KOR. JOUR. MICROBIOL. September, 1987 p. 165-172 Vol. 25, No. 3

Construction of an expression vector with SV40 DNA in a mammalian cell

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SV 40 DNA를 이용한 포유동물의 유전자 운반체 개발

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ABSTRACT: An expression vector in a mammalian cell was constructed using the origin of replication (OR) and the promoters of SV40. The plasmid pSVOE was constructed by inserting SV40 DNA fragment (1,118 bp) containing SV40 OR and promoters into pBR322-1, and then a multiple cloning sequence was inserted at the immediate downstream of the late promoter of SV40 in the pSVOE vector. The plasmid was named pSVML. As a selection marker, thymidine kinase gene of herpes simplex virus with its promoter was inserted into EcoRI site of pSVML and the recombinant was named pSVML-TKp.

To test the expression capacity of foreigen gene inserted at the multiple cloning site of pSVML, the thymidine kinase gene without its own promoter was inserted at the BamHI site of pSVML. The recombinant was named pSVML-TK. These plasmids, pSVML-TKp and pSVML-TK, were transfected into COS cells with calcium phosphate precipitation method. The thymidine kinase activity was significantly increased in both transfected cells.

KEY WORDS□SV40 promoter, expression vector, animal cell.

Much progress has been made in the development of hybrid plasmid vectors. The capacity of propagating and screening these vectors in bacterial systems gives them a significant advantage for isolation of cloned eukaryotic genes. However, bacterial gene expression system has limitations in the synthesis of the eukaryotic proteins. In particular, secretion, glycosylation, proteolytic processing may occur unsuitably or may not occur in bacteria (Mulligan *et al.*, 1981). Therefore, it is necessary to reintroduce eukaryotic genes into the eukaryotic cells, if it is to be expressed.

For this purpose, SV40 has been most commonly used for the construction of eukaryotic vectors. Its replication and regulation of gene expression were well studied (Gluzman, Y. *et al.*, 1981), and complete nucleotide sequence of the genome is known (P. Berg, 1981). In addition, it has a wide host range, and is transcriptionally active in many cell types. For these reasons, SV40 vector system is suitable for expression and amplification of foreign genes (Subramani *et al.*, 1983). Eukaryotic gene expression vectors involve a marker gene for easy selection of eukaryotic transformants. The thymidine kinase (TK) gene of the herpes simplex virus (HSV) has been studied as an ideal genetic marker in gene expression system (Wigler *et al.*, 1977; Roizman B. *et al.*, 1984).

In this paper, we report the construction of an mammalian expression vector by using the origin of replication and promoters of SV40. This vector contains the multiple cloning site and HSV-tk gene as a selection marker.

MATERIALS AND METHODS

Enzymes and chemicals

Enzymes including restriction endonucleases, Klenow fragment and phage T4 ligase were obtained from New England Biolabs and Bethesta Research Laboratories (BRL). Dulbeco's modified eagle's medium, fetal bovine serum and antibiotics used in mammalian cell culture were purchased from Gibco.

Bacterial strains and mammalian cells

E. coli K-12 strain HB101 (F^-, r^-, m^-, recA13) was used as host cell for all bacterial transformations. Bacteria were grown in LB medium (10g of Bacto trypton, 5g of yeast extract and 10g of NaCl per liter). COS cells were grown in Dulbeco's modified eagle's medium supplemented with 10% fetal bovine serum, penicillin G at 250 units/ml, and streptomycin at 0.2 μg/ml, and the cultures were maintained at 37°C in a moist atmosphere containing 5% CO_2.

Preparation of plasmid DNA

Plasmid DNAs were isolated according to the procedure of Birnboim & Doly (1979), and purified by CsCl/ethidium bromide equilibrium density gradient centrifugation (Clewell and Hellinsky, 1969). Plasmids were routinely checked by agarose gel electrophoresis.

Enzymatic reactions

Enzymatic digestions were carried out at 37°C for 1 hr in common digestion buffer (33 mM Tris-HCl pH 7.0, 66 mM KOAc, 10 mM $Mg(OAc)_2$, 0.5 mM dithiothreitol). The ligation of DNA fragment with cohesive ends as carried out at 12-14°C, and that with blunt ends as performed overnight at 18-25°C.

Transformation and transfection

Transformation of *E. coli* strain HB 101 was performed by the method of Kushner (Maniatis *et al.*, 1982). Mammalian cell transfection was carried out by calcium phosphate precipitation method (Graham and Van der Eb, 1973).

Preparation of COS cell extract

To prepare the COS cell extract for thymidine kinase (TK) assay, the cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS) solution. The cell pellet was then suspended in a sonication buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 5 μM thymidine. The cell suspension was sonicated and the supernatant was used to assay the cytoplasmic TK in the extract.

Thymidine kinase (TK) assays

The enzyme assays described previously for HSV-tk (Jamieson & Suback Sharpe, 1974) was modified as follows. The assay mixture contained 50 mM Tris-HCl (pH 7.5),10 mM $MgCl_2$, 10 mM ATP, 0.2 mM CTP, 5 μCi ^{3}H-thymidine (20 Ci/mmol) and each cell extract in a total volume of 100 μl. Reaction mixtures were incubated at 37°C for 1 hr and the reactions stopped by brief boiling followed by quick cooling on ice. Phosphorylated thymidine was retained from unphosphorylated nucleoside onto Whatman DE 81 paper discs by spotting 25 μl aliquots, in triplicate, of the assay supernatant. Discs were washed with cold 30 mM ammonium formate for 30 min, with 4 mM ammonium formate/5 μM thymidine for 3 times each for 30 min and with cold distilled water, rinsed with absolute alcohol, dried and counted in PPO-POPOP scintillation cocktail, toluene solution of 2,5-diphenyloxazole and 1,4-bis [2-(5-phenyloxazoyl)] benzene.

RESULTS AND DISCUSSION

Construction of pSVOE

To express the foreign genes in mammalian cells, eukaryotic vector pSVOE was constructed as shown in Fig. 1. pSVOE consists of eukaryotic and bacterial DNA sequences which are derived from SV40 and pBR322, respectively. The 1,118 bp HindIII fragment of SV40 comprises the sequences capable of promoting the synthesis of mRNA in both the early and the late directions (Crowley *et al.*, 1983), increasing transcriptional

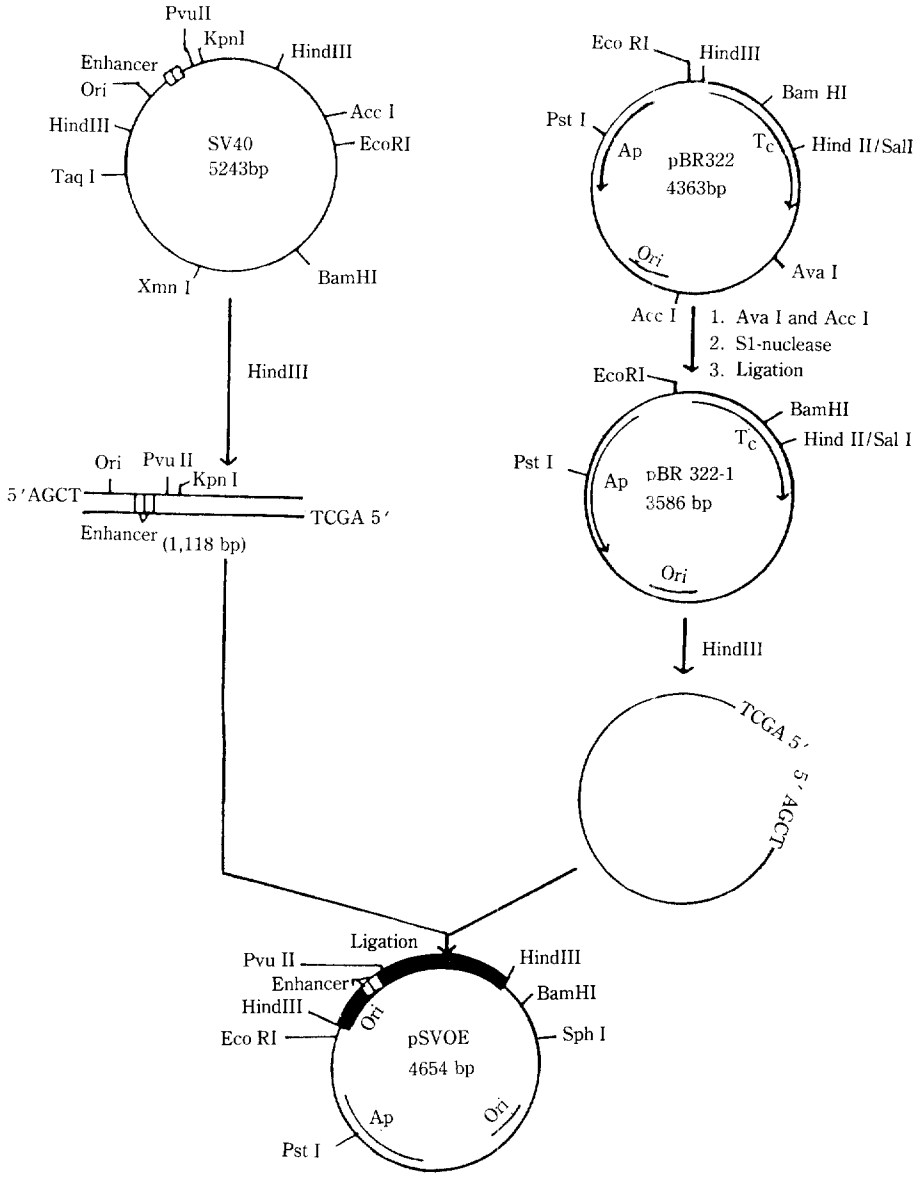

Fig. 1. *The strategy for the construction of a eukaryotic vector pSVOE.*
HindIII fragment of SV40 (1,118 bp) was inserted into the same site of pBR322-1. Ap. ampicilline; Ori (solid box), the SV40 replication origin; open box, 72 bp repeats of SV40.

efficiency, and serving as an origin of replication of SV40. This DNA fragment was obtained among six fragments of SV40 DNA digested by HindIII and used for the construction of pSVOE. The bacterial sequences of pSVOE are originated from pBR322-1 that is a pBR322 derivative lacking sequences which inhibit replication in monkey cells.

The procedure for the construction of pBR322-1 is as follows. The larger fragment (3.5 kb) produced by AvaI and AccI codigestion of pBR322 was treated with SI nuclease to produce blunt ends and recircularized to make pBR322-1. Then the 1,118 bp HindIII fragment of SV40 was inserted into the HindIII site of pBR322-1. The resulted plasmid

was named pSVOE and characterized by restriction endonuclease analysis (data not shown).

Construction of pSVML

Previously constructed pSVOE has only a few cloning sites behind both early and late promoters of SV40. To insert foreign genes with variable restriction ends into the downstream region of the late promoter of SV40, pSVOE was modified as follows.

The general approach for the modification of pSVOE involved the incorporation of multiple cloning sequence of M13mp18 into pSVOE as shown in Fig. 2. First, pSVOE and M13mp18 were codigested with KpnI and SalI, respectively. A DNA fragment (27 bp) of M13mp18 containing eight cloning sites was ligated with the larger fragment of pSVOE. The resulting plasmid was named pSVML and characterized by restriction endonuclease analysis (data not shown). pSVML has many cloning sites for the insertion of foreign genes, and then it can replicate to high copy number in COS cells constitutively producing the large T antigen of SV40.

Construction of expression vector pSVML-TKp

In order to distinguish the transfected cells from nontransfected ones when the eukaryotic vector pSVML is transfected to the mammalian cells, the selective marker gene was introduced in-

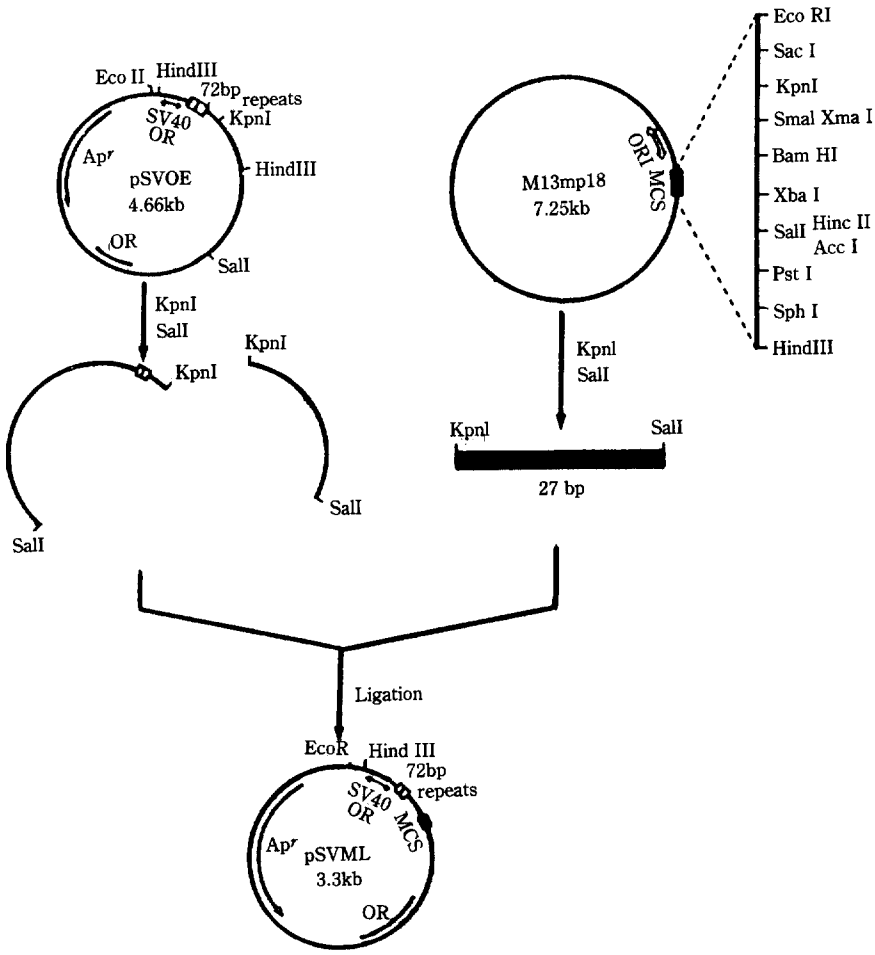

Fig. 2. *The strategy for the construction of pSVML.*
pSVML was constructed by inserting KpnI/SalI fragment of M13mp18 into pSVOE. Solid box; multiple cloning site.

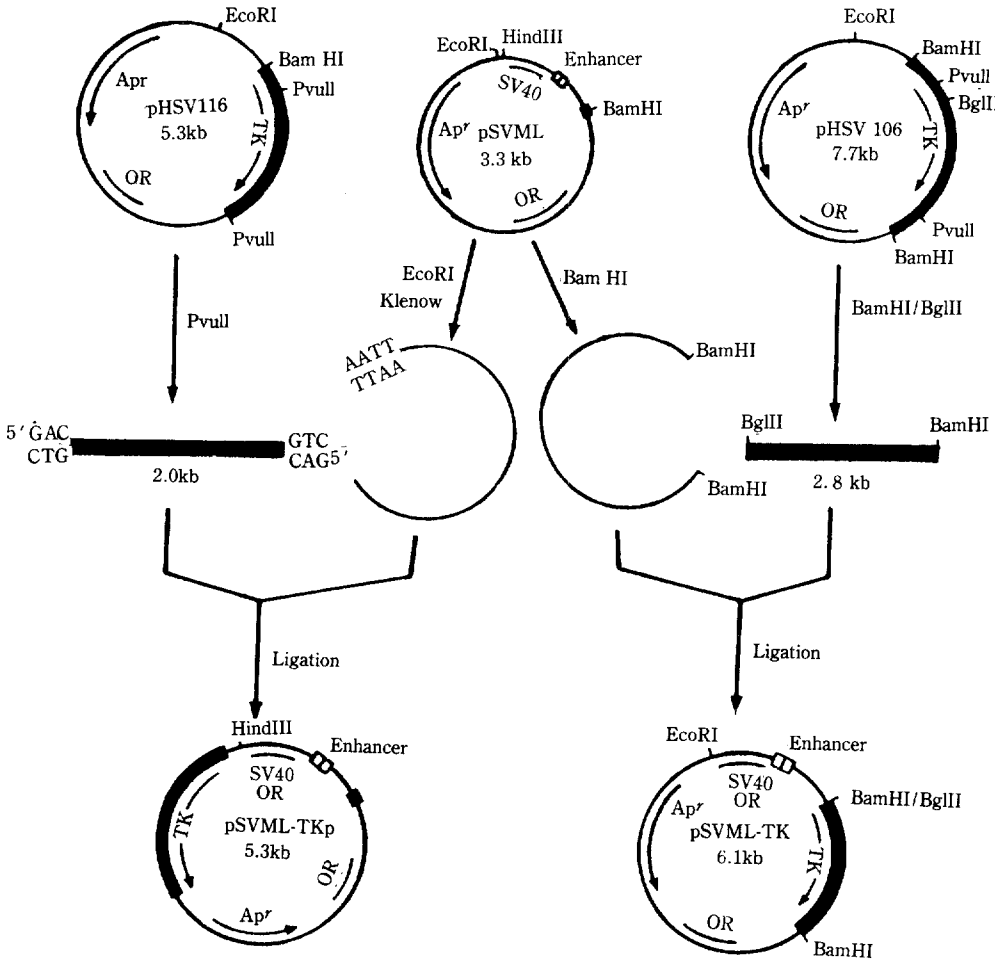

Fig. 3. *The strategy for the constructions of pSVML-TKp and pSVML-TK.*
The thymidine kinase gene of Herpes simplex virus was inserted into either EcoRI site (pSVML-TKp) or BamHI site (pSVML-TK) of pSVML, respectively. The direction of transcription is shown by arrows.

to pSVML. The thymidine kinase (TK) gene of Herpes simplex virus (HSV) was chosen as the selective marker gene and obtained from pHSV 116 which is the recombinant plasmid comprising BamHI-HSV DNA fragment with TK gene. The 2.0 Kb DNA fragment of TK gene with its promoter was isolated from pHSV116 by PvuII digestion (Fig. 3). pSVML was linearized by cutting with EcoRI and its ends were filled in with klenow fragment. PvuII fragment of TK gene was ligated to linearized pSVML by blunt end ligation. Through the transformation of HB101 with the ligation mixture and the selection of colonies showing resistance against the antibiotics, ampicillin, the recombinant plasmid pSVML-TKp was isolated and characterized as Fig. 4. As pSVML-TKp was codigested with BamHI and BglII to identify the orientation of the inserted TK gene, two bands of 4.6 kb and 0.7 kb DNA fragment were detected through 0.8% agarose gel electrophoresis (Fig. 4, lane 3). This result represents that the transcriptional direction of TK gene inserted in pSVML-TKp is the same as β-lactamase gene in pSVML-TKp. When pSVML-TKp is introduced in TK^- cells, one can select the transformants (TK^+ cell) and study a lot of aspects in long-term trans-

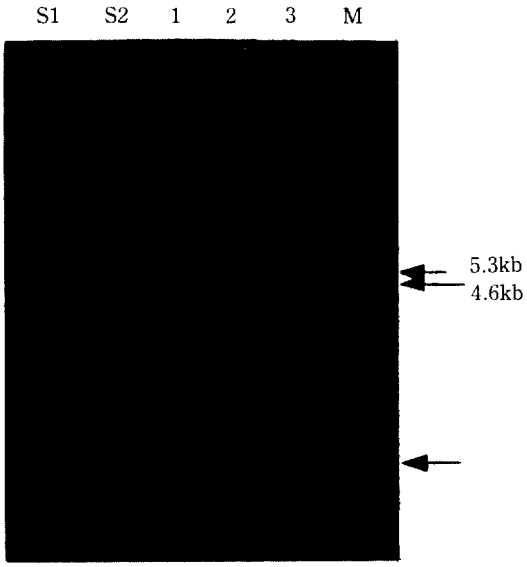

Fig. 4. *Restriction endonuclease analysis of pSVML-TKp.*
Lane S1, standard DNA of pHSV 116: S2, pSVML-TKp: 1, pSVML-TKp + HindIII: 2, pSVML-TKp + BamHI: 3, pSVML-TKp + BamHI + BglII: M, λ-EcoRI size marker.

formed cells.

Construction of pSVML-TK

To investigate the expression capacity of the eukaryotic vector pSVOE for the cloned foreign genes, the TK structural gene without its promoter was incorporated downstream of the late promoter of SV40 in pSVML. The recombinant plasmid was named pSVML-TK and the procedure for the construction of pSVML-TK represented in Fig. 3. The TK structural gene without its promoter was obtained from BglII-BamHI digestion of pHSV106 as described (Wigler, M.J. *et al.*, 1981). The 2.8 kb DNA fragment was inserted into BamHI site of pSVML. As a result, this test gene located behind the SV40 late promoter in the same orientation with respect to transcriptional direction is under the control of SV40 late promoter and enhancer. Fig. 5 represents the characterization of pSVML-TK by restriction analysis.

To identify the orientation of cloned TK structural gene pSVML-TK was digested with PvuII (Fig. 5, lane 2). The TK structural gene was cloned to the correct orientation as described previously.

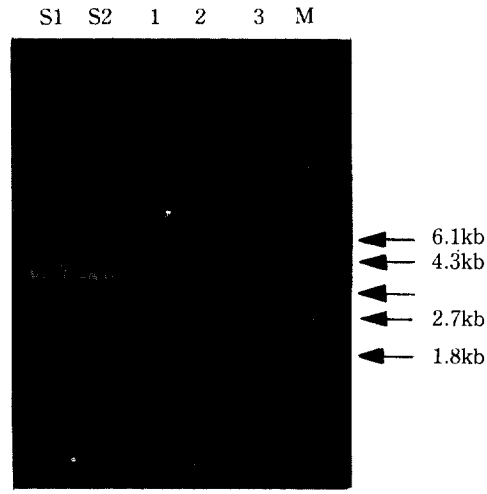

Fig. 5. *Restriction analysis of pSVML-TK.*
Lane S1, standard DNA of pSVBS; S2, pSVML-TK; 1, pSVML-TK + EcoRI; 2, pSVML-TK + PvuII; 3, pSVML-TK + BamHI; M, λ-HindIII size marker.

Expression of the thymidine kinase (TK) gene in the COS cells

We analyzed the expression level of the TK gene under transcription unit of pSVML and the probability of the TK gene to use as a selective marker gene. First, COS cells were transfected with the plasmid pSVML-TK and pSVML-TKp by calcium phosphate precipitation technique, respectively, and then the levels of TK activity in COS cell extract were measured 3 days later. COS cells (5×10^5 cell/dish) were exposed to a calcium phosphate coprecipitates formed with either 5 μg of pSVML-TKp or 5 μg of pSVML-TK and 5 μg of calf thymus carrier DNA each as described in Materials and Methods. As a control, mock transfected cells that were transfected with 10 μg of carrier DNA were used in the same manner. Fig. 6 represents that the TK enzymatic activity versus the μg of total protein concentration in the cell extract. To observe the effect of protein concentration on TK activity, protein concentration was measured by Bradford method (1976) and radioactivity was measured for different protein concentration. When dose-response curves of transfected cells and mock-transfected cells are com-

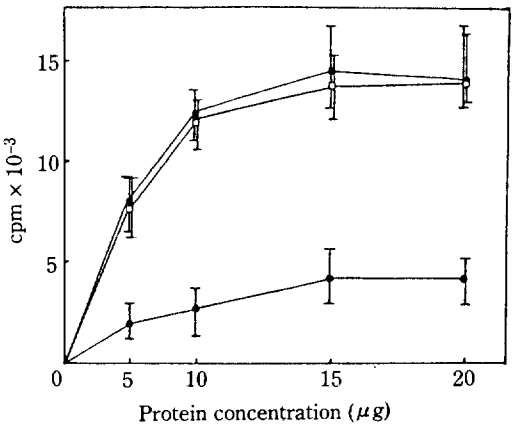

Fig. 6. *TK enzymatic activity versus the μg of total protein concentration in the cell extract.*
The assay extract was prepared from COS cells 3 days after transfection with pSVML-TK (□), pSVML-TKp (■), and with carrier DNA (●), respectively.

pared, TK activity was found to be significantly increased in both pSVML-TKp and pSVML-TK transfected cells. This result demonstrates that a significant increase of the TK activity in the transfected COS cells results from the TK gene expression of the plasmid pSVML-TKp and pSVML-TK in COS cells. In this result, the difference of the TK activity values in pSVML-TKp transfected cells from the pSVML-TK transfected cells is not shown. We don't know the reason of this result well. But probably the TK gene expression in pSVML-TKp may be controlled under the early transcription unit of SV40 rather than under the promoter of TK gene itself. In this assay of TK activity, mock-transfected COS cells were shown residual activity and we are at present unable to detailed explanation for various factors encounted in any quantitative measurement. However, dose-response curves of Fig. 6 represent that an increase in TK activity in the transfected COS cells as compared with mock-transfected cells results from the expression of the TK gene in COS cells transfected with pSVML-TKp and pSVML-TK, respectively. Therefore we can suggest that the eukaryotic expression vector constructed in this report would be suitable for mammalian gene expression system and useful for analysis of gene regulation in mammalian cells.

적 요

고등동물세포에서 외래 유전자의 발현 및 조절기작을 연구하기 위하여 SV 40의 복제원점과 프로모터를 포함하며, multiple cloning site 부위를 갖는 유전자 운반체를 개발하였다.

SV 40 DNA와 pBR 322 DNA의 두 복제원점이 다 포함된 유전자 운반체 pSVO를 이용하여, multiple cloning site를 도입하고(pSVML), 표지유전자로써 Herpes simplex virus에서 thymidine kinase(TK) 유전자를 분리하여 삽입하였다(pSVML-TKp). 또한 multiple cloning site에 재조합된 외래 유전자의 발현성을 보기 위하여 프로모터가 없는 TK 구조 유전자만을 pSVML에 삽입하였다(pSVML-TK).

이와같이 만든 재조합 플라스미드(pSVML-TKp, pSVML-TK)를 칼슘, 인산염 방법으로 COS 세포에 형질전환시키고 TK 활성을 조사한 결과, 대조구와 비교할 때 모두 TK 활성이 현저하게 높은 것으로 나타났다.

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(Received Aug. 6, 1987)