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Inhibition of influenza A virus reproduction by a ribozyme targeted against PB1 mRNA

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Abstract

A ribozyme gene directed at a specific cleavage of mRNA coding for PB1 protein, a component of RNA-dependent RNA-polymerase of influenza A virus, was constructed. The avian adenovirus CELO virus-associated RNA (VA RNA CELO) promoter and human cytomegalovirus (CMV) promoter were used for the permanent expression of the ribozyme in cell lines. The cells were infected with influenza A virus strains A/Singapore/1/57 and A/WSN/33, and the suppression of the virus reproduction and virus-specific protein synthesis was measured. The maximal level of the inhibition of virus reproduction as compared to the reproduction in non-transformed cells was 93.5%. Defective recombinant adenoviruses were constructed carrying the genes of functional and non-functional ribozymes under the control of human cytomegalovirus (CMV) promoter. The reproduction of A/WSN/33 virus in CV-1 cells preinfected with recombinant adenoviruses was shown to be suppressed.

Keywords: Ribozyme; Influenza A; Adenovirus

1. Introduction

The creation of novel approaches to the prevention and control of virus infections, tumors and hereditary diseases includes the development of efficient specific agents for the abolishment of the expression of viral and cellular genes. Ribozymes, small RNA molecules able to catalyze the reac-

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tion of specific endonucleolytic RNA cleavage, may be regarded as such agents (Symons, 1992, 1994).

The use of the ribozymes to suppress virus infections is of special interest. Extensive studies on the inhibition of HIV by ribozymes were performed (Kijima et al., 1995). The ribozymes were also used for the suppression of replication of bovine leucosis virus (Cantor et al., 1996), hepatitis C virus (Lieber et al., 1996; Sakamoto et al., 1996) and Epstein-Barr virus (Huang et al., 1997).

Theoretically, single-strand or double-strand RNA viruses are ideal targets for ribozymes. Influenza A virus, an important pathogen of humans, mammals and birds, is a negative-strand RNA virus with a segmented genome. In the present studies we explore the biological activity of the ribozymes directed against mRNA of PB1, a subunit of influenza A virus RNA-dependent RNA polymerase. The ribozymes were either permanently expressed in the transformed continuous cell lines, or they were expressed by recombinant adenoviruses in the infected cells.

In an earlier attempt to use a ribozyme that suppresses influenza A virus reproduction (Tang et al., 1994), RNA segment 5 (coding for virus nucleoprotein) was used as a target. We preferred to choose as a target a region of PB1 mRNA. PB1 is a subunit of influenza virus polymerase; it plays an important role in the transcription and replication of the virus genome (Huang et al., 1990). The PB1 gene is highly conservative: influenza A viruses of humans, mammals and birds have long homologous regions in this RNA segment, so that PB1-directed ribozyme may be expected to cleave mRNA of widely different virus strains.

In order to assess adequately the input of the antisense effect in the inhibition of virus replication by the ribozyme, we synthesized a ribozyme gene with a nucleotide substitution in the catalytic domain. The ribozyme transcript produced by this gene was inactive in RNA cleavage in vitro.

In any attempt to use ribozymes as antiviral agents one encounters the problem of the most efficient way of delivery of the ribozyme genes into the cell. The use of virus vectors, especially the vectors on the basis of human adenoviruses, seems to be the most promising. Adenovirus vectors have been used for the expression of ribozymes directed against conservative regions of hepatitis C virus genome RNA (Lieber et al., 1996), and against mRNA of the nuclear antigen of Epstein-Barr virus (Huang et al., 1997). In the present studies we used for the first time a defective adenovirus vector on the basis of human adenovirus type 5 for the delivery and expression of ribozyme genes with the aim to specifically inhibit influenza A virus reproduction in cell culture. The results suggest that specific ribozymes directed against influenza A virus mRNAs may be regarded as potential antiviral agents.

2. Materials and methods

2.1. Cells and viruses

Continuous cell lines CV-1 and MDCK were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal bovine serum with penicillin (100 U/ml) and streptomycin (100 ug/ml). Influenza virus strains A/Singapore/1/57 and A/WSN/33 were obtained from the repository of the D.I. Ivanovsky Institute of Virology. The viruses were propagated in 10-day old embryonated chicken eggs. The virus-containing allantoic fluid was collected and stored at 4°C.

2.2. Construction of ribozyme genes and recombinant plasmids

A GUC site at position 1568 in PB1 mRNA of influenza virus A/Kiev/59/79 (H1N1) (Petrov et al., 1987) was chosen as the ribozyme target site (numbering according to GenBank accession number M38376). Oligodeoxyribonucleotides were synthesized corresponding to the 'hammerhead' ribozyme gene (Haseloff and Gerlach, 1988). They contained the sequence corresponding to the catalytic domain (24 nucleotides) and flanking antisense sequences complementary to the RNA target (12 nucleotides on both sides of the catalytic domain) (Fig. 1A). For the assessment of the 'antisense effect', oligodeoxyribonucleotides were synthesized corresponding to the gene of a defective ribozyme containing a single nucleotide substitution (G/A) in the catalytic domain critical for the specific endonu-

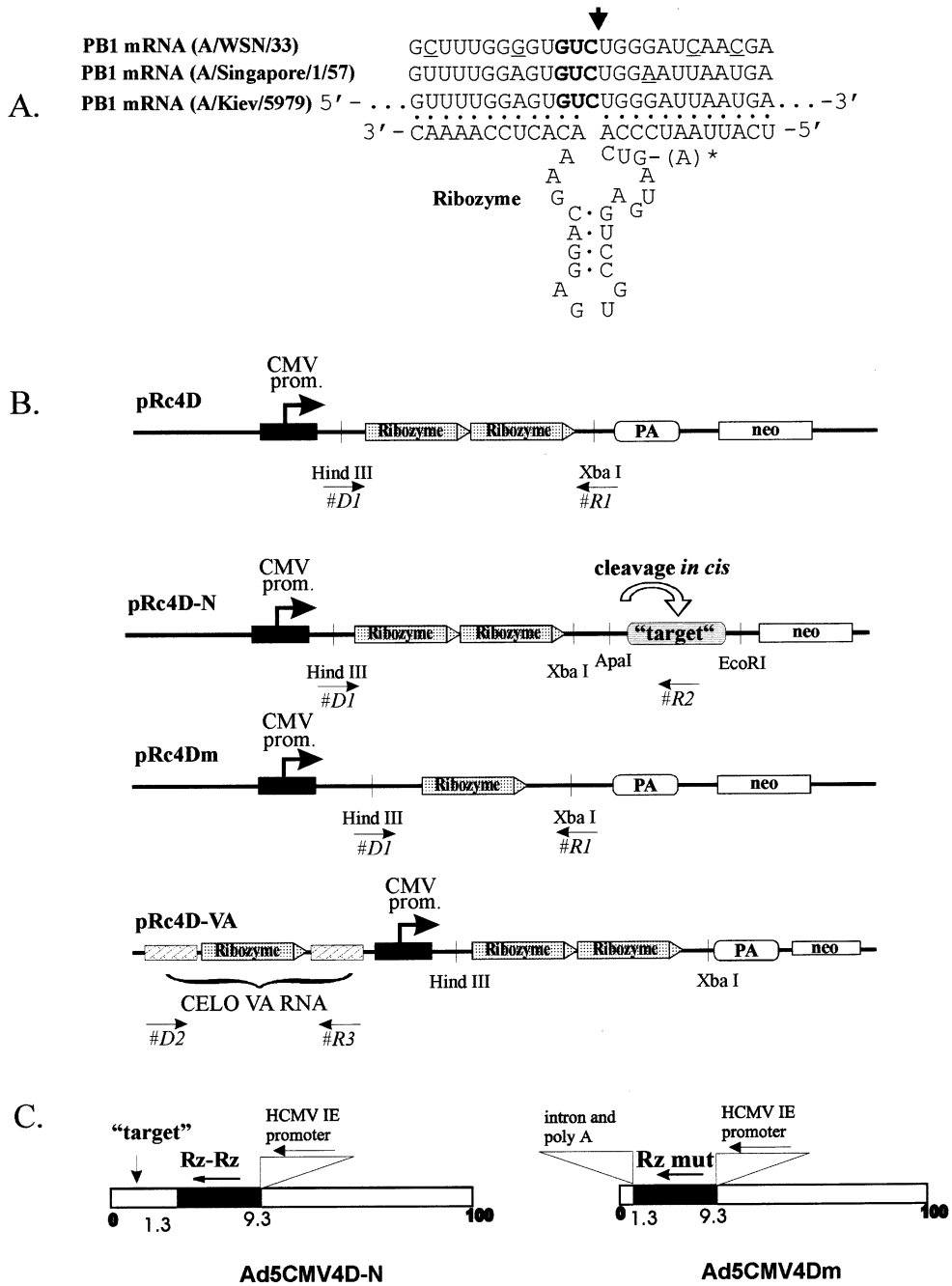

Fig. 1. (A) Sequence and secondary structure of the ribozyme directed against influenza virus PB1 mRNA. The interaction with the complementary target sequences is presented, and the cleavage site is shown in bold letters. The nucleotides differing from the nucleotides in the respective positions in the sequence of A/Kiev/59/79 strain (used for the preparation of cDNA) are underlined. The nucleotide substitution in the catalytic domain making the ribozyme non-functional is shown by an asterisk. (B) Diagrams of ribozyme expression units under the control of HCMV and VA RNA CELO promoters for in vivo studies. The position and orientation of primers for reverse transcription and PCR amplification are indicated at the bottom of each plasmid's diagram. PA-polyadenylation site(s); neo-neomycin resistance gene; 'target'-oligonucleotide sequence corresponding to a region of PB1 gene of influenza A/Kiev/59/79 virus. (C) Schematic representation of recombinant adenoviruses Ad5CMV4D-N and Ad5CMV4Dm. White boxes, Ad5 sequences. Black boxes, plasmid sequences. Rz-Rz, ribozyme gene dimer, Rz mut, the non-functional ribozyme gene. Numbers represent the units of Ad5 genome physical map.

cleolytic activity of the ribozyme (Fig. 1A, indicated by an asterisk).

The oligodeoxyribonucleotides were cloned into pRc/CMV plasmid ('Invitrogen') at the sites HindIII/XbaI under the control of HCMV promoter. The clones bearing a plasmid containing head-to-tail oriented dimer of the functional ribozyme gene (pRc4D plasmid), as well as the clones bearing a plasmid containing the gene of the mutant (non-functional) ribozyme (pRc4Dm plasmid), were selected. Oligodeoxyribonucleotides corresponding to the target region (5′-GGCCTGGAGTGTCTGGGATTA and 5′-AATTTAATCCCAGACACTCCA) including the ribozyme cleavage site were hybridized, phosphorylated and cloned into pRc4D plasmid at ApaI–EcoRI sites. This ensured the intramolecular cleavage of the full-length transcript concomitantly with the transcription, since both the ribozyme and the target were parts of the same molecule (cis-ribozyme). The resulting plasmid was designated pRc4D-N. For the expression of the ribozyme gene under the control of the promoter of the virus-associated (VA) RNA of the avian adenovirus type 1 (CELO), ribozyme genes were cloned into the unique BspMII site of pCVA plasmid (Zakharchuk et al., 1995). The resulting plasmid was designated pCVArib4. BamHI-fragment of pCVArib4 plasmid was cloned into BglII site of pRc4D plasmid. The resulting plasmid was designated pRc4D-VA. The plasmid constructions are presented in Fig. 1.

2.3. Creation of cell lines constantly expressing ribozyme genes

CV-1 cells were seeded and incubated in 6 cm-diameter plastic dishes. Semi-confluent (80–85% of the dish surface) monolayers were used for transfection with plasmid DNA by calcium–phosphate technique (Graham and van der Eb, 1973). The efficiency of transfection as assessed by the marker gene beta-gal was 15–20%. Plasmids pRc4Dm, pRc4D, pRc4D-N and pRc4D-VA were used. Selective medium (1XDMEM with 10% FBS and 400 μg/ml of Gibco-G418) (Santerre et al., 1991) was added 48 h post transfection, G-418-resistant colonies were picked and propagated.

2.4. Determination of ribozyme gene expression in the cell lines by reverse transcription-PCR technique

Total cell RNA was extracted (Chomczynsky and Sacchi, 1987) and used in reverse transcription (RT) reaction with the use of primers specific for bacterial SP6 promoter (#R1 5′-ATTTAGGTGACACTATAGAA) and M13 origin (#R2 5′-AGATCTACGTACGAGCTCGC) of pRc/CMV plasmid, and for the sequence adjacent to promoter box B of VA RNA CELO gene (#R3 5′-CGGAGGTGGAGGTAATACAT). 'Reverse Transcription System' kit ('Promega') was used for RT reaction. After the completion of RT reaction the primers for PCR specific for bacterial promoter T7 (#D1 5′-TAATACGACTCACTATAGGG) and for the sequence adjacent to promoter box A of VA RNA CELO gene (#D2 5′-GATCAAGATCGACAGTGTAG) were added to RT mixture. The location of the primers is shown in Fig. 1. PCR products were detected by electrophoresis in 3% agarose (FMS, Rockland, ME) gel.

2.5. Assessment of influenza virus accumulation by plaque titration

Ribozyme-expressing cells and the original CV-1 cells were infected with influenza A/Singapore/1/57 or A/WSN/33 virus at a multiplicity of infection (m.o.i.) of 1 or 10 PFU/cell. After 35 min of virus adsorption, the cells were washed twice with PBS, the maintenance medium (DMEM containing 0.2% bovine serum albumin) was added and the cells were incubated at 37°C. Virus-containing culture fluid was collected 8 h post infection (p.i.) and treated for 1 h at 37°C with trypsin (3.0 μg/ml). The concentration of infectious virus was determined by plaque titration in MDCK cells grown in 35 cm-diameter dishes. MDCK cells were inoculated with serial dilutions of the virus-containing culture fluid, washed twice after virus adsorbtion with PBS, and overlayed with 0.7% agarose in DMEM containing 0.001% trypsin. The cells were incubated

for 3 days at 37°C and stained with neutral red (Flow Laboratories) to reveal the plaques.

2.6. Measurement of influenza virus-specific protein synthesis in the infected cells

Ribozyme-expressing cells and the original CV-1 cells were infected with influenza virus, as described in the previous section, and incubated 8 or 16 h at 37°C. The cells were washed with methionine-free Minimal Essential Medium (Eagle), the same medium containing 50 μCi/ml of ^{35}S-methionine was added, the cells were incubated for 2 h at 37°C, lysed in lysis buffer (0.125 M Tris–HCl pH 6.8, 4% SDS, 50 mM DTT, 0.01% bromophenol blue, 20% glycerol), and analysed in polyacrylamide gel electrophoresis at 15% acrylamide concentration. The gel slabs were dried, autoradiographed and scanned in a densitometer.

2.7. Construction of recombinant adenoviruses

Recombinant adenoviruses were produced as described by McGrory et al. (1988). Regions of DNA of pRc4D-N and pRc4Dm plasmids containing the sequences of human cytomegalovirus promoter and the genes of the functional and non-functional ribozymes were subcloned in a plasmid carrying Ad5 sequence with the prevalent part of E1 region deleted. The resulting plasmids and the plasmid pJM17 (McGrory et al., 1988) were introduced as DNA into the cells of 293 line by the calcium–phosphate precipitation technique (Graham and van der Eb, 1973). Individual virus plaques were picked 12–14 days after transfection, and the virus was propagated in the cells of the continuous 293 cell line. The recombinant adenoviruses were designated Ad5CMV4D-N (the virus containing the dimer of the functional ribozyme gene) and Ad5MV4Dm (the virus containing the mutant ribozyme gene). The recombinant adenovirus constructs are represented in Fig. 1C. CV-1 cells were infected with the recombinant adenoviruses at the multiplicities of 1 and 10 PFU/cell. At 12 h p.i. the cells were superinfected with influenza A/WSN/33 virus (1 PFU/cell). At 8 h after the superinfection the culture fluid was used for virus infectivity titration by plaque technique in MDCK cells. Human adenovirus type 5 and recombinant adenovirus Ad5neo (van Doren et al., 1984) were used as control.

3. Results

3.1. Ribozyme RNA synthesis in the transfected cells

CV-1 cells transfected with pRc4Dm, pRc4D, pRc4D-N and pRc4D-VA plasmids (lines CV-Dm, CV-D, CV-N and CV-VA, respectively) were characterized with respect to the expression of ribozyme RNA. Ten clones of each cell line were used. In order to detect ribozyme-specific mRNA, total cellular RNA was extracted from the cells and analyzed by RT-PCR using specific primers (see Section 2.4). The specificity of PCR products was confirmed by hybridization with labelled ribozyme sequence-containing probe (data not shown). For each line three clones with the maximum level of the signal were chosen for further studies. The results of the analysis of PCR products of the selected clones in gel electrophoresis are presented in Fig. 2.

3.2. Inhibition of virus protein synthesis in cell lines permanently expressing ribozyme genes

The cell lines were infected with influenza viruses (strains A/Singapore/1/57 and A/WSN/33) and labelled with ^{35}S-methionine 8 or 16 h p.i. The autographs of polyacrylamide gel electrophoresis slabs revealed a typical pattern of influenza A virus-specific proteins with a characteristic shut-off of cellular protein synthesis. The results for CV-1, CV-Dm and CV-D cells are presented in Fig. 3. To assess the extent of inhibition the autoradiographs were scanned in a densitometer, the areas under the peaks corresponding to NP and NS1 proteins were measured, and per cent inhibition in ribozyme-expressing lines as compared to CV-1 cells was calculated. In the cells infected with A/Singapore/1/57 strain the maximal level of inhibition (90.5

and 88.7% for NP and NS1, respectively) was noted in the CV-VA line, whereas in the cell line expressing the functionally inactive mutant ribozyme the level of inhibition was 11.8 and 3.6% for NP and NS1, respectively. With A/Singapore/1/57 virus the extent of inhibition for all cell lines tested was lowered at 16 h p.i. as compared to the extent of inhibition at 8 h. In the cells infected with A/WSN/33 virus again the maximal level of inhibition was observed in the CV-VA line (89.6% at a m.o.i. of 1 PFU/cell and 85.6% at a m.o.i. of 10 PFU/cell for NP protein), and the minimal inhibition was observed in the line expressing the non-functional ribozyme (4.5% at a m.o.i. of 10 PFU/cell 16 h p.i.). The results of the assessment of inhibition of virus protein synthesis for the clones with the highest inhibitory effect are summarized in Table 1.

3.3. Suppression of influenza virus reproduction in ribozyme-expressing cell lines

Cell lines expressing functional or non-functional ribozyme were infected with A/Singapore/1/57 or A/WSN/33 virus, and the level of the inhibition of virus accumulation in culture fluid was determined by plaque titration, as described in Section 2. In the cells infected with A/Singapore/1/57 at a m.o.i. of 1 PFU/cell the maximal level of inhibition was registered in CV-N and CV-VA lines (92.3 and 90.4%, respectively). In the line expressing the non-functional ribozyme it was 44.5%. In the cells infected with A/WSN/33 virus the maximal level of inhibition was observed in the CV-VA line (93.5% at a m.o.i. of 1 PFU/cell). It is noteworthy that in A/WSN/33 virus-infected cell line expressing the non-functional ribozyme the level of inhibition was 63.0% at a m.o.i. of 1 PFU/cell, whereas it was three times lower (22.2%) at a m.o.i. of 10 PFU/cell. The data on the inhibition of virus reproduction are summarized in Table 2.

3.4. Inhibition of influenza A/WSN/33 virus reproduction in CV-1 cells preinfected with recombinant adenoviruses

Recombinant adenoviruses Ad5CMV4D-N and Ad5CMV4Dm were used for the preinfection of CV-1 cells. Adenoviruses have the ability to infect a wide range of different cell types. Using recombinant adenovirus, expressing beta-gal gene, we confirmed the ability of human adenovirus to efficiently infect CV-1 cells (data not shown). The cells were superinfected with influenza A/WSN/33 virus 12 h after the preinfection.

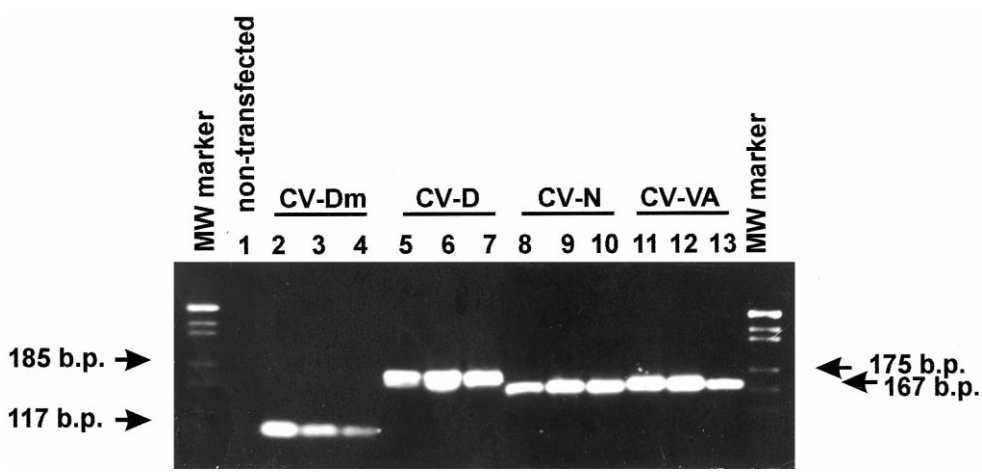

Fig. 2. RT-PCR amplification of ribozyme RNA expressed in individual clones of permanent cell lines. Lane 1, RNA from non-transfected CV-1 cells. Lanes 2, 3 and 4, RNA from clones 1, 4 and 2 of cell line CV-Dm (amplified fragment 117 b.p.). Lanes 5, 6 and 7, RNA from clones 8, 5 and 2 of cell line CV-D (amplified fragment 185 b.p.). Lanes 8, 9 and 10, RNA from clones 5, 7 and 1 of cell line CV-N (amplified fragment 167 b.p.). Lanes 11, 12 and 13, RNA from clones 6, 5 and 3 of cell line CV-VA (amplified fragment 175 b.p.). M, molecular weight marker. The specificity of PCR products was confirmed by hybridization with a labelled ribozyme-sequence containing probe.

Table 1
Per cent inhibition of influenza virus protein synthesis in cell lines expressing the ribozyme targeted against mRNA of PB 1 gene[a]

Influenza virus strain	A/Singapore/1/57				A/WSN/33							
Time after infection	8 h		16 h		8 h				16 h			
Multiplicity of infection	1 PFU/cell		1 PFU/cell		1 PFU/cell		10 PFU/cell		1 PFU/cell		10 PFU/cell	
Protein	NP	NSI	NP	NSI	NP	NSI	NP	NSI	NP	NSI	NP	NSI
CV-Dm	38.4	32.0	11.8	3.6	42.4	46.3	11.4	16.8	26.8	46.0	4.5	3.3
CV-D	72.2	84.3	68.9	39.4	72.7	47.8	60.6	39.5	62.4	58.8	35.5	34.7
CV-N	81.8	85.9	75.8	58.2	79.4	64.2	67.5	72.1	84.7	76.4	48.2	51.0
CV-VA	90.5	88.7	81.2	67.0	86.2	69.0	85.9	87.1	89.6	87.1	83.4	41.0

[a] With respect to the synthesis in the non-transfected CV-1 cells. The data are presented for one clone of each cell line exhibiting the suppression of virus protein synthesis.

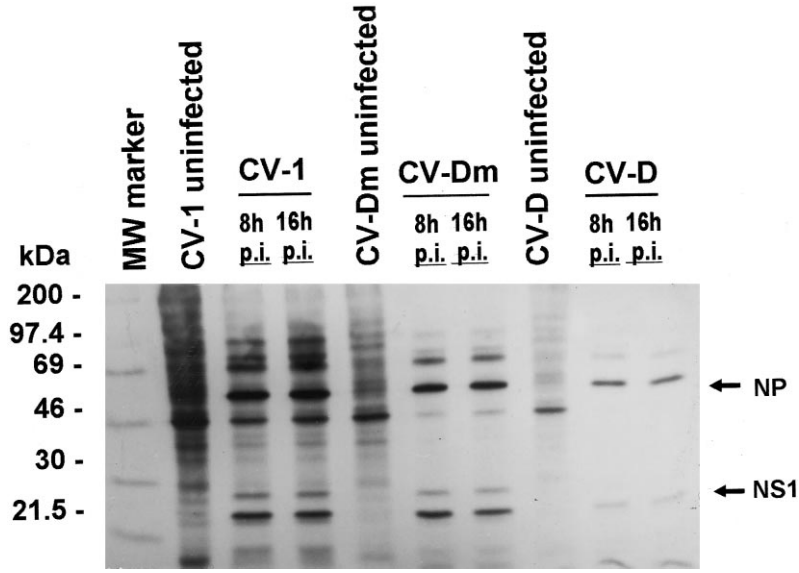

Fig. 3. Polyacrylamide gel electrophoresis analysis of virus-specific proteins synthesized in the cells infected with influenza virus A/Singapore/1/57. 35S-methionine added at 8 or 16 h p.i. m.o.i.: 1 PFU/cell. Acrylamide concentration 15%. Lane 1, molecular weight marker (Rainbow TM (14C) methylated protein). Lanes 2, 5 and 8, mock-infected cell lines CV-1, CV-Dm and CV-D, respectively. Lanes 3 and 4, CV-1 cells at 8 and 16 h p.i., respectively. Lanes 6 and 7, CV-Dm cells at 8 and 16 h p. i., respectively. Lanes 9 and 10, CV-D cells at 8 and 16 h p.i., respectively.

The maximum level of the inhibition (91.4%) of influenza A/WSN/33 virus reproduction was observed after the preinfection of CV-1 cells with Ad5CMV4D-N, that is, with the virus expressing the gene of the functional ribozyme. In the cells pre-infected with the recombinant adenovirus expressing the gene of the non-functional ribozyme the level of inhibition was 21.5% at a m.o.i. of 1 PFU/cell, and 43.7% at a m.o.i. of 10 PFU/cell. The degree of inhibition increased with the multiplicity of adenovirus infection. To a small extent (10–15%) the inhibition was also observed in the cells preinfected with the wild-type adenovirus, or with the recombinant Ad5neo adenovirus. The results of the experiments are summarized in Table 3.

4. Discussion

We have constructed a hammerhead-type ribozyme directed at a specific cleavage of PB1 mRNA of influenza A virus. The in vitro activity of this ribozyme had been described in our earlier publication (Zakharchuk et al., 1996).

When ribozymes are used to inhibit the expression of viral or cellular genes, the access to the target and the biological significance of the target molecule play a major role. Data were reported (Tang et al., 1994) on the use of a ribozyme directed against influenza virus genomic RNA segment 5 coding for the virus nucleoprotein (NP). The use of the genomic RNA as a target has some advantages. However, one has to take into consideration that genomic segments are combined with viral proteins, and this may hamper the interaction between the ribozyme and the target. (Bertrand and Rossi, 1994). Therefore we have chosen as the target a region of virus-specific mRNA. It is important that the chosen mRNA codes for PB1 protein, a crucial component of the machinery involved in the transcription and replication of the virus genome: it is responsible for the initiation and elongation of nascent RNA strands (Krug et al., 1989). Besides, PB1 gene is one of the least variable influenza A virus genes. A comparison of nucleotide sequences of PB1 genes of 15 strains suggested that the ribozyme should bind and cleave their mRNAs. Our results demonstrate that it is active against

A/Singapore/1/57 and A/WSN/33 influenza virus strains. The data allow to presume that the ribozyme is able to inhibit the reproduction of a wide range of influenza A viruses.

To analyze the ribozyme activity in cell culture we conctructed a series of recombinant plasmids containing the ribozyme gene under the control of the human cytomegalovirus (HCMV) promoter or the VA RNA promoter of CELO virus. Plasmid pRc4D contained a dimer of identical ribozyme genes under HCMV promoter, whereas pRc4D-VA plasmid contained, in addition to this dimer construction, also a monoribozyme under the control of the VA RNA CELO promoter. It was reported (Ohkawa et al., 1993) that polyribozymes have a higher activity as compared to monoribozymes.

In another construction, pRc4D-N, the polyadenylation signal was replaced by oligonucleotides complementary to the target sequence in the mRNA of PB1 gene. This led to an intramolecular cleavage of the transcript (Zakharchuk et al., 1996): the absence of poly(A) at the end of the transcript had to ensure its predominant accumulation in the nucleus. This is important, since the transcription and replication of influenza virus genome occur in the nucleus. With a goal to assess the biological activity of the ribozymes in cell culture, we used the plasmid constructions for the transformation of CV-1 cells in order to obtain cell lines permanently expressing the ribozyme genes.

PB1 protein is a viral protein of low abundance in the infected cell. Therefore it was difficult to assess properly the extent of inhibition of PB1 gene expression directly, by the measurement of PB1 synthesis. On the other hand, this protein is crucial for the amplification of synthesis of influenza virus proteins, since it is indispensable for the virus genome replication (Huang et al., 1990). For this reason, it is possible to use the extent of inhibition of the production of major virus proteins as a measure of the suppression of virus protein synthesis. We assessed the synthesis of NP, a structural virus protein forming the ribonucleoprotein complex with virus RNA segments, and the synthesis of NS1 protein, a component involved in the transport of host RNA, in RNA splicing, and in the translation of virus mRNA.

The assessment of virus protein synthesis showed that the maximum level of inhibition of the synthesis of NP and NS1 proteins (90.5 and 88.7%, respectively) occured in the cell lines CV-N and CV-VA (Table 1).

The virus accumulation in cell culture as measured by plaque titration of the virus yield in the cell lines permanently expressing the genes of the functional and non-functional ribozymes also suggested that the maximum level of inhibition occured in the lines CV-N and CV-VA. (Table 2).

A greater extent of inhibition of two influenza virus strains in CV-VA and CV-N cell lines as compared to CV-D line may be ascribed to the expression of an additional ribozyme gene in CV-VA cells, and to the accumulation of the ribozyme in the cell nucleus (as a result of the absence of poly(A) signal) in CV-N cells.

It should be noted that the inhibition of virus reproduction may result from a non-specific interferon-mediated effect (Leiter et al., 1989). In our cell lines permanently expressing ribozyme genes, the difference in the extent of inhibition among three different clones of the same cell line did not exceed 10%.

Table 2
Per cent inhibition of influenza A virus reproduction in cell lines expressing the ribozyme targeted against PB1 mRNA as determined by plaque titration of the virus accumulated in culture fluid with respect to the virus accumulation in CV-1 cell line

Influenza virus strain	A/Singapore/1/57	A/WSN/33	
Multiplicity of infection	1 PFU/cell	1 PFU/cell	10 PFU/cell
CV-Dm	44.5 ± 4.9	63.0 ± 1.4	22.2 ± 6.3
CV-D	81.6 ± 1.5	81.4 ± 2.7	71.9 ± 4.1
CV-N	92.3 ± 4.0	87.5 ± 3.7	82.8 ± 5.4
CV-VA	90.4 ± 5.1	93.5 ± 1.8	75.2 ± 8.7

Table 3
Per cent inhibition of influenza A/WSN/33 virus reproduction in CV-1 cells preinfected with recombinant ribozyme-expressing adenoviruses[a]

Recombinant adenoviruses	Multiplicity of adenovirus infection (PFU/cell)	Per cent inhibition of influenza virus reproduction
Ad5CMV4D-N	1	67.6 ± 2.9
	10	91.4 ± 3.6
Ad5CMV4Dm	1	21.5 ± 1.7
	10	43.7 ± 5.3
Ad5neo	1	15.3 ± 1.9
Ad5	1	10.7 ± 0.7

[a] With respect to the reproduction of influenza A/WSN/33 influenza virus in CV-1 cells not preinfected with adenoviruses. The virus reproduction was assessed by plaque titration of virus.

To make the transfer and expression of ribozyme genes in cell lines more efficient, we used the recombinant adenovirus on the basis of human adenovirus type 5.

Since the adenovirus genome contained the ribozyme gene (under the control of CMV promoter) in the deleted E1 region, the recombinant adenovirus could not replicate in the cells (other than 293 line), but it could infect cells and persist in them for a long time, while expressing the cloned genes (Lieber et al., 1996).

The replication of influenza A/WSN/33 virus in CV-1 cells was inhibited when the cells were preinfected with the recombinant ribozyme-expressing adenoviruses. The maximal level of inhibition as compared to the non-preinfected cells was 91.4% (Table 3). The extent of inhibition depended on the multiplicity of infection with the recombinant adenovirus. Such a correlation is to be expected, since the efficiency of the inhibition of the gene expression by ribozymes had been shown to depend directly on the excess of the ribozyme over the substrate (Cameron and Jennings, 1989). It should be noted that the extent of inhibition of gene expression by ribozymes may also vary depending on the relation between the rate of transcription and the rate of decay of the target mRNA.

A major problem in the use of ribozymes for the inhibition of gene expression is an adequate assessment of the antisense effect of the flanking sequences complementary to the target RNA. This effect may vary from 9 (Steinecke et al., 1992) to 100% (Saxena and Ackerman, 1990). In an attempt to evaluate the input of the antisense effect, we constructed a non-functional ribozyme gene with a nucleotide change in the catalytic domain.

The results of the measurement of the inhibition of virus protein synthesis in the cell line expressing the non-functional ribozyme showed that the 'antisense effect' decreased more sharply (as compared to the effect of the ribozyme-induced cleavage) with the increase of the influenza virus multiplicity of infection (Table 2). Accordingly, in the experiments using the recombinant adenoviruses, the 10-fold increase in the multiplicity of infection by the recombinant adenovirus expressing the gene of the non-functional ribozyme, resulted in a 2-fold increase in the extent of inhibition (21.5% at 1 PFU/ml and 43.7% at 10 PFU/cell) (Table 3). Thus, the inhibition of influenza A virus reproduction with our ribozyme construction results mostly from the effect of RNA cleavage, and only to a small extent from the 'antisense' effect of the flanking complementary sequences.

In conclusion, we have constructed and expressed a ribozyme able to suppress influenza A virus infection in cell culture through the cleavage of a virus-specific mRNA. The data may be regarded as a basis for future experiments on the protection against influenza infection by the antiviral effect of ribozyme genes in transgenic animals.

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