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High-Efficiency Formation of Influenza Virus Transfectants

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cDNA-derived RNAs were introduced into the genomes of influenza viruses by using an improved ribonucleoprotein (RNP) transfection protocol. Up to 10^5 viral transfectants with a novel neuraminidase gene could be obtained by using a 35-mm dish (10^6 cells) for RNP transfection. In addition to genes coding for surface proteins (hemagglutinin and neuraminidase), we also exchanged a gene coding for nonsurface proteins. The cDNA-derived influenza A/PR/8/34 virus NS gene was introduced into a temperature-sensitive mutant with a defect in this gene. We suggest that the term influenza virus transfectant be used for those viruses which are made by RNP transfection with cDNA-derived RNA.

We recently succeeded in introducing site-specific mutations into the genome of influenza A virus (3). This was achieved by reconstituting in vitro an active ribonucleoprotein (RNP) complex from RNA and purified viral proteins. This RNP complex was then transfected into cells which had been infected with helper virus. Although this technique allowed the rescue of a virus containing a neuraminidase (NA) gene with site-specific mutations, the efficiency of rescue was low. Starting with a 35-mm dish (10^6 cells), a total of 2.5×10^2 transfectants were obtained under optimal conditions (3).

In this report, we describe an improved RNP transfection protocol which increases the yield of transfectants by a factor of at least 100. This was made possible by coupling the in vitro transcription from cDNA with the actual assembly of the RNP. We used this protocol to rescue influenza viruses with a novel NA gene and to replace the hemagglutinin (HA) gene of influenza virus with a cDNA-derived HA RNA. In the latter case, an antibody screen was used for selection of the transfectants. The higher efficiency of the new RNP transfection protocol then allowed us to use a temperature-sensitive mutant as a helper virus and to introduce a cDNA-derived gene coding for the nonstructural proteins NS1 and NS2 into the genome of influenza virus.

We suggest the term influenza virus transfectant for these rescued viruses to indicate that the resulting virus derives RNA from cloned cDNA. Thus, influenza virus transfectants may contain mutations or chimeric genes which are not present in natural variants or reassortant viruses.

Improved transfection method. Compared with the previous method, the major difference of the new transfection protocol is that the viral proteins are present during the in vitro transcription of RNA. This is done to allow the formation of an RNP complex in statu nascendi. It was hoped that these conditions would favor the formation of biologically active RNP complexes. Initial experiments revealed that the chloramphenicol acetyltransferase (CAT) activity in RNP-transfected cells was indeed increased by using the new RNP transfection technique (Table 1). Optimization of the technique suggested that 15 min of T7 polymerase incubation was maximal and that the system depended on the presence of influenza viral proteins. It was also found that an increase of the total reaction mixture did not lead to an increase of transfection activity, i.e., by increasing all components by a factor of two (including the volume of the mixture), the CAT activity remained the same (data not shown).

Exchange of NA gene. We then proceeded to apply the new transfection protocol to the rescue of infectious influenza virus with cDNA-derived RNA. The WSN NA gene was introduced into the genome of influenza virus by RNP

TABLE 1. Optimization of RNP transfection system with CAT RNA[a]

Step	CAT activity
Previously published system	1
New system	210
Incubation at 31°C	70
Incubation for 30 min	90
20 μl of influenza virus proteins	100
4 μl of T7 RNA polymerase	200
Minus DNase	90
Minus DNA	<0.05
Minus influenza virus proteins	<0.05

[a] The viral nucleoprotein and RNA polymerase proteins were purified from influenza A/PR/8/34 virus. Virus was first detergent disrupted, and the RNP fraction was isolated by glycerol centrifugation. The purified RNP was then centrifuged through a CsCl-glycerol gradient. Fractions containing the nucleoprotein and polymerase proteins were pooled and dialyzed (8). Before the transfection experiment, these viral proteins were pretreated by UV irradiation (for 2 min on ice at 10-cm distance with an 8-W germicidal UV lamp G8T5/GL-8) which destroyed the viral RNA but had no effect on the biological activity of the viral proteins. Plasmid DNA (1 μg), 10 μl of influenza virus polymerase and nucleoprotein (1 μg of total protein), and 2 μl of bacteriophage T7 RNA polymerase (50 U/μl; Stratagene) were incubated at 37°C for 15 min in the presence of 40 mM Tris-hydrochloride (pH 8.0). A concentration of 0.5 mM each of the four deoxynucleoside triphosphates, 10 mM dithiothreitol, 8 mM $MgCl_2$, 50 mM NaCl, and 2 mM spermidine was mixed in a total volume of 50 μl. DNA was then digested with 2 μl of RQ1 DNase (1 u/μl; Promega Corp.) at 37°C for 5 min. This reaction mixture was then diluted with 100 μl of phosphate-buffered saline containing 0.1% gelatin and immediately used for transfection into cells. The DEAE-dextran RNP transfection protocol was identical to that described previously (4). The previously published system involves the use of 1 μg of purified RNA which is added to 20 μl (2 μg) of viral protein. The resulting RNP was then transfected into cells as described previously (4).

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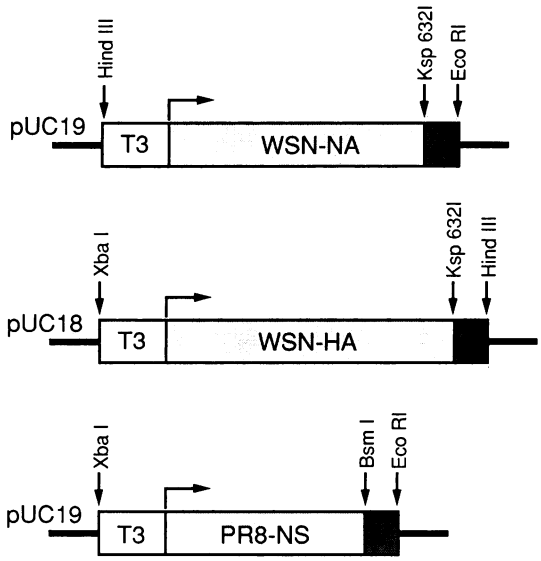

FIG. 1. Diagram of plasmids coding for influenza virus NA, HA, and NS genes. The pUC19-derived plasmids containing a truncated bacteriophage T3 RNA polymerase promoter, an influenza WSN virus NA or HA gene, and the restriction enzyme site *Ksp* 632I were used for T3 polymerase runoff transcription. pT3/WSN-NA was previously described (3). pT3/WSN-HA was constructed by polymerase chain reaction with the primer pair 5'-GCGCGCTCTA GAAATTAACCCTCACTAAAGTAGAAACAAGGGTGTTTT TCC-3' and 5'-GCGCGCAAGCTTCTCTTCGAGCAAAAGCAGG GGAAAATAAAAAC-3' and viral RNA following procedures described previously (3). The pT3/PR8-NS, which contains the phage T3 RNA polymerase promoter and the influenza A/PR/8/34 virus NS cDNA followed by the *Bsm*I restriction enzyme site, was constructed in the same way with primers 5'-GCGCGCTCTAGAAT TAACCCTCACTAAAGTAGAAACAAGGGTGTTTTTTATTA TTA-3' and 5'-GCGCGCGAATTCGAATGCGAGCAAAAGCAG GGTGACAAAGACAT-3'.

transfection with pT3/WSN-NA (Fig. 1). The new technique resulted in the formation of 5×10^4 to 1×10^5 transfectants, which formed plaques in MDBK cells, while the previous protocol yielded only 1×10^2 to 2.5×10^2 PFU (Table 2). The efficiency of rescue was thus at least 100-fold higher than previously reported. The identity of the transfectants was not studied because it had previously been demonstrated that only viruses containing the WSN NA gene would form plaques in MDBK cells (9). Similar results but lower yields were obtained by using the infectious center assay (Table 2).

Exchange of HA gene. To introduce a novel HA gene, we did an RNP transfection experiment using plasmid pT3/WSN-HA and HK-WSN virus as the helper virus. The latter virus was obtained in our laboratory by coinfection of cells with HK and WSN virus and was shown to derive all genes from WSN virus except for the HK HA gene (7). The helper virus was grown in 10-day-old chicken eggs at 37°C for 2 days (9). The RNP transfection experiment was performed in MDBK cells. After 16 h, the yield was plaqued on fresh MDBK cells in the presence of 0.1% anti-HK antiserum which was prepared as previously reported (9). Up to 10^4 transfectants were obtained with a 35-mm dish (Table 2). Virus from three plaques was amplified, and the presence of the WSN HA protein was confirmed in these viruses by hemagglutination inhibition tests with anti-WSN and anti-HK antisera (9). The yield of HA transfectants was lower than that of NA transfectants. This may have been because the antiserum used for selection suppressed the isolation of phenotypically mixed HA transfectants.

TABLE 2. Rescue of infectious influenza virus

Step	Transfectants[a]
WSN NA transfection (old system)[b]	2×10^2
WSN NA transfection (new system)[c]	8×10^4
Incubation at 31°C	2×10^4
Incubation for 30 min	4×10^4
20 μl of influenza virus proteins	4×10^4
4 μl of T3 RNA polymerase	8×10^4
Minus DNase	4×10^4
Minus DNA	ND[d]
Minus influenza virus proteins	ND
Infectious center assay[e]	2×10^3
WSN HA transfection (new system)[f]	1×10^4
PR8 NS transfection (new system)[g]	1×10^3

[a] The number of transfectants obtained from a 35-mm dish.
[b] Previously published RNP transfection system (4).
[c] Complete new RNP transfection system described in Table 1. pT3/WSN-NA and pT3/WSN-HA DNAs were digested with restriction enzyme *Ksp* 6321 (Boehringer Mannheim) and end filled with Klenow enzyme (Bethesda Research Laboratories) before use.
[d] ND, Not detectable.
[e] After RNP transfection, 10^6 cells were treated with 0.5 ml of 0.1% trypsin and 0.01% EDTA in phosphate-buffered saline at 37°C for 10 min and suspended in 1 ml of REM medium (11) containing 10% fetal calf serum. A portion of this cell suspension was mixed with 10^7 uninfected MDBK cells, poured onto 10-cm-diameter dishes, and incubated at 37°C for 3 h. Medium was then carefully removed, and the cells were overlaid with fresh medium containing 0.6% agar.
[f] The lower yield of HA transfectants compared with the yield of NA transfectants may be due to the use of antibody for selection.
[g] The low yield of NS transfectants may be due to the use of MDCK cells for RNP transfection and/or the use of a different helper virus (see text).

Exchange of NS gene. We then proceeded to introduce a gene coding for a nonsurface protein. The PR8 NS gene transcribed from plasmid pT3/PR8-NS was transfected at 33°C into MDCK cells which were infected with the temperature-sensitive host-range influenza virus mutant PD-11C-1/1-1-3P1 E2 (P1 virus), which was kindly provided by F. H. Maassab. This virus derives the HA, NA, NP, and M genes from influenza A/CAM/46 virus and the PB1, PB2, PA, and NS genes from the temperature-sensitive host-range mutant CR43-3 (5, 6). The NS gene of CR43-3 was previously shown to contain a 36-nucleotide deletion (2) and was found to be associated with the host-range-dependent temperature sensitivity of the virus (10). After RNP transfection and incubation at 33°C for 20 h, transfectants were selected by plaquing at 38°C. In a typical experiment, 10^3 transfectants were obtained from a 35-mm dish (Table 2). The viral RNA of a transfectant was characterized by RNA gel electrophoresis and shown to contain the slow-moving PR8-derived NS RNA (Fig. 2). The efficiency of RNP transfection in MDCK cells appears to be lower than that in MDBK cells. Previously, all rescue experiments were done in MDBK cells. However, since the helper virus P1 does not replicate in MDBK cells, we had to use MDCK cells for the above experiment. Surprisingly, transfection in the absence of plasmid DNA gave a low background of transfectants (total of 10^2 PFU). This most likely resulted from the presence of contaminating NS gene-containing RNP molecules in the viral protein preparation. This background could be eliminated by pretreatment of viral protein preparations by UV irradiation, which destroys the RNA but has no effect on the biological activity of the viral proteins.

The efficiency of the RNP transfection system now makes

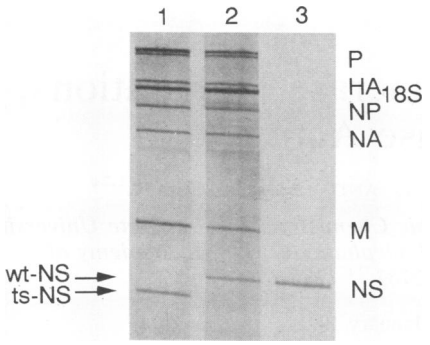

FIG. 2. Polyacrylamide gel electrophoresis of RNA purified from NS transfectant. RNAs were separated in 3% acrylamide–0.08% *N,N'*-methylenebisacrylamide gels containing 7.7 M urea in Tris-borate-EDTA buffer and were visualized by silver staining as described previously (3). Lane 1, 50 ng of viral RNA obtained from P1 helper virus grown in embryonated eggs. Lane 2, 50 ng of viral RNA of transfectant rescued by PR8 NS gene transfection. Lane 3, 10 ng of PR8 NS viral RNA synthesized in vitro by using pT3/PR8-NS plasmid and T3 RNA polymerase. RNAs encoding polymerase (P), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural proteins (NS) are indicated at right. The viral RNA preparation contains contaminating 18S rRNA. wt, Wild type; ts, temperature sensitive.

it possible to isolate transfectants with replacements in all the genes. In addition, the protocol is rapid since it avoids the step of isolating in vitro-transcribed RNA. The increase in efficiency of this new protocol is more than 100-fold compared with the previously published technique, and the present technology should thus allow the isolation of viruses without a selection screen. This will be important if the resulting transfectant (with a site-directed mutation) has no growth advantage over the helper virus used in the experiment and when chimeric genes are introduced into the genome.

The RNP transfection efficiency is still 10- to 100-fold lower than the optimal level for reassortment between genes of coinfecting influenza viruses (11). However, it is also clear that only RNP transfection (and not reassortment) provides a means to introduce site-specific mutations into the genome of a given influenza virus. The improved RNP transfection technology now makes it easier to modify different genes of influenza virus and to construct viruses with chimeric genes or altered transcription, replication, and packaging signals. Based on this technology, which was developed for influenza viruses, and a recent breakthrough by Ballart et al. (1) that allows isolation of infectious measles virus from cDNA-derived RNA, new avenues have been opened for the study of negative-strand viruses.

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