Acta virologica **54**: 91 – 98, 2010 doi:10.4149/av_2010_02_91

# Analysis of Chinese Rabies virus isolates from 2003–2007 based on P and M protein genes

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*Received August 17, 2009; accepted March 15, 2010*

**Summary. –** Phylogenetic analysis of nucleotide sequences or deduced amino acid sequences of phosphoprotein (P protein), matrix (M) protein, and glycoprotein (G protein) genes of 18 Chinese isolates of Rabies virus (RABV) from 2003–2007 showed that these isolates formed a separate monophyletic lineage consisting of sub-lineages A and B. Compared with laboratory-fixed strains, recent Chinese isolates of sub-lineage B contained Val or Ala instead of Met69 in P protein, which is involved in generating truncated P proteins. In addition, one of these isolates CHpg3 had Pro instead of Ser63 and Leu instead of Ser64. Importantly, all functional domains of P and M proteins of all recent Chinese isolates were similar to those of laboratory-fixed strains. This study showed that although the recent Chinese RABV isolates belonged to a distinct lineage, their functional domains of P and M proteins were highly conserved.

**Keywords:** rabies virus; glycoprotein; phosphoprotein; matrix protein; China

## Introduction

Rabies virus (the genus *Lyssavirus*, the family *Rhabdoviridae*) is the etiological agent of rabies encephalitis that is enzootic in most regions of the world. It forms a bullet-shaped virion consisting of five proteins – nucleoprotein (N protein), P, M, G, and polymerase protein (L protein). The viral genome encapsulated by N protein forms a ribonucleoprotein (RNP) that interacts with P and L proteins, while M protein takes part in linking of RNP complex to the viral envelope. The G protein is glycosylated and forms spikes on the viral surface. It is involved in the host cell receptor recognition and membrane fusion (Gaudin *et al*., 1991; Thoulouze *et al*., 1998; Tuffereau *et al*., 1998). Recently, P and M proteins have been found to affect not only viral propagation, but also host-viral interactions. P protein contributes to the viral transcription and replication in conjunction with the L and N proteins of RNP (Chenik *et al*., 1998; Jacob *et al*., 2001). In addition, P protein interacts with a nascent and free nucleoprotein ($N^0$ protein) and delivers it to the newly synthesized viral genome (Mavrakis *et al*., 2006). The motif DKSTQT of P protein also contributes to the viral transcription (Poisson *et al*., 2001). Phosphoacceptors are involved in the alternation of P protein (Gupta *et al*., 2000). Furthermore, four additional N-terminal truncated P proteins (P2, P3, P4, and P5) are generated from the internal methionin residues and they show different intracellular distributions (Chenik *et al*., 1995). The cytoplasmic distribution of the full-length P and P2 proteins is due to the presence of a nuclear export signal (NES), whereas the nucleoplasmic distribution of P3, P4, and P5 proteins is due to the presence of a nuclear localization signal (NLS), (Pasdeloup *et al*., 2005). These P variants counteract interferon (IFN)-induced host cell responses (Brzózka *et al*., 2006; Vidy *et al.,* 2007).

M protein plays an important role in viral budding and the motif PPxY (x can be any amino acid) is critical in this

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**Abbreviations:** G protein = glycoprotein; IFN = interferon; L protein = polymerase protein; M = matrix; N protein = nucleoprotein; $N^0$ = nascent and free nucleoprotein; NES = nuclear export signal; NLS = nuclear localization signal; P protein = phosphoprotein; RABV = Rabies virus; RNP = ribonucleoprotein

**Table 1. Chinese RABV isolates from Guangxi Province, China**

| Isolate | Host | Region of isolation | Year | GenBank Acc. No. | | |
|---|---|---|---|---|---|---|
| | | | | G gene | P gene | M gene |
| CHdg2 | dog | Linzhou | 2005 | AB428308 | AB428326 | AB428317 |
| CHpg3 | pig | Nanning | 2005 | AB428309 | AB428327 | AB428318 |
| CHdg4 | dog | Guizin | 2004 | AB428310 | AB428328 | AB428319 |
| CHdg5 | dog | Wuzhou | 2004 | AB428311 | AB428329 | AB428320 |
| CHdg6 | dog | Baise | 2003 | AB428312 | AB428330 | AB428321 |
| CHbv7 | cow | Laibi | 2004 | AB428313 | AB428331 | AB428322 |
| CHdg8 | dog | Congzuo | 2003 | AB428314 | AB428332 | AB428323 |
| CHbv9 | cow | Nanning | 2005 | AB428315 | AB428333 | AB428324 |
| CHdg10 | dog | Qinzhou | 2006 | AB428316 | AB428334 | AB428325 |
| CHdg11 | dog | Liuzhou | 2007 | AB458790 | AB458799 | AB505882 |
| CHdg12 | dog | Qinzhou | 2007 | AB458791 | AB458800 | AB505883 |
| CHdg13 | dog | Nanning | 2007 | AB458792 | AB458801 | AB505884 |
| CHdg14 | dog | Nanning | 2007 | AB458793 | AB458802 | AB505885 |
| CHdg15 | dog | Liuzhou | 2007 | AB458794 | AB458803 | AB505886 |
| CHdg17 | dog | Nanning | 2007 | AB458795 | AB458804 | AB505887 |
| CHdg18 | dog | Hechi | 2007 | AB458796 | AB458805 | AB505888 |
| CHdg19 | dog | Yulin | 2007 | AB458797 | AB458806 | AB505889 |
| CHdg20 | dog | Nanning | 2007 | AB458798 | AB458807 | AB505890 |

process (Mebatsion *et al*., 1999; Wirblich *et al*., 2008). Additionally, M as well as P protein regulates the viral RNA synthesis and M protein has been identified as an apoptosis factor in RABV-infected neuronal cells (Finke *et al*., 2003a; Kassis *et al*., 2004; Mita *et al*., 2008).

In China, rabies encephalitis is becoming a serious public health problem and since 1997 the number of rabies-related human deaths has increased. In 2006, a high number of new cases (3,293) were reported representing a 30% increase compared to the cases reported in 2005 (Zhang *et al*., 2009). The G protein of Chinese RABV has been antigenically characterized and its antigenic sites are conserved among Chinese RABV isolates and similar to those of laboratory-fixed strains (Meng *et al*., 2007). Additionally, a phylogenetic analysis based on the G gene showed the existence of two distinct lineages among RABV isolates. lineage China + SE Asia consists mainly of the isolates collected from China and Southeast Asia. European lineage consists of the isolates originated from Europe including well-known laboratory-fixed strains (Zhang *et al*., 2009).

Although P and M proteins are significantly involved in the important viral functions, there are only a few studies dealing with these proteins derived from field RABV isolates (Nadin-Davis *et al*., 1997, 2002, 2006; Kobayashi *et al*., 2007). Moreover, P and M proteins of Chinese RABV isolates have not yet been appropriately characterized.

To prevent and control rabies in China effectively, it is important to understand the characteristics of P and M proteins of Chinese RABV isolates. The present study analyzed the genetic characteristics of P and M proteins of recent Chinese isolates in comparison with older Chinese isolates as well as with other isolates from the rest of the world.

## Materials and Methods

*Specimens.* 18 RABV samples were obtained from 15 dogs, 2 cows, and 1 pig in Guangxi, a district that has one of the highest prevalence rates of rabies in China (Table 1). The brain specimens were determined to be RABV-positive by RT-PCR following the extraction of total RNA using a QIAamp Viral RNA mini kit (Qiagen).

*RT-PCR and sequencing.* RT-PCR and sequencing were performed as described previously (Sato *et al*., 2004). Primers are described in Table 2. Nucleotide sequences were determined by ATGC-WIN software ver. 4.0.2 (GENETYX).

*Sequence and phylogenetic analyses.* Multiple alignments were performed using ClustalW multiple sequence alignment program (Thompson *et al*., 1994). Phylogenetic trees based on the complete nucleotide sequences of G, P, and M genes were generated by the neighbor-joining method with bootstrap analysis (1000 replicates) under the Kimura-2 parameter model using MEGA software ver. 4.0 (Tamura *et al*., 2007). To confirm the phylogenetic relationship, the recent Chinese RABV isolates were analyzed with representative Chinese RABV isolates studied previously and laboratory-fixed strains. Multiple alignments of the deduced P and M amino acid sequences were generated using BioEdit software to characterize

**Table 2. Primers used for RT-PCR and sequencing**

| | Gene | Primer | Sequence | Position (nt)[a] |
|---|---|---|---|---|
| PCR and sequence primer | N | CHIDN-S1 (F) | 5'-TGAGGAAGAGATAAGAAGAATG-3' | 865–886 |
| | L | L5914-5895 (R) | 5'-TACCTCCCAAATGCCTTATC-3' | 5914–5895 |
| | P | N8 (R) | 5'-AGTTTCTTCAGCCATCTC-3' | 1585–1568 |
| | P | Psense2 (F) | 5'-CAAATAGTCAGACAAATGA-3' | 1820–1838 |
| | P | CHIDP-H2 (R) | 5'-GGACCAGAGGCAGTTTG-3' | 2055–2039 |
| | P | Panti3 (R) | 5'-AAGCTCTCAGCAATCTGGTGAGC-3' | 2139–2117 |
| | P | Panti2 (R) | 5'-AAGTTCCTCATGTTCTTCTTGC-3' | 2653–2632 |
| | M | CHIDM-S1 (F) | 5'-CTTCTCCAAGAAATACAAATTTCC-3' | 2137–2160 |
| Sequence primer | M | CHIDM-S2 (F) | 5'-GTCCCGCTGAAAGAACTC-3' | 2610–2627 |
| | M | CHIDM-H1 (R) | 5'-ATCCAAGAGGCACAAAATG-3' | 3275–3257 |
| | G | CHIDG-F868 (F) | 5'-CTTGTTGTGGAGGAGTTGG-3' | 4185–4202 |
| | G | Ga3883 (F) | 5'-CAACYAACCAYGATTACAC-3' | 3883–3901 |
| | G | CHIDG-R3 (R) | 5'-CACTTAAACGTGGTGGTGAC-3' | 3625–3608 |
| | G | CHIDG-R758 (R) | 5'-CTAAGTCCAAGAACCCCAC-3' | 4075–4057 |
| | G | CHIDG-R877 (R) | 5'-ACCACTCCTCCACAACAAG-3' | 4203–4186 |
| | G | CHIDG-R2 (R) | 5'-GCACCGTTAGTCACTGGAAC-3' | 5109–5089 |

[a]Numbering based on the strain PV/M13215.

the specific amino acids, motifs, domains, and similarities among Chinese RABV isolates (Hall, 1999).

## Results

*Phylogenetic analysis of the G, P, and M genes of RABV isolates.* The phylogenetic analysis of G gene showed that the recent Chinese isolates belonged to the monophyletic China + SE Asia lineage. In addition, these isolates could be classified into two sub-lineages, China + SE Asia A and China + SE Asia B (Fig. 1). The phylogenetic trees based on P and M genes indicated the same topology (Fig. 2).

*Sequence analysis of the P and M genes of RABV isolates.* ORF of the Chinese RABV P gene contains 894 nts encoding 297 aa. Within the Chinese field isolates, the nucleotide similarity was 86.2% and the amino acid similarity 91.2%. The China + SE Asia A sub-lineage possessed the conserved Asp51, Asn67, Ser134, Thr135, Pro140, Val149, and Lys151, while the other isolates belonging to the China + SE Asia B sub-lineage contained conserved Ser67, Met130, and Thr131. Furthermore, Gly90, Pro158, and Ser161 were unique to both sub-lineages (Fig. 3). The L, $N^0$, and N binding domains at aa 1–19, 4–40, and 186–297, respectively, were highly conserved among the P proteins of recent Chinese RABV isolates (Fig. 3). Three phosphoacceptor sites, Ser162, Ser210, and Ser271 were found in all Chinese field isolates. Regarding two of the phosphoacceptor sites, most of the Chinese field isolates contained Pro63 and Ser64 however, in the strain CHpg3, Pro63 and Leu64 were found. The DKSTQT motif (aa 143–148) was completely conserved within all field

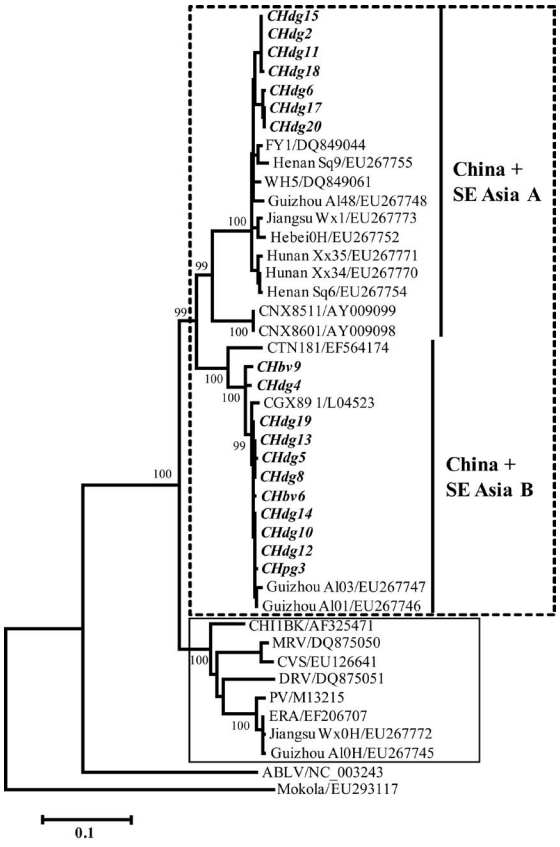


**Fig. 1**

**Phylogenetic tree of RABV isolates based on the G gene**

Recent Chinese isolates (italics), China + SE Asia lineage (broken-line box), and European lineage (solid-line box).

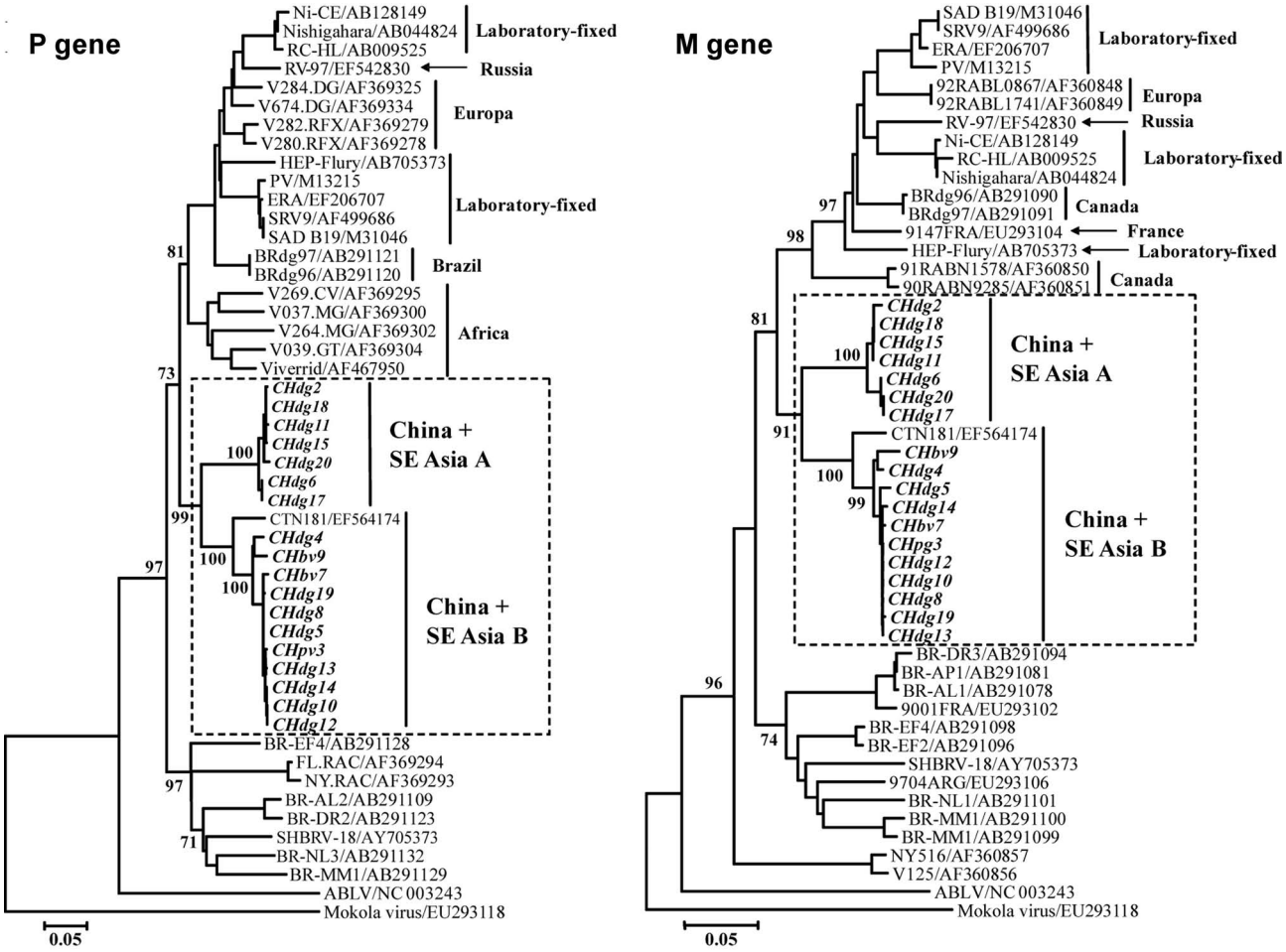


**Fig. 2**

**Phylogenetic tree of RABV isolates based on the P and M genes**

Recent Chinese isolates (italics) and China + SE Asia lineage (broken-line boxes).

isolates analyzed in this study. Three of the four methionine internal residues (Met20, Met53, and Met83) were conserved, whereas $Met_{69}$ of the China + SE Asia B sub-lineage was replaced with val or ala. the strain CTN181 is a vaccine strain derived from a Chinese field isolate retained Met69 and belongs to the sub-lineage China + SE Asia B. The isolates of the China + SE Asia A sub-lineage possessed a unique Asp51 within the NES (LxxxxxxLxL at aa 49–58), whereas the NLS (KKYK at aa 211–214 and Arg260) was completely conserved within the Chinese isolates.

ORF of the Chinese RABV M gene contains 609 nts encoding 202 aa. Within the Chinese field isolates, the nucleotide similarity was 89.1% and the amino acid similarity 96.5%. The multiple alignment of the deduced amino acid sequences showed that the isolates of China + SE Asia A sub-lineage had specific amino acids, Gln27 and Phe30 (Fig. 4). No unique amino acid was found in the sequences of the China + SE Asia B sub-lineage. In the Chinese isolates, the PPxY motif was highly conserved. In addition, the RNA synthesis regulating site represented Glu58 and the cytopathic determinant site Val95.

## Discussion

Previous molecular epidemiological reports based on the G gene have shown that the China + SE Asia lineage is a monophyletic lineage distinct from other lineages and is predominant in the current rabies epidemic in China (Zhang *et al.*, 2009). Chinese field isolates analyzed in this study belonged to the monophyletic China + SE Asia lineage with high bootstrap values in the phylogenetic tree based on the G gene. Thus,

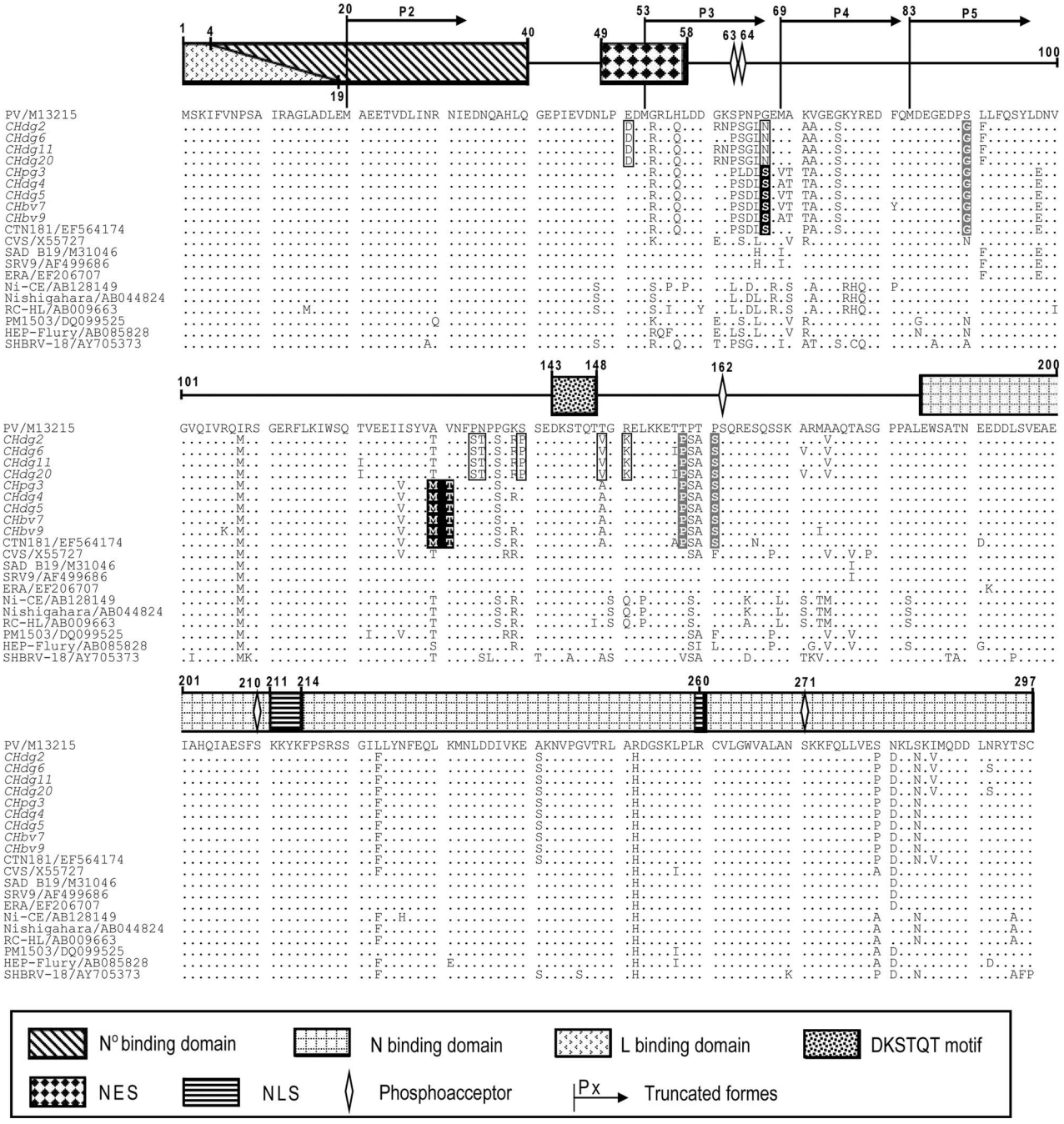


**Fig. 3**

**Multiple alignments of the deduced P protein sequences of RABV isolates**

Recent Chinese isolates (italics), amino acids specific for the China + SE Asia lineage (gray boxes), China + SE A sub-lineage (solid-line boxes), and China + SE B sub-lineage (black boxes).

the properties of the P and M genes characterized in this study indicated the general characteristics of Chinese RABV.

P protein that is involved in viral transcription and replication, associates with N and L proteins via their respective binding domains (Albertini *et al.*, 2008). Previous study has shown that the P protein has two N binding domains (Chenik *et al.*, 1998; Mavrakis *et al.*, 2006). One of them located close to the C-terminus (aa 186–297) has been dem-

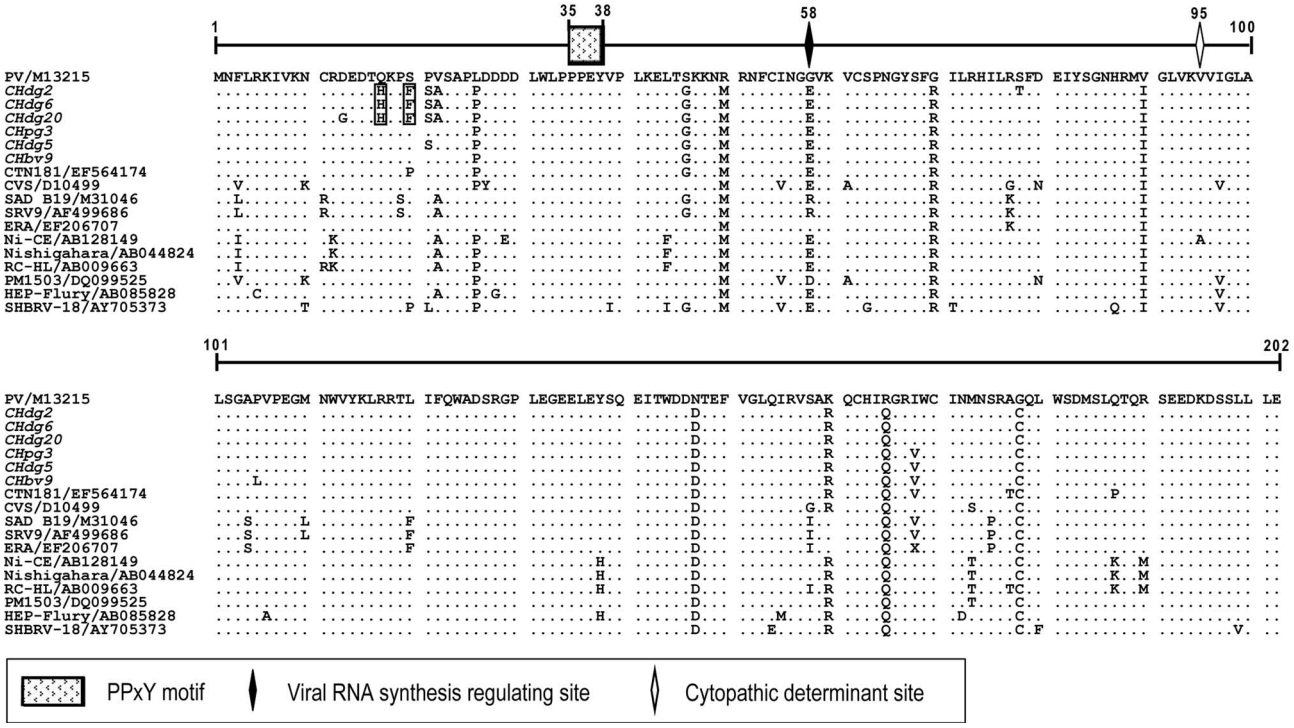


**Fig. 4**

**Multiple alignments of the deduced M protein sequences of RABV isolates**

Recent Chinese isolates (italics) and amino acids specific for China + SE Asia A sub-lineage (solid-line boxes).

onstrated using PV strain and the other one located close to the N-terminus (aa 4–40) binds to $N^0$ protein that was demonstrated using CVS strain (Jacob *et al*., 2001; Mavrakis *et al*., 2006). The L binding domain (aa 1–19) is partially wrapped in $N^0$ binding domain and consequently, $N^0$ and L proteins bound to the P protein are competing with each other. This interaction is essential for mediating the transcription and replication of RABV (Mavrakis *et al*., 2006). Two N and one L binding domain within the P protein were highly conserved suggesting that the P protein of Chinese RABV might be responsible for the transcription and replication of viral RNA similar to the laboratory-fixed strains.

There are five phosphoacceptors within the P protein that interact with two protein kinases. Ser162, Ser210, and Ser271 are associated with the protein kinase C, while Ser63 and Ser64 are associated with the RABV protein kinase (Gupta *et al*., 2000). These phosphoacceptors, especially RABV protein kinase-related phosphoacceptors, are involved in the conformation of RABV P protein and its interaction with RNP suggests that the phosphorylated forms of P protein may play an important role in the viral replication (Takamatsu *et al*., 1998; Gupta *et al*., 2000; Eriguchi *et al*., 2002). The aa 64 in most of RABV isolates analyzed in this study encoded serine however, aa 63 and 64 of the strain CHpg3 were not serines, what suggested that the aa 63 and 64 did not have to be serines for viral replication.

In addition to the full-length P protein, four N-terminal truncated P proteins are generated by the leaky scanning system. In this system a ribosomal failure to initiate translation at the first AUG codon would result in the continued scanning downstream to the next available AUG (Chenik *et al*., 1995). Furthermore, the P variants migrate into the cytoplasm and nucleoplasm of the infected cell due to the presence of NES and NLS (Pasdeloup *et al*., 2005). Previous experiments using the strains SAD-L16 and CVS have shown that P protein variants inhibit IFN signaling by binding to a signal transducer and activator of transcription (STAT) in both the cytoplasm and nucleoplasm (Brzózka *et al*., 2005, 2006; Vidy *et al*., 2005, 2007). The NES and NLS were highly conserved in all of isolates analyzed in this study, suggesting that the P variants generated from Chinese RABV might have the ability to be distributed in the nucleoplasm or cytoplasm. However, in the isolates belonging to the China + SE Asia B sub-lineage, excluding the strain CTN181, Met69 in the leaky scanning system was replaced with valine or alanine. On the other hand, strain SAD-derived laboratory-fixed

strains generated only four truncated P proteins in the absence of internal Met69, suggesting that the isolates belonging to the China + SE Asia B sub-lineage might not be able to generate the intranuclear-type truncated P, P4 (Brzózka *et al.*, 2006). Conversely, the strain CTN181 might be able to generate P4.

M protein is required for the viral assembly and budding and is involved in the generation of the typical bullet-shaped RABV virion (Mebatsion *et al.*, 1999). The PPxY motif has been reported as important for the virus release from RABV-infected cells (Wirblich *et al.*, 2008). The Chinese field isolates analyzed in this study encoded the PPxY motif. Moreover, M protein regulates the viral transcription and replication and its determinant residue aa 58 is critical for the viral RNA synthesis. It was reported that the viral RNA synthesis was higher in the strain SAD-L16 containing Arg58 than in the strain CVS containing Glu58 suggesting that the Chinese field isolates might be similar to the CVS strain concerning to the viral RNA expression (Finke *et al.*, 2003a,b).

M protein induces apoptosis of neuroblastoma cells by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) independent of tumor necrosis factor α (Kassis *et al.*, 2004). Recently, aa 95 within the M protein has been identified as a cytopathic determinant relating to the apoptosis (Mita *et al.*, 2008). A strong cytopathic effect is caused by Ala95 encoded in M protein of the strain Ni-CE derived from Nishigahara strain, while the Nishigahara strain containing Val95 has weak cytopathic effect (Mita *et al.*, 2008). All of the Chinese field isolates contained Val95 suggesting that the isolates might have weak cytopathic effects similar to that of Nishigahara strain.

The present study elucidated the genetic character of the RABV isolates in China based on the P and M genes. In previous reports the Chinese field isolates isolated from the current epidemic were connected to a monophyletic lineage that was fairly distant from other lineages. Therefore, it was predicted that these Chinese isolates might have substitutions within their functional domains. Nevertheless, the functional domains of the recent Chinese isolates were similar to those of laboratory-fixed strains and were highly conserved within these lineages as found in previously analyzed field strains (Nadin-Davis *et al.*, 1997, 2002, 2006; Kobayashi *et al.*, 2007). Moreover, this study elucidated the substitution relating to the mechanism that blocked the host IFN signaling between street RABVs and laboratory-fixed strains including the Chinese vaccine strain. The present genetic data could contribute to the further studies dealing with the pathogenicity of Chinese field isolates, field isolates from different parts of the world and laboratory-fixed strains.

**Acknowledgments.** This work was supported by the Grants for Research on Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labour, and Welfare of Japan, by the grant No. 2005CB523003, 973 Program, from the National Basic Research Program, China, and by the grant (1998)-679 from the Scientific Research Foundation for Returned Overseas Chinese Scholars, State Education Ministry of China.

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