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Importance of both the Coding and the Segment-Specific Noncoding Regions of the Influenza A Virus NS Segment for Its Efficient Incorporation into Virions

Ken Fujii,[1,2] Yutaka Fujii,[3] Takeshi Noda,[4] Yukiko Muramoto,[4] Tokiko Watanabe,[1,2] Ayato Takada,[1,2] Hideo Goto,[1,2] Taisuke Horimoto,[1,2] and Yoshihiro Kawaoka[1,2,5]*

Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo,[1] Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Saitama,[2] Department of Virology, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima,[3] and Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo,[4] Japan, and Department of Pathological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin[5]

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The genome of influenza A virus consists of eight single-strand negative-sense RNA segments, each comprised of a coding region and a noncoding region. The noncoding region of the NS segment is thought to provide the signal for packaging; however, we recently showed that the coding regions located at both ends of the hemagglutinin and neuraminidase segments were important for their incorporation into virions. In an effort to improve our understanding of the mechanism of influenza virus genome packaging, we sought to identify the regions of NS viral RNA (vRNA) that are required for its efficient incorporation into virions. Deletion analysis showed that the first 30 nucleotides of the 3′ coding region are critical for efficient NS vRNA incorporation and that deletion of the 3′ segment-specific noncoding region drastically reduces NS vRNA incorporation into virions. Furthermore, silent mutations in the first 30 nucleotides of the 3′ NS coding region reduced the incorporation efficiency of the NS segment and affected virus replication. These results suggested that segment-specific noncoding regions together with adjacent coding regions (especially at the 3′ end) form a structure that is required for efficient influenza A virus vRNA packaging.

The genetic information that characterizes all life systems must be transmitted to each generation. For viruses, this is achieved by amplification of the genome within cells and its subsequent packaging into progeny virions. The influenza A virus genome is fragmented into eight single-strand negative-sense RNA segments that encode at least 11 proteins (3, 10). These viral RNAs (vRNAs) form the ribonucleoprotein (RNP) complex with heterotrimeric viral polymerase subunit proteins (PB2, PB1, and PA) and nucleoprotein (NP) (10). After introduction into cells via receptor-mediated endocytosis, the viral RNPs are transported into the nucleolus, where viral mRNAs are transcribed and translated into viral proteins (10). The vRNAs are replicated and, together with the viral proteins, form the RNP complexes (10), which are then exported to the cytoplasm in a CRM-1-dependent manner (4, 14, 15, 18, 21, 26). The final step is packaging of the RNP complexes into progeny virions at the plasma membrane (10).

Influenza A virus RNA contains noncoding regions at both ends of its coding region (10). These noncoding regions are composed of sequences that are conserved among eight vRNAs (U13 and U12) as well as sequences specific for each segment that are located inside the terminal noncoding region (10). These noncoding regions regulate replication of the viral genome and transcription (22). Luytjes et al. (13) generated an artificial viral RNA comprising the chloramphenicol acetyltransferase (CAT) gene flanked by the noncoding regions of an NS segment. They showed that this RNA was incorporated into influenza virus virions, using the CAT activity of virus-infected cells as an indicator of segment incorporation. However, the CAT activity gradually decreased upon passaging. Recently, we showed that both ends of the coding sequences of the hemagglutinin (HA) (28) and neuraminidase (NA) (6) segments of influenza A virus, in addition to the noncoding regions, were necessary for efficient vRNA incorporation into virions, although the 3′ ends of these segments are more important than the other ends for efficient packaging. Although the sequences downstream and upstream of the conserved end sequences in the noncoding regions are segment specific, their contribution to vRNA packaging remains unknown.

To further understand packaging of vRNA segments into influenza virus virions, here we asked whether both ends of the coding regions are important for incorporation of NS vRNA segments into progeny virions, as we have shown for the HA and NA vRNAs. To this end, we generated a series of mutant vRNAs that possessed coding regions of different lengths derived from the NS segment and tested the efficiency with which they were incorporated into influenza virus virions. In addition, we assessed the contributions of the segment-specific noncoding regions to NS vRNA incorporation into virions.

* Corresponding author. Mailing address: Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-03-5449-5310. Fax: 81-03-5449-5408. E-mail: kawaoka@ims.u-tokyo.ac.jp.

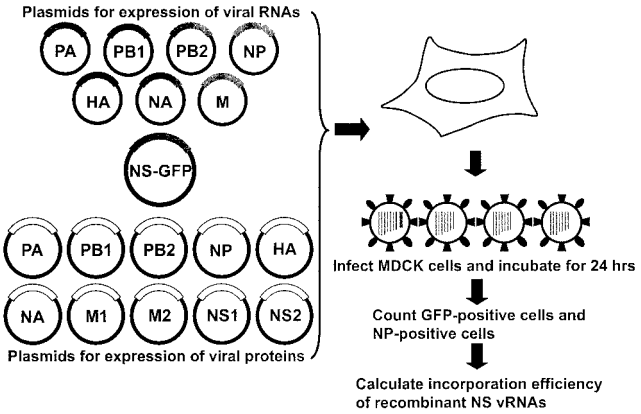

FIG. 1. Schematic representation of the system used to determine the efficiency of vRNA incorporation into virions. 293T cells were transfected with plasmids for production of influenza virus VLPs. Forty-eight hours later, aliquots of the supernatant were used to infect MDCK cells. To determine efficiency of incorporation of mutant NS vRNA segments, GFP- and NP-expressing cells were counted at 24 h postinfection.

MATERIALS AND METHODS

Cells. 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) containing 5% newborn calf serum and antibiotics. All cells were maintained at 37°C with 5% CO_2.

Construction of plasmids. Construction of plasmids (referred to as PolI plasmids) for the expression of vRNAs of A/WSN/33 (H1N1) virus by cellular RNA polymerase I following transfection into cells has been described previously (17).

A series of plasmids, pPolI/NS-GFP, containing a gene cassette for the open reading frame of enhanced green fluorescence protein (GFP) (Clontech) flanked by different lengths of the coding and noncoding regions of the NS segment under the control of the RNA polymerase I promoter and terminator was constructed as follows. pPolINS(0)GFP(0) (to express negative-sense RNA containing the 3′ noncoding sequence of the NS vRNA, the complementary coding sequence of GFP, and the 5′ noncoding sequence of the NS vRNA) was produced by PCR amplification of the GFP gene with primers containing a BsmBI site and the 3′ or 5′ noncoding region of NS, digestion of the product with BsmBI, and cloning it into the BsmBI site of the PolI plasmid (17). Introduction of this plasmid into cells yielded an RNA that contained the GFP-coding sequence in the negative-sense orientation flanked by the 3′ and 5′ noncoding sequences of the NS vRNA.

To construct plasmids for the expression of mutant NS vRNA with deletions in the coding regions, the sequence of NS vRNA was cloned into pUC19 (pUC19/NS) and deletions were introduced. This pUC19/NS was amplified by inverse PCR (20) by use of the back-to-back primers NSUP-1 (5′-GAATTCGAAGACTAATAAGGTCAGAAGTTTGAAGAAATAAGATGG-3′) and NSDW-1 (5′-GAATTCGAAGACTACCATAAGAGTGCTGCCTCTTCCTCTTAGG-3′). The PCR product was digested with BbsI, and the GFP gene was amplified with primers containing BbsI and was then inserted into the BbsI sites, resulting in pUC19/NS(150)GFP(150). The DNA fragment containing the NS and GFP sequences was obtained from pUC19/NS(150)GFP(150) by digestion with BsmBI and then was inserted between the BsmBI sites of a PolI plasmid. The resultant plasmid, pPolINS(150)GFP(150), was used to produce negative-sense RNA containing the 3′ noncoding region and 150 nucleotides of the 3′ coding region of the NS vRNA, the GFP coding sequences, 150 nucleotides of 5′ coding region, and the 5′ noncoding region of the NS vRNA. A series of NS coding deletion mutants was also produced by inverse PCR in the same manner. These mutants were designated according to the number of nucleotides derived from the NS coding region; for example, the NS(30)GFP(150) RNA segment contains the 3′ noncoding region and 30 nucleotides of the coding region of the NS vRNA, GFP sequence, 150 nucleotides of 3′ coding region, and the 5′ noncoding region of the NS vRNA.

pPolINS(Δ30)GFP(150) was made by using inverse PCR with the back-to-back primers NSUP-10 (5′-AATTCGAAGACTAGTAGATTGCTTTCTTTGGCAT-3′) and NSDW-10 (5′-GAATTCGAAGACTACTACCATTATGTCTTTGTCAC-3′) in combination to amplify pUC19/NS(150)GFP(150). The PCR product was digested with BbsI and self-ligated. The resultant plasmid, pUC19/NS(Δ30)GFP(150), was digested with BsmBI and inserted between the BsmBI sites of a PolI plasmid. The resultant plasmid, pPolINS(Δ30)GFP(150), was then used to produce negative-sense RNA containing the 3′ noncoding region and 120 nucleotides containing nucleotides 31 to 150 of the 3′ coding region of the NS vRNA, the GFP coding sequence, 150 nucleotides of 5′ coding region, and the 5′ noncoding region of the NS vRNA.

To generate plasmid pPolIm1-G, which encodes the recombinant NS-GFP segment with silent mutations in the 3′ end of the NS coding region, PCR was performed with plasmid pUC19/NS(150)GFP(150) as a template and back-to-back primers NSmUP-1 (5′-GAATTCGAAGACAACGTTTCTAGTTTCCAGGTAGATTGCTTTCTTTGGCATGTCC-3′) and NSmDW-1 (5′-GAATTCGAAGACAAAACGGTATTCGGGTCCAT TATGTCTTTGTCACCCTGC-3′). The PCR products were self-ligated, resulting in pUC19/m1-G. The DNA fragment containing the NS and GFP sequences was obtained from pUC19/m1-G by digestion with BsmBI and then inserted between the BsmBI sites of a PolI plasmid, resulting in pPolIm1-G. A series of NS segments containing introduced point mutations was also produced by PCR in the same manner.

To construct plasmid pPolINSΔNTR such that it expresses the recombinant NS segment without segment-specific noncoding regions, PCR was performed with plasmid pUC19/NS(150)GFP(150) as a template and primers NS2UP-6-2 (5′-GAATTCGAAGACATCAGGGTGATGGATCCAAACACTGTGTC-3′) and NS2DW-6 (5′-GAATTCGAAGACATAAGGGTGTTTTTTAAATAAGCTGAAACGAGAAAG-3′). The PCR product was digested with BsmBI and cloned into the BsmBI site of the pPolI plasmid containing the sequences of U12 and U13. A series of NS segment-specific noncoding deletion mutants was also produced by PCR in the same manner.

To generate a plasmid that expresses the WSN NS1 protein, PCR was performed with plasmid pUC19/NS as a template and primers NS1UP1 (5′-GGAATTCATGGATCCAAACACTGTGTCAAG-3′) and NS1DW1 (5′-GAAGATCTTCAATCAACCATCTTATTTCTTCAAAC-3′) in combination. The PCR products were digested with EcoRI and BglII and inserted between the EcoRI and BglII sites of plasmid pCAGGS/MCS (9, 19), resulting in pCAGGS/NS1.

Generation of influenza virus or virus-like particles (VLPs). 293T cells (10^6 cells) were transfected with 18 plasmids (Fig. 1) by use of Trans IT 293 (Panvera, Madison, Wis.) according to the manufacturer's instructions. Briefly, DNA and transfection reagent were mixed (2 μl of Trans IT 293 per μg of DNA), incubated at room temperature for 10 min, and then added to the cells. At 6 h posttransfection, the DNA-transfection reagent mixture was replaced with Opti-MEM (GIBCO-BRL) containing 1% fetal calf serum. Forty-eight hours later, aliquots of the supernatant were used to infect MDCK cells. GFP-positive cells were counted at 24 h postinfection, fixed, and processed for indirect immunostaining.

Replicative properties of transfectant viruses. MDCK cells in triplicate wells of 24-well plates were infected with wild-type or mutant viruses, overlaid with MEM containing 0.5 μg of trypsin per ml, and incubated at 37°C. At different times, supernatants were assayed for infectious virus by plaque assay on MDCK cells.

Real-time quantitative reverse transcription-PCR. Total RNA in 293T cells transfected with PolI plasmids was extracted by use of the Isogen RNA extraction kit (Nippon Gene, Tokyo, Japan) at 48 h posttransfection. The extracted RNAs were digested with DNase I (Takara) at 37°C for 30 min to eliminate the transfected plasmid DNA, and the enzyme was then inactivated at 80°C for 15 min. The vRNA of the NS-GFP or NP segment was reverse transcribed by using TaqMan reverse transcription reagents (Applied Biosystems) with a strand-specific primer for the NS-GFP segment (NSNTR-UP3, 5′-AGCAAAAGCAGGGTG-3′) or NP segment (WSNNP1, 5′-AGCAAAAGCAGGGTAGATAATCACTC-3′). vRNA was quantified by using the ABI PRISM 7000 sequence detection system with the TaqMan universal PCR master mix (Applied Biosystems), specific primers, and a TaqMan probe. The set of primers and probe for detection of the NS-GFP segment are TQMG1-N (5′-AGCAAAGACCCCAACGAGAA-3′), TQMG-1C (5′-GGCGGCGGTCACGAA-3′), and G646T (5′-CGCGATCACATGGTCCTGCTGG-3′). These primers specifically detect the NS-GFP segment but not the authentic NS segment. The set of primers and probe for detection of the NP segment are TQNP2-F (5′-CGGCCCCAGACCTCCTA-3′), TQNP2-R (5′-CTTGTTCGCACAGGAATGGA-3′), and NP1014T (5′-ACCCTGCATCAGTGAGCACATCCTGG-3′).

Immunostaining assay. All staining procedures were carried out at room temperature. VLP-infected cells were fixed for 30 min with methanol and then permeabilized with 0.1% Triton X-100 for 30 min. The samples were incubated with anti-NS2 antibody or anti-NP antibody at 1:1000 dilutions in phosphate-

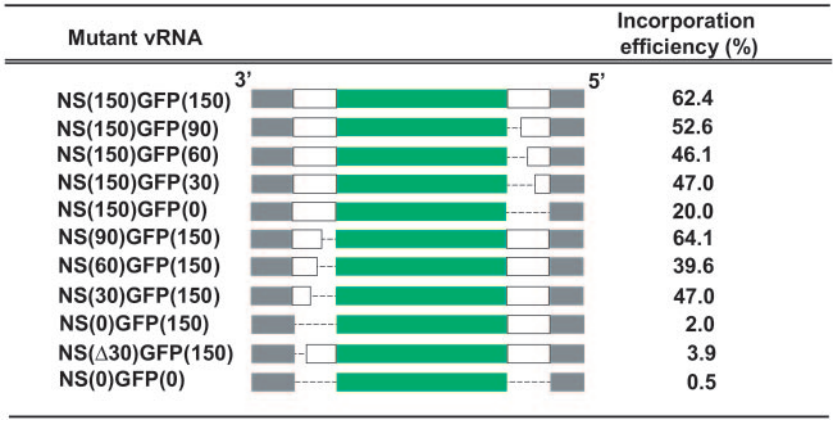

FIG. 2. Efficiency of incorporation of recombinant NS segments. Incorporation efficiencies of recombinant NS segments with different lengths of NS coding regions were determined as described in the legend to Fig. 1. The noncoding and coding regions of the NS vRNA are represented by grey and white bars, respectively, while the green bars represent the coding region of the GFP gene. The dotted line represents deleted sequences of the NS coding region. The results shown are representative data from three independent experiments.

buffered saline (PBS) for 30 min. After being washed three times with PBS, the samples were incubated with biotinylated antibody (Vector Laboratories) at 1:100 dilutions in PBS for 30 min and then treated with Vectastain Elite ABC reagent (Vector Laboratories) according to the manufacturer's instructions. For visualization, the samples were mixed with diaminobenzidine tetrahydrochloride (Sigma).

Alignment of incorporation signals of influenza viruses. NS sequences were obtained from the influenza A virus sequence database at Los Alamos National Laboratory. The sequence alignment was calculated with GENETYX-WIN (Software Development Co., Ltd.).

RESULTS

A system to determine the efficiency of NS vRNA incorporation into virions. To determine the efficiency of incorporation of NS vRNA segments into virions, we used a reverse genetic system (Fig. 1). The pPolI/NS-GFP plasmids, upon transfection into cells, produced virus-like RNAs with a GFP open reading frame. VLPs were produced when 293T cells were transfected with both the plasmids designed to express the influenza vRNAs (pPolI plasmids) and those designed to express all of the viral proteins (pCAGGS plasmids) (Fig. 1). On harvesting of the VLPs at 48 h posttransfection and incubation of the VLP supernatant with MDCK cells for 24 h, the incorporation efficiency of the mutant NS vRNA segments was calculated as a ratio of the number of NP-positive cells (i.e., the number of total VLPs) and the number of GFP-positive cells (i.e., the number of VLPs containing mutant NS vRNA). Under these conditions, the packaging efficiency of the full-length NS segment was approximately 70 to 80%.

Importance of the coding region for efficient incorporation of NS vRNA into virions. For the HA and NA segments of influenza A virus, both ends of the coding region are important for incorporation of the segments into virions (6, 28). To determine whether this concept extends to the NS segment, we produced plasmids encoding 11 mutant NS vRNAs, each of which differed in the length of its coding region (Fig. 2). These mutant NS vRNAs expressed fusion proteins possessing the amino terminus of NS1 and GFP. Upon transfection of each of these plasmids into 293T cells, together with plasmids for the production of influenza virus VLPs, approximately 3×10^5 infectious units of VLPs per ml were detected in the supernatants. Under these conditions, the incorporation efficiency of a mutant NS vRNA without the NS coding sequence [NS(0) GFP(0)] was 0.5%, while that of a mutant that maintained 150 nucleotides at each end of the NS coding sequence [NS(150) GFP(150)] was 62.4%, indicating that the coding region is indeed important for efficient NS vRNA incorporation, consistent with the findings for incorporation of HA and NA vRNAs.

Relative contributions of the 3′ and 5′ ends of the coding region to efficient incorporation of NS vRNA. Next, we investigated whether the 3′ end, the 5′ end, or both ends of the NS vRNA coding sequences are critical for NS vRNA incorporation. As the length of the 3′ or the 5′ NS coding region was reduced, the vRNA incorporation efficiency also gradually diminished [Fig. 2, compare NS(150)GFP(150), NS(150)GFP (90), NS(150)GFP(60), NS(150)GFP(30), NS(90)GFP(150), NS(60)GFP(150), and NS(30)GFP(150)]. Although deletion of either the 3′ or the 5′ end appreciably reduced vRNA incorporation [Fig. 2, NS(0)GFP(150) and NS(150)GFP(0)], the effect was more drastic for the former. These data indicate that both ends of the NS vRNA coding region are necessary for efficient NS vRNA incorporation into virions and that the requirement for the 3′ NS coding sequence is of more importance than that for the 5′ end.

The drastic reduction of NS vRNA incorporation upon deletion of the 3′ NS coding sequences prompted us to produce a mutant NS vRNA that lacked the first 30 nucleotides of the 3′ NS coding sequence. This mutant NS vRNA [NS(Δ30)GFP (150)] was not efficiently incorporated into virions, whereas deletion of the region downstream of the first 30 nucleotides of the 3′ NS coding sequence had a limited effect [Fig. 2, NS(30)GFP(150)]. These results indicate that the first 30 nucleotides of the NS sequence are critical for efficient incorporation of the NS vRNA segment.

Effect of nucleotide substitutions in the coding regions on efficient incorporation of the NS segment. To confirm that nucleotides in the coding regions per se are important for efficient NS vRNA incorporation, we introduced silent mutations in the NS coding sequence of NS(150)GFP(150) vRNA

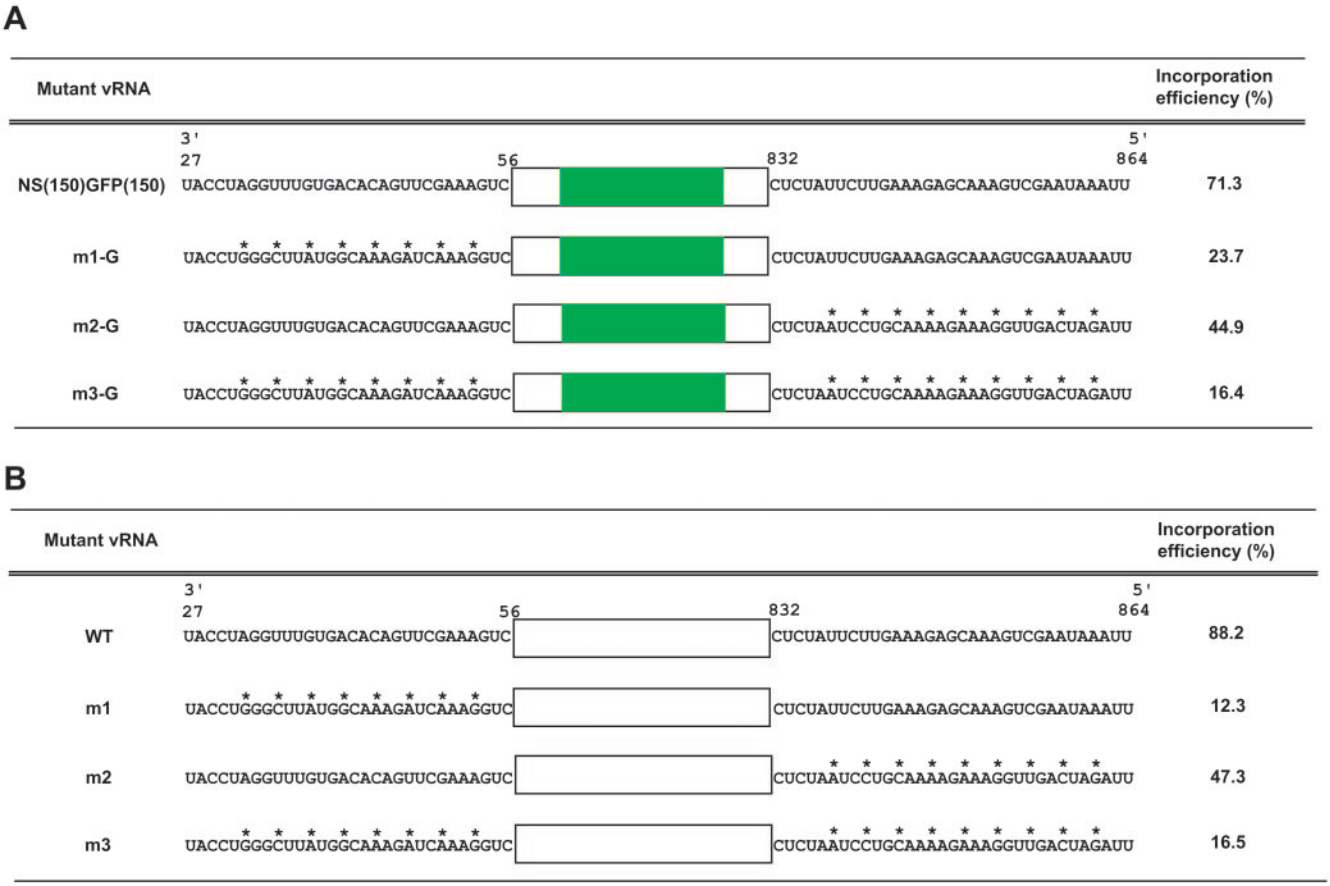

FIG. 3. Efficiencies of incorporation of NS segments with nucleotide substitutions in the NS coding regions. The nucleotide sequences at the 3′ (positions 27 to 56) or 5′ (positions 832 to 864) end of the NS coding region are shown. The silent mutations introduced are shown by asterisks. The coding regions of the NS vRNA and GFP are represented by white and green bars, respectively. The results shown are representative data for three independent experiments. (A) Efficiencies of incorporation of the NS-GFP segment with nucleotide substitutions in the NS coding regions. Incorporation efficiencies were determined as described in the legend to Fig. 1. (B) Efficiencies of incorporation of the NS segment with nucleotide substitutions in the NS coding regions. To determine the incorporation efficiency of mutant NS vRNA segments, NS2- and NP-expressing cells were counted at 24 h postinfection.

(Fig. 3A). m1-G vRNA possesses silent mutations at the 3′ end, m2-G vRNA possesses silent mutations at the 5′ end, and m3-G possesses silent mutations at both ends of the coding regions important for vRNA incorporation (i.e., positions 27 to 56 at the 3′ end and 832 to 864 at the 5′ end). We then tested the efficiency of virion incorporation of these mutant NS vRNAs. Introduction of silent mutations at the 3′ end (m1-G) reduced vRNA incorporation more profoundly than did that at the 5′ end (m2-G), while mutation at both ends (m3-G) reduced vRNA incorporation to 16.4%.

Next, we investigated whether the silent mutations that affected NS-GFP vRNA incorporation affect incorporation of authentic NS vRNA (Fig. 3B). The incorporation efficiency of the authentic NS segment was determined by dividing the number of NS2-positive cells (i.e., the number of viral particles containing the NS segment) by the number of NP-positive cells (i.e., the total number infectious virions) at 24 h postinfection of cells with virus released from plasmid-transfected cells. The effect of the silent mutations on the authentic NS vRNA segment on virion incorporation was similar to that found with NS-GFP vRNA, although the 3′-end silent mutant had a more profound effect than its NS-GFP counterpart (compare m1-G in Fig. 3A and m1 in Fig. 3B). These results indicate the importance of specific nucleotides in the coding region for efficient NS vRNA incorporation.

Growth properties of m1, m2, and m3 mutant viruses in tissue culture. To test the effects of the reduction of virion incorporation of NS vRNA on viral replication, we compared the growth properties of wild-type and mutant viruses with silent mutations in the NS coding regions in MDCK cells (Fig. 4). Cells were infected at a multiplicity of infection of 0.001 PFU/ml, and yields of virus in the culture supernatant were determined at different times postinfection at 37°C. The growth of m2 mutant virus was similar to that of wild-type virus. Although m1 mutant virus produced a lower titer than the wild-type virus at 12 and 18 h postinfection, its maximum titer at 36 h was almost the same as that of the wild-type virus. By contrast, m3 mutant virus, possessing mutations at both ends of the coding region, grew slower than wild-type virus; its titer at 30 h was 2 log units lower than that of wild-type virus. These results indicate that the reduction of NS vRNA virion incorporation affects viral replication.

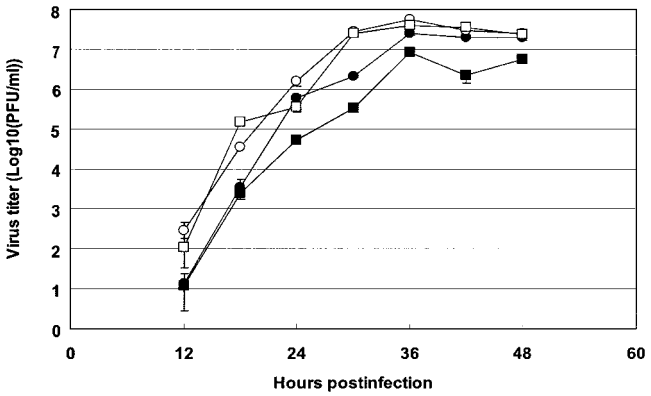

FIG. 4. Growth properties of mutant viruses containing silent mutations in the coding regions of the NS segment. MDCK cells were infected with virus at an multiplicity of infection of 0.001. At the indicated times after infection, the virus titer in the supernatant was determined. The values are means and standard deviations from triplicate experiments. ●, m1; ○, m2; ■, m3; □, wild type.

Sequences of the NS segment-specific noncoding region important for NS vRNA incorporation into virions. Although the noncoding regions of NS vRNA alone did not allow efficient incorporation of NS vRNA into virions [Fig. 2, NS(0)GFP(0)], this result does not necessarily mean that this region is not required for vRNA incorporation. For this reason, we investigated the sequences of the noncoding region to determine whether they influenced the efficiency of incorporation of the NS vRNA segment. The 3′ and 5′ ends of the vRNAs contain 12 and 13 conserved nucleotides, respectively (Fig. 5A, U12 and U13) (23). Downstream and upstream, respectively, of U12 and U13, segment-specific noncoding sequences exist. Although the 5′ ends of these sequences contain the conserved poly(U) signal and is thought to regulate viral transcription (11, 12), its role in viral replication remains unknown. Because our approach for evaluating segment incorporation is based on GFP expression from test vRNAs, the poly(U) signal (Fig. 5A) is necessary because it allows for addition of the poly(A) tail to the viral mRNAs (11, 12). Therefore, to determine the importance of the segment-specific noncoding regions in virion incorporation of vRNA, we constructed plasmids designed to express mutant NS vRNAs that had deletions in these regions but retained the poly(U) signal (Fig. 5A).

Deletion of both the 3′ and 5′ segment-specific noncoding sequences drastically reduced the incorporation efficiency of NS vRNA (Fig. 5B, NSΔNTR), demonstrating that segment-specific noncoding sequences are important for NS vRNA incorporation. To determine the relative importance of the 3′ and 5′ segment-specific noncoding sequences, we generated two deletion mutants, one lacking the 3′ and the other lacking the 5′ segment-specific noncoding sequence. Deletion of the 5′ segment-specific noncoding sequence resulted in a moderate reduction in NS vRNA incorporation, whereas deletion of the

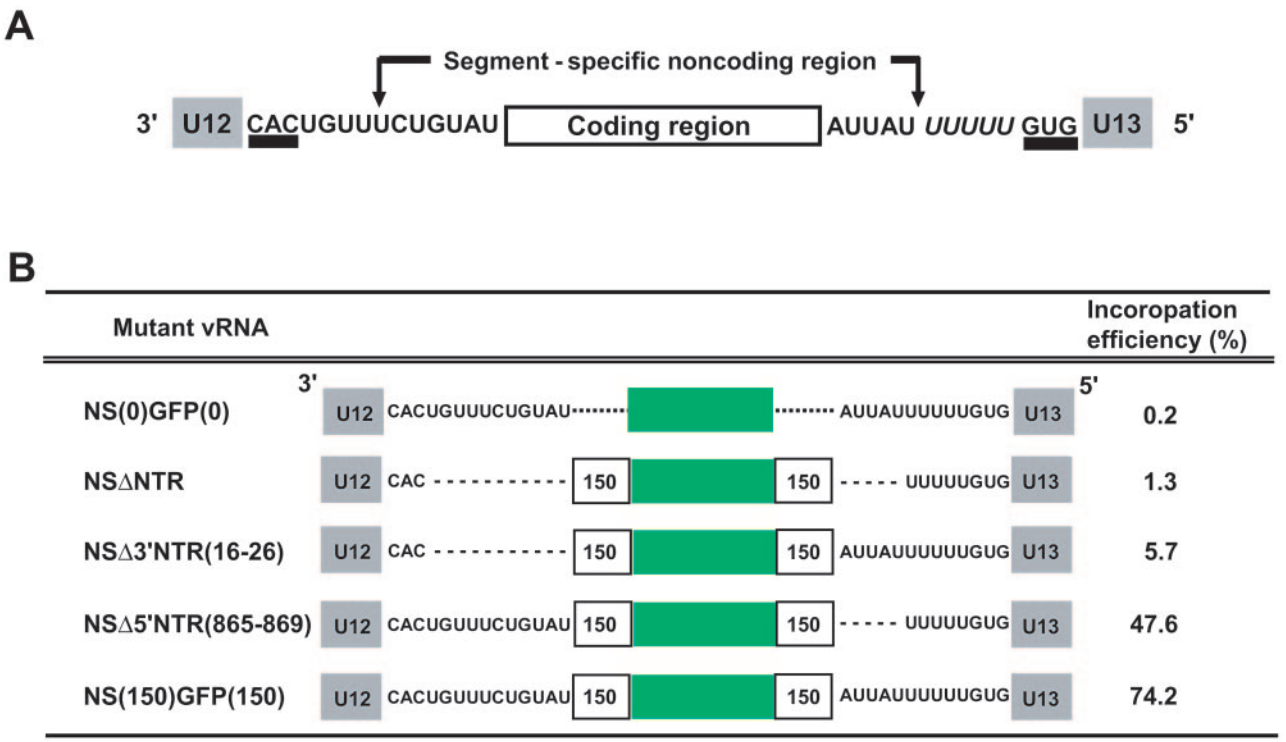

FIG. 5. Importance of NS segment-specific noncoding regions for incorporation of recombinant NS segments. (A) Schematic diagram of the NS vRNA. Segment-specific complementary sequences are underlined. The poly(U) sequence for the addition of the poly(A) tail to the mRNA is shown in italics. The grey boxes represent U12 and U13. (B) Efficiencies of incorporation of recombinant NS segments with deletions in the NS segment-specific noncoding regions. U12 and U13 are represented by grey boxes. The coding regions of the NS vRNA and GFP gene are represented by white and green bars, respectively. The dotted lines represent sequences deleted from the NS segment. The results shown are representative data from three independent experiments.

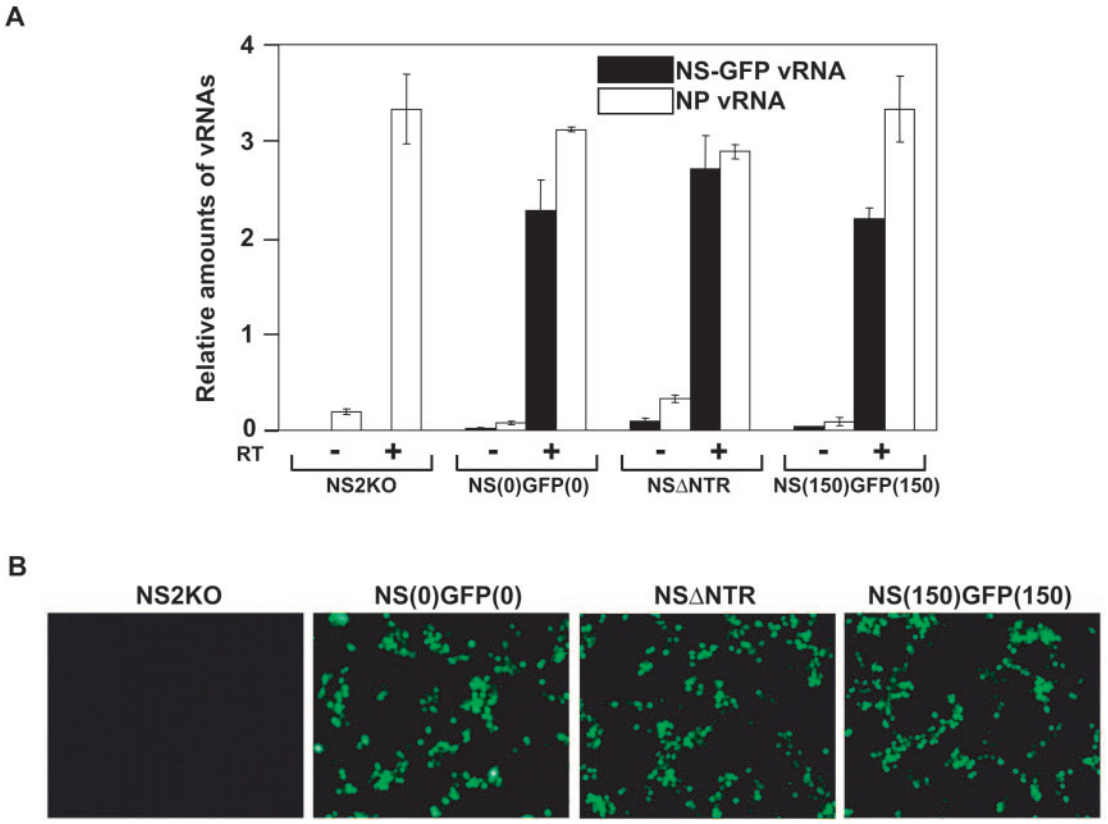

FIG. 6. Comparison of test NS vRNA amounts and GFP expression levels in plasmid-transfected cells. (A) Quantitative analysis of NS(0)GFP(0), NSΔNTR, and NS(150)GFP(150) vRNAs produced from PolI plasmids in 293T cells by real-time quantitative reverse transcription-PCR. 293T cells (10^6 cells) were transfected with 18 plasmids. At 48 h posttransfection, total RNA was extracted from 293T cells and quantified by real-time PCR with GFP and NP sequence-specific primers and probes as described in Materials and Methods. The primers and probes for the NS-GFP segments detect NS-GFP but not the authentic NS segment. Experiments were performed in the presence (+) or absence (−) of reverse transcriptase (RT) to demonstrate that the results are not affected by the plasmids used for the experiments. NS2KO represents NS2 knockout virus, which does not produce NS2(NEP) protein and does not undergo multiple cycles of replication (27). It therefore serves as a negative control for detection of NS-GFP and a positive control for the NP segment. The results shown are representative data from three independent experiments. Error bars indicate standard deviations. (B) Comparison of GFP expression levels in PolI plasmid-transfected 293T cells by fluorescence microscopy. At 48 h after transfection, the cells were observed by fluorescence microscopy.

3′ segment-specific noncoding sequence drastically reduced incorporation efficiency [Fig. 5B, NSΔ5′NTR(865–869) and NSΔ3′NTR(16–26)]. These results indicate that the 3′ segment-specific noncoding sequence plays a crucial role in the incorporation of NS vRNA into virions.

The length of recombinant NS-GFP vRNA does not affect intracellular amounts of vRNA or GFP expression levels. Differences in the lengths of the test vRNAs may affect transcription efficiency and thus the amounts of vRNAs in cells, which in turn could influence the efficiency of vRNA incorporation into virions. We therefore compared the amounts of NS(0)GFP(0), NSΔNTR, and NS(150)GFP(150) vRNAs in plasmid-transfected cells by real-time PCR analysis with GFP and NP sequence-specific primers and probes (Fig. 6A). The amounts of these vRNAs in transfected cells did not differ from each other to an extent that would affect incorporation efficiency, indicating that the lengths of the test NS-GFP vRNAs did not affect their transcription efficiency.

To further confirm this observation, we compared GFP expression levels in the plasmid-transfected cells by fluorescence microscopy, since the differences in the lengths of the test vRNAs may affect the translation efficiency of GFP. Under these conditions, we did not observe an appreciable difference in GFP expression among the NS(0)GFP(0), NSΔNTR, and NS(150)GFP(150) vRNA segments (Fig. 6B).

DISCUSSION

Here we show that both segment-specific noncoding and coding regions of the NS vRNA segment of influenza A virus are important for efficient incorporation of the NS segment into virions, suggesting that these regions likely form a structure that is required for this activity. Moreover, silent mutations in the coding regions that are important for NS vRNA incorporation reduced the replicative ability of the virus. Our results confirm and extend the finding of Luytjes et al. that the NS noncoding region possesses a signal for the packaging of NS vRNA (13). These data also explain why the artificial vRNA segment possessing the CAT sequence was not efficiently maintained in virions during repeated passages; the CAT segment tested by Luytjes et al. (13) lacked the coding regions important for efficient incorporation, and thus the

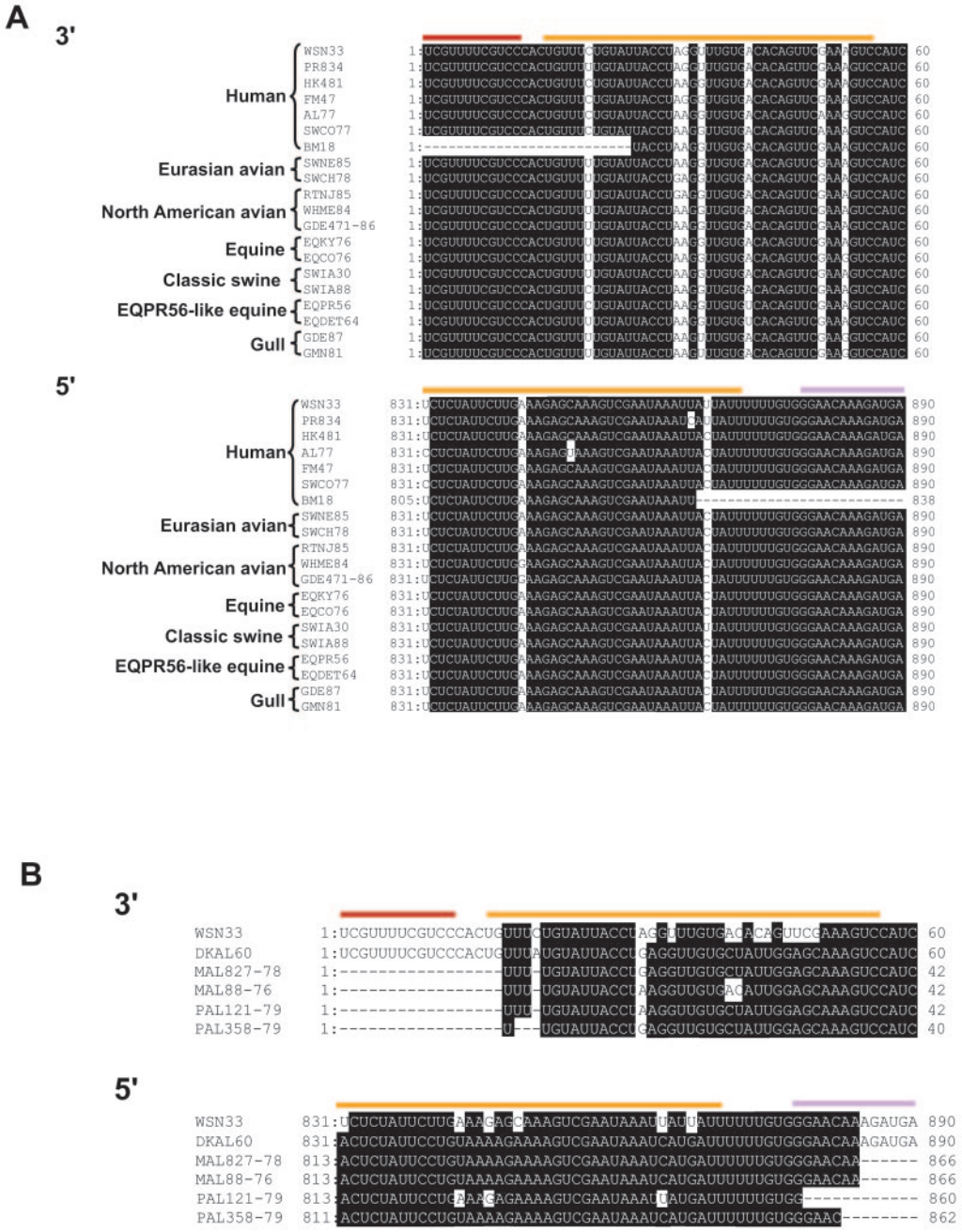

FIG. 7. Sequence alignment of the NS incorporation signals among influenza A viruses. The red, orange, and purple lines represent U12, incorporation signal, and U13, respectively. The conserved sequences among all strains are shown in black. The NS sequences were obtained from the influenza A virus sequence database at Los Alamos National Laboratory. (A) Comparison of the incorporation signals among allele A NS segments. Human viruses: WSN, A/WSN/33; PR834, A/Puerto Rico/8/34; HK481, A/Hong Kong/481/97; FM47, A/Fort Monmouth/1/47; AL77, A/Alaska/6/77; SWCO77, A/swine/Colorado/1/77; BM18, A/Brevig Mission/1/18. Eurasian avian viruses: SWNE85, A/swine/Netherlands/12/85; SWCH78, A/swine/China/8/78. North American avian viruses: RTNJ85, A/ruddy turnstone/New Jersey/47/85; WHME84, A/whale/Marine/328/84; GDE471-86, A/gull/Delaware/471/86. Equine viruses: EQKY76, A/equine/Kentucky/76; EQCO76, A/equine/Cordaba/4/76. Classic swine viruses: SWIA30, A/swine/Iowa/15/30; SWIA88, A/swine/Italy/671/87. EQPR56-like equine viruses: EQPR56, A/equine/Prague/1/56; EQDET64, A/equine/Detroit/3/64. Gull viruses: GDE87, A/gull/Delaware/2838/87; GMN81, A/gull/Minnesota/1352/81. (B) Comparison of the incorporation signals among allele B NS segments. DKAL60, A/duck/Alberta/60/76; MAL827-78, A/mallard/Alberta/827/78; MAL88-76, A/mallard/Alberta/88/76; PAL121-79, A/pintail/Alberta/121/79; PAL358-79, A/pintail/Alberta/358/79. The sequence data for BM18, MAL827-78, MAL88-76, PAL121-79, and PAL358-79 are not complete in the influenza virus database.

CAT segment did not have sufficient signal for efficient packaging.

Previously, we have shown the importance of the coding regions in the HA and NA segments of influenza A virus for their incorporation into virions (6, 28). Although we did not examine the contribution of the segment-specific noncoding regions of these segments to this activity, it is likely that these regions are also important for segments other than the NS.

Indeed, it may be that in all segments a structure formed by both segment-specific noncoding and adjacent coding regions plays a critical role in incorporation into virions. The mechanism by which the regions we have identified contribute to virion incorporation and function in packaging remains unknown. However, in the segments we have examined thus far (HA [28]; NA [6]; NS [this study]; PA, PB2, and PB1 [Y. Muramoto and Y. Kawaoka, unpublished data]; and M [J. Maeda and Y. Kawaoka, unpublished data]), the 3′ ends of the segments are more important than the other sequences for this event. Thus, there is a polarity in the packaging of the gene segments. Further studies are needed to reveal the role of these regions in genome packaging.

Two models have been proposed to explain the mechanism by which the influenza virus genome is packaged. The random-packaging model suggests that each viral RNA segment possesses a common packaging signal that allows random incorporation of RNA segments into virions (2). The selective-packaging model suggests that there are unique packaging signals in each viral RNA segment that lead to the incorporation of a set of all eight RNA segments into a virion (16). In all viral RNA segments examined thus far (i.e., HA [28]; NA [6]; NS [this study]; PA, PB1, and PB2 [Y. Muramoto and Y. Kawaoka, unpublished data]; and M [J. Maeda and Y. Kawaoka, unpublished data]), the nucleotide sequences important for efficient genome packaging into virions differ from one another (i.e., each viral segment has an individual signal for incorporation into virions). Such segment-specific incorporation signals might be used to form a multisegment macromolecule that facilitates the incorporation of a set of eight RNA segments into virions, thus supporting the selective-packaging model.

NS segments of influenza A viruses are phylogenetically separated into two groups, alleles A and B (1, 8, 25). Alignment of the nucleotide sequences of several influenza A viruses revealed that the sequences important for efficient incorporation of NS segment (nucleotides 16 to 56 or 841 to 870 in the vRNA sense orientation) are relatively conserved (Fig. 7). This means that NS segment incorporation likely imposes few constraints on reassortment. The potentially promiscuous nature of the NS segment has important implications for the emergence of highly virulent influenza viruses, since some of the NS1 proteins are responsible for high virulence (7, 24); theoretically, however, the NS segments of nonpathogenic influenza viruses could easily be replaced with that of a lethal virus.

The promoter activity of NS vRNA is higher than that of other segments (5). Given this knowledge, together with the information we now have on segment incorporation, one could explore the possibility of using the NS segment to incorporate foreign genes (6, 28), making influenza virus an attractive gene expression vector.

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