



HOKKAIDO UNIVERSITY

Title	EXPRESSION OF THE ENDOGENOUS MAREK'S DISEASE VIRUS ICP4 HOMOLOG (MDV ICP4) GENE IS ENHANCED IN LATENTLY INFECTED CELLS BY TRANSIENT TRANSFECTION WITH THE RECOMBINANT MDV ICP4 GENE
Author(s)	ENDO, Daiji; IKEGAWA, Satoshi; KON, Yasuhiro et al.
Citation	Japanese Journal of Veterinary Research, 43(3-4), 109-124
Issue Date	1995-12-27
DOI	https://doi.org/10.14943/jjvr.43.3-4.109
Doc URL	https://hdl.handle.net/2115/2516
Type	departmental bulletin paper
File Information	KJ00002398192.pdf



EXPRESSION OF THE ENDOGENOUS MAREK'S DISEASE VIRUS ICP4 HOMOLOG (MDV ICP4) GENE IS ENHANCED IN LATENTLY INFECTED CELLS BY TRANSIENT TRANSFECTION WITH THE RECOMBINANT MDV ICP4 GENE

Daiji ENDOH¹, Satoshi IKEGAWA¹, Yasuhiro KON²,
Masanobu HAYASHI³ and Fumiaki SATO¹

(Accepted for publication: Nov. 14, 1995)

ABSTRACT

The ICP4 homolog of Marek's disease virus (MDV ICP4) is a possible candidate for the transactivator of the early genes. We transfected MDCC-MSB-1 (MSB-1) tumor cells with plasmid including a coding region of MDV ICP4 using cationic liposome. As carriers for intranuclear transport, high mobility group -1 and -2 proteins were bound to the plasmid DNA before forming liposomes. We detected transcripts from the plasmid 2 hr after transfection by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. We also detected abundant transcripts of endogenous ICP4 2-96 hr after transfection. These data suggested that expression of introduced MDV ICP4 gene enhanced the expression of endogenous MDV ICP4. On the other hand, quantitative PCR analysis for virus genome DNA indicated no significant alteration of copy number of virus genome in transfected MSB-1 cells, suggesting that reactivation of virus requires more than turning on MDV ICP4 gene.

Key Words: Marek's disease virus, ICP4 homolog, High mobility group proteins, Cationic liposomes, Virus replication

INTRODUCTION

Marek's disease virus (MDV) is a cell-associated herpesvirus that causes T cell lymphoma and demyelinating peripheral neuropathy in chicken⁶⁾. MDV has some similarity with gammaherpesviruses such as Epstein-Barr virus (EBV) based on its biological properties²⁴⁾. On the other hand, based on its structure and homology of

¹ Laboratory of Radiation Biology and ² Experimental Animal Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060.

³ Department of Veterinary Radiology, Faculty of Veterinary Medicine, Rakunogakuen University, Ebetsu 069.

genes, MDV is similar to alphaherpesvirus such as Herpes Simplex virus type-1 (HSV-1) and varicella-zoster virus (VZV)²⁶⁾. Restriction enzyme map analysis confirmed that the structure of MDV DNA is indeed similar to that of HSV-1, consisting of a unique long (U_L) and a unique short (U_S) region both flanked by a set of inverted repeats (TR_L , IR_L , IR_S and TR_S) (Fig. 1)¹⁰⁾. Amino acid sequences predicted from MDV genes also indicate a closer relationship to alphaherpesviruses^{4,25-27)}.

Although *in vitro* transformation is not easily achieved and the fragments of the genome responsible for transformation have not yet been identified, lymphoblastoid cell lines have been established from Marek's disease lymphomas⁷⁾. The virus can be obtained from most lymphoblastoid cell lines by co-cultivation with susceptible cells such as chicken embryo fibroblast (CEF) cells or by inoculation with tumor cells into chickens. Viral antigens are not detectable in these cell lines by immunofluorescence (IF) tests using conventional anti-MDV sera. On the other hand, a phosphorylated polypeptide was demonstrated after treatment of the cells with 5-iodo-2-deoxyuridine (IUdR), using monoclonal antibodies¹¹⁾. These results indicate that the lymphoblastoid cells were latently infected⁷⁾.

Previous studies have shown that gene expression of MDV in many cell lines is mainly restricted to the repeat regions of the genome and the majority of the transcripts are those of immediate-early (IE) genes^{28,29)}, indicating that IE genes should play a role in maintenance of latency and of the transformed state. On the other hand, IE genes are required for the efficient expression of the subsequently early (E) and late (L) genes in lytic infection²⁴⁾. One of the HSV-1 IE gene products, ICP4, is indispensable for the growth of HSV-1 because it activates transcription of most of the essential genes of the virus^{16,20,23)}. As the MDV ICP4 shows similarity in gene structure and protein structure with ICP4²⁾, the MDV ICP4 protein should have similar functions with ICP4 such as transactivation of E and L genes.

Pratt et al.²²⁾ indicated that pp38 gene expression was upregulated in lymphoblastoid cells in which MDV was latently infected and MDV ICP4 gene was constitutively transfected. As they did not detect the transcripts of plasmid-coding MDV ICP4 gene, they did not show the direct evidence for the transactivation by MDV ICP4. The main objective of the present work to observe the direct influence of the expression of MDV ICP4 on the expression of MDV genes in lymphoblastoid cell line MSB-1. To achieve the high efficiency of transfection, we used cationic liposome and high mobility group (HMG) proteins which are carriers of the fast transport of introduced plasmid into intranuclear region. The fast transport resulted in the expression of MDV ICP4 in a short range of time. Using reverse-transcriptase polymerase chain reaction (RT-PCR) method, we detected the expression of plasmid-coding MDV ICP4 gene and the enhancement of expression of endogenous MDV ICP4 gene. On the other hand, quantitative PCR of virus DNA showed no enhancement of viral replication. The results of this study suggest that reactivation of MDV requires

more than turning on MDV ICP4 gene.

MATERIALS AND METHODS

Cells and cell cultures. MSB-1 MD tumor cells¹⁾ were propagated at 37°C in 5% CO₂-95% air in Daigo-T medium (Nihon Pharmaceutical Co. Ltd., Osaka, Japan), supplemented with 5% fetal bovine serum (GIBCO BRL Life Technology Inc., Gaithersburg, MD, U.S.A.), glutamine and antibiotics. CEF cells were cultured in 9-cm dishes and infected with MDV serotype 1 strain CVI-988 as previously described⁹⁾.

Construction of MDV ICP4 plasmids. A 4320 base pair (bp) DNA fragment including the entire coding region was amplified with pfu polymerase (Promega Biotec, Madison, WI U.S.A.) as described below and cloned into pSV- β containing the β -galactosidase gene under the control of the SV 40 early promoter/enhancer unit¹⁸⁾, which was obtained from RIKEN DNA Bank, Tsukuba, Japan (Fig. 1). Sequences of oligonucleotides obtained from Science Tanaka, Ebetsu, Japan, which were used for PCR, are listed in Table 1. The oligonucleotide sequences of the MDV ICP4 gene are those reported by Anderson et al.²⁾ and McKie et al.¹⁹⁾. A clone of *Bam*HI-A fragment of MDV was kindly provided by Dr. M. Nonoyama¹⁰⁾.

The PCR mixture was brought to volume of 100 μ l containing 10 μ l of 10x buffer (200 mM Tris, pH 8.4, 500 mM KCl and 15 mM MgCl₂), 20 nmol of each deoxyribonucleoside triphosphate (dNTP, GIBCO BRL Life Technology Inc.), 100 pmol of each Ip1 and Ip2 primer (Table 1), 1 ng of the *Bam*HI-A fragment and 2.5 U of pfu polymerase (Promega Biotec). Amplifications were performed on a DNA thermal cycler (Astec Industrial Co. Ltd, Tokyo, Japan) with denaturation for 4 min at 94°C, annealing for 4 min at 60°C, and extension for 15 min at 72°C for 33 cycles. The PCR fragment was ligated with *Not* I linkers and cloned into the *Not* I site pSV- β vector by substitution with β -galactosidase. This plasmid, in which the coding region of MDV ICP4 gene was cloned in the sense direction, was designated pSV-ICP4S (Fig. 1).

Transfection of plasmid. Gene transfer with DNA-enclosed liposomes was performed essentially according to the method of Kaneda et al.¹⁴⁾, except that we used a cationic lipid (Gene Transfer, Wako Pure Chemicals Co. Ltd, Osaka, Japan) to make liposomes and did not use Sendai virus for the fusion with recipient cells because of the high efficiency of the cationic liposome for fusion with cells. To make the DNA-protein complex, the plasmid (20 μ g) was mixed with 6.5 μ g of high-mobility group (HMG)-1 and 2 solution (Wako Pure Chemicals Co. Ltd), stood at room temperature for 1 hr and diluted to 1 ml with PBS. Diluted plasmid-HMG solution was transferred into a tube containing the dried film of Gene Transfer and vortexed for 2 min. Subsequently, the mixture was transferred into a 3 cm-diameter-dish containing 10⁵ MSB-1 cells in 2 ml of T-medium. Medium was changed 24 hr after transfection and culture was maintained for 4 days.

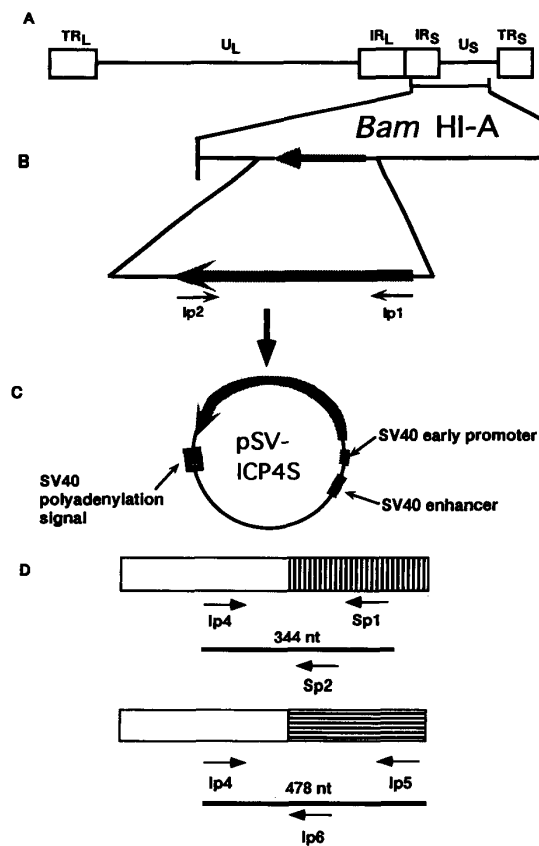


Fig. 1 (A) Diagram of the MDV genome showing the unique long region (U_L) and unique short region (U_S) flanked by repeat regions. TR_L , terminal repeat flanking U_L ; IR_L , internal repeat flanking U_L ; IR_S , internal repeat flanking U_S ; TR_S , terminal repeat flanking U_S . (B) Expanded map of the *Bam*HI-A genomic fragment. The large arrow indicating the predicted coding region, small arrows indicate the directions and relative positions of PCR primers. Primer pair Ip_1 and Ip_2 was used for amplification of the coding region. (C) Construction of pSV-ICP4S, which was designed for expression of recombinant MDV ICP4 in transfected cells. (D) Detailed drawings of amplified fragments of recombinant and endogenous MDV ICP4 transcripts. Open boxes indicate the coding region of MDV ICP4 in both transcripts. Different designs of hatched boxes to the left of open boxes indicate the 5'-untranslated region of the recombinant or endogenous MDV ICP4 transcript. Different designs indicate that the nucleotide sequences of the 5'-untranslated region were different from each other. Below each box, arrows indicate the directions and relative positions of PCR primers (Sp_1 , Ip_4 and Ip_5) and probes (Sp_2 and Ip_6).

Table 1. Primers and probes.

Gene/Transcript	Oligo-nucleotide ^a	Sequence (5' to 3')	Product Size (bp)	
Chicken β -actin ^b	Ap1 ^U	TGTGATGGTTGGTATGGGCC	443	
	Ap2 ^D	AGGTAGTCCGTAGGTCACG		
	Ap3 ^I	CTCAGGGTCAGGATACCTC		
MDV ICP4 ^c	(Coding region)	Ip1 ^U	GATGGTCGAGTTGGGGTTCC	4320
		Ip2 ^D	CAGATTGGAGGGCGAATTGA	
		Ip3 ^R	AAGTGCTTGTCGATGCCAC	
	(RT Primer) (Coded in pSV-ICP4S)	Sp1 ^{U,d}	AAAAGCTCCTCGAGGAACTG	344
		Ip4 ^D	TCCATATTGGCGTTGCAACGG	
		Sp2 ^{I,d}	AGCTGCGGAATTGTACCCGC	
	(Endogenous)	Ip5 ^U	GATGGAAGCACCAATCCCTG	478
		Ip4 ^D	TCCATATTGGCTGGTCAACGG	
		Ip6 ^I	TCCAACAACCTCGCTATCGCG	
Promoter region of the meq gene ^e	Mp1 ^U	AATTCGGTGATATAAAGACG	248	
	Mp2 ^D	TCCCCGAACCAAATAATAA		

^a Suffixing indicates the following : U, upstream primer ; D, downstream primer ; I, internal probe ; R, primer for reverse transcriptase.

^b Sequences are as reported by Kost et al. ¹⁵⁾.

^c Sequences are as reported by Anderson et al. ²⁾.

^d Sequences are reported by MacGregor and Caskey ¹⁸⁾.

^e Sequences are as reported by Jones et al. ¹³⁾.

To observe the time course of gene expression of introduced plasmid, MSB-1 cells were transfected with pGV-C vector (Tokyo Ink Co. Ltd, Tokyo, Japan), which contained the beetle luciferase gene under the control of the SV40 early promoter/enhancer unit, and the efficiency of transfer was assessed by measuring the luciferase activity in cell extracts. Mock transfection was performed using pSV- β vector, which contained the β -galactosidase gene instead of the luciferase gene. Expression of luciferase was measured using a luciferase assay-kit (PicaGene, Tokyo Ink Co. Ltd.) according to manufacturer's instructions. Briefly, transfected cells were lysed in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1, 2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol and 1% Triton X-100) for 15 min. The lysis solution was then mixed with a luciferin-ATP-acetyl-coenzyme A mixture (20 mM Tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μ M coenzyme A, 470 μ M luciferin and 530 μ M

ATP). Ten seconds after mixing, chemiluminescence was measured with an LSC-700 liquid scintillation counter (Aloka Co. Ltd., Tokyo, Japan) for 1 min.

Preparation of DNA and RNA from MSB-1 cells. Pelleted cells were suspended in TRIZOL (GIBCO BRL Life Technology Inc.) with pipettes of a microdispenser and stored at -80°C till the series of samples were collected. Series of samples were defrozen simultaneously and extracted with chloroform once. RNAs in the collected aqueous phase were precipitated with isopropanol, washed with 70% ethanol, and dissolved in RNase-free distilled water (DW). Sequentially, poly(A)⁺ RNA was purified with BioMag oligo(dT)₂₀ beads (Perspective Diagnostic, Concord, MA, U.S.A.) according to manufacturer's instructions. Briefly, poly(A)⁺ RNA was bound to oligo(dT)₂₀ beads in 0.15 M NaCl solution, washed with 0.1 M NaCl solution and eluted in DW containing 0.01% sodium azide at 60°C for 10 min.

DNAs of transfected MSB-1 cells were extracted with DNA Isolator genomic DNA extraction reagent (Genosys Biotechnologies, Inc., The Woodlands, TX, U.S.A.) according to manufacturer's instructions. Briefly, pelleted MSB-1 cells were homogenized in 1 ml of the reagent. The reagent was extracted with chloroform. DNA was isolated into the aqueous phase, precipitated with ethanol and dissolved in DW. To quantitate the amount of high molecular weight genomic DNA, an aliquot of DNA electrophoresed in 0.8% agarose gel. The high molecular weight fraction of the DNA was isolated with a JET sorb DNA extraction kit (Genomed GmbH, Oeynhausen, Germany) and eluted into 1 ml of DW. The concentration of high molecular weight DNA was measured as OD₂₆₀.

RT-PCR. Reverse transcription was performed essentially as by Kramer and Coen¹⁶). Following an initial denaturation step of 10 min at 95°C , poly(A)⁺RNA was hybridized with downstream primers Ap2 and Ip3 (Table 1), for 5 min at 65°C in a 12 μl volume containing 3 pmol of each downstream primer, 250 mM KCl, 10 mM Tris (pH 8.3), and 1 mM EDTA and then covered with mineral oil. After phase inversion with chloroform, half (6 μl) of each sample was added to each of two reaction mixtures containing 12 μl of 1.5x RT buffer (1.5 mM of each dNTP, 7.5 mM dithiothreitol, 15 mM MgCl₂, 90 mM KCl and 0.63 mM Tris, pH 8.3). Reaction mixtures were incubated with or without 10 U of Superscript II RT (GIBCO BRL Life Technology Inc.) at 42.5°C for 1 hr and then subjected to heat denaturation at 95°C for 5 min. The resulting cDNA samples were stored at -80°C .

PCR mixtures were brought to a volume of 20 μl containing 2 μl of 10x buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1.0% TritonX-100), 20 pmol of each upstream and downstream primer (Table 1), 0.6 μl of 50 mM MgCl₂, 0.4 μl of 10 mM dNTPs, 0.5 U of Taq polymerase (GIBCO BRL Life Technology Inc.) and the cDNA sample. The PCR mixtures were constructed on ice (cold-start method) and covered with mineral oil. The thermal cycle consisted of denaturation for 2 min at 94°C , annealing for 2 min at the temperature listed in Table 2 and extension for 1 min

Table 2. PCR conditions.

PCR Product	[Mg ²⁺] (mM)	Annealing temp (°C) ^a	No. of cycles ^b
Chicken β -action	1.5	54	25
Recombinant MDV ICP4	3.0	60	30
Endogenous MDV ICP4	3.0	55	27
Promoter region of the meq gene	1.5	55	28

^a In each case, the denaturation was performed at 94°C for 2 min and the extension was performed at 72°C for 1 min. For annealing, each temperature was held for 2 min.

^b The cycle number reflects the relative abundance of a given RNA species.

at 72°C for the total number of 28 cycles. Reaction mixtures were stored at 4°C. When recombinant or endogenous MDV ICP4 transcripts were amplified and quantitated, 5'-noncoding sequences were used to distinguish these transcripts (Fig. 1D). Thus, although the downstream primer Ip4 was hybridized to both transcripts, forward primers and internal probes hybridized only to specific transcripts (Fig. 1D). Sp1 and Sp2 were hybridized to recombinant MDV ICP4 and Ip5 and Ip6 were hybridized to endogenous MDV ICP4.

RNA and DNA standards. To quantitate the amount of the gene-specific transcript, cRNAs were synthesized as described below. Amplified fragments of the PCR products using Ap1/Ap2, Ip4/Ip5, Sp1/Ip6 and Mp1/Mp2 were cloned into pGEM-T vector (Promega Biotec.) according to manufacturer's instructions. Selected plasmids were constructed so that sense strand was transcribed with T7 RNA polymerase. RNA transcription *in vitro* was performed by using 1 μ g of linearized plasmid DNA in 25- μ l reaction volumes. Following treatment RNase-free DNase (Promega Biotec), phenol-chloroform extraction, and ethanol precipitation, the samples were resuspended in 100 μ l of RNase-free DW and 10- μ l samples were taken to measure the amount of synthesized RNA with OD₂₆₀. These synthetic cRNA stocks were serially diluted to 0.0166, 0.166, 1.66 and 16.6 fmol/l (10², 1,0³, 10⁴ and 10⁵ molecules per 10 μ l) were mixed with 10 μ l yeast transfer RNA (Boehringer Mannheim GmbH, Mannheim, Germany), 3 pmol of Ap2, 3 pmol of Ip3 and 10x annealing buffer (2.5 M KCl, 10 mM Tris, pH 8.3 and 1 mM EDTA). After annealing of primers, cDNAs were synthesized, amplified and hybridized with the gene-specific probes as described above.

Detection and quantification of PCR products. Reaction products (10 μ l) were separated by electrophoresis on a 2% agarose gel (AgaroseX, Nippon Gene, Toyama, Japan). Gels were stained with ethidium bromide and visualized under UV light. Separated products were capillary blot transferred to a nylon filter (BioDyne B, Pall Process Filtration Ltd, Portsmouth, United Kingdom), UV cross-linked for 2 min, and prehybridized in hybridization buffer (50 mM potassium phosphate, pH 7.0, 50 μ g/ml of

denatured salmon sperm DNA, 2% blocking reagent (Boeringer Mannheim GmbH.), 7% SDS, and 5×SSC). Probes (Table 1) were end labeled with polynucleotide kinase (New England BioLabs., Inc., Beverly, MA, U.S.A.) in the presence of [γ - 32 P]ATP (Institute of Isotopes Co. Ltd., Budapest, Hungary). Hybridization was performed in the same buffer as the prehybridization. After hybridization, filters were washed with 1×SSC at 65°C and exposed to an IP plate (Fuji Photo Film Co., Ltd., Tokyo, Japan) for 1 to 24 hr. Radioactivity of the hybridized probe was measured with a BAS1000 BioImaging analyzer (Fuji Photo Film Co., Ltd.) and indicated as PSL, a radioactivity unit for luminescent counting directly proportional to radioactivity over 5 orders of magnitude.

RESULTS

At first, we observed the time course of gene expression after transfection using a luciferase-containing vector (Fig. 2). When pGV-C was introduced with an HMG-1

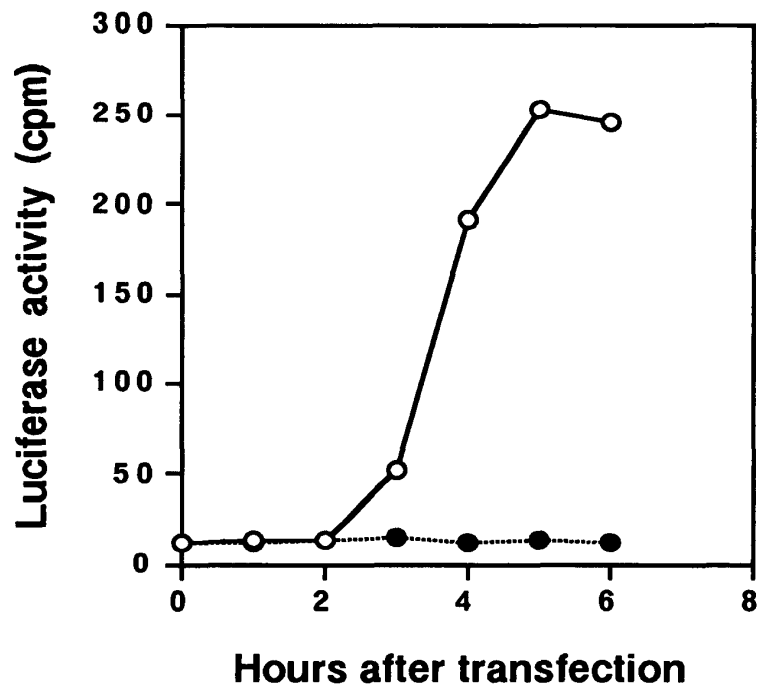


Fig. 2 Time course of expression of the luciferase gene introduced into MSB-1 MD tumor cells. Luciferase activity is expressed as the scintillation count (cpm). MSB-1 cells were incubated with 0.1 ml of liposome containing 2 μ g pGV-C (○) and 0.65 μ g of the HMG-1 and -2 mixture for 1–6 hr. Mock transfection (●) was performed with the same liposome mixture except that pGV-C was replaced with pSV- β

and-2 mixture, luciferase activity was first detected in MSB-1 cells 4 hr after transfection and reached a maximum within 5 hr. These data indicated that the intranuclear-transport of the plasmid and transcription of the transfected plasmid began within 4 hr after transfection.

Second, we applied a PCR-based RNA assay for specific sequences (QR-PCR method) to measure levels of transcripts arising from the β -actin gene (Fig. 3A) used as a standard, the recombinant MDV ICP4 gene (Fig. 3B) and endogenous MDV ICP4 gene (Fig. 3C). A linear relationships between log(PSL) and log(RNA molecules) was shown (Fig. 3D) in the range of 10^2 – 10^4 copies of the target RNA molecules in the PCR assay.

Using the transfection and QR-PCR method described above, we detected transcripts of the recombinant MDV ICP4 gene, which was coded on the plasmid pSV-ICP4S (Fig. 4B). Transcripts of recombinant MDV ICP4 were detected 2 hr after transfection at a level of 1.7 molecules per MSB-1 cell (8.3×10^3 molecules in 5×10^3 cells). These transcripts were not detected on the samples collected at 5–96 hr in the MSB-1 cells after transfection of pSV-ICP4S. Normalized intensity of hybridized signals with the β -actin gene showed a constant yield of total mRNAs extracted from MSB-1 cells (Fig. 4A, 1,000–2,000 molecules per cell). Additionally, the PCR product was the predicted size for this primer pair (Table 1) and hybridized specifically with the oligonucleotide probe (Fig. 4B). The cells immediately after the transfection also showed no MDV ICP4 signal (Fig. 4B). These data showed that the recombinant MDV ICP4 was expressed transiently at 2 hr after transfection.

On the other hand, endogenous MDV ICP4 coded on the MDV genome was expressed abundantly (Fig. 4C). From the radioactivity on the filter the level of transcripts of endogenous MDV ICP4 was calculated to be 21.3 molecules per cell (Fig. 4C). Although the intensity of MDV ICP4 in lytically infected CEF on the hybridized filter was weaker than that in the transfected MSB-1 cells (Fig. 4C), an almost equal amount of mRNA as those of the transfected MSB-1 cells was calculated to exist after normalization with β -actin and number of cells contributed to each lane (Fig. 4A). MDV ICP4 was not detected in mock-transfected MSB-1 cell. These data showed that the endogenous MDV ICP4 was upregulated after transfection of the plasmid pSV-ICP4S.

Increased expression of IE genes ordinarily starts the cascade of the lytic infection cycle, which results in an increase of the copy number of the MDV genome. Abundant transcription of the endogenous MDV ICP4 suggested the possible start of the lytic infection cycle in pSV-ICP4S-incorporated MSB-1 cells. However, the copy number of the MDV genome did not increase in MSB-1 cells throughout the experiment (Fig. 5C), when referred to the standard curve (Fig. 5A and B). The copy number of the MDV genome was calculated to be 4.3 copies per MSB-1 cell and 137.2 copies per CVI-988-infected CEF cell. These data indicated that the upregulated

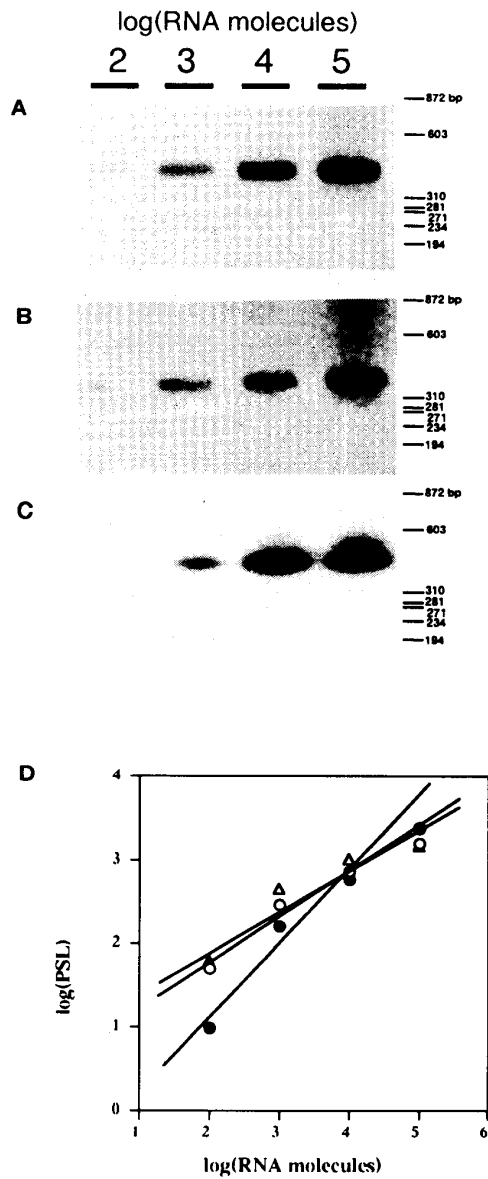


Fig. 3 Standard curves for QR-PCR assay. Autoradiography of β -actin (A), recombinant MDV ICP4 (B) and endogenous MDV ICP4 transcripts (C). These standard reactions were assayed starting with 10-fold dilutions of synthetic transcript preparations, RT-PCR and detection was performed as described in materials and methods. (D) The radiolabeled blots of the standards shown in panels A-C were quantified by using a BAS-1000 BioImage analyzer, and the log (PSL) units were plotted against the log(RNA molecules) of each standard (\circ , β -actin; \bullet , recombinant MDV ICP4; \triangle , endogenous MDV ICP4). Lines are made by linear regression analysis.

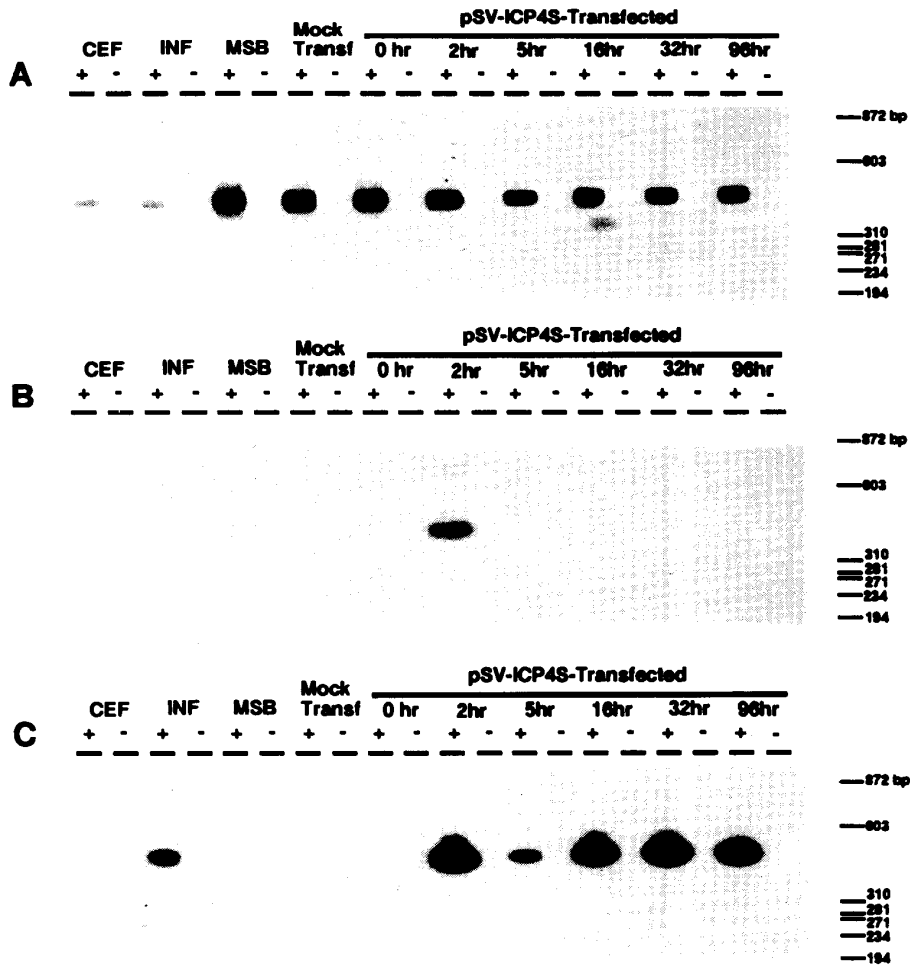


Fig. 4 Gene- or transcript-specific RNA in MDV-infected CEF and pSV-ICP4S-transfected MSB-1 cells. Autoradiograms of a probed blot of β -actin (A), recombinant MDV ICP4 (B) and endogenous MDV ICP4 (C) are shown. RNAs extracted from each sample of cells (RNA extracted from 50 cells per lane for panel A, RNA extracted from 5,000 cells per lane for panel B and C) were reacted with (+) or without (-) RT and displayed in adjacent lanes. CEF, uninfected chicken embryo fibroblast; INF, MDV (CVI-988 strain)-infected CEF; MSB, MSB-1 cells with no treatment; mock transf, MSB-1 cells 2 hr after transfection with plasmid pSV- β ; pSV-ICP4S-transfected, MSB-1 cells transfected with plasmid pSV-ICP4S. For pSV-ICP4S-transfected cells, the time course is displayed below each indicated sample name (0-96 hr). The 443 bp β -actin-specific, 344 bp recombinant MDV-ICP4 transcript-specific and 478 bp endogenous MDV ICP4-specific signals were detected by probing with oligonucleotide Ap3 (A), Sp2 (B) or Ip6 (C).

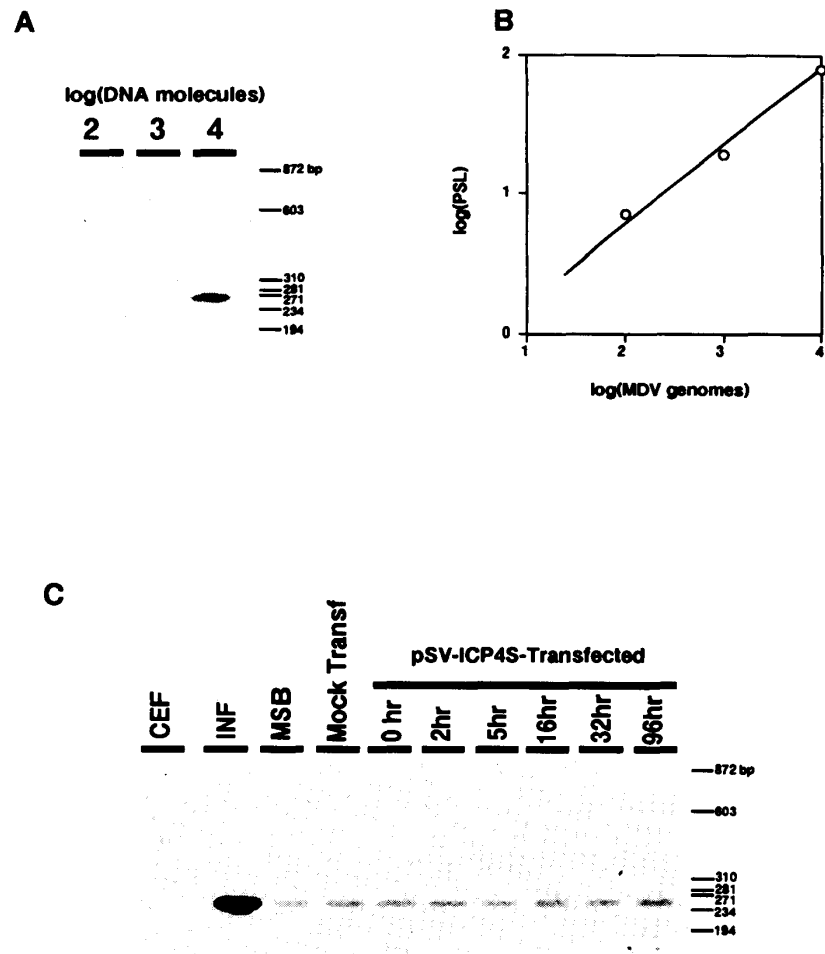


Fig. 5 Quantitative detection of MDV genome DNA. (A) Autoradiography of the MDV genome standard curve. Standard reactions were assayed starting with 10-fold dilutions of meq-promoter containing a plasmid (Pr-meq-pBC). The number of DNA molecules is expressed as \log_{10} . The amplified standard sets were electrophoretically separated, transferred to nylon filters and hybridized to radiolabeled cRNA probes. (B) The radiolabeled blots of the standards shown in panel A were quantified by using a BAS-1000 BioImage analyzer, and the $\log(\text{PSL})$ were plotted against $\log(\text{DNA molecules})$. Line is made by linear regression analysis. (C) MDV genome-specific DNA in MDV-infected CEF and pSV-ICP4S-transfected MSB-1 cells. Autoradiograms of a probed blot of meq-promoter is shown. Five hundred pg of the high-molecular weight DNAs are displayed in adjacent lanes. Abbreviations above the lanes are the same as Fig. 4. The 248 bp meq-promoter specific signal was detected by probing with ^{32}P -labelled cRNA.

MDV ICP4 did not enhance replication of the MDV genome and lytic infection cycle.

DISCUSSION

We developed a transient transfection assay system for the detection and quantification of MDV ICP4 transcripts. We found that (i) using a cationic liposome and HMG proteins, the recombinant MDV ICP4 gene was expressed at 2 hr after transfection, (ii) the transcript containing the whole coding region of MDV ICP4 upregulated endogenous MDV ICP4 transcription, and (iii) neither expression of the ICP4-encoding plasmid nor upregulated expression of endogenous ICP4 enhanced viral replication. Next we will discuss the potential significance of our findings for MDV ICP4 in reactivation of latently infected cells.

Pratt et al.²²⁾ transfected MSB-1 cells with MDV ICP4-expressing plasmid but they did not report the expression of the recombinant MDV ICP4. On the other hand, we detected transcripts of recombinant MDV ICP4 at 2 hr after transfection (Fig 4B). This rapid expression of recombinant MDV ICP4 might be the effect of HMG proteins which is suggested to work as a carrier. Kaneda et al.¹⁴⁾ reported a rapid gene-transfer with liposomes having HMG proteins. In their reports, the liposome had a neutral surface and was covered with Sendai virus to assist the fusion of the liposome with cells^{14, 30)}. Cationic liposomes are reported to have a level of function affinity similar to that of Sendai virus-coated liposomes^{5,12)}. In this study a marker gene expressed rapidly (Fig. 2). This is the first report of gene-transfer using a DNA-HMG protein complex in a cationic liposome.

To quantitate transcripts, we chose a QR-PCR assay with external standards rather than utilizing a competitive strategy. Competitive approach requires dividing each sample into many portions to quantify a single gene sequence, while the external standard approach permits dividing each sample into several portions for measurement of many different gene sequences and therefore yields more information per individual sample. Reconstituting synthetic cRNA standards with nonspecific RNA controlled for nonspecific amplification of sequences (Fig. 3). Use of a radiolabeled probe maximized detection of a specific PCR product (Figs. 3 and 4). Although Kramer and Coen¹⁶⁾ originally used radiolabeled internal oligomers as probes as we used for detection of transcripts (Figs. 3 and 4), we showed that a full length cloned PCR fragment could also be used as a probe (Fig. 5). With β -actin, the level of transcripts was calculated to 850–1,300 per cell from the standard curve and RT-PCR of samples (Fig. 4A). This copy number of β -actin agreed with a report using RNase protection assay³⁾. This agreement indicated the reliability of our QR-PCR experiments.

Using the QR-PCR method, we predicted 1.7 copies of the recombinant MDV ICP4 transcript per cell from the standard curve (Fig. 4B). This copy number was 10 times smaller than of the endogenous MDV ICP4 in the lytically infected cells (Fig.

4C). Three possible reasons for the low frequency of expression may be: first, the pSV-ICP4S plasmid is unstable in MSB-1 cells. Second, the main transcripts from pSV-ICP4S have no or a loose poly(A) tail. Third, although the luciferase gene of the pGV-C vector was expressed under the control of the SV40 early promoter/enhancer, that promoter/enhancer is not efficient for MDV ICP4. The first reason could be discounted because we detected pSV-ICP4S in the DNA extracted from the pSV-ICP4S-transfected-MSB-1 cells 96 hr after transfection (data not shown). To ascertain the second reason, we are now trying to detect the transcripts of recombinant MDV ICP4 from total RNA of pSV-ICP4S-transfected MSB-1 cells. The matching of promoters must be surveyed with various constructions, regardless of expression of luciferase, of the pGV-C vector.

Comparing with the recombinant MDV ICP4, endogenous MDV ICP4 was abundantly transcribed in the transfected MSB-1 cells (Fig. 4C). Although we did not confirm that the primer for RT (Ip3) hybridized to the mRNA of endogenous MDV ICP4, Northern blot analyses reported by Pratt²²⁾, Cantello⁸⁾ and Li¹⁷⁾ suggested that the main transcript to which primer Ip3 hybridized was full-length mRNA of endogenous MDV ICP4. Thus our results suggested that the expression of the recombinant MDV ICP4 gene enhanced the expression of full-length mRNA (Fig. 4C). Although it has been suggested that IE gene expression may substitute a switch initiating the lytic cascade leading to reactivation, upregulated expression of endogenous MDV ICP4 gene did not enhance viral replication (Fig. 5). It is suggested that reactivation of virus requires more than turning on MDV ICP4 gene. Similar phenomena were reported with the BZLF1 gene of EBV²¹⁾. An introduced BZLF1 gene enhanced the expression of the endogenous BZLF1 gene in EBV-transformed cells but did not induce the full lytic infection. The BZLF1 gene is similar to the MDV ICP4 gene in 2 points: products of both genes are important positive regulators of their virus, and antisense-transcripts are transcribed from their coding regions. Prang et al.²¹⁾ speculated that the expression of high numbers of recombinant BZLF1 RNAs saturated inhibitory factors for BZLF1 and that the decrease in active inhibitory factors resulted in the enhancement of the endogenous BZLF1 gene expression. Our data suggested that, although the recombinant MDV ICP4 RNAs were not abundant (Fig. 5B), the upregulation mechanism should be studied further in accordance with their speculation.

REFERENCES

- 1) AKIYAMA, Y. & KATO, S.: Two cell lines from lymphomas of Marek's disease. *Biken J.* 17: 105-116, 1974.
- 2) ANDERSON, A. S., Francesconi, A. & MORGAN, R. W.: Complete nucleotide sequence of the Marek's disease virus ICP4 gene. *Virology* 189: 657-667, 1992.
- 3) ARMSTRONG, D. G. & HOGG, C. O.: Type-I insulin-like growth factor receptor gene expression in the chick. Developmental changes and the effect of selection for

- increased growth on the amount of receptor mRNA. *Endocrinology* **12**: 3–12, 1994.
- 4) BECKER, Y., ASHER, Y., TABOR, E., DAVIDSON, I. & MALKINSON, M. : Open reading frames in a 4556 nucleotide sequence within MDV-1 BamHI-D DNA fragment : evidence for splicing of mRNA from a new viral glycoprotein gene. *Virus Genes* **8** : 55–69, 1994
 - 5) BRAZOLOT, C. L., PETITTE, J. N., ETCHES, R. J. & VERRINDER, G. A. : Efficient transfection of chicken cells by lipofection, and introduction of transduced blastodermal cells into the embryo. *Mol. Reprod. Dev.* **30** : 304–312, 1991.
 - 6) CALNEK, B. W. : Marek's disease-a model for herpesvirus oncology. *Crit. Rev. Microbiol.* **12** : 293–320, 1986.
 - 7) CALNEK, B. W. & SCHAT, K. A. : Proliferation of chicken lymphoblastoid cells after in vitro infection with Marek's disease virus. *Avian Dis.* **35** : 728–737, 1991.
 - 8) CANTELLO, J. L., ANDERSON, A. S. & MORGAN, R. W. : Identification of latency-associated transcripts that map antisense to the ICP4 homolog gene of Marek's disease virus. *J. Virol.* **68** : 6280–6290, 1994.
 - 9) ENDOH, D., NIKURA, M., HIRAI, K., INAGAKI, N., HAYASHI, M., NAKAJIMA, K. & SATO, F. : Expression and purification of recombinant Marek's disease virus serotype 1 specific phosphorylated protein pp38 in *E. coli*. *J. Vet. Med. Sci.* **56** : 823–826, 1994.
 - 10) FUKUCHI, K., SUDO, M., LEE, Y. S., TANAKA, A. & NONOYAMA, M. : Structure of Marek's disease virus DNA : detailed restriction enzyme map. *J. Virol.* **51** : 102–109, 1984.
 - 11) IKUTA, K., NAKAJIMA, M., NAITO, S. H., UEDA, S., KATO, S. & HIRAI, K. : Identification of Marek's disease virus specific antigen in Marek's disease lymphoblastoid cell line using monoclonal antibody against virus-specific phosphorylated polypeptides. *Int. J. Cancer* **35** : 257–264, 1985.
 - 12) JARNAGIN, W. R., DEBS, R. J., WANG, S. S. & BISSELL, D. M. : Cationic lipid-mediated transfection of liver cells in primary culture. *Nucleic Acids Res.* **20** : 4205–4211, 1992.
 - 13) JONES, D., LEE, L., LIU, J. L., KUNG, H. J. & TILLOTSON, J. K : Marek disease virus encodes a basic-leucine zipper gene resembling the fos/jun oncogenes that is highly expressed in lymphoblastoid tumors. *Proc. Natl. Acad. Sci. U. S. A.* **89** : 4042–4046, 1992.
 - 14) KANEDA, Y., IWAI, K. & UCHIDA, T. : Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science* **243** : 375–378, 1989.
 - 15) KOST, T. A., THEODORAKIS, N. & HUGHES, S. H. : The nucleotide sequence of the chick cytoplasmic beta-actin gene. *Nucleic. Acids Res.* **11** : 8287–8301, 1983.
 - 16) KRAMER, M. F. & COEN, D. M. : Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. *J. Virol.* **69** : 1389–1399, 1995.
 - 17) LI, D. S., PASTOREK, J., ZELNIK, V., SMITH, G. D. & ROSS, L. J. : Identification of novel transcripts complementary to the Marek's disease virus homologue of the

- ICP4 gene of herpes simplex virus. *J. Gen. Virol.* **75**: 1713–1722, 1994.
- 18) MACGREGOR, G. R. & CASKEY, C. T. : Construction of plasmids that express E. coli betagalactosidase in mammalian cells. *Nucleic Acids. Res.* **17**: 2365, 1989.
 - 19) MCKIE, E. A., UBUKATA, E., HASEGAWA, S., ZHANG, S. J., NONOYAMA, M. & TANAKA, A. : The transcripts from the sequences flanking the short component of Marek's disease virus during latent infection form a unique family of 3'-coterminal RNAs. *J. Virol.* **69**: 1310–1314, 1995.
 - 20) METZLER, D. W. & WILCOX, K. W. : Isolation of Herpes simplex virus regulatory protein ICP4 as a homodimeric complex. *J. Virol.* **55**: 329–337, 1985.
 - 21) PRANG, N., WOLF, H. & SCHWARZMANN, F. : Epstein-Barr virus lytic replication is controlled by posttranscriptional negative regulation of BZLF1. *J. Virol.* **69**: 2644–2648, 1995.
 - 22) PRATT, W. D., CANTELLO, J., MORGAN, R. W. & SCHAT, K. A. : Enhanced expression of the Marek's disease virus-specific phosphoproteins after stable transfection of MSB-1 cells with the Marek's disease virus homologue of ICP4. *Virology* **201**: 132–136, 1994.
 - 23) RIVERA, G. R., IMBALZANO, A. N., GU, B. & DELUCA, N. A. : The role of ICP4 repressor activity in temporal expression of the IE-3 and latency-associated transcript promoters during HSV-1 infection. *Virology* **202**: 550–564, 1994.
 - 24) ROIZMAN, B. : The family herpesviridae: general description, taxonomy, and classification. In: B. Roizman ed., *The Herpesviruses*, pp. 1–23, Plenum Press, New York, 1982.
 - 25) ROSS, L. J. & BINNS, M. M. : Properties and evolutionary relationships of the Marek's disease virus homologues of protein kinase, glycoprotein D and glycoprotein I of herpes simplex virus. *J. Gen. Virol.* **72**: 939–942, 1991.
 - 26) ROSS, L. J., BINNS, M. M. & PASTOREK, J. : DNA sequenced and organization of genes in a 5.5 kbp EcoRI fragment mapping in the short unique segment of Marek's disease virus (strain RB1B). *J. Gen. Virol.* **72**: 949–954, 1991.
 - 27) SAKAGUCHI, M., URAKAWA, T., HIRAYAMA, Y., MIKI, N., YAMAMOTO, M. & HIRAI, K. : Sequence determination and genetic content of an 8.9-kb restriction fragment in the short unique region and the internal inverted repeat of Marek's disease virus type 1 DNA. *Virus Genes* **6**: 365–378, 1992.
 - 28) SCHAT, K. A., BUCKMASTER A. & ROSS, L. J. N. : Partial transcription map of Marek's disease herpesvirus in lytically infected cells and lymphoblastoid cell lines. *Int. J. Cancer* **44**: 101–109, 1989.
 - 29) SUGAYA, K., BRADLEY, G., NONOYAMA, M. & TANAKA, A. : Latent transcripts of Marek's disease virus are clustered in the short and long repeat regions. *J. Virol.* **64**: 5773–5782, 1990.
 - 30) TOMITA, N., HIGAKI, J., MORISHITA, R., KATO, K., MIKAMI, H., KANEDA, Y. & OGIHARA, T. : Direct *in vivo* gene introduction into rat kidney. *Biochem. Biophys. Res. Commun.* **186**: 129–134, 1992.