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Exploitation of Nucleic Acid Packaging Signals To Generate a Novel Influenza Virus-Based Vector Stably Expressing Two Foreign Genes

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At the final step in viral replication, the viral genome must be incorporated into progeny virions, yet the genomic regions required for this process are largely unknown in RNA viruses, including influenza virus. Recently, it was reported that both ends of the neuraminidase (NA) coding region are critically important for incorporation of this vRNA segment into influenza virions (Y. Fujii, H. Goto, T. Watanabe, T. Yoshida, and Y. Kawaoka, Proc. Natl. Acad. Sci. USA 100:2002-2007, 2003). To determine the signals in the hemagglutinin (HA) vRNA required for its virion incorporation, we made a series of deletion constructs of this segment. Subsequent analysis showed that 9 nucleotides at the 3′ end of the coding region and 80 nucleotides at the 5′ end are sufficient for efficient virion incorporation of the HA vRNA. The utility of this information for stable expression of foreign genes in influenza viruses was assessed by generating a virus whose HA and NA vRNA coding regions were replaced with those of vesicular stomatitis virus glycoprotein (VSVG) and green fluorescent protein (GFP), respectively, while retaining virion incorporation signals for these segments. Despite the lack of HA and NA proteins, the resultant virus, which possessed only VSVG on the virion surface, was viable and produced GFP-expressing plaques in cells even after repeated passages, demonstrating that two foreign genes can be incorporated and maintained stably in influenza A virus. These findings could serve as a model for the construction of influenza A viruses designed to express and/or deliver foreign genes.

Viruses generally initiate their life cycle by attaching to host cell surface receptors, entering the cells, and uncoating their viral nucleic acid, followed by replication of the viral genome. After new copies of viral proteins and genes are synthesized, these components assemble into progeny virions, which then exit the cell (14). During the assembly step, the progeny virus must select its genomic nucleic acid efficiently from a large pool of viral and cellular nucleic acids present in the cytoplasm. The packaging of viral genomes into virions typically involves recognition by viral components of a *cis*-acting sequence in the viral nucleic acid, the so-called "packaging signal." Defining such signals is important for understanding the viral life cycle and provides us with information that could be used to construct viral vectors for the expression of foreign proteins. Indeed, the utility of retroviruses as vehicles for gene delivery vectors for the expression of foreign proteins can be attributed in large measure to the well-established knowledge of the process of their vRNA packaging into progeny virions (5, 18). The genomic packaging signals of other RNA viruses are poorly understood, impeding progress in their use as vectors for the expression and delivery of foreign genes.

Influenza A virus is an enveloped negative-strand RNA virus whose segmented genome has a coding capacity for 11 viral proteins (2, 19). This virus has two membrane-spanning glycoproteins, hemagglutinin (HA) and neuraminidase (NA), on the envelope. The HA protein binds to sialic acid-containing receptors on the host cell surface and mediates fusion of the viral envelope with endosomal membrane after receptor-mediated endocytosis (19). In contrast, the NA protein plays a crucial role late in infection by removing sialic acid from sialyloligosaccharides, thus releasing newly assembled virions from the cell surface and preventing the self-aggregation of virus particles. Within the envelope, the viral genome, comprising eight different viral RNA (vRNA) segments, is tightly linked to the nucleoprotein (NP) and polymerase proteins (PA, PB1, and PB2), forming the ribonucleoprotein complexes (19). A *cis*-acting signal necessary for viral genome packaging was believed to reside in the 3′ and 5′ ends of the noncoding region of each RNA segment, which also control vRNA transcription and replication by the RNA polymerase proteins (20). However, a foreign gene flanked by the 3′ and 5′ noncoding regions of influenza vRNA was lost after several viral passages (20), suggesting that these regions alone may not be sufficient for the incorporation of RNA segments into virions.

Recently, we found that both ends of the NA vRNA coding region are critically important for the incorporation of this segment into virions (6), providing us with a clue to the mechanisms of influenza virus genome packaging. As described here, we next determined the HA vRNA sequences required for virion incorporation by making a series of deletion constructs. The information obtained from this and previous (6) studies was then used to produce a virus in which the HA coding region was replaced with that of vesicular stomatitis virus glycoprotein (VSVG), while the majority of the NA coding region was replaced with that of green fluorescent protein

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(GFP). Our findings should prove useful in the development of influenza virus-based vectors for expression and/or delivery of foreign genes.

MATERIALS AND METHODS

Cells and viruses. 293T human embryonic kidney cells (a derivative of the 293 cell line into which the gene for simian virus 40 T antigen was inserted [4]) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS). For baby hamster kidney (BHK) and Chinese hamster ovary (CHO), Dulbecco modified Eagle medium containing 5% FCS or 10% newborn calf serum, respectively, was used. Minimal essential medium (MEM) containing 5% newborn calf serum was used for Madin-Darby canine kidney (MDCK) cells. All cells were maintained at 37°C in 5% CO_2. A/WSN/33 (H1N1) (WSN) virus was generated by reverse genetics as described previously (23) and propagated in MDCK cells. VSV Indiana strain generated by reverse genetics (which was kindly provided by M. Whitt) was propagated in BHK cells.

Reverse genetics. To generate influenza virus-like particles (VLPs) and mutant influenza A viruses, we used plasmids possessing the cDNA of WSN viral genes under control of the human RNA polymerase I promoter and the mouse RNA polymerase I terminator (referred to as PolI plasmids) and the eukaryotic protein expression vector pCAGGS/MCS (controlled by the chicken β-actin promoter) (23, 24). Briefly, PolI plasmids and protein expression plasmids were mixed with a transfection reagent, Trans-IT LT-1 (Panvera, Madison, Wis.), incubated at room temperature for 15 min, and added to 10^6 293T cells cultured in Opti-MEM I (Gibco-BRL). Six hours later, the DNA-transfection reagent mixture was replaced with Opti-MEM I containing 0.3% bovine serum albumin and 0.01% FCS. Forty-eight hours later, VLPs or mutant influenza A viruses were harvested from the supernatant. Transfectants generated in the present study contain a mutant HA vRNA segment, together with other vRNA segments of WSN virus, and are designated by the name of the mutant HA vRNA segment [e.g., a VLP containing the HA(0)GFP(0) RNA segment is designated HA(0)GFP(0) VLP].

Construction of plasmids. pPolIHA(0)GFP(0) was used to produce negative-sense RNA containing the 3′ noncoding region of HA vRNA, the complementary coding sequence of enhanced GFP (Clontech), and the 5′ noncoding region of HA vRNA. Briefly, the GFP gene was amplified by PCR with primers containing the *Bsm*BI sites and the 3′ or 5′ noncoding sequence of HA, digested with *Bsm*BI, and cloned into the *Bsm*BI site of the PolI plasmid (23). Introduction of this plasmid into cells yielded an RNA containing the GFP-coding sequence in negative-sense orientation flanked by the 5′ and 3′ noncoding regions of HA vRNA.

pPolIHA(468)GFP(513) was made as follows. pPolIHA for the production of WSN vRNA (23) was first amplified by inverse PCR (25) by using the back-to-back primers Bam500R (5′-GCGGATCCTCCCCTATGGGAGCATGATAC-3′) and Xba1218F (5′-GCTCTAGAAACTCTGTTATCGAGAAAATG-3′). The PCR product was digested with *Bam*HI and *Xba*I, and the GFP gene was then cloned into the *Bam*HI site and the *Xba*I site. The resultant plasmid, pPolIHA(468)GFP(513), was used to produce negative-sense RNA containing the 3′ noncoding region and 468 bases of the 3′ coding region of HA vRNA, the GFP coding sequence, 513 bases of the 5′ coding region, and the 5′ noncoding region of HA vRNA. A series of HA deletion mutants was also produced by inverse PCR in the same manner. The mutants were designated according to the number of nucleotides derived from the HA coding region; for example, the HA(9)GFP(80) RNA segment contains the 3′ HA noncoding region, 9 nucleotides from the HA coding sequence corresponding to the N-terminal region, the GFP open reading frame, 80 nucleotides from the HA coding sequence corresponding to the C-terminal region, and the 5′ HA noncoding sequence. All plasmid constructs were sequenced to ensure that unwanted mutations were not introduced by PCR.

pPolIHA(0)VSVG(0), produced by PCR, was used to generate negative-sense RNA containing the 3′ noncoding region of HA vRNA, the complementary coding sequence of VSVG, and the 5′ noncoding region of HA vRNA. Briefly, the VSVG gene was amplified by PCR with pCAGGS-VSVG (35) as a template and primers containing the *Bsm*BI sites and the 3′ or 5′ noncoding sequence of HA. The PCR product was then digested with *Bsm*BI and cloned into the *Bsm*BI site of the PolI plasmid (23). pPolIHA(9)VSVG(80) was made by cloning the coding sequences of VSVG into the *Bam*HI site and the *Xba*I site of pPolIHA(9)GFP(80).

pPolINA(183)GFP(157) containing the 3′ noncoding ends of NA vRNA and a complementary sequence encoding a fusion protein possessing 61 N-terminal NA codons and GFP, two consecutive stop codons (TAA-TAG), and 185 bases of the 5′ end of NA vRNA was produced as follows. The region corresponding to nucleotides 203 to 1109 (positive sense) of WSN NA gene in pT7Blue-NA was first replaced with a *Bgl*II site by inverse PCR. The GFP gene was then cloned into this *Bgl*II site and *Stu*I site at position 1226 (in the wild-type NA gene) in frame with the NA protein. The NA(183)GFP(157) gene was then inserted into the *Bsm*BI site of the PolI plasmid (23). pPolINA(183)GFP(157)Met(−), used to produce negative-sense NA (183)GFP(157)Met(−) RNA, which lacks the start codon for the NA protein, was generated as follows. The ATG initiation codon and another ATG at the 15th codon of the NA(183)GFP(157) gene in pPolINA(183)GFP(157) was changed to GCG by in vitro site-directed mutagenesis (GeneEditor; Promega). The resultant construct, pPolINA (183)GFP (157)Met(−), contained the 3′ NA noncoding region (19 nucleotides), 183 nucleotides corresponding to the N-terminal NA coding region, the GFP open reading frame, two consecutive stop codons (TAA-TAG), 157 nucleotides corresponding to the C-terminal NA coding region, and the 5′ NA noncoding region (28 nucleotides).

Immunostaining assay. At 16 h after infection with influenza VLPs, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde (in PBS) for 20 min at room temperature, followed by treatment with 0.1% Triton X-100, and then processed as described previously (22). To examine the efficiency of VLP generation, we inoculated 10^6 cells with 0.1 ml of the culture supernatant of plasmid-transfected 293T cells and recorded the number of NP-positive cells (determined with the immunostaining assay) at 16 h postinfection.

Western blotting. The VLPs or mutant viruses were spun down for 1.5 h at 50,000 × *g* at 4°C. Concentrated VLPs or viruses were resuspended in lysis buffer (0.6 M KCl, 50 mM Tris-HCl [pH 7.5], 0.5% Triton X-100). The lysates were then placed on sodium dodecyl sulfate–10% polyacrylamide gels, electrotransferred to a polyvinylidene difluoride membrane, blocked overnight at 4°C with 5% skimmed milk in PBS, and incubated with anti-WSN virus polyclonal antibody, anti-HA monoclonal antibody, or anti-VSVG monoclonal antibody for 1 h at room temperature. The membrane was washed three times with PBS containing 0.05% Tween 20. Bound antibodies were detected with a Vectastain ABC kit (Vector, Burlingame, Calif.) and a Konica immunostaining kit.

Northern hybridization. vRNA present in 293T cells transfected with PolI plasmids was extracted with the Isogen RNA extraction kit (Nippon Gene, Tokyo, Japan) at 24 h posttransfection. RNAs were glyoxalated in glyoxal-dimethyl sulfoxide-phosphate buffer at 50°C for 1 h and separated by electrophoresis on 1.0% agarose gel in 10 mM phosphate buffer (pH 7.0). RNAs were blotted onto nylon membranes and hybridized with an oligonucleotide probe complementary to the GFP sequence (ATGGCCGACAAGCAGAAGAACGG CATCAAGG; 10 pmol), which was labeled by using a DIG Oligonucleotide Tailing Kit (Roche) at 37°C for 30 min. Hybridization was performed overnight with the GFP probe in Easy-Hyb (Roche) at 42°C. The RNA bands were detected by using the DIG Nucleic Acid Detection Kit (Roche). Briefly, the hybridized membrane was washed with a 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 anti-digoxigenin antibody (1:5,000) conjugated with alkaline phosphatase for 30 min at room temperature. The membrane was then washed with the wash buffer and incubated with nitroblue tetrazolium chloride–BCIP (5-bromo-4-chloro-3-indolylphosphate) in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl [pH 9.5]) at room temperature in the dark.

Electron microscopy. Viruses centrifuged through 20% sucrose were stained with 2% phosphotungstic acid solution (PTA) and examined with a JEM-1200 electron microscope at 80 kV. For immune electron microscopy, the sample was absorbed to Formvar-coated nickel grids and washed with PBS containing 1% bovine serum albumin. The grids were then treated with mouse anti-VSVG monoclonal antibody and rinsed six times with PBS, followed by incubation with a goat anti-mouse immunoglobulin conjugated to 15-nm gold particles (1:50 dilution; BB International). After a washing step, the samples were fixed for 10 min in 2% glutaraldehyde and negatively stained with 2% PTA.

Replicative properties of transfectant viruses. BHK, CHO, or MDCK cells in duplicate wells of 24-well plates were infected with a virus, overlaid with MEM containing 0.01% FCS, and incubated at 37°C. At different times, supernatants were assayed for infectious virus in plaque assays on MDCK cells.

RESULTS

The coding region of HA vRNA is required for incorporation of the HA vRNA into virions. To test the prediction—developed from insight gained from an earlier study of the NA vRNA (6)—that the coding region of HA vRNA may be

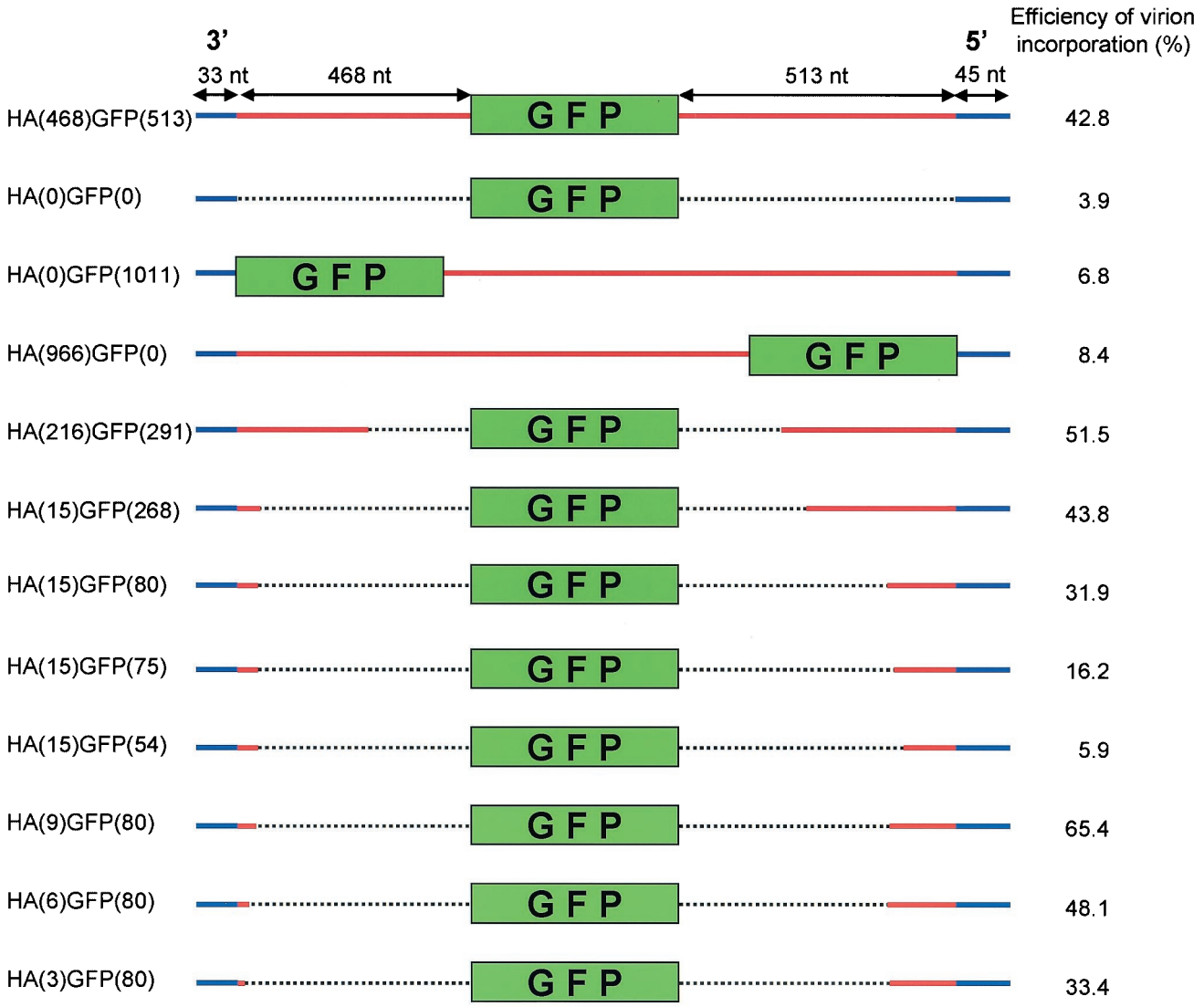

FIG. 1. Schematic diagram of mutant HA vRNAs and their efficiency of virion incorporation. All mutant HA RNAs are shown in the negative-sense orientation. Each mutant contains the GFP open reading frame (inserted in frame with the HA open reading frame) flanked by a stop codon, 33 nucleotides of the 3′ noncoding region, and 45 nucleotides of the 5′ noncoding region of HA vRNA. The lengths of the regions are not drawn to scale. Mutants were designated according to the number of nucleotides derived from the HA coding regions (orange bars). Horizontal broken lines indicate a deletion. The efficiency of incorporation of mutant HA vRNA into VLPs was determined by dividing the number of cells expressing GFP by that of cells expressing NP in the VLP-infected cells after the cells were fixed at 16 h postinfection. The levels of GFP expression were comparable among the constructs tested. The means of three experiments are shown.

needed for its virion incorporation, we first constructed two plasmids: pPolIHA(0)GFP(0), which contains only the 3′ and 5′ noncoding regions of HA vRNA and the GFP coding sequence, and pPolIHA(468)GFP(513), which contains the GFP coding sequence inserted into the HA gene in frame after deletion of the HA sequence at nucleotide positions 500 to 1218 (in positive-sense orientation) (Fig. 1). The latter construct possesses the 3′ HA noncoding region (33 nucleotides), 468 nucleotides corresponding to the N-terminal HA coding region, the GFP open reading frame with a stop codon, 513 nucleotides corresponding to the C-terminal HA coding region, and the 5′ HA noncoding region (45 nucleotides). The resultant fusion protein contains the N-terminal 156 amino acids of the HA and the entire GFP sequence. To generate VLPs possessing these mutant HA vRNAs, we transfected 293T cells with pPolIHA(0)GFP(0) or pPolIHA(468)GFP (513), and seven RNA PolI plasmids for the production of the remaining influenza viral RNA segments and protein expression plasmids for nine viral proteins (i.e., PA, PB1, PB2, NP, HA, NA, M1, M2, and NS2). At 48 h posttransfection, VLPs in the supernatants of 293T cell cultures were harvested and used to infect MDCK cells. Since the resultant VLPs possessed mutant HA vRNA instead of normal HA vRNA, they infected cells and expressed GFP along with other viral proteins (except HA) without generating infectious progeny virus (data not shown). The efficiency of mutant HA vRNA incorporation into virions was determined by dividing the number of cells expressing GFP (i.e., the number of VLPs that possessed the segment

encoding the GFP gene) by that of cells expressing NP (i.e., the number of all infectious VLPs) at 16 h postinfection. The titer of infectious VLPs in the culture supernatant of 293T cells transfected with pPolIHA(468)GFP(513) was 7.4×10^5 infectious VLPs/ml, whereas that of VLPs containing HA(468)GFP(513) RNA was 3.2×10^5 VLPs/ml. These results indicate that 42.8% of all infectious VLPs harbored mutant HA vRNA, whereas only 3.9% of VLPs possessed the HA(0)GFP(0) RNA segment (Fig. 1), a finding similar to that obtained with the NA segment (6). Thus, the coding region of HA vRNA appears to have been required for the incorporation of the HA segment into influenza virions.

Both the 3′ and 5′ ends of the HA vRNA coding region are important for incorporation of the HA segment into virions. Since our previous study showed that the 3′ end of the NA vRNA coding region plays a more crucial role in virion incorporation than does the 5′ end (6), we next determined whether the 3′ end, the 5′ end, or both ends were required for virion incorporation of the HA vRNA segment. To address this issue, we generated the HA(0)GFP(1011) gene, which lacks the 3′ terminus of the HA vRNA coding region, and the HA(966)GFP(0) gene, which lacks the 5′ terminus of the HA vRNA coding region (Fig. 1) and then examined virion incorporation of these HA vRNAs, as described above. Although the amounts of both vRNAs in plasmid-transfected cells were comparable to that of HA(468)GFP(513) vRNA (data not shown), the efficiencies of segment incorporation of HA(0)GFP(1011) and HA(966)GFP(0) were only 6.8 and 8.4%, respectively (Fig. 1), indicating that both the 3′ and the 5′ termini of the HA vRNA coding region play an important role in virion incorporation of the HA segment.

The critical signaling region in the HA vRNA was refined by generating a series of VLPs that possessed truncated HA vRNAs with further deletion in the 3′ and/or 5′ coding region (Fig. 1) and examining the incorporation efficiency of mutant HA vRNA into VLPs. Since further deletion in the 3′ end leaving only 15 nucleotides and of the 5′ end leaving 268 nucleotides did not affect the efficiency of HA vRNA incorporation [compare HA(468)GFP(513) with HA(15)GFP(268)], we made additional deletion constructs by using pPolIHA(15)GFP(268), which possessed 15 nucleotides of the 3′ end of the HA coding region and 268 nucleotides of the 5′ end. Although the extent of vRNA incorporation was reduced gradually as the extent of deletions increased, 80 nucleotides in the 5′ HA coding region seemed minimally required for efficient virion incorporation of the HA vRNA [compare HA(15)GFP(80) with HA(15)GFP(75)]. Further deletion analysis demonstrated that HA(9)GFP(80) with 9 nucleotides at the 3′ end of the HA coding region resulted in efficient virion incorporation of the HA vRNA (>65%); however, the level of HA(9)GFP(80) vRNA present in transfected cells did not differ appreciably from that of HA(0)GFP(0) vRNA (Fig. 1 and 2), and thus both RNAs were equally available for packaging into virions. These results indicate that 9 nucleotides in the 3′ end and 80 nucleotides in the 5′ end of the HA coding region are required for efficient HA vRNA incorporation into virions.

Generation of a novel influenza A virus whose HA and NA genes contain the coding sequences of foreign genes. Having determined the sequences required for HA segment incorporation into virions, we next examined whether a foreign gene flanked by these regions could be incorporated into influenza A viruses and maintained during repeated passages. As a model foreign gene, the VSVG coding sequence was inserted into the *Bam*HI and *Xba*I sites of pPolIHA(9)GFP(80) instead of GFP sequence. The resultant construct was designated pPolIHA(9)VSVG(80), which possesses the 3′ HA noncoding region (33 nucleotides), 9 nucleotides corresponding to the N-terminal HA coding region, the VSVG open reading frame with a stop codon (1,552 nucleotides), 80 nucleotides corresponding to the C-terminal HA coding region, and the 5′ HA noncoding region (45 nucleotides). As a control, we constructed pPolIHA(0)VSVG(0), which possesses the 3′ and 5′ noncoding region but not the coding region of HA vRNA, as described in Materials and Methods. Since VSVG protein should substitute for both HA and NA proteins, the NA coding region can be substituted with a foreign gene. We therefore constructed pPolINA(183)GFP(157)Met(−) for production of a recombinant NA RNA segment containing the GFP coding sequence and the portion of NA coding sequences required for the efficient virion incorporation of the NA segment. In this construct, the initiation codon for the NA open reading frame was destroyed by changing ATG to GCG. Thus, the GFP open reading frame would be translated from its own initiation codon. We then transfected 293T cells with plasmids for the production of both recombinant HA(9)VSVG (80) and NA(183)GFP(157)Met(−) segments and the remaining six viral RNA segments, as well as plasmids for the expression of influenza virus polymerase proteins and NP, M1, M2, NS2, and VSVG. At 72 h after transfection, we harvested the supernatants of 293T cells and performed plaque assays with MDCK cells. A transfectant virus [VSVG(HA)GFP(NA)] harboring two recombinant RNA segments, HA(9)VSVG(80) and NA(183)GFP(157)Met(−), was viable and produced plaques in the absence of trypsin (Fig. 3). The expression of VSVG, but not HA, in MDCK cells was confirmed by immunostaining (Fig. 3). Cells infected with VSVG(HA)GFP(NA) virus, but not with control WSN virus, also expressed GFP (Fig. 3). More than 70% of the plaques produced by this virus expressed GFP.

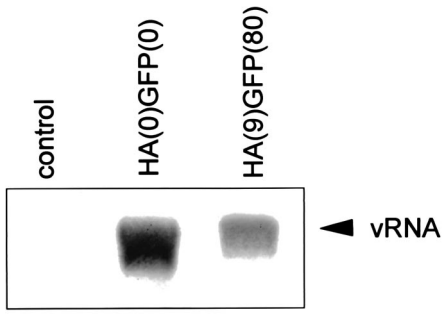

FIG. 2. The vRNA levels in 293T cells transfected with plasmids expressing mutant HA vRNAs. 293T cells were transfected with pPolIHA(0)GFP(0) or pPolIHA(9)GFP(80) and plasmids expressing PA, PB1, PB2, and NP. At 24 h posttransfection, vRNA present in transfected cells was extracted, and glyoxalated RNA was separated by electrophoresis on 1.0% agarose gel in 10 mM phosphate buffer. RNAs were blotted onto nylon membranes and hybridized with a digoxigenin-labeled probe complementary to the GFP sequence for overnight at 42°C. The RNA bands were detected by using DIG Nucleic Acid Detection Kit (Roche). Control RNA was extracted from mock-transfected 293T cells.

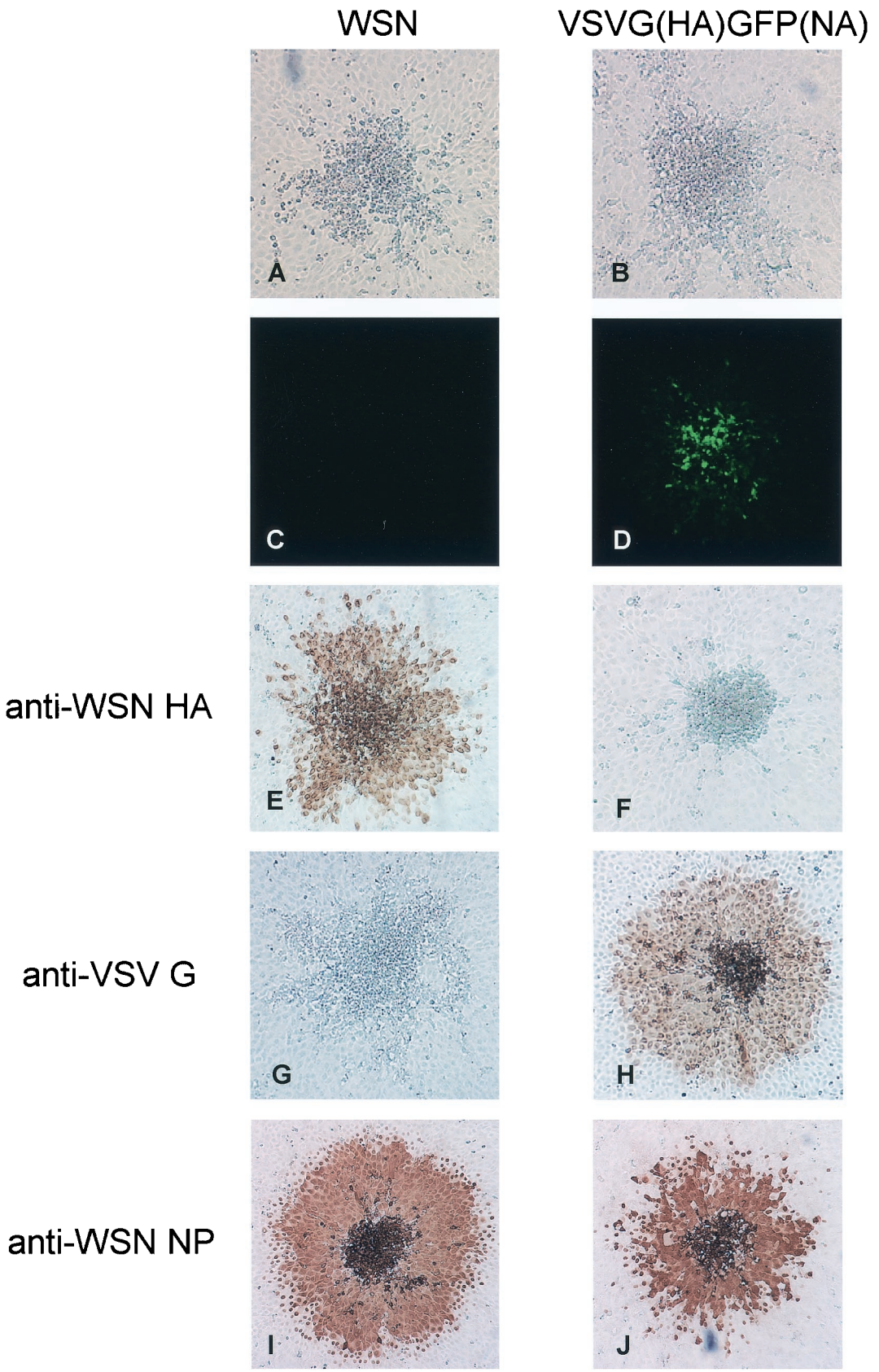

FIG. 3. VSVG(HA)GFP(NA) virus-infected cells express VSVG and GFP. MDCK cells were infected with VSVG(HA)GFP(NA) virus or WSN virus and overlaid with 1.0% agarose. The infected cells were incubated for 48 h at 37°C, and representative plaques were photographed under normal light (A and B) and under fluorescent light (C and D). The cells were fixed and treated with 0.1% Triton X-100 in 3% formaldehyde solution. The viral proteins were detected by immunostaining with anti-WSN HA monoclonal antibody (E and F), anti-VSVG monoclonal antibody (G and H), or anti-WSN NP monoclonal antibody (I and J) as the primary antibody and biotinylated secondary antibody by using the Vectastain ABC kit.

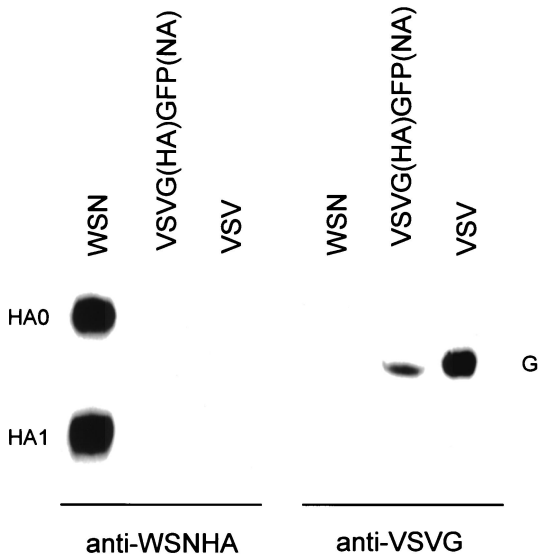

FIG. 4. Incorporation of the VSVG protein into VSVG(HA)GFP(NA) virus. Concentrated WSN and VSVG(HA)GFP(NA) viruses, and VSV were lysed in a sample buffer. Viral proteins were treated with 2-mercaptoethanol, separated by SDS–10% polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and incubated with anti-VSVG monoclonal antibody or anti-WSN HA monoclonal antibody.

In contrast, plaques were not observed when the pPolIHA(0) VSVG(0) plasmid was used instead of pPolIHA (9)VSVG(80), although single cells expressing GFP and/or NP protein were detected in MDCK cells (data not shown). We also observed that both VSVG and GFP continued to be expressed in MDCK cells infected with the VSVG(HA)GFP(NA) virus after five consecutive passages (data not shown). VSVG(HA)GFP(NA) virus, which had been passaged five times, was used as stock virus for subsequent experiments.

The HA region of the HA(9)VSVG(80) RNA segment of the VSVG(HA)GFP(NA) virus did not acquire mutations after five passages, but a Gln-to-stop mutation at position 499 was identified in the amino acid sequence of VSVG. Although wild-type VSVG protein has 29 residues in its cytoplasmic domain, the last 13 residues were deleted due to the Gln-to-stop mutation at position 499. When we produced the VSVG (HA)GFP(NA) virus in a separate experiment, a virus possessing the Arg-to-stop mutation at position 498 became dominant by the third passage in MDCK cells. These findings indicate that the tail truncation in the VSVG protein promotes its functionality in the context of influenza A viral components.

Biological properties of VSVG(HA)GFP(NA) virus. To determine whether VSVG protein is indeed incorporated into virions composed of other influenza viral proteins, we performed Western blot analysis on concentrated VSVG(HA)GFP(NA) and WSN viruses and on VSV. As shown in Fig. 4, VSVG protein, but not HA, was detected in VSVG(HA)GFP (NA) virions, confirming the virion incorporation of VSVG protein. Examination by electron microscopy further demonstrated that the VSVG(HA)GFP(NA) virions indeed contained VSVG protein on the surface (Fig. 5).

Finally, we examined the growth properties of VSVG(HA) GFP(NA) virus in BHK, CHO, or MDCK cells. Cells were infected at a multiplicity of infection of 0.001, and yields of

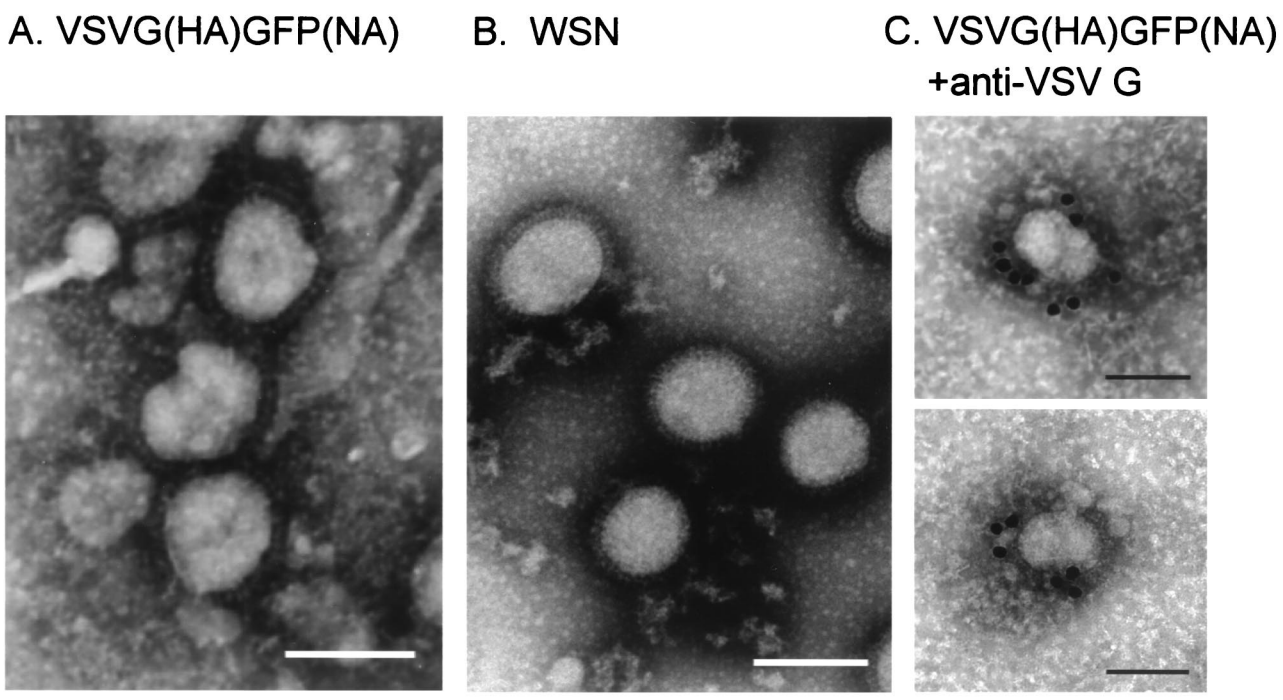

FIG. 5. Electron microscopy of VSVG(HA)GFP(NA) virus. VSVG(HA)GFP(NA) (A) and WSN (B) viruses were centrifuged through 20% sucrose, and the pelleted materials were then negatively stained with 2% PTA. (C) Pelleted VSVG(HA)GFP(NA) virus was also immunolabeled with anti-VSVG monoclonal antibody conjugated to 15-nm gold particles. Bar, 100 nm.

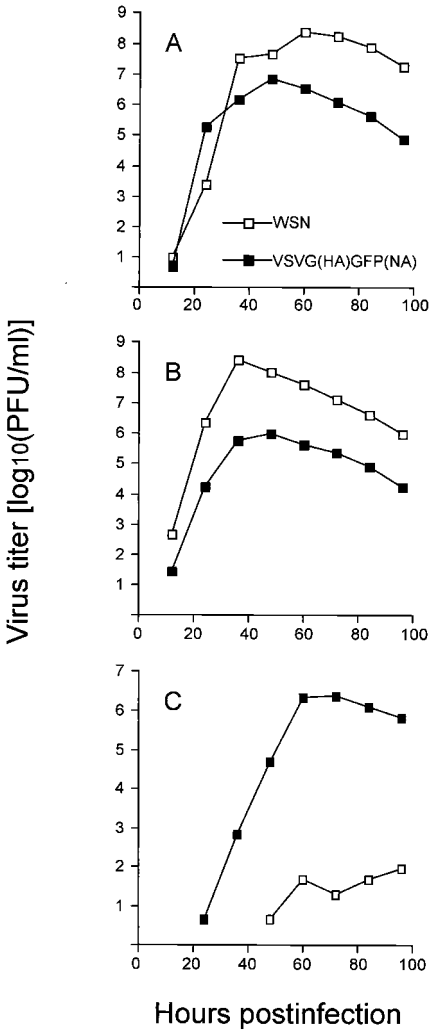

FIG. 6. Growth curves of VSVG(HA)GFP(NA) virus in BHK, CHO, and MDCK cells. BHK (A), MDCK (B), and CHO (C) cells were infected with virus at a multiplicity of infection of 0.001. At the indicated times after infection, the virus titer in the supernatant was determined by using MDCK cells. The values are means of duplicate experiments.

virus in the culture supernatant were determined at different times postinfection at 37°C by plaque assay on MDCK cells. Although lower than the results for WSN virus, the maximum titer of VSVG(HA)GFP(NA) virus in both BHK and MDCK cells reached at least 10^6 PFU per ml (Fig. 6A and B). In contrast to the poor growth of WSN virus in CHO cells, VSVG(HA)GFP(NA) grew to a titer similar to that in the other two cell lines tested (Fig. 6C). Moreover, cells infected with VSVG(HA)GFP(NA) virus expressed GFP.

These results indicate that both the HA(9)VSVG(80) and NA(183)GFP(157)Met(−) segments were efficiently incorporated into influenza virions, demonstrating that two foreign genes can be stably maintained in influenza A virus during repeated passages.

DISCUSSION

Knowledge of genome packaging mechanisms is critical to understanding the life cycle of influenza virus, as well as for the development of influenza virus-based vectors for the expression of foreign proteins. In the present study, we demonstrated that sequences in both the 3′ and the 5′ ends of the coding regions in the HA vRNA were required for efficient incorporation of this segment into virions. Moreover, by using the information obtained here and from a previous report (6), we generated a novel influenza-based virus possessing two recombinant RNA segments containing the coding sequences of VSVG and GFP flanked by sequences necessary for virion incorporation of HA vRNA and NA vRNA, respectively, demonstrating stable expression of two foreign genes.

Several approaches have been advocated for the development of influenza A virus-based vaccine vectors for the expression of genes or portions of genes from unrelated infectious agents (8, 27, 28, 34, 40). In one, short polypeptides are inserted into the antigenic sites of HA, resulting in positive immune responses against the inserted peptides (28, 34, 40). For the expression of longer polypeptides and proteins, the foreign genes are inserted into one of the influenza virus genes, and the foreign proteins are expressed by utilizing internal ribosomal entry sites (8) or the foot-and-mouth disease virus 2A protease (27). The system we describe could be used to express a foreign protein by exploiting *cis*-acting virion incorporation signals in the NA and HA vRNAs. The ability of influenza virus-based constructs to incorporate more than 1.5 kb of a foreign gene (e.g., VSVG) underscores the potential of this strategy. For example, replication-incompetent influenza VLPs, which have shown considerable efficacy as a vaccine (39), could be modified to include a recombinant RNA segment containing a gene from an unrelated pathogen. The resultant vaccine would be especially attractive for use against human immunodeficiency virus infection, foot-and-mouth disease, and other infections in which reversion of a live vaccine virus to wild type would be absolutely unacceptable or in which the efficacy of inactivated vaccines may be limited due to inadequate induction of mucosal immunity and cytotoxic-T-lymphocyte responses.

Although vectors derived from adenoviruses and retroviruses efficiently introduce foreign genes into target cells (1, 5, 18), they contain DNA or have DNA replication intermediates that could be integrated into the host chromosome, increasing the risk of an adverse outcome. The likelihood of such integration would be essentially eliminated by the use of influenza viruses, which lack a DNA replication phase in infected cells. Moreover, since VSVG(HA)GFP(NA) virus does not require trypsin for HA cleavage, unlike normal influenza viruses, it may be effective in a broader range of cell types; the cell tropism of a recombinant virus could be specified by altering a glycoprotein on the virion surface. Thus, by utilizing *cis*-acting signals in vRNA segments for virion incorporation, it should be possible to design recombinant influenza virus-based vectors capable of delivering an assortment of foreign genes into target cells.

The assembly and release of some viruses from epithelial cells are polarized, occurring selectively at either the apical or the basolateral surface. Polarized virus budding is thought to play a role in determining the pathogenesis of viral infections (3). Influenza A virus buds apically from infected epithelial cells, and individually expressed HA, NA, and M2 proteins are targeted to the apical surface of the cells (13, 15, 30, 31). On

the other hand, VSV is released from the basolateral surface of infected cells, and VSVG protein is transported to the basolateral surface (7, 9, 29). In the current study, a recombinant VSVG(HA)GFP(NA) virus, possessing VSVG instead of the HA and NA proteins, was successfully generated. However, we found that the VSVG protein of this recombinant virus lacked the last 13 residues of the cytoplasmic domain due to a point mutation. Deletion of these 13 residues in the cytoplasmic domain is known to yield a protein that is more efficiently transported to the apical surface than the basolateral surface (37). Hence, the mutation introduced into the VSVG tail in VSVG(HA)GFP(NA) virus likely promoted its efficient transport to the apical surface, leading to efficient budding of VSVG(HA)GFP(NA) virus. In addition, it is possible that shortening of the cytoplasmic tail may favor efficient incorporation of VSVG protein into influenza virions.

Influenza pandemics usually arise from a reassortant virus whose HA and/or NA is immunologically distinct from those of the previously circulating strain. We have demonstrated that sequences in the 3′ and 5′ ends of the coding regions within the HA (the present study), NA (6), M (J. Maeda and Y. Kawaoka, unpublished data), and NS (K. Fujii and Y. Kawaoka, unpublished data) vRNAs are required for their efficient incorporation into virions, suggesting that packaging of vRNA segments (most likely as a viral ribonucleoprotein complex) is mediated by RNA-RNA interactions occurring in *trans* among the viral RNA segments. If so, specific incorporation signals within each segment may restrict reassortment, just as functional interactions (e.g., formation of the polymerase complex [12], HA-NA [12, 16, 21, 32, 33, 38], and cleavable HA-M2 functional associations [10, 11, 26, 36]) restrict the random reassortment of viral proteins. In this context, it is interesting that in both the 1957 and 1968 pandemics, the PB1, HA, and/or NA genes were introduced into human viruses from avian viruses (17), suggesting a possible link between the HA and PB1 RNA segments. Further characterization of the signals that drive incorporation of RNA segments into virions may provide clues to the mechanisms of vRNA reassortment, eventually enabling the prediction of new pandemic strains of influenza A virus.

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