Expression of antisense RNA fails to inhibit influenza virus replication

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Summary

Cell lines were constructed which permanently express influenza virus-specific RNA. Two approaches were followed. C127 cells were transformed with bovine papilloma virus (BPV) vectors and the resulting cell lines were found to inhibit the replication of influenza virus at low multiplicity of infection (MOI 0.05). However, examination of cellular RNA using single-stranded probes revealed the presence of both (+)sense and antisense RNA transcripts (45–70 copies per cell). In this BPV-based system the inhibitory activity appeared to be associated with a non-specific, interferon (IFN)-mediated effect. In the second approach, an expression system was used which involved 293 cells, a chimeric human cytomegalovirus (CMV)/human immunodeficiency virus (HIV) promoter, and methotrexate- (Mtx)-mediated gene amplification. Cells were found to express up to 7500 copies of influenza virus-specific RNA per cell at a steady state level. In this system no RNA transcripts of the opposite orientation were found. However, all cell lines permanently expressing either (−)sense or (+)sense viral RNA failed to reduce influenza virus titers in a multi-cycle replication experiment (MOI 0.01).

Antisense RNA; Influenza virus

Introduction

Antisense RNA, i.e. RNA complementary to mRNA, has been shown to act as a regulatory element in certain prokaryotic systems and has been used to inhibit gene

expression in prokaryotic as well as eukaryotic cells (for review: Weintraub et al., 1985; Green et al., 1986; Melton, 1988; van der Krol et al., 1988). Furthermore, the use of antisense RNA expression has been suggested as a potentially powerful tool to prevent expression of harmful genes, including oncogenes and viral genes (Green et al., 1986). In one study, expression of phage SP antisense RNA effectively blocked replication of phage SP in *E. coli* cells, but it had no effect on the growth of related phages GA and Qβ (Hirashima et al., 1986). With respect to animal viruses it has been reported that expression of the envelope (env) gene product from a transfected plasmid can be inhibited by antisense RNA, preventing rescue of a defective Rous sarcoma virus from this transformed quail cell line (Chang and Stoltzfus, 1985; Chang and Stoltzfus, 1987). However, this experiment was designed to allow only for transient expression of antisense RNA, and it was not determined whether such an approach could lead to permanent resistance of cells against virus infection. More recently, a permanent cell line was established which was able to inhibit replication of a retrovirus via antisense RNA expression (To et al., 1986). The retrovirus was constructed to contain a neomycin resistance (neo^R) gene and its replication was inhibited by expression of neo^R sequences in the opposite sense in these cells. Also, von Rüden and Gilboa (1989) have shown that expression of antisense RNA could reduce replication of human T-cell leukemia virus type 1 in primary human T-lymphocytes. Another approach to inhibit virus replication has been through the use of antisense oligonucleotides (reviewed by Stein and Cohen, 1988). Treatment of cells with these compounds was reported to specifically inhibit replication not only of retroviruses (Zamecnik and Stephenson, 1978; Matsukura et al., 1987; Agrawal et al., 1988; Goodchild et al., 1988), but also of herpes (Smith et al., 1986), vesicular stomatitis (Agris et al., 1986; Lemaitre et al., 1987), and influenza viruses (Zerial et al., 1987).

The goal of the present study was to determine whether replication of influenza virus can be inhibited by expression of virus-specific antisense RNA in mammalian cells. We describe the successful development of cell lines which permanently express high levels of influenza virus specific RNA in the (+)sense or in the antisense orientation. Experiments were then performed to determine the effect of this high level of expression of viral RNAs on viral replication.

Materials and methods

Viruses and cells

Influenza A/WSN/33 (ts^+) virus was grown in MDCK or MDBK cells as described (Sugiura et al., 1972). The attenuated influenza virus laboratory recombinant R19 (Rott et al., 1979) was kindly provided by Dr C. Scholtissek and adapted for growth in C127 cells by multiple passages. Murine C127 cells (C127I) were initially obtained from the American Type Culture Collection (CRL 1616) and grown as described (Krystal et al., 1986). Human 293 cells (Graham et al., 1977)

were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5 units/ml penicillin, and 5 μg/ml streptomycin.

Expression of viral RNA in C127 cells and replication of virus

Construction of BPV-based expression vectors and transformation of C127 cells were carried out as previously described (Krystal et al., 1986). The cDNA clone of the NS gene of R19 virus was synthesized using purified viral RNA, reverse transcriptase, and synthetic oligonucleotide primers as described previously (Baez et al., 1980). A tandem copy of the NS gene was inserted into the unique *Xho*I-site of expression vector pBMT3X, which contains the human metallothionein-1A gene as a selectable marker (Krystal et al., 1986). Transformed cells were selected in the presence of up to 20 μM CdCl$_2$. C127 cells were infected with influenza A/R19 virus or VSV/Indiana at 37° C (MOI 0.05). Cells were washed two times with phosphate-buffered saline, maintenance medium containing 0.1% trypsin was added, and cells were incubated at 37°C for two to three days. At various times after infection, samples of medium were taken for titration of infectious virus. In some experiments, polyclonal antiserum to interferon was added to the maintenance medium one hour post infection (final concentration, 25 U/ml). Rabbit antibodies to mouse IFN-α and -β (10,000 U/ml) were obtained from Lee Biomolecular Research (San Diego, CA).

Construction of plasmids used for expression of antisense RNA in 293 cells

Plasmid πH3 (Aruffo and Seed, 1987) contains a chimeric CMV/HIV promoter, followed by various cloning sites and the simian virus 40 (SV40) splice and polyadenylation signals (Fig. 1). An SV40 origin permits replication of plasmid πH3 in COS cells, and the pBR322 origin and the supF gene allow amplification in MC1061/p3 bacteria (Aruffo and Seed, 1987). Plasmids pGEM3/NSm and pGEM3/NSv (Fig. 1), containing the NS gene of influenza A/WSN/33 virus (Buonagurio et al., 1986), were made by cloning virus-specific cDNA into plasmid pGEM3 (Promega Biotec, Madison, WI) using synthetic *Xba*I-linkers as described (Baez et al., 1980).

In order to insert the DHFR gene into plasmids πFR, πFRNSm, and πFRNSv (Fig. 1), the coding region of a chinese hamster dihydrofolate reductase (DHFR) minigene was removed from plasmid pDCH0 (Venolia et al., 1987) by cutting with *Avr*II and *Bam*HI. This fragment was then inserted into the *Xba*I/*Bam*HI-window of pUC19, resulting in plasmid pUCFR. For construction of plasmid πFR, πH3 was digested with *Xho*I, then treated with the Klenow fragment of *E. coli* DNA polymerase I and subsequently cut with *Hin*dIII. The linearized vector, containing a *Hin*dIII cohesive end and a blunt end, was then used for ligation with the *Hin*dIII to *Sma*I fragment of plasmid pUCFR. A similar approach was taken to construct plasmids πFRNSm and πFRNSv, except that an *Ava*I/*Sal*I-fragment containing the NS gene was used in a trimolecular ligation reaction. Depending on the orientation of the NS cDNA in pGEM3, the final construct had the NS gene either
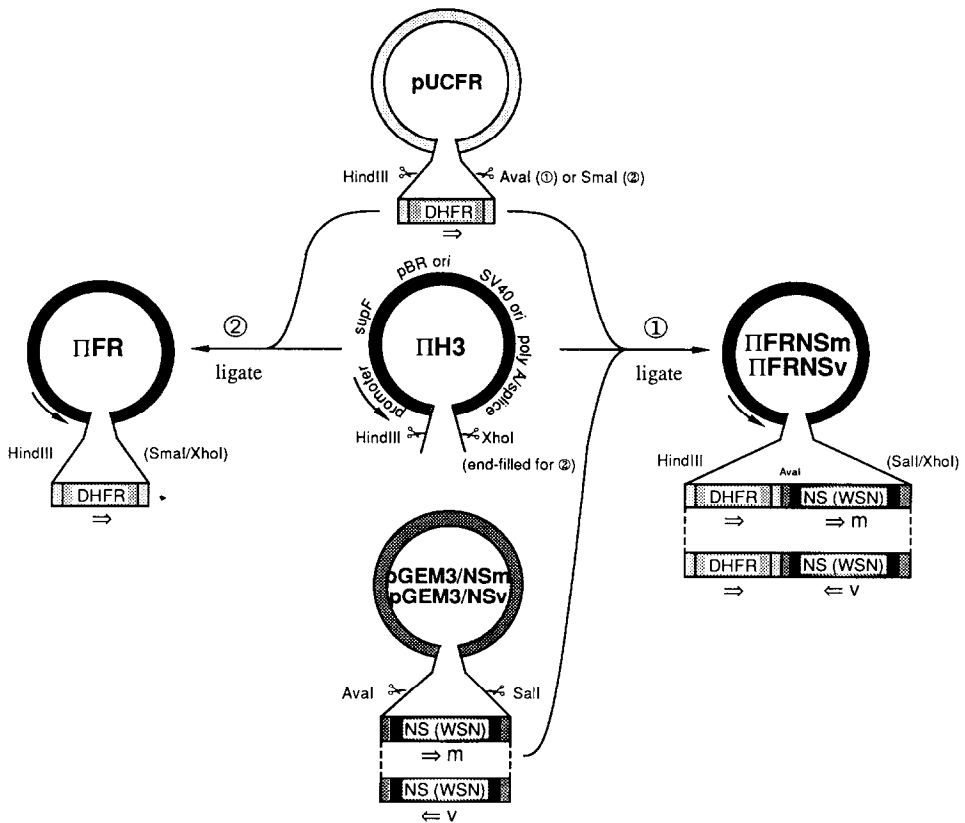
Fig. 1. Construction of expression vectors πFR, πFRNSm, and πFRNSv. The NS cDNA of influenza A/WSN/33 virus was cloned in both orientations into the unique XbaI-site of pGEM3. The DHFR gene was obtained from pDCH0 (Venolia et al., 1987) and inserted into pUC19 yielding pUCFR, as described in Materials and Methods. Plasmid πFRNSm was obtained in a trimolecular ligation reaction (①) using the HindIII to AvaI DHFR-fragment from pUCFR, the AvaI to SalI NS-fragment from pGEM3/NSm, and the HindIII/XhoI-digested vector πH3. Similarly, plasmid πFRNSv was made by using the NS-fragment from pGEM3/NSv. Control plasmid πFR was constructed by ligating (②) the HindIII to SmaI DHFR-fragment from pUCFR with the HindIII/end-filled-XhoI linearized vector πH3. The pBR322 origin of replication (pBR ori), the SV40 origin of replication (SV40 ori), the synthetic suppressor tRNA element supF, the CMV/HIV-promoter, and SV40 splice/termination/polyadenylation signals, derived from plasmid πH3 (Aruffo and Seed, 1987), are indicated. Short open arrows indicate sequences in the (+)sense (→) or antisense (←) orientation relative to the CMV/HIV promoter of πH3 or relative to the T7 promoter of pGEM3.

in the sense or in the antisense orientation relative to the CMV/HIV promoter and the DHFR gene.

DNA transfection and methotrexate selection of 293 cells

For construction of recombinant cell lines, 293 cells were transfected with expression vectors using the modified calcium phosphate precipitation procedure

described by Chen and Okayama (1987). Briefly, each 10 cm dish, containing 5×10^5 cells, received 1 µg of p342-12ΔBPV DNA and 14 µg of either πFR, πFRNSm, or πFRNSv DNA. Plasmid p342-12ΔBPV (Li et al., 1989) expresses a bacterial enzyme which inactivates the drug G418 (Southern and Berg, 1982). Twenty hours after transfection, cells were trypsinized and split 1 : 10 into medium containing 600 µg G418/ml. Four days later the medium was exchanged to contain 10% dialyzed FBS, 600 µg/ml G418 and 80 nM Mtx. This medium was replaced every four to seven days and three to four weeks after transfection large colonies were picked and cells expanded. For amplification, $2-4 \times 10^5$ recombinant cells were seeded onto 10 cm plates in media containing different concentrations of Mtx (up to 16 µM). Mtx-resistant clones were isolated three weeks later.

Virus infection of 293 cells

Trypsinized 293 cells were resuspended in Minimum Essential Medium Eagle with Earle's salts and 0.2% bovine albumin (MEM-BA). Into 24-well multidishes (NUNC, Thousand Oaks, CA) 5×10^5 cells/well were seeded, coated with 8 µg Cell-Tak (BioPolymers, Farmington, CT) per well (1.9 cm^2). After 30–60 min, the medium was replaced with DMEM containing 10% FBS and cells were allowed to grow overnight. The next day, cells were washed once with MEM-BA and 250 µl of virus sample (2×10^4 PFU/ml, in MEM-BA) were added to each well. After adsorption for 40 minutes at 37°C, 1 ml of MEM-BA containing 1 µg/ml trypsin was added per well. Samples of supernatant were taken at various times after infection for hemagglutination titrations, which were performed as previously described (Vlasak et al., 1988). All experiments were performed in duplicate.

Transient expression in COS cells and immunofluorescence

To test the vectors for expression of NS1 protein, we introduced the DNA into COS-1 cells for expression and subsequently visualized the gene product by immunofluorescence, as described elsewhere (Greenspan et al., 1987). Briefly, COS-1 cells, which were seeded onto coverslips, were transfected with 15–20 µg πNSm or πFRNSm DNA per 6 cm tissue culture dish. Two days after transfection, cells were fixed and stained by incubation with rabbit anti-NS1 serum, followed by treatment with fluorescein-conjugated goat anti-rabbit immunoglobulin G.

RNA analyses

RNA was prepared from recombinant 293 cells or transformed C127 cells by extraction using guanidinium isothiocyanate and pelleting through a cesium chloride step gradient (Glisin et al., 1974). Single-stranded RNA probes were transcribed by T7 polymerase using conditions which were previously described (Melton et al., 1984). The DNA templates contained the influenza virus NS cDNA in the (+)sense or antisense orientation or hamster actin cDNA (Dodemont et al., 1982) in the antisense orientation relative to the bacteriophage T7 promoter. T7 RNA poly-

merase was isolated from *E. coli* strain BL21 as previously described (Davanloo et al., 1984). DNA probes were prepared by the random primer extension method (Feinberg and Vogelstein, 1983) using restriction enzyme fragments of cloned hamster actin cDNA (Dodemont et al., 1982) or the coding region of a DHFR gene (Venolia et al., 1987). Dot-blots were performed by denaturing serial dilutions of each RNA sample in formaldehyde and filtration through a manifold (Schleicher and Schuell, Keene, NH) onto nitrocellulose (Chien and Dawid, 1984). Filters were hybridized overnight in 50% formamide, $6 \times$ SSC, $5 \times$ Denhardt's solution, 0.2 mg/ml salmon sperm DNA, 0.2 mg/ml yeast tRNA, and 1 mM EDTA using ^{32}P-labeled RNA probes at $50\,^{\circ}$C or ^{32}P-labeled DNA probes at $37\,^{\circ}$C. Filters were subsequently washed in 0.1x SSC and 0.1% SDS at $65\,^{\circ}$C (RNA probes) or $42\,^{\circ}$C (DNA probes). Ribonuclease protection analysis was done as described (Zinn et al., 1983). Briefly, 10 μg of total cellular RNA were hybridized with 1×10^{6} cpm of ^{32}P-labeled probe (1×10^{8} cpm/μg). Unprotected probe was removed by treatment with RNase A (40 μg/ml) and RNase T1 (2 μg/ml). After inactivation of ribonucleases by proteinase K treatment and phenol:chloroform extraction, samples were analyzed on a 4% acrylamide/8M urea gel.

Radioimmunoassay (RIA) for NS1 protein

Recombinant 293 cells were lysed by repeated freezing and thawing in 0.05% Tween 20. Lysates were vortexed for one minute and clarified by centrifugation at 15 000 g prior to use. Microtiter plates were coated with a rat anti-mouse kappa monoclonal antibody (mAb) (Dr. Thomas Moran, Mt. Sinai School of Medicine, New York). Wells were then covered with the mouse anti-NS1 mAb 1A7 (Dr. Jonathan Yewdell, N.I.H.) and lysates were added at a 1:100 dilution. The plates were subsequently incubated with polyclonal rabbit anti-NS1 serum (1:250) and finally treated with ^{125}I-labeled goat anti-rabbit IgG (NEN DuPont). Wells were extensively washed with PBS between incubations. All dilutions were done using 20% goat serum/1% BSA/0.05% Tween 20/PBS. In preliminary experiments the assay was optimized by using dilutions of the reagents which yielded a linear increase in cpm with increasing amounts of lysate.

Results

Viral RNA expression in BPV transformed C127 cells

Previously, cell lines have been established by transfection of C127 cells with BPV vectors containing cloned influenza virus genes (Krystal et al., 1986; Li et al., 1989). These cell lines were shown to express virus-specfic RNA and proteins permanently and they were successfully used to complement the growth of temperature-sensitive influenza virus mutants at the non-permissive temperature (Krystal et al., 1986; Li et al., 1989). Using the same approach, we first transfected C127 cells with a BPV vector which contained a tandem of the NS gene downstream of the
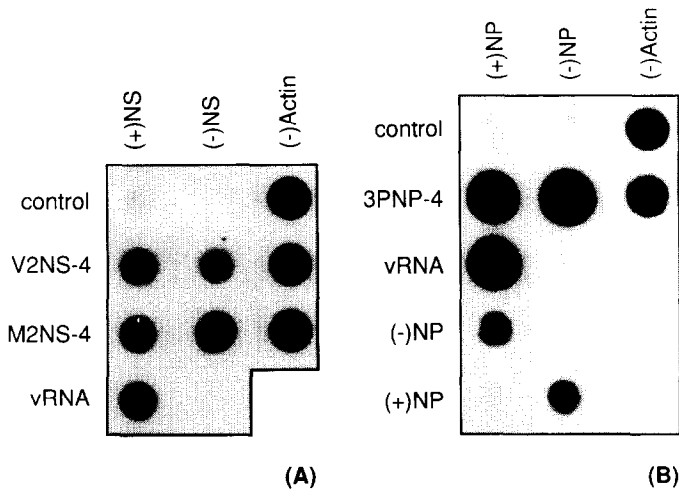
Fig. 2. Dot-blot analysis of influenza virus-specific RNA expressed in BPV-transformed cells. RNA was prepared from BPV-transformed cell lines (V2NS-4, M2NS-4, 3PNP-4), control cells, purified influenza A/WSN/33 virus (vRNA), or by in-vitro transcription of NP-cDNA templates using SP6 polymerase ((−)NP, (+)NP). RNA preparations were denatured in formaldehyde and filtered through a manifold onto nitrocellulose. Hybridization was performed using single-stranded ^{32}P-labeled probes, as indicated above each set. (Exposure times for filters using actin-probes were approximately 20-fold shorter than for filters using influenza virus-specific probes). A: probes were hybridized to 45 µg of total cellular RNA (control, V2NS-4, M2NS-4) or 1.5 ng vRNA. B: probes were hybridized to 45 µg of total cellular RNA (control, 3PNP-4), 1.5 ng vRNA, 50 pg NP (+)sense RNA [(+)NP], or 50 pg NP (−) sense NP RNA [(−)NP].

murine metallothionein-1 promoter. The construct was obtained by cloning a tandem of the NS gene of influenza A/R19 virus into plasmid pSPR1 (Krystal et al., 1986) and subsequently into the BPV vector pBMT3X. This construct was chosen because each transcript should yield two copies of the NS gene in the antisense orientation. In order to enable cadmium selection, the vector was initially constructed to contain the human metallothionein-1A gene (Krystal et al., 1986). Six cell lines were obtained which were expected to express the NS sequences in the antisense orientation and they were screened for expression by RNA dot-blot analysis. The average copy number per cell of virus-specific RNA was estimated using purified virion RNA as a standard. Cell line V2NS-4 was found to produce the highest levels of NS antisense RNA (approx. 70 copies/cell). Surprisingly, when the RNA of these cells was examined using single-stranded probes, both (+)sense and antisense NS sequences were detected (Fig. 2A). Similar results, i.e. virus-specific transcripts of both orientations, were obtained for the M2NS-4 cell line. These cells were transformed with a vector that contains the tandem of the NS gene in the (+)sense orientation, and therefore only the expression of (+)sense NS transcripts was anticipated (Fig. 2A). This result suggested that cell lines obtained by transfection with this BPV vector expressed the expected virus-specific RNA, but in addition, transcripts in the opposite orientation were observed. Confirmation that

TABLE 1

Replication of influenza A/R19 virus and VSV in BPV-transformed cell line permanently expressing the influenza virus NS gene

Cell line [b]	Titer (pfu/ml) [a]	
	Influenza A/R19	VSV
C127	5×10^7	7×10^5
V2NS-4	5×10^4 (3×10^7) [c]	4×10^2

[a] Viral titers were obtained from supernatants of infected cells (MOI 0.05), 43 h post infection.

[b] The parental cell line C127 was transformed with a BPV vector containing the NS gene of influenza R/19 virus to yield cell line V2NS-4.

[c] The value in parenthesis indicates virus titer obtained in the presence of 25 U/ml rabbit anti-mouse interferon antibodies.

BPV vectors may direct transcription of RNA in both orientations was obtained by analysis of yet another cell line. 3PNP-4 cells were previously shown to express the nucleoprotein of influenza virus (Li et al., 1989) and they were now found to express approximately 45 copies per cell of NP (−)RNA and comparable amounts of NP (+)RNA transcripts (Fig. 2B).

Virus replication in BPV-transformed C127 cells

Single-cycle replication of influenza A virus was not inhibited in BPV-trans-formed cells which express influenza virus proteins (Krystal et al., 1986; Li et al., 1989). Similarly, the V2NS-4 cell line constructed to express the NS gene in the antisense did not reveal inhibition of influenza virus in a single-cycle (data not shown). However, a single-cycle replication assay would not detect small differences in replication efficiencies. Therefore infections were performed at low multiplicity (multi-cycle replication) as even small reductions during each replication step would be amplified over several cycles. Interestingly, inhibition of influenza virus repli-cation under these multi-cycle conditions was observed using cell line V2NS-4, compared to the parental cell line C127 (Table 1). Although infection of the V2NS-4 cells at low MOI (0.05) resulted in a 1000-fold reduction of viral titers, a similar reduction was observed following infection with VSV at low multiplicity (Table 1). Similar results were obtained using BPV transformed cell lines which were con-structed to express the polymerase PA and BP2 genes in the antisense orientation (data not shown). Since VSV replication was also inhibited we tested whether the effect was IFN-mediated by adding anti-IFN (α and β) antibodies to the medium 1 h after virus infection. Following infection at low multiplicity (0.05), viral titers were restored to levels seen in control cultures lacking anti-IFN antibodies. Taken together, our data suggest firstly that transcription of both kinds of RNA ((+)sense and antisense) takes place in BPV transformed C127 cells and secondly that selective repression of a specific viral protein via antisense RNA expression is most likely not a major contributing factor in the establishment of the antiviral state in this system. Therefore, we decided to develop an alternative system which expresses

excess levels of antisense RNA (in the absence of (+)sense transcripts) and at the same time supports a productive influenza virus infection.

Construction of an alternative expression system

In initial experiments we tested different cell lines for transfection efficiency and for their ability to allow replication of influenza virus. 293 cells were found to readily support multicycle replication of influenza A/WSN/33 virus. In addition, following transfection of the appropriate DNA, they expressed the human growth hormone gene (Selden et al., 1986) more efficiently than MDCK cells or DHFR⁻ VERO cells (data not shown). Thus, a new set of plasmids was constructed using the eukaryotic expression vector πH3 (Aruffo and Seed, 1987) which contains a chimeric CMV/HIV promoter, multiple cloning sites, and SV40 splice and poly-adenylation signals (Fig. 1). An SV40 origin of replication allows for transient expression in COS cells which permits testing of DNA constructs prior to establishing permanent cell lines. Vectors πFRNSm and πFRNSv contain a DHFR gene followed by the NS gene sequence derived from influenza A/WSN/33 virus (Fig. 1). In both constructs the DHFR fragment was inserted in the (+)sense orientation relative to the CMV/HIV promoter. Successfully transfected cells thus became resistant to the drug Mtx by expressing high levels of the enzyme DHFR. Since the influenza virus sequences are expected to be on the same RNA transcript as the DHFR coding region, Mtx selection should result in cell lines expressing high levels of influenza virus specific RNA. Prior to establishing permanent cell lines we tested the vectors in a transient expression assay. The dicistronic plasmid πFRNSm and a monocistronic control, πNSm, were used to transfect COS-1 cells. Expression of NS1-protein was analyzed two days later by indirect immunofluorescence. Upon transfection with πNSm, which contains the NS gene but lacks the DHFR gene, the NS1 protein was found in the nuclei of the cells (Fig. 3A). When COS-1 cells were transfected with plasmid πFRNSm, which contains the additional DHFR sequence 5' of the NS sequence, no NS1 protein could be detected by immunofluorescence (Fig. 3B). This result suggests that the second cistron downstream of the DHFR coding region is not utilized efficiently for translation in this construct.

RNA expression in recombinant 293 cells

For the construction of permanent cell lines, 293 cells were transfected with either plasmid πFRNSv, πFRNSm, or the control plasmid πFR. In addition, a plasmid containing the neo^R gene was used for co-transfection and selection (see Materials and Methods for details). This was done because parental 293 cells have endogenous DHFR activity and are therefore resistant to low levels (less than 50 nM) of Mtx. Transformants were selected in medium containing 80 nM Mtx as well as 600 μg/ml G418. Initially, 13 cell lines expressing antisense NS RNA and 8 cell lines expressing (+)sense NS RNA were obtained. These cell lines were tested for expression of influenza virus-specific RNA by dot-blot analysis. Out of these 21 cell lines three clones, two expressing antisense RNA and one expressing (+)sense
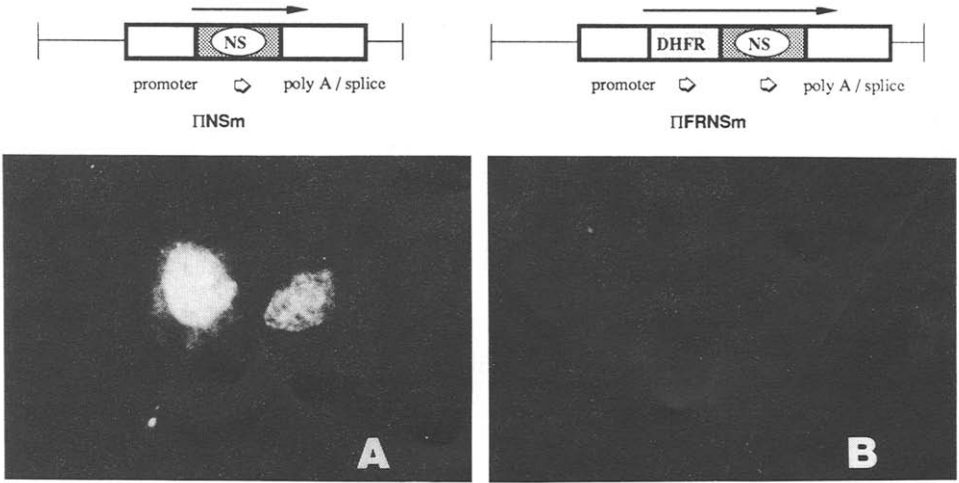
Fig. 3. Immunofluorescence of πNSm- or πFRNSm-transfected COS-1 cells. Cells were transfected, fixed, and stained as described in Materials and Methods. Linear maps of the expression vectors used for transfection are shown above each photograph. Plasmid πNSm was obtained by direct insertion of the WSN NS gene into the *Xba*I-site of πH3 in the (+)sense orientation. The construction of πFRNSm is shown in Fig. 1. Long thin arrows ($\rightarrow$) show predicted primary transcripts. Short open arrows ($\Rightarrow$) indicate orientation of coding regions. (A) Immunofluorescence of πNSm-transfected cells incubated with anti-NS1 serum. (B) Immunofluorescence of πFRNSm-transfected cells incubated with anti-NS1 serum.
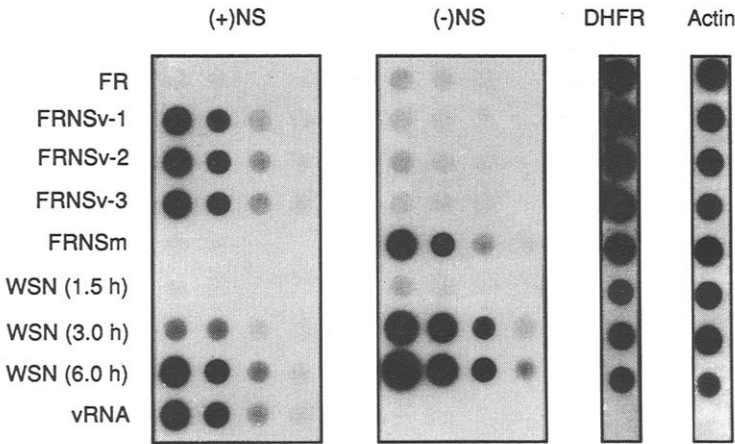

Fig. 4. Dot-blot analysis of influenza virus-specific RNA expressed in recombinant 293 cells. RNA was prepared from recombinant cell lines (FR, FRNSv-1, FRNSv-2, FRNSv-3, FRNSm), influenza A/WSN/33 virus-infected FR-2 cells (at 1.5, 3 and 6 h p.i.), or purified influenza A/PR/8/34 virus (vRNA), as indicated on the left side of the panel. Four-fold dilutions of each RNA preparation were denatured in formaldehyde and filtered through a manifold onto nitrocellulose, beginning with 25 μg of total cellular RNA or 100 ng of virion RNA. Hybridization was performed using either an NS (+)sense RNA probe ((+)NS), an NS (-)sense RNA probe ((-)NS), a DHFR DNA probe (DHFR), or an actin DNA probe (Actin), as indicated. Using the vRNA sample as a standard we estimated the steady-state levels of virus-specific RNA per cell, as described in the text.

RNA, were chosen for further amplification and subcloning using Mtx concentrations up to 16 μM. Fifteen clones which were resistant to the higher Mtx concentrations were picked and screened for specific RNA synthesis. All fifteen clones produced influenza virus-specific RNA, but cell lines differed in the level of expression. Cell lines FRNSv-1, FRNSv-2, and FRNSv-3 showed the highest steady-state level of expression with approximately 7500 copies of NS RNA per cell (Fig. 4). After amplification and subcloning, one cell line, NSm, was obtained and it was found to express approximately 1200 copies/cell. In contrast to BPV-transformed C127 cells, significant levels of transcripts in the opposite orientation were not detected in this system (Fig. 4). Eight control cell lines were obtained using vector πFR for transfection of 293 cells. One of these cell lines, FR, was passaged in increasing concentrations of Mtx (up to 1 μM) and found to express high levels of DHFR-specific RNA (Fig. 4). Another control cell line, FR-2, was infected with influenza A/WSN/33 virus (MOI 5). We then compared the levels of virus-specific RNA expressed in cell lines FRNSv-1, FRNSv-2, FRNSv-3, and FRNSm with those
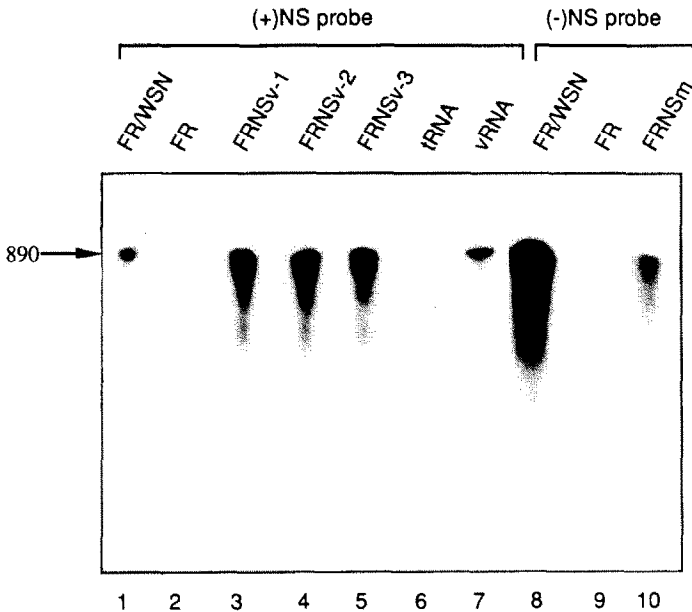

Fig. 5. Ribonuclease protection of influenza virus-specific RNA transcripts. In vitro transcription using T7 polymerase yielded a ^{32}P-labeled single-stranded NS probe (1×10^8 cpm/μg) which was hybridized to RNA isolated from 293 cells or to virion RNA. Subsequently, free probe was removed by treatment with RNAse A and RNase T1. Samples were analyzed on a 4% acrylamide/8 M urea gel. Lanes 1–7: (+)sense RNA probe (1×10^6 cpm) was hybridized to: 10 μg RNA from cells infected with influenza A/WSN/33 virus, 3 h p.i. (lane 1); 10 μg RNA from FR cells (lane 2); 10 μg RNA from antisense RNA expressing cell lines FRNSv-1 (lane 3), FRNSv-2 (lane 4), or FRNSv-3 (lane 5); 10 μg tRNA (lane 6); 10 ng influenza A/WSN/33 virus RNA and 9 μg tRNA (lane 7). Lanes 8–10: (−)sense RNA probe (1×10^6 cpm) was hybridized to: 10 μg RNA from cells infected with influenza A/WSN/33 virus, 3 h p.i. (lane 8); 10 μg RNA from FR cells (lane 9); 10 μg RNA from FRNSm cells (lane 10).

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found in virus-infected FR-2 cells. As shown in Fig. 4, the level of antisense RNA expressed in cell lines FRNSv-1, FRNSv-2, and FRNSv-3 is comparable to the levels of (−)RNA found in virus infected cells six hours after infection. Levels of (+)sense RNA made in FRNSm cells are comparable to those seen in virus-infected cells three hours after infection.

In order to characterize the RNA expressed in these cell lines, ribonuclease protection experiments were performed. RNA was isolated from five antisense RNA expressing cell lines, two (+)sense RNA expressing cell lines, the control cell line FR, virus-infected cells, and purified influenza A/WSN/33 virus. Single-stranded probes were obtained using linearized plasmids pGEM3/NSm or pGEM3/NSv (Fig. 1) and T7 polymerase. After hybridization of the transcribed probes to the RNAs, samples were treated with ribonucleases A and T1 and subsequently analyzed on a denaturing gel. Results using RNA from cell lines FRNSv-1, FRNSv-2, FRNSv-3, FRNSm, and controls are shown in Fig. 5. The major band in lanes 3, 4, 5 and 10 is seen at a position of 890 nucleotides which corresponds to
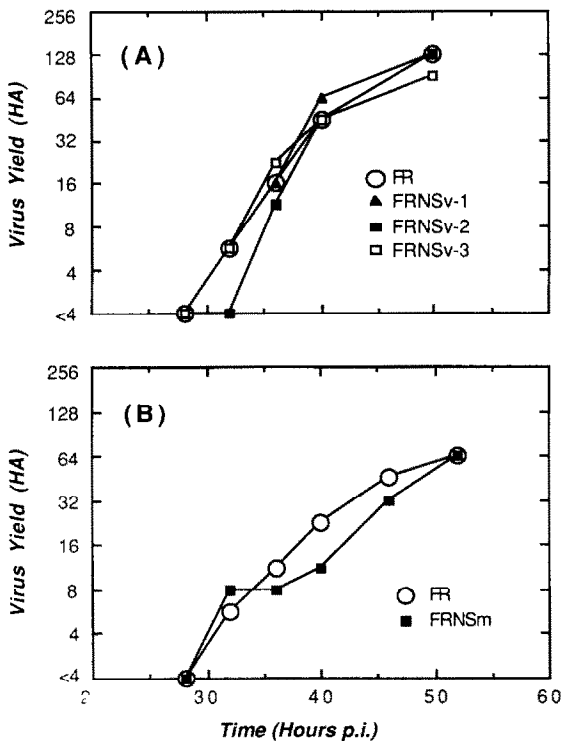

Fig. 6. Influenza virus replication in recombinant 293 cell lines; 5×10^5 cells were infected with 5×10^3 PFU of influenza A/WSN/33 virus. At the times indicated samples of supernatant were taken for determination of virus yield by hemagglutinin (HA) titration. A: 293 cells expressing antisense RNA. FRNSv-1, FRNSv-2, and FRNSv-3, antisense RNA expressing cell lines. FR, control cell line. B: 293 cells expressing (+)sense RNA. FRNSm, (+)sense RNA expressing cell line. FR, control cell line.
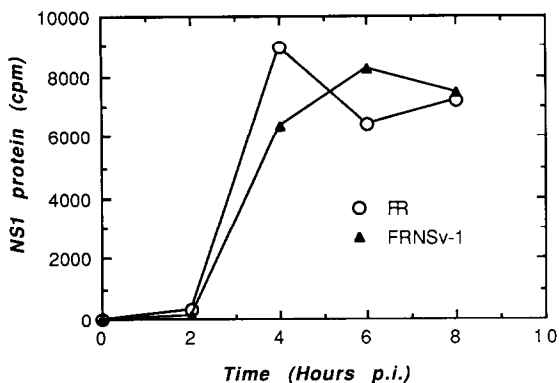
Fig. 7. NS1 protein synthesis in influenza virus infected 293 cells. Recombinant 293 cells were infected with influenza A/WSN/33 virus (MOI 5). Lysates were obtained from control cells (FR) and antisense RNA expressing cells (FRNSv-1) at various times after infection. RIA for NS1 protein was performed as described in Materials and Methods. CPM values given were calculated by subtracting the background which was obtained from uninfected FR cells (3539 cpm) or uninfected FRNSv-1 cells (3290 cpm). The data shown represent the mean of quadruplicate values.

RNA from virus-infected cells (lanes 1 and 8) or virion RNA (lane 7). The smaller fragments are most likely break-down products since they were not always observed but we cannot exclude early termination of some transcripts. In summary, we were able to establish permanent cell lines which express high levels of full-length antisense NS RNA or full-length (+)sense NS RNA, in the absence of detectable complementary RNA sequences.

Influenza virus replication in recombinant 293 cells

We next asked the question whether cells permanently expressing high levels of influenza virus-specific RNA are able to inhibit viral replication. Control cells and antisense RNA expressing cell lines were infected at low MOI (0.01) with influenza A/WSN/33 virus. Samples were taken at different times after infection and virus yields were determined by hemagglutination assay. Ten antisense RNA expressing cell lines and six (+)sense RNA expressing cell lines were tested. Fig. 6 shows the results of a typical experiment. No inhibition of virus replication could be observed in either the antisense RNA expressing cell lines (Fig. 6A) or the (+)RNA expressing cell line (Fig. 6B). When infected cells were assayed for NS1 protein synthesis by RIA no significant difference between antisense RNA expressing cells and control cells was found (Fig. 7). The same result was obtained in a separate experiment using an immuno dot-blot technique (data not shown).

Discussion

In this paper we report on the expression of influenza virus specific RNA in permanent cell lines and study their ability to inhibit viral replication. Initially, we

used BPV vectors to express influenza virus-specific RNA in C127 cells. We chose this system because it had been successfully used to express viral proteins and complement the growth of influenza virus mutants at the nonpermissive tempera- ture (Krystal et al., 1986; Li et al., 1989). Cell lines were designed to express the NS gene of an influenza A virus in the antisense orientation. We chose the NS gene as our primary target for the following reasons: First, the NS gene codes for two different mRNA molecules (Lamb, 1983) and antisense NS RNA may thus prevent synthesis of two viral proteins, NS1 and NS2. Secondly, the availability of many different influenza virus strains and the known sequence of their NS genes (Buona- gurio et al., 1986) would allow us to determine the necessary degree of sequence identity required for an antisense RNA to be effective –provided a specific effect on viral replication can be demonstrated. Finally, several lines of evidence suggest that the NS gene is indeed essential for influenza virus replication: (a) All influenza virus strains analyzed so far possess the NS gene; (b) Several temperature-sensitive mutants were isolated which were found to contain a defective NS gene (for review: Mahy, 1983); (c) A deletion in the NS gene was described to result in a restricted host range of the affected virus (Buonagurio et al., 1984).

BPV-transformed C127 cells (V2NS-4) were found to express only low levels of influenza virus specific RNA, but they were shown to inhibit growth of influenza A virus in a multi-cycle replication experiment as compared to the parental C127 cells (Table 1). Another control cell line which was obtained upon transfection with the BPV vector alone (i.e., lacking influenza virus sequences) also had no inhibitory effect on viral replication compared to C127 cells (not shown). However, this does not necessarily reflect a sequence-specific mode of action of the influenza virus RNA expressing cells since the transcriptional activity of the BPV vector lacking influenza virus sequences may be different. When VSV was used for infection of V2NS-4 cells, a decrease in virus yield similar to that observed following infection with influenza virus was observed (Table 1), indicating a non-specific mechanism of action. The situation was further complicated when the analysis of cellular RNA using single-stranded probes revealed the presence of both (+)sense and antisense RNA transcripts in these cell lines (Fig. 2). The latter phenomenon may be the result of promoters which are present in both orientations. Adding anti-IFN antibodies to the medium during influenza virus infection restored viral titers to control levels. Since double-stranded RNA is a known inducer of IFNs (for review, Lengyel, 1982), we speculate that the presence of double-stranded RNA induces an IFN-mediated antiviral state in this system. Since the observed effect appeared to be non-specific we decided to abandon these experiments and search for an alternative expression system.

We hoped that a different combination of cells and vectors would allow us to construct cell lines that (a) express high levels of antisense RNA and (b) do not synthesize (+)sense transcripts. For construction of expression vectors, we followed the approach of Kim and Wold (1985), who put antisense TK sequences down- stream of the DHFR coding region. This resulted in bifunctional RNA transcripts consisting of a DHFR portion and the antisense RNA sequence located down- stream of the selectable gene. Since expression of DHFR renders successfully

transfected cells resistant to the drug Mtx, the antisense RNA domain is always present in transformed cells and its concentration can be greatly increased by selection for high-dose Mtx resistance. Using the chimeric CMV/HIV promoter present in plasmid πH3 (Aruffo and Seed, 1987), we constructed vectors which we found to be particularly efficient in 293 cells. Using virion RNA from influenza A/WSN/33 virus as a standard, we estimate that one cell contains approximately 7500 copies of NS specific antisense RNA molecules. This level of expression is about 100-fold higher than that achieved in the C127/BPV system. Furthermore, complementary transcripts could not be detected in recombinant 293 cells (Fig. 4).

However, our data show that in this CMV/HIV/293 system the expression of high levels of either antisense or (+)sense RNA do not inhibit growth of influenza virus (Fig. 6). Also, as shown in Fig. 7 the antisense RNA expressing cell line fails to block expression of NS1 protein following virus infection. The failure to inhibit protein synthesis cannot easily be explained by the loss of important virus-specific sequences during selection of cell lines, since the length of the antisense RNA, as determined by RNase mapping, is very similar to that of virion RNA or to RNA isolated from infected cells (Fig. 5). It is also unlikely that a significant number of cells lost their ability to express antisense RNA. As described in the Results section, RNA was isolated from several independent cell populations after repeated subcloning and in every case the clones were found to make the desired RNA. Also, it is unlikely that a significant portion of antisense RNA molecules is rapidly degraded upon virus infection since we found no major decrease in hybridizable sequences in virus-infected FR-2 cells using a DHFR probe (Fig. 4).

It is evident from looking at other antisense RNA systems (e.g., Kim and Wold, 1985) that a large excess of antisense RNA over mRNA is a prerequisite for inhibition of gene expression. In our study, the levels of antisense RNA were found to be at least as high as the levels of virus-specific (+)RNA in infected cells, 3 h after infection (Fig. 4). Since cells were infected at low MOI, not more than one infectious virus particle per cell should be expected at the beginning of infection. The small number of mRNA molecules initially being synthesized (see Fig. 4, RNA from virus-infected cells 1.5 h p.i.) would be met by a large excess of antisense RNA. Similarly, in the case of cells permanently expressing (+)sense RNA, the vRNA of the incoming virus particle or synthesized following infection should be met by an excess of (+)sense RNA. Still no antiviral effect was observed in this system (Fig. 6). Although this result is puzzling we should consider that even in the most effective antisense RNA systems studied, the level of reduction in gene expression is at best 80–90% of the wild-type level (e.g., Kim and Wold, 1985). Therefore, viruses such as influenza virus, which are able to replicate effectively in a short period of time, may not be a suitable target for antisense RNA inhibition. If 10–20% of the viral genes are expressed at the beginning of infection, very soon the concentration of target RNA may be too high for inhibition by antisense RNA.

Other systems have been described in which excess antisense RNA failed to inhibit expression of specific genes (Salmons et al., 1986; Rebagliati and Melton, 1987; Bass and Weintraub, 1987; Kerr et al., 1988). Two groups have identified an RNA duplex unwinding activity in Xenopus embryos which was suggested to

prevent injected antisense RNA from forming stable hybrids with endogenous mRNA (Rabagliati and Melton, 1987; Bass and Weintraub, 1987). Although the presence of an unwinding activity may counteract the effects of antisense RNAs, the situation is complex. As the RNA is unwound it is modified in such a way that it can no longer hybridize to complementary sequences (Bass and Weintraub, 1988). The modification of mRNA should result in nonfunctional protein and thereby facilitate the antisense RNA effect (Bass and Weintraub, 1988). Hence, the failure to inhibit gene expression in these experiments (Rabagliati and Melton, 1987; Bass and Weintraub, 1987) cannot be explained by the presence of an RNA unwinding/modifying activity and other unknown factor(s) were postulated to be operating (Bass and Weintraub, 1988).

An alternative explanation for the failure of antisense RNA or (+)sense RNA to inhibit influenza virus replication in our system may be the following. During replication of influenza virus, both (+)stranded RNAs and (−)stranded RNAs are present during the entire cycle and one may speculate that regulatory mechanisms are operating which prevent a stable hybridization of the two complementary strands of RNA. For example, double-stranded RNA could be separated by a virus induced unwinding activity and/or vRNA may not be accessible for stable hybridization because it is never free as naked RNA but is always protected by viral protein. Finally, it is possible that the DHFR portion in the fused DHFR-NS gene influences the stability of virus specific hybrids in vivo and that other antisense constructs would be more successful. In this context it should be noted that the DHFR-NS constructs were designed following the successful approach used by Kim and Wold (1985).

In summary, based on our present experience it is unlikely that expression of full length antisense RNA or (+)sense RNA effectively inhibits influenza virus replication but we cannot exclude that antisense oligonucleotides may be effective for inhibition of virus growth (Zerial et al., 1987). Short oligonucleotides may be able to hybridize to small regions of virus-specific RNA which are not protected by proteins and long-term hybridization may not be required if the RNA-DNA duplex is rapidly cleaved by RNase H resulting in the loss of functional virus-specific RNA.

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