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A Reverse Genetics Approach for Recovery of Recombinant Influenza B Viruses Entirely from cDNA

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The recovery of recombinant influenza A virus entirely from cDNA was recently described (9, 19). We adapted the technique for engineering influenza B virus and generated a mutant bearing an amino acid change E116G in the viral neuraminidase which was resistant in vitro to the neuraminidase inhibitor zanamivir. The method also facilitates rapid isolation of single-gene reassortants suitable as vaccine seeds and will aid further investigations of unique features of influenza B virus.

Influenza illness, a cause of more than 20,000 excess deaths in epidemic years in the United Kingdom (7), is attributable to infection with one of two subtypes of influenza A virus or with influenza type B virus (8). Thus, influenza B virus surface antigens are an essential component of any vaccine effective in reducing influenza morbidity. Influenza B viruses, like their type A counterparts, are orthomyxoviruses with genomes comprised of eight segments of negative-sense single-stranded RNA. In general, they encode proteins with homology to those encoded by influenza A virus, although there are notable and interesting differences, particularly in RNA segments 6 and 7 (16). In addition, a recently described additional open reading frame within RNA segment 2 present in many influenza A virus strains is absent from influenza B viruses (5). The reasons for and biological consequences of the genetic differences between influenza A and B viruses are of considerable academic interest and may have implications for understanding the limited host range for influenza B virus as well as for developing antiviral strategies for these orthomyxoviruses.

The manipulation of the influenza virus genome was first described by Palese and coworkers in 1989 (17), and the development of the technique to site-specifically change an infectious influenza B virus followed shortly after (1). However, this method, which utilized a helper virus and appropriate selection system, suffered from the limitation that engineering of all RNA segments was not possible. Since 1999 it has been possible to recover recombinant influenza A viruses entirely from cDNAs (9, 13, 19). Even within the short time this system has been available, it has revolutionized the potential for influenza virus research by facilitating a reverse genetic approach to the study of all the influenza A virus genes (3, 5, 11, 12, 20, 27). The process involves the in situ generation of virus RNAs transcribed from eight separate plasmids in which the influenza virus sequences are placed downstream of polymerase I promoters. In addition, expression of the four proteins which comprise the viral polymerase, NP, PB1, PB2, and PA, is achieved by placing their coding sequences under the control of a polymerase II promoter either in separate expression plasmids or in bidirectional plasmids (13). In this report we describe the adaptation of reverse genetics technology for engineering recombinant influenza B viruses.

The strategy we applied is based upon that described for recovery of recombinant influenza A viruses (9, 19). We used a cassette vector, pPRG, which allows cloning of the segments of the influenza B virus genome so that they are flanked by a human polymerase I promoter at the 5’ terminus and the hepatitis delta virus (HDV) antigenic ribozyme at the 3’ terminus, such that their transcription results in negative-sense RNAs with exact viral-like termini. The polymerase I promoter was cloned from a human genomic library by PCR using primers based on published sequences (15). The ribozyme sequence was derived from the antigenomic sense ribozyme of HDV (kindly provided by A. Ball). The plasmid pPRG was previously used for the rescue of influenza virus A/Victoria/3/75 H3N2 from cloned DNA (J. Daly, A. Cadman, W. Snowden, M. Tisdale, and T. Zurcher, Abstr. Options Control Influenza IV, abstr. W23-5, 2000). We inserted sequences encoding the influenza B virus-like model RNA HABCAT (1) and cotransfected this plasmid, pPRGCAT, with four plasmids directing expression of the influenza B virus polymerase complex. These helper plasmids were based on vector pCI (Invitrogen) and had coding sequences for NP, PB1, PB2, and PA derived from B/Panama/45/90 virus (14) inserted downstream of a cytomegalovirus (CMV) promoter. As a control we inserted HABCAT sequences into the pPolIRRTSapI vector, described and kindly provided by Fodor et al. (9), to create pPRFCAT. We sequenced both polymerase I promoters and found no nucleotide differences between them, although they differed from published sequences (15). However, it should be noted that pPRFCAT contains the HDV genomic sense ribozyme. The data in Fig. 1 indicate that chloramphenicol acetyltransferase (CAT) expression following transfection of each construct varied. It may be that the efficiency of cleavage by either ribozyme affects expression. However, we cannot exclude the possibility that different sequences surrounding the promoter and ribozyme in the two vectors affect their efficiencies. Since the pPRG vector resulted in the most CAT protein produced, we chose this for insertion of the influenza B virus gene segments. We also tested whether polymerase proteins from a different
strain of influenza B virus, B/AA/66 (25), or from influenza A virus could support replication of the HABCAT viral-like RNA. Figure 1 shows that both supported replication of the model RNA, although less well than the B/Panama polymerase proteins. It is well established that influenza A virus polymerases replicate model RNAs containing influenza B virus promoters (6, 14, 18, 28). The differences we observed in replication efficiency by influenza B virus polymerases might be due either to sequence, since they are from different virus strains, or to the nature of the expression plasmid in which they were cloned. In virus rescue experiments we used B/Panama/45/90 polymerases (Table 1).

The influenza B virus gene segments were cloned by reverse transcription-PCR (RT-PCR), using appropriate primers complementary to the segment termini (26) (primer sequences available on request) that introduced BsmBI or SapI restriction enzyme sites to facilitate their insertion into pPRG cassette vectors containing these sites. Six of the RNA segments were derived from influenza virus B/Beijing/1/87. Segment 1 was derived from a cDNA clone of the PB2 gene from influenza virus B/Panama/45/90 (14). Segment 5 was obtained by RT-PCR from influenza virus B/Lee/40 viral RNA (vRNA). This cDNA was inserted into a bidirectional construct containing both polymerase I and polymerase II promoters by subcloning the polymerase I and ribosome sequences into pcDNA3 (Invitrogen) (Table 1). Aliquots of 0.5 μg of each of the eight plasmids were cotransfected into 293T cells with 0.5 μg of pCIPB1, 0.5 μg of pCIPB2, 0.5 μg of pCIPA, and 1 μg of pCIIP. The pCI plasmids express B/Panama/45/90 polymerase and NP proteins. pPRGCA was also cotransfected with plasmids expressing the A/Victoria/3/75 PB1, PB2, PA, and NP proteins or with pcDNA3-based plasmids expressing the same proteins from the B/Ann Arbor/60 virus. Transfections were performed in duplicate using the FuGENE 6 transfection reagent (Roche). After 48 h cells were lysed and cell lysates were used in a CAT enzyme-linked immunosorbent assay (Roche).

FIG. 1. Replication and expression of HABCAT influenza B virus model RNAs. CAT protein synthesized from two constructs containing an influenza B vRNA-like CAT reporter gene was quantified. pPRGCA and pPRFCAT contain the CAT gene in a negative-sense orientation flanked by the influenza B virus HA gene noncoding regions between a human RNA polymerase I promoter and an HDV ribozyme terminator. One microgram of each construct was cotransfected into 5 × 10⁵ 293T cells with 0.5 μg of pCIPB1, 0.5 μg of pCIPB2, 0.5 μg of pCIPA, and 1 μg of pCIIP. The pCI plasmids express B/Panama/45/90 polymerase and NP proteins. pPRGCA was also cotransfected with plasmids expressing the A/Victoria/3/75 PB1, PB2, PA, and NP proteins or with pcDNA3-based plasmids expressing the same proteins from the B/Ann Arbor/60 virus. Transfections were performed in duplicate using the FuGENE 6 transfection reagent (Roche). After 48 h cells were lysed and cell lysates were used in a CAT enzyme-linked immunosorbent assay (Roche).

We observed that the rescued virus contained a gene constellation that is easily identified since it differs from other influenza B virus NP genes in that it contains a single EcoRI restriction site, whereas other sequences contain two or more, and it lacks a BamBI restriction site whereas this site is present at nucleotide 1370 in other influenza B viruses. Moreover, influenza virus B/Panama/45/90 virus has never been propagated in our laboratory, and the sequence of RT-PCR products from the recombinant virus illustrates that it contains a B/Panama/45/90 PB2 segment (data not shown).

We observed this to be the first successful recovery of recombinant influenza B virus entirely from cDNA. Although the technique we have used is an adaptation of that previously developed for influenza A viruses, we have made some important and possibly significant improvements. Firstly, as discussed above, the plasmid vector and polymerases used in the system differ from those used in other laboratories. Secondly, the genetic constellation of the virus we rescued is unusual (Table 1). Six of the segments are from influenza virus B/Beijing/1/87 virus, but PB2 is from B/Panama/45/90 and NP is from the highly laboratory-adapted strain B/Lee/40. This was useful for identifying the recovered virus and also may contribute to our success, as it results in a virus which replicates very well in MDCK cells, so even if transfection efficiency was low recovered virus would be readily amplified following coculture. Thirdly, we utilized a bidirectional vector for generation of segment 5 RNA. We do not know at present whether this is essential for virus recovery. However, subcloning of segment 5 cDNAs into the pPRG vector did not allow recovery of virus. Interestingly, although the bidirectional plasmid generates NP that can support HABCAT model RNA replication (data not shown), omission of the pCIIP plasmid did not allow virus recovery, although substitution with pcDNA3NP from B/AA/66 did.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Origin of viral gene</th>
<th>Promoter</th>
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<tbody>
<tr>
<td>pPRPB1</td>
<td>B/Beijing/1/87</td>
<td>Human RNA polymerase I</td>
</tr>
<tr>
<td>pPRPB2</td>
<td>B/Panama/45/90</td>
<td>Human RNA polymerase I</td>
</tr>
<tr>
<td>pPRPA</td>
<td>B/Beijing/1/87</td>
<td>Human RNA polymerase I</td>
</tr>
<tr>
<td>pPRHA</td>
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<td>Human RNA polymerase I</td>
</tr>
<tr>
<td>pPRNP</td>
<td>B/Lee/40</td>
<td>Human RNA polymerase I and CMV RNA polymerase II</td>
</tr>
<tr>
<td>pPRNA</td>
<td>B/Beijing/1/87</td>
<td>Human RNA polymerase I</td>
</tr>
<tr>
<td>pPRM</td>
<td>B/Beijing/1/87</td>
<td>Human RNA polymerase I</td>
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<td>CMV RNA polymerase II</td>
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<td>B/Panama/45/90</td>
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<td>B/Panama/45/90</td>
<td>CMV RNA polymerase II</td>
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<tr>
<td>pCIIP</td>
<td>B/Panama/45/90</td>
<td>CMV RNA polymerase II</td>
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TABLE 1. Plasmids used for rescue of recombinant influenza B virus entirely from cDNA
We engineered a mutation into the pPRNA plasmid to alter amino acid residue 116 of the B/Beijing/1/87 neuraminidase (NA) protein from glutamic acid to glycine (Fig. 2A). This mutation has been independently identified in two laboratory isolates which acquired resistance to the NA inhibitor zanamivir (2, 24). A virus was recovered that grew well in MDCK cells. Figure 2B illustrates the multicycle growth characteristics in comparison with the recovered wild-type virus with the same genetic constellation. As predicted, the E116G mutant displayed a drug-resistant phenotype in plaque reduction assays. At a concentration of 0.03 μg of zanamivir/ml, the size and number of plaques formed by wild-type virus were greatly reduced but the E116G mutant was unaffected. Sequencing of RT-PCR products confirmed that the E116G mutation was present, as was a silent mutation in the preceding codon engineered into the cDNA as a tag (Fig. 2A).

We next assayed the inhibition of mutant NA enzyme activity by zanamivir. Infected cell supernatants containing wild-type or E116G virus were adjusted for equivalent NA activity and then subjected to the 4-methylumbelliferyl-β-D-acetylneuraminic acid (MUNANA) enzyme assay in the presence of different concentrations of drug. A greater amount of virus was needed to assay the mutant enzyme, indicating that it had a reduced NA activity. Indeed, hemagglutination elution at 37°C (assumed to be mediated by NA activity) was not observed for the mutant virus. Moreover, the E116G mutant NA was only inhibited by high concentrations of zanamivir. The 50% inhibitory concentration (IC50) was approximately 1 nM for wild-type virus but increased to over 8 μM for the mutant (Fig. 3). These results correlate well with IC50 values previously obtained for wild-type and E116G B/Beijing/1/87 viruses (2).

Since both influenza B viruses previously described which contain the E116G mutation also contained additional hemagglutinin (HA) mutations (2, 24), we sequenced RT-PCR products derived from the entire HA gene of the mutant virus. No changes from the wild-type B/Beijing/1/87 HA sequence were found. Thus, the reverse genetics procedure has allowed the recovery of a genetically defined virus altered in a single gene segment which can help to understand the contribution of single point mutations to a drug resistance phenotype. It has been particularly difficult to study the contribution of NA mutations to resistance to the NA inhibitors in the context of live virus when using a traditional genetic approach, since NA mutations are always accompanied by changes in the HA gene both in vitro and in vivo (2, 10, 24). In studies with influenza A virus mutants, the contributions of the NA and HA sequence changes to the drug-resistant phenotype have been separated by generating single-gene reassortants (4). The reverse genetics technology allows the analysis of individual mutations in separate gene segments without needing to resort to such methods. The NA E116G mutant demonstrates that the
drug-resistant phenotype to an NA inhibitor can be conveyed by a single amino acid change in the NA gene. Interestingly, the E116G virus displayed efficient replication in MDCK cells. However, it has been shown previously that NA mutants with reduced enzyme activity may grow well in MDCK cells but have reduced infectivity in vivo (11). Carr, I. J., Ives, N. A. Roberts, C. Y. Tai, D. B. Mendel, L. Kelly, R. Lambkin, and J. Oxford, Abstr. 2nd Int. Symp. Influenza Other Respir. Viruses, 1999. It may be that the reduction in NA activity is not sufficient to significantly disrupt the balance between HA affinity and NA activity in MDCK cells. Changes in HA leading to altered receptor binding and antigenic properties of influenza B viruses upon adaptation to cell culture and eggs have been documented (22, 23).

We replaced the pPRHA plasmid with pPRHA2X, which encodes the HA gene from influenza virus B/Md/59 genetically tagged with an XhoI restriction enzyme site (1). A recombinant virus was rescued which contained the genetic tag and displayed similar growth kinetics to the wild type and the drug-resistant mutants described above (Fig. 2B).

The ability to generate antigenic variants of influenza B virus within a high-growth background of internal genes may have important consequences for generation of vaccine strains. In recent years the World Health Organization (WHO)-recommended strain of influenza B virus has grown poorly in eggs. During 1998 to 2002, alternative influenza B virus strains which resembled the WHO strain antigenically but grew to higher titers in eggs were selected for use in EU vaccines (John Wood, personal communication). Even so, in some cases growth of the chosen virus was still inefficient, causing a potential reduction in the number of available doses. Since typically influenza A virus high-growth reassortants increase yield approximately fourfold over that of wild-type strains, the ability to generate similar high-growth strains of influenza B virus to order will be a useful addition to the influenza virus reverse genetic repertoire.

We thank Andrew Ball, Ervin Fodor, Juan Ortin, Peter Palese, and Augustin Portela for providing plasmids. We also thank Philip Yates for help with the MUNANA assay and Mark Stevens and John Wood for useful discussion.

ADDITION IN PROOF

Since submission of this article, Hoffmann et al. have reported the rescue of recombinant influenza B virus using a similar approach (E. Hoffmann, K. Mahmood, C.-F. Yang, R. G. Webster, H. B. Greenberg, and G. Kemble, Proc. Natl. Acad. Sci. USA 99:11411–11414, 2002).

REFERENCES