大韓바이러스學會誌 : 第26卷 第1號 1996
J. of Kor. Soc. of Virology, Vol. 26, No. 1, 1996

Cloning of Thymidine Kinase Gene of *Herpes simplex* Virus Type-1

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=국문초록=

Herpes simplex Virus Type-1 Thymidine Kinase 유전자의 크로닝

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Herpes simplex virus type-1의 vero 세포에서의 증식기작을 규명하기위해 전자현미경으로 관찰하였고, 유전학적 특성을 규명하기위해 유전자도서관을 작성하였고, thymidine kinase (TK) 유전자를 클로닝을하였다. 감염 48시간 후 많은 수의 바이러스 nucleocapsid가 핵 뿐만 아니라 세포질에서 관찰되었다. 바이러스는 세포에 감염된 후 핵내에서 복제증식한 후 세포질내로 이동하였으며, 이때 핵막을 통과하면서 외투막을 갖고 세포질로 이동하여 세포밖으로 나가는 것을 관찰할 수 있었으며, 또한 일부 nucleocapsid는 세포막을 출아하여 비리온으로 출아되었다. HSV-1의 DNA를 *Bam*HI과 *Bgl*II 제한효소로 각각 절단하여 DNA의 절단 양상을 조사하였다. *Bam*HI에 의해 절단된 단편은 27개 이었고, 그들 분자량의 범위는 1.1 - 14 kb이었으며, *Bgl*II에의해 절단된 단편은 16개 이었고, 분자량의 범위는 4.5 - 20.1 kb이었다. Southern blot 방법으로 TK유전자를 포함하고 있는 단편을 확인하였는데, pHLA-12와 pHLB-14클론에 포함되어 있었고 각 단편의 분자량은 3.74와 6.41 kb이었다.

Key Words : *Herpes simplex* virus type-1, thymidine kinase, genome library

INTRODUCTION

The herpesvirus family consist of Cytomegalovirus, Epstein Barr virus, Varicella-Zoster virus, *Herpes simplex* virus, B virus and Human Herpesvirus-6 [1]. There are two types of *Herpes simplex* virus type 1 (HSV-1) and type 2 (HSV-2). These types can be dis-tinguished by their antigenic, biological and biochemical characteristics [2,3]. HSV-1 is primarily associated with oral and ocular lesions and is transmitted in oral and respira-tory secretion, whereas HSV-2 is isolated pri-mary from genital and anal lesion, and passed through sexual contact [4].

The herpes virion consists of a double stranded DNA core and an icosahedral capsid with a memb-rane envelope, and the HSV-1 and HSV-2 DNAs are linear double-stranded molecules with approximately 150 kilobase pairs. It consists of two covalently linked components, designated as L (long) and S (short). Each component consists of unique sequences bracke-ted by inverted repeats [1, 5-7].

The thymidine kinase (TK) gene is an early

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gene of HSV. Viral TK phosphorylates deoxynucleoside as substrate. Aacyclovir competes as a substrate with deoxynucleoside and is selectively phosphorylated by the viral TK [8]. Viral TK is far more effective than cellular TK in phosphorylating acyclovir. Therefore the active drug targets infected as opposed to uninfected cells. Acyclovir monophosphate is converted to its di- and triphosphate derivatives by cellular enzymes [9]. Acyclovir triphosphate acts by competing selectively with the deoxynucleoside triphos-phates for viral DNA polymerase [10] and more importantly by its inhibitory effect on DNA polymerase [8]. The substrate activity of acyclovir triphosphate is self-limiting, as the incorporated acyclovir monophosphate lacks a 3'-OH group, which results in DNA chain termination [10]. Tthe viral TK may be acted as an antiviral action, therefore TK gene is useful to study antiviral action and anti-cancer mechanisms. For the reasons we undertook this study.

In this article, we report the multiplication pattern of HSV in vero cell, restriction pattern of HSV type-1 genomic DNA digested with *Bam*HI and *Bgl*II restriction enzymes and its construction of gene library, and cloning of thymidine kinase gene of HSV-1.

MATERIALS AND METHODS

Viruses, cells and media

Herpes simplex virus type 1 (HSV-1) F strain (ATCC VR-733) was obtained from Korean AIDS (Acquired Immune Deficiency Syndrome) center, and vero cells (African green monkey kidney cell, ATCC-CCL 81) were obtained from Korean Type Culture Collec-tion (KTCC). The viruses were purified by plaque assay. The vero cells were cultured in plastic tissue culture flasks (Nunc) with Eagle's minimum essential medium (MEM, GIBCO) containing 10% fetal bovine serum (GIBCO), 0.22% sodium bicarbonate (Sigma), 50μg/ml gentamycin (GIBCO) and routinely maintained in MEM with 2% fetal bovine serum.

Bacterial strains and plasmids

E. coli XL-1 Blue and HB 101 strains (Research Institute for Genetic Engineering, Konkuk university, Seoul) were used as a cloning host. pBluescript KS (+) and pBacPAK 9 plasmids (Stratagene, La Jolla, CA, USA) and Clontech (Palo Alto, CA, USA), were used for construction of the genomic DNA library.

Cell culture

Vero cells were cultured at 37℃, 5% CO_2 incubator in plastic tissue flask (75cm^2) (Nunc) with Eagle's salts (GIBCO), supplemented with fetal bovine serum (GIBCO BRL) to a final concentration of 10% and 50μg/ml gentamycin (GIBCO BRL). The vero cells were monolayered as a number of 2×10^7cells in 75cm^2 tissue flask. In order to transfer cell monolayer, the vero cells were washed with PBS (phosphate buffered solution) (a) and were treated with 2ml of 0.05% trypsin-EDTA (GIBCO BRL) for 30 sec. After the removal trypsin-EDTA, cells were separated from the bottom of flask using 5ml of 10% MEM by pipetting. The cells in one flask were transfered into each of two flasks and 15ml of new medium was added.

Observation of vero cells infected with HSV-1 by electron microscopy

Vero cells were propagated at 37℃, 5% CO2 incubator in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (GIBCO), 0.22% sodium bicarbonate (Sigma) and 50μg of gentamicin (GIBCO) per ml. The cultures were infected with 0.1 M.O.I. of HSV-1 at 37℃, 5% CO_2 incubator for 48h. Infected cells with HSV-1 were fixed with 2.5% glutaraldehyde and 2% paraaldehyde solution for 24h. After primary fixation, fixed samples were wased with PBS (pH 7.4) [10] and were fixed with 1% osmium tetroxide (OsO_4) for 1h. Fixed samples were sequentially dehydrated with first 60% (v/v) ethanol and finally 100%

ethanol. After embedded in Epoxin resin for 60, thin sections cut with a microtome (Sorvall MT-600) were stained with uranyl acetate and lead citrate for 30min and 60min, respectively. The grids were observed and photographed with TEM (Hitachi H- 600, Japan) at 75 kV.

Plaque assay and isolation of virus clone

Lee and Miller [11] procedure was modified. Vero cell monolayers in tissue flasks ($75cm^2$) were inoculated with HSV-1 of approximately 0.1 M.O.I. The virus was absorbed to cell for 1 h at 37℃ in a 5% CO_2 incubator, and then 15ml of 2% MEM was added to the tissue flask. Cultures were incubated at 37℃ until advanced cytopathic effect was observed. When a complete cytopathic effect was observed in the infected cell, they were scraped into the media with policeman. Cell and virus fluid were centrifuged at 30,000 x g for 30 min at 4℃. Cell debris was removed and supernant was used for an inoculum.

Approximately 3×10^6 vero cell supplemented with 10% MEM, were transferred to petri dishes (60×15mm, Nunc) and allowed to culture in a monolayer overnight. The medium was removed and the virus, diluted in PBS, was innoculated to the cell monolayer. The virus was allowed to absorb in the cell at 37℃, 5% CO_2 incubator for 1 h with intermittent tilting for uniform distribution. The cells were then overlaid with 5ml of supplemented 5% MEM-containing 0.9% agarose. The overlay media was prepared by autoclaving 9g of low melting temperature agarose in 91.0ml of distilled water, cooling the agarose solution to 45℃ and diluted it 1:10 in 2% MEM preheated to 40℃. The cells were incubated at 37℃, 5% CO_2 incubator in saturated humidity for 2 to 3 days. The pla-ques were visualized by staining with a 0.01% solution of neutral red (GIBCO) in 2% MEM overlay media for 2h. Plaques were picked from petri dish infected 10^{-4} diluted virus. Plaque was delivered into 0.5ml of PBS and pipetted gently to release virus from the agarose overlay and was used for inoculation of cell monolayers to develop a virus stock for second plaque assay purification step. The supernatant was stored as virus stock at -70℃ deep freezer.

Restriction endonuclease digestion of viral DNA and electrophoresis

*Bam*HI and *Bgl*II restriction enzymes (Amersham) were used for digestion of viral DNA. The HSV-1 genomic DNA digested with restriction enzymes was electrophoresed on 0. 6% agarose gel for 16 h and the molecular size of each DNA fragment was determined by comparing its mobility with *Hind*III-digested phage λ DNA fragments (POSCO). The reactions were stopped by addition of 1/10 volume of 7M urea, 50% sucrose, 0.1mM EDTA and 0. 1% Bromophenol Blue.

Cloning of viral genomic DNA digested with restriction enzyme

HSV-1 gene library was constructed (11). HSV-1 genomic DNA fragments digested with *Bam*HI and *Bgl*II restriction enzyme, were cloned into the pBluescript SK (+) and pBacPAK 9 vec-tors (Boeringer Mannheim) in 10×ligation buffer containing 10mM ATP. The ligation mixture was mixed with $CaCl_2$ treated *E.coli* XL-1 Blue strain to transform. *E.coli* XL-1 Blue was kept on ice for 1h and then was heat-shocked at 42℃ for 90 sec. After the aliquot was placed on ice again for 5min, directly smeared on LA medium (1% NaCl, 0.5% yeast extract, 1% bacto-trypton, 1.8% bacto-agar) containing ampicillin (50μg/ml) for selection of transformed colonies. This plate was incubated at 37℃ for overnight. The recombinant plamid was cultured in LB broth at 37℃ and the plasmid DNA was purified by Birnboim and Doly [15] procedure.

Cloning confirmation of HSV-1 TK gene by Southern blot

Southern blotting for detection of the HSV-

1 TK gene was performed by Maniatis *et al.*, [12]. Recombinant plasmid containing probe DNA (pHLB-4) was digested with *Asp*-I. Digested pHLB-4, probe DNA (200ng) was boiled for 5mins, followed by cooling on ice for 5 min, labelled *in vitro* by ECL kit (Amersham), using 20μl (300ng) of Horse radish peroxidase and 20μl of glutaraldehyde. The total mixture (60μl) was incubated for 10 min at 37℃, and held on ice for 10-15min. Transfer to Hybond-N^+ membranes (Amersham) was done by standard method [13]. The blot was hybridized to labelled probe DNA in the hybri-dization gold buffer (Amersham), 0.5M NaCl and 5% blocking agent. The incuba-tion was continued with agitation at 42℃ overnight. Primary washes of the Southern blots were washed twice at 42℃ for 20 min in primary wash buffer containing urea (6M Urea, 0.4% SDS, 0.5×SSC : 1×SSC is 150mM NaCl plus 15mM sodium citrate). Secondary washes were twice with an excess of secondary wash buffer (4×SSC). Incubation with agitation was for 5 minutes at room temperature. Following incubation of the blot in detection reagent I and II (Amersham) was exposed to Hyperfilm-ECL. DNA side of membrane was covered with equal volumes of detection reagent I and II to give sufficient to cover the blot. The blot drained on 3mm paper was wrapped in Saran WrapTM and exposed DNA side to autoradiography film (Hyperfilm-ECL). The film was developed following the proce- . dures suggested by Kodak.

RESULTS AND DISCUSSION

Multiplication of HSV-1

HSV-1 was infected and multiplicated on vero cell monolayer in petri dish (6015×mm) at 37℃, purified by a plaque assay and then cloned a plaque which was named HL-1 clone. The selected clone was reinfected on vero cell monolayer in plastic tissue flask (25cm^2). The purified plaque was replaqued and titered by the plaque assay. The viral titer was 4×105 pfu per ml.

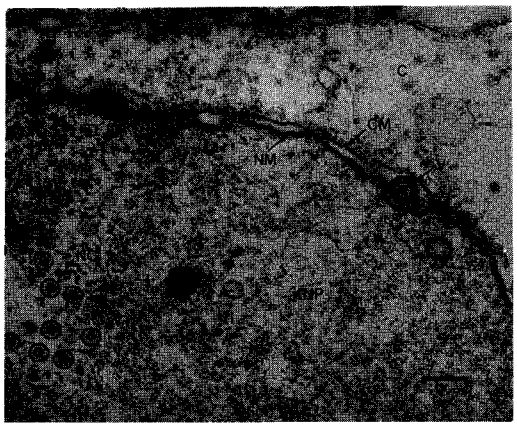

Fig. 1. Cytoplamic area of Vero cell infected with HSV-1 at 48 h p.i. Abbreviations; NC:nucleocapsids, C:cytoplasm, NM:nuclear membrane, NP:nucleoplasm, and V:nucleocapsids budding out. The black bar represents 1 μm.

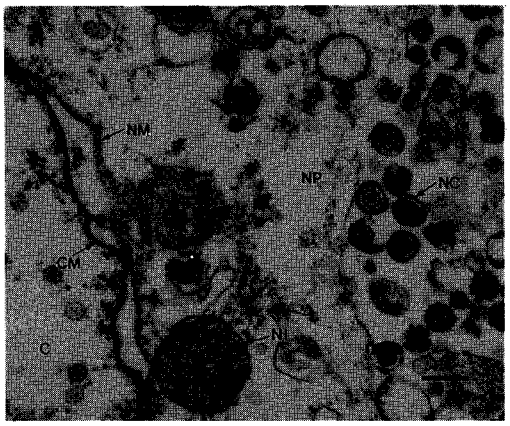

Fig. 2. Nuclear area of Vero cell infected with HSV-1 at 48 h p.i. Abbreviations; NC:nucleocapsids, C:cytoplasm, NM:nuclear membrane, NP:nucleoplasm, and NI:nuclear inclusion body. The black bar represents 1 μm .

Vero cells infected with HSV-1 HL-1 clone were observed with electron microscope. At 48 h postinfection a number of icosahedral nucleocapsids were found in nucleus and cytoplasm, and scme of the nucleocapsids were budding through nuclear membrane (Fig. 1, 2 and 3). Also matured nuclear inclusions were found (Fig. 2 and 3). Some of the nucleocapsids were enveloped through vacuole and cytoplasmic

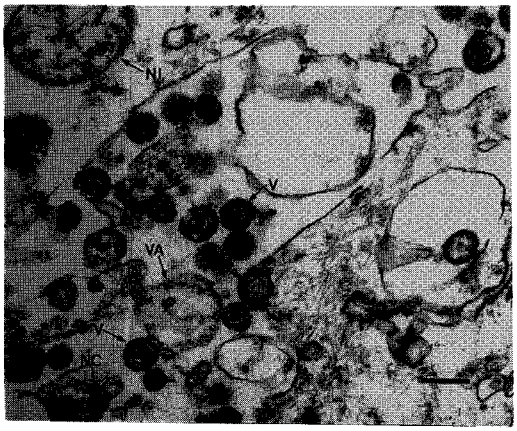

Fig. 3. Nuclear area of Vero cell infected with HSV-1 at 48 h p.i. Abbreviations; NC:nucleocapsids, NI:nuclear inclusion body, VA:vacuole, and V: matured virion. The black bar represents 1 μm.

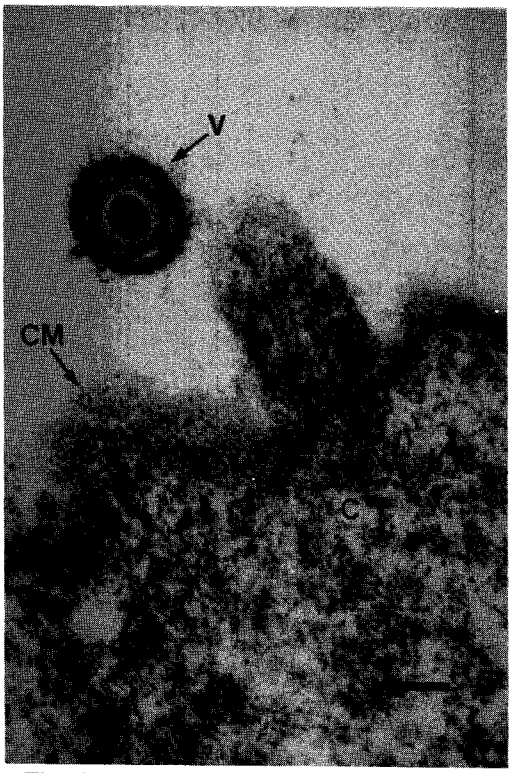

Fig. 4. Cellular membrane area of Vero cell infected with HSV-1 at 48 h p.i. Virion was budding out from the cytoplasmic membrane. Abbreviations: V: matured virion. CM: cell membrane. C: cytoplasm. The black bar represents 1 μm.

membrane, and were budding out through cytoplasmic membrane (Fig. 4). As the number of

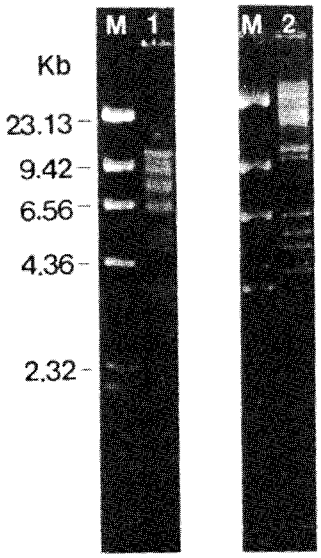

Fig. 5. Restriction enzyme patterns of HSV-1 genomic DNA. The digested DNAs were electrophoresed on 0.6% agarose slab gel. Lanes M: λphage DNA digested with *Hind*III enzyme, 1: HSV-1 DNA digested with *BamH*I, and 2: the DNA digested with *Bgl*II enzyme.

nucleocapsids increased, chromatin density were gradually decreased (Fig. 2). When nucleocapsids were replicated in the nucleus and moved to cytoplasm, the nucleo-capsids were enveloped through nuclear membrane and moved to cytoplasm (Fig. 4). A similar result was reported that FL cell (hu-man amnion cells) infected with HSV-1 appea-red nucleocapsids with highly electron-dense at 40 h p.i. by Nii [12].

HSV-1 HL-1 strain genome library

To construct genome library of HSV-1 HL-1 clone, the purified HSV-1 HL-1 clone genomic DNA was digested with *Bam*H I or *Bgl* II restriction enzymes, and then the digested DNA fragments were electrophoreised on 0.6% agarose gel (Fig. 5). The genomic DNA digested with *Bam*H I or *Bigl* II was cleaved into twenty-seven and sixteen, respectively. The molecular sizes of the latter were ranged from 1.1 to 14 Kb and that of the former were ranged from 4.

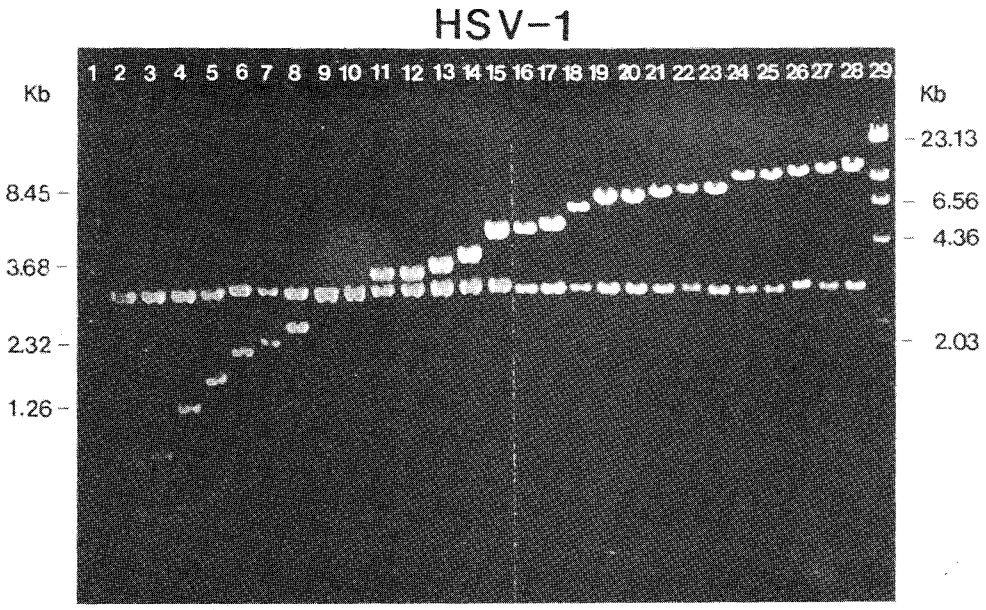

Fig. 6. Confirmation of the cloned DNA fragments in pBluescript SK (+). The recombinant plasmids were digested with *Bam*HI enzyme and electrophoresed. In the center there are linear vector DNAs, and upper and lower bands are the *Bam*HI DNA fragments inserted in the plasmids. Lanes 1:λphage DNA digested with *Bst*EII, 2:pHLA-1, 3:pHLA-2, 4:pHLA-3, 5:pHLA-4, 6:pHLA-5, 7:pHLA-6, 8:pHLA-7, 9:pHLA-8, 10:pHLA-9, 11:pHLA-10, 12:pHLA-11, 13:pHLA-12, 14:pHLA-13, 15:pHLA-14, 16:pHLA-15, 17:pHLA-16, 18:pHLA-17, 19:pHLA-18, 20:pHLA-19, 21:pHLA-20, 22:pHLA-21, 23:pHLA-22, 24:pHLA-23, 25:pHLA-24, 26:pHLA-25, 27:pHLA-26, 28:pHLA-27, 29:pHLA-28 and 29:λphage DNA with *Hind*III.

5 to 20.10 Kbs (Fig. 5 and Table 1). Each DNA fragments were numerically numbered on the basis of molecular sizes from the largest one to the small one.

To confirm the cloned DNA fragments the recombinant plasmids were redigested with *Bam*H I or *Bgl* II restriction enzymes and then their electrophoretic mobilities in agarose gel were observed (Fig. 6 and 7). Each clones were named through an arabic numerals designation on the basis of molecular sizes such that the largest insertional fragment which was digested with *Bam*H I is named pHLA-1, the second largest, pHLA-2 (Fig. 6 and Table 1) and that the largest insertional fragment which was digested with *Bgl* II is named pHLB-1, the second largest, pHLB-2, etc (Fig. 7 and Table 1). In the Figures 6 and 7 the original *Bam*HI or*Bgl*II DNA fragments and linear form of their host vector DNAs were observed. The linear forms of their host vector DNA horizonally appeared

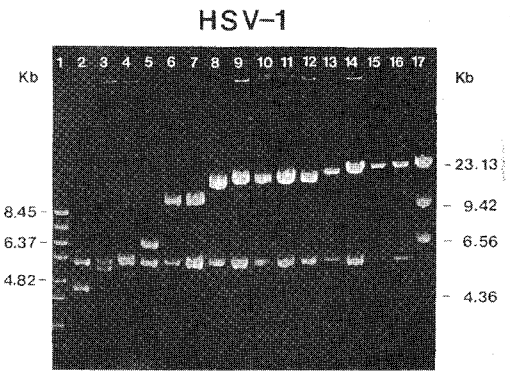

Fig. 7. Confirmation of the cloned DNA fragments in pBakPAK9 vector. The recombinant plasmids were digested with *Bgl*II enzyme and electrophoresed. In the center there are linear vector DNAs, and upper and lowere bands are the *BamH*I DNA fragments inserted in the plasmids. Lanes 1: λphage DNA digested with *Bst*EII enzyme, 2:pHLB-1, 3:pHLB-2, 3:pHLB-2, 4:pHLB-3, 5:pHLB-4, 6: pHLB-5, 7:pHLB-6, 8:pHLB-7, 9:pHLB-8, 10: pHLB-9, 11:pHLB-10, 12:pHLB-11, 13:pHLB-12, 14:pHLB-13, 15:pHLB-14, 16:pHLB-15 and 17: λphage DNA digested with *Hind*III.

Table 1. Sizes and recombinant clones of HSV-1 genome DNA digested with *Bam*H I or *Bgl* II resriction enzymes

Oder of fragments	*Bam*H I		*Bgl* II	
	sizes	clones	sizes	clones'
1	1.13	pHLA-1	4.55	pHLB-1
2	1.17	pHLA-2	4.92	pHLB-2
3	1.42	pHLA-3	5.55	pHLB-3
4	1.71	pHLA-4	6.41	pHLB-4
5	1.97	pHLA-5	8.90	pHLB-5
6	2.17	pHLA-6	9.35	pHLB-6
7	2.41	pHLA-7	9.47	pHLB-7
8	2.76	pHLA-8	9.55	pHLB-8
9	2.83	pHLA-9	9.63	pHLB-9
10	3.23	pHLA-10	9.72	pHLB-10
11	3.49	pHLA-11	10.03	pHLB-11
12	3.74	pHLA-12	11.02	pHLB-12
13	3.98	pHLA-13	13.05	pHLB-13
14	4.61	pHLA-14	15.02	pHLB-14
15	5.26	pHLA-15	20.10	pHLB-15
16	5.45	pHLA-16		
17	6.43	pHLA-17		
18	6.51	pHLA-18		
19	6.56	pHLA-19		
20	7.40	pHLA-20		
21	7.82	pHLA-21		
22	7.92	pHLA-22		
23	9.58	pHLA-23		
24	9.64	pHLA-24		
25	9.99	pHLA-25		
26	12.01	pHLA-26		
27	13.87	pHLA-27		
Total	150.04	147.47		

Sizes: kilobases

on the gel center, however the restriction fragments were presented according to their sizes.

Cloning of TK gene of HSV-1

A novel thymidine kinase (TK:ATP:thymidine 5'-phosphotransferase, EC.2.7.1.21) gene is encoded in cells were infeced with HSV-1 [14]. The TK gene is an early gene of HSV. It has become a target for antiviral therapy because the viral TK gene has a much broader range of phosphorylation substrates than does the cellular enzyme [16]. The TK gene is stably and heritably incorporated into the genome of the cell infected and is necessary for normal virus multiplication in experimental infections [17]. This characteristic enabled us to identify the TK gene. In this study, the HSV-1 genomic DNA fragments which were digested with BamHI restric-tion en-zyme was clon-ed to pBluescript KS (+) plasmid, and the HSV-1

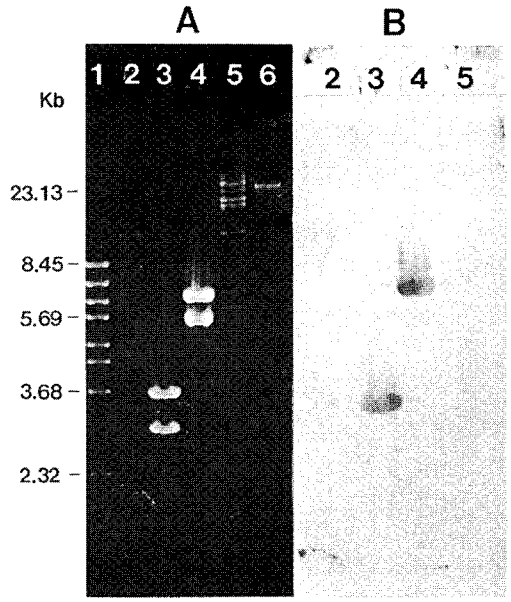

Fig. 8. Identification of HSV-1 thymidine kinase gene on recombinant plasmids, pHLA-12 and pHLB4. Lanes 1:λphage DNA digested with *Bst*EII enzyme, 2:λDNA digested with *Bam*HI enzyme, 3: pHLA-12 clone digested with *Bam*HI, 4:pHLB-4 clone digested with *Bgl*II, 5:HSV-1 DNA digested with *Bgl*II, and 6:λphage DNA digested with *Hin-d*III enzyme. B is the Southern blot of the panel A. The black bands on the B indicate the presence of the thymidine kinase gene.

genomic DNA fragments which were digested with Bgl II restriction enzyme was cloned to pBacPAK 9 plasmid. The recom-binant pHLA-12 plamid containing whole part of the TK gene was confirmed by Southern blotting [13] with the 1.59 kb fragment of pHLB-4 recombi-nant DNA probe, which was digested with *Asp* I . The 1.59 kb fragment con-tained a part of TK gene. The recom-binant pHLB-4 plamid containing a part of TK gene was confirmed by Southern blotting with the 1.59 kb fragment of pHLB-4 recombinant DNA probe, which was digested with *Asp* I (Fig. 8). The molecular sizes of the fragment which contains TK gene were 3.74 kb in pHLA-12 and 6.41kb in pHLB-4 recom-binant plasmids, respectively (Fig. 8). However the result of Colbere-Garapin *et al.* [18] showed that the TK gene of HSV-1 macroplaque strain was located within 3.4kb DNA fragment that was digested with *Bam*H I and residues fully within the 2.03kb *Pvu* II fragment.

SUMMARY

Multiplication of *Herpes simplex* virus type-1 was observed by electronmicroscopy, a gene library of the genome was constructed and thymidine kinase gene was cloned. Vero cells infected with the virus were lysed 48 h p.i. and multinucleated giant cells were observed approximately at 72 h p.i. The nucleocapsids were observed in nuclei and cytoplasm, and the assembled nucleocapsids were budded out through the vacuole and cytoplasmic membranes, and then virions were released from the cells. HSV-1 genome DNA was digested with *Bam*HI and *Bgl*II enzymes and then the gene library of the genome fragments were constructed. The *Bam*HI cleaved the genome DNA into twenty-seven fragments in the range of 1.1 - 14 kb, and *Bgl*II cleaved the genome DNA into sixteen fragments in the range of 4.5～20.1 kb. The pHLA-12 and pHLB-4 recombinant plasmids were contained TK gene by Southern blot analysis. The molecular sizes of the fragments which contained the TK gene were 3.74 in pHLA-12 and 6.41kb in pHLB-4 recombinant plasmid, respectively.

Acknowledgement

This work was supported by Ministry of Welfare and Health, Korea.

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