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Thymidine kinase gene mutation leads to reduced virulence of pseudorabies virus

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Abstract **To explore correlation between the tk gene structure of pseudorabies virus (PRV) and its virulence, to study the effect of the gene mutation on PRV biological properties, and to investigate mechinism of reduced virulence, thymidine kinase (TK)-deficient mutant of pseudorabies virus strain Hubei (PRV HB) was isolated by selection for resistance to 5-bromodeoxyuridine. The tk genes of PRV HB and its TK⁻ mutant were cloned and sequenced. 1587 base pairs of the tk gene and flanking regions of wild-type (wt) virus were sequenced, which included an open reading frame (ORF) of 1098 bp encoding a protein of 366 amino acids. The ORF contained two 137-bp repeated sequences, which were connected by an adenosine. 1458 bp of the tk and flanking regions of TK⁻ mutant were sequenced. Analysis of the tk gene sequence of TK⁻ mutant indicated that one of 137 bp repeated sequence and the connecting adenosine in the tk gene of the wt virus was deleted and a repeated sequence of 8 nucleotides (GCGCGCC) was inserted. All other nucleotides of TK⁻mutant were identical to that of wt virus. Deletion and insertion of the nucleotide sequence resulted in a frameshift and a premature chain termination, and the resultant TK protein was not active. Analysis of the amino acid sequence revealed that TK protein of PRV HB contained the conserved consensus sequence of herpesviral TKs and an additional conserved-DHR-motif. The results of this work also indicated that TK⁻ mutant was genetically stable. Compared to PRV HB, virulence of TK⁻ mutant was greatly decreased. Mice vaccinated with TK⁻ mutant were completely protected against a lethal challenge with virulent PRV (HB).**

Keywords: pseudorabies virus, thymidine kinase, nucleotide sequence, amino acid sequence, TK⁻ mutant.

Herpesvirus thymidine kinases (TKs) are products of early viral gene (thymidine kinase, tk) expression[1]. The TK activity is non-essential for virus replication in cell cultures, but is essential for virulence, and the enzyme appears to be important in the establisnment and maintenance of neural latency[2,3]. The TK catalyses the conversion of thymidine/pyrimidine deoxyribonucleotides to their monophosphate forms as part of the "salvage pathway" of nucleotide synthesis. Viral TK can phosphorylate some nucleotide analogues to cause cytotoxicity and termination of DNA elongation at the viral DNA polymerase. These nucleotide analogues have been used in gene therapy for cancer to achieve selective toxicity after introduction of the viral TK gene into tumour cells[4], and have also been employed in viral studies of TK⁻mutant[5]. TK-deficient mutants of herpesvirus can be isolated in the presence of nucleotide analogues, and the precise nature of mutational events leading to the TK⁻ phenotype has been the subject of interest for many years. Studies on a series of bromodeoxyuridine (BUdR)-selected HSV mutants revealed that the TK polypeptide was absent or, in some cases, of lower molecular weight[6]. TK⁻mutants of BHV-1 were a frameshift and a premature chain termination due to deletion or insertion of a single base, or to deletion of three bases encoding a single amino acid located in the enzyme activity center[7]. The PRV TK⁻ selected by BUdR and arabinosylthymine (araT) were avirulent for mice and young pigs and induced complete protection against the lethal challenge with virulent PRV, however, the precise nature of mutational events to the TK⁻mutant is unknown.

PRV is the causative agent of Aujeszky's disease, one of the most serious infectious diseases of pigs which results in significant economic losses. PRV belongs to the alphaherpesvirinae, and contains a 150 kb, double-stranded, linear DNA genome, which at least consists of 70 genes. Similar to that of other herpesviruses, the tk gene of PRV is an early viral, virulent gene, and is non-essential for virus replication in cell cultures. The TK⁻ mutant of pseudorabies virus strain Hubei (PRV HB) was isolated by selection for resistance to 5-bromodeoxyuridine. The tk genes of PRV HB and its TK⁻mutant were cloned and sequenced. Biological properties such as propagation, virulence and immunity of PRV TK⁻ mutant were investigated. These results might be contributive to the study of the molecular mechanism of PRV virulence and the development of an genetically engineered TK-deficient PRV vaccine.

1 Materials and methods

(ⅰ) Viruses and cells. Pseudorabies virus strain Hubei (PRV HB, HS9403) was from the area of Hubei Province, China. Baby hamster kidney (BHK-21) cells, human 143 TK⁻ cells and African green monkey kidney (Vero) cells, kindly supplied by China Center for Type Culture Collection (CCTCC), were grown in MEM or DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% non-essential amino acids and 10% newborn calf serum. The virus was propagated on BHK-21 cells, PRV TK⁻mutant was selected on 143 TK⁻ cells, and isolated on Vero cells.

(ⅱ) Isolation of TK-deficient mutant of PRV. PRV strain HB was passaged thrice on 143 TK⁻ cells in presence of 25 μg/mL 5-bromodeoxyuridine (BUdR), using virus multiplicities of 1—5 pfu/cell. Plaque purification

was performed after the third passage by selection of BUdR, and was repeated three times. TK^- mutant was propagated on BHK-21, and the tk gene structure and biological properties were further analysed.

(iii) Amplification, cloning and sequencing of the tk gene of PRV HB and TK^- mutant. The tests were performed as described in ref. [8]. PCR primers were designed on the basis of NIA-3 tk sequence[9]. Sense primer: 5′-GAACA- CCAGCAGGGGCACGAGC-3′, anti-sense primer: 5′- GGGCACGGCAAACTTTATTGGG-3′. Nucleotide sequence was determined by the dideoxynucleotide chain termination method on automatic DNA sequencer type 377(ABI). Both strands were sequenced by the strategies of constructing subclone and walking method, respectively. Analysis of the nucleotide and predicted amino acid sequence was carried out using DNASIS v2.5 software on computer.

(iv) Thymidine kinase activity[10]. The TK activity was tested by thymidine plaque autoradiographic experiment. Viral DNA was labeled with ^{3}H-thymidine.

(v) Virulence and protective efficacy of PRV TK^- mutant in mice[10]. 18—22 g female mice were randomly divided into seven groups (5 mice/group). Experimental mice were inoculated intraperitoneally (IP) with 0.05 mL of the wt virus or TK^- mutant. The control group was inoculated IP with 0.05 mL of physiological salt solution. The mice survived after inoculation with TK^- mutant were again immunized with 10^7 pfu TK^- mutant in the fourth week. Mice vaccinated were challenged IP with 100 LD50 virulent PRV HB after three weeks. The death number of each group mice was recorded daily for 3 weeks.

2 Results

(i) Nucleotede and predicted amino acid sequence of the tk gene of PRV strain Hubei. The region of DNA sequenced was 1578 bp, with G+C content of 72.7%. Codon ATG located in 358 nucleotides of the sequence might be initiation codon, which exhibited typical features of an eucaroytic translation initiation signal[11]. An open reading frame of 1098 bp located in 358—1456 nucleotide encoded a protein of 366 amino acids, with a molecular weight of 40 ku. A typical TATA box was not found, however, the distal transcription signals GGGCGGG and CCGCCC of the herpesviral tk gene were located at 160, 192 and 340 nucleotides, upstream of the initiation codon, respectively. A typical AATAAA polyadenylation signal sequence was present at 111 nucleotide, downstream of a termination codon. Nucleotides of 611—748 and those of 750—886 were two perfect repeated sequences, connected by an adenosine. The protein sequence encoded by the tk gene of PRV HB was 46 amino acids longer than that of NIA-3 (fig.1).

(ii) Changes in the tk gene structure of TK^- mutant. A DNA region of tk gene of TK^- mutant cloned and sequenced was 1458 bp by using the same PCR primers and sequencing strategies for that of the wt virus. Compared to the nucleotide sequence of the wt virus, TK^- mutant missed the 137 bp repeated sequence and the connecting adenosine. However, an eight nucleotides (GCGCGCCC) insertion occurred between 991 (C) and 992 (G) nucleotide in the tk gene of TK^- mutant. All other nucleotides of the genes of the wt virus and TK^- mutant were identical. Deletion and insertion of the nucleotide sequence resulted in a frameshift mutation and a premature termination codon TGA located 1079 nucleotides and the active TK protein was not produced (fig. 1 and table 1).

(iii) TK activity and growth properties of TK^- mutant. The results of thymidine plaque autoradiography experiments indicated that the plaques made by PRV HB on BHK-21 cells, labeled with ^{3}H-TdR, formed dark rims on radiographic film, but plaques made by TK^- mutants on 143 TK^- or BHK-21 cells could not, showing that TK^- mutant lacks TK-producing activity. TK^- mutant could propagate and form CPE on Vero and BHK-21 cells, with a slower propagation rate and a 6—12 h delay of CPE formation in comparison with the wt virus. BUdR inhibited the growth of the wt virus on cell cultures, but did not inhibit that of TK^- mutant. The growth properties of TK^- mutant were not changed after 12 passages on BHK-21 cells.

(iv) Virulence and induction of protective immunity of TK^- mutant in mice. The results of animal experiments showed that the median lethal dose (LD50) for mice inoculated IP with parental PRV HB was 56 pfu/mouse, but $>10^7$ pfu/mouse for TK^- mutant. Virulence of TK^- progeny virus of 12 passages on BHK-21 cells was not changed in mice. Mice vaccinated with 10^6 and 10^7 pfu of mouse were fully protected against a lethal challenge with virulent PRV HB (table 2).

3 Discussion

To investigate the molecular background of PRV HB and to construct a genetically engineered TK-deficient PRV vaccine, the tk gene of PRV HB was cloned and sequenced. Analysis of DNA sequence revealed that the ORF of the tk gene was composed of 1098 bp, which contained two adjacent repeated sequence of 137 bp connected by an adenosine (insertion of a total 138 bp). The GC box structure of an eucaroytic promoter was found in upstream of the ORF, without TATA box. Adenosine located at 163 nucleotide of the tk gene was changed into Guanosine, which led to form another CCGCCC structure. Molecular mechanism and biological significance of these changes is to be elucidated.

Analysis of the structure helps to explain the structural and functional evolution of the protein. Analysis of

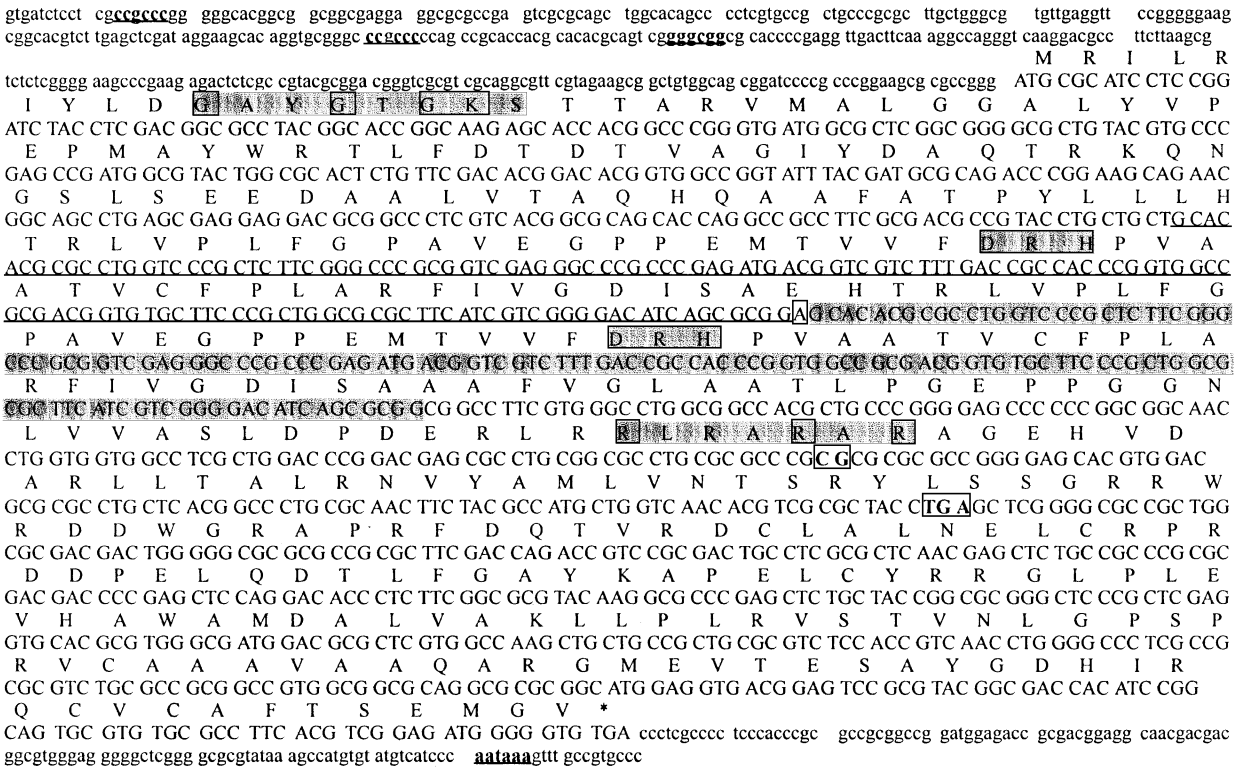

Fig. 1. The nucleotide and predicted amino acid sequence of PRV strain HB. Repeated nucleotides are indicated by underlining and shading. Connecting "A" is shown boxed. Black letters underlined mark the upstream transcription signals sequences of the initiation codon and downstream polyadenylation signal sequence of the termination codon, respectively. Black letters GC and TGA boxed indicate the insertion-site of 8 nucleotides and a premature termination codon of TK$^-$ mutant, respectively. Shaded amino acids indicate the conserved functional motifs and boxed amino acids are completely conserved in the herpesviral TK.

Table 1 Comparison of tk gene structure of PRV HB and TK$^-$ mutant

PRV	Change and site of nucleotides	Length of ORF (bp)	Amino acid residue No.	Position of stop codon
Wild type	–	1098	366	1456
Mutant	① deletion of 138 bp located in 612-749 / 748-885 nucleotides ② insertion of 8 bp in 991-992 site	594	197	1079

Table 2 Virulence of PRV HB and TK$^-$ mutant and protection induced by TK$^-$ mutant in mice

Viruses	PRV HB			TK$^-$ mutant			Control
Dose (pfu/mouse)	10^1	10^2	10^5	10^5	10^6	10^7	—
route		(IP)			(IP)		
Mortality (5 mice)	1/5	4/5	5/5	0/5	0/5	0/5	—
LD50 (pfu)		56			$>10^7$		—
No. of survivors	—	—	—	4/5	5/5	5/5	0/5

the primary structure showed that the PRV HB TK included the conserved structural and functional regions of other herpesviral TKs[12]. The major conserved structural and functional regions were in the following: First, the conserved motif -GAYGTGKS-, the consensus sequence of the herpesviral TKs was -GXXGXGKT/S- (X represents various amino acids). The motif of the herpesviral TKs was considered to participate in binding ATP. All of three glycines of the motif were very important to TK activity. The substitution of aspartic acid for the first glycine in the motif -GXXGXGKT/S- resulted in the loss of TK activity of PRV NIA-3[9]. Site-directed mutagenesis of HSV-1 TK showed that TK activity was lost when anyone of the glycines in the motif -GXXGXGKT/S- was mutated to valine, or if the lysine was mutated to isoleucine and when the threonine was mutated to alanine. However, TK activity was retained if the threonine was mutated to serine, though the km values for substrates were different[13]. It has been suggested that the small-sized glycine could form a flexible tertiary structure involved in the access and the correct orientation of enzyme to substrates, while the hydroxyl group of threonine (and of serine) reacted directly with the nucleotide phosphoryl group. Second, the conserved motif -DHR-, the most conserved motif in the herpesviral TKs, was considered to be involved in thymidine recognition of the enzyme[14]. There were two motifs -DHR- in TK of PRV HB. Mechanism and significance of the structural change is unknown. Third, the conserved arginine-rich motif -RLRARAR-, the consensus sequence -RXXXRXR- of the herpesviral TKs, was considered to bind substrate phosphoryl groups. It has

been suggested that an arginine-rich motif moves towards the nucleotide-binding site (-GXXGXGKT/S-) in the presence of substrate[15]. Recently, the high-resolution X-ray study showed that some amino acids of HSV-1 TK, such as lysine of the motif -GXXGXGKT/S- and aspartic acid and arginine of the motif -DHR- could directly interact via hydrongen bonds with the nucleotide analogues[16].

Studies on the molecular mechanism of the reduced virulence have the important significance to instruct the reconstruction of the virus genome and to develop the engineered vaccine. PRV HB TK^- mutant was isolated by selection of BUdR. The tk gene of TK^- mutant was cloned and sequenced using the same strategy as described for the wt virus. Analysis of nucleotide sequence revealed that the 138 bp inserted sequence in the tk gene of PRV HB was deleted and the following 8 nucleotides (GCGCGCCC) were inserted in the tk gene of TK^- mutant. The 8 nucleotides were identical to adjacent 8 nucleotides. Deletion and insertion of the nucleotide sequence resulted in a frameshift mutation, and a premature termination codon TGA located in 1079 nucleotides, and the inactive TK protein of 179 amino acids was produced. Insertion of nucleotides existed in ORFs of the wt virus and TK^- mutant, and both the inserted sequences are adjacent tandem repeats. However, insertion in the tk gene of the wt virus did not change its ORF and an active TK protein was produced, but insertion in the tk gene of TK^- mutant changed the ORF of the tk gene, resulting in the inactive TK. Significance of the gene evolution of insertion and deletion of the tandem sequence in the virus gene needs to be explored. Moreover, PRV HB TK^- mutant was genetically stable, and was avirulent in mice and induced the protection against a lethal challenge with virulent PRV in mice. The results of this work laid the experimental foundation of developing the avirulent engineered PRV TK-deficient vaccine. The tk gene cloned and sequenced has been used for construction of transfer vector[17] and development of the engineered PRV vaccine.

References

1. Preston, C. M., Control of herpes simplex virus type 1 mRNA synthesis in cells injected wild-type virus or the temperature-sensitive mutant tsK, J. Virol., 1979, 29: 275.
2. Field, H. J., Wildy, P., The pathogenesis of thymidine kinase-deficient mutants of herpes simplex virus in mice, J. Hyg., 1978, 81: 267.
3. Field, H. J., Resistance and latency, British Medical Bulletin, 1985, 41: 345.
4. Black, M. E., Newcmb, T. G., Wilson, H. M. P. et al., Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy, Proc. Natl. Acad. Sci. USA, 1996, 93: 3525.
5. Borelli, E., Heyiuan, R., His, M. et al., Targeting of an inducible toxic phenotype in animal cells, Proc. Natl. Acad. Sci. USA, 1988, 85: 7572.
6. Sumers, W. P., Wagner, M., Summers, W. C., Possible chain termination mutants in thymidine kinase gene of a mammalion virus, herpes simplex virus, Proc. Natl. Acad. Sci. USA, 1975, 72: 5081.
7. Mittal, S. K., Field, K. H. J., Analysis of the bovine herpesvirus type 1 thymidine kinase (TK) gene from wild-type virus and TK-deficient mutants, J. Gen. Virol., 1989, 70: 901.
8. Sambrook, J., Fritsch, E. F., Maniantis, T., Molecular Cloning: A Laboratory Manual, 2nd ed., New York: Cold Spring Harbor Laboratory Press, 1989.
9. Priete, J., Hernandez, A. M. M., Tabares, E., Loss of pseudorabies virus thymidine kinase activity due to a single base mutation and amino acid substitution, J. Gen. Virol., 1991, 72: 1435.
10. Kit, S., Kit, M., Pirtle, E. C., Attenuated properties of thymidine kinase-negative deletion mutant of pseudorabies virus, Am. J. Vet. Res., 1985, 46: 1359.
11. Kozak, M., The scanning model for translation: An update, J. Cell. Biol., 1989,108: 229.
12. Balasubramaniam, N. K., Veerisetty, V., Gentry, G. A., Herpesviral deoxythymidine kinases contain a site analogous to the phosphoryl-binding arginine-rich region of porcine adenylate kinase; comparison of secondary structure predictions and conservation, J. Gen. Virol., 1990, 71: 2979.
13. Liu, Q., Summers, W. C., Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: effects on catalytic activity, Virology, 1988, 163: 638.
14. Darby, G., Larder, B. A., Inglis, M. M., Evidence that the "active centre" of herpes simplex virus thymidine kinase involves an interaction between three distinct regions of the polypeptide, J. Gen. Virol., 1986, 67: 753.
15. Pai, E. F., Sachsenheimer, E., Schirmer, R. et al., Substrate positions and induced-fit in crystalline adenylate kinase, J. Mol. Bio., 1977, 114: 71.
16. Bennett, M. S., Wien, F., Champness, J. N. et al., Structure to 1.9Å resolution of a complex with herpes simplex virus type-1 thymidine kinase of a novel, non-substrate inhibitor: X-ray crystallographic comparison with binding of aciclovir, FEBS Letters, 1999, 443: 121.
17. Pan, Z., Zhang, C., Zhao, W. et al., Construction of the pseudorabies virus transfer vector and the expression of *E. coli* β-galactosidase, J. Wuhan Univ. (Nat Sci Ed) (in Chinese), 2000, 46: 717.

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