ELSEVIER

Virus Research 67 (2000) 41–48

Virus Research

www.elsevier.com/locate/virusres

Rescue of synthetic RNAs into Thogoto and influenza A virus particles using core proteins purified from Thogoto virus

Paulino Gómez-Puertas [a], Michael B. Leahy [b], Patricia A. Nuttall [b], Agustín Portela [a,*]

[a] *Centro Nacional de Biología Fundamental, Instituto de Salud Carlos III, Majadahonda 28220, Madrid, Spain*
[b] *NERC Institute of Virology and Environmental Microbiology, Oxford OX1 3SR, UK*

Received 2 November 1999; received in revised form 20 January 2000; accepted 20 January 2000

Abstract

The ribonucleoprotein (RNP) complexes of Thogoto virus (THOV), a tick-borne orthomyxovirus, have been purified from detergent-lysed virions. The purified RNPs were then disrupted by centrifugation through a CsCl-glycerol gradient to obtain fractions highly enriched in nucleoprotein (NP) and virtually devoid of viral genomic RNA. When these NP-enriched fractions were incubated with a synthetic THOV-like RNA, and the mixtures were transfected into THOV-infected cells, the synthetic RNA was expressed and packaged into THOV particles. Similarly, hybrid mixtures containing purified THOV NP and influenza A virus synthetic RNAs (either a model CAT RNA or a gene encoding the viral neuraminidase), were prepared and transfected into influenza A virus-infected cells. The synthetic CAT RNA, was shown to be expressed and packaged into virus particles, and the neuraminidase gene was rescued into influenza virions. These data are discussed in terms of the similarities observed between THOV and influenza A virus and the potential application of the THOV purified proteins for rescuing synthetic genes into infectious viruses.

Keywords: Thogoto virus; Influenza virus; RNP transfection; Synthetic RNAs

1. Introduction

The family *Orthomyxoviridae* comprises the genera *Influenzavirus A*, *B*, and *C*, and the genus *Thogotovirus*, whose prototype member is Thogoto virus (THOV), an arbovirus which replicates both in ticks and vertebrates (Nuttall et al., 1995; Mayo and Pringle, 1998). All members of this family are enveloped and possess a segmented genome made up of 6–8 single-stranded RNA segments of negative polarity (Clerx et al., 1983; Lamb and Krug, 1996). The ends of the viral RNA segments show partial complementarity and those of THOV and influenza viruses show se-

* Corresponding author. Tel.: +34-91-5097904; fax: 34-91-5097919.
E-mail address: aportela@isciii.es (A. Portela)

0168-1702/00/$ - see front matter
PII: S0168-1702(00)00127-1

quence similarity (Desselberger et al., 1980; Clerx et al., 1983).

A large body of evidence has demonstrated that the templates for influenza A virus RNA synthesis are the ribonucleoprotein (RNP) complexes (Krug et al., 1989; Lamb and Krug, 1996). These complexes are made up of the genomic RNA associated with four virus-encoded proteins: the nucleoprotein (NP), which encapsidates the viral genome, and the three subunits of the viral polymerase. A number of findings have demonstrated the similarities between influenza A virus and THOV in the process of virus-specific RNA synthesis. Indeed, THOV encodes four polypeptides, which show sequence similarity with the four protein components of influenza A virus RNPs (Weber et al., 1996; Leahy et al., 1997b). For both influenza A virus and THOV, these four core proteins are the minimum set of viral polypeptides required for expression of the viral genome (Huang et al., 1990; Weber et al., 1998). Moreover, replication and transcription of the influenza A virus genome takes place in the nucleus (Herz et al., 1981) and it appears that the THOV life cycle involves a nuclear phase since the THOV NP protein accumulates in the nucleus of infected cells (Portela et al., 1992). Furthermore, influenza A virus and THOV use a cap-snatching mechanism for mRNA transcription initiation (Plotch et al., 1981; Albo et al., 1996; Weber et al., 1996; Leahy et al., 1997c).

In vitro polymerase assays using synthetic RNAs and core proteins obtained from virions and/or infected cells have been developed for influenza A virus (Parvin et al., 1989; Martín et al., 1992; Seong and Brownlee, 1992). The four viral core proteins have been isolated from RNPs, which have been depleted of their RNA component by either treatment with micrococcal nuclease (Seong and Brownlee, 1992) or by centrifugation in CsCl-glycerol gradients (Parvin et al., 1989; Martín et al., 1992). The RNP complexes reconstituted from purified proteins and synthetic RNAs can transcribe the encapsidated RNA in vitro (Parvin et al., 1989; Martín et al., 1992; Seong and Brownlee, 1992). Moreover, the reconstituted complexes are biologically active in vivo since the encapsidated RNA can be expressed and incorporated into new viral particles when transfected into cells which had been infected with a helper influenza virus (Luytjes et al., 1989; Martín et al., 1992; Seong and Brownlee, 1992). This methodology has been used to rescue a number of synthetic genes into influenza virions (reviewed in Palese et al., 1996 and in García-Sastre, 1998).

An in vitro polymerase assay using synthetic RNAs has also been developed for THOV (Leahy et al., 1997a,c). The strategy followed to prepare the THOV RNP core fraction, unlike that used with influenza A virus, did not involve treatment specifically aimed at disrupting the THOV RNPs into its protein and RNA components. Instead, THOV was disrupted with detergent and centrifuged through two glycerol gradients to obtain a fraction capable of transcribing short synthetic THOV-like RNA templates in vitro. Moreover, this THOV core preparation also functioned efficiently to transcribe in vitro model templates containing the influenza A virus RNA conserved ends (Leahy et al., 1997a). However, it was not determined whether this THOV core preparation was competent to reconstitute RNPs that were biologically active in vivo.

Using a procedure originally established for influenza virus that involves centrifugation of purified RNP complexes through a CsCl-glycerol gradient (Honda et al., 1988; Parvin et al., 1989; Martín et al., 1992), we report the preparation of THOV RNP core proteins free of viral RNA. The THOV purified proteins allowed rescue of synthetic RNAs into both THO virions and influenza A virus particles.

THOV was grown in BHK-21 cells and purified as indicated in the legend to Fig. 1. As previously described (Portela et al., 1992), the purified THO virions contain three major structural polypeptides, a single glycoprotein (gp75) and two internal components the NP (52-kDa) and matrix (29-kDa) proteins (Fig. 1(A)). The virion also contains three minor protein components (of 72-, 82-, and 90-kDa) which presumably correspond to the subunits of the viral polymerase (Portela et al., 1992; Leahy et al., 1997b). A standard procedure for purifying influenza virus RNPs was used to isolate the THOV RNPs by centrifuging deter-

gent-lysed virions through a glycerol gradient (33–70%). As shown in Fig. 1(A, left panel), the upper fractions of this gradient contained the THOV glycoprotein and matrix proteins, whereas the NP protein was distributed through the middle and lower part of the gradient with a peak in fraction 5. The fractions of this gradient were also analyzed for their viral RNA content by primer extension using an oligonucleotide corresponding to THOV RNA segment 4, which encodes the viral glycoprotein, gp75 (Fig. 1(B)). There was a clear overlapping of the fractions containing the NP and those including the viral RNA, thus demonstrating that the NP-containing fractions contained the RNP complexes. The fractions enriched in RNPs were then centrifuged through a CsCl-glycerol gradient, following protocols routinely used to isolate influenza virus core proteins (Honda et al., 1988; Parvin et al., 1989; Martín et al., 1992). Under these centrifugation conditions, the THOV RNP components dissociated from each other, with the NP sedimenting to the central

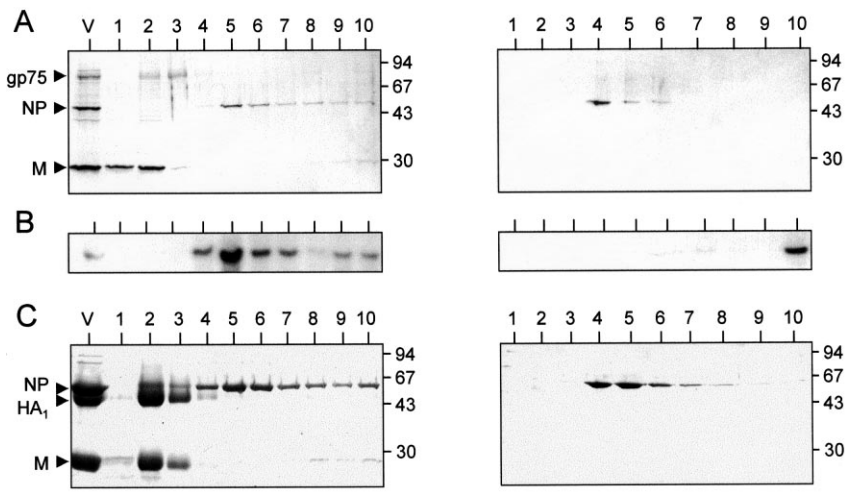

Fig. 1. Purification of THOV core proteins. (A). THOV (isolate SiAr/126/72) was grown in BHK-21 cells, which were maintained in medium M-199. The supernatant from infected cells was clarified by low-speed centrifugation, and centrifuged for 90 min at 14°C and 40 000 rpm in a 45Ti rotor to pellet the virions. The virus pellet was resuspended in 4 ml of TNE (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA), loaded onto a discontinuous sucrose gradient (50 and 33% w/v) and subjected to centrifugation for 60 min at 14°C and 40 000 rpm in a SW40 rotor. The virus present in the sucrose interface was collected, diluted in TNE buffer and pelleted as indicated above in a SW40 rotor. The pellet was resuspended in 1 ml of a solution containing 1.5% Triton-N-101, lysolecithin (10 mg/ml), 100 mM Tris–HCl (pH 8.1), 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol, and 1.5 mM dithiothreitol and incubated for 25 min at 30°C. The virus lysate was loaded onto a discontinuous glycerol gradient containing four steps of 1 ml of 70, 60, 40 and 33% glycerol (in 50 mM Tris–HCl pH 7.8, 150 mM NaCl, 1 mM dithiothreitol) and centrifuged for 4 h at 4°C and 45 000 rpm in a SW55 rotor. Fractions of the gradient were collected from the top (fraction 1) and analyzed by SDS-PAGE and Coomassie blue staining (left panel). Fractions 5 and 6 were pooled and loaded onto a discontinuous CsCl-glycerol gradient, which had four steps of 1 ml of 3 M CsCl and 45% glycerol, 2.5 M CsCl and 40% glycerol, 2 M CsCl and 35% glycerol and 1.5 M CsCl and 30% glycerol (all steps buffered with Tris–HCl pH 7.6). The sample was centrifuged for 25 h at 4°C and 45 000 rpm in a SW55 rotor, and fractions were harvested from the top (fraction 1) and analyzed by SDS-PAGE and Coomassie blue staining (right panel). Fractions 4–6 of this latter gradient, were pooled, dialyzed against a glycerol-containing buffer as described by Parvin et al. (1989) and stored frozen at −80°C until use. Lane V, purified THOV. The positions of the major structural THOV proteins are indicated on the left and the position of molecular weight markers are indicated in thousands on the right on each panel. (B). Detection of THOV RNA segment four. Fractions of the gradient were deproteinized by extraction with a phenol–chloroform mixture, and incubated with reverse transcriptase and a positive sense ^{32}P-labeled oligonucleotide (5′GGTCAAATCTAAAGACGCGCTCATCCAGAC 3′) (positions 202 to 231 of Thogoto virus RNA segment 4) as described (Albo et al., 1996). The extension products were analyzed by electrophoresis in a 4% polyacrylamide gel containing 8 M urea and autoradiography. (C). Purification of influenza virus core proteins. Purified egg-grown influenza virus (strain A/Puerto Rico/8/34) was processed as indicated in part A for THOV.

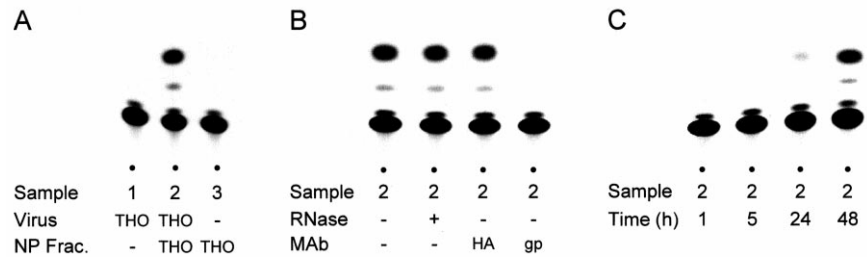

Fig. 2. (A) Expression of a model CAT RNA in THOV-infected cells. Plasmid pTHONPCAT (a gift from J. Ortín) was linearized with Ear I and transcribed in vitro with T7 RNA polymerase to generate a negative-sense RNA (THO-CAT RNA) containing the CAT gene flanked by the non-coding regions of THOV NP gene (Weber et al., 1998). Cultures of BHK-21 cells (10^6 cells) were mock-infected or infected with THOV (multiplicity of infection -MOI- of 1) as indicated (Virus). One hour later, these cultures were transfected, using cationic liposomes, with mixtures prepared in 100 μl of 100 mM Tris–HCl pH 7.4, 100 mM NaCl and 10 mM $MgCl_2$. These mixtures contained 1 μg of THO-CAT RNA and had been incubated for 30 min at 37°C in the presence or absence of 10 μl of purified THOV NP, as indicated (NP frac.). Cell extracts were prepared at 48 h p.i., and samples corresponding to 500 000 cells were assayed for CAT activity using [^{14}C]chloramphenicol and chromatography on thin layer plates as described previously (Martín et al., 1992). (B and C) Packaging of the THO-CAT RNA into THOV particles. Aliquots of the medium harvested from sample 2 were left untreated (part C) or incubated with RNase A or neutralizing MAbs specific for the influenza virus HA (MAb 234/1/F4) or THOV gp75 (MAb 17) (Portela et al., 1992) as indicated in part B. These supernatants were then used to infect fresh BHK-21 cultures (10^6 cells) and cell extracts were prepared 48 h later (B) or at the indicated time postinfection (C). Samples corresponding to 500 000 cells were assayed for CAT activity.

region of the gradient, and the RNA to the bottom (Fig. 1(A), right panel, and Fig. 1(B), right panel). Similar results have been described for influenza virus RNPs (Honda et al., 1988; Parvin et al., 1989; Martín et al., 1992). The THOV NP-enriched fractions were dialyzed and used for the analyses described below. Due to the low abundance of the THOV polymerase proteins, it was not possible to determine the localization of these proteins in the fractions of the two gradients shown in Fig. 1(A).

Following the same procedure, a NP-enriched fraction was obtained from virions of the influenza A virus A/Puerto Rico/8/34. As shown in Fig. 1(A and C), the distribution of the THOV and influenza A viral proteins followed the same pattern in the two gradients.

To determine whether the THOV NP-enriched RNA-free preparations could reconstitute biologically active RNPs, a synthetic negative-sense THOV-like RNA (THO-CAT RNA) encoding a chloramphenicol acetyltransferase (CAT) gene was used. As shown in Fig. 2(A), expression of the reporter CAT gene was detected in extracts obtained from BHK-21 cells, which had been infected with THOV and transfected with mixtures containing the THO-CAT RNA and the THOV NP-enriched fraction (sample 2). However, no reporter gene activity was observed with either mock-infected cells (sample 3) or when the naked RNA was transfected (sample 1). The situation is thus similar to that observed for influenza virus, in that naked RNA was not expressed in virus infected cells, and superinfection with a helper virus was required for expression of the in vitro reconstituted RNPs (Luytjes et al., 1989; Martín et al., 1992). To determine whether following expression, the CAT RNA was packaged into THO virions, the supernatant from the transfection-infection experiments was used to infect fresh BHK-21 cell cultures. As observed in Fig. 2(B and C), significant levels of CAT activity were detected in these cultures, and this activity was abolished by pretreatment of the supernatant with a neutralizing monoclonal antibody (MAb) raised against the gp75 protein of THOV. However, CAT activity was unaffected following incubation of the supernatant with RNase A or with a control MAb (anti-HA of influenza A virus). Furthermore, CAT expression was only detected at later times post-infection (Fig. 2C), indicating that the virions transferred the CAT RNA rather than the CAT enzyme. Altogether, these results demonstrated that CAT transmission was in fact medi-

ated by THOV particles that enclosed CAT RNPs, and the full functionality of the THOV core protein to rescue genes into THOV infectious particles.

Considering that THOV cores can transcribe synthetic short influenza A virus-like templates in vitro (Leahy et al., 1997a), we asked the question whether THOV purified proteins would allow expression of a synthetic influenza A virus-like RNA into influenza A virus-infected cells. We reasoned that the reconstituted hybrid RNPs containing a negative-sense influenza A virus RNA and THOV core proteins could synthesize replicative intermediate (cRNA) molecules when transfected into mammalian cells. We hypothesized, that if these products were produced in influenza A virus-infected cells, the viral proteins provided by the helper infection would allow these cRNA templates to be encapsidated, amplified and finally packaged into influenza virions.

The initial experiments were carried out using a synthetic negative-sense influenza A virus-like RNA (FLU-CAT RNA) encoding the CAT gene. As shown in Fig. 3(A), CAT activity was detected in influenza A-virus infected cultures that had been transfected with mixtures containing the FLU-CAT RNA and either the influenza A virus (sample 2) or the THOV (sample 3) purified core proteins. Moreover, a series of experiments similar to those described in Fig. 2(B) demonstrated that in both cases the FLU-CAT RNA was in fact packaged into the influenza virions released from the transfected-infected cells (Fig. 3(B)). Among the experiments shown in Fig. 3(B), the effect of trypsin treatment of the supernatant harvested from the transfected-infected cells was examined. Trypsin incubation is required to activate the HA of the helper A/Victoria/3/5 influenza virus, but this treatment is not needed to grow and plaque THOV (Clerx et al., 1983). As shown in Fig. 3(B), transmission of the FLU-CAT RNA to fresh cultures required trypsin treatment of the supernatant, and it was not inhibited by treatment of the supernatant with a neutralizing anti-gp75 MAb. These results ruled out the possibility that CAT RNA transmission was mediated by THOV particles, which could have arisen from THOV RNPs that may have been contaminating the THOV NP-enriched fractions.

The above experiments, which involved model CAT genes, did not provide quantitative data on the efficiency of the THOV proteins in rescuing synthetic genes. To address this question, purified THOV proteins were tested for their ability to rescue the full-length influenza virus WSN NA gene into the genome of the influenza virus WSN-

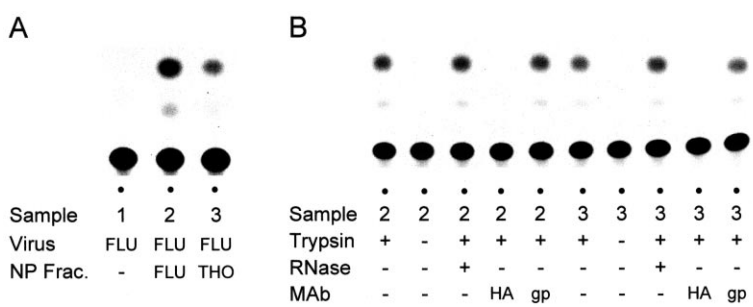

Fig. 3. Expression of a model CAT RNA in influenza A virus-infected cells. A negative sense influenza A virus-like RNA containing the CAT gene flanked by the untranslated sequences of RNA segment eight of influenza A virus (FLU-CAT RNA) was transcribed in vitro from plasmid pIVACAT1/S (a gift from P. Palese)(Piccone et al., 1993). Cultures of COS-1 cells (10^6 cells) were infected with influenza A/Victoria/3/75 virus (MOI of 1) as indicated (Virus). One hour later, the cultures were transfected (as described in Fig. 2) with mixtures, which contained 1 μg of FLU-CAT RNA and 10 μl of the purified NP fraction from either THOV or influenza A virus, as indicated (NP frac.). Cell extracts from these cultures were prepared at 24 h p.i., and assayed for CAT activity as indicated in Fig. 2A. (B) Packaging of the FLU-CAT RNA into influenza virus particles. Aliquots of the medium harvested from samples 2 and 3 were incubated with or without trypsin, and either RNase A or neutralizing MAbs specific for influenza virus HA or THOV gp75, as indicated in the figure. These supernatants were then used to infect fresh MDCK cultures (10^6 cells), which were tested, at 24 h p.i., for CAT expression as indicated in Fig. 2B.

HK. The reassortant, WSN-HK, which derives its NA from A/Hong Kong/8/68 strain and the rest of its genes from WSN virus, does not plaque on MDBK cells in the absence of trypsin, whereas transfectant viruses that rescue the WSN NA gene can form plaques in these cells (Schulman and Palese, 1977; Enami et al., 1990). To rescue the synthetic WSN NA gene we used the plasmid pT3NAM1 (a gift form P. Palese), which contains the NA gene under transcriptional control of a T3 RNA polymerase promoter (García-Sastre et al., 1994), and the protocol described by Enami and Palese (1991). Mixtures containing 1 μg of plasmid pT3NAM1 (pre-digested with restriction enzyme Bbs I and treated with DNA Polymerase I-Klenow fragment-), T3 RNA polymerase (from the MAXIscript T3 Kit, Ambion), and 3 μl of the protein fractions isolated from either influenza virus or THOV, were incubated at 37°C for 15 min in the presence of the four ribonucleoside triphosphates in the buffer included in the transcription kit. The reaction mixture was then transfected into WSN-HK infected MDBK cells following the DEAE-dextran transfection protocol described by Luytjes et al. (1989). Eighteen hours after transfection, the cell supernatant was harvested and one third of it was treated with trypsin and plaqued on MDBK cells in the presence of fetal calf serum to detect the transfectant viruses. The number of virus plaques observed in three experiments were 61, 81 and 88, when using influenza virus proteins, and 32, 40 and 50, when using THOV-derived proteins. No plaques were observed in cells that were transfected with mixtures that lacked the NA gene but contained either the influenza- or the THOV-purified proteins. To determine unambiguously the identity of the NA gene of the viruses selected on MDBK cells, six virus plaques were grown up and a fragment of the NA gene (corresponding to the 5′ terminal 460 nucleotides of the vRNA segment) was amplified by RT-PCR. Sequencing of the amplified fragment confirmed that the NA gene derived from the synthetic WSN NA RNA.

All NA gene rescue experiments included 3 μl of the NP-enriched fraction, from either THOV or influenza virus, in the transfection mixture since it was observed that adding higher volumes of the protein fraction reduced the efficiency of rescue. This 3 μl sample corresponded to $\sim$300 ng of the influenza virus NP and to $\sim$60 ng of the THOV NP, which suggests that there are more viruses rescued per ng of NP protein when using the THOV fraction. However, these figures do not indicate that the THOV preparation was more effective than the influenza virus preparation in rescuing synthetic genes, since we and others have observed that there is no direct correlation between the amount of influenza A virus NP in the transfection mixture and the rescue efficiency (Castrucci et al., 1992; and data not shown). Nevertheless, the data shown here indicate that THOV and influenza virus NP fractions are similarly active at rescuing synthetic genes into influenza A particles.

As indicated above, it has been shown that THOV cores could transcribe influenza A model RNAs in vitro (Leahy et al., 1997a). However, we demonstrated that the FLU-CAT RNA was not expressed in cells which synthesized the THOV core proteins from cloned cDNAs (Weber et al., 1998). It should be noted, however, that the latter system, by analogy to an equivalent system using influenza A virus components (Huang et al., 1990), is very demanding since CAT activity detection is dependant on replication (amplification) of the input RNA, a situation which is not required for detection of RNA transcripts produced in vitro. Therefore, these two sets of data are not contradictory, but they suggest that THO virus polymerase can copy influenza A virus templates although it is inefficient at amplifying them. In agreement with this interpretation, we suggest that the fact that the THO virus core preparation allowed rescue of influenza A virus genes indicates that the THOV proteins encapsidated and copied the influenza A RNAs to produce a replicative intermediate (cRNA) in transfected cells. The cRNA molecules would then be recognized and efficiently amplified not by the THOV core proteins but through the proteins provided by the helper influenza A virus infection.

In summary, we have demonstrated that THO virus RNPs can be isolated and dissociated into

their components following procedures established for influenza A virus, an observation which adds further support to the classification of THO and influenza A viruses within the same family. In addition, we have demonstrated here the ability of the THOV purified RNP proteins to rescue synthetic genes into both THO and influenza A viruses. Thus, this methodology offers a means of obtaining genetically modified tick-borne viruses, which has applications for understanding how members of the *Orthomyxoviridae* have adapted to different modes of transmission. Recently, Neumann et al. (1999) and Fodor et al. (1999) have described the generation of influenza A virus using exclusively plasmidic DNA. Although it can be anticipated that this procedure will be widely used to rescue modified genes into the influenza virus genome, the procedure using RNPs reconstituted from viral proteins will still be useful in some instances. For example, when rescuing only one gene into a specific viral strain, it may be simpler to substitute that particular gene by means of the RNP-transfection procedure rather than cloning all genes of the viral strain in order to rescue the virus entirely from cDNAs. However, it has been observed when using influenza virus-derived proteins for reconstituting RNPs, the progeny of a transfection-infection experiment may include viruses that have incorporated a segment from the virus used to prepare the NP protein fraction (Subbarao et al., 1993; Li et al., 1995). The alternative use of the THO virus proteins would eliminate this problem since none of the THOV segments are likely to substitute for any of the influenza virus genes.

Acknowledgements

This work was supported by Fondo de Investigaciones Sanitarias (Grant 98/0315). P. Gómez-Puertas was supported by a fellowship from 'Instituto de Salud Carlos III'. We thank T. Zürcher and J. Ortín for useful discussions during the development of this work and we also thank J. Ortín and P. Palese for the reagents provided.

References

Albo, C., Martín, J., Portela, A., 1996. The 5′ ends of Thogoto virus (*Orthomyxoviridae*) mRNAs are homogeneous in both length and sequence. J. Virol. 70, 9013–9017.

Castrucci, M.R., Bilsel, P., Kawaoka, Y., 1992. Attenuation of influenza A virus by insertion of a foreign epitope into the neuraminidase. J. Virol. 66, 4647–4653.

Clerx, J.P.M., Fuller, F., Bishop, D.H.L., 1983. Tick-borne viruses structurally similar to Orthomyxoviruses. Virology 127, 205–219.

Desselberger, U., Racaniello, V.R., Zarza, J.J., Palese, P., 1980. The 3′ and 5′-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene 8, 315–328.

Enami, M., Luytjes, W., Krystal, M., Palese, P., 1990. Introduction of site-specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA 87, 3802–3805.

Enami, M., Palese, P., 1991. High-efficiency formation of influenza virus transfectants. J. Virol. 65, 2711–2713.

Fodor, E., Devenish, L., Engelhardt, O.G., Palese, P., Brownlee, G.G., García-Sastre, A., 1999. Rescue of influenza A virus from recombinant DNA. J. Virol. 73, 9679–9682.

García-Sastre, A., Muster, T., Barclay, W.S., Percy, N., Palese, P., 1994. Use of a mammalian internal ribosomal entry site element for expression of a foreign protein by a transfectant influenza virus. J. Virol. 68, 6254–6261.

García-Sastre, A., 1998. Negative-strand RNA viruses: applications to biotechnology. Trends Biotechnol. 16, 230–235.

Herz, C., Stavnezer, E., Krug, R.M., 1981. Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. Cell 26, 391–400.

Honda, A., Uéda, K., Nagata, K., Ishihama, A., 1988. RNA polymerase of influenza virus: role of NP in RNA chain elongation. J. Biochem. 104, 1021–1026.

Huang, T.-S., Palese, P., Krystal, M., 1990. Determination of influenza virus proteins required for genome replication. J. Virol. 64, 5669–5673.

Krug, R.M., Alonso-Caplen, F.V., Julkunem, I., Katze, M.G., 1989. Expression and replication of the influenza virus genome. In: Krug, R.M. (Ed.), The Influenza Viruses. Plenum Press, New York, pp. 89–152.

Lamb, R.A., Krug, R.M., 1996. *Orthomyxoviridae*: the viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, Third edn. Lippincott-Raven, Philadelphia, PA, pp. 1353–1395.

Leahy, M.B, Dessens, J.T., Nuttall, P.A., 1997a. Striking conformational similarities between the transcription promoters of the Thogoto and influenza A viruses: evidence for intrastrand base-pairing in the 5′ promoter arm. J. Virol. 71, 8352–8356.

Leahy, M.B., Dessens, J.T., Weber, F., Kochs, G., Nuttall, P.A., 1997b. The fourth genus in the *Orthomyxoviridae*: sequence analyses of Thogoto virus polymerase proteins and comparison with influenza viruses. Virus Res. 50, 215–224.

Leahy, M., Dessens, J.T., Nuttall, P.A., 1997c. In vitro polymerase activity of Thogoto virus: evidence for a unique cap-snatching mechanism in a tick-borne orthomyxovirus. J. Virol. 71, 8347–8351.

Li, S., Xu, M., Coelingh, K., 1995. Electroporation of influenza virus ribonucleoprotein complexes for rescue of the nucleoprotein and matrix genes. Virus Res. 37, 153–161.

Luytjes, W., Krystal, M., Enami, M., Parvin, J.D., Palese, P., 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell 59, 1107–1113.

Martín, J., Albo, C., Ortín, J., Melero, J.A., Portela, A., 1992. In vitro reconstitution of active influenza virus ribonucleoprotein complexes using viral proteins purified from infected cells. J. Gen. Virol. 73, 1855–1859.

Mayo, M.A, Pringle, C.R., 1998. Virus taxonomy-1997. J. Gen. Virol. 79, 649–657.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., Hobom, G., Kawaoka, Y., 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. USA 96, 9345–9350.

Nuttall, P.A., Morse, M.A., Jones, L.D., Portela, A., 1995. Adaptation of members of the *Orthomyxoviridae* family to transmission by ticks. In: Gibbs, A.J., Calisher, C.H., García-Arenal, F. (Eds.), Molecular Basis of Virus Evolution. Cambridge University Press, Cambridge, pp. 416–425.

Palese, P., Zheng, H., Engelhardt, O.G., Pleschka, S., García-Sastre, A., 1996. Negative-strand RNA viruses: genetic engineering and applications. Proc. Natl. Acad. Sci. USA 93, 11354–11358.

Parvin, J.D., Palese, P., Honda, A., Ishihama, A., Krystal, M., 1989. Promoter analysis of influenza virus RNA polymerase. J. Virol. 63, 5142–5152.

Piccone, M.E., Fernánzdez-Sesma, A., Palese, P., 1993. Mutational analysis of the influenza virus vRNA promoter. Virus Res. 28, 99–112.

Plotch, S.J., Bouloy, M., Ulmanen, I., Krug, R.M., 1981. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 23, 847–858.

Portela, A., Jones, L.D., Nuttall, P.A., 1992. Identification of viral structural polypeptides of Thogoto virus (a tick-borne orthomyxo-like virus) and functions associated with the glycoprotein. J. Gen. Virol. 73, 2823–2830.

Schulman, J.L., Palese, P., 1977. Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. J. Virol. 24, 170–176.

Seong, B.L., Brownlee, G.G., 1992. A new method for reconstituting influenza polymerase and RNA in vitro: a study of the promoter elements for cRNA and vRNA synthesis in vitro and viral rescue in vivo. Virology 186, 247–260.

Subbarao, E.K., Kawaoka, Y., Murphy, B.R., 1993. Rescue of an influenza A virus wild-type PB2 gene and a mutant derivative bearing a site-specific temperature-sensitive and attenuating mutation. J. Virol. 67, 7223–7228.

Weber, F., Haller, O., Kochs, G., 1996. Nucleoprotein viral RNA and mRNA of Thogoto virus: a novel 'cap-stealing' mechanism in tick-borne orthomyxoviruses? J. Virol. 70, 8361–8367.

Weber, F., Jambrina, E., González, S., Dessens, J.T., Leahy, M., Kochs, G., Portela, A., Nuttall, P.A., Haller, O., Ortín, J., Zürcher, T., 1998. In vivo reconstitution of active Thogoto virus polymerase: assays for the compatibility with other orthomyxovirus core proteins and template RNAs. Virus Res. 58, 13–20.