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Efficient formation of influenza virus-like particles: dependence on the expression levels of viral proteins

Paulino Gómez-Puertas,[1] Ignacio Mena,[1]† Mar Castillo,[1] Amparo Vivo,[2] Esperanza Pérez-Pastrana[2] and Agustín Portela[1]

Centro Nacional de Biología Fundamental[1] and Centro Nacional de Microbiología[2], Instituto de Salud Carlos III, Majadahonda 28220, Madrid, Spain

It has previously been demonstrated in this laboratory that an influenza virus-like chloramphenicol acetyltransferase (CAT) RNA could be expressed in COS-1 cells that synthesized all ten influenza A virus-encoded proteins from recombinant plasmids. It was also shown that supernatant fluids harvested from these cultures contained virus-like particles (VLPs) that could deliver an enclosed CAT RNA to MDCK cells. Here, it is shown that the levels of expression of the reporter gene in the COS-1 and/or MDCK cells can be altered drastically by modifying the concentrations of the recombinant plasmids transfected in the COS-1 cells. Thus, it was observed that overexpression of NS2 reduced CAT expression in COS-1 cells, whereas overexpression of M2 and NS1 proteins dramatically decreased transmission of the CAT RNA to the MDCK cultures. These results are discussed with reference to the roles of these proteins during virus replication. From these experiments, a ratio of transfected plasmids was found that increased the efficiency of the previously described system by 50–100-fold. Under these optimized conditions, it was demonstrated that VLPs can be formed in the absence of neuraminidase expression and that these VLPs remained aggregated to each other and to cell membranes. Moreover, it is shown that CAT RNA transmission was dependent on specific interactions of the ribonucleoprotein complex with other viral structural polypeptides. These data demonstrate the usefulness of this encapsidation–packaging system for the study of different aspects of the influenza virus life-cycle.

Introduction

Influenza A virions are pleomorphic, enveloped particles with a diameter of 80–120 nm. The viral genome, which consists of eight negative-sense, single-stranded RNAs, has a coding capacity for ten polypeptides. The virion contains three integral membrane proteins, haemagglutinin (HA), neuraminidase (NA) and the M2 ion channel protein. Six other viral proteins are found within the virion membrane. Four of them [nucleoprotein (NP), PB1, PB2 and PA] are associated with the viral genome to form ribonucleoprotein (RNP) complexes and the other two polypeptides, M1 and NS2 [also called NEP; O'Neill *et al.* (1998)], interact with each other and with the RNPs. The NS1 protein is the only non-structural component of the virus (Lamb, 1989; Lamb & Krug, 1996).

Author for correspondence: Agustín Portela.
Fax +34 91 5097918. e-mail aportela@isciii.es

†**Present address:** Department of Neuropharmacology, CVN-9, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

The virus is internalized by receptor-mediated endocytosis and, after fusion of the viral and endosomal membranes, the infecting RNPs are transported from the cytosol to the cell nucleus (Martin & Helenius, 1991), where replication and transcription of the viral genome takes place (Herz *et al.*, 1981). The newly synthesized RNPs are then exported from the cell nucleus to the cytoplasm (Martin & Helenius, 1991; Whittaker *et al.*, 1996; O'Neill *et al.*, 1998) and should reach the proximity of the cellular membrane, where virus budding occurs (Compans & Dimmock, 1969).

Although the interactions between the virus components that govern formation of virion particles are poorly understood, it is thought that contacts between the RNPs and other virus components are critically important. In fact, it has been shown that interactions between the RNPs and the M1 and NS2 proteins modulate the nuclear–cytoplasmic transport of RNPs (Martin & Helenius, 1991; Whittaker *et al.*, 1996;

O'Neill *et al.*, 1998) and morphological (Murti *et al.*, 1992) and biochemical (Zvonarjev & Ghendon, 1980; Zhirnov, 1992) observations suggest that M1 protein associates with the RNPs in the virion. Despite these reports, functional evidence demonstrating the importance of the interactions between RNPs and other virus factors for the formation of infectious particles is still lacking.

Contacts between the cytoplasmic tails of the virus membrane proteins and the virion internal components are also important for formation of the budding particle. Thus, viruses lacking the cytoplasmic tail of HA or NA or both have reduced infectivity and show alterations in their morphology (Jin *et al.*, 1994, 1997; García-Sastre & Palese, 1995; Mitnaul *et al.*, 1996). The role of NA in the formation of infectious virions has been the subject of a number of studies. It has been shown that NA-deficient viruses produce particles that form large aggregates on the cell surface (Palese *et al.*, 1974; Liu *et al.*, 1995) and that these particles, when released from the cell, can complete a round of replication in animals and cell culture (Liu *et al.*, 1995). These results indicate, therefore, that NA activity is needed to prevent formation of virus aggregates and to allow the release of fully assembled virus particles from the cell surface. It should be mentioned that the NA-deficient mutants, which were selected in the presence of bacterial NA, contained a deleted NA segment that retains the capacity to code for an N-terminal NA peptide (Yang *et al.*, 1997). It is, however, not known whether expression of this N-terminal sequence, which includes the membrane-anchoring region of NA, is required for formation of virions.

Recently, we described a system in which a synthetic influenza A virus-like chloramphenicol acetyltransferase (CAT) RNA could be encapsidated, replicated and packaged into virus-like particles (VLPs) in cells expressing all virus-encoded polypeptides from plasmids (Mena *et al.*, 1996). This system is analogous to those described for the negative-sense RNA viruses vesicular stomatitis virus (Pattnaik & Wertz, 1991), rabies virus (Conzelmann & Schnell, 1994), Bunyamwera virus (Bridgen & Elliott, 1996) and human respiratory syncytial virus (Teng & Collins, 1998). The influenza virus rescue system was, however, very inefficient with respect to transmission of the CAT RNA by the VLPs. Therefore, the system was not suitable for systematic studies of the roles played by the viral proteins during virus replication.

Here, we describe the optimization of the rescue system by a factor of $\sim$ 50–100-fold and show the usefulness of this system for the study of the role of NA in formation of VLPs and to demonstrate that interactions of RNP components with other virus factors are required for formation of functional VLPs.

Methods

■ **Cell cultures and viruses.** COS-1 and MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum. Recombinant vaccinia virus (vTF7-3), which expresses T7 RNA polymerase, was kindly provided by B. Moss (National Institutes of Health, Bethesda, MA, USA) (Fuerst *et al.*, 1986). The influenza virus strains used were A/Victoria/3/75 (H3N2), A/Puerto Rico/8/34 (H1N1) and B/Panamá/45/90.

■ **Plasmids and RNAs.** Plasmids pGEM-PB1, pGEM-PB2, pGEM-PA, pGEM-NP, pGEM-HA, pGEM-NA, pGEM-M1, pGEM-M2, pGEM-NS1 and pGEM-NS2, encoding the influenza virus polypeptides PB1, PB2, PA, NP, HA, NA, M1, M2, NS1 and NS2, respectively, from the A/Victoria/3/75 strain, have been described previously (Mena *et al.*, 1994, 1996). In these plasmids, the virus genes are cloned downstream of the T7 promoter of plasmids pGEM-3 or pGEM-4 (Promega). Plasmid pGEM-M3-1, which contains a cDNA copy of $mRNA_3$ (derived from the M segment) (Lamb *et al.*, 1981) cloned under the control of the T7 promoter of plasmid pGEM-3, was derived by RT–PCR from mRNA isolated from cells infected with influenza virus A/Victoria/3/75. Plasmids pGB-PB1-89.1, pGB-PB2-2, pGB-PA-4 and pGB-NP-7, encoding the influenza virus proteins PB1, PB2, PA and NP, respectively, from the B/Panamá/45/90 strain, have been described previously (Jambrina *et al.*, 1997). Plasmids pIVACAT1/S (Piccone *et al.*, 1993) and pT7NSBCAT (Barclay & Palese, 1995) were kindly provided by P. Palese and W. S. Barclay (Mount Sinai School of Medicine, New York, USA). These plasmids were used to generate influenza A and B virus-like model RNAs after transcription with T7 RNA polymerase. These RNAs contain the CAT gene in negative polarity flanked by the 5′ and 3′ untranslated sequences of the RNA segment encoding the NS proteins of the corresponding influenza A or B virus. Plasmid concentrations were estimated spectrophotometrically by assuming that an A_{260} of 1 corresponded to 50 μg/ml DNA.

■ **Antibodies and immunoblotting.** Monoclonal antibodies (MAbs) M/58/p51/G, HA1-50 and M/234/1/F4, which recognize the A/Victoria/3/75 NP and HA proteins, and a rabbit antiserum that recognizes the C terminus of NP have been described previously (Arrese & Portela, 1996; López *et al.*, 1986; Sánchez-Fauquier *et al.*, 1987). Goat antiserum against M2 protein was a gift from Alan Hay (National Institute for Medical Research, London, UK). A polyclonal antiserum against M1 protein was obtained by immunizing rabbits with a denatured, histidine-tagged M1 protein. Western blotting analysis with antisera to M2, M1 or NP and MAb HA1-50 was carried out as described previously (Arrese & Portela, 1996).

■ **Construction of plasmids pGEM-M1Δ, pGEM-M2Δ and pGEM-NS2Δ.** Plasmid pGEM-M2 contains a cDNA copy of the M2 mRNA cloned in the polylinker of plasmid pGEM-3 (Promega). In this plasmid, the nucleotide sequence following the T7 promoter is GGGAGACCGGAATTCGAGCTCGGTACCCTCTTCAagcaaacgcag-gtagataatcgaaag*atg* … , where the pGEM-3 vector sequence is shown in capitals and the influenza virus-derived sequence is shown in lower case. The *Eco*RI site present in the vector is underlined and the ATG initiation codon of the M2 protein is indicated in italics. A new *Eco*RI site was introduced in this plasmid by converting the sequence ta into at. This mutagenesis step was carried with the Transformer site-directed mutagenesis kit (Clontech). The mutagenized plasmid was then digested with *Eco*RI and circularized to yield plasmid pGEM-M2Δ. This plasmid contains the sequence GGGAGACCGGAATTCgaaag*atg* … following the T7 promoter and therefore lacks most of the virus sequences present upstream of the ATG codon in plasmid pGEM-M2.

An analogous strategy was used to generate plasmids pGEM-M1Δ and pGEM-NS2Δ from plasmids pGEM-M1 and pGEM-NS2, respectively. The only difference was that an *Xba*I site, instead of the *Eco*RI site, was introduced to prepare plasmid pGEM-M1Δ, since plasmid pGEM-M1 was derived from the pGEM-4 vector. The sequences downstream of the T7 promoter in these plasmids were GGGAGACCGGAAGCTT-

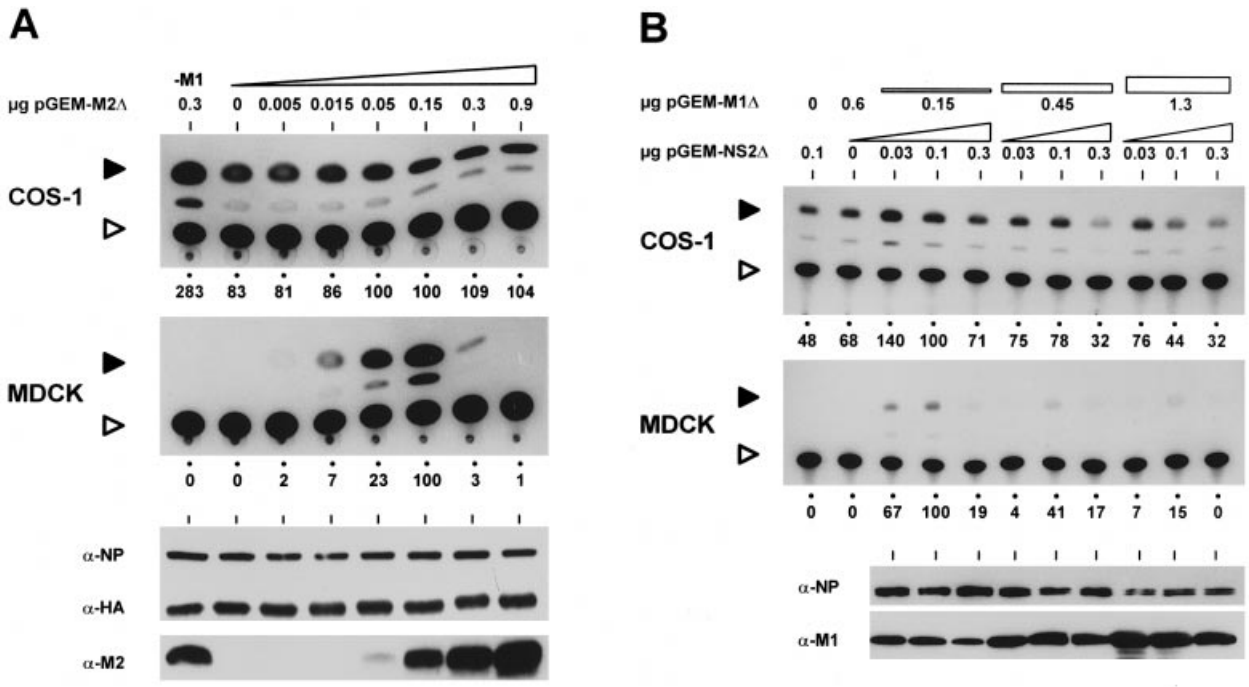

Fig. 1. Optimization of input amounts of plasmids pGEM-M2Δ, pGEM-M1Δ and pGEM-NS2Δ. (A) COS-1 cells were infected with vTF7-3 and transfected with plasmids encoding proteins PB1, PB2, PA, NP, HA, NA, M1 and NS2 (the amounts are indicated in the text) and the indicated amounts (μg) of plasmid pGEM-M2Δ. The cultures were transfected 5 h later with a synthetic CAT RNA. The supernatants from these cultures were harvested 72 h post-infection and added to MDCK cells that were superinfected with a helper influenza virus as detailed in Methods. Aliquots of the COS-1 and MDCK cell extracts were assayed for CAT activity and autoradiographs of the corresponding TLC plates are shown in the figure, with the positions of [^{14}C]chloramphenicol and 3-acetylated [^{14}C]chloramphenicol indicated by open and filled arrowheads, respectively. CAT activities in MDCK cells were expressed as a percentage of that obtained in the sample yielding the highest activity (as shown bellow each lane on the TLC). CAT activities in COS-1 cells were expressed as a percentage by considering the activity of the COS-1 cell sample corresponding to that yielding the highest CAT level in MDCK cells as 100%. The relative CAT activities are shown below each of the samples of the corresponding TLC plate. The lower panel corresponds to a Western blot of the COS-1 cell extracts probed with antibodies (α) against the proteins indicated. In sample −M1, the plasmid pGEM-M1Δ was not included in the transfection mixture. (B) The experiment was performed as described in (A) except that the amount of plasmid pGEM-M2Δ included in the transfection mixture was 150 ng and the concentrations of pGEM-M1Δ and pGEM-NS2Δ were varied as indicated.

GCATGCCTGCAGGTCGACTCTAGAaag*atg* … (pGEM-M1Δ) and GGGAGACCGGAATTCgacata*atg* … (pGEM-NS2Δ).

■ **Assay for detection of functional VLPs.** The standard protocol was carried out basically as described by Mena *et al.* (1996). The major modifications were (i) that plasmids pGEM-M1Δ, pGEM-M2Δ and pGEM-NS2Δ were used instead of plasmids pGEM-M1, pGEM-M2 and pGEM-NS2 and (ii) that the influenza virus strain A/Puerto Rico/8/34 substituted for A/Victoria/3/75 as helper virus, since the former grows to higher titres in cell culture. Briefly, the protocol was as follows. Cultures of COS-1 cells (10^6 cells) growing in 35 mm diameter dishes in the presence of DMEM-Ara-Ant (DMEM containing 40 μg/ml cytosine β-D-arabinofuranoside, 100 U/ml penicillin and 100 μg/ml streptomycin) were infected with vTF7-3 (m.o.i. of 5). After virus adsorption, the cultures were incubated with 1 ml DMEM-Ara-Ant that was supplemented with a 100 μl mixture that contained cationic liposomes and the plasmids indicated in each case. After the optimization experiments, the optimal amounts of the plasmids in the transfection mixture were: pGEM-PB1 (0·6 μg), pGEM-PB2 (0·6 μg), pGEM-PA (0·2 μg), pGEM-NP (2 μg), pGEM-HA (0·6 μg), pGEM-NA (0·6 μg), pGEM-M1Δ (0·15 μg), pGEM-M2Δ (0·15 μg) and pGEM-NS2Δ (0·1 μg) (see text for details). After 5 h incubation with the plasmids, the cells were transfected again with a mixture containing 0·5 μg synthetic CAT RNA. After incubation overnight, the medium was replaced with 1 ml DMEM-Ara-Ant and the cultures were incubated for an additional 48 h. Cell supernatants were then harvested and cell extracts were prepared. Aliquots of the cell extracts were used for Western blotting and for determination of CAT activity (see below).

The supernatant collected from the COS-1 cells was clarified by centrifugation for 15 min in a microcentrifuge and subjected to three cycles of freezing and thawing. To test for the presence of VLPs, an aliquot of the supernatant (typically 400 μl) was incubated with trypsin (2·5 μg/ml) for 15 min at 37 °C and added to 10^6 MDCK cells, growing in 35 mm diameter dishes in the presence of DMEM-Ara-Ant. After 1 h incubation, the cells were superinfected with influenza virus A/Puerto Rico/8/34 (or B/Panamá/45/90 when indicated) at an m.o.i. of 5 and the cultures were incubated for 17 h. Cell extracts were then prepared for determination of CAT activity. Routinely, for these assays a sample corresponding to 5000 COS-1 cells and to 50000 MDCK cells was incubated for 1 h at 37 °C with [^{14}C]chloramphenicol as described previously (Mena *et al.*, 1996). Quantification of CAT activity was performed by phosphorimaging of the acetylated spots detected on TLC plates by using a Fujix Bas 1000 phosphorimager and the software PCBAS v2.09.

■ **Electron microscopy.** Transfected cultures were pre-cooled for 15 min at 4 °C and incubated for 30 min with the anti-HA MAb M/234/1/F4 (diluted 1:5 in DMEM containing 5% sheep serum). After washing with DMEM–5% sheep serum, the samples were incubated for 30 min with a secondary antibody (10 nm gold-labelled, goat anti-mouse IgG; Auroprobe EM GAM IgG G10; Amersham) diluted 1:20 in DMEM. After washing with Tris-buffered saline (25 mM Tris–HCl, pH 7·4, 2·68 mM KCl, 137 mM NaCl), the cells were fixed with 2% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4) and post-fixed with 1% osmium tetroxide prepared in the same buffer for 1 h at 4 °C. The fixed cells were then dehydrated and embedded in epoxy resin EPON 812. Sections prepared with an LKB Ultratome IV were post-stained in 1% aqueous uranyl acetate for 15 min and then 3 min in lead citrate and were visualized with a Philips 400T electron microscope at 80 kV.

Results

Optimization of the CAT rescue system

As detailed previously (Mena *et al.*, 1996), the protocol followed to generate and detect recombinant VLPs included two steps. In the first step, COS-1 cells were infected with vTF7-3 and transfected with the pGEM-derived plasmids encoding all influenza virus polypeptides and with a synthetic, negative-sense CAT RNA. In the second step, the supernatant fluids collected from the transfected COS-1 cells were treated with trypsin and incubated with fresh MDCK cultures that were then superinfected with a wild-type influenza helper virus. Detection of CAT activity in COS-1 cells demonstrated expression of the model CAT RNA by the recombinant polymerase, whereas detection of CAT activity in the MDCK cell extracts indicated that the COS-1 cell supernatant contained functional VLPs, i.e. VLPs competent to deliver an enclosed CAT RNA into MDCK cells.

It has been shown for a number of systems in which a model RNA is replicated and/or packaged that obtaining the highest replication and/or packaging levels depends on finding the optimal ratio of transfected plasmids (Pattnaik & Wertz, 1990; Dunn *et al.*, 1995; Sanz-Ezquerro *et al.*, 1995; Teng & Collins, 1998). It was therefore decided to test the concentration-dependent effects of the transfected plasmids on the formation of influenza VLPs by following the protocol outlined above. The experiments were initiated with only the nine plasmids that encode the viral structural components, since we had demonstrated previously that formation of VLPs does not require expression of NS1 protein (Mena *et al.*, 1996). The concentrations of the plasmids encoding the virus core proteins were those found previously to be optimal for expression of a synthetic CAT RNA (Sanz-Ezquerro *et al.*, 1995) and the starting concentrations of the remaining plasmids were those used in the previous report (1 μg of each plasmid).

The first experiment was to optimize the concentration of plasmid pGEM-M2Δ (Fig. 1A), since preliminary assays indicated that the concentration of the M2 protein drastically affected the CAT activity detected in MDCK cells (not shown). It was observed that increasing the amount of transfected M2 plasmid resulted in greater accumulation of the M2 protein in COS-1 cells, as expected from the transient expression system used (Fuerst *et al.*, 1986). To detect alterations in the expression levels of the other recombinant proteins, which could arise as a result of transfecting different doses of a particular plasmid, we routinely checked for the accumulation of NP and/or HA in the different cell samples. As can be observed in Fig. 1(A), there were no significant changes in the accumulation of these two proteins in any of the samples analysed. Moreover, the reporter gene activity measured in COS-1 cells was not affected by the level of expression of M2, indicating that this protein does not modify the functionality of the core proteins to express the input CAT RNA. However, the concentration of the M2 plasmid affected formation of functional VLPs drastically. Transfection of very small amounts of the plasmid (5 ng) were sufficient to allow detection of functional VLPs and maximum detection was achieved after transfection of 150 ng plasmid. From that point on, CAT activity in MDCK cells decreased until it reached virtually background values with transfection of 0·9 μg plasmid. On the basis of these results, all subsequent transfection experiments were carried out with 150 ng pGEM-M2Δ.

It was next decided to titrate the plasmids encoding the M1 and NS2 proteins. In preliminary tests, it was observed that transfecting more than 300 ng pGEM-NS2Δ had an inhibitory effect on CAT expression in COS-1 cells (data not shown, see below), whereas transfection of small amounts of the M1 plasmid (50 ng) resulted in low levels of CAT expression in the MDCK cultures (data not shown). Since M1 and NS2 proteins appear to interact with each other (Yasuda *et al.*, 1993; O'Neill *et al.*, 1998), it was decided to carry out co-transfection experiments with the plasmids encoding these two proteins. On the basis of preliminary experiments, the doses chosen for these analyses varied from 150 to 1300 ng for pGEM-M1Δ and from 30 to 300 ng for pGEM-NS2Δ (Fig. 1B). For each of the three doses of pGEM-M1Δ tested, 100 ng of the NS2 plasmid always yielded the highest CAT activities in MDCK cells; the sample transfected with the smallest amount of M1 plasmid was the one which yielded the highest reporter gene activity in MDCK cells. It was again observed that there was a direct correlation between the amount of plasmid pGEM-M1Δ transfected and the concentration of M1 protein in the COS-1 cultures. The accumulation of NS2 protein was not tested because of the lack of an appropriate immunological reagent.

Once the optimal amounts of the M1 (150 ng) and NS2 (100 ng) plasmids were determined, the dose-dependent effects of HA and NA plasmids in the CAT rescue system were investigated. As shown in Fig. 2(A), the different amounts of plasmids tested did not have any significant effect on the CAT activity obtained in COS-1 cells and the doses of these two plasmids that yielded the highest level of CAT expression in MDCK cells were 600 ng.

It was then decided to study the effect of NP concentration on the system and to confirm the inhibitory effect (mentioned

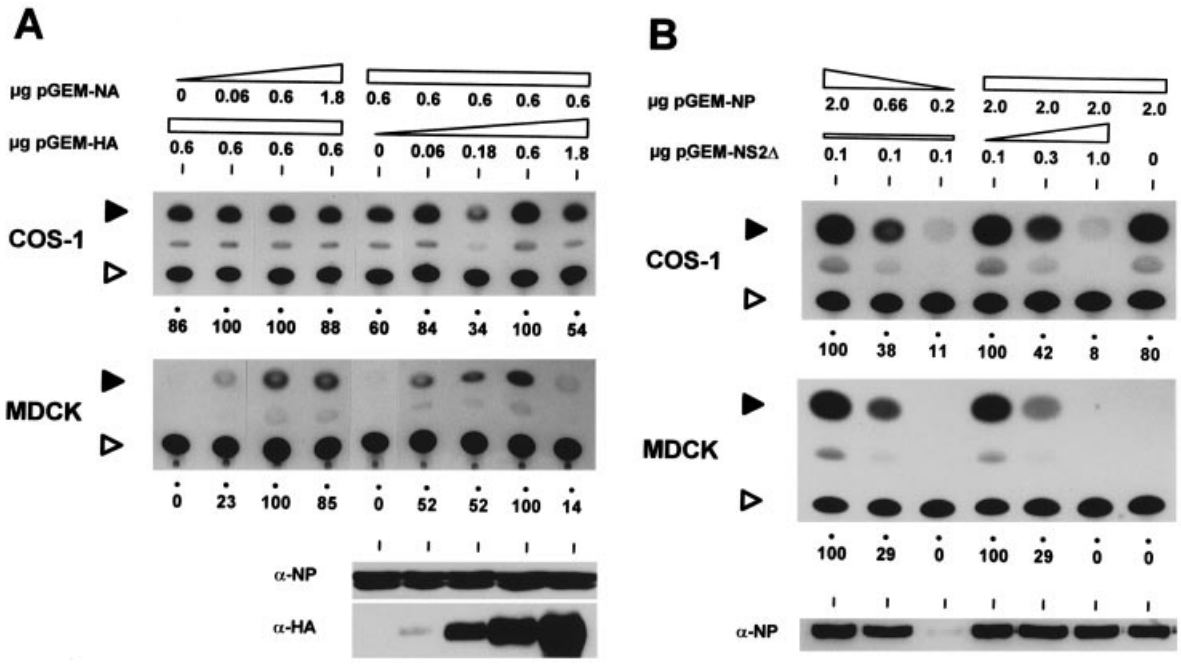

Fig. 2. Optimization of input amounts of plasmids pGEM-HA, pGEM-NA, pGEM-NP and pGEM-NS2Δ. (A) The experiment was performed as described in Fig. 1 (B) except that the amounts of plasmids pGEM-M1Δ and pGEM-NS2Δ included in the transfection mixture were 150 and 100 ng, respectively. The concentrations of pGEM-HA and pGEM-NA were varied as indicated. (B) The experiment was performed as described in (A) except that the amounts of pGEM-HA and pGEM-NA included in the transfection mixture were 600 ng each. The concentrations of plasmids pGEM-NP and pGEM-NS2Δ were varied as indicated.

above) of NS2 on expression of the CAT RNA in COS-1 cells (Fig. 2B). It was observed that reducing the amount of NP plasmid led to a reduction in the level of CAT expression in COS-1 cells. A similar effect was observed by increasing the amount of plasmid pGEM-NS2Δ in the transfection mixture. In both cases, the CAT activity detected in MDCK cells was roughly proportional to that detected in COS-1 cells. One interpretation of these results would be that either small amounts of NP or large amounts of NS2 reduced the production of viral RNPs (containing the CAT RNA) in transfected cells. Consequently, less viral RNP would be packaged into VLPs and lower CAT activity would be detected in the MDCK cell extracts. On the basis of the results obtained, the doses of plasmids encoding NP and NS2 were maintained at 2 μg and 100 ng, respectively.

Once the system had been optimized in the absence of NS1 protein, we re-examined the effect of this protein on formation of VLPs. It was confirmed that NS1 protein was not required for efficient transmission of CAT RNA to MDCK cells (Fig. 3 A) (Mena *et al.*, 1996). Strikingly, it was observed that increasing the amount of NS1 plasmid from 100 ng to 2 μg resulted in a ~ 100-fold reduction in the CAT activity detected in MDCK cells, whereas within this dose range, there was only a ~ 3-fold reduction in the CAT activity observed in the COS-1 cell extracts.

Finally, the possible effect of $mRNA_3$ on the rescue system was tested. $mRNA_3$ is a spliced product derived from RNA segment 7 and it contains an open reading frame for a 9 amino acid peptide that has never been found in infected cell cultures (Lamb *et al.*, 1981). As shown in Fig. 3 (B), expression of $mRNA_3$ did not have a significant effect on the reporter gene activities reached in COS-1 and MDCK cells. It should be pointed out that the M1 mRNA (derived from plasmid pGEM-M1Δ) will not be spliced to generate $mRNA_3$, since it lacks the 5′ splice site. Moreover, it is worth mentioning that the T7-derived transcripts produced in the vaccinia T7 system are not expected to be spliced, since they are synthesized in the cytoplasm.

From the above experiments, we determined the optimal concentration of the nine plasmids encoding the virus structural polypeptides that allowed efficient formation of functional VLPs. By considering the amounts of the MDCK cell extract tested in the CAT assays (5% as compared to 20–50% in the previous report) as well as the CAT activities observed, it was calculated that the optimized rescue system was 50–100-fold more efficient than the previous one (Mena *et al.*, 1996) for CAT transmission to the MDCK cells.

CAT transmission to MDCK cells is mediated by VLPs

Using the optimized conditions, a series of experiments identical to those described in the previous report (Mena *et al.*, 1996) were carried out to characterize the particles that transmitted the CAT RNA. It was confirmed, with three different plasmid preparations, that expression of all viral

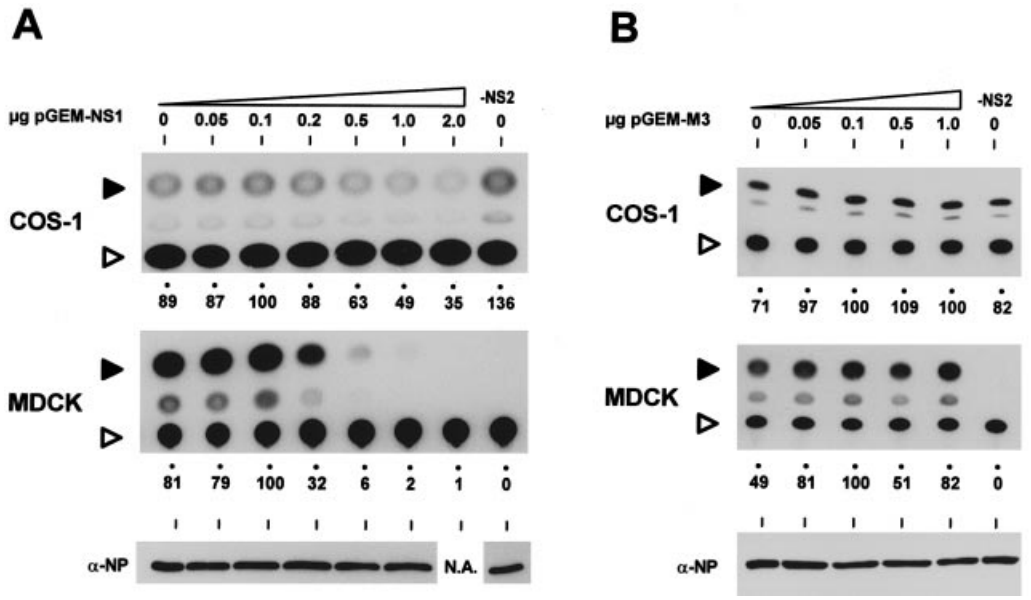

Fig. 3. Optimization of input amounts of plasmids pGEM-NS1 and pGEM-M3. The experiment was performed as described in Fig. 2 (B). The concentrations of plasmids pGEM-NS1 (A) and pGEM-M3-1 (B) were varied as indicated. N.A., Not analysed. In sample −NS2, the plasmid pGEM-NS2Δ was not included in the transfection mixture.

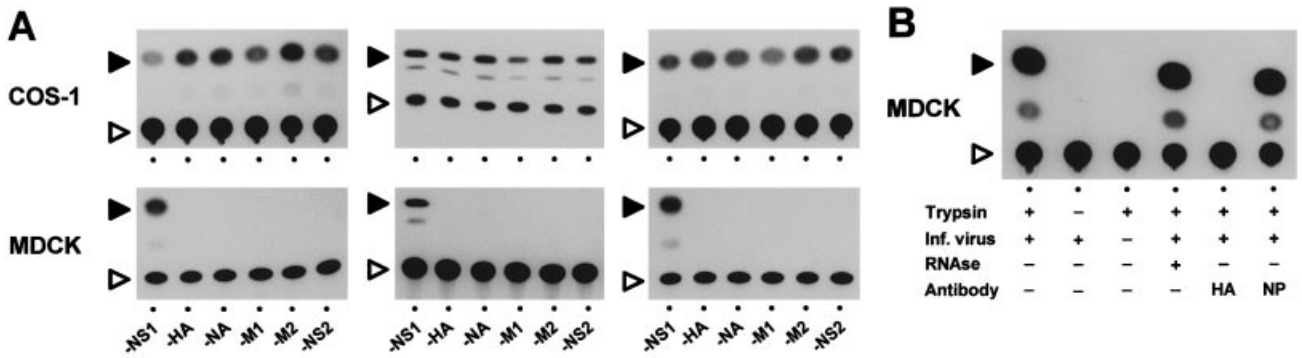

Fig. 4. Viral proteins required for transmission of CAT RNA and characterization of the VLPs that transmit the CAT RNA to MDCK cells. (A) COS-1 cells were infected with vTF7-3 and transfected with the nine plasmids encoding the structural viral polypeptides (sample −NS1) or with only eight of them (samples −HA, −NA, −M1, −M2 and −NS2, where the plasmid encoding the indicated gene product was also omitted). The cultures were then transfected with a CAT RNA and the supernatants from these cultures were tested on MDCK cells. Aliquots of the COS-1 and MDCK cell extracts were assayed for CAT activity as detailed in Methods. (B) The supernatant obtained from COS-1 cells expressing the nine structural virus polypeptides and transfected with a CAT RNA was harvested. Aliquots of this supernatant were then incubated with trypsin, RNase A or MAbs specific for influenza virus HA (MAb 234/1/F4) or NP (MAb M58/p51/G) as indicated. The treated supernatants were next added to MDCK cells, which were then mock-infected or infected with the influenza virus strain A/Puerto Rico/8/34 as indicated (Inf. virus). Cell extracts were prepared and assayed for CAT activity as described above.

structural proteins was required for detection of CAT activity in MDCK cells (Fig. 4A). Moreover, it was demonstrated that treatment of the COS-1 cell supernatant with trypsin and superinfection with an influenza helper virus were absolute requirements for detection of reporter gene activity in MDCK cells (Fig. 4B). In addition, it was shown that transmission of the CAT RNA to MDCK cells could be abolished by incubation of the COS-1 cell supernatant with a neutralizing anti-HA MAb but that it was unaffected by treatment of this supernatant with a MAb to NP, an antiserum to vaccinia virus or RNase A (Fig. 4B and data not shown). Taken together, these results allowed us to conclude that the CAT activity detected in the MDCK cell extracts was transmitted by VLPs enclosing the CAT RNA and that these VLPs should resemble authentic influenza virions, since expression of all virus structural proteins was required for CAT RNA transmission.

Electron microscopy of VLPs

In an attempt to visualize the recombinant VLPs, thin sections of COS-1 cells expressing all virus structural proteins were incubated with an anti-HA MAb and examined by

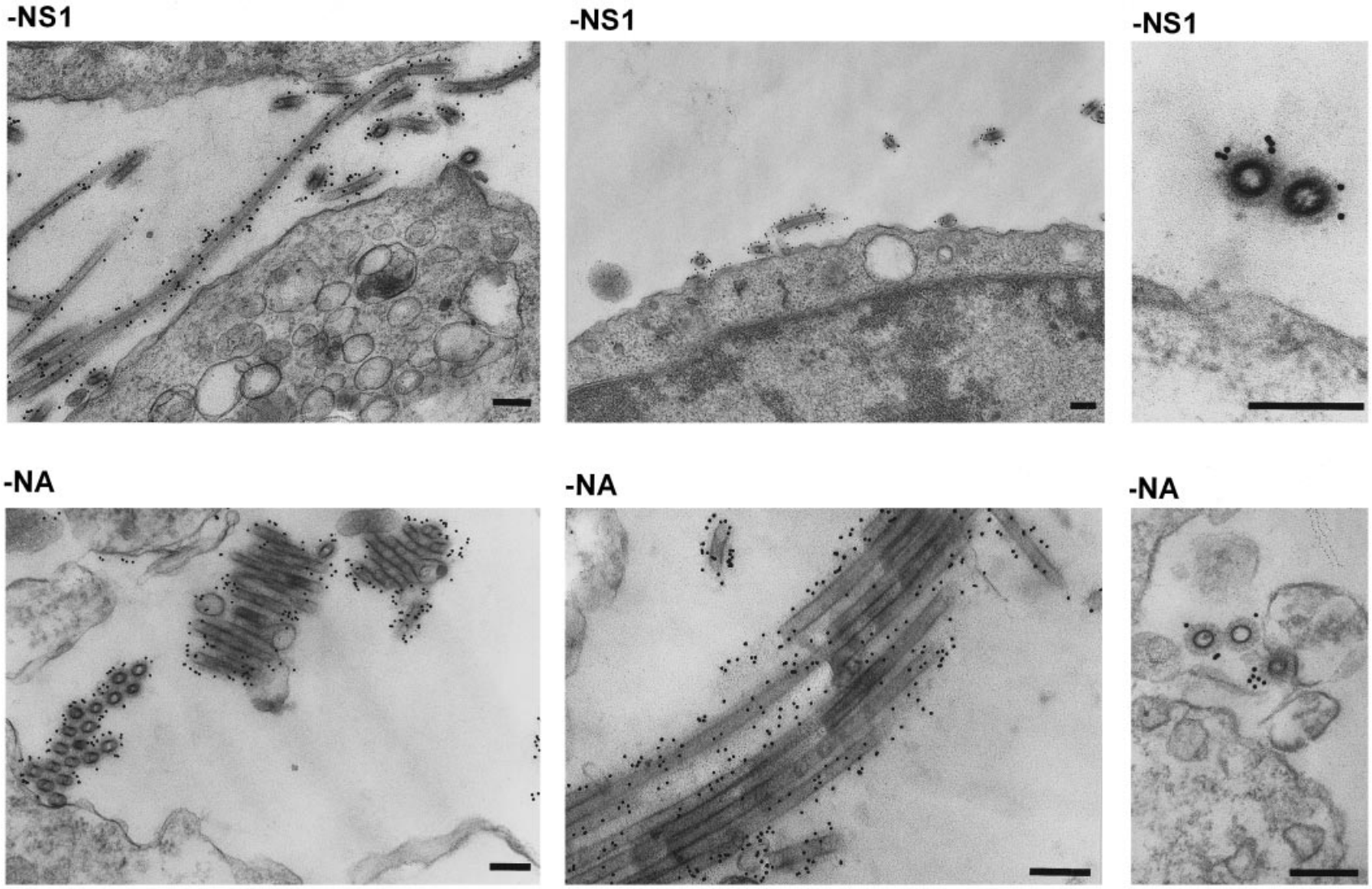

Fig. 5. Immunoelectron microscopy of transfected COS-1 cells. COS-1 cells were infected with vTF7-3 and transfected with the nine plasmids encoding all the influenza virus structural proteins (—NS1) or with a similar mixture that also lacked the NA plasmid (—NA). At 60 h post-infection, cells were incubated with an anti-HA MAb (M234/1/F4) and decorated with a 10 nm immunogold conjugate before fixation as described in Methods. Bars represent 200 nm.

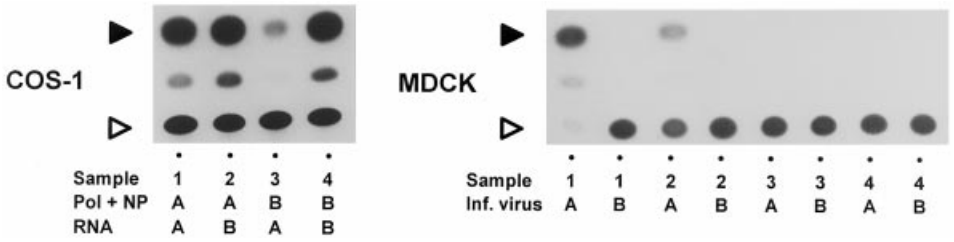

Fig. 6. Formation of heterotypic VLPs. Four COS-1 cell cultures (samples 1–4) were infected with vTF7-3 and then transfected with DNA mixtures containing the pGEM-derived plasmids corresponding to the influenza A virus proteins HA, NA, M1, M2 and NS2. The DNA mixtures contained, in addition, four plasmids encoding the three subunits of the polymerase and NP from either A/Victoria/3/75 (A) or B/Panamá/45/90 (B) influenza viruses (as indicated under Pol + NP). The amounts of type B plasmids included in the transfection mixture were: 1 μg of pGB-PB1-89.1, 0·5 μg of pGB-PB2-2, 0·5 μg of pGB-PA-4 and 3 μg of pGB-NP-7. The cells were transfected again 5 h later with an NS-CAT RNA containing the conserved ends of either type A or B virus (indicated in RNA). At 72 h post-infection, cell supernatants were harvested and the COS-1 cell extracts were assayed for CAT (left panel). Supernatants from COS-1 samples 1–4 were divided into two aliquots and added to MDCK cultures that were then infected with either influenza virus A/Puerto Rico/8/34 (A) or B/Panamá/45/90 (B) as indicated (Inf. virus). Cell extracts were prepared 17 h later and assayed for CAT expression (right panel).

electron microscopy (Fig. 5, samples −NS1). A few filamentous particles were observed in the proximity of 5–30% of the cells examined (routinely more than 100) and similar structures were not observed in cultures that did not receive plasmid pGEM-M1Δ. These particles resembled authentic influenza virions in size and morphology and could be labelled specifically with an anti-HA antibody. It was therefore concluded that the particles detected corresponded to the VLPs that transmitted the CAT RNA.

We have demonstrated that the supernatant from COS-1 cells expressing all structural proteins except NA was unable to transmit the CAT RNA to MDCK cells (Fig. 4A). One explanation of this result would be that VLPs were not formed in the absence of the NA protein. Alternatively, on the basis of the results obtained with the NA-deficient viruses (see Introduction), it is possible that budded particles were formed but that they remained aggregated and attached to the surface of the COS-1 cells. To distinguish between these two possibilities, COS-1 cultures not expressing NA were examined by electron microscopy (Fig. 5, samples −NA). Particles that had apparently completed budding were observed in 5–30% of the cells examined. Virtually all particles observed were found in aggregates or associated with the cell surface singly or in small groups. The aggregated particles could be decorated with an anti-HA MAb and structures of this kind were not observed in cultures expressing all structural proteins except NA and M1. On the basis of these findings, we concluded that the filamentous, membrane-associated particles were in fact VLPs lacking NA.

Interactions between RNPs and other viral proteins are essential for formation of functional VLPs

It was then decided to test whether the rescue system could provide functional evidence of the importance of interactions between RNPs and other viral proteins for the formation of mature virions. To this end, transfection mixtures containing plasmids encoding either the influenza A or B virus core proteins and, in addition, plasmids expressing the influenza A virus HA, NA, M1, M2 and NS2 proteins were prepared. These mixtures were transfected into COS-1 cells that were then further transfected with either an influenza A or B virus-type CAT RNA. The supernatants from these cultures were then assayed for CAT RNA transmission to MDCK cells by the standard protocol, infecting the cultures with either influenza A or B virus. The results obtained in a representative experiment are shown in Fig. 6. As we have described previously (Jambrina *et al.*, 1997), both the influenza A and B core proteins were capable of replicating/amplifying the heterotypic RNAs. However, CAT expression in MDCK cells was only observed in the samples in which all recombinant proteins and the helper virus were from the type A virus, independent of the transfected CAT RNA. These results suggest that virus type-specific contacts between the RNPs and other viral polypeptides are required for formation of functional VLPs.

Discussion

We have described here an optimized system that allows efficient rescue of synthetic CAT RNA into functional VLPs. Several interesting observations were made during the optimization experiments. Overexpression of the NS2 protein had an inhibitory effect on CAT expression in COS-1 cells, suggesting that this protein reduced the level of transcription and/or replication of the input CAT RNA. It is suggested that the observed effect may involve interactions of NS2 with virus factors other than the core proteins, since other authors did not observe such an effect in cells expressing exclusively NS2 and the four core proteins (Huang *et al.*, 1990; Enami *et al.*, 1994). It remains to be tested whether the NS2 effect may have something to do with the poorly characterized role of the protein in replication of viral RNAs (Odagiri & Tobita, 1990)

and/or with the role of NS2 in transport of RNPs from the nucleus to the cytoplasm (O'Neill *et al.*, 1998).

Overexpression of M2 completely blocked CAT RNA transmission to MDCK cultures. M2 functions as an ion channel protein (Sugrue *et al.*, 1990; Pinto *et al.*, 1992) and it has been shown that, when this protein is overexpressed, the intracellular transport of co-expressed HA is inhibited and the accumulation of HA at the plasma membrane is reduced by 75–80% (Sakaguchi *et al.*, 1996; Henkel & Weisz, 1998). It is suggested that a similar effect occurs in the rescue system, so that, by overexpressing M2, the accumulation of virus membrane proteins at the plasma membrane is reduced and hence there is a drastic reduction in the number of functional VLPs produced.

The situation with NS1 is peculiar, in that the protein was not needed for formation of functional VLPs but functional VLPs were not detected when the protein was overexpressed. The NS1 protein is an RNA-binding protein that has been found associated with RNPs (Marión *et al.*, 1997) and that inhibits nucleocytoplasmic transport of RNAs, stimulates translation of viral mRNAs and modulates splicing of influenza virus mRNAs (Nemeroff *et al.*, 1998; reviewed in Lamb & Krug, 1996; Ortín, 1998). We hypothesize that, when NS1 protein is overexpressed, the CAT RNPs are sequestered into the cell nucleus and therefore cannot be packaged into VLPs.

It should be mentioned that the three proteins (NS1, NS2 and M2) that most dramatically affected CAT expression are translated in infected cells from spliced mRNAs (M2 and NS2) or from an mRNA that is a substrate for splicing (NS1) (Lamb & Lai, 1980; Lamb *et al.*, 1981). This is consistent with previous evidence indicating that splicing of viral mRNAs is tightly regulated in infected cells (Alonso-Caplen & Krug, 1991; Valcárcel *et al.*, 1991; Shih *et al.*, 1995).

We have shown here, using three different plasmid preparations, that all virus structural proteins were required for formation of functional VLPs. It should be mentioned that in our previous report (Mena *et al.*, 1996), similar results were obtained with two plasmid preparations but in the case of a third, it was observed that expression of M2 was not required for formation of functional VLPs. We found here that transfecting very small amounts of the M2 plasmid was sufficient for a positive CAT signal to be detected in the MDCK cells. It is therefore suggested that the results obtained previously with one of the plasmid preparations were probably due to minor contamination with the M2 plasmid.

It was demonstrated by electron microscopic analysis that particles similar to wild-type virions were formed in the absence of NA expression. As indicated above, the NA-deficient mutants described previously, which were selected in the presence of bacterial NA, retained the capacity to encode an N-terminal NA peptide (Yang *et al.*, 1997). Our data indicate that expression of this NA fragment is not essential for virus particle formation, although we cannot rule out that its expression could confer a selective advantage to the NA-deficient virus. It has been described that viruses lacking the cytoplasmic tail of NA have a higher tendency to be filamentous (Mitnaul *et al.*, 1996; Jin *et al.*, 1997). This observation has been made with the WSN strain, which produces primarily spherical particles. It has not been possible to determine whether a similar effect occurs with the A/Victoria/3/75 VLPs formed in the absence of NA, since both the wild-type virions and the VLPs containing NA appear mainly as filamentous viruses.

Functional VLPs were not detected when the core proteins were derived from an influenza B virus and the rest of the structural proteins from a type A virus. This result suggests strongly that critical virus type-specific interactions between the RNP components and other viral proteins take place during virion assembly. These interactions probably involve protein–protein rather than RNA–protein contacts, since an influenza B virus RNA could be transmitted to MDCK cells by the type A proteins. In the RNP complexes, the polymerase complex holds the RNA ends together, whereas NP interacts with the rest of the RNA, exposing the RNA bases to the solvent (Klumpp *et al.*, 1997). We propose that the interactions that affect RNP packaging involve the NP polypeptide, since the polymerase subunits are minor components of the RNPs. An interesting observation made during the experiments involving heterotypic virus components was that the type A CAT RNPs were expressed in MDCK cells infected with an influenza A virus but not when the cells were infected with an influenza B virus (samples 1 and 2, Fig. 6). We had hypothesized (Mena *et al.*, 1996) that the CAT RNPs delivered into MDCK cells are capable, in the absence of helper virus infection, of primary transcription, but that detection of CAT activity depends on the replication (amplification) of the CAT RNPs, mediated by the proteins provided in *trans* by the helper virus. The lack of expression of the type A CAT RNPs in influenza B virus-infected cells indicates that the influenza B virus proteins cannot switch the type A CAT RNPs from primary transcription to replication.

In summary, we have developed an efficient encapsidation–packaging system and we have presented evidence that shows its usefulness in the characterization of the roles of viral proteins during the influenza virus life-cycle.

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