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Rescue of a Synthetic Chloramphenicol Acetyltransferase RNA into Influenza Virus-Like Particles Obtained from Recombinant Plasmids

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We have shown previously that COS-1 cells infected with a vaccinia virus recombinant (vTF7-3) which expresses the T7 RNA polymerase gene and then transfected with four pGEM-derived plasmids encoding the influenza A virus core proteins (nucleoprotein, PB1, PB2, and PA polypeptides) can express a synthetic influenza virus-like chloramphenical acetyltransferase (CAT) RNA (I. Mena, S. de la Luna, C. Albo, J. Martín, A. Nieto, J. Ortín, and A. Portela, J. Gen. Virol. 75:2109–2114, 1994). Here we demonstrate that by supplying the vTF7-3-infected cells with plasmids containing cDNAs of all 10 influenza virus-encoded proteins, the transfected CAT RNA can be expressed and rescued into particles that are budded into the supernatant fluids. The released particles can transfer the enclosed CAT RNA to MDCK cultures and resemble true influenza virions in that they require trypsin treatment to deliver the RNA to fresh cells and are neutralized by a monoclonal antibody specific for the influenza A virus hemagglutinin. Moreover, analysis by electron microscopy showed that the culture medium harvested from the transfected cells contained vesicles that could be labeled with an anti-HA monoclonal antibody and that were similar in size and morphology to authentic influenza virus particles. It is also shown that detection of recombinant particles capable of transmitting the CAT RNA does not require expression of the influenza virus nonstructural protein NS1. All of these data indicate that influenza virus-like particles enclosing a synthetic virus-like RNA can be assembled in cells expressing all viral structural components from recombinant plasmids.

Influenza A viruses are enveloped orthomyxoviruses whose genomes consist of eight negative-sense single-stranded RNA segments. The six largest RNA molecules each code for one polypeptide (PB1, PB2, PA, nucleoprotein [NP], hemagglutinin [HA], and neuraminidase [NA]), whereas each of the two smallest genomic RNAs gives rise to two coding mRNAs, one collinear with the viral RNA and the other derived by splicing. Thus, segment 7 encodes the proteins M1 and M2, and segment 8 encodes the proteins NS1 and NS2 (reviewed in reference 22).

The HA mediates attachment of the viral particle to the plasma membrane of the target cell and promotes fusion of the viral and endosomal membranes (reviewed in reference 51). The membrane fusion event requires a proteolytically cleaved HA (20, 25) and is activated by conformational changes in the HA at the acidified pH found in the interior of the endosomal vesicles (48). The NA protein displays an enzymatic activity that hydrolyzes sialic acid from HA receptors on the cell surface, and this activity seems to promote the release of virus particles from the cell membrane of infected cells (26, 35). The M2 polypeptide is a membrane protein (23) that has ion channel activity (40). One of its functions is to modulate the pH in the Golgi apparatus and thus prevent the acid pH transition of intracellularly cleaved HAs during its transport from the rough endoplasmic reticulum to the cell membrane (50). The M1 and NS2 proteins are also structural components of the virus (4, 53). The NP and the three subunits of the viral polymerase (PB1, PB2, and PA) are complexed with the genomic viral RNA (vRNA) to form ribonucleoprotein (RNP) structures which are the active templates for viral RNA synthesis (reviewed in reference 21). The NS1 protein is the only nonstructural component of the virus. It has been shown that this protein, when expressed from a cloned cDNA, blocks mRNA nucleocytoplasmic transport (10, 28), alters splicing of pre-mRNAs (10, 28), and stimulates translation of some viral mRNAs (6, 8).

Following fusion of viral and endosome membranes, the RNP complexes are released into the cytoplasm and migrate to the cell nucleus, where transcription and replication of the viral genome occur (15, 18). The incoming RNPs are first transcribed into monocistronic mRNAs that are translated to yield viral proteins that are required for replication of the viral genome (2, 14). Replication of the viral genome involves copying of the vRNA molecules into complementary RNA templates which in turn are used for synthesis of new vRNAs (reviewed in reference 21). Newly synthesized vRNAs are finally exported from the cell nucleus to the cytoplasm and associate with the other structural viral components, by a poorly understood process, to form virus particles that are budded at the plasma membrane and released into the cell medium.

Our understanding of the roles and functional interactions of the influenza virus polypeptides has been greatly enhanced by studies in which cloned viral genes have been expressed (individually or in combination) in mammalian cells. More recently, reverse genetics technology by which RNA molecules derived from cloned genes can be incorporated into influenza virions (29; reviewed in reference 12) is expanding our knowledge of the roles played by the viral proteins during virus replication.

For the two nonsegmented negative-sense RNA viruses vesicular stomatitis virus (VSV) and rabies viruses, it has been possible to reconstitute the entire viral replicative cycle in cells expressing all virus-encoded polypeptides from recombinant cDNAs (5, 36, 38). For these two viruses, it has been shown that synthetic RNA analogs can be encapsidated, replicated,

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and packaged into recombinant virus-like particles (VLPs) in cells expressing all virus-encoded polypeptides from plasmids. In this study, we have analyzed whether a similar system can be established for influenza virus. Huang et al. (16) were the first to show that cells expressing the influenza virus PB1, PB2, PA, and NP proteins from cloned genes could support expression of a synthetic virus-like RNA molecule. We have also described an alternative system in which expression of an influenza virus-like chloramphenicol acetyltransferase (CAT) RNA is achieved in cells infected with recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase, and transfected with four pGEM-derived plasmids encoding the four influenza A virus core proteins (32,33). Here we show that when the vTF7-3-infected cells are transfected with pGEM recombinant plasmids encoding all 10 viral proteins, the CAT RNA is not only expressed but also rescued into VLPs that resemble authentic influenza virions.

MATERIALS AND METHODS

Cell cultures and viruses. COS-1 and MDCK cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. vTF7-3 (11), which expresses T7 RNA polymerase, was kindly provided by B. Moss. The influenza virus strains used were A/Victoria/3/75 (H3N2) and A/Puerto Rico/8/34 (H1N1).

Plasmids and cDNA clones. All influenza virus cDNAs derived from the viral strain A/Victoria/3/75 and were cloned into pGEM-3 or pGEM-4 vectors (Promega) downstream from the T7 RNA polymerase promoter. Plasmids pGEM-PB1, pGEM-PB2, pGEM-PA, and pGEM-NP, encoding the influenza virus PB1, PB2, PA, and NP polypeptides, respectively, have been described elsewhere (32, 33). The origins of influenza virus HA, M1, and NS1 cDNAs were plasmids pSVa 970 (42), pSVa 982 (42), and pSLVa 232N (43), respectively. The corresponding cDNA inserts were excised from these plasmids and subcloned, according to standard protocols, into pGEM vectors to generate plasmids pGEM-HA, pGEM-M1, and pGEM-NS1. Plasmid pGEM-NA, which encodes the NA gene, was obtained from plasmid pSVa1000 (42) and plasmid pBGS-NA5 (31). Plasmids pGEM-M2 and pGEM-NS2, which contain the M2 and NS2 genes, respectively, were derived by reverse transcription-PCR using specific primers and mRNA isolated from influenza virus-infected cells.

Plasmids pPB2CAT9 and pIVACAT1/S (39) were kindly provided by M. Krystal and P. Palese, respectively. These plasmids contain the coding sequence of the CAT gene (in negative polarity) flanked by the 5′ and 3′ nontranslated sequences of the influenza virus segment encoding either the PB2 (pPB2CAT9) or the NS (pIVACAT1/S) proteins. The CAT gene is located downstream from the T7 RNA polymerase promoter.

Synthesis of influenza virus-like CAT RNAs. Plasmids pPB2CAT9 and pIVACAT1/S were digested with the restriction enzyme *Hga*I and transcribed in vitro with T7 RNA polymerase, using a MEGAscript T7 kit (Ambion). Following transcription, the reaction mixture was treated with DNase I, phenol extracted, and precipitated with ethanol. The RNAs were then recovered by centrifugation, resuspended in water, and stored at −80°C until used.

Antibodies and antisera. Monoclonal antibodies (MAbs) M/234/1/F4, M/58/p51/G, M234/1/G10, HA1-50 (provided by J. A. Melero), and 14C2 (a gift from B. Lamb), which recognize the HA, NP, NA, HA, and M2 proteins of influenza virus A/Victoria/3/75, respectively, have been described elsewhere (27, 44, 54). Rabbit antisera against NS1 and NS2 proteins were provided by J. Ortín. Neutralizing anti-vaccinia virus antiserum (a gift from B. García-Barreno) was obtained from rabbits hyperimmunized with vTF7-3.

Immunochemical techniques. (i) Immunoprecipitation. COS-1 cells were infected with vTF7-3 and transfected with the appropriate pGEM plasmids (4 μg), using Lipofectin reagent (Gibco-BRL). After 24 h of incubation, cells were labeled with 50 μCi of Tran^{35}S-label (ICN) for 2 h. Total cell extracts were prepared and immunoprecipitated with MAbs or polyclonal sera as previously described (1).

(ii) Immunofluorescence. Cell cultures were infected with vTF7-3 and transfected as described above. Cells were fixed at 24 h postinfection (p.i.) in cold methanol for 20 min and incubated sequentially with the appropriate primary antibody and with fluorescein-conjugated immunoglobulins containing the nuclear Hoechst dye (0.5 μg/μl).

(iii) Western blotting (immunoblotting). Cell extracts were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to Immobilon-P paper, and developed with the appropriate antibody by using an ECL kit (Amersham).

Infection of COS-1 cell cultures with vTF7-3 and transfection with plasmid DNAs and CAT RNA. The standard protocol (outlined in Fig. 3) was done as follows. Confluent cultures of 10^6 COS-1 cells growing in 35-mm-diameter dishes were incubated in DMEM-Ara (serum-free DMEM containing 40 μg of cytosine β-D-arabinofuranoside [AraC] per ml and lacking antibiotics) for 1 h. The medium was then aspirated, and cell cultures were inoculated with vTF7-3 (multiplicity of infection of 5) diluted in DMEM-Ara. One hour later, the inoculum was removed, and the cultures were washed once with DMEM-Ara and incubated with 500 μl of DMEM-Ara supplemented with 100 μl of a Lipofectin–DNA–DMEM-Ara mixture. This mixture contained 50 μl of DMEM-Ara, 20 μl of Lipofectin reagent, 2 μg of plasmid pGEM-NP, 1 μg of plasmids pGEM-PB1, pGEM-PB2, pGEM-HA, pGEM-NA, pGEM-M1, pGEM-M2, pGEM-NS1, and pGEM-NS2, and 200 ng of plasmid pGEM-PA. After 5 h of incubation, the medium was replaced with 500 μl of DMEM-Ara containing 1 μg of CAT RNA, 4 μg of yeast tRNA, and 15 μl of Lipofectin. Following overnight incubation, the medium was removed and 1 ml of DMEM-Ara-Ant (DMEM containing 100 U of penicillin, 100 μg streptomycin, and 40 μg per ml of AraC) was added. At the indicated times (usually 48 or 72 h p.i.), the cell supernatants were harvested and cell extracts were prepared as described below.

Incubation of MDCK cell cultures with the supernatant obtained from transfected COS-1 cells and superinfection with influenza virus. The culture medium harvested from the transfected COS-1 cultures was centrifuged for 1 min in a microcentrifuge to remove floating cells and stored frozen at −20 or −80°C. Supernatant aliquots were subjected to one to three freezing (−80°C)-thawing (37°C) cycles and clarified by centrifugation in a microcentrifuge for 5 to 15 min. The clarified supernatant was then incubated at 37°C with trypsin (final concentration, 2.5 μg/ml) for 15 min, and aliquots (100 to 400 μl) of this treated supernatant were added to 10^6 MDCK cells (growing in 35-mm-diameter petri dishes) which had been incubated in DMEM-Ara-Ant for 1 h. Cells were incubated with the trypsin-treated supernatant for 30 to 60 min at 37°C with occasional rocking before the medium was removed. Cell cultures were washed with 1 ml of DMEM-Ara-Ant, and cells were infected with influenza virus A/Victoria/3/75 at a multiplicity of infection of 1. At the indicated times (usually 18 h p.i.), cell extracts were prepared as described below.

Preparation of MDCK and COS-1 cell extracts. Cell cultures were scraped off the plates, and cells were collected by a brief spin in a microcentrifuge. The pelleted cells were washed with 1× TNE (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA), resuspended in 100 μl of 0.25 M Tris (pH 7.5), and lysed by three cycles of freezing and thawing. Cellular debris were removed by centrifugation for 5 min in a microcentrifuge, and the supernatant was used for determination of CAT activity as described by Gorman et al. (13). Briefly, aliquots of the cell extracts (routinely 0.1 μl of COS-1 cell extracts and 20 to 50 μl of MDCK cell extracts) were incubated in a 150-μl mixture containing 0.5 mM acetyl coenzyme A and 0.1 μCi of [^{14}C]chloramphenicol. Following overnight incubation at 37°C, the mixture was extracted with ethyl acetate, and the organic phase was lyophilized, resuspended in 20 μl of ethyl acetate, and spotted onto a thin-layer plate. This plate was developed in a chromatographic tank with chloroform-methanol (19:1) and exposed for autoradiography for 1 or 2 days.

EM. For negative staining, a drop of the cell culture supernatant was applied to carbon-coated Formvar copper grids, and the preparations were stained for 40 s at room temperature with 2% phosphotungstic acid (pH 7.2). The samples were examined in a Philips 400T electron microscope. For immunogold labeling, aliquots of culture medium were adsorbed onto carbon-coated grids. The grids were then incubated sequentially at room temperature with buffer A (1% bovine albumin–5% goat serum in phosphate-buffered saline) for 10 min, with MAb M234/1/F4 (anti-HA) diluted in buffer A for 90 min, and with immunogold conjugate electron microscopy (EM) goat anti-mouse immunoglobulins G and M (10 nm; Biocell) diluted in buffer A for 2 h and then were washed with buffer A and with water. Finally, the preparations were negatively stained and visualized as indicated above.

RESULTS

Expression of all influenza virus-encoded polypeptides from pGEM recombinant plasmids. Plasmids containing the genes that encode the PB1, PB2, PA, NP, NA, HA, M1, and NS1 proteins of influenza virus A/Victoria/3/75 were obtained by transferring previously cloned cDNAs to plasmid pGEM-3 or pGEM-4 downstream from the T7 RNA polymerase promoter (Fig. 1). The transient expression system based on a vaccinia virus recombinant (vTF7-3) that expresses the T7 RNA polymerase (11) was used for expression of the influenza virus cloned genes. In this system, transcription of the target cloned gene by the T7 RNA polymerase takes place in the cell cytoplasm, and hence the T7-derived transcripts are not spliced. Therefore, pGEM-derived plasmids containing cDNAs of the influenza virus spliced mRNAs coding for the NS2 and M2 proteins were also obtained (Fig. 1). It should be noted that none of the recombinant plasmids was expected to yield authentic cRNA molecules in the vTF7-3-infected cells since the T7-derived transcripts would contain additional non-virus-en-

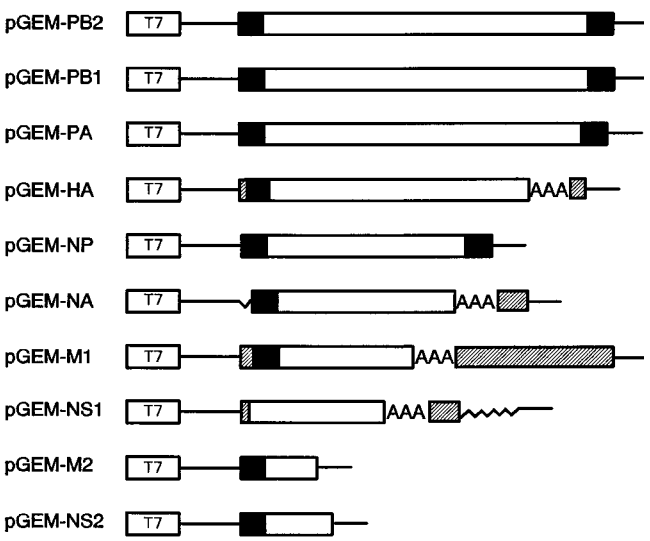

FIG. 1. Structures of the pGEM-derived plasmids containing cDNAs of all 10 influenza virus-encoded polypeptides. The cDNAs corresponding to the M1 and NS1 genes were cloned in plasmid pGEM-4, and the rest of the viral cDNAs were cloned in plasmid pGEM-3. Symbols: T7, T7 RNA polymerase promoter; —, pGEM sequences; □, influenza virus nucleotide sequences corresponding to each of the 10 viral genes (drawn to scale); AAA, poly(A) tail; ▨, simian virus 40-derived sequences (drawn to scale); ∿, pBR322-derived sequences (drawn to scale); ■, conserved nontranslated nucleotide sequences at the ends of the influenza virus genomic RNA segments.

coded nucleotides (derived from plasmid sequences) at both the 5′ and 3′ ends of the corresponding influenza virus RNA segment. Moreover, the cDNAs encoding the HA, NA, M1, M2, NS1, and NS2 proteins were derived from viral mRNAs and therefore do not contain the 3′ nontranslated nucleotide sequences present in the corresponding cRNA molecules.

Functional expression of the NP and the three P proteins from plasmids pGEM-NP, pGEM-PB1, pGEM-PB2, and pGEM-PA has already been demonstrated (32, 33). The expression of the other cloned viral gene products was tested in cells infected with vTF7-3 and transfected with the pGEM-derived plasmids by immunofluorescence and/or immunoprecipitation tests (Fig. 2). As shown in Fig. 2A, labeled bands which comigrate with the influenza virus M1 (lane 3), NS1 (lane 4), NS2 (lane 5), and HA (lane 6) polypeptides were detected in ^{35}S-labeled cell lysates obtained from cell cultures transfected with the corresponding pGEM recombinant plasmids. These protein bands were not detected in cells infected with vTF7-3 but not transfected (lane 2). The identities of the HA, NS1, and NS2 protein bands were confirmed in immunoprecipitation assays using specific antibodies (Fig. 2B). Expression of the M2 and NA polypeptides from plasmids pGEM-M2 and pGEM-NA, respectively, was demonstrated in immunofluorescence assays (Fig. 2C and D). Immunostaining of cells was specific for the corresponding viral protein, since (i) the antibodies used did not label mock-transfected cells (data not shown) and (ii) as can be observed in Fig. 2 (compare panel C with C′ and panel D with D′), not all cells from the transfected cultures were detected by indirect immunofluorescence.

Rescue of synthetic CAT RNAs. To investigate whether coexpression of all 10 influenza virus-encoded proteins would allow a synthetic CAT RNA transfected into these cells to be expressed and rescued into influenza virus-like particles, the protocol outlined in Fig. 3 was followed (details are given in Materials and Methods). Detection of CAT activity in COS-1 cell extracts would demonstrate expression of the CAT RNA mediated by the recombinant viral polymerase, and detection of CAT activity in the MDCK cell extracts would be an indication of the presence of particles enclosing the synthetic CAT RNA in the supernatant fluids harvested from the COS-1 cell cultures.

As indicated in Fig. 3, the culture medium recovered from the transfected COS-1 cells was routinely incubated with trypsin since the A/Victoria/3/75 HA must be cleaved by this enzyme for the virions to be fully infective. As also noted in Fig. 3, the MDCK cells were incubated with the trypsin-treated supernatant and then infected with a wild-type influenza virus. Infection with wild-type virus would provide in *trans* the viral proteins needed for amplification and secondary transcription of the rescued CAT RNA molecules (2, 14). It should also be mentioned that COS-1 and MDCK cell cultures were maintained throughout the experiment in medium containing AraC, an inhibitor of vaccinia virus replication and late gene expression (19). AraC, however, did not reduce the plaquing efficiency of influenza virus A/Victoria/3/75 and did not significantly affect expression of a CAT RNA in COS-1 cells coexpressing the PB1, PB2, PA, and NP proteins of influenza virus from recombinant plasmids (data not shown).

In the first experiment, two different RNA molecules were tested. These RNA templates contained the CAT gene in negative polarity flanked by the nontranslated nucleotide sequences of the influenza virus segments encoding either the PB2 (PB2-CAT RNA) or NS (NS-CAT RNA) protein (Fig. 4A to C). The CAT RNAs were transfected into COS-1 cell cultures that expressed, from recombinant pGEM plasmids, all 10 influenza virus-encoded proteins (samples 1 and 2) or only 9 of them (all but the HA) (sample 3). Aliquots of the supernatant fluids from these cultures were harvested at 24 and 48 h p.i. and assayed for the presence of viral recombinant particles as summarized in Fig. 3. Although efficient expression of the CAT reporter enzyme was detected in the COS-1 cell extracts of the three samples (Fig. 4A), CAT activity was detected in MDCK cultures of samples 1 and 2 when supernatants collected at 48 h p.i. were used (Fig. 4C) but not when the MDCK cells were incubated with the supernatants collected at 24 h p.i. (Fig. 4B). Since the COS-1 cultures from sample 3 did not express HA and this protein is essential for infectivity of influenza virus particles, these results suggested the presence of HA-containing particles in the supernatant fluids harvested from samples 1 and 2. On the basis of the results shown in Fig. 4A to C, the NS-CAT RNA was selected for further experiments, and the supernatants from the COS-1 cells were always harvested at 48 to 72 h following infection with vTF7-3. It should be mentioned that the efficiency of the system, as regarding the level of CAT activity detected in the MDCK cultures, was usually very low. In fact, in most of the experiments shown in this report, the CAT activities detected in the MDCK cells were obtained following incubation of the MDCK cells with one-third of the culture medium harvested from transfected cells and upon testing of one-third of the MDCK cell extracts in the CAT assay (see details in Materials and Methods).

The CAT RNA is packaged into HA-containing particles. To determine whether the CAT activity detected in the MDCK cultures was mediated by HA-containing particles, a series of control experiments was conducted (Fig. 4D). Detection of CAT activity in MDCK cell extracts was abolished by incubation of the supernatant obtained from the COS-1 cells with an anti-HA MAb that neutralizes influenza A/Victoria/3/75 virions. Furthermore, the absence of CAT expression in those extracts was not due to a residual activity of the antibody on the helper A/Victoria/3/75 virus, since a similar result was obtained when the viral strain A/Puerto Rico/8/34, which is not

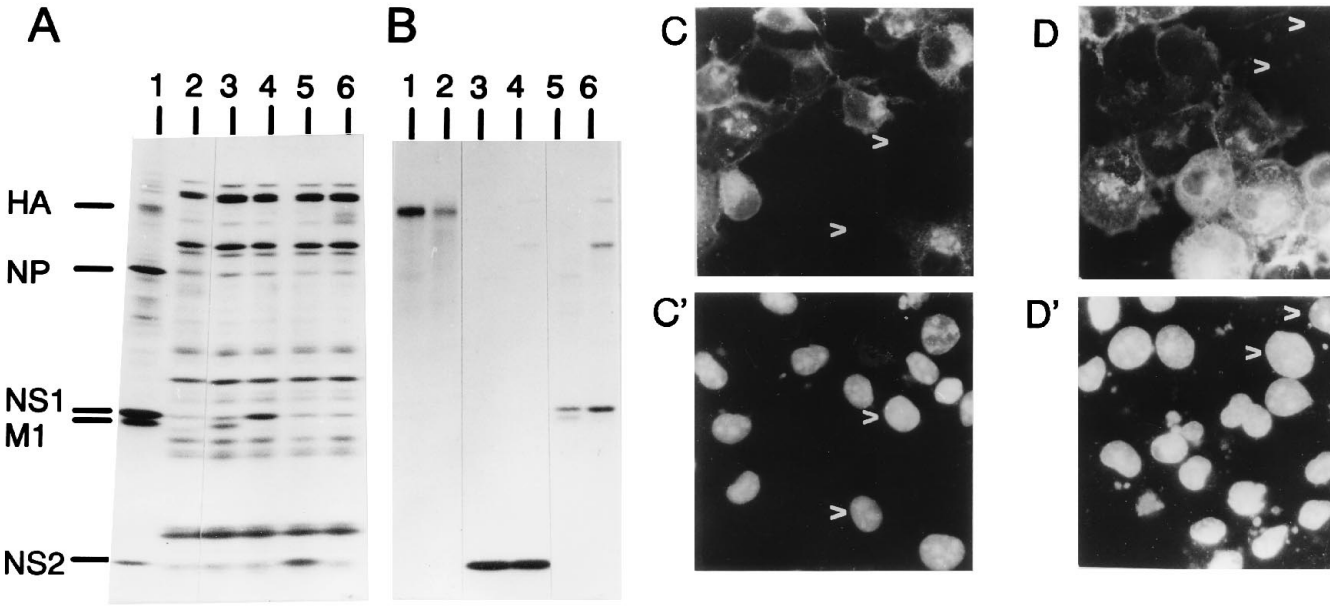

FIG. 2. Expression of influenza virus proteins from cloned cDNAs. (A) COS-1 cells were infected with influenza virus A/Victoria/3/75 (lane 1) or with vaccinia virus vTF7-3 (lanes 2 to 6). Cells infected with vTF7-3 were then mock transfected (lane 2) or transfected with plasmid pGEM-M1 (lane 3), pGEM-NS1 (lane 4), pGEM-NS2 (lane 5), or pGEM-HA (lane 6) as described in Materials and Methods. Twenty hours later, cell cultures were labeled with Tran^{35}S-label for 2 h, and cell extracts were prepared. The proteins present in the extracts were resolved in an SDS–5 to 20% gradient polyacrylamide gel and visualized by fluorography. Positions of influenza virus proteins are indicated at left. (B) The labeled extracts from cells infected with influenza virus (lanes 1, 3, and 5) and from COS-1 cultures infected with vTF7-3 and transfected with plasmid pGEM-HA (lane 2), PGEM-NS2 (lane 4), or pGEM-NS1 (lane 6) were immunoprecipitated with specific antibodies raised against the HA (lanes 1 and 2), NS2 (lanes 3 and 4), or NS1 (lanes 5 and 6) polypeptide. Antibody-bound proteins were resolved by SDS-PAGE and visualized by autoradiography. (C and D) COS-1 cells were infected with vTF7-3 and transfected with plasmid pGEM-NA (C) or pGEM-M2 (D). Twenty hours later, cell cultures were fixed, and the influenza virus proteins were localized by indirect immunofluorescence using antibodies to the NA and M2 polypeptides, respectively (C and D). The same fields were visualized by staining with the Hoechst nuclear dye (C′ and D′). Arrowheads show cells that were not stained with the antibodies used.

neutralized by the anti-HA MAb used, was the superinfecting viral strain. CAT activity in MDCK cell extracts was, however, unaffected by pretreatment of the COS-1 cells supernatant with (i) RNase A, (ii) an anti-NP MAb that inhibits the transcriptase activity associated with purified RNPs (1), or (iii) an anti-vaccinia virus antiserum that reduces 100-fold the infectivity of vTF7-3 in plaque assays. In addition, and as predicted, trypsin treatment of the supernatant was an absolute requirement for detection of CAT expression in MDCK cells. Taken together, these results demonstrate that the CAT activity detected in MDCK cells was mediated by HA-containing vesicles and not by vaccinia virus particles, RNP complexes, or naked CAT RNA molecules. Moreover, the HA-containing particles enclosed a CAT RNA and not CAT enzyme, since (i) infection with a helper virus was needed to detect CAT activity in the MDCK cells and (ii) CAT activity was not detected in MDCK cell extracts prepared at early times following influenza virus infection but was detected at later times, presumably as a consequence of the amplification of the CAT RNA (Fig. 4D).

Viral proteins required for detection of recombinant particles. It was of interest to know whether any of the influenza virus-encoded proteins were dispensable for formation of particles capable of transmitting the CAT RNA to fresh cells. To this end, experiments were performed as indicated in Fig. 3, using COS-1 cell cultures that had been transfected with either all 10 pGEM-derived plasmids or only 9 of them. The corresponding COS-1 cell samples were designated ALL (transfected with the 10 plasmids) and -HA, -NA, -M1, -M2, -NS1, and -NS2 (where a dash denotes the missing gene product). First, the transfected COS-1 cells were examined for accumulation of various recombinant proteins by Western blotting. As shown in Fig. 5, similar amounts of NP and HA proteins accumulated in all extracts transfected with the corresponding plasmids. Similar results were obtained when the extracts were analyzed for accumulation of the PA polypeptide (data not shown). These results therefore showed that transfections with different combinations of plasmids do not drastically affect the accumulation of the recombinant proteins analyzed. It is worth mentioning that the HA protein detected in the transfected cells has a mobility corresponding to that of the uncleaved HA0 precursor (Fig. 5B). Therefore, the particles present in the supernatant from the COS-1 cultures would contain uncleaved HA, and hence the strict requirement of trypsin treatment for detection of recombinant particles is explained (Fig. 4D). We have also noted that the HA0 precursor migrates more slowly in SDS-polyacrylamide gels when the M2 protein is not expressed. This effect is barely visible in the SDS–10% polyacrylamide gel shown in Fig. 5, but it was clearly apparent when the proteins were resolved in a lower-percentage polyacrylamide gel (data not shown).

A total of six independent experiments using three different plasmid preparations (A, B, and C) were carried out to analyze the abilities of the supernatants collected from cells transfected with different combinations of plasmids to transmit the synthetic CAT RNA to MDCK cells. The results of four of these experiments, in which CAT expression was also monitored in the COS-1 cell extracts, are shown in Fig. 6. In all experiments, efficient expression of the reporter gene was observed in the COS-1 cell cultures. However, there were differences among the various cultures analyzed within the same experiment. These differences are due, at least in part, to the variability of the transfection process because they were not consistently detected in all experiments. Importantly, the differences in CAT expression detected in COS-1 cells did not

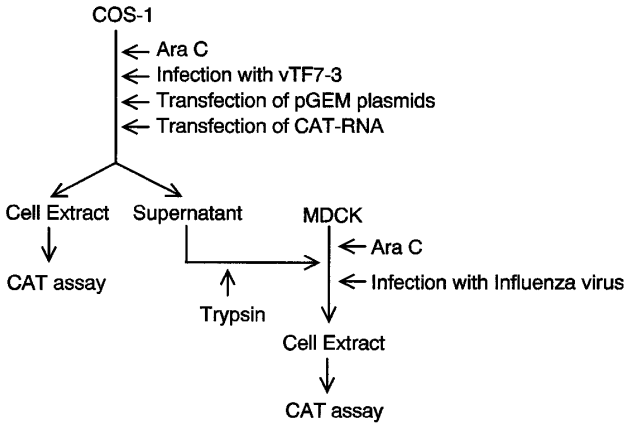

FIG. 3. Scheme of the protocol used to detect influenza VLPs in the supernatant from COS-1 transfected cultures.

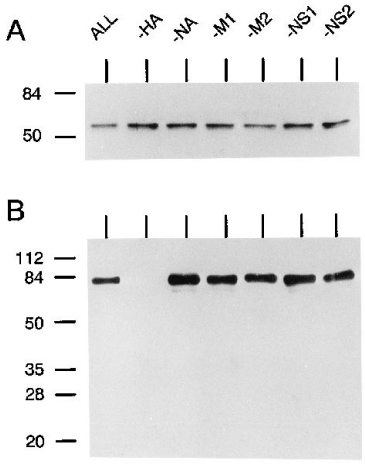

FIG. 5. Western blotting analysis of COS-1 cell cultures transfected with different combinations of plasmids. COS-1 cells were infected with vTF7-3 and transfected with the combinations of plasmids indicated at the top (see text). At 72 h p.i., cell extracts were prepared and resolved by electrophoresis in an SDS–10% polyacrylamide gel. The proteins were then electroblotted onto Immobilon-P paper and developed with a rabbit anti-NP antiserum (A) or an anti-HA MAb (MAb HA1–50) (B). Molecular weights of prestained markers are indicated on the left in thousands.

strictly correlate with those observed in the corresponding MDCK cell extracts.

In the six experiments done, the supernatants from cultures -HA and -NA never transmitted detectable levels of CAT RNA to MDCK cultures, whereas in the six same experiments, particles enclosing the CAT RNA were detected in the supernatants from cultures -NS1. In all but one of the six experiments (experiment 2 in Fig. 6), the supernatants from cultures ALL were positive for CAT RNA transmission and yielded, in MDCK cells, CAT expression levels similar to those of the corresponding -NS1 samples.

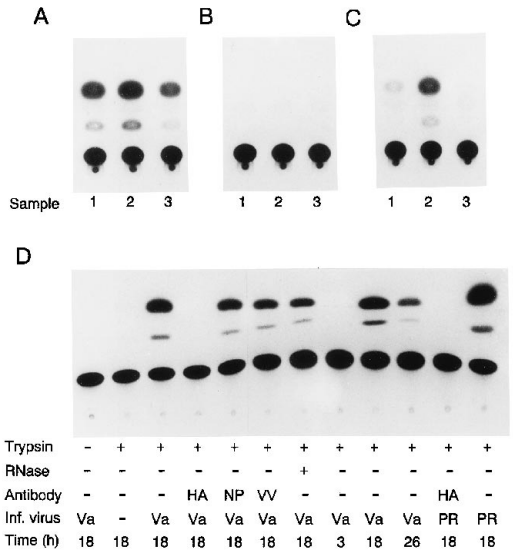

FIG. 4. Transmission of CAT RNA to MDCK cells. COS-1 cells were infected with vTF7-3 and transfected with the 10 pGEM recombinant plasmids encoding all influenza virus genes (samples 1 and 2) or with only 9 of them (all but plasmid pGEM-HA) (sample 3). Five hours later, cells were transfected again with either PB2-CAT RNA (sample 1) or NS-CAT RNA (samples 2 and 3). Supernatants from these cultures were collected at 24 and 48 h p.i. and processed as indicated in Fig. 3. Aliquots of the COS-1 cell extracts (A) and of the MDCK cultures (B and C) obtained when supernatants harvested at 24 (B) or (C) 48 h p.i. were used were assayed for CAT activity, using [^{14}C]chloramphenicol and chromatography on thin-layer chromatography plates. (D) The supernatant obtained from COS-1 cells infected with vTF7-3 virus and transfected with all 10 pGEM recombinant plasmids was harvested. Aliquots of this supernatant were then incubated with trypsin, RNase A, an MAb specific for the HA (MAb 234/1/F4) (HA) or the NP (MAb M58/p51/G) (NP) protein of influenza virus, or a neutralizing anti-vaccinia virus antiserum (VV). The treated supernatants were then added to MDCK cells, which were then mock infected or infected with the influenza virus strain A/Victoria/3/75 (Va) or A/Puerto Rico/8/34 (PR). Cells extracts were prepared at the indicated times p.i. (expressed in hours) and assayed for CAT activity as described above.

The results obtained with the supernatant from cultures -M2 varied depending on the plasmid preparation used. With plasmid preparation A (experiments 1 and 2 in Fig. 6), significant CAT expression levels were consistently detected in MDCK extracts, whereas with plasmid preparations B (experiments 3 and 4) and C (not shown), no CAT activity or background signals (less than 10% of the activity in sample ALL) were observed. It was verified by immunofluorescence, that none of the plasmids of preparation A was contaminated with a plasmid expressing the M2 protein (the level of detection being 1%). Therefore, the results obtained in experiments 1 and 2, with respect to the requirement of M2 protein for transmission of the CAT RNA, reflect a feature intrinsic to plasmid preparation A.

The supernatant fluids harvested from samples -M2 and -NS1 were examined as indicated in Fig. 4D for the supernatant from culture ALL. It was observed that the recombinant particles present in supernatant from samples -M2 and -NS1 behave like those present in the supernatant from culture ALL, since transmission of the CAT RNA to MDCK cells (i) required treatment of supernatant with trypsin and superinfection with an influenza helper virus and (ii) was abolished by incubation of the supernatant with a neutralizing anti-HA MAb but was unaffected by incubation with an anti-NP MAb, anti-VTF7-3 antiserum or RNase A (data not shown).

The supernatants from cultures -M1 and -NS2 either were negative for CAT RNA transmission (in four experiments) or yielded background CAT expression levels (10 to 20% of the level in sample ALL) (in two experiments). It should be noted, however, that in experiment 4 (Fig. 6), in which background signals were detected, the corresponding COS-1 cell extracts yielded levels of CAT expression higher than those observed in cultures ALL and -NS1. Thus, if the CAT signals detected in MDCK cell extracts were corrected by taking into account

those observed in the transfected cells, the efficiency of rescuing the CAT RNA with supernatants -M1 and -NS2 would be much smaller than reflected by direct examination of the CAT activities detected in MDCK cell extracts.

From all these experiments, it was concluded that expression of all structural components of the viral particle, with the possible exception of M2, is required for efficient transmission of the CAT RNA to MDCK cells.

Visualization of particles by EM. Influenza virions have a peculiar morphology characterized by a lipid envelope surrounded by spikes that correspond to the viral glycoproteins HA and NA. The particles are pleomorphic and have a diameter of 80 to 120 nm, and some appear as long filaments (reference 7 and references therein; 47). To determine whether influenza VLPs could be detected in the culture medium of transfected cells, the supernatants from COS-1 cultures ALL, -M1, -HA, and -NS1 were examined by negative staining and EM. Particles resembling influenza virions with respect to morphology and size were detected in the culture media collected from cultures ALL and -NS1 (Fig. 7) but not in those harvested from cultures -HA and -M1. Importantly, the particles contain HA at their surface, since they were labeled with an anti-HA MAb (representative micrographs are shown in Fig. 8) but not with an unrelated MAb (data not shown).

DISCUSSION

In this report, we have shown that the culture medium harvested from cells that were transfected with a synthetic CAT RNA and that express all influenza virus-encoded proteins from recombinant plasmids can transmit the synthetic RNA to MDCK cultures. Efficient transmission of the CAT RNA (i) depended on trypsin treatment of the culture medium, (ii) was abolished by preincubation of the medium with an anti-HA MAb, and (iii) required expression of all viral structural components, with the possible exception of M2 (see below). Moreover, the supernatants from cultures -M1 and -NS2, both of which express the HA and NA proteins, either were negative for CAT RNA transfer (four experiments) or yielded background CAT activities in MDCK cells (two experiments). These results indicate that transmission of the CAT RNA by supernatants from cultures expressing all viral structural components is not mediated by vesicles containing only the HA and NA proteins. Furthermore, EM studies showed that the supernatant collected from transfected cultures contains vesicles that can be labeled with an anti-HA MAb and that resemble, in size and morphology, authentic influenza virus particles. Taken together, these data strongly suggest that transmission of the reporter RNA molecule is mediated by recombinant influenza VLPs enclosing the CAT RNA. The system reported here is thus similar to those described for the enveloped negative-sense RNA viruses VSV and rabies virus (5, 36, 38). It should be noted, however, that influenza virus, unlike VSV and rabies virus, has a segmented RNA genome and that the complexity of the viral particle in terms of structural proteins is higher; influenza virions contain nine polypeptides, whereas VSV and rabies virus particles possess only five proteins.

It is unclear whether there is a mechanism by which viral particles containing the eight genomic RNAs are preferentially assembled in influenza virus-infected cells. Although the findings reported here do not preclude the existence of such a mechanism, they show that packaging of all eight vRNAs is not a strict requirement for formation of influenza VLPs. This interpretation is in consonance with data that indicate that virions containing more or less than the eight vRNA segments are released from influenza virus-infected cells (9, 30).

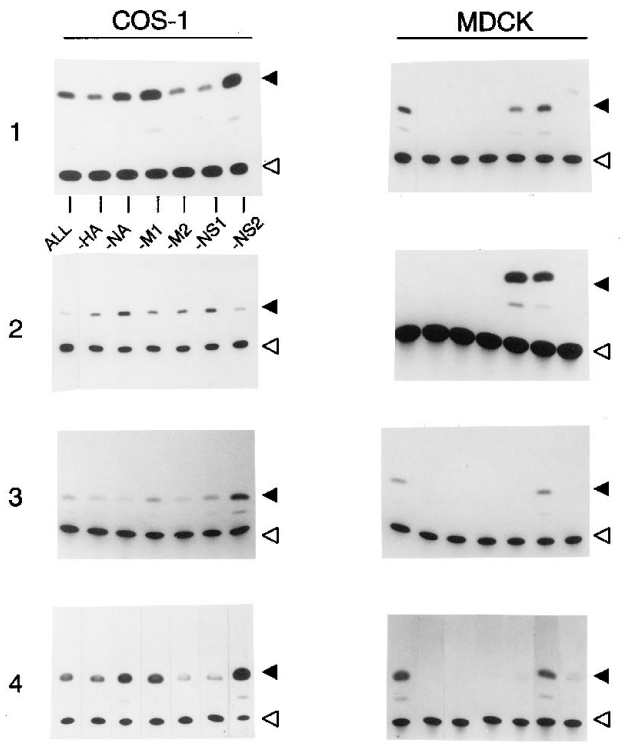

FIG. 6. Analyses of the supernatants obtained from COS-1 cells transfected with different combinations of plasmids for transmission of the CAT RNA to MDCK cells. COS-1 cells cultures were infected with vTF7-3, transfected with the plasmids indicated (see text), and assayed for CAT RNA expression (COS-1). The supernatants from these cultures were tested on MDCK cells as schematized in Fig. 3, and the corresponding MDCK cell extracts were assayed for CAT activity (MDCK). Experiments 1 and 2 were carried out with plasmid preparation A; experiments 3 and 4 were carried out with preparation B. In all panels, the order of the samples is as indicated for the COS-1 cell extracts in experiment 1. The positions of [^{14}C]chloramphenicol (open arrowheads) and 3-acetylated [^{14}C]chloramphenicol (black arrowheads) are indicated.

Interestingly, expression of NS1 was dispensable for detection of recombinant VLPs in biochemical tests (detection of CAT activity in MDCK cells) and in EM studies. This observation, which agrees with the fact that NS1 has never been detected in virus particles, shows that the roles played by NS1 protein during the viral replicative cycle are not needed in the system described here. The NS1 protein inhibits nucleocytoplasmic transport of mRNAs (10, 28), and it has been postulated that this activity increases the availability of capped mRNAs in the nuclei of infected cells to be used for the generation of capped primers that are needed for synthesis of virus-specific mRNAs (41). In the vaccinia virus/T7 expression system used here, the viral proteins are translated from T7-derived transcripts made in the cytoplasm. Therefore, the lack of NS1 expression should not affect the levels of mRNAs encoding the viral proteins, although it may affect the CAT mRNA levels since these transcripts are synthesized by the recombinant influenza virus polymerase in the cell nucleus. The NS1 protein has also been shown to be involved in increasing the translation efficiency of some viral mRNAs (6, 8) and in modulating splicing of influenza virus mRNAs (10, 28). It can be suggested that these two functions are dispensable for formation of recombinant particles because (i) an intrinsic feature of the vaccinia virus/T7 system is high expression levels of the target genes and (ii) the influenza virus spliced mRNA gene products (NS2 and M2) are synthesized in the cytoplasm

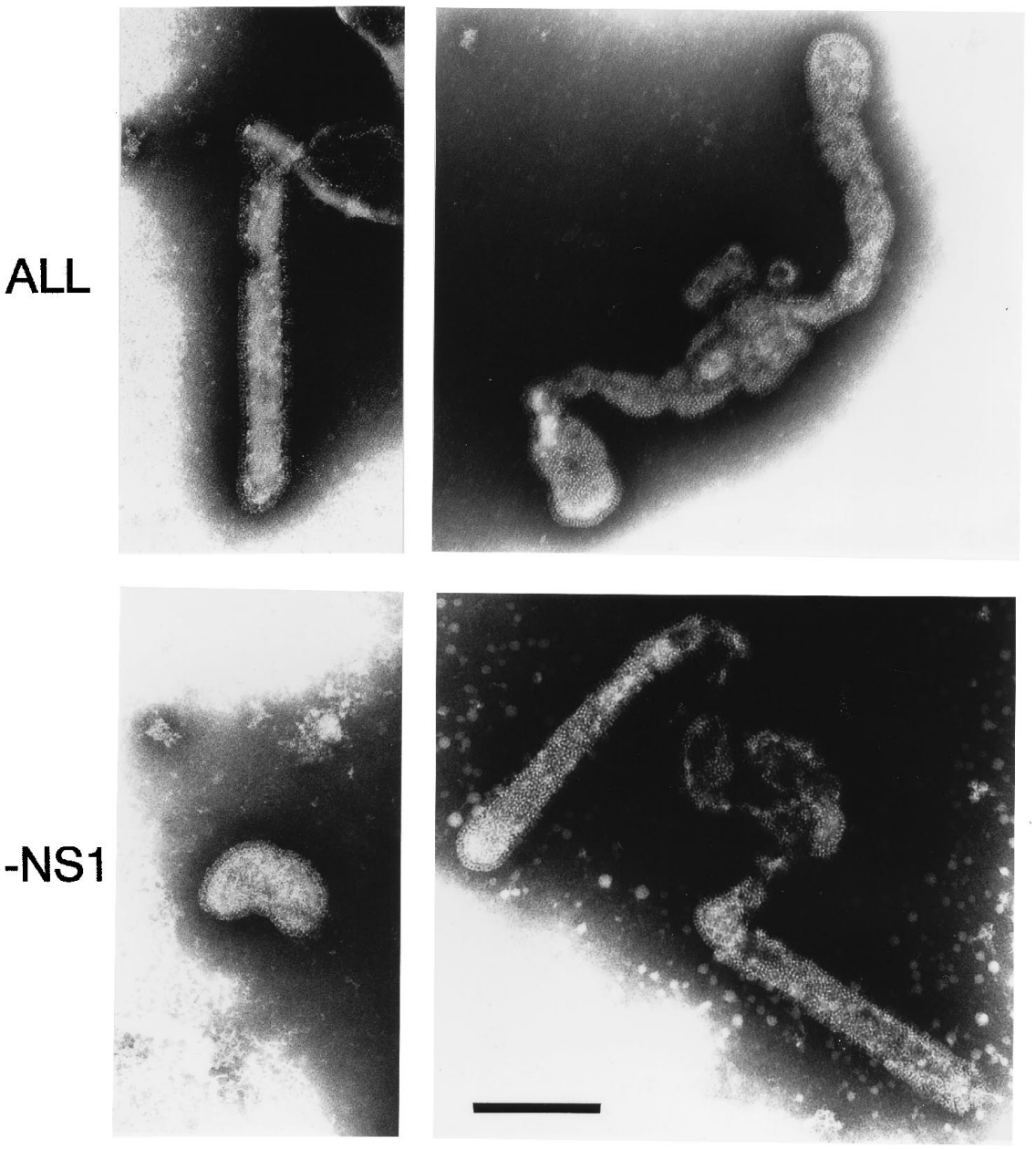

FIG. 7. Electron micrographs of the particles detected in the supernatant harvested from COS-1 transfected cells. Aliquots of the supernatants obtained from cultures ALL and -NS1 were adsorbed onto carbon-coated grids and negatively stained with phosphotungstic acid as described in Materials and Methods. Representative micrographs of the VLPs detected in the supernatant fluids are shown. The bar represents 200 nm.

from plasmids different from those yielding the nonspliced NS1 and M1 mRNAs.

Different results, which correlated with the plasmid preparation used, were obtained regarding the requirement of M2 expression for detection of VLPs in the biochemical tests. One of the functions of M2 is to regulate the pH in the Golgi complex and thus prevent, in viruses whose HA is cleaved by intracellular proteases, the pH-sensitive conformational changes of the HA during its transport to the cell membrane (50). Considering that the A/Victoria/3/75 HA is not cleaved by intracellular proteases, it may be suggested that expression of M2 is not needed for accumulation of functional HA at the COS-1 cell membrane (34). However, it appears that M2 is required for the generation of infectious virus particles, since this protein is involved in organization of virion particles (17, 55) and in an early step following virus infection (3, 30, 40, 49). We can only speculate that the different results obtained when independent plasmid preparations were used are due to differences in the amounts (or ratios) of viral proteins being expressed with each preparation. It may be that with one of the plasmid preparations, particles containing a particular ratio of protein components (such as the NP/M1 ratio) that compensate for the lack of M2 were formed. Further experiments are needed to confirm this hypothesis.

The system described here opens the possibility of studying the role of each of the influenza virus proteins in virus repli-

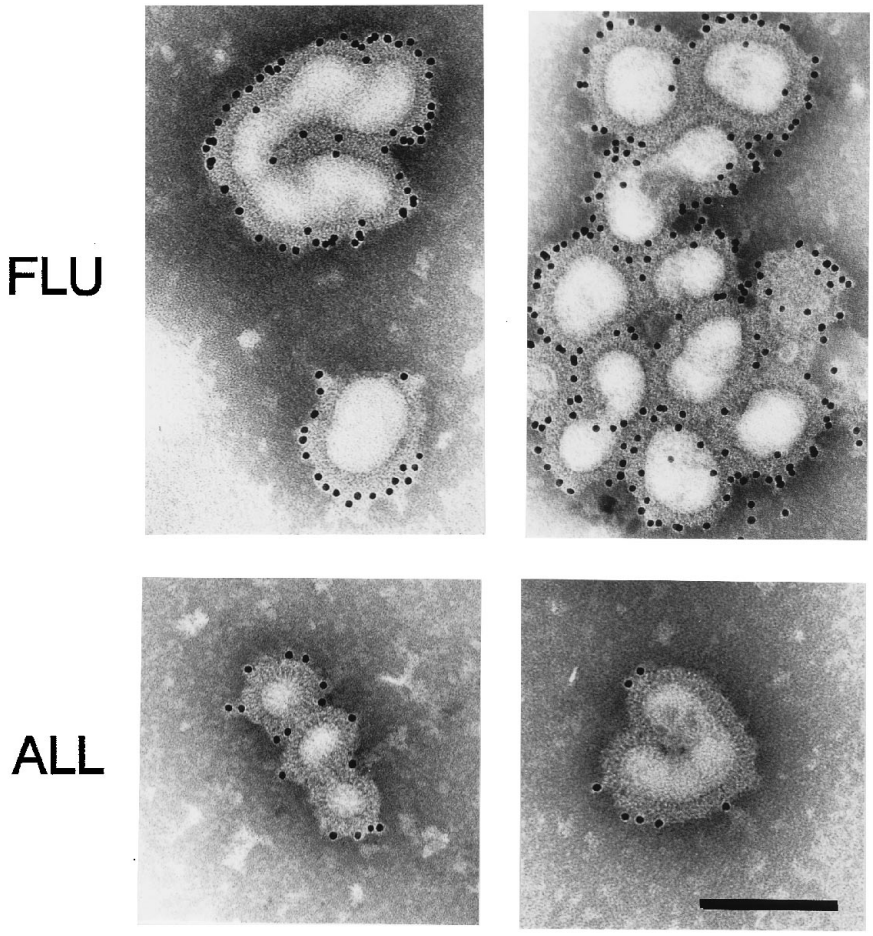

FIG. 8. Immunoelectron microscopy of wild-type influenza virus A/Victoria/3/75 particles and recombinant particles obtained from COS-1 transfected cells. Aliquots of the supernatants harvested from either influenza virus-infected cells (FLU) or COS-1 cells transfected with the 10 pGEM recombinant plasmids (ALL) were absorbed onto carbon-coated grids and incubated with the anti-HA MAb M234/1/F4 and with goat anti-mouse antibodies coupled to 10-nm colloidal gold. Prior to examination, the preparations were negatively stained with phosphotungstic acid. Representative micrographs of the particles detected are shown. The bar represents 200 nm.

cation. However, to perform these studies systematically, the efficiency of the system must be increased. As indicated above, when the supernatant harvested from culture ALL was used, the levels of CAT activity detected in MDCK cells were usually very close to the limit of detection of the CAT assays, and in ~25% of the experiments, we have failed to detect transmission of the CAT reporter gene to MDCK cells. In this regard, it is worth mentioning that more than 90% of the transfected cells expressed the recombinant proteins, whereas only 1 to 5% of them were stained with an antiserum raised against CAT enzyme. It is thus reasoned that if the number of cells expressing the CAT RNA is increased, the efficiency of the system described here would most likely be higher. Similarly, we think that the efficiency of the system will increase upon finding an optimal ratio of transfected plasmids as has been shown to be the case in systems in which replication and transcription of viral replicons are achieved in cells expressing viral proteins from recombinant plasmids (37, 45).

Influenza viruses in which one of their segments has been replaced by transcripts derived from recombinant plasmids have been obtained (29; reviewed in reference 12). However, unlike VSV and rabies virus (24, 46, 52), an influenza virus derived completely from cDNA has not yet been described. To reach this goal, an important limitation is that the eight RNA segments have to be introduced into the same cell. The system described here offers a way of overcoming this problem. Recombinant VLPs, enclosing each of the vRNA segments, could be obtained from independent cultures, and mixtures of the resulting VLPs could then be used to infect fresh MDCK cultures. In this way, the eight RNPs would be delivered in the same cell following the natural process taken place in a viral infection.

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