Transcription of a recombinant influenza virus RNA in cells that can express the influenza virus RNA polymerase and nucleoprotein genes

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A new transfection system for influenza virus was developed using the clone 76 cell line, in which the viral RNA polymerase and nucleoprotein (NP) genes can be expressed in response to dexamethasone. Ribonucleoprotein (RNP) complexes were reconstituted by expressing proteins from a chimeric NS–chloramphenicol acetyltransferase (CAT) RNA consisting of the full-length negative-strand RNA of the CAT gene positioned between the 5′- and 3′-terminal sequences of influenza virus RNA segment 8, and purifying NP from an NP gene-expressing Escherichia coli strain. When the reconstituted RNP 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.

Introduction

Influenza virus contains a genome consisting of eight segments of negative-strand RNA. Transcription and replication of the influenza virus genome are catalysed by a virus-encoded RNA-dependent RNA polymerase (for reviews see Ishihama & Nagata, 1988; Lamb, 1989). The RNA polymerase has been purified from virus particles and found to be composed of the three P proteins, PB1, PB2 and PA (Honda et al., 1990). In virus particles, the genomic RNA segments are associated with the RNA polymerase subunits and the nucleoprotein (NP), which together form ribonucleoprotein (RNP) complexes. Biochemical and genetic analyses have revealed that the PB2 protein recognizes and binds to the cap-1 structure of primer RNA, and that the PB1 protein is involved in transcription initiation and RNA chain elongation (reviewed in Krug et al., 1989). The PA protein is present in the elongation complex with the PB1 and PB2 proteins during transcription, but its function is not known (Krug et al., 1989). NP is a major component of the RNP complex and is required for efficient elongation of RNA chains (Honda et al., 1988).

Naked RNA from negative-sense RNA viruses, unlike that of positive-sense RNA viruses, was not infectious when introduced into susceptible cells, but was considered to become infectious when RNA polymerase was introduced simultaneously. Recently, this has been confirmed by successful RNA polymerase purification (Honda et al., 1990) and reconstitution of the infectious nucleocapsid structure (Luytjes et al., 1989; Yamanaka et al., 1991). The establishment of a transfection system enabled genetic manipulation to be used to gain a detailed understanding of the structure and function of individual virus proteins in transcription and replication. Furthermore, influenza virus RNA could be used as an RNA vector for propagation and expression of any RNA molecule.

The transfection system depends upon the availability of RNA-free RNA polymerase. To avoid this difficulty, we have recently established a mouse cell line, clone 76, in which the genes for all three polymerase proteins (PB1, PB2 and PA) and NP could be expressed in response to dexamethasone (Nakamura et al., 1991). In this report, we describe evidence indicating that a chimeric NS–chloramphenicol acetyltransferase (CAT) RNA containing the CAT gene positioned between the 5′- and 3′-terminal sequences of RNA segment 8 [non-structural (NS) protein gene] of influenza virus A/PR/8/34 (Luytjes et al., 1989; Yamanaka et al., 1991) can be transcribed in this cell line following transfection in the absence of RNA polymerase.

Methods

Cells. The murine C127 cell line and its derivative, clone 76, (Nakamura et al., 1991), were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS).
Fig. 1. Insertion of the influenza virus NP gene into pOTSV expression vector. (a) Construction of plasmid pOTSV-NP as described in Methods. The sequence of the cII ribosome-binding site (cII R.B. site) and of the formylmethionine initiation codon (fMet) of pOTSV are indicated. The EcoT14I site located downstream of the initiation codon of the NP gene is also shown. The modified NP gene thus constructed is illustrated. (b) Structure of plasmid pOTSV-NP. Nut, N utilization site; to, transcription termination; ori, origin of replication of pOTSV plasmid; Amp', ampicillin resistance gene.

Construction of recombinant plasmid pOTSV-NP and expression of NP. An expression vector, the pOTSV plasmid (Shatzman & Rosenberg, 1986), was cleaved at the translation initiation codon using BamHI, and its ends were filled using dGTP, dATP and TTP (Fig. 1a). The plasmid was then digested with XbaI, and a 5.8 kb fragment containing the λ phage Pλ promoter, the cII ribosome-binding site and the transcription termination codon was isolated. The pSPNP-I plasmid, carrying a cDNA copy of the influenza A/PR/8/34 virus NP gene, was partially cleaved with EcoT14I, the ends were filled using TTP and dCTP, and it was then digested with XbaI. A 1.5 kb fragment containing the NP coding region was isolated and ligated to the 5.8 kb fragment of the pOTSV vector to generate plasmid pOTSV-NP (Fig. 1b). The pOTSV-NP plasmid contains the complete NP coding region, except that it lacks three nucleotides and contains a substitution of two nucleotides, resulting in an amino acid change at position 2 and a missing amino acid at position 3, near the N terminus of NP (Fig. 1a).

Purification of NP produced in bacteria. A 1 L culture of Escherichia coli strain AR120 carrying plasmid pOTSV-NP was grown at 37 °C to an optical density at 610 nm of 0.4. Nalidixic acid (60 mg) was then added to induce the expression of the NP gene and cultivation was continued for 8 h. The bacteria were harvested and washed once with PBS, and the bacterial pellet was frozen at −80 °C, thawed and resuspended in 30 ml of buffer A (50 mM-Tris–HCl pH 8.0, 2 mM-EDTA, 0.1 mM-DTT and 5% v/v glycerol). After the addition of 0.2 mg/ml of lysozyme, the suspension was incubated on ice for 20 min and centrifuged for 30 min.
at 12000 g. The pellet was resuspended in 30 ml of buffer A and sodium deoxycholate was added to a final concentration of 0.05% (w/v). The mixture was then homogenized in a Dounce homogenizer (15 strokes) and incubated at 15 °C for 30 min. The proteins were pelleted with 30 to 50% ammonium sulphate and resuspended in 10 ml of 50 mM-Tris-HCl pH 7.5. The proteins were fractionated by Sephadex G-200 column chromatography (bed volume, 490 ml) and each fraction was analysed for NP by 10% SDS-PAGE. Fractions 3 to 11, containing NP, were pooled and fractionated by DEAE-Sepharose CL-6B ion-exchange column chromatography (bed volume, 30 ml) in a 0 to 2 M-NaCl linear gradient. NP eluted at approximately 0-7 M-NaCl. The NP fraction from each purification step is shown in Fig. 2. The amount of NP induced was estimated at approximately 5 to 6% of the total cellular protein (Fig. 2, lane 2) and the NP preparation purified in this way was approximately 50 to 60% pure as determined by densitometric analysis (CS-9000; Shimadzu) (see Fig. 2, lane 5). NP was used for reconstitution and transfection of nucleotides.

In vitro RNA synthesis. Plasmid pOUMS101 was cleaved with *MboII* and purified in a NACS PREPAC ion-exchange minicolumn (BRL, 1525NP). A T7 polymerase reaction was carried out using the standard procedure (Davanloo et al., 1984) in the presence of [α-32P]UTP (Amersham) and RNasin (Takara Shuzo). Template DNA was removed by treatment with DNase I (Takara Shuzo). After electrophoresis on a 3% polyacrylamide gel in the presence of 7 M-urea, the RNA transcript was eluted from the gel and used for the following experiments.

Filter binding assay. A 32P-labelled RNA transcript (3 ng) was incubated with purified NP or NS1 (Young et al., 1983) protein at 0 °C for 10 min and then at 30 °C for 10 min in 10 mM-HEPES-NaOH pH 7.0, 25 mM-NaCl, 5 mM-MgCl₂, 0.5 mM-EDTA, 20% (v/v) glycerol and 2.5 mM-DTT. The mixture was filtered through a nitrocellulose filter (HAWP, Millipore) and 32P-labelled RNA retained on the filter was counted using a liquid scintillation counter.

RNA transfection of clone 76 and C127 cells. For RNA transfection, 50 ng of RNA and 4.5 μg of the purified NP (RNA:NP, 1:500 molar ratio) were incubated at 0 °C for 10 min and then at 30 °C for 10 min. After addition of 15 μg of lipofection reagent [N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA); Boehringer Mannheim], the mixture was incubated at room temperature for 10 min and DMEM containing 0-21% bovine albumin (BA) was added to 1 ml. This mixture was used for transfection. Approximately 50% confluent cells grown in 35 mm dishes (for CAT assay) or on glass coverslips in 35 mm dishes (for immunofluorescence analysis) were used for transfection. The cells were treated with 10-6 M-dexamethasone for 24 h at 37 °C or left untreated, and then transfected with the RNA/NP/DOTMA complexes described above. At 6 h after transfection, 1-5 ml of DMEM containing 0-21% BA was added and the cells were cultivated for a further 15 h at 37 °C in the absence of dexamethasone. The medium was changed to fresh DMEM containing 10% FCS only and the cells were incubated further at 37 °C.

CAT assay. Cells were harvested at various times after transfection and the CAT assay was carried out according to the method of Gorman et al. (1982). Approximately 100 μg of protein was used for each assay.

Immunofluorescence. At various times after transfection, the cells were washed with PBS and fixed with acetone at −20 °C for 30 min. The cells were treated with 100 μl rabbit anti-CAT antibody (5 prime → 3 prime) or rabbit anti-NP antibody at a 1:100 dilution for 30 min at 37 °C. The cells were washed three times with PBS and stained with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-rabbit IgG and IgL (Tago) at a 1:200 dilution at 37 °C for 30 min. After washing with PBS, the cells were observed in a fluorescence microscope (Olympus).

Results

RNA binding activity of NP

NP produced in *E. coli* carried an amino acid deletion and an amino acid substitution at the N terminus (see Fig. 1a). To examine the RNA-binding activity of purified NP, a filter binding assay was carried out using 32P-labelled NS–CAT chimeric RNA prepared by transcribing the *MboII*-digested pOUMS101 plasmid.
Fig. 3. Filter binding assay of NP binding to RNA. Radioactive NS-CAT chimeric RNA (3 ng) was incubated with NP (●), purified as described, or the NS1 protein (○), purified by the method of Young et al. (1983) at the molar ratio indicated at 0 °C for 10 min and then 30 °C for 10 min before filtration through a nitrocellulose filter. Filter-bound 32P radioactivity was counted.

Using T7 RNA polymerase (Yamanaka et al., 1991; also see below). As shown in Fig. 3, the amount of the NP-RNA complex formed increased with an increasing amount of purified NP. The complex began to be formed at a molar ratio of about 10:1 (NP:RNA), and its formation was nearly saturated at a ratio of 250:1. On the other hand, NS1 protein purified from E. coli expressing a cDNA copy of influenza A/PR/8/34 virus segment 8 from the plasmid vector pAS1 (Young et al., 1983) did not bind efficiently. Furthermore, the RNA-binding activity of NP was confirmed by a gel retardation assay. The size of the RNA-NP complexes gradually increased concomitantly with the increase in the amount of NP (data not shown). These results indicate that NP carries a high RNA-binding activity, although it was only 50 to 60% pure.

Expression of the CAT gene in cells that can express the three polymerase and the NP genes

Plasmid pOUUMS101 digested with MboII directs the synthesis of an NS-CAT chimeric RNA transcript when using T7 RNA polymerase. Yamanaka et al. (1991) reported that when the NS-CAT chimeric RNA is mixed with NP purified from virions and introduced into cells together with RNP complexes as a helper, the CAT gene is expressed from the negative-strand RNA. In this study, we tested whether the same chimeric RNA can be transcribed in our established cell line (clone 76 cells), which can express the three polymerase and NP genes in response to dexamethasone (Nakamura et al., 1991). For this purpose, the RNA was mixed with purified NP and the resulting reconstituted RNP complexes were introduced into clone 76 cells treated with dexamethasone by liposome-mediated transfection. CAT activity was detected at 10 h after transfection (Fig. 4, lane 3), and reached a maximum at 29 h (lane 5). Upon further incubation, CAT activity gradually decreased (lanes 6 and 7). The RNA polymerase proteins were transiently expressed in clone 76 cells by treatment with dexamethasone and then gradually degraded because dexamethasone was not added after transfection. No CAT activity was detected in either the mock-transfected clone 76 cells (lane 1) or the NS-CAT RNA-transfected parental mouse C127 cells (data not shown).

CAT activity in the NS-CAT RNA-transfected clone 76 cells was also examined after infection with influenza A/WSN/33 virus. After treatment with dexamethasone for 24 h, clone 76 cells were transfected with the RNA for 6 h and then superinfected with A/WSN/33 virus. At 7 h after infection, CAT activity in virus-infected cells (Fig. 4, lane 9) was almost the same as that in clone 76 cells transfected with reconstituted RNP complexes alone for 29 h (lane 5). These results suggest that the transcription activity of the three polymerase proteins induced in clone 76 cells in response to dexamethasone is almost the same as that in virus-infected cells, although the time taken to reach a maximum level of CAT activity in RNA-transfected virus-infected clone 76 cells was shorter than that in transfected cells (compare lanes 9 and 5). On the other hand, when parental mouse C127 cells were transfected with NS-CAT RNA and then superinfected with influenza virus, CAT activity was not detected (data not shown). To analyse the amount of transfected RNA, the NS-CAT RNA in dexamethasone-treated
Fig. 5. Immunofluorescence analysis of CAT protein. The NS-CAT chimeric RNA assembled with NP was transfected into dexamethasone-treated clone 76 cells. After transfection, the cells were fixed with acetone at 20 h (b), 29 h (c) and 52 h (d) and indirect immunofluorescence staining was carried out. (a) Mock-transfected clone 76 cells.

Clone 76 cells and mouse C127 cells transfected for 6 h was examined by using reverse transcription and the polymerase chain reaction using synthetic oligomers as primers. The NS-CAT RNA was detected in clone 76 cells but not in C127 cells (data not shown). This result suggests that by the time when helper virus was added for the superinfection the transfected RNA had been degraded in C127 cells because the RNA could be detected in clone 76 cells but not in C127 cells.

CAT protein in the transfected cells was also examined by immunostaining with anti-CAT antibody, followed by staining with FITC-conjugated second antibody. The CAT protein was detected in only a small population of cells at 20 h after transfection (Fig. 5b), but the proportion of fluorescence-positive cells increased significantly at 29 h (Fig. 5c), in good agreement with the increase in CAT activity (Fig. 4). However, CAT protein was detected in only 30 to 40% of cells. The fraction of fluorescence-positive cells and the intensity of fluorescence remained almost constant thereafter (Fig. 5d), suggesting that the CAT protein was rather unstable in clone 76 cells, being inactivated after prolonged incubation (compare Fig. 5 to Fig. 4). No immunofluorescence was observed in mock-transfected clone 76 cells (Fig. 5a) or NS-CAT RNA-transfected C127 cells (data not shown).

We subsequently analysed the relationship between CAT expression and the P/NP expression. Clone 76 cells were fixed with acetone after treatment with dexamethasone for 24 h and examined for fluorescence-positive cells by staining with anti-NP antibody and FITC-conjugated second antibody. Approximately 30 to 40% of the cell population expressed detectable levels of NP (Fig. 6b). The intensity of fluorescence derived from the three polymerase proteins expressed in clone 76 cells treated with dexamethasone was very weak (data not shown). To examine the titre of the anti-polymerase antibodies, virus-infected MDCK cells were analysed in indirect immunofluorescence experiments using these antibodies. The fluorescence attributable to the three polymerase proteins was very weak (data not shown), indicating that the titres of these antibodies are not high. Although the level of expression of and the cell population expressing the three polymerases could not be analysed, the fraction of cells expressing NP was consistent with that expressing CAT protein (compare
Finally, the stability of NS-CAT chimeric RNA in clone 76 cells was examined. Clone 76 cells were treated with dexamethasone for 24 h and transfected with the RNA for 21 h. The cells were then passaged three times and a CAT assay was performed each time. Although CAT activity decreased with passage, significant levels of CAT activity were detected in first and second passage cells (data not shown). These results indicate that the NS-CAT RNA is relatively stable in clone 76 cells.

Discussion

Positive-strand RNA transcripts derived from the full-length cDNA of poliovirus (Kaplan et al., 1985; van der Werf et al., 1986), human rhinovirus (Mizutani & Colonna, 1985), Sindbis virus (Rice et al., 1987), black beetle virus (DasMahapatra et al., 1986), brome mosaic virus (Ahlquist et al., 1984), tobacco mosaic virus (Dawson et al., 1986; Meshi et al., 1986) and cowpea chlorotic mottle virus (Allison et al., 1988) have been shown to be infectious when transfected into cells in the absence of added viral proteins. In contrast, the RNA genomes of negative-sense RNA viruses must be transcribed by virus-specific RNA polymerases. It has been reported recently that cDNA-derived RNA transcripts of influenza virus are transcribed and replicate when transfected together with virus core proteins. However, these systems require either helper virus (Luytjes et al., 1989; Enami et al., 1990) or infectious helper RNP cores (Yamanaka et al., 1991). Since reconstituted RNP complexes are fully active in transcription in vitro (Honda et al., 1988; Parvin et al., 1989), it is not clear why coinfection of either helper viruses or helper RNP cores is needed for transfection. Although these systems are useful for analysis of the promoter and origin regions (cis-acting signals for transcription and replication), and the packaging signal(s) for viral RNA genome assembly by using site-directed mutagenesis of cDNA, it is not so easy to analyse the functions of individual proteins required for transcription and replication.

To overcome such limitations of the transfection system, we used clone 76 cells, in which all three polymerase genes and the NP gene are expressed in response to dexamethasone (Nakamura et al., 1991). Clone 76 cells are able to complement the growth of three temperature-sensitive polymerase protein mutants efficiently at the non-permissive temperature after induction of these genes by treatment with dexamethasone (Nakamura et al., 1991). Using clone 76 cells and the NS–CAT chimeric RNA we found that NP alone is sufficient to reconstitute the RNP complexes recognized by the influenza virus RNA polymerases expressed in clone 76 cells in response to dexamethasone. Huang et al. (1990) have reported that after reconstitution of RNP complexes with purified RNA polymerases and NP, the transfected synthetic NS–CAT chimeric RNA is expressed in mouse C127 cells after infection with vaccinia virus recombinants expressing the RNA polymerase and NP genes. Our results are consistent with their data.

Although the NP expressed in E. coli is different from native NP, carrying an amino acid substitution and a deletion at positions 2 and 3, respectively (see Fig. 1), it seemed fully active in RNA binding. Yamanaka et al. (1990) have reported that a 3600-fold excess of NP purified from virions was needed to produce 50% of the maximal binding activity, as judged by a filter binding assay. This level of activity was observed at an NP:RNA molar ratio of approximately 25 : 1 (see Fig. 3). Judging from these results, the NP expressed in E. coli possesses greater RNA-binding activity than that prepared from virions. The value is consistent with a previous report that NP binds to the RNA genome at intervals of approximately 20 nucleotides in virus particles (Compans & Choppin, 1975). However, the binding curve is not displaced from the expected value with increasing amounts of NP (see Fig. 3) because NP may form aggregates before binding all the RNA at higher concentrations. Harley et al. (1990) reported that NP can be purified from an NP-expressing E. coli strain; the binding activity of NP with RNA was analysed by the filter binding assay and suggested that NP is self-
aggregating. Although the NP used is only 50 to 60% pure, we did not assess contamination by an E. coli RNA-binding protein because our aim was to use NP only to protect RNA molecules against ribonucleases during transfection. Once RNA molecules enter cells, the NP induced in clone 76 cells would bind to them. CAT activity was also obtained when naked RNA was transfected into clone 76 cells pretreated with dexamethasone (Fig. 7). However, the time taken to reach the maximum level of CAT activity was 8 h more than that required using reconstituted RNP complexes (compare Fig. 7, lane 3 to Fig. 4, lane 5). This result indicates that RNA reconstituted with NP is better protected against ribonucleases during transfection than naked RNA.

The newly developed transfection system described here is a convenient and useful artificial system in which cDNA-directed RNA transcripts can be transcribed in cells provided with a continuous supply of influenza virus RNA polymerases and NP simply by induction with dexamethasone. We are currently studying whether the transfected synthetic RNA molecules replicate in this cell line.

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References


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