In vitro reconstitution of active influenza virus ribonucleoprotein complexes using viral proteins purified from infected cells

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A procedure to obtain RNA-free preparations containing the nucleoprotein (NP) and polymerase (P) proteins from influenza virus-infected cell extracts has been developed. The influenza virus P proteins present in these preparations copied small synthetic RNA molecules derived from plasmid sequences. In addition, RNA molecules encapsidated with the NP and P proteins were amplified and packaged into virus particles when transfected into influenza virus-infected cells. Thus, the preparations of NP and P proteins display features similar to those isolated from purified influenza virions, and represent an alternative for the preparation of active influenza virus P protein.

Several laboratories have recently reported procedures that allow cDNA-derived RNA molecules to be introduced into the genome of animal viruses containing negative-sense ssRNA (Luytjes et al., 1989; Yamanaka et al., 1991; Park et al., 1991). For influenza viruses, the methodology described makes use of functional ribonucleoprotein (RNP) complexes reconstituted in vitro from synthetic RNA templates and RNA-free protein preparations [hereafter referred to as the NP-Ps fraction(s)], isolated from egg-grown influenza virus and containing nucleoprotein (NP) and traces of the three polymerase (P) proteins (Honda et al., 1988, 1990; Parvin et al., 1989). Nucleocapsids reconstituted with these NP-Ps fractions and synthetic RNA are functional in vitro because the encapsidated RNA can be transcribed by the virus-specific replicase (Parvin et al., 1989; Yamanaka et al., 1991). However, since RNP cores isolated from virions synthesize only positive-sense RNA (Bishop et al., 1971; Plotch & Krug, 1977; Bouloy et al., 1978; Braam et al., 1983), this polymerase might not be suitable for analysing templates other than the negative-sense viral RNA. However, when RNP complexes assembled in vitro are transfected into susceptible cells previously infected with influenza virus, the encapsidated RNA can be rescued into influenza virus particles. In this way it has been possible to package a synthetic RNA encoding the chloramphenicol acetyltransferase (CAT) enzyme flanked by the non-translated sequences of viral segment 8 RNA into influenza virions (Luytjes et al., 1989), as well as to exchange segments 4, 6 and 8 of influenza A virus (Enami et al., 1990; Enami & Palese, 1991; Muster et al., 1991).

In this report we describe an alternative method for the preparation of active NP-Ps fractions. The major difference from procedures described previously lies in the active fractions being prepared from influenza virus-infected cell extracts instead of from purified virus. We reasoned that infected cell extracts could be a good source for preparing NP-Ps fractions because large amounts of active RNP complexes are formed in infected cells and cell extracts have been shown to be active in synthesizing virus-specific RNA in vitro (Beaton & Krug, 1984; Del Rio et al., 1985; Shapiro & Krug, 1988; López de Turiso et al., 1990). Accordingly, a homogenate obtained from infected MDCK cells lysed...
in hypotonic buffer (prepared as described by Dignam et al., 1983) was used as the starting material. RNP cores present in the homogenate were pelleted at high speed and dissociated by centrifugation through two consecutive CsCl-glycerol step gradients [following a procedure similar to that described by Honda et al. (1988) and Parvin et al. (1989)]. Experimental details are given in the legend to Fig. 1.

Typically we started with $2 \times 10^8$ cells infected with either A/PR/8/34 virus (PR8) or VP virus [a reassortant virus containing the haemagglutinin (HA) and neuraminidase segments from A/Victoria/3/75 virus and the other segments from PR8] (P. Suárez, unpublished results). Cells were lysed in hypotonic buffer to yield a cellular homogenate which was clarified by low speed centrifugation to remove nuclei and cell debris. The resulting clear supernatant was subjected to high speed centrifugation to pellet RNP cores. Under these conditions virtually all the NP (approximately 500 µg) was pelleted, indicating that this polypeptide was in the form of RNP cores or protein aggregates. The pellet from this step was then resuspended, loaded onto a CsCl-glycerol step gradient and centrifuged for 24 h. The distribution of the proteins along this gradient is shown in Fig. 1 (a). Most of the cellular and viral proteins were located in the top half of the gradient (fractions 1 to 7). However, almost all the NP was found in the middle and lower part of the gradient, fractions 7 to 9 being most highly enriched in this polypeptide. The two bands of 89K to 100K (indicated by Ps in the figure) detected in the pellet were tentatively identified as the influenza virus P polypeptides based on their electrophoretic mobility in the gel and their absence from a similar gradient prepared from non-infected cells (data not shown). Although P polypeptides were mainly found in the pellet fraction, significant amounts were also detected in fractions from the lower half of the gradient.

The RNA content of the gradient was analysed by agarose gel electrophoresis and ethidium bromide staining (Fig. 1 b). Most of the RNA present in the extract was
in the lowest fractions, and fractions enriched in NP were practically devoid of RNA molecules. The distribution of proteins and RNA in this gradient was not a peculiarity of PR8 virus-infected cell extracts because similar results were obtained when a homogenate from cells infected with the A/Victoria/3/75 strain of virus was used (data not shown). In this case the amount of viral genomic RNA present in the different fractions of the CsCl gradient was estimated by dot hybridization using an oligodeoxynucleotide specific for segment 4 as a probe. It was determined that a sample analogous to fraction 8 in Fig. 1(a) contained less than 50 pg of negative-sense HA RNA per µg of NP, whereas the same amount of NP in the RNP core pellet contained 5000 pg of this RNA.

To isolate the NP from other cellular proteins, fraction 8 (Fig. 1a) was subjected to a second centrifugation in another CsCl–glycerol step gradient. Again, NP–Ps fractions were identified by SDS–PAGE and Coomassie blue staining. Usually, two distinct fractions, A and B, were harvested, the first corresponding to the fraction containing the largest amount of NP and the second being a pool of the next two fractions from the gradient. Both fractions were dialysed independently and stored frozen at −80 °C. Typically, 200 µl of fraction A and 500 µl of fraction B were recovered. In Fig. 1(c), the protein composition of these two fractions (lanes A and B) was compared to that of a NP–Ps fraction prepared from 4 mg of purified virus by the procedure of Parvin et al. (1989). Although cell-derived fractions contained small amounts of cellular proteins, they were enriched in NP and contained significant amounts of P polypeptides. It was estimated, by comparison with BSA electrophoresed as a standard in the same gel, that the concentration of NP in fractions A and B was 1 and 0.2 µg/µl, respectively.

To determine whether the influenza virus P proteins present in these fractions were able to copy small RNA templates derived from plasmid-encoded sequences, plasmid pT7HA3′ was prepared. This plasmid contains the last 73 nucleotides of the HA gene from PR8 virus so that after digestion with Hgal and transcription with T7 RNA polymerase, RNA molecules 87 nucleotides in length (ending with the last 73 nucleotides from the 3′ end of the HA gene) were obtained. This RNA template was mixed with cell-derived NP–Ps fractions and incubated in the presence of [α-32P]CTP; the reaction products were analysed in a denaturing polyacrylamide gel (Fig. 2a). A band comigrating with an 87 nucleotide RNA marker was observed when the dinucleotide ApG was included as a primer for the influenza virus replicase (Plotch & Krug, 1977; Bouloy et al., 1978). This 87 nucleotide band was not detected when either the template RNA or ApG were absent. When ApG was included in the reaction a general background of bands was detected which probably correspond to RNA products transcribed from endogenous viral RNAs not removed during the purification of the NP–Ps fraction. These endogenous RNA molecules probably remained bound to the polymerase complex and thus functioned more efficiently than the added RNA template. Although no further attempt was made to characterize the
87 nucleotide product, it was assumed (based on the results obtained previously by Parvin et al., 1989) that this product corresponded to a full-length copy of the template RNA made by the influenza virus replicase.

To determine whether the RNA encapsidated in vitro could be amplified and rescued into influenza virions, plasmid pB2CAT9 (a gift from M. Krystal) was used to rescue CAT activity. This plasmid, which mirrors pIVACAT1 (Luytjes et al., 1989), contains the CAT gene flanked by the non-translated regions of the PB2 gene so that after transcription of the Hgal-digested plasmid with T7 RNA polymerase, negative-sense RNA molecules containing 5' and 3' sequences identical to the influenza virus RNA ends are obtained. Reconstituted RNP complexes obtained from this RNA template and cell-derived NP-Ps fractions were used to transfect WSN virus-infected MDBK cells. Cell extracts were then harvested and tested for CAT activity (Fig. 2b). The minus-sense PB2CAT9 RNA was amplified and transcribed because high levels of CAT activity were found when using either fraction A or B. However, no CAT activity was detected when any components of the reaction mixture (NP-Ps fraction, T7 RNA polymerase, plasmid DNA or helper virus) were omitted (data not shown).

The level of CAT activity present in the cell extracts was quantified by determining the number of pmol of [3H]acetyl-CoA transferred to chloramphenicol in 1 h, when 15 μg of protein of the cell extract was incubated under the conditions described in Portela et al. (1985). The results obtained varied depending on the experiment and the enzyme preparation. When using cell-derived NP-Ps fractions, values ranging between 47 and 1661 pmol of acetyl-CoA transferred were obtained. The high value obtained in three independent experiments when using the same virion-derived NP-Ps fraction was 77 pmol. The variability of the transfection assays precludes a precise quantification of the efficiency of both types of preparations. Nevertheless, the data showed that NP-Ps fractions prepared from infected cell extracts function at least as efficiently as those prepared from purified virions in amplifying the CAT gene in vivo.

To examine whether the synthetic CAT gene was encapsidated, a cell monolayer was infected with the virus supernatant recovered from the transfection experiment and tested for the presence of CAT activity (Fig. 2c). High levels of CAT activity were detected when cells were infected with the virus inoculum, and this activity was abolished by pretreatment of the supernatant with anti-WSN virus antiserum, but not by RNase.

These two sets of experiments demonstrate that cell-derived NP-Ps fractions are able to reconstitute active RNP complexes that are active both in copying in vitro and in rescuing synthetic RNA molecules into influenza virions.

In conclusion, we have shown that cell extracts are a good source from which to prepare NP-Ps fractions that display properties the same as those obtained from purified influenza virions (Parvin et al., 1989; Luytjes et al., 1989; Yamanaka et al., 1991). Therefore the procedure described is an alternative method which circumvents the manipulation of large amounts of live virus required to prepare virion-derived NP-Ps fractions. In addition, because cellular extracts have been shown to be active in transcribing both positive- and negative-sense viral RNA (Beaton & Krug, 1984; Del Río et al., 1985; Shapiro & Krug, 1988; López de Turizo et al., 1990), it is conceivable that cell-derived NP-Ps fractions harvested at different times post-infection could display different properties regarding their template requirements for in vitro RNA synthesis. This feature, if proven, could contribute to characterizing cis-acting sequences involved in the transcription–replication processes of the influenza virus genome.

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References


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