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EXPRESSION OF FUNCTIONAL INFLUENZA VIRUS A POLYMERASE PROTEINS AND TEMPLATE FROM CLONED cDNAS IN RECOMBINANT VACCINIA VIRUS INFECTED CELLS

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Summary: cDNAs containing the coding sequences of influenza type A virus polymerase proteins (PB1, PB2 and PA) and nucleoprotein (NP) have been expressed in mammalian cells by T7 polymerase provided by a recombinant vaccinia virus. The resulting proteins are able to form a complex that can copy a negative sense influenza-like RNA, transcribed from input DNA by the T7 polymerase, into a positive sense RNA that is translated into active chloramphenicol acetyltransferase (CAT). In this system there is no requirement for helper virus or purified viral core proteins, thus it will allow manipulation of all proteins as well as template for studies of replication in influenza virus.

The genome of influenza virus A consists of eight segments of negative polarity RNA. The three largest segments encode an RNA-dependent RNA polymerase complex composed of three polypeptides, PB1, PB2 and PA. This RNA-dependent RNA polymerase complex has multiple functions such as endonucleolytic cleavage of newly synthesized host cell mRNAs, elongation of influenza capped RNA-primed mRNAs, addition of poly(A) to growing ends of mRNAs, primer-independent synthesis of full-length copies (cRNA) of viral RNA (vRNA) and cRNA-dependent synthesis of vRNA (reviewed in 1 and 2). It has been found that PB2 recognizes and binds to the cap-1 structure at the 5' end of the newly synthesized host-cell RNA and PB1 is responsible for the chain initiation and elongation. PA is a component of the virus polymerase complex but its function is unknown. Up to now it is not clear which of the proteins constitutes the endonuclease to cleave the host-cell RNA primer 10-13 nucleotides from the cap structure and which protein is involved in poly(A) addition to mRNAs. The inhibition of one or more of these multiple functions might be a feasible approach toward developing antiviral compounds.

The difficulties in studying replication of negative-strand viruses are that the naked virus RNA is not infectious when introduced into cells and the requirements for transcription and replication are not yet known. The difficulty in modifying the genomes

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of negative-strand RNA viruses has severely limited progress in understanding the replication of these viruses. To study the structure-function relationships of the influenza virus polymerase complex, and to understand the mechanisms responsible for the regulation of RNA transcription and replication, a variety of systems have been used to express influenza virus polymerase genes from cDNA copies. Recently an experimental approach was developed to study the replication of influenza viruse by reconstituting *in vitro* an active ribonucleoprotein (RNP) complex from antisense RNA for CAT gene and purified influenza virus polymerase proteins. When this RNP complex was transfected into cells which had been infected by helper virus the CAT gene was expressed, amplified and packaged into infectious virus particles (3-6). This method made it possible to construct viruses with modified genes or altered transcription, replication and packaging signals. Based on this method genetically altered influenza viruses have been obtained which exhibit unique biological and antigenic characteristics (7).

An alternative approach to study replication of a negative-sense RNA virus was developed using recombinant vaccinia virus to express mixtures of vesicular stomatitis viral proteins together with a template RNA. This system has allowed studies of virus replication, assembly and growth (8-10). A recombinant vaccinia virus vector-driven replication system was also developed for influenza virus RNA. In that system mouse fibroblast C127 cells were infected with a mixture of recombinant vaccinia viruses containing cDNAs for PB1, PB2, PA and NP (nucleoprotein) and transfected one hour later with RNP formed by mixing purified influenza virus polymerase proteins and the NS-like viral RNA containing the antisense sequence of the coding region of the CAT gene. The minimum subset of influenza virus proteins needed for specific replication and expression of the viral RNP was found to be the three polymerase proteins and NP (11). Another transfection system for influenza virus using a continuous cell line (clone 76), which expresses the viral RNA polymerase proteins and NP in response to dexamethasone, was also reported (12). When an RNP complex, reconstituted from the NS-like viral RNA containing CAT gene in a negative sense and purified NP, was transfected into clone 76 cells, CAT was produced only when the synthesis of the three RNA polymerase subunits and NP was induced by treatment with dexamethasone (12). Simian virus 40 (SV40) recombinant viruses were also used to express influenza virus polymerase complex. Naked influenza virus RNA was expressed upon transfection into cells co-expressing the polymerase proteins and NP from SV40 recombinant viruses (13).

Here we have established an in vivo system using T7 polymerase from recombinant vaccinia virus (vvTF7-3) to express all the influenza virus A polymerase proteins, NP and template. Mutagenesis of the polymerase proteins may become a feasible method to study the functions of these proteins in influenza A virus RNA expression and replication.

MATERIALS AND METHODS

Cell culture and virus: The baby hamster kidney (BHK-21) cell line was obtained from the American Type Culture Collection (ATCC). Cell monolayers were grown in Eagle's minimum essential medium (MEM, GIBCO) supplemented with 2 mM L-glutamine, nonessential amino acids, penicillin, streptomycin, and 10% fetal bovine (calf) serum. The recombinant vaccinia virus, vvTF7-3, which expresses phage T7 DNA-dependent RNA polymerase (14) was obtained from Dr. B. Moss. Recombinant vaccinia virus that expresses phage T3 RNA polymerase (VV-T3pol, 15) was obtained from Dr. M. Esteban.

Plasmid construction: Plasmids containing cDNAs for polymerase proteins of influenza virus A/NT/60/68 in pAT153 (16-19) were kindly provided by Dr. G.G. Brownlee. cDNA inserts corresponding to PB1, PB2, PA and NP were transferred from pAT153 to pBluescript (pBS, Stratagene) by standard methods. The resulting plasmids were designated as pBS-PB1, pBS-PB2, pBS-PA and pBS-NP (under control of T7 promoter) or pBS-SK-PB1, pBS-SK-PB2, pBS-SK-PA and pBS-SK-NP (under control of T3 promoter).

Infection and transfection: Confluent BHK cells in 35mm plates were infected with vvTF7-3 diluted in CaMg-PBS containing 0.1% BSA at m.o.i. of 5 PFU/cell. After adsorption for 60 min at 37°C, cells were washed with serum-free DMEM (Dulbecco's modified Eagle medium, GIBCO) and transfected with plasmid DNAs (5μg each for pBS-PB1, pBS-PB2 and pBS-PA and 25μg for pBS-NP) and Lipofectin (12μl, BRL) in serum-free DMEM. After 4 hour incubation at 37°C, the mixture of 2.5μg DNA (pPB2CAT, kindly provided by Dr. M. Krystal) digested with HgaI and the ends filled-in, medium and Lipofectin (4μl) was added to cells to bring the final volume to 1ml. Cells were further incubated for 40 hours and then lysed for CAT enzyme assay.

Labeling and immunoprecipitation: For labeling and immunoprecipitation BHK cells in 35 mm plates were infected with vvTF7-3 or VV-T3pol at m.o.i. of 5 PFU/cell and transfected with 15μg each for pBS-PB1, pBS-PB2, pBS-PA and pBS-NP or 10μg each for pBS-SK-PB1, pBS-SK-PB2, pBS-SK-PA and pBS-SK-NP and 12μl Lipofectin. At 20 hours post-transfection, cells were starved for 1 hour at 37°C and labeled for 1 hour with EXPRE^{35}S^{35}S-Protein Labeling Mix (NEN) at 37°C. The labeled cells were scraped, lysed with Triton X-100 and immunoprecipitated (20) with rabbit antiserum to core proteins for 30 min at 37°C. Immunoprecipitated proteins were recovered by protein A-Sepharose (30 min at RT), and the SDS-eluted samples were analyzed on a 9% Laemmli gel (21).

Northern blot analysis: *In vitro* transcribed RNA (0.5μg each) and total RNAs (10μg each) prepared from BHK cells and treated with RQ1 DNase (Promega) were electrophoresed in a 4% polyacrylamide-urea (7M) gel, transferred onto a Nylon membrane (Gelman Sciences Inc.) and hybridized with [^{32}P]-labeled PCR amplified DNA containing the CAT gene.

CAT assay: Cells were harvested at 40 hours post-transfection, suspended in 100μl 0.25M Tris-HCl and lysed by three freeze-thaw cycles. 50μl of cell extract was used for CAT assay based on the method of Gorman et al. (22).

RESULTS AND DISCUSSION

The cDNAs containing the full length sequences of influenza virus A/NT/60/68 polymerase proteins (PB1, PB2 and PA) and nucleoprotein (NP) were expressed by the bacteriophage T7 RNA polymerase. To assay the expressed proteins in the recombinant vaccinia virus system antisera against core proteins of influenza virus A/PR/8/34 were made by immunization of rabbits. These antisera against core proteins of influenza virus A/PR/8/34 can recognize polymerase proteins and NP of A/NT/60/68 (data not shown). Figure 1 shows total labeled cell lysates from BHK cells infected by

recombinant vaccinia virus (vvTF7-3) and transfected with 15μg of individual plasmid (pBS) containing cDNAs for PB1, PB2, PA or NP. Protein bands corresponding to PB2 and NP (as marked) can be easily seen. PB1 and PA could not be seen because they comigrate with vaccinia virus protein bands (Fig.1). To visualize these proteins labeled cell lysates from BHK cells infected by VV-T3pol and transfected with 10μg of individual plasmid (pBS-SK) containing cDNAs for PB1, PB2, PA or NP were immunoprecipitated with rabbit anti-core antiserum. As shown in Figure 2, PB1, PB2, PA and NP were precipitated by the core protein specific antiserum (as marked). Cells infected with VV-T3pol (lane vv) were used as the negative control. These data indicate that influenza virus polymerase proteins and NP can be expressed in the recombinant vaccinia virus system. Of interest is that the expression efficiency of NP is much higher than that of polymerase proteins (Fig.1), reflecting the natural influenza virus infection.

To determine whether the expressed proteins in this system could form an active RNA-dependent RNA polymerase, CAT gene was used as a reporter gene because of its high sensitivity. The plasmid DNA (pPB2CAT) contains the antisense sequence of the coding region of CAT gene in the place of the antisense coding region for PB2 under the control of a truncated T7 promoter. When pPB2CAT is linearized with HgaI and end is filled in, the T7 run-off transcripts should have the

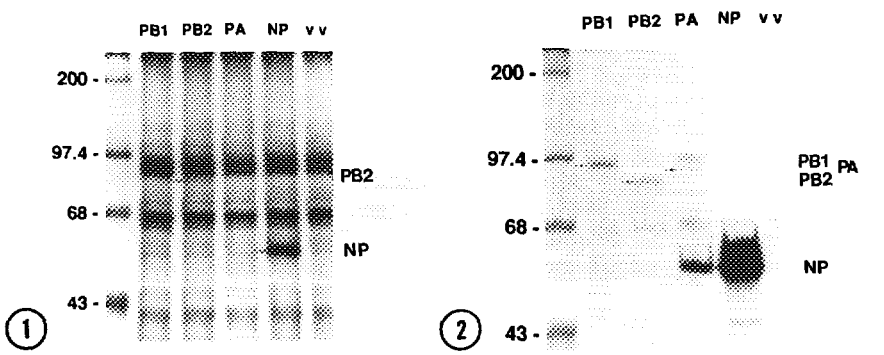

Figure 1.
Protein synthesis in vvTF7-3 infected and transfected BHK cells. BHK cells were labeled with EXPRE^{35}S^{35}S at 20 hours post-transfection as described under MATERIALS and METHODS. The labeled cells were lysed and 7 μl of labeled cell lysate was boiled for 3 min with 10μl urea-SDS sample buffer before loading onto a 9% Laemmli gel. An autoradiography of labeled proteins is shown. Protein bands corresponding to PB2 and NP are indicated. vv, cells infected by vvTF7-3 only.

Figure 2.
Immunoprecipitation of expressed influenza virus A/NT/60/68 polymerase proteins and NP in recombinant vaccinia virus (VV-T3pol) infected and transfected BHK cells. At 20 hours post-transfection, the cells were labeled with EXPRE^{35}S^{35}S. The labeled cells were scraped, lysed and immunoprecipitated with rabbit antiserum to core proteins (A/PR/8/34). Immunoprecipitated proteins were recovered by Protein A-Sepharose, and the SDS-eluted samples were loaded onto a 9% Laemmli gel. vv, cells infected by VV-T3pol.

exact termini as the authentic influenza RNA segments. To make sure that the *in vivo* transcribed RNAs for the reporter gene have the same size as those transcribed *in vitro* , total RNA was isolated from cells infected by vvTF7-3 and transfected by a PCR amplified DNA containing T7 promoter and CAT gene in a negative sense and used for Northern blot analysis. Figure 3 shows that the *in vivo* transcribed RNAs have the same size as the *in vitro* transcribed RNAs from the same DNA template. To express the influenza virus A polymerase complex BHK cells were infected by vvTF7-3 and transfected with a mixture of plasmid DNAs containing cDNAs for PB1, PB2, PA and NP (3P+NP) and 4 hour later with pPB2CAT (HgaI cut). Forty hours post-transfection cell extracts were made by freezing and thawing and CAT activity was assayed by thin layer chromatography (TLC). As shown in Figure 4A, CAT activity was detected only in the cells co-expressing influenza virus A polymerase proteins and NP and transfected with a digested plasmid DNA containing CAT gene (lane 5). If the plasmide DNAs for 3P and NP or CAT were omitted no CAT activity was detected (Fig.4A, lanes 3 and 4). A dose-response for pPB2CAT (HgaI cut) is shown in Figure 4B. Maximal CAT expression was seen when 5μg of that DNA was used for transfection (Fig.4B, lane 4).

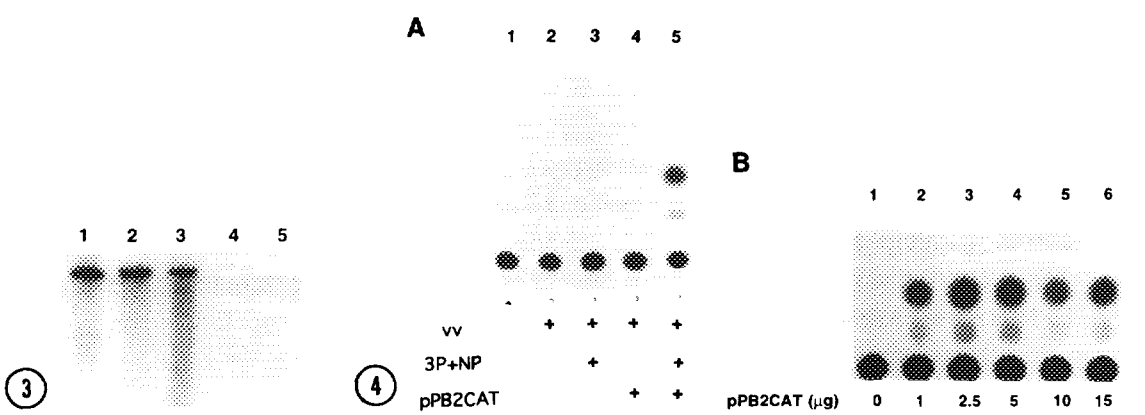

Figure 3.
Northern blot analysis to show the size of the *in vivo* transcribed reporter RNA. Lane 1 is the *in vitro* transcribed RNA from pPB2CAT (HgaI cut) and lane 2 is the *in vitro* transcribed RNA from PCR amplified pPB2CAT. Lane 3 is the total RNA isolated from cells infected by vvTF7-3 and transfected with PCR amplified pPB2CAT. Lanes 4 and 5 are the total RNAs isolated from vvTF7-3 infected and uninfected BHK cells. The RNAs were transferred from a 4% polyacrylamide-urea (7M) gel to a Nylon membrane and probed with [^{32}P]-labeled PCR DNA containing CAT gene.

Figure 4.
(A) CAT expression in recombinant vaccinia virus infected and transfected BHK cells. Forty hours post-transfection cells were harvested and lysed in 100 μl Tris-HCl by three times freezing and thawing. 50 μl of the cell extract was used for CAT assay. vv, cells infected by vvTF7-3; 3P+NP, cells transfected with a mixture of four plasmid DNAs (pBS-PB1, pBS-PB2, pBS-PA and pBS-NP); pPB2CAT, cells transfected with pPB2CAT (HgaI cut and filled-in ends). (B) Titration series comparing relative CAT expression in BHK cells infected by vvTF7-3 and transfected by a mixture of four plasmid DNAs and the indicated amounts of pPB2CAT (HgaI cut and filled-in ends).

The difference of this system from the previous systems was that all the polymerase proteins, NP and the reporter gene were expressed from plasmid DNAs in the absense of viral components, *in vitro* transcribed RNA or RNP. Upon transfection into recombinant vaccinia virus infected cells the DNA fragment for CAT gene could be transcribed into a virus-like template RNA, with 3' ends functional for initiation of mRNA synthesis. The results reported here demonstrate that the functional influenza virus A polymerase complex expressed from cDNAs encoding PB1, PB2, PA and NP in the cells expressing T7 RNA polymerase is competent for transcription of a functional mRNA.

The advantages of the vaccinia virus system (14) are that the expression of the viral proteins and template is completely artificial and all components can be tested by mutagenesis studies for viral RNA expression and replication. In this system there is no requirement for helper influenza virus or the purified viral proteins. In particular, mutagenesis of the polymerase proteins becomes a feasible and powerful method of further analyzing structure-function relationships of the viral polymerase proteins. It may eventually be possible to rescue infectious influenza virus by transfection of plasmid DNAs containing the sequences for all segments of the influenza virus genome, in either positive and negative senses.

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