A host restriction-based selection system for influenza haemagglutinin transfectant viruses

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During the 1996 influenza epidemic in Vienna we obtained influenza A virus specimens (Vienna/47/96, Vienna/81/96) which grow efficiently in African green monkey kidney (Vero) cells but not in embryonated chicken eggs. Amplification of the specimens in Vero cells resulted in progeny that agglutinated human but not chicken erythrocytes. Reassortment analysis suggested that the haemagglutinin (HA) might be responsible for the host restriction. Vero cells were infected with the Vienna/47/96 virus and then transfected with reconstituted ribonucleoprotein complexes containing HA genes from egg-adapted strains. Subsequent selective passages in embryonated chicken eggs resulted in selection of transfected viruses, growing in eggs and containing the transfected HAs. The results demonstrate that host restriction of the Vero-adapted Vienna/47/96 virus is due to its HA. Moreover, the experiments showed that the Vienna/47/96 strain can be used as helper virus for reverse genetics experiments.

Influenza A viruses are negative-strand RNA viruses. The virus contains a lipid envelope in which the haemagglutinin (HA), neuraminidase (NA) and M2 proteins are anchored. The matrix protein (M1) forms a protein layer beneath the envelope and inside the M1 protein layer are ribonucleoprotein complexes (RNPs), which consist of RNA segments associated with the nucleoprotein (NP) and the polymerase subunits PB1, PB2 and PA (Lamb & Krug, 1996).

It is possible to introduce mutations into the genome of influenza viruses by site-directed mutagenesis (Enami et al., 1990). For example, genetically manipulated influenza virus can be rescued by transfecting in vitro-reconstituted RNP complexes into cells that have been previously infected with an influenza helper virus. The RNPs are formed by incubating plasmid-derived RNA with purified NP and polymerase subunits (for a review see García-Sastre & Palese, 1995). Alternatively, RNPs can be reconstituted in vivo upon expression of plasmids encoding the NP, polymerase proteins and viral RNA in the cells. Cells are then infected with influenza helper virus. Subsequent selection permits the isolation of transfectant virus containing the plasmid derived RNP (Pleschka et al., 1996). Both the RNP transfection technology and the plasmid-derived reverse genetics system rely on the use of a helper virus. Therefore, it is important to have an efficient selection system that permits viruses containing the cDNA derived gene segment to be distinguished from the parental helper virus.

Several selection strategies have been established. For example, synthetic neuraminidase (NA) genes that allow influenza virus to grow in the absence of a protease were transfected into cells that were infected with protease-dependent strains. Subsequent selection in the absence of a protease resulted in transfectant viruses in which the viral NA-RNA segment was exchanged for the synthetic NA-RNA (Enami et al., 1990). In other experiments, viruses containing cDNA-derived HA-RNA segments were rescued. In this case, the selection was done via the use of neutralizing antibodies that specifically recognize the HA protein of the helper virus but not the transfected synthetic HA (Enami & Palese, 1991; Li et al., 1992; Muster et al., 1994). A selection strategy for HA transfectant viruses using an immunocapture procedure followed by selection with monoclonal antibodies was also successful (Horimoto & Kawaoka, 1994).

In this study, we describe a new rescue system for HA transfectant viruses. As a helper virus for the transfection experiment we used an influenza virus which was isolated on Vero cells and shown to be restricted for growth in...
embryonated eggs. The Vero cell line has been used for the production of vaccines against a variety of diseases including measles, polio and rabies (WHO Study Group, 1987). Vero cells have recently been shown to be permissive for influenza virus (Gorvako et al., 1996). The selection of HA transfectant viruses, described in this report, is based on the host restriction of the Vero-isolated helper virus.

During the 1996 influenza epidemic in Vienna we isolated two influenza A viruses on both Vero and Madin–Darby canine kidney (MDCK) cells. Specifically, two clinical specimens (Vienna/47/96 and Vienna/81/96) were used for infection of MDCK cells, Vero cells or embryonated chicken eggs. One egg or $3 \times 10^6$ cells were infected with 50 µl of the specimen, either undiluted or diluted 1:10. Three days after infection, the supernatants and the allantoic fluids were harvested and used as inoculum for a second passage in the corresponding host.

The isolates were subtyped to be H3N2 A/Johannesburg/94-like by haemagglutination inhibition (HI) assay with ferret reference antisera at the Centers for Disease Control (Atlanta, Georgia, USA). Viruses isolated on Vero cells agglutinated human erythrocytes, but did not agglutinate chicken erythrocytes. While viruses amplified in Vero cells retained their host restriction phenotype, viruses isolated in MDCK cells became infectious for chicken eggs. Further passages of the Vero cell-derived A/Vienna/47/96 variant showed that host restriction and agglutination properties of the virus were stable at least during five passages on Vero cells. No egg-growing variants were obtained even after two blind passages of the A/Vienna/47/96 Vero isolate in eggs.

A Vienna/Singapore(7/1) reassortant virus, containing the NA gene from the influenza A/Singapore/57 strain and all other genes from the Vienna/47/96 virus, showed growth and agglutination properties identical to those of the Vienna/47/96 host-restricted variant (data not shown). This observation allowed us to exclude the NA as the responsible host restriction factor, and suggested the HA as a target for further investigation.

In order to determine the role of the HA in limiting the host range of the Vienna/47/96 isolate, transfection experiments were performed. In addition, we wanted to establish a rescue system for chimeric HA-transfectant viruses on cell lines that are licensed for vaccine production. For this purpose, two chimeric HA plasmids were constructed. Bj-2F5-1 corresponds to the HA of an egg-passaged influenza A/Beijing/32/92 virus with an insertion of the amino acid sequence LELDKWA.
in antigenic site B, including a unique SacI restriction site as a marker. A cDNA derived from an egg-passaged influenza A/Beijing/32/92 (H3N2) virus was used as template for PCR and as a vector for cloning (Muster et al., 1994). Construct WSN-IA3 was generated by cloning a PCR product into the plasmid pT3HA/M, which encodes the WSN-HA (Li et al., 1992). WSN-IA3 contained the human major gastrointestinal tumour-associated protein-specific amino acid sequence QKTQNDV (Szala et al., 1990) in the antigenic site B with a unique MaeI restriction site as marker. The plasmid pT3HA/M containing the cDNA derived from an egg-permissive A/WSN/33 (H1N1) virus (Li et al., 1992) was used as template for PCR and as vector for cloning.

For the transfection experiment approximately 10^6 Vero cells were infected with the host-restricted influenza Vienna/47/96 strain at an m.o.i. of 0.1 with virus from the third passage on Vero cells. RNP complexes containing the chimeric HA-RNAs, which were derived from cDNAs of the egg-permissive strains, were transfected into the cells according to the protocol previously described (Enami et al., 1990). Subsequently, transfaction supernatants were used undiluted and 1:10 diluted to infect embryonated chicken eggs. After two passages in chicken eggs, allantoic fluids were harvested (Fig. 1a). In order to obtain pure clones the allantoic fluids containing infectious viruses were passed three times with limited dilutions in embryonated chicken eggs. Then RT–PCR was performed to determine whether the synthetic HA genes were transfected into the Vienna/47/96 virus. Viral RNA was extracted as previously described (Luytjes et al., 1989). For RT–PCR, the full-length HA gene of either chimeric or wild-type virus was amplified using the primers 5′ AGCAAAAAGCGGGGATAATTTC 3′ and 5′H3 antisense 5′ TAATACCTCAAATGCAAATG 3′ for H3 viruses and 5′H1 sense 5′ AGCAAAAAGCGGGGAAAATA 3′ and 5′H1 antisense 5′ AGTAGAAACAAGGGTATTTC 3′ for H1 viruses. HA PCR-products from rescued viruses were digested with restriction enzymes specific for the inserted foreign restriction sites. Amplified HAs of H3 viruses were digested with restriction enzyme SacI (Fermentas, Lithuania), and H1-type HAs were digested with MaseI (Boehringer Mannheim).

### Table 1. HI titres of transfectant viruses

<table>
<thead>
<tr>
<th>Transfectant influenza virus</th>
<th>H3-specific HI titre*</th>
<th>H1-specific HI titre*</th>
<th>2F5-specific HI titre†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bj-2F5-1</td>
<td>160</td>
<td>&lt; 10</td>
<td>128</td>
</tr>
<tr>
<td>WSN-IA3</td>
<td>&lt; 10</td>
<td>320</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

* Antisera were obtained by immunizing mice with either A/Shandong/93 (H3N2) or A/WSN/33 (H1N1) virus.
† Initial concentration of hMAb 2F5 was 200 µg/ml.

In addition, the antigenicity of the transfectant viruses was characterized by an HI assay. Viruses were adjusted to 4 haemagglutination units and incubated for 1 h with 2-fold reciprocal dilutions of either hMAb 2F5, mouse anti-WSN and mouse anti-H3 serum starting from a 1/10 dilution. The inhibition of agglutination of chicken erythrocytes is shown in Table 1. Transfectant Bj-2F5-1 reacted with the H3 serum and the hMAb 2F5, which specifically binds to the HIV-1 neutralizing epitope ELDKWA (Muster et al., 1993). The IA3 transfectant virus reacted with an influenza A/WSN/33 antiserum which does not recognize the antigenically distinct helper virus. The data from the restriction analysis and the HI test were confirmed by sequencing of the transfectant HA genes.

Yielding up to 2 × 10^8 transfectants per 35 mm dish, the efficiency of the transfection system of Vero cells was comparable to previously described transfection methods for HA genes (Enami & Palese, 1991). A benefit of this positive selection method is that it is less time-consuming when compared to negative selection in the presence of antibodies against the helper virus.

The successful rescue experiment showed that the HA is responsible for the host-restricted growth of the Vienna/47/96 virus isolated on Vero cells. We were therefore interested in the molecular basis of this phenomenon. Usually, mutations involved in modulation of receptor specificity are observed in the HA1 subunit of the HA. Therefore, we compared the HA1 sequence of the Vero-isolated Vienna/47/96 and Vienna/81/96 strains with that of the Vienna/47/96 variant, which was isolated on MDCK cells. Several differences between the Vero variants and the MDCK variant were found near the receptor-binding site. Asparagine at amino acid (aa) position 98 was observed instead of tyrosine for the Vienna/47/96 MDCK variant. The Vienna/47/96-MDCK variant contained arginine instead of threonine at aa position 128 in the Vienna/47/96 Vero variant. This destroyed the glycosylation site at aa position 126 in the MDCK variant. Previous studies by other workers have also noted changes in glycosylation involved in egg adaptation (Oxford et al., 1991; Rota et al., 1989; Wang et al., 1989). The asparagine at aa position 145 in the Vero variants was found to be lysine in the MDCK variant. Outside the receptor-binding site phenylalanine at aa position 257 in the Vero variants was changed to tyrosine in the MDCK variant (Table 2). It has been reported that passaging of influenza virus in embryonated eggs may select for viruses containing glutamine at position 226 (Williams & Robertson, 1993; Skehel, 1993). This position was reported to be important for receptor-binding specificity (Rogers et al., 1983). However, the agglutination phenotype and the host-restricted growth of our isolates are not associated with a mutation at this position, suggesting that these virus isolates belong to a new clade of H3N2 viruses which was...
recently described (Lindstrom et al., 1996). Further studies to understand the molecular basis of the host-restricted phenotype of the Vienna/47/96 virus are in progress. It is also important to investigate the influence of the host-restriction phenotype on virulence and immunogenicity of these viruses.

The ability to genetically manipulate influenza virus allows its use as a vector for foreign antigens. For example, recombinant influenza viruses expressing epitopes of the circumsporozite protein of Plasmodium yoelii resulted in protective immune responses in the mouse model (Rodrigues et al., 1994). In addition, long-lasting immune responses against HIV-1 at the systemic and the mucosal level were induced by a chimeric influenza virus expressing the HIV-1 neutralizing epitope ELDKW (Muster et al., 1995). These promising results suggested that influenza viruses may be used as a vector for immunization against foreign pathogens.

So far, the results with influenza virus vector were obtained solely in the mouse model. Therefore it is important to substantiate the mouse data with human data. However, currently described HA transfection systems are dependent on cell lines that are not licensed for the production of vaccines, like Madin–Darby bovine kidney cells (MDBK) and MDCK cells. Vero cells and embryonated chicken eggs, which were used for the generation of transfectant viruses in our study, have been licensed for the production of vaccines for humans. Therefore, the immunogenicity and safety of chimeric influenza viruses generated by the described rescue system could be analysed in human clinical trials also.

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