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Cloning, Sequencing and Expression in *Escherichia coli* of *Herpes simplex* virus Type-1 Thymidine Kinase Gene

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=Abstract=

Cloning, sequencing and expressing in *E. coli* of the thymidine kinase (TK) gene of *Herpes simplex* virus type-1 (HSV-1) strain F was investigated. The TK gene, located in the *Bam*HI 3.74 kb DNA fragment of the plasmid pHLA-12, was amplified by polymerase chain reaction (PCR). The 1,131 kb PCR product was cloned into the *Bam*HI and *Eco*RI sites of pBacPAK9 plasmid and then named pBac-TK recombinant. The TK gene was subcloned into the *Bam*HI and *Bgl*II sites of pQE-30, and named pQE-TK recombinant. The nucleotide sequence of the 1,131 kb TK gene was determined, and the GC content was 65.13%. There were deduced 367 amino acid residues with a total molecular weight of 43 kDa. The weight was confirmed by the protein produced by *E. coli* M15/pQE-TK on the SDS-PAGE and Western blot. The production of the TK protein in the IPTG induced cells was measured over 4 h. At the end of 1, 2 and 3 h the level increased by 146, 204 and 242%, respectively. The amount of the protein at the highest fraction purified with Ni-NTA resin chromatography was 0.68 μg per ml. The soluble state TK protein was present in the cytoplasm. In these results the F strain was different in base sequence and amino acid sequence from that of the CL101 strain, which caused difference in their strains.

Key Words: *Herpes simplex* virus type 1, Thymidine kinase gene

INTRODUCTION

Herpes simplex virus type-1 (HSV-1) belongs to the family *Herpesviridae* [27], contains double-stranded linear DNA molecules with molecular weight $100x10^6$ at the central genomic core, and encodes at least 70 polypeptides [10]. The DNA core is surrounded by a capsid containing 162 capsomers, and the nucleocapsids are enveloped [25]. During the lytic cycle, HSV genes are expressed in a three phase cascade; immediate early (α), early (β), and late (γ). The biosynthesis in the three phases occurs in a highly regulated fashion [12]. The β proteins include several regulatory proteins and enzymes such as thymidine kinase (TK) and DNA polymerase that are essential for DNA replication. The TK gene and TK encoded by HSV is of great interest for several reasons. First, as an early gene of HSV, the TK gene may be a model of gene regulation in infected cells because TK transcription is regulated both positively and negatively by other viral products [12]. Second, viral TK phosphorylates deoxynucleoside as a substrate [6]. Therefore, the

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Table 1. List of primers used in this work

Primer	Polarity	Sequence (5' → 3')
k-1	+	TTTGGCAAGGGATCCGTAGAAGCGCGTATG
k-2	-	CCCGAATTCAGTTAGCCTCCCCCACTCCCC

expression of the TK gene of HSV-1 endows the host cell with a conditional lethal phenotype whose characteristics depend on the presence of nucleoside analogues metabolized by this enzyme into toxic inhibitors of DNA replication. Acyclovir is a base analogue, competes as a substrate with deoxynucleoside, and is selectively phosphorylated by the viral TK. Viral TK is far more effective than cellular TK in phosphorylating acyclovir [27]. Third, TK is amenable to biochemical and genetic analysis because the TK gene is not an essential viral gene. The easy transfer of the TK gene to cells has facilitated the study of integration and retention of viral sequences into chromosomal DNA and has provided a versatile method for introducing other foreign genes into eukaryotic cells [8, 17, 27, 32]. HSV-1 strain F [9] is a pathogen to human. Because its TK gene and TK are not yet illustrated we undertook this research to characterize the TK gene and protein. We constructed the genome library of the HSV-1 and searched the TK gene location [15], Further studies are necessary to characterize the gene and protein and used them for beneficial purposes.

In this report, we describe studies on cloning, nucleotide sequencing and expressing in *E. coli* of the TK gene of *Herpes simplex* virus type-1 strain F. The TK gene of the plasmid pHLA-12 was amplified by PCR, The 1,131 kb PCR product was obtained and used for cloning, sequencing, and expressing.

MATERIALS AND METHODS

1. Bacterial strains and plasmids

pQE-30 plasmid in *E. coli* M15 [4], pBacPAK9 vector [16] and pBluescript II SK(*) in *E. coli* XL1-Blue [30] were used for cloning in this work.

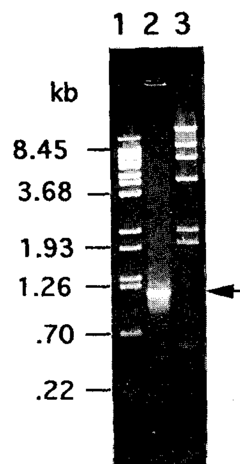

Fig. 1. cDNA of TK gene of HSV-1 strain F produced by PCR. Arrow indicates the PCR product cDNA, whose molecular size is approximately 1.13 kb. The fragment was created by using 5'-end specific primer k-1 and 3'-end specific primer k-2.

2. Synthetic oligomers

Synthetic oligomers (primers) listed in Table 1 were deduced from sequences of the TK gene of the HSV-1 CL101 strain [34] and then synthesized at the Korea Biotech. Inc. Daejon, Korea. The primer k-1 contained one *Bam*HI site and start codon ATG at the 5' end, and the primer k-2 contained one *Eco*RI site and stop codon TGA at the 3' end.

3. Radioisotopes and antibodies

The [α-^{35}S]dATP for nucleotide sequencing came from Amersham (Arlington Height, IL), and the secondary antibodies for immunoblotting and enzyme-linked immunosorbent assay

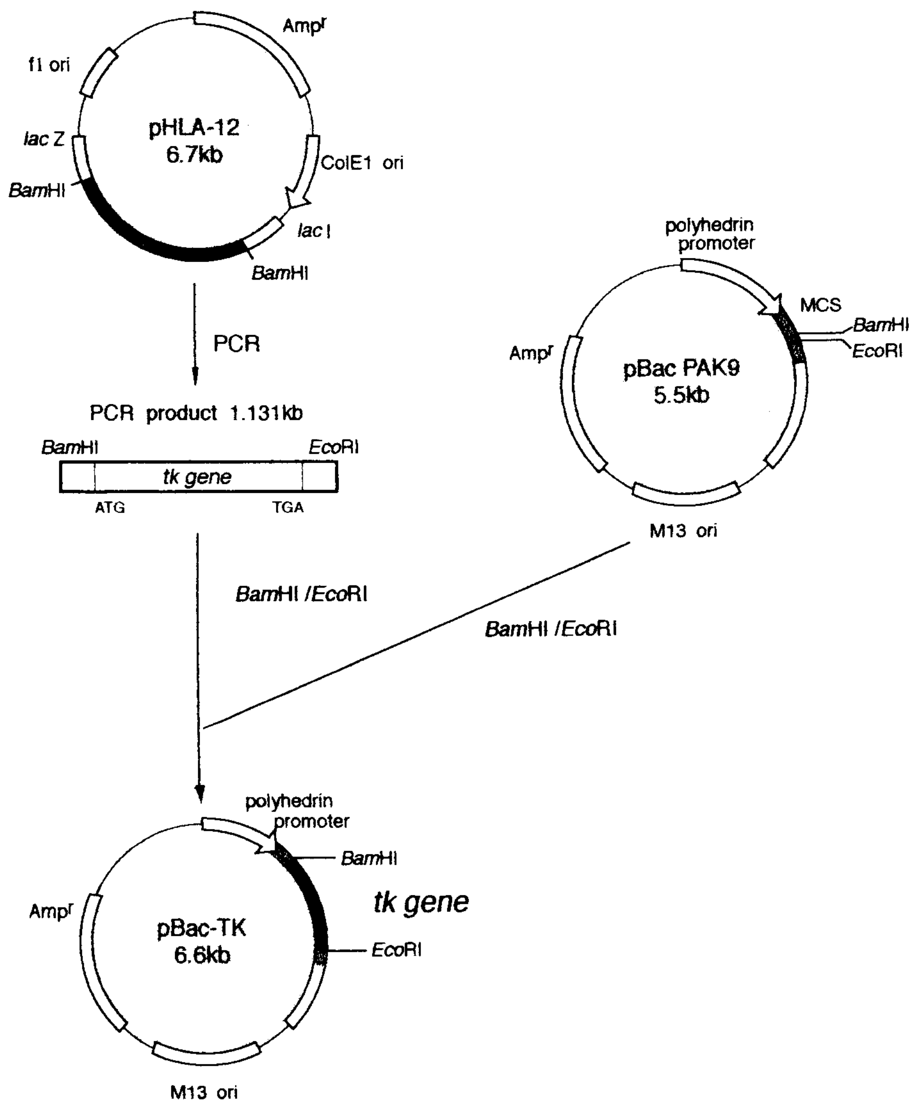

Fig. 2. Construction strategy of pBac-TK recombinant plasmid. The 1.13 kb TK gene cDNA was cloned into *Bam*HI and *Eco*RI sites in the pBac-PAK9 vector, and the resulting plasmid was named pBac-TK.

came from Boehringer Mannheim (Indianapolis, IN).

4. Isolation of plasmid DNA and agarose gel electrophoresis

Plasmid DNAs were isolated using the alkaline lysis procedure described by Birnboim and Doly [1]. Digestion of 1 μg plasmid DNA was performed with 1 unit restriction enzyme for 2 h, and then run on 0.8% agarose gel [20, 21].

5. Amplification of TK gene by polymerase chain reaction

The TK gene on the plasmid was amplified by polymerase chain reaction (PCR) system 2400 (Perkin-Elmer, Foster, CA) as described by Jung *et al.*, [14] and Chung *et al.*, [7].

6. Cloning of TK gene in vector

Cloning techniques [5, 19, 23] were used

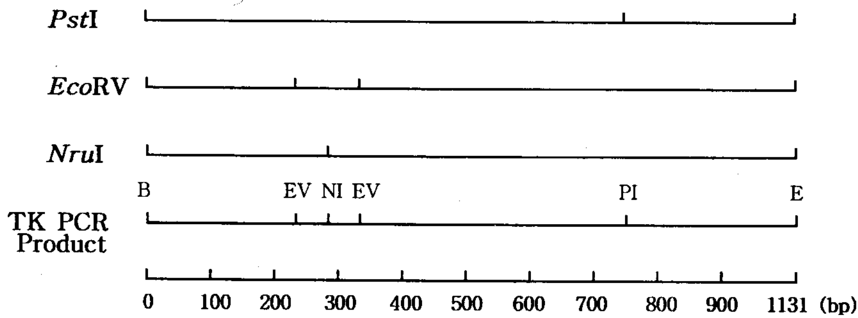

Fig. 3. Physical map of 1,131 kb TK gene DNA of HSV-1. The horizontal lines refer to the size of the gene and the vertical lines indicate restriction sites. Abbreviations: B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; NI, *Nru*I and PI, *Pst*I.

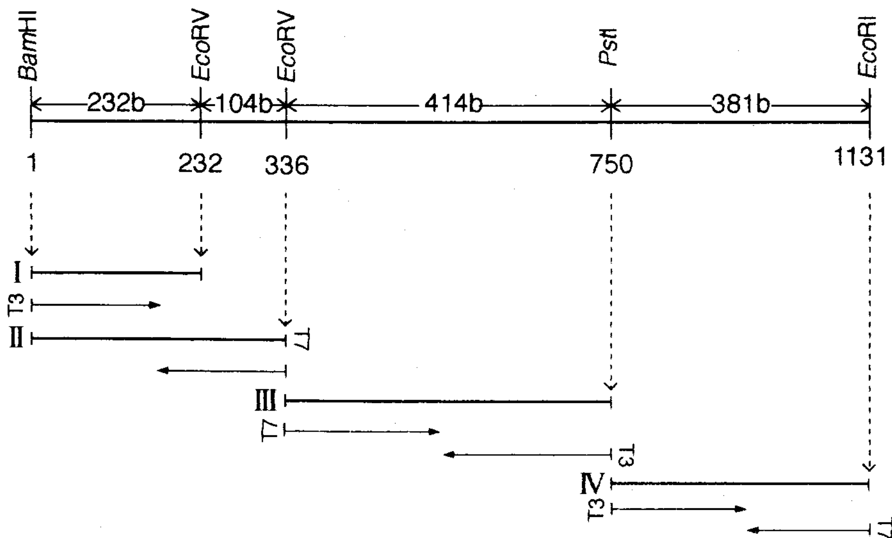

Fig. 4. Strategy for TK gene sequencing of HSV-1. The TK gene DNA was digested with restriction enzymes and cloned into pBluescript II SK(*), and their sequences were determined by the dideoxy chain termination procedure. Vertical lines indicate the ends of the cloned fragments. Arrows denote the length and direction of sequencing. Roman numerals indicate the base sequence of the region and the name of the subclone recombinant plasmids. T3 and T7 primers were used.

throughout these experiments. The amplified TK gene was digested with appropriate restriction enzymes (Promega, Madison, WI) and cloned into the enzyme sites of vectors. The recombinant plasmid was transformed in *E. coli* [22], and the insertion was confirmed by Southern blot analysis [31].

7. Nucleotide sequencing of TK gene

Dideoxy chain termination sequencing [19, 29] was done on polyacrylamide sequencing gel using approximately 8 μg/μl of double-stranded DNA templates and 100 pmol/μl of limiting primer. The DNA fragments were labeled with ^{35}S-ATP and sequenced with a 7-deaza-dGTP sequencing kit with Sequenase version 2.0 T7 DNA polymerase (United State Biochemical, Cleaveland, OH), using pBluescript T3 primer, T7 DNA primer and M13 reverse primer.

```
1
ATG GCT TCG TAC CCC TGC CAT CAA CAC GCG TCT GCG TTC GAC CAG GCT GCG CGT
 M   A   S   Y   P   C   H   Q   H   A   S   A   F   D   Q   A   A   R
55
TCT CGC GGC CAT AGC AAC CGA CGT ACG GCG TTG CGC CCT CGC CGG CAG CAA GAA
 S   R   G   H   S   N   R   R   T   A   L   R   P   R   R   Q   Q   E
109
GCC ACG GAA GTC CGC CTG GAG CAG AAA ATG CCC ACG CTA CTG CGG GTT TAT ATA
 A   T   E   V   R   L   E   Q   K   M   P   T   L   L   R   V   Y   I
163
GAC GGT CCT CAC GGG ATG GGG AAA ACC ACC ACC ACG CAA CTG CTG GTG GCC CTG
 D   G   P   H   G   M   G   K   T   T   T   T   Q   L   L   V   A   L
217
GGT TCG CGC GAC GAT ATC GTC TAC GTA CCC GAG CCG ATG ACT TAC TGG CAG GTG
 G   S   R   D   D   I   V   Y   V   P   E   P   M   T   Y   W   Q   V
271
CTG GGG GCT TCC GAG ACA ATC GCG AAC ATC TAC ACC ACA CAA CAC CGC CTC GAC
 L   G   A   S   E   T   I   A   N   I   Y   T   T   Q   H   R   L   D
325
CAG GGT GAG ATA TCG GCC GGG GAC GCG GCG GTG GTA ATG ACA AGC GCC CAG ATA
 Q   G   E   I   S   A   G   D   A   A   V   V   M   T   S   A   Q   I
379
ACA ATG GGC ATG CCT TAT GCC GTG ACC GAC GCC GTT CTG GCT CCT CAT ATC GGG
 T   M   G   M   P   Y   A   V   T   D   A   V   L   A   P   H   I   G
433
GGG GAG GCT GGG AGC TCA CAT GCC CCG CCC CCG GCC CTC ACC CTC ATC TTC GAC
 G   E   A   G   S   S   H   A   P   P   P   A   L   T   L   I   F   D
487
CGC CAT CCC ATC GCC GCC CTC CTG TGC TAC CCG GCC GCG CGA TAC CTT ATG GGC
 R   H   P   I   A   A   L   L   C   Y   P   A   A   R   Y   L   M   G
541
AGC ATG ACC CCC CAG GCC GTG CTG GCG TTC GTG GCC CTC ATC CCG CCG ACC TTG
 S   M   T   P   Q   A   V   L   A   F   V   A   L   I   P   P   T   L
595
CCC GGC ACA AAC ATC GTG TTG GGG GCC CTT CCG GAG GAC AGA CAC ATC GAC CGC
 P   G   T   N   I   V   L   G   A   L   P   E   D   R   H   I   D   R
649
CTG GCC AAA CGC CAG CGC CCC GGC GAG CGG CTT GAC CTG GCT ATG CTG GCC GCG
 L   A   K   R   Q   R   P   G   E   R   L   D   L   A   M   L   A   A
703
ATT CGC CGC GTT TAC GGG CTG CTT GCC AAT ACG GTG CGG TAT CTG CAG GGC GGC
 I   R   R   V   Y   G   L   L   A   N   T   V   R   Y   L   Q   G   G
757
GGG TCG TGG CGG GAG GAT TGG GGA CAG CTT TCG GGG ACG GCC GTG CCG CCC CAG
 G   S   W   R   E   D   W   G   Q   L   S   G   T   A   V   P   P   Q
811
GGT GCC GAG CCC CAG AGC AAC GCG GGC CCA CGA CCC CAT ATC GGG GAC ACG TTA
 G   A   E   P   Q   S   N   A   G   P   R   P   H   I   G   D   T   L
865
TTT ACC CTG TTT CGG GCC CCC GAG TTG CTG GCC CCC AAC GGC GAC CTG TAT AAC
 F   T   L   F   R   A   P   E   L   L   A   P   N   G   D   L   Y   N
919
GTG TTT GCC TGG GCC TTG GAC GTC TTG GCC AAA CGC CTC CGT CCC ATG CAC GTC
 V   F   A   W   A   L   D   V   L   A   K   R   L   R   P   M   H   V
973
TTT ATC CTG GAT TAC GAC CAA TCG CCC GCC GGC TGC CGG GAC GCC CTG CTG CAA
 F   I   L   D   Y   D   Q   S   P   A   G   C   R   D   A   L   L   Q
1027
CTT ACC TCC GGG ATG GTC CAG ACC CAC GTC ACC ACC CCC GGC TCC ATA CCG ACG
 L   T   S   G   M   V   Q   T   H   V   T   T   P   G   S   I   P   T
1081
ATC TGC GAC CTG GCG CGC ACG TTT GCC CGG GGA GTG GGG GAG GCT AAC TGA
 I   C   D   L   A   R   T   F   A   R   G   V   G   E   A   N   *
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Fig. 5. Nucleotide and deduced amino acid sequences of thymidine kinase gene of HSV-1. The numbers on the left indicate nucleotide numbers. The number 1 is the location of the translation initiation codon, ATG and the termination codon is shown(*), TGA. The putative ORF of the TK gene of HSV-1 has 1,131 kb. The one letter symbols of the standard amino acid shorthand were used.

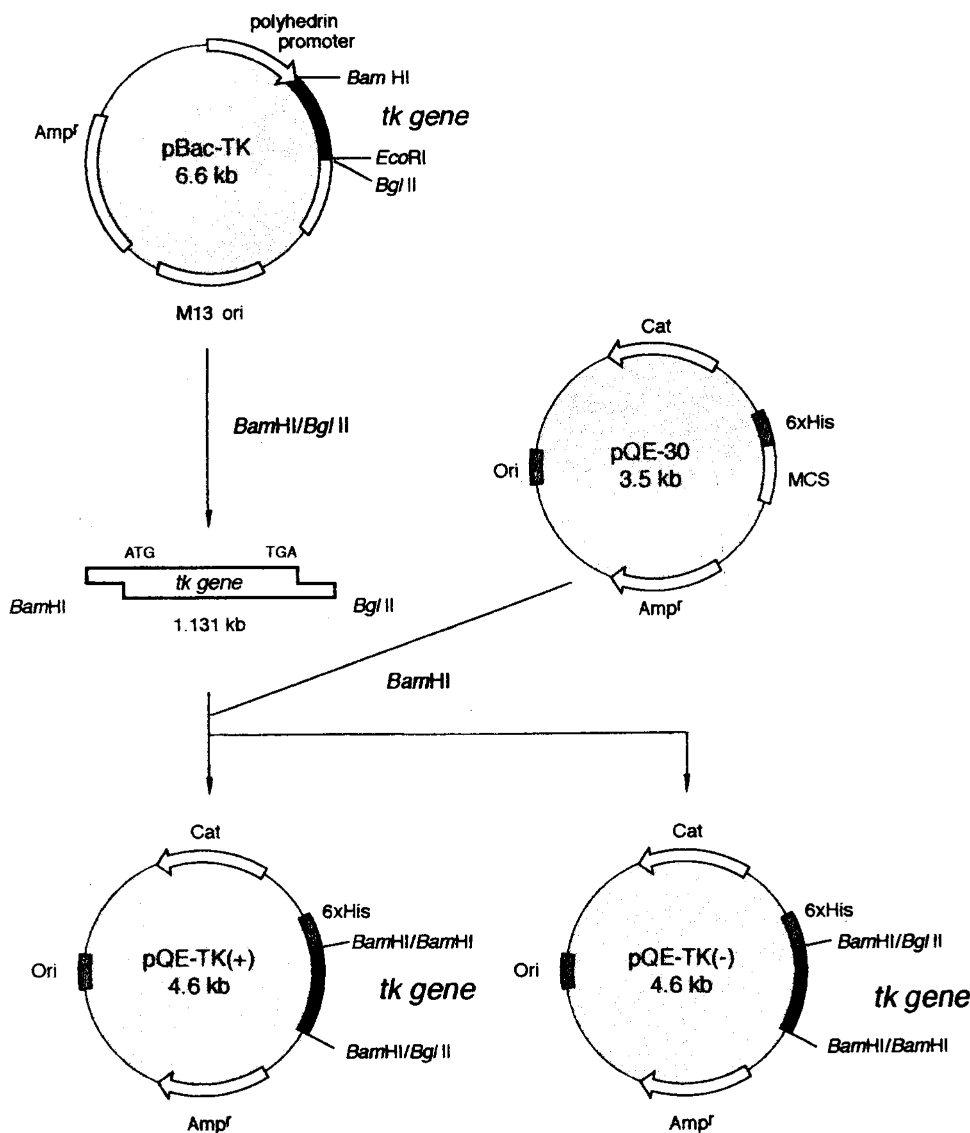

Fig. 6. Construction scheme of pQE-TK recombinant plasmid. The TK gene sequence in the pBac-TK was transferred into the *Bam*HI and *Bgl*II sites of pQE-30 vector, and named pQE-TK (+) or pQE-TK (−). (+) indicates that the TK gene is inserted in the right orientation to the promoter and (−) means the inverted orientation.

8. Purification of TK protein by affinity chromatography

TK protein was purified by immobilized metal affinity chromatography with the procedure described by Hochuli et al., [11], Janknecht et al., [13] and Lee et al., [19]. Protein concentrations were determined by the method of Bradford [3] using bovine serum albumin as a standard.

9. Preparation of TK antiserum

TK antiserum was prepared with the following procedures. A 0.5~50 μg of TK fusion protein and an equal volume of complete Freund's adjuvant (GIBCO) were mixed and in-

jected into a peritoneal cavity of a mouse. After 3 weeks, 0.5~50 μg of fusion protein and an equal volume of incomplete Freund's adjuvant (GIBCO) were mixed and also injected into the peritoneal cavity. To obtain an antiserum from the immunized mice at 10~14 days, blood was collected from the heart and incubated at 4℃ for 18 h. After centrifuging at 4,000 x g for 10 min, the supernatant was stocked at -20℃. The titer of the antibody was measured by an enzyme linked immunosorbent assay (ELISA) as described by Thiermann and Garrett [33].

10. Detection of TK protein in *E. coli* cells

TK protein produced in cells was detected with vertical slab SDS-PAGE and Western blot assays as described by Bollag and Edelstein [2], Laemmli [18] and Lee et al., [19].

11. Examination of TK protein location in *E. coli* cell

The *E. coli* with the pQE-TK clone was cultured in a medium. Then production and locations of the TK protein within the cells were examined as described by Lee et al., [19].

RESULTS AND DISCUSSION

1. Cloning and sequencing of the TK gene of HSV-1

Previously, Kang et al., [15] reported that the 3.74 kb *Bam*HI fragment of HSV-1 strain F was cloned and named pHLA-12 plasmid. Using two primers, k-1 with *Bam*HI site and k-2 with *Eco*RI site, the TK gene in the plasmid pHLA-12 was amplified by PCR and cloned into the pBacPAK9 plasmid. The 1,131 kb PCR product of the TK gene (Fig. 1) was cloned into the *Bam*HI and *Eco*RI sites of pBacPAK9 plasmid, and named pBac-TK recombinant (Fig. 2). The insertion was confirmed by Southern blot (Fig. 8 lane 5). The cDNA was mapped using restriction enzymes. The cDNA was cleaved into 2 fragments with 750 and 381 bases by *Pst*I; 3 fragments with 232, 104 and 795 bases by *Eco*RV; and 2 fragments with 838 and 293 bases by *Nru*I. The physical maps of the restriction cleaves are shown in Fig. 3.

The cDNA of the TK gene was sequenced by the strategy shown in Fig. 4. The cDNA fragments were digested with *Bam*HI, *Eco*RV, *Pst*I, and *Eco*RI enzymes, and its sequence was determined by the dideoxy chain termination method [19, 21]. The DNA sequences are shown in Fig. 5. The open reading frame (ORF) of TK gene of the putative HSV-1 strain F had 1,131 bases (Fig. 5). There were deduced 376 amino acid residues with a predicted molecular weight of 43 kDa. The GC content was 65.13%.

The ORF of the TK gene of HSV-1 strain F shared a 99.4% similarity with that of the TK gene of HSV-1 strain CL101 [34] (Table 2).

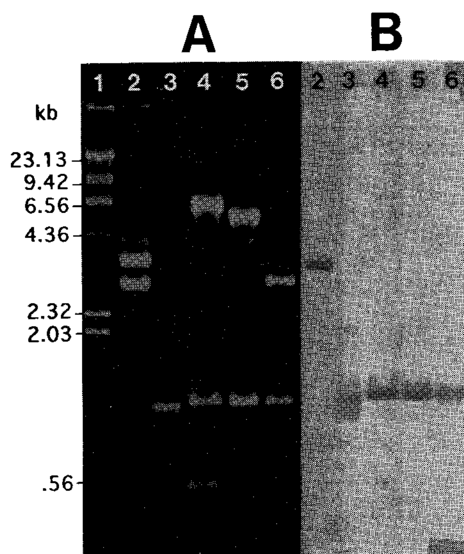

Fig. 7. Confirmation of the recombinant plasmids containing TK genes by Southern blot analysis. A is electrophoresed DNA patterns and B is the Southern blot of the A with pHLA-12 DNA probe. Lanes 1, λ phage DNA digested with *Hin*dIII; 2, pHLA-12 digested with *Bam*HI; 3, PCR product 1,131 kb of the TK gene of the HSV-1; 5, pBac-TK plasmid digested with *Bam*HI and *Eco*RI; and 6: pQE-TK (+) digested with *Bam*HI and *Eco*RI.

Table 2. Nucleotide and amino acid changes in TK gene between HSV-1 CL101 and F strains

Differences in the strains, CL101 → F			
Nucleotide sequence		Amino acid sequence	
Region	Bases change	Region	Change
427	G→A	143	V→I
447	T→C	–	–
766	T→C	256	W→R
1065	A→C	–	–
1112	A→G	371	E→G
1113	G→A	371	E→G
1114	A→G	372	M→V

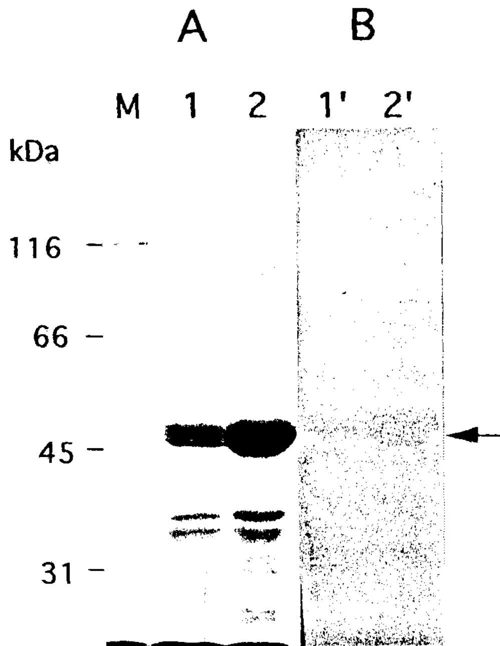

Fig. 8. Immunoblotting of 6xHis-tagged thymidine kinase produced by pQE-TK clone. His antibody used for the detection of the fused proteins containing the epitope His. A is the electrophoresed proteins and B is the Western blot of the A. Lanes M, molecular weight standards; 1, *E. coli*/pQE-TK lysate; 2, IPTG-induced *E. coli*/pQE-TK lysate. Arrow indicates TK protein and its blot.

There were a few intra-typic changes between the two strains of HSV-1. Within the coding region, there were 7 nucleotide changes, which resulted in four different amino acid changes in the TK protein. The nucleotides in the structural genes between the two HSV-1 strains varied 0.62% which caused a 1.1% difference in the amino acid sequence between the two strains. These results indicated that the change might be related differences between in the strains.

Table 3. Relative density percents of 6xHis-tagged thymidine kinase by the induced time

Time (h) induced	Relative density (%)	Increased density (%)
0	2.6	100
1	3.8	146
2	5.3	204
3	6.3	242
4	6.3	242

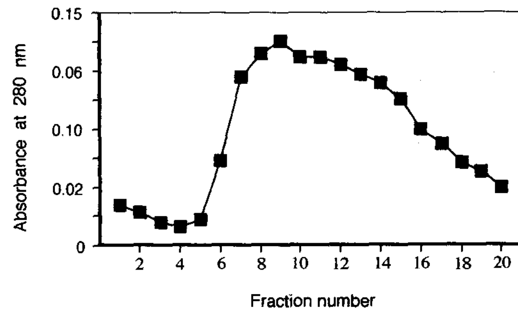

Fig. 9. Purification of 6xHis-tagged TK protein by Ni-NTA chromatography. The TK protein produced by *E. coli*/pQE-TK clone was purified by the chromatography. The soluble protein solution was fractionated in tubes with 3 ml each, and measured at 280 nm.

The TK gene sequence in the pBac-TK plasmid was digested with *Bam*HI and *Bgl*II, inserted into the *Bam*HI site of pQE-30 vector, and named pQE-TK (Fig. 6). The pQE-TK(+) DNAs digested with *Bam*HI and *Bgl*II restriction enzymes were electrophoresed and confirmed by Southern blot analysis (Fig. 7 lane 6). The results indicated that the TK gene sequence was inserted into the recombinant plasmid.

2. Identification of the TK protein produced in *E. coli*

The IPTG-induced *E. coil* M15 with the pQE-TK clone produced the 6xhistidine-tagged TK fusion protein, having a molecular weight of approximately 43 kDa, was estimated with the 10% SDS-polyacrylamide gel and then confirmed by Western blot analysis (Fig. 8).

Production of the TK protein in *E. coli*/pQE-TK was induced with 2 mM IPTG for 1, 2, 3, and 4 h. Their density percentages measured at each time intervals, increased 146, 204, 242, 242 and 242% respectively (Table 3). The highest amount of the protein was measured after 3 h. The TK proteins produced by pQE-TK clone were purified using Ni-NTA resin-affinity chromatography and measured at 280 nm [11, 13] (Fig. 9). The amount of the protein at the highest fraction purified with Ni-NTA resin chromatography was 0.68 μg per ml. Whether the fusion protein in *E. coil* M15 was soluble or insoluble, and cytoplasmic or periplasmic were examined. The TK proteins were present in the soluble cytoplasmic protein.

Cloning, sequencing and expressing in *E. coli* of TK gene of HSV-1 strain F was investigated and the information will be provided a basis for further understanding the molecular biology of the viral gene.

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