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Expression of a Foreign Protein by Influenza A Virus

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In this report we describe the rescue of a transfectant influenza A virus which stably expresses a heterologous protein, bacterial chloramphenicol acetyltransferase (CAT). The foreign sequences encoding CAT are expressed as part of an essential influenza virus segment, that coding for the neuraminidase (NA) protein. The novel way by which this was achieved involved inserting in frame the 16-amino-acid self-cleaving 2A protease of foot-and-mouth disease virus between the CAT and the NA coding sequences. The resultant gene produces a polyprotein which is proteolytically cleaved to release both CAT and NA. The intramolecular cleavage occurs at the C terminus of the 2A sequence between a glycine-proline dipeptide motif such that the released NA protein has an additional N-terminal proline residue. The transfectant virus is stable upon passage in tissue culture. CAT activity is expressed at high levels in cell culture supernatants and in the allantoic fluid of infected eggs. Since the chimeric segment must maintain the heterologous reading frame to retain viability, the virus stability is dependent upon concomitant synthesis of the heterologous protein. This design may be particularly appropriate for utilization of influenza virus as a mammalian expression vector.

Influenza A viruses are segmented negative-stranded RNA viruses belonging to the family *Orthomyxoviridae*. The genomes of influenza A viruses, comprising approximately 14,000 nucleotides, are distributed in eight different RNA segments (18), with each segment coding for at least one viral protein (10). Genetic manipulation of influenza A viruses is now possible because of the development of a reverse genetics system for introducing synthetic RNAs into the viral genome (5, 6). This is achieved by reconstituting active ribonucleoprotein (RNP) complexes from synthetic RNAs transcribed in the presence of purified viral polymerases in vitro and using these complexes to transfect influenza virus-infected cells.

The RNP transfection technology has allowed the characterization of the molecular mechanisms underlying the control of transcription and replication of the genome of influenza A virus. The chloramphenicol acetyltransferase (CAT) reporter gene flanked by influenza virus noncoding regions was used as the model RNA, and an evaluation of the effects of specific mutations has led to the identification of *cis*-acting sequences essential for transcription, replication, and polyadenylation of influenza virus RNAs (12, 15, 19, 20). However, the incorporation of a synthetic nonessential RNA segment into influenza virions is transient. The foreign RNA segment is packaged as a defective RNA into the viral particles but, being nonessential, is rapidly segregated out within a few virus generations. Foreign sequences might be permanently expressed in influenza virus if they were incorporated as part of an essential viral protein. Short foreign sequences have been introduced into the open reading frames of neuraminidase (NA) or hemagglutinin, resulting in viruses displaying dual antigenicity and immunogenicity (2, 11). However, the latter application is limited because of size and sequence restrictions on inserted sequences. Since the viral proteins themselves are being altered, large foreign sequences can severely perturb the protein structures and affect their biological activity. Even so, influenza virus RNAs can tolerate and replicate additional noncoding sequences. Recently, we have shown that the influenza A/WSN/33 (WSN) virus NA segment can stably maintain an additional 900 nucleotides over several generations in tissue culture (8).

In this paper we describe the rescue of a transfectant influenza A virus which stably expresses the bacterial CAT protein. The foreign CAT sequences are expressed as part of the NA segment essential for influenza virus viability. Insertion of the self-cleaving 2A protease of foot-and-mouth disease virus (FMDV) in frame between the CAT and the WSN virus NA coding sequences produces a polyprotein which is proteolytically cleaved to release both a CAT2A fusion protein and the NA. The high-level CAT activity expressed from the transfectant virus is stable upon passage in tissue culture. This report may have important implications for the use of influenza virus as an expression vector, particularly in the development of live-attenuated chimeric influenza virus vaccines to protect against infection by other pathogens.

MATERIALS AND METHODS

Cells and viruses. Transfectant viruses and WSN virus were grown in Madin-Darby bovine kidney (MDBK) cells in reinforced minimal essential medium. Influenza A/PR/8/34 virus and WSN-HK virus, a reassortant influenza virus containing seven genes from WSN virus and the NA gene from influenza A/HK/8/68 virus, were grown in 10-day-old embryonated chicken eggs. MDBK cells were used for transfection experiments and for selection and plaque purification of rescued transfectant viruses (6).

Construction of plasmids. Figure 1 shows the construction of parent plasmid pT3CAT2A/NA. The initial plasmid pIVACAT1/S has been previously described (20). It contains the CAT gene flanked by the noncoding sequences of the NS gene of influenza A virus. A unique *SalI* restriction enzyme site had been created by introducing silent mutations in codon 11 (GTT to GTC) and codon 12 (GAT to GAC) (20). The first intermediate in the construction of plasmid pT3CAT2A/NA was plasmid pIVACAT*HpaI*, in which the stop codon of CAT

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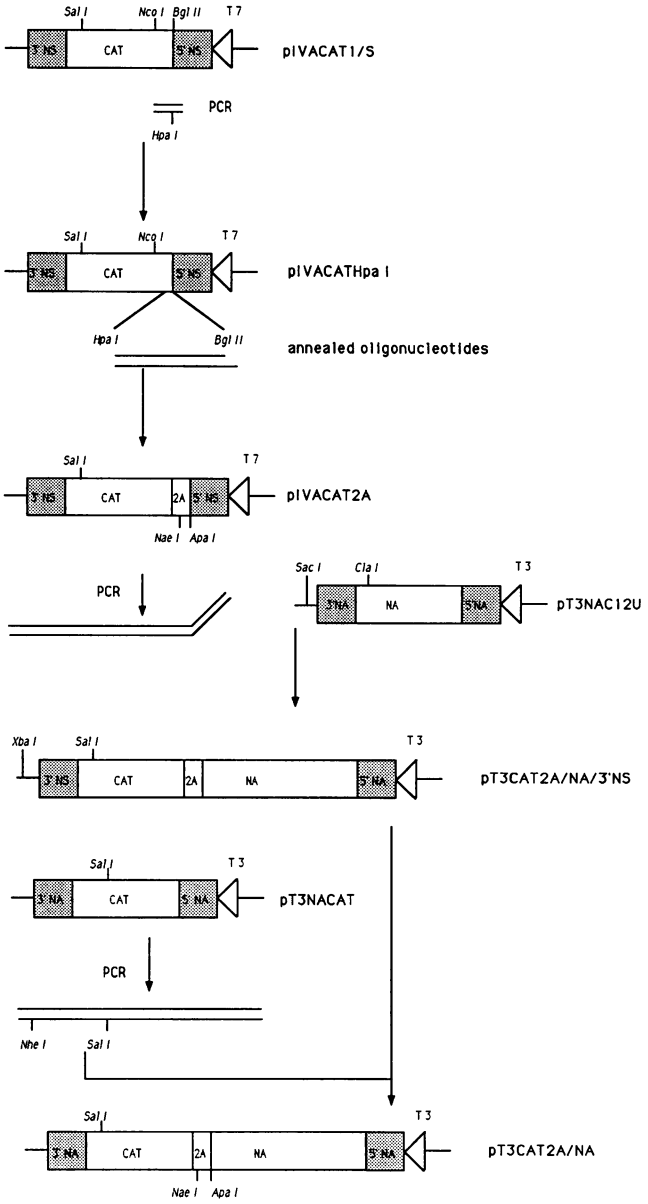


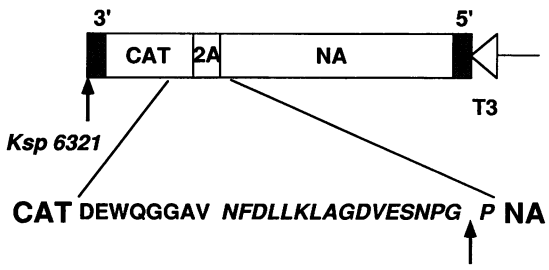
FIG. 1. Construction of parent plasmid pT3CAT2A/NA. All genes were cloned into the multiple cloning site of pUC19. The noncoding regions of influenza virus RNAs are represented as shaded boxes and marked NS or NA depending on whether sequences are derived from the NS or the NA gene. Transcriptional promoters for T3 or T7 RNA polymerase are indicated by triangles. PCR products or annealed oligonucleotides are drawn as double lines. Relevant restriction enzyme sites are shown in italics. The sequences of primers and a detailed description of the cloning steps are given in Materials and Methods.

has been replaced by a valine residue which is followed by an AAC triplet to form a unique *Hpa*I site (GTTAAC) at the C terminus of CAT. This was achieved by cloning the product of a PCR performed with primers 5′-TTTTCACCATGGGCAA ATATT-3′ (nucleotides 508 to 528 in CAT, encompassing the *Nco*I site) and 5′-TTTTTCAGATCTGCGGCCGCGTTAA CCGCCCCGCCCTGCCACT-3′ (complementary to 17 3′ nucleotides of the CAT gene and *Hpa*I and *Bgl*II sites) and pIVACAT1/S as the template. The PCR product was digested with *Nco*I and *Bgl*II and cloned into pIVACAT1/S digested with the same enzymes (the *Bgl*II site is located after the stop codon of CAT in the 5′ noncoding region of pIVACAT1/S). Next, the sequence encoding the 2A protease of the O1K strain of FMDV was inserted in frame with the CAT sequences by ligation of annealed oligomers 5′-AACTTTGACCTTCTCAA GTTGGCCGGCGACGTCGAGTCCAACCCA-3′ and 5′-G ATCTGGGTTGGACTCGACGTCGCCGGCCAACTTGA GAAGGTCAAAGTT-3′ into pIVACAT*Hpa*I digested with *Hpa*I and *Bgl*II to form pIVACAT2A. The codon usage for the 2A sequence created unique *Nae*I and *Apa*I sites to facilitate future cloning steps. Plasmid pT3NA*Cla*I contains the complete NA gene of WSN virus downstream of a truncated T3 promoter (3a) and was derived from pT3NAv, which has been described previously (5). The unique *Cla*I restriction site had been introduced into the NA coding sequence requiring two silent mutations in codon 11 (GGG to GGA) and codon 12 (TCA to TCG). Sequences encoding the CAT2A fusion were inserted into pT3NAC12U(30), a derivative of pT3NA*Cla*I, by cloning the PCR product obtained by using the primer 2ANA (5′-CATACAGATCGATCCAATGGTTATTATTTTCTG GTTGGGCCCTGGGTTGGACTCGACGTCGCCGGC-3′), which is complementary to 27 nucleotides of the 2A sequences and contains a further 39 nucleotides of the NA gene extending past the *Cla*I site, and the pUC universal primer (5′-GTA AAACGACGGCCAGT-3′) with pIVACAT2A as the template. The 750-bp PCR product was digested with *Sac*I and *Cla*I and ligated into pT3NAC12UI cut with the same enzymes. The resultant plasmid (pT3CAT2A/NA/3′NS) contains the coding information to produce the chimeric CAT2A/NA polyprotein but contains the 3′ noncoding region of the NS gene. The 3′ noncoding region was exchanged with that of the NA gene by first generating a PCR product by using the plasmid pT3NACAT (13) as template, primer M3 (5′-CGCG GCTAGCCTCTTCGAGCGAAAGCAG-3′), and the reverse primer of pUC (5′-TTCACACAGGAAACAG-3′). From this 750-bp product, a 70-nucleotide *Nhe*I-*Sal*I fragment was excised and ligated into pT3CAT2A/NA/3′NS digested with *Xba*I and *Sal*I to produce pT3CAT2A/NA. The amino acid sequence at the carboxy region of CAT fused to the 2A protease is shown in Fig. 2A..

The third amino acid of the wild-type NA protein of WSN virus is a proline. Since the 2A protease specifically cleaves between glycine-proline pairs, the construct described above had been designed to exploit the presence of a proline in the amino terminus of NA for use as part of the 2A cleavage motif. Thus, the first two amino acids (methionine and asparagine) are deleted from the NA (Fig. 2B). Two alternative versions of the CAT2A/NA plasmid, pT3CAT2A/NAmodI and pT3CAT2A/NAmodII, were engineered (Fig. 2B). These were constructed by cloning PCR products amplified by using the primer 5′-CAGATCGATCCAATGGTTATT-3′ (complementary to NA nucleotides 40 to 60) with either primer 5′-TTTAAAGGGC **CCAAT**CCAAACCAGAAAATAAC-3′ or primer 5′-TTTA AAGGG**CCCATGAAT**CCAAACCAGAAAATAAC-3′ containing the additional nucleotides (in boldface) encoding a proline (for 2A cleavage) and the asparagine residue (position 2 of the NA) or a proline and the asparagine and the initiator methionine. The PCR products were digested with *Apa*I and *Cla*I and ligated into pT3CAT2A/NA which was restricted with the same enzymes. The sequences at the amino termini of the NA proteins encoded by the new plasmids, pT3CAT2A/NAmodI and pT3CAT2A/NAmodII, are shown in Fig. 2B.

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. All constructs were confirmed by direct

A



B

plasmid	Sequence at NA amino terminus	virus
pT3NACla	MNPNQK	+
pT3CAT2A/NA	*P* NQK	–
pT3CAT2A/NAmodI	*P* NPNQK	+
pT3CAT2A/NAmodII	*P* MNPNQK	+

FIG. 2. Plasmids used to direct expression of CAT2A/NA polyprotein within influenza virus segments. (A) The genes to express a CAT2A/NA fusion polyprotein are flanked by the noncoding regions of the NA segment (shaded boxes). Transcription is directed from the modified T3 promoter (triangle). Plasmid DNAs are linearized with *Ksp* 6321 so that the synthetic RNAs have a 3′ end identical to that of viral RNA. The amino acid sequence at the carboxy region of CAT is shown below fused with the sequence of the 2A protease (in italics). The site of protease cleavage is indicated by the arrow, and the resulting amino-terminal proline added to the NA protein is shown after the arrow. (B) The three versions of the pT3CAT2A/NA plasmid differ in the sequence of the NA amino terminus. The wild-type sequence is shown here for comparison. Viruses containing novel RNA segments were isolated after transfection of plasmids indicated by +. The additional proline required for 2A cleavage is shown in italics.

plasmid DNA sequencing with Sequenase (United States Biochemical).

RNP transfection of MDBK cells. Influenza virus RNA polymerases were isolated from influenza A/PR/8/34 virus as described previously (19). RNP transfection was performed with MDBK cells according to the method of Enami and Palese (6) with WSN-HK as helper virus. Briefly, plasmids used in transfections were digested with *Ksp* 6321 and purified by phenol extraction. Five hundred nanograms of linearized plasmid was transcribed with T3 RNA polymerase (Stratagene) in the presence of purified influenza virus polymerase preparations. The resultant RNP complexes were DEAE-dextran transfected into WSN-HK-infected MDBK cells. Following blind liquid passage in MDBK cells, rescued transfectant viruses were plaque purified three times in MDBK cells in the absence of trypsin in the overlay.

Sequence analysis of transfectant viral RNA. Virus was purified through a 30% sucrose layer, and viral RNA was isolated by phenol extraction. The sequence at the 3′ region of the NA segment was obtained as follows. RNA was transcribed into cDNA by using avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories) and the primer EKFlu (5′-GCGCGAATTCCTCTTCGAGCAAAAGCAG-3′), which contains the first 12 nucleotides of the WSN virus NA gene with additional *Eco*RI and *Ksp* 6321 sites. The primers for subsequent PCR amplification of the cDNA were 5′-AGAGATGAATTGCCGG-3′ (corresponding to positions 229 to 243 of the WSN virus NA gene) and the EKFlu primer. The purified PCR product was directly sequenced by using 5′-TATTGAGATTATATTTCC-3′ (corresponding to positions 98 to 115 of the WSN virus NA gene) as primer and Sequenase with dimethyl sulfoxide according to the method of Seto (22).

Growth curves of transfectant viruses. Confluent monolayers of MDBK cells in 35-mm-diameter dishes were infected with either wild-type (NA*Cla*I) or transfectant viruses at a multiplicity of infection (MOI) of 0.001. At 12-h intervals, supernatants were harvested and PFU titers were determined in MDBK cells.

CAT assay. Confluent MDBK cells were infected with transfectant virus at an MOI of 2 or with the virus released from 1 plaque. Cells were harvested into 100 μl of 0.25 M Tris-HCl (pH 7.5) and lysed by three rounds of freeze-thawing. Cell debris were removed by microcentrifugation for 15 min at 10,000 × *g*. To analyze CAT enzyme activities released from infected cells, cell culture supernatants or allantoic fluids were diluted in 0.25 M Tris-HCl (pH 7.5).

CAT assays were done according to standard procedures adapted from those described by Gorman et al. (9). The 2-h assays were performed by using 2 μl of [^{14}C]chloramphenicol (58 mCi/mmol, 0.05 mCi/ml; Du Pont/NEN), 20 μl of 40 mM acetyl coenzyme A (Boehringer Mannheim), and 50 μl of cell extract in 0.25 M Tris-HCl (pH 7.5). When necessary, cell extracts were diluted in the same extraction buffer.

To quantify CAT activity, thin-layer chromatography plates were dissected and radioactivity was counted by scintillation. To estimate units of CAT enzyme produced at various times after infection, it was assumed that 1 U is the amount of enzyme which acetylates 1 nmol of [^{14}C]chloramphenicol in 1 min under the conditions described above. As a standard, CAT enzyme obtained from Boehringer Mannheim (catalog no. 874 434) was assumed to be 100% pure.

Immunoprecipitation of labelled proteins from infected cells. Confluent MDBK cells were infected with either wild-type (NA*Cla*I) or transfectant viruses at a multiplicity of 2. Cells at 5 h postinfection were labelled with L-[^{35}S]cysteine for 45 min and chased with cold medium for 1 h. For immunoprecipitation, labelled cells were lysed in 0.15 M NaCl–20 mM Tris-HCl (pH 7.4) containing 1.5% *n*-octylglucoside and 1 mM $CaCl_2$. Proteins were immunoprecipitated by overnight incubation with rabbit polyclonal anti-WSN serum, monoclonal antibodies 10C9 and 3C8 (specific for WSN NA), or rabbit polyclonal anti-CAT antiserum (5 Prime-3 Prime Inc.) followed by 1 h of incubation with protein G-Sepharose. After the bound Sepharose was washed four times in the *n*-octylglucoside buffer, immunoprecipitated proteins were analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography following fluorography using Amplify (Amersham).

RESULTS

Rescue of transfectant viruses with CAT2A coding sequences. In order to stably express a heterologous gene in influenza virus, we chose to encode the foreign protein as part of an essential influenza virus RNA. The target influenza virus segment was that encoding NA, since exchange of this segment with synthetic RNAs is facilitated by an efficient selection system (5). The foreign protein (CAT) was expressed as part of

a polyprotein with the NA which would be proteolytically cleaved to release both CAT and the NA protein in the infected cell. To achieve this, we inserted the self-cleaving 2A protease of FMDV in frame between the CAT and NA open reading frames (21) (Fig. 2A). For the initial construct, pT3 CAT2A/NA, use was made of the natural proline residue at position 3 of the NA to form one half of the G-P dipeptide motif required by the 2A protease for self-cleavage. Thus, the NA protein produced from this construct would lack the first two amino acids at the amino terminus (Fig. 2B). Because of the striking sequence conservation among strains of influenza A viruses within this region (17), we constructed two additional plasmids (pT3CAT2A/NAmodI and pT3CAT2A/NAmodII) to express NA proteins which retained the position 2 asparagine (modI) or kept both the methionine and asparagine residues of the cytoplasmic tail (modII). Thus, in the polyprotein encoded by pT3CAT2A/NAmodI, the initiator methionine residue of wild-type NA would be exchanged for a proline required for 2A protease cleavage, and in that encoded by pT3CAT2A/NAmodII, an additional proline would precede the authentic cytoplasmic tail sequence (Fig. 2B).

RNP transfection was performed with MDBK cells according to the method of Enami and Palese (6) with WSN-HK as helper virus. Following overnight incubation, 500 μl of supernatant was passaged onto confluent 60-mm-diameter MDBK dishes. Seven days later the progeny from these passages was assayed in MDBK cells. Plaques were obtained only from the control transfection using pT3NA*Cla*I and from pT3CAT2A/NAmodI and pT3CAT2A/NAmodII RNP transfections (Fig. 2B). Despite repeated attempts, no virus could be obtained following transfections of RNPs derived from pT3CAT2A/NA. Transfectant viruses were plaque purified three times and amplified in MDBK cells.

Analysis of CAT2A/NAmodI and CAT2A/NAmodII transfectant viruses. To verify that the transfectant viruses contained the chimeric RNA, amplified virus was purified through a 30% sucrose layer and viral RNA was isolated by phenol extraction. PAGE of RNAs purified from transfectants indicated that in both viruses the NA segment was larger than that of wild-type virus and migrated to the same position as synthetic RNA derived by in vitro transcription of the pT3CAT2A/NAmodI or -modII DNA templates. Also, the new segment was packaged in an amount equimolar to that of the other segments (data not shown). In order to confirm the sequence of the CAT2A/NAmodI and CAT2A/NAmodII RNAs, PCR products were obtained by reverse transcription of viral RNA and amplification of the resultant cDNAs, using primers which flank the inserted sequences (described in Materials and Methods). Direct sequencing of these DNA products showed that the entire CAT and 2A sequences were present, with no mutations at the 2A/NA fusion junction (data not shown).

Growth characteristics of CAT2A/NAmodI and CAT2ANA/modII viruses. MDBK cells were infected with either NA*Cla*I, CAT2A/NAmodI, or CAT2A/NAmodII transfectant viruses at an MOI of 0.001. The growth kinetics of the chimeric transfectant viruses are compared with those of the wild type virus in Fig. 3. Peak yields of virus were attained at 52 h after infection. The peak titer of CAT2A/NAmodII virus was approximately 1 $\log_{10}$ lower than that of wild-type virus. On the other hand, the titer of the CAT2A/NAmodI virus increased more slowly, and the maximum yield was at least 2 $\log_{10}$ lower than that of the wild-type virus. Correspondingly, the plaque sizes of the transfectant viruses were smaller than that of the wild-type virus; in particular, CAT2A/NAmodI showed tiny, nondistinct plaques (data not shown).

Expression of CAT by transfectant viruses. Following their initial isolation, the transfectant viruses were plaque purified three times in MDBK cells. To test the stability of CAT expression, individual plaques were then passaged in tissue culture with liquid overlay, and the yield was plaqued once more. Ten individual plaques were then picked and used to infect 35-mm dishes of MDBK cells. CAT assays were performed on extracts of these cells after they showed cytopathic effects (1 to 2 days postinfection). Plaques of transfectant virus CAT2A/NAmodII all showed high-level CAT expression under these conditions, whereas only 8 of the 10 CAT2A/NAmodI plaques gave rise to CAT expression (data not shown). For this reason further characterization of CAT expression from transfectant viruses was performed solely with the CAT2A/NAmodII virus. The reasons for loss of CAT activity from two of the CAT2A/NAmodI plaques were not further investigated.

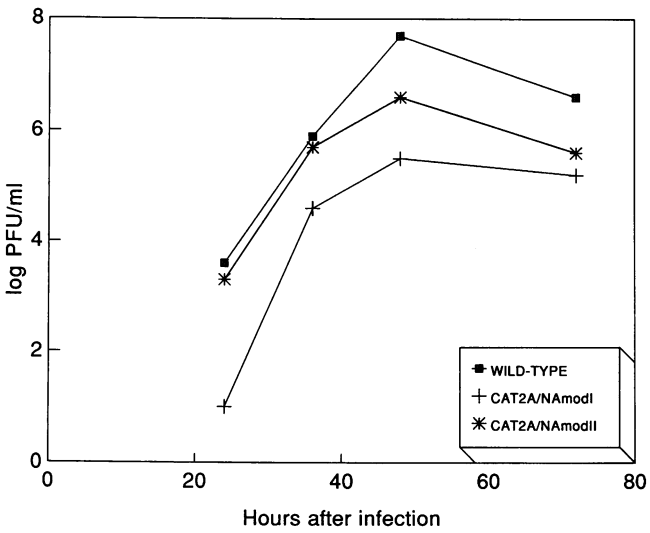


FIG. 3. Growth curves of CAT2A/NA transfectant viruses. MDBK cells were infected with transfectant or wild-type viruses at an MOI of 0.001. At various times after infection with wild-type, CAT2A/NAmodI, or CAT2A/NAmodII virus, cell supernatants were quantified by plaque assay in MDBK cells.

Kinetics of CAT production in infected cells. The CAT production in extracts of CAT2A/NAmodII-infected cells increased with time up to 12 h after infection at an MOI of 2 (Fig. 4A). We estimate that 1 μg of CAT enzyme is produced in 10^7 cells infected with CAT2A/NAmodII virus. This calculation is based on the assumption that the CAT2A fusion protein has the same specific activity as the commercially available CAT enzyme (see Materials and Methods). CAT activity was undetectable in supernatants at 6, 8, and 9 h after infection. However, at 24 h after infection, CAT enzyme was released into infected-cell supernatants at a level approximately 40% of that detected in cell extracts at that time (data not shown). We also tested whether the foreign protein was expressed in the allantoic fluids of infected eggs. Two eggs were infected with CAT2A/NAmodII virus and incubated at 37°C for 2 days. The allantoic fluids contained high levels of CAT enzyme. Figure 4B shows the CAT conversion with 50 μl of allantoic fluid (1/200 of the total yield from 1 egg).

2A cleavage in transfectant-infected cells. To confirm that the 2A protease was actually cleaving the recombinant polyprotein, we analyzed the size of the protein produced in cells infected with transfectant virus. MDBK cells were in-

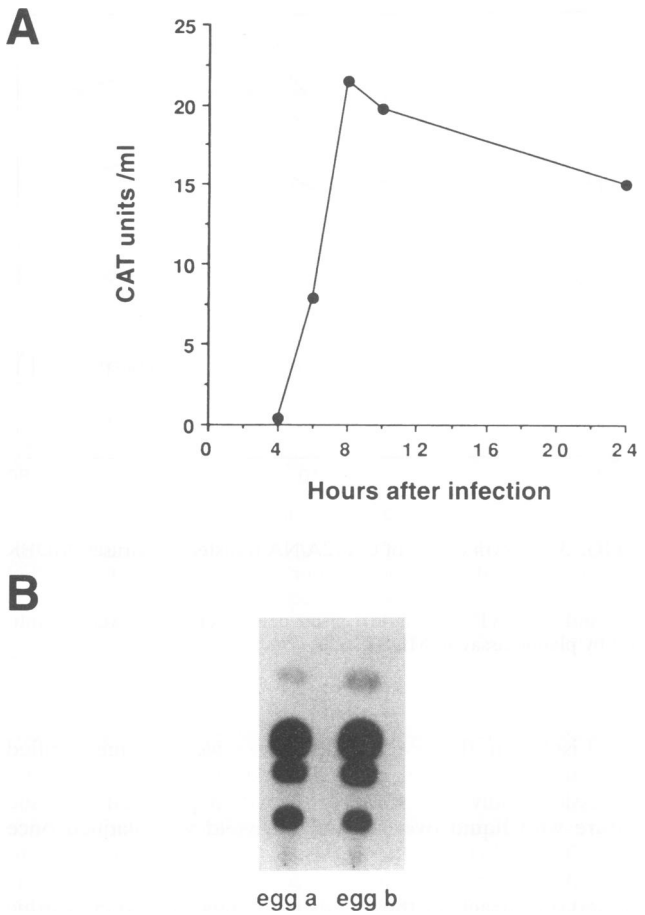


FIG. 4. CAT activity following infection with CAT2A/NAmodII transfectant virus. (A) Time course of CAT activity in cell extracts after infection of MDBK cell monolayers with CAT2A/NAmodII virus (MOI = 2). Units of CAT enzyme activity are described in Materials and Methods. (B) CAT activity in allantoic fluids of eggs infected 2 days previously with 10^5 PFU of CAT2A/NAmodII virus. The assay shows activity with 50 μl of allantoic fluid.

fected with either NA*Cla*I or CAT2A/NAmodII transfectant at an MOI of 2. Proteins were labelled at 5 h postinfection. Figure 5 shows the SDS-PAGE analysis of labelled viral proteins immunoprecipitated with either polyclonal antiviral serum, NA-specific monoclonal antibodies, or a CAT polyclonal antibody. The NA-specific monoclonal antibodies precipitated a protein from CAT2A/NAmodII-infected cells which had the same apparent size as the reactive band from wild-type virus-infected cells. This indicates that cleavage at the 2A/NA junction is highly efficient, since a larger reactive product is not observed. In addition, a protein reactive with polyclonal CAT antiserum was released from CAT2A/NA modII-infected cells but not from cells infected with wild-type virus (Fig. 5). The size of this product (26 kDa) is identical to that expected for the CAT2A fusion protein. The intensity of the band precipitated by the CAT-specific polyclonal serum was less than that of the band precipitated by the NA-specific monoclonal antibodies. This may be because the affinity of the monoclonal antibody is higher than that of the polyclonal preparation for their respective antigens or, alternatively, simply because there are fewer cysteine residues in the CAT protein than in the NA (5 versus 19).

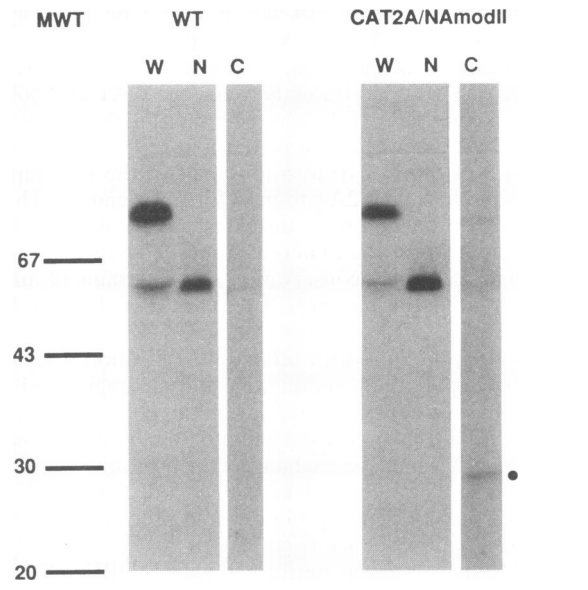


FIG. 5. Immunoprecipitation of NA and CAT proteins from cells infected with CAT2A/NAmodII virus. Cells were labelled with [^{35}S]cysteine at 5 h postinfection with wild-type (WT) or transfectant (CAT2A/NAmodII) virus at an MOI of 2. Immunoprecipitation was with antibodies to whole virus (W), NA (N), or CAT (C). The detection of CAT protein (indicated by a dot) required a longer exposure of the autoradiograph than that of NA or total viral proteins (7 days versus 1 day). Numbers on the left are molecular weights (MWT) in thousands.

DISCUSSION

Foreign sequences might be permanently expressed in an influenza virus genome if they were incorporated as part of a viral segment. This could be mediated by creating bicistronic segments which utilize multiple splicing strategies or internal initiation of translation (7). Another approach is to exchange a region of the coding information of the influenza virus genome with foreign sequences to produce a chimeric protein. To minimize structural disruption, the foreign sequences could be expressed at one of the termini of the protein. Furthermore, by insertion of protease recognition sequences, the foreign protein may be excised from the polyprotein. In this paper we describe the rescue of such transfectant influenza A viruses which stably express the bacterial CAT gene expressed as part of the NA segment essential for influenza virus viability. This has been achieved by inserting the 2A protease of FMDV in frame between the CAT and NA coding sequences.

Since the 2A self-cleavage occurs between the amino acids glycine and proline (21), the released NA protein possesses a proline at its amino terminus. The wild-type amino-terminal cytoplasmic tail of the NA consists of six amino acids (MNPNQK) (Fig. 2B). This sequence is conserved among all the NAs of influenza A viruses examined to date (17). In contrast, the cytoplasmic tail of influenza B virus NA has seven residues which differ at all positions from those of the influenza A virus NA except for the proline at position 3 and the initiator methionine. The effects of changes in this region of WSN NA were recently analyzed by using reverse genetics techniques to recover transfectant viruses with altered NA cytoplasmic tails (1). This study demonstrated that the sequence in this region was flexible except for position 3. No viruses which lacked a proline at this position were recovered. In the present study we designed several versions of the plasmid to direct expression of

CAT2A/NA polyproteins (Fig. 2B). The first construct, pT3CAT2A/NA, utilized the proline at position 3 of the NA as part of the 2A protease motif. The resulting NA protein would lack the first two residues. We were unable to rescue a virus following transfection of RNPs from pT3CAT2A/NA. On the other hand, viruses could be rescued from RNPs derived from plasmids pT3CAT2A/NAmodI and -modII. Virus from the modI construct appears to be unstable. It showed a very small plaque morphology, and not all of the plaques from this virus expressed CAT. Although the cytoplasmic tail is the same length as that of the wild-type NA, the initiator methionine has been replaced by a proline residue. It is possible that the two proline residues in close proximity impose conformational constraints on the cytoplasmic domain and thereby disrupt the function of the NA. The nature of the reversion by which CAT2A/NAmodI virus loses the ability to produce CAT remains unknown.

In contrast, the virus CAT2A/NAmodII was stable upon passage. This virus has a cytoplasmic tail which is one residue longer than that of wild-type NA. Bilsel et al. (1) have also isolated two transfectant WSN viruses with seven residues in the NA cytoplasmic tail. These viruses (CYT 7B and FLUB-CYT) were attenuated compared with wild-type WSN for growth in tissue culture and replication in mice. The basis of attenuation appears to be that these mutant NAs were less efficiently incorporated into virions, and this might also be the reason for the small plaques displayed by our modII virus.

The amount of CAT enzyme detected in 10^7 cells infected with CAT2A/NAmodII virus was estimated to be 1 μg. This number of cells infected with WSN virus yields approximately 2 to 4 μg of NA protein. Considering that the molecular weight of CAT protein is about half that of NA protein, we would expect 1 to 2 μg of CAT protein to have been produced. It would seem probable that the number of CAT protein molecules in a cell infected with the CAT2A/NAmodII virus would be equal to the number of NA molecules, since both are transcribed and translated from the same promoter sequences. We chose here to express CAT from the NA segment since transfectant viruses altered in NA are currently the most efficiently isolated. However, greater expression of foreign proteins from influenza RNAs may eventually be achieved by incorporation into more highly expressed segments such as those encoding NS, NP, hemagglutinin or M protein.

Influenza virus NA is a type II integral membrane protein. The presence of a large nonviral sequence inserted at the NA amino terminus did not adversely affect the insertion of the NA protein into the membrane or its transport to and expression at the cell surface. This implies that the CAT2A/NA polyprotein is cleaved cotranslationally. Indeed, Drew and Ryan have recently extended their studies of this unique enzyme activity to demonstrate that the 2A protease is able to function with high efficiency in a heterologous context (4), and our own studies presented here confirm this observation. Since the CAT protein is not itself a membrane protein, the orientation of insertion of the NA in the membrane is not affected. A situation could be envisaged in which the two proteins expressed by the polyprotein are both membrane proteins, and in this case care must be taken in the design and placement of signal peptides.

The ability to stably maintain heterologous sequences is desirable for the construction of influenza virus vectors expressing foreign polypeptides. This report may have important implications for the use of influenza virus as an expression vector to induce immunity against other pathogens. By inserting a small autocatalytic protease between a foreign protein and an essential influenza virus protein, we have successfully isolated influenza A viruses which stably express additional genetic material. Extensive analysis of the 2A protease has recently revealed that the core protease is functional in a heterologous context when only 13 residues are included (4). We intend to exploit the versatility of this small, highly efficient enzyme to express a variety of foreign sequences from different influenza virus segments.

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