Comparative Evaluation of Seven Oligonucleotide Analogues as Potential Antisense Agents

F. Morvan,† H. Porumb,§ G. Degols,‡ I. Lefebvre,† A. Pompon,† B. S. Sproat,∥ B. Rayner,† C. Malvy,§ B. Lebleu,‡ and J.-L. Imbach*

Laboratoire de Chimie Bio-Organique, URA 488 du CNRS, et Laboratoire de Biochimie des Protéines, URA 1191 du CNRS, Université de Montpellier II, 34095 Montpellier Cédex 5, France, Laboratoire de Biochimie Enzymologie et de Physico-chimie Macromoléculaire, URA 147 du CNRS, Institut Gustave Roussy, 94800 Villejuif, France, and European Molecular Biology Laboratory, Postfach 10. 2209, W-6900 Heidelberg, Germany

Received July 17, 1992

12-Mer analogues, representative of seven different classes of structurally modified oligonucleotides and complementary to the same target, have been compared for their binding affinity for both single-stranded DNA and RNA, resistance to hydrolysis by nucleases in culture medium (RPMI 1640 + 10% inactivated fetal calf serum), and inhibition of HIV-1 replication in de novo infected MT4 T lymphocytes. The viral target was the splice acceptor site of the premessenger coding for the regulatory protein *tat*.The oligo(2′-*O*-alkyl)ribonucleotides (β-2′*O*-allyl-RNA and β-2′OMe-RNA) were shown to form the most stable hybrids with complementary RNA strands whereas the α-anomeric oligodeoxynucleoside phosphorothioate analogue displayed the highest stability in the culture medium. All the modified oligonucleotides examined in the present study exhibited a sequence-nonspecific inhibitory effect on HIV-1 replication, the phosphorothioate analogues being the most active ones ($ED_{50} < 1\ \mu M$).

Introduction

The development of oligonucleotides as potential therapeutic agents has received increasing attention during the past years, as these compounds can interfere specifically with transcription, mRNA processing, RNA stability, or translation.[1,2] The main hurdles in the therapeutic development of oligonucleotides are sequence-nonspecific interactions, sensitivity to nucleases, and low cellular delivery. To overcome these limitations numerous structural variations have been proposed ranging from phosphate backbone (i.e. methylphosphonate,[3] phosphorothioates[4]) to sugar (i.e. 2′-substituted ribonucleosides,[5] α-configuration[6]) modifications.

All these oligonucleotide analogues are more resistant to nuclease degradation than their unmodified counterparts, bind specifically to complementary nucleic acid strands, and exhibit some biological activity when incubated in cell-free systems or injected into cells. Up to now, few studies comparing various analogues have been performed;[7-9] moreover none of them attempted to correlate hybridization properties, nuclease resistance, and biological efficiency as done here on a series of seven different species of oligonucleotide analogues.

The choice of the common target is of prime importance as this comparative study includes antisense species which do not induce the target RNA cleavage by RNase H (i.e. α-oligos[10,11] and methylphosphonate oligos[12]). We selected as a common target a splice site. Oligos binding to such a site may prevent the interaction of the necessary factors and hence inhibit the splicing reactions and subsequent formation of the mature mRNA. In this respect the *tat* splice acceptor site has been previously shown to be one of the most active sites on HIV for RNase H-dependent[13] as well as for nondependent oligos.[14,15] In addition, as it has been demonstrated that oligos rapidly diffuse and concentrate within the nucleus when microinjected,[16,17] likewise oligonucleotides added in the culture medium accumulate in cell nuclei.[18] One can expect a nuclear target to be more effectively addressed than those located in the cytoplasm.

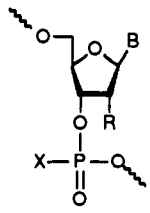
Antisense length should achieve specificity under physiological conditions.[19] It has been calculated[1] that a 11–15 mer sequence (depending on its base composition) can only be represented statistically once in the mRNA population of higher eukaryotes. The effect of mismatches on the binding energy indeed decreases when oligonucleotide length increases. In addition, since the oligo targets are intracellular, oligos must be able to traverse the cellular membrane and as their uptake is length dependent, short oligos are again to be preferred since they are more readily taken up than longer ones.[21] Therefore, we compared the behavior of a series of 12-mer modified oligonucleotides (Figure 1) all directed at this specific *tat* target (Table I).

As the high stability of duplex formed with the complementary nucleic acid sequence is essential for the eventual use of oligos as antisense agents, the melting temperature (Tm) of these compounds was determined upon hybridization with complementary synthetic DNA or RNA strands. Surprisingly, previous studies made use of DNA targets most commonly and in some cases of synthetic RNA targets[20] while the target is obviously a cellular or viral RNA with its own secondary structure. It seemed important therefore to determine the thermoelution temperature (Tc) of these oligos with a long *tat* RNA fragment (259 nucleotides) transcribed in vitro from pMG755 plasmid.

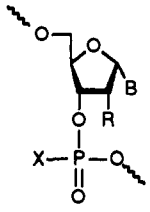
The stability of the oligonucleotides against nucleases in culture medium and host cells is another important factor to be determined. All the modified oligonucleotides in the present study exhibit an increased or total resistance to nuclease degradation, suggesting that they would be good candidates for antisense experiments. However, the

* Laboratoire de Chimie Bio-Organique, URA 488 du CNRS, Université Montpellier II, Place E.-Bataillon, 34095 Montpellier Cédex 5, France.
† Laboratoire de Chimie Bio-Organique.
‡ Laboratoire de Biochimie des Protéines.
§ Institut Gustave Roussy.
∥ European Molecular Biology Laboratory.

0022-2623/93/1836-0280$04.00/0



1 β-DNA: X = O^-, R = H
4 β-2′-OMe-RNA: X = O^-, R = OCH_3
5 β-2′-O-allyl-RNA: X = O^-, R = $OCH_2CH{=}CH_2$
6 β-Me-P-DNA: X = CH_3, R = H
7 β-S-DNA: X = S^-, R = H



2 α-DNA: X = O^-, R = H
3 α-RNA: X = O^-, R = OH
8 α-S-DNA: X = S^-, R = H

Figure 1. Structure of the oligonucleotide analogues.

Table I. Sequences of the Different Oligonucleotide Analogues Complementary to HIV-1 *tat* Gene and Control Sequences[a]

genomic RNA: 5′...^{5357}AGAAUUGGGUGU^{5368}...3′

β-configuration: antiparallel orientation	entries	α-configuration: parallel orientation	entries
antisense sequences		antisense sequences	
5′ACACCCAATTCT3′	1, 6, 7	5′TCTTAACCCACA3′	2, 8
5′ACACCCAAUUCU3′	4, 5	5′UCUUAACCCACA3′	3
control sequences		control sequences	
5′CCCCTTAAATCA3′	1, 6, 7	5′ACTAAATTCCCC3′	2, 8
5′CCCCUUAAAUCA3′	4, 5	5′ACUAAAUUCCCC3′	3

[a] The control sequence, with the same base composition, was determined in order to have minimum complementarity to the HIV BRU sequence (see Experimental section).

previous enzymatic stability data are incomplete either because only purified enzymes were used or because gel electrophoretic analysis of ^{32}P-labeled oligos allows only the detection of 5′-labeled fragments. Therefore, instead of evaluating oligo stability using the previously reported approaches, we determined their comparative enzymatic behavior in culture medium using an original HPLC technique that we have recently introduced.[22] This procedure, which does not require any sample pretreatment, permits the detection of any oligo fragment thus allowing a more realistic evaluation of the oligos enzymatic stability in biological milieu.

Finally, we determined the ability of these analogues to inhibit HIV-1 replication using a well established de novo infection assay in MT4 T lymphocytes.

Results and Discussion

The applicability of antisense oligonucleotides as inhibitors of gene expression is limited by various factors ranging from solubility, hybridization with complementary targets, and sensitivity to degradation by nucleases to cellular uptake. Up to now, no antisense analogue yet developed has fulfilled all the criteria of an ideal antisense effector, and improvement in one particular property has often been counteracted by losses in another. With the exception of the methylphosphonate oligos, all the antisense oligonucleotides of the present study (see Figure 1 for structures and Table I for sequences) bear a polyanionic backbone which ensures their aqueous solubility.

The first key factor examined was their capacity to form stable heteroduplexes with the target. The overwhelming majority of antisense molecules are designed to exert their effect upon hybridizing to RNA species. However, in many cases, the hybridization ability of antisense oligonucleotides has been assessed on the basis of Tm measurements with unmodified complementary oligodeoxynucleotides (DNA) rather than the less accessible oligoribonucleotides (RNA). This may lead to erroneous conclusions as the stability of antisense/DNA and antisense/RNA heteroduplexes may be considerably different. For example, it has been reported[23] that progressive replacement of phosphodiester with methylphosphonate linkages leads to progressive and significant decrease of Tm of oligodeoxynucleotide/RNA heteroduplexes and furthermore that 2′-*O*-methyl oligoribonucleotides hybridize to RNA more strongly than to DNA of the same sequence.[24] Thus, we decided to evaluate the hybridization properties of all the antisense oligos against synthetic DNA and RNA complementary strands. In addition, it seemed worthwhile to confirm the stability of the antisense/RNA duplexes using thermoelution measurements. In this case the target within a 259 nucleotide long RNA obtained from an appropriate plasmid may be closer in its secondary structure to that present in vivo with the full length pre-mRNA.

A comparison of the Tm data shown in Table II and obtained from melting curves at high (1 M NaCl) and low (0.1 M NaCl) salt concentration confirms the importance of the ionic strength on the duplex stability of charged oligos. As expected, the neutral methylphosphonate oligo presents roughly the same Tm values at both ionic strengths, which reflects the lack of charge–charge repulsion for duplex formation. It is also worth noting a second transition at 44 °C observed with α-RNA at 1 M NaCl, which corresponds to a 2α/1β stoichiometry.[25]

The duplexes formed with either β-DNA or α-DNA present roughly the same stability whether their complementary counterpart is a DNA or a RNA (Table II, condition 2). However, the α-DNA heteroduplexes are slightly less stable than the β-DNA ones. This difference may be connected to the number of α-Pyr/β-Pur base pairs as commented on in a previous work.[26] The introduction of a sulfur atom on the phosphate backbone decreases the stability of the corresponding heteroduplexes by about 3 °C in the α-DNA but by 10 °C in the β-DNA as expected. Under low salt conditions, destabilization observed with the α-RNA heteroduplexes may be tentatively explained by steric effects between the 2′-OH group and the nucleobase giving rise to less stable Watson–Crick base pairing.

Noteworthy is the behavior of the two β-2′-substituted RNA analogues. Both heteroduplexes formed with RNA are much more stable than with DNA by about 19 °C, the β-2′-OMe-RNA heteroduplexes being slightly more stable than the corresponding β-2′-*O*-allyl-RNA. In contrast, the β-Me-P-DNA oligo hybridized poorly to RNA. The decrease in the Tm value is about 13 °C relative to the DNA heteroduplex. These observations demonstrate conclusively that the interaction of modified oligos with DNA is not a good model for their interaction with RNA. In other words, the well accepted assumption that DNA can be used as a model for the mRNA sequence may give

Table II. Thermal Stabilities of Hybrids Formed between Antisense Oligomers and DNA or RNA Target

		melting temperature (Tm)[a] (±0.5 °C)			
		condition 1	condition 2		thermoelution (Tc)[b]
entry	oligos	vs 12-mer DNA	vs 12-mer DNA	vs 12-mer RNA	(±1 °C) vs transcribed RNA
1	β-DNA	57.0	47.6	46.1	39
2	α-DNA	58.5	42.8	43.1	33
3	α-RNA	25.5 44.0	14.3	16.5	53
4	β-2′OMe-RNA	55.0	39.0	58.4	55
5	β-2′*O*-allyl-RNA	ND	35.7	54.0	54
6	β-Me-P-DNA	40.8	40.3	27.4	ND
7	β-S-DNA	ND	37.9	36.1	33
8	α-S-DNA	ND	40.3	40.3	30

[a] Tm Condition 1: 10 μM for each synthetic strand in 1.0 M NaCl, 10 mM Na cacodylate pH 7.0. Condition 2: 3 μM for each synthetic strand in 0.1 M NaCl, 10 mM Na cacodylate pH 7.0. [b] Tc: labeled oligonucleotides hybridized with a 259 nucleotide long RNA (from pMG755) containing the target, immobilized on a nitrocellulose membrane, were thermoeluted in 6xSSC (0.9 M NaCl, 90 mM Na citrate pH 7.2).

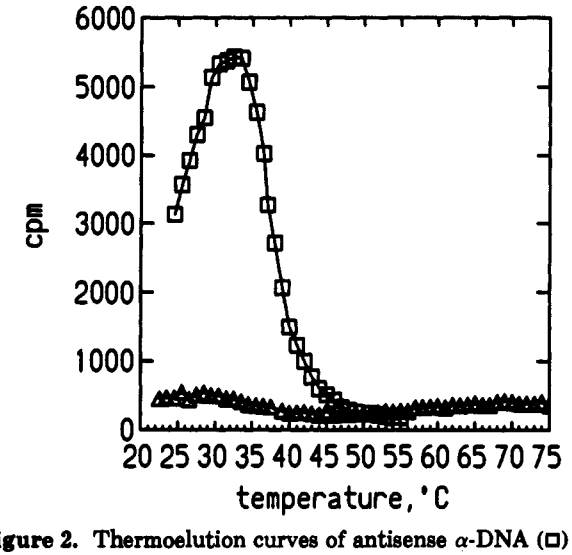


Figure 2. Thermoelution curves of antisense α-DNA (□) and control α-DNA (△).

rise to misleading conclusions. In addition, with the exception of α-RNA which presents unsolved structural problems, all phosphodiester and phosphorothioate analogues deserve a general comment concerning the stability of their duplexes. By averaging the melting temperatures, the values of 38, 42, and 56 °C are obtained respectively for the DNA/RNA, DNA/DNA, and RNA/RNA duplexes under conditions 2. These values are in accordance with recent data[27,28] and may be related to the fact that RNA/RNA duplexes are in the more stacked A form.

The thermoelution results confirm the validity of the above findings for a target which might be expected to adopt a secondary structure closer to reality. It was first ascertained that (i) the antisense oligonucleotide generated, in each case, a well-defined elution peak, whereas the control sequence containing the same bases in a different order, showed no annealing (Figure 2, Table II); (ii) the antisense oligonucleotides did not hybridize to the noncomplementary RNA strand obtained by transcription from the plasmid in reverse direction (not shown). Qualitative tests, by Northern blotting, also confirmed the specificity of the oligonucleotides solely for the RNA molecule bearing the target (not shown). It is clear from the last columns of Table II that the oligos of RNA type (entries 3, 4, and 5) have the most favorable elution temperatures, higher than 50 °C under the present experimental conditions. The elution temperatures of the oligos derived from DNA were lower by 14 or even 25 °C. It is not known whether the strong binding of α-RNA, which was in a high ionic strength environment close to condition 1 of Tm experiments, was due to the formation of a 2α/1β complex. If this was the case, the shoulder present on the low temperature side of the elution curve (not shown) could then be assigned to the classical duplex.

Table III. Half-life of Oligonucleotide Analogues and Their First Degradation Product (11-mer) in RPMI 1640 Medium Supplemented with 10% Inactivated Fetal Calf Serum

		$t_{1/2}$	
entry	oligos	12-mer	11-mer
1	β-DNA	31 min	13 min
2	α-DNA	41 min	14 h
3	α-RNA	14 min	45 h
4	β-2′OMe-RNA	68 min	58 min
5	β-2′*O*-allyl-RNA	60 min	ND
6	β-Me-P-DNA	ND	ND
7	β-S-DNA	45 h	30 h
8	α-S-DNA	10 days[a]	>100 days[a]

[a] Extrapolated value.

The second key factor which was important to evaluate was the comparative enzymatic stability of these oligo series as some of them have been described as nuclease resistant.[4,29-31] Since we planned to investigate the biological activities of these oligos in cell culture, we determined their half-life in culture medium. For this purpose, the ISRP on-line HPLC technique was used.[22] The oligos half-life (based upon the disappearance of the starting oligos) in culture medium are presented in Table III. It is apparent that all the phosphodiester derivatives (entries 1–5) were half degraded within 1 h. The phosphorothioate oligos (entries 7 and 8) were, as expected, very resistant to degradation, the α-S-DNA (entry 8) being the most stable with a half-life of more than 10 days. Noteworthy is the pattern of decomposition of these oligos which depends on the anomeric configuration of the constituent nucleosides. For all the β-anomeric oligomers (entries 1, 4, 5, and 7) only a processive hydrolysis from the 3′ end was observed. Figure 3 presents the HPLC analysis of the antisense β-2′-*O*-allyl-RNA degradation products in culture medium and the corresponding kinetic curves of the different species. Under the same conditions, the behavior of the three α-anomeric oligomers (entries 2, 3, and 8) was surprising as each of them was degraded to a single shorter entity which was then very stable (Figure 4). We have as yet no explanation for this degradative process except that it is a 3′-end degradation. Work is in progress to elucidate the exact mechanism of hydrolysis of the α-series and to identify the exact nature of their degradation products, but one has to keep in mind that the enzymatic behavior of α-oligos always differs from that of the β series.[32]

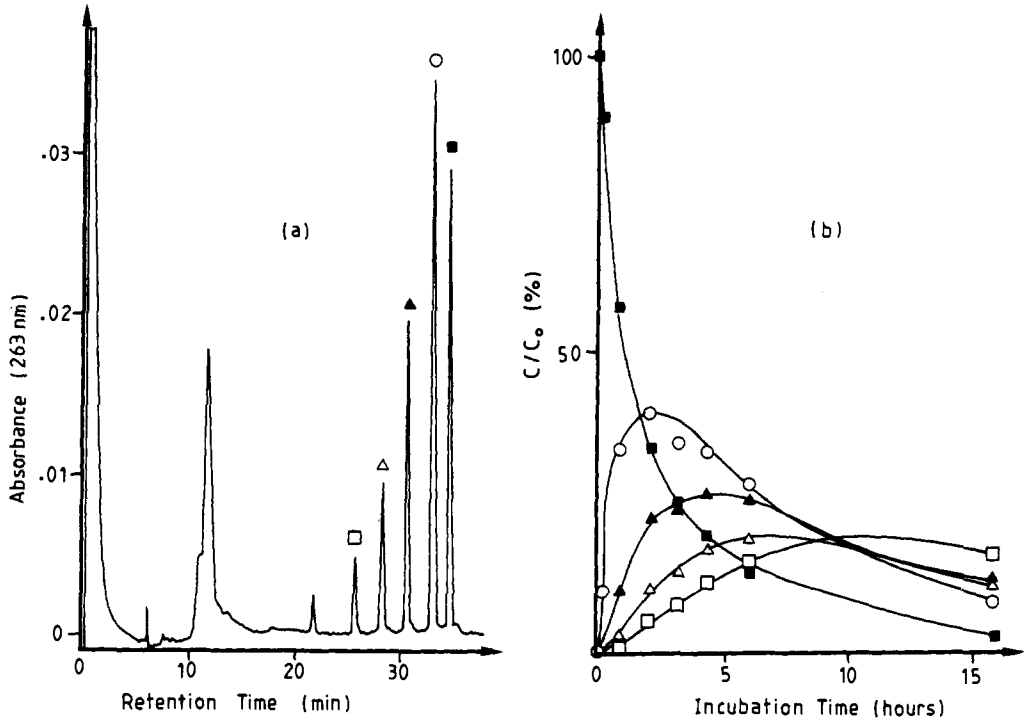


Figure 3. Degradation at 37 °C of antisense β-2′*O*-allyl-RNA in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum. Initial concentration was 10^{-6} M [(■) parent oligomer, (○) 11-mer, (▲) 10-mer, (△) 9-mer, (□) 8-mer]. (a) HPLC analysis of a crude sample after 2-h incubation. Signals with retention time lower than 15 min correspond to compounds already present in the culture medium; (b) relative concentrations versus time of incubation.

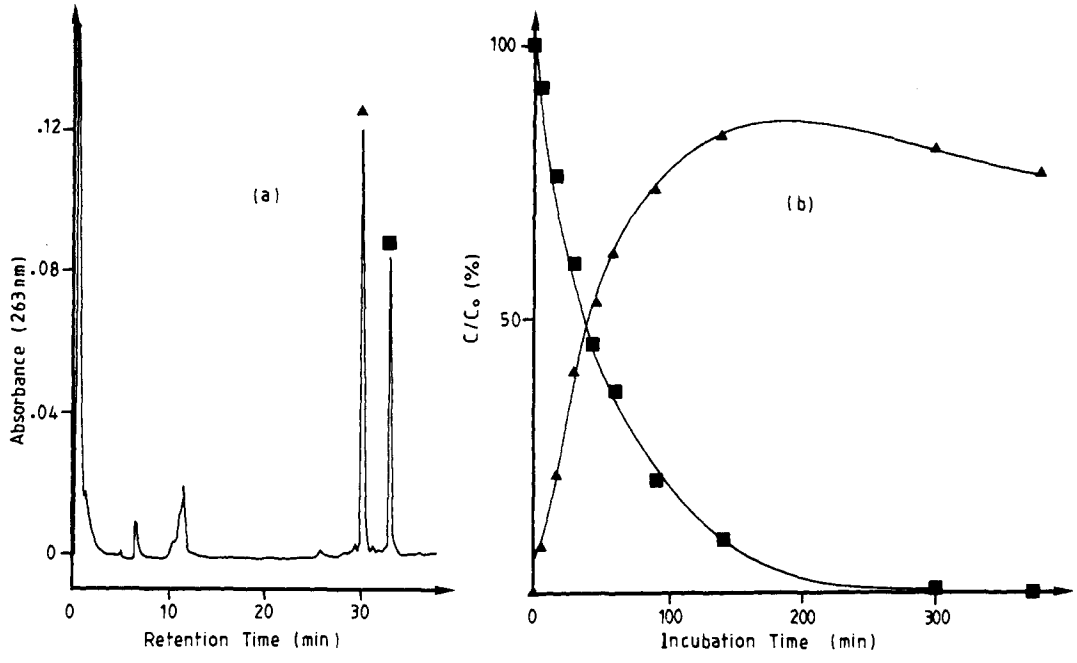


Figure 4. Degradation at 37 °C of antisense α-DNA in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum. Initial concentration was 10^{-6} M [(■) parent oligomer, (▲) 11-mer]. (a) HPLC analysis of a crude sample after 4-h incubation. Signals with retention time lower than 15 min correspond to compounds already present in the culture medium; (b) relative concentrations versus time of incubation.

Coming back to the β-anomeric oligonucleotide series the following comments can be made. The observed processive decomposition pattern is in accordance with 3′-exonuclease activities and corroborates previous independent reports indicating that in serum[31-34] and in plasma[35] 3′-phosphodiesterases play a predominant role in the breakdown of oligos. In this work neither endonucleolytic nor 5′-exonuclease activities were detected, which is in accordance with Hoke's and Eder's data[35] but differs from data reported in another study where both a 3′-exonuclease and a substantial endonuclease activity were reported in heat-inactivated fetal calf serum (50 °C, 30 min).[28] It is noteworthy that our experiments were conducted in culture medium containing 10% heat-inactivated fetal calf serum (65 °C, 30 min), a treatment which has been shown to inactivate endonuclease but not exonuclease activities.[36] Our data confirm that heat inactivation does not destroy all nucleases but only reduces the rate of oligo degradation.[21,22] However, the results obtained with inactivated sera do not totally account for

Table IV. Comparative Inhibition Efficiencies of the Different Oligonucleotide Analogues against HIV-1 Infection

genomic RNA: 5′...^{5357}AGAAUUGGGUGU^{5368}...3′

entry	oligos	ED_{50} (μM) in MT4 cells[a] antisense	control
1	β-DNA	>100	nd
2	α-DNA	60	70 ± 5
3	α-RNA	10–20	10–20
4	β-2′OMe-RNA	20 ± 3	>50
5	β-2′O-allyl-RNA	4 ± 0	10 ± 1
6	β-Me-P-DNA	38 ± 7	nd
7	β-S-DNA	0.8 ± 1.5	0.2 ± 0.1
8	α-S-DNA	0.25 ± 0	0.6 ± 0

[a] MT4 cells were infected with HIV-1 BRU as described in Experimental Section. ED_{50} were estimated from RT values in three independent experiments.

the stability of oligonucleotides in cells. Indeed, Hoke et al. suggested that endonucleases may be the predominant nuclease activity in intact cells.[34]

Inhibition of HIV replication with these oligonucleotide analogues targeted at the *tat* splice acceptor site[13,37] is presented in Table IV where the data are expressed in terms of ED_{50} values. Sequences of the same length and with the same base composition were used as control oligomers. These control sequences have been checked by computer search not to correspond to any other target in the HIV genome. In addition, we have verified that they do not bind to the 259 base long RNA (data not shown).

From the data presented in Table IV, the following comments can be made: (i) all the antisense oligos exhibit a nonspecific inhibitory activity with the control oligomers producing roughly the same effect; (ii) no clear correlations can be drawn between the oligo inhibitory activity and their hybridization capacities (Table II). Enzymatic stability (Table III) and antiviral activity (Table IV) do correlate to a large extent. For instance, the absence of an inhibitory effect for the 12-mer β-DNA may be tentatively related to its rapid degradation. It is noteworthy that the α-RNA exhibits the shortest half-life ($t_{1/2}$ = 14 min) but in contrast to the β-oligos, the resulting 11-mer is very stable (vide supra); (iii) the two phosphorothioate analogues, in α or β configuration, produced the greatest effects (about 1 log of difference for the ED_{50} as compared to the phosphodiester oligos). Noteworthy is the activity of the β-2′-OMe-RNA which has been previously described as inactive when directed against the same RNA target.[15] However, the corresponding phosphorothioate oligos have been previously reported to inhibit HIV expression.[15] The stronger inhibitory effect observed with phosphorothioate oligos could be related to an increase in lipophilicity resulting from the replacement of the oxygen atom by sulfur and thereby giving rise to a greater affinity for membrane proteins and/or for reverse transcriptase.[21,38,39] In keeping with this, it has been reported that phosphorothioate oligos are more strongly associated with cell membranes than the corresponding phosphodiester ones.[21,40] A nonspecific inhibition of the viral cytopathic effect is not limited to HIV-infected cells but has also been shown for other virus-infected cells. It has been reported that phosphorothioate oligos can inhibit in vitro reverse transcription.[41] Furthermore, it has been proposed that phosphorothioate oligos can block gp 120-CD4 binding, hence suggesting that they may exert their cytoprotective effects by interfering with the binding of HIV to the target cells.[42] Inhibition of interactions with other sites of virus replication cannot be excluded as well. In the experimental protocol which has been used here (see Assays for HIV Inhibition in Experimental Section) the oligonucleotides have been added after adsorption of the virus thereby limiting but by no way excluding interference at this stage of infection. The inhibitory activity of these various analogues on purified recombinant HIV reverse transcriptase is under way in our laboratory.

Conclusion

It has been shown in this study that evaluation of the ability of oligo analogues to be used as potential antisense agents should be undertaken under more appropriate experimental conditions than those previously used. In this respect hybridization experiments (Tm determination) must be conducted against a RNA target and not against a DNA oligomer as the latter may give rise to misleading results. In addition, the determination of the thermoelution stability of oligos using long RNA fragments is required to support the oligos stability data obtained from the Tm experiments. Furthermore, nuclease resistance of oligos can be accurately evaluated using an HPLC technique. Seven antisense analogues directed at a common target—the HIV *tat* splice acceptor site—have been evaluated comparatively. The inhibitory effect of these various oligomers was determined on HIV-infected MT4 cells and it was shown that all the oligo analogues studied exhibit a nonspecific inhibitory effect on the de novo infection. This non-sequence-specific effect may be related to binding of the oligomers to membrane proteins thereby interfering with gp 120-CD4 interaction in accordance with that that was previously proposed for the phosphorothioate oligonucleotides.

Experimental Section

Oligonucleotides. The methylphosphonate oligonucleoside was kindly supplied by G. Zon (Applied Biosystems Inc., Foster City, CA). α-DNA,[43] α-RNA,[25] β-2′-OMe, and β-2′-O-allyl RNA[44,45] dodecamers were synthesized with an Applied Biosystems 381 A synthesizer with use of the phosphoramidite chemistry and characterized according to already published procedures. Oligonucleoside phosphorothioate analogues were obtained by substituting iodine solution by a solution of 0.05 M 3H-1,2-benzodithiol-3-one 1,1-dioxide[46] in acetonitrile during the assembling on the synthesizer. All these oligonucleotides were purified by C_{18} reverse-phase HPLC and their homogeneity was ascertained by gel filled capillary (Micro-Gel_{100}, Applied Biosystems Inc.) electrophoresis analysis. The control sequence was determined thanks to a quick basic program written for a PC computer by Dr. G. Muzard (Villejuif, France). This program generates random sequences and looks for the number of complementary bases within the HIV genome. It selects sequences which have the lowest complementary. The sequence CCCUUAAAUCA was selected after the generation of 6800 random sequences with the anti-*tat* oligonucleotide base composition: A(4); U or T(3); C(5). After checking a few sequences with the CITI 2 program and gene bank, only one sequence was found to present as low as eight points of recognition (for 21 different sites).

Melting Temperature. Melting curves were recorded on a UVIKON 810 spectrophotometer (Kontron) interfaced to an IBM PC compatible computer. The temperature control was through a HUBER PD 415 temperature programmer connected to a refrigerated water bath (Huber ministat). Experiments were performed with equimolar mixtures of complementary oligonucleotides each at a concentration of 10 μM in 1.0 M NaCl, 10 mM sodium cacodylate, pH 7 (condition 1), or of 3 μM in 0.1 M NaCl, 10 mM sodium cacodylate, pH 7 (condition 2). The cell

compartment was flushed with dry nitrogen during measurements below ambient temperature. The heating rate was 0.5 °C min^{-1}. Each sample was heated to 80 °C and slowly cooled before recording the melting curve. Tm values were determined from the maxima of first derivative plots of absorbance versus temperature.

Plasmid Manipulations. The plasmid pMG755, containing the fragment EcoRI (5750) to Sac I (6009) of HIV-1 (Arv 2) in pBluescript KS^- (Stratagene) has been obtained from M. Gait (M. R. C. Laboratory of Molecular Biology, Cambridge, UK). The sequence which contained a Sac I fragment (684–6014) of HIV-1 (ARV-2) had been cloned in pUC19 by T. Kimura of that laboratory. Following linearization of the plasmid by Sac I, the T3 polymerase transcript is the sense strand, containing 259 nucleotides of the acceptor splice junction of the HIV-1 pre-mRNA with an additional 12 bases at the 5′ end being derived from the polylinker. Linearization by Eco RI and transcription by the T7 polymerase generates the (−) RNA strand. The plasmid identity was tested by restriction mapping. Following linearization by either of the two restriction enzymes, the plasmid was purified by electroelution from agarose gel, Sephadex G-50 gel-filtration and ethanol precipitation. The size and quality of the sense and antisense RNA transcripts were verified by 4% (w/v) polyacrylamide/8 M urea denaturing gel electrophoresis. The presence of the target sequence and the specificity of the interaction with the oligonucleotides were confirmed by formaldehyde/0.8% agarose denaturing gel electrophoresis, Nothern blotting onto a nylon membrane, and hybridization to *β-tat* sense and antisense 12-mers in the presence of 6xSSC-10xDenhardt's/0.1% (w/v) SDS medium. The level of nonspecific binding was lower than 0.1%.

Thermoelution. The oligonucleotides were phosphorylated with T4 polynucleotide kinase (Biolabs), except for the methylphosphonate, which is not a substrate of this enzyme. Gel electrophoresis in 20% (w/v) polyacrylamide indicated that the label had been incorporated to better than 90% in all samples. The oligonucleotides were phenol-extracted, gel-filtered in spin columns containing Sephadex G-10, heated to 80 °C, and cooled on ice, and then 20 pmol of termini were transferred to the hybridization medium (6xSSC-10xDenhardt's medium) and allowed to hybridize overnight at room temperature to RNA (about 5 pmol target sequence), previously immobilized at 80 °C on a nitrocellulose membrane. To confirm the persistance/integrity of the oligonucleotides during hybridization, aliquots of the hybridization medium, at the end of the incubation, were run on a 20% (w/v) polyacrylamide electrophoresis gel. All initial radioactivity was accounted for by free oligonucleotides in the hybridization medium and by the oligonucleotide in the thermoelution peaks (see below). No degradation was noted. The membrane was mounted in the filter support of the thermoelution apparatus[47] and the column was filled with 6xSSC buffer at 18 °C (0.9 M NaCl, 0.09 M Na citrate, pH 7.2). This buffer was pumped across the membrane and 300-μL fractions were collected every minute. After the tenth fraction, a temperature gradient of 72 °C/h was imposed and monitored by a thermocouple in contact with the membrane. The radioactivity of each fraction was counted. The delay due to the length of the tubing through which was collected the eluent was taken into account and this enabled the assignment of a temperature to each fraction. The critical elution temperature corresponds to the fraction where maximum dehybridization occurred. Elution curves (radioactivity vs temperature) were plotted pairwise at the same scale for the targeted and the corresponding control (random) oligonucleotide eluted from the same RNA, to emphasize the specificity of binding. (A broadening of the base of the elution peak in the case of the *β*-2′-*O*-allyl-RNA and *β*-2′-OMe-RNA was found to be associated with the presence of a slowly-migrating aggregate of unknown nature). Further control experiments included the thermal elution of the antisense oligonucleotides from the (−) RNA strand and the comparison of their elution peaks to the peak obtained with an oligonucleotide complementary to this RNA. All oligonucleotides used in this study were labeled to the same specific radioactivity and all membranes contained identical amounts of RNA to enable an eventual analysis of the amplitudes of the elution peaks should this parameter become better understood.

Oligonucleotide Stability in Cell Culture Medium. HPLC was performed on a Waters-Millipore instrument (Bedford, MA), equipped with a M712 autosampler and a M990 diode-array UV detector. A six-port 4010 Rheodyne valve allowed switching between the precolumn and either the detector or the column. Precolumn and column were purchased from SFCC/Shandon (Eragny, France). Distilled water was purified on a Milli-Q system (Millipore). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and potassium chloride were pro analysi grade (Merck, Darmstadt, Germany). Cell culture medium RPMI 1640 (ref 041-02401) and fetal calf serum were purchased from Gibco BRL (Uxbridge, U.K.). Antibiotics were purchased from Bohringer (Mannheim, Germany). A stock solution of cell culture medium containing 10% (v/v) heat-inactivated serum (30 min at 56 °C), penicillin (20 IU/mL), and streptomycin (20 μg/mL) in RPMI was filtered (0.22 μm, Millex GS) and then divided into 1.5-mL sterile containers which were stored at −20 °C. For each kinetic study, one A_{260} unit of oligonucleotide was diluted to 1 mL with one thawed aliquot of culture medium in an ice bath, vortex-mixed, and divided up into 10 conic inserts used in the autosampler. Inserts were tightly stopped and stored immediately at −20 °C. Sterile procedures were used for all handlings. For each measure, one insert was quickly thawed, vortex-mixed, incubated at 37 °C for the required duration, and frozen again. Immediately before HPLC analysis, an insert was quickly thawed and vortex-mixed, and 50 μL were injected without further treatment. Since important variations in oligomer stability were observed with different batches of serum, the same batch of serum was used for all the oligonucleotide analogues, and samples were subjected to the same cycle of cooling–heating, with a view of minimizing possible variations of the phosphodiesterase activity.

The recently described "On-line Internal-Surface Reversed-Phase (ISRP) Cleaning" technique which allows the direct HPLC analysis of drugs and metabolites in biological fluids was used in this work: the crude sample was injected into a short precolumn of ISRP materials (Ultrabiosep C_8, 10 μM, 4.6 × 10 mm). According to the properties of ISRP materials, proteins, salts, and other unwanted compounds were quickly eluted by means of a buffer A (20 mM potassium phosphate, 0.2 M potassium chloride, pH 6.0), while retaining the oligonucleotides and their degradation products. These were next transferred and concentrated at the front of an analytical column which combines ion-exchanger and reversed-phase properties (Ultranucleotide 4000-10, 10 μm, 4.6 × 100 mm). Transfer occurred by means of an intermediate solvent B (20% acetonitrile (v/v) in 20 mM potassium phosphate, 0.2 M potassium chloride, during 3 min), when actuating the valve. At last, oligonucleotides were chromatographed according to their charge and lipophilic contents when increasing the ionic strength of the solvent: linear gradient in 30 min from B to C (as B, except 0.4 M potassium chloride).

Assays for HIV Inhibition. The MT4 cell line (human T cell leukemia virus type 1 (HTLV-1)-transformed human leukemic $CD4^+$ cell line provided by N. Yamamoto, NIH, Tokyo, Japan[48]) was maintained at 37 °C and 5% CO_2 in RPMI medium supplemented with 10% (v/v) decomplemented fetal calf serum, 2 mM glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin. These cells, harvested during the exponential growth phase, were concentrated to 3 × 10^6 cells/mL, infected with an equal volume of HIV-1 BRU[49]-containing medium (1000 $TCID_{50}$), and incubated for 30 min at 4 °C. Cells were washed, diluted to 3 × 10^5 cells/mL, and incubated at 37 °C in the presence of oligomers. Five days after infection, culture samples were removed to determine syncytia formation and reverse transcriptase (RT) activity. RT assays were performed upon 1-mL samples of infected cell supernatant as already described.[50] Cytotoxicity of oligomers was monitored by trypan blue exclusion 2 days after infection.

Acknowledgment. We are particularly indebted to Dr. J.-J. Toulmé for having permitted us to reproduce his thermal elution system. We also thank Dr. G. Zon and Drs. M. Gait and T. Kimura for having kindly provided us, respectively, methylphosphonate oligonucleoside and the plasmid pMG755, and Dr D. M. Tidd for careful reading of the manuscript. This work was supported by

grants from Centre National de la Recherche Scientifique, Association pour le Developpement de la Recherche sur le Cancer, and Agence Nationale de la Recherche sur le SIDA.

References

(1) Hélène, C.; Toulmé, J.-J. Control of Gene Expression by Oligodeoxynucleotides Covalently Linked to Intercalating Agents and Nucleic Acid-cleaving Reagents. In *Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression*; Cohen, J. S. Ed.; Macmillan Press: London, 1989; pp 137–172.

(2) Uhlmann, E.; Peyman, A. Antisense Oligonucleotides: A New Therapeutic Principle. *Chem. Rev.* **1990**, *90*, 543–584.

(3) Miller, P. S.; Ts'O, P. O. P. Oligonucleotide Inhibitors of Gene Expression in Living Cells: New Opportunities in Drug Design. *Annu. Rep. Med. Chem.* **1988**, *23*, 295–304.

(4) Stein, C. A.; Cohen, J. S. Oligodeoxynucleotides as Inhibitors of Gene Expression: a Review. *Cancer Res.* **1988**, *48*, 2659–2668.

(5) Sproat, B. S.; Lamond, A. I.; Beijer, B.; Neuner, P.; Ryder, U. Highly Efficient Chemical Synthesis of 2′-O-Methyloligoribonucleotides and Tetrabiotinylated Derivatives: Novel Probes that are Resistant to Degradation by RNA or DNA Specific Nucleases. *Nucl. Acids Res.* **1989**, *17*, 3373–3386.

(6) Imbach, J.-L.; Rayner, B.; Morvan, F. Sugar-Modified Oligonucleotides: Synthesis, Physicochemical and Biological Properties. *Nucleosides Nucleotides* **1989**, *8*, 627–648.

(7) Cazenave, C.; Stein, C. A.; Loreau, N.; Thuong, N. T.; Neckers, L. M.; Subasinghe, C.; Hélène, C.; Cohen, J. S.; Toulmé, J.-J. Comparative Inhibition of Rabbit Globin mRNA Translation by Modified Antisense Oligodeoxynucleotides. *Nucl. Acids. Res.* **1989**, *17*, 4255–4273.

(8) Matsukura, M.; Shinozuka, K.; Zon, G.; Misuya, H.; Reitz, M.; Cohen, J. S.; Broder, S. Phosphorothioate Analogs of Oligodeoxynucleotides: Inhibitors of Replication and Cytopathic Effects of Human Immunodificiency Virus. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7706–7710.

(9) Chang, E. H.; Yu, Z.; Shinozuka, K.; Zon, G.; Wilson, W. D.; Strekovska, A. Comparative Inhibition of *ras* p21 Protein Synthesis with Phosphorus-Modified Antisense Oligonucleotides. *Anti-Cancer Drug Des.* **1989**, *4*, 221–232.

(10) Gagnor, C.; Bertrand, J.-R.; Thenet, S.; Lemaître, M.; Morvan, F.; Rayner, B.; Malvy, C.; Lebleu, B.; Imbach, J.-L.; Paoletti, C. α-DNA VI: Comparative Study of α- and β-Anomeric Oligodeoxyribonucleotides in Hybridization to mRNA and in Cell Free Translation Inhibition. *Nucl. Acids Res.* **1987**, *15*, 10419–10436.

(11) Boiziau, C.; Kurfurst, R.; Cazenave, C.; Roig, V.; Thuong, N. T.; Toulmé, J.-J. Inhibition of Translation Initiation by Antisense Oligonucleotides via an RNase H Independent Mechanism. *Nucl. Acids Res.* **1991**, *19*, 1113–1119.

(12) Furdon, P. J.; Dominski, Z.; Kole, R. RNase H Cleavage of RNA Hybridized to Oligonucleotides Containing Methylphosphonate, Phosphorothioate and Phosphodiester Bonds. *Nucl. Acids Res.* **1989**, *17*, 9193–9204.

(13) Zamecnik, P. C.; Goodchild, J.; Taguchi, Y.; Sarin, P. S. Inhibition of Replication and Expression of Human T-Cell Lymphotropic Virus Type III in Cultured Cells by Exogenous Synthetic Oligonucleotides Complementary to Viral RNA. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4143–4146.

(14) Zaia, J. A.; Rossi, J. J.; Murakawa, G. J.; Spallone, P. A.; Stephens, D. A.; Kaplan, B. E.; Eritja, R.; Wallace, R. B.; Cantin, E. M. Inhibition of Human Immunodeficiency Virus by Using an Oligonucleoside Methylphosphonate Targeted to the *tat*-3 Gene. *J. Virol.* **1988**, *62*, 3914–3917.

(15) Shibahara, S.; Mukai, S.; Morizawa, H.; Nakashima, H.; Kobayashi, S.; Yamamoto, N.; Inhibition of Human Immunodeficiency Virus (HIV-1) Replication by Synthetic Oligo-RNA Derivatives. *Nucl. Acids Res.* **1989**, *17*, 239–252.

(16) Chim, D. J.; Green, G. A.; Zon, G.; Szoka, F. C.; Straubinger, R. M. Rapid Nuclear Accumulation of Injected Oligonucleotides. *New Biologist* **1990**, *9*, 1091–1100.

(17) Leonetti, J.-P.; Mechti, N.; Degols, G.; Gagnor, C.; Lebleu, B. Intracellular Distribution of Microinjected Antisense Oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 2702–2706.

(18) Geselowitz, D. A.; Neckers, L. M. Analysis of Oligonucleotide Binding, Internalization and Intracellular Trafficking Utilizing a Novel Radiolabeled Crosslinker. *Antisense Res. Dev.* **1992**, *2*, 17–25.

(19) Hélène, C. Rational Design of Sequence-Specific Oncogene Inhibitors Based on Antisense and Antigene Oligonucleotides. *Eur. J. Cancer* **1991**, *27*, 1466–1471.

(20) Stein, C. A.; Subasinghe, C.; Shinozuka, K.; Cohen, J. S. Physicochemical Properties of Phosphorothioate Oligodeoxynucleotides. *Nucl. Acids Res.* **1988**, *16*, 3209–3221.

(21) Crooke, S. T. Therapeutic Applications of Oligonucleotides. *Annu. Rev. Pharmacol. Toxicol.* **1992**, 329–376.

(22) Pompon, A.; Lefebvre, I.; Imbach, J.-L. On-line Internal Surface Reversed-Phase Cleaning: the Direct HPLC Analysis of Crude Biological Samples. Application to the Kinetics of Degradation of Oligonucleotides in Cell Culture Medium. *Biochem. Pharmacol.* **1992**, *43*, 1769–1775.

(23) Giles, R. V.; Tidd, D. M. Enhanced RNase H Activity with Methylphosphonodiester/phosphodiester Chimeric Antisense Oligodeoxynucleotides. *Anti-Cancer Drug Des.* **1992**, *7*, 35–46.

(24) Inoue, H.; Hayase, Y.; Iwai, S.; Ohtsuka, E. Sequence Dependent Hydrolysis of RNA Using Modified Oligonucleotide Splints and RNase H. *FEBS Lett.* **1987**, *215*, 327–330.

(25) Debart, F.; Rayner, B.; Degols, G.; Imbach, J.-L. Synthesis and Base-Pairing Properties of the Nuclease-Resistant α-Anomeric Dodecaribonucleotide α-[r(UCUUAACCCACA)]. *Nucl. Acids Res.* **1992**, *20*, 1193–1200.

(26) Paoletti, J.; Bazile, D.; Morvan, F.; Imbach, J.-L.; Paoletti, C. α-DNA VIII Thermodynamic Parameters of Complexes Formed between the Oligo-Alpha-Deoxynucleotides: α-d(GGAAGG) and α-d(CCTTCC) and their Complementary Oligo-Beta-Deoxynucleotides: β-d(CCTTCC) and β-d(GGAAGG) are Different. *Nucl. Acids Res.* **1989**, *17*, 2693–2704.

(27) Hall, K. B.; McLaughlin, L. W. Thermodynamic and Structural Properties of Pentamer DNA.DNA, RNA.RNA, and DNA.RNA Duplexes of Identical Sequence. *Biochemistry* **1991**, *30*, 10606–10613.

(28) Freier, S. M.; Lima, W. F.; Sanghri, Y. S.; Vickers, T.; Zounes, M.; Cook, P. D.; Ecker, D. J. Thermodynamics of Antisense Oligonucleotide Hybridization. In *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson, R. P., Izant, J. G., Eds.; Raven Press, Ltd: New York, 1992; pp 95–107.

(29) Morvan, F.; Rayner, B.; Imbach, J. L.; Thenet, S.; Bertrand, J. R.; Paoletti, J.; Malvy, C.; Paoletti, C. α-DNA II. Synthesis of Unnatural α-Anomeric Oligodeoxyribonucleotides Containing the Four Usual Bases and Study of Their Substrate Activities for Nucleases. *Nucl. Acids Res.* **1987**, *15*, 3421–3437.

(30) Bacon, T. A.; Morvan, F.; Rayner, B.; Imbach, J.-L.; Wickstrom, E. α-Oligodeoxynucleotide Stability in Serum Subcellular Extracts and Culture Media. *J. Biochem. Biophys. Methods* **1988**, *16*, 311–317.

(31) Tidd, D. M.; Warenius, H. M. Partial Protection of Oncogene, Antisense Oligodeoxyribonucleotides against Serum Nuclease Degradation Using Methylphosphonate Groups. *Br. J. Cancer* **1989**, *60*, 343–350.

(32) Morvan, F.; Rayner, B.; Imbach, J.-L. α-Oligonucleotides: A Unique Class of Modified Chimeric Nucleic Acids. *Anti-Cancer Drug Des.* **1991**, *6*, 521–529.

(33) Saw, J. P.; Kent, K.; Bird, J.; Fishback, J.; Froehler, B. Modified Deoxyoligonucleotides Stable to Nuclease Degradation in Serum. *Nucl. Acids Res.* **1991**, *19*, 747–750.

(34) Hoke, G. D.; Draper, K.; Freier, S. M.; Gonzalez, C.; Driver, V. B.; Zounes, M. C.; Ecker, D. J. Effects of Phosphorothioate Capping on Antisense Oligonucleotide Stability, Hybridization and Antiviral Efficacy Versus Herpes Simplex Virus Infection. *Nucl. Acids Res.* **1991**, *19*, 5743–5748.

(35) Eder, P. S.; DeVine, R. J.; Dagle, J. M.; Walder, J. A. Substrate Specificity and Kinetics of Degradation of Antisense Oligonucleotides by a 3′-Exonuclease in Plasma. *Antisense Res. Dev.* **1991**, *1*, 141–151.

(36) Tidd, D. M. Unpublished results.

(37) Agrawal, S.; Sarin, P. S. Antisense Oligonucleotides: Gene Regulation and Chemotherapy of AIDS. *Adv. Drug Delivery Rev.* **1991**, *6*, 251–270.

(38) Crooke, R. M. In Vitro Toxicology and Pharmacokinetics of Antisense Oligonucleotides. *Anti-Cancer Drug Des.* **1991**, *6*, 609–646.

(39) Cohen, J. S. Chemically Modified Oligodeoxynucleotide Analogs as Regulators of Viral and Cellular Gene Expression. In *Gene Regulation: Biology of Antisense RNA and DNA*; Erickson, R. P.; Izant, J. G., Eds.; Raven Press, Ltd: New York, 1992, pp 247–259.

(40) Boiziau, C.; Toulmé, J. J. Modified Oligonucleotides in Rabbit Reticulocytes: Uptake, Stability and Antisense Properties. *Biochimie* **1991**, *73*, 1403–1408.

(41) Majumdar, C.; Stein, C. A.; Cohen, J. S.; Broder, S.; Wilson, S. H. Stepwise Mechanism of HIV Reverse Transcriptase: Primer Function of Phosphorothioate Oligodeoxynucleotides. *Biochemistry* **1989**, *28*, 1340–1346.

(42) Stein, C. A.; Neckers, L. M.; Naire, B. C.; Mumbaner, S.; Hoke, G.; Pol, R. Phosphorothioates Oligodeoxycytidine Interferes with Binding of HIV-1 gp120 to CD4. *J. Acquired Immune Defic. Syndr.* **1991**, *4*, 686–693.

(43) Morvan, F.; Rayner, B.; Leonetti, J.-P.; Imbach, J.-L. α-DNA VII. Solid Phase Synthesis of α-Anomeric Oligodeoxyribonucleotides. *Nucl. Acids Res.* **1988**, *16*, 833–847.

(44) Sproat, B. S.; Lamond, A. I.; Beijer, B.; Neumer, P.; Ryder, U. Highly Efficient Chemical Synthesis of 2′-O-Methyl-oligoribonucleotides and Tetrabiotinylated Derivatives; Novel Probes that Are Resistant to Degradation by RNA or DNA Specific Nucleases. *Nucl. Acids Res.* **1989**, *17*, 3373–3386.

(45) Sproat, B. S.; Iribarren, A. M.; Garcia, R. G.; Beijer, B. New Synthetic Routes to Synthons Suitable for 2′-O-Allyloligoribonucleotide Assembly. *Nucl. Acids Res.* **1991**, *19*, 733–738.

(46) Iyer, R. P.; Egan, W.; Regan, J. B.; Beaucage, S. L. 3H-1,2-Benzodithiole-3-one 1,1-Dioxide as an Improved Sulfurizing Reagent in the Solid-Phase Synthesis of Oligodeoxyribonucleoside Phosphorothioates. *J. Am. Chem. Soc.* **1990**, *112*, 1253–1254.

(47) Toulmé, J.-J.; Krisch, H. M.; Loreau, N.; Thuong, N. T.; Hélène, C. Specific Inhibition of mRNA Translation by Complementary Oligonucleotides Covalently Linked to Intercalating Agents. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1227–1231.

(48) Harada, S.; Koyanagi, Y.; Yamamoto, N. Infection of HTLV III/LAV in HTLV I Carrying Cells MT2 and MT4 and Application in a Plaque Assay. *Science* **1985**, *229*, 563–566.

(49) Barré-Sinoussi, F.; Chermann, J.-C.; Rey, F.; Nygere, M. T.; Chamaret, S.; Gruest, J.; Dauguet, C.; Axler-Blin, C.; Brun-Vezinet, F.; Rouzioux, C.; Rosenbaum, W.; Montagnier, L. Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immunodeficiency Syndrome (AIDS). *Science* **1983**, *220*, 868–871.

(50) Rey, M. A.; Spire, B.; Dormont, D.; Barré-Sinoussi, F.; Montagnier, L.; Cherman, J.-C. Characterization of the RNA Dependent DNA Polymerase of a New Human T-Lymphotropic Retrovirus (LAV). *Biochem. Biophys. Res. Commun.* **1984**, *121*, 126–133.