Replacement of Internal Protein Genes, with the Exception of the Matrix, in Equine 1 Viruses by Equine 2 Influenza Virus Genes during Evolution in Nature

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ABSTRACT. To establish the evolutionary association between the equine 1 H7 HA and M genes, phylogenetic analyses of the six internal gene segments of equine 1 influenza viruses (H7N7 subtype) were performed using partial nucleotide sequences. The results demonstrated that five internal genes (PB1, PB2, PA, NP and NS) of equine 1 viruses isolated after 1964 were replaced by those of equine 2 H3N8 viruses. However, the M gene was maintained during the evolution of these equine 1 viruses. These findings suggest a functional association between equine H7 HA and M gene products, most likely M2 protein.—KEY WORDS: equine influenza, hemagglutinin, matrix protein.

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Equine 1 influenza viruses (H7N7 subtype) were first isolated from horses in Prague, Czechoslovakia, in 1956 [12], while antigenically distinct equine 2 (H3N8) viruses were isolated in Florida in 1963 [17]. These two types of viruses cocirculated in the equine population until at least 1977, when the equine 1 viruses disappeared [16]. Competitive RNA-RNA hybridization [4] and nucleotide sequence analysis [6] showed that some equine 1 viruses contained the equine 2 genes encoding internal proteins (i.e. PB1, PB2, PA, and NP genes), indicating that genetic reassortment occurred between the two viruses in nature.

The M2 protein functions as an ion channel that permits protons to enter the virion interior to disrupt low pH-labile interactions between M1 and the ribonucleoprotein core, facilitating the uncoating process prior to fusion of the viral membrane with the endosomal membrane. In addition, the M2 ion channel is thought to increase the intralumenal pH of the trans Golgi network, preventing the low pH-induced conformational change of the intracellularly cleaved fowl plague HA [13]. In fact, there is empirical evidence suggesting a functional association between M2 and HA. For example, the preferential association between HA and M genes was demonstrated [15] when reassortants between virulent A/chicken/Pennsylvania/1370/83 (H5N2), whose HA is intracellularly cleaved, and avirulent avian viruses were made: all but one out of 20 reassortants bearing the virulent H5 HA contained the M gene from the virulent parent. Such HA-M gene association has been found only in virulent avian viruses with intracellularly cleaved HAs.

The HAs of equine 1 (H7N7) influenza viruses are also intracellularly cleaved [5, 8]. However, an *in vitro* study showed that unlike the fowl plague virus HA, a low pH-induced conformational change was not detected in the equine H7 HA expressed on the cell surface even without the concomitant expression of the M2 [14]. Thus, the aim of this study is to investigate whether the equine 1 M gene is functionally associated with its HA gene, and thus maintained in the equine 1 virus during evolution.

The influenza viruses used in this study were from the repositories at the Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University and the Department of Veterinary Public Health, Faculty of Agriculture, Tottori University. The viruses used are as follows: A/equine/Prague/1/56 (H7N7; EQPR56), A/equine/Detroit/3/64 (H7N7; EQDET64), A/equine/London/1416/73 (H7N7; EQLD73), A/equine/New York/49/73 (H7N7; EQNY73), A/equine/Kentucky/1/75 (H7N7; EQKY75), A/equine/Cordova/4/76 (H7N7; EQCOR76), A/equine/Kentucky/1/77(H7N7; EQKY77), A/equine/Miami/1/63 (H3N8; EQMI63), A/equine/Tokyo/2/71 (H3N8; EQTKY71), A/equine/Fontainbleau/2/79 (H3N8; EQFONT79), A/equine/Kentucky/5/86 (H3N8; EQKY86), and A/equine/Tennessee/2/86 (H3N8; EQTN86). These viruses were grown in 11-day-old embryonated chicken eggs. Virion RNA was isolated by treatment of infected chorioallantoic fluid with proteinase K and sodium dodecyl sulfate, followed by extraction with phenol:chloroform (1:1) as described previously [3].

To determine the phylogenetic relationship of the genes encoding the internal proteins, partial nucleotide sequences were determined by direct sequencing of RT-PCR products using viral RNA as a template and oligonucleotide primers for the following regions of the genes: PB1, positions 66–234; PB2, positions 45–233; PA, positions 76–201; NP, positions 274–485; M, positions 725–878; and NS, positions 610–812. The nucleotide sequences, together with those from GenBank, were analyzed by the Neighbor-Joining method [11].

To establish the evolutionary association between the

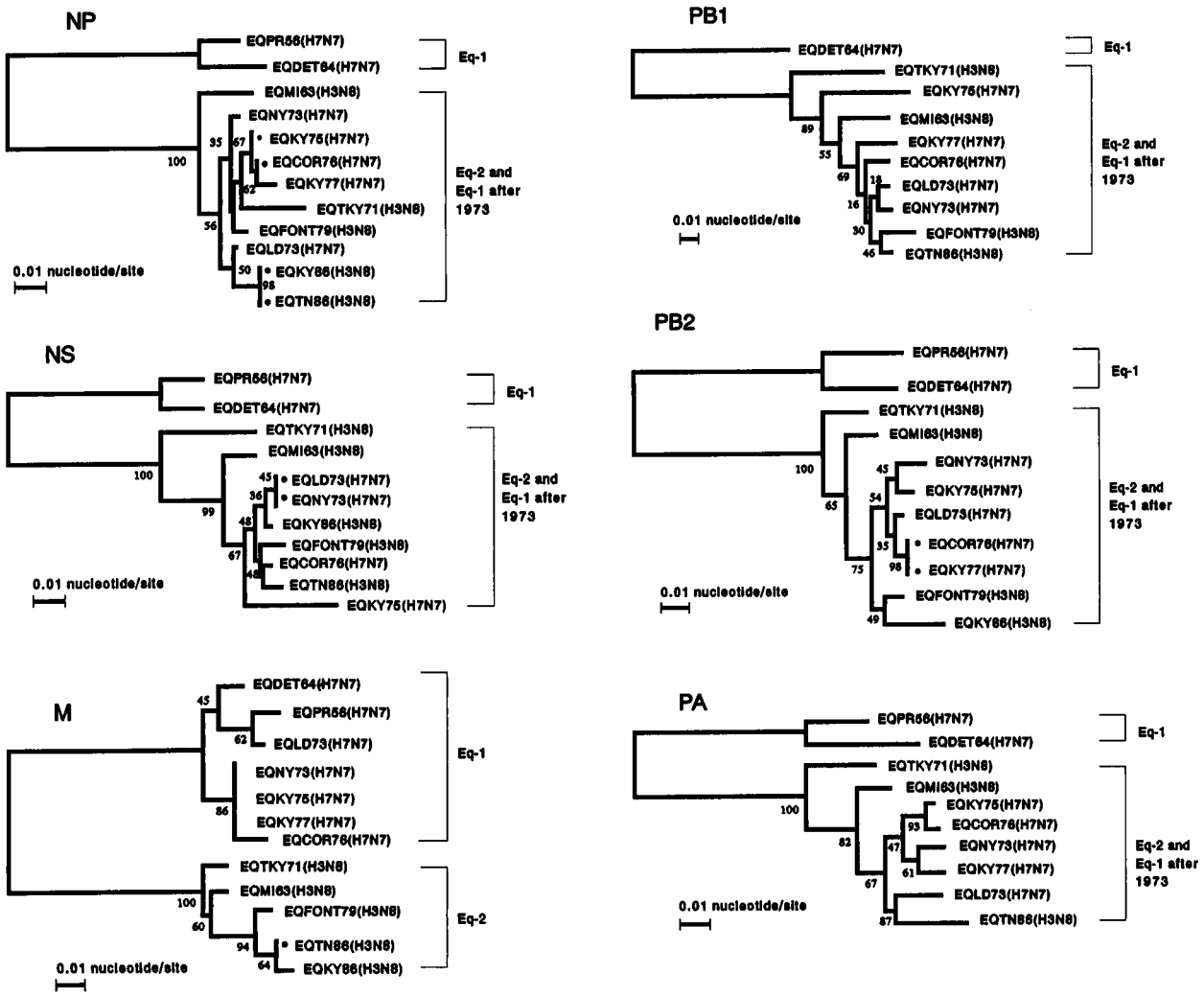

Fig. 1. Phylogenetic trees for equine influenza A virus 6 internal genes. The following regions of the genes: PB1, positions 66–234; PB2, positions 45–233; PA, positions 76–201; NP, positions 274–485; M, positions 725–878; and NS, positions 610–812, were analyzed by the Neighbor-Joining method [11]. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and NP or M sequences. Vertical lines are for spacing branches and labels. Bootstrap values (1000 replications) are presented for each node. Abbreviations for viruses may be found in Materials and Methods.

equine 1 HA and M genes, we phylogenetically analyzed the partial nucleotide sequences of the six internal gene segments of seven equine 1 viruses along with published equine 2 genes. The six internal gene trees are shown in Fig. 1. The evolutionary analysis showed that all but the M gene of equine 1 viruses isolated after 1964 belonged to the equine 2 virus lineage, indicating that genetic reassortment between equine 1 and equine 2 viruses occurred sometime between 1973 and 1964, and that this reassortant replaced the previously circulating equine 1 non-reassortants. By contrast, the M gene of the equine 2 viruses was not introduced into equine 1 viruses. Bootstrapping to calculate confidence limits on each evolutionary tree strongly supports these findings (Fig. 1). Namely, bootstrap values are 89–100% at each node between equine 1 genes before and after 1973.

Previous phylogenetic comparison of influenza virus genes revealed a recent introduction of the NS, M and PB2 genes into horses from avian sources by genetic reassortment [9]. This discrepancy may due to the limited number of strains analyzed. Lindstrom *et al.* [10] reported that the NS gene of an H7 virus from 1977 was very similar to that of a 1979-H3 virus, while the M gene was closest phylogenetically to that of the earliest H7 virus isolated in 1956. These findings support the notion that genetic reassortment occurred between the two viruses in nature, although only two equine H7 strains were analyzed. Adeyafa *et al.* [1] also demonstrated genetic reassortment in equine 1 viruses by partial sequence comparison, although a phylogenetic analysis was not done. Their results, and

ours, suggest that the equine 1 HA is functionally associated with M gene products, most likely the M2 protein, during the evolution of equine 1 viruses.

Reassortants possessing the HA gene from A/equine/London/1416/73 (H7N7) (Eq/LD) and five or more genes from A/chicken/Pennsylvania/1370/83 (H5N2) (Ck/Penn), including the M gene, were lethal in chickens, indicating that the Ck/Penn M2 was functional for the Eq/LD HA [2]. Are there specific residues of the M2 associated with intracellular cleavage of HA? Alignment of M2 protein sequences revealed no obvious residues that suggest functional differences among M2 proteins [7].

Takeuchi *et al.* [14] reported that equine 1 influenza virus does not require functional M2 protein ion channel activity for maintenance of the native form of HA expressed from cDNA in MDCK cells. The optimal pH for transition of equine 1 HA to the low-pH form was determined to be lower than pH 5.3. Thus, it can be inferred that the intralumenal pH of the trans Golgi network does not fall below pH 5.3 in MDCK cells. It is of interest whether equine 1 viruses require functional M2 in the epithelial cells of equine host trachea, although no information is available on the intralumenal pH of the trans Golgi network of equine host cells.

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