KOR. JOUR. MICROBIOL, September, 1988 p. 162-166 Vol. 26, No. 3

Inhibition of SV40 DNA Replication in Simian Cell by Bacteriophage M13 DNA sequences

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원숭이 신장 세포에서 M13 DNA에 의한 SV40 DNA 복제 억제 현상에 대하여

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ABSTRACT: Bacteriophage M13 DNAs carrying the wild type or base substituted SV40 DNA replication origins were used for replication assay. *In vivo* and *in vitro* assay with African green monkey cell line COS-1 showed that the replication of M13-SV40 recombinant DNAs was restricted like a pBR322-SV40 recombinant DNA(Lusky and Botchan, 1981). Furthermore, recombinant phage DNAs isolated from the transfected siminan cells subsequently show a reduced ability to retransform *E. coli*. But pATSV-W(Kim *et al.*, 1988) was replicated in COS-1 cells normally. We think that a poison sequence may exist on bacteriophage M13 DNA like pBR322.
KEY WORDS □ SV40 replication origin, M13 DNA, poison sequence.

The mechanisms regulating eukaryotic DNA replication are not well understood. To study this problem the simian virus 40(SV40) and the bovine papilloma virus(BPV) provide a unique and simple system for analyzing replication control. While bacterial DNA replication is regulated by positive elements necessary for the initiation of DNA replication(Abeles *et al.*, 1984, Itoh and Tomizawa, 1980, Lin and Meyer, 1984, Shafferman *et al.*, 1982) the replication control of eukaryotic genome poses a fundamentally different problem. Eukaryotic chromosomes are organized into multiple replication units, approximately ten thousand per vertebrate genome. Despite this complexity there is the absolute requirement that not only must each DNA sequence be replicated once within each cell cycle, but it must be replicated only once. There must be a mechanism to prevent the reinitiation of replication on DNA that has been previously replicated.

Earlier SV40 DNA replication studies revealed that specific pBR322 DNA sequences(poison sequences) (Lusky and Botchan, 1981) and BPV DNA sequences(Robert and Weintraub, 1986) inhibited SV40 DNA replication in monkey cells. In studies for SV40 DNA replication(Kim *et al.*, 1988), we have found that bacteriophage M13 DNA carrying the SV40 origin region was replicated poorly if at all *in vitro* and *in vivo*. Such results suggest that bacteriophage M13 DNA may involve a cis-acting poison DNA sequences as that of pBR322 plasmid.

In this paper, we describe some results that obtained from SV40 DNA replication experiments

with the plasmids pATSV-W and phage DNAs, M13SV-2 and MuSV-series(Kim *et al.*, 1988).

MATERIALS AND METHODS

Cells and all DNA templates(Fig. 1) for DNA replication have been described previously(Kim *et al.*, 1988).

Preparation of cell extracts

Cellular extracts were prepared and DNA replication was carried out under conditions similar to those described by Yamaguchi and DePhamphilis(1986), but with modifications. CV-1 cells in 10-cm dish were infected with SV40 wt830 (100 PFU/cell), and 36 hr post-infection those were taken into ice box, and washed twice with 5 m*l* of ice-cold hypotonic buffer(10 mM HEPES, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT), and the excess buffer was removed. The swollen cells were then scraped into a Dounce homogenizer with 10 strokes of tight-fitting pestle B, and the total volume was adjusted to 0.2 m*l* per dish. The lysate was incubated on ice for 40 min, centrifuged at 10,000 × g for 10 min, and the clarified lysate was stored at −70 °C.

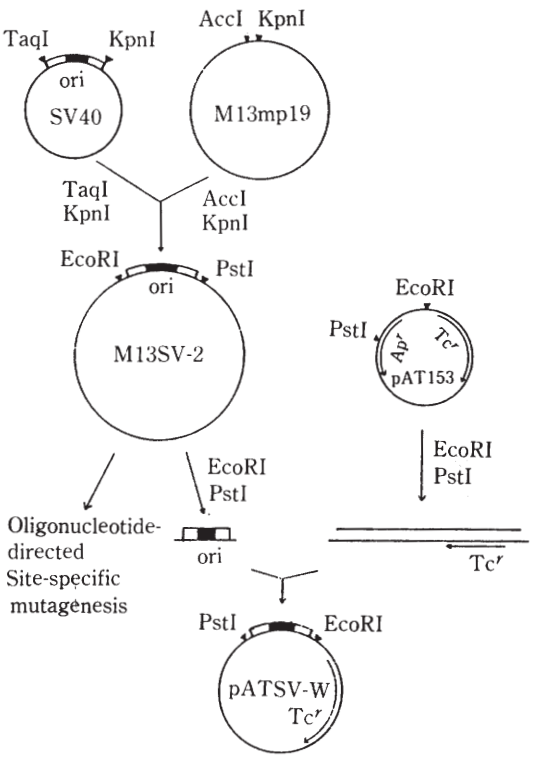

Fig. 1. *Diagrammatic representation of M13SV-2 and pATSV-W. M13SV-W was constructed by insertion of a 0.8kb(SV40 nucleotide 4,740 to 299) ori-containing segment into the AccI site and the KpnI site of M13mp19 DNA. Four kinds of mutant (MuSV-A, -B, -C, and -D) were introduced into this recombinant phage DNA, M13SV-2, by oligonucleotide-directed site-specific mutagenesis. pATSV-W was subcloned from M13SV-2 by insertion of a EcoRI-PstI SV40 ori-containing fragment into the pAT153(Twigg and Sherratt, 1980) which is a poison sequence-lacking derivative of pBR322.*

Purification of T antigen

SV40 large T antigen was purified from COS-1 cells infected with SV40 cs1085[Kindly provided by K.W.C. Peden] at 20 PFU per cell. The purification included immunoaffinity chromatography according to the procedure of Dixon and Nathans(1985) using a monoclonal antibody against SV40 T antigen, PAb419[Kindly provided by E. Harlow] (Harlow *et al.*, 1981).

Conditions for *in vitro* DNA synthesis

The DNA replication assay(50 u*l*) contained 42 mM Hepes(pH 7.8), 102 mM potassium acetate, 7 mM Mg acetate, 1 mM EGTA, 0.8 mM DTT, 4 mM ATP, 0.2 mM each of CTP, GTP, and GTP, 0.1 mM each of dATP, dGTP, dTTP, and 100 uM of [α-^{32}P]dCTP(15 uCi; 3,000 Ci/mmol), 40 mM phosphocreatine, 10 ug of creatine phosphokinase, 0.5 ug of DNA, 20 u*l* of cell extract(150 ug of proteins), and 0.5 ug of SV40 T Ag. Reaction mixtures were incubated 2.5 hr at 30 °C and then adjusted to 0.5% sodium dodecyl sulfate / 15 mM EDTA/tRNA(200 ug/m*l*)/proteinase K(100 ug/m*l*), and incubated 30 min at 37 °C. DNA was purified and digested with a excess of restriction enzyme EcoRI. DNA samples were fractionated by electrophoresis in agarose gels and ^{32}P-labeled DNA bands were visualized by autoradiography.

DNA replication assay *in vivo*

In vivo DNA replication assay was carried out by the method described in previous paper(Kim *et al.*, 1988). Replicated in COS-1 cells and Hirt extracted DNA samples were digested with DpnI

and used to transform competent *E. coli* JM103. Intact supercoiled DNA, DpnI resistant DNA, represent the replicated DNA in COS-1 cells, and the number of transformants or phage plaques are utilized to calculate the replication efficiency of the SV40 origin-carrying plasmid or phage DNA.

Transformation of *E. coli* JM105 by phage DNAs extracted from COS-1 cells

Subconfluent monolayers of COS-1 cells were transfected with bacteriophage M13mp19 DNA and recombinant bacteriophage MuSV-A DNA and the low molecular weight DNAs extracted 48 h later. To quantitate the amounts of phage DNA present in these extracts, samples of the Hirt supernatants were subjected to Dot hybridization along with standards of 0.025, 0.25, 10, and 50 ng of purified phage DNAs. After autoradiography, the amounts of radioactivity were measured by Cerenkov counting. The sample DNA concentrations were determined from those radioactivities. Within each experiment the counting response was leanear with respect to the standards. We then used equal quantities to transform *E. coli* JM105, selecting for plaques.

RESULTS AND DISCUSSION

Suppression of DNA replication of phage M13 DNA carrying SV40 origin *in vitro*

M13SV-2 and MuSV-series were tested for their ability to replicate when incubated in an extract of SV40-infected CV-1 cells containing T antigen to support normal SV40 DNA replication. M13SV-2 is a bacteriophage M13 DNA containing the wild-type SV40 replication origin, and MuSV-series are M13 DNAs containing the base-substituted SV40 replication origin. DNA replication was detected by incorporation of [α-^{32}P]dCTP followed by EcoRI digestion and agarose gel electrophoresis. When cut with EcoRI , we observed that there were only infected form III SV40 DNA and small amount of replicating DNA intermediates(RI) but SV40 origin-carrying M13 DNA(Fig. 2). We also incubated the reaction mixtures for long time(3 hr), but M13 DNAs were not replicated(data not shown).

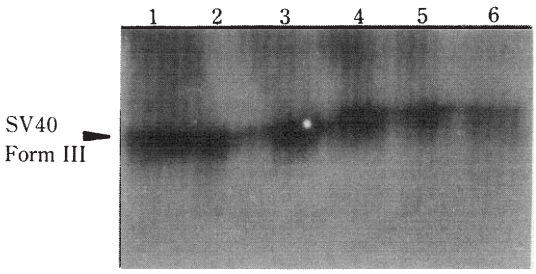

Fig. 2. *In vitro replication of M13 DNA containing the SV40 origin of replication. DNA was synthesized in standard reaction mixture containing extract from CV-1 infected with SV40 wt830 and purified T Ag. As indicated above each lane, the reaction mixtures were incubated for 2 hr 30 min at 37°C in the absence of added DNA(lane 1) or in the presence of M13SV-2(lane 2), MuSV-A(lane 3), MuSV-B(lane 4), MuSV-C(lane 5), and MuSV-D (lane 6). The redioactive reaction products were electrophoresed in a 0.5% agarose gel after endonuclease digestion and the gel was then autoradiographed. The arrow marks the position of the endogenous SV40 form III DNA. But the M13-SV40 series were not replicated.*

Comparison of *in vivo* replication of plasmids and phage DNAs by transformation of bacteria

The plasmid DNA and the phage DNA isolated by Hirt procedure(Hirt, 1967) were digested with excess DpnI restriction endonuclease. Parent and replicated daughter DNA molecules are distinguishable by their differential sensitivity to the methylation sensitive enzyme DpnI(Kim *et al.*, 1988). The restriction enzyme digestions are used to transform competent *E. coli* JM103(Hanahan, 1983). DpnI resistant DNA molecules representing the replicated fraction transform *E. coli* efficiently and are thus a measure of replication.

We also observed that the phage DNAs extracted from simian cells transformed *E. coli* 50- to 100-fold less efficiently than the same phage DNAs extracted from *E. coli*(Table 1). Table 2 shows that SV40 replication origins cloned into bacteriophage M13 DNA were restricted in their ability to replicate. In four independent experiments, M13 DNAs containing SV40 origin replicated on average only 1-2% compared with pATSV-W,. In another set of experiments by *in vivo* labelling of the replicating DNAs with

Table 1. *Transformation of E. coli JM105 by bacteriophage DNAs.*

DNA	Transformants per μg DNA	R*
purified from *E. coli*		
M13mp19 DNA	0.82–1.06 × 10^6	
MuSV-A DNA	3.38–4.06 × 10^6	
extracted from COS-1		
M13mp19 DNA	1.19–3.87 × 10^3	0.002
MuSV-A DNA	4.15–9.22 × 10^4	0.015

*R(Relative transformation efficiency) = the ratio of the Number of transformants by DNA extracted from COS-1 cells to the Number of transformants by DNA purified from *E. coli*

trithium-labelled thymidine(purchased from Amersham), we gained the same results(data not shown). Therefore, we concluded that the full-length of M13 DNA restricts the replication of SV40 DNA in simian cell.

Lusky and Botchan(1981) reported that pBR322-SV40 recombinant DNAs propagated in *E. coli* replicate poorly if at all after transfection of simian cells. Furthermore, such recombinant plasmids isolated from the transfected simian cells subsequently show a reduced ability to retransform *E. coli*(Hanahan *et al.*, 1980). Hanahan *et al.* (1980) have proposed that DNA modifications produced in the simian cell may influence replication elongation rate in simian cell and reestablishment of the plasmid in *E. coli.* But Lusky and Botchan (1981) have proposed that nic/bom site modifications of pBR322-SV40 plasmids may occur in the simian cell which influence the establishment of these DNAs in bacterial cells. And they have

Table 2. *Comparison of in vivo replication of plasmids and phage DNAs by transformation of E. coli***.*

DNA	Number of transformants	P(%)*
uncut DNA(total DNA extracted from COS-1 cells)		
pATSV-W	1.1 × 10^4	
M13SV-2	1.2 × 10^4	
DpnI-digested DNA		
pATSV-W	1.9 × 10^3	17.2
M13SV-2	3.2 × 10	0.27
MuSV-A	2.8 × 10	0.23
MuSV-B	2.6 × 10	0.22
MuSV-C	2.8 × 10	0.23
MuSV-D	2.4 × 10	0.20

*P(Percent of the replicated products) = the ratio of the Number of the transformants by DpnI-digested DNA to the Number of the transformants by uncut DNA.
**Full-length M13 DNA inhibits SV40 origin-dependent DNA replication. After DpnI digestion, Hirt extracted pATSV-W was utilized to transform *E. coli* JM103 and the transformed cells were plated on agar supplemented with tetracyclines. In case of phage DNAs, transformed cells were mixed with plating culture of *E. coli* JM103 in soft agar, poured on L-agar, incubated overnight at 37 °C. Colonies and plaques were counted mannually.

characterized the poison sequence (Lusky and Botchan, 1981) which functions as cis-acting sequence involved in inhibition of SV40 replication and overlaps the nic/bom site in pBR322.

At present, we do not know why is the M13-SV40 recombinant DNA replication restricted in simian cell. Whether a cis-acting sequence like a poison sequence in pBR322 exists in a bacteriophage M13 DNA remains to be determined.

적 요

야생형의 복제 원점 부위와 복제 원점 부위에 point mutation(base substitution)이 일어난 SV40 DNA 절편을 박테리오파지 M13 DNA에 삽입한 뒤 원숭이 신장 세포주 COS-1 system을 이용하여 *in vivo*와 *in vitro* 상태에서 복제효율을 측정하였다. COS-1 세포주에서 SV40 DNA 복제기능이 정상인 플라스미드 pATSV-W를 대조구로 사용하였을 때 M13-SV40 재조합 DNA는 원숭이 신장 세포주에서 복제가 억제됨을 보여주었다. 이는 M13 DNA상에 SV40 DNA의 복제를 억제시킬 수 있는 cis-acting sequence의 존재를 시사한다.

ACKNOWLEDGEMENTS

The authors thank Dr. E. Harlow at Oncogene Science, INC. and Dr. K.W.C. Peden at University of John's Hopkins for kindly gift of PAb419 and SV40 cs1085.

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(Received May 6, 1988)