Genetic Variation of Envelope gp90 Gene of Equine Infectious Anemia Virus Isolated from an Experimentally Infected Horse

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ABSTRACT. Six strains of equine infectious anemia virus (EIAV) were recovered from febrile and non-febrile stages of a horse experimentally infected with the P337-V70 strain given once to a horse. The *env* gp90 genes of the isolates, the P337-V70 and P337-V26, avirulent virus derived from the P337-V70 strain, were sequenced. A comparison of the gp90 gene sequences revealed that amino acid variations among the viruses tested showed as high as 8.2 to 11.5%. In addition, the comparison also indicated that the isolates that recovered from the non-febrile stage were contained in nucleotide insertions in the principal neutralizing domain (PND) region. The insertions were arranged regularly with smaller segments. The nucleotide sequence of the P337-V26 gp90 gene was found to contain a six-nucleotides insertion and seven nucleotide substitutions outside the PND region, when compared with that of the P337-V70 strain.
— KEY WORDS: *env* gp90 gene, equine infectious anemia virus, mutation, nucleotide sequence.

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Equine infectious anemia virus (EIAV) is a member of the genus *Lentivirus* of the family *Retroviridae*, to which the human immunodeficiency virus (HIV) belongs [2, 22]. The virus causes a clinical disease characterized by cyclical episodes of fever, weight loss and anemia. Febrile cycles are associated with the emergence of novel antigenic variants of EIAV which are thought to arise due to selective immune pressure [12, 14, 15, 21]. Envelope glycoprotein of EIAV is a target recognized by neutralizing antibody, and it has been suggested that alterations in the envelope enable an antigenically novel virus to escape the host immunosurveillance, resulting in a new round of viral replications and clinical diseases [10, 12, 15].

Kono [10] and Kono *et al.* [12] found and described the antigenic variation of EIAV, in which viruses isolated from the sequential febrile periods could be antigenically distinguished by autologous neutralizing antibody. Sequence analyses have shown variations widely throughout the genome of the *env* gene of EIAV. These envelope alterations give rise to an antigenic divergence which can circumvent the current immune status in horses. Ball *et al.* [1] reported that the correlation of EIAV gp90 and HIV-1 gp120 structural properties allows the prediction of critical functional domains in EIAV *env* gene, such as a principal neutralization domain (PND) equivalent to the HIV-1 V3 loop, which is the CD4-binding domain [4].

To characterize the EIAV genome associated with persistent infection and genetic variation, the *env* gp90 gene sequences of the viruses recovered from the experimentally EIAV-infected horse were sequenced and analyzed in this study.

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MATERIALS AND METHODS

Test horse and viruses: The P337-V70 strain of EIAV [11] was grown once in a horse and the virus that recovered from the serum in horse leukocyte cultures was used as an inoculum. A healthy, 17-years-old crossbred female horse (H115) was shown to be sero-negative by immunodiffusion test for EIAV, and inoculated subcutaneously with 10^4 $TCID_{50}$ of the virus. The rectal temperature was recorded twice per day, erythrocytes and leukocytes were counted at least once per week throughout the infection period to document the kinetics of their decline and rebound. The virus titers in serum were determined during repeated febrile periods from 9 days of post inoculation (pi) to 65 days of pi.

Virus isolation and cell cultures: Serum samples collected from the H115 horse were used as material for virus isolation. To grow these viruses, primary horse leukocyte cultures were prepared by a method described elsewhere [9].

Primers: Primer pairs for the polymerase chain reaction (PCR) were prepared on the basis of the sequence of the Wyoming strain of EIAV [7]. The sequence of a forward primer is 5'-GGTAACATGGTCAGCATCGC-3' (F6; nucleotides (nt) 5306 to 5325 of the Wyoming strain sequence) and that of a reverse primer is 5'-TAGCGGCTACAATAGCTGCC-3' (R22; nt 6685 to 6666). PCR amplification using these primers was expected to

amplify the 1379 base pair fragment covering the complete gp90 gene. For one isolate, we synthesized an outer primer set for nested PCR, a forward primer: 5'-CAACAAGGAAGACAACCTC-3' (F70; nt 5242 to 5261) and a reverse primer: 5'-AGACATAGTAGCGCTAGCAG-3' (R51; nt 6714 to 6695).

DNA isolation and PCR amplification: Cellular DNA was extracted from the horse leukocyte cells inoculated with the viruses using a Sepe-Gene DNA extraction kit (Sanko Junyaku, Japan). The DNA samples obtained were dissolved with a TE buffer (10 mM Tris-HCl, pH 80 and 0.1 mM EDTA), and used for PCR amplification. PCR amplification was carried out in a 50 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.7 mM $MgCl_2$, 1% dimethyl sulfoxide, 200 mM of each deoxynucleoside triphosphate, 1.5 units of Taq polymerase (Takara Shuzo, Japan), 0.4 μM of each primer and 0.5 to 1.0 μg of a DNA template. PCR amplification was carried out in a DNA thermal cycler (Perkin-Elmer, U.S.A.) by 25 cycles of template denaturation for 1 min at 94°C, the primer annealing for 2 min at 50°C and polymerization for 3 min at 72°C and final a cycle gradually extended for 10 min at 72°C.

Cloning and sequencing: The PCR products were directly cloned into a cloning vector, pCR II (Invitrogen, U.S.A.), according to the manufacturer's instruction. Bacterial cells transformed were plated onto LB agar plates containing ampicillin (50 μg/m*l*), 5-bromo-4-chloro-3-indolyl β-D-galactoside (36 μg/m*l*) and isopropyl β-D-thiogalactoside (40 μg/m*l*). After determining the insert sizes by agarose gel electrophoresis, recombinant plasmids containing the desired insert were prepared by Wizard Midipreps (Promega, U.S.A.). The DNAs inserted were sequenced by the 373A automated DNA sequencer (Applied Biosystems, U.S.A.) using the dideoxy chain termination method with the dye primer cycle sequencing kit or the dye terminator cycle sequencing kit (Applied Biosystems, U.S.A.). At least five clones were sequenced and sequences were compared and analyzed by the GENETYX program (Software Development, U.S.A.).

Phylogenetic analysis: Nucleotide and amino acid distances were estimated with full-length gp90 sequences. The dataset of nucleotide sequence distances was estimated with Kimura's method using the program DNADIST from the PHYLIP package [6]. Multiple sequence alignments and evolutionary distances between amino acid sequences were estimated with the PAM 250 matrix which weights amino acid change according to empirically determined probabilities of change [5].

Phylogenetic tree for full-length gp90 sequences were constructed with the DNAML program implemented in the PHYLIP software package (version 3.4), using the maximum likelihood method and setting for the transitions and transversions ratio was 2:1 [6].

RESULTS

Virus isolation: The H115 horse developed an acute disease, characterized by high fever, depression, anemia, and viremia. Five remarkable febrile episodes (15 to 74 days) were observed (Fig. 1B). Finally, the horse was sacrificed on the 112 days of pi because of weight loss and

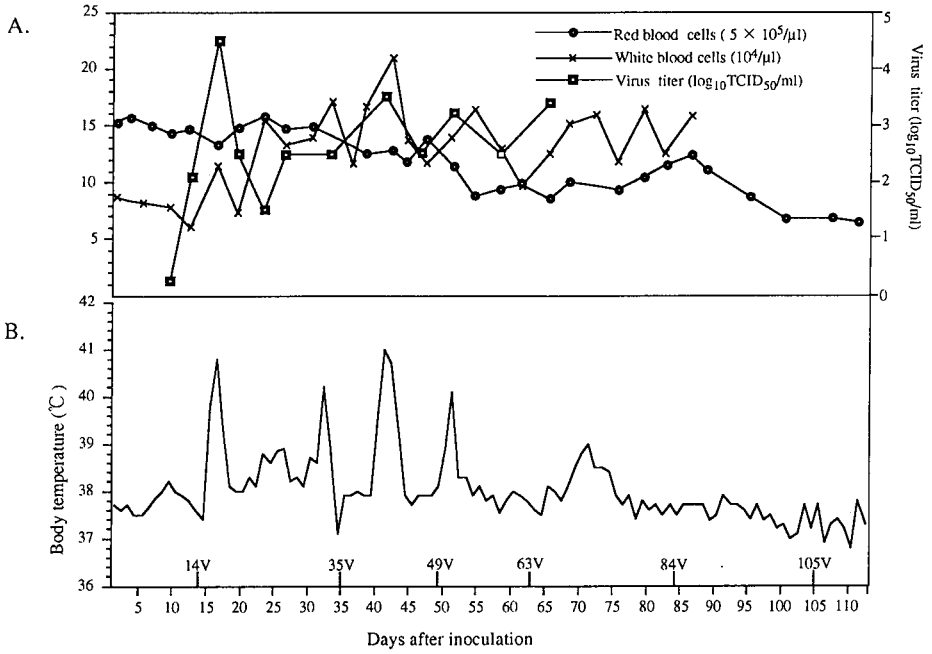

Fig. 1. A clinical examine index of the H115 horse infected with P337-V70 strain. A; Red blood cell count showed a remarkable decline daily, while the white blood cell count showed a sizable increase, and the virus titers in plasma with 50% tissue culture infective doses ($TCID_{50}$) were determined until 65 days of post inoculation. B; Rectal temperature profile.

severe anemia. The virus titer in the serum of the first febrile episode reached $10^{4.5}$ $TCID_{50}$/ml (Fig. 1A). Three of six serum specimens were sampled at 35, 49 and 63 days of pi during the febrile period, one was on the 14 day of pi (before developing its first high fever on 15 day of pi). The others were collected on the 84 and 105 day of pi in the non-febrile period. After inoculation of the serum samples, the viruses, which showed a cytopathic effect (CPE), were harvested and named 14V, 35V, 49V, 63V, 84V and 105V according to their isolation date.

Sequence alignments: The nucleotide sequence of the gp90 region from the total nine viruses, the inoculated virus, the P337-V70 strain, the P337-V26 avirulent strain [11] and six isolates from the virus-infected H115 horse, could be determined. PCR amplification were performed by the one-step method with the F6 and R22 primer pair. However, 105V could not be amplified sufficiently. Thus, the outer primer pair (F70 and R51) was used for nested PCR amplification of 105V.

The nucleotide sequences of the gp90 gene exhibited extensive variations due to nucleotide substitutions and insertion-deletion events. The nucleotide sequence of gp90 gene of the inoculated virus was completely identical with that of the P337-V70 strain. Both the P337-V26 and Wyoming strains were found to have a six nucleotide insertion located at position 115 from the initiation codon of the gp90 gene, when compared with the P337-V70 strain (Fig. 2). There were six nucleotide substitutions between the P337-V70 and P337-V26 strains, and two of them were non-synonymous substitutions (Figs. 2 and 3). Among the inoculated virus and the six isolates, there were 92.0 to 98.0% amino acid sequence homologies (Fig. 3). Among the three of the six isolates, the 63V (isolated from the last febrile episode) and 84V and 105V (both isolated from the long non-febrile period), contained nucleotide insertions to as many as 51, 39 and 51, respectively, in the PND region. However, the other three (14V, 35V and 49V) had no insertion throughout the gp90 gene (Fig. 3).

Although the insertions of 63V and 105V showed a very similar sequence except for four nucleotide substitutions and that of 84V was considerably different from these two isolates (Fig. 4A). It should be noted that the insertion observed in 63V consisted of two long nucleotide repeats (21 nucleotides) and one short fragment (9 nucleotides) (Fig. 4B). The other regions in the *env* gene sequences were generally of the same length. In contrast, the insertion of 84V showed a partially overlapping sequence (Fig. 4C).

Genetic distances: Nucleotide and amino acid distances between the pairs of sequences for the dataset of the gp90 gene was estimated, and presented in Table 1. The Wyoming strain is clearly much most distant from each other, and is 4.10 and 4.58% divergent from the P337-V70 and P337-V26 strains at nucleotide sequence level and 8.12 and 8.63% in amino acid sequence, respectively. The six isolates showed 0.08 to 0.63% nucleotide and 0.22 to 2.24% amino acid sequence diversity when compared to the inoculated virus. Between the P337-V70 and P337-V26 strains, there were only 0.47% (nucleotide) and 0.44% (amino acid) differences. From the phylogenetic tree analysis, it is suggested that the Wyoming strain distantly related to the viruses isolated in Japan which formed a cluster. The tree also indicated that the inoculated virus was the most distant from the 84V strain (data not shown).

DISCUSSION

Although the antigenic drift nature and inability to develop effective vaccines of EIAV are widely accepted [3, 10, 12], little is still known about the mechanism of the recurrence of the disease and viral persistence. To systematically investigate the genetic variation of the virulent P337-V70 strain isolated formerly in Japan, a horse (H115) was experimentally infected with the virus grown once in a horse, and viruses were isolated periodically from the infected horse. All isolates have been grown in leukocyte cultures to obtain a predominant viral population. The gp90 gene was amplified by the one-step PCR method with a primer pair (F6 and R22) to investigate the genetic variations. All the viruses except 105V were sufficiently amplified, and to obtain the 105V proviral DNA, we designed an outer primer set for the nested PCR. Finally, amplification of the 105V proviral DNA could be accomplished, suggesting that replication of 105V in horse leukocyte cultures was very low just as described by Kim and Casey [8] in which EIAV proviral DNA is sometimes present in low copy numbers.

When the gp90 sequences of the P337-V70 and avirulent P337-V26 strains were compared, the P337-V26 strain was found to contain only a six nucleotide insertion and two amino acid substitutions throughout the gp90 gene. This two amino acids insertion was found in the Wyoming strain gp90 sequence (Fig. 3). On the contrary, the predicted amino acid sequences showed a drastic difference among the P337-V70 strain and the six isolates from the infected horse, confirming the previous reports. Most of the amino acid sequence variations were concentrated in two regions, the PND and hyper variable regions (HVR) described by Payne *et al*. [18].

Three of the six isolates contained long nucleotide insertions in the PND region. It is unlikely that the insertion-deletion events observed among the isolates are due to the *Taq*-induced misincorporations, because Lichtenstein *et al*. [13] estimated the *Taq* DNA polymerase error rate to be 0.06% for the EIAV template. Such long insertions in the PND region observed in the three viruses isolated from the late stage of the infection have not been reported so far, and differed remarkably from the previous study [19]. Pathak and Temin [16, 17], and Pulsinelli and Temin [20] reported that transitions, transversions, deletions, and deletions and insertions could be occurred during a single cycle of retroviral replication by the action of error-prone retroviral reverse transcriptase. They also reported that the region which can form the potential secondary structure is the hot spot of deletion. We have found no

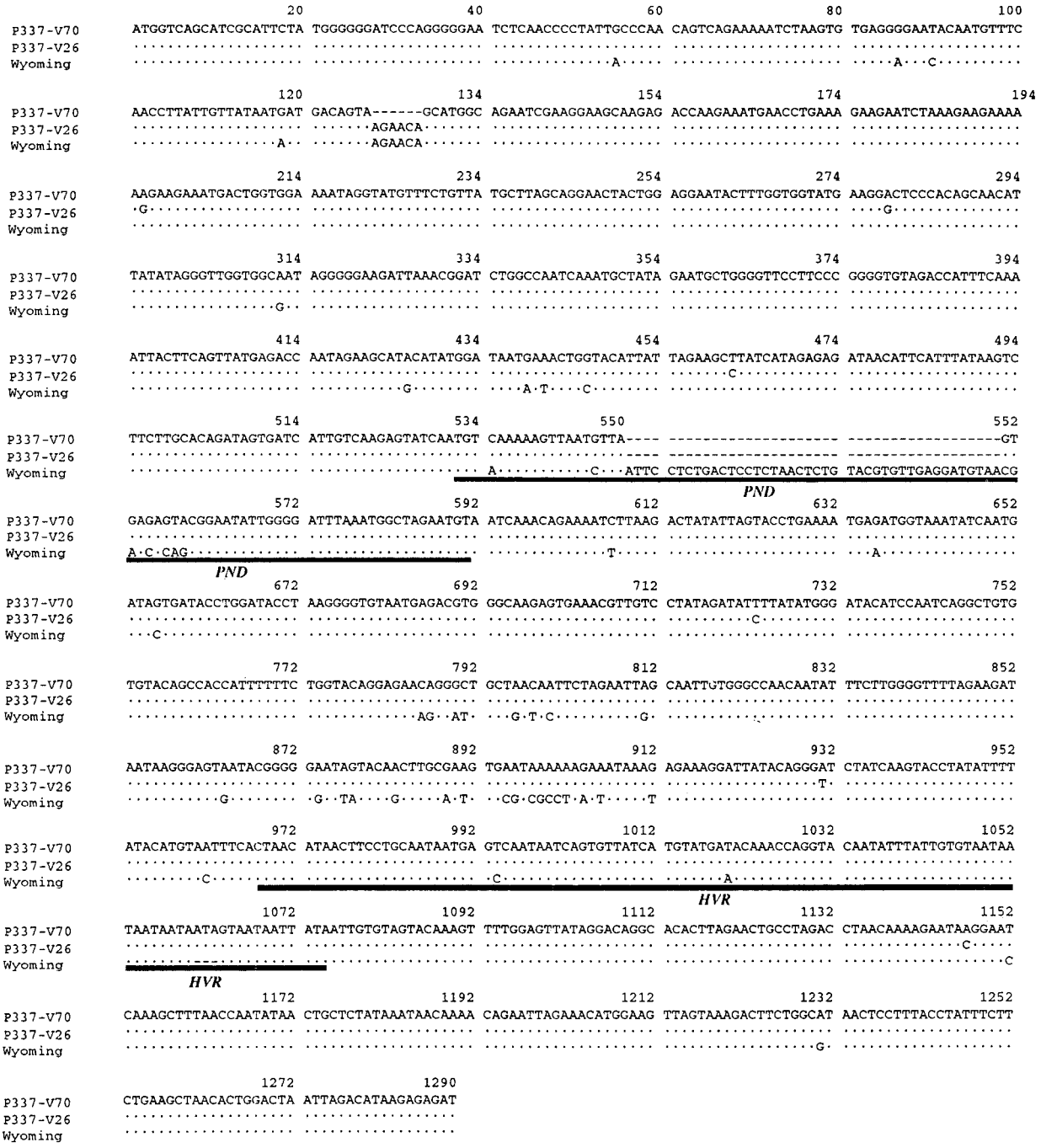

Fig. 2. Alignment of nucleotide sequence covering gp90 gene of the P337-V70, P337-V26, and the cell-adapted Wyoming strains. The dots denote the identical nucleotide with the P337-V70 strain. Gaps inserted during alignment are indicated by the dashes. The principal neutralizing domain (PND) and hypervariable region (HVR) are underlined. The sequences of P337-V70 gp90 gene were numbered from the initiation codon (ATG).

significant secondary structures in the neighborhood of the PND region using computer analysis. Thus, the mechanism(s) of such long in-frame insertions is still unclear. However, a question arises whether such insertions are generated by rapid evolution in the virus-infected horse, or whether such diversity may have been originally present in the virus inoculum. To answer this question, the sequences of the gp90 gene multiplying in a virus-infected horse has to be directly analyzed.

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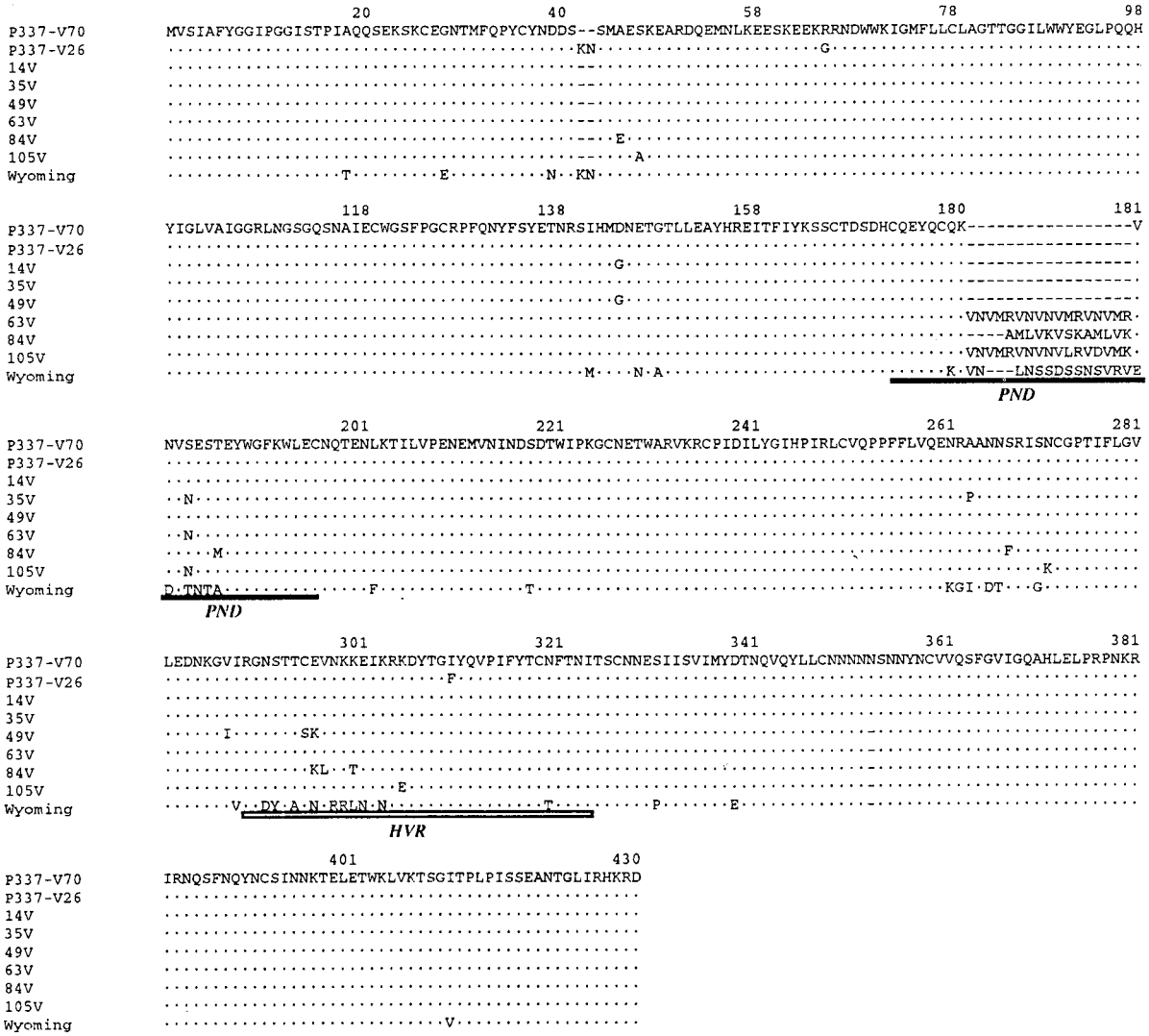

Fig. 3. Alignment of the predicted amino acid sequences of the six isolates from the H115 horse infected with P337-V70 strain, compared with the P337-V70, P337-V26 and Wyoming strains. The dots denote the identical amino acid with the P337-V70 strain. Gaps inserted during alignment are indicated by the dashes. The amino acids of P337-V70 gp90 were numbered from the amino termini.

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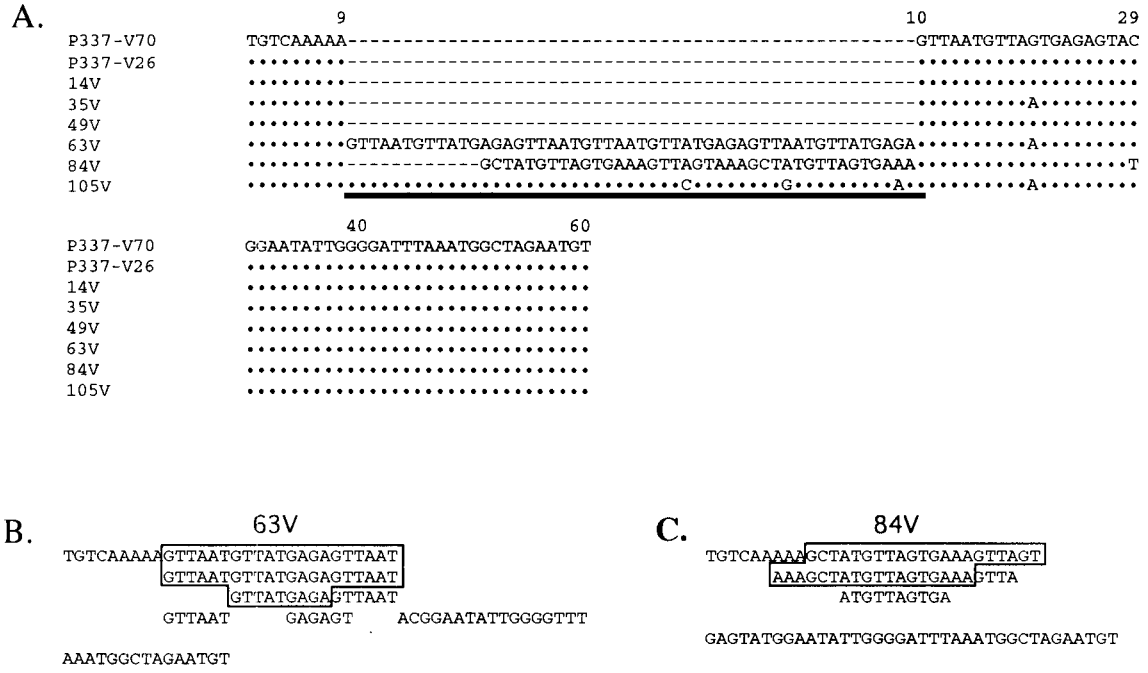

Fig. 4. A; Alignment for the nucleotide sequence of the PND region. B; A model of the arranged pattern of the insertion in the PND region of 63V. The framed nucleotide sequences are inserted. Abreast sequences indicated the nucleotide repeats of the insertion. C; A model of arranged pattern of 84V.

Table 1. Pairwise distances between *env* full-length gp90 sequences of EIAV isolates[a)]

	Wyoming	P337-V70	P337-V26	14V	35V	49V	63V	84V	105V
Wyoming		0.0812	0.0863	0.0838	0.0816	0.0882	0.1038	0.1150	0.1111
P337-V70	0.0410		0.0044	0.0022	0.0044	0.0089	0.0022	0.0224	0.0089
P337-V26	0.0458	0.0047		0.0067	0.0089	0.0134	0.0067	0.0271	0.0134
14V	0.0418	0.0008	0.0054		0.0066	0.0066	0.0044	0.0247	0.0111
35V	0.0419	0.0023	0.0054	0.0031		0.0133	0.0022	0.0269	0.0089
49V	0.0427	0.0031	0.0078	0.0023	0.0055		0.0111	0.0269	0.0178
63V	0.0571	0.0008	0.0055	0.0016	0.0016	0.0039		0.0440	0.0127
84V	0.0591	0.0063	0.0094	0.0070	0.0070	0.0078	0.0115		0.0464
105V	0.0587	0.0039	0.0086	0.0047	0.0047	0.0070	0.0053	0.0138	

a) Pairwise distances are presented as a triangular matrix. Nucleotide distances are showed in the lower half and aminoacid distances in the upper half of each matrix.

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