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Nonconserved Nucleotides at the 3′ and 5′ Ends of an Influenza A Virus RNA Play an Important Role in Viral RNA Replication

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The genome of influenza A viruses is composed of eight negative-strand RNA segments which contain short noncoding regions at their 3′ and 5′ ends. The signals required for replication, transcription, and packaging of the viral RNAs are thought to be located in these regions. The highly conserved noncoding nucleotides, which form "panhandle" or "fork" structures by partial complementarity, are important for the transcriptional activity of the viral RNA polymerase. In contrast, the nonconserved noncoding nucleotides located close to the open reading frame of the viral RNAs had not been implicated in RNA transcription. Using a reverse-genetics system, we have now rescued influenza A/WSN/33 viruses whose NA-specific RNA segments have deletions in these nonconserved noncoding regions. Deletion either of the nucleotide residues between the poly(U) stretch and the stop codon at the 5′ end or of the nucleotides between position 15 and the start codon at the 3′ end did not affect the amount of NA-RNA species found in virions or infected cells. However, a combination of deletions at both the 3′ and the 5′ ends decreased by 60 times the levels of NA-specific viral RNA found in infected cells at late periods of infection and in virions. This double deletion was also responsible for a fourfold reduction of the steady-state levels of the NA-specific mRNA in infected cells. Viruses whose NA-specific open reading frames were flanked by the noncoding regions of the PB1- or the NS-RNA segments of influenza A/WSN/33 virus also showed a reduction in the NA-specific viral RNA in virions and in infected cells. The present results demonstrate that the nonconserved nucleotides at the 3′ and 5′ ends of the NA-RNA segment of influenza A virus play an important role in the replication of this segment.

INTRODUCTION

Influenza A viruses are negative-strand RNA viruses whose genome consists of eight different RNA segments. The genomic RNAs are found in virions and infected cells together with the viral NP and P (PB1, PB2, and PA) proteins as ribonucleoprotein (RNP) complexes. The viral RNAs (vRNAs) contain one or more open reading frames (ORF) which are flanked by short noncoding sequences at both ends of the gene (for reviews see Lamb, 1989; Lamb and Horvath, 1991). These noncoding sequences are believed to contain the signals required for the replication, transcription, and packaging of the vRNAs (Luytjes *et al.,* 1989). The noncoding sequences of the different RNA segments of all influenza A virus strains share several striking characteristics: (1) The first 12 and 13 nucleotides (nt) at the 3′ and 5′ ends of the vRNAs, respectively, are highly conserved among different RNA segments and virus strains. (2) A stretch of 5–7 uridine residues is located 15–16 nt away from the 5′ end. (3) The U-stretch is juxtaposed to a panhandle structure which is formed as a result of the partial complementarity between the 12 and 13 conserved nt at the 3′ and the 5′ ends of the vRNAs (Hsu *et al.,* 1987). In addition, the panhandle may be extended by nonconserved nt at the 3′ and 5′ ends, sometimes even involving a portion of the U-stretch at the 5′ end. Thus, nt 14, 15, and 16 at the 5′ end of the vRNAs are in general complementary to nt 13, 14, and 15 at the 3′ end. These nt positions, although nonconserved among the different vRNA segments, are in most instances highly conserved for the same segment of different influenza virus strains (Desselberger *et al.,* 1980; Stoeckle *et al.,* 1987).

Once the viral RNPs enter the nucleus of the infected cell, transcription and replication of the viral genome is achieved by a viral-coded RNA-dependent RNA polymerase. RNA replication takes place through replicative intermediate RNAs (cRNAs), which are full-length copies of the vRNAs (for a review see Krug *et al.,* 1989). The promoters for RNA synthesis are thought to be formed by the 12 and 13 conserved nt at the 3′ and 5′ ends of the vRNAs and cRNAs. Reverse genetics and *in vitro* studies confirmed that the conserved nt at the 3′ end are indeed part of the RNA promoter (Parvin *et al.,* 1989; Yamanaka *et al.,* 1991; Li and Palese, 1992; Seong and Brownlee, 1992a,b; Piccone *et al.,* 1993; Neumann and Hobom, 1995). However, the viral RNA polymerase binds

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preferentially to the conserved 5′ end of the RNA (Fodor *et al.,* 1994; Tiley *et al.,* 1994) and it is now accepted that a panhandle or fork RNA structure is needed for optimal vRNA and cRNA promoter activity *in vitro* (Fodor *et al.,* 1994, 1995; Pritlove *et al.,* 1995).

The vRNAs are used as templates for the synthesis of mRNAs and cRNAs. In contrast to cRNA formation, mRNA synthesis terminates at the U-stretch of the vRNAs by addition of a poly(A) tail. The RNA polymerase is thought to stutter at the U-stretch due to the panhandle structure located next to the U-stretch. Accordingly, both the U-stretch and the panhandle structure are needed for mRNA synthesis (Luo *et al.,* 1991; Li and Palese, 1994). Another difference between influenza cRNAs and mRNAs is that the latter are capped. Transcription of the mRNAs is primed by short capped oligonucleotides which are thought to be cleaved from cellular mRNAs by the PB2 protein, a subunit of the viral RNA polymerase. The 5′ end of the vRNA stimulates the binding of the viral polymerase to capped mRNAs, but both 5′ and 3′ ends of the vRNA are needed for activation of the endonuclease activity (Hagen *et al.,* 1994; Cianci *et al.,* 1995).

While the conserved features of the influenza virus RNAs are known to be involved in polymerase binding, promoter activity, polyadenylation, and endonuclease activity, a function has not yet been determined for the nonconserved noncoding sequences which are located between the panhandle structure and the start codon at the 3′ end and between the poly(U) stretch and the stop codon at the 5′ end of the vRNAs. These sequences are of different length and nt composition among different RNA segments and among different influenza virus strains. One might have expected that these nonconserved sequences would not play a role in the replication cycle of the virus. In the present report we demonstrate, using a reverse genetics approach (Enami *et al.,* 1990), that a change in these nt can dramatically affect the levels of vRNA and mRNA in infected cells, and that the presence of specific coding sequences in the NA-specific RNA segment appears to have an effect on its RNA levels.

MATERIALS AND METHODS

Viruses and cells

WSN-HK virus, a reassortant influenza virus containing seven genes from influenza A/WSN/33 virus (WSN) and the NA gene from influenza A/HK/8/68 virus, was grown in 10-day-old embryonated chicken eggs. WSN virus and transfectant viruses were grown in Madin–Darby bovine kidney (MDBK) cells in reinforced minimal essential medium. MDBK cells were also used for transfection experiments and for selection and plaque purification of rescued transfectant viruses. Sucrose-gradient-purified 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.

Construction of plasmids

pT3NAM1 contains the complete NA gene of WSN virus flanked by the T3 RNA polymerase promoter and *Bbs*I restriction enzyme site (García-Sastre *et al.,* 1994). Two silent nt changes were introduced into the NA-specific cDNA creating a *Cla*I site at nt positions 52–57. For the construction of pT3NA3D, a PCR product was made using pT3NAM1 as template and the following primers: 5′-GGCCATCGATCCAATGGTTATTATTTCTGGTTT-GGATTCATACTCCTGCTTTTGCTCGAAGAG-3′ and 5′-GTTTTCCCAGTCACGAC-3′. The PCR product was digested with *Eco*RI and *Cla*I restriction enzymes and it was cloned into pT3NAM1 cut with the same restriction enzymes. Another PCR was performed in order to construct the plasmids pT3NA5D and pT3NAdD, using pT3NAM1 as template and two oligonucleotides 5′-GGC-CAAGCTTTAAATTAACCCTCACTAAAAGTAGAAACA-AGGAGTTTTTTCTACTTGTCAATGGTG-3′ and 5′-TGG-ACTAGTGGGAGCATCAT-3′ as primers. The PCR product was digested with *Spe*I and *Hin*dIII restriction enzymes and cloned into *Spe*I/*Hin*dIII-digested pT3NAM1 or pT3NA3D.

pT7CASCAT contains the complete sequence of the CAT ORF flanked by the noncoding regions of the NS-segment of WSN virus (N. Percy, unpublished). pT3NA/NS was constructed as follows: first, in order to construct pT3NA/3NS5NA, a PCR product was made using pT7CASCAT as the template and the two oligonucleotides, 5′-GGCCATCGATCCAATGGTTATTATTTTCTGGT-TTGGATTCATTATGTCTTTGTCACCC-3′ and 5′-TCCCA-GTCACGACGT-3′, as primers. This PCR product was digested with *Eco*RI and *Cla*I restriction enzymes and cloned into pT3NAM1 cut with *Eco*RI and *Cla*I. Then, a second PCR was done using pT3NAM1 as template and two oligonucleotides, 5′-GGCCAAGCTTATTAACCCTCA-CTAAAAGTAGAAACAAGGGTGTTTTTTATTATTACTTGT-CAATGGTG-3′ and 5′-TGGACTAGTGGGAGCATCAT-3′, as the primers. The product was digested by *Hin*dIII and *Spe*I restriction enzymes and cloned into pT3NA/3NS5NA cut with *Hind*III and *Spe*I.

For the construction of pT3NA/PB1, two silent mutations were introduced into pT3NAM1 at nt 1358 (T to C) and 1360 (G to C) of the NA-specific cDNA to create a *Sac*I site. pT3CAT/PB1 contains the complete sequence of the CAT ORF flanked by the noncoding regions of the PB1-segment of WSN virus (M. Bergmann, unpublished). Using this plasmid as template, a PCR product was made with the following two primers: 5′-GGCCGAGCTCC-CGTTCACCATTGACAAGTAGTGAATTTAGCTTGTCC-3′ and 5′-GGAATTGTGAGCGGATAAC-3′. This product was cut with *Sac*I and *Hin*dIII restriction enzymes and cloned

into the modified pT3NAM1 digested with *Sac*I/*Hin*dIII to construct pT3NA/3NA5PB1. A second PCR was done by using pT3CAT/PB1 as template and oligonucleotides 5′-GGCCATCGATCCAATGGTTATTATTTTCTGGTTTGGATTCATTCAAATGGTTTGCC-3′ and 5′-TCCCAGTCACGACGT-3′ as primers. Then the PCR product was cut with *Eco*RI and *Cla*I restriction enzymes and cloned into pT3NA/3NA5PB1 digested with *Eco*RI and *Cla*I.

Presence of the desired 3′ and 5′ noncoding sequences in all these plasmids was confirmed by sequencing with a DNA sequencing kit (United States Biochemical Corporation, Cleveland, OH).

Ribonucleoprotein transfection

The influenza virus RNA polymerase and NP protein complex was isolated from influenza X-31 virus as described (Parvin *et al.,* 1989). RNP transfection was performed in MDBK cells according to Enami and Palese (1991) using WSN-HK as helper virus. Rescued transfectant viruses were plaque purified three times in MDBK cells covered with agar overlay media.

Sequence of the NA genes of transfectant viruses

Sequences at the 3′ ends of the NA genes of transfectant viruses NA3D, NA5D, NAdD, NA/NS, and NA/PB1 were confirmed as follows: first, RNA extracted from virions was 3′-polyadenylated using poly(A) polymerase (Gibco BRL, Gaithersburg, MD). Then, polyadenylated RNA was reverse transcribed using the primer 5′-TCTAGATTTTTTTTTTTTTTTTAGC-3′ and Superscript reverse transcriptase (Gibco BRL). The cDNA was PCR amplified using the primers 5′-GCGCAAGCTTCTAGATTTTTTTTTTTTTT-3′ and 5′-GCGCAAGCTTTATTGAGATTATATTTCC-3′, the latter containing nt positions 115–98 of the NA gene of WSN. The PCR product was digested with *Xba*I and *Hin*dIII restriction enzymes, ligated into *Xba*I/*Hin*dIII-digested pUC19, and sequenced with a DNA sequencing kit (United States Biochemical Corporation). For the sequencing of the 5′ end of the NA gene of the transfectant viruses, a reverse transcription–PCR product was obtained using the oligonucleotide 5′-TGGACTAGTGGGAGCATCAT-3′ (complementary to nt positions 1280 to 1299 of the WSN NA gene) as reverse transcription and first PCR primer, and the oligonucleotide 5′-GCGCAAGCTTAGTAGAAACAAGG-3′ (which contains the last 13 nt at the 5′ end of the influenza A virus vRNAs) as the second PCR primer. The PCR products were cut with *Spe*I and *Hin*dIII restriction enzymes, ligated into *Xba*I/*Hin*dIII-digested pUC19, and sequenced.

RNAs were isolated by phenol extraction from viruses purified through a 30–60% sucrose gradient (Luo *et al.,* 1992).

Growth curve of the transfectant viruses

Confluent monolayers of MDBK cells in 35-mm dishes were infected with NA3D, NA5D, NAdD, NA/NS, or NA/PB1 transfectant viruses at a multiplicity of infection (m.o.i.) of 0.001. At 12-hr intervals, supernatants were harvested and hemagglutination (HA) titers and plaque forming unit (PFU) titers were determined using chicken erythrocytes or MDBK cells, respectively. WSN virus was used as a wild-type virus control.

RNA electrophoresis

vRNAs extracted from sucrose-gradient-purified influenza viruses were electrophoresed on a 2.8% polyacrylamide gel containing 7.7 *M* urea at 150 V for 1 hr 50 min. RNA molecules were visualized by silver staining, as described previously (Luo *et al.,* 1992).

RNA primer extension

The vRNAs of the NA- and NS-segments of WSN virus and the NA3D, NA5D, NAdD, NA/NS, and NA/PB1 transfectant viruses were quantitated by primer extension, as previously described (Luo *et al.,* 1992). The NS-specific primer, 5′-GGGAACAATTAGGTCAGAAGT-3′, is complementary to the region between nt 695 and 715 of the NS-specific vRNA. The NA-specific primer, 5′-GTGGCAATAACTAATCGGTCA-3′, is complementary to the region between nt 1151 and 1171 of the NA-vRNA. One hundred nanograms of RNA extracted from sucrose-purified viruses or 5 μg of total RNA isolated from infected MDBK cells was reverse transcribed in the presence of 3×10^5 cpm of the end-labeled NS- and NA-specific primers. The extended products were analyzed on a 6% polyacrylamide gel containing 7 *M* urea. The amount of product was quantitated by a phosphorimaging system (Molecular Dynamics, CA). For the isolation of vRNA from infected cells, MDBK cells were infected with either WSN virus or transfectant viruses at an m.o.i. of 2 and harvested at the indicated time points. Cells were lysed with guanidinium isothiocyanate, and total RNA was purified by cesium chloride ultracentrifugation, as previously described (Luo *et al.,* 1992).

RNase protection assay

The NA- and NS-specific mRNAs in MDBK cells infected by WSN virus or NA3D, NA5D, or NAdD transfectant viruses were measured by RNase protection assay, as per the manufacturer's instructions (Ambion, Inc., Austin, TX). Negative-sense RNA probes which hybridize to the NA- and NS-specific positive-sense RNAs were made from plasmids deIT3NAv and pIBI30-NS, respectively (Luo *et al.,* 1992). The NA-specific probe was 403 nt long and contained the last 313 nt of the NA-specific vRNA. Thus, the probe has the potential to protect two frag-

ments of 313 and 298 nt corresponding to the NA-specific cRNA and mRNA, respectively. The NS-specific probe was 470 nt long and contained the last 425 nt of the NS-specific vRNA. This probe was designed to protect fragments of 425 and 409 nt corresponding to the NS-specific cRNA and mRNA, respectively. NA- and NS-specific probes were labeled in the presence of 4 μl of [α-^{32}P]UTP (800 Ci/mmol; DuPont NEN, Boston, MA) as previously described (Luo *et al.,* 1992). Five micrograms of total RNA isolated from virus-infected cells and 5×10^4 cpm of the negative-sense NA- and NS-specific probes were incubated at 45° for 36 hr. The hybridization mixture was then digested with RNases A and T1 at 37° for 30 min. The digested products were analyzed on a 6% polyacrylamide gel containing 7 *M* urea and quantitated by a phosphorimaging system (Molecular Dynamics). Since cRNA synthesis was found to be only 3 to 5% of that of mRNA synthesis, we could only quantify the NA-specific mRNA levels under the assay conditions (Luo *et al.,* 1992).

Analysis of NA protein synthesis in infected cells

MDBK cells grown in 35-mm dishes were infected with either WSN virus or the transfectant viruses at an m.o.i. of 2. At the indicated time points, the cells were labeled using medium containing 25 μl of [^{35}S]cysteine (1027 Ci/mmol; DuPont NEN) for 15 min. Cells were then lysed in 100 μl of 0.15 *M* NaCl, 20 m*M* Tris–HCl (pH 7.4) containing 1.5% *n*-octylglucoside and 1 m*M* $CaCl_2$ (lysis buffer) after being chased with fresh medium for 30 min. Five microliters of cell extracts was used for immunoprecipitation with a mixture of monoclonal antibodies 10C9 and 3C8 (specific for WSN NA protein) or with monoclonal antibody HT-103 (specific for WSN NP protein) as described (Percy *et al.,* 1994). Immunoprecipitated proteins were analyzed on a sodium dodecyl sulfate–10% polyacrylamide gel and quantitated by a phosphorimaging system (Molecular Dynamics).

RESULTS

Rescue of transfectant influenza A viruses NA3D, NA5D, and NAdD

We have engineered three WSN NA genes in which portions of the NA-specific noncoding regions were deleted (Fig. 1A). The NA3D (3′ deletion) gene lacks 4 nt (AAUU) at positions 16–19 of the 3′ end adjacent to the start codon of the NA gene. Seven nucleotides between the poly(U) stretch and the stop codon (GAACAAA, positions 22–28 of the 5′ end) are deleted in the NA5D (5′ deletion) gene. The NAdD (double deletion) gene has a combination of both deletions at the 3′ and 5′ ends. RNP transfection of the engineered NA genes into WSN-HK-infected MDBK cells resulted in the rescue of transfectant viruses. After three plaque to plaque passagings of the transfectant viruses, a single plaque was used for amplification in MDBK cells. The resulting virus preparations were used for further analysis. The identity of the transfectant viruses was confirmed by sequencing the 3′ and 5′ ends of their NA-RNA segments, as described under Materials and Methods. Sequences were identical to those of the corresponding transfected genes.

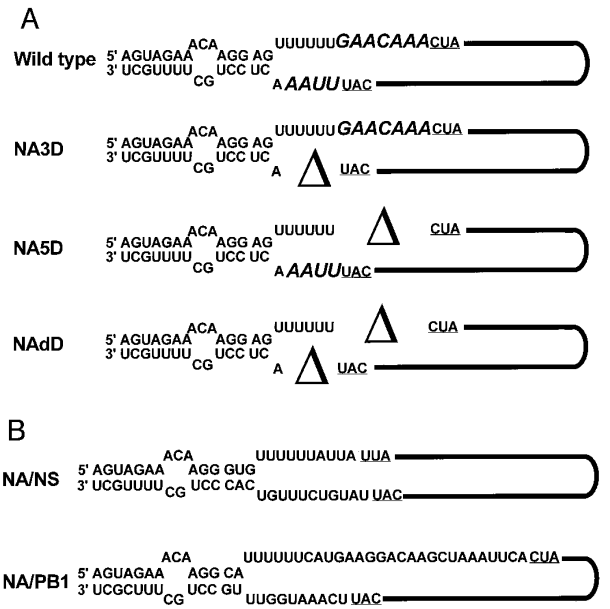

FIG. 1. Schematic representation of influenza virus NA-specific vRNAs used in rescue experiments. All sequences are shown in a (hypothetical) panhandle configuration. The start and stop codons of the NA genes are underlined. The heavy lines represent the coding sequences (ORF) of the NA gene. (A) Wild-type sequence of the noncoding regions of the NA gene of influenza A/WSN/33 virus is shown on the top. Sequences of the noncoding regions of the NA3D, NA5D, and NAdD vRNAs used in transfection experiments are also shown. Nucleotides that were deleted are shown in italics in the wild-type sequence (top). Δ indicates deletions in the mutants. (B) Sequences of the noncoding regions of the NA/NS- and NA/PB1-vRNAs. These sequences correspond to the wild-type noncoding sequences of the NS- and PB1-specific vRNAs of influenza A/WSN/33 virus.

NA-specific vRNA levels in purified NA3D, NA5D, and NAdD transfectant viruses

Viral RNA isolated from purified wild-type and transfectant viruses was used for quantitation of the levels of the NA-specific vRNA segment in virions. One hundred nanograms of RNA from sucrose-purified transfectant viruses was analyzed by PAGE, and a dramatic reduction in the amount of NA-RNA in NAdD virions was observed in the silver-stained polyacrylamide gel, but not in that of the NA-RNA of NA3D and NA5D viruses (Fig. 2). NA-specific vRNA levels were quantified by primer extension analysis using the NS gene as internal control. Levels of the NA-vRNA segment in NAdD virions were approxi-

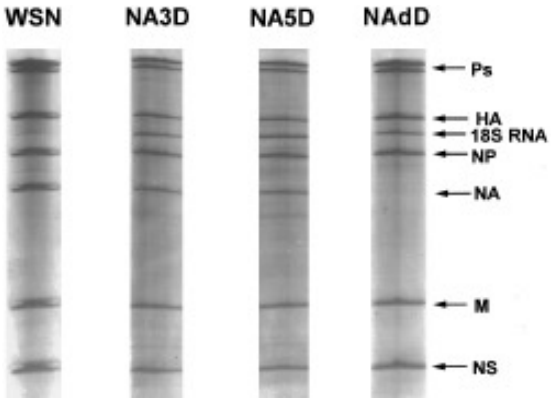

FIG. 2. Polyacrylamide gel electrophoresis of RNAs extracted from purified WSN, NA3D, NA5D, and NAdD viruses. RNAs were separated in a 2.8% polyacrylamide gel containing 7.7 *M* urea and visualized by silver staining. RNAs that encode polymerase proteins (Ps), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M), and nonstructural proteins (NS) are indicated. 18S ribosomal RNA, which often appears in viral RNA preparations as contaminant cellular RNA, is also indicated.

mately 60 times reduced compared to those of wild-type virus (Fig. 3A). However, the amount of NA-vRNA in the single deletion mutants NA3D and NA5D was similar to that of wild-type WSN virus. It should be noted that the NA-specific extention products in Fig. 3A are slightly shorter for the NA5D and NAdD viruses, due to the deletion of seven nt at the 5′ end of the NA-vRNA. Weak additional bands present in Fig. 3A may represent vRNA degradation products and/or premature termination products of the reverse-transcription reaction.

NA-specific vRNA levels in NA3D, NA5D, and NAdD transfectant virus-infected cells

The lower representation of the NA-specific vRNA segment in purified NAdD viruses could result from a lower replication of the segment in virus-infected cells or from a defect in the packaging of the NA-vRNA into budding viruses or both. In order to distinguish among these possibilities, we performed a primer extension analysis of the NA-specific vRNA segment in infected cells (Fig. 3B). MDBK cells were infected with wild-type or transfectant viruses at an m.o.i. of 2, and total RNA was isolated at different times postinfection. NA-vRNA levels in NAdD virus-infected cells were about 5, 30, and 60 times lower than those of WSN virus-infected cells at 6, 7.5, and 9 hr postinfection, respectively. Those of single deletion mutants NA3D and NA5D were similar to those of WSN virus. Thus, at late times of infection, the amounts of NA-vRNA in NAdD virus-infected cells and in purified viruses are comparable. The results indicate that the lower representation of NA-vRNA in NAdD purified viruses correlates with a lower efficiency in NA-vRNA replication. The additional bands present in Fig. 3B represent nonspecific annealing of the primers to cellular RNAs, since they were also detected when the cellular extracts were made 1.5 hr postinfection (data not shown).

NA-specific mRNA levels in NA3D, NA5D, and NAdD transfectant virus-infected cells

Since vRNA serves as the template for mRNA synthesis, we measured the mRNA levels in MDBK cells infected with the NA-deletion mutants. Cell monolayers were infected with wild-type or transfectant viruses at an m.o.i. of 2. Total RNA from infected cells was extracted at 1.5, 3.0, 4.5, 6.0, and 7.5 hr postinfection. The amount of NA-specific mRNA was then determined by RNase protection assay as described under Materials and Methods. The NS-specific mRNA was used as internal control. Figure 4 shows the PAGE analysis of the RNase digested products. Under the present conditions, the NA- and NS-specific cRNA signals are too low to be detected or are obscured by the mRNA signals. NA-specific mRNA levels in NAdD virus-infected cells at the peak of expression (4.5 hr) were approximately four times lower than those of WSN virus when standardized versus NS-specific mRNA levels. However, NA-specific mRNA levels of the single-deletion mutants were similar to those of WSN.

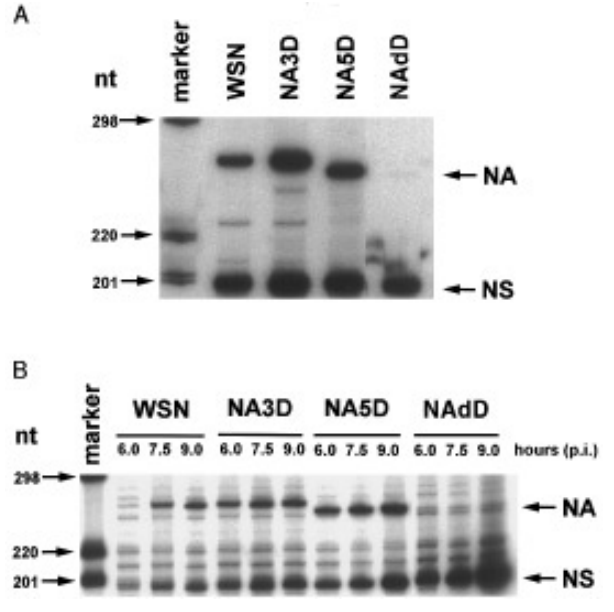

FIG. 3. Measurement of the NA-specific vRNA levels in purified WSN, NA3D, NA5D, and NAdD viruses and in infected cells. (A) RNAs were extracted from purified viruses and subjected to primer extension, using primers specific for the NA- and NS-vRNAs. Extended products were analyzed in a 6% polyacrylamide gel containing 7.7 *M* urea. (B) MDBK cells were infected with the indicated viruses at an m.o.i. of 2. Total RNA was extracted from virus-infected cells at the indicated time points postinfection (p.i.) and subjected to primer extension, using primers specific for the NA- and NS-vRNAs. Extended products were analyzed in a 6% polyacrylamide gel containing 7 *M* urea. Molecular weight markers are indicated on the left.

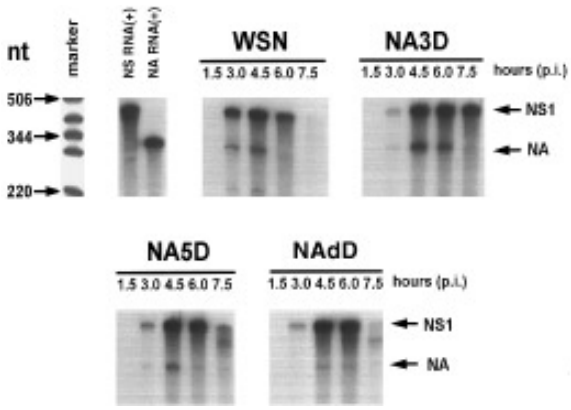

FIG. 4. NA-specific mRNA levels in WSN, NA3D, NA5D, and NAdD virus-infected cells. MDBK cells were infected with the indicated viruses at an m.o.i. of 2. Total RNA was extracted from virus-infected cells at the indicated time points postinfection (p.i.) and subjected to RNAse protection analysis, using NS- and NA-specific probes (minus sense). Protected fragments were analyzed in a 6% polyacrylamide gel containing 7 *M* urea. Signals derived from NS1- and NA-specific mRNAs are indicated. A control using positive-sense *in vitro*-transcribed NS- and NA-RNAs (cRNA-like) is shown on the left. Molecular weight markers are also indicated on the left.

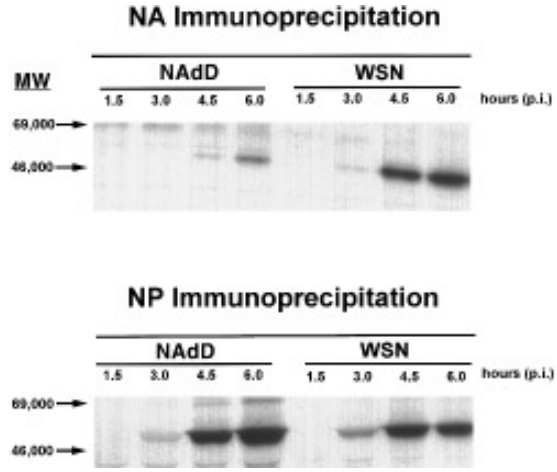

FIG. 5. Characterization of the NA protein expression levels in NAdD virus-infected cells. MDBK cells were infected with NAdD or WSN viruses at an m.o.i of 2. Infected cells were subsequent pulsed with [^{35}S]cysteine for 15 min and chased for another 30 min, at the indicated time points postinfection (p.i.). Cell extracts were immunoprecipitated with a mixture of NA-specifc monoclonal antibodies or with an NP-specific monoclonal antibody. Immunoprecipitated products were analyzed by SDS–polyacrylamide gel electrophoresis. Molecular weight markers (MW) are indicated on the left.

NA expression in NAdD transfectant virus-infected cells

In order to investigate if the reduction in NA-specific mRNA levels correlated with a reduction in NA levels, we measured the expression of NA in NAdD virus-infected cells at different times postinfection. MDBK cells were infected with NAdD or WSN viruses at an m.o.i. of 2. Cells were then pulse labeled at 1.5, 3.0, 4.5, and 6.0 hr postinfection. After a 30-min chase, cell lysates were obtained and the amounts of NA were compared by immunoprecipitation using NA-specific monoclonal antibodies. For control, the NP protein was also immunoprecipitated with an NP-specific antibody. Although the amount of NP protein was similar in NAdD and WSN virus-infected cells, the amount of NA protein was about 10 times lower in NAdD-infected cells when labeled at both 4.5 and 6.0 hr postinfection (Fig. 5). A similar reduction in the amount of NA was found in purified NAdD viruses compared to that of wild-type viruses (data not shown). We think that the differences found between the levels of expression of NA protein (10 times lower) and NA-specific mRNA (4 times lower) in NAdD virus-infected cells are not significant.

NAdD transfectant virus is attenuated in tissue culture

Growth properties of transfectant viruses NA3D, NA5D, and NAdD were analyzed in MDBK cells. For this purpose, confluent MDBK cell monolayers were infected at low m.o.i. (0.001) and virus production was assayed at different time points by plaque assay in MDBK cells (Fig. 6). WSN virus was also included in the assay as positive control. The maximum PFU titer of NAdD virus was approximately 2 logs lower than that of wild-type virus. However, single deletion NA mutants were not significantly affected in multicycle infectious virus production compared to WSN virus. HA titers of the NAdD virus were reduced only twofold (data not shown), indicating that a large percentage of NAdD viruses is not infectious. Thus, the NAdD virus is, in this respect, phenotypically similar

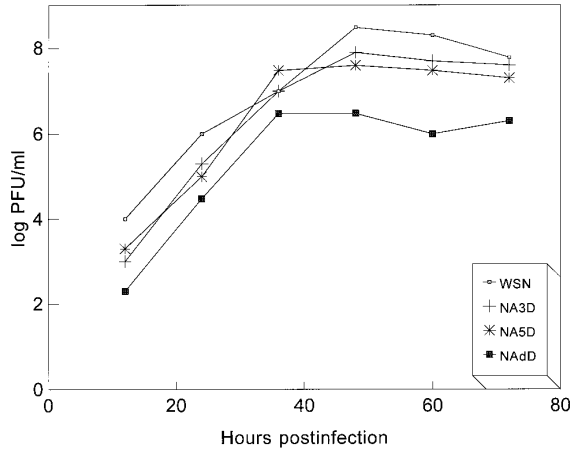

FIG. 6. Growth curves of the transfectant influenza viruses NA3D, NA5D, and NAdD on MDBK cells. Confluent MDBK cells in 35-mm dishes were infected with influenza A/WSN/33 (WSN) virus or with the transfectant NA3D, NA5D, or NAdD viruses at an m.o.i. of 0.001. At 12-hr intervals, infectious particles present in the media were titrated by plaque assay in MDBK cells.

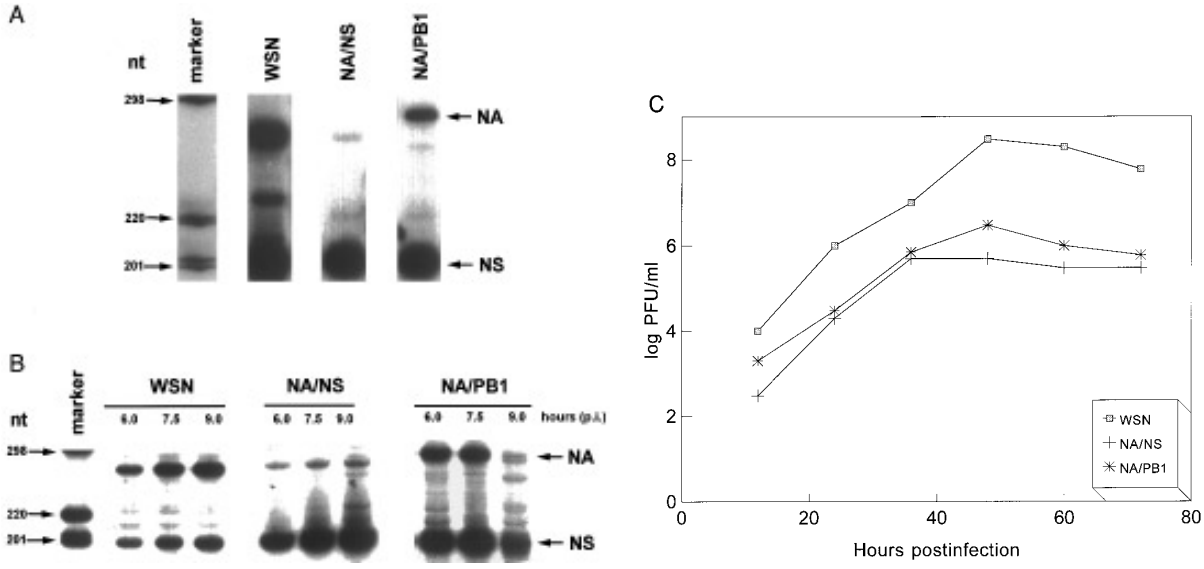

FIG. 7. Analysis of the transfectant viruses NA/NS and NA/PB1. (A) Characterization of the NA-specific vRNA levels in purified NA/NS and NA/PB1 viruses. RNAs were extracted from purified viruses and subjected to primer extension, using primers specific for the NA- and NS-vRNAs. Extended products were analyzed in a 6% polyacrylamide gel containing 7.7 *M* urea. (B) Characterization of the NA-specific vRNA levels in NA/NS and NA/PB1 virus-infected cells. MDBK cells were infected with the indicated viruses at an m.o.i. of 2. Total RNA was extracted from virus-infected cells at the indicated time points postinfection (p.i.) and subjected to primer extension, using primers specific for the NA- and NS-vRNAs. Extended products were analyzed in a 6% polyacrylamide gel containing 7 *M* urea. Molecular weight markers are indicated on the left. (C) Growth curves of the NA/NS and NA/PB1 viruses on MDBK cells. Confluent MDBK cells in 35-mm dishes were infected with influenza A/WSN/33 (WSN) virus or with the transfectant NA/NS or NA/PB1 viruses at an m.o.i. of 0.001. At 12-hr intervals, infectious particles present in the media were titrated by plaque assay in MDBK cells.

to the previously described NA/B-NS transfectant virus (Muster *et al.,* 1991; Luo *et al.,* 1992). Both of these viruses have a low replication level of their NA gene when compared to that of wild-type WSN virus, resulting in a large population of noninfectious viruses lacking the NA gene. However, the NAdD virus is more drastically affected by the introduced mutations than the NA/B-NS virus.

Rescue and characterization of transfectant viruses containing NA-RNA segments whose noncoding sequences are derived from heterologous RNA segments

We have also engineered two WSN NA-RNA segments in which the noncoding sequences at the 3′ and 5′ ends are substituted by the corresponding noncoding sequences of the PB1- and NS-RNA segments (Fig. 1B). RNP transfection of these two RNAs resulted in the rescue of the transfectant viruses NA/NS and NA/PB1. The sequences of the 3′ and 5′ ends of the NA-specific vRNAs isolated from these viruses were identical to those of the transfected RNAs. Primer extension analysis revealed that the NA-specific vRNA levels in NA/NS and NA/PB1 transfectant viruses were about 50 and 5 times lower, respectively, than those of wild-type WSN virus (Fig. 7A). As in the case with the NAdD virus, a significant reduction of the NA-specific vRNAs was also found in NA/NS and NA/PB1 virus-infected MDBK cells (Fig. 7B). The NA-specific vRNA levels of NA/NS virus-infected cells were reduced by factors of 14, 40, and 50 at 6, 7.5, and 9.0 hr postinfection, respectively, compared with WSN virus-infected cells. For the NA/PB1 virus, these levels were reduced by factors of 4, 10, and 14 at 6, 7.5, and 9.0 hr postinfection, respectively. These results indicate that the low representation of the NA-specific vRNA in purified transfectant viruses correlates with the respective levels of NA-specific vRNA in infected cells at late times. As expected, both NA/NS and NA/PB1 transfectant viruses were attenuated in MDBK cells (Fig. 7C).

DISCUSSION

We have studied the contribution of the nonconserved nt in the noncoding region of the NA-specific RNA segment of influenza A virus on influenza viral RNA replication and transcription. For this purpose, we constructed transfectant viruses in which we deleted nt positions 16–19 at the 3′ end of the NA-specific vRNA and/or nt positions 22–28 at the 5′ end. These nt are variable not only among different vRNA segments of the same influenza virus strain, but also among NA-RNA segments of different influenza virus strains. In addition, a number of *in vitro* transcription experiments have failed to provide evidence that these regions are important for transcriptional activity. Thus, one might predict that deletion of these nt would not affect virus replication. This is in fact the case when a single deletion is made at either the 3′

or the 5′ end of the NA-specific segment (viruses NA3D and NA5D). These viruses are similar to wild-type virus with respect both to their NA-specific vRNA and mRNA levels and to their growth properties in tissue culture. Single deletions also did not affect the packaging of the NA-specific vRNA segments into virions. However, when both single deletions were combined in the same virus, NAdD, we found that the level of the NA-specific vRNA segment was dramatically reduced (60 times) in virions. This reduction does not seem to be due to a packaging defect of the segment, since a similar reduction of the NA-specific vRNA segment is found in virus-infected cells at late times of infection. Also, the levels of NA-specific mRNA — and subsequently of the NA protein — were reduced in virus-infected cells, although not to the same degree as the NA-specific vRNA. At 4.5 hr postinfection, mRNA levels of the mutant gene were 4 times lower than wild-type levels. This difference between mRNA and vRNA levels may indicate a preferential defect of the NA-specific RNA synthesis at late times of infection. More likely, the higher reduction in vRNA levels at 9 hr postinfection is due to an intrinsic amplification effect during the replication of the vRNA. Thus, since the expression levels depend on the amount of available template, one would expect an exponential decrease of the amount of vRNA with time. For example, let us assume that a wild-type virus quadruples the number of vRNA molecules every hour and that a mutant virus defective in replication only doubles its number. In this case, starting with one NA-specific vRNA molecule, the wild-type virus will have synthesized 4 molecules and the mutant virus only 2 molecules 1 hr later (twofold difference). However, 2 hr later, the wild-type virus will have synthesized 16 molecules and the mutant virus only 4 molecules (fourfold difference).

There are several possible explanations for the phenotype of the NAdD transfectant virus. One possibility is that the NA-specific vRNA and/or cRNA has a reduced promoter activity. *In vitro* experiments have confined the region of the promoter to the conserved nt at the 3′ and 5′ ends (Yamanaka *et al.,* 1991; Li and Palese, 1992; Seong and Brownlee, 1992b; Piccone *et al.,* 1993; García-Sastre and Palese, 1993). However, it is also known that the panhandle/fork structure of the vRNA and cRNA plays an important role in RNA promoter activity *in vitro* (Fodor *et al.,* 1994, 1995; Pritlove *et al.,* 1995). Nucleotides close to this structure may enhance or inhibit the formation of the panhandle structure, either by directly interacting with the conserved nt or by interacting with internal sequences. In this respect, it is interesting to note that the panhandle structure of the NAdD sequence is the least stable structure predicted by the method of Zuker (Zuker and Stiegler, 1981) among all the NA genes which were used in the study (data not shown). It is also possible that the nonconserved nt are involved in the formation of more complex RNA structures, such as pseudoknots, which might be required for interaction with cellular or viral factors during RNA replication.

There are other explanations which could account for the lower NA-specific RNA levels in NAdD infected cells: (1) The stability of NadD-specific vRNA or cRNA may be lowered compared to that of the wild-type molecule. We do not favor this hypothesis since it is unlikely that small deletions in the NA-specific RNA segment would affect RNA stability in such a dramatic way. (2) It is possible that the NP protein shows reduced binding activity to the mutant NA-RNA. This would result in a lower rate of RNP formation, which is the actual template for RNA transcription and replication. Since the NP shows nonspecific binding activity to single-stranded RNA (Albo *et al.,* 1995), the above-mentioned explanation does not appear to be convincing. (3) The P proteins may have a reduced binding activity to the mutant RNA. However, the deleted nt in the NAdD-specific RNA segment do not appear to be important for binding to the P proteins *in vitro* (Fodor *et al.,* 1993, 1994; Tiley *et al.,* 1994; Pritlove *et al.,* 1995). (4) The mutant NA-RNA could have a tendency to produce defective interfering (DI) RNAs by forming internal deletions of the segment during RNA replication. We do not favor this possibility, since we did not detect abnormally high levels of DI RNAs in our purified NAdD virus preparations. (5) An intriguing possibility is that the mutant NA-RNA is defective in antitermination activity. Antitermination is required in order to skip the polyadenylation signal during the synthesis of cRNA from vRNA. The presence of soluble NP seems to be required for this activity (Beaton and Krug, 1986), but the *cis*-acting signals in the RNA responsible for this activity are not known. A defect in antitermination activity would preferentially affect the cRNA and vRNA levels, but the decrease of vRNA template would also result in lower levels of mRNA. Indeed, after several passages of the NAdD virus in MDBK cells, there is in infected cells an accumulation of vRNA molecules with an increased number of U residues at the poly(U) stretch (unpublished). This finding could indicate that the viral polymerase has a tendency to stutter at the poly(U) stretch before copying the 5′ end of the vRNA during cRNA synthesis. Again, it is not obvious why changes at both the 3′ end and the 5′ end are required to influence antitermination. Further molecular and biochemical studies will be needed in order to distinguish among these different explanations.

The characterization of the NAdD transfectant virus led us to the conclusion that the nonconserved nt located at the 3′ and 5′ noncoding ends play an important role in the RNA replication. Deletion of these nt in the NA gene results in an attenuated virus with low levels of NA-specific vRNA, mRNA, and protein in infected cells. In order to address the question of whether the coding sequences also influence RNA replication, we engi-

neered two NA-RNA segments whose noncoding ends were substituted by the corresponding noncoding ends of the PB1- or the NS-RNA segments. In the silver-stained polyacrylamide gels, the viral RNA segments of the wild-type WSN virus appear to be represented at equimolar levels. Thus, if the signals required for optimal replication and packaging of the influenza virus RNAs are contained within the noncoding regions of the segment, one would predict that an NA-segment with heterologous noncoding regions, derived from another vRNA segment, would be found at wild-type levels in virions. However, transfectant viruses containing an NA-RNA segment with PB1- or NS-specific noncoding regions show a reduction in NA-specific vRNA levels of about 10 and 50 times, respectively, both in virions and in infected cells. The levels of both the PB1 and the NS genes of the transfectants were found to be unaffected, as measured by the intensity of silver staining in polyacrylamide gels (data not shown). These results suggest that RNA synthesis is influenced by both the coding and the noncoding regions of the viral RNA. The noncoding regions of the influenza virus RNAs have probably evolved in such a way that every segment contains a noncoding region that matches its corresponding coding region. This would ensure the optimal replication and transcription of each of the eight RNA segments. In conclusion, we have demonstrated for the first time that the replication and transcription of influenza virus RNAs *in vivo* is modulated by nt outside the conserved sequences and motifs of the viral RNA segments. Currently, we are attempting to define the minimal region that is needed for optimal RNA replication of the NA-segment and the contribution of both the coding and the noncoding sequences of the influenza virus genes to RNA replication and protein expression.

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