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Mechanism of Attenuation of a Chimeric Influenza A/B Transfectant Virus

GUANGXIANG LUO, MICHAEL BERGMANN, ADOLFO GARCIA-SASTRE,† AND PETER PALESE*

Department of Microbiology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, New York 10029-6574

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The ribonucleoprotein transfection system for influenza virus allowed us to construct an influenza A virus containing a chimeric neuraminidase (NA) gene in which the noncoding sequence is derived from the NS gene of influenza B virus (T. Muster, E. K. Subbarao, M. Enami, B. P. Murphy, and P. Palese, Proc. Natl. Acad. Sci. USA 88:5177–5181, 1991). This transfectant virus is attenuated in mice and grows to lower titers in tissue culture than wild-type virus. Since such a virus has characteristics desirable for a live attenuated vaccine strain, attempts were made to characterize this virus at the molecular level. Our analysis suggests that the attenuation of the virus is due to changes in the *cis* signal sequences, which resulted in a reduction of transcription and replication of the chimeric NA gene. The major finding concerns a sixfold reduction in NA-specific viral RNA in the virion, causing a reduction in the ratio of infectious particles to physical particles compared with the ratio in wild-type virus. Although the NA-specific mRNA level is also reduced in transfectant virus-infected cells, it does not appear to contribute to the attenuation characteristics of the virus. The levels of the other RNAs and their expression appear to be unchanged for the transfectant virus. It is suggested that downregulation of the synthesis of one viral RNA segment leads to the generation of defective viruses during each replication cycle. We believe that this represents a general principle for attenuation which may be applied to other segmented viruses containing either single-stranded or double-stranded RNA.

Influenza, an acute infectious respiratory disease, remains a constant worldwide threat to human health and, in the absence of effective antiviral agents, can best be prevented or limited by vaccination (1, 13). The influenza virus vaccines currently in use in the United States are derived from inactivated influenza virus, and they provide only limited protection in vaccinees (2, 13). An alternative approach would involve the development of safe live attenuated vaccines; these vaccines should be longer-lasting and afford greater protection (1). An ideal way to generate such an attenuated vaccine would be to use genetic engineering techniques. However, until recently, influenza virus—like all other negative-strand RNA viruses—was not amenable to site-specific genetic manipulation. The situation has changed for influenza virus, because synthetic RNAs reconstituted in vitro with purified viral polymerase complex can now be rescued into infectious influenza virus particles (5, 10, 12).

Applying this novel reverse genetic approach, we generated a transfectant influenza virus which possesses a chimeric neuraminidase (NA) gene containing the coding sequence of the NA gene of influenza A/WSN/33 virus and the noncoding sequence from the NS segment of influenza B/Lee virus. The virus was shown to be attenuated in mice and to induce a protective immune response against challenge virus. These features suggested that the virus has many properties desirable for an attenuated live influenza virus vaccine (14). In order to understand the molecular characteristics responsible for the attenuation of this transfectant virus, we carried out a series of experiments to analyze the virus at the molecular level. The data presented in this report suggest that attenuation results from the effect of the altered *cis* elements on the replication of the chimeric NA gene. A low level of the NA gene in the virus preparation results in a higher proportion of defective particles than is found in a wild-type virus preparation. In addition, the data support a random mechanism for the packaging of virion RNAs (vRNAs) into influenza virus particles.

MATERIALS AND METHODS

Viruses and cells. The stocks of influenza A/WSN/33 virus and the NA/B-NS transfectant virus were prepared from purified plaques by growing them in Madin-Darby bovine kidney (MDBK) cells in reinforced minimal essential medium containing 2 μg of trypsin per ml. MDBK cells were used for the plaquing of viruses and the study of virus-specific RNA synthesis.

Plasmids. In order to generate riboprobes, several plasmids were constructed. pSP64-NS DNA contains the entire NS segment of A/WSN/33 virus inserted into the *Sal*I site of the pSP64 vector (Promega, Madison, Wis.) in the orientation by which positive-sense NS RNA can be made by using SP6 RNA polymerase. To create plasmid pIBI30-NS, the entire NS DNA fragment derived from pSP64-NS was inserted between the *Hin*dIII and *Eco*RI sites of the pIBI30 vector (IBI, New Haven, Conn.) in the orientation by which negative-sense NS RNA can be generated by using T7 RNA polymerase. In order to obtain plasmid ΔT3NAv, which produces the negative-sense NA-specific RNA probe, the DNA in the pT3NAv plasmid (5) was shortened by deleting nucleotides 1 to 1096 of the NA vRNA. In addition, we created a plasmid, designated IBI30-NA, by inserting the entire NA fragment derived from pT3NAv (5) into the *Xba*I

* Corresponding author.
† On leave of absence from the Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Salamanca, Salamanca, Spain.

and *Eco*RI sites of the IBI30 vector. This plasmid can produce positive-sense NA-specific RNA by T7 RNA polymerase transcription.

Virus purification and RNA extraction. Influenza A/WSN/33 virus and NA/B-NS transfectant virus were grown in MDBK cells and then purified by 30 to 60% sucrose gradient centrifugation as described previously (5). Virus purified from four 175-cm^2 flasks of MDBK cells was resuspended in 0.3 ml of TMK buffer (10 mM Tris [pH 7.5], 1.5 mM $MgCl_2$, 10 mM KCl) and disrupted by incubation with 9 μl of 10% sodium dodecyl sulfate (SDS) and 7.5 μl of proteinase K (10 mg/ml) at 56°C for 10 min, followed by addition of 35 μl of SLN buffer (5% SDS, 1.4 M LiCl, 100 mM sodium acetate [pH 7.0]). Virion RNAs were extracted with phenol-chloroform and collected by ethanol precipitation. For isolating viral RNAs from infected cells, MDBK cells were infected with either influenza A/WSN/33 virus or NA/B-NS transfectant virus at a multiplicity of infection of 1 and harvested at the indicated time points. Cells were washed twice with ice-cold phosphate-buffered saline and lysed with 4 M guanidinium isothiocyanate (Sigma). Total RNA was then purified by equilibrium centrifugation in 5.7 M cesium chloride (Fisher) (9).

Determination of the ratio of infectious and physical particles. In order to determine the total number of physical particles of influenza A/WSN/33 virus and NA/B-NS transfectant virus, the virus preparations were mixed with an equal volume of a suspension of carboxylate polystyrene beads (0.1 μm in diameter) at a concentration of 4.5×10^9 particles per ml (Polyscience, Inc., Warrington, Pa.) and then stained with phosphotungstic acid. The ratio of virus and polystyrene beads was determined by counting the two different particles under the electron microscope. For measuring the number of infectious particles in the preparations, the virus stocks were serially diluted and plaqued in MDBK cells.

RNA electrophoresis. RNAs extracted from influenza A/WSN/33 virus and NA/B-NS transfectant virus were electrophoresed on a 3% polyacrylamide gel containing 7.7 M urea at 150 V for 2 h. The RNA segments were visualized by silver staining as described previously (5).

RNase protection assay. The RNase protection assay was used for quantitation of virion RNA in viral particles as well as for the measurement of viral mRNAs and cRNAs in infected MDBK cells. The NS segment was chosen as an internal control. For measuring virion RNAs, positive-sense NS- and NA-specific RNA probes were generated by run-off transcription with phage SP6 or T7 RNA polymerase and plasmid pSP64-NS DNA linearized by *Nco*I (nucleotide [nt] 265) or plasmid IBI30-NA digested with *Fok*I (nt 240), respectively. The NS-specific RNA probe spans the region between nt 1 and 265 of the NS cRNA, and the NA-specific RNA probe covers nt 1 to 240 of the NA cRNA.

To determine the amounts of mRNAs and cRNAs, negative-sense NS- and NA-specific RNA probes were transcribed from *Dde*I (nt 465)-digested IBI30-NS DNA and *Pvu*II (nt 306 in pUC19)-cut ΔT3NA DNA, respectively, with phage T7 or T3 RNA polymerase, respectively. The negative-sense NS-specific RNA probe is 470 nt long, containing nt 465 to 890 of the NS vRNA and a vector sequence of 45 nt. The negative-sense NA-specific RNA probe is 403 nt long, covering the region between nt 1096 and 1409 of the NA vRNA and containing a vector sequence of 90 nt.

The RNA probes were labeled with [α-^{32}P]UTP (800 Ci/mmol; Du Pont, NEN, Boston, Mass.). In general, 50 ng of virion RNA extracted from purified virus was hybridized to 5×10^4 cpm each of the positive-sense NS- and NA-specific probes, or 5 μg of total RNA isolated from virus-infected cells was hybridized to 5×10^4 cpm each of the negative-sense NS- and NA-specific probes. After 12 h of incubation at 45°C, the hybridization mixture was digested with RNases A and T_1, following the manufacturer's instruction (Ambion Inc., Austin, Tex.), and the resulting products were analyzed on a 6% acrylamide denaturing gel (9).

Primer extension. The genomic RNAs (vRNAs) of the NA and NS segments of influenza A/WSN/33 virus and of NA/B-NS transfectant virus were quantitated by primer extension (11). The primers for detecting NS- and NA-specific vRNA are 21 nt long and are complementary to negative-sense vRNA. The NS primer, 5′-GGGAACAATT AGGTCAGAAGT-3′, spans the region between nt 695 and 714 of the NS cRNA. The NA primer, 5′-GTGGCAATAAC TAATCGGTCA-3′, covers nt 1151 to 1171 of the NA cRNA. Both the NS and NA primers were 5′-end labeled by incubation with [γ^{32}P]ATP (3,000 Ci/mmol; Du Pont, NEN) and T4 DNA kinase (Biolabs, Beverly, Mass.). Then, 100 ng of RNA extracted from virus or 5 μg of total RNA isolated from infected MDBK cells was reverse transcribed by RNase H-minus murine leukemia virus reverse transcriptase (BRL, Gaithersburg, Md.) in the presence of 3×10^5 cpm each of the 5′-end-labeled NS and NA primers. After incubation at 37°C for 2 h, the reaction was stopped by addition of EDTA to 10 mM, and the reaction mix was subjected to phenol-chloroform extraction and alkali treatment (11). The products were analyzed on a 6% polyacrylamide gel containing 7 M urea. The amount of product was measured by directly counting the radioactivity of the gel piece corresponding to each band on the film.

Neuraminidase assay and Western immunoblot analysis. For the assay of neuraminidase activity of the influenza A/WSN/33 and NA/B-NS transfectant viruses, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Sigma) was used as the substrate. The reaction mixture consisted of 25 μl of 2 mM substrate, 25 μl of virus, and 50 μl of 0.2 M phosphate buffer (pH 6.0) containing 2 mM $CaCl_2$. After incubation at 37°C for 10 min, the reaction was stopped by addition of 2 ml of 0.5 M glycine-NaOH buffer (pH 10.6), and then the neuraminidase activity was determined by measuring the fluorescence with excitation at 365 nm and emission at 450 nm, with methylumbelliferone as a standard. The protein concentration of the viruses was measured with the Bio-Rad protein assay kit. For the Western analysis of the NA and HA proteins of the WSN/33 and NA/B-NS viruses, viral proteins were electrophoresed on a 10% polyacrylamide Laemmli gel (7) and subsequently transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.). A monoclonal antibody directed against carbohydrates was used to detect the NA and HA glycoproteins of the viruses. The Western blot was developed with a rat antibody against mouse kappa chains, which was labeled with ^{125}I and was generously provided by Thomas Moran.

Analysis of protein synthesis in infected cells. MDBK cells (35-mm dish) were infected with either WSN/33 virus or NA/B-NS transfectant virus at a multiplicity of infection of approximately 3. This multiplicity was used because the NA/B-NS transfectant virus did not grow to higher titer. At indicated times, the proteins were labeled in cysteine-free medium with [^{35}S]cysteine (1,027 Ci/mmol; Du Pont, NEN) at 100 μCi/ml of medium for 30 min. The cells were then washed twice with ice-cold phosphate-buffered saline and lysed in 150 μl of lysis buffer containing 1% Nonidet P-40,

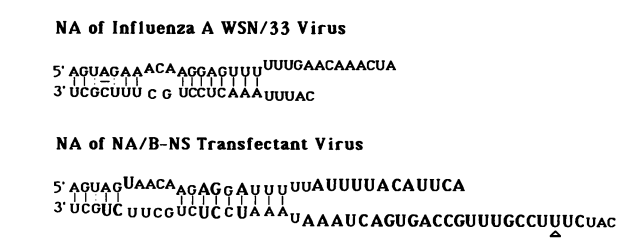

FIG. 1. Noncoding sequences of the NA segments of influenza A/WSN/33 virus and the NA/B-NS transfectant virus. The 5'- and 3'-terminal sequences are drawn in a panhandle structure, which consists of two base-paired stems and one mismatched internal loop in the middle. The noncoding nucleotides of the chimeric NA gene of the NA/B-NS virus are derived from the NS gene of influenza B/Lee virus. The large letters indicate nucleotides in the 5'- and 3'-terminal regions which are different for the two NA genes. The open triangle marks the altered Kozak sequence in the NA gene of the NA/B-NS virus.

150 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 1 mM phenylmethylsulfonyl fluoride. About 1/20 of the sample was loaded onto a 10% polyacrylamide Laemmli gel (7).

RESULTS

Ratio of infectious to physical particles. The genome of the transfectant NA/B-NS virus differs from that of wild-type influenza A/WSN/33 virus only in the noncoding region of the NA gene (Fig. 1). It is thus likely that the altered biological properties of the transfectant virus are the result of altered *cis* signals located in the noncoding region of the chimeric NA gene (9, 15, 18). Specifically, it was noted earlier that the transfectant virus grew to lower titers than wild-type virus in MDBK cells, MDCK cells, and mice. In addition, the low-multiplicity growth curves in tissue culture were significantly delayed relative to those of wild-type virus (14). We then proceeded to examine whether the transfectant virus had a temperature-sensitive phenotype, which could explain the altered growth characteristics. However, the pattern of the growth curve at 30, 33, 37, and 38°C for NA/B-NS virus was not different from that of wild-type virus at the corresponding temperatures. We next asked whether defective particles were present in the NA/B-NS virus preparation. The virus was characterized by counting the physical particles under the electron microscope and comparing this number with the PFU of the preparation. Interestingly, the NA/B-NS transfectant virus showed a similar number of physical particles as the wild-type virus but consistently lower PFU titers (14) (Table 1). Thus, the NA/B-NS virus grown in MDBK cells shows at least a 5- to 10-fold-lower infectious particle-to-physical particle ratio than is seen with the WSN/33 wild-type virus.

Characterization of the RNA of the NA/B-NS virus. Since the NA/B-NS virus contains many defective particles, the viral RNA was examined for the presence of defective RNAs. Following extraction from purified virus, the genomic RNA was separated on a 3% polyacrylamide gel containing 7.7 M urea. As shown in Fig. 2A, the chimeric NA RNA of the NA/B-NS transfectant virus is almost invisible on the gel, whereas the other seven segments are present in approximately equimolar concentrations. When the amount of RNA on the gel was increased, the chimeric NA RNA was shown to migrate at the same position as the control RNA in lane 3 (data not shown). However, electrophoresis and silver staining did not permit the quantitation of the chimeric NA RNA packaged in virions. For this purpose, the RNase protection assay and primer extension experiments were performed.

TABLE 1. Ratio of infectious particles to physical particles of influenza A/WSN/33 virus and NA/B-NS transfectant virus

Virus	Physical particles (pp) (no./ml)	Infectious particles (ip) (no./ml)	Ratio, pp/ip
WSN/33	1.8×10^9	1.2×10^8	15
NA/B-NS	1.2×10^9	1.0×10^7	120

Positive-sense NS-specific and NA-specific probes were hybridized in the same reaction to purified virion RNA from either WSN/33 virus (Fig. 2B, lane 3) or NA/B-NS transfectant virus (Fig. 2B, lane 4) and then digested with RNases A and T_1. The resulting products were analyzed on a 6% polyacrylamide gel containing 7 M urea (9), and the amounts of vRNA were calculated by counting the radioactivity of the gel slices corresponding to the bands on the film. As shown in Fig. 2B, the probe protected by the chimeric NA RNA migrates faster than that of the wild-type gene because the 5 noncoding sequences are different in the two NA genes. Compared with the NA segment of WSN/33 virus, the amount of chimeric NA RNA in the transfectant virus is about six times lower, with the NS gene used as an internal control. The primer extension experiment, as shown in Fig. 2C, shows a similar reduction in the amount of chimeric NA RNA packaged in virions relative to the NS gene, suggesting a specific lower representation of the chimeric NA gene in the transfectant virus preparation.

Characterization of the protein of the NA/B-NS virus. The next question was whether the NA/B-NS transfectant virus also contained less neuraminidase protein. We carried out neuraminidase assays and a Western analysis of the NA protein in virus particles. For the enzymatic assay of the neuraminidase, the total concentration of viral proteins was first determined by the protein assay, and then the same amount of purified virus was used. The NA/B-NS transfectant virus showed only a twofold-lower NA activity than the WSN/33 wild-type virus. A similar finding was obtained by Western analysis, which showed 1.9-fold less NA protein but the same amount of HA protein in virions of the NA/B-NS transfectant virus compared with those of WSN/33 virus (see Fig. 5A).

Virus-specific RNA synthesis in infected cells. Based on the vRNA analysis of the NA/B-NS transfectant virus, there is little doubt that less chimeric NA RNA is contained in the viral particles. The lower level of the chimeric NA RNA in the virions could be caused either by a change in the packaging signal, leading to less efficient packaging of the NA RNA, or by a change in the synthesis of genomic NA RNA, which could be the result of inefficient recognition of the influenza B virus-specific promoter by the influenza A viral polymerase. To pinpoint the exact mechanism, we examined the vRNA synthesis of the chimeric NA gene in infected MDBK cells. Considering that vRNA is mainly synthesized in the late phase of infection (19), we used RNA extracted from cells at 7, 8.5, and 10 h postinfection. Total RNA (5 μg) extracted from virus-infected cells was used for the primer extension analysis. The data in Fig. 3 show that vRNA synthesis of the chimeric NA gene was remarkably decreased relative to that of control virus. Compared with

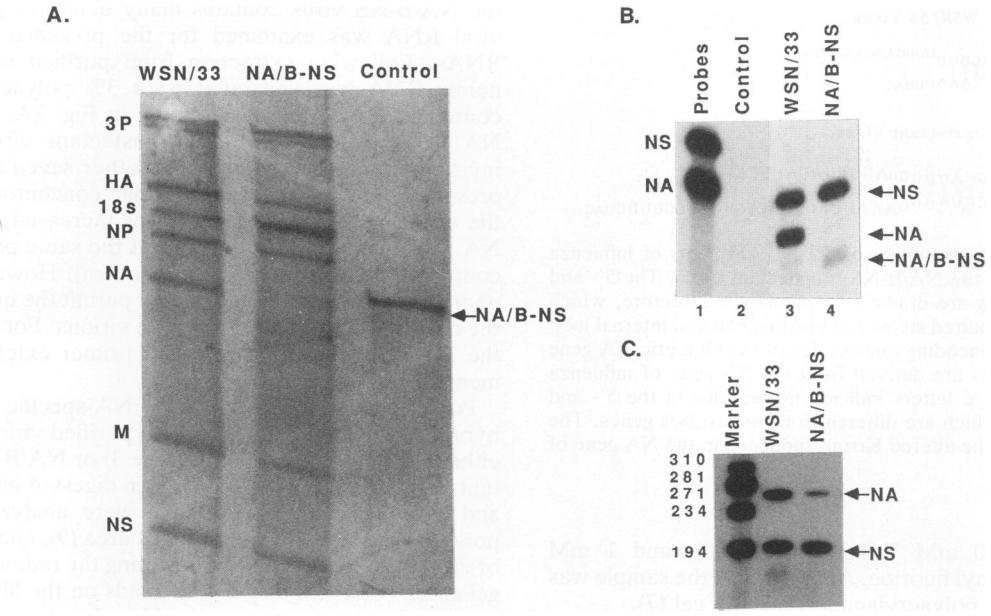

FIG. 2. Characterization of the RNA of the NA/B-NS virus. (A) RNA electrophoresis. The RNAs extracted from purified viruses were analyzed on a 3% polyacrylamide gel containing 7.7 M urea and visualized by silver staining. Lane 1, RNA of influenza A WSN/33 virus; lane 2, RNA of NA/B-NS transfectant virus; lane 3, RNA obtained by run-off transcription from plasmid pT_7NA/B-NS, which produces the chimeric NA RNA. (B) Analysis of NA RNA in virions by the RNase protection assay. RNA (50 ng) extracted from purified virus was used in the hybridization reaction with positive-sense NS- and NA-specific riboprobes as described in Materials and Methods. The protected probes were electrophoresed on a 6% acrylamide gel containing 7 M urea. Lane 1, riboprobes without RNase A or T_1 digestion; lane 2, riboprobes following RNase A and T_1 digestion; lane 3, riboprobes protected by the RNA of influenza A/WSN/33 virus; lane 4, riboprobes protected by the RNA of NA/B-NS transfectant virus. (C) Quantitation of NA-specific RNA in virus by primer extension. The vRNA extracted from either WSN/33 virus (lane 2) or NA/B-NS transfectant virus (lane 3) was reverse transcribed by RNase H-minus reverse transcriptase with NS and NA segment-specific primers as described in Materials and Methods. The products for the NS RNAs are 195 nt long, and those for the NA RNAs are approximately 260 nt long. The products were analyzed on a 6% polyacrylamide gel containing 7 M urea. Size markers are shown on the left (in nucleotides).

the synthesis of the NA RNA of WSN/33 virus, synthesis from the chimeric NA gene is reduced by a factor of 9, 8, and 8 at 7, 8.5, and 10 h postinfection, respectively. However, no reduction of vRNA synthesis was observed with respect to the NS segment of the NA/B-NS transfectant virus. This result suggests that the reduction of the chimeric NA RNA in transfectant virus is similar to the reduction in synthesis observed in infected cells. Thus, the lower representation of the chimeric NA segment in virus particles is most likely not due to a defect in the packaging of the chimeric NA RNA.

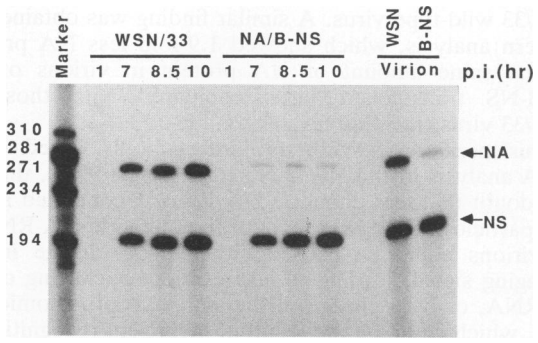

FIG. 3. Analysis of NA-specific vRNA synthesis in infected cells by primer extension. RNA isolated from infected cells was reverse transcribed with reverse transcriptase and NA and NS vRNA-specific primers, as described in Materials and Methods. vRNAs extracted from purified virus were used as a control (right). The resulting products were displayed on a 6% acrylamide denaturing gel. The reverse transcripts of the NS RNAs are 195 nt long, and those of the NA-specific segments are approximately 260 nt long, as indicated by arrows at the right. The numbers on the top indicate times postinfection. Virion represents the RNA obtained from purified virus. Size markers are shown in nucleotides.

In order to determine the level of mRNA synthesis of the chimeric NA gene, MDBK cells were infected with either WSN/33 virus or NA/B-NS transfectant virus, and total RNA was then isolated from virus-infected cells at different times postinfection. Subsequently, the level of virus-specific mRNAs was quantitated by the RNase protection assay. Again, the NS segment was used as a control. From the time course, it is apparent that the level of chimeric NA mRNA is markedly reduced relative to that of the wild-type virus and that it only increases slightly with time (Fig. 4A). At 5 h postinfection, the level of chimeric NA mRNA was fivefold less than that of WSN/33 virus (Fig. 4B), whereas the NS-specific mRNA synthesis of the NA/B-NS transfectant virus was similar to that of WSN/33 virus at the indicated times (Fig. 4C). It should be noted that the NS-specific mRNA synthesis appears earlier and is more efficient than NA-specific mRNA synthesis in both WSN/33 and NA/B-NS transfectant virus-infected cells (probes of similar activity were used in the experiment). This finding is in good agreement with previous reports (4) and suggests that the mRNA synthesis of different influenza virus RNA segments is differentially regulated. The conditions of the assay did not

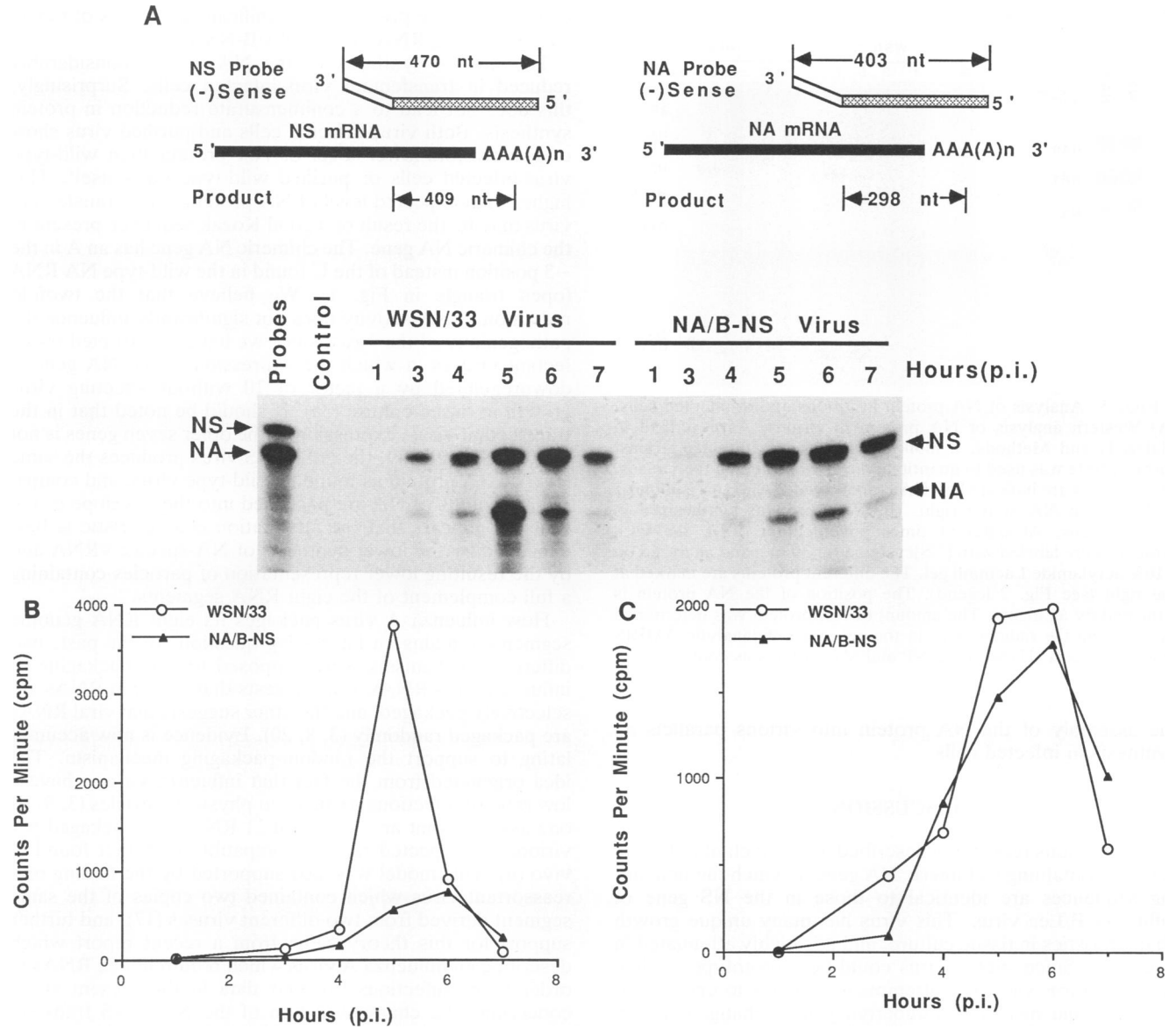

FIG. 4. Time course of mRNA synthesis in MDBK cells. (A) Quantitation of NA- and NS-specific mRNAs at different times postinfection (p.i.) by the RNase protection assay. The diagram on the top schematically illustrates the procedure. Both the NS and NA probes are negative sense and contain sequences corresponding to the 3'-terminal side of the cRNA, flanked by vector sequences at the 3' terminus, as indicated by the open rectangles. The sizes of the probes are shown on the top, and the sizes of the resulting products are indicated at the bottom of the diagram. The time points are indicated on the top. The positions of probes and products on the gel are indicated by arrows at the left and right, respectively. (B) Time course of NA-specific mRNA synthesis. The amount of the NA mRNA was measured by directly counting the radioactivity (counts per minute) in the corresponding band excised from the gel shown in panel A. (C) Comparison of NS-specific mRNA synthesis of transfectant virus and A/WSN/33 virus in infected cells. The amount of mRNA for each time point was determined as described for panel B.

permit us to quantitate the level of cRNA of the chimeric NA gene because the cRNA synthesis was found to be only 3 to 5% of that of mRNA synthesis (10a).

Reduction of NA protein synthesis in vivo. The NA protein was found to be reduced by a factor of 2 in virions of the NA/B-NS transfectant virus, whereas the synthesis of chimeric NA mRNA was reduced by much more. The question then arose whether the NA protein encoded by the chimeric NA mRNA inside cells parallels the amount of its mRNA, or whether there is a selective incorporation of the neuraminidase into the viral envelope. In order to answer this question, we measured the synthesis of NA protein in infected cells. Viral proteins were labeled with [^{35}S]cysteine at 1, 3, 5, and 7 h postinfection (Fig. 5B). The cell lysates were then analyzed on a 10% polyacrylamide Laemmli gel (7), and the proteins were quantitated by an AMBIS radioanalytic imaging system (11). Synthesis of the NA protein of the NA/B-NS transfectant virus was measured and shown to be two times lower than that of wild-type virus at both 5 and 7 h postinfection. From this experiment, we can conclude that

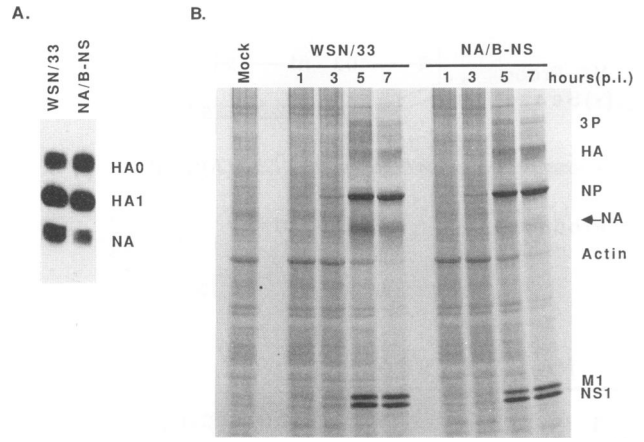

FIG. 5. Analysis of NA protein in virions and in infected cells. (A) Western analysis of NA protein in virions. As described in Materials and Methods, a monoclonal antibody directed against carbohydrate was used to quantitate the glycoprotein in the viruses. The proteins are indicated by HA0 (uncleaved HA), HA1 (subunit 1 of HA), and NA at the right. (B) Viral proteins synthesized in infected cells. At different times postinfection (p.i.), the viral proteins were labeled with [^{35}S]cysteine for 30 min and analyzed on a 10% acrylamide Laemmli gel. The different proteins are marked at the right (see Fig. 2 legend). The position of the NA protein is indicated by an arrow. The amount of NA protein was determined by counting the radioactivity in the gel in a radioanalytic AMBIS imaging system (11) with the NP and M1 proteins as controls.

the assembly of the NA protein into virions parallels its synthesis in infected cells.

DISCUSSION

In a previous report, we described a transfectant influenza A virus containing a chimeric NA gene in which the noncoding sequences are identical to those in the NS gene of influenza B/Lee virus. This virus has many unique growth characteristics in tissue culture, and it is highly attenuated in mice (14). Since such a virus could be a prototype for live influenza virus vaccines, attempts were made to understand the molecular mechanism underlying these changed growth characteristics. Several lines of evidence obtained from these experiments suggest that the *cis* elements derived from the influenza B/Lee virus gene are responsible for the dramatic effects on transcription and replication of the chimeric NA gene of the NA/B-NS transfectant virus. It was found that the NA gene had a sixfold-lower representation in the purified viral preparation than did the remaining seven RNAs. This strikingly lower representation of one RNA is compatible with the finding that the NA/B-NS transfectant virus has an approximately 5- to 10-fold-lower infectious particle-to-physical particle ratio than wild-type virus.

It is assumed that an infectious virus would require the presence of a full complement of all eight influenza virus RNA segments. Many of the NA/B-NS progeny virus, however, lack an NA gene, so that more defective particles are formed than is the case in a wild-type virus infection. It is not clear whether this 5- to 10-fold reduction in titer is only a reflection of the lower representation of the NA gene or whether other factors also play a role. For example, some viruses may contain defective interfering RNAs which would lower the infectivity titer of the preparation. However, in this context it should be noted that there is no evidence for the presence of significant quantities of defective interfering RNAs in the NA/B-NS virus.

The mRNA synthesis of the NA is also considerably reduced in transfectant virus-infected cells. Surprisingly, this does not lead to a commensurate reduction in protein synthesis. Both virus-infected cells and purified virus show only a twofold-lower level of NA protein than wild-type virus-infected cells or purified wild-type virus itself. This higher-than-expected level of NA protein in the transfectant virus may be the result of a good Kozak sequence present in the chimeric NA gene. The chimeric NA gene has an A in the −3 position instead of the U found in the wild-type NA RNA (open triangle in Fig. 1). We believe that the twofold reduction in NA activity does not significantly influence the pathogenicity of the virus, since we have constructed transfectant viruses in which the expression of the NA gene is downregulated by a factor of 10 without affecting virus growth in tissue culture (3a). It should be noted that in the transfectant virus, expression of the other seven genes is not altered. Specifically, the NA/B-NS virus produces the same level of HA protein as found in wild-type virus, and comparable amounts of HA are packaged into the envelope of the virus. It appears that the attenuation characteristic is best explained by the lower synthesis of NA-specific vRNA and by the resulting lower representation of particles containing a full complement of the eight RNA segments.

How influenza A virus packages its eight RNA genome segments remains an interesting question. In the past, two different mechanisms were proposed for the packaging of influenza virus RNAs: one suggests that the eight RNAs are selectively packaged, and the other suggests that viral RNAs are packaged randomly (3, 8, 20). Evidence is now accumulating to support the random-packaging mechanism. The idea originated from the fact that influenza viruses have a low ratio of infectious particles to physical particles (3, 8). If one assumes that an average of 11 RNAs are packaged per virion, the expected ratio is compatible with that found in vivo (6). This model was also supported by the finding of a reassortant virus which contained two copies of the same segment derived from two different viruses (17), and further support for this theory came from a recent report which described an influenza A virus which required nine RNAs in order to be infectious (6). Our data in the present study concerning the characterization of the NA/B-NS transfectant virus also seem to favor the random-packaging mechanism rather than the selective one. The lower level of chimeric NA RNA found in virions is consistent with the reduction of its synthesis in infected cells. In a selective packaging model, it would be expected that approximately equimolar amounts of chimeric NA RNA would be packaged into virus particles.

In summary, the present data suggest that influenza viruses for which the synthesis of a vRNA segment is downregulated produce defective particles during replication. Since the proteins of this virus are unaltered compared with those of wild-type virus, attenuation must be the result of inefficient *cis*-acting signals. We believe that this principle of attenuation can be applied to other viruses with segmented genomes. For example, the introduction of modifications into the noncoding sequences of rotavirus genes or of genes of other segmented double-stranded RNA viruses (16) should also allow the pathogenicity of these viruses to be altered. With the influenza virus system, we now plan to identify the nucleotide sequences in the NA/B-NS virus which are responsible for the regulation of the synthesis of vRNA and mRNA. This should help us to precisely deter-

mine the *cis* elements required for the replication of influenza viruses.

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