# PARTIAL INHIBITION OF YELLOW FEVER VIRUS REPLICATION *IN VITRO* WITH DIFFERENT PHOSPHOROTHIOATE OLIGODEOXYRIBONUCLEOTIDES

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**Summary.** – Phosphorothioate oligodeoxyribonucleotides targeted to different regions of the viral genome were synthesized and used in two kinds of experiments testing their activity against yellow fever virus (YFV) replication in cultured cells. We found that oligonucleotides complementary to the 3'-end or to the coding region of the viral RNA were regularly active in plaque reduction assay, although with inconstant efficiency. Oligonucleotides targeted to the 5'-end or to the initiation codon region exhibited lesser activity. Homologous oligonucleotides targeted to dengue virus RNA had no detectable inhibitory activity against dengue virus replication. However, in YFV production reduction assay, a non-specific inhibitory activity of a random oligonucleotide was observed. Taken as a whole, our results indicate that flaviviruses present detectable but heterogeneous sensitivity to phosphorothioate inhibition. Possible explanations are discussed.

**Key words:** antisense phosphorothioate oligodeoxyribonucleotides; flaviviruses; inhibition of replication *in vitro*

## Introduction

Antisense oligodeoxyribonucleotides targeted to viral RNAs have been demonstrated during the last few years to inhibit *in vitro* replication of viruses of different families, including Rous sarcoma virus (Zamecnik *et al.*, 1978), vesicular stomatitis virus (Agris *et al.*, 1986), human immunodeficiency virus (Agrawal *et al.*, 1989) and herpes simplex virus (Smith *et al.*, 1986). Possible therapeutic applications have been proposed (Cohen, 1991; Crooke, 1992). The antisense activity relies on the ability of oligonucleotides to bind specifically to complementary sequences on the target RNA and interfere directly with various molecular mechanisms such as RNA splicing, translation or reverse transcription (Cazenave and Hélène, 1991; Boiziau *et al.*, 1994). Alternatively, for oligonucleotides with no direct inhibitory activity, RNase-H degradation of the annealed RNA, when this activity is present, may mediate indiscernible effects.

However, the use of oligonucleotides as research tools or therapeutic agents is greatly impaired by their rapid degradation in extracellular media and their poor cellular uptake, making it difficult to reach and maintain efficient concentrations in cells. The replacement of an oxygen atom with a sulphur on the phosphate group, first proposed by De Clercq *et al.* (1969), produces oligonucleotides with improved stability and cell penetration due to resistance to nuclease degradation and to facilitation of transport through cell membranes (Stein *et al.*, 1988; Temsamani *et al.*, 1994). Such phosphorothioate oligonucleotides are more efficient than unmodified ones in most of the systems studied, being commonly active at concentrations lower than 1 μmol/l in culture medium. They also have been reported to promote various non-sequence-specific effects, due in part to specific protein binding (Brown *et al.*, 1994). Some of these effects may contribute to antiviral activity (Perez *et al.*, 1994; Zelphati *et al.*, 1994). Concentrations higher than 1 μmol/l are known to favour non-specific mechanisms (Cazenave

**Abbreviations:** FCS = foetal calf serum; nt = nucleotide; TBE = tick-borne encephalitis virus; YFV = yellow fever virus

and Hélène, 1991). They can also inhibit RNase-H (Gao *et al.*, 1992), the number of phosphorothioate linkages being critical.

Sufficient stability of the oligonucleotide-RNA hybrid is also important for the antisense activity. It relies basically on base pairing between complementary sequences, and consequently on base composition and length of the oligonucleotide. However, environmental parameters, such as temperature or ionic strength, can alter the stability of the hybrid. Furthermore, target RNA sequence engaged in stable secondary structure requires the use of an oligonucleotide with strong affinity. Phosphorothioates 17-20 nucleotides (nt) long have been demonstrated to be optimal for both affinity and reducing side effects (Cazenave and Hélène, 1991).

Viruses of the genus *Flavivirus*, with about 70 identified species, represent an important cause of human illnesses. YFV is still responsible for severe outbreaks, despite the existence of a very efficient and safe attenuated vaccine, strain 17D. A comparable vaccine does not exist yet against dengue viruses which cause dengue haemorrhagic fever and dengue shock syndrome, the severe forms of dengue fever, with growing frequency (Halstead, 1988). Furthermore, there is no specific therapy available.

Although representing an alternative and potentially powerful approach, the use of antisense strategy with flaviviruses is poorly documented (Nomokonova *et al.*, 1993; Raviprakash *et al.*, 1995). In our laboratory, preliminary experiments were designed to screen for possible oligonucleotides active against YFV replication in cultured Vero cells. We found that soluble unmodified oligonucleotides had no detectable specific effect, even at concentrations up to 50 μmol/l in the culture medium. However, we demonstrated that calcium phosphate precipitation protected the oligonucleotides from nuclease degradation and greatly favoured their penetration into cells (Tolou, 1993). Using this technique, we observed that oligonucleotides targeted to the initiation codon region as well as to the 3'-end of the viral RNA were able to reduce virus production in infected Vero cells in a specific manner, whereas an oligonucleotide directed to the coding region and control oligonucleotides had no detectable inhibitory effect (Tolou, 1992, 1993, and unpublished results). However, these results were poorly reproducible. Reduction of the viral production, as determined by plaque titration, was variable, depending in particular on the origin and passage number of Vero cells.

In an attempt to confirm or invalidate our first observations, we synthesized phosphorothioate analogues of the oligonucleotides mentioned above and used them in new experiments against YFV (strain 17D). We demonstrate here that significant activity on YFV replication can be achieved with oligonucleotides targeted to different regions of the viral RNA, possibly by different mechanisms. An oligonucleotide complementary to the 3'-end of the RNA was constantly most effective.

## Materials and Methods

*Cells and viruses.* Vero cells were from Eurobio or ICN. They were tested for absence of mycoplasma with Hoechst 33258 and cultivated in Medium 199 containing 4 μmol/l L-glutamine (Gibco) and 5% foetal calf serum (FCS, D. Dutscher) at 37°C in 5% $CO_2$ atmosphere. Viruses (YFV, strain 17D and dengue 2, strain New Guinea) were stored at -80°C as homogenates of infected suckling mouse brains ($10^7$ PFU/ml) and diluted in Medium 199 without FCS for infection of cells.

*Phosphorothioate oligodeoxyribonucleotides.* 20 or 21 nt long, were synthesized in 329B Applied Synthesizer using cyanoethylphosphoramidite chemistry and tetraethylthiuram disulfide (Applied Biosystems) as sulphurizing agent. Products were ethanol-precipitated twice and checked for purity and homogeneity by capillary electrophoresis in gel-filled capillaries (Microgel 100, Applied Biosystems). The sequence of the different oligonucleotides is presented in Table 1. In the designation of oligonucleotides, the first two letters indicate virus (FJ = YFV 17D strain; D2 = dengue 2 virus New Guinea strain), the third letter indicates polarity (C = antisense; D = sense), and the number indicates position of the 5'-nucleotide on the genomic (sense) RNA. FJ/C/121 is complementary to the initiation codon region for YFV and FJ/C/10862 and D2/C/10723 are complementary to the 3'-end of the viral genomes according to published sequences (Rice *et al.*, 1985; Deubel *et al.*, 1986, 1988). The M13 sequencing primer, 21 nt long, was taken as a control (M13/21).

**Table 1. Sequence of tested antisense phosphorothioate oligodeoxyribonucleotides**

| Name | Sequence |
|---|---|
| FJ/D/1 | 5'-AGTAAATCCTGTGTGCTAAT-3' |
| FJ/C/20 | 5'-ATTAGCACACAGGATTTACT-3' |
| FJ/C/121 | 5'-CATGTTCTGGTCAGTTCTCT-3' |
| FJ/C/2499 | 5'-CCGCACTTGAGCTCTCTCTT-3' |
| FJ/C/10862 | 5'-AGTGGTTTTGTGTTTGTCATC-3' |
| D2/C/20 | 5'-GTCCACGTAGACTAACAACT-3' |
| D2/C/10723 | 5'-AGAACCTGTTGATTCAACAG-3' |
| M13/21 | 5'-CGTTGTAAAACGACGGCCAGT-3' |

Nucleotides complementary to the initiation codon are underlined.

*Inhibition of plaque formation.* Vero cells were cultivated to confluence in 35 mm wells. After medium was removed, cells were infected with 0.5 ml of titrated YFV suspension giving 100 plaques per well (30 mins at 37°C). The inoculum was then discarded and the cells were covered with 2 ml of Medium 199 containing 0.8% gum tragacanth (Sigma) and phosphorothioate oligonucleotide (1 μmol/l) when needed. Cells were incubated at 37°C for 5 days

in 5% $CO_2$ atmosphere before plaques were stained with crystal violet in formaldehyde.

*Reduction of virus production.* Confluent Vero cells in 15 mm wells were inoculated with YFV at multiplicity of infection of 1 PFU per cell. After 45 mins of incubation, the inoculum was discarded and the cells were covered with 0.5 ml of Medium 199 containing an oligonucleotide (1 μmol/l or 5 μmol/l) or no oligonucleotide as control. Cells were incubated for 48 hrs in $CO_2$ atmosphere. Then virus released into the medium was titrated.

*Plaque titration.* Infective supernatants were serially diluted in Medium 199 and inoculated to confluent Vero cell monolayers in 35 mm wells. Cells covered with Medium 199 supplemented with 0.8% gum tragacanth were incubated for 5 days in 5% $CO_2$ atmosphere and then plaques were stained with crystal violet in formaldehyde.

## Results

Two kinds of experiments were designed for testing potential inhibitory activity of antisense phosphorothioate oligonucleotides in flavivirus infection.

### *Direct effect on plaque formation*

Confluent Vero cell monolayers infected with 100 PFU of YFV were treated with different oligonucleotides. Controls received no oligonucleotide, an oligonucleotide without complementarity to YFV RNA, or an oligonucleotide of the same polarity as the viral genomic RNA (FJ/D1). After incubation, plaques were counted and subjected to microscopic examination. As shown in Fig. 1, three oligonucleotides (FJ/C/20, FJ/C/2499 and FJ/C/10862) were found to give significant reduction of YFV plaque number. They are complementary to 5'- or 3'-ends of YFV RNA, or to the coding region of the RNA. An oligonucleotide targeted to the translation initiation region (FJ/C/121) gave inconstant reduction of plaque number. The different control oligonucleotides had no detectable activity in repeated experiments.

Besides being less numerous, most of the plaques formed in the presence of FJ/C/20 and especially of FJ/C/10862 were smaller than those with other oligonucleotides. This aspect was confirmed by microscopic examination of the plaques: in the presence of an active antisense molecule, plaques were generally smaller and contained more surviving cells (Fig. 2). The inhibitory effect of FJ/C/10862 on plaque number and size was not reinforced using the oligonucleotide in higher concentration (10 μmol/l) (data not shown).

As we had previously observed, our results could not be reproduced with all lines of Vero cells purchased from different suppliers. These "insensitive" cells were not further characterized and were excluded from our study. Such a variation of the activity of antisense oligonucleotides with cell lines of various history or culture conditions has been reported also by Wagner (1994). It clearly represents a limitation of the use of phosphorothioate oligonucleotides.

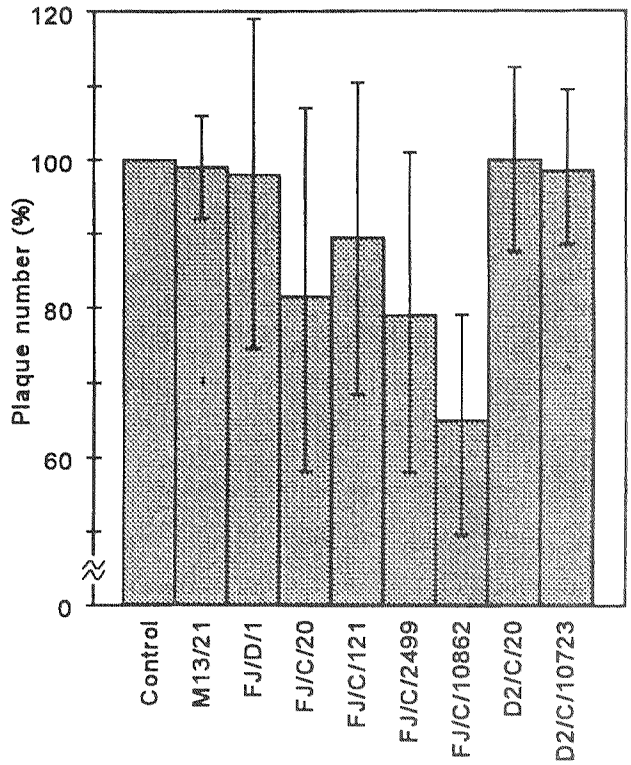


**Fig. 1**
**Direct inhibition of YFV plaque formation by different phosphorothioate oligonucleotides**
Each column corresponds to the average of 7 – 9 experiments. Plaque reduction promoted by oligonucleotides FJ/C/10862, FJ/C/2499 and FJ/C/20 was found significant ($p < 0.05$) using the Student's t-test. Error bars correspond to SD.

In order to test the specificity of the inhibition promoted by oligonucleotides FJ/C/20 and FJ/C/10862, we performed a few experiments with dengue 2 virus. Homologous oligonucleotides targeted to the dengue 2 virus RNA, D2/C/20 and D2/C/10723, were used. We observed that both YFV and dengue 2 virus-specific oligonucleotides were ineffective in promoting a reduction of dengue 2 virus plaque number (Fig. 3).

### *Effect on virus production*

In these experiments YFV-infected Vero cells were incubated in Medium 199 with or without an oligonucleotide. At 48 hrs post infection, supernatants were taken for virus titration. The results are shown in Fig. 4. All these experiments were performed with Vero cells of the same origin,

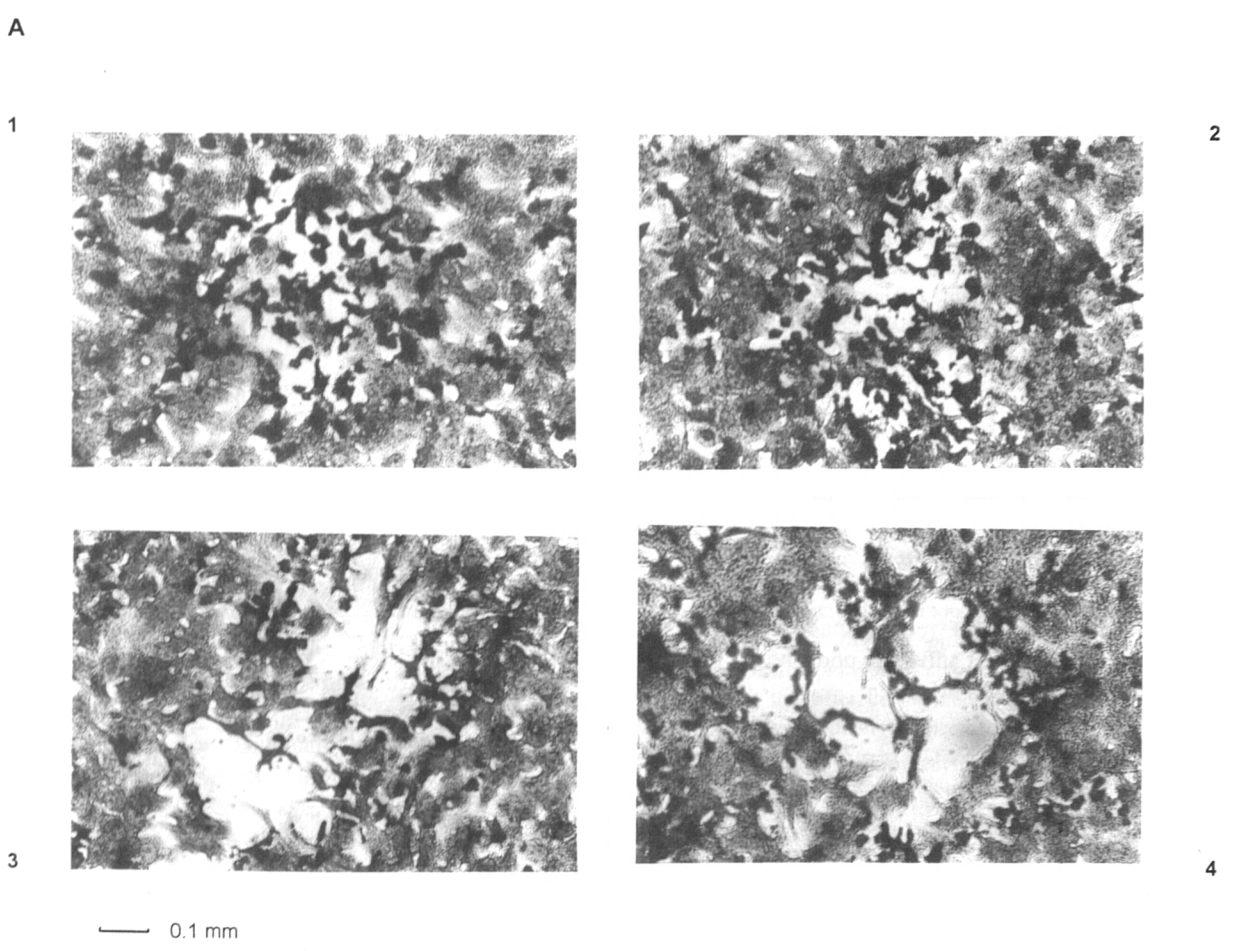


**Fig. 2**
**YFV plaques on Vero cells**
Microscopic (A) and macroscopic (B) appearance. FJ/C/10862 (1,2), M13/21 (3) and no oligonucleotide (4).

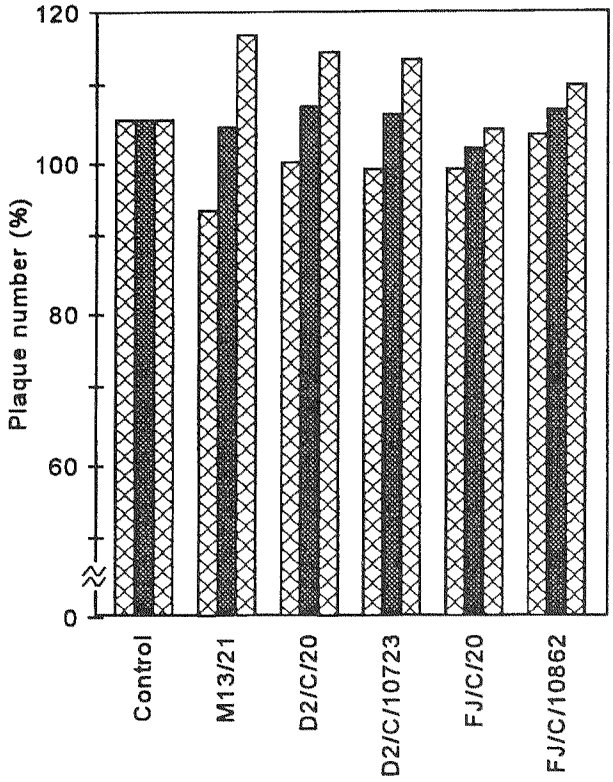


Fig. 3
**Direct inhibition of dengue 2 virus plaque formation by different phosphorothioate oligonucleotides**
Dark columns correspond to averages of two experiments, indicated as crossed columns.

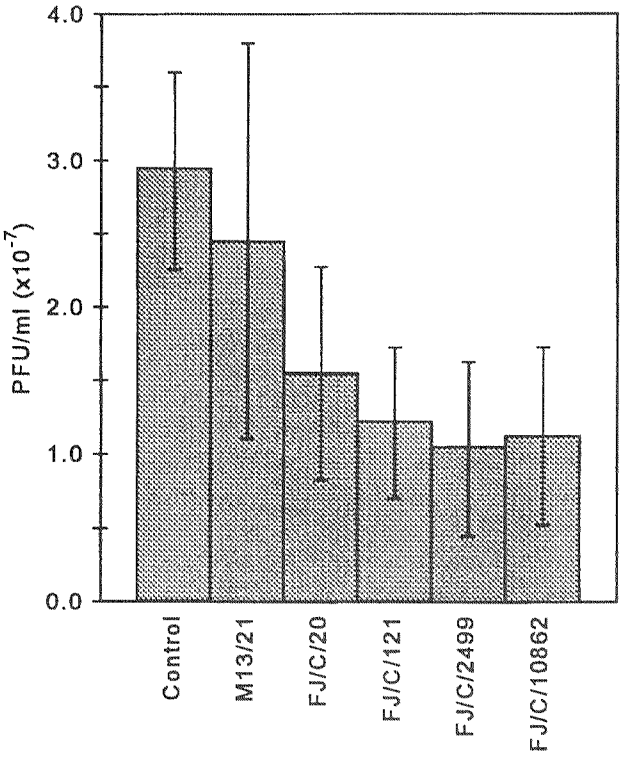


Fig. 4
**YFV yields in Vero cells treated with different phosphorothioate oligonucleotides**
Averages of three (FJ/C/20 and FJ/C/2499) or four (others) idependent experiments are shown. Error bars correspond to SD.

the same ones with which plaque reduction was achieved. When used at 1 μmol/l concentration, oligonucleotides FJ/C/121, FJ/C/2499 and FJ/C/10862 appeared regularly active. Oligonucleotide FJ/C/20 exhibited a weaker activity. Surprisingly, oligonucleotide M13/21, a random oligonucleotide used as a negative control, promoted a noticeable reduction of virus titer, despite it had no extended homology with viral RNA as determined by computer analysis. This "non-complementarity-dependent effect" was reinforced at higher concentration of M13/21 (5 μmol/l), giving even higher titer reduction than the specific oligonucleotides (data not shown).

## Discussion

Although successful in many systems, the use of antisense strategy against flaviviruses has been rarely reported. Our preliminary experiments with unmodified oligonucleotides targeted to the YFV RNA gave indecisive results. However, in a reticulocyte lysate system, oligonucleotides are quite active in inhibiting translation of short flavivirus RNAs. Nomokonova *et al.* (1993) reported that unmodified oligonucleotides targeted to the coding region of tick-borne encephalitis (TBE) virus RNA inhibited its translation, provided RNase-H was present in the assay. Working with two RNAs, 1500 and 2300 nt long, both corresponding to the 5'-end of the genomic dengue 2 RNA, we observed that unmodified oligonucleotides complementary to the 5'-end or the initiation codon region could completely block the *in vitro* translation in a reticulocyte lysate system. The inhibition was fully reversed when oligonucleotides complementary to the active ones were added at the same concentration, proving that the inhibition was specific. RNase-H was probably not involved in this case, as it was not added and an oligonucleotide complementary to the coding region was ineffective. We consider these *in vitro* systems poorly representative for the mechanisms that take place in the flavivirus-infected cell.

The phosphothioate modification is known to improve the antisense activity of oligonucleotides in living cells through different mechanisms (Cazenave and Hélène, 1991;

Crooke, 1992; Temsamani *et al.*, 1994). Therefore, phosphorothioate oligonucleotides may be currently regarded as a reference in the antisense strategy. In this work, we used phosphorothioate oligonucleotides complementary to YFV RNA in two different experiments in order to define conditions for the antisense activity assay and to identify the RNA sequences to be targeted. In optimal conditions, as apparently realized in our plaque reduction assay, phosphorothioate oligonucleotides inhibited YFV replication in a sequence-specific manner, while dengue 2 virus appeared insensitive. Low concentrations of oligonucleotides complementary to the 5'- and 3'-ends or to the coding region of the YFV genome were effective. The inhibitory activity of the oligonucleotide targeted to the initiation codon region was weaker and less reproducible. These effects appeared specific, as different control oligonucleotides used in our experiments failed to promote a plaque count reduction. Moreover, two of the oligonucleotides active agaisnt YFV replication were inefficient against dengue 2 virus in the same cell system, indicating that the antiviral activity was not mediated by a general effect on cellular metabolism.

Things were less clear in the assay of the effect on virus production. In this assay, the inhibitory activities of oligonucleotides FJ/C/20, FJ/C/121, FJ/C/2499 and FJ/C/10862 appeared equivalent, but a non-specific activity was found for oligonucleotide M13/21 which gave similar reduction of virus titer in some experiments. Curiously, even at higher concentration (10 µmol/l), M13/21 had no detectable effect in the plaque reduction assay. The reasons for these phenomena were so far not investigated. M13/21 was arbitrarily taken as a negative control because of absence of homology with YFV RNA. No cytopathic effect was detected in cells exposed to concentrations up to 10 µmol/l during 5 days in the plaque reduction assay. However, specific interaction of M13/21 with some cellular or viral component cannot be ruled out, as the phosphorothioate modification is known to favour such mechanism. The non-specific inhibitory activity of phosphorothioate oligonucleotides is now largely documented (Wagner, 1994; Gura, 1995). The activity of M13/21 exemplifies our yet incomplete understanding of so-called antisense effect and the difficulty to identify a true sequence-pairing-dependent ones. Nevertheless, it may be of interest in the search for antiviral compounds.

Phosphorothioate oligonucleotide concentrations up to 20 µmol/l or more in culture medium have been reported to be necessary and efficient in some experiments (Leiter *et al.*, 1990). However, in our experience with YFV, an inhibitory activity was detectable already at a concentration of 1 µmol/l. At 5 µmol/l, non-specific effects were detected without enhancement of the specific ones. Thus, higher concentrations were not tested.

Recently, Raviprakash *et al.* (1995) reported that both the unmodified and phosphorothioate oligonucleotides were inefficient in promoting significant inhibition of dengue 2 virus replication in cultured cells. Here, we report that YFV behaved in a different way, being sensitive to phosphorothioate oligonucleotides targeted to multiple regions of the genomic RNA. Although statistically significant, the inhibition observed in our experiments was incomplete. Taken together, the results of Raviprakash *et al.* (1995), Nomokonova *et al.* (1993) and ours indicate that flaviviruses vary in their sensitivity to antisense oligonucleotides. The reason for this is not elucidated. As a possible target, flavivirus RNA translation is not known to involve specific mechanisms accounting for particular resistance to the antisense mechanism. However, Raviprakash *et al.* (1995) suggest that the affinity of phosphorothioate oligonucleotides to their target sequences is not sufficient to cause dengue 2 virus inhibition. Some viral features, such as stable structure of genomic RNA and poor accessibility or strong interaction of the RNA with proteins in the infected cell may account for this phenomenon and for the different behaviour of YFV and dengue 2 virus. Indeed, the secondary structures in the 5'-non-coding region of RNA have been proposed to prevent anti-translational activity of the oligonucleotides complementary to the 5'-end or to the initiation codon region in the case of TBE virus (Nomokonova *et al.*, 1993). Nevertheless, we found homologous oligonucleotides active against dengue 2 virus RNA translation, but not against virus replication in infected cells. Working in *in vitro* assays with short RNAs, possibly poorly structured, might be an explanation for these conflicting results. As we observed differences between Vero cells of various history, cellular factors might play a role in this phenomenon.

As indicated by the activity of the oligonucleotide targeted to the coding region of the YFV RNA, RNase-H-mediated cleavage of the RNA probably supports in part the effect of the different oligonucleotides used in our experiments. Alternatively, direct inhibition of RNA translation or transcription might take place for oligonucleotides complementary to the 5'- and 3'-regions of the RNA.

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