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Native Oligodeoxynucleotides Specifically Active against Human Immunodeficiency Virus Type 1 In Vitro: a G-Quartet-Driven Effect?

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Among a series of unmodified phosphodiester (PO)-oligodeoxynucleotides (PO-ODNs) complementary to some of the human immunodeficiency virus type 1 (HIV-1) regulatory genes, several PO-ODN sequences complementary to the *vpr* gene (PO-ODNs–a-*vpr*, where a-*vpr* is the antisense *vpr* sequence) emerged as potent inhibitors (at concentrations of 0.8 to 3.3 μM) of HIV-1 multiplication in de novo infected MT-4 cells, while they showed no cytotoxicity for uninfected cells at concentrations up to 100 μM. Unlike phosphorothioate counterparts, PO-ODN–a-*vpr* sequences were not inhibitory to HIV-2 multiplication in de novo infected C8166 cells and neither prevented the fusion between chronically infected and bystander $CD4^+$ cells nor inhibited the activity of the HIV-1 reverse transcriptase in enzyme assays. Moreover, they were not inhibitory to HIV-1 multiplication in chronically infected cells. Delayed addition experiments showed that PO-ODNs–a-*vpr* inhibit an event in the HIV-1 replication cycle following adsorption to the host cell, but preceding reverse transcription. Structure-activity relationship studies indicated that the antiviral activity of the test PO-ODN–a-*vpr* sequences is not related to an antisense mechanism but to the presence, within the active sequences, of contiguous guanine residues. Physical characterization of the test PO-ODNs suggested that the active structure is a tetramer stabilized by G quartets (i.e., four G residues connected by eight hydrogen bonds).

In recent years there has been an increasing search for oligonucleotides capable of blocking viral infections by the antisense methodology (13, 20, 26, 32, 35). This approach involves the binding of a synthetic oligodeoxynucleotide (ODN) to a complementary sequence in the target nucleic acid through a highly selective process governed by hydrogen bond formation between complementary bases and stacking interactions between base pairs. When the target is an mRNA, as in the majority of the reported examples, translation is prevented. Therefore, because of the very nature of the process, if the ODN is directed against a sequence that is present only in the viral genome, the treatment with an antisense ODN is, in principle, devoid of toxicity for the host.

Both native and chemically modified antisense ODNs have been investigated as human immunodeficiency virus (HIV) inhibitors. The former have turned out to be very sensitive to nucleases. The latter, although quite effective, have shown a number of drawbacks, from poor aqueous solubility (methylphosphonate-ODNs) to aspecific interactions with proteins (phosphorothioate-ODNs [PS-ODNs]), and it has been proved that they possess non-sequence-specific modes of action, from interference with virus adsorption to inhibition of reverse transcriptase and RNase H (16–19, 27, 29, 30).

More recently, ODNs which aggregate into higher-order inter- or intramolecular structures have been developed. Among them are G-rich sequences that form tetramers stabilized by G quartets (four G residues connected by eight hydrogen bonds) or that fold to form structures stabilized by intramolecular G tetrads (21, 23, 34).

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On the basis of the assumption that because of the less abundant level of their mRNAs, virus regulatory genes could be more suitable targets for antisense ODNs than structural genes, we have synthesized antisense sequences to the following HIV type 1 (HIV-1) genes: *vif*, which is responsible for the infectivity of cell-free virus; *vpr*, which is a weak transcriptional activator; *rev*, which is necessary for exporting viral mRNAs into the cytoplasm; and *nef*, which is required for efficient in vivo viral replication and pathogenicity (3, 6, 11, 12). When prepared as PS, some of the sequences of the genes listed above have been found to be very potent inhibitors of the HIV-1 and HIV-2 multiplication in acutely infected cells. However, their anti-HIV activities, although highly selective, were non-sequence specific (5).

Here we report the results obtained when the sequences described above, synthesized as native phosphodiester (PO)-ODNs, were tested in cells acutely and chronically infected with either HIV-1 or HIV-2. Also presented in this report are the results of experiments designed to obtain a clearer insight into the mode of action of the antisense *vpr* sequence (a-*vpr*), the sole unmodified PO-ODN that showed activity against HIV-1.

MATERIALS AND METHODS

ODNs used in the study. The following antisense PO-ODNs and their target sites on the HIV-1 genome of the III_B strain) were used in the study: a-*vif* (positions 5040 to 5058), a-*vpr* (positions 5561 to 5579), a-*rev* (positions 5895 to 6000), and a-*nef* (positions 8793 to 8810). Antisense sequences to *vif*, *rev*, and *nef* encompass the translational AUG start codon sequence that has been indicated by Ghosh and Cohen (8) to be one of the most appropriate targets for a-ODNs. The PS-ODNs antisense *vpr*, antisense *vif*, and sense *vpr*, which, we have reported, are active in acute infection assays (5), were used as reference inhibitors.

Synthesis and purification of ODNs. The synthesis of PO-ODNs was carried out on a Gene Assembler Plus automatic synthesizer (Pharmacia) by the phosphoramidite method (10-μM scale). Commercial controlled pore glass (50.0-nm) supports and 2′-deoxynucleotide phosphoramidites were purchased from Chem-

TABLE 1. Cytotoxicities and anti-HIV-1 activities of PO-ODN-a-*vpr*-related sequences[a]

ODN	Sequences (5′→3′)[b]	EC_{50} (μM)[c]		
		FCS (CPE)	FCSi	
			CPE	p24
1	GGTCTTCTGGGGCTTGTTC	2.5	1.6	3.4
2	CTTGGTCTTCTGGGGCTTGTTCC	3.0	0.7	2.1
3	GGTCTTCTGGGGCTTGTTCCAT	3.3	2.4	3.1
4	**C**GGTCTTCTGGGGCTTGTT**CC**	16	11	14
5	**C**TTGGTCTTCTGGGGCTTGTT**CC**	0.9	0.5	1.7
6	CCTTGTTCGGGGTCTTCTGGTTC	2.3	1.2	3.3
7	CTT**N**GTCTTCT**NGN**GCTTGTTCC	>20	>20	>20
8	CT**N**GGTCTTC**N**GGGGC**N**TGTTCC	0.8	0.5	1.2

[a] Data represent mean values for two separate experiments. Variation among duplicate samples was less than 15%. The concentration of compound required to reduce the viability of mock-infected MT-4 cells by 50% was ≥20 μg/ml for all compounds.

[b] Boldface letters C in ODN-4 and -5 indicate L-dC; boldface letters N in ODN-7 and -8 indicate an equimolar mixture of natural dA and dC.

[c] EC_{50}, compound concentration required to achieve 50% protection of MT-4 cells against the HIV-1-induced cytopathogenicity or to reduce p24 levels by 50%.

Genes Corporation. In the case of L-modified ODNs, the corresponding L-phosphoramidites were synthesized as described previously (25). After deblocking (16 h at 50°C in 30% NH_4OH), the ODN solutions were lyophilized and purified by anion-exchange chromatography (DEAE Sephacel from Parmacia; TEAB (triethyl ammonium bicarbonate) gradient from 0.1 to 1.8 M [pH 7.0]). The fractions with high-pressure liquid chromatography titers greater than 85% (RP18 Hypersil column; CH_3CN gradient in aqueous 0.05 M KH_2PO_4 [pH 4.5]) were collected, coevaporated with water several times to remove the excess buffer, converted to the sodium salt (Dowex 50 WX8 resin, Na^+ form), and then lyophilized.

CD spectroscopy. Circular dichroism (CD) spectra were recorded on a Jasco J-700 spectropolarimeter interfaced to a computer (International Business Machines). Single-stranded solutions (3 μM) of the ODNs were prepared in 0.1 M NaCl–0.1 M Tris-HCl (pH 7.0), heated at 90°C for 15 min, slowly cooled to room temperature, and incubated at 4°C for 15 min before the CD experiments. The temperature was maintained by placing the samples in a 1-cm-path-length cell holder surrounded by an external jacket connected to a circulating water bath. The spectra are the average of four scans taken at the same temperature, minus the average spectrum (four scans) of the buffer alone.

Cells. $H9/III_B$, CEM, MT-4, and C8166 cells (grown in RPMI 1640 containing 10% fetal calf serum [FCS], 100 IU of penicillin G per ml, and 100 μg of streptomycin per ml) were used for anti-HIV assays. Cell cultures were checked periodically for mycoplasma contamination with a MycoTect Kit (Gibco).

Viruses. HIV-1 ($HTLV/III_B$ strain) and HIV-2 (ROD strain; kindly provided by L. Montagnier) were obtained from supernatants of persistently infected $H9/III_B$ and CEM cells, respectively. HIV-1 and HIV-2 stock solutions had titers of 4.5×10^6 and 1.4×10^5 50% cell culture infective doses per ml, respectively.

Chronic infection assay. Chronically infected $H9/III_B$ and CEM cells were extensively washed to remove previously produced virus particles and were resuspended at 10^5 cells per ml in the absence or presence of serial dilutions of the test compounds. After incubation at 37°C for 96 h, the infectious HIV yield was determined by endpoint titration of the supernatants (see below). Cell numbers were determined with a Coulter counter, and the values were corrected for viability, as determined by the trypan blue dye exclusion test. The concentrations of compound required to reduce the HIV yield by 90% and the 50% cytotoxic concentrations were determined by linear regression analysis.

HIV-1 titration. Titration of HIV-1 and HIV-2 was performed in C8166 cells by the standard limiting dilution method (dilution, 1:2; four replica wells per dilution) in 96-well plates. Infectious virus titers were determined by light microscope scoring of the syncytia after 4 days of incubation. Virus titers were expressed as 50% cell culture infective doses per milliliter by the Reed and Muench method.

Acute infection assay. The activities of the compounds against HIV multiplication in acutely infected cells were based on the inhibition of virus-induced cytopathogenicity in MT-4 cells. Brielfly, 50 μl of culture medium containing 10% FCS (either normal FCS or FCS inactivated at 65°C for 30 min [FCSi]) and 10^4 MT-4 cells (infected de novo with HIV-1 or HIV-2 at the multiplicities of infection specified below) were added to each well of flat-bottom microtiter trays containing 50 μl of medium with or without serial dilutions of the test compounds. After 4 days of incubation at 37°C, the number of viable MT-4 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (22). The cytotoxicities of the compounds were evaluated in parallel with their antiviral activities and were based on the viabilities of mock-infected cells, as monitored by the MTT method.

Syncytium assay. Extensively washed $H9/III_B$ cells (10^3 cells) were cocultured with uninfected C8166 cells (10^4 cells) in the absence or presence of test drugs. The number of syncytia was determined with a light microscope after incubation for 24 h.

HIV-1 reverse transcriptase assay. The effects of the compounds on HIV-1 recombinant reverse transcriptase activity were evaluated with purified enzyme. Assays were performed at 37°C for 30 min in a 50-μl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 80 mM KCl, 6 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, 10 mM [^{3}H]dTTP (1 Ci/mmol), 0.05 units of poly(rA)-oligo$(dT)_{10}$ at an optical density of 260 nm per ml, and 1.6×10^{-3} U of enzyme [a unit was defined as the amount of enzyme necessary to incorporate 1 nmol of [^{3}H]dTMP into the poly(rA)-oligo$(dT)_{10}$ template in 1 min at 37°C]. Aliquots of 40 μl were spotted onto glass fiber filters (Whatman GF/A), and the filters were processed for determination of trichloroacetic acid-insoluble radioactivity.

RESULTS

In vitro anti-HIV activities of a-ODNs. The effects of single treatments with PO-ODN-a-*vif*, -*vpr*, -*rev*, and -*nef* (for nucleotide coordinates, see Materials and Methods) on HIV-1 and HIV-2 multiplication were evaluated in cells infected at different multiplicities of infection, whereas the cytotoxicity was evaluated in parallel samples of mock-infected MT-4 cells.

As determined by the MTT assay, none of the ODNs was cytotoxic for MT-4 cells at concentrations up to 20 μM; in particular, PO-ODN-a-*vpr* was found to be noncytotoxic at 100 μM. When evaluated for the capability of preventing virus-induced cytopathogenicity (cytopathic effect) under conditions of multiple growth cycles of the viruses (multiplicity of infection = 0.01), the sole effective PO-ODN was a-*vpr*, which proved to be both potent (50% effective concentration, 2.5 μM) and selective (selectivity index, ≥40) against HIV-1 but totally ineffective against HIV-2. On the contrary, reference PS-ODN–*vif* and –*vpr* (see Materials and Methods) were confirmed to be very potent and selective inhibitors of both HIV-1 and HIV-2, independently of the target gene and the sequence used, i.e., antisense or sense sequence (data not shown).

Like its thioate derivative and zidovudine (AZT), PO-ODN–a-*vpr* was also active in cultures infected at high multiplicities of infection (0.1 and 1.0), at which it exhibited a potency two- to sixfold lower than that at a multiplicity of infection of 0.01 (data not shown).

The effect of PO-ODNs on HIV-1 and HIV-2 multiplication in chronically infected cells was evaluated in $H9/III_B$ and CEM cells, respectively. At concentrations of 20 μM, none of the ODNs, including PO-ODN–a-*vpr*, was inhibitory to cell proliferation or p24 antigen production (data not shown). This suggested that the target of PO-ODN–a-*vpr* is a step of the HIV-1 multiplication cycle preceding integration.

Features of PO-ODNs related to a-*vpr*. In the attempt to obtain PO-ODNs with higher potencies and to gain more insights into both the target and mode of action of PO-ODN–a-*vpr* (ODN-1), we synthesized the following ODNs (Table 1): (i) ODN-2 and ODN-3 are versions of PO-ODN–a-*vpr* slightly extended at the 5′ or 3′ end, or both ends, so that ODN-3 could encompass the translational AUG start codon sequence; (ii) ODN-4 and ODN-5 are counterparts of ODN-1 and ODN-2, respectively, capped at both ends with L-dC residues to make them resistant to degradation by nucleases (4); (iii) ODN-6 is a version of ODN-2, but with reversed polarity, and represents the more appropriate control when sequences containing homoguanine tracts are used (28); and (iv) ODN-7 and ODN-8 are new versions of ODN-2 in which dA or dC substitute for the original nucleosides inside (ODN-7) or outside (ODN-8) guanine stretches.

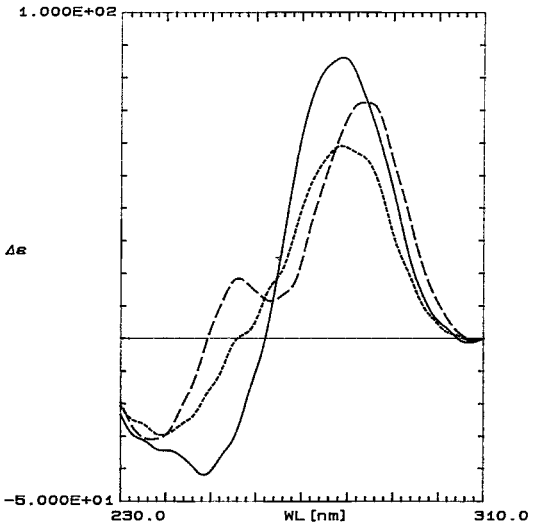

FIG. 1. CD spectra of ODNs 2 and 7. — — —, ODN-2 at 37°C; ----, ODN-2 at 80°C; ___, ODN-7 at 37°C. Δε, differential molar extinction coefficient; WL, wavelength.

Anti-HIV-1 activity of PO–a-*vpr* and related sequences. The effects of single treatments with the PO-ODNs listed above on HIV-1 multiplication in acutely infected cells were evaluated by measuring both protection from a virus-induced cytopathic effect and p24 antigen levels under conditions of multiple growth cycles of the virus. In order to evaluate the stability of the PO-ODNs to serum nucleases, the assays were carried out in both normal FCS and FCSi. The effects of the ODNs on cell proliferation were evaluated in parallel samples of mock-infected cells.

None of the compounds (Table 1) was cytotoxic for MT-4 cells at the highest dose tested (20 μM). With the exception of ODN-7, all the ODNs were capable of reducing both the HIV-1-induced cytopathic effect and p24 antigen levels. Moreover, they were as potent in medium containing normal serum as in medium containing FCSi, thus showing a somewhat intrinsic stability to serum nucleases.

The lengthening of the a-*vpr* sequence (ODNs 2 and 3) did not improve the potency, suggesting that in order to have antiviral activity, it is not necessary to encompass the translational AUG start codon sequence (8). An alternative possibility was that the modes of action of these ODNs are other than through an antisense mechanism.

While the capping of ODN-1 with L-dC resulted in an unexpected, sharp decrease in potency in both normal FCS and FCSi (ODN-4), the capping of ODN-2 proved to be valuable in slightly increasing the potency in normal serum (compare the activity of ODNs 2 and 5 in FCS). Because ODNs 4 and 5 differ at their 5′ ends in the number of nucleotides separating the C residue from the G_2 sequence, and since L-nucleosides alter the geometry of the adjacent residues (1), the findings presented above were taken as an indication that the G_2 motif could play a role in the anti-HIV-1 activity of PO-ODN-a-*vpr*.

Interestingly, the ODN-6 showed a potency comparable to that of ODN-2, although its binding to the putative target was expected to lead to the formation, if any, of very unstable parallel-stranded duplexes (7). Therefore, its antiviral activity was more likely related to the presence of the G_2 or G_4 segment, or both, rather than to an antisense mechanism.

Further proof of the involvement of G tracts in the antiviral activity of PO-ODN–a-*vpr* came from the results obtained with ODNs 7 and 8. ODN-7, which is an equimolecular mixture of eight sequences, with dA or dC residues replacing three of the original guanosines, was devoid of anti-HIV activity. The latter, which contains dA and dC residues replacing nucleosides other than guanosines, showed potent anti-HIV-1 activity.

Structure-activity relationships of a-*vpr* and related sequences. Recently, the phosphorothioate $T_2G_4T_2$ sequence has been reported to inhibit HIV infection in vitro, and the active structure has been shown to be a G_4-stabilized tetramer that loses its antiviral properties upon heat denaturation (34).

To establish whether similar intermolecular complexes could also be held responsible for the anti-HIV-1 activities of our PO-ODNs, the structure in solution of some of them was investigated by CD spectroscopy, a technique widely used to study the association of homoguanylates and G-rich sequences (2, 9, 14). The CD spectra of active ODNs 2 and 8 and of inactive ODN-7 were recorded at different temperatures in 0.1 M NaCl–0.1 M Tris (pH 7.0). At 37°C, the CD spectra of ODN-2 (Fig. 1) and ODN-8 (data not shown) presented a positive band characteristic of parallel-stranded G-quartet structures in the region of 250 to 260 nm (28). This band disappeared at high temperature, giving rise to a spectrum very similar to that obtained at 37°C with inactive ODN-7.

Furthermore, when a stock solution (1.3 mM) of ODN-2 was heated at 85°C for 20 min, chilled on ice, diluted in cold medium, and then added to HIV-1-infected cell cultures, it failed to exhibit antiviral activity even at concentrations as high as 40 μM (data not shown). Altogether, these findings suggest that the anti-HIV-1 activities of ODNs 2 and 8 are strictly dependent on the ability to self-assemble into G_4-stabilized tetramers.

Effect of a-*vpr* on virus-mediated cell fusion. The phosphorothioate $T_2G_4T_2$ sequence has been reported to block the binding of HIV to cells and virus-mediated cell fusion (34). Therefore, we evaluated the capabilities of PO-ODNs 1, 5, and 6 to prevent syncytium formation between chronically infected and normal T lymphocytes, which is mediated by a gp120-CD4 interaction similar to that leading to HIV-1 adsorption to target cells. Unlike dextran sulfate and PS-ODN-a-*vpr*, which were used as reference drugs, none of the test PO-ODNs

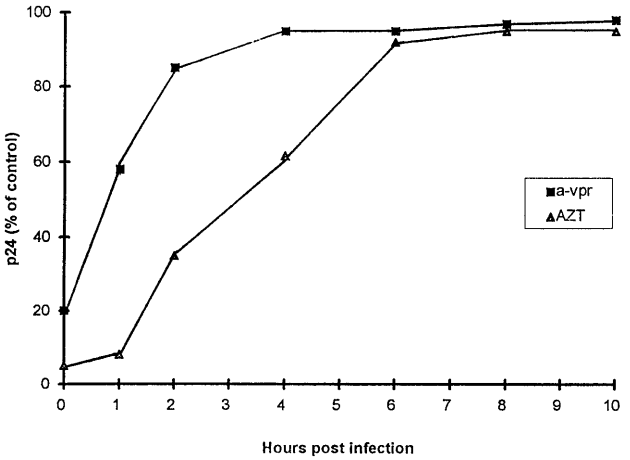

FIG. 2. Variation of expression of p24 antigen with the time of addition of PO-ODN–a-*vpr* and AZT. MT-4 cells were infected for 1 h at 20°C with HIV-1 at a multiplicity of infection of ≥1. Then, unadsorbed virus was removed by extensive washings, and PO-ODN–a-*vpr* (50 μM) or AZT (2.5 μM) was added to duplicate sets of cultures at different times (0, 1, 2, 4, 6, 8 and 10 h) postinfection. The levels of p24 antigen were determined at the end of 72 h of incubation at 37°C.

TABLE 2. Effect of ODNs on HIV-1 recombinant reverse transcriptase activity[a]

Compound	IC_{50} (μM)
1	>10
5	>10
6	>10
PS-ODN–a-*vpr*	0.05 ± 0.008
AZTTP	0.04 ± 0.009

[a] Data represent means ± standard deviations for three separate experiments. IC_{50}, compound concentration required to inhibit the enzyme activity by 50%. Poly(rA)-oligo$(dT)_{12}$ was used as the template-primer.

inhibited cell fusion (data not shown). This fact, coupled with the inactivity of PO-ODNs against HIV-2, suggested a lack of interference with receptor binding-related events.

Effect of delayed additions of a-*vpr*. In order to obtain more insights into the mode of action of PO-ODN–a-*vpr*, the effect of its addition at different times after the infection of MT-4 cells with HIV-1 was compared with that of AZT. As shown in Fig. 2, PO-ODN–a-*vpr* was effective in inhibiting viral multiplication only when it was added immediately after the end of the infection period, whereas AZT was significantly active even when it was added 2 to 3 h postinfection. These results suggest that PO-ODN–a-*vpr* interferes with an event of the HIV-1 replication cycle following adsorption but preceding reverse transcription.

Effects of ODNs on the HIV-1 recombinant reverse transcriptase activity. The effects of PO-ODNs 1, 5, and 6 on the activity of the HIV-1 recombinant reverse transcriptase were evaluated. PS-ODN-a-*vpr* and AZT triphosphate (AZTTP) were used as reference compounds. The results (Table 2) indicated that, contrary to PS-ODN–a-*vpr* and AZTTP, which were confirmed to be very potent inhibitors of the RNA-dependent DNA polymerase function, PO-ODNs 1, 5, and 6 were totally ineffective.

DISCUSSION

Among a series of unmodified PO-ODNs with sequences antisense to the sequences of selected regions of HIV-1 regulatory genes, a sequence complementary to the 5′ end of the *vpr* gene (PO-ODN–a-*vpr*) emerged as a potent inhibitor of HIV-1 replication in acutely infected cells. It did not inhibit the multiplication of HIV-2 in a de novo infection or the multiplication of HIV-1 and HIV-2 in chronically infected cells.

A peculiar structural feature of PO-ODN–a-*vpr* and the other active ODNs reported here was the presence of G_2 and G_4 clusters separated by six T or C nucleotides. ODNs containing G clusters have been reported to spontaneously form inter- and intramolecular superstructures held together by G-quartet planes, i.e., four guanine residues connected by eight hydrogen bonds (24, 27, 31, 33). Evidence indicating the presence of such a superstructural motif in the PO-ODNs described here comes from the CD spectra of the active compounds, which show a positive signal at 250 to 260 nm that is the marker of parallel-stranded guanine aggregates (15). It remains to be established whether the stabilizing G-quartet planes are contributed by both the G_2 and G_4 segments. Nevertheless, the G_2 segment seems necessary for the anti-HIV-1 activity; in fact, when its native geometry is distorted by an adjacent L-dC residue (as in ODN-4), the resulting sequence shows a significantly lower potency. Moreover, the persistence of the antiviral effect throughout the 4-day incubation period in medium containing FCS that is not heat inactivated suggests that the superstructure described above contributes to the stability of the test ODNs against nuclease degradation.

As far as the mode of action is concerned, all the available evidence indicates that the inhibition of HIV-1 multiplication by the test PO-ODNs is not due to an antisense mechanism. In fact, inversion of strand polarity (as in ODN-6) and base substitutions outside the G clusters (as in ODN-8) do not affect the anti-HIV-1 potency. On the other hand, a complete loss of activity is obtained following distruption of the G clusters in the primary sequence (as in ODN-7) and heat denaturation before treatment, suggesting the need for the maintenance of the three-dimensional structure.

To the best of our knowledge, this is the third example of G-rich ODNs whose in vitro anti-HIV activities have been ascribed to their ability to form three-dimensional structures. However, although inhibition of HIV-1 replication has been found, as in the present study, but only in acutely infected cells, both the proposed modes of action and the structures of the assembled ODNs seem to be different.

In the first example, the phosphorothioate $T_2G_4T_2$ (34) forms tetramers containing four G-quartet planes that bind to the V3 loop of the viral protein gp120, like longer PS-ODNs do (10). In this case, both the G-quartet structure and the sulfur-containing backbone seem to be necessary for the antiviral effect, which is due to inhibition of virus-to-cell and cell-to-cell infection.

In the second example, several PO-ODNs composed only of thymidine and dG have been found to be inhibitory to HIV-1 (21). The most effective sequence is a 17mer with four G clusters that give a monomeric fold-back structure containing two stacked G-quartet planes. This compound has been reported not to interfere with virus adsorption but to inhibit viral transcription (23).

In our case, ODNs have neither a thioate backbone nor a primary sequence compatible with the formation of intramolecular fold-back structures, and although further studies are needed to define their three-dimensional structures and targets, they seem to specifically interfere with an event(s) of the HIV-1 life cycle following the adsorption step and preceding reverse transcription.

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