Selective incorporation of influenza virus RNA segments into virions

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The genome of influenza A virus is comprised of eight viral RNA (vRNA) segments. Although the products of all eight vRNA segments must be present for viral replication, little is known about the mechanism(s) responsible for incorporation of these segments into virions. Two models have been proposed for the generation of infectious virions containing eight vRNA segments. The random-incorporation model assumes a common structural feature in all the vRNAs, enabling any combination of vRNAs to be incorporated randomly into virions. The selective-incorporation model predicts the presence of specific structures in each vRNA segment, leading to the incorporation of a set of eight vRNA segments into virions. Here we demonstrate that eight different vRNA segments must be present for efficient virion formation and that sequences within the coding region of (and thus unique to) the neuraminidase vRNA possess a signal that drives incorporation of this segment into virions. These findings indicate a unique contribution from individual vRNA segments and thus suggest a selective (rather than random) mechanism of vRNA recruitment into virions. The neuraminidase vRNA incorporation signal and others yet to be identified should provide attractive targets for the attenuation of influenza viruses in vaccine production and the design of new antiviral drugs.

The influenza A virus genome consists of eight segments of single-stranded RNA with negative polarity (complementary to mRNA). Each viral RNA (vRNA) segment resides within a complex [the viral ribonucleoprotein complex (vRNP)] of nucleoprotein and three polymerase subunits designated PA, PB1, and PB2. After transport of M1 and NS2 proteins into the nucleus, vRNPs formed in this compartment are exported to the cytoplasm, where presumably they interact with viral membrane-associated proteins including hemagglutinin (HA), neuraminidase (NA), and M1 to ensure the correct assembly of virions and their release from the host cell. Although all eight vRNPs must be present for efficient viral replication, little is known about the mechanism(s) responsible for incorporation of vRNA segments into virions and their stable maintenance during repeated cycles of replication.

Two models have been proposed for the generation of infectious virions containing eight vRNA segments. The random-incorporation model assumes a common structural feature in all the vRNPs, enabling them to be incorporated randomly into virions. Support for this model comes from the observation that an influenza A virion can possess more than eight vRNPs (1). The selective-incorporation model predicts the presence of specific structures in each vRNA segment, leading to their individual incorporation into virions. This hypothesis was suggested by data showing that the presence of excess amounts of internally deleted segments encoding polymerase proteins resulted in a corresponding reduction of full-length segments in virions (2, 3).

Air and coworkers (4–6) produced an influenza A virus with a large internal deletion in the NA vRNA segment by growing the virus in the presence of a bacterial sialidase and an antibody to viral NA. We generated a similar virus and adapted it to growth in cell culture, embryonated eggs, and mice without exogenous sialidase (7). Interestingly, even after extensive passaging these mutant viruses still maintained an internally truncated NA vRNA segment (4–7), suggesting that the altered NA segment participates in viral replication and carries structural features required for its incorporation into virions. Here we demonstrate a requirement for individual vRNA segments in efficient virion production and identify distinct NA coding sequences that seem to facilitate recruitment of this segment into virions. Our results clearly support a selective mechanism of vRNA recruitment during virion assembly.

Materials and Methods

Cells. 293T human embryonic kidney cells were maintained in Dulbecco's medium supplemented with 10% FCS, and Madin–Darby canine kidney (MDCK) cells were maintained in Eagle's medium supplemented with 5% newborn calf serum.

Plasmid-Based Reverse Genetics. Influenza A viruses were generated with plasmids possessing the cDNA of A/WSN/33 (H1N1) viral genes under the control of an RNA polymerase I promoter and terminator (referred to as PolI plasmids) and the eukaryotic protein expression vector pCAGGS/MCS (controlled by the chicken β-actin promoter; refs. 8 and 9) as described (10).

Plasmids. pPolI-NAFLAG was used to produce negative-sense NAFLAG RNA that contains the 3′ noncoding region of NA vRNA (19 nt), 153 nt of the NA coding region corresponding to the cytoplasmic tail (6 aa), transmembrane (29 aa) and stalk (16 aa) regions, and nucleotides for the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) in the negative sense, two sequential stop codons (TAA-TAG) in the negative sense, and 185 nucleotides of 5′-terminal sequence of NA vRNA. pPolI-NAFLAG-Met(−), used for the production of negative-sense NAFLAG-Met(−) RNA, which lacks the start codon for the NA protein, was generated by changing the ATG initiation codon and another ATG at the 15th codon of the truncated NAFLAG protein to GCG by *in vitro* site-directed mutagenesis (GENEEDITOR, Promega).

pPolINA(183)GFP(157), used for the production of negative-sense RNA and containing the 3′ noncoding ends of NA vRNA and a complementary sequence encoding a fusion protein with 61 N-terminal NA codons, enhanced GFP (CLONTECH), two consecutive stop codons (TAA-TAG), and 185 bases of the 5′ end of NA vRNA, was produced by replacing nucleotides 203–1,109 (positive sense) of the WSN NA gene in pT7Blue-NA with a *Bgl*II site by inverse PCR (11). The GFP gene was cloned into this *Bgl*II site and the *Stu*I site at position 1,226 (in the wild-type NA gene) in frame with the NA protein. The NA(183)GFP(157) gene then was inserted into the *Bsm*BI site of pHH21. The series of deletion mutants described was produced

Abbreviations: vRNA, viral RNA; vRNP, viral ribonucleoprotein complex; HA, hemagglutinin; NA, neuraminidase; MDCK, Madin–Darby canine kidney; PolI, RNA polymerase I.

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by PCR mutagenesis. All plasmid constructs were sequenced to ensure that unwanted mutations were not introduced by PCR.

pPolI-HAstop for generating vRNA that does not encode functional HA protein was made by inverse PCR as follows: The HA coding sequence starting from the initiation codon, ATG-AAG-GCA, was changed to ATG-TGA-TGA-G to convert the codons immediately downstream of the initiation codon to stop codons and to cause a frame shift. The lack of HA protein expression from this construct was confirmed by immunofluorescent assays in 293T cells transfected with this plasmid together with plasmids for the expression of PA, PB1, PB2, and NP.

pPolI-NAMet(−) for generating vRNA that does not encode functional NA protein was also made by inverse PCR as follows: The initiation codon and the next methionine codon in the same frame were changed to GCG, and the codon immediately downstream of the second methionine was changed to a stop codon, TAA. Lack of NA protein expression from this construct was confirmed as described above for pPolI-HAstop.

pPolI-WSN-NSΔsplice, which contains a point mutation in the splice donor sequence (G56A, with reference to WSN-NS cRNA) and a stop signal at codon 17 of NS2 (C548A), was generated previously (12). Because of these mutations, vRNA generated from this plasmid does not encode a functional NS2 protein. pPolI-HAstop, pPolI-NAMet(−), and pPolI-WSN-NSΔsplice were used only for the experiments presented in Fig. 1.

Immunostaining. Cells were fixed with 3% formaldehyde solution and permeated with 0.1% Triton X-100 in 3% formaldehyde solution. Antigens were detected with anti-FLAG monoclonal antibody M2 (Sigma) or rabbit antiserum against influenza WSN virus used as the primary antibody and biotinylated anti-mouse IgG for the FLAG epitope or biotinylated anti-rabbit IgG for viral antigens used as the secondary antibody (VECTASTAIN ABC kit, Vector Laboratories).

***In Situ* Hybridization.** Cells were fixed with 3% formaldehyde solution, permeated by 0.1% Triton X-100 in 3% formaldehyde solution, and prehybridized at 65°C for 30 min in prehybridization buffer [5× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0)/1% blocking reagent/DIG nucleic acid-detection kit (Roche Diagnostics, Indianapolis)/0.1% *N*-lauroylsarcosine/0.02% SDS containing 0.1 mg/ml poly(A) DNA from the DIG-detection kit)]. An oligonucleotide probe complementary to the FLAG sequence (10 pmol) labeled by using the DIG oligonucleotide-tailing kit (Roche) at 37°C for 6 h was added to the prehybridization buffer and hybridized at 55°C for 1 h. The hybridized cells were washed for 5 min with wash buffer (0.1 M maleic acid/0.15 M NaCl/0.3% Tween 20, pH 7.5), blocked with 1% blocking reagent for 30 min at room temperature, and incubated with antidigoxigenin antibody (1:500) conjugated with alkaline phosphatase for 30 min at room temperature. Cells then were washed with the wash buffer and incubated with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate in the detection buffer (0.1 M Tris·HCl/0.1 M NaCl, pH 9.5) at room temperature for 3 h in the dark.

Competitive Passages. NAFLAG or NAFLAGMet(−) virus (300 plaque forming units) was mixed with 3×10^4 plaque-forming units of NA(−) virus and used to infect subconfluent MDCK cells (multiplicity of infection, 0.01) and incubated for 72 h in medium containing 0.5 μg/ml trypsin and 100 microunits/ml *Vibrio cholerae* sialidase. The viruses harvested were used to infect MDCK cells. This process was repeated five times.

Quantification of vRNA in PolI Plasmid-Transfected Cells. vRNA in 293T cells transfected with PolI plasmids was extracted with the Isogen RNA-extraction kit (Nippon Gene, Tokyo) at 48 h posttransfection. The extracted RNAs were digested with DNase at 37°C for 30 min to eliminate the transfected plasmid DNA, and the enzyme was inactivated by heating at 80°C for 15 min. The vRNA of the NA-GFP segment was reverse-transcribed by using TaqMan reverse-transcription reagents (Applied Biosystems) with a strand-specific primer for the NA segment and quantified by using the ABI PRISM 7000 sequence-detection system with the TaqMan universal PCR Master mix (Applied Biosystems), specific primers, and TaqMan probe (13).

Prediction of Secondary Structure of vRNA. RNA-folding calculations were performed with the program MFOLD in the GCG WISCONSIN 10.2 package (Accelrys, San Diego) (14).

Results

vRNA Segments Contribute Individually to Virion Production. The conservation of a truncated NA vRNA during long-term viral replication suggested that individual vRNA segments might contribute to efficient virion formation. To investigate this possibility, we compared the production of infectious virions among those possessing six, seven, or eight vRNA segments. To generate an eight-segment virus (wild type), we transfected 293T cells with protein expression plasmids for all nine structural proteins and with eight plasmids for all vRNA segments, exactly as required for normal virus production (10). Seven-segment viruses were produced by eliminating the plasmids for either HA or NA vRNA, whereas for a six-segment virus, the plasmids for both the HA and NA vRNA were eliminated. To prevent the viruses generated in 293T cells from undergoing multiple cycles of replication, we used a plasmid for mutant NS vRNA possessing two mutations that eliminate production of the NS2 protein (12). We also used plasmids in which the HA and NA vRNAs contained mutations that block the production of HA and NA proteins such that the effects of eliminating particular gene segments would not extend to the gene products.

To compare the efficiency of virion production by these viruses, we harvested virions released from plasmid-transfected 293T cells and titrated them by immunostaining virus-infected MDCK cells with an antiserum to the influenza A/WSN/33 virus at 24 and 48 h postinfection. As shown in Fig. 1, the efficiency of infectious virion production at either time point correlated with the number of different vRNA segments; that is, the higher the number of different vRNA segments the higher the efficiency of virion production. These results suggest a unique contribution from the HA, NA, and possibly other vRNA segments to efficient virion assembly and thus a selective mechanism of vRNA recognition and incorporation.

Importance of the NA vRNA in Efficient Viral Growth. To obtain further evidence that individual vRNAs contribute differently to efficient virus production, we tested the growth properties of eight- and seven-segment viruses in competitive-replication experiments. For this purpose, we generated a virus with a truncated NA vRNA and a seven-segment virus lacking the NA vRNA [designated NA(−)]. We first designed a plasmid that produces the NA vRNA segment with an internal deletion (similar to that in the naturally occurring truncated NA vRNA) and a FLAG epitope sequence (designated NAFLAG), which was introduced to allow detection of the truncated NA protein in cells (Fig. 2). This plasmid was modified further to produce NAFLAGMet(−) RNA, which does not express the truncated NA protein, by changing both the NA start codon and an ATG codon (the 15th codon) in frame with the NA protein from ATG to GCG, while leaving the FLAG sequence intact. Viruses possessing NAFLAG or NAFLAGMet(−) segments together with other influenza vRNA segments were produced. These and the NA(−) viruses all produced plaques in the presence of bacterial sialidase that reacted with an antiserum to WSN virus

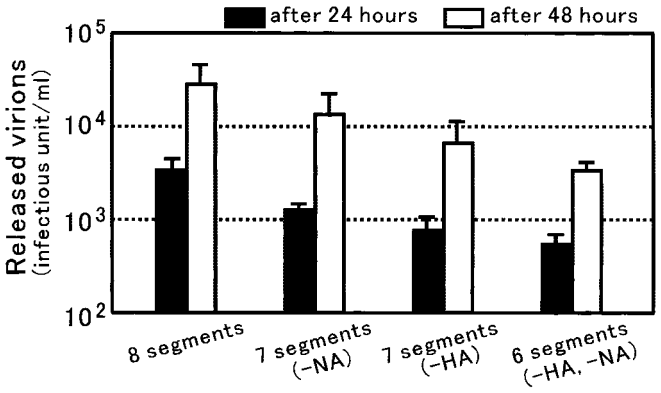

Fig. 1. Effect of the number of RNA segments on the efficiency of virion production. 293T cells were transfected with protein expression plasmids for nine structural proteins and plasmids for the production of eight, seven, or six different vRNA segments. The plasmid for NS vRNA production possesses two mutations to eliminate NS2 protein production (12) such that the resultant virus does not undergo multiple cycles of replication. The plasmids for HA and NA vRNA production contain mutations that eliminate protein production, thus avoiding the effects of these proteins expressed from vRNA. To produce a virus with seven segments, we eliminated the plasmid for HA (−HA) or NA (−NA) vRNA production, whereas for the production of a six-segment virus we omitted the plasmids for both the HA and NA vRNA. Released virions were harvested at 24 (black bar) or 48 h (white bar) posttransfection and titrated by immunostaining virus-infected MDCK cells with antiserum to the influenza WSN strain.

(Fig. 3*A*, *a–c*), but only those of the NAFLAG virus reacted with an anti-FLAG antibody (*d–f*), indicating that the NAFLAG virus possesses the truncated NA vRNA, whereas the NAFLAG-Met(−) virus does not express the truncated NA protein. To ensure that the NAFLAGMet(−) mutant virus carried the truncated NA vRNA segment, we performed *in situ* hybridizations on plaques produced by this virus by using a FLAG sequence-specific probe. Plaques produced by the NAFLAG-Met(−) and NAFLAG (positive control) viruses reacted with the probe, whereas the NA(−) virus (negative control) did not

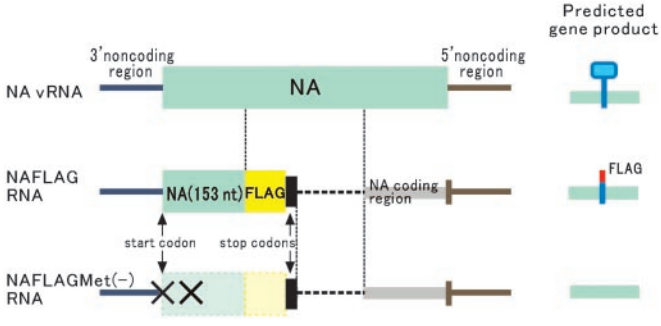

Fig. 2. Schematic diagram of mutant vRNAs. NAFLAG RNA contains the 3′ noncoding region of NA vRNA (19 nucleotides), 153 nucleotides of the NA coding regions, corresponding to the cytoplasmic tail (6 aa), the transmembrane (29 aa) and stalk (16 aa) regions, and nucleotides for the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) in the negative sense, two sequential stop codons (TAA TAG) in the negative sense, 185 nucleotides corresponding to the NA C terminus, and the 5′ noncoding region of NA vRNA. NAFLAGMet(−) RNA has changes in both the NA start codon and a downstream ATG codon (the 15th codon) in frame with the NA reading frame from ATG to GCG (in the positive sense) such that a protein is not produced from the reading frame, as indicated by a box with dashed lines. Both of these RNAs are shown in the negative-sense orientation. The horizontal broken line indicates a deletion. The lengths of the regions are not to scale. Predicted gene products from individual RNA segments are shown on the right.

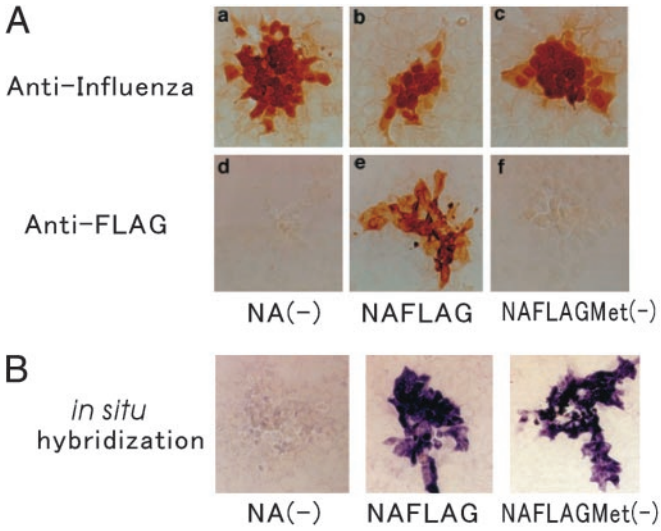

Fig. 3. Confirmation of virus production with truncated NA RNA segments. (*A*) MDCK cells were infected with NA(−) (*a* and *d*), NAFLAG (*b* and *e*), or NAFLAGMet(−) (*c* and *f*) viruses and overlaid with 0.6% agarose. After incubation for 48 h at 37°C, the cells were fixed and permeated with 0.1% Triton X-100 in 3% formaldehyde solution. The viral proteins or FLAG epitope were detected by immunostaining with antiserum to influenza WSN strain (*a–c*) or anti-FLAG monoclonal antibody (*d–f*) as the primary antibody and biotinylated secondary antibody with the VECTASTAIN ABC kit (Vector Laboratories). (*B*) MDCK cells infected with NA(−), NAFLAG, or NAFLAGMet(−) viruses were incubated, fixed, and permeated as described above. The FLAG sequence in mRNA was detected by *in situ* hybridization with a digoxigenin-labeled probe specific for the sequence and visualized with the DIG nucleic acid-detection kit (Roche).

(Fig. 3*B*), confirming the presence of the NAFLAGMet(−) RNA segment in the virus.

Using a competitive-replication assay, we then tested whether the truncated NA vRNA promotes viral growth. In this assay, we mixed NA(−) and NAFLAG or NAFLAGMet(−) viruses at a ratio of 100:1 and incubated the mixture on MDCK cells. At 72 h postinfection, the supernatant was removed and passaged in MDCK cells. A portion of the supernatant was used to produce plaques, which were immunostained with an anti-WSN antibody. Plaques produced by NAFLAG or NAFLAGMet(−) virus were detected either by immunostaining with anti-FLAG monoclonal antibody or by *in situ* hybridizations with the FLAG sequence-specific probe, respectively.

As shown in Fig. 4, the plaques produced by the viruses containing a truncated NA vRNA [NAFLAG or NAFLAG-Met(−)] gradually increased over five passages, reaching >70% by the last passage. None of the plaques produced by NAFLAG-Met(−) virus reacted with anti-FLAG antibody at the fifth passage, indicating that the virus did not revert to one expressing the truncated NA protein (data not shown). Thus, the truncated NA vRNA segment itself seems to play an important role in efficient viral replication. However, the rate at which the NAFLAGMet(−) virus became dominant in the mixed infection was slower than that of the NAFLAG virus, suggesting some effect of the truncated NA protein on viral replication.

The NA vRNA Coding Region Is Required for Incorporation of the NA Segment into Virions. Maintenance of the truncated NA vRNA during extensive passaging in different hosts (4, 7) and the mechanism of specific vRNA segment recognition suggested by the results shown in Figs. 1 and 4 led us to conjecture that the truncated NA vRNA retained one or more structural features required for its incorporation into virions. The ability to generate

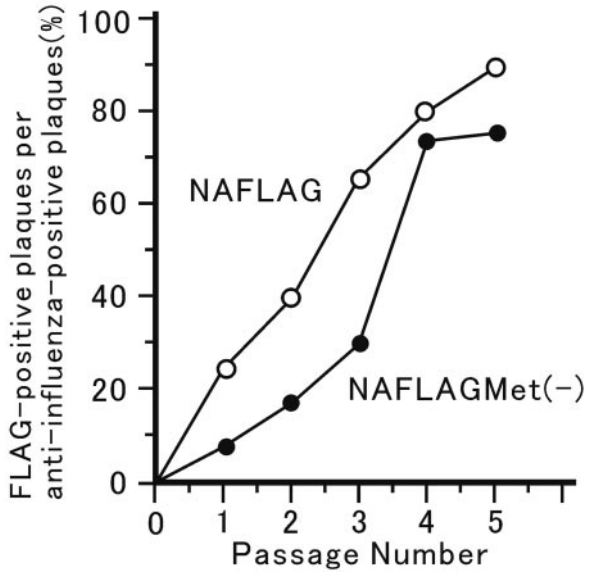

Fig. 4. Effect of truncated NA vRNA on efficiency of viral growth. Three hundred plaque-forming units of NAFLAG (○) or NAFLAGMet(−) (●) virus were mixed with 3×10^4 plaque-forming units of NA(−) virus, used to infect subconfluent MDCK cells (multiplicity of infection, 0.01), and incubated for 72 h at 37°C in the presence of *V. cholerae* sialidase. The virus in the supernatant was used to infect MDCK cells. This procedure was repeated four more times. In each passage, virus in the supernatant was used to perform plaque assays and determine the percentage of plaques positive for anti-FLAG epitope by immunostaining (NAFLAG) with anti-FLAG monoclonal antibody or by *in situ* hybridization [NAFLAGMet(−)] for the FLAG sequence with a probe specific for this sequence.

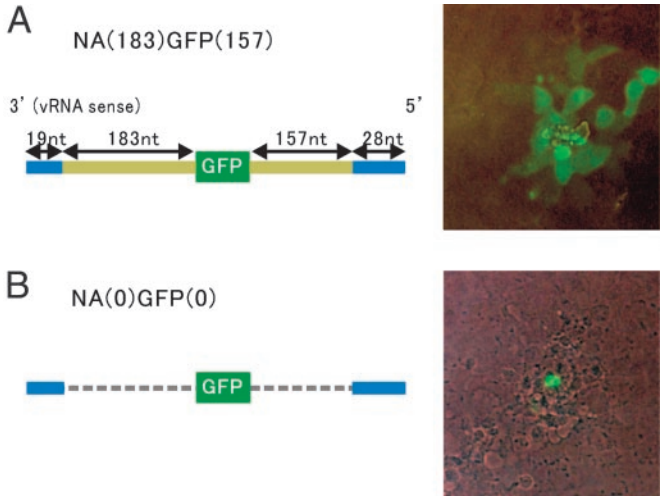

Fig. 5. The coding region is important for NA vRNA virion incorporation. MDCK cells were infected with NA(183)GFP(157) (*A*) or NA(0)GFP(0) (*B*) virus and overlaid with 0.6% agarose. The infected cells were incubated for 48 h at 37°C, and the plaques were photographed under fluorescent light together with limited normal light to identify plaques. The lengths of the regions in the RNA constructs are not to scale. Note the limited number of GFP-positive cells in the plaque produced by the NA(0)GFP(0) mutant contrasted with the abundance of GFP-expressing cells in plaques produced by NA(183)GFP(157).

replication-competent viruses with a truncated NA segment afforded the opportunity to test this prediction. We first inserted a GFP coding sequence into the truncated NA gene in frame with the deleted NA sequence. This recombinant segment, designated NA(183)GFP(157) (Fig. 5), possesses the 3′ NA noncoding region (19 nucleotides), 183 nucleotides corresponding to the N-terminal coding region, the GFP ORF, two consecutive stop codons, 157 nucleotides corresponding to the C-terminal NA coding region, and the 5′ NA noncoding region (28 nucleotides). The resultant fusion protein contains the N-terminal 61 aa of the NA and the entire GFP sequence. We then produced a virus harboring NA(183)GFP(157) RNA instead of normal NA vRNA and performed plaque assays. Over 90% of the plaques expressed GFP (Fig. 5*A*). Moreover, this virus maintained NA(183)GFP(157) RNA after five consecutive passages in cell culture (data not shown), indicating that the NA(183)GFP(157) segment was incorporated into virions and maintained stably during viral replication.

To determine whether the sequences critical for virion incorporation reside in the coding region, we constructed a gene, NA(0)GFP(0) (Fig. 5*B*), that contained the GFP coding sequence (with a stop codon) flanked by the 3′ and 5′ NA noncoding regions, thus lacking the NA coding sequence. In cells transfected with the PolI plasmid encoding the NA(0)GFP(0) gene, together with protein expression plasmids encoding PA, PB1, PB2, and NP, the levels of GFP expression were equivalent to those seen in cells transfected with the PolI plasmid encoding NA(183)GFP(157)(data not shown). This result indicates that deletion of the 3′ and 5′ NA coding regions does not appreciably affect the formation of RNPs *per se*. Although the virus bearing this RNA segment produced plaques, only a few (1.1%) contained GFP-expressing cells, and only one or two cells in these plaques expressed GFP (Fig. 5*B*), indicating that the NA(0)GFP(0) RNA segment was not maintained stably during viral replication.

To ensure that equivalent amounts of NA(0)GFP(0) and NA(183)GFP(157) RNAs were being produced and were available for packaging into virions, we quantified the amounts of these RNAs in plasmid-transfected cells by a real-time quantitative PCR assay (13). The amount of vRNA present for the former construct was 68% of the latter (data not shown). Thus, our finding that only 1.1% of plaques produced by NA(0)GFP(0) virus contained GFP-positive cells cannot be attributed to lower levels of NA(0)GFP(0) RNA in plasmid-transfected cells and likely reflects the absence of structural features critical for virion incorporation. These results indicate that certain sequences in the NA vRNA coding region are critically important for the incorporation of this segment into virions.

Both the 3′ and 5′ Ends of the NA vRNA Coding Region Contribute to Virion Incorporation, Although the 3′ End Is More Important. To refine the critical virion incorporation site(s) in NA vRNA, we made a series of viruses possessing the truncated NA vRNA segment, with further deletion in the 3′ or 5′ (vRNA sense) coding region (Fig. 6). Approximately 40% of plaques produced by the NA(183)GFP(0) virus, which lacks the 5′ terminus of the NA coding region, expressed GFP, in contrast to 1.8% of plaques produced by the NA(0)GFP(157) virus, which lacks the 3′ terminus of the NA coding region. These data indicate that the 3′ end of the NA vRNA coding region plays a more important role in virion packaging than does the 5′ end. The critical region of the 3′ end of the coding region was refined further by using a construct that lacks the 5′ end of the NA coding region. Although the extent of vRNA incorporation did not change appreciably until all but seven codons of the 3′ end of the NA coding region were deleted [NA(21)GFP(0) virus], deletion of a single additional codon [NA(18)GFP(0)] resulted in a drastic reduction of vRNA incorporation, indicating that at least 21 nucleotides in the 3′ end of the NA coding region are required for efficient vRNA incorporation into virions.

Because deletion of the entire 3′ end resulted in extensive reduction in vRNA incorporation [NA(0)GFP(157) virus], we

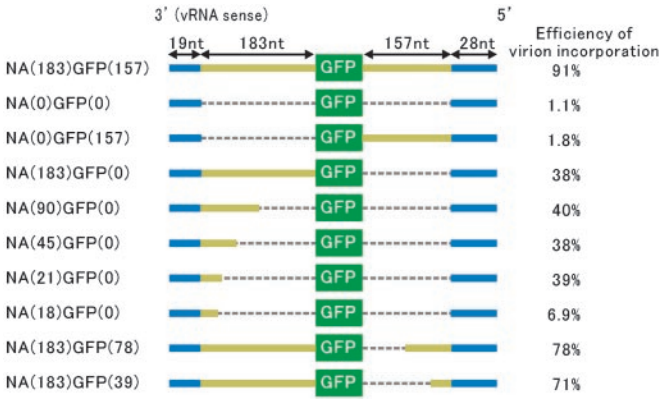

Fig. 6. Identification of the region critical for vRNA incorporation into virions. Each mutant contains the GFP ORF (inserted in frame with the NA ORF) flanked by stop codons, 19 nucleotides of the 3′ noncoding region, and 28 nucleotides of the 5′ noncoding region of NA vRNA. The NA coding regions are shown as beige bars. The virion incorporation efficiency of each NA-GFP RNA was calculated as the percentage of GFP-positive plaques among total plaques.

studied the minimal requirement for the 5′ end sequence for vRNA incorporation by using a construct possessing 183 nucleotides of the 3′ NA coding region. Deletion of 118 nucleotides (leaving 39 residues) in the 5′ NA coding region resulted in an ≈20% reduction in vRNA incorporation [compare NA(183)GFP(39) with NA(183)GFP(157) in Fig. 6]; however, this mutant was still incorporated into virions more efficiently than the construct lacking the entire 5′ NA coding region [compare NA(183)GFP(39) with NA(183)GFP(0)]. These results demonstrate that both the 3′ and 5′ ends of the NA coding region present in NA(183)GFP(157) are needed for maximally efficient vRNA incorporation into virions, with 21 nucleotides in the 3′ coding region seeming critical for this process. Identification of virion incorporation signals in the NA coding region, which therefore are unique to this segment, supports a selective mechanism of vRNA recruitment into virions.

Discussion

We have exploited a truncated NA vRNA segment to elucidate the mechanism of vRNA segment incorporation into virions of influenza A virus. Influenza virions were produced most efficiently in the presence of eight different vRNA segments followed by seven and then six. Because the HA and NA vRNA segments used in this experiment did not encode functional proteins, we attribute these results to incorporation signals unique to these segments. Moreover, other segments could not compensate efficiently for the absence of the NA and HA vRNAs, suggesting that specific recognition of influenza vRNA segments is integrally linked to efficient virion formation. Thus, our findings support the mechanistic model (3), which predicts selective incorporation of vRNAs into virions, as opposed to the random-incorporation model (1).

Recently, Bancroft and Parslow (15) reported that influenza A vRNA segments compete for packaging in a random, nonspecific manner. This conclusion was based on competitive virion incorporation experiments with RNA segments containing GFP or a yellow fluorescent protein ORF fused to the noncoding regions of different influenza vRNA segments; the vRNA coding region was absent. These authors did not compare the efficiency of virion incorporation using segments with and without the coding regions and therefore could not appreciate the requirement for incorporation signals residing in the coding sequences. In our experiments, the incorporation efficiency of segments lacking the viral coding region is <2% of that of a segment containing coding regions derived from the NA (this study), HA (T.W. and Y.K., unpublished data), M (J. Maeda and Y.K., unpublished data), or NS segment (K. Fujii and Y.K., unpublished data). Thus, the mechanism described by Bancroft and Parslow (15) does not represent authentic vRNA incorporation but rather the marginal (<2%), arbitrary incorporation of vRNA that escaped selective incorporation mediated by signals within the coding region.

What is the specific role in virion incorporation of the 21 nucleotides at the 3′ end of the NA vRNA coding region? One possibility is that this short sequence is recognized directly by viral protein, leading to recruitment of the segment for virion formation. If, however, each vRNA segment possesses a unique packaging signal, this recruitment mechanism would entail recognition of multiple signal sequences by a limited number of viral proteins. Alternatively, the short sequence in the 3′ end of the NA vRNA coding region may specifically associate with another vRNA segment through a base-pairing mechanism. In fact, a predicted secondary structure of the 3′ end of NA vRNA suggests that the region corresponding to the seventh codon is available for such base pairing (data not shown). Support for this model comes from flock house virus and red clover necrotic mosaic virus, in which intersegmental association through base pairing of short sequences is critical for RNA synthesis (16, 17). Thus, in influenza A virus, the eight vRNA segments may form a multisegmental macromolecule (most likely as vRNPs) through base pairing that can be recognized by the limited repertoire of viral and/or cellular proteins embedded in the plasma membrane, leading to virion formation and budding of the viruses from the membrane. Studies to identify regions critical for virion incorporation of vRNA segments other than the NA, test the intersegmental association model, and determine the cellular location and viral and/or host proteins involved in formation of a multisegmental macromolecule may provide further insight.

Several strategies have been used to incorporate foreign cDNA into influenza virions (18–25). Here we generated an influenza A virus that stably maintained the GFP gene, suggesting the potential of this system for gene delivery and expression in diverse applications, including vaccine production. Vaccines including a live attenuated, cold-adapted, and temperature-sensitive influenza virus are being tested in clinical trials (26). Although such viruses are safe and efficacious, the limited number of amino acid replacements has raised concerns over their genetic stability. Although no reversion to the wild-type phenotype has been reported to date, the introduction of additional mutations to stabilize the attenuated phenotype further would be desirable. We suggest that the vRNA packaging signals described in this article would afford targets for attenuating mutations.

Two classes of antiinfluenza drugs with different inhibitory mechanisms are currently available (reviewed in ref. 27): M2 ion channel inhibitors (amantadine and rimantadine) and NA inhibitors (zanamivir and oseltamivir). Rapid emergence of resistance associated with amino acid substitutions in the transmembrane portion of the M2 protein has been a consistent problem in patients treated with the former compounds. Although clinical resistance to the NA inhibitors has been infrequent (28), mutations in or near the HA receptor-binding site or amino acid substitutions at conserved residues in the NA enzyme active site have been observed in cell culture and may restrict the clinical usefulness of these drugs in the future. Thus, it is important to identify alternative targets for the development of antiinfluenza drugs. We suggest that sequences critical for efficient vRNA incorporation into virions would be excellent candidates for this role.

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