

## Attenuation of Influenza A Virus mRNA Levels by Promoter Mutations

ERVIN FODOR,<sup>1†</sup> PETER PALESE,<sup>1\*</sup> GEORGE G. BROWNLEE,<sup>2</sup>  
AND ADOLFO GARCÍA-SASTRE<sup>1</sup>

*Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029,<sup>1</sup>  
and Sir William Dunn School of Pathology, University of Oxford,  
Oxford OX1 3RE, United Kingdom<sup>2</sup>*

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**We have engineered influenza A/WSN/33 viruses which have viral RNA (vRNA) segments with altered base pairs in the conserved double-stranded region of their vRNA promoters. The mutations were introduced into the segment coding for the neuraminidase (NA) by using a reverse genetics system. Two of the rescued viruses which share a C-G→A-U double mutation at positions 11 and 12' at the 3' and 5' ends of the NA-specific vRNA, respectively, showed approximately a 10-fold reduction of NA levels. The mutations did not dramatically affect the NA-specific vRNA levels found in virions or the NA-specific vRNA and cRNA levels in infected cells. In contrast, there was a significant decrease in the steady-state levels of NA-specific mRNAs in infected cells. Transcription studies in vitro with ribonucleoprotein complexes isolated from the two transfectant viruses indicated that transcription initiation of the NA-specific segment was not affected. However, the majority of NA-specific transcripts lacked poly(A) tails, suggesting that mutations in the double-stranded region of the influenza virus vRNA promoter can attenuate polyadenylation of mRNA molecules. This is the first time that a promoter mutation in an engineered influenza virus has shown a differential effect on influenza virus RNA transcription and replication.**

Influenza A virus is a negative-strand RNA virus belonging to the orthomyxovirus family. It has a segmented genome consisting of eight single-stranded RNA molecules (27). During the replication cycle of the virus, the viral genome (vRNA) is transcribed into mRNA and replicated into cRNA molecules, which in turn are used as templates for vRNA synthesis (16). These processes are known to be catalyzed by the viral polymerase complex consisting of three subunits, PB1, PB2, and PA (13). mRNA synthesis is initiated by capped RNA primers which are cleaved from host cell mRNA by an endonuclease associated with the viral polymerase complex. The synthesis of mRNA is prematurely terminated at a run of five to seven uridines 16 or 17 nucleotides (nt) away from the 5' end of the vRNA template, and subsequently a poly(A) tail is added. On the other hand, cRNA synthesis is believed to be initiated in the absence of primer resulting in a full-length precise copy of vRNA. The nucleoprotein (NP) has been implicated as a switching factor by acting as an antiterminator during cRNA synthesis (1).

The development of ribonucleoprotein (RNP) reconstitution and transfection systems allowed detailed characterization of the RNA signals involved in the regulation of transcription initiation, termination, and polyadenylation (4, 23–25, 28, 35, 37). All of these signals are known to reside in the terminal sequences of vRNA segments (22). The 5' and 3' ends contain 13 and 12 conserved nt, respectively, which have the ability to form a partially double stranded panhandle/RNA-fork or corkscrew structure (6, 8, 14). Initial in vitro transcription studies with model RNA templates implied that vRNA and cRNA promoters were located exclusively in the 3'-terminal se-

quences (28, 35), and the panhandle had no apparent role in the initiation of transcription in vitro. However, detailed mutagenesis studies of the terminal sequences showed that the 5' end forms an integral part of the promoter. These findings were based on binding experiments of the RNA polymerase to the putative promoter RNA (8, 36) and, more importantly, on in vitro transcription studies with mutant model template RNAs (8, 9, 31). In addition, activation of the viral polymerase-associated endonuclease requires interaction of the polymerase complex with the 5' - as well as the 3'-terminal sequences of vRNA segments (2, 12).

The polyadenylation site for mRNAs has been mapped to the sequence of five to seven uridines near the 5' end of vRNA (34). Since the stretch of U residues is adjacent to the panhandle/RNA-fork structure, it has been postulated that polyadenylation occurs by stuttering of the viral polymerase at the RNA duplex and repeated copying of the U sequence. Initial studies confirmed that the RNA duplex structure adjacent to the stretch of U residues is indeed required for polyadenylation (19, 21). Identification of a major polymerase binding site at the 5' end of vRNA led to the proposal of an alternative model for polyadenylation of mRNA (8, 36). According to the new model, during mRNA elongation the RNA polymerase remains bound to the 5' end of the template vRNA. Upon reaching the U stretch, "stuttering addition" of a poly(A) sequence occurs by the polymerase trying to transcribe the 5' end to which it is bound. Recent findings that a functional polymerase binding site at the 5' end is required for polyadenylation to occur support this hypothesis (32).

In the present report, we describe the effect of base pair mutations in the conserved double-stranded region of the RNA-fork in the context of infectious viruses. Using the rescue system for the neuraminidase (NA) gene (4), we introduced double mutations into the NA segment of influenza A/WSN/33 virus. We found that a double mutation at positions 11 and 12' resulted in attenuated NA-specific mRNA levels, but vRNA and cRNA levels were not dramatically affected.

\* Corresponding author. Mailing address: Department of Microbiology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029. Phone: (212) 241-7318. Fax: (212) 722-3634. E-mail: ppalese@smtplink.mssm.edu.

† Permanent address: Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic.

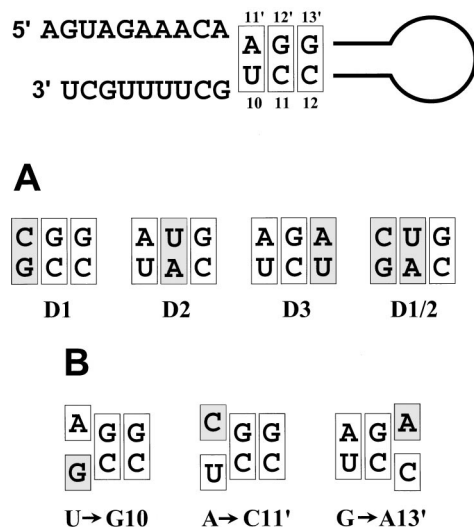


FIG. 1. Representation of the conserved sequences of influenza A virus vRNA in the panhandle/RNA-fork conformation (8, 14). Conserved base pairs in the double-stranded region of the RNA-fork, involving both the 5' and 3' ends of the RNA segment, are boxed. Numbering of residues starts from the 3' end and from the 5' end. The 5'-end numbers are distinguished by primes. (A) Base pairs of D1, D2, D3, and D1/2 transfectant viruses in the conserved double-stranded region. Changed base pairs are highlighted. (B) Sequences of single-nucleotide mutants.

## MATERIALS AND METHODS

**Cells and viruses.** Influenza A/WSN/33 wild-type virus and transfectant viruses were grown in Madin-Darby bovine kidney (MDBK) cells in reinforced minimal essential medium. Influenza WSN-HK virus, a reassortant virus containing seven genes from influenza A/WSN/33 virus and the NA gene from influenza A/HK/8/68 virus, was grown in 10-day embryonated chicken eggs. Influenza X-31 virus, a reassortant of influenza A/HK/8/68 and A/PR/8/34 viruses, was supplied by Evans Biological, Ltd., Liverpool, England. RNP transfections, selection and plaque purification of rescued transfectant viruses, and plaque assays were performed on MDBK cells.

**Construction of plasmids.** Plasmid pT3NAM1 contains the full-length cDNA of the wild-type NA gene of influenza A/WSN/33 virus flanked by a bacteriophage T3 RNA polymerase promoter at one end and a unique *Bbs*I restriction site at the other end (10). To construct plasmids encoding NA genes with mutations in the terminal noncoding sequences, PCR products were made by using pT3NAM1 as the template and the following primers with mutations as specified in Fig. 1: 5'-CGGAATTGGAAGAGCGAGCAAAAGCAGGAGTTTAAATGAATCC-3' and 5'-CCAAGCTTATTAACCTCACTAAAAGTAGAACAAGGAGTTTGAAC-3' (the residues at which mutations were introduced are underlined). The PCR products were digested with restriction enzymes *Eco*RI and *Hind*III and cloned into pT3NAM1 cut with the same enzymes. NA genes and the flanking sequences in the modified plasmids were sequenced with an automated sequencer (Applied Biosystems).

**RNP transfection.** The transfectant viruses were prepared as described earlier (5). Briefly, synthetic RNAs were obtained by T3 RNA polymerase transcription of modified pT3NAM1 plasmids linearized with restriction enzyme *Bbs*I. RNAs were reconstituted into RNP complexes by using RNA polymerase and NP isolated from influenza X-31 virus and were transfected by the DEAE-dextran transfection method into MDBK cells infected with influenza WSN-HK helper virus. Rescued transfectant viruses were plaque purified three times in MDBK cells.

**Sequencing of the NA genes of transfectant viruses.** Viral RNA for sequencing was isolated by phenol-chloroform extraction from transfectant viruses purified through a 30% sucrose cushion. In some cases, total RNA isolated with RNazol B (Tel-Test, Inc., Friendswood, Tex.) from infected cells was used. Sequences of the 5' end were obtained either by direct RNA sequencing or by rapid amplification of 5' DNA ends (5' RACE). Direct sequencing of the 5' ends was performed by using a primer complementary to nucleotide positions 1280 to 1299 (5'-TG GACTAGTGGGAGCATCAT-3') of the WSN NA gene and an RNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) as instructed by the manufacturer. For 5' RACE, vRNA was reverse transcribed by using a primer complementary to nt 879 to 898 (5'-GGGTGTCCTTCGACCAAAAC-3') of the WSN NA gene. The reverse transcription product was extended with terminal deoxynucleotidyltransferase (Gibco BRL, Gaithersburg, Md.) and amplified by PCR with the primer used for direct RNA sequencing (see above) and the 5' RACE abridged anchor primer (Gibco BRL). PCR products cut with

restriction enzyme *Spe*I were cloned into the *Xba*I site of pUC18 and sequenced with a DNA sequencing kit (United States Biochemical). To sequence the 3' end of the NA gene of transfectant viruses, viral RNA was 3' polyadenylated by using poly(A) polymerase (Gibco BRL). The polyadenylated RNA was reverse transcribed by using the primer 5'-GCGCAAGCTTCTAGATTATTTTATTTT-3', and the cDNA was amplified by PCR with a primer containing nucleotides corresponding to positions 115 to 98 (5'-GCGCAAGCTTTATTGAGATTATA TTTCC-3') of the WSN NA gene and the primer used for reverse transcription. PCR products digested with *Hind*III were cloned into pUC18 and sequenced with the DNA sequencing kit.

**Virus purification and PNGase F digestion.** Influenza A/WSN/33 and transfectant viruses were grown in MDBK cells and purified by 30 to 60% sucrose gradient ultracentrifugation. About 10 µg of viral proteins was denatured with 0.5% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol at 100°C for 10 min and digested with 400 U of peptide *N*-glycosidase F (PNGase F; New England Biolabs, Inc., Beverly, Mass.) for 20 h at 37°C in a reaction buffer containing 50 mM sodium phosphate (pH 7.5), 1% Nonidet P-40 (NP-40), and 5 mM Pefabloc (Boehringer Mannheim Corporation, Indianapolis, Ind.). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide gel and staining with Coomassie brilliant blue.

**NA activity determination.** About 2, 0.5, 0.125, and 0.031 µg (fourfold dilutions) of proteins from purified virus were incubated for 10 min at 37°C in 150 mM phosphate buffer (pH 6.0)–1 mM CaCl<sub>2</sub> containing 50 nmol of 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MU-NANA) as the substrate in a total volume of 100 µl (30). Then 2 ml of stop buffer (0.5 M glycine-NaOH [pH 10.4]) was added, and the released 4-methylumbelliferone was determined by spectrofluorometry. A 0.1 mM solution of 4-methylumbelliferone was used as a standard control. NA activity was expressed as nanomoles of 4-methylumbelliferone released in 1 min per microgram of viral proteins.

**RNA primer extension.** vRNA from wild-type and transfectant viruses purified through a 30% sucrose cushion was extracted with phenol-chloroform. To isolate total RNA from infected cells, MDBK cells were infected with either wild-type or transfectant virus at a multiplicity of infection (MOI) of 2, and RNA was extracted from cells with RNazol B (Tel-Test) at the indicated time points postinfection. Primer extension analysis of NA and nonstructural protein (NS) vRNA levels was performed as previously described (20). Briefly, 100 ng of viral RNA or 5 µg of total RNA was transcribed with 200 U of SuperScript (Gibco BRL) for 1 h at 42°C in the presence of 3 × 10<sup>5</sup> cpm of <sup>32</sup>P-labeled NA- and NS-specific primers. The NA-specific primer, 5'-GTGGCAATAACTAATCGG TCA-3', is complementary to nt 1151 to 1171 of the NA vRNA. The NS-specific primer, 5'-GGGAACAATTAGGTCAGAGT-3', is complementary to positions 695 to 715 of the NS vRNA. Primer extension analysis of NA and hemagglutinin (HA) mRNA and cRNA levels in total RNA from infected cells was performed under the same conditions as described above. The primer for NA-specific mRNA and cRNA, 5'-GCGCAAGCTTTATTGAGATTATATTTC-3', contains 18 nt (underlined) corresponding to positions 115 to 98 of the NA gene. The primer for the extension of HA-specific mRNA and cRNA, 5'-CAT ATTGTGTCTGCATCTGTAGCT-3', corresponds to positions 94 to 71 of the HA gene. Primer extension reactions were stopped by adding equal volume of 90% formamide and 10 mM EDTA followed by heating to 95°C for 3 min. Extension products were analyzed on 5% polyacrylamide gels in the presence of 7 M urea and quantitated by PhosphorImager (Molecular Dynamics) analysis of dried gels.

**Isolation of RNP complexes for in vitro transcription.** Wild-type influenza A/WSN/33 virus and D2 and D1/2 transfectants were grown in MDBK cells and purified on a 30% sucrose cushion. Twelve 15-cm dishes were used for each virus. The purified viruses were resuspended in 200 µl of phosphate-buffered saline and disrupted by addition of 50 µl of 5× disruption buffer (500 mM Tris-HCl [pH 7.4], 500 mM NaCl, 25 mM MgCl<sub>2</sub>, 5 mM dithiothreitol [DTT], 25% glycerol, 2.5% NP-40, 2.5% Triton X-100, 50 mg of lysocleithin ml<sup>-1</sup>) and incubation at 37°C for 30 min. The disrupted viruses were fractionated by centrifugation on a discontinuous glycerol gradient (70, 50, and 30%, 150 µl of each) in 100 mM Tris-HCl (pH 7.4)–100 mM NaCl–5 mM MgCl<sub>2</sub>–1 mM DTT. The gradients were centrifuged for 4 h at 15°C in 0.8-ml tubes at 45,000 rpm in a Beckman SW55 rotor with adapters. Fractions collected from the bottom of the tubes were analyzed by SDS-PAGE (12% gel), and those enriched in RNPs were used in transcription assays.

**In vitro transcription of RNP complexes and oligo(dT)-cellulose chromatography.** Transcription reactions were performed by using 6 µl of RNPs in a total reaction volume of 20 µl containing 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM ATP, 0.5 mM each GTP and CTP, 50 µM UTP, 0.1 µM [α-<sup>32</sup>P]UTP (3,000 Ci mmol<sup>-1</sup>), 20 U of RNase inhibitor (Boehringer Mannheim Corporation, Indianapolis, Ind.), and 0.6 µg of rabbit globin mRNA (Gibco BRL). After incubation at 31°C for 1.5 h, transcription products were extracted with phenol-chloroform and precipitated in the presence of 5 µg of carrier yeast RNA. Oligo(dT)-cellulose separation of transcription products was performed as described previously (32). Briefly, transcription products dissolved in water were diluted to 200 µl in binding buffer (10 mM Tris-HCl [pH 7.5], 500 mM NaCl) and then mixed with 50 mg of oligo(dT)-cellulose, and the mixture was incubated with agitation at room temperature for 1 h. Oligo(dT)-cellulose was pelleted by microcentrifugation, and the supernatant containing the poly(A)<sup>−</sup> fraction was collected. The pellet was washed twice

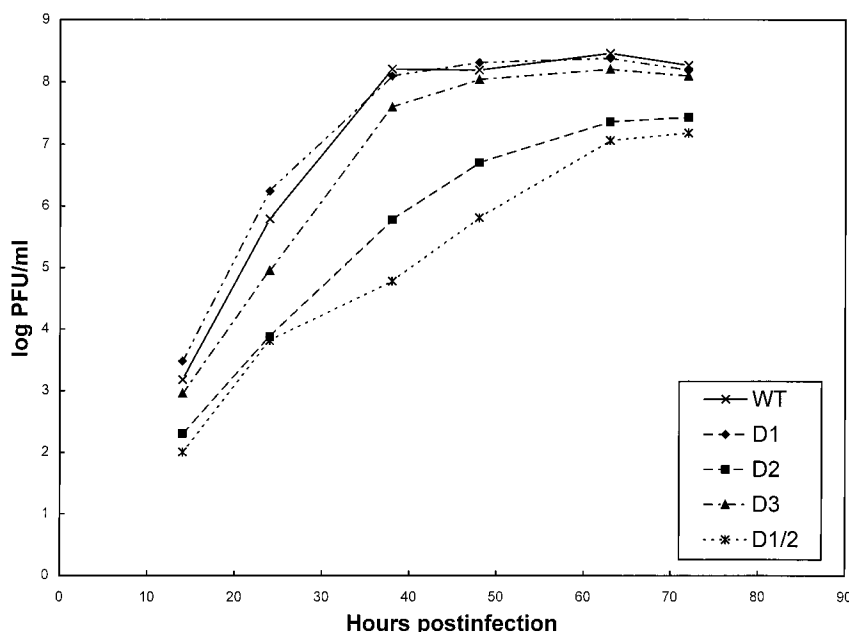


FIG. 2. Growth curves of transfectant influenza viruses on MDBK cells. Confluent MDBK cells in 35-mm-diameter dishes were infected with wild-type (WT) influenza A/WSN/33 virus and with the transfectant D1, D2, D3, and D1/2 viruses at an MOI of 0.01. At the indicated time points, infectious particles present in the media were titrated by plaque assay in MDBK cells. The presented values are averages from duplicate experiments.

in 1 ml of binding buffer, followed by incubation in 0.2 ml of low-salt buffer (10 mM Tris-HCl [pH 7.5], 250 mM NaCl) at 37°C for 15 min. The pellet was washed with an additional 0.2 ml of low-salt buffer, and the poly(A)<sup>+</sup> fraction was eluted with 0.2 ml of 10 mM Tris-HCl (pH 7.5) at 50°C for 15 min. Poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions were recovered by precipitation in the presence of 5 µg of carrier yeast RNA. RNA pellets were dissolved in 90% formamide–10 mM EDTA, heated to 95°C for 2 min, and analyzed on a 3% polyacrylamide gel containing 7 M urea.

## RESULTS

**Rescue of influenza viruses containing promoter mutations in the NA segment and growth characteristics.** Influenza virus transfectants were constructed with mutations in the double-stranded region of the panhandle/RNA-fork (8, 14) in order to study the effects of mutations on viral RNA transcription and replication in the context of infectious viruses. The following double mutations were introduced into the NA gene of influenza A/WSN/33 virus: U-A→G-C(10–11') (mutant D1), C-G→A-U(11–12') (mutant D2), and C-G→U-A(12–13') (mutant D3) (Fig. 1). These mutations were selected based on the results of a previous *in vivo* analysis of the double-stranded region of the vRNA promoter by using the chloramphenicol acetyltransferase (CAT) reporter gene (15). In addition, six NA genes with the corresponding single mutations (U→G10, A→C11', C→A11, G→U12', C→U12, and G→A13') were constructed. NA-specific RNP complexes were reconstituted *in vitro* and transfected into MDBK cells infected with A/WSN-HK helper virus (5). Transfection of all three NA genes with double mutations resulted in rescue of transfectant viruses (D1, D2, and D3). On the other hand, only three of the six single-mutation constructs, carrying mutations at positions 10, 11', and 13', were rescued (Fig. 1). In three attempts, none of the other three constructs (with mutations at positions 11, 12, and 12') was rescued.

The transfectant viruses were plaque purified three times on MDBK cells, and a single plaque was used for preparing a stock virus for further analysis. The presence of the double mutations in the D1, D2, and D3 transfectants was confirmed

by sequence analysis of the 3'- and 5'-terminal sequences of the NA gene. The mutation was also confirmed in the 5' end of the G→A13' transfectant virus with a single mutation. The 3' end of this transfectant was wild type as expected. Confirmation of mutations in the two other single-mutant transfectants was more difficult since they were unstable. Specifically, cloning of the 3' end of the NA vRNA of the U→G10 mutant resulted in one clone with mutant and two clones with wild-type sequences. Direct RNA sequencing of the 5' end of the NA-specific vRNA from the purified A→C11' transfectant, following three plaque-to-plaque passages, revealed a wild-type sequence. However, when NA-specific vRNA from MDBK cells infected with the original plaque of this transfectant was sequenced, the presence of the mutation was confirmed. Thus, it seems likely that the transfectant reverted to wild type during the plaque purification steps. This interpretation is supported by the observation that the transfectant initially produced small plaques but showed larger plaques upon passaging. Taken together, sequencing data for the single mutants show that transfectant viruses with single mutations, at least those with mutations at positions 10 and 11', are unstable. Although in all cases reversion of the single mutants resulted in wild-type sequences, we cannot exclude that phenotypic reversion may also occur by other compensatory mutations, since we did not analyze additional revertants. Because of the unstable nature of the single mutants, further work was focused on the double mutants.

Growth properties of transfectant viruses D1, D2, and D3 were analyzed on MDBK cells. Confluent monolayers of MDBK cells were infected at a low MOI (0.01), and the amount of infectious virus released into the medium was assayed at different time points by plaque assay on MDBK cells (Fig. 2). The D2 transfectant virus showed an approximately 1-log reduction in plaque titer compared to the wild-type virus. However, D1 and D3 transfectant viruses were not significantly affected by the mutations. Consistently, the plaque size of D2 was reduced,



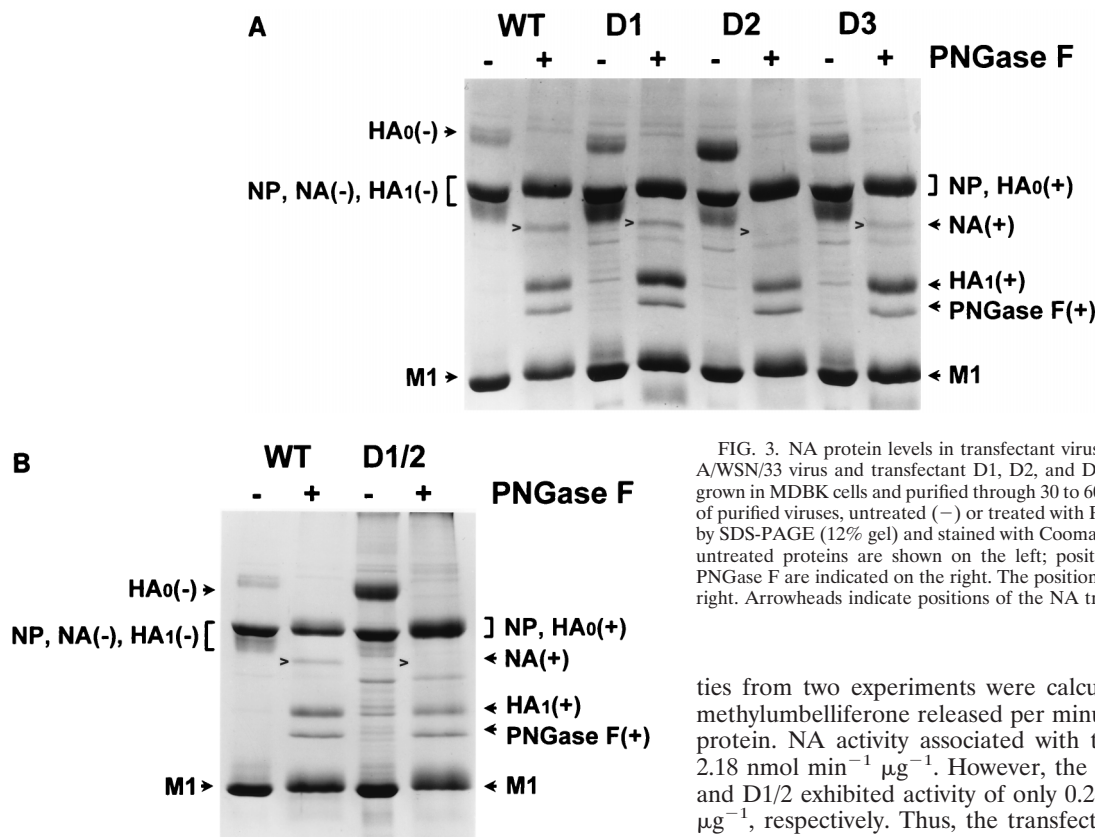


FIG. 3. NA protein levels in transfectant viruses. Wild-type (WT) influenza A/WSN/33 virus and transfectant D1, D2, and D3 (A) or D1/2 (B) virus were grown in MDBK cells and purified through 30 to 60% sucrose gradients. Proteins of purified viruses, untreated (–) or treated with PNGase F (+), were separated by SDS-PAGE (12% gel) and stained with Coomassie brilliant blue. Positions of untreated proteins are shown on the left; positions of proteins treated with PNGase F are indicated on the right. The position of PNGase F is shown on the right. Arrowheads indicate positions of the NA treated with PNGase F (17).

but both D1 and D3 viruses showed plaque sizes similar to that of the wild type.

Since the presence of single-base-pair mutations did not result in severe impairment of viral growth, an attempt was made to introduce multiple double mutations into the same region of the NA gene. A construct incorporating double mutations from both D1 and D2 transfectants was successfully rescued (D1/2) (Fig. 1) into infectious virus. The D1/2 transfectant was plaque purified three times, and the presence of mutations was confirmed by sequencing. This virus showed similar reduction in plaque titers (Fig. 2) and plaque size on MDBK cells as the D2 transfectant. The effect of the D1/2 mutations on viral growth was more dramatic on MDCK and Vero cells, where reductions of 3 to 4 logs in plaque titers were observed (data not shown).

**NA levels in transfectant viruses.** We examined whether the observed reduction in growth of the D2 and D1/2 viruses corresponds to lower NA levels expressed by these viruses. First, the protein composition of virions was determined. Viruses grown in MDBK cells and purified on sucrose density gradients were analyzed by SDS-PAGE, and the proteins were visualized by staining with Coomassie brilliant blue (Fig. 3). To obtain a better resolution of the NA band, which migrates closely to the NP and HA<sub>1</sub> bands on gels, virions were treated with PNGase F to remove N-linked carbohydrate chains from NA and HA. Both D2 and D1/2 virions showed a dramatic reduction in NA content compared to that of the wild-type virus or the D1 and D3 transfectants (Fig. 3).

To quantitate NA levels of the D2 and D1/2 viruses, NA activity was measured. Purified virus was incubated with MU-NANA as the substrate, and the released 4-methylumbelliferone was measured by spectrofluorometry. Average NA activi-

ties from two experiments were calculated as nanomoles of methylumbelliferone released per minute per  $\mu\text{g}$  of total viral protein. NA activity associated with the wild-type virus was  $2.18 \text{ nmol min}^{-1} \mu\text{g}^{-1}$ . However, the transfectant viruses D2 and D1/2 exhibited activity of only  $0.24$  and  $0.25 \text{ nmol min}^{-1} \mu\text{g}^{-1}$ , respectively. Thus, the transfectant viruses showed approximately a 10-fold reduction in NA activity compared to the wild-type virus, which is in agreement with the reduced NA levels observed in SDS-PAGE (see above).

**NA-specific vRNA levels in purified transfectant viruses.** vRNAs purified from wild-type and transfectant viruses were analyzed by PAGE, and the RNA segments were visualized by silver staining (Fig. 4). The NA segment was present in all transfectant viruses at levels comparable to that of the wild-

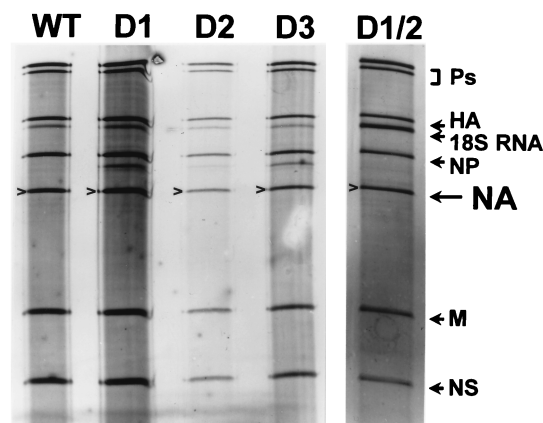


FIG. 4. vRNA of purified transfectant D1, D2, D3, and D1/2 viruses. RNAs extracted from purified wild-type (WT) A/WSN/33 and transfectant D1, D2, D3, and D1/2 viruses were separated in a 2.8% polyacrylamide gel containing 7 M urea and visualized by silver staining. The positions of RNAs that encode the polymerase proteins (Ps), HA, NP, NA, M1 and M2 proteins (M), and NS1 and NS2 (NS) are indicated. The position of 18S RNA, a contaminating cellular RNA often present in viral RNA preparations, is also indicated. Arrowheads indicate the position of the NA-specific vRNA segments. The origin of the faint band in lanes D1, D2, and D3, moving slightly faster than the NP, is unknown.

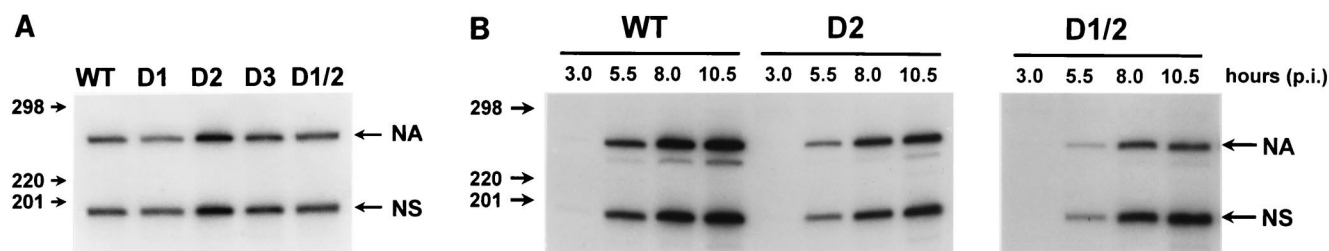


FIG. 5. NA-specific vRNA levels in purified transfectant D1, D2, D3, and D1/2 viruses (A) and in cells infected with transfectants D2 and D1/2 (B). (A) Viral RNA was extracted from wild-type (WT) A/WSN/33 virus and the D1, D2, D3, or D1/2 transfectant purified through a 30% sucrose cushion. (B) Total RNAs from MDBK cells infected at an MOI of 2 with wild-type (WT) A/WSN/33 virus and the D2 or D1/2 transfectant were extracted at the indicated time points postinfection (p.i.). Both viral and total RNAs were subjected to primer extension analysis, using primers specific for the NA and NS vRNAs, and the primer extension products were analyzed on a 5% polyacrylamide gel containing 7 M urea. The expected length of the primer extension products for the NA segment is 259 nt; that for the control NS segment is 196 nt. Size markers in nucleotides are shown on the left.

type virus. To quantify NA-specific vRNA levels, a primer extension analysis was performed with vRNA extracted from purified viruses (Fig. 5A). The NS gene was used as an internal control. The amounts of NA-specific vRNA segments in the transfectant viruses were similar ( $\pm 20\%$ ) to that of the wild-type virus in two experiments as determined by PhosphorImager analysis.

**NA-specific vRNA levels in cells infected with the D2 or D1/2 transfectant virus.** MDBK cells were infected with wild-type or transfectant virus at an MOI of 2, and total RNA was isolated from cells at 3.0, 5.5, 8.0, and 10.5 h postinfection. NA-specific vRNA levels in total RNA were measured by primer extension assay using the NS gene as an internal control. Cells infected with the D2 transfectant virus contained NA-specific vRNA levels similar ( $\pm 10\%$ ) to those infected with the wild-type virus (Fig. 5B). Although cells infected with the D1/2 transfectant virus showed a 28 to 53% reduction in NA-specific vRNA levels (results obtained by PhosphorImager analysis in two experiments at 5.5, 8.0, and 10.5 h postinfection), this decrease cannot account for the 10-fold reduction of NA protein levels.

**NA-specific mRNA and cRNA levels in cells infected with the D2 or D1/2 transfectant virus.** Since NA-specific vRNA levels were not dramatically affected by the mutations in the D2 and D1/2 transfectant viruses (Fig. 5), the 10-fold reduction in NA levels (see above) could result from a reduction in mRNA levels and/or from a defect in translation. To distinguish between these possibilities, the amounts of NA-specific mRNA in cells infected with the D2 or D1/2 transfectant virus were measured by using a primer extension assay (Fig. 6). MDBK cells were infected at an MOI of 2 with wild-type or transfectant virus, and total RNA was isolated at 3.0, 4.5, 6.0,

and 7.5 h postinfection. The primer extension assay was performed with an NA-specific primer and an HA-specific primer as an internal control. Since total RNA from infected cells contains both mRNA and cRNA, which differ only at their termini, signals for both species of RNAs were expected in the same primer extension assay. Due to the presence of a heterologous 10- to 15-nt-long capped primer at the 5' end of mRNA molecules, the signal for mRNA on gel appears as a multiple band containing DNA species of different sizes (Fig. 6). On the other hand, the signal for cRNA appears as a single band, which is approximately 10 to 15 nt shorter than the signal for mRNA. NA-specific mRNA levels in cells infected with either D2 or D1/2 transfectant virus were below detection levels (Fig. 6). PhosphorImager scanning of the gel shown in Fig. 6 suggests that the NA-specific mRNA levels were at least eightfold lower in the transfectant virus-infected cells than in wild-type virus-infected cells. However, NA-specific cRNA levels were apparently unaffected in these transfectant virus-infected cells. The additional band running slightly faster than the NA-specific cRNA band, detected in all samples, represents a nonspecific signal, since it was also detected in RNAs extracted from uninfected cells (data not shown).

**In vitro transcription of NA-specific RNP complexes.** Primer extension analysis of mRNA levels in infected cells showed that NA-specific mRNA levels are lower in cells infected with the D2 or D1/2 transfectant than in cells infected with the wild-type virus (see above). In theory, the reduction of mRNA levels could have been caused by a decrease in mRNA stability or by a decrease in mRNA synthesis. The interference with mRNA synthesis may occur at the point of initiation; e.g., capped RNA primer binding or endonuclease activity could be

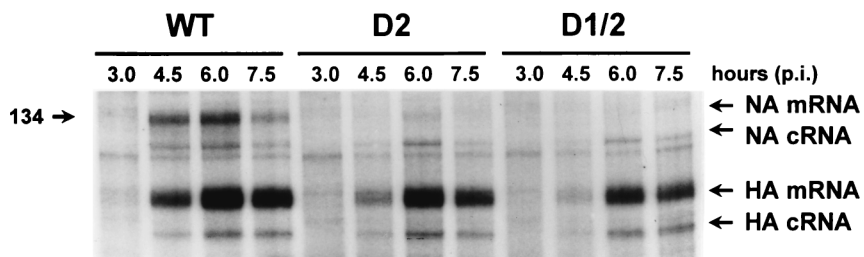


FIG. 6. NA-specific mRNA and cRNA levels in cells infected with transfectant D2 or D1/2. Total RNAs from MDBK cells infected at an MOI of 2 with wild-type (WT) A/WSN/33 virus or the D2 or D1/2 transfectant were extracted at the indicated time points postinfection (p.i.) and subjected to primer extension analysis, using primers specific for the positive-sense NA and HA RNAs. Primer extension products were analyzed on a 5% polyacrylamide gel containing 7 M urea. The expected length of the primer extension products for the NA cRNA is 125 nt; that for the NA mRNA is about 135 to 140 nt due to the presence of host cell mRNA-derived heterogeneous capped RNA primers at the 5' end of viral mRNAs. The expected length of HA-specific extension products is 94 nt for the cRNA and about 104 to 109 nt for the mRNA. Size markers in nucleotides are shown on the left.

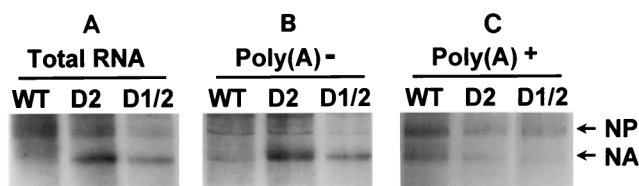


FIG. 7. In vitro transcription of NA-specific RNP complexes and analysis of the transcription products on oligo(dT)-cellulose. In vitro transcription reactions with RNPs isolated from wild-type (WT) A/WSN/33 virus and D2 or D1/2 transfectant virus were performed with globin mRNA as primer (for details see Materials and Methods). One third of the transcription products was directly analyzed (A) on a 3% polyacrylamide gel in 7 M urea. Two-thirds of the transcription products were separated on oligo(dT)-cellulose, and fractions depleted of (B) and enriched in (C) poly(A)-containing molecules were analyzed on the same gel. A longer exposure of the D1/2 products enriched in poly(A)-containing molecules is shown. The positions of the NP- and NA-specific transcription products are indicated.

inhibited. Alternatively, termination or polyadenylation of viral mRNA could be affected. To distinguish between all of these possibilities, in vitro transcription assays were performed. RNP complexes were isolated from purified D2 and D1/2 transfectants and from wild-type A/WSN/33 virus, and in vitro transcriptional activity was measured by using globin mRNA as primer. Figure 7A shows that NA-specific transcription products were synthesized from both the wild-type and the transfectant RNPs. However, there was a significant difference in the pattern of the bands. The wild-type NA-specific transcription product appeared as a wide band corresponding to RNA species with poly(A) tails of different sizes. On the other hand, the NA-specific transcription products of both the D2 and D1/2 transfectants produced less diffuse bands, which implied that these products might not be polyadenylated. To characterize the transcription products, they were analyzed by oligo(dT)-cellulose chromatography. The fractions depleted of poly(A)-containing molecules (Fig. 7B) showed higher levels of NA-specific transcription products for the D2 and D1/2 transfectants but lower levels for the wild-type control. On the other hand, fractions enriched in poly(A)-containing molecules (Fig. 7C) showed lower levels of the NA-specific transcription products for the D2 and D1/2 transfectants but higher levels for the wild-type virus. This result seems to confirm that there is a large proportion of NA-specific transcription products of the D2 and D1/2 transfectants which lack poly(A) tails (Fig. 7B).

## DISCUSSION

In this study, we have investigated the effect of mutations in the double-stranded region of the RNA-fork/panhandle of influenza A virus vRNA in the context of infectious viruses. The postulated double-stranded region of the vRNA promoter consists of five to eight base pairs in different vRNA segments (3). The first three base pairs, those formed by nt 11' to 13' at the 5' end and nt 10 to 12 at the 3' end, are strictly conserved among different vRNA segments of all influenza A viruses. Using a reverse genetics approach, we have introduced double mutations at the three conserved base pairs into the NA gene of influenza A/WSN/33 virus and studied their effects on viral growth. Interestingly, double mutations at positions 10 and 11' and at positions 12 and 13' had no detectable effect on viral growth (D1 and D3 transfectant viruses). On the other hand, a double mutation at positions 11 and 12' resulted in a transfectant (D2) which showed a 1-log reduction in plaque titers on

MDBK cells. The reduction in growth was most likely due to the reduced levels of neuraminidase produced by this virus.

It should be noted that the D2 transfectant, as part of the base pair mutation, has a C→A change at position 11 in the 3' end, which creates an alternative AUG initiation codon in the corresponding mRNA (Fig. 1). Since this initiation codon is upstream of the one for the NA and the two are not in frame, it is possible that the observed reduced NA levels are due to preferred initiation at the alternative AUG resulting in a 23-amino-acid peptide. However, we observed reduced steady-state levels of NA-specific mRNA in cells infected with this transfectant, which suggests that the lower amount of mRNA is the cause for reduced NA levels. This interpretation is supported by the rescue of another transfectant (D1/2), which incorporates the double mutations from transfectant D1 and D2 viruses. The D1/2 transfectant virus shows growth characteristics very similar to those of the D2 transfectant. It grows as slowly as the D2 transfectant on MDBK cells and produces reduced levels of NA and NA-specific mRNA. However, due to the presence of the additional mutation at position 10, the alternative AUG initiation codon is disrupted, thus eliminating the possibility of translation of an incorrect open reading frame. Taken together, the data suggest that both D2 and D1/2 transfectant viruses produce reduced levels of NA due to reduced NA-specific mRNAs.

Interestingly, reduced NA-specific mRNA levels did not correlate with reduced NA-specific vRNA levels for either of the transfectant viruses. Both D2 and D1/2 purified virions contained NA-specific vRNA levels similar to the levels of other segments. Also, in cells infected with D2 transfectant, we found NA-specific vRNA levels corresponding to those found in cells infected with the wild-type virus. In cells infected with the D1/2 transfectant, NA-specific vRNA levels showed a 1.5- to 2-fold reduction. However, this cannot account for the severe reduction of NA-specific mRNA levels. We also did not see any significant alteration of cRNA levels in cells infected with the D2 or D1/2 transfectant compared to wild-type virus-infected cells.

To further investigate whether mRNA synthesis was affected, we performed transcription studies in vitro with RNPs isolated from the D2 and D1/2 transfectant viruses, using capped RNA primer. The experiments showed that initiation of transcription and elongation were not affected by the mutations, but analysis of the transcription products by oligo(dT)-cellulose chromatography implied that the majority of the NA-specific transcription products of the D2 and D1/2 transfectants were not polyadenylated. This result suggests that the mutations interfere with polyadenylation of mRNA transcripts. The observed low levels of mRNA in infected cells (Fig. 6) are fully consistent with these results, since nonpolyadenylated capped transcripts are most likely rapidly degraded in the cell (33). Lack of polyadenylation may be due to the inability of the RNA polymerase to polyadenylate after stopping at the stretch of U residues of the vRNA template. Alternatively, the RNA polymerase may not stop at the poly(U) stretch, resulting in the synthesis of a capped full-length, nonpolyadenylated transcript. It should be noted that efficient RNA synthesis in both mutant and wild-type virus-derived RNPs was dependent on the addition of primer (data not shown).

We have rescued four transfectant viruses with different base pair mutations. Neither of the mutations in the D1 and D3 transfectant viruses exhibited a significant effect on transcription or replication of the NA gene, as measured by determining NA and vRNA levels in virions. On the other hand, two of the transfectants (D2 and D1/2) which had the same C-G→A-U(11-12') double mutation had a defect in mRNA synthesis,



although initiation of transcription was not affected, as determined by the *in vitro* transcription assay (Fig. 7). The fact that the initiation of transcription and replication were not dramatically affected in any of the four transfectant viruses with different double mutations suggests that the studied positions are not crucial for initiation as long as the double-stranded structure is maintained. The importance of complementarity between the 5' and 3' ends in this region of the vRNA promoter has been demonstrated in several studies performed *in vitro* (8, 9) and *in vivo* (15, 21, 25). In agreement with the requirement for complementarity, we were not able to rescue viruses with single mutations at all of the conserved positions involved in base pairing. Those which we have rescued were unstable, rapidly reverting to a wild-type sequence upon passaging. Previous findings that single-point mutations at positions 10 to 12 in the 3' end and at positions 11' to 13' in the 5' end eliminated or decreased reporter activity *in vivo* (15, 18, 25, 29) are also in agreement with the importance of complementarity. After replication of vRNA into cRNA, the double mutations will also be present in the cRNA promoter. It has been shown *in vitro* that the cRNA promoter functions as a panhandle (31). The fact that we did not see dramatic effects of mutations on the replication of vRNA in infected cells indicates that double mutations do not interfere with the function of the vRNA or cRNA promoter in RNA replication. However, we cannot exclude the possibility that different base pair mutations in the duplex region would have different effects.

The observed attenuation of NA-specific mRNA levels in cells infected with the D2 transfectant is in agreement with previous findings (15) that an A-U(11–12') base pair mutation in the context of a vRNA-like CAT reporter gene resulted only in 22% reporter activity compared to a wild-type control. However, the G-C(10–11') and U-A(12–13') base pair mutations, which had no effect on the expression levels of the NA of the D1 and D3 transfectants, resulted in only 20 and 31% activities, respectively, in a CAT reporter gene system (15). This result suggests that base pair mutations in the context of a CAT reporter gene and an NA gene might have different effects (38).

The question arises as to the mechanism of attenuation of polyadenylation of NA-specific mRNAs in the D2 and D1/2 viruses. Recent findings suggest that poly(A) addition occurs by a stuttering polymerase which is bound to the 5' end of the vRNA template (8, 32, 36). It is possible that mutations in the D2 and D1/2 transfectants interfere with the stabilization of polymerase binding to the 5' end of vRNA, which might be important for the polymerase to remain bound to the 5' end of vRNA during polyadenylation.

The results presented in this paper show that by introducing double mutations into an influenza A virus segment, we can attenuate mRNA levels and consequently reduce protein levels encoded by the mutated gene. We believe that transfectant viruses with double mutations should be stable since two specific mutations would have to occur simultaneously in order to revert to the wild-type sequence. We were unable to rescue any transfectant viruses with C→A11 or G→U12' single mutation, which suggests that such viruses might be severely impaired or not viable at all. In addition, the double mutation in the D2 transfectant was preserved during 10 passages on MDBK cells (7). It remains to be seen whether additional mutations in the NA or perhaps in the HA (11) gene may occur to compensate, at least partially, for the reduced NA levels produced by this virus.

In summary, we have rescued and characterized four transfectant viruses with promoter mutations. The mutations in these viruses did not dramatically interfere with the replication

of vRNA, but a C-G→A-U(11–12') mutation in two of the transfectants affected their mRNA levels, most likely by interfering with the efficiency of polyadenylation. This is the first example of an engineered influenza virus with a mutation in the conserved region of the vRNA promoter which does not affect the initiation of transcription or RNA replication but severely impairs polyadenylation and, thus, mRNA synthesis.

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