ELSEVIER

Antiviral Research 44 (1999) 133–141

Antiviral Research

www.elsevier.com/locate/antiviral

Short communication

Heterotypic inhibition of foot-and-mouth disease virus infection by combinations of RNA transcripts corresponding to the 5′ and 3′ regions

Paloma Bigeriego [a,b], Maria Flora Rosas [b], Eva Zamora [b], Encarnación Martínez-Salas [a], Francisco Sobrino [a,b,*]

[a] *Centro de Biología Molecular, Severo Ochoa (CSIC-UAM), 28049 Cantoblanco, Madrid, Spain*
[b] *Centro de Investigación en Sanidad Animal, INIA, 28130 Valdeolmos, Madrid, Spain*

Received 3 December 1998; accepted 28 July 1999

Abstract

Strategies to inhibit RNA virus multiplication based on the use of interfering nucleic acids have to consider the high genetic polymorphism exhibited by this group of viruses. Here, we report high levels of heterotypic inhibition of foot-and-mouth disease virus (FMDV) infective particle formation in cotransfection experiments of susceptible cell lines with infections viral RNA and combinations of viral transcripts. The interfering molecules used include the following regions on type C FMDV RNA: (i) sequences from the 5′ region, spanning the proximal part of the internal ribosome entry site element and the two functional initiator AUGs; and (ii) the 3′ terminal region including the 3′ end of 3D gene and the complete 3′ non-coding region. Combination of 5′ *antisense* RNA molecules with either *sense* or *antisense* RNA molecules from the 3′ region resulted in inhibition of up to 90% of the infectivity of homologous type C FMDV RNA. The inhibition was dose-dependent and specific, as no reduction was observed in the plaque-forming units recovered from RNA of swine vesicular disease virus, a related picornavirus. Interestingly, high levels of intertypic inhibition, about 60% or higher, were observed when viral RNAs of serotypes O and A were analysed. These levels of inhibition are consistent with the levels of nucleotide homology exhibited by the viruses analysed in the target sequences. Inhibition of virus yield was also observed in FMDV-infected cells transiently expressing the interfering RNAs. Thus, transcripts of the FMDV RNA corresponding to the 5′ and 3′ regions specifically inhibit FMDV particle formation in a serotype-independent manner.

Keywords: FMDV; Viral inhibition; Heterotypic; Transcripts combination; Genetic variability

* Corresponding author. Tel.: +34-91-6202-300; fax: +34-91-6202-247.
E-mail address: sobrino@samba.cnb.uam.es (F. Sobrino)

Nucleic acids complementary to both *sense* and *antisense* viral sequences can block specific steps in viral infection (reviewed by Cohen, 1991;

0166-3542/99/$ - see front matter
PII: S0166-3542(99)00057-1

Agrawal, 1992; Bischofberger and Wagner, 1992). Consequently, synthetic oligonucleotides and RNA transcripts have been widely used as tools to interfere virus multiplication (Kim et al., 1995; Hanecak et al., 1996; Yao et al., 1996). In some instances, transgenic animals expressing viral subgenomic RNAs have been shown to exhibit a decreased susceptibility to virus infection (Mizutani et al., 1993). Foot-and-mouth disease virus (FMDV) leads to a devastating disease and is a major animal health problem world-wide (Bachrach, 1968; Domingo et al., 1990). Efficiency of immunization campaigns with chemically inactivated virus vaccines (Barteling and Vreeswijk, 1991) is still problematic in many areas of the world due to the high costs, the instability of the vaccines and the extensive antigenic diversity exhibited by the virus, which is reflected in the seven serotypes identified to date (Pereira, 1981). Therefore, alternative antiviral strategies as those based on the specific inhibition of FMDV multiplication are of considerable interest, provided they can be effective against a virus with a high potential for genetic variation (reviewed by Domingo et al., 1992).

FMDV belongs to the *Picornaviridae* family and its genome consists of a positive strand RNA molecule of about 8500 nt that contains two non-coding regions (NCR) flanking a unique open reading frame (ORF). Replication and translation (Sangar, 1979) occur in the cytoplasm of infected cells (Arlinghaus and Polatnick, 1969). The cap-independent translation initiation of the FMDV RNA (Belsham and Brangwyn, 1990; Kühn et al., 1990; Martínez-Salas et al., 1993) starts at two AUG codons (Beck et al., 1983b), following ribosome recognition of the upstream internal ribosome entry site (IRES). The IRES element is composed of about 465 bases from the 5′NCR of FMDV RNA; this region is predicted to form extensive secondary structures (Pilipenko et al., 1989) and contains several unused AUG codons. A highly ordered structure is also predicted at the 3′NCR which contains a genome-encoded poly-(A) tail. The evidence available for other picornaviruses indicates that the 3′NCR is involved in the interaction with cellular and/or viral proteins during RNA replication (Todd et al., 1995; Todd and Semler, 1996; Mellits et al., 1998).

We have previously reported that type C RNA molecules containing the 3′ region in both *antisense* and *sense* orientations were able to inhibit infective particle formation in BHK-21 cells, following cotransfection with the homologous infectious viral RNA. Likewise, *antisense*, but not *sense*, transcripts from the 5′ region, including the proximal part of the IRES element and the two functional initiator AUGs, were also inhibitory (Gutiérrez et al., 1993). These inhibitions were dose-dependent and increased when the annealing between the two RNAs was favoured. In this report, and as a further step in assessing the antiviral effect of nucleic acids containing the 5′ and 3′ FMDV RNA regions, we have analysed the inhibitions exerted by combinations of transcripts from these two regions on the infectivity of the homologous type C RNA as well as of heterologous RNAs of serotypes O and A.

The FMDV transcripts used in this study corresponded to the sequence of C-S8 type C isolate (Martínez-Salas et al., 1985, 1993). They were synthesized in vitro from plasmids p-5′ and p-3′2, derivatives of plasmid PT7tk (Gutiérrez et al., 1994; Martínez-Salas and Domingo, 1995). SP6 RNA polymerase transcription, following linearization of these plasmids with *Sac*I, rendered *sense* molecules (5′S and 3′S), while linearization with *Xba*I and T7 RNA polymerase transcription yielded *antisense* molecules (5′AS and 3′AS). The RNA transcribed from plasmid p-5′ (156 nt) contained 63 nt from 3′ end of the IRES element and the first 93 nt of the ORF, including the two functional initiator AUGs. The RNAs transcribed from plasmid p-3′2 (397 nt) spanned 302 nt of the 3′ end of the ORF (3D polymerase gene) and 95 nt of the 3′NCR. The inhibitions exerted by these transcripts were estimated by the reduction of the plaque forming units (PFU) recovered upon their cotransfection with infectious viral RNA in cell lines susceptible to FMDV infection. Cotransfections were carried out as previously reported (Gutiérrez et al., 1994), using lipofectine (DDAB-DOPE) prepared according to Rose et al. (1991). Briefly, triplicates of cell monolayers in 35 mm-diameter dishes (about 80% confluent) were cotransfected with different amounts of FMDV transcripts and about 10 pg of purified viral

RNA, with or without a previous incubation under renaturing conditions to favour annealing. The infection was allowed to proceed in the presence of 0.7% agar. The PFU recovered at 24 h postinfection were determined after crystal violet staining. The amount of infectious viral RNA used was shown to produce 50–100 PFU when transfected either alone or in the presence of 0.3–30 ng of an unrelated RNA obtained upon transcription of pGEM plasmid (RGpct, Promega) (control assay). The percentages of inhibition were calculated as the differences between the number of PFU recovered in the control assay and that obtained from the RNA-transcript mixture, divided by the number of PFU in the control assay. No significant differences were noticed in the PFU recovered from transfections that included viral RNA alone or in combination with control RNA at 1:20 to 1:2000 molar ratio.

The inhibitory effects obtained upon cotransfection of viral RNA and the different transcripts are shown in Fig. 1A. In all cases, a reduction in the percentage of inhibition was observed, this reduction being higher for transcript combinations 5′AS-3′AS and 5′AS-3′S.

We then studied the inhibition of the PFU produced in BHK-21 cells by the individual transcripts or the combination of 5′AS with either 3′AS or 3′S, under renaturing conditions. As shown in Fig. 1B, no significant inhibition was observed with RNA 5′S, as previously reported (Gutiérrez et al., 1994). Among the interfering RNAs, the highest inhibition values corresponded to 5′AS, (above 60%). All inhibitions were dose-dependent; they were observed at an RNA/transcript molar ratio higher than 1:20. The percentage of inhibition increased when combinations of RNAs 5′AS-3′AS and 5′AS-3′S were used. In both cases, inhibitions higher than 60% were obtained at a molar ratio of 1:200, and the inhibitions were up to 90% at a ratio of 1:2000. The use of an RNA/transcript ratio of 1:20 000 did not result in a further increase of the PFU inhibition (data not shown). No toxic effect was observed on the cell monolayers transfected with any of the different RNA/transcript combinations. For all the transcripts, the number of PFU recovered in the assay did not increase when the monolayers were stained 48 h (instead of 24 h) post-infection, supporting a permanent inhibition of viral RNA infectivity The increase of the percentage of inhibition when previous annealing was favoured is consistent with the finding that the inhibitions observed depend on efficient hybridisation between viral RNA and transcripts.

The inhibition induced by the FMDV transcripts was specific, since no reduction in the PFU was observed after cotransfection of any of the interfering molecules studied with RNA from swine vesicular disease virus (SVDV) strain UK (Seechurn et al., 1990) (Fig. 1C). SVDV is a picornavirus belonging to the enterovirus genus that produces a disease clinically indistinguishable from FMD (Knowles and Sellers, 1994). In this case, cotransfections were performed in IBRS-2 cells, as SVDV does not productively infect BHK-21 cells. In IBRS-2 cells, derived from swine epithelia, the FMDV transcripts inhibited PFU formation to a similar level as observed in BHK-21, hamster kidney cells. Among the individual RNAs, 5′AS was the one that induced higher inhibition (80%), and values close to 90% were found with the combinations 5′AS-3′S and 5′AS-3′AS. (Fig. 1C).

The capacity of the FMDV transcripts to inhibit the infectivity of viral RNAs from serotypes other than C was then investigated. For this purpose, RNAs from isolates of serotype O, O1Kb (Forss et al., 1984) and serotype A, A5Ww (Beck et al., 1983a), were used in cotransfection experiments of BHK-21 cells with the different transcripts, under conditions that favoured the previous annealing. The inhibitions observed in the infectivity of type O and type A RNAs were close to those found for type C RNA (Fig. 2A,B). No significant PFU reduction was noticed with RNA 5′S, and the highest inhibition was produced by 5′AS (60% for type O and 50% for type A). Likewise, the combination of 5′AS with either 3′S or 3′AS made inhibition higher than 60%.

The magnitude of these inhibitions was compared with the nucleotide homologies shown by the viruses studied at the RNA regions corresponding to the transcripts used. Therefore, we determined the sequences of A5Ww RNA that span the transcripts 5′S and 3′S (sequences de-

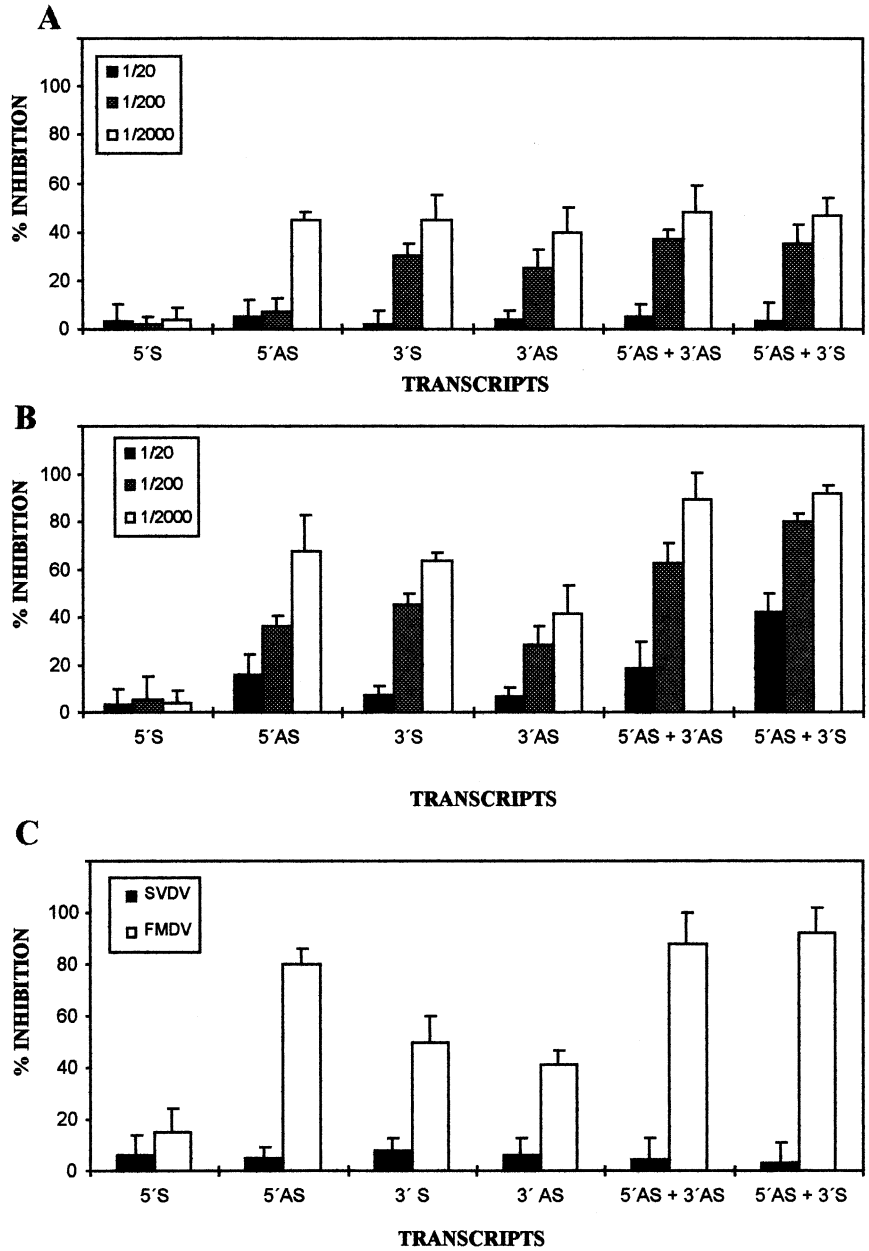

Fig. 1. Inhibition of PFU in cells cotransfected with viral RNA and FMDV transcripts. Infectious FMDV RNAs were phenol extracted and ethanol precipitated from viral preparations purified through a cesium chloride gradient (Sáiz et al., 1989). SVDV RNA was extracted from virions purified in a similar manner through a 10–35% (w/w) sucrose gradient (E. Brocci, personal communication). Infectious RNA, producing 50–100 PFU, was used to cotransfect cells in the presence of different molar ratios of type C FMDV subgenomic RNAs (see text for details). (A) Percentage of inhibition of PFU in BHK-21 cells cotransfected with type C FMDV RNA and *sense* and *antisense* transcripts at the indicated molar ratios. RNA mixtures were cotransfected in the absence of previous RNA-transcript annealing. (B) Percentage of inhibition of PFU in BHK-21 cells cotransfected with RNA-transcript mixtures that were denatured and then incubated at room temperature for 15 min, prior to transfection. (C) Percentage of inhibition of PFU in IBRS-2 cells cotransfected, as in (B), with SVDV RNA and FMDV transcripts at a molar ratio of 1:2000. The inhibition obtained in parallel with type C FMDV RNA and the transcripts is included for comparison. Data correspond to the mean values of three independent experiments and their standard deviations.

posited in the EMBL Data Bank, accession numbers AJ010870 and AJ010871, respectively). This analysis was performed from RT-PCR amplified products, obtained using oligonucleotides spanning nt positions 895–917 and 7588–7615, and complementary to positions 1185–1207 and 8103–8122 (according to Martínez-Salas et al., 1985, 1993). DNA sequences were obtained using the fmol sequencing kit (Promega), as described previously (Sáiz et al., 1993). The alignment of the sequence of transcript 5′AS with those corresponding to RNA of isolates O1Kb (Forss et al., 1984) and A5Ww revealed homologies of 84 and 79%, respectively (data not shown). These homologies compared favorably with the inhibitions produced by 5′AS in RNAs of type O ($59 \pm 5\%$) and type A ($60 \pm 6.5\%$). The lack of inhibition of SVDV RNA infectivity was also in agreement with the low homology, 20%, shown by its RNA in this region with the FMDV transcript (data not shown). A similar correlation was observed in the comparison of the sequences corresponding to transcripts 3′S and 3′AS. In this case, the nucleotide homologies were of 91% for both type O

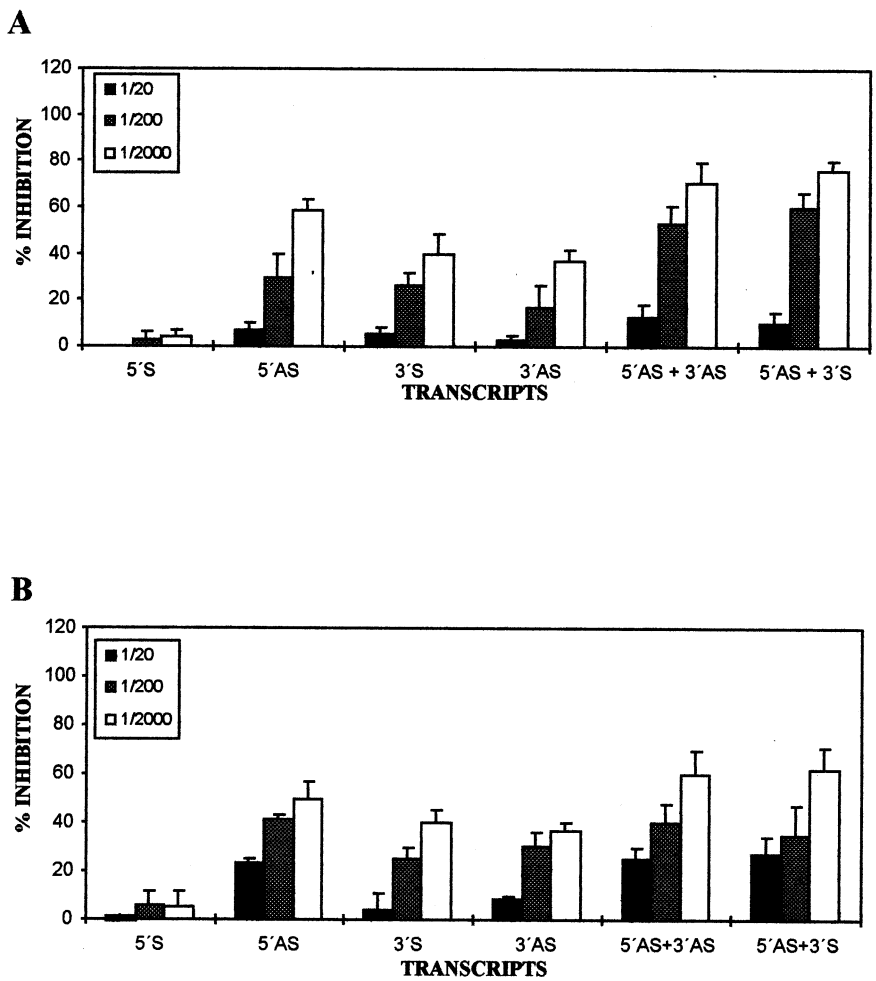

Fig. 2. Inhibition of PFU in BHK-21 cells cotransfected with heterotypic FMDV RNAs and viral transcripts. Experiments were carried out as detailed in the legend to Fig. 1B. (A) type O1Kb RNA. (B) type A5Ww RNA. Data correspond to the mean values of three independent experiments and their standard deviations.

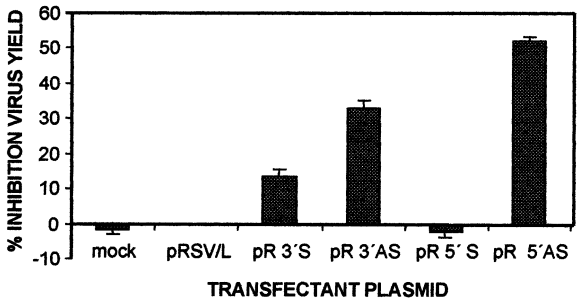

Fig. 3. Inhibition of virus yield in BHK-21 cells transiently expressing different FMDV transcripts. Cells were independently transfected with each of the plasmids indicated using lipofectamine plus (Gibco, BRL), as described in the text. After 24 h, cells were infected with C-S8 FMDV at a m.o.i. of 0.1. The number of PFU in the supernatants were determined at 24 h post-infection, using triplicates for each dilution. The percentage of inhibition of the virus yield was calculated from the mean of three independent determinations with respect to the PFU recovered from cells transfected with plasmid pRSV/L, as described in the text. A student's t analysis of the PFU means obtained with cells transfected with plasmids pR5′AS, pR3′AS and pR3′S indicated that these values were significantly different ($P = 0.001$) from that obtained with cells transfected with plasmid pRSV/L.

and type A (data not shown), which was consistent with the inhibition observed, 38–40%, for each of the two serotypes. The low level of homology between FMDV type C and SVDV, 27% (data not shown), correlated with the absence of significant inhibition in SVDV RNA infectivity.

Efficient inhibition of viruses lacking a nuclear stage is hard to accomplish because of the cytoplasm compartmentalisation (Helené and Toulmé, 1990; Gutiérrez et al., 1993). In many cases, *antisense* molecules are thought to specifically hybridize to their viral target sequences and thus interfere with viral functions. *Sense* sequences may cause an indirect inhibition of gene expression by their interaction or competition with proteins involved in nucleic acid metabolism. The lack of inhibition of SVDV, as well as of encephalomyocarditis virus (Gutiérrez et al., 1994), argues against an indirect effect as responsible for the FMDV inhibition observed. Due to the close relationship among these three picornaviruses, it is unexpected that effects such as competition for cellular or viral components required for viral infection will affect the infectivity of only one of the viruses studied and not the others. Therefore, we favor the hypothesis of transcript-RNA hybrid formation as the key factor responsible for viral interference. We previously reported that 5′AS, but not 3′S or 3′AS RNAs, inhibit in vitro translation of FMDV RNA (Gutiérrez et al., 1994). Thus, the inhibition mediated by 5′AS is likely to be contributed by a blockade of viral translation initiation. Conversely, 3′S and 3′AS were both predicted to distort highly ordered structural motifs present at the 3′NCR. Therewith consistent, the secondary structure of several picornavirus 3′NCRs has been shown to play an important role in viral replication (Rohll et al., 1995; Mellits et al., 1998).

The inhibitions exerted by combinations of FMDV transcripts 5′S-3′AS and 5′S-3′S depend, to some extent, on the efficient hybridisation between transcripts and viral RNA. An increase from 50 to 85% was observed in the inhibition of PFU when an annealing step between transcripts and viral RNA was performed. Thus, this increase of the interfering capacity is probably due to the ability of these transcripts to efficiently disrupt RNA structures (Graessmann and Graessmann, 1989) and to produce stable hybrids with viral RNA, even in the presence of intertypic levels of nucleotide variation.

A wide antigenic and/or genetic spectrum of protection is crucial in designing any anti FMDV strategy (Barteling and Woortmeyer, 1987), as this virus exhibits a considerable potential for virus variation (Domingo et al., 1992). Consequently, the control of FMD by vaccination requires serotype-specific vaccines formulated with virus strains that have to be closely antigenically related to the viruses circulating in the field. In addition, selection of virus variants from the heterogeneous FMDV populations may constitute a drawback for the efficacy of new synthetic vaccines (Domingo and Holland, 1992; Taboga et al., 1997). We report here that combinations of interfering molecules containing sequences from the 3′ and 5′NCR of FMDV produced high levels of inhibition of viral infectivity in BHK-21 cells. Interestingly, the inhibitions observed for viral RNA belonging to other serotypes were consider-

ably high, reaching percent of inhibition above 60%. Furthermore, similar inhibitions have been obtained in the cell line IBRS-2, derived from epithelium of swine, a natural FMDV host. Since epithelial cells have been reported to be the main target during the replication of this virus in natural infections (Yilma, 1980), our results reinforce the potential of viral transcripts as anti-FMD tools. The results described above were obtained using an experimental approach in which the viral RNA and the different transcripts were allowed to interact prior to its delivery in the cell, and therefore do not ensure that such interaction may occur during a natural infection. Favoring this possibility, previous findings indicated that cells transfected with 5′AS transcript showed a significant reduction of plaque-forming ability during the course of an experimental FMDV infection (Gutiérrez et al., 1994). Along these lines, we studied the effect of the transient expression of the interfering transcripts, under the control of the Rous sarcoma virus (RSV) LTR promotor, on the virus yield recovered upon FMDV infection of BHK-21 cells. For this purpose, the cDNA sequences corresponding to transcripts 5′S, 5′AS, 3′S and 3′AS were subcloned in the *Hin*dIII, *Sma*I sites of plasmid pRSV/L (de Wet et al., 1987), substituting the luciferase ORF by that of the desired transcript, as will be described in detail elsewhere. FMDV transcripts were detected in BHK-21 upon 24 h of transfection with each of the resulting plamids (pR5′S, pR5′AS, pR3′S and pR3′AS) (La Rosas et al., manuscript in preparation). Consistently with the inhibitions observed in the cotransfection experiments in the absence of previous RNA/transcript annealing (Fig. 1A), cells infected following 24 h of their transfection with plasmid pR5′AS showed a significant inhibition, around 50%, of the virus yield, when compared with that observed in cells transfected with control plasmid pRSV/L expressing the luciferase gene (Fig. 3). Transfection of cells with plasmid pR5′S did not reduce the viral yield. Plasmids pR3′S and pR3′AS induced significant inhibitions around 15 and 30%, respectively, that were lower than those observed in the cotransfection experiments. These differences may be due, among other possibilities, to the presence, in our experimental conditions, of lower intracellular concentrations of these transcripts or to a decreased accessibility of their viral RNA targets during a natural infection. Implementation of *antisense* strategies requires efficient delivery systems. Therefore, to gain useful information on this aspect of *antisense* performance, we are now preparing transformed culture cells, which constitutively express the interfering molecules, as a further step to confirm the therapeutic potential inhibitory effects exerted by FMDV transcripts. The achievement of significant levels of virus inhibition using this strategy may open the possibility of producing transgenic animals expressing these interfering molecules, as a therapeutic alternative against FMD.

Acknowledgements

We thank M. Sáiz for critical reading of the manuscript. We also thank C. Gómez-Tejedor for kindly providing us with an SVDV isolate. This work was supported by CICYT (grant BIO96-0400-C02-01), CAM (grants 062/1996 and 08.2/0024/1997), DGES (grant PM95.0001) and by Fundación Ramón Areces.

References

Agrawal, S., 1992. Antisense oligonucleotides as antiviral agents. TIBTECH 10, 152–158.

Arlinghaus, R.B., Polatnick, J., 1969. The isolation of two enzyme-ribonucleic acid complexes involved in the synthesis of foot-and-mouth disease virus ribonucleic acid. Proc. Natl. Acad. Sci. USA 26, 821–829.

Bachrach, H.L., 1968. Foot-and-mouth disease virus. Annu. Rev. Microbiol. 22, 201–244.

Barteling, S.J., Vreeswijk, J., 1991. Developments in foot-and-mouth disease vaccines. Vaccine 9, 75–88.

Barteling, S.J., Woortmeyer, R., 1987. Multiple variants in foot-and-mouth disease virus (FMDV) populations: the Achilles heel for peptide and rec. DNA vaccines? Dev. Biol. Stand. 66, 511–520.

Beck, E., Feil, G., Strohmaier, K., 1983a. The molecular basis of antigenic variation of foot-and-mouth disease virus. EMBO J. 2, 555–559.

Beck, E., Forss, S., Strebel, K., Cattaneo, R., Feil, G., 1983b. Structure of the FMDV translation initiation site and of the structural proteins. Nucleic Acids Res. 11, 7873–7885.

Belsham, G.J., Brangwyn, J.K., 1990. A region of the 5′ noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. J. Virol. 64, 5389–5395.

Bischofberger, N., Wagner, R.W., 1992. Antisense approaches to antiviral agents. Semin. Virol. 3, 57–66.

Cohen, J.S., 1991. Antisense oligodeoxynucleotides as antiviral agents. Antiviral Res. 16, 121–133.

de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R., Subramani, S., 1987. Firefly luciferase yen: structure and expression in mammalian cells. Mol. Cell. Biol. 7, 725–737.

Domingo, E., Escarmís, C., Martínez, M.A., Martínez-Salas, E., Mateu, M.G., 1992. Foot-and-mouth disease virus populations are quasispecies. Curr. Top. Microbiol. Immunol. 176, 33–47.

Domingo, E., Holland, J.J., 1992. Complications of the RNA heterogeneity for the engineering of virus vaccines and antiviral agents. In: Setlow, J.K. (Ed.), Genetic Engineering, Principles and Methods, vol 14. Plenum, New York, pp. 13–32.

Domingo, E., Mateu, G., Martínez, M.A., Dopazo, J., Moya, A., Sobrino, F., 1990. Genetic variability and antigenic diversity of foot- and-mouth disease virus. In: Kurstak, E., Marusyk, R.G., Murphy, S.A., Van Regenmortel, M.H.V. (Eds.), Applied Virology Research, Virus Variation and Epidemiology, vol. II. Plenum, New York, pp. 233–266.

Forss, S., Strebel, K., Beck, E., Schaller, H., 1984. Nucleotide sequence and genome organization of FMDV. Nucleic Acids Res. 12, 6587–6601.

Graessmann, M., Graessmann, A., 1989. The biological activity of early SV40 antisense RNA and DNA molecules. Curr. Top. Microbiol. Immunol. 44, 97–103.

Gutiérrez, A., Martínez-Salas, E., Pintado, B., Sobrino, F., 1994. Specific inhibition of aphthovirus infection by RNAs transcribed from both 5′ and 3′ noncoding regions. J. Virol. 68, 7426–7432.

Gutiérrez, A., Rodríguez, A., Pintado, B., Sobrino, F., 1993. Transient inhibition of foot-and-mouth disease virus infection of BHK-21 cells by antisense oligonucleotides directed against the second functional initiator AUG. Antiviral Res. 22, 1–13.

Hanecak, R., Brown-Driver, V., Fox, M.C., Azad, R.F., Furusako, S., Nozaki, C., Ford, C., Sasmor, H., Anderson, K.P., 1996. Antisense oligonucleotide inhibition of hepatitis C virus gene expression in transformed hepatocytes. J. Virol. 70, 5203–5212.

Helené, C., Toulmé, J.J., 1990. Specific regulation of gene expression by antisense, sense and antisense nucleic acids. Biochim. Biophys. Acta 1049, 99–125.

Kim, S.G., Hatta, T., Tsukahara, S., Nakashima, H., Yamamoto, N., Shoji, Y., Takai, K., Takaku, H., 1995. Antiviral effect of phosphorothioate oligodeoxyribonucleotides complementary to human immunodeficiency virus. Bioorg. Med. Chem. 3, 49–54.

Knowles, N.J., Sellers, R.F., 1994. Swine vesicular disease. In: Beran, G.W. (Ed.), Handbook of Zoonoses, Section B: Viral, 2nd edn. CRC Press, Boca Raton, FL, pp. 437–444.

Kühn, R., Luz, N., Beck, E., 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. J. Virol. 64, 4625–4631.

Martínez-Salas, E., Domingo, E., 1995. Effect of expression of the aphthovirus protease 3C on viral infection and gene expression. Virology 212, 111–120.

Martínez-Salas, E., Ortín, J., Domingo, E., 1985. Sequence of the viral replicase gene from foot-and-mouth disease virus C1-Santa Pau (C-S8). Gene 35, 55–61.

Martínez-Salas, E., Sáiz, J.C., Dávila, M., Belsham, G.J., Domingo, E., 1993. A single nucleotide substitution in the internal ribosome entry site of foot-and mouth disease virus leads to enhanced cap-independent translation in vivo. J. Virol. 67, 3748–3755.

Mellits, K.H., Meredith, J.M., Rohll, J.B., Evans, D.J, Almond, J.W., 1998. Binding of a cellular factor to the 3′ untranslated region of the RNA genomes of entero and rhinoviruses plays a role in virus replication. J. Gen. Virol. 79, 1715–1723.

Mizutani, T., Hayashi, M., Maeda, A., Sasaki, N., Yamashita, T., Kasai, N., Namioka, S., 1993. Inhibition of mouse hepatitis virus multiplication by antisense oligonucleotide, antisense RNA, sense RNA and ribozyme. Adv. Exp. Med. Biol. 342, 129–135.

Pereira, H.G., 1981. Foot-and-mouth disease. In: Gibbs, E.P.J. (Ed.), Virus Diseases of Food Animals, vol. I. Academic Press, London, pp. 333–363.

Pilipenko, E.V., Blinov, V.H., Chernov, B.K., Dimitrieva, T.M., Agol, V.I., 1989. Conservation of secondary structure elements of the 5′ untranslated region of cardio and aphthovirus RNAs. Nucleic Acids Res. 17, 5701–5711.

Rohll, J.B., Moon, D.H., Evans, D.J., Almond, J.W., 1995. The 3′ untraslated region of picornavirus RNA: features required for efficient genome replication. J. Virol. 69, 7835–7844.

Rose, J.K., Buonocore, L., Whitt, M.A., 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. Biotechniques 10, 520–525.

Sáiz, J.C., González, M.J., Morgan, D.O., Card, J.L., Sobrino, F., Moore, D.M., 1989. Antigenic comparison of different foot-and-mouth disease virus types using monoclonal antibodies defining multiple neutralizing epitopes on FMDV A5 subtypes. Virus Res. 13, 45–60.

Sáiz, J.C., Sobrino, F., Dopazo, J., 1993. Molecular evolution of foot-and-mouth disease virus type O. J. Gen. Virol. 74, 2281–2285.

Sangar, D.V., 1979. The replication of picornavirus. Virology 45, 1–11.

Seechurn, P., Knowles, N.J., McCauley, J.W., 1990. The complete nucleotide sequence of a pathogenic swine vesicular disease virus. Virus Res. 16, 255–274.

Taboga, O., Tami, C., Carrillo, E., Núñez, J.I., Rodríguez, A., Sáiz, J.C., Blanco, E., Valero, M.L., Roig, X., Camarero, J.A., Andreu, D., Mateu, M.G., Giralt, E., Domingo, E.,

Sobrino, F., Palma, E.L., 1997. A large scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of scape mutants. J. Virol. 71, 2606–2614.

Todd, S., Nguyen, J.H., Semler, B.L., 1995. RNA-protein interactions directed by the 3′ end of human rhinovirus genomic RNA. J. Virol. 69, 3605–3614.

Todd, S., Semler, B.L., 1996. Structure-infectivity analysis of the human rhinovirus genomic RNA 3′ non-coding region. Nucleic Acids Res. 24, 2133–2142.

Yao, Z., Zhou, Y., Feng, X., Chen, C., Guo, J., 1996. In vivo inhibition of hepatitis B viral gene expression by antisense phosphorothioate oligodeoxynucleotides in athymic nude mice. J. Viral Hepat. 3, 19–22.

Yilma, T., 1980. Morphogenesis of vesiculation in foot-and-mouth disease virus. Am. J. Vet. Res. 41, 1537–1542.