Persistent influenza C virus possesses distinct functional properties due to a modified HEF glycoprotein

Manfred Marschall,1* Georg Herrler,2 Christoph Böswald,1 Gisela Foerst1 and Herbert Meier-Ewert1

1 Abteilung für Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Technische Universität München, Biedersteiner Straße 29, DW-80802 München and 2 Institut für Virologie, Philipps-Universität Marburg, DW-35037 Marburg, Germany

A model of long term viral persistence has been established by selecting a spontaneous mutant strain of influenza C/Ann Arbor/1/50 virus in a permanent carrier culture of MDCK cells. Infectivity and cell tropism are mainly determined by the multifunctional viral membrane glycoprotein (HEF). HEF analysis was aimed at identifying a putative correlation between sequence and function, i.e. receptor binding, enzymatic activity, antigenicity and rate of infection. The current experimental picture is summarized by the following findings: (i) C/Ann Arbor/1/50 persistent virus carries a modified receptor-binding sequence, (ii) receptor-binding activity is altered, as indicated by a higher efficiency in recognizing low amounts of the receptor determinant N-acetyl-9-O-acetylneuraminic acid, (iii) direct attachment to cell surfaces differs from that of wild-type virus, as measured by slower kinetics of viral elution, (iv) receptor-destroying enzymatic activity is diminished, (v) characteristic features of virion surface elution are altered or unstable, (vi) persistent-type HEF epitopes are distinguishable by monoclonal antibodies from wild-type and (vii) viral infectivity is intensified for cells bearing a low number of receptors. The sum of these changes highlights a structurally and functionally modified HEF glycoprotein that allows long term viral persistence. In order to clarify which of the described points are required for the persistent viral phenotype, a working concept is presented.

Introduction

Persistent infections with RNA viruses are supposed to be achieved by different viral strategies, e.g. restricted viral replication, interference with cell regulation and escape from immune surveillance (reviewed by Oldstone, 1991). The support of survival and longevity of infected cells is a critical prerequisite for viral persistence in culture models (Celma & Fernandez-Muñoz, 1992; Urabe et al., 1992), as well as in the natural host (Kandolf et al., 1991). In order to maintain the infectious process over long periods the selection of low-cytotoxicity variants and reduced virus shedding appear to be essential (Clavo et al., 1993; Urabe et al., 1993). Events stabilizing virus-cell interactions, such as the inhibition of differentiation and of programmed cell death (Montgomery et al., 1991; Levine et al., 1993), as well as alteration of the tissue tropism (Shioda et al., 1992; Subbarao et al., 1993), have been repeatedly observed.

In influenza C virus infection, vital functions for infectivity are served by the single viral surface glycoprotein (HEF). The functions of HEF include receptor binding, receptor-destroying activity and membrane fusion (reviewed by Herrler & Klenk, 1991). Point mutations in the predicted receptor-binding pocket have been demonstrated to change the binding preference for modified receptors (Szepanski et al., 1992) and, consequently, to influence the reproduction rate in cell culture (Umetsu et al., 1992). The receptor-destroying function of HEF was proposed to be required for entry into target cells at a step prior to fusion (Strobl & Vlasak, 1993). The fusion efficiency between viral and cellular membranes was attributed to pH-dependent, conformational changes of HEF (Formanowski et al., 1990). In the as yet unexplained phenomenon of persistent infection, HEF seems to undergo structural and functional variation. This was proposed after initial studies on virus antigenicity, haemagglutination inhibition and plaque morphology (Camilleri & Maassab, 1988).

Given this background we sought to correlate HEF variability with the persistence of infection. HEF sequence studies were carried out in parallel with experiments on its function and were supplemented by data on the structure and infectivity of virions. Here we present evidence that influenza C virus persistence is
associated with specific functional changes displayed on the virion surface.

Methods

Virus and cell culture. Influenza C virus strains Ann Arbor/1/50 wild-type (C/AA-wt) or a persisting variant (C/AA-pi) were grown in MDCK cells or embryonated eggs as described previously (Formanowski & Meier-Ewert, 1988). MDCK cells and persistently infected MDCK cells were grown in Dulbecco's minimum essential medium containing 10% (v/v) fetal calf serum. The persistently infected culture was maintained at 33 °C with biweekly feedings by replacing 50% of the growth medium.

Amplification of virus sequences by PCR. Virion RNA was extracted from infectious allantoic fluids according to the guanidinium thiocyanate protocol (Chomczynski & Sacchi, 1987). One μg of RNA was reverse-transcribed in 10 μl of 100 mM-Tris-HCl pH 8.3, 10 μM-MgCl₂, 140 mM-KCl, 10 mM-DTT, 400 μM-dNTPs and 3 pmol of influenza virus universal primer Unil (5' AGCAAAGACGGG 3') (Robertson, 1979) using avian myeloblastosis virus reverse transcriptase (12.5 U; Boehringer Mannheim). The mixture was incubated at 42 °C for 60 min and the reaction terminated by boiling for 1 min. Subsequent PCR was performed in a 30 cycle programme as described by Manuguerra et al. (1992) with temperature levels of 50 °C for annealing (1 min), 72 °C for polymerization (3 min) and 94 °C for denaturation (1 min). The verification of amplification products was achieved by agarose gel electrophoresis. Oligonucleotide primers for the HEF-encoding segment 4 were chosen from nucleotide positions 22 to 52 (5' ATG-TTTTTTCTCATTACTCTTGATGTTGGGCC 3') and 1024 to 1048 (3' CCGTCTCTTAGACTGGTACGTCACC 5') (position numbering according to Buonagurio et al., 1985).

Nucleotide sequencing. PCR products were purified using NICK columns (Sephadex G-50), as described by the manufacturer (Pharmacia) and used directly as sequencing templates. The nucleotide sequences of both strands were determined by the dideoxynucleotide chain termination method (Sanger et al., 1977). Primers were between 15 and 17 nucleotides in length. They were synthesized using a Bioscience Award synthesizer and were desalted with NAP-5 columns (Sephadex G-25; Pharmacia). Sequencing reaction products were separated on 6 to 8% urea/polyacrylamide gels in a Bio-Rad Sequi-Gen sequencing cell.

Resialylation of erythrocytes. Erythrocytes from 1-day-old chicks were treated with neuraminidase and resialylated using α2,3- and α2,6-sialyltransferases (Boehringer Mannheim) as described elsewhere (Schulz et al., 1990; Szepanski et al., 1992). The enzyme substrate, CMP-activated N-acetyl-9-O-acetylmuraminic acid (CMP-Neu5,9Ac₂), was kindly provided by Professor Dr Dr R. Brossmer. Using different amounts of substrate, batches of erythrocytes were obtained that differed in the level of surface-bound Neu5,9Ac₂. Following incubation for 3 h at 37 °C, the erythrocytes were washed, resuspended in PBS to a final concentration of 1% and used for haemagglutination assays.

Attachment assay. Two-million trypsinized MDCK cells per reaction were rinsed with PBS and incubated with 50 haemagglutinating units of influenza C virus in a 50 μl volume. Virus attachment to cell surfaces was achieved by incubation for 30 min at room temperature. The cells were then rinsed again and incubated in 50 μl of PBS at 37 °C for the times indicated in order to allow the membrane-attached virus to elute or penetrate. The process was stopped by non-detergent formaldehyde fixation onto glass slides (3% in PBS, 15 min at room temperature). Surface fluorescence study of bound particles was performed by staining with influenza C virus-specific rabbit antisera in a standard protocol for indirect immunofluorescence (Marshall et al., 1989).

Acetylesterase assay. Virus was pelleted from allantoic fluids, rinsed and resuspended in PBS. The protein concentration was determined with a conventional test kit (Bio-Rad). One μg of the prepared virus was used to measure viral acetylesterase activity by incubation with the substrate analogue p-nitrophenyl acetate (1 mm in PBS) in a final volume of 0.5 ml. The turnover of the colour substrate at 37 °C was monitored for the times indicated by measurement of the A₄₀₀ of the reaction (Marshall et al., 1993).

Determination of infectivity. Chick embryo fibroblasts (CEF) were prepared from 10-day-old fertilized eggs. After mechanical grinding, embryo tissues were incubated in 0.25% (w/v) trypsin for 1 h at 37 °C and filtered through a sterile gauze mesh. The solubilized cells were pelleted and seeded in 24-well microtitre plates (Nunc) in minimal essential medium (MEM) containing 10% fetal calf serum. When the cells became confluent, adhering fibroblasts were washed to remove remaining cell debris and then utilized for infectivity assays. Two sublines of Madin-Darby canine kidney cells, MDCK I cells (high levels of virus receptor) and MDCK II (low levels of virus receptor), were seeded as described above, but grown in Dulbecco's MEM. Infections were performed with serial dilutions of influenza C virus for 1 h at 33 °C. The virus inoculum was removed, followed by two washes with PBS. Incubation post-infection (p.i.) was performed at 33 °C for 3 days with reduced fetal calf serum (2% v/v). Virus yield was determined from the supernatant by standard haemagglutination microtitration using 1% chicken erythrocytes.

Electron microscopy. Cell culture-grown virus was pelleted from the supernatant (90000 g for 2 h) after removal of cell debris (3000 g for 15 min). Pellets were rinsed in PBS pH 7.2 and resuspended by mild sonication on ice. Using carbon-coated grids, the samples were negatively stained with 2% (w/v) potassium phosphotungstic acid 60 for 15 s and analysed immediately.

Results

Persistent virus carries a modified receptor-binding sequence that is associated with an enhanced binding efficiency

In order to characterize critical steps in the persistent virus cycle, we investigated its receptor binding properties in resialylation experiments (Fig. 1a and b). Neuraminidase-treated erythrocytes from 1-day-old chicks were incubated with CMP-activated 9-O-acetylated sialic acid and one of the two different sialyltransferases. In this way erythrocytes were obtained bearing Neu5,9Ac₂ bound to surface glycoconjugates either in α2,3 linkage to Gal/I1,3GalNAc or in α2,6 linkage to Gal/I1,4GlcNAc. Further variation was obtained by varying the amount of CMP-Neu5,9Ac₂. As shown in Fig. 1, persistent virus was more efficient in recognizing 9-O-acetylated sialic acid in both the α2,3 (a) and α2,6 linkage (b) than the parental wild-type virus. The only case where haemagglutination was effected by both viruses to the same titre is shown in Fig. 1(b); in this case erythrocytes were incubated with α2,6-sialyltransferase and 50 pmol of CMP-activated sialic acid. When the amount of CMP-Neu5,9Ac₂ was lowered
Influenza C virus persistence

Fig. 1. Virus-receptor binding studies. The ability of influenza C/Ann Arbor/1/50 virus wild-type (C/AA-wt) and the persistent variant (C/AA-pi) to use 9-O-acetylated sialic acid in two different linkage types as a receptor determinant was tested. Neuraminidase-treated erythrocytes were resialylated by incubation with either Ga1β3GalNAcβ2,3-sialyltransferase (a) or Ga1β4GlcNAcβ2,6-sialyltransferase (b) in the presence of different amounts of CMP-Neu5,9Ac₂. Following sialylation, the erythrocytes were used to determine the haemagglutinating titre of C/AA-wt (dark bars) and C/AA-pi (light bars). Agglutination of erythrocytes from 1-day-old chickens (c) was determined for C/AA-pi (light bars), C/AA-wt (medium bars) and as a standard virus control C/JHB/1/66 (dark bars). Neuraminidase-treated erythrocytes (Asialylated) were included as a negative control.

Table 1. HEF sequence variation with respect to receptor binding and cell tropism

<table>
<thead>
<tr>
<th>Influenza C virus isolates</th>
<th>Amino acid sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/AA/1/50 (wild-type)†</td>
<td>275-SPYT GNSDTPT MQCD- 290</td>
</tr>
<tr>
<td>C/AA/1/50 (persisting variant)</td>
<td>I</td>
</tr>
<tr>
<td>C/JHB/1/66 (receptor-binding variant)‡</td>
<td>N</td>
</tr>
<tr>
<td>C/YA/4/88 (cell-adapted variant)¶</td>
<td>N</td>
</tr>
<tr>
<td>C/YA/4/88 (neutralization-resistant variant)¶</td>
<td>N</td>
</tr>
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</table>

* The bold-print sequence represents the receptor-binding pocket, as determined by analogy with influenza A virus (Weis et al., 1988) and with the variants aligned below.
† The region shown for C/AA/1/50 (wild-type) is totally conserved in different strains, e.g. C/Johannesburg/1/66, C/Yamagata/10/81 or C/California/78 (Buonagurio et al., 1985).
‡ Sequence comparison between C/AA/1/50 (persisting variant) and the depicted isolates was based on publications by Szepanski et al. (1992), Umetsu et al. (1992) and Matsuzaki et al. (1992), respectively.

Fig. 2. Viral RDE activities were determined by haemagglutination elution (a) and acetyl esterase assay (b). (a) Conventional haemagglutination reactions, using 1% chicken erythrocytes and equivalent dilutions of egg-grown influenza C/Ann Arbor/1/50 virus were assayed for 30 min at room temperature. Thereafter the microtitre plates were shifted to 37 °C in a water bath and observed for RDE-mediated elution. The continued reversion of haemagglutination to elution is expressed as percentage relative to the initial haemagglutination titre. (b) Acetyl esterase activity of particles was quantified in vitro by the colour reaction with a substrate analogue. Reactions were performed in triplicate for control and quickly measured at fixed time points. The profiles of the test shown relate to 1 µg of total protein each.
fivefold, the receptors generated by the transferase reaction were insufficient for haemagglutination by C/AA-wt, whereas the persistent virus still showed activity, even when the amount of substrate was reduced 20-fold. Moreover, persistent virus also had a significant advantage over wild-type when the haemagglutination test was performed with erythrocytes containing 9-O-acetylated sialic acid in an α2,3 linkage (Fig. 1 a). These results indicate that C/AA-pi has an increased affinity for Neu5,9Ac₂ compared to that of the parental virus.

The high efficiency of receptor-binding by C/AA-pi is also evident from its unexpected ability to agglutinate erythrocytes from 1-day old chicks (Fig. 1 c). It is important to note that these erythrocytes contain 9-O-acetylated sialic acid in such a low amount that they are resistant to the haemagglutinating activity of all influenza C virus strains analysed so far (Herrler & Klenk, 1987a). The results shown in Fig. 1 (c) indicate that the persistent virus is unique among influenza C viruses in its ability to utilize these low amounts of Neu5,9Ac₂ for the agglutination of erythrocytes.

Nucleotide sequence analysis (Table 1) revealed a change of two amino acids located close to one another within the predicted receptor-binding pocket of HEF at positions 284 (Thr → Leu) and 286 (Thr → Ile). This alteration was demonstrated repeatedly in several samples of persistent genomes isolated and amplified independently (i.e. different cell culture and egg passages).

Wild-type virus, conversely, was found to be genetically homogeneous with respect to the conserved receptor-binding sequence as shown by a strain-specific PCR technique. Since residue 284 (Thr → Leu) is altered by a double point mutation in two neighbouring nucleotides, we positioned the extreme 3’ end of the respective PCR primer exactly at this genome location and hence successful amplification was strictly reserved for the persistent type (Marschall & Meier-Ewert, 1993).

Viral receptor-destroying enzyme (RDE) activity and the rates of cell surface elution

The RDE of influenza C virus is a neuraminate-O-acetylesterase (Herrler et al., 1985). RDE activity counteracts the effect of haemagglutination. We attempted to find strain-specific differences in the RDE activity by the reversal of haemagglutination (Fig. 2a). The kinetics of elution from erythrocytes, comparing wild-type and persistent virus, revealed a constant retardation of about 70 min in the case of persistent-type virions, regarding complete RDE-mediated haemagglutination clearance.

Quantification of the enzymatic HEF activity was accomplished by an in vitro acetylesterase assay (Fig. 2b). Virus preparations, equivalent in amounts of total protein, were tested and the results corroborated the previous findings. Persistent virus was reduced in
substrate reactivity as shown by a lowered signal at the turnover saturation point (60 min).

These inherent features of persistent virus were confirmed at the level of cell surface reactivity. Both types of virions were allowed to attach to MDCK cells and were subsequently tested for the continuous presence of membrane-bound viral antigen (Fig. 3). The efficiency of binding and elution was quantified by microscopic cell counting with respect to the rate of specific fluorescence. Virus clearance occurred with an efficiency of approximately 90% after 120 min at 37 °C, in the case of wild-type virus. Persistent virus, however, was clearly retained on the cell surface after the same incubation period (reduction of only 10%). As a control, the reaction was carried out on ice and no difference was observed between the two strains, i.e. bound viral particles of both types remained constantly detectable. This prompted us to test the effectiveness of a potent RDE inhibitor, 3,4-dichloroisocoumarin (Vlasak et al., 1989), in our assay. The presence of 500 μM of inhibitor during the 120 min incubation at 37 °C reduced the wild-type specific surface clearance by approximately 50%, indicating that acetyl-esterase activity is involved in this process but is not the sole cause of the effects observed. The constant binding of persistent virus was unaffected under these conditions (data not shown).

Structural divergence of persistent virions

The surface of influenza C virus particles is typically organized in a highly ordered HEF glycoprotein structure, i.e. hexagons (Flewett & Apostolov, 1967). Electron microscopic analysis of C/AA-wt demonstrated a hexagonal pattern of spikes on the virion surface (Fig. 4).

Table 2. Virus variant-specific antibody reactivity

<table>
<thead>
<tr>
<th>Influenza C virus-specific antibodies*</th>
<th>Haemagglutination inhibition titres†</th>
<th>C/AA-wt</th>
<th>C/AA-pi</th>
<th>Ratio‡</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>H1/66§</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FC1-16:3-3¶</td>
<td>128000</td>
<td>64000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FCGB4-6:3¶</td>
<td>6400</td>
<td>12800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FCD4D-1¶</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA2-6:4¶</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DC6-6-7-2¶</td>
<td>5</td>
<td>40</td>
</tr>
</tbody>
</table>

* All antibodies tested were raised against virus strain C/JHB/1/66.
† Absolute values represent the maximum dilution factor of the respective antibody achieving inhibition in the standard haemagglutination inhibition tests.
‡ Relative values represent the ratio between the antibody’s reactivity towards wild-type and persistent virus, respectively.
§ Polyspecific rabbit antiserum used as a positive control.
|| Non-immune rabbit serum used as a negative control.
¶ HEF glycoprotein-specific MAbs.

Fig. 4. Virion morphology. Influenza C/Ann Arbor/1/50 virus wild-type (a and b) and its persistent variant (c and d) were freshly prepared from infected cell supernatants by ultracentrifugation. Negatively stained particles were photographed. Note the differences in conservation of the hexagonal surface pattern. Bar marker in (d) represents 400 nm in (b) and (d) and 250 nm in (a) and (c).
Contrastingly, the persistent variant was shown to be largely devoid of the wild-type morphology, showing a strikingly smooth appearance. The paucity of hexagons was noticed in independent virion specimens. The influences of storage, preparation or staining conditions on this structural aberration have still to be determined.

In order to gain insight into the consequences of structural variation, we tested influenza C virus strains for antibody binding measured by haemagglutination inhibition (Table 2). Monoclonal antibodies (MAbs) directed against HEF (strain C/JHB/1/66; kindly provided by Dr R.W. Compans) displayed variable reactivities on a scale from very high (1:128000) to almost ineffective (1:5) with both viruses tested. There was no total loss of any of the epitopes tested for in the persistent-type virus. However, an intriguing finding is that there is at least one MAb (FCDD4.1) which reacted to a significantly higher (16:1) ratio in favour of persistent virus. This effect indicates a decisive modification in HEF epitopes and antigenicity.

Infectivity profiles and virus quantities in these cells were mostly identical, which is in direct contrast to the situation in MDCK II cells. This cell-specific discrepancy argues strongly against a general, low permissiveness of MDCK cells or a defective wild-type virus preparation.

### Discussion

In this study of a persistent influenza C virus strain, we describe mutations in the putative receptor-binding pocket of HEF in association with unusual receptor-binding, RDE and structural properties. Furthermore an increase in infectivity under conditions of low cell receptor concentrations was noted and the possibility of host preference, concerning the cell tropism, is evident.

Specific binding to cells carrying a limited number of receptor sites will consequently lead to the specialization for a subpopulation of target cells. Analogous receptor-binding variants of influenza C virus with mutations in the same position or in the vicinity of the ones depicted here have been published (Table 1) and the importance...
of receptor determinants for the cell tropism is well characterized (Herrler & Klenk, 1987b). A reinforcing effect, illustrating the idea of virus benefit at low receptor concentrations, is the impairment of RDE activity. Persistent virus might be handicapped in dealing with high cellular receptor levels, in terms of lack of elution (i.e. inefficient cleavage from invalid receptors on non-permissive cells or binding to lysed membranes by default). Cells of a low receptor number type appear to become profitable targets for this virus variety.

Importantly, RDE activity is considered to be a rate-limiting factor in influenza C virus reproduction. Recently this function was shown to be required for entry into target cells (Strobl & Vlasak, 1993), therefore an impairment might contribute to the self-limitation of the viral cycle under persistence. Serine 71 of HEF was described as being located in the RDE active site, between amino acids 68 and 74, and is highly conserved among influenza C virus strains (Herrler et al., 1988). We also found this site to be conserved in persistent virus by nucleotide sequencing (data not shown). Since this sequence was claimed to be essential for enzymatic activity, dramatic changes would be predicted to lead to a complete loss of function. The measured reduction in RDE activity, however, is more likely to be the result of an altered conformation in adjacent tertiary structures as proposed by Table 2 and Fig. 4: changes in epitope accessibility were shown to be concomitant with alterations in virion morphology (at least under stressed conditions, e.g. microscopy staining). The significance of these structural effects has still to be clarified in detail, but information from a persistence model with lymphocytic choriomeningitis virus (LCMV) illustrates this point: a single amino acid change in the LCMV glycoprotein was shown to be associated with an unusual epitope reactivity and with persistence in vivo (Salvato et al., 1991). The effects of alterations in the virus surface have also been discussed in a report about two mutated capsid genes of poliovirus which were sufficient to confer a persistent phenotype (Calvez et al., 1993).

Nucleotide sequencing and functional analysis of persistent-type HEF were performed with virus derived from different hosts, i.e. cell culture and embryonated hen eggs. Persistently infected MDCK cells have been cultured for more than 5 years and virus isolation from the supernatant was carried out several times. Thus the propagation of cell-grown C/AA-pi in fertilized eggs was achieved by serial allantoic inoculation and all virus used in these studies originated from passage levels four and five. The conservation of the persistent viral phenotype was proven by the re-infection and re-establishment of long term persistence in fresh MDCK cells (unpublished observation). Furthermore, in the biochemical tests described, persistent virus appeared to be invariable.

These findings suggest that the determinant for persistence is genetically stable and that no host-specific selection of revertants is evident.

These results imply a discrete effect of the described changes in HEF on the establishment of influenza C virus persistence. Given this, our hypothesis to explain the trigger for viral persistence includes several further observations. We propose the possible involvement of more than one critical determinant. (i) Divergence of the virion glycoprotein seems to confer a distinct functional prerequisite for a non-cytocidal, persistent viral life cycle. The selection of moderately or low productive strains, evolving by long term persistence, has been reported for other virus systems, e.g. measles virus (Celma & Fernandez-Muñoz, 1992; Hirano et al., 1993) and influenza B virus (Clavo et al., 1993). These characteristics in virus replication accompany an adaptation to suitable cellular subpopulations. (ii) The connection between cell tropism and an extreme variation of viral glycoproteins is impressively illustrated by persistence of human immunodeficiency virus (Shioda et al., 1992). Yet, apart from this, (iii) we have experimental evidence for the elusive stability of viral RNA during non-productive phases (Marshall et al., 1993) and for the ability to maintain RNA persistence only in certain cell types (M. Marshall, G. Foerst & H. Meier-Ewert, unpublished results). In line with our observations are studies showing the extraordinarily long half-life of inactivated influenza A viral RNA segments (Cane & Dimmock, 1990) and on the characterization of a unique virus regulatory protein from persistent infection (Lucas et al., 1988). Considering these aspects we favour the notion that besides HEF variation, additional regulatory functions of other virus genes might be involved. For C/AA-pi virus, investigations of the non-structural coding region and gene products are under way.

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