Alternate Strand DNA Triple Helix-mediated Inhibition of HIV-1 U5 Long Terminal Repeat Integration in Vitro*

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Integration of the human immunodeficiency virus (HIV) DNA into the host genome is an obligatory process in the replicative life cycle of the virus. This event is mediated in vitro by integrase, a viral protein which binds to specific sequences located on both extremities of the DNA long terminal repeats (LTRs). These sites are highly conserved in all HIV genomes and thus provide potential targets for the selective inhibition of integration. The integrase-binding site located on the HIV-1 U5 LTR end contains two adjacent purine tracts on opposite strands, 5′ ... GGAATAATTCTT-3′/3′-CTCTTTTAGAGA ... 5′, in parallel orientations. A single strand oligonucleotide 5′-GGTTTTTGTGT-3′ was designed to associate with these tracts via its ability to form a continuous alternate strand DNA triplex. Under neutral pH and physiological temperature, the oligonucleotide, tagged with an intercalator chromophore oxazolopyridocarbazole, formed a stable triplex with the target DNA. The occurrence of this unusual triplex was demonstrated by both DNase I footprinting and electron microscopy. The triplex inhibits the two steps of the integrase-mediated reactions, namely, the endonucleolytic cleavage of the dinucleotide 5′-GT-3′ from the 3′ end of the integration substrate and the integration of the substrate into the heterologous target DNA. The midpoints for both inhibition reactions were observed at oligonucleotide concentrations of 50–100 nM. We believe that these results open new possibilities for the specific targeting of viral DNA LTR ends with the view of inhibiting integration under physiological conditions.

The integration of the human immunodeficiency virus (HIV)1 genome into the host genome is mediated by the viral protein integrase (IN) (1). After the reverse transcription of the HIV genomic RNA, two reactions occur, catalyzed by the viral IN enzyme, a site-specific removal of two nucleotides (5′-GT-3′) from the 3′ ends of the long terminal repeats (LTR) of the viral DNA and the integration of the recessed viral DNA into the host genome (2, 3). Efficient methods have been reported to investigate integrase activity by analyzing the in vitro reaction products (4), and several families of compounds that inhibit integrase activity have now been identified (5–13). However, none of them displayed a strong and/or selective inhibitory effect. Our approach toward obtaining more potent inhibitors involves the targeting of the LTR extremities that contain the DNA sequences required for correct integration, further shown to be binding sites for integrase (14). These sequences can be considered as a potential target for the selective inhibition of integration by double-stranded DNA binding ligands. Depending on the sequence context, selective recognition of double-stranded DNA can be chiefly achieved either with minor groove-binding oligopeptides or with triple helix-forming oligonucleotides (15). For instance, we previously demonstrated that the minor groove binder netropsin selectively binds to an (A + T)-rich sequence located in the MMLV LTR end and consequently inhibits the in vitro integration process (9). Furthermore, a derivative of netropsin was capable of blocking early steps of MMLV replication in vivo, most likely by interfering with integration of proviral DNA (16). Recently, we have extended this approach to HIV. The HIV U3 LTR end contains a short purine-pyrimidine which could be selectively targeted by a purine 7-mer triple helix-forming oligonucleotide coupled to the intercalating chromophore oxazolopyridocarbazole (OPC) (10, 17). However, theoretical considerations indicate that 7-mer oligonucleotides are only poorly selective, thus preventing the use of too short TFOs for in vivo experiments (18, 19). To overcome this limitation, it was necessary to increase the length of the target sequence. We noticed that the IN-binding site located near the US LTR HIV-1 end contains two adjacent purine tracts oriented in parallel or opposite DNA strands, i.e. 5′-GGAAATATTCTT-3′/3′-CTCTTTTAGAGA-5′. Theoretical (20) and experimental data (21, 22) have pointed out that TFO with natural 5′-3′ phosphodiester bonds can recognize the two alternate purine tracts simultaneously by switching from one strand to the other and thus providing alternating triplexes of opposite polarities, parallel and antiparallel ones (20).

The present work shows that a 11-mer oligonucleotide-intercalator conjugate (OPC linked to the 5′-end of 5′-GGTTTTTGTTGTT-3′) (i) readily forms a stable triplex with a DNA fragment containing the US LTR end sequence at neutral pH and physiological temperature and (ii) selectively inhibits the IN-catalyzed integration of the US LTR end into heterologous DNA in vitro. The formation of this unusual triplex is demonstrated by both footprinting assay and electron microscopy.

MATERIALS AND METHODS

Synthesis of TFO-OPC—Oligonucleotides were synthesized on Applied Biosystems model 381A DNA synthesizer. For modified oligonucleotides, the 5′ linker was obtained with the amino link 2 phosphor-
amidite base. The OPC-derived oligonucleotides were prepared essentially as described by Gautier et al. (23). Briefly, peroxidase was added to a solution of the oligonucleotide linker and 2-methyl-9-hydroxylcarnitine acetate in 50 mM phosphate buffer, pH 7.4, in the presence of 20 mM hydrogen peroxide (24). Conjugates were purified by desalting gel electrophoresis, visualized by UV shadowing and direct fluorescence. The oligonucleotides were deprotected by chromatography on the Sephadex G-10 phase. The concentration of conjugates was determined spectrophotometrically.

Footprinting Experiments—A 0.1-μg portion of the 42-mer oligonucleotide 5'-AGAATTTTGTCACCTGCTGAGATTTTCCACACGAT-3' was labeled by polynucleotide kinase and annealed to the complementary strand at 40 μl in order to obtain target DNA for footprinting. The footprinting was performed in a buffer containing 20 mM Tris-HCl, pH 7.0, 5 mM MgCl₂, 2 mM MnCl₂, 0.5 mM spermine, target DNA (10 nM), and additional nonspecific unlabeled DNA (60 ng). In the standard assay, TFO-OPC was added to the reaction mixture at different concentrations (see figure legends), and the mixture was incubated at 30°C for 30 min. Digestion was carried out by addition of DNase I (3 units/ml) and after digestion for 2 min by adding EDTA (10 mM), sodium acetate (0.3 M), and carrier RNA (5 μg). Products of the reaction were subsequently precipitated with ethanol, dried, and resuspended in formamide/EDTA gel-loading buffer. The cleavage patterns were loaded on 18% denaturing gel and visualized by autoradiography.

Oligonucleotide Sequencing—Maxim-Gilbert sequencing reactions were performed using G, A, C and T ladders of end-labeled DNA (25). For G reactions, 9 μl of end-labeled DNA in TE buffer were incubated for 10 min at room temperature with 1 μl of 1/100 dimethyl sulfate in water. For the (G + A) reaction, 9 μl of labeled DNA in TE buffer (10 mM Tris, 1 mM EDTA) were mixed with 1 μl of 1 mM piperidine formate (pH 2.0) and incubated for 5 min at 65°C. For T reactions, 9 μl of labeled DNA were heated at 90°C for 2 min, cooled quickly to room temperature, mixed with 1 μl of 3 mM KMeO₂, and incubated further for 7 min at room temperature. The reaction was finally quenched with 1 μl of 1 mM alkyll alcohol. Then all three mixtures were treated for 15 min at 90°C, dried, and resuspended in formamide/EDTA gel-loading buffer.

HIV-1 LTRs Integration Reaction—Double-stranded oligonucleotides were used as HIV-1 DNA substrates for the integration assay. Sequences (21-mer) corresponding to the U3 and U5 LTR ends were 5'-GAGGATATTCCCTTCA-3' and 5'-GTTGGATAAATCTC-3', respectively. They were 5'-labeled by polynucleotide kinase and annealed to their unlabeled complementary strands thus giving the desired substrates (called LTR U3 and LTR U5, respectively). For the integration reactions, LTR U3-GT or LTR U5-GT (LTR U3 and LTR U5 lacking the terminal dinucleotides 5'-GT-3', respectively) were used as substrates. HIV-1 IN was expressed in E. coli. The bacterial strain carrying the expression vector was kindly provided by guest on July 18, 2018

RESULTS AND DISCUSSION

Formation of a triple helix by oligonucleotides in a sequence-specific manner is limited to polypurine tracts of duplex DNA. Recent theoretical and experimental work has demonstrated the ability of oligonucleotides to bind to oligopurine sequences which alternate on the strands of duplex DNA (15, 20–22, 31–37). Three different types of alternate triple helix-forming oligonucleotides have been described: (i) two pyrimidine oligonucleotides can be linked either by their 3' or by their 5' ends to allow the recognition of alternating polypurine sequences (31, 32); (ii) a purine oligonucleotide that binds in antiparallel orientation can be linked to a pyrimidine oligonucleotide which binds in an opposite orientation with respect to the oligopurine target (33–37); and (iii) a single (T/G)-containing oligonucleotide can interact with two oligopurine tracts that alternate on both strands of the target DNA. Indeed, (G/T)-containing oligonucleotides may adopt either a parallel or an antiparallel orientation with respect to the oligopurine target depending upon the sequence considered and, in particular, the number of GpT-3' and 5'-TpG-3' steps present in the sequence (38). We adopted this strategy and synthesized an 11-mer oligonucleotide 5'-GGTTTTGTGT-3' designed in order to create a stable alternate strand DNA triplex with the 5'-GGAAATCTTCT-3' motif located at the extremity of the HIV-1 U5 LTR (Fig. 1). It was assumed that this oligonucleotide will form two mini triple helices, the first one involves antiparallel binding of the third strand to the 5'-AGAGA-3' motif (referred as antiparallel domain), and the second involves parallel binding to the 5'-GAAAA-3' motif (referred as parallel domain) of the LTR extremity (Fig. 1B). Despite the fact that the parallel mini helix contains GQC triplets in a noncanonical orientation, the orientation of the third strand was chosen to be consistent with empirical rules for design of the (G/T)-containing third strand (38). According to these rules, two parameters were taken into consideration for the stability of these mini helices: (i) there is only one GpT step in the 5'-GGTTTTG motif and (ii) more than 50% of the putative mini triple helix is composed of TAT triplets. Initial results indicated that the association of the oligonucleotide alone with the DNA target resulted in the formation of a single helix. The complex, however, was poorly stable even after an overnight incubation at 4°C. In order to increase the stability of the triple helix, the oligonucleotide was conjugated with the intercalating chromophore OPC. The resulting com-
pound, termed HIVS-OPC, is shown in Fig. 1A. As a control, an alternative 5′-GGTTTTTGTTT-3′ oligonucleotide was also conjugated to OPC (termed HIVT-OPC, Fig. 1A) to be used for its inability to form a stable triplex with the U5 LTR.

The ability of the HIVS-OPC conjugate to bind to double-stranded DNA via the formation of a triple helix has been initially examined by gel-retardation experiments (data not shown). Results from a 21-mer LTR US substrate provided evidence for the formation of a stable complex between the partners. These were confirmed by DNase I footprinting using a labeled 42-mer target at different HIVS-OPC concentrations (Fig. 2). The footprinting experiments were performed on the two strands of the DNA target. These were involved in the formation of either antiparallel or parallel triplets. The results are presented on Fig. 2, A and B, respectively. The antiparallel domain which interacts with the 3′-end of HIVS-OPC was fully protected for concentrations of HIVS-OPC above 10 nM. The protection spanned over the entire length of the target sequence and extended to the parallel domain (Fig. 2A). Simultaneously, the second strand was itself also protected over the whole length of both domains in the same range of the concentrations of HIVS-OPC (Fig. 2B). Control experiments were performed with conjugate HIVT-OPC. This oligonucleotide contained the intact parallel binding domain but was scrambled within the antiparallel binding domain (see Fig. 1) so that two mismatched triplets would be formed at positions 7 and 10 (from the 5′ end) upon its binding to the target. Actually no protection was observed even at micromolar concentrations (data not shown). Taken together, these results provided strong evidence for the necessity of simultaneous binding of the (G/T)-containing oligonucleotide-OPC conjugate to both target domains in order to give rise to a stable triple-helix complex. It is worthy to note that (G/T)-containing conjugate HIVS-OPC was not optimized for the junction step. In particular, the oligonucleotide moiety was not deleted at the junction to accommodate crossing of the major groove as suggested by Beal and Dervan (33). This alteration appears therefore dispensable in the context of (G/T) oligonucleotides. However, we cannot rule out that a punctual deletion at the junction may improve the overall stability of the triple helical complex.

In parallel, we used electron microscopy to detect and localize the binding of the HIVS-OPC conjugate on its target DNA sequence. Previously, this technique has been applied successfully to detect triplexes formed between DNA and either biotinylated TFO or peptide nucleic acid oligomers with streptavidin used as a label; triplexes are visualized readily as streptavidin beads on DNA molecules (27, 39, 40). The electron microscopy

![Figure 1: Structure of triple helix-forming oligonucleotides conjugated with OPC and schematic presentation of the triplex. A, HIVS-OPC is a oligonucleotide conjugate whose binding oligopurine-oligopyrimidine sequence is located in the U5 LTR HIV-1 end region. HIVT-OPC was synthesized to serve as a control conjugate. B, schematic representation of the alternate strand DNA triplex formed between HIVS-OPC and the target sequence.](http://www.jbc.org/)

![Figure 2: DNase I footprinting of a 42-base pair fragment in the presence of HIVS-OPC. A, protection of the oligonucleotide containing 5′-AGAGATTGT-3′ site as a function of HIVS-OPC concentration. B, protection of the complementary strand as a function of HIVS-OPC concentration. Lanes 1, 2, and 3, G, (G + A), and T ladders, respectively; lanes 4–9, decreasing concentrations of HIVS-OPC from 2 μM to 10 nM, respectively; lane 10, control of digestion; lane 11, the target fragment only.](http://www.jbc.org/)
micrographs presented in Fig. 3 were obtained with the 3'-biotinylated HIVS-OPC conjugate and plasmid DNA containing a cloned U5 copy of the terminal sequences. They confirm the formation of a highly specific and selective complex, located 263 bp from the nearest end of DNA. This coincides well within experimental error (21 bp) with the position of the target sequence 273–283 bp from the same end of DNA molecule.

To demonstrate that the oligonucleotide-OPC conjugate could inhibit the processing and integration of the U5 LTR, we applied a quantitative assay as described previously (17). The assay involves synthetic double-stranded oligonucleotide which match one of the HIV-1 LTR extremities as the strand transfer substrate and a heterologous plasmid DNA as a target substrate. It concerns the first step of integration, namely, endonucleolytic cleavage, where integrase removes the dinucleotide 5'-GT-3' from the 3' end of LTR U5 (Fig. 4A, lane 9). Increasing the HIVS-OPC concentration from 10 nM to 2 μM in the reaction mixture resulted in a notable inhibition of the endonucleolytic cleavage (Fig. 4A, lanes 1–8). In contrast, the control oligonucleotide Hivot-OPC, even at high concentrations, had no effect on this activity (Fig. 4B). As expected, the LTR U3 substrate was normally processed by integrase (Fig. 5A, lane 9) in the presence of either HIVS-OPC or HIVT-OPC, thus reflecting the absence of triplex formation with this segment of DNA (Fig. 5A and B, respectively).

To further evaluate the influence of HIVS-OPC and HIVT-OPC on integration, the LTR U5-GT and LTR U3-GT oligonucleotides were used as substrates and the pSP65 vector as a target DNA. In the absence of the TFO conjugate, integrase yielded an integration of about 20% for U5-GT and of 5–10% for U3-GT, consistent with a previous report (41). Adding HIVS-OPC into the integration mixture resulted in a strong inhibition of the reaction with a midpoint of integration efficiency corresponding to 60 nM HIVS-OPC (Fig. 6A). Noteworthy, the midpoint of inhibition for the endonucleolytic cleavage occurred at the same range of HIVS-OPC concentrations (Fig. 4A, lanes 5–8). With the control HIVT-OPC, the integration processed normally at any concentration tested (Fig. 6B). As expected, neither HIVS-OPC nor HIVT-OPC had a visible effect on the integration of LTR U3-GT into plasmid DNA (Fig. 7).

The results presented here clearly demonstrate through two independent methods the occurrence of an alternate strand DNA triplex near the integrase-binding site of the U5 LTR HIV-1 end. Our DNase I footprinting experiments show that binding of the designed oligonucleotide to a 42-mer target sequence of LTR U5 was confirmed by electron microscopy visualization (Fig. 3A and C). The sites of triplex formation are seen as beads corresponding to the streptavidin molecules (see C). A, micrographs of the complexes bio-3'-GGTTTTTGTGT-5'OPC-pU5/Nhel plasmid DNA-streptavidin. The arrows indicate the streptavidin molecules. Micrographs were taken in an annular dark-field mode on rotary-shadowed molecules. B, histogram of the distribution of bound streptavidin on 49 DNA molecules. The position of the peak center is 263 bp from the nearest end. The arrow indicates the position of the U5 LTR target site. C, schematic illustrating our approach.
sequence results in the formation of a triplex that is stable at neutral pH and physiological temperature. Electron micrographic data, obtained with the same target DNA cloned within a plasmid DNA, confirm the high selectivity of the triplex formation as an uniquely positioned complex over the whole sequence of plasmid DNA. The inhibitory effect of the triplex formation was proven through the inhibition of the two HIV-1 integrase-mediated reactions, namely, the endonucleolytic cleavage of the substrate and its subsequent integration into the heterologous DNA. This inhibition was observed at rela-
tively low concentrations of HIVs-OPC (50–100 nM) probably due to the beneficial influence of the 3'-end-conjugated intercalator chromophore.

Together with our previous findings on the in vitro inhibition of U3 LTR HIV-1 end integration via a canonical triplex, this work extends the range of possible targets on HIV DNA. It may also constitute a new basis for a pharmacological strategy against the propagation of AIDS.

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