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Transient inhibition of foot-and-mouth disease virus infection of BHK-21 cells by antisense oligonucleotides directed against the second functional initiator AUG

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Summary

The antiviral activity of antisense oligonucleotides corresponding to different regions of foot-and-mouth disease virus (FMDV) genome has been assessed in BHK-21 cells. The locations of the oligonucleotides used were: (i) two regions within the internal ribosome entry site (IRES), involved in the regulation of the translation initiation of the viral polyprotein; (ii) each of the two functional initiator AUGs; (iii) an internal sequence of P2A gene; and (iv) a region at the 3′ end non-coding region. Cytoplasmic microinjection of oligodeoxyribonucleotides and oligoribonucleotides complementary to the second AUG resulted in a transient inhibition of viral VP1 expression in infected cells. Significant inhibitions, ranging from 35 to 52%, were obtained at 5 h post-infection using oligonucleotide concentrations of 125 μM and higher. The extent and duration of this inhibition seemed to be mediated by both a rapid transport to the nucleus and the short half-life of the oligonucleotide. This inhibition of FMDV protein synthesis was correlated with a reduction of virus yield of about 50%, as observed after the addition to the cell culture of an oligodeoxyribonucleotide phosphorothioate complementary to the second AUG.

FMDV; Antisense oligonucleotides; Microinjection; Translation inhibition

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Introduction

The use of antisense nucleic acids to block the biological activity of viral RNAs offers exciting prospects for the molecular analysis of the viral cycle, and constitutes a promising approach for the treatment of a variety of viral infections as well as for obtaining transgenic animals with modified susceptibilities to viral infections (reviewed in Cohen, 1991; Bischofberger and Wagner, 1992 and Agrawal, 1992). These molecules can interfere with transcription, message processing, RNA stability or translation (reviewed in Hélene and Toulmé, 1990). Two approaches have been described to interfere specifically with viral gene expression: (i) in situ production of antisense RNA from recombinant vectors, and (ii) introduction of exogenous oligonucleotides (ODNs) complementary to the coding strand, by microinjection or transfection. Several oligonucleotide derivatives have been used to improve the uptake and the intracellular stability of ODNs. The ability of phosphorothioate analogs (S-ODNs) to inhibit viral infection have been demonstrated for herpes simplex virus (Smith et al., 1986; Kulka et al., 1989), human immunodeficiency virus (Agrawal, 1989; Matsukura, 1987), human papillomavirus (Storey, 1991) and influenza virus (Leiter et al., 1990).

Foot-and-mouth disease (FMD) is a major animal health problem worldwide (Bachrach, 1968; Pereira, 1981). Efficiency of immunization campaigns with chemically inactivated virus (Barleling and Vreeswijk, 1992) is still a problem 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 described (reviewed in Domingo et al., 1990). FMD virus (FMDV) belongs to the Picornaviridae family and its genome consists of a positive RNA of approx. 8500 nucleotides, that encodes a unique polyprotein. Replication and translation (reviewed in Sangar, 1979) occur in the cytoplasm of infected cells (Arlinghaus et al., 1969).

The CAP-independent initiation of the FMDV polyprotein synthesis (reviewed in Jackson et al., 1990) occurs at two AUG codons (Beck et al., 1983), following ribosome recognition of the adjacent internal ribosome entry site region (IRES) (Belsham and Brangwyng, 1990; Khun et al., 1990). The IRES element is composed of about 450 bases from the 5′ non-coding region of FMDV RNA, which display a high degree of secondary structure (Pilipenko et al., 1989). Mutations in two separate regions within the IRES element have been shown to diminish the efficiency of the in vitro FMDV RNA translation (Kuhn et al., 1990).

As a first step in the definition of FMDV RNA regions that could act as target for viral cycle inhibition by antisense molecules, we have explored the antiviral activity in BHK-21 cells of different synthetic ODNs by cytoplasmic microinjection and addition to the culture medium. The ODNs used were complementary to regions potentially involved in regulatory processes, such as initiation of translation or replication, as well as internal regions within FMDV polyprotein. Oligonucleotides complementary to the second functional AUG

codon resulted in a transient inhibition of FMDV multiplication in BHK-21 cells.

Materials and Methods

Oligonucleotides

Oligodeoxyribonucleotides (O-ODNs), oligoribonucleotides (O-ORNs), and oligodeoxyribonucleotides phosphorothioate (S-ODNs), were supplied by Isogen (Bioscience). O-ODNs labelled with fluorescein isothiocyanate (FITC) were supplied by Bio Synthesis (Madrid, Spain). Before use, O-ORNs were treated to remove the protective group, as specified by the manufactures, and then ethanol precipitated. Oligonucleotides were dissolved in sterile water and their concentrations were estimated by measuring the A_{260}. In the case of FITC oligonucleotides, free fluorescent dye was removed by three serial ethanol precipitations.

Cells and viruses

BHK-21 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cultures were incubated at 37°C in presence of 5% CO_2. Cells were infected with FMDV strain O1 Kaufbeuren (O1Kb).

FMDV infectious yield assay

Fresh monolayers of BHK-21 cells (5×10^4 cells per well), grown in 24 well tissue culture plates, were covered with 1 ml of culture medium containing the appropriate oligonucleotide. In each case, three wells per ODN were used. As control, wells were kept without addition of ODNs. At 2 h after incubation, the medium was removed and 50 µl of FMDV were added into each well at multiplicity of infection (m.o.i.) of about 0.001 plaque forming unit/cell (p.f.u./cell). Virus and cells were incubated for 1 h at 37°C. Following viral adsorption, monolayers were washed with DMEM and cells were incubated with 1 ml of medium supplemented with 0.2% fetal calf serum and the corresponding ODN. Samples of medium were taken at various times after infection. All replicates were harvested and frozen at −70°C. Viral quantitation was performed by plaque assay on BHK-21 cells. For each ODN relative antiviral activity, expressed as the percentage of viral yield inhibition (PI), was calculated as follows:

$$PI = \frac{\text{pfu/ml of virus in ODNs untreated sample} - \text{pfu/ml of virus in ODNs treated sample}}{\text{pfu/ml of virus in ODNs untreated sample}} \times 100$$

Inhibition of FMDV VP1 protein synthesis assay

BHK-21 cells grown for 2 days on glass coverslips (9 × 9 mm) were used. Before microinjection, growth medium was supplemented with 20 mM Hepes

(Gibco, Glasgow, UK). Dilutions of ODNs (dissolved in water) were supplemented with 0.5% of the fluorescence marker FITC-dextran, MW 150 000. Microinjection was performed as described by Graessmann and Graessmann (1986). Cells were microinjected ($3–5 \times 10^{-14}$ l. per cell) with a Zeiss manual micromanipulator, using a semiautomatic microinjector (Eppendorf microinjector 5242, Hamburg, Germany) under phase-contrast microscopy. An injection of 50–100 hPa/0.5 s was used. Cell survival was around 90%.

In each experiment, 100 cells were injected in triplicate. Immediately after microinjection, cells were infected with FMDV (m.o.i. = 2 pfu/cell) as described above. At different times post-injection, cells were fixed with 3.5% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 15 min at 37°C, washed two times with PBS, covered with 10 mM glycine (2×5 min) and washed again with PBS. Finally cells were treated with 0.1% Triton X-100 in PBS, and incubated for 60 min with rabbit anti-FMDV VP1 serum (diluted 1:300) (Strebel et al., 1986). Coverslips were incubated 45 min with Texas-red coupled with donkey anti-rabbit serum (diluted 1:30) (Amersham, UK), and washed extensively with PBS. The coverslips were mounted in Moviol 4.88 (Hoechst AG, Frankfurt, Germany) on microscope slides.

Every field of microinjected cells on the coverslip was photographed at different times post-infection using a fluorescence microscope, first with a fluorescein excitation filter and second with a Texas-red excitation filter. A control of cells injected only with FITC-dextran was included. The number of green cells (injected) and red cells (infected) was scored, and the percentage of inhibition (PI) of VP1 protein synthesis in injected cells was calculated as described above from the percentages of injected and control cells expressing VP1.

Intracellular distribution of microinjected ODNs

Different concentrations of a O-ODN-4 FITC derivative were used to microinject the cytoplasm of BHK-21 cells. The intracellular distribution of the fluorescence was followed either directly or after ethanol fixation.

Results

Selection of the ODNs used in this study

To assess the effect of antisense hybridization on the viral life-cycle, the following regions of FMDV O1Kb RNA were selected (Fig. 1). Target regions 1 and 2 corresponded to sequences located within the IRES element, at the 5′ non-coding region, for which mutations were reported to decrease the efficiency of the in vitro translation of the viral polyprotein (Kuhn et al., 1990); regions 3 and 4 spanned each of the two functional AUG of the FMDV polyprotein, respectively; region 5 was complementary to an internal region on

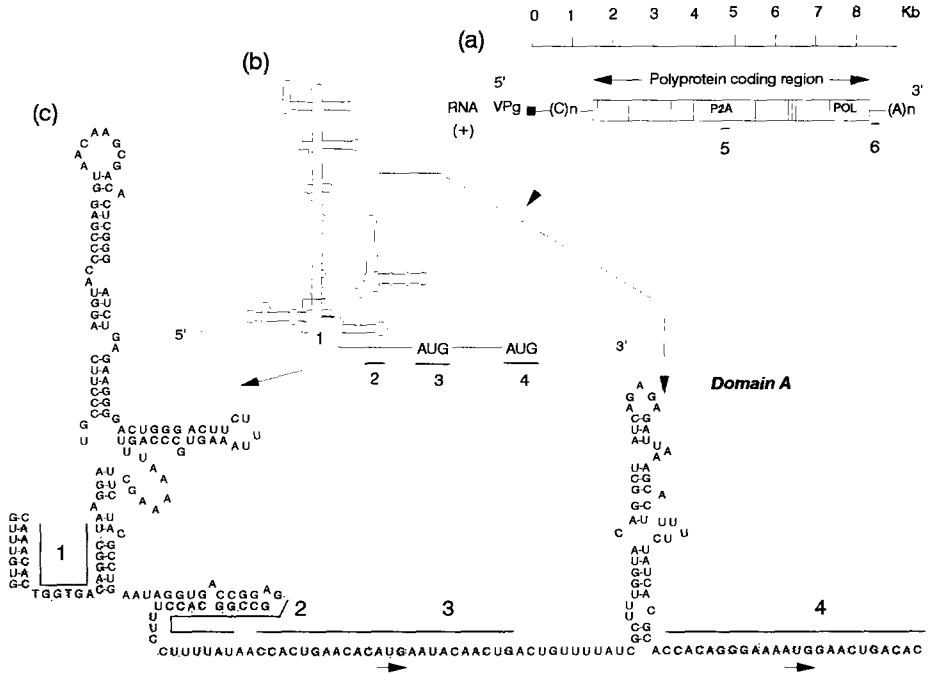

Fig. 1. Location of the O1Kb FMDV RNA regions selected as target for antisense inhibition. The sequences spanned by each of the selected regions are indicated and numbered from 1 to 6. (a) Representation of the FMDV genome including the genome-linked protein (VPg) at the 5′ end of the RNA, the poly cytidilic tract (C)n, the coding region, and the poly adenylic tail (A)n. (b) Detail of the putative secondary structure of the IRES element of FMDV, according to the model of Pilipenko et al., 1989. (c) Detail of the nucleotide sequence from the 3′ end of the IRES element to the second translation initiation codon (AUG). The secondary structure shown for Domain A was predicted using the computer program RNAFOLD from PC GENE 6.5.

TABLE 1

Description of the antisense sequences selected and of the ODNs used in this study

	Target sites[a]	Antisense sequence	Oligonucleotides used[b]		
			O-ODN	O-ORN	S-ODN
1-	636–654	5′ AGCCTGTCACCAGTGTTT 3′	+		
2-	767–788	5′ ATAAAAGGAAAGGTGCCGGCC 3′	+		
3-	794–818	5′ CAGTTGTATTCATGTGTTCAGTGG 3′	+	+	+
4-	878–902	5′ GTGTCAGTTCCATTTTCCCTGTGG 3′	+	+	+
5-	3648–3669	5′ GAAGGGCCCAGGGTTGGACTC 3′	+		
6-	7803–7824	5′ AGCCCGGCTGTCGTGGCCTCT 3′	+		
7-	Control[c]	5′ TCGTCTTGTGCGCCTTAGTCAGTT 3′	+		+

[a]Numbering corresponds to that of O1kb RNA (Forss et al., 1984). [b]Plus denotes those ODNs used in this study. O-ORN contain U instead of T. [c]This ODN was synthesized with identical base composition, although a different sequence than ODN-4. O-ODN: oligodeoxyribonucleotide; O-ORN: oligoribonucleotide; and S-ODN: oligodeoxyribonucleotide phosphorothioate.

P2A gen (Beck and Strohmaier, 1987); and region 6 was located at the beginning of the 3′ non-coding region.

Table 1 shows the sequences and locations of the different ODNs and derivatives used in this study.

Effect of microinjection of ODNs on the synthesis of FMDV proteins

As a first screening of antiviral activity, we determined the effect of ODN microinjection in FMDV protein synthesis.

Concentrations of ODNs (20 to 250 μM) were microinjected into the cytoplasm of BHK-21 cells. After infection with FMDV O1Kb, the expression of VP1 protein at 5 h post-infection (h.p.i.) was used as an assay of viral protein

TABLE 2

Inhibition of FMDV VP1 expression in cells microinjected with antisense oligonucleotides

OLIGO	Conc.[a] (μM)	% of cells expressing VP1[b]		PI[c]	X^2
		Injected with ODNs	Control		
O-ODN-1	125	32.21	38.98	17.36	0.38
	250	35.22	44.11	20.17	0.12
O-ODN-2	125	39.92	41.41	3.62	0.63
	250	24.57	26.08	5.78	0.79
O-ODN-3	125	26.69	29.45	9.20	0.50
	250	25.45	25.00	−1.80	0.95
O-ODN-4	125	12.70	26.65	52.34	<0.001
	250	19.33	29.75	35	<0.05
O-ODN-4 + 3	125	18.46	36.06	48.81	<0.001
	250	17.06	35.38	51.78	<0.001
O-ODN-4 + 1	125	21.16	37.42	43.47	<0.001
	250	17.77	32.80	47.43	<0.001
O-ODN-5	125	30.40	26.80	−0.13	0.70
	250	26.06	27.86	0.06	0.80
O-ODN-6	125	24.58	25.83	0.05	0.90
	250	25.19	27.00	0.06	0.70
O-ODN-7	125	35.00	31.06	−12.68	0.48
	250	31.54	30.25	4.26	0.70
O-ORN-3	125	22.79	26.69	14.61	0.39
	250	26.66	26.08	0.02	1
O-ORN-4	125	16.77	27.94	39.98	<0.05
	250	11.26	27.86	59.58	<0.01
O-ORN-4 + 3	125	16.98	35.11	51.58	<0.001
	250	12.76	31.06	58.92	<0.001
S-ODN-3	125	30.76	34.00	9.53	0.38
	250	37.50	38.02	1.37	0.70
S-ODN-4	125	21.43	42.14	49.15	<0.001
	250	21.31	48.63	56.18	<0.001
S-ODN-7	125	24.47	25.5	4.04	0.70
	250	25.90	27.86	7.04	0.60

[a]In coinjections, the concentration shown correspond to that of the two ODNs. [b]Cells were microinjected and scored as described in Materials and Methods. [c]Percentages of inhibition were calculated as described in Materials and Methods.

synthesis. In none of the cases an inhibition of FMDV translation was observed when using concentrations of 20 μM, which correspond to 7×10^5 molecules injected per cell. As shown in Table 2, a statistically significant percentage of inhibition (PI) of VP1 expression of about 35–52% was obtained for O-ODN-4 at concentrations of 125 and 250 μM. The sequence specificity of the antisense inhibition exerted by O-ODN-4 was confirmed by the lack of inhibition obtained when a random oligonucleotide, with an identical base composition but different sequence (O-ODN-7), was microinjected (Table 2). The sequence spanned by ODN-4 was complementary to the second functional AUG of the FMDV polyprotein. The remaining PIs, including those corresponding to O-ODN-1 (17%), were not statistically significant. Co-microinjection of O-ODN-4 with either O-ODN-1 or -3 resulted in PIs within the same range of those obtained with O-ODN-4 alone.

Microinjection in BHK-21 cells of O-ORNs 4, and 3, as well as S-ODNs 3, 4 and 7, resulted in PIs similar to those obtained for the corresponding O-ODNs (see Table 2).

When VP1 protein synthesis was assessed at 7 h.p.i. no significant difference was found between those cells microinjected with each of the ODNs and the control cells (results not shown). Thus, our results indicate that microinjection of oligonucleotides spanning the second functional AUG into the cytoplasm of BHK-21 cells produced a transient inhibition of FMDV protein synthesis.

Intracellular distribution of microinjected O-ODNs

Different concentrations (20, 125 and 250 μM) of an FITC derivative of O-ODN-4 were used to microinject the cytoplasm of BHK-21 cells. The intracellular distribution of the fluorescence was determined at different times post-infection (p.i.). As early as 1 min p.i. the fluorescence present in the cells injected with a concentration of 20 μM and fixed with ethanol was only detected in the nucleus. The direct observation of the microinjected cells confirmed the rapid transport of the oligonucleotide to this cell compartment and revealed that the fluorescence remained at 3 h.p.i. (Fig. 2) and 5 h.p.i., but was not detectable at 7 h.p.i. (data not shown). Nuclear uptake of O-ODN seems to be a saturable process since cells microinjected with concentrations of 125 and 250 μM showed cytoplasmic fluorescence at 15 min, 3 h.p.i. (Fig. 2), and 5 h.p.i. (data not shown).

Effect of the addition of ODNs to the cell culture medium on the production of infective FMDV

To explore if the inhibition observed in VP1 protein synthesis could be correlated with a decrease in the production of infective virus, we studied the effect of the addition of ODNs to the cell culture medium on the infective particle formation in BHK-21 cells.

We first tested the ability of unmodified O-ODNs (1 to 7) to interfere with the production of infective FMDV when present in the culture medium (see Materials and Methods). Addition of O-ODNs at concentrations that ranged

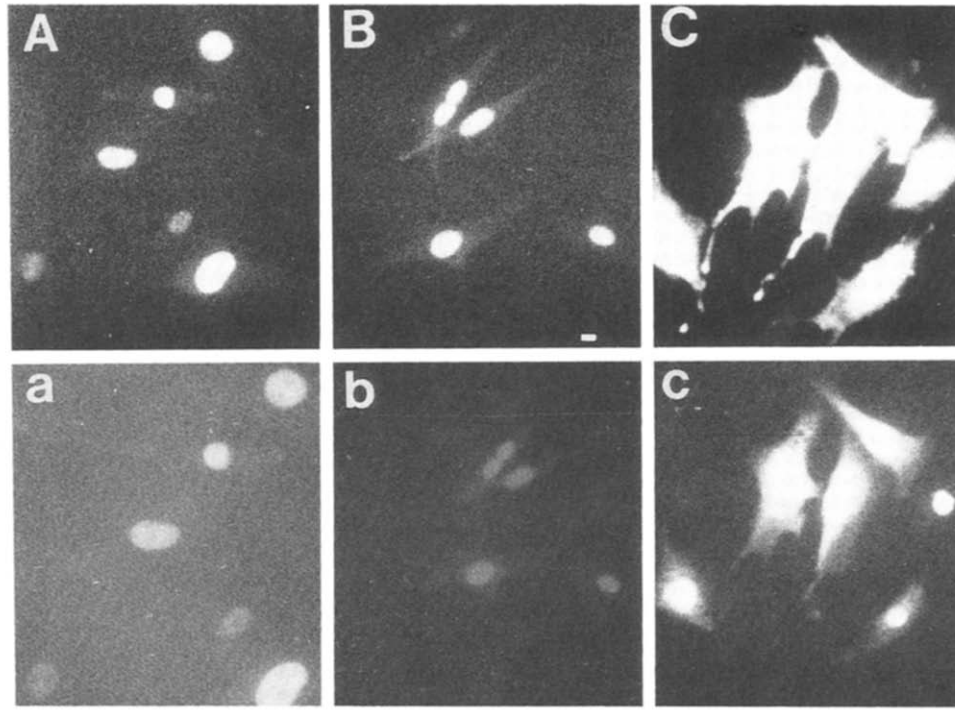

Fig. 2. Intracellular distribution of FITC-conjugated O-ODN-4 injected in the cytoplasm of BHK-21 cells. Fluorescence photomicrographs of the same group of living cells at 15 min (capital letter) or 3 h (small letter) after microinjection (see Materials and Methods for details). A: Cells microinjected with 20 μM of FITC labelled O-ODN-4, B: 125 μM and C: 250 μM.

from 1 to 100 μM did not result in any significant reduction of the p.f.u. recovered from the culture medium (Fig. 3A).

When phosphorothioate derivatives S-ODN-3 and S-ODN-4, were used in this assay, and in agreement with the previous results, S-ODN-4 but not S-ODN-3 reduced the titer of p.f.u. recovered. The sequence specificity of the antisense inhibition of p.f.u. formation by S-ODN-4 was confirmed by the lack of inhibition obtained when a control oligonucleotide (S-ODN-7) was used. The result of a typical experiment is shown in Fig. 3A. S-ODN-4 was active at concentrations greater than 20 μM, and the inhibition observed decreased with time (Fig. 3B). No BHK-21 cytotoxic effect was observed as monitorized by phase-contrast microscopy and cell counting.

Our results indicate that the inclusion of S-ODN-4 in the culture medium induced a reduction in FMDV infective particle formation, the compound still being active at 19 h.p.i.

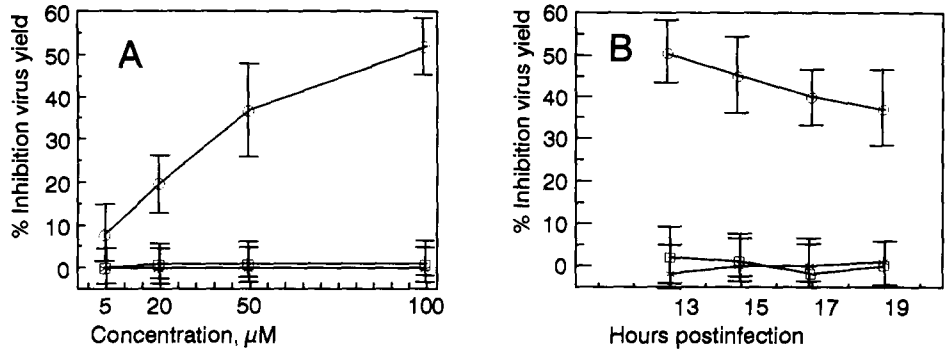

Fig. 3. Effect on the FMDV yield of the addition of O-ODNs and S-ODNs to the cell culture of BHK-21 cells. Percentage inhibition of virus yield (p.f.u. formation) was determined as described in Materials and Methods. A. Dose-effect of different ODNs on the inhibition of FMDV p.f.u. formation at 13 h post-infection. B. Kinetic of the FMDV yield inhibition by a concentration of 100 μM of different ODNs. Circle, S-ODN-4; square, S-ODN-3 and -7; and asterisk, rest of O-ODNs.

Discussion

Efficient inhibition of viral multiplication by antisense nucleic acids has been described to be mediated by molecules complementary to the initiation translation codon (Melton, 1985; Agris et al., 1986) as well as to other genomic regions (reviewed in Bischofberger and Wagner, 1992). Nevertheless, there is not a general rule for the prediction of effectivity of selected antisense regions as inhibitors of viral multiplication in infected cells (Bischofberger and Wagner, 1992). In the present report we have assessed the ability of oligonucleotides targeted to regions of viral RNA involved in viral reproductive processes to inhibit multiplication of FMDV in BHK-21 cells. Besides two internal ODNs, the regions selected included each of the two functional AUG as well as another two regions involved in the regulation of the translation initiation. We first assayed the antiviral activity of ODNs after microinjection, since this strategy allowed us to select effective ODNs using low amounts of these molecules. Selected ODNs were confirmed and further analyzed by external addition to the cell culture medium. The results obtained indicate that only ODN-4, which is complementary to the second AUG of the FMDV polyprotein, induces a transient inhibition of both viral protein synthesis and p.f.u. formation. The sequence specificity of these antisense effect has been confirmed by the lack of inhibition induced by either non-effective ODNs complementary to other regions of FMDV or random ODNs.

Efficacy of inhibition may be improved by using more than one antisense ODN. However, co-microinjection of O-ODN-4 with either O-ODNs 1 or 3, although resulting in more significant values, did not substantially increase the percentage of the viral inhibition. Similar results have been obtained when the corresponding antisense RNA molecules (O-ORNs), reported as effective translation inhibitors in another system (Strickland et al., 1988) were used. The

results obtained suggest that the inhibition observed is exerted at the initiation of FMDV translation. This step appears to be regulated by the IRES element. It has been described that ribosomes bind most likely to an internal entry site at the 3′ end of IRES, from where they are translocated to the authentic initiator codon (Pelletier and Sonenberg, 1989; Belsham, 1992). The ability of ODN-4 but not ODN 3 to block FMDV multiplication may be explained by the observation made by Belsham (1992) that in absence of the IRES, both initiation sites are efficiently used, while the initiation preferentially occurs at the second site when the IRES element is present. ODNs 1 and 2 are complementary to regions that seems to be highly structured, and that could be a reason of their inability to inhibit viral translation.

As described in other systems (Kim and Wold, 1985), the effectiveness of antisense inhibition of FMDV translation requires a considerable excess of antisense ODN versus FMDV RNA molecules. Inhibition is only observed when approx. 5×10^6 molecules (corresponding to those injected using a concentration of 125 μM) or more are microinjected. In addition, and probably due to the cytoplasmic location of FMDV life-cycle, antisense inhibition seems to largely depend on the stability and distribution in the cell of the antisense molecule. In fact, a correlation between the presence of ODN-4 in the cytoplasm and the inhibition of FMDV multiplication has been observed. The study of the intracellular distribution of O-ODN-4 shows that it is rapidly and preferentially exported to the nucleus (Fig. 2). This observation is in agreement to that reported by Leonetti et al. (1991), which observed absence of competition for nuclear import in cells microinjected with labelled O-ODNs (30 μM) in the presence of a 10-fold excess of unlabelled O-ODN. In our experiment, only labelled O-ODN were microinjected, and the process was likely to be saturable since microinjection of 125 μM and higher concentrations of ODN-4 rendered detectable levels of this molecule in the cytoplasm, at a time (5 h.p.i.) at which inhibition of FMDV translation was observed. The rapid transport to the nucleus of the ODNs used, makes it difficult to estimate the antisense molecule actually present in the cytoplasm.

The potential of antisense molecules complementary to the second FMDV AUG to interfere with the viral multiplication has been confirmed by the transient reduction in the viral yield observed after addition of a phosphorothioate derivative of ODN-4 (S-ODN-4) to BHK-21 cells. This modification increases the resistance to degradation by nucleases (Agrawal, 1992) and has been widely used to improve the efficiency of antisense inhibitor. In this case, the unmodified O-ODN-4 failed to induce a reduction in the p.f.u. titre probably due to extracellular and/or intracellular degradation. The concentrations needed to obtain antisense inhibition were higher when S-ODN-4 was microinjected (125 μM) than when the compound was externally added (20 μM). This difference is probably increased by the low efficiency reported for cellular uptake of S-ODNs (Thierry and Dritschilo, 1992). As these authors have pointed out, the difference observed in our experiment can be due to the different distribution of S-ODNs in the cell, rapidly transported to the nucleus

when microinjected (Chin et al., 1990), while mainly present in cytoplasmic vesicles, at least during 24 h, when externally added (Thierry and Dritschilo, 1992). Although the inhibition of FMDV translation by microinjection of ODNs cannot be directly compared with that observed after addition of ODNs to the culture medium (Zamecnik et al., 1986; Matsukura et al., 1987), the main features of both inhibitions are similar, and they probably reflect a temporal decrease in the efficiency of FMDV translation.

The extent of the inhibitions observed is probably limited by the rapid exportation to the nucleus of the ODNs microinjected (Chin et al., 1990; Leonetti et al., 1991). The use of antisense molecules of higher size can constitute an alternative to improve the extent and duration of the antisense inhibition (Strickland et al., 1988). Therefore we are now exploring the inhibitions achieved with larger RNA antisense molecules.

In summary, a transient antisense inhibition of FMDV multiplication in infected cells has been obtained using exogenous O-ODNs, O-ORNs and S-ODNs complementary to the second functional AUG. This inhibition is sequence-specific, and it is likely to be exerted at the level of initiation of translation. At least in the case of microinjection, the extent and duration of this inhibition seems to be limited by the rapid exportation to the nucleus and the short half-life of the antisense molecules used.

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