Rescue of Influenza A Virus from Recombinant DNA

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We have rescued influenza A virus by transfection of 12 plasmids into Vero cells. The eight individual negative-sense genomic viral RNAs were transcribed from plasmids containing human RNA polymerase I promoter and hepatitis delta virus ribozyme sequences. The three influenza virus polymerase proteins and the nucleoprotein were expressed from protein expression plasmids. This plasmid-based reverse genetics technique facilitates the generation of recombinant influenza viruses containing specific mutations in their genes.

Reverse genetics for negative-strand RNA viruses, first developed for influenza virus (8, 22), has dramatically changed our understanding of the replication cycles of these viruses. In addition, this methodology has allowed genetic manipulation of viral genomes in order to generate new viruses, which can be used as live, attenuated vaccines or vectors to express heterologous proteins (12). The past 5 years have witnessed the rescue of most of the important nonsegmented, negative-strand RNA viruses from recombinant DNA. First, Schnell et al. (30) succeeded in the recovery of rabies virus from cloned DNA. Shortly after, rescue systems were developed for vesicular stomatitis virus (21, 32), respiratory syncytial virus (5, 18a), measles virus (29), Sendai virus (14, 20), and more recently for human parainfluenza type 3 (7, 17), rinderpest virus (1), simian virus 5 (16), bovine respiratory syncytial virus (4), and Newcastle disease virus (27). Bridge and Elliott (3) succeeded in rescuing a segmented, negative-strand RNA virus, a bunyavirus, from cDNA. In general, all these methods rely on intracellular reconstitution of RNPs complexes from RNA and viral proteins, i.e., nucleoprotein and RNA-dependent RNA polymerase, which are introduced into cells by a variety of techniques. Generally, a recombinant vaccinia virus expressing T7 RNA polymerase is used to drive transcription of antigenomic positive-sense RNA as the template in order to initiate the replication cycle. Alternatively, transcription is driven by a T7 RNA polymerase, which is constitutively expressed in specific cell lines. Successful recoveries have also been reported by directly transfecting naked RNA (plus sense or minus sense) into cells expressing the essential proteins for encapsidation, transcription, and replication (20). Although reverse genetics techniques allowing genetic manipulation of negative-strand RNA viruses were established for influenza A virus before other negative-strand RNA viruses (8, 22, 31), full recovery of infectious influenza virus from cDNA without the use of helper virus has proved to be technically more difficult.

The genome of influenza A virus consists of eight segments of single-stranded, negative-sense RNA (25). The minimal set of viral proteins required for encapsidation, transcription, and replication of the viral genome are the three subunits of the viral RNA-dependent RNA polymerase complex (PB1, PB2, and PA) and the nucleoprotein (NP) (18). Initially, in order to manipulate the genome of influenza virus, RNP complexes were reconstructed in vitro from RNA transcribed from plasmid DNA in the presence of polymerase proteins and NP isolated from purified influenza virus (8, 9). The in vitro-reconstituted RNPs were transfected into cells infected with a helper influenza virus, which provided the remaining viral proteins and RNA segments, resulting in the generation of transfectant viruses. This technique has been extremely useful in advancing our understanding of the molecular biology and pathogenicity of influenza viruses. However, it relies on highly specialized selection methods to isolate the transfectant viruses from the helper virus, which restricts its use to certain RNA segments of a limited number of viral strains.

More recently, alternative methods for introducing influenza virus RNPs into cells have been developed, based on intracellular reconstitution of RNPs from in vivo-transcribed RNA and intracellularly expressed viral proteins (23, 24, 28, 33). We showed that the three polymerase proteins (PB1, PB2, and PA) and the nucleoprotein (NP) expressed from recombinant plasmids could encapsidate, transcribe, and replicate an influenza virus viral RNA (vRNA)-like RNA containing a chloramphenicol acetyltransferase (CAT) reporter gene in transfected human 293 cells (28). This vRNA-like reporter gene was introduced into the cells by transfection of a plasmid DNA (pPOLI-CAT-RT) with a truncated human RNA polymerase I (Pol I) promoter (nucleotides [nt] −250 to −1) positioned upstream of the vRNA-coding region. The sequence of the hepatitis delta virus genomic ribozyme was positioned downstream of the vRNA-coding region in order to ensure that RNA processing gave the correct 3′ end of the vRNA. It has also been demonstrated that, by replacing the plasmid coding for the CAT reporter gene with a plasmid encoding an authentic influenza vRNA segment, intracellularly reconstituted RNP complexes could be rescued into transfectant viruses upon infection of the transfected cells with an influenza helper virus. Thus, helper virus-based rescue systems using the RNA Pol I promoter-driven reverse genetics technique have been established for the segments encoding the neuraminidase (NA), the hemagglutinin (HA), the NS1 and NEP proteins, and the polymerase 2 basic protein (PB2) (11, 13, 26, 28). These results suggested that coexpression of the eight vRNA segments of influenza virus with the three polymerase proteins and the NP might allow rescue of infectious influenza virus from plasmid DNA.

In this report we describe the rescue of influenza A virus
from recombinant DNA. The system is entirely plasmid driven and does not involve the use of any helper or heterologous virus (Fig. 1). In order to recover infectious influenza virus from cloned cDNA, we analyzed two of the eight vRNA segments into which genetic tags were introduced. The HA segment contained a mutation of 6 nucleotides near the 3' end of the vRNA (26). Nucleotides 31 to 35 from the 3' end (3'-UUUUG-5') were replaced with 3'-AAAAC-5', resulting in an amino acid substitution at amino acid 4 (K→F) and at amino acid 5 (L→V) near the N terminus of HA within the signal peptide. In addition, a silent C→U mutation was created at nucleotide 40. These changes introduced several new restriction sites, including an SpeI site. The NA segment contained two silent mutations at nucleotides 1358 and 1360, introducing a novel SacI restriction site (28). Medium from MDBK cells infected with the rescued transfectant virus was used to isolate vRNA. Short regions of the HA and NA vRNA containing the genetic tags were amplified by reverse transcription-PCR (RT-PCR) and then analyzed by digestion with SpeI and SacI restriction enzymes, respectively. As a control, the same regions of the HA and NA segments were amplified from vRNA isolated from authentic A/WSN/33 virus using the same RT-PCR primers. As expected, the PCR products obtained from both viruses had identical sizes (Fig. 2, compare lanes 2 and 5 and lanes 7 and 10). Those originating from the HA and the NA segments of the rescued transfectant virus could be digested with SpeI and SacI, respectively (lanes 3 and 8). However, the PCR products from the authentic A/WSN/33 virus were, as expected, not digested (lanes 6 and 11). The omission of reverse transcriptase in control RT-PCR reactions resulted in no visible PCR products (lanes 4 and 9). It should be pointed out that we have succeeded in recovering influenza virus from plasmids expressing negative-sense vRNA. This seems to contradict some earlier studies, which...
emphasized the importance of using positive-strand RNA for rescuing negative-strand RNA viruses (3, 29a, 30). However, more recent successful recoveries of negative-strand RNA viruses from negative-sense RNA have been also reported (7, 20). Since at early stages posttransfection, positive-sense mRNA from the four protein expression plasmids coexists with naked negative-sense genomic vRNA transcribed from the transcription plasmids, inevitably double-stranded RNA can form. Formation of double-stranded RNA could lead to the induction of interferon-mediated antiviral responses and consequently to suppression of the growth of any rescued virus. Therefore the use of a cell line, such as Vero, which is known to be deficient in interferon expression (6), might be an important factor for successful virus rescue. Further work, however, is needed to prove this.

At present, we are able to rescue 1 to 2 infectious viral particles from about 10^6 transfected cells, which corresponds with the “average” recoveries obtained for other negative-strand RNA viruses. By increasing transfection efficiencies and optimizing the ratio of transfected plasmids it might be possible to obtain higher recoveries of virus. Recently, Gómez-Puertas et al. (15) demonstrated that by optimizing plasmid ratios they can significantly increase the formation of influenza virus-like particles from expressed viral proteins. In addition, cell lines expressing essential proteins for encapsidation, transcription, and replication of viral genomic RNA (PB1, PB2, PA, and NP) could help to reduce the number of plasmids needed and thus increase the efficiency of rescue.

In summary, we have rescued a recombinant influenza A virus by cotransfecting eight transcription plasmids for the individual vRNA segments and four protein expression plasmids, entirely from cDNA in the absence of any helper virus. The identity of the rescued virus was confirmed by providing evidence for the presence of two genetic tags in two different genome segments. The development of an entirely plasmid-based rescue system for influenza A virus opens the way for the study of different aspects of influenza virus replication and its interactions with the host cell. In addition, it allows full manipulation of the genome of the virus, which might result in the development of new vaccine strains not only for influenza, but for other infectious agents by introducing specific foreign epitopes into influenza virus proteins. In contrast to the earlier helper virus-based rescue techniques, the plasmid-based system can easily be used for the generation of infectious influenza viruses containing multiple mutations in several different genes at the same time.

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ADDENDUM IN PROOF


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