Synthesis of biologically active influenza virus core proteins using a vaccinia virus–T7 RNA polymerase expression system

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An in vivo system in which expression of a synthetic influenza virus-like chloramphenicol acetyltransferase (CAT) RNA is driven by influenza virus proteins synthesized from cloned cDNAs has been developed. Expression of the four influenza virus core proteins (nucleoprotein, PA, PB1 and PB2) was performed by transfection of four pGEM recombinant plasmids, each containing one of the four viral genes, into cell cultures previously infected with a vaccinia virus recombinant encoding the T7 RNA polymerase (vTF7-3). When a naked negative-sense influenza virus-like CAT RNA was transfected into cells expressing the four influenza virus proteins, CAT activity was detected in the cell extracts, demonstrating that the expressed proteins had RNA-synthesizing activity. In this system, CAT RNA templates containing additional nucleotides at the 3′ end were also expressed, resulting in CAT activity. This showed that the influenza virus polymerase can recognize its promoter when located internally on an RNA template. In influenza virus-infected cells however, CAT activity was detected only when the CAT RNA contained the viral promoter at the exact 3′ end and was transfected as in vitro assembled ribonucleoprotein. These results are discussed in terms of the different requirements of the two helper systems for expression of an exogenously added RNA.

Our knowledge of the RNA replication and transcription processes of negative-sense ssRNA viruses has been limited by the lack of systems in which the functionality of viral replicase genes expressed from cloned cDNAs could be tested. In the last few years however, several such systems have been described for members of the Orthomyxoviridae (Huang et al., 1990; Kimura et al., 1992; de la Luna et al., 1993), Rhabdoviridae (Pattnaik & Wertz, 1990; Pattnaik et al., 1992), Paramyxoviridae (Curran et al., 1991; Calain et al., 1992) and Bunyaviridae (Jin & Elliot, 1991) families. Thus, it has been possible to define the cis-acting RNA regions and viral proteins required for virus-specific RNA synthesis.

In the case of influenza virus, several procedures that allow in vitro reconstitution of influenza virus-like ribonucleoprotein (RNP) complexes using synthetic RNAs and purified viral proteins have been described (Parvin et al., 1989; Luytjes et al., 1989; Yamanaka et al., 1991; Seong & Brownlee, 1992a,b; Piccone et al., 1993). It is, however, not clear whether the viral promoter has to be located at the extreme 3′ end of the RNA template. Parvin et al. (1989) reported that an RNA template containing five extra nucleotides at the 3′ end of the influenza A virus vRNA promoter was recognized and copied in vitro, although at one-third the efficiency of the wild-type RNA, by the influenza virus replicase. Similarly, templates containing one, five and 13 nucleotides added at the 3′ end of a synthetic chloramphenicol acetyltransferase (CAT) cRNA retained 55, 26 and 6% of the promoter activity in in vitro assays respectively (Li & Palese, 1992). However, when these CAT cRNAs were tested in vivo by transfection into influenza virus-infected cells, only the template with one additional nucleotide was functional (20% of the wild-type RNA; Li & Palese, 1992). Piccone et al. (1993) showed that RNA templates containing 30 extra nucleotides were not functional in either of the assays.

Three systems where expression of synthetic influenza virus-like RNAs was driven by influenza virus proteins expressed from cloned cDNAs have been described (Huang et al., 1990; Kimura et al., 1992; de la Luna et al., 1993). In all three cases, the influenza virus nucleoprotein (NP), PA, PB1 and PB2 polypeptides were
**Fig. 1.** Expression of the influenza virus proteins as analysed by SDS-PAGE and immunoprecipitation. (a) COS-1 cells were infected with influenza virus at an m.o.i. of 1 (lane 1), vTF7-3 at an m.o.i. of 5 (lane 2) or with vTF7-3 at an m.o.i. of 5 and transfected with the four pGEM recombinant plasmids encoding the influenza virus PA, PB1, PB2 and NP proteins (0.5 μg of each plasmid) using lipofectin (lane 3). At 20 h post-transfection, cell cultures were labelled with Tran35S-label for 2 h. Cell extracts were then prepared, and the proteins solubilized and resolved by SDS-PAGE. Positions of Mr markers are indicated on the left. (b) [35S]Methionine-labelled extracts, either from vTF7-3-infected cells (lanes 1, 3, 5 and 7) or from cells infected with vTF7-3 and transfected with the four pGEM recombinant plasmids (lanes 2, 4, 6 and 8) were immunoprecipitated (Portela et al., 1992) with specific monoclonal antibodies raised against the NP, PA and PB2 polypeptides, or with a control monoclonal antibody (C) as indicated.

the minimum set of viral components required for efficient expression of synthetic RNAs. In the system described by Huang et al. (1990) the influenza virus proteins were supplied through infection with four vaccinia virus recombinants, each containing one of four influenza virus genes. Kimura et al. (1992) reported functional expression of the influenza virus polymerase in a cell line that expresses the proteins in response to dexamethasone. In the system described by de la Luna et al. (1993) the viral proteins were provided by co-infection of cell cultures with a set of four simian virus (SV) 40 recombinant viruses. In the latter two systems, unlike the one described by Huang et al. (1990), naked RNA templates were expressed efficiently upon transfection into the cells.

Here we describe a system based on a vaccinia virus recombinant expressing the T7 RNA polymerase (vTF7-3; Fuerst et al., 1986) and four pGEM recombinant plasmids, which encode the four influenza virus core proteins, that allows expression of a synthetic influenza virus-like CAT RNA. In this system, naked RNA molecules were expressed efficiently and RNA templates containing non-influenza virus nucleotides added at the 3' terminus were also transcribed by the influenza virus replicase.

Four cDNAs encoding the influenza virus PB1, PB2, PA and NP polypeptides of strain A/Victoria/3/75 (de la Luna et al., 1989) were transferred from pUC18 plasmids to the polylinker of pGEM-3z (Promega) vectors, downstream of the T7 RNA polymerase promoter, to generate plasmids pGEM-PB1, pGEM-PB2, pGEM-PA and pGEM-NP respectively. To determine whether the plasmids directed the expression of cloned genes, COS-1 cells were infected with vTF7-3 and subsequently transfected with the four pGEM plasmids. The cells were then labelled with Tran35S-label (ICN) and either whole cell lysates or proteins immunoprecipitated from the cell extracts were fractionated by SDS-PAGE (Fig. 1). In crude lysates from cells infected with vTF7-3 and transfected with the plasmids, 35S-labelled bands with the expected electrophoretic mobility for the influenza virus NP and PB2–PA proteins were observed (Fig. 1a). To confirm the identity of these bands, immunoprecipitation experiments were performed with monoclonal antibodies to the influenza virus NP, PA and PB2 polypeptides. As shown in Fig.
Influenza virus-like CAT RNA in COS-1 cells. COS-1 cells (1 x 10^6) were infected with influenza virus at an m.o.i. of 1, vTF7-3 virus at an m.o.i. of 5 and transfected with different combinations of pGEM recombinant plasmids or with a recombinant vaccinia virus that expresses the F protein of RS virus (vWF) at an m.o.i. of 5 (Portela et al., 1989) and transfected with the four pGEM plasmid DNAs, as indicated. After 6 h incubation, all cell cultures were transfected with either naked (N) or encapsidated (E) CAT RNA (prepared as described below) and 18 h later cell extracts were prepared and CAT activity was assayed by chromatography on TLC plates (Gorman et al., 1982). The CAT RNA template was prepared by incubating 0.5 µg of Hgal-digested pPB2CAT9 plasmid with 15 units of T7 RNA polymerase in buffer (Promega). The transcription reaction was carried out for 15 min either in the presence or absence of purified influenza virus core proteins (Martin et al., 1992) to yield naked and encapsidated RNAs respectively. Transcription mixtures were then incubated for 15 min with DNase I (1 unit; Promega), mixed with lipofectin reagent and transfected into infected cell cultures. In lane C, the in vitro transcription reaction took place in the absence of the T7 enzyme.

<table>
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<th>Helper virus</th>
<th>Influenza</th>
<th>vWF</th>
<th>vTF7-3</th>
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<tbody>
<tr>
<td>pGEM-PB1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGEM-PB2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pGEM-PA</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pGEM-NP</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CAT RNA</td>
<td>N E N N</td>
<td>N N</td>
<td>C N E</td>
</tr>
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</table>

(VWF) was added to the system reported here and in those reported by Kimura et al., 1989) instead of the T7 enzyme. In addition, in vitro transcription of Hgal-digested pPB2CAT9 plasmid with the T7 RNA polymerase was an absolute requirement for CAT detection. It was therefore concluded that CAT activity detected in the cell extracts was mediated by influenza virus components expressed from the cloned viral genes. Therefore, the NP and the three subunits of the influenza virus polymerase are the minimum set of proteins required for expression of the influenza virus genome, as shown previously (Huang et al., 1990; Kimura et al., 1992; de la Luna et al., 1993).

When influenza virus was used as a helper (Fig. 2), reporter gene activity was only detected if the CAT RNA was supplied as an in vitro assembled RNP, as reported by Luytjes et al. (1989). However, in the expression system reported here and in those reported by Kimura et al. (1992) and de la Luna et al. (1993), naked RNAs were efficiently transcribed in vivo by the influenza virus replicase. These results contrast with the expression system based on four vaccinia virus recombinants (Huang et al., 1990) in which naked RNA was not expressed. The reason for the different behaviour of the latter expression system is not clear but it could be due to different expression levels of cloned influenza virus genes. In any case, the results shown here strongly suggest that the negative results obtained with naked RNA in that system were not a consequence of the vaccinia virus infection.

Precise quantification of CAT activity present in the cell extracts was carried out by calculating the number of pmol of labelled chloramphenicol obtained when aliquots of the cell extracts were incubated with chloramphenicol and [3H]acetyl-CoA for 1 h under the conditions described by Portela et al. (1985). When naked RNA
(0.5 μg) was transfected into cells expressing the influenza virus proteins from pGEM plasmids, 20 to 80 pmol of acetyl groups was transferred to chloramphenicol per μg of cell extract. These values were similar to those obtained using a set of four SV40 recombinant viruses that direct the expression of the same influenza virus genes (de la Luna et al., 1993). When encapsidated RNA was transfected into cells expressing the viral polymerase from recombinant plasmids the amount of CAT activity detected varied depending on the experiment, probably because of differences in the transfection efficiency of a mixture containing protein–RNA complexes. Nonetheless, naked RNA always yielded higher CAT activity levels than encapsidated RNA.

It was then decided to test whether templates containing additional nucleotides at the 3' end of the vRNA promoter could be functional in the vaccinia virus–T7 system. For this purpose plasmid pPB2CAT9 was digested separately with XhoI, XbaI and HindIII and transcribed in vitro with T7 RNA polymerase to yield RNA molecules predicted to contain five, 13 and more than 2600 heterologous nucleotides (corresponding to the sequences of pUC19 plasmid) added to the 3' end of the viral RNA sequences. To confirm that the RNA transcripts were of the predicted length the T7 transcription reaction was carried out in the presence of [α-32P]CTP and the transcription products were resolved by electrophoresis in agarose and polyacrylamide gels. As shown in Fig. 3, one major single-sized RNA product was obtained from each DNA template. Moreover, the mobility of the RNA transcripts was reduced according to their expected lengths when compared to the Hgal-digested CAT RNA.

These RNA templates (0.5 μg) were then transfected as naked molecules into cells expressing the four influenza virus proteins from pGEM plasmids and the levels of CAT activity were measured. As summarized in Table 1, significant levels of CAT activity were detected with all the templates in several independent experiments. However, reporter gene expression was progressively reduced as the length of the RNA template was increased. The amount of CAT activity detected in cells transfected with the RNA containing 13 extra nucleotides ranged from 33 to 74% of that obtained with the wild-type RNA, and only low levels (2%) of CAT activity were detected with the longest template. To demonstrate that the results obtained were not peculiar to the vaccinia virus system, the same templates were tested in cells that expressed the same influenza virus proteins from four SV40 recombinant viruses (de la Luna et al., 1993). As shown in Table 1, results similar to those obtained with the vaccinia virus–T7 system were observed, with the three

![Fig. 3. Analyses of the RNA transcripts containing extensions at the 3' end of vRNA sequences by agarose and acrylamide gel electrophoresis. Plasmid pPB2CAT9 was digested separately with Hgal (lane 1), XhoI (lane 2), XbaI (lane 3) and HindIII (lane 4), and transcribed in vitro with T7 RNA polymerase in the presence of [α-32P]CTP. The reaction products were resolved in a 1% agarose gel (a) and in a 4% acrylamide-7 M-urea gel (b). Lane M contains a mixture of the three RNA transcripts loaded in lanes 1, 2 and 3.](image)

Table 1. CAT activity in COS-1 cells transfected using CAT RNAs with additional 3' nucleotides

<table>
<thead>
<tr>
<th>Helper system</th>
<th>Influenza virus*</th>
<th>vTF7-3 and pGEM</th>
<th>SV40 recombinants</th>
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<tbody>
<tr>
<td>pPB2CAT9 digested with</td>
<td>3' nucleotides present after transcription</td>
<td>CAT activity (%)†</td>
<td>Positive assays/total‡</td>
</tr>
<tr>
<td>Hgal</td>
<td>5'..UUUCGCU 3'</td>
<td>100</td>
<td>3/3</td>
</tr>
<tr>
<td>XhoI</td>
<td>5'..UUUCGCUCUCGA 3'</td>
<td>ND§</td>
<td>0/2</td>
</tr>
<tr>
<td>XbaI</td>
<td>5'..UUUCGCCUCGAGGCUAAG 3'</td>
<td>ND</td>
<td>0/3</td>
</tr>
<tr>
<td>HindIII</td>
<td>5'..UUUCGCUCUC.. (2-6 kb)..AGCU 3'</td>
<td>ND</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* The transfected RNAs were previously encapsidated in vitro with NP and P proteins.
† The amount of acetylated chloramphenicol was calculated by liquid scintillation counting of acetylated [14C]chloramphenicol removed from TLC plates. The sp. act. of wild-type RNA (plasmid digested with Hgal) was taken as 100% activity.
‡ An experiment was scored as positive when the sp. act. of the acetylated product was at least five times background level.
§ ND, Not detected (less than 1% of wild-type).
templates being competent to yield significant CAT activity levels. However, as expected from previous reports (Li & Palese, 1992; Piccone et al., 1993), no CAT activity was detected when templates with added nucleotides at the 3' end were transfected, as in vitro reconstituted RNPs, into influenza virus-infected cells (Table 1).

It was concluded, from the data shown in Table 1, that in influenza virus-infected cells only the RNA template containing the viral promoter at the 3' end was functional, whereas in cells expressing the viral replicase from cloned cDNAs, RNAs containing heterologous nucleotides at the 3' terminus were transcribed by the viral polymerase to some extent. It should be pointed out that the results obtained with the vaccinia virus–T7 and SV40 helper systems agree with in vitro studies, which showed that templates containing additional nucleotides at the 3' end were also copied by the viral replicase (Parvin et al., 1989; Li & Palese, 1992).

An important point to be considered in interpreting the results reported here is that in influenza virus-infected cells the transfected RNA has to compete with wild-type RNPs for a limited amount of polymerase, whereas in the expression systems there is no such RNA competitor. Hence an explanation for the negative results obtained when influenza virus was used as helper could be that RNAs containing 3' heterologous sequences are competed out by the wild-type RNPs. Alternatively, other virus-induced proteins, which are only present in cells infected with influenza virus, could affect the expression of transfected templates. Either explanation would also account for the observation that naked RNAs were not transcribed when the influenza virus polymerase was provided through infection with the homologous virus.

It is not known whether the CAT activity detected in the cell extracts is produced through transcription from the input RNA or through transcription from amplified RNA (necessitating RNA replication). It should be noted that only if CAT activity were the result of primary transcription would the differences in CAT expression observed with the different templates exactly reflect the efficiencies with which each template is used by the viral polymerase. Notwithstanding this point, the template that did not have 3'-terminal extensions consistently led to the highest levels of CAT activity, strongly suggesting that the viral replicase prefers templates containing the promoter at the extreme 3' end.

The observation that the influenza virus polymerase can recognize its promoter when located internally on an RNA template is not a peculiar feature of this enzyme. There have been a number of reports showing rescue of ssRNA viruses from cDNA-derived RNAs containing non-viral nucleotides at the 3' and 5' ends (van der Werf et al., 1986; Shaklee, et al., 1988; Dzianott & Bujarski, 1989; Pattnaik et al., 1992). In all cases analysed, these extra nucleotides were not retained in the progeny virus, showing that the viral replicase initiated RNA synthesis at the correct site to remove the extra nucleotides.

Currently, specific changes in each of the cloned influenza virus genes are being introduced to study the role of the viral proteins during virus-specific RNA synthesis. In this regard, the vaccinia virus–T7 expression system described here offers a major advantage over the previously described systems since it is not necessary to transfer the mutated cDNAs into a viral vector to assess the mutation effects on CAT expression.

In summary, we have developed an in vivo transient expression system that supports expression of an influenza virus-like CAT RNA and we have shown that in this system the viral polymerase can recognize RNA templates containing additional nucleotides at their 3' end.

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References


De la Luna, S., Martín, J., Portela, A. & Ortín, J. (1993). 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. Journal of General Virology 74, 535–539.


KIMURA, N., NISHIDA, M., NAGATA, K., ISHIHAMA, A., ODA, K. &
RNA in cells that can express the influenza virus RNA polymerase


polyadenylation signal of influenza virus RNA involves a stretch of
uridines followed by the RNA duplex of the panhandle structure.

LUYTJES, W., KRISTAL, M., ENAMI, M., PARVIN, J. D. & PALESE, P.

In vitro reconstitution of active influenza virus ribonucleoprotein
complexes using viral proteins purified from infected cells. Journal of
General Virology 73, 1855–1859.

PARVIN, J. D., PALESE, P., HONDA, A., ISHIHAMA, A. & KRISTAL, M.

of defective interfering particle RNAs of vesicular stomatitis
virus in cells expressing viral proteins from vectors containing cloned
cDNAs. Journal of Virology 64, 2948–2957.

(1992). Infectious defective interfering particles of VSV from

analysis of the influenza virus vRNA promoter. Virus Research 28,
99–112.

ORTÍN, J. (1985). Regulation of gene amplification and expression in
cells that constitutively express a temperature sensitive SV40 T-

PORTELA, A., RODRÍGUEZ, J. R., RODRÍGUEZ, D., RODRÍGUEZ, F.,
Expression of the fusion (F) protein of human respiratory syncytial
virus using an attenuated strain of vaccinia virus. In Genetics and
Pathogenicity of Negative Strand Viruses, pp. 74–78. Edited by D.

structural polypeptides of Thogoto virus (a tick-borne orthomyxo-
like virus) and functions associated with the glycoprotein. Journal of
General Virology 73, 2823–2830.

reconstituting influenza polymerase and RNA in vitro: a study of the
promoter elements for cRNA and vRNA synthesis in vitro and viral

SEONG, B. L. & BROWNLEE, G. G. (1992b). Nucleotides 9 to 11 of the
influenza A viron RNA promoter are crucial for activity in vitro.
Journal of General Virology 73, 3115–3124.

SHAKLEE, P. N., MIGLIETTA, J. J., PALMBERG, A. C. & KAESSBERG, P.
(1988). Infectious positive- and negative-strand transcript RNAs

VAN DER WERF, S., BRADLEY, J., WINTER, E., STUDIER, F. W. & DUNN,
RNA polymerase. Proceedings of the National Academy of Sciences,

YAMANAKA, K., OGAWARA, N., YOSHIIWA, H., ISHIHAMA, A. &
influenza virus RNA genome using a transfection system with an
engineered RNA. Proceedings of the National Academy of Sciences,
U.S.A. 88, 5369–5373.

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