JOURNAL OF VIROLOGY, Oct. 1994, p. 6254–6261
0022-538X/94/$04.00+0

Vol. 68, No. 10

Use of a Mammalian Internal Ribosomal Entry Site Element for Expression of a Foreign Protein by a Transfectant Influenza Virus

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Received 30 March 1994/Accepted 30 June 1994

The ribonucleoprotein transfection system for influenza virus allowed us to construct two influenza A viruses, GP2/BIP-NA and HGP2/BIP-NA, which contained bicistronic neuraminidase (NA) genes. The mRNAs derived from the bicistronic NA genes have two different open reading frames (ORFs). The first ORF encodes a foreign polypeptide (GP2 or HGP2) containing amino acid sequences derived from the gp41 protein of human immunodeficiency virus type 1. The second ORF encodes the NA protein; its translation is achieved via an internal ribosomal entry site which is derived from the 5′ noncoding region of the human immunoglobulin heavy-chain-binding protein mRNA. The GP2 (79 amino acids) and HGP2 (91 amino acids) polypeptides are expressed in cells infected with the corresponding transfectant virus. The HGP2 polypeptide, which contains transmembrane and cytoplasmic domains identical to those of the hemagglutinin (HA) protein of influenza A/WSN/33 virus, is packaged into virus particles. This novel influenza virus system involving an internal ribosomal entry site element may afford a way to express a variety of foreign genes in mammalian cells.

Influenza A viruses are negative-strand RNA viruses which have a segmented genome with a coding capacity for 10 polypeptides. The virus genome is composed of eight different RNA segments which are tightly associated with the viral nucleoprotein and polymerases in ribonucleoprotein (RNP) complexes (14). Because of the noninfectious nature of the genomic RNAs, influenza viruses were until recently not amenable to standard techniques for genetic manipulation. The development of a new transfection methodology, based on the in vitro reconstitution of RNPs using synthetic RNAs, has now made it possible to construct influenza viruses containing plasmid-derived RNA segments (5, 19; for a review, see reference 8). This system allows the study of *cis*-acting sequences in the viral RNAs as well as the functional analysis of the viral proteins. In addition, this methodology has been used to generate recombinant influenza virus vectors expressing foreign sequences.

A foreign gene flanked by the 3′ and 5′ noncoding sequences of influenza virus RNA segments can be amplified, expressed, and packaged into virus particles after RNP transfection into influenza virus-infected cells. However, the recombinant (nonessential) gene is lost after several viral passages. The generation of stable influenza virus vectors expressing foreign amino acid sequences was achieved by inserting the foreign sequence into an essential viral gene. The hemagglutinin (HA) and neuraminidase (NA) are spike-like proteins on the influenza virus envelope; they are also expressed on the surface of influenza virus-infected cells. For these reasons, they are attractive proteins for the presentation of foreign B-cell and T-cell epitopes. Transfectant influenza viruses which contain short foreign polypeptides as part of their HA or NA proteins have been constructed (4, 15, 16, 24). Several of these recombinant viruses induce a strong immune response in mice against the inserted epitope. However, the insertion of a foreign epitope into the amino acid sequence of a viral protein can sometimes alter the properties of the protein sufficiently to prevent the generation of viable viruses.

We therefore tried to find a way to increase the coding capacity of influenza viruses without altering the structure of the viral proteins. This could be accomplished by the construction of bicistronic influenza virus RNA segments. In a previous report, we showed that influenza A viruses tolerate heterologous insertions of as much as 1 kb into the NA gene (9). However, we were not able to demonstrate the expression of a reporter gene from a bicistronic influenza virus NA segment containing an encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) (9). In this report, we show that the IRES element derived from the 5′ noncoding region of the human immunoglobulin heavy-chain-binding protein (BiP) mRNA (20) is able to promote translation of a downstream open reading frame (ORF) in influenza virus-infected cells. Specifically, we have constructed transfectant influenza viruses containing bicistronic NA segments which express a foreign polypeptide on the surface of infected cells in addition to the NA protein. The foreign polypeptide is translated in infected cells from the bicistronic mRNA via cap-dependent initiation of translation. The NA is translated via internal binding of the ribosome to the bicistronic mRNA, which contains the BiP IRES element. Furthermore, we show that a foreign polypeptide is incorporated into the virus particle.

MATERIALS AND METHODS

Viruses and cells. Influenza A virus strain X-31, which is a reassortant of influenza A/HK/68 and A/PR/8/34 viruses, was grown in the allantoic fluid of embryonated chicken eggs, purified by sucrose density gradient centrifugation, and supplied by Evans Biological Ltd., Liverpool, England. Influenza A/WSN/33 (WSN) virus was grown in Madin-Darby bovine

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kidney (MDBK) cells in reinforced minimal essential medium (REM). WSN-HK virus, a reassortant influenza virus which derives the NA gene from influenza A/HK/8/68 virus and the seven remaining RNA segments from WSN virus, was grown in the allantoic fluid of 10-day-old embryonated chicken eggs (5). MDBK cells were used in RNP transfection experiments and for the selection and plaque purification of transfectant viruses. Madin-Darby canine kidney (MDCK) cells were infected with transfectant viruses for use in immunostaining experiments.

Construction of plasmids. Plasmids were constructed by standard techniques (21). pT3NACAT(wt) contains the chloramphenicol acetyltransferase (CAT) gene in negative polarity flanked by the 3′ and 5′ noncoding regions of the WSN NA gene, under the transcriptional control of a truncated T3 promoter (2a). pT3NA/BIP was constructed as follows. First, a PCR product was obtained by using oligonucleotides 5′-GGCCACTAGTAGGTCGACGCCGGC-3′ and 5′-GCGCT GGCCATCTTGCCAGCCA-3′ as primers and a plasmid containing the 5′ noncoding region of the BiP gene (kindly provided by P. Sarnow, University of Colorado Health Sciences Center) as a template. This PCR product was digested with restriction enzymes *Msc*I and *Spe*I and cloned into *Msc*I- and *Xba*I-digested pT3NA/EMC (9). The resulting plasmid, pT3NA/BIP, contains the NA ORF followed by the IRES sequences of the BiP gene (nucleotides [nt] 372 to 592 of GenBank database entry HUMGRP78). pT3NA/BIP-CAT contains, in addition, the CAT ORF following the BiP IRES-derived sequences. pT3NA/BIP-CAT was constructed by inserting into *Msc*I-digested pT3NA/BIP the PCR product which was obtained by using primers 5′-AGAAAAAAATCACT GGG-3′ and 5′-TTACGCCCCGCCCTGCC-3′ and template pIVACAT1/S (30). A fragment of approximately 920 nt derived from the NA ORF was deleted from pT3NA/BIP-CAT by digestion with *Ppu*MI and *Spe*I, trimming, and religation of the plasmid. The resulting plasmid was called pT3delNA/BIP-CAT. To construct pT3BIP-NA, the PCR product obtained by using primers 5′-GCGCATCGATAGGTCGACGCCGG-3′ and 5′-GGCCATCGATCCAATGGTTATTATTTTCTGGT TTGGATTCATCTTGCCAGTTGGG-3′ and a plasmid containing the 5′ noncoding region of the BiP gene as a template was digested with *Cla*I and inserted into *Cla*I-digested pT3NAM1. pT3NAM1 contains the NA gene of WSN virus in which a *Cla*I site has been created at nt 52 to 57 by two silent changes (4a). The resulting plasmid, pT3BIP-NA, has the BiP IRES-derived sequences in front of the NA ORF. To construct pT3GP2/BIP-NA, a PCR product was obtained by using oligonucleotides 5′-ATGACTGGATCCGCTAGCATGGCC ATCATTTATCTCATTCTCCTGTTCACAGCAGTGAG AGGGGACCAGATAGAAGAATCGCAAAACCAGC-3′ (L primer) and 5′-ATGACAGAATTCGTCGACTTATCTATT CACTACAGAAAG-3′ (M primer) as primers and a plasmid containing the DNA copy of the genome of the human immunodeficiency virus type 1 (HIV-1) isolate BH10 (GenBank database entry HIVBH102) as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI-*Eco*RI-digested pGEX-2T (Pharmacia). This clone was used as template for the generation of a PCR product with primers M and 5′-GCGCGAAGACGCAGCAAAAGCAGG AGTTTAAGCTAGCATGGCCATCATTTATC-3′. The resulting PCR product was digested with *Bbs*I and *Sal*I and ligated into *Bbs*I-*Sal*I-digested pT3BIP-NA. The resulting plasmid, pT3GP2/BIP-NA, has an ORF in front of the BiP IRES sequences which codes for a gp41-derived polypeptide, containing 38 amino acids (aa) of the ectodomain of gp41, the 22 aa of the transmembrane domain, and 2 aa of the cytoplasmic tail of gp41. This sequence is preceded by the signal peptide (15 aa) and the first 2 aa of the HA of influenza A/Japan/305/57 virus. For the construction of pT3HGP2/BIP-NA, a PCR product containing the sequences encoding the transmembrane and cytoplasmic tail of the HA of WSN virus was obtained by using primers 5′-CGATGGATCCGCTAG CTTGGAATCGATGGGGGTGTATC-3′ and 5′-ATCGA TGAATTCGTCGACTCAGATGCATATTCTGCAC-3′ and template pT3/WSN-HA (6). This PCR product was digested with restriction enzymes *Bam*HI and *Sal*I and subcloned into *Bam*HI-*Sal*I-digested pGEX-2T. A second PCR product was inserted into this subclone between the *Bam*HI and *Cla*I restriction sites. The second PCR product was obtained by using oligonucleotides L and 5′-ATGACTGTCGACCCATG GAAGTCAATCGATGTTATGTTAAACCAATTCCAC-3′ as primers and the plasmid containing the DNA copy of the HIV-1 genome as a template. From this plasmid, an *Nhe*I-*Sal*I fragment was recloned into *Nhe*I-*Sal*I-digested pT3GP2/BIP-NA, and the resulting plasmid was called pT3HGP2/BIP-NA. The first ORF of pT3HGP2/BIP-NA codes for a polypeptide which has an ectodomain (39 aa, of which 31 are derived from the gp41 protein ectodomain), followed by the transmembrane and cytoplasmic domains of the HA of influenza WSN virus (37 aa). Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer. The presence of the appropriate sequences in the plasmid DNAs was confirmed by sequencing with a DNA sequencing kit (United States Biochemical Corporation).

RNP transfections. Influenza virus RNA polymerase was isolated from influenza virus strain X-31 as previously described (27). RNP transfections were performed in influenza virus-infected MDBK cells as described by Enami and Palese (6). Briefly, plasmids used in transfections were digested with restriction enzyme *Ksp*632I or *Bbs*I, and 500 ng of linearized plasmid was transcribed with T3 RNA polymerase (Stratagene) in the presence of purified influenza virus polymerase. The resultant RNP complexes were transfected with DEAE-dextran into WSN- or WSN-HK-infected MDBK cells.

CAT assays. After RNP transfection of 10^6 WSN-infected MDBK cells in a 35-mm-diameter dish, cells were further incubated at 37°C for 16 h in the presence of 1.5 ml of REM; after that period, cells were harvested with a rubber policeman. Following low-speed centrifugation, cell pellets were resuspended in 100 μl of 0.25 M Tris-HCl buffer (pH 7.5), and CAT activity in these samples was determined as previously described (17).

Selection of influenza virus transfectants. RNP transfections were performed as described before for CAT expression except that MDBK cells were infected with WSN-HK helper virus (6). Medium from transfected cells was harvested 18 h after transfection and used for infection of a subconfluent monolayer of MDBK cells in an 80-cm^2 flask. Infected cells were incubated for 4 days at 37°C in REM, and transfectant viruses released to the medium were plaque purified three times in MDBK cells covered with agar overlay medium.

Virus purification. WSN virus and transfectant viruses were grown in MDBK cells after infection at a multiplicity of infection (MOI) of 0.01. Medium from infected cells was harvested 2 days after infection and clarified by two 30-min centrifugations at 3,000 and 10,000 rpm, respectively. Viruses were purified from supernatants by pelleting in a Beckman SW27 rotor at 25,000 rpm (90 min) through a 10-ml 30% sucrose cushion in NTE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Virus pellets were resuspended in NTE, and the protein concentration in purified virus samples was determined by the Bio-Rad protein assay. To test

the purity of the samples, 100 ng of protein was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a 10% polyacrylamide gel as described by Laemmli (13), and protein bands were visualized by silver staining (22). In all cases, the viral proteins in the samples represented more than 90% of the total amount of protein.

RNA extraction, electrophoresis, and sequencing. RNAs were extracted from purified viruses as previously described (18). Approximately 100 ng of virion RNAs was electrophoresed on a 2.8% polyacrylamide gel containing 7.7 M urea at 150 V for 110 min. The RNA segments were visualized by silver staining (5). The NA RNA segment of transfectant viruses was sequenced as follows. First, 100 ng of virion RNAs was used for a reverse transcription reaction using the primer 5′-GCGCGAATTCTCTTCGAGCAAAAGCAGG-3′ (EKFLU, annealing to the last 12 nt at the 3′ end of the influenza A virus RNAs) and SuperScript reverse transcriptase (Gibco-BRL). The obtained cDNAs were PCR amplified by using the primers EKFLU and 5′-AGAGATGAATTGCCGGTT-3′ (corresponding to nt 243 to 226 of the NA gene). PCR products were cloned into pUC19 (New England Biolabs) and sequenced with a DNA sequencing kit (United States Biochemical).

Immunostaining of infected cells. Confluent MDCK monolayers in a 96-well plate were infected with transfectant or wild-type influenza virus at an MOI of ≥2. Nine hours postinfection, cells were washed with phosphate-buffered saline (PBS) and fixed with 25 μl of 1% paraformaldehyde in PBS. Then, cells were incubated with 100 μl of PBS containing 0.1% bovine serum albumin (BSA) for 1 h, washed with PBS three times, and incubated for 1 h with 50 μl of PBS–0.1% BSA containing 2 μg of the human monoclonal antibody 2F5 per ml. This antibody recognizes the amino acid sequence Glu-Leu-Asp-Lys-Trp-Ala (ELDKWA), which is present in the ectodomain of gp41 of HIV-1 (25). After three washes with PBS, 2F5-treated cells were incubated with 50 μl of PBS–0.1% BSA containing a 1:100 dilution of a peroxidase-conjugated goat antibody directed against human immunoglobulins (Boehringer Mannheim). Finally, cells were washed with PBS three times and stained with a peroxidase substrate (AEC chromogen; Dako Corporation).

Western immunoblot analysis. Confluent monolayers of MDBK cells in 35-mm-diameter dishes were infected with transfectant or wild-type influenza virus at an MOI of ≥2. Eight hours postinfection, infected cells were lysed in 100 μl of 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 1% Nonidet P-40, and 1 mM Pefabloc (Boehringer Mannheim). Five microliters of these cell extracts or 2 μg of purified viruses was subjected to SDS-PAGE on a 5 to 20% polyacrylamide gradient gel as described by Fairbanks et al. (7). Proteins were subsequently transferred to a nitrocellulose membrane, and monoclonal antibody 2F5 was used to detect the gp41-derived polypeptides. The Western blot was developed with an alkaline phosphatase-coupled goat antibody against human immunoglobulins (Boehringer Mannheim).

RESULTS

A synthetic influenza virus-like gene is expressed under the translational control of the BiP IRES element in influenza virus-infected cells. pT3delNA/BIP-CAT (Fig. 1A) was constructed in order to study an influenza virus gene whose second ORF is preceded by the BiP IRES element. This plasmid contains the CAT ORF downstream of the BiP IRES sequence. Since the BiP IRES-derived sequences do not contain any initiation codon, a short ORF (110 aa) derived from the

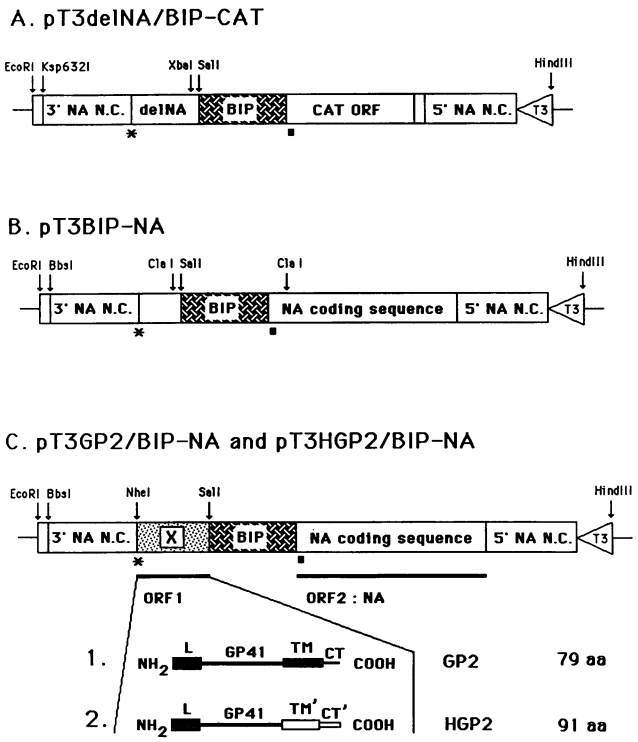

FIG. 1. Schematic representation of plasmids used in RNP transfection experiments. All plasmids are pUC19 derivatives. The arrows indicate selected restriction sites of the constructs. Asterisks represent the first ATG at the 5′ end of the encoded mRNA. Closed squares represent the first ATG downstream of the BiP IRES sequences. Domains of the plasmids are indicated as follows: 3′ NA N.C. and 5′ NA N.C., 3′ and 5′ noncoding regions of the influenza A/WSN/33 virus NA gene (10); delNA, small ORF (110 aa) derived from the influenza A/WSN/33 virus NA coding sequences; BIP, IRES sequences derived from the 5′ untranslated region of the BiP mRNA; CAT ORF, coding sequences of the CAT gene; T3, truncated T3 RNA polymerase promoter; NA coding sequence, NA coding sequence of the influenza A/WSN/33 virus NA gene; X, coding sequence of the foreign recombinant protein GP2 or HGP2. ORFs 1 and 2 are indicated by a line below the pT3GP2/BIP-NA and pT3HGP2/BIP-NA plasmid representations. GP2 and HGP2 polypeptide domains are indicated at the bottom. L, leader peptide (15 aa) derived from the HA protein of influenza A/Japan/305/57 virus; GP41, ectodomain derived from the ectodomain of the gp41 protein of HIV-1; TM and TM′, transmembrane domains derived from the gp41 protein (22 aa) of HIV-1 and from the HA protein (27 aa) of influenza A/WSN/33 virus, respectively; CT and CT′, cytoplasmic tails derived from the truncated cytoplasmic domain of gp41 (2 aa) of HIV-1 and from the HA protein (10 aa) of influenza A/WSN/33 virus, respectively. The total lengths in amino acids of the encoded GP2 and HGP2 proteins, including the leader peptide, are indicated on the right.

NA gene (delNA) was inserted upstream of the IRES. We used the delNA ORF instead of the full-length NA to reduce the size of the synthetic gene, since the RNP transfection efficiency decreases with the length of the transfected gene (9). Recognition of the delNA/BIP-CAT gene by the influenza virus transcription machinery was achieved by flanking the gene with the 3′ and 5′ noncoding regions of the influenza virus NA gene. pT3delNA/BIP-CAT was linearized with *Ksp*632I to allow runoff transcription in vitro, using T3 RNA polymerase. The resulting delNA/BIP-CAT RNA was incubated with purified influenza virus polymerases to form RNP complexes which were transfected into WSN-infected MDBK

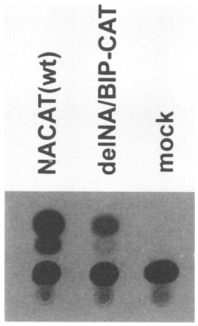

FIG. 2. CAT assays of MDBK cells that were RNP transfected with NACAT(wt) or BIP-NA RNA. Cells were RNP transfected 1 h after virus infection, harvested 16 h posttransfection, and assayed for CAT activity. mock, mock-transfected cells.

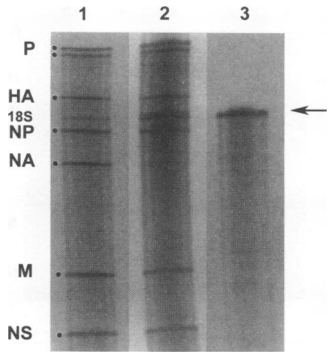

FIG. 3. PAGE of RNAs extracted from BIP-NA transfectant influenza virus. RNAs were visualized by silver staining. RNAs that encode polymerase proteins (P), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M), and nonstructural proteins (NS) are indicated on the left. BIP-NA RNA is indicated by the arrow. 18S rRNA is also indicated. Lanes: 1, influenza A/WSN/33 virus RNA; 2, BIP-NA virus RNA; lane 3, RNA transcribed in vitro from pT3BIP-NA. To facilitate the comparison of the RNA patterns, lane 2 has a shorter photographic exposure time than lanes 1 and 3.

cells. Cells were collected 16 h posttransfection, and extracts were assayed for CAT enzyme (Fig. 2). For comparison, an RNP transfection was performed with NACAT(wt) RNA, which contains the CAT gene flanked by the noncoding sequences of the NA gene of WSN. As shown in Fig. 2, the delNA/BIP-CAT RNA was transcribed in influenza virus-infected cells, and the resulting mRNA was translated into the CAT protein. It is thus likely that CAT expression from the delNA/BIP-CAT mRNA started by internal binding of the ribosomes to the BiP IRES sequences and that initiation of translation began at the ATG of the CAT ORF. The CAT activity of delNA/BIP-CAT was approximately 10-fold lower than that of NACAT(wt) (data not shown). This could be due to (i) different transfection efficiencies of the RNAs, delNA/BIP-CAT RNA being longer than NACAT(wt) RNA, or (ii) a lower translation efficiency of the CAT ORF in the context of the delNA/BIP-CAT construct. We have not investigated this question further. Also, no attempt was made to determine whether the truncated NA protein was expressed.

Rescue of a transfectant influenza virus in which the BiP IRES element was inserted upstream of the NA ORF. CAT expression from delNA/BIP-CAT RNA in influenza virus-infected cells suggested that the BiP IRES could be used for the construction of transfectant influenza viruses containing a bicistronic gene. Such a gene could direct from the same mRNA the synthesis of both a foreign protein and an essential virus protein. Thus, we attempted to rescue a transfectant virus whose NA-specific ORF was preceded by the BiP IRES element. Plasmid pT3BIP-NA was engineered (Fig. 1B) to direct the synthesis of an RNA which contains the NA ORF of WSN virus downstream of a BiP IRES element, both in negative polarity, and flanked by the 3′ and 5′ noncoding regions of the NA RNA segment of WSN virus. As in delNA/BIP-CAT RNA, a short ORF (42 aa) derived also from the NA gene was introduced upstream of the IRES sequences. Thus, initiation of translation of the NA protein by ribosomal scanning of the 5′ end of the BIP-NA-derived mRNA is unlikely. RNP transfection of BIP-NA into WSN-HK-infected MDBK cells resulted in the rescue of infectious virus. To confirm the presence of the transfected gene in the rescued BIP-NA virus, viral RNA was extracted from purified virions and analyzed by PAGE (Fig. 3). The RNA preparation of the transfectant virus did not show an RNA band at the position of the wild-type NA gene and contained a new RNA segment whose length was identical to that of the in vitro-synthesized pT3BIP-NA RNA. Confirmation of the presence of the transfected gene in the BIP-NA virus was obtained by PCR amplification and sequencing of the 3′ end of the NA gene as described in Materials and Methods. The sequence was found to be identical to that of the corresponding plasmid.

Rescue of transfectant influenza viruses whose bicistronic NA genes contain a foreign ORF. Since we were able to rescue the transfectant BIP-NA virus, the next step was to attempt to insert a foreign ORF upstream of the BiP IRES element. pT3GP2/BIP-NA and pT3HGP2/BIP-NA were constructed (Fig. 1C). The GP2/BIP-NA RNA contains two ORFs. The first ORF, GP2, codes for a polypeptide containing 38 aa of the ectodomain of the gp41 protein of HIV-1, the complete transmembrane domain, and the first 2 aa of the cytoplasmic tail of gp41. To target the GP2 polypeptide to the endoplasmic reticulum, the leader peptide-coding sequence of the HA protein of influenza A/Japan/305/57 virus was fused in frame to the GP2 ORF. The second ORF of the GP2/BIP-NA RNA codes for the WSN NA protein. This second ORF is under the translational control of the BiP IRES element. The HGP2/BIP-NA RNA is identical to the GP2/BIP-NA RNA except that 7 aa of the ectodomain and the transmembrane and cytoplasmic domains of the encoded GP2 polypeptide were replaced by 6 aa of the ectodomain and by the transmembrane and cytoplasmic domains of the HA protein of the influenza WSN virus. RNP transfection of GP2/BIP-NA and HGP2/BIP-NA RNAs into WSN-HK-infected MDBK cells resulted in the rescue of these RNAs into transfectant viruses. The presence of the transfected gene in GP2/BIP-NA and HGP2/BIP-NA transfectant viruses was confirmed by PAGE of viral RNAs isolated from purified virions (Fig. 4) and by PCR amplification and sequencing of the 3′ end of the NA RNA segments of the transfectants. GP2/BIP-NA and HGP2/BIP-NA transfectant viruses grew to 0.5- and 1.5-log-lower titers, respectively, than wild-type transfectant virus (data not shown).

GP2 and HGP2 expression in transfectant virus-infected cells. Since the NA is required for viral infectivity in MDBK cells, transfectant viruses GP2/BIP-NA and HGP2/BIP-NA were able to express the NA protein in infected cells from their bicistronic NA genes. To study the expression of the GP2 and HGP2 polypeptides in transfectant virus-infected cells, we immunostained infected MDCK cells by using the human monoclonal antibody 2F5. This antibody is specific for the amino acid sequence ELDKWA of gp41, which is present in

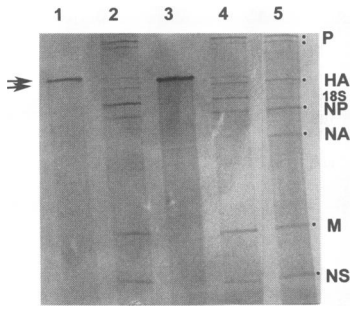

FIG. 4. PAGE of RNAs extracted from GP2/BIP-NA and HGP2/BIP-NA transfectant viruses. RNAs were visualized by silver staining. RNAs that encode polymerase proteins (P), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M), and nonstructural proteins (NS) are indicated on the right. The positions of GP2/BIP-NA and HGP2/BIP-NA RNAs are indicated by arrows. 18S rRNA is also indicated. Lanes: 1 and 3, RNAs transcribed in vitro from pT3HGP2/BIP-NA and pT3GP2/BIP-NA, respectively; 2, HGP2/BIP-NA virus RNA; 4, GP2/BIP-NA virus RNA; lane 5, influenza A/WSN/33 virus RNA. Lane 5 has a shorter photographic exposure time than lanes 1 to 4.

the GP2 and HGP2 polypeptides. The results are shown in Fig. 5. Wild-type WSN virus-infected cells did not stain with the gp41-specific antibody 2F5. In contrast, both GP2/BIP-NA and HGP2/BIP-NA virus-infected cells showed positive staining with antibody 2F5. These results indicate that the first cistron (GP2 or HGP2) of the NA RNA segment of these two transfectant viruses is expressed in infected cells. The patterns of immunostaining were different for GP2/BIP-NA and HGP2/BIP-NA virus-infected cells. Most of the staining in HGP2/BIP-NA-infected cells was localized on the cell surface, specifically at the junction between cells. In contrast, cytoplasmic structures, possibly corresponding to the endoplasmic reticulum or the Golgi complex, are strongly stained in GP2/BIP-NA-infected cells. This finding might indicate that the GP2 protein is transported to the membrane at a slower rate than HGP2 protein. Alternatively, the GP2 protein may be retained in the endoplasmic reticulum or the Golgi complex. Although we did not use a conventional reagent for cell permeabilization in these experiments, it is assumed that cell fixation with 1% paraformaldehyde permeabilizes the cells to some extent, allowing cytoplasmic staining.

The HGP2 polypeptide is incorporated into virus particles. We analyzed the presence of the GP2 and HGP2 polypeptides in GP2/BIP-NA and HGP2/BIP-NA virus-infected MDBK cells and in purified virions by Western immunoblotting with antibody 2F5. It was shown previously that fusion proteins containing the amino acid sequence ELDKWA can be recognized by antibody 2F5 in a Western blot assay (25). A low-molecular-weight polypeptide was detected in GP2/BIP-NA and HGP2/BIP-NA virus-infected cells (Fig. 6A, lanes 2 and 3) and not in wild-type WSN virus-infected cells (Fig. 6A, lane 1). The presence of an additional protein band in HGP2/BIP-NA-infected cells (Fig. 6A, lane 3) might be attributed to different levels of glycosylation of the HGP2 protein. A putative glycosylation site, Asn-X-Thr, is present in both the GP2 and HGP2 polypeptides. When the infected cell extracts were incubated with peptide *N*-glycosidase (New England Biolabs) prior to electrophoresis, only one band was detected (data not shown). In addition, a gp41-derived polypeptide was also detectable in purified HGP2/BIP-NA (and not in in GP2/BIP-NA or WSN) virions (Fig. 6B). These results indicate

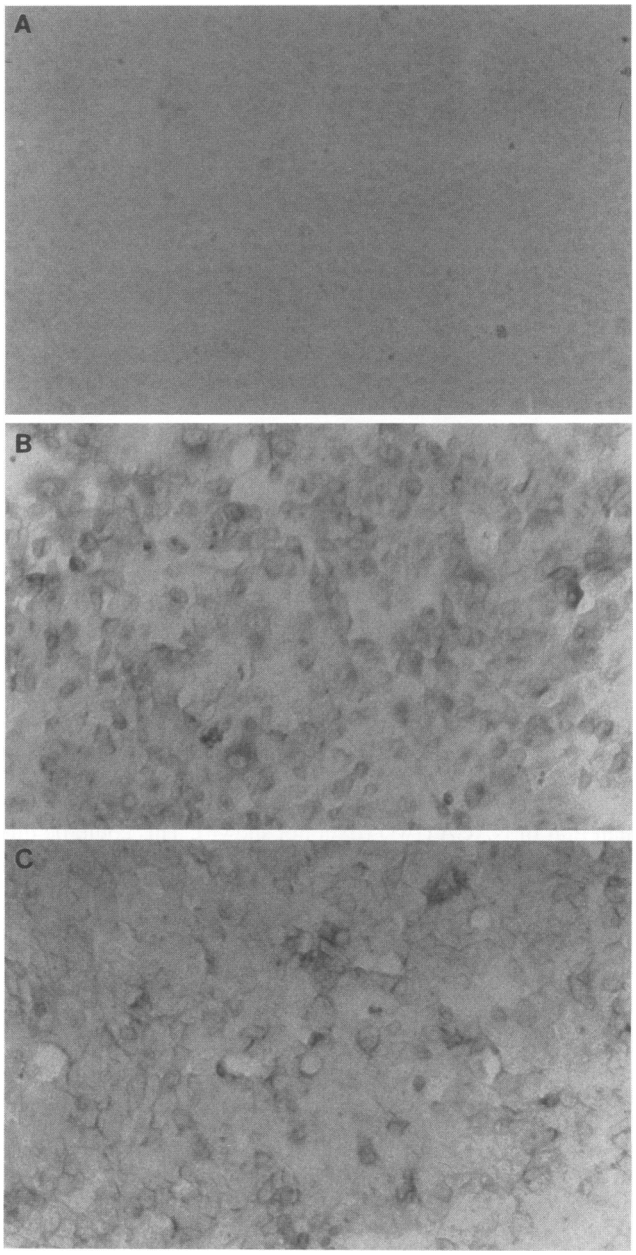

FIG. 5. Immunostaining of influenza virus-infected MDCK cells. MDCK monolayers were infected at an MOI of ≥2 with influenza A/WSN/33 virus (A) or with the transfectant virus GP2/BIP-NA (B) or HGP2/BIP-NA (C). At 9 h postinfection, cells were fixed and stained with a specific monoclonal antibody (2F5) directed against gp41 as described in Materials and Methods.

that both the GP2 and HGP2 polypeptides are expressed in cells infected with the corresponding transfectant viruses. However, only the HGP2 polypeptide, which contains the transmembrane and the cytoplasmic tail of the WSN HA protein, is incorporated into virus particles.

DISCUSSION

Viral vectors suitable for expression and/or delivery of foreign proteins are important tools for both basic and clinical

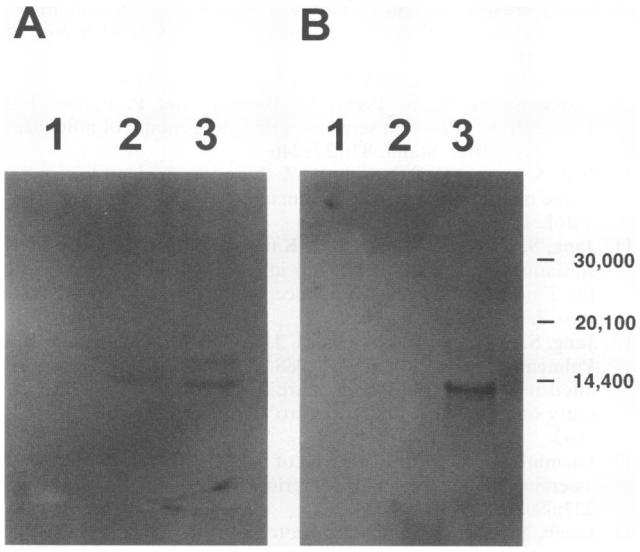

FIG. 6. Western blot analysis of the GP2 and HGP2 proteins in infected cell extracts and in purified virions. (A) MDBK cells were infected at an MOI of ≥2 with influenza A/WSN/33 virus or with GP2/BIP-NA or HGP2/BIP-NA transfectant virus. At 8 h postinfection, cells were lysed in Nonidet P-40 lysis buffer, and cellular extracts were subjected to SDS-PAGE. The monoclonal antibody 2F5 was used to detect the recombinant proteins GP2 and HGP2 in the Western blot analysis. Lanes: 1, influenza A/WSN/33 virus-infected cells; 2, GP2/BIP-NA virus-infected cells; 3, HGP2/BIP-NA virus-infected cells. (B) Two micrograms of purified virus was analyzed by the same technique. Lanes: 1, influenza A/WSN/33 virus; 2, GP2/BIP-NA virus; 3, HGP2/BIP-NA virus. Molecular weight markers are indicated on the right.

research (31). Several DNA viruses and positive-strand RNA viruses have been genetically manipulated to allow the expression of novel genes of interest in target cells. Since it is now possible to genetically manipulate influenza A virus, a negative-strand RNA virus, we attempted to develop new methodologies which allow the expression of foreign polypeptides by this virus. We succeeded in generating transfectant influenza viruses containing bicistronic NA genes. These bicistronic genes are maintained in the virus population after passaging, since the gene is required for expression of the essential viral NA protein. In addition, these transfectant viruses direct the synthesis of foreign polypeptides.

For the construction of a bicistronic influenza virus gene, we have taken advantage of a mammalian IRES sequence. IRES elements are RNA sequences which have been shown to promote the internal initiation of translation from mRNAs. IRES sequences were first discovered in the nontranslated regions of picornavirus mRNAs (11, 12, 28) derived from EMCV, poliovirus, rhinovirus, or foot-and-mouth disease virus. Although the EMCV IRES has been used for the construction of bicistronic genes in chimeric retroviruses and polioviruses (1, 2, 23), we were unable to detect expression of a reporter gene engineered downstream of an EMCV IRES in a synthetic influenza virus gene (7a, 9). We therefore considered using nonviral IRES elements. In this report, we describe the generation of functional bicistronic genes by using the IRES element derived from the human BiP mRNA. Other strategies to express foreign polypeptides involve the use of a fusion protein containing a protease signal (29), but these approaches result in the expression of altered proteins due to the presence of the specific protease signal in the polyproteins.

The BiP IRES element, which allows the expression of a second independently translated cistron, was attractive to us for two reasons. First, it only has about 220 nt and is thus shorter than the functionally equivalent picornavirus sequences. This is a desired characteristic, since we have previously found limitations with respect to the length of the influenza virus RNAs that can be transfected. Second, the BiP IRES element has no sequence or structural homology with the picornavirus elements, which is desirable since the latter appear to have only low activity in influenza virus-infected cells (7b). The hepatitis C virus IRES element (32) would be another attractive candidate for the construction of bicistronic influenza virus genes for the same reasons, but we did not include this IRES element in the present study.

We first tried to determine if the BiP IRES element was active in influenza virus-infected cells after RNP transfection using the CAT reporter system. Our experiments demonstrated that the BiP IRES element (in pT3delNA/BIP-CAT RNA) can initiate translation of the CAT gene in influenza virus-infected cells (Fig. 2). Similar CAT constructs which contained the EMCV IRES or the rhinovirus 14 IRES instead of the BiP-derived sequence did not express CAT protein in influenza virus-infected cells (see above). Next, we extended our investigations by constructing an influenza virus (BIP-NA) whose NA protein was translated from an internal ORF preceded by the BiP IRES element. The fact that this virus was viable indicates that the NA expression achieved by using the IRES element was sufficient for the generation of an infectious influenza virus. Finally, we generated two transfectant influenza viruses, GP2/BIP-NA and HGP2/BIP-NA, whose NA mRNAs directed translation of a foreign cistron (GP2 or HGP2, containing sequences derived from gp41 of HIV-1) by a conventional cap-dependent scanning mechanism and of a second cistron (NA) by internal initiation from the BiP IRES. The results are in agreement with the reported IRES activity of the nontranslated region of the BiP mRNA (20). The experiments described above do not rule out the possibility that translation of the NA ORF in these bicistronic genes is achieved via resumption of scanning by the ribosomes. Specifically, there is no initiation codon between the stop codon of the first ORF and the ATG of the NA ORF. However, we have rescued a transfectant influenza virus containing a bicistronic NA gene in which nine independent initiation codons are engineered between the stop codon of the first cistron and the BiP NA sequence. In addition, there are several stop codons in each of the three reading frames between the first cistron and the NA ORF (8a). This result strongly supports the notion that internal initiation of translation is the operative mechanism for translation of the second ORF in the delNA/BIP-CAT, BIP-NA, GP2/BIP-NA, and HGP2/BIP-NA constructs.

Although we did not attempt to quantify the proteins, we assume that the levels of expression of the GP2 and HGP2 proteins in transfectant virus-infected cells are similar to those of the NA in wild-type virus-infected cells, since the foreign recombinant ORFs are in the same background as the ORF of the wild-type NA. It is likely that the levels of expression of the foreign protein could be increased by using other influenza virus genes, such as the HA gene, which on a molar level appears to express 5 to 10 times more protein than the NA gene. Although we did not quantify the expression of the NA protein in transfectant virus-infected cells, we might expect lower levels than in wild-type virus-infected cells, since the transfectant viruses are attenuated in tissue culture. Also, RNP transfection experiments using the construct delNA/BIP-CAT showed lower CAT expression levels than the control involving the wild-type construct NACAT(wt) (Fig. 2).

Surface expression of a foreign protein may be desirable for the induction of a humoral immune response against the protein (3). We attempted to direct surface expression of both gp41-derived polypeptides by fusing in frame the coding sequence for the leader peptide of the HA of influenza A/Japan/305/57 virus with the coding sequence of the foreign protein. Surface expression was detected by immunostaining of infected cells with antibody 2F5, which is specific for a linear epitope in the ectodomain of gp41. In addition, we were able to direct the packaging of the HGP2 polypeptide into virus particles by including the transmembrane and cytoplasmic domains of the influenza virus HA. (It should be noted, however, that initial experiments following infection of mice with HGP2/BIP-NA virus did not show a vigorous immune response directed against the gp41-derived sequences.) In contrast to our finding with HGP2, the GP2 polypeptide, which contains the transmembrane domain derived from the HIV-1 gp41, was not packaged into virus particles. Recently, Naim and Roth (26) reported that an HA-specific transmembrane domain is required for incorporation of the HA into influenza virions. Our results are in agreement with this finding, and they also demonstrate that the transmembrane and cytoplasmic domains of the HA protein contain all of the signals required for incorporation of a protein into influenza virus envelopes. In summary, the use of the BiP IRES element for the construction of bicistronic influenza virus vectors may represent a new methodology for expressing foreign genes which should have practical applications in molecular and medical virology.

ACKNOWLEDGMENTS

We thank Peter Sarnow for providing the cDNA of the BiP IRES element. We also thank Michael Bergmann, Mehmet Doymaz, and Thomas Zürcher for helpful discussion and sharing of plasmids and oligonucleotides.

This work was supported by an MEC/Fulbright scholarship from the Spanish Government (A.G.-S.), the Austrian Fonds zur Förderung der wissenschaftlichen Forschung project P09775-MOB (T.M.), the American Foundation for AIDS research (W.S.B.), and the NIH (P.P.).

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