维普资讯 http://www.cqvip.com

第6卷第4期 中国病毒学 Vol.6 No.4
1991年12月 VIROLOGICA SINICA Dec. 1991

人巨细胞病毒形态转化区的鉴定

Identification of the Morphological Transforming Regions of Human Cytomegalovirus

Leonard J Rosenthal Shang Shi-zhang* Anita Inamdar

(*Department of Microbiology, Georgetown University Medical Center, Washington, D.C. 20007, USA*)

关键词: 人巨细胞病毒 转化活性 DNA序列分析 Southern印迹 S_1核酸酶分析 启动子活性 氯霉素乙烯转移酶测定 NIH 3T3 细胞

Key words: Human Cytomegaloviruses Transformation Activity DNA Sequence Analysis Southern Blot Hybridization S_1 Nuclease Analysis NIH 3T3 Cells Promoter Activity Chloramphenicol Acetyltransferase Assay

DNA of human cytomegalovirus (HCMV) contains three transforming fragments, which have been mapped in the long unique region of the viral genome (see Fig.1). A minimal region of 558 base pairs (bp) (pCM4127) was localized in the XbaI-HindIII fragment of HCMV strain AD169 (map unit 0.123—0.140) and designated morphological transforming region I (mtr I). Mtr I was reported to cause one-step focal transformation of primary Wistar rat embryo cells and NIH 3T3 mouse cells[6]. This sequence was noncoding and contained a stem-loop structure analogous to an insertion-like element[1].

Another transforming region was mapped in the 20-kilobase (kb) Xba I fragment E (Xba I-E) of HCMV Towne DNA (map unit 0.680—0.770). The Xba I-E fragment immortalized diploid Syrian hamster embryo cells and induced neoplastic transformation of established rodent cell lines[2]. Subsequently, within Xba I-E, a 3kb Xba I-BamHI EM segment and a 7.6 kb XbaI-BamHI EJ segment were each independently capable of inducing tumorigenic transformation of established rodent cells[3]. Cell lines transformed individually by EJ or EM and their tumor derivatives retained EM but not EJ sequences[3]. Similarly, only EM sequences were retained in the transformed and tumor-derived cells induced by cotransfection of cloned EJ and EM sequences[3].

* Shang Shi-zhang (尚诗樟), Visiting Scholar, Department of Histology and Embryology, Jinan University Medical College, Guangzhou, PRC

These data suggested that EM sequences were required both to initiate and to maintain the transformed phenotype. In contrast, EJ sequences were required only for the initiation of transformation, perhaps by a hit-and-run mechanism analogous to the BglII-N fragment of herpes simplex virus 2[7] or to pCM4127 of HCMV AD169[1].

Thus far, transforming domains among the herpesviruses have been localized to small non-coding DNA fragments[1,4,8,9]. To date, no transforming polypeptide has been identified in HCMV-induced transformation. As a result, viral DNA elements have been proposed to be responsible for transformation through modulation of cellular genes by transcriptional activation[10], genetic recombination and rearrangement[11] or DNA amplification[11]. In this review, we propose that mtrII of HCMV Towne may code for a transforming polypeptide. Our identification of the presence of the 79aa ORF in transforming strains Towne and AD169 and its absence in transformation-defective Tanaka strain implies its possible role in HCMV transformation. Our analysis of HCMV lytically infected cells has identified two mtrII-specific early RNA transcripts, one of which is large enough to code for the 79 aa ORF. Furthermore, the cloning of mtrII upstream of a promoter-less reporter gene has enabled us to demonstrate the promoter activity of mtrII.

Localization of Transforming Activity within the HCMV EM Fragment

Culture conditions for NIH 3T3 mouse cells, DNA transfection, transformation assays of focus-formation, and anchorage-independence were performed as previously described[8]. To define the transforming region of Towne EM, recombinant subclones of EM were generated and assayed for transforming activity in NIH 3T3 cells. The EM clone in pACYC184 as well as the PstI and XhoI subclones were used in transformation assays. Results shown in Table 1 demonstrated that only the 1.5-kb PstI-XhoI subfragment of EM showed transforming activity. No activity was detected with the XbaI-PstI or XhoI-BamHI subfragments of EM.

To delineata further the transforming region within the 1.5 kb PstI-XhoI subfragment, a 980-bp subclone extending from the BanII to the XhoI site was constructed. The BanII site is located 610 bp to the right of the PstI site. When tested for transforming activity, the BanII-XhoI clone induced foci at a transforming efficiency identical to that of EM (Table 1). The restriction enzyme BglII cuts once within the BanII-XhoI fragment. When a clone extending from the BglII site to the BamHI site was tested, a signifi-

维普资讯 http://www.cqvip.com

Table 1. Transformation Activity of Towne EM and Subfragments in NIH 3T3 cells

表 1　Towne株 EM 区段和亚区段对 NIH3T3 细胞的转化活性

Source of DNA DNA 来源	Restriction Endonuclease 限制性核酸内切酶	DNA (μg/dish) DNAμg/培养皿	Foci/no. of dishes 转化灶数/培养皿数	Transformation Frequency (10^{-6}) 转化率，10^{-6}
Towne 3 kb-EM	XbaI-BamHI	15.0	34/5	1.13
Towne 株 EM 段 3000 碱基对	〃	〃	21/6	0.70
Towne 1.5 kb-EM	PstI-XhoI	〃	34/6	1.13
Towne 株 EM 段 1500 碱基对	〃	〃	25/5	1.00
Towne 980 bp Towne 株 980 碱基对	BanII-XhoI	〃	34/5	1.13
Towne 1.54 kb Towne 株 1540 碱基对	BglII-BamHI	〃	13/5	0.55
Towne 1.0 kb-EM	XhoI-BamHI	〃	4/6	0.13
Towne 株 EM 段 1000碱基对	〃	〃	1/6	0.03
Towne 0.5 kb-EM	XbaI-PstI	〃	0/6	>0.03
Towne 株 EM 段 500 碱基对	〃	〃	0/6	>0.03
Salmon testes	......	20.0	0/6	>0.03
鲑鱼睾丸	......	〃	0/8	>0.03

Data taken from El-Beik et al.[3] and Razzaque et al.[4].

资料来自El-Beik 等[3]和Razzaque等[4]

cant reduction in focus formation was detected. These data suggested that the BanII-XhoI region was responsible for transformation. Similar studies and results were also obtained by transfecting Rat-2 cells with the EM subclones (data not shown, 3, 4). Focal-derived lines obtained following transfection with EM or the BanII-XhoI subfragment were tumorigenic in immunocompetent syngeneic rats[4].

Retention of EM in Rat Tumor Lines

When 2×10^6 cells of EM-induced focal lines were subcutaneously inoculated into 5-week-old immunocompetent Fisher rats, palpable tumors developed at the site of inoculation. Tumors were excised, trypsinized, and cell lines were established in culture. Cell line RBM 2T1 derived from an EM-induced tumor was examined for the presence of HCMV DNA sequences by Southern blot hybridization (Fig.2). Genomic DNA from RBM 2T1 was first restricted with XbaI-BamHI, the terminal restriction sites of the EM fragment, and another sample of the same digest was further restricted with XhoI and PstI to liberate the three subfragments of EM. As a control, genomic DNA from non-transformed Rat-2 cells was similarly digested

维普资讯 http://www.cqvip.com

and used. One genome equivalent of EM DNA as well as the 1.5, 1.0 and 0.5 kb subfragments of EM were used as markers. Hybridization using ^{32}P-labeled EM DNA was carried out under stringent conditions. Intense hybridization representing multiple copies was detected in RBM 2Tl at a position comigrating with the 3.0 kb EM marker. A number of additional bands was also observed. Bands higher than 3 kb may represent integrated EM lacking either the XbaI or BamHI site, whereas bands lower than 3 kb may represent extensively deleted EM sequences. In the lane with 4-cut DNA, multiple copies of virus-specific sequences comigrating at positions corresponding to the 1.5, 1.0 and 0.5 kb EM subfragment markers were detected. Normal Rat-2 DNA exhibited faint multiple bands hybridizing to the EM probe, however, this was later shown to be due to cell-related sequence homology in the 1.0-kb XhoI-BamHI subfragment of EM[3].

DNA Sequence Analysis of the mtrII and Expression in Lytically Infected Cells

The complete nucleotide sequence of the BanII-XhoI fragment has been reported elsewhere[4]. Analysis of the mtrII sequence sevealed several unique features which are shown schematically in Fig. 3. These include three small ORFs of 79, 83, and 34 amino acid residues located at mp 294 to 530, 612 to 860, and 622 to 723. The 79-residue ORF contains the initiation sequence CGGTGATGC and the 34-residue ORF contains the initiation sequence GCG-TCATGG. These initation motifs have 55% or 78% identity respectively with the Kozak consensus translational initiation sequence of CCA/GCCATGG. A polyadenylation signal, AATAAA, is located approximately 500 bp downstream of the first termination codon TGA at mp 530, within the XhoI-BamHI fragment. Moreover, sequences at the 5′ terminus of the ORFs contain several transcriptional regulatory sequences. For example, CAAT sequences have been identified at mps 96, 153, 160, 201 and 215. In addition, three potential binding sites for transcription factor Spl are located at mps 75—82, 264—269, and 276—281. In addition, six copies of the heptanucleotide sequence GGTGA/GTG are present in the upstream region at mps 62, 65, 68, 71, 290, and 302. These repeats have approximately 75% identity with the SV40 enhancer core consensus sequence. No consensus splice donor or acceptor signals are present in the sequence.

There are several ORFs in the opposite DNA strand. However, the translational initiation sequence of each of these peptides is weaker than those discussed above. In addition, there are no potential regulatory signals that

维普资讯 http://www.cqvip.com

would serve as a transcriptional promoter element.

To determine if mtrII transcripts were expressed in HCMV lytically infected cells, the Sl nuclease analysis was carried out as previously described[4]. When the BanII-XhoI probe, representing the entire 980 bp DNA fragment, was used, the Sl nuclease analysis revealed several distinct early RNA species. The transcripts P1 and P2 were of particular interest because the length of the protected DNA fragments would position the 5′ end of the RNA upstream of the putative ORFs. The P1 transcript was determined to be approximately 720 nucleotides in length and, depending upon the exact position of the 5′ end, may contain the coding sequences for the 79, 83-and 34-residue polypeptides. The major RNA transcript, P2, was determined to be 410 nucleotides in length. Identically sized protected DNA fragments were detected with both BanII-XhoI and BglII-XhoI probes, demonstrating the validity of the S1 mapping technique. This would position the 5′ end of the RNA at approximately 570±10, 30—40 nucleotides upstream of the AUG initiation codons for the 83-and 34-residue polypeptides, respectively. These results indicated that at least two early RNA transcripts were mapped to mtrII and were expressed in lytically infected cells.

Comparison of Transforming Activity of Towne mtrII with the Colinear Fragments in HCMV Strains AD169 and Tanaka

A comparison of transforming activity of mtrII from HCMV strains Towne and Tanaka as well as the colinear 2.2 kb XbaI-BamHI fragment from strain AD169 was carried out on immortalized NIH 3T3 cells (Table 2). The results showed that compared to Towne, the colinear region in AD169 had the similar transforming activity. The numbers of large foci, previously shown to correlate with tumorigenic potential, as well as the colony efficiency in 0.3% agar were similar for the Towne mtrII and the AD169 2.2 kb fragment. By contrast, Tanaka mtrII showed 25% of the overall transforming activity and only 4.3 to 5.5% of the activity for large-focus formation. Evidence of the importance of the unique BglII restriction site within Towne mtrII is shown. The restriction enzyme BglII cuts once within Towne mtrII specifically at nucleotide 436 within the 79aa ORF. Dishes transfected with BglII-digested Towne mtrII showed a 56% reduction in transforming potential compared to intact Towne mtrII.

To ascertain what may be responsible for the differences in transforming potential, sequencing of the Tanaka mtrII was carried out and compared to the published sequences of Towne mtrII and the colinear region in strain

维普资讯 http://www.cqvip.com

Table 2. Transforming potential of HCMV mtrII in established NIH 3T3 cells

表 2. HCMV mtrⅡ 区对已建立的 NIH 3T3 细胞的转化性能

Transfected DNA 转染的DNA种类	Focus formation[a] 转化灶数[a]	Cloning in agarose[b] 在琼脂中的克隆性能[b]	
		Colony efficiency 克隆效率	Colony size 克隆大小
Towne mtrII (980 bp) Towne株mtrⅡ区980碱基对	43(23 L(大),11 M(中), 9S(小))	1.4—3.5	Macro(大)
Tanaka mtrII (980 bp) Tanaka株mtrⅡ区980碱基对	11(1 L(大),6 M(中), 4 S(小))	0.005—0.01	Micro(小)
Towne mtrII (BglII digsted) Towne株mtrⅡ区BglⅡ消化段	19(10 L(大),5 M(中), 5 S(小))	NT(未做)	
AD169 colinear 2.2-kb region AD169株相当的2200碱基对线形区	43(18 L(大),14 M(中), 11 S(小))	0.48—1.09	Macro(大)
Salmon testes 鲑鱼睾丸	2(1 M(大),1 S(小))	0.003	Micro(小)
Control 对照	2(1 M(大),1 S(小))	0.003	Micro(小)

a：由2个分开的实验所获的每100mm直径培养皿内转化灶的平均数值。按过去所述方法[3]，应用15μg量每一种HCMV的DNA与5μg量鲑鱼睾丸DNA（载体）联合转染NIH3T3细胞。48小时后，细胞用胰酶消化，并以1比3比例作分离传代接种。5至6周后，记录形态学转化细胞灶数。转化灶的直径："L"；示大于2.5mm；"M"为1.5至2.5mm；"S"为大于1.0mm。

b：细胞接种于0.3%琼脂培养基中，细胞的接种数为10^5/60mm直径培养皿。3至4周后，记录形成的克隆数。克隆效率规定为："（克隆数×100）/（接种的细胞数）"，所示资料为全部被测转化细胞系克隆效率的变动范围。克隆直径大小："Micro（小）"为0.1至0.25mm；"Macro（大）"为大于0.5mm。NT：未测定。资料来自Jahan等[6]。

AD169[5]. A comparison of the three sequeuces revealed that the only major change in strain Tanaka compared to the other two strains was a single-base deletion at position 360 which led to a frame-shift and the truncation of the 79 aa ORF. The upstream regulatory regions in all three strains were similar.

Assessment of Promoter Activity of Towne mtrII

The chloramphenicol acatyltransferase (CAF) *in vivo* transient expression system was used to assess whether mtrII had promoter activity. The 980 bp fragment (mtrII) was cloned in front of the CAT gene in both the positive and the negative orientations with reference to the ORF. The data are shown in Fig.4. When cloned in the sense or positive orientation in front of the CAT gene, Towne mtrII exhibited promoter activity that is almost 40-fold higher than the promoter-less CAT plasmid, pA10CAT3M. In the negative orientation Towne mtrII exhibited negigible activity and was similar to the CAT plasmid. The data support our sequence analysis of this fragment, which indicated the presence of transcriptional control elements in the positive but

维普资讯 http://www.cqvip.com

not in the negative orientation[4]. Furthermore, our demonstration of early RNA species mapping within mtrII suggests that Towne mtrII functions as a *promoter and may encode a transforming polypeptide involved in* HCMV *transformation*.

Summary and Conclusions

Transforming domains of herpesviruses have been mapped to small DNA fragments[1,4,8,9]. Sequence analysis have revealed these transforming domains to be noncoding, which would suggest no role for virally encoded polypeptides in transformation. However, we have reported[4] the localization and DNA sequence analysis of HCMV Towne mtrII. In contrast to other herpesvirus transforming domains, mtrII was retained in transformed and tumor-derived cell lines and was found to contain ORFs of 79, 83, and 34 aa. Furthermore, S1 analysis identified RNA transcripts in cells lytically infected with HCMV which were large enough to code for the ORFs.

The significance of these ORFs was made by comparing transforming activities of mtrII colinear regions in strains AD169 and Tanaka. In contrast to Towne mtrII and AD169 colinear transforming fragments, the Tanaka mtrII had substantially reduced transforming activity and produced significantly reduced numbers of focal lines. Nucleotide sequence comparisons of the ORFs in strains Towne, AD169, and Tanaka are consistent with the hypothesis that the 79 aa ORF may play a role in transformation.

The current identification of promoter activity within mtrII supports the *in vivo* studies which mapped the P1 RNA transcript upstream of the 79, 83, and 34 aa ORFs and the P2 transcript upstream of the 83 and 34 aa ORFs. The demonstration of mtrII promoter activity together with the presence of the 79 aa ORF in the transforming Towne and AD169 strains and its absence in the transformation-defective Tanaka strain further suggests the role of the 79 aa ORF in transformation. Further studies with expression vector constructs containing the 79 aa ORF will be required to directly demonstrate the role of the mtrII 79 aa ORF in HCMV transformation.

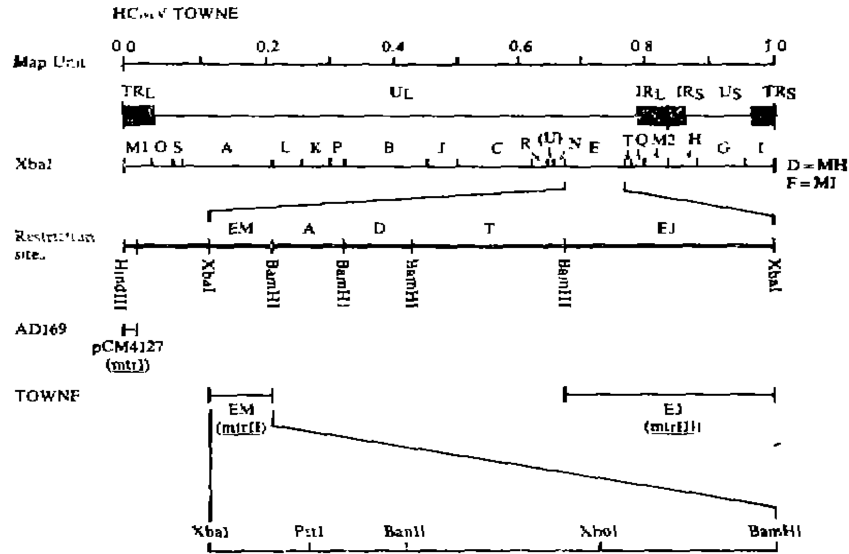

Fig. 1. XbaI restriction map of HCMV DNA and the BamHI subfragments contained within the Towne XbaI-E fragment. The XbaI-BamHI EM subfragment is shown and mtrII is located from the BanII-XhoI region. The unique BglII site is located 440 nucleotides downstream of the BanII site.

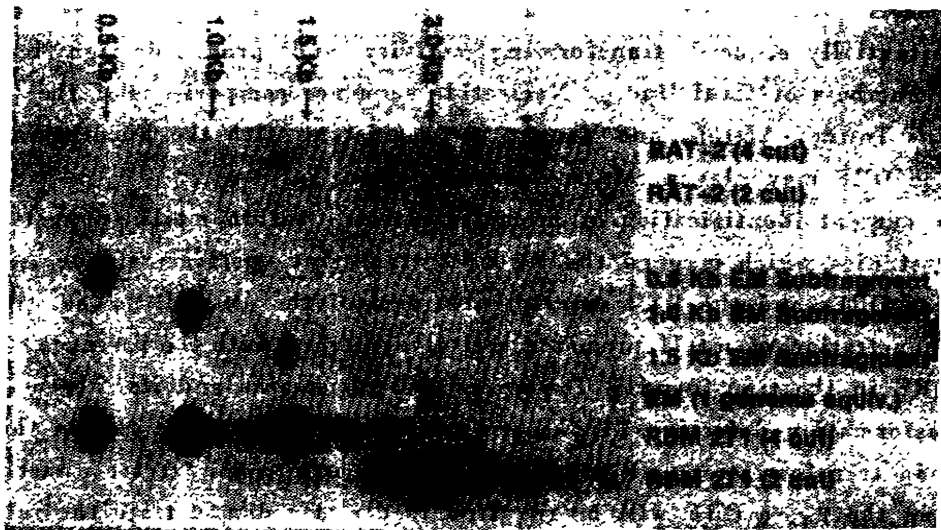

Fig. 2. Southern hybridization of XbaI-BamHI EM-transformed Rat-2 tumor-derived cellular DNAs to ^{32}P-labeled XbaI-BamHI EM DNA probe. Normal Rat-2 and tumor DNAs (RBM 2T1) (10 μg each) were digested with two enzymes (XbaI and BamHI) (2 cut) or four enzymes (XbaI, BamHI, PstI, and XhoI) (4 cut) and hybridized. EM subfragments of 1.5, 1.0 and 0.5 kb and one genome equivalent of 3.0 kb DM DNA were used as markers. Data taken from El-Beik *et al*.[13].

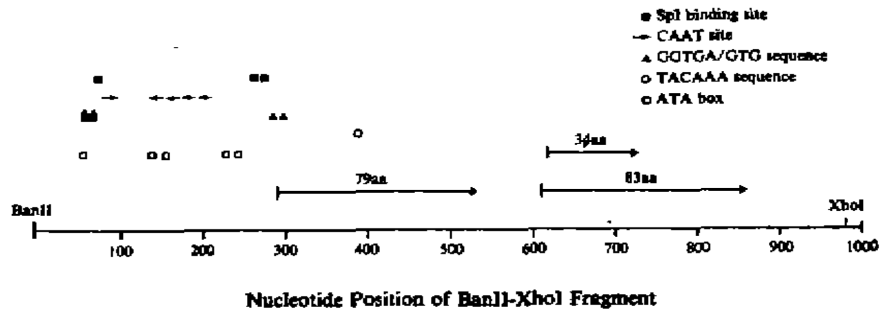

Fig. 3.　Map positions of three putative ORFs of 79, 34, and 83 amino acids (aa) and the upstream regulatory sequences located in HCMV mtrII. Data taken from Jahan *et al.* [5].

A)

Plasmid Designation	Plasmid Construct	% Acetylated Cm
pSV2CAT	SV40 promoter-enhancer-CAT	21.9
pA10CAT3M	CAT	0.4
TOWNE 980	BanII ⟹⟹ BglII ›–›–›–›–›–›– XhoI CAT	15.5
TOWNE 980	XhoI ‹–‹–‹–‹–‹–‹– BglII ⟸⟸ BanII CAT	0.6

B)

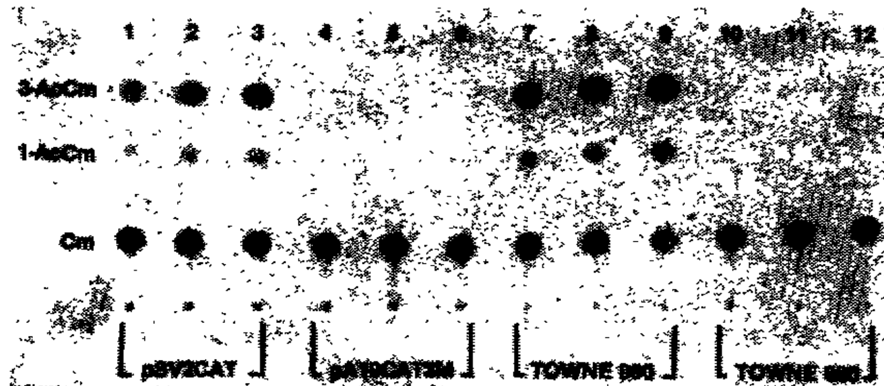

Fig. 4.　Promoter activity of mtrII. A) The BanII-XhoI sites of mtrII were converted to XbaI sites and ligated into the promoter-less plasmid pA10CAT3M. Clones representing the positive and negative orientations of 980 bp to the CAT gene were selected with BglII. CV-1 cells were transfected with 10 μg of each indicated plasmid DNA together with 15 μg carrier DNA according to the Chen-Okayama protocol [12]. At 48 hr after transfection, cell extract were assayed for CAT activity as described [13]. The data presented are for 1 μl of the pSV2CAT cell extract and 10 μl for all others cell extracts. B) CAT activity is presented as % conversion of C^{14}-chloramphenicol (Cm) to 1'-acetyl-Cm and 3'-acetyl-Cm forms. Lanes 1—3 represent 0.5, 1.0, and 1.5 μl samples of pSV2CAT respectively; Lanes 4—6: 20, 30, and 40 μl of pA10CAT3M; Lanes

维普资讯 http://www.cqvip.com

7—9 : 20, 30, and 40 μl of Towne 980 (+orientation) ; Lanes 10—12 : 20, 30, and 40 μl Towne 980 (—orientation) .

References

〔1〕 Nelson, J.A. et al., 1984, *J.Virol.*49 : 109.

〔2〕 Clanton, D.J. et al., 1983, *Proc. Natl. Acad. Sci.* USA 80 : 3826.

〔3〕 El-Beik, T. et al., 1986, *J. Virol.* 60 : 645.

〔4〕 Razzaque, A. et al., 1988, *Proc. Natl. Acad. Sci. USA* 85 : 5709.

〔5〕 Jahan, N. et al., 1989, *J. Virol.* 63 : 2866.

〔6〕 Nelson, J.A. et al., 1982, *J. Virol.* 43 : 83.

〔7〕 Galloway, D.A. and McDougall, J.K., 1983, *Nature* 302 : 21.

〔8〕 Galloway, D.A. et al., 1984, *Proc. Natl. Acad. Sci. USA* 81 : 4736.

〔9〕 Jones, C. et al., 1986, *Proc. Natl. Acad. Sci. USA* 83 : 7855.

〔10〕 Galloway, D.A. et al., 1986, in *Cancer Cells* Vol 4 : DNA Tumor Viruses. Eds M. Botcham, T. Grodzicker and P.A. Sharp, Cold Spring Harbor Lab. New York, p355.

〔11〕 Jariwalla, R. et al., 1986, *Proc. Natl. Acad. Sci. USA* 83 : 1738.

〔12〕 Chen, C. and Okayama, H., 1987, *Mol. and Cell.Biol.*7 : 2745.

〔13〕 Gorman, C. et al., 1982, *Mol. and Cell. Biol.* 2 : 1044.