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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). Bridgen 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 ribonucleoprotein (RNP) 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, RNPs were reconstituted 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

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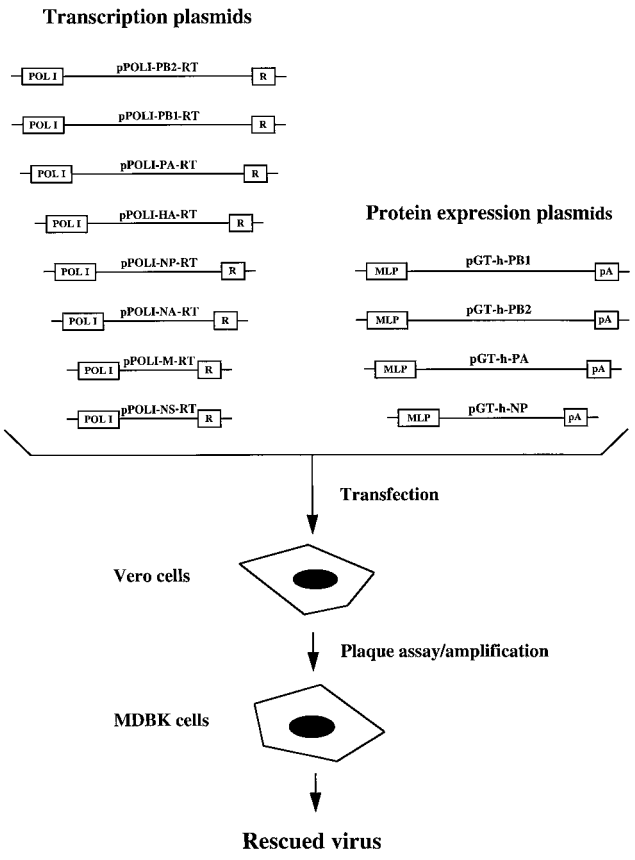

FIG. 1. Schematic representation of the plasmid-based rescue system for influenza A virus. The pGT-h set of protein expression plasmids was constructed by inserting the open reading frames of PB1, PB2, PA, and NP proteins into the *Bcl*I cloning site of the pGT-h plasmid (2). The PB1 and PA genes were derived from influenza A/WSN/33 virus. The PB2 and NP genes were derived from influenza A/PR/8/34 virus. The viral genomic sequences of influenza A/WSN/33 virus were cloned into pUC18- or pUC19-based plasmids between a truncated human RNA Pol I promoter (nt −250 to −1) (19) and sequences of the hepatitis delta virus ribozyme in an analogous way as described for pPOLI-CAT-RT (28). Genetic tags were inserted into the HA- and NA-encoding plasmids by using conventional mutagenic techniques. For viral rescue, 5 μg of each of the polymerase protein expression plasmids (pGT-h-PB1, pGT-h-PB2, and pGT-h-PA), 10 μg of the NP-expressing pGT-h-NP, and 3 μg of each of the eight vRNA-coding transcription plasmids (pPOLI-PB2-RT, pPOLI-PB1-RT, pPOLI-PA-RT, pPOLI-HA-RT, pPOLI-NP-RT, pPOLI-NA-RT, pPOLI-M-RT, and pPOLI-NS-RT) were diluted to a concentration of 0.1 μg/μl in 20 mM HEPES buffer (pH 7.5). The DNA solution was added to diluted DOTAP liposomal transfection reagent (Boehringer) containing 240 μl of DOTAP and 720 μl of 20 mM HEPES buffer (pH 7.5). The transfection mixture was incubated at room temperature for 15 min, mixed with 6.5 ml of MEM containing 0.5% fetal calf serum, 0.3% bovine serum albumin, penicillin, and streptomycin and added to near-confluent Vero cells washed with phosphate-buffered saline in 8.5-cm dishes (about 10^7 cells). At 24 h after transfection, the transfection mixture was removed and the cells were incubated with 8 ml of fresh medium, which was replaced daily for 4 days. The harvested medium from transfected dishes was screened for rescued influenza virus by plaquing and amplification on MDBK cells. POL I, truncated human RNA polymerase I promoter; R, genomic hepatitis virus ribozyme; MLP, adenovirus type 2 major late promoter; pA, polyadenylation sequence from SV40.

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 used a mixture of eight plasmids expressing the individual vRNA segments of influenza A/WSN/33 virus from a truncated human Pol I promoter. We also replaced the previously used four protein expression plasmids, which expressed PB1, PB2, PA, and NP under the control of a mouse hydroxymethylglutaryl-coenzyme A reductase (HMG) promoter (28), with plasmids expressing the same proteins under the control of the adenovirus type 2 major late promoter (pGT-h-PB1, pGT-h-PB2, pGT-h-PA, and pGT-h-NP) (2). Cotransfection of these four plasmids with pPOLI-CAT-RT into 293 or Vero cells resulted in CAT expression (results not shown). We decided to use Vero cells for the virus rescue, since they support the growth of influenza A/WSN/33 virus better than 293 cells (about 1 log difference in maximum viral titers) (results not shown).

For virus rescue, near-confluent Vero cells in 8.5-cm-diameter dishes, were cotransfected with the four protein expression plasmids and the eight vRNA transcription plasmids (pPOLI-PB2-RT, pPOLI-PB1-RT, pPOLI-PA-RT, pPOLI-HA-RT, pPOLI-NP-RT, pPOLI-NA-RT, pPOLI-M-RT, and pPOLI-NS-RT). After 24 h, the transfection medium was removed from the cells and it was replaced with 8 ml of fresh medium (MEM) containing 0.5% fetal calf serum, 0.3% bovine serum albumin, penicillin, and streptomycin. The transfected cells were maintained for at least 4 days after transfection. Every day, the medium from the transfected cells was collected and assayed for the presence of influenza virus by plaquing a 0.5-ml aliquot on MDBK cells by standard methods. The rest of the medium was transferred into 75-cm^2 flasks of subconfluent MDBK cells for amplification of any rescued virus. This procedure resulted in the recovery of infectious influenza virus on day 4 posttransfection. We obtained about 10 to 20 PFU of virus from an 8.5-cm dish containing approximately 10^7 cells. The rescued virus showed a specific property which is characteristic of influenza A/WSN/33 virus, i.e., it formed plaques on MDBK cells in the absence of trypsin. The plaques formed by the rescued virus were comparable in size to those formed by an authentic wild-type influenza A/WSN/33 virus grown on the same MDBK cells (results not shown).

To formally prove that the viral plaques observed on MDBK cells were formed by virus derived from the 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 *Spe*I site. The NA segment contained two silent mutations at nucleotides 1358 and 1360, introducing a novel *Sac*I 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 *Spe*I and *Sac*I 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 *Spe*I and *Sac*I, 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

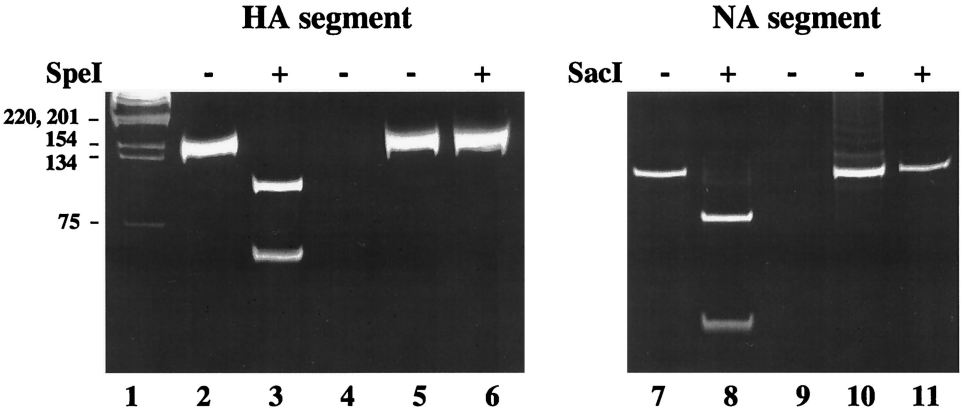

FIG. 2. Demonstration of the presence of genetic tags in the HA and NA vRNA segments of the rescued virus by RT-PCR and restriction enzyme analysis. vRNA of the rescued virus was isolated from medium of infected MDBK cells. One hundred microliters of the medium was treated with 5 U of RNase-free DNase to remove any residual plasmid DNA carried over. After 15 min at 37°C, vRNA was isolated by using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. vRNA from authentic wild-type A/WSN/33 virus was isolated from purified virus as described previously (10). The first 149 nt at the 3′ end of the HA vRNA were amplified by RT-PCR using oligonucleotide primers 5′-GCGCTCTAGAGCAAAAGCAGGGGAAAATAA-3′ (corresponding to nt 1 to 21) and 5′-CGCGAAGCT TCTCGAATATTGTGTCAAC-3′ (corresponding to nt 129 to 149), resulting in a 165-nt-long PCR product. To amplify the sequence containing the genetic tag from the NA segment, primers 5′-TGGACTAGTGGGAGCATCAT-3′ (corresponding to nt 1280 to 1309) and 5′-GAACAAACTACTTGTCAATGGT-3′ (corresponding to nt 1367 to 1388) were used in RT-PCR to produce a 108-nt PCR product. The HA- and NA-specific PCR products were incubated for 2 h at 37°C in the presence (+) or absence (−) of 10 U of *Spe*I and *Sac*I restriction enzymes, respectively. Samples were analyzed on 16% polyacrylamide gels and stained with ethidium bromide. Lanes: 1, DNA size markers (sizes in nucleotides are indicated); 2, 3, 7, and 8, PCR products from the rescued virus; 4 and 9, control reactions omitting reverse transcriptase; 5, 6, 10, and 11, PCR products from the authentic wild-type A/WSN virus.

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

Following submission of this paper, Neumann et al. (G. Neumann, T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka, Proc. Natl. Acad. Sci. USA **96:**9345–9350, 1999) provided independent evidence for a plasmid-based rescue of influenza A virus.

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