Control of gene expression in viruses and protozoan parasites by antisense oligonucleotides

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SUMMARY

Chemically-modified oligonucleotides are now routinely used to prevent gene expression in cell-free media and in cultured cells. The binding of an antisense sequence to a complementary RNA target may lead to the selective inhibition of the encoded information. This may occur at different levels: splicing; transport of the mature RNA from the nucleus to the cytoplasm; translation. Antisense oligonucleotides constitute an interesting tool to shed some light on gene function. They are also potential new therapeutic agents against pathogenic organisms. This review discusses the rules that guide the design of an antisense oligomer and the choice of a target sequence. Examples of the potential use of antisense oligonucleotides in the fields of virology and parasitology, in particular in relation to trypanosomatids, are described.

Key words: antisense oligonucleotides, gene expression, translation, virus, trypanosomatid, mini-exon sequence.

INTRODUCTION

Synthetic oligonucleotides are now routinely used to regulate gene expression in cell-free extracts, in cultured cells as well as *in vivo*. The most popular strategy is certainly the use of antisense oligonucleotides: strictly speaking, an antisense oligomer is complementary to part of an RNA, the sense sequence. The formation of a sense/antisense duplex prevents reading of the genetic information encoded in the target RNA. Depending on the RNA function, pre-RNA, mRNA or retroviral RNA gene expression can be inhibited at the splicing, translation, replication or reverse transcription level. This strategy can be implemented for any gene whose sequence is known: an antisense oligomer is rationally designed using the Watson–Crick pairing rules. This concept was suggested more than 30 years ago (Belikova, Zarytova & Grineva, 1967) and its potential was experimentally demonstrated in the pioneering works of Ts'o and Miller (Barrett, Miller & Ts'o, 1974; Miller, Braiterman & Ts'o, 1977) and of Zamecnik (Stephenson & Zamecnik, 1978; Zamecnik & Stephenson, 1978), in the late seventies.

Besides the antisense approach, several other possibilities can be exploited to down-regulate the expression of a gene of interest with synthetic oligonucleotides (see Table 1). In the triplex or 'antigene' strategy an oligomer binds in the major groove of double-stranded DNA, potentially leading to the inhibition of different processes. For example, transcription association of a DNA binding factor or enzymatic modification of the DNA (see Hélène & Toulmé, 1990; Thuong & Hélène, 1993 for reviews). Alternatively, sense oligomers can be used to trap nucleic acid-binding proteins; thus, a double-stranded decoy, mimicking the binding site of a transcriptional activator, will decrease the transcriptional efficiency when added in excess, as the protein bound to the sense oligomer will not play its physiological role (Bielinska *et al*. 1990; Clusel *et al*. 1993). This is restricted to those genes under the control of a factor whose sequence requirement for binding has been identified. However, the use of *in vitro* selection procedures to isolate aptamers from randomly synthesized DNA or RNA libraries can circumvent this limitation. This strategy is not restricted to nucleic acid binding proteins. Aptamers have been characterized which display selective affinity for various kinds of ligands: proteins, nucleoside triphosphate, amino acids, nucleic acids (see Gold et al. 1995; Kumar & Ellington, 1995 for

Table 1. Strategies using oligonucleotides for modulating gene expression

Antisense (ribozyme)	RNA	Translation, splicing, reverse transcription.
Triplex	DNA	Transcription, replication.
Sense	Protein	Transcription, translation, reverse transcription.
Aptamer	Protein, DNA, RNA, any ligand	Transcription, translation, other processes …

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Parasitology (1997), **114**, S45–S59. Printed in the United Kingdom
https://doi.org/10.1017/S0031182097001005 Published online by Cambridge University Press

reviews). Lastly, ribozymes which are catalytic 'antisense RNA' enzymes can also be used although they are generally derived *in situ* from the transcription of an appropriate construct and not delivered as a chemical entity (Bartolomé, Madejon & Carreno, 1995; Symons, 1992). These different approaches (triplex, sense, aptamers, synthetic ribozymes), which share a few properties with antisense oligonucleotides, will not be discussed in detail here. This review focuses on the design of synthetic antisense oligomers, their use to study the expression of genes from pathogen agents and the attempts made to design antiviral and antiparasite drugs.

BACKGROUND TO THE DESIGN OF ANTISENSE OLIGONUCLEOTIDES

Affinity and selectivity of the oligonucleotide for its target are primarily driven by the length and the sequence of the antisense oligomer. In a human genome, a 17 nucleotide long target is statistically unique (Hélène & Toulmé, 1989). Although it might be tempting to lengthen the antisense strand to increase the affinity of the oligomer for its target and better guarantee the uniqueness of the binding site, this will generally result in a decreased specificity as this will lead to stable mismatched duplexes with non-target sites. Conventional unmodified oligodeoxynucleotides are not attractive antisense sequences for use in biological environments, i.e. with cultured cells or *in vivo*. Nuclease susceptibility and limited uptake by live cells are the major weaknesses of regular DNA oligomers.

Chemically-modified oligomers

Chemists have designed numerous oligonucleotide analogues that fulfil the major requirements for antisense purposes (Beaucage & Iyer, 1993; Milligan, Matteucci & Martin, 1993). Nuclease-resistant derivatives have been obtained with modified internucleoside linkages, substituted sugar or anomer of the nucleoside unit.

Contrary to what was generally admitted in the early days of antisense oligomer research, these molecules do penetrate the cell membrane, as unambiguously demonstrated by the numerous positive results obtained both *in vitro* and *in vivo*. However, the uptake efficiency is limited. The major route for oligonucleotide entry is fluid phase and adsorptive endocytosis even though membrane proteins have been described which can play a role in the process (Vlassov, Balakireva & Yakubov, 1994). This leads to the accumulation of oligonucleotides within the lysosomal compartment from which they have to escape to reach their target either in the cytoplasm or in the nucleus. Limited knowledge of intracellular trafficking is presently available. Several solutions are offered which significantly improve the uptake and subsequently the efficiency of antisense oligomers: attachment of a hydrophobic tail such as cholesterol or a polycation such as poly (L-lysine) to the end of the oligonucleotide are effective means to increase the intracellular pool of antisense sequences. Alternatively, oligomers can be delivered by various carriers: liposomes, nanoparticles and lipoproteins have been successfully used (Léonetti *et al*. 1993). It should be noted that strategies aimed at improving uptake can lead to an additional benefit with respect to the pharmacological half-life of the oligonucleotide. Once encapsulated into a vehicle or associated with a carrier, the oligomer will be much less susceptible to nucleases. Moreover, as 3′ exonucleases are the major DNases (Verspieren *et al*. 1987), conjugating a cholesterol residue or a poly (L-lysine) chain at the 3′ end of the oligonucleotide will slow down the degradation.

Mechanisms responsible for antisense effects

Antisense oligonucleotides were initially seen as road blocks, the antisense/sense duplex preventing the genetic information from being read by the cell machinery. Hence, the name 'hybrid-arrest' used for a while. However, it was quickly recognized that the target RNA did not always remain intact in the process, suggesting that the oligodeoxynucleotide mediated the degradation of the complementary RNA by ribonucleases H (RNases H) which are ubiquitous enzymes responsible for the degradation of the RNA strand of RNA/DNA heteroduplexes. Their involvement in antisense effects was demonstrated about ten years ago in cell-free extracts and in injected *Xenopus* oocytes (Cazenave *et al*. 1987; Walder & Walder, 1988) and more recently in permeabilized human leukaemia cells (Giles, Spiller & Tidd, 1995). Indirect evidence strongly suggests that RNase H contributes to the inhibition induced by antisense oligonucleotides in intact cells. Not every chemically-modified oligomer forms an RNase H substrate upon association to its target RNA sequence. Phosphorothioate oligomers elicit RNase H activity whereas other routinely used nuclease-resistant analogues do not: this includes methylphosphonates, alpha isomers, 2′-*O*-methyl oligoribonucleotide and Peptide Nucleic Acid (Toulmé & Tidd, 1997). Interestingly, a phosphorothioate 20-mer, targeted to the 3′ untranslated region of ICAM-1 mRNA, was inhibitory in cultured human lung carcinoma cells whereas its 2′-*O*-methyl analogue was not, suggesting that this antisense sequence was active via an RNase H-dependent mechanism (Chiang *et al*. 1991).

The major differences between the two inhibitory processes (hybrid arrest, and induced degradation)

https://doi.org/10.1017/S0031182097001005 Published online by Cambridge University Press

resides in the fact that RNase H-mediated inhibition inactivates permanently the target RNA, and that antisense oligonucleotides in combination with the enzyme can work catalytically as long as the oligomer remains intact in the process; in other words, a single antisense molecule can induce the cleavage of multiple RNAs which will no longer support gene expression. In contrast, the RNase H-independent mechanism is stoichiometric and transient: the dissociation of the antisense/sense complex allows gene expression.

Ribonucleases H are able to cleave mismatched heteroduplexes. This might explain the non-specific effects of oligonucleotides. An oligomer may induce the inhibition of non-target genes following its binding to a partially complementary sequence. This effect is more important if RNase H is able to cleave the duplex. The affinity of the antisense sequence for its target (primarily its length) should be carefully adjusted; ideally a single mismatch should lead to non-stable complexes under the conditions of interest. As a consequence, oligomer analogues which do not elicit RNase H activity can induce more specific effects as shown with 2′-*O*-methyl oligoribonucleotides targeted to the rabbit α-globin mRNA in cell free assays (Larrouy *et al*. 1995). In order to take full advantage of the various properties of oligonucleotide analogues, different chemistry can be combined within an antisense sequence. For instance, methylphosphonate-phosphodiester chimeras are exonuclease-resistant analogues which allow RNase H activity; such compounds have been shown to display a higher selectivity both in cell-free assays and in cultured cells (Giles *et al*. 1995; Giles & Tidd, 1992; Larrouy *et al*. 1992).

In addition to these RNase H-dependent and RNase H-independent mechanisms for antisense effects, it is worth mentioning that non-antisense effects, both sequence-dependent and sequence-independent, have been reported. For instance, GT-rich oligomers or phosphorothioate analogues display particular properties in cells. Some of them might be of interest: phosphorothioate oligonucleotides are inhibitors of the HIV reverse transcriptase (Majumdar *et al*. 1989).

Selection of a target sequence

As mentioned above, any RNA can be targeted for regulating gene expression. Indeed, pre-RNA splicing (Dominski & Kole, 1993) as well as reverse transcription (Boiziau, Thuong & Toulmé, 1992) have been inhibited by oligonucleotides complementary to introns and RNA template, respectively. But most of the work has been performed with antisense oligonucleotides designed to interact with mRNA. However, in culture assays it is generally not known whether inhibitory effects are related to the binding of the oligomer to the intended message, in the cytoplasm or in the nucleus, or to the pre-mRNA in the nucleus.

Molecular mechanisms responsible for the inhibition of translation are clearly identified (see Toulmé & Tidd, 1997, for a review): oligonucleotides complementary to the coding region cannot block translation unless they induce cleavage by RNase H (Cazenave *et al*. 1989). Therefore, chemically-modified oligonucleotides which do not allow RNase H activity are inactive when targeted downstream of the AUG initiation codon: the elongating ribosome is endowed with an unwinding activity which displaces antisense sequences (Boiziau *et al*. 1991*b*). In contrast, oligonucleotides can prevent the initiation of translation when targeted to the 5′ untranslated leader as demonstrated for alpha, 2′-*O*-methyl and PNA analogues (Bertrand *et al*. 1989; Johansson *et al*. 1994; Larrouy *et al*. 1995). According to these results, most of antisense experiments are carried out with oligonucleotides complementary to the initiator AUG region.

Antisense effects are directly related to the formation of oligonucleotide/RNA complexes. Therefore, weak effects will be observed with oligomers targeted to a folded RNA region due to the competition between intramolecular and inter-molecular base-pairing (Verspieren *et al*. 1990; Vickers *et al*. 1991). Unfortunately, RNAs adopt multiple secondary and tertiary structures which are not easily predicted. Therefore, the selection of a target site for antisense sequences can be essentially a hit and miss process.

ANTIVIRAL APPLICATIONS

The expression of a gene of a pathogen can be prevented with antisense oligonucleotides. If the gene product is crucial for this organism, this will constitute a means to control its development and there will be no detrimental effect to the host if the target sequence is not present in the host genome. Therefore antisense oligonucleotides constitute prototypes for new therapeutic agents.

A few studies have been performed with prokaryotes; methyl phosphonate and carbamate analogues targeted against *Escherichia coli* 16S rRNA have been used (Jayaraman *et al*. 1981; Rahman *et al*. 1991). Psoralen-oligonucleotide conjugates have successfully inhibited the plasmid-borne ampicillin resistance in heat-shocked *E. coli* (Gasparro *et al*. 1991). Non sequence-specific inhibition of the *lux* gene in *Vibrio fisheri* by phosphorothioate oligonucleotides has been described (Chrisey, Pazirandeh & Liss, 1995). Interestingly no permeabilisation of the cells was required. Recently, the antimycobacterial activity of oligonucleotides was reported using oligomers conjugated to various ligands in order to promote the uptake of the antisense sequence (Rapaport *et al*. 1996).

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In contrast, there have been numerous attempts to interfere with viral gene expression using antisense oligomers. This is undoubtedly related to the need for efficient antiviral drugs, as highlighted by the AIDS pandemic. It was probably fortuitous that one of the first antisense experiments described the use of oligomers to control the production of Rous Sarcoma Virus in cultured chicken fibroblasts (Zamecnik & Stephenson, 1978). Since then many viruses have been targeted *in vitro*: the vesicular stomatitis virus (VSV), the simian virus 40, the influenza virus, the hepatitis virus, the cytomegalovirus, the human papilloma virus, the herpes simplex viruses (HSV) and, last but not least, the human immunodeficiency virus (HIV).

Several reviews have been recently devoted to antisense oligonucleotides as antiviral agents (Cohen, 1991; Cowsert, 1993; To, 1992; Whitton, 1994). Here a few studies that highlight questions of general interest for the design of antisense oligonucleotides, and their mode of action against viruses, will be mentioned.

The first two examples demonstrate that oligonucleotides distribute within the cell, both in the cytoplasm and in the nucleus. An antisense oligomer targeted to the message coding for the N protein of VSV (a virus which develops exclusively in the cytoplasm of the host cell), inhibited the multiplication of the virus in cultured L929 fibroblasts. The antisense efficiency was largely improved upon conjugating the oligonucleotide to poly (L-lysine), 50% reduction of virus titre being achieved in the 10–100 nM range (Lemaître, Bayard & Lebleu, 1987). A methylphosphonate oligomer complementary to the splice acceptor site of an immediate early gene of HSV-1 reduced the *in vitro* infection of Vero cells. The antiviral activity was improved by two orders of magnitude using a psoralen-methylphosphonate oligomer derivative (Kulka *et al*. 1989). Upon u.v. irradiation, a covalent photoproduct is formed between the antisense sequence and the target RNA, leading to the permanent inactivation of the cross-linked RNA species, i.e. to splicing inhibition. The effect was selective: anti-HSV1 oligomers had no effect on HSV2 development, due to a different exon sequence at the splice junction. Coincidentally, this constitutes a better control for specificity than varying the oligonucleotide sequence as non-antisense effects can also be sequence-dependent. A similar approach has been used for HIV (Degols *et al*. 1992) and for influenza virus (Zerial, Thuong & Hélène, 1987): oligonucleotides were shown to be active against a given strain but not against another one for which the target sequence was mutated. Unfortunately, such a control is not always possible.

The urgent need for therapeutic agents against HIV has led to numerous studies with antisense oligonucleotides. Almost all available chemically-modified analogues have been tested. Both translation and splicing events have been subject to antisense studies. The best studied compounds are a 27mer targeted to the 5′ end of the *rev* mRNA and a 25mer targeted to the initiation codon of the *gag* message, both used as phosphorothioate derivatives. Selective effects have generally been reported with phosphorothioate oligomers in the μM range in chronically infected T-lymphocytes whereas non-selective inhibition was reported for *de novo* infected cells (Agrawal & Tang, 1992; Matsukura *et al*. 1989). Retroviruses offer additional opportunities for their control with antisense sequences: the inhibition of reverse transcription would prevent the integration of the viral genome into the host DNA. RNA-dependent cDNA synthesis can be blocked by two different mechanisms reminiscent of translation inhibition. Antisense oligonucleotides bound to the template can block the scanning reverse transcriptase (hybrid-arrest) or induce the destruction of the RNA template by the RNase H activity associated with the DNA polymerase activity (Boiziau *et al*. 1992). The relative contribution of the two mechanisms will essentially depend on the chemical nature of the antisense sequence. In our hands, in the test tube, unmodified oligodeoxynucleotides act essentially via the RNase H-dependent mechanism, whereas hybrid-arrested cDNA synthesis was observed with 2′-*O*-alkyl oligoribonucleotides (Boiziau *et al*. 1995; Boiziau *et al*. 1992). Triple-helices can be formed from homopurine-homopyrimidine double-stranded sequences (see below). Such a motif is present in the HIV-1 sequence and can be targeted at the RNA level, using a clamp oligonucleotide, to prevent reverse transcription (Volkmann *et al*. 1995). Alternatively, triplex-forming oligomers can bind to this sequence on the proviral DNA (Giovannangeli *et al*. 1992).

Phosphorothioate oligomers display additional properties, largely sequence-independent: they are inhibitors of cDNA synthesis due to direct competitive inhibition of substrate binding to the reverse transcriptase (Matsukura *et al*. 1987). They also interfere with the gp120-CD4 interaction (Stein *et al*. 1991). Therefore these compounds are non-specific inhibitors, in *de novo* HIV-infected cells. The contribution of sequence-dependent (antisense) and sequence-independent (non-antisense) effects was evaluated by a rational comparison between phosphodiester and phosphorothioate oligomers complementary to the *rev* mRNA, delivered to either *de novo* or chronically infected cells. In particular, encapsulation of phosphorothioate analogues into liposomes prevented the interference with virus-mediated cell fusion (Zelphati *et al*. 1994). The non-antisense effects are not restricted to HIV: phosphorothioate derivatives inhibited the herpes simplex virus-induced DNA polymerase and the *in vitro* growth of the virus (Gao *et al*. 1989, 1990). Other

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non-antisense effects have been described: oligonucleotides composed entirely of guanosine and thymidine display antiviral properties against HIV-1 and HSV in culture (Fennewald *et al*. 1995; Wyatt *et al*. 1994). Such sequences can form guanosine-quartet structures which might, in part, be responsible for their activity and their nuclease resistance when used unmodified. However, it does not matter which way oligonucleotides are actually acting as long as their effect is restricted to viruses. Interestingly, phosphorothioate oligomers have a lower inhibitory activity on human DNA polymerases α, β and γ than on HSV-2 polymerase or HIV-1 reverse transcriptase (Gao *et al*. 1989).

In vivo experiments have been carried out for several viruses. The methylphosphonate oligonucleotide complementary to the acceptor splice junction of an immediate early gene of HSV-1 caused a significant decrease of the virus growth in intradermally infected BALB/c mice (Kulka *et al*. 1993). Topical applications of the oligomer in a modified aqueous polyethylene glycol cream during 3–5 days post-infection led to a significant reduction of the virus titre. This effect was sequence-dependent: this oligomer had a lower effect on HSV-2-infected mice in agreement with the different splice junction sequence. In addition, a non-complementary oligomer did not show any anti-viral property. Phosphorothioate oligomers have been successfully used to block hepatitis B virus (HBV) infection in Pekin ducklings (Offensperger *et al*. 1993). A daily intravenous injection of an antisense 18mer, 2 weeks post-infection of 1-day-old duckling, led to a dose-dependent and sequence-dependent reduction of viral replication. A nearly complete elimination of viral DNA from liver cells was achieved after a 10 day treatment at a daily dose of 20 μg/g body weight. These very encouraging data obtained on animal models opened the way to human clinical trials. Several phosphorothioate antisense oligonucleotides are in phase I to phase III trials against human papilloma virus (genital warts) and cytomegalovirus (AIDS-related retinitis) (Zon, 1995).

ANTISENSE OLIGONUCLEOTIDES TARGETED TO PARASITE GENES

Cell-free translation experiments

The first use of antisense oligonucleotides in parasitology aimed at evaluating the occurrence, in kinetoplastid mRNAs, of a sequence termed the mini-exon or spliced leader. This sequence was found at the 5′ non-translated end of messengers coding for variant-specific surface glycoproteins, the surface coat proteins of African trypanosomes, and in a subset of other mRNAs, in both *Trypanosoma brucei* and *T. cruzi*. Independently, two teams performed *in vitro* translation of trypanosome mRNA in the presence of oligonucleotides complementary to the mini-exon sequence. Antisense oligomers inhibited the translation of all trypanosome mRNA in wheat germ extract (Cornelissen *et al*. 1986) or in reticulocyte lysates (Walder *et al*. 1986) whereas these oligomers had no effect on the synthesis of proteins directed by non-related mRNA such as Brome Mosaic Virus mRNA which did not contain the target sequence. This demonstrated that the mini-exon sequence was a characteristic of trypanosomatid mRNA and that *trans*-splicing, the process through which the mini-exon RNA piece is acquired, is a general feature for mRNA maturation in these organisms.

A similar approach was used to demonstrate the presence of a mini-exon sequence in *Leishmania* (Pascolo *et al*. 1993) and in *Crithidia* (Gabriel, Sisodia & Cleveland, 1987). This suggests that anti-mini-exon oligonucleotides might be of interest for controlling the development of parasitic trypanosomatidae and of some nematodes; indeed, it was demonstrated that *trans*-splicing was responsible for the presence of a 22 nucleotide leader sequence at the 5′ end of the nematodes *Ascaris suum* and *Haemonchus contortus* (Bektesh, Van Doren & Hirsh, 1988).

Antisense oligomers were also evaluated as potential inhibitors of *Plasmodium* genes, namely hypoxanthine-guanine phosphoribosyl transferase (HPRT) and dihydrofolate reductase/thymidylate synthetase (DHFR/TS). A 24mer covering the AUG initiator of HPRT gene inhibited *in vitro* translation of an HPRT transcript in rabbit reticulocyte lysate in a dose- and sequence-dependent manner, 80% inhibition being achieved at an oligonucleotide concentration of 5 μM (Dawson *et al*. 1993). The *in vitro* inhibition of DHFR/TS was observed only with long oligomers (30–50 nucleotides long) at very high concentrations: several sequences complementary to the translation initiation region were used at about 150 μM for reducing 50% of the protein synthesis from total RNA prepared from parasites (Sartorius & Franklin, 1991). This might suggest that the target site is poorly accessible to the antisense sequence. Surprisingly, a 49mer complementary to the coding region was the best inhibitor among the antisense sequences tested: 6 μM elicited a 50% reduction in protein synthesis, whereas the message remained intact. This constitutes an indication of an RNase H-independent inhibition of translation elongation by an antisense oligomer in the cell-free extract. However, it is known that clamp oligonucleotides can block an elongating ribosome; therefore, it remains to be demonstrated that the DHFR/TS mRNA-antisense 49mer complex, responsible for hybrid-arrested translation, is a regular Watson–Crick duplex. Long oligomer sequences can form more

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sophisticated structures which might not be unwound by the translation machinery.

Cultured parasites

Oligonucleotides have to be taken up by the cells to exert their regulatory-antisense-effect. Alternatively, membranes can be permeabilized to promote the entry of the antisense sequence. Trypanosomes were made permeable, by lysolecithin, to direct cleavage of a target RNA by *E. coli* RNase H and synthetic oligonucleotides when added to the milieu. This allowed the analysis of the role of UsnRNA in trypanosomes: degradation of U_2, U_4 or U_6 snRNA by antisense-directed cleavage abolished *trans*-splicing of the mini-exon sequence (Tschudi & Ullu, 1990). Such permeabilized trypanosomes were used to determine accessible regions of the pre-mini-exon RNA that interfere with *trans*-splicing, using complementary 2′-*O*-methyl oligoribonucleotides which bind strongly to RNA but do not elicit RNase H cleavage (Ullu & Tschudi, 1993). Studies on live non-permeabilized trypanosomatids in culture have also been carried out in our laboratory and are summarized in the next section.

Antisense oligonucleotides have also been evaluated against *Plasmodium falciparum*. Most of the studies used phosphorothioate derivatives against the DHFR/TS gene. Oligonucleotides of various lengths, from 18 to 30 nt, were targeted to the translation initiation codon. Results from three different teams demonstrated a significant effect on parasite growth following the addition of 1–10 μM oligonucleotide to the culture medium (Clark *et al*. 1994; Ramasamy *et al*. 1996; Rapaport *et al*. 1992). Different strains, either chloroquine sensitive (FCR-3, D6) or drug resistant (W2, MDR-K) as well as one isolated from a cerebral malaria patient (CM 87), were susceptible to phosphorothioate oligomers in a dose-dependent manner. This is an interesting result, as this mRNA region was previously shown to be insensitive to antisense sequences in a cell-free study (Sartorius & Franklin, 1991). However, *in vitro* translation experiments do not accurately reflect the intracellular process. The anti-*Plasmodium* activity was dependent on the size of the oligomer used, but the selectivity of the effect was questionable and controversial. In two studies (Clark *et al*. 1994; Ramasamy *et al*. 1996) the authors concluded that the observed activity was sequence-independent as antisense, sense and homo-oligomers were inhibitory. Similar results were obtained with phosphorothioate oligonucleotides targeted to the choline phosphate cytidylyltransferase (Yeo and Vial, personal communication). In contrast, Zamecnik and co-workers (Rapaport *et al*. 1992) observed some selectivity for complementary oligonucleotides although a non-related oligomer was as efficient as antisense sequences. A similar conclusion was reached from an independent study (Dawson *et al*. 1993) on *P. falciparum* FCQ-27D10 strain in which phosphorothioate 24mers targeted against the AUG start codon of the HPRT message were used. Recently, a careful analysis of the effect induced by about 25 different antisense sequences on the growth of line W2 was carried out (Barker *et al*. 1996) From this work, it turns out that the antimalarial activity of phosphorothioates, 25–30 nucleotides long, is essentially non-specific above 1 μM. In contrast, at concentrations below 0·1 μM, parasite growth inhibition was sequence-dependent. The most efficient oligomer, complementary to a conserved sequence (nt 1153–1179) of the DHFR gene, had an IC_{50} below 0·05 μM. According to what is known about the antisense mechanisms, this suggests that this oligonucleotide targeted to the coding sequence of the message is working through the RNase H-mediated cleavage of the DHFR mRNA. The non-specific effects were ascribed to the inhibition of red blood cell invasion. This is reminiscent to what has been described for HIV (as described above), phosphorothioate oligonucleotides preventing the adsorption of the retrovirus on the host cell.

It is of interest for the further application of antisense oligonucleotides against intracellular pathogens to note that uptake of the oligonucleotides might be increased in infected cells. *Plasmodium*-parasitized erythrocytes are accessible to oligonucleotides whereas uninfected ones do not permit any entry (Rapaport *et al*. 1992). A similar observation has been reported for the Friend retrovirus: the internalization of oligomers associated with liposomes or nanoparticles is correlated to virus budding (Ropert, Malvy & Couvreur, 1993; Ropert *et al*. 1996). These differences between infected and uninfected cells could be related to the effect that the pathogen exerts on the host cell membrane. Indeed, it has been reported that *Plasmodium* induces changes in erythrocyte permeability and that uptake of molecules increases as the parasite matures. Membrane fluidity is also known to vary in retrovirus-infected cells. Besides the direct benefit for the inhibition of the target gene by oligonucleotides, these properties of infected cells add one level of specificity, the antisense oligonucleotide being preferentially distributed in those cells where it is required. This might also reduce the cytotoxicity toward uninfected cells.

TARGETING THE MINI-EXON SEQUENCE OF TRYPANOSOMATIDS WITH ANTISENSE OLIGONUCLEOTIDES

In the Kinetoplastida every messenger RNA is synthesized by discontinuous transcription of two separate genes followed by a *trans*-splicing event (reviewed in Agabian, 1990; Borst, 1986; Laird, 1989). Other organisms (nematodes, trematodes and

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Euglena) carry out *trans*- as well as *cis*-splicing (Davis *et al*. 1995; Nilsen, 1993; Tessier *et al*. 1991), whereas no *cis*-splicing has been described in trypanosomatids. In these organisms the maturation process of pre-mRNAs through the joining of two independent transcripts accounts for the acquisition of a 39 nucleotide-long sequence (known as the mini-exon or spliced leader sequence) at the 5′ end of all nuclear mRNAs. This sequence is derived from the mini-exon-donor RNA (medRNA), a small (85–140 nucleotides) nuclear RNA. *Trans*-splicing allows the production of individual mRNA from polycistronic precursors, providing for each mature mRNA a functional cap. The 5′ end of medRNA in *T. brucei*, *T. cruzi* and *Leishmania amazonensis* and very likely in all other trypanosomatids bears a 'cap4' structure consisting of a 7 methylguanosine cap and four modified nucleosides: ^{7}Gpppm$_{2}^{6}$A(2′Om)A(2′Om)-C(2′Om)m^{3}U(2′Om) (Bangs *et al*. 1992; Freistadt, 1988; Perry, Watkins & Agabian, 1987). In addition, the A residue in the 6th position is a 2′-*O*-methyl ribonucleoside (Perry *et al*. 1987). The pattern of methylation of the cap4 structure plays a key role for *trans*-splicing (Ullu & Tschudi, 1991; Ullu & Tschudi, 1993).

Cell-free studies

The mini-exon sequence is an exquisite target for antisense oligonucleotides: a single oligomer sequence will bind to every message of the trypanosomatid and to medRNA, and consequently will potentially interfere with the expression of all genes, as demonstrated by cell-free translation studies (Cornelissen *et al*. 1986; Walder *et al*. 1986). The inhibition is sequence-specific: as the mini-exon sequence varies among species, oligonucleotides complementary to *T. brucei* displayed lower inhibitory efficiency against *Leishmania* mRNA than against *T. brucei* mRNA in cell-free translation (Verspieren *et al*. 1990).

The amplitude of the inhibition is dependent on the length of the antisense sequence, that is on the affinity of the oligomer for its target (Cornelissen *et al*. 1986; Toulmé *et al*. 1990*b*; Verspieren *et al*. 1990). Alternatively, rather than lengthening the antisense sequence one can increase its affinity for the complementary RNA sequence by using chemically-modified analogues. Attaching an intercalating agent such as an acridine derivative to the oligomer generates additional stacking interactions that stabilize the complexes. A nonamer, complementary to the *T. brucei* mini-exon sequence, covalently linked at its 3′ end to 2-methoxy,6-chloro,9-amino-acridine (Acr-9mer) was an efficient inhibitor of *in vitro* translation of *T. brucei* mRNA (Verspieren *et al*. 1987) without any effect on translation extracts programmed with Brome Mosaic Virus mRNA (Verspieren *et al*. 1988). Similar experiments were performed with *L. amazonensis* mRNA. However, in this case, the acridine-linked 12-mer was not more efficient than the unmodified oligomer at inhibiting *in vitro* protein synthesis, in contrast to what was observed with *T. brucei*. Stability measurement of oligonucleotide-RNA complexes failed to show any stabilization of the acridine-conjugate suggesting that the dye did not contribute to the binding (Pascolo *et al*. 1993). This might be related either to a sequence peculiarity or to the attachment site of the intercalating agent: 3′ for the anti-*T. brucei* sequence, 5′ for the anti-*Leishmania* sequence.

Antisense oligonucleotides can also be turned into active (or activatable) compounds. Following their binding to the target sequence, a chemical reaction leads to a permanently damaged RNA, no longer able to support gene expression. Both cleaving and cross-linking reagents have been used. We targeted *T. brucei* and *L. amazonensis* mRNA by antisense oligomers conjugated either to an alkylating group (Boiziau *et al*. 1991*a*) or to a psoralen derivative (Pascolo *et al*. 1994). In both cases a covalent adduct between the RNA and the antisense oligonucleotide was formed. This approach is of particular interest when an oligomer complementary to the coding region is used under conditions that do not allow RNase H cleavage. The modifying oligomer makes RNase H activity dispensable.

Cultured parasites

Antisense oligonucleotides are now routinely used to block gene expression in cultured cells. Antisense sequences complementary to the mini-exon have been used *in vitro* with live parasites, trypanosomes as well as leishmanias.

Acr-9mer, the oligomer which reduced the protein synthesis of *T. brucei* mRNA in cell-free assays, was of interest for studies with intact trypanosomes. First, the acridine derivative was expected to stabilize the antisense oligonucleotide-RNA duplex, as shown with purified molecules. Second, the presence of the dye at the 3′ end of the oligomer prevented its trimming by 3′ exonucleases. Indeed, the lifetime of the acridine derivative is about 6 times higher in growth medium containing 10% fetal calf serum than that of the unmodified homologous 9mer (Verspieren *et al*. 1987). Third, the uptake of the acridine conjugate by the parasites is faster than that of the unmodified oligonucleotide (Fig. 1); this might be related to hydrophobic interactions between the acridine ring and the parasite membrane. The addition of 100 μM Acr-9mer to *T. brucei* procyclics led to extensive changes in the morphology of the parasites. After about 30 h, the cells were vacuolized and had a reduced mobility. No live parasites were detected after 48 h incubation in the presence of the acridine oligomer (Verspieren *et al*.

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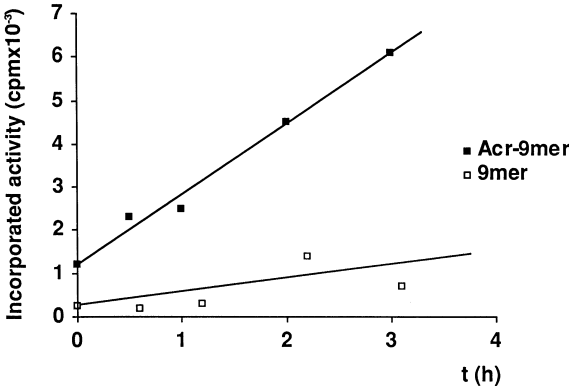

Fig. 1. Oligonucleotide uptake by cultured *T. brucei*. ^{32}P end-labelled oligomers were added to parasites (10^6 cells/ml) in SPG buffer (Verspieren *et al*. 1987). The radioactivity associated to the cells after washes was measured by scintillation counting. (□) unmodified 9mer 5′ TAATAGCGT, (■) Acr-9mer; (2-methoxy, 6-chloro, 9-aminoacridine was linked at the 3′ end of the oligonucleotide via a pentamethylene tether).

1987). The trypanocidal effect was dependent on the oligonucleotide sequence and, therefore, possibly resulted from the association with the mini-exon sequence: non-complementary 9mers, linked to the acridine derivative, did not induce any cytotoxicity. Moreover, the antisense activity was related to the amount of bound oligomer. Firstly, a dose dependent effect of Acr-9 mers was observed. Secondly a modified complementary hexamer, Acr-6 mer, which has a lower affinity than Acr-9 mer for its target did not show any trypanocidal activity. These results rule out a direct effect of the acridine. Last, unmodified oligonucleotides complementary to the mini-exon sequence did not kill the trypanosomes. This might be related either to a short lifetime or a limited uptake. A weak binding is not the likely reason for the failure as longer unmodified complementary oligonucleotides (up to 16 mers) did not display any activity.

Antisense oligonucleotides have also been used against *L. amazonensis* in culture. Phosphorothiate oligomers complementary to the mini-exon sequence of this parasite were prepared and added to infected murine peritoneal macrophages. After about 24 h incubation in the presence of a 16mer complementary to nucleotides 8–23 (16 PS), the treated cells showed fragmented parasitophorous vacuoles. Parasite remnants were detected in a few macrophages, indicative of a lethal process. About 40% of the macrophages were cured at 25 μM oligonucleotide, compared to untreated cells (Ramazeilles *et al*. 1994). Moreover, the parasitic load was much lower in the treated culture than in the untreated one. However, even at a higher dose of 16PS it was not possible to cure more than 50% of the cells. This was not due to resistant parasites as an experiment carried out with macrophages infected by parasites prepared from previously 16PS-treated cells led to the same result. The effects were sequence-specific: 4 non-complementary phosphorothioate 16mers were without significant effect (Fig. 2).

Attempts to improve the uptake of antisense oligonucleotides by parasites led to the synthesis of various conjugates. No trypanocidal effect was observed with a 9mer conjugated to poly L-lysine (Toulmé *et al*. 1990*a*) although such conjugates were reported to have increased antiviral efficiency (Lemaître *et al*. 1987). In contrast, a significant improvement of leishmanicidal activity was achieved with oligonucleotides linked to a palmitate chain and delivered by low density lipoproteins (LDL) (Mishra, Le Tinévez & Toulmé, 1995). On the average, 15 oligomers were loaded per LDL particle. About 40% *L. amazonensis*-infected macrophages were cured upon incubation in the presence of 2·5 μM 16PS associated to oxidized LDL (through hydrophobic-interaction with the palmitate tail); thus this resulted in a 10-fold decrease in the oligonucleotide concentration required compared with the free non-conjugated 16PS. This result is possibly due to an improved uptake of the antisense sequence as the 16PS-palmitate conjugate had similar properties to non-conjugated 16PS in cell-free translation assays. It is worth noting that the effect was sequence-dependent and possibly represented a true antisense effect. Interestingly, the palmitate conjugate in the absence of LDL showed a slight but reproducible improvement over the non-conjugated antisense oligomer. The hydrophobic tail could promote the interaction with the cell membrane, as shown for cholesterol-oligonucleotide conjugates (Boutorin *et al*. 1989).

The use of various chemically-modified oligonucleotides brings some information on the mechanism responsible for the antiparasite properties of antisense oligonucleotides. The methylphosphonate, as well as the phosphorothioate or the alpha analogue sequences of Acr-9mer, were inactive against cultured procyclic trypanosomes, although all these derivatives were resistant to nucleases (Toulmé *et al*. 1990*a*). In the case of *Leishmania*, neither 2′-*O*-methyl oligoribonucleotides nor an alpha oligodeoxynucleotide, 16 nt long, complementary to the mini-exon sequence displayed any effect (Ramazeilles *et al*. 1994). One does not expect a significant difference for uptake between these compounds. Whereas the alpha oligomer is a weak binder, 2′-*O*-methyl RNAs have a higher affinity for RNA than unmodified or PS oligodeoxynucleotides. This makes it unlikely that the binding constant of the antisense sequence for its target is the key parameter for the observed differences. But, neither 2′-*O*-methyl RNA nor alpha-DNA elicit RNase H activity in contrast to phosphothioate oligomers. This might suggest that the anti-mini-exon sequences are acting through an RNAse H-dependent mechanism. If this were true, one might expect that 'sandwich' oligomers made of

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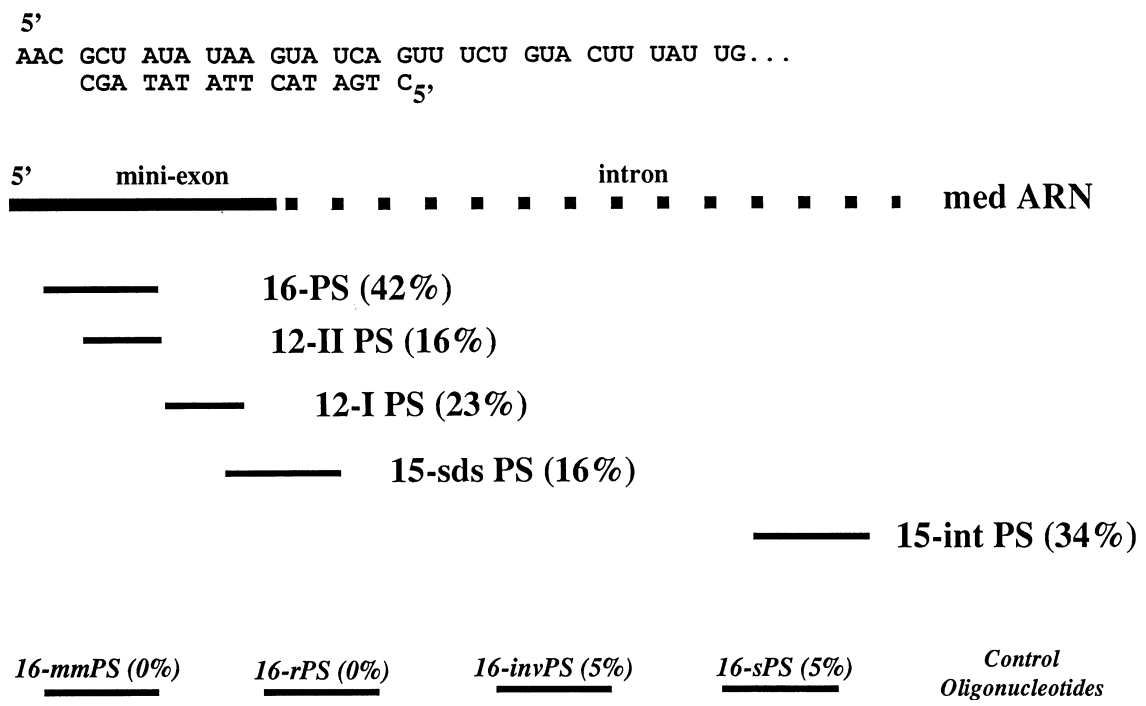

Fig. 2. Leishmanicidal properties of phosphorothioate oligonucleotides targeted to the med RNA sequence of *L. amazonensis*. Murine peritoneal macrophages were infected as described in (Ramazeilles *et al*. 1994) and incubated at 34 °C for 48 h in the presence of 25 μM oligonucleotide. The percentage of cured macrophages is indicated in parentheses for oligomers complementary to the mini-exon (16PS, 12IPS or 12IIPS), to the exon-intron junction (15-sdsPS) or the intron part (15-intPS). Non-complementary phosphorothioate 16mers, mismatched (mm), random (r), inverted (inv) or scrambled (s), have been used as control sequences. A portion of the mini-exon sequence is given at the top of the figure along with the complementary 16PS. (Adapted from Ramazeilles *et al*. 1994.)

two blocks of 2′-*O*-methyl RNA flanking a stretch of regular DNA would be active as long as the stretch is long enough to allow RNase H to cleave, i.e. 4–5 nucleotides. Such experiments are underway in our laboratory and might provide an indication about the role played by RNase H in antisense effects.

The oligomer 16PS was not the only sequence active against *L. amazonensis*. Phosphorothioate oligonucleotides targeted to other parts of the medRNA displayed leishmanicidal efficiency. This included a 15mer complementary to the intron-exon junction and a second one complementary to the very 3′ end of the medRNA (Fig. 2). This indicates that antisense oligonucleotides can interfere with the *trans*-splicing event. This also means that part of the intracellular pool of oligonucleotides has a nuclear location. Indeed, fluorescence microscopy of trypanosomes incubated in the presence of the inactive Acr-6mer indicated the presence of the dye both in the cytoplasm and in the nucleus (Verspieren *et al*. 1987).

In summary, antisense oligonucleotides complementary to the mini-exon sequence can prevent: (1) the translation of the message through binding to mRNAs in the cytoplasm; (2) the transport from the nucleus to the cytoplasm; and (3) *trans*-splicing following the association with the medRNA. This latter possibility could be either related to the splicing event itself or to the modification of the cap4 structure as shown with permeabilized trypanosomes (Ullu & Tschudi, 1991; Ullu & Tschudi, 1995).

Targeting folded RNA

The availability of a target sequence is a prerequisite for binding a complementary oligonucleotide and subsequently observing antisense effects. RNA secondary and tertiary structures will compete with the formation of oligonucleotide-RNA complexes and therefore weaken the antisense efficiency. The mini-exon sequence of *L. amazonensis* can adopt a hairpin structure (Pascolo *et al*. 1994; Verspieren *et al*. 1990). medRNAs from various trypanosomatids can fold into secondary structures containing several stem-loops (Bruzik *et al*. 1988; Harris, Crothers & Ullu, 1995; Lecuyer & Crothers, 1993). Oligonucleotides with a high binding constant can be used to unfold the RNA target. However, this might generate non-specific effects due to the formation of stable mismatched duplexes formed with non-target RNA. Alternatively, one can rationally accommodate the folded motif into a complex. This requires the detailed knowledge of the biologically active RNA structure(s). Lastly, one can use *in vitro* selection procedures to identify oligonucleotide sequences able to recognize the three-dimensional network of interacting groups in the folded RNA. In this case one does not need to know how the RNA is

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organized. In an effort to design oligomers able to bind to structured targets we undertook to investigate several possibilities to recognize the folded mini-exon sequence (reviewed in Toulmé, Le Tinévez & Brossalina, 1996).

Nucleic acid base analogues could promote the invasion of double-stranded region (Woo, Meyer & Gamper, 1996) or increase stacking interactions (Michel *et al*. 1995; Wagner *et al*. 1993). Oligonucleotides able to bind in the major groove of homopurine-homopyrimidine double-stranded nucleic acids, giving rise to triple helices can also be prepared (reviewed in Thuong & Hélène, 1993). Two major types of triplets have been identified: in the Py-Pu∗Py motif ('∗' denotes the interaction of the third strand) the oligopyrimidine third strand is parallel to the purine strand of the duplex whereas in the Py-Pu∗Pu motif the two purine stretches are antiparallel. In addition, the cytosine residues in the third strand of the Py-Pu∗Py motif have to be protonated; consequently, these triple helices are more stable at acidic pH. We took advantage of such a mode of binding to design oligonucleotides able to interact with hairpin structures. The antisense oligomer forms a double-strand with a single-stranded region of the target, at the bottom of the stem, and then folds back on itself to generate a local triple helix, giving rise to a so-called double hairpin complex. Such a structure was formed at pH 6·0 with an oligopyrimidine targeted to a DNA hairpin (Brossalina & Toulmé, 1993), inhibiting the digestion of the hairpin by a restriction enzyme (Brossalina, Pascolo & Toulmé, 1993).

Unfortunately, the putative hairpin structure formed by the *Leishmania* mini-exon sequence does not show an appropriate nucleic acid base distribution for triple helix formation, involving canonical triplets T-A∗T and C-G∗C^+. The formation of a double hairpin complex required to accommodate inverted base pairs, i.e. to 'read' six interspersed pyrimidines out of 15 bases. We designed a 29mer oligonucleotide able to form 10 Watson-Crick base pairs and 15 triplets which included 4 non-isomorphic A-U∗G and 2 G-C∗T triplets in addition to 10 canonical triplets. Electrophoretic mobility shift assays and footprinting studies demonstrated that this antisense oligomer binds to either the DNA or the RNA version of the *Leishmania* mini-exon sequence, giving rise to a double hairpin complex at pH 6·0, despite the presence of 6 non-canonical triplets (Pascolo & Toulmé, 1996). This demonstrates the possibility to target folded RNA with antisense oligomers. Oligonucleotides able to bind at neutral pH can be obtained by using derivatives such as 5-methyl- or 5-propynyl-cytosine (Wagner *et al*. 1993; Xodo *et al*. 1991).

The screening of large libraries comprising billions of different nucleic acid sequences allows the selection of oligonucleotides recognizing any kind of predetermined target (see Ellington & Conrad, 1995; Gold *et al*. 1995, for reviews). We used *in vitro* selection procedures to identify oligonucleotides able to bind to a hairpin structure. Starting from a DNA library made of oligonucleotides with 16 random positions (containing therefore 4^{16} [about 4 billion] different sequences) we identified, after 4 rounds of selection, three oligomers that recognized a DNA hairpin (Mishra & Toulmé, 1994). Examination of the selected sequences did not detect any complementarity between the random stretch and the target. Structural analysis of the complexes formed by the aptamers (the selected oligomers) and the target revealed an overall organisation similar to a double hairpin complex. However, in contrast to what was obtained with oligopyrimidines forming a Py-Pu∗Py triple helical structure, aptamers were bound at neutral pH (Mishra, Le Tinévez & Toulmé, 1996). This combinatorial strategy might allow the design of oligonucleotides able to recognize a folded RNA motif. Importantly, one does not need to know the structure of the target to perform *in vitro* selection.

CONCLUSION

Antisense oligonucleotides hold great promise as inhibitors of gene expression at post-transcriptional level. Chemical modifications introduced in the molecules improve nuclease resistance and uptake efficiency. Efforts are being made to understand better the mechanisms responsible for the observed biological effects. This will undoubtedly result in the improvement of specificity and cell-compartment targeting. *In vitro* selection procedures may lead to the development of new strategies to take into account peculiarities of the target. Oligonucleotides are currently powerful tools to delineate gene function. Pharmacological and toxicological studies for these compounds are underway. The first clinical trials in humans will soon tell us whether new drugs can be made from these compounds.

ACKNOWLEDGEMENTS

This work was supported in part by the 'Pôle Médicament Aquitaine', by the 'Direction des Recherches, Etudes et Techniques', and by an East–West contract from INSERM. L. Yurchenko was the recipient of a P.A.S.T. position at the University Victor Segalen Bordeaux 2.

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https://doi.org/10.1017/S0031182097001005 Published online by Cambridge University Press