosphate buffered saline (PBS) at 37 ° C for 40 min and added to cell suspension containing 10^6 of HL-60 cells cultured in RPMI1640 medium supplemented with 10 % fetal calf serum. At 40 hr after transfection, the low-molecular-weight DNAs were extracted by the Hirt procedure, deproteinized with proteinase K, and digested with DpnI and EcoRI. The DNA fragments were separated on 1 % agarose gel, transferred to a nitrocellulose filter (8), and hybridized with a ^{32}P-labelled pBR322 probe. The hybridization was carried out in a mixture containing 3 x SSC (1 x SSC contains 1.5 M NaCl and 0.15 M sodium citrate), 50 mM Tris -HCl (pH 7.5), 1 mM EDTA, 1 x Denhardt's solution (1 x Denhardt's solution contains 0.02 % Ficoll, 0.02 % bovine serum albumin, and 0.02 % polyvinylpyrrolidone), 20 μg of tRNA per ml, 20 μg of heat-denatured salmon testis DNA per ml, ^{32}P-labelled pBR322, 0.1 % sodium dodecylsulfate, and 50 % formamide. After 20 hrs at 42° C, the filter was washed twice with 3 x SSC-0.1 % sodium dodecylsulfate at 37 ° C for 30 min, then twice with 0.1 x SSC-0.1 % sodium dodecylsulfate at 37 ° C for 30 min, and finally with 0.1 x SSC. The filter was autoradiographed at -80 ° C.

Antibodies

Two independent anti-human c-myc antibodies were used in this study. The c-myc specific monoclonal antibody, IF7, was well characterized previously (9). The other antibody, a sheep polyclonal one, was prepared by Oncor Inc., U.S.A., using a synthetic oligopeptide sepcific for human c-myc protein, and it was confirmed by the company that the antibody reacts with purified bacterially expressed human c-myc protein. This antibody was also characterized by us (2). First, the proteins in nuclear extract from HL-60 cells was precipitated with anti-c-myc antibody, followed by a suspension of formaldehyde-fixed Staphylococcus aureus containing protein A, and transferred to nitrocellulose filter. The filters were reacted with biotinylated anti-sheep IgG and then with ^{125}I-labelled

streptavidin and washed with PBS. Second, HL-60 cells were labelled with [^{35}S]methionine for 4 hrs and the proteins were immunoprecipitated with anti-c-myc antibody. Third, S. frugiperdacells were infected with Ac337/hc-myc which is specifically expressing human c-myc protein and labelled with [^{35}S]methionine. The labelled proteins were immunoprecipitated with anti-c-myc antibody. All the experiments gave only one band of about 60,000 dalton protein on polyacrylamide gel as monoclonal antibody IF7 used instead in the same experiments.

RESULTS

Replication of SV40 origin-containing plasmid in human cells

The pSVO contains the SV40 origin region spanning from PvuII site (nucleotide number 270) to HindIII site (nucleotide number 5171) (Fig. 1). HL-60 (the human promyelocytic leukemia cell line), U937 (the human histiocytic lymphoma cell line), and Raji (the human Burkitt lymphoma cell line) cells were transfected with pSVO or pBR322 by a liposome-mediated gene transfer technique (7). At 40 hrs after transfection, the DNA in the

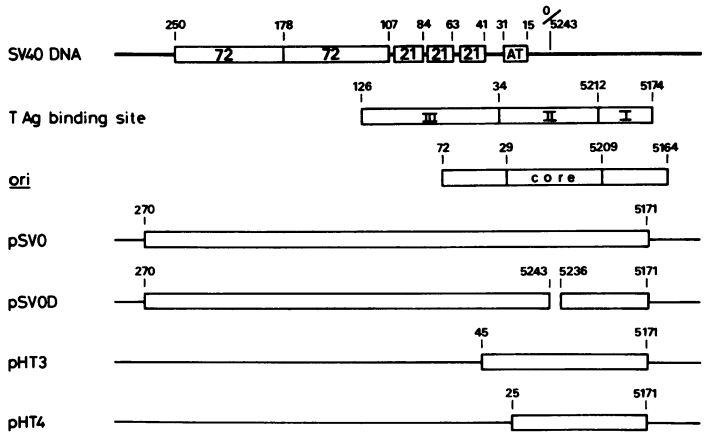


Fig. 1. Schematic drawing of SV40 origin region and regions contained in the plasmids used in this experiment.
The origin region of SV40 DNA is drawn in top of the figure. The boxed numbers of 72 or 21 represent the repeated sequences. The plasmids containing various regions of SV40 ori sequences are shown in lower part of the figure where SV40-derived sequences are drawn as open boxes.

Hirt supernatant of the cells were analyzed by Southern blot hybridization after the DNA was digested with DpnI and EcoRI to eliminate the input DNA and to linearlize the replicated DNA (Fig. 2). The pSV0 or other plasmid DNAs grown in a dam$^+$ Escherichia coli strain were methylated and thus rendered sensitive to cleavage by DpnI. Since mammalian cells do not contain this methylase, DNA replication produces hemimethylated and unmethylated DNA, both of which are insensitive to cleavage by DpnI. The results showed that replicated pSV0 was detected only in HL-60 cells, not in U937 cells. The pBR322 replicated in neither cells (data not shown and in Fig. 4). Both HL-60 and U937 cells do not produce SV40 T antigen essential to SV40 DNA replication. However, HL-60 and Raji cells produce the c-myc

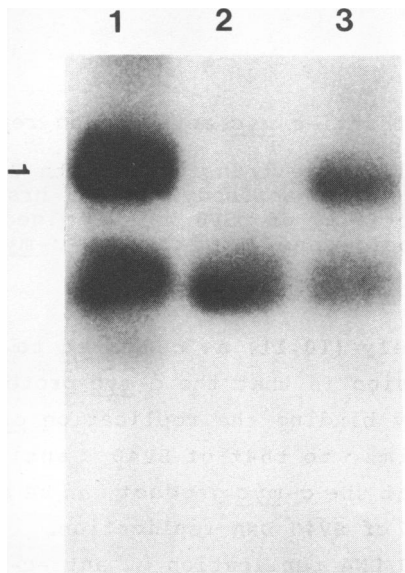


Fig. 2. Southern blot analysis of the DNA from Hirt supernatants of various transfected cells.

Samples of 10^6 cells were transfected with 7 μg of pSV0. At 40 hrs after transfection, the low-molecular-weight DNA was extracted from Hirt supernatants, digested with DpnI and EcoRI, electrophoresed on 1.2 % agarose gel, transferred to nitrocellulose filters by the method of Southern (8), and then hybridized with ^{32}P-labelled pBR322 probe. Lane 1, HL-60; lane 2, U937; lane 3, Raji. The arrow indicates the position of replicated pSV0.

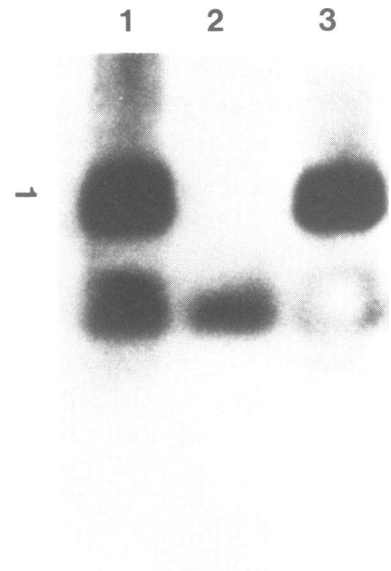


Fig. 3. Effect of anti-c-myc antibody on replication of pSV0 in HL-60 cells.
The HL-60 cells were transfected with 7 μg of pSV0 together with or without 2 μg of antibody. At 40 hrs after transfection, the replicating activity of pSV0 was examined as in Fig. 2. Lane 1, without antibody; lane 2, with anti-c-myc antibody; lane 3, with non-specific human IgG.

protein extensively (10,11) as compared to that in U937 cells. Our recent conclusion is that the c-myc protein promotes cellular DNA replication by binding the replication ori, the mechanism of which is quite mimic to that of SV40 T antigen (2). Therefore, it is possible that the c-myc product can be substituted for SV40 T antigen in case of SV40 DNA replication.

Inhibition of SV40 DNA replication by anti-c-myc antibody

To know the possible participation of c-myc protein in DNA replication, anti-c-myc antibody was added to HL-60 cells transfected with pSV0. The anti-c-myc antibody used in this experiment was prepared against synthetic oligopeptide specific for human c-myc protein. This antibody does precipitate approximately 60,000 dalton c-myc protein in HL-60 cells and c-myc protein in insect cells infected with baculovirus vector expressing human c-myc protein (2) (See 'MATERIALS AND METHODS').

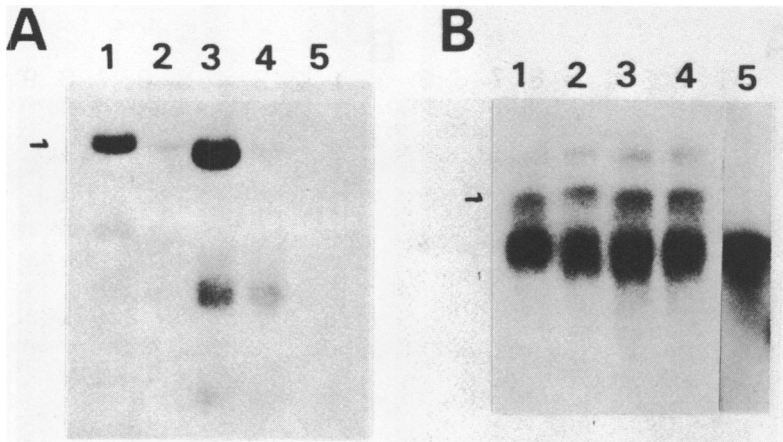


Fig. 4. Replicating activity of various deletion mutant of SV40 DNA in monkey Cos-I or HL-60 cells.

The plasmids containing the various region of SV40 origin region as described in Fig. 1. were transfected into monkey Cos-I cells (A) or human HL-60 cells (B). At 40 hrs after transfection, the replicating activity of plasmids was examined as in Fig. 2. Lane 1, pSV0; lane 2, pSV0D; lane 3, pHT3; lane 4, pHT4; lane 5, pBR322. The exposure time was 2 hrs and 20 hrs for experiment A and B, respectively. The arrow indicates the position of replicated DNA in transfected cells.

After co-transfection of anti-c-myc antibody with pSV0 into HL-60 cells, the replication of pSV0 was hardly detected, while control non-specific antibody did not affect (Fig. 3). This effect was also observed by using other antibody, human c-myc specific monoclonal antibody, IF7 (9) (data not shown). These results indicate that the replication of pSV0 in HL-60 cells is dependent upon c-myc protein, or proteins reacted with used anti-c-myc antibody.

Replication of mutant SV40 DNA in HL-60 cells

It is known that the regions necessary for replication of SV40 DNA in monkey cells are 65 nucleotides in length, spanning from 29 to 5209 (Fig. 1) (12). The various kinds of plasmid DNAs containing the origin region of SV40 DNA replication were constructed (Fig. 1). To examine the replication activity of these DNAs, the cloned DNA was transfected into monkey CosI cells which were producing SV40 T antigen constitutively (5). At 40 hrs after transfection, the low-molecular-weight DNA was

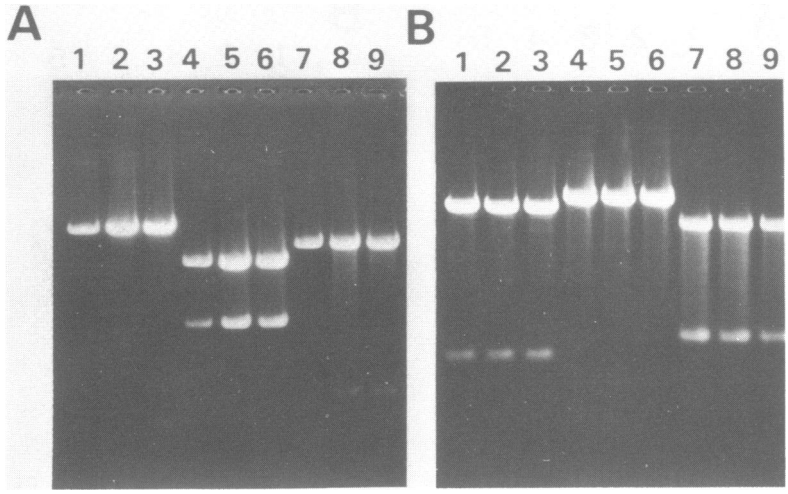


Fig. 5. Ethidium bromide staining patterns of DNA derived from Hirt supernatant of transfected HL-60 cells.

The low-molecular-weight DNA was extracted from the Hirt supernatants of HL-60 cells transfected with pSV0 (A) or pSV0D (B), and digested with DpnI completely. E. coli C600 was transformed to the ampicillin-resistant phenotype with the above DNAs. The plasmid DNAs were extracted from E. coli transformants, analysed by agarose gel electrophoresis, and visualized under UV light. Lanes 1, 4, and 7, pSV0 (A) or pSV0D (B); lanes 2, 5, and 8, DNA from clone 1; lanes 3, 6, and 9, DNA from clone 2. Lanes 1, 2, and 3, digested with HindIII and PvuII; lanes 4, 5, and 6, digested with BglI; lanes 7, 8, and 9, digested with PstI.

extracted from the transfected CosI cells by Hirt procedure (13) and analyzed by Southern blot hybridization after the DNA was digested with DpnI and EcoRI to eliminate the input DNA and to linearlize the replicated DNA (Fig. 4A). As expected, the level of replication of DNA was dependent upon the region contained in the plasmid. The pSVO containing the region spanning nucleotide number (Nn) 270 to Nn 5171 and the pHT3 containing the region from Nn 45 to Nn 5171 of SV40 genome replicated well in transfected CosI cells. The pHT4 containing the region spanning from Nn 25 to Nn 5171 and the pSVOD which is pSVO lacking 4 base pairs at BglI site could replicate only to a quite less amount in CosI cells. When these four plasmid DNAs were transfected into human HL-60 cells, on the other hand, the four plasmid DNAs also

replicated, to a lower extent than in CosI cells, and the difference of the levels of replication among these four plasmids was not observed here (Fig. 4B). The pBR322 as a negative control, however, could replicate neither in CosI cells nor in HL-60 cells. These results suggest that the c-myc protein functions as a replication protein in SV40 DNA replication in stead of T antigen and that the recognizing specificity of the c-myc protein which can also recognizes the origin sequences of SV40 DNA replication is not so strict as that of SV40 T antigen.

Recovery of pSVO from transfected HL-60 cells

To confirm that SV40 origin-containing plasmids could replicate in HL-60 cells, pSVO, oSVOD or pBR322 were transfected into HL-60 cells. At two days after transfection, the low molecular weight DNA was extracted from Hirt supernatant of HL-60 cells and digested with DpnI to eliminate input DNA. E. coli C600 was transformed with above DNAs and plasmid DNA was extracted from ampicillin resistant colony of E. coli. Both pSVO and pSVOD gave thousands of colonies, while pBR322 gave no colonies in agar plate containing ampicillin. The extracted DNA was analyzed by digestion with several restriction enzymes (Fig. 5). The structures of recovered DNAs from Hirt supernatant were exactly the same as compared with original pSVO or pSVOD, indicating that both pSVO and pSVOD could replicate in HL-60 cells without any rearrangement of DNA.

DISCUSSION

It is generally known that SV40 DNA replicates in dependently upon viral gene product, T antigen (1). In addition to above function, T antigen plays variety of roles in cellular and viral gene regulation (1,12). The c-myc gene product, the cellular protooncogene product, seems to be a multi-functional protein as the SV40 T antigen (14). Recently, we have obtained the results that c-myc gene product can promote cellular DNA replication through binding its putative initiation site (2), and it was also reported that anti-c-myc antibody inhibits DNA replication carried out in isolated nuclei, suggesting that the c-myc protein may participate in elongation of cellular DNA replication (15). These are quite reminiscent of the function of

SV40 T antigen. In this manuscript, it was shown that the c-myc gene product may be substituted for SV40 T antigen in SV40 DNA replication. The SV40 origin-containing plasmid could replicate in transfected human cells that do not produce SV40 T antigen at all. And the level of replication was almost paralell to that of c-myc expression and the replication was inhibited by the anti-c-myc antibody. However, the copy number of replicated plasmid in HL-60 cells was lower at two order magnitude than that in Cos-I cells that constitutively produce SV40 T antigen (the copy numbers of replicated pSV0 in CosI cells were approximately 5 x 10^4 to 1 x 10^5 copies per cell). Therefore, it is presumable that there exists the strict specificity between SV40 T antigen and SV40 origin of DNA replication, whereas there is loose specificity between c-myc product and replication origins of both viral and cellular DNA.

It is not clear at present that c-myc protein is working in the cells that produce SV40 T antigen such as Cos-I cells or SV40 infected cells. We have recently reconstituted the SV40 DNA replication system and revealed that at least four proteins from uninfected cells are necessary (16). One protein of those was DNA-binding protein and bound the region near SV40 origin. The molecular weight of this protein was approximately 60 - 65 k dalton. So, it is possible that this protein might be c-myc protein. In order to clarify this possibility, completely purified and reconstituted system of SV40 DNA replication should be needed.

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