Influenza virus naked RNA can be expressed upon transfection into cells co-expressing the three subunits of the polymerase and the nucleoprotein from simian virus 40 recombinant viruses

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The functionality of the influenza virus polymerase subunits and the nucleoprotein expressed from simian virus 40 (SV40) recombinants has been tested by their ability to direct the in vivo expression of influenza virus-like RNAs. These RNAs, which contained either the chloramphenicol acetyltransferase (CAT) or haemagglutinin (HA) genes, were synthesized and reconstituted in vitro into viral ribonucleoproteins with a polymerase/ribonucleoprotein mixture purified from influenza virus-infected cells. Only the coinfection with SV40 recombinant viruses expressing the three polymerase subunits and the nucleoprotein allowed the expression of the transfecting CAT or HA RNAs, confirming that this set of viral genes is the minimal requirement for viral gene expression. Unexpectedly, transfection of the corresponding naked RNAs into SV40 recombinant-infected cells was as effective in directing the synthesis of CAT or HA proteins as the standard reconstituted ribonucleoprotein transfection. These results may be important for the genetic analysis of trans-acting factors involved in influenza virus transcription and replication and may open the way to rescuing influenza viruses in the absence of a helper virus.

Influenza virus RNA transcription and replication take place in the nucleus of the infected cell (Herz et al., 1981; Jackson et al., 1982) in ribonucleoprotein (RNP) complexes consisting of at least the three polymerase subunits (PB1, PB2 and PA) and the nucleoprotein (NP) in addition to positive or negative polarity RNA segments (Beaton & Krug, 1986; Detjen et al., 1987; Honda et al., 1988; van Wike et al., 1981). In spite of the development of several in vitro transcription and replication systems derived from infected cells (Beaton & Krug, 1984; del Rio et al., 1985; Lopez-Turiso et al., 1990; Shapiro & Krug, 1988; Takeuchi et al., 1987), several questions remain unresolved relating to the control of the transcription-replication and cRNA–vRNA synthesis switches, as well as the structure of the transcription/replication complex(es).

In the last few years, an experimental approach has been developed for the rescue of artificial influenza virus RNAs into infectious virus (Enami & Palese, 1991; Luytjes et al., 1989; Yamanaka et al., 1991). This system involves the in vitro reconstitution of RNPs, using synthetic RNA and a mixture of polymerase subunits and nucleoprotein purified from virions, and its transfection into cells infected by a helper virus. Its use has allowed the introduction of defined mutations into viral RNA (Enami et al., 1990) and the analysis of the structure of the viral promoter (Li & Palese, 1992; Seong & Brownlee, 1992; Yamanaka et al., 1991). However, the requirement for a helper virus infection has precluded the study of trans-acting factors involved in the transcription and replication processes. An important breakthrough in that direction was the description of a completely artificial system in which defined vaccinia virus recombinants substituted for the helper virus infection (Huang et al., 1990). Using this system, it was concluded that the three polymerase subunits and the nucleoprotein constitute the minimal requirements for viral RNA transcription and replication (Huang et al., 1990). In spite of previous evidence for the role of the non-structural proteins NS1 and NS2 in viral RNA synthesis (Koenecke et al., 1981; Odagiri & Tobita, 1990; Shimizu et al., 1982; Snyder et al., 1990) and their location in the nucleus of the infected cells (Briedis et al., 1981; Greenspan et al., 1985), no effect could be seen when they were expressed in addition to the minimal replication system (Huang et al., 1990).

In previous studies we have expressed a number of

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Fig. 1. Biological activity of the SV40 recombinants expressing the polymerase subunits and NP. Cultures of $3 \times 10^6$ COS-1 cells were infected with the SV40 recombinants, with influenza virus or mock-infected. Independent controls were carried out to ensure that each SV40 recombinant virus infected most of the cells in the culture. At 48 h post-infection, the cultures were transfected by the DEAE-dextran/DMSO method with the T7 RNA polymerase transcript of plasmid pPB2CAT9. Transcription was carried out in the presence of polymerase/NP mixture purified from PR/8 influenza virus-infected cells. At 20 h after transfection, total cell protein extracts were prepared and assayed for CAT activity. (a) Assays were carried out by the TLC procedure using 50 µg of protein, except for the sample of cells infected by all four SV40 recombinants that had 10 µg, and an incubation time of 4 h. (b) Assays were carried out by the PE method using 1 h incubation time and two doses of protein to ensure that the assay was not saturated. The values given indicate pmol of $[^3H]$acetate transferred to chloramphenicol per µg of protein lysate. SVPOLs indicates a mixture of SVPB1, SVPB2 and SVPA recombinant viruses. ND, Activity not detectable.
were carried out by the PE method as in Fig. 1. The presence or absence of polymerase/NP mixture are shown. The assays recombinants and transfected with CAT RNA transcribed in the cell protein extracts were prepared and assayed for CAT activity. (a) Assays were carried out by the TLC procedure using 20 μg of protein and an incubation time of 4 h. (b) The results of four independent experiments in which the cultures were infected by all four SV40 recombinants and transfected with CAT RNA transcribed in the presence or absence of polymerase/NP mixture are shown. The assays were carried out by the PE method as in Fig. 1.

Fig. 2. Expression of naked influenza virus-like CAT RNA. Cultures of COS-1 cells were infected with SV40 recombinants as described in Fig. 1. Infected cell cultures were transfected by the lipofectin method with CAT RNA transcribed, as indicated in Fig. 1, in the presence or absence of purified polymerase/NP mixture. At 20 h after transfection, total cell protein extracts were prepared and assayed for CAT activity. (a) Assays were carried out by the TLC procedure using 20 μg of protein and an incubation time of 4 h. (b) The results of four independent transfection experiments, the activities obtained with naked RNA were 1-2- to 24-fold higher than those observed in parallel standard RNP transfections. The basis for the differences among the various expression systems is not apparent. It is conceivable that the former transfections demand a more effective protection of the transfecting RNA than the latter. Conversely, the higher concentration of polymerase subunits provided by the SV40 expression system (Nieto et al., 1992) and, more importantly, the fact that they are free to complex the incoming RNA, might lead to a more effective intracellular formation of transcription complexes. In this respect, the SV40-based system is similar to the one recently described by Kimura et al. (1992) and, in addition, it offers an easier way of expressing different combinations of proteins by means of infection with the corresponding SV40 recombinants.

To check further the efficiency of the system in directing influenza virus RNA expression, cell cultures were infected with SVPB1, SVPB2, SVPA and SVNP recombinant viruses and transfected with a negative-polarity RNA corresponding to segment 4 of WSN influenza virus. This RNA was obtained by T3 RNA polymerase transcription of plasmid pT3/WSN-HA digested with Ksp632I and filled in with Klenow polymerase (Enami & Palese, 1991), both in the presence and in the absence of purified polymerase–NP mixture. Sixteen hours after transfection, cell monolayers were fixed and analysed by immunofluorescence. Immunostaining with a specific polyclonal antibody showed that haemagglutinin (HA) was detected after transfection of either naked HA RNA or reconstituted HA RNP into cells infected with SV40 recombinants expressing the three subunits of the polymerase and NP, but not in mock-infected, in mock-transfected cells or in cells expressing the three subunits of the polymerase (Fig. 3). The expression of HA was observed in 10 to 20% of the transfected cells and its accumulation was comparable to that observed during a normal influenza virus infection.

In conclusion, using influenza virus gene products expressed by means of SV40 recombinants in COS-1 cells, we have confirmed that in this system the three subunits of the polymerase and the nucleoprotein constitute the minimal gene complement for the expression of a foreign, influenza virus-like, gene. Unexpectedly, however, the in vitro reconstitution of the
Mock Mock/T SVPOLs

Fig. 3. Expression of influenza virus HA naked RNA. Cultures of COS-1 cells were infected as described in Fig. 1. At 48 h post-infection, cells were mock-transfected or transfected by the lipofectin method with a T3 polymerase transcript of plasmid pT3/WSN-HA prepared in the presence or absence of the purified polymerase/NP mixture. At 16 h after transfection, cultures were fixed with methanol and stained with an anti-PR/8 virus rabbit antisemur and Texas red-tagged goat anti-rabbit IgG serum. SVPOLs indicates a mixture of SVPB1, SVPB2 and SVPA viruses. Mock/T indicates transfection on a mock-infected culture.

transfected RNA into viral RNP was not required for its expression. This result might be relevant from a practical point of view, since it eliminates the necessity for purification of the polymerase/NP mixture from either virions or infected cells (Luytjes et al., 1989; Martin et al., 1992). In addition, the possibility of expressing viral genes after transfection of naked RNA eliminates the problems that the incoming, transfecting viral proteins might introduce in the interpretation of the phenotype of viral gene mutants being assayed in the system. Furthermore, this possibility may open the way for a clearer procedure by which to rescue transfectant viruses, since the purified polymerase/NP mixture, in spite of being practically devoid of viral RNA, can produce infectious virus when complemented in trans by SV40 recombinant viruses expressing the polymerase and the NP (data not shown).

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