Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3802–3805, May 1990
Microbiology

Introduction of site-specific mutations into the genome of influenza virus

(negative-sense virus/rescue of synthetic RNA/viral selection system)

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Communicated by Robert M. Chanock, March 12, 1990

ABSTRACT **We succeeded in rescuing infectious influenza virus by transfecting cells with RNAs derived from specific recombinant DNAs. RNA corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 (WSN) virus was transcribed *in vitro* from plasmid DNA and, following the addition of purified influenza virus RNA polymerase complex, was transfected into MDBK cells. Superinfection with helper virus lacking the WSN NA gene resulted in the release of virus containing the WSN NA gene. We then introduced five point mutations into the WSN NA gene by cassette mutagenesis of the plasmid DNA. Sequence analysis of the rescued virus revealed that the genome contained all five mutations present in the mutated plasmid. The ability to create viruses with site-specific mutations will allow the engineering of influenza viruses with defined biological properties.**

Influenza viruses are negative-strand RNA viruses that possess a segmented genome (1). Although most DNA viruses (2–4), as well as most positive-strand RNA viruses (5–7), can be altered through site-specific mutagenesis using recombinant DNA, the negative-strand viruses have proven refractory to this approach. The (naked) RNA of negative-sense RNA viruses, unlike that of the positive-sense RNA viruses, is noninfectious when introduced into cells and until now virus-recovery ("rescue") experiments involving cDNA-derived RNAs were not feasible. This difficulty in modifying the genomes of negative-strand RNA viruses has slowed our progress in understanding the replication and the pathogenicity of the negative-strand virus groups.

In previous experiments we have shown that the 3′-terminal 15 nucleotides of negative-strand influenza virus RNAs are sufficient to allow transcription *in vitro* with purified influenza virus polymerase proteins (8). In addition, our studies using a reporter gene encoding chloramphenicol acetyltransferase have shown that the 5′-terminal 22 nucleotides and the 3′-terminal 26 nucleotides of the viral RNAs contain all the signals necessary for transcription, replication, and packaging of influenza virus RNAs (9). In this paper we describe the rescue of synthetic RNAs, derived from appropriate recombinant plasmid DNAs, into stable and infectious influenza viruses.

MATERIALS AND METHODS

Viruses and Cells. Influenza A/WSN/33 (WSN) virus was grown in Madin–Darby canine kidney (MDCK) cells (10). Influenza A/WSN-HK (WSN-HK) virus, a reassortant containing seven genes from WSN virus and the neuraminidase (NA) gene from influenza A/HK/8/68 virus, was grown in embryonated chicken eggs (11). Influenza A/PR/8/34 virus was also grown in embryonated eggs. Madin–Darby bovine kidney (MDBK) cells were used for the transfection experiments and for selection of rescued virus (9, 10).

Construction of Plasmids. The pT3NAv, pT3NAv mut1, and pT3NAv mut2 plasmids were constructed by DNA polymerase chain reaction (PCR)-directed mutagenesis using a cloned copy of the WSN NA gene, which was obtained by standard procedures (11). To construct pT3NAv, the PCR primers were 5′-CGGAATTCTCTTCGAGCGAAAGCAGGAGTT-3′ and 5′-CCAAGCTTATTAACCCTCACTAAAAGTAGAAACAAGGAGTTT-3′. After 35 cycles in a thermal cycler (Coy Laboratory Products, Ann Arbor, MI), the PCR product was digested with *Eco*RI and *Hin*dIII and cloned into pUC19. Plasmid pT3NAv mut1 was constructed in a similar fashion except that the sequence of the primer was altered (Fig. 1). Plasmid pT3NAv mut2 was constructed by cassette mutagenesis through the digestion of pT3NAv with *Pst* I and *Nco* I and religation in the presence of the synthetic oligonucleotides 5′-CATGGGTGAGTTTCGACCAAAATCTAGATTATAAAATAGGATACATATGCA-3′ and 5′-TATGTATCCTATTTTATAATCTAGATTTTGGTCGAAACTCACC-3′. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. The final clones pT3NAv, pT3NAv mut1, and pT3NAv mut2 were grown up and the DNAs were partially sequenced starting from the flanking pUC19 sequences and reaching into the coding sequences of the NA gene. The mutations in pT3NAv mut2 were also confirmed by sequencing.

Purification of Influenza A Virus RNA Polymerase and Ribonucleoprotein (RNP) Transfection of MDBK Cells. The RNA polymerase complex was purified from influenza A/PR/8/34 virus as described (8) and was then used for RNP transfection of MDBK cells. The transfection procedure followed the protocol provided earlier (9) except that WSN-HK virus was used as a helper virus at a multiplicity of infection of 1. RNAs used for RNP transfection were obtained by phenol extraction of purified virus or by transcription (using bacteriophage T3 polymerase) of pT3NAv, pT3NAv mut1, and pT3NAv mut2 (8, 9). All plasmids were digested with *Ksp*632I, end-filled by Klenow enzyme (BRL), and then transcribed in a runoff reaction (9).

RESULTS

Rescue of Infectious Influenza Virus from MDBK Cells Transfected with RNA Derived from Recombinant Plasmid DNA. A plasmid, pT3NAv, was constructed to contain the complete NA gene of influenza WSN virus downstream of a truncated

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Abbreviations: NA, neuraminidase; RNP, ribonucleoprotein; PCR, polymerase chain reaction; MDCK, Madin–Darby canine kidney; MDBK, Madin–Darby bovine kidney; WSN virus, influenza A/WSN/33 virus; WSN-HK virus, reassortant virus containing seven genes from WSN virus and the NA gene from influenza A/HK/8/68 virus.

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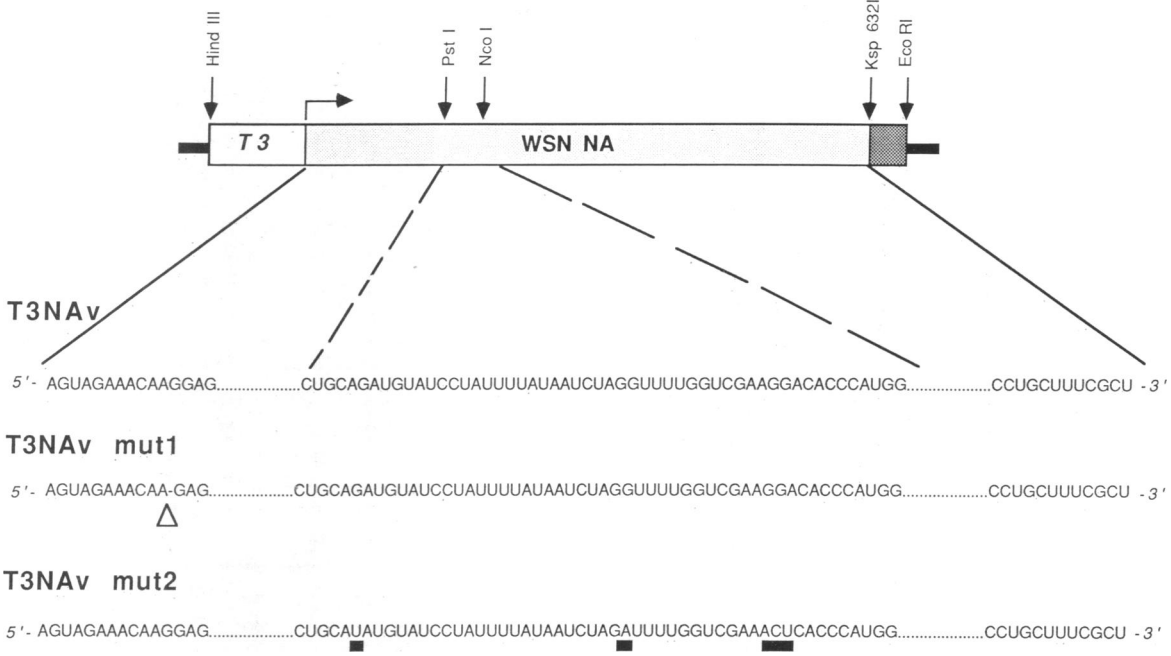

FIG. 1. Relevant portions of the NA gene-containing plasmids used for transfection experiments. The pUC19-derived plasmid pT3NAv contains the influenza WSN virus NA gene and a truncated promoter specifically recognized by bacteriophage T3 RNA polymerase. The T3 promoter was truncated so that the initial transcribed nucleotide (an adenine) corresponded to the 5′ adenine of the WSN NA gene. At the 3′ end of the cDNA copy of the NA gene, a *Ksp*632I restriction enzyme site was inserted so that the cleavage site occurred directly after the 3′ end of the NA gene sequence. A 1409-nucleotide-long transcript was obtained following *Ksp*632I digestion and transcription by T3 RNA polymerase of pT3NAv (see *Materials and Methods*). The 5′-terminal 15 nucleotides, the 52 nucleotides corresponding to the region between the *Nco* I and *Pst* I sites, and the 3′-terminal 12 nucleotides are shown. The transcript of pT3NAv mut1 is identical to that of pT3NAv except for a single deletion, 11 nucleotides downstream from the 5′ end of the wild-type RNA. The transcript of pT3NAv mut2 is identical to that of pT3NAv except for five mutations located in the central region (indicated by bars below the sequence). These five mutations do not change the amino acid sequence in the open reading frame of the gene. The serine codon UCC at positions 887–889 (plus-sense RNA) was replaced with the serine codon AGU in the same frame. The numbering of nucleotides follows ref. 13.

T3 promoter (Fig. 1). Runoff transcription of the plasmid, cut at the *Ksp*632I site, yielded an RNA identical in length to the true genomic NA gene of the WSN virus (Fig. 2, lane 3). This RNA was then incubated with purified polymerase (8) and used in an RNP transfection experiment to allow the rescue of infectious virus from cells infected with helper virus. The choice of WSN-HK virus as helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, it was shown (12) that the WSN-HK virus can form plaques in MDBK cells only when protease is added to the medium. This is in marked contrast to WSN virus (isogenic to WSN-HK helper virus except for the NA gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (10). MDBK cells were infected with the WSN-HK helper virus and then RNP-transfected 1 hr later. Following overnight incubation in the presence of plasminogen (20 μg/ml), supernatant from these cells was then amplified and plaqued in MDBK cells in the absence of protease in the medium. The appearance of plaques in MDBK cells (12) suggested the presence of virus that contained the WSN virus NA gene, since supernatant from control experiments of cells infected only with the WSN-HK virus did not produce plaques. In a typical experiment involving the use of a 35-mm dish for the RNP transfection, 2.5×10^2 plaques were observed.

In another control experiment, synthetic NA RNA was used that was derived from plasmid pT3NAv mut1 (Fig. 1). This RNA differs from the wild-type NA RNA derived from pT3NAv by a single nucleotide deletion in the nontranslated region at the 5′ end (Fig. 1). RNP transfection of MDBK cells with this RNA and superinfection with WSN-HK virus did not result in the formation of rescued virus. This negative result was not unexpected, since we had shown earlier that the essential sequences for the recognition of viral RNA by viral polymerases as well as the packaging signals are located within the 3′ and 5′ terminal sequences of the viral RNAs (8, 9). However, at this time we cannot exclude the possibility that rescue of virus with this mutated RNA does occur, but at a frequency too low for detection.

RNA Analysis of Rescued Virus. Virus obtained in the rescue experiment was plaque-purified and amplified in MDBK cells, and RNA was extracted from this preparation. The RNA was then analyzed by electrophoresis in a polyacrylamide gel. Fig. 2 shows the RNA of the helper virus WSN-HK (lane 1) and the synthetic NA RNA (lane 3), which was transcribed by T3 polymerase from plasmid pT3NAv. The migration pattern of the RNAs of the rescued virus (lane 2) was identical to that of control WSN virus (lane 4). Also, the NA RNAs in lanes 2 and 4 migrated at the same position as the NA RNA derived from cDNA (lane 3) and faster than the HK virus NA band in the helper WSN-HK virus (lane 1). These experiments thus suggest that as a result of the RNP transfection, infectious virus was formed containing WSN virus NA RNA derived from cDNA.

Rescue of Infectious Influenza Virus After Transfection of Virion RNA. In another transfection experiment we used RNA extracted from purified WSN virus. When this naked RNA was transfected together with the polymerase proteins into helper virus-infected cells, rescue of WSN virus capable of replicating in MDBK cells was observed. When RNA isolated from an amplified plaque in this experiment was analyzed (Fig. 2, lane 5) it showed a pattern indistinguishable from that of the control WSN virus (lane 4).

Introduction of Site-Specific Mutations into the Viral Genome. The experiments described so far involved the rescue of influenza WSN virus. Since the synthetic RNA used in

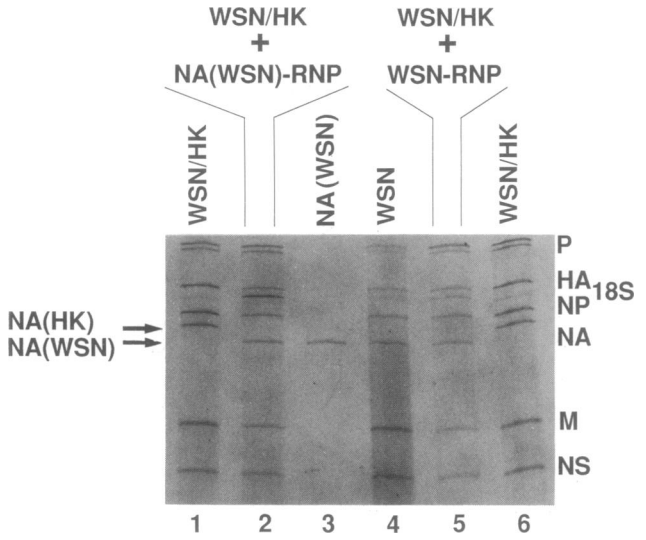

FIG. 2. Polyacrylamide gel electrophoresis of RNAs purified from rescued influenza viruses. One microgram of RNA transcript of pT3NAv (Fig. 1) or of phenol-extracted RNA derived from WSN virus was mixed with purified polymerase preparations according to the protocol of Luytjes *et al.* (9). These reconstituted RNPs were then transfected into MDBK cells that had been infected 1 hr earlier with WSN-HK helper virus. The medium, containing plasminogen at 20 μg/ml, was harvested after 16 hr and yielded a hemagglutination titer of 128, corresponding to ≈10^6 plaque-forming units (12) when measured in MDCK cells. Virus was then plaqued on MDBK cells in the absence of protease and further amplified in MDBK cells, and RNA was phenol-extracted from purified virus preparations (8–10). RNAs were separated in 2.8% acrylamide/0.075% *N,N'*-methylenebisacrylamide gels containing 7.7 M urea in Tris/borate/EDTA buffer and were visualized by silver staining (8). Lanes 1 and 6, WSN-HK virus RNA; lane 2, RNA of virus rescued from MDBK cells following RNP transfection with pT3NAv-derived NA RNA and infection with WSN-HK helper virus; lane 3, NA RNA transcribed *in vitro* from pT3NAv; lane 4, RNA of control WSN virus; lane 5, RNA of virus rescued from MDBK cells following RNP transfection with phenol-extracted WSN virus RNA and infection with WSN-HK helper virus. RNAs that encode polymerase proteins (P), hemagglutinin (HA), nucleoprotein (NP), matrix proteins (M), and nonstructural proteins (NS) are indicated at right.

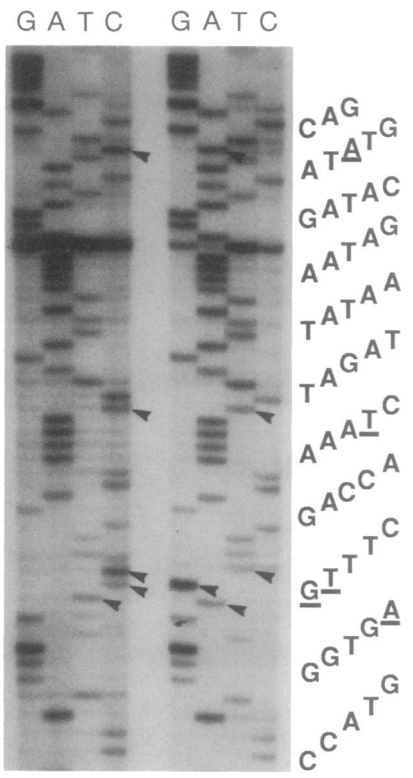

FIG. 3. Sequence analysis of RNA obtained from rescued influenza virus containing five site-specific mutations. Following infection with the WSN-HK helper virus, MDBK cells were RNP-transfected with T3NAv mut2 RNA obtained by transcription from pT3NAv mut2. After overnight incubation in the presence of plasminogen (20 μg/ml), medium was used for propagation and plaquing on MDBK cells in the absence of protease. Virus from plaques was then amplified and RNA was obtained after phenol extraction of purified virus. Rescue of the mutant NA gene into virus particles was verified through direct RNA sequencing using 5'-TACGAGGAATGTTCCTGTTA-3' as primer (corresponding to positions 800–819) (13) and reverse transcriptase (14). Sequences shown correspond to positions 878–930 in the NA gene (13). The arrowheads and the underlined nucleotides indicate the changes in the mutant RNA compared to the wild-type RNA. Left: Control RNA obtained from influenza A/WSN/33 virus. Right: RNA of mutant virus rescued from MDBK cells which were RNP-transfected with T3NAv mut2 RNA and infected with helper virus WSN-HK.

these experiments was identical to the authentic WSN NA gene, the unlikely possibility of contamination by wild-type WSN virus could not be rigorously ruled out. Therefore, we introduced five silent point mutations into the coding region of the NA gene in plasmid pT3NAv. These mutations were introduced by cassette mutagenesis through replacement of the short *Nco* I–*Pst* I fragment of the NA cDNA. The five mutations in the cDNA included a C → T change at position 901 and a C → A change at position 925, creating a new *Xba* I site and destroying the original *Pst* I site, respectively. In addition, the entire serine codon at positions 887–889 of the cDNA clone was replaced with an alternative serine triplet (Fig. 1). RNP transfection of this mutagenized RNA (pT3NAv mut2) into helper virus-infected MDBK cells again resulted in the rescue of a WSN-like virus that grew in MDBK cells in the absence of added protease. When the RNA of this virus was examined by sequence analysis, all five point mutations present in the plasmid DNA (Fig. 1) were observed in the viral RNA (Fig. 3). Since it is extremely unlikely that these mutations evolved in the wild-type influenza WSN virus, we conclude that successful rescue of infectious influenza virus containing five site-specific mutations was achieved via RNP transfection of engineered RNA.

DISCUSSION

The present experiments extend our earlier studies that defined the cis-acting sequences required for transcription, replication, and packaging of influenza virus RNAs (8, 9). We now demonstrate that recombinant DNA techniques can be used to introduce site-specific mutations into the genomes of infectious influenza viruses.

Synthetic RNAs derived by transcription of plasmid DNA *in vitro* were used in RNP-transfection experiments to rescue infectious influenza virus. To enable selection of this virus, we chose a system that required the presence of a WSN-like NA gene in the rescued virus. Viruses containing this gene can grow in MDBK cells in the absence of protease in the medium (12). The helper virus WSN-HK does not grow under these circumstances. Clearly, alternative selection systems exist. For example, antibody screens or conditionally lethal mutants could be used to isolate rescued viruses containing RNAs derived from plasmid DNAs. In the present experiments we recovered viruses that were WSN virus-like. The WSN NA gene was derived from plasmid DNAs or from purified WSN virion RNA (Fig. 2, lanes 2 and 5). In the latter case, using whole virion RNA for the RNP transfection, we do not know whether other genes were also transferred to the rescued virus, since the helper virus shares the remaining seven genes with WSN virus. The rescued viruses had the expected RNA patterns (Fig. 2) and grew to titers in MDBK

or MDCK cells that were indistinguishable from those of the wild-type WSN virus (data not shown). It should be noted that rescue of an NA RNA containing a single nucleotide deletion in the 5′ nontranslated region was not possible. This again illustrates the importance of regulatory sequences present in the nontranslated regions of influenza virus RNAs (8, 9).

We also rescued virus using RNA that was engineered to contain 5 nucleotide changes in a 39-nucleotide region (Fig. 1). We verified the presence of these mutations in the rescued mutant virus by direct sequencing of the RNA (Fig. 3). These mutations did not result in any amino acid change in the NA protein and thus were not expected to change the biological properties of the virus. Although this virus was not extensively studied, its plaquing behavior and its growth characteristics were indistinguishable from those of wild-type WSN virus.

The introduction of mutations that change the biological characteristics of influenza viruses would help to define the precise functions of all the viral proteins, including those of the nonstructural proteins. The nontranslated regions of the genome could also be studied by mutagenesis, leading to a better understanding of the regulatory signals present in viral RNAs. Another area of great interest concerns the development of the influenza virus system as a vaccine vector, which will require the introduction of foreign genes into the influenza virus genome. Clearly, there will be limitations in this system, including the size of the foreign genes and the stability of such chimeric constructs. Nevertheless, the current approach has immediate promise in several areas, particularly with respect to the study of the function of viral proteins as well as of the signals involved in transcription, replication, poly(A) addition, and packaging of influenza viral RNAs.

We thank Kazue Enami for her superb technical assistance and Wayne Pan for help in the original cloning of the influenza WSN virus NA gene. This work was supported by Merit Award AI18998 (P.P.) and by U.S. Public Health Service Grants AI2663 (M.K.), AI24460 (P.P.), and AI11823 (P.P.) from the National Institutes of Health. M.K. is also supported by funds from the New York Lung Association and an Irma T. Hirschl Scholar Award.

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