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Segment-Specific Noncoding Sequences of the Influenza Virus Genome RNA Are Involved in the Specific Competition between Defective Interfering RNA and Its Progenitor RNA Segment at the Virion Assembly Step

TAKATO ODAGIRI[1]* AND MASATO TASHIRO[2]

Department of Virology, Jichi Medical School, Minami-Kawachi, Tochigi 329-04,[1] and Department of Virology 1, National Institute of Health, Tokyo 162,[2] Japan

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The generation of influenza A virus defective interfering (DI) particles was studied by using an NS2 mutant which produces, in a single cycle of virus replication, a large amount of DI particles lacking the PA polymerase gene. The decrease in PA gene replication has been shown to occur primarily at the cRNA synthesis step, with preferential amplification of PA DI RNA species present in a marginal amount in the virus stock. In addition, at the assembly step the PA DI RNAs were preferentially incorporated into virions, resulting in selective reduction in the packaging of the PA gene into virions. Similarly, in cells dually infected with the NS2 mutant and wild-type viruses, packaging of the wild-type PA gene was also greatly suppressed. In contrast, incorporation of other RNA segments, i.e., the PB2 and NS genes, was not affected, suggesting that the PA DI RNAs competed only with the PA gene in a segment-specific manner. Experiments involving rescue of recombinant chloramphenicol acetyltransferase (CAT) RNA flanked by the noncoding regions of the PA (PA/CAT RNA) and PB2 (PB2/CAT RNA) genes into viral particles showed that only PA/CAT RNA was not rescued by infection with the NS2 mutant virus containing the PA DI RNAs. However, recombinant PA/CAT RNA in which either the 3′ or 5′ noncoding region was replaced with that of the PB2 gene was rescued by the NS2 mutant. These results suggest that the noncoding regions of the PA gene are responsible for the competition with PA DI RNA species at the virus assembly step and that coexistence of the both noncoding regions would be a prerequisite for this phenomenon. Decreased packaging of the progenitor RNA by the DI RNA, in addition to the suppression of cRNA synthesis, is likely involved in the production of DI particles.

The genome of influenza A virus consists of eight RNA segments of negative polarity (18, 33). The 3′- and 5′-terminal noncoding regions of the genome RNA, consisting of 12 and 13 nucleotides, respectively, are highly conserved among the eight segments, and the two regions are partially complementary to each other, forming a panhandle structure (7, 13, 17, 35, 41). The 3′ noncoding regions contain the promoter sequences responsible for genome replication and transcription (12, 14, 16, 21, 23, 34, 38). By the reverse genetics system, it has been shown that the 3′-terminal 26 nucleotides and 5′-terminal 22 nucleotides of the NS genome RNA were necessary for packaging of the RNA into virions (23). A set of eight RNA segments is required for an influenza virus particle to express infectivity. However, the mechanism of assembly or packaging of the RNA segments into an infectious particle remains unknown. Two models have been proposed for the packaging. One is the random-packaging model, in which genome RNA segments are picked up randomly among a pool of eight different RNAs bearing a common packaging signal(s). This is based on the observation that the ratio of infectious viral particles to noninfectious ones is estimated to be as low as 10% (6, 15) and on the calculation that the proportion of infectious particles containing the full complement of eight RNA segments is increased by incorporation of extra RNA segments into virions (20). Rescue of transfectants containing two NS genes (10) and of reassortants with two copies each of RNA segments 2 and 6 (37) has suggested that an influenza virus particle has the capacity to contain more than eight RNA segments.

A second model depicts selective packaging of each RNA segment into a virion. This is based on the findings that the eight RNA segments are present in an infectious particle in approximately equimolar quantities (24) and that the relative amount of each genome RNA segment in infected cells does not reflect that in virions (3, 40). Furthermore, the selective-packaging model has been favored by analyses of defective interfering (DI) preparations of influenza virus, in which the presence of short DI RNAs, with internal deletions in the coding region, is always accompanied by a specific reduction of their progenitor genes (8, 27–29, 30).

The influenza A virus mutants Wa-182 and its subclone A3/e-3, which have mutated NS2 nonstructural proteins, produce large amounts of DI particles lacking the PA gene after a single cycle of replication at a high multiplicity of infection (MOI) (30, 32). The mutated NS2 protein has been shown to be responsible for the preferential replication of DI RNA species derived from the PA gene (PA DI RNAs) which are present in a marginal amount in the virus stock and for the selective suppression of the standard PA gene replication at the step of cRNA synthesis (32). Furthermore, we observed that the relative amount of DI RNA species of the NS2 mutants was much larger in virions than in infected cells when compared with those of standard genome RNA (32), suggesting that the DI RNAs were preferentially incorporated into virions.

On the basis of these findings, we have attempted to deter-

* Corresponding author. Mailing address: Department of Virology, Jichi Medical School, Minami-Kawachi, Tochigi 329-04, Japan. Phone: 81/285-44-2111. Fax: 81/285-44-4981. E-mail: todagiri@jichi.ac.jp.

mine the step at which DI RNAs become predominant over the full-length PA gene (PA vRNA) in their transportation pathway in infected cells and to identify the signal(s) which is responsible for competition between the DI RNA and its progenitor RNA segments. Here we show that the PA DI RNAs were preferentially incorporated into virions at the step of virion assembly, so that the packaging of PA vRNA was selectively decreased. Furthermore, by use of a reverse genetics system, we show that the 3′ and 5′ nonconserved untranslated regions of the PA gene were responsible for recognition as the selective competitor by the PA DI RNAs.

MATERIALS AND METHODS

Viruses and cells. The NS2 mutants of influenza A virus, A3/e-3 (32) and 182/A-4, are subclones of Wa-182, which contain the mutated NS gene derived from A/Aichi/2/68 virus and the other seven genes derived from WSN/33 virus. These mutants produce a large amount of DI particles lacking the PA gene after a single cycle of replication at a high MOI (30, 32). The DI particles of A3/e-3 contain two PA DI RNAs (561 and 463 nucleotides) with internal deletions corresponding to residues 205 to 1876 and 208 to 1977, respectively (32). The DI particles of 182/A-4 contain three PA DI RNAs (438, 432, and 400 nucleotides) with internal deletions from residues 268 to 2062, 185 to 1985, and 150 to 1982, respectively. AwNS/2, used as a wild-type (wt) virus, is also a reassortant containing the wt NS gene of Aichi virus and the other seven genes of WSN virus (32). This progeny virus grown at a high MOI contains a negligible amount of PA DI RNAs, which are detectable only after amplification by PCR (32).

MDCK cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Construction of plasmids. Plasmids pPA/CAT and pPB2/CAT contained the coding region of the chloramphenicol acetyltransferase (CAT) gene flanked by the noncoding sequences of the WSN PA and PB2 genes, respectively (see Fig. 5A). pPA/CAT was made by cloning of the PCR product (36) made with the *Hin*dIII fragment of the CAT gene derived from pCM7 (Pharmacia, P-L Biochemicals) as a template and with primers 5′-GCGCGCGGATCCCTCTTCGAGCGAAAGCAGGTACTGATTCAAAATGGAGAAAAAATCACTGGGTATA-3′ (3′PA/CAT) and 5′-GCGCGCAAGCTTAATACGACTCACTATAAGTAGAAACAAGGTACTTTTTTGGACAGTATGGATAGCAAATAGTAGCATTGCCACAATTACGCCCCGCCCTGCCACTCATC-3′(5′PA/T7CAT). pPB2/CAT was made in a similar way by using primers 5′-GCGCGCGGATCCCTCTTCGAGCGAAAGCAGGTCAATTATATTCAATATGGAGAAAAAAATCACTGGGTATA-3′ (3′PB2/CAT) and 5′GCGCGCAAGCTTAATACGACTCACTATAAGTAGAAACAAGGTCGTTTTTAAACTATTCGACATTACGCCCCGCCCTGCCACTCATC-3′ (5′PB2/T7CAT). pPA/CAT and pPB2/CAT contained a T7 RNA polymerase promoter upstream and an *Ear*I site downstream of each noncoding sequence, so that viral sense RNA transcripts were generated when the plasmids were digested with *Ear*I, filled in with Klenow fragment, and transcribed with T7 RNA polymerase.

pPA/PB2 and pPB2/PA were constructed by replacing the 5′ noncoding regions of pPA/CAT and pPB2/CAT, respectively, with that of the other plasmid (see Fig. 5A). pPA(7PB2 int) contained chimeric noncoding sequences constructed by replacing part of the noncoding regions of pPA/CAT with the corresponding sequences of the PB2 gene (see Fig. 5A), made by PCR with the pPA/CAT template and primers 5′-GCGCGCGGATCCCTCTTCGAGCGAAAGCAGGTCAATTATCAAAATGGAG (3′PA/7mut) and 5′-GCGCGCAAGCTTAATACGACTCACTATAAGTAGAAACAAGGTCGTTTTTTGGACAGTAT (5′PA/3mutT7). All plasmid DNAs used in the present study were partially sequenced from the flanking pUC19 vector sequences to the CAT gene. No deviation from the expected sequences was found.

RNP transfection to MDCK cells. Nucleoprotein and polymerase protein were purified from influenza virus A/PR/8/34 as previously described (34). A ribonucleoprotein (RNP) complex was prepared by transcribing pPA/CAT, pPB2/CAT, pPA/PB2, pPB2/PA, or pPA(7PB2 int) with T7 RNA polymerase in the presence of nucleoprotein and polymerase protein after the plasmids were digested with *Ear*I and filled in with Klenow fragment as described previously (11). The RNP complex was then transfected into 60% confluent MDCK cells by the DEAE-dextran method (11); the cells were infected with 182/A-4 at an MOI of 5 PFU/cell 1 h before transfection. After incubation for 15 h at 37°C, transfectant viruses produced in the culture medium were purified by centrifugation through a 30% sucrose cushion for 90 min at 35,000 rpm in an SW55 rotor. The purified virions were then treated for 30 min with 1 mg of RNase A per ml on ice, and the virion RNAs (vRNAs) were extracted by acid guanidine thiocyanate-phenol-chloroform extraction (4). The CAT gene constructs incorporated into the virions and the NS RNA segment of transfectant virus were detected by reverse transcription-PCR (RT-PCR) for 25 cycles with the [γ-^{32}P]ATP-labeled CAT gene-specific primers 5′-CAGTCAGTTGCTCAATGTACCTATAACCAGAC and 5′-GAACCTGAATCGCCAGCGGCATCAGCACCTTGTCG and the NS-specific primers 5′-GTGACAAAGACATAATG and 5′-AGTAGAAACAAGG GTGTTT after reverse transcription of 100 ng of vRNA with Superscript II (Gibco BRL) with the primer 5′-AGCAAAAGCAGG. The ^{32}P-labeled PCR products were analyzed by electrophoresis on a 4% polyacrylamide gel. The radioactivity of the PCR products was measured with a BAS 2000 Bioimaging Analyzer (Fuji Film).

Cell fractionation. MDCK monolayer cells were infected with virus at 10 PFU/cell and incubated at 34°C. At 10 h after infection, the cells were treated for 30 min with 3 U of *Clostridium perfringens* neuraminidase per ml to remove completely virions associated on the cell surface. The cells were harvested by being scraped from a 50-cm^2 culture dish after treatment for 3 min with a mixture of 0.025% trypsin and 0.01% EDTA in phosphate-buffered saline deficient in Mg^{2+} and Ca^{2+}. The collected cells were incubated for 10 min in 0.5 ml of ice-cold buffer A (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and homogenized in a glass Dounce homogenizer on ice after addition of an equal volume of buffer B (0.5 M sucrose and 0.2% Triton X-100 in buffer A). After centrifugation through a 0.5 M sucrose cushion for 10 min at 3,000 × *g*, the nuclei and cytoplasm were recovered as the pellet and supernatant fractions, respectively. The RNA species in each cell fraction were extracted as described above.

Quantitative analysis of vRNAs. PA vRNAs and PA DI vRNAs in each cell fraction and in the virions were quantified by an RNase protection assay (RPA) with a [^{32}P]UTP (30 TBq/mmol)-labeled positive-sense riboprobe, corresponding to nucleotides 8 to 581 of the PA mRNA of Wa-182 (32). Similarly, for quantitation of NS vRNA and PB2 vRNA, ^{32}P-riboprobes were transcribed from pNS/14 linearized with *Nru*I, corresponding to nucleotides 1 to 287 of the NS mRNA of Aichi wt virus, and from pPB2/49 linearized with *Taq*I, corresponding to nucleotides 4 to 392 of the PB2 mRNA of A3/e-3, respectively. The protected RNAs, after digestion with a mixture of RNases A and T_1, were analyzed by electrophoresis on a 5% polyacrylamide gel containing 8 M urea (32). The radioactivities of the RNA bands were measured as described above after exposure to an imaging plate for 4 h. In our previous experiments, the linearity of the RPA response was ensured, with an average error of 14.6% (32).

RESULTS

Quantitation of PA vRNA and PA DI RNAs in infected cells and virions. In our previous study using the NS2 mutant A3/e-3, we observed that the ratio of the PA genomic RNA (PA vRNA) to the PA DI RNAs in the progeny virions was much smaller than that in infected cells (32). We reexamined this with the RNA species extracted from cells infected with A3/e-3 at a high MOI and from the virions released. Since the amount of vRNA packaged into progeny virions would reflect the level of intracellular vRNA species synthesized prior to the time of virion assembly, we collected infected cells at 6 h postinfection (p.i.) and virions at 8 h p.i. The PA vRNA and PA DI RNAs in those preparations detected by RPA are shown in Fig. 1. Although A3/e-3 actually produces two PA DI RNAs (32), they were detected as a single band in an RPA gel because of only a 3-nucleotide difference at the 3′ deletion site. In infected cells the ratio of PA vRNA to PA DI RNAs was found to be 20% (Fig. 1A, lane C), whereas in virions it was greatly decreased, to 2.3% (lane V). These results were consistent with our previous observation (32) and suggested that at the virion assembly step the PA DI RNA species were preferentially incorporated into virions over the PA vRNA.

Quantitation of PA vRNA and PA DI RNAs in the nuclei and cytoplasm of infected cells and in virions. To determine whether transportation of PA vRNA from the nucleus to cytoplasm in infected cells is affected by PA DI RNA, A3/e-3-infected cells were fractionated into the nucleus and cytoplasm as described in Materials and Methods. We examined the purity of each cell fraction by two procedures. Possible contamination of the cytoplasm in the nuclear fraction was monitored by counting the number of nuclei associated with the cytoplasmic membrane under a phase-contrast microscope. This contamination was evaluated as being less than 9.4% (Fig. 2A, panel N). On the other hand, nuclear contamination in the cytoplasmic fraction was evaluated by detecting influenza virus cRNA species in the cytoplasmic fraction by RPA, since the cRNA remains in the nucleus throughout the infection cycle (39). By cell fractionation, 6.2 and 67.3% of total RNA were recovered as the nuclear and cytoplasmic RNAs, respectively.

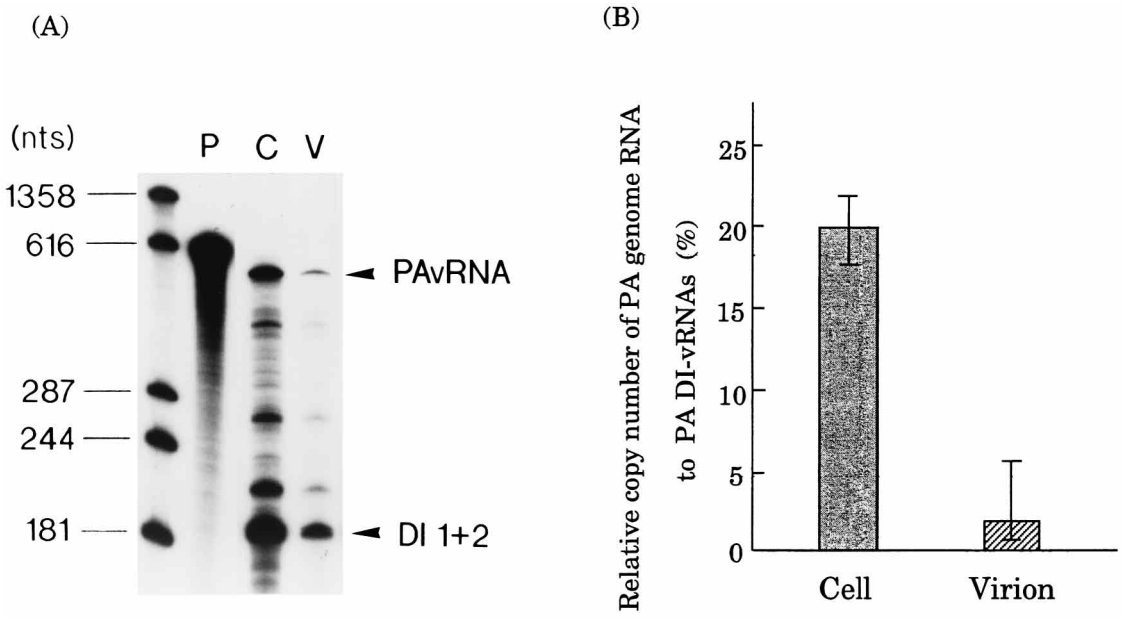

FIG. 1. Quantitation of PA vRNA and PA DI vRNA of the NS2 mutant A3/e-3 in infected cells and virions. (A) The PA gene-specific RNAs in 6 ng of vRNA extracted from the purified virions harvested at 8 h p.i. (lane V) and in 1 μg of total RNA extracted from infected cells collected at 6 h p.i. (lane C) were analyzed by the RPA with the ^{32}P-labeled positive-sense riboprobe specific for the PA gene. Lane P, probe. Size markers are shown on the left. nts, nucleotides. (B) The radioactivities of undigested bands in the RPA gel were measured with a BAS 2000 Bioimaging Analyzer after exposure for 4 h on an imaging plate. The graph indicates the ratios of PA vRNA to PA DI RNA in infected cells and virions. Error bars indicate standard errors.

Figure 2B shows PA cRNA in a poly(A)$^-$ RNA preparation of each cell fraction. Although the PA cRNA species were found in both the nuclear and cytoplasmic fractions, 78.1% of the total cRNA was recovered from the nuclear fraction (Fig. 2B, lane N), while only 21.9% of it was contaminating the cytoplasm (lane C).

Figure 3A shows the PA vRNA and PA DI RNAs, in addition to the NS vRNA, in the nuclear and cytoplasmic fractions

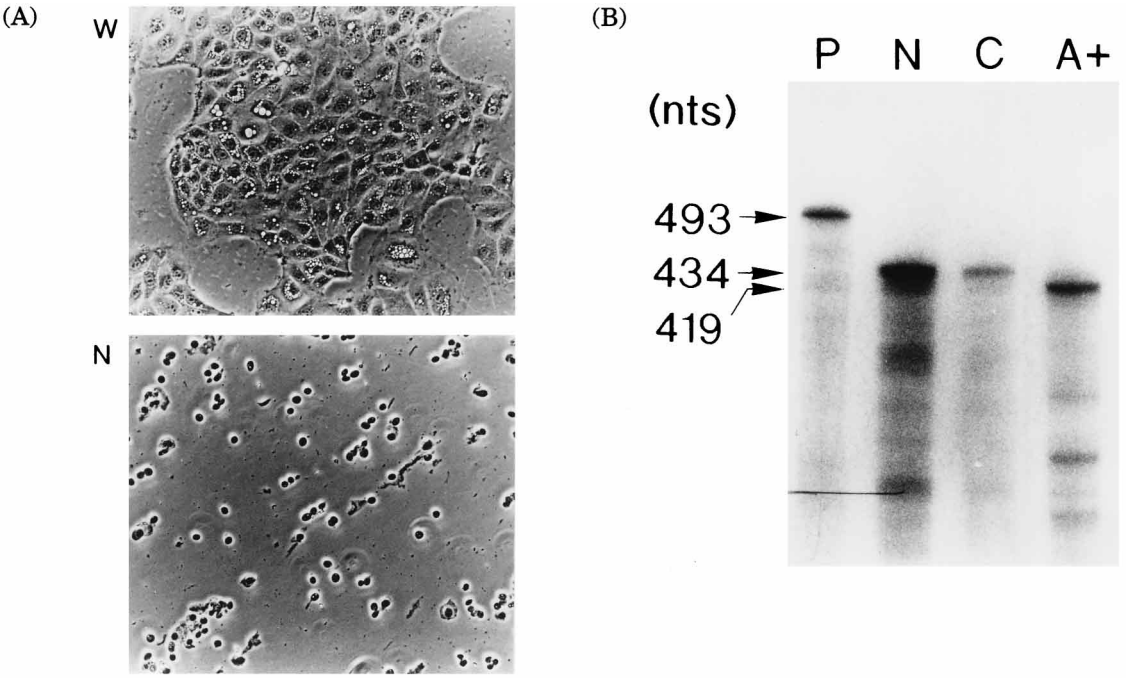

FIG. 2. Determination of purity of the nuclear and cytoplasmic fractions obtained from infected MDCK cells. (A) Phase-contrast micrographs of infected cells harvested at 4 h p.i. W, whole cells; N, nuclear fraction. (B) At 4 h p.i., cells infected with wt virus were harvested and fractionated as described in the text. RNA species extracted from the nuclear (lane N) and cytoplasmic (lane C) fractions were separated into poly(A)$^-$ and poly(A)$^+$ RNAs. The cRNA specific for the PA gene (434 nucleotides [nts]) in 300 ng of poly(A)$^-$ RNA was quantified by RPA with the ^{32}P-labeled negative-sense riboprobe as described previously (32). The PA-specific mRNA (419 nucleotides) in 50 ng of cytoplasmic poly(A)$^+$ RNA is shown as a size marker (lane A+). Lane P, probe.

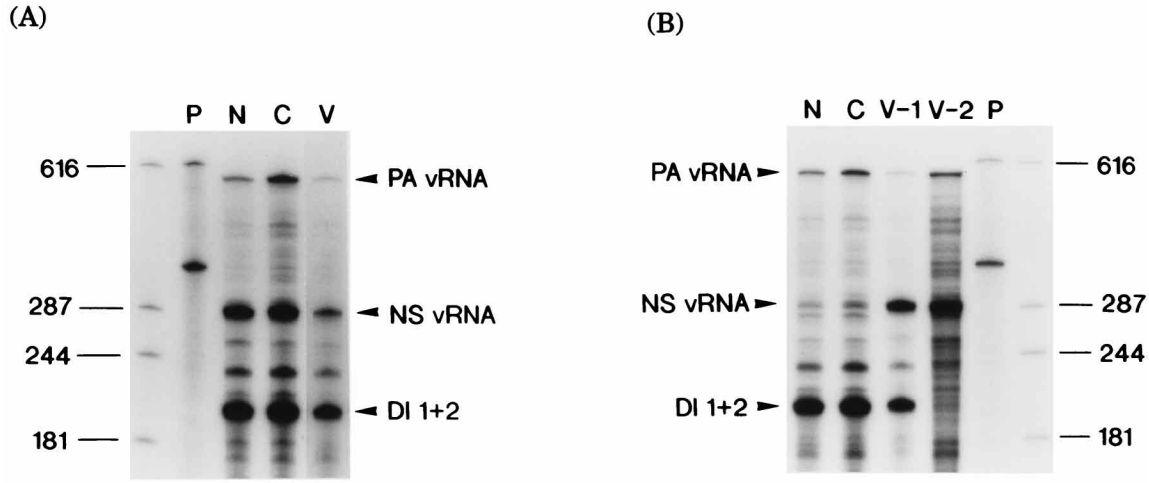

FIG. 3. Quantitation of the PA and NS gene-specific RNAs detected in the nuclei and cytoplasm of virus-infected cells. (A) A3/e-3-infected cells collected at 10 h p.i. were fractionated into the nuclei (lane N) and cytoplasm (lane C), while the virions were harvested at 13 h p.i. (lane V). Seven nanograms of virion RNA, 200 ng of nuclear RNA, and 100 ng of cytoplasmic RNA were subjected to RPA with a mixture of ^{32}P-riboprobes for the PA and NS genes. The X-ray films of the cell fractions and virions were exposed for 22 and 34 h, respectively. (B) Cells infected with a mixture of the NS2 mutant and the wt virus AwNS/2 were collected at 10 h p.i. The PA-specific RNAs in the nuclei (lane N) and cytoplasm (lane C) were examined by RPA with ^{32}P-riboprobes specific for the PA gene. The progeny virions and the wt virions were harvested at 15 h p.i., and the purified vRNAs were subjected to RPA with a mixture of ^{32}P-riboprobes for the PA and NS genes. Lane V-1, virion RNA obtained from the dually infected cells; lane V-2, virion RNA from wt virus; lanes P, a mixture of probes for the PA and NS genes. Size markers (nucleotides) are shown on the left and right.

prepared at 10 h p.i. and in virions collected at 13 h p.i. These RNA species were quantified three times by using independently prepared RNAs, and the results are summarized in Table 1. In the nucleus the PA DI RNAs were predominant over the PA vRNA, and the relative amount of PA vRNA compared to PA DI RNAs was less than 10% (Fig. 3A, lane N, and Table 1). This was consistent with the finding that in mutant-infected cells, synthesis of PA DI RNAs was greatly enhanced at the late phase of infection, and thereby synthesis of PA vRNA was strikingly suppressed (32). In the cytoplasm, however, the content of PA vRNA was increased 2 to 5 times over that in the nucleus, although the PA DI RNAs were still predominant over the PA vRNA (Fig. 3A, lane C, and Table 1). In contrast, in virions only a small amount of PA vRNA was detected (Fig. 3A, lane V, and Table 1). To determine the distribution of the PA vRNA, we calculated the ratios of the PA vRNA contents in the nucleus, cytoplasm, and virions to the sum of the PA vRNA contents detected in infected cells and virions. Similarly, the distributions of PA DI RNA and NS vRNA were also calculated (Table 1). By 10 h p.i., most of the PA vRNA as well as the PA DI RNAs and NS vRNA was transferred from the nucleus to the cytoplasm. Consequently, it was clear that the nuclear-cytoplasmic transport of the PA vRNA occurred normally. However, only 4% of the PA vRNA was incorporated into virions, whereas a quarter of PA DI RNA and NS vRNA synthesized was incorporated. From these results, we concluded that the small amount of PA vRNA in virions was the consequence of both the decreased synthesis of the PA vRNA in the nuclei of infected cells and the less efficient packaging of the PA vRNA at the virus assembly step at the plasma membrane.

TABLE 1. Quantitative analysis of PA vRNA, PA DI RNA, and NS vRNA segments in the nuclei and cytoplasm of NS2 mutant-infected cells collected at 10 h p.i. and in virions produced by 13 h p.i.

Fraction	PA vRNA/PA DI RNA ratio in expt:			Relative content[a]		
	1	2	3	PA vRNA	PA DI RNA	NS vRNA
Nuclei	0.05	0.07	0.07	0.05	0.03	0.04
Cytoplasm	0.24	0.17	0.15	0.91	0.72	0.68
Released virions	0.03	0.04	0.04	0.04	0.25	0.28

[a] The content of each RNA species was calculated as a ratio of the RNA in each fraction to the total RNA detected in the cells and virions. The values are averages from three experiments.

We next examined whether the PA DI RNAs of A3/e-3 affect the packaging of the wt PA gene in cells infected with a mixture of A3/e-3 and wt viruses. As shown in Fig. 3B, the PA DI RNAs were synthesized predominantly, and thereby the synthesis of PA vRNA again was greatly affected. The contents of these RNA species in each cell fraction were not significantly different from those in cells infected with A3/e-3 alone. Furthermore, the defect in packaging of the PA vRNA was also observed, so that only a trace amount of PA vRNA was detected in virions (Fig. 3B, lane V-1). This was in great contrast to the PA vRNA content in the wt virions, although the amount of NS vRNA was almost the same in both (compare Fig. 3B, lanes V-1 and V-2). These results therefore indicate that the preferential amplification and packaging of the PA DI RNAs of A3/e-3 virus coincided with a striking reduction of the wt PA vRNA in both the replication and packaging processes.

Packaging of other RNA segments of the NS2 mutant. To examine whether the PA DI RNA species affect the packaging of RNA segments other than the progenitor PA vRNA, we also quantified the smallest RNA segment, the NS gene, and another polymerase gene, PB2, of the NS2 mutant and wt viruses. At 10 h p.i. the amounts of NS vRNA of the mutant virus in each cell fraction and in virions were essentially identical to those of wt virus (Fig. 4A). This was consistent with the result shown in Fig. 3B. Similarly, incorporation of the PB2 vRNA into virions was not significantly different between the viruses (Fig. 4B). These results suggest that the PA DI RNAs com-

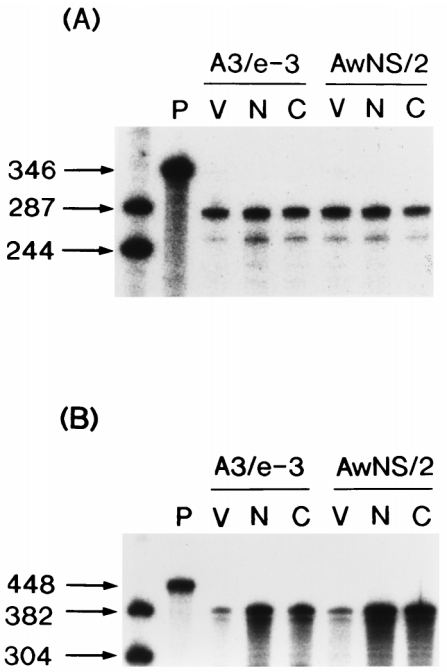

FIG. 4. Quantitation of the NS vRNA (A) and PB2 vRNA (B) of the NS2 mutant A3/e-3 and the wt virus AwNS/2. The infected cells and the progeny virions were harvested at 10 h p.i., and the vRNA species in the virions (lanes V), nuclei (lanes N), and cytoplasm (lanes C) were subjected to RPA as described in the legend to Fig. 3. Lane P, probe. Size markers (nucleotides) are shown on the left.

peted only with the progenitor PA gene and did not interfere with the replication and packaging of other RNA segments.

Rescue into virions of the transfected chimeric genome-like CAT RNAs containing the untranslated terminal sequences of the PA and PB2 genes. The specific competition between the PA DI RNAs and their progenitor PA gene occurring in the packaging process suggested that a given DI RNA species can recognize the competitor progenitor gene among eight different RNA segments through a specific signal(s) common to the DI RNA and the competitor RNA. We inferred that such segment-specific recognition signal, if any, should be located in the 3′ and 5′ untranslated regions, excluding the 3′-terminal 12 nucleotides and 5′-terminal 13 nucleotides, which are conserved among all eight segments. This seems likely because (i) these noncoding regions are always retained among DI RNA species, although the deletion sites of DI RNAs vary from species to species (19, 28, 29, 32); (ii) the PA DI RNA of the NS2 mutant used in the present study lacks approximately 90% of the coding region of the PA gene (see Materials and Methods); and (iii) the untranslated regions provided the packaging signals of RNA segments even when the coding region was replaced with that of the CAT gene (2, 23, 25) or with that of another segment (5, 26, 43). To verify this possibility, we constructed plasmid DNAs which can synthesize in vitro influenza virus-like CAT RNAs flanked by the 3′ and 5′ untranslated regions of the PA (PA/CAT) and PB2 (PB2/CAT) genes, as shown in Fig. 5A. We then examined whether these CAT RNAs are rescued by the NS2 mutant virus possessing the PA DI RNAs after RNP transfection to the infected cells. Because there was no selective pressure to isolate transfectant virus containing the CAT RNA, the rescued CAT RNAs were detected by RT-PCR with ^{32}P-labeled CAT gene-specific primers and the total vRNA extracted from the purified virions followed by RNase A treatment. In addition, the NS RNA segment of the transfectant virus was simultaneously detected as an internal control for the relative quantity of the rescued CAT RNA by using ^{32}P-labeled NS-specific primers.

From the wt-virus-infected cells, the PB2/CAT and PA/CAT RNAs were rescued with similar efficiencies when compared with each NS RNA segment (Fig. 5B, lanes 1 and 2). Since these CAT RNAs were detected by RT-PCR, there was a possibility that the PCR products of the CAT RNAs were contaminants of input residual plasmid DNA or free RNP. To exclude this, the PB2/CAT RNA was transfected to cells without virus infection and the culture medium was processed as described above. No PCR product was detected from this sample, as shown in Fig. 5B, lane C. In addition, when the vRNA of helper virus produced from the cells without the CAT RNA transfection was subjected to RT-PCR, only the PCR product of the NS gene was detected (Fig. 5B and C, lanes H). Consequently, the CAT RNAs detected by the PCR were neither contaminants nor nonspecific reactants. Furthermore, we observed that when the culture media of wt-virus-infected cells after transfection with PB2/CAT and PA/CAT were passaged to new cells after RNase A treatment, CAT activities expressed by the CAT RNAs were detected in the cell lysate by superinfection with the wt virus (data not shown). The results obtained from the RT-PCR and CAT assay revealed that the transfected CAT RNAs were packaged into wt virus particles.

The NS2 mutant, on the other hand, rescued the PB2/CAT RNA as efficiently as did wt virus (Fig. 5C, lane 1) but failed to rescue the PA/CAT RNA (lane 2). These results were reproducibly found in two independent experiments. Since the NS2 mutant virus contains PA DI RNAs, these results suggest that the PA DI RNAs competed with the PA/CAT RNA but not with the PB2/CAT RNA, so that packaging of PA/CAT RNA did not occur. Since nucleotides 13 to 24 and 2176 to 2220 of PA/CAT RNA were entirely different from those of PB2/CAT RNA (19, 31), as shown in Fig. 5A, these sequences would be involved in discrimination of PA/CAT RNA from PB2/CAT RNA.

We next attempted to identify which site of the noncoding sequences of the PA/CAT RNA is responsible for the specific competition with the PA DI RNA. To do this, we constructed plasmid DNAs which synthesized recombinant CAT RNAs containing the 3′ PA and 5′ PB2 noncoding sequences (3′PA/5′PB2) and the 3′ PB2 and 5′ PA noncoding sequences (3′PB2/5′PA) (Fig. 5A). These recombinant CAT RNAs were rescued by wt virus like the PB2/CAT and PA/CAT RNAs (Fig. 5B, lanes 3 and 4), demonstrating that the RNA segment possessing the 3′ and 5′ noncoding regions each derived from different segments is capable of being packaged into viral particles.

The recombinant CAT RNAs were also rescued by the NS2 mutant virus, although the efficiency was not as high as that of the PB2/CAT RNA (Fig. 5C, lanes 3 and 4). Interestingly, the CAT RNA containing the chimeric PA noncoding regions, where both nucleotides 13 to 19 and 2213 to 2220 were simultaneously replaced with the corresponding sequences of the PB2 gene [Fig. 5A, PA(7PB2 int)], was rescued as efficiently as the PB2/CAT vRNA by the NS2 mutant (Fig. 5C, lane 5). This result implies that the coexistence of those terminal sequences in the PA-specific noncoding regions would be required for recognition as a target of the competitor by PA DI RNA species.

DISCUSSION

In the present study, using the NS2 mutant A3/e-3 of influenza virus, we have shown that production of the DI virus lacking the PA gene was the consequence of suppression of the

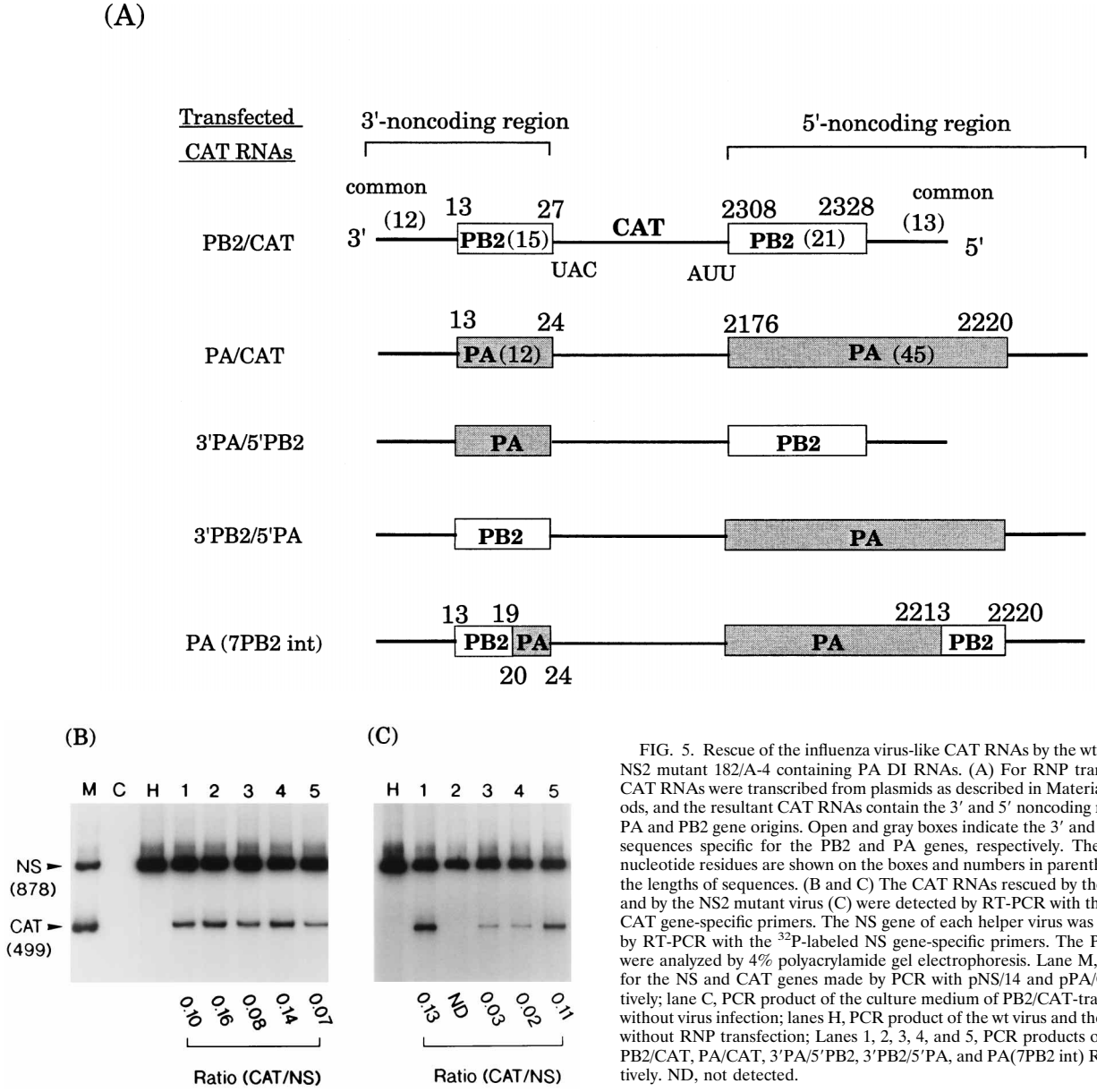

FIG. 5. Rescue of the influenza virus-like CAT RNAs by the wt virus and the NS2 mutant 182/A-4 containing PA DI RNAs. (A) For RNP transfection, the CAT RNAs were transcribed from plasmids as described in Materials and Methods, and the resultant CAT RNAs contain the 3′ and 5′ noncoding regions of the PA and PB2 gene origins. Open and gray boxes indicate the 3′ and 5′ noncoding sequences specific for the PB2 and PA genes, respectively. The numbers of nucleotide residues are shown on the boxes and numbers in parentheses indicate the lengths of sequences. (B and C) The CAT RNAs rescued by the wt virus (B) and by the NS2 mutant virus (C) were detected by RT-PCR with the ^{32}P-labeled CAT gene-specific primers. The NS gene of each helper virus was also detected by RT-PCR with the ^{32}P-labeled NS gene-specific primers. The PCR products were analyzed by 4% polyacrylamide gel electrophoresis. Lane M, size markers for the NS and CAT genes made by PCR with pNS/14 and pPA/CAT, respectively; lane C, PCR product of the culture medium of PB2/CAT-transfected cells without virus infection; lanes H, PCR product of the wt virus and the NS2 mutant without RNP transfection; Lanes 1, 2, 3, 4, and 5, PCR products of the rescued PB2/CAT, PA/CAT, 3′PA/5′PB2, 3′PB2/5′PA, and PA(7PB2 int) RNAs, respectively. ND, not detected.

PA gene occurring in two different steps, vRNA replication in the nucleus and assembly of vRNA segments at the cell membrane. These suppressions were caused in parallel with the preferential amplification and packaging of the PA DI RNA species. Although the suppression of PA vRNA replication occurs primarily at the level of cRNA synthesis (32), this was not lethally strong, since the PA vRNA was still synthesized at one-fifth as much as PA DI RNAs in infected cells (Fig. 1). In contrast, only a small amount of the PA vRNA was detected in released virions (Fig. 1 and 3). These inconsistent proportions of PA vRNA in infected cells and in virions suggest that the striking suppression of the PA vRNA occurs after the vRNA replication step.

It is unlikely, however, that the transport of PA vRNA from the nucleus to the cytoplasm was prevented by the PA DI RNAs. In cell fractionation experiments with NS2 mutant-infected cells, more than 90% of PA vRNA synthesized in the nucleus was detected in the cytoplasm, and only 5% remained in the nucleus (Table 1). This revealed that most of the PA vRNA was exported to the cytoplasm. The PA DI RNA and the NS vRNA segment also showed similar behavior. Consequently, it is clear that the nuclear-cytoplasmic transport of PA vRNA took place normally. However, most of the transported PA vRNA was left in the cytoplasm, and only 4% of it was incorporated into virions. This was in great contrast to the packaging efficiency of PA DI RNA and NS vRNA, since about 25% of these RNAs was incorporated into virions (Table 1). Since 20% of the vRNA of influenza virus synthesized after 4 h p.i. is known to be incorporated into virions (40), the remarkably low packaging efficiency of the PA vRNA of A3/e-3 is considered to be crucial for the production of DI particles lacking the PA gene.

With respect to the packaging of each RNA segment into virions, two different observations have been reported so far. One is that the content of each vRNA segment in virions correlates with the level of vRNA synthesis in infected cells,

suggesting that no selection occurs in vRNA packaging into virions. This was based on the analysis of transfectants containing chimeric NA genes that showed low levels of the chimeric RNAs both in infected cells and in virions (26, 43). Preferential packaging of the PA DI RNAs of A3/e-3 can be explained by this model. They were synthesized to have a content 0.9 to 2.6 times greater than that of the NS vRNA in infected cells (Fig. 3A), and their content in virions was approximately 2.5 times greater than that of the NS and NP vRNAs, as found when the ^{32}P-end-labeled vRNA segments in the A3/e-3 virion were analyzed by polyacrylamide gel electrophoresis (data not shown). These results were consistent with the finding that the PA DI RNA and NS vRNA were packaged into virions with similar efficiencies (Table 1).

Nevertheless, difference in the proportions of PA vRNA in infected cells and in virions cannot be interpreted without considering regulation at the packaging process. In fact, the efficiency of PA vRNA packaging was six times lower than that for PA DI RNA and NS vRNA (Table 1). Smith and Hay have shown that the relative abundances of vRNAs in infected cells do not reflect the composition of the vRNA segments in virions (40), suggesting that there is selection in the incorporation of vRNAs into virion. Furthermore, studies of transfectant viruses with the mutated NA genes NA/X and NA/Y show that the NA RNA is synthesized at a low level in infected cells but at a normal level in virions (3). Conversely, for an avian DI virus defective RNA segment 1, vRNA synthesis occurs normally in infected cells, but the packaging of RNA segment 1 was selectively inhibited (8). Suppression of the PA vRNA of A3/e-3 occurred independently in both the RNA synthesis and the RNA packaging steps. If the packaging of RNA segments of influenza virus is caused only by random selection as described previously (10), such differences should not be observed. Consequently, it is likely that the signals which regulate the vRNA replication are distinct from those responsible for regulating the amount of the vRNA in virions.

In most of DI viruses, DI RNA species specifically interfere with their progenitor RNA segments (1, 8, 27, 30, 32), although in one case a DI RNA, whose origin was not identified precisely by the sequencing, did not interfere with its progenitor RNA segment but with a different RNA segment (42). In the case of NS2 mutant A3/e-3, the target of interference was restricted to the PA vRNA segment and did not include other segments, i.e., NS and PB2. Interestingly, from the cells dually infected with A3/e-3 and the wt virus, the DI particle lacking the PA gene was produced again (Fig. 3B). This indicates that packaging of the wt PA vRNA as well as the A3/e-3 PA vRNA was also specifically affected in the presence of the PA DI RNAs and implies that there is a specific correlation between the target RNA segment and DI RNA species. Although it remains uncertain that the DI RNA species directly suppress the packaging of the target RNA segment, we have inferred that DI RNA species can distinguish the target and competitor RNAs from the other RNA segments through a segment-specific signal(s) which must reside on individual RNA molecules.

By rescue experiments with the influenza virus-like CAT RNAs, we showed that in the presence of PA DI RNAs, the PA/CAT RNA was not rescued into the NS2 mutant virus but the PB2/CAT RNA was rescued (Fig. 5C). Since these CAT RNAs were efficiently rescued into the wt virus (Fig. 5B), it is unlikely that the replication of PA/CAT RNA was decreased in the transfected cells, resulting in the failure of rescue of the PA/CAT RNA into the NS2 mutant virions. Thus, it is conceivable that the PA DI RNAs specifically competed with the PA/CAT RNA and impeded its packaging. The 3′ and 5′ noncoding regions of PA DI RNA were identical to those of PA/CAT RNA, while the segment-specific noncoding sequences in the PA/CAT RNA, i.e., nucleotides 13 to 24 at the 3′ end and 2176 to 2220 at the 5′ end, were entirely different from those of the PB2/CAT RNA (19, 31). Consequently, the signal(s) responsible for the specific competition between PA/CAT RNA and PA DI RNA would reside on the segment-specific sequences. This was supported by rescue of the chimeric CAT RNAs (Fig. 5C). Modification of the noncoding regions of PA/CAT RNA resulted in a great difference in the rescue efficiency by the NS2 mutant. The recombinant PA/CAT RNAs, whose 3′ or 5′ noncoding regions were replaced with those of the PB2 gene (3′PA/5′PB2 and 3′PB2/5′PA), were rescued, but with a low efficiency. This suggests that the recombinant CAT RNAs containing only one site of the PA noncoding region partially competed with the PA DI RNAs in the NS2 mutant, but the packaging was not strikingly impeded as it was with the PA/CAT RNA. This was consistent with the finding that the deletion of both sites of the nonconserved terminal sequences of NA gene affected RNA synthesis but that the deletion of only one site did not (43). In contrast, when the terminal 7 or 8 nucleotides of the both PA-specific noncoding regions were simultaneously replaced with those of the PB2 gene, the CAT RNA containing such chimeric noncoding regions, PA(7PB2 int), was rescued efficiently like the PB2/CAT RNA. The results suggest that coexistence of both terminal sequences on the same RNA molecule is required for recognition as a competitor of PA DI RNAs. Recently, segment-specific noncoding sequences of influenza virus genome RNA have been shown to influence packaging of the hemagglutinin gene into virions (2), NA vRNA synthesis in infected cells (43), and translation initiation of the M1 protein mediated by the NS1 protein (9, 22). Therefore, the segment-specific noncoding regions play important roles in replication, expression, and packaging of genome RNAs.

In conclusion, we have shown in the present study that the packaging of DI RNA species into virions resulted in the selective reduction of the packaging of their progenitor vRNA segments and that the 3′ and 5′ segment-specific noncoding regions were involved in the specific recognition between the competitor RNA and the DI RNA species. If the packaging of influenza virus genome RNA occurs only by a random process, then the selective decrease of a certain genome RNA segment should not be found in DI viral particles. However, most DI viruses lack an RNA segment corresponding to the progenitor of the DI RNA species. These observations therefore imply that packaging of virus genome RNAs does not occur simply by random selection.

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REFERENCES

1. **Akkina, R. K., T. M. Chambers, and D. P. Nayak.** 1994. Mechanism of interference by defective-interfering particles of influenza virus: differential reduction of intracellular synthesis of specific polymerase proteins. Virus Res. **1:**687–702.
2. **Barclay, W. S., and P. Palese.** 1995. Influenza B viruses with site-specific mutations introduced into the HA gene. J. Virol. **69:**1275–1279.
3. **Bergmann, M., and T. Muster.** 1995. The relative amount of an influenza A

virus segment present in the viral particle is not affected by a reduction in replication of that segment. J. Gen. Virol. **76:**3211–3215.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Claas, E. C. J., and Y. Kawaoka.** 1994. Rescue of influenza A neuraminidase mutant viruses containing the non-coding region of other gene segments, abstr. 45, p. 64. *In* Abstracts of the Ninth International Conference on Negative Strand Viruses.
6. **Compans, R. W., and P. W. Choppin.** 1975. Reproduction of myxoviruses, p. 179–252. *In* Comprehensive virology, vol. 4. Plenum Press, New York, N.Y.
7. **Desselberger, U. V., R. Racaniello, J. J. Zazra, and P. Palese.** 1980. The 3′ and 5′ terminal sequences of influenza A, B, and C virus RNA segments are highly conserved and show partial inverted complementary. Gene **8:**315–328.
8. **Duhaut, S. D., and J. W. McCauley.** 1996. Defective RNAs inhibit the assembly of influenza virus genome segments in a segment-specific manner. Virology **216:**326–337.
9. **Enami, K., T. Sato, S. Nakada, and M. Enami.** 1994. Influenza virus NS1 protein stimulates translation of the M1 protein. J. Virol. **68:**1432–1437.
10. **Enami, M., G. Shirma, C. Benham, and P. Palese.** 1991. An influenza virus containing nine different RNA segments. Virology **185:**291–298.
11. **Enami, M., and P. Palese.** 1991. High-efficiency formation of influenza virus transfectants. J. Virol. **65:**2711–2713.
12. **Fodor, E., B. L. Seong, and G. G. Brownlee.** 1993. Photochemical cross-linking of influenza A polymerase to its virion RNA promoter defines a polymerase binding site at residues 9 to 12 of the promoter. J. Gen. Virol. **74:**1327–1333.
13. **Fodor, E., D. C. Pritlove, and G. G. Brownlee.** 1994. The influenza virus panhandle is involved in the initiation of transcription. J. Virol. **68:**4092–4096.
14. **Fodor, E., D. C. Pritlove, and G. G. Brownlee.** 1995. Characterization of the RNA-fork model of virion RNA in the initiation of transcription in influenza A virus. J. Virol. **69:**4012–4019.
15. **Hirst, G. K., and M. W. Pons.** 1973. Mechanism of influenza recombination. II. Virus aggregation and its effect on plaque formation by so-called noninfective virus. Virology **56:**620–631.
16. **Honda, A., K. Ueda, K. Nagata, and A. Ishihama.** 1987. Identification of the RNA polymerase binding site on genome RNA of the influenza virus. J. Biochem. **102:**1241–1249.
17. **Hsu, M., J. D. Parvin, S. Gupta, M. Krystal, and P. Palese.** 1987. Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc. Natl. Acad. Sci. USA **84:**8140–8144.
18. **Inglis, S. C., A. R. Carroll, R. A. Lamb, and B. W. J. Mahy.** 1976. Polypeptide specified by the influenza virus genome. 1. Evidence for eight distinct gene products specified by fowl plague virus. Virology **74:**489–503.
19. **Kaptein, J., and D. P. Nayak.** 1982. Complete nucleotide sequence of the polymerase 3 (P3) gene of human influenza virus A/WSN/33. J. Virol. **42:**55–63.
20. **Lamb, R. A., and P. W. Choppin.** 1983. The gene structure and replication of influenza virus. Annu. Rev. Biochem. **52:**467–506.
21. **Li, X., and P. Palese.** 1992. Mutational analysis of the promoter required for influenza virus virion RNA synthesis. J. Virol. **66:**4331–4338.
22. **Luna, S. L., P. Fortes, A. Beloso, and J. Ortin.** 1995. Influenza virus NS1 protein enhances the rate of translation initiation of viral mRNAs. J. Virol. **69:**2427–2433.
23. **Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese.** 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell **59:**1107–1113.
24. **McGeoch, D., P. Fellner, and C. Newton.** 1976. Influenza virus genome consists of eight distinct RNA species. Proc. Natl. Acad. Sci. USA **73:**3045–3049.
25. **Mena, I., A. Vivo, E. Perez, and A. Portela.** 1996. Rescue of a synthetic chloramphenicol acetyltransferase RNA into influenza virus-like particles obtained from recombinant plasmids. J. Virol. **70:**5016–5024.
26. **Muster, T., E. K. Subbarao, E. Enami, B. R. Murphy, and P. Palese.** 1991. An influenza A virus containing influenza B virus 5′ and 3′ noncoding regions on the neuraminidase gene is attenuated in mice. Proc. Natl. Acad. Sci. USA **88:**5177–5181.
27. **Nakajima, K., M. Ueda, and A. Sugiura.** 1974. Origin of small RNA in von Magnus particles of influenza virus. J. Virol. **29:**1142–1148.
28. **Nayak, D. P., N. Sivasubramanian, A. R. Davis, R. Cortini, and J. Sung.** 1982. Complete sequence analysis show that two defective interfering influenza viral RNAs contain a single internal deletion of a polymerase gene. Proc. Natl. Acad. Sci. USA **79:**2216–2220.
29. **Nayak, D. P., T. M. Chambers, and R. M. Akkina.** 1989. Structure of defective-interfering RNAs of influenza viruses and their role in interference, p. 269–317. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York, N.Y.
30. **Odagiri, T., and K. Tobita.** 1990. Mutation in NS2, a nonstructural protein of influenza A virus, extragenically causes aberrant replication and expression of the PA gene and leads to generation of defective interfering particles. Proc. Natl. Acad. Sci. USA **87:**5988–5992.
31. **Odagiri, T., and K. Tobita.** 1990. Nucleotide sequence of the PA gene of influenza A/WSN/33 (H1N1). Nucleic Acids Res. **18:**654.
32. **Odagiri, T., K. Tominaga, T. Tobita, and S. Ohta.** 1994. An amino acid change in the non-structural NS2 protein of an influenza A virus mutant is responsible for the generation of defective interfering (DI) particles by amplifying DI RNAs and suppressing complementary RNA synthesis. J. Gen. Virol. **75:**43–53.
33. **Palese, P., and J. L. Schulman.** 1976. Differences in RNA patterns of influenza A viruses. J. Virol. **17:**876–884.
34. **Parvin, J. D., P. Palese, A. Honda, A. Ishihama, and M. Krystal.** 1989. Promoter analysis of the influenza viral RNA polymerase. J. Virol. **63:**5142–5152.
35. **Robertson, J.** 1979. 5′ and 3′ terminal nucleotide sequences of the RNA genome segments of influenza virus. Nucleic Acids Res. **6:**3745–3757.
36. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
37. **Scholtissek, C., W. Rohde, E. Harms, R. Rott, M. Orlich, and C. B. Bochek.** 1978. A possible partial heterozygote of an influenza A virus. Virology **89:**506–516.
38. **Seong, B. L., and G. G. Brownlee.** 1992. Nucleotides 9 to 11 of the influenza A virus RNA promoter are crucial for activity in vitro. J. Gen. Virol. **73:**3115–3124.
39. **Shapiro, G. I., T. Gurney, Jr., and R. M. Krug.** 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. J. Virol. **61:**764–773.
40. **Smith, G. C., and A. J. Hay.** 1982. Replication of the influenza virus genome. Virology **118:**96–108.
41. **Stoeckle, M. Y., M. W. Shaw, and P. W. Choppin.** 1987. Segment-specific and common nucleotide sequences in the noncoding regions of influenza B virus genome RNAs. Proc. Natl. Acad. Sci. USA **84:**2703–2707.
42. **Ueda, M., K. Nakajima, and A. Sugiura.** 1980. Extra RNAs of von Magnus particles of influenza virus cause reduction of particular polymerase genes. J. Virol. **34:**1–8.
43. **Zheng, H., P. Palese, and A. G. Sastre.** 1996. Nonconserved nucleotides at the 3′ and 5′ ends of an influenza A virus RNA play an important role in viral RNA replication. Virology **217:**242–251.