Disruption of HIV-1 Integrase−DNA Complexes by Short 6-Oxocytosine-Containing Oligonucleotides[†,‡]

Priscille Brodin,[§,||] Marina Pinskaya,[⊥] Malcolm Buckle,[§] Uwe Parsch,[@] Elena Romanova,[⊥] Joachim Engels,[@] Marina Gottikh,[⊥] and Jean-François Mouscadet*[,§]

Institut Gustave Roussy, UMR 8532, 39 rue Camille Desmoulins, 94805 Villejuif, France, Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119899 Moscow, Russia, and Institut für Organische Chemie, Johann-Wolfgang-Goethe Universität, Marie-Curie Strasse 11, 60439 Frankfurt, Germany

Received August 29, 2001; Revised Manuscript Received November 29, 2001

ABSTRACT: We recently found that oligonucleotides containing the 6-oxocytosine heterocyclic base are efficient inhibitors of the HIV-1 integrase in vitro [Brodin, P., et al. (2001) *Nucleosides Nucleotides Nucleic Acids 20*, 481−486]. In this report, we demonstrate that the inhibition arises from a noncompetitive mechanism in which the modified oligonucleotide attacks the integrase−DNA complex, leading to its active disruption. This conclusion is based on the following results. First, despite the fact that the respective affinities of a 6-oxocytosine-containing oligonucleotide and of its nonmodified counterpart for integrase were identical, only the modified compound inhibited the enzyme activities. Second, DNA binding and UV cross-linking assays indicated that the 6-oxocytosine-containing oligonucleotide prevented the formation of a stable integrase−DNA complex. Third, the kinetics of the dissociation of the integrase−DNA complex were dramatically accelerated in the presence of the modified ODN, whereas the nonmodified counterpart did not influence the dissociation. This mechanism was supported by the ability of the 6-oxocytosine-containing oligonucleotide to inhibit the strand transfer activity of HIV-1 preintegration complexes in vitro. Disruption of integrase−DNA complexes by 6-oxocytosine-containing oligonucleotides constitutes an original mechanism of integration inhibition, therefore suggesting a strategy for searching for inhibitors of the HIV-1 preintegration complexes.

Acquired immunodeficiency syndrome (AIDS) could theoretically be treated with potent antiviral agents, thus warranting the interest for the search for new powerful inhibitors of replication of the virus. The recent use of drug combinations has permitted the durable suppression of HIV-1[1] replication (*1*). However, drug toxicity and the emergence of drug-resistant isolates necessitate the search for new drugs that can be added to the multidrug regime termed the highly active antiretroviral therapy (HAART). HIV replication depends on the viral molecular engine consisting of three viral enzymes: the reverse transcriptase, the protease, and the integrase (IN). Drugs included in the HAART regime are mainly directed against the reverse transcriptase (RT) and the protease (PR). The third enzyme, IN, carries out the integration of the viral DNA into the host cell genome, a critical step for HIV-1 replication as it ensures expression and perpetuation of the viral genome (*2*). Therefore, IN constitutes an attractive target for new pharmacological approaches. In vitro, IN is the only factor that is necessary to carry out the integration reaction which can be modeled with purified recombinant integrase and a short dsDNA substrate (*3*−*5*). The integration process is composed of two steps called 3′-processing and strand transfer, both involving the nucleophilic attack of an internucleotide phosphate by a hydroxyl group (*6*). Both reactions require only short dsDNA substrates that mimic the ends of the unintegrated viral DNA and a divalent metal ion cofactor (*7*). IN can also carry out a disintegration reaction which is the reverse of the strand transfer step. It is a nonphysiological reaction that can be obtained with the isolated catalytic core, whereas 3′-processing and strand transfer require the whole enzyme (*8*). Development of these assays has made possible the identification of several classes of integrase inhibitors, among them nucleotides and oligonucleotides (for a recent review, see ref *9*). With regard to the oligonucleotides, most studies focused on structure-forming oligonucleotides such as tetrad-forming oligonucleotides (*10*−*12*), structured branched compounds (*13*), and triplex-forming oligonucleotides (*14*,

[†] This work was supported by funds from INTAS Grant 96-1216, from Russian Foundation for Basic Research Grant 02-04-48797, from the Agence Nationale de Recherche sur le Sida (ANRS), and from SIDACTION. P.B. and M.P. gratefully acknowledge fellowship support from SIDACTION and from INTAS Grant YSF-00-88, respectively. M.B. is grateful for the support of the Fondation de la Recherche Medicale (FRM) for the award of a grant for the creation of a new laboratory.

[‡] Preliminary accounts of this work were presented at the XIV[th] Roundtable on Nucleosides, Nucleotides and Their Biological Applications (*24*).

* To whom correspondence should be addressed: Institut Gustave Roussy, PR2, UMR 8532 CNRS, 39 rue Camille Desmoulins, 94805 Villejuif cedex, France. Telephone: 33 (0)1 42 11 50 43. Fax: 33 (0)1 42 11 52 76. E-mail: jfm@igr.fr.

[§] UMR 8532.

[||] Present address: Unité de génétique moléculaire bactérienne, Institut Pasteur, 28, rue du Dr Roux, 75724 Paris cedex 15, France.

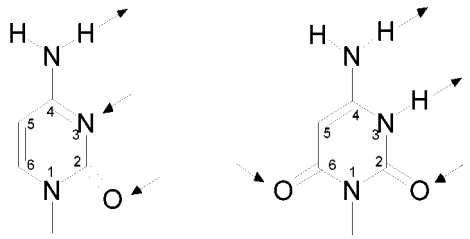
[⊥] Lomonosov Moscow State University.

[@] Johann-Wolfgang-Goethe Universität.

[1] Abbreviations: DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HIV-1, human immunodeficiency virus, type 1; IN, integrase; LTR, long terminal repeat; ODN, oligodeoxynucleotide; PIC, preintegration complex.

10.1021/bi015732y CCC: $22.00
Published on Web 01/11/2002

Chart 1: Structures of Cytidine (**1**) and 6-Oxocytidine (**2**)[a]



1 **2**

[a] Arrows indicate the potential hydrogen bonding pattern of the bases.

15). The fact that these oligonucleotides were capable of forming either a self-structure or an intermolecular structure with the DNA substrate played a key role in their inhibitory effect. The active site of IN which accepts a variety of structurally dissimilar phosphoryl donor and acceptor substrate molecules was proposed to be the actual target of tetrad-forming oligonucleotides (*16*). On the other hand, single-stranded oligodeoxynucleotides that do not form such structures may also interact with integrase (*17*−*19*). In this category, those ODNs that mimic the LTR sequence are the most active, suggesting that IN may accommodate a single-stranded LTR extremity. Accordingly, a mononucleotide binding site was identified as containing the three lysines of the catalytic core domain, Lys-156, Lys-159, and Lys-160 (*20*). Modified and phosphorylated nucleotides as well as dinucleotides have been reported to exhibit antiviral properties against integrase by binding to this site (*21*−*23*). Together, these observations suggest that modified bases may also improve the inhibitory activity of single-stranded oligonucleotides. For this reason, a set of 11-mer oligonucleotides containing 6-oxocytosine, 8-oxoguanine, and 7-deazaguanine were synthesized and tested. We observed that oligonucleotides harboring 6-oxocytosine are integrase inhibitors with IC_{50} values in the micromolar range (*24*). In this report, we investigate their mechanism of inhibition. We demonstrate that they act by disrupting the IN−DNA nucleoprotein, thereby inhibiting the PIC-mediated integration reaction.

EXPERIMENTAL PROCEDURES

Oligonucleotides. Oligodeoxyribonucleotides U5B (5′-GTGTGGAAAATCTCTAGCAGT-3′), U5B-2 (5′-GTGTGGAAAATCTCTAGCA-3′), U5A (5′-ACTGCTAGAGATTTTCCACAC-3′), U3B (5′-AGTGAATTAAGCCCTTCCAGT-3′), U3A (5′-ACTGGAAGGGCTTAATTCACT-3′), and DHIV38 (5′-TGCTAGTTCTAGCAGGCCCTTGGGCCGGCGCTTGCGCC-3′) were synthesized by Eurogentec and purified on 18% polyacrylamide/7 M urea gels. 6-Oxo-2′-deoxycytidine-3′-*O*-phosphoramidite was synthesized as described in ref *25* and incorporated in 11-mer oligodeoxynucleotides using phosphoramidite chemistry by standard techniques with an automatic DNA synthesizer (Applied Biosystems model ABI 380B) (the nucleotide sequence of all oligonucleotides is shown in Chart 1). After a mild deprotection procedure (concentrated aqueous ammonium, room temperature, 36 h), modified oligonucleotides were precipitated by ethanol and purified on a 20% polyacrylamide/7 M urea gel.

Assays for Integrase 3′-Processing, Strand Transfer, and Disintegration Activities. Oligonucleotides U5B, U3B, U5B-2, and DHIV38 were labeled at their 5′-end by T4 polynucleotide kinase (Biolabs) using [γ-^{32}P]ATP (specific activity of 3000 Ci/mol of NEN). The three different integrase activities were assayed with the following substrates. (i) For 3′-end processing, 21-mer duplexes U5 and U3 corresponding to the sequences of the HIV-1 long terminal repeats (LTR) were used as DNA substrates. They were prepared by annealing ^{32}P-labeled U5B or U3B with their complementary strand (U5A or U3A, respectively) in 100 mM NaCl, and the resulting duplexes were finally desalted on a ChromaSpin-10 column (Clontech). (ii) For strand transfer, to assay only the strand transfer, a preprocessed substrate (U5B-2/A) corresponding to the viral U5 LTR end after 3′-end processing was used. The ^{32}P-labeled oligonucleotide U5B-2 was annealed to U5A and desalted as described above. (iii) For disintegration, DHIV38 was heated to 90 °C for 5 min and then slowly cooled to 4 °C to form the dumbbell structure. Recombinant full-length and (50−212) integrases were purified as previously described (*26*). The same reaction conditions were used for 3′-processing, strand transfer, and disintegration. The ^{32}P-labeled DNA substrate (0.025 pmol) was incubated in the presence of 1.3 pmol of integrase in a buffer containing 20 mM Hepes (pH 7.2), 20 mM NaCl, 1 mM DTT, and 10 mM $MgCl_2$ at 37 °C. The reaction was stopped by adding 80 μL of a stop solution [0.3 M sodium acetate, 10 mM Tris (pH 7.5), and 1 mM EDTA]. The reaction products were phenol-extracted, precipitated with ethanol, and resuspended in 7 M urea. Products were separated on 18% polyacrylamide/7 M urea gels. Phosphorimages were recorded on a STORM 840 Phosphorimager (Molecular Dynamics) and quantified using ImageQuant software. Data of three independent experiments were averaged, and isotherm binding and inhibition curves were fitted with Prism3.0 software.

Assays for Integrase Binding to Its Substrate. The reaction conditions of integrase activity assay were also used for the integrase binding assays. Integrase and the U5 substrate were incubated at 25 °C for 20 min. Samples were loaded on an 8% nondenaturing acrylamide gel containing 0.2% glycerol. Electrophoresis was performed with a running buffer containing 50 mM Tris-Borate (pH 7.5) and 1 mM EDTA at 4 °C (5.6 V/cm). Analysis and quantification were performed on a Molecular Dynamics STORM 840 Phosphorimager.

Laser-Mediated UV Cross-Linking. Rapid pulse UV cross-linking was performed using a high-energy light source emitted by a Nd:YAG laser (DCR-3G, Spectra Physics) as described previously (*27*). DNA binding reactions were performed as described above prior to exposure to a single 5 ns pulse of high-energy monochromatic (266 nm) light. Adduct formation was monitored by 12% SDS−polyacrylamide gel electrophoresis on a Mini-Protean II system (Bio-Rad).

Assay of Preintegration Complexes (PICs). Preintegration complexes were purified from HIV-infected cells according to the method of ref *28*. Target DNA (312 bp) was obtained by PCR amplification of the pGEM-3Z plasmid (Promega) with primers Biospic1 (5′-GCTTAATCAGTGAGGCACCTA-3′) and Biospic2 (5′-GTAGCAATGCCAACAACGTTG-3′). PIC integration reactions were carried out by incubating 10 μL of extract containing PICs with 100 ng of the 312 bp

Table 1: Inhibitory Effect of Different Oligonucleotides Containing a 6-Oxocytosine-Modified Base (C*)

compound	sequence (5′−3′)	IC_{50} (μM)[a]
$TC*_{11}$	AGAGATTTTC*C*	0.5
$AC*_{11}$	GGAAAATC*TC*T	1.5
$C*T_{11}$	C*C*TTTTAGAGA	0.3
$C*A_{11}$	TC*TC*TAAAAGG	3
CT_{11}	CCTTTTAGAGA	>10

[a] IC_{50} values were determined in a strand transfer assay using the U5-2 substrate.

target DNA in 20 μL of buffer containing 10 mM Hepes (pH 7.5), 1 mM DTT, and 10 mM $MgCl_2$ at 37 °C for 1 h. The DNA fragments were first recovered after proteolysis with 2 μL of proteinase K (Qiagen, 20 mg/mL) for 10 min at 56 °C and then were phenol-purified and precipitated with ethanol. Integrated fragments are PCR-amplified with ^{32}P-labeled primer U5B-2 (5′-GTGTGGAAAATCTCTAGCA-3′), primer Biospic1 (5′-GCTTAATCAGTGAGGCACCTA-3′), and Taq Gold star (Perkin-Elmer). The cycling method was as follows: 94 °C for 10 min and 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min. DNA fragments were then suspended in 7 M urea and separated on an 8% polyacrylamide/7 M urea gel. Finally, gels were analyzed on a STORM 840 Phosphorimager (Molecular Dynamics).

RESULTS

A Short Oligonucleotide Containing 6-Oxocytosine Inhibits the HIV-1 Integrase. Oligonucleotides containing modified heterocyclic bases were synthesized and tested as inhibitors of integrase-mediated reactions in vitro (*24*). Integrase inhibition assays were performed with recombinant integrase and double-stranded oligonucleotide substrate U5 mimicking the U5 viral DNA end. Only 6-oxocytosine (C*)-containing oligonucleotides were found to be inhibitors of U5 LTR integration. Among the oligonucleotides that were studied, two ($TC*_{11}$ and $AC*_{11}$) mimicked internal sequences of the U5 integrase binding site whereas two others ($C*T_{11}$ and $C*A_{11}$) displayed the inverted polarity (Table 1). Their effect on the strand transfer reaction was assayed using a preprocessed duplex (U5B-2) as the IN substrate.

Strand transfer is a transesterification resulting in the insertion of one 3′-processed oligonucleotide into another oligonucleotide, yielding higher-molecular weight products with a slower migration than the 19-mer U5B-2 on a 15% denaturing acrylamide gel. As shown in Figure 1A, the modified oligonucleotide $C*T_{11}$ efficiently inhibited the strand transfer reaction. The concentration dependence of the inhibition fitted well to a sigmoidal dose−response curve, yielding an IC_{50} equal to 0.3 μM (Figure 1B). In contrast, the nonmodified ODN CT_{11} did not exhibit any effect up to 10 μM, thus indicating that the inhibitory effect was correlated with the presence of the modified base. Furthermore, the position of 6-oxocytosine was found to be important for the inhibitory effect since oligonucleotides containing two neighboring C* nucleosides at their 3′- or 5′-end displayed the strongest activity (see Table 1).

The 11-mer C*C*TTTTAGAGA mimicking the viral LTR U5 in the inverted polarity was the most potent compound with an IC_{50} of 0.3 μM. The modified nucleoside 6-oxo-2′-deoxycytidine (C*) demonstrated a slight inhibitory effect, although in the millimolar concentration range (*24*). The fact that the inhibition depends on the position of the modified heterocyclic base position suggests that 6-oxocytosine-containing oligonucleotides may display a specific effect against integrase. This conclusion is supported by the fact that $C*T_{11}$ did not show any inhibitory effect on either the Klenow fragment of DNA polymerase or *Escherichia coli* RNAse H up to 20 μM (data not shown), even though these enzymes share structural similarities with the viral integrase (*29*, *30*). Finally, it is worth noting that a low concentration (1 μM) of the nonmodified CT_{11} oligonucleotide repeatedly led to stimulation of the integrase activity (Figure 1C).

Contradictory results are found in the literature regarding the inhibition of integrase by short oligonucleotides. While short nonmodified oligonucleotides (l < 21) were repeatedly

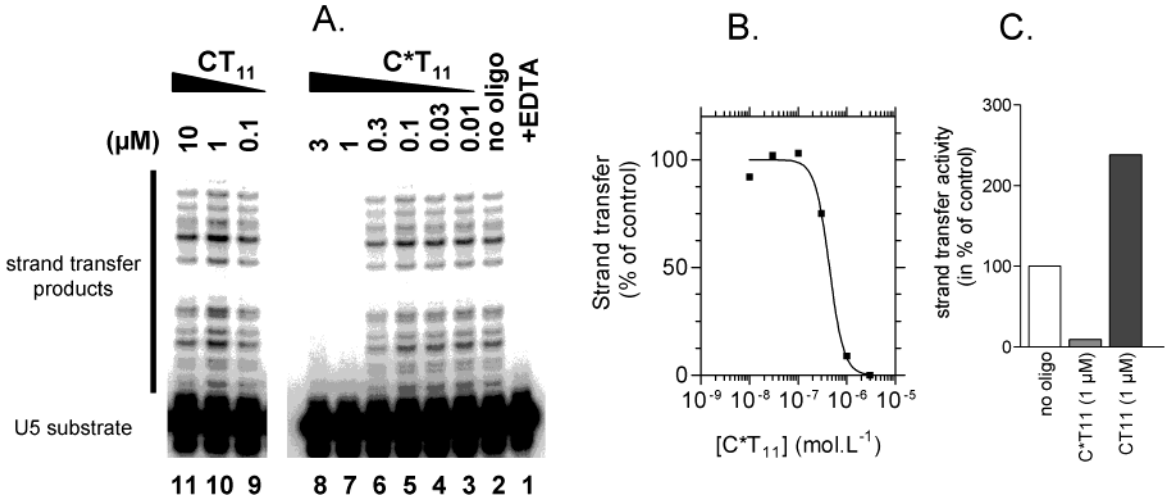


FIGURE 1: Inhibition of the strand transfer activity of HIV-1 integrase by the $C*T_{11}$ oligonucleotide. (A) Dose−response effect of modified and nonmodified ODNs on strand transfer activity. The 5′-end-labeled U5 substrate (1.25 nM) was incubated for 1 h at 37 °C with 65 nM IN: lane 1, negative control in the presence of 20 mM EDTA; lane 2, no inhibitor; lanes 3−8, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, and 3 μM $C*T_{11}$ ODN, respectively; and lanes 9−11, 100 nM, 1 μM, and 10 μM nonmodified CT_{11} ODN. (B) Quantification of experimental data obtained in the presence of the $C*T_{11}$ ODN. Data from three independent experiments were averaged and fitted on a sigmoidal dose−response model using Prism3.0 software. (C) Graphical comparison of the strand transfer activity of IN in the absence of a short ODN (white) and in the presence of either 1 μM $C*T_{11}$ (gray) or 1 μM CT_{11} (black) ODNs.

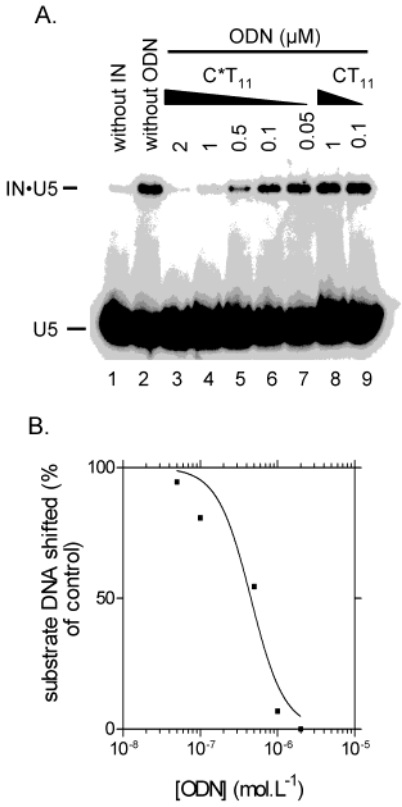


FIGURE 2: Inhibition of the formation of the stable IN•S complex by the C^*T_{11} ODN. (A) Dose−response effect of modified and nonmodified ODNs as measured by an electrophoretic mobility shift assay. IN (100 nM) was incubated with 10 nM U5 target for 20 min at 25 °C in the presence of increasing concentrations of C^*T_{11} or CT_{11} ODNs. Complexes were separated on an 8% native polyacrylamide gel: lane 1, control without IN; lane 2, positive control without ODN; lanes 3−7, 2, 1, 0.5, 0.1, and 0.05 μM C^*T_{11}, respectively; and lanes 8 and 9, 1 and 0.1 μM CT_{11}, respectively. (B) Quantitative analysis of complex formation in the presence of the C^*T_{11} ODN. Data were fitted to a sigmoidal dose−response model.

found to be inactive against HIV integrase in agreement with our own results (*27*), Caumont and colleagues (*17*) reported that ODNs mimicking the LTR sequence competitively inhibited integrase activity. We therefore decided to investigate the mode of inhibition of integrase activity by 6-oxocytosine-containing ODNs. To determine how the short modified ODN impaired the function of the integrase, we examined its effect on the formation of the complex between the integrase (IN) and its U5 DNA substrate (S). This complex will be termed the IN•S complex from this point on.

The Presence of the 6-Oxocytosine-Containing ODN Impairs the Formation of an IN•S Complex. To determine whether 11-mer oligonucleotides were able to influence the binding of integrase to DNA, we used the property of IN of forming insoluble complexes with DNA that can be isolated by either low-speed centrifugation or acrylamide gel electrophoresis. For these experiments, the U5 DNA substrate and the short oligonucleotide inhibitor were added to the integrase solution simultaneously in a buffer corresponding to standard reaction conditions. As shown in Figure 2A, the complex of integrase with its ^{32}P-labeled U5 LTR DNA remained on top of an 8% acrylamide gel running under nondenaturing conditions, whereas the nonbound substrate migrated faster.

Addition of increasing concentrations of C^*T_{11} resulted in a decrease in the amount of the ^{32}P-labeled IN•S complex. A 50% binding inhibition was observed at 0.3 μM (Figure 2B), in agreement with the IC_{50} value. Conversely, the nonmodified oligonucleotide CT_{11} did not influence integrase binding up to 1 μM. These results demonstrated that the 6-oxocytosine-containing oligonucleotide was a strong inhibitor of formation of a stable IN•S complex.

Three different mechanisms can be postulated to explain the effect of the 6-oxocytosine-modified oligonucleotide. First, the short ODNs may bind to the substrate DNA by forming an intermolecular structure occluding the binding site of IN. This mechanism was previously demonstrated for modified triple-helix-forming oligonucleotides (*14*, *15*). Second, IN may bind to the 6-oxocytosine ODN, thus preventing formation of the IN•S complex. In this case, a putative secondary structure formed by 6-oxocytosine-containing oligonucleotides due to an unusual base pairing of modified nucleosides,may be recognized by IN as demonstrated for the DNA quadruplex. Such a competitive mechanism implies that IN shows more affinity for the modified ODN than for the nonmodified one since the latter did not influence the formation of the IN•S complex. Finally, the modified ODN may directly attack the IN•S complex, resulting in its disruption.

6-Oxocytosine-Containing Oligonucleotide C^*T_{11} Inhibits Integration of both U5 and U3 Viral Substrates. The sequence of the oligonucleotide C^*T_{11} may potentially lead to the formation of a very short triplex with the U5 LTR DNA. However, the fact that the oligonucleotide TC^*_{11} incapable of triplex formation due to its inverted polarity inhibited U5 LTR integration with approximately the same efficiency ($IC_{50} = 0.5$ μM) does not support this mechanism. To definitively rule out this possibility, we determined the effect of the oligonucleotides using different substrates. Indeed, IN is capable of using either U5 or U3 LTR extremities as viral DNA substrates (see Experimental Procedures for sequences). Since both sequences display no homology, an inhibitory effect due to the formation of a sequence-specific intermolecular structure with the U5 LTR will be absent when using the U3 substrate. The oligonucleotides were tested against the 3′-processing reaction which liberates the 3′-terminal dinucleotide from either U5 or U3 double-stranded 21-mer substrates. As shown in Figure 3, the addition of increasing concentrations of 6-oxocytosine-containing oligonucleotide C^*T_{11} led to a significant inhibition of the 3′-processing using either the U3 or U5 (Figure 3A) substrate. In both cases, a sigmoid inhibition curve was obtained, yielding an IC_{50} value of 8×10^{-7} or 4×10^{-7} M, respectively (Figure 3C). Furthermore, integrase activity was not impaired by the presence of the nonmodified ODN CT_{11}. Instead, the integrase 3′-processing activity was stimulated again at a low concentration (1 μM) of the nonmodified CT_{11} oligonucleotide (Figure 3C).

The 6-Oxocytosine-Containing Oligonucleotide Inhibits the (50−212) Core Domain-Mediated Disintegration. A similar result was obtained when the inhibitory activity of the oligonucleotides was assayed on the catalytic core-mediated disintegration. Indeed, the (50−212) fragment of IN is capable of performing the disintegration reaction using a dumbbell DNA substrate which lacks the purine stretch that forms a potential binding site for a triple-helix-forming

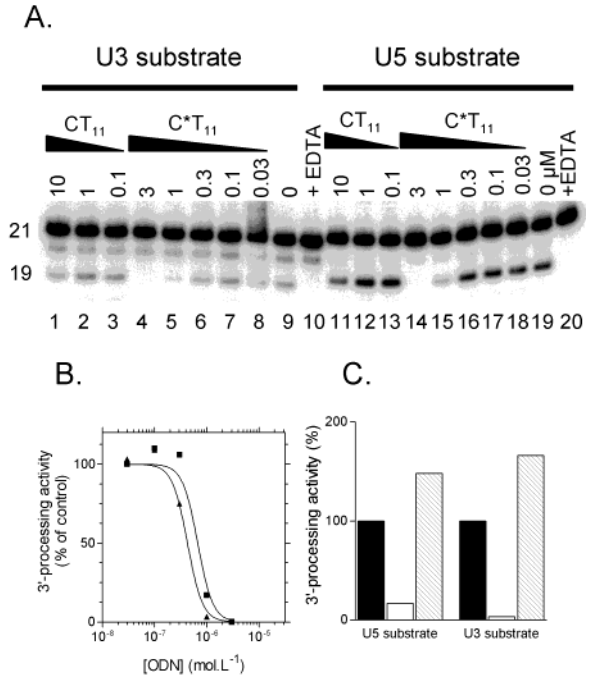


FIGURE 3: Inhibition of the 3′-processing activity of HIV-1 integrase by the C*T_{11} oligonucleotide. (A) Dose−response effect of C*T_{11} and CT_{11} ODNs on the 3′-processing activity of either U3 or U5 substrate. The 5′-end-labeled U5 substrate (0.025 pmol) was incubated for 1 h at 37 °C with 1.3 pmol of IN: lanes 1−3 and 11−13, 10, 1, and 0.1 μM CT_{11}, respectively; lanes 4−8 and 14−18, 3, 1, 0.3, 0.1, and 0.03 μM C*T_{11}, respectively; lanes 9 and 19, control without ODN; and lanes 10 and 20, 20 mM EDTA. (B) Graphical representation of experimental data obtained in the presence of the C*T_{11} ODN: (■) U5 substrate and (▲) U3 substrate. Data were fitted to a sigmoidal dose−response model. (C) Graphical comparison of the strand transfer activity of IN in the absence of short ODN (black bars) and in the presence of either C*T_{11} (white bar) or CT_{11} (cross-hatched bar) ODNs at 1 μM.

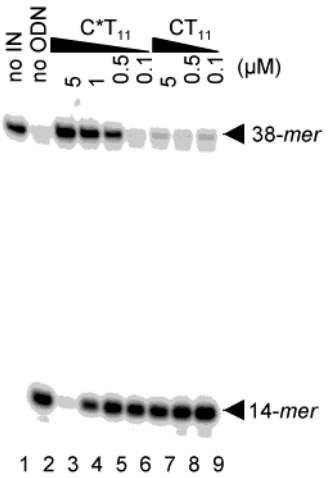


FIGURE 4: Inhibition of the (50−212) core domain-mediated disintegration by the C*T_{11} oligonucleotide. Dose−response effect of C*T_{11} and CT_{11} ODNs on disintegration with the dumbbell substrate. The 5′-end-labeled DHIV38 substrate (0.025 pmol) was incubated for 1 h at 37 °C with 2 pmol of the (50−212) core domain: lane 1, negative control without integrase; lane 2, without inhibitor; lanes 3−6, 5, 1, 0.5, and 0.1 μM C*T_{11} ODN, respectively; and lanes 7−9, 5, 0.5, and 0.1 μM nonmodified CT_{11} ODN, respectively. The numbers 38 and 14 are the lengths of the substrate and the disintegration product, respectively, in nucleotides.

oligonucleotide (*8*). The disintegration liberates the viral DNA moiety of the 38 bp dumbbell substrate, yielding 14 bp products.

As shown in Figure 4, the addition of increasing concentrations of 6-oxocytosine-containing oligonucleotide C*T_{11} led to a significant inhibition of the disintegration reaction. Integrase activity was not affected by the presence of the nonmodified ODN CT_{11}. We conclude that the inhibition mechanism does not involve an intermolecular structure between the substrate and the inhibitor, thus indicating that the actual target of the ODN was either the IN itself or the IN·S complex. To discriminate between these two hypotheses, the affinities of the enzyme for both the modified C*T_{11} and the unmodified CT_{11} ODNs were compared by performing UV cross-linking experiments. Modified as well as control oligonucleotides were ^{32}P-labeled at their 5′-extremities. Under standard integration reaction conditions, samples were irradiated in the presence of increasing concentrations of integrase with a single 5 ns pulse of high-energy 266 nm UV light. Adduct formation was monitored by SDS−PAGE. This approach was validated by determining the affinity of integrase for the 21-mer duplex U5 substrate (Figure 5A and dotted curve on panel B). Apart from the excess of ^{32}P-labeled modified oligonucleotides, one major cross-linked product was observed. Its apparent molecular mass corresponded to a complex between one integrase and one oligonucleotide. The amount of this retarded band compared to free oligonucleotide increased as a function of integrase concentration. As previously demonstrated, the dissociation constant (K_d) corresponding to the binding affinity of the integrase can be obtained from fractional saturation curves regardless of the absolute value of DNA cross-linking (*27*, *31*). If it assumed that, under our conditions, the apparent K_d is the value of the concentration of total integrase at which half-saturation was reached, it allowed an estimation for K_d of 2×10^{-7} M. These results are in good agreement with those previously reported (*27*). Similar binding curves were obtained with the single-stranded ODNs (Figure 5B, bold curves). However, first, the dissociation constants were significantly higher with either the modified or the nonmodified ODNs, thus indicating a preferential binding of IN to the double-stranded substrate DNA, and second, the experiment failed to reveal a preferential binding of integrase for the modified oligonucleotide C*T_{11} as compared to the nonmodified control.

Consequently, a higher affinity of IN for the C*T_{11} ODN cannot alone account for the selective inhibitory effect of this compound. The fact that UV-melting and CD spectroscopy studies failed to reveal any secondary structure formed by either CT or C*T oligonucleotides (data not shown) is another argument for the absence of specific binding to the C*T ODN.

The 6-Oxocytosine-Modified Oligonucleotide Disrupts the IN·S Complex. Since the modified oligonucleotide did not exhibit preferential binding either to integrase or to the substrate DNA, we investigated a potential interaction with the IN·S complex by determining whether 11-mer oligonucleotides were capable of dissociating preformed integrase−viral DNA substrate complexes.

Again, we used rapid pulse UV-laser cross-linking to quantify the amount of IN·S complex. Integrase, the ^{32}P-labeled U5 substrate, and 11-mer oligonucleotides (C*T_{11} or CT_{11}) were immediately incubated at 37 °C for 15 min before irradiation. As shown in Figure 6A (■), the C*T_{11}

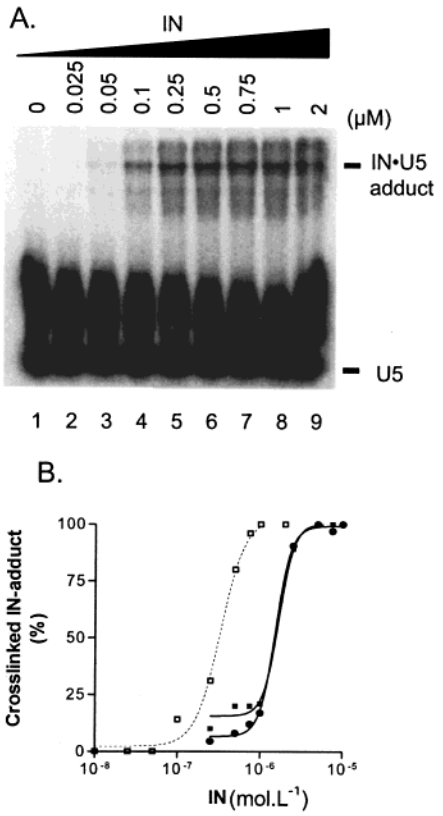


FIGURE 5: Comparison of cross-linking of IN to the U5 substrate and short single-stranded oligonucleotides. (A) Integrase was incubated with 0.4 pmol of labeled LTR U5 substrate for 20 min at 25 °C prior to exposure to a 5 ns pulse of 266 nm UV light. Cross-linked adducts were resolved by SDS−polyacrylamide gel electrophoresis. The phosphorimage shows the results of a typical titration experiment performed by varying the integrase concentration: lane 1, no IN; and lanes 2−9, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM, 1 μM, and 2 μM IN, respectively. (B) Comparison of IN binding to U5 (□) vs either C*T_{11} (■) or CT_{11} (●). Data points represent the quantification by phosphorimage densitometry of gels such as the one shown in panel A. Saturation binding curves were fitted with Prism3.0 software.

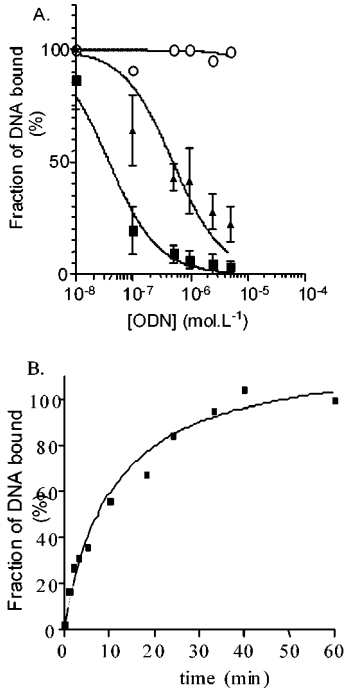


FIGURE 6: Disruption of preassembled IN·DNA complexes by C*T_{11}. Integrase−DNA complexes were allowed to form for 20 min at 25 °C. (A) Effect of increasing concentrations of C*T_{11} added before (■) or after (▲) preassembly of the complex. Absence of the effect of the CT_{11} ODN added prior to the preassembly (○). Data correspond to the means of three independent experiments. (B) Typical association curve for integrase and the U5 substrate as a function of time.

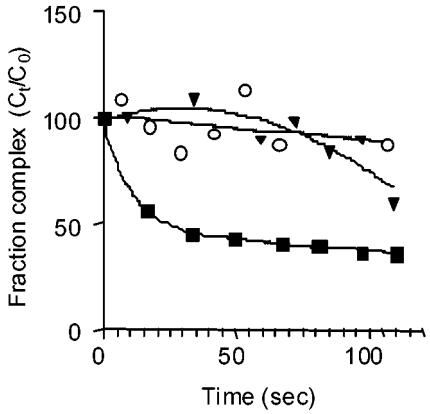


FIGURE 7: Kinetics of dissociation of IN·DNA complexes in the presence of the C*T_{11} ODN. Integrase was preincubated with the U5 LTR for 20 min at 25 °C prior to dilution in the presence of cold U5 (▼), CT11 (○), or C*T11 (■) ODNs. The time reported for the dissociation is with respect to the dilution. The amount of residual complex expressed as the ratio of the amount of complex at time t (C_t) over the initial amount (C_0) was measured by laser-mediated UV cross-linking.

ODN proved to be a very efficient inhibitor of the formation of the IN·S complex since a dose−response inhibition was obtained when increasing concentrations of the C*T_{11} ODN were added, thus confirming the previous observation from the mobility shift experiments. In the next experiment, the IN−substrate complex was allowed to form for 20 min before being challenged with the 11-mer oligonucleotides.

As shown in Figure 6B which displays a typical association curve under these conditions, more than 75% of the integrase−substrate complex was preformed after 20 min at 25 °C. The result of this experiment in the presence of increasing concentrations of the modified oligonucleotide C*T_{11} is shown in Figure 6A (▲). It turned out that the modified ODN was capable of efficiently dissociating the integrase−DNA complex. In sharp contrast, no disruption of the preformed complex was observed with the nonmodified oligonucleotide CT_{11} [Figure 6A (○)].

Altogether, these observations support a noncompetitive mechanism in which the short modified ODN attacks the IN·S complex leading to its active disruption. This mechanism implies that the dissociation rate of the IN·S complex is accelerated by the presence of the inhibitor. Thus, the measurement of the dissociation rate of the complex was conducted by rapid-pulse UV cross-linking in the absence or presence of the 11-mer ODNs. After a 20 min preformation of the integrase−substrate complex at 25 °C, the inhibitor was added and the mixture was irradiated at different times. As shown in Figure 7, incubation with 5 μM 6-oxocytosine-containing oligonucleotide leads to 50% dissociation after 20 s (■), whereas incubation with the nonmodified oligonucleotide (▲) or the nonlabeled substrate (○) had no effect on the dissociation rate constant of the preformed complex.

Although the displacement was not total, it can be clearly attributed to the dissociation of the IN·S complex since more than 75% of this complex was actually preformed after the preincubation period (see Figure 6B). This experiment provides compelling evidence that the inhibitory activity of the C*T_{11} oligonucleotide is a consequence of its interaction with integrase−substrate complexes and their subsequent disruption.

Dissociation of the IN·S Complex May Occur before the 3′-Processing. We have shown that the modified ODN C*T_{11} disrupted the IN·S complex and consequently inhibited IN. Since the ODN inhibited the 3′-processing activity of IN as efficiently as strand transfer activity, then the formation of the unstable ternary complex may occur early in the reaction, i.e., before 3′-processing can take place.

To give support to this, the effect of the C*T_{11} against the 3′-processing was assayed on preformed complexes and compared to the inhibition obtained when the ODN was present prior to complex formation. IN was preincubated with the U5 DNA substrate for 20 min. These conditions were chosen to allow the formation of more than 75% of the complex (see Figure 6). Any 3′-processing activity that could have taken place during this preincubation period was estimated by a control experiment in which EDTA was added to the IN·DNA solution after the preincubation period. The results of this experiment, shown in Figure 8, demonstrate that the C*T_{11} ODN was capable of blocking the 3′-processing activity of the preformed complex. In conclusion, the attack of the modified ODN on the IN·S complex occurred early in the reaction, after binding of IN to S, thus indicating that the inhibitor can act either on the IN·U5 complex or on the processed IN·U5-2 complex.

Inhibition of PIC Integration by the 6-Oxocytosine-Containing Oligonucleotide. The fact that the 6-oxocytosine-containing oligonucleotide was capable of inhibiting the integration upon disruption of preformed complexes hinted at the possibility that it may inhibit the integration activity of purified preintegration complexes (PICs). PICs are composed of the integrase bound to the viral DNA as well as other viral and cellular proteins (*32*, *33*). PICs purified from infected cells can be used as a source for in vitro assays of integration activity (*28*, *34*, *35*). Events of integration of viral DNA into an heterologous target can be revealed by a PCR amplification of viral−target junctions.

In this reaction, one primer was complementary to the U5 LTR and the second primer was located in the DNA target. Under standard reaction conditions, PICs were incubated with a heterologous 312 bp DNA target and the integrated products were detected after a PCR amplification round using the ^{32}P-labeled 19-mer U5B-2 oligonucleotide as a viral primer. Amplification products were separated on an 8% denaturing polyacrylamide gel. Integration events at a particular site gave rise to a unique labeled band. We compared the pattern of amplified products obtained in the presence of modified and nonmodified short ODNs. Results of a typical experiment are shown in Figure 9A. Addition of the C*T_{11} oligonucleotide led to a decrease in the amount of integrated products, suggesting an inhibitory effect of this compound on the strand transfer activity of the PIC. In comparison, no inhibition was seen in the presence of the CT_{11} oligonucleotide. Quantification of two independent experiments is given in Figure 9B. In conclusion, C*T_{11} inhibited the PIC-mediated integration reaction, thus providing further evidence that the IN·DNA complex was the actual target of this compound.

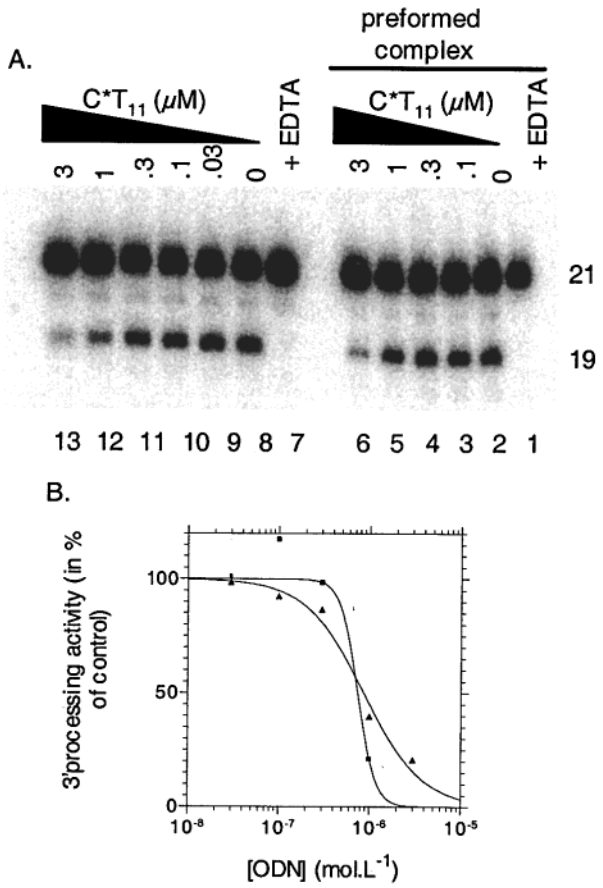


FIGURE 8: C*T_{11} inhibition of 3′-processing of preassembled complexes. (A) Phosphorimage of the dose−response effect of C*T_{11} on 3′-processing activity. The 5′-end-labeled U5 substrate (0.025 pmol) was incubated for 20 min at 25 °C with 1.3 pmol of IN in the absence or presence of increasing concentrations of C*T_{11}. The 3′-processing reaction was started by placing the samples at 37 °C. The reaction was allowed to proceed for 1 h at 37 °C in the presence of 20 mM EDTA (lanes 1 and 7), no inhibitor (lanes 2 and 8), or 10 nM, 30 nM, 100 nM, 1 μM, and 3 μM C*T_{11} ODN (lanes 2−6 and 9−13, respectively). (B) Graphical representation of experimental data. Data were fitted to a sigmoidal dose−response model: (▲) preformed IN·U5 complex and (■) absence of complex preformation.

DISCUSSION

Since the natural substrate of IN is DNA, nucleotides, oligonucleotides, and polynucleotides would all be expected to interfere with IN activity. Inhibition by these compounds has already been demonstrated (see ref *9* for a review), thus indicating that integrase may possess several binding modes with nucleic acids. This is not in itself surprising since integrase has been shown to specifically cleave a substrate DNA, 3′ of a conserved CA, and to covalently insert the processed ends into nonspecific target DNA. Thus, at some point during the integration process, the protein has to bind simultaneously to both a specific viral DNA end and a nonspecific target DNA. Although the role of these interactions during the overall integration process is not yet precisely known, pharmacological studies have demonstrated that integrase can accommodate a variety of structurally dissimilar

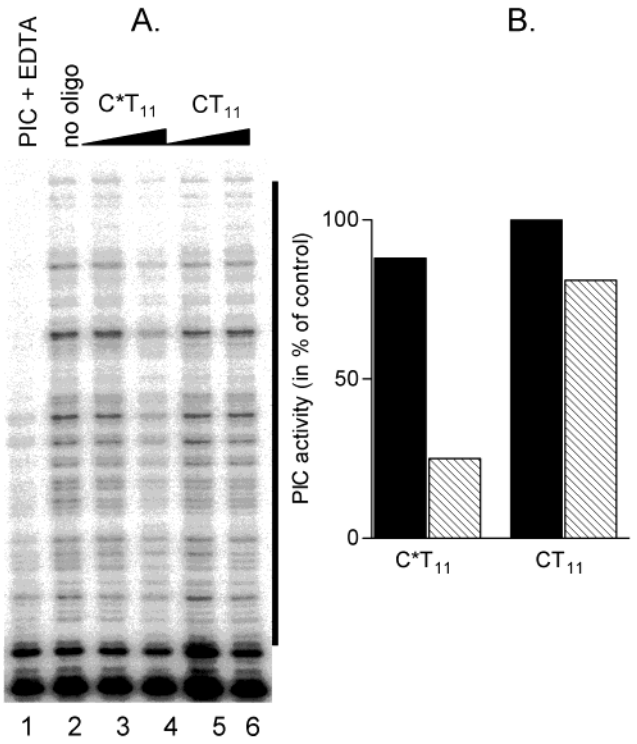


FIGURE 9: Inhibition of PIC-mediated integration by C^*T_{11}. (A) PIC-containing cell extracts were incubated for 1 h at 37 °C with a 300 bp linear DNA target in the absence or presence of the inhibitor. Integration events were PCR-amplified as described in Materials and Methods: lane 1, with 20 mM EDTA; lane 2, PIC activity in the absence of inhibitor; lanes 3 and 4, with 1 and 10 μM C^*T_{11}, respectively; and lanes 5 and 6, with 1 and 10 μM CT_{11}, respectively. The black line indicates the integration events that were used for quantification. (B) Quantification of two independent experiments: (black bars) 1 μM ODNs and (cross-hatched bars) 10 μM ODNs.

phosphoryl donor and acceptor nucleotide compounds. The characterization of the anti-integrase properties of certain mono- or dinucleotides led to the identification of a nucleotide binding site which was mapped close to the active site and involves residues Lys-156, Lys-159, and Lys-160 (*10*, *20*−*23*, *36*). These compounds displayed competitive inhibition with moderate apparent IC_{50} values in vitro. Single-stranded oligonucleotides (ODNs) are also inhibitors of HIV-1 integrase (*17*, *18*). The most potent ODNs are 17-mer self-structured G-rich ODNs that bind tightly to integrase. In this specific case, the interaction with the active site is stabilized by secondary interactions with distal domains of the protein (*37*). Larger oligonucleotides have also been found to inhibit integrase in vitro (*17*, *27*). However, they displayed a different mode of inhibition since they acted by attacking the preformed IN·DNA complex (IN·S). This effect was strongly dependent on the length of the oligonucleotide, and disruption of complexes was not observed for oligonucleotides shorter than 21 nucleotides (*27*).

Since inhibition by mono- and dinucleotides appeared to depend more on the nature of the bases than on modification of the sugar moieties (*23*), we introduced modified bases with the aim of increasing the affinity of short ODNs for IN. We observed that a 11-mer ODN modified with 6-oxocytosine but not its unmodified counterpart disrupted complexes between HIV-1 integrase and its cognate DNA substrate. A number of important differences were noted in the inhibitory effects of 6-oxocytosine-containing oligonucleotides compared to mononucleotides, which are competitive inhibitors of integrase. First, although the respective affinities of the modified and the control ODN for the enzyme were comparable, only the modified oligonucleotide inhibited integrase activities in vitro. Second, the inhibition was correlated with the ability of the ODN to prevent the formation of a stable IN·S complex. Third, the kinetics of IN·S complex dissociation were dramatically accelerated in the presence of the modified ODN. It is interesting to note that the incomplete dissociation of the complex in the presence of the inhibitor confirms the presence of a different challenge-resistant complex which forms and the amount of which increases slowly over time in vitro after the initial binding of IN to DNA (*27*). The presence of this resistant complex gave rise to biphasic dissociation kinetics. The challenge-sensitive complex dissociated quickly ($k_{obs} = 0.1$ s^{-1}), indicating that the half-life of the ternary intermediate was very short. From the dissociation rate, a half-life of approximately 7 s at 25 °C can be estimated. Since the resistant complex appears only after the formation of the sensitive complex, then the short half-life of the sensitive complex in the presence of the C*T ODN accounts for the complete inhibition of complex formation when the integrase, the substrate, and the inhibitor are mixed simultaneously.

Finally, the modified ODN was capable of inhibiting the strand transfer activity of purified preintegration complexes (PICs). These observations strongly support a model in which the short modified ODN actively dissociates the IN·S complex. It is unlikely that a simple electrostatic interaction between the single-stranded ODN and integrase is sufficiently robust to explain the effect reported here. Indeed, weak complexes formed between integrase and nonmodified single-stranded ODNs result in the slight stimulation of both IN activities in the micromolar concentration range (ref *17* and this report) and inhibition at higher concentrations (*17*). On the contrary, disruption of the IN·S complex by an inhibitor would require that the interaction with the enzyme occur to actively disfavor the original nucleoprotein geometry and thus enhance subsequent dissociation of the complex. Consequently, the inhibitor would need to establish either hydrogen or hydrophobic interactions with the complex. Since no inhibition was observed with the nonmodified ODN, this function is clearly associated with the presence of the modified heterocyclic base. Moreover, the nature of the modified base is crucial as the inhibitory effect was not observed with 8-oxoguanosine, 7-deazaguanosine (*24*), or 5-methylcytosine (data not shown). Examination of the structure of the modified base yields some clues concerning the nature of this relatively strong interaction. First, the acceptor−donor (A−D) hydrogen bonding pattern of 6-oxocytosine 2 is different from that of cytosine 1 since protonation of position 3 shifts the pattern from D−A−A to D−D−A (see Chart 1).

Furthermore, the presence of an oxygen at position 6 introduces the potential for a supplementary hydrogen bond involving this acceptor group. Finally, the modification also increases the acidity at the 3-NH and 4-NH_2 groups, thus stabilizing potential hydrogen bonds formed by these groups (*25*). We would suggest therefore that binding of the ODN to the integrase in the context of the IN·S complex involves

hydrogen bonding between integrase and the 6-oxocytosine, giving rise to a local deformation of the protein and a subsequent disruption of the complex. The fact that the presence of two contiguous 6-oxocytosines gave rise to a more pronounced inhibition adds further support to this hypothesis. We also suggest that the ODN first interacts with the enzyme through an electrostatic interaction. This is consistent with the fact that neither sequence nor orientation was a key factor for inhibition. The subsequent formation of more stable complexes arises through the establishment of stronger interactions with the modified bases of the inhibitor.

Our results indicate that this stabilization can take place only after the integrase was bound to its substrate. In other words, the inhibitor binds to a configuration of the integrase unique to the integrase·DNA complex. This property has been seen recently for integrase inhibitors that bind specifically to the IN·S complex (*38*, *39*). Our experimental data indicate that both the 3′-processing and strand transfer were strongly inhibited and furthermore that the inhibition of 3′-processing was observed when the ODN was added even after preformation of the IN·S complex. This result demonstrates that the modified ODN binding site which was occluded on the protein alone was unmasked by the binding of IN to its substrate. This site is part of the central core domain since neither the C- nor the N-end domains were required to obtain the inhibition of disintegration. What could be the potential role of this site on the IN·S complex?

To address this question, we can use the three-step mechanism previously postulated to describe the overall integration reaction (*27*). The formation of the IN−LTR complex may be represented by the reaction $IN_n + S \rightarrow IN_n \cdot S$, where *n* is the number of IN protomers associated in the oligomer bound to the viral DNA (*27*). A considerable amount of data indicates that IN undergoes a conformational change upon binding to its DNA substrate (*40*). Thus, a maturation step of the IN·S complex, leading to the formation of an intermediary mature complex, the "so-called" ISC complex, was proposed (*41*). The cleavage reaction is a property of the ISC complex, giving rise to the processed complex PSC (ISC → PSC). Finally, the PSC complex captures a target DNA and undergoes the strand transfer reaction (PSC + DNA → strand transfer products). Extrapolating from this idea, we suggest that the conformational change of IN allows the protein to accommodate short single-stranded DNA bearing modified heterocyclic bases. In support of this hypothesis, the disruption of the terminal base pair of retroviral DNA has been shown to be required during integration (*42*). Furthermore, the disruption of base pairing at the site of cleavage may account for the ability of integrase to act specifically at the ends of viral DNA, whereas identical sequences at internal sites are not substrates of the reaction. A putative role for DNA unpairing is also supported by the fact that integration in a plasmid target in vitro is favored in stem−loop structures resembling fraying DNA ends (*43*).

The comparison of protein·DNA contacts between the integrase and either the target dsDNA or the modified single-stranded ODN should provide clues about the nature of the interacting surfaces involved in both cases. This work is currently under way.

In conclusion, we have shown that a short modified ODN can disrupt the integrase DNA complex. Since no secondary structure of the oligonucleotide was detected, the unique determinant of this effect was the presence of the modified base. The fact that the 6-oxocytosine-containing oligonucleotide was capable of inhibiting the integration upon disruption of preformed complexes suggests a strategy for searching for inhibitors of the HIV-1 preintegration complexes (PICs), which are more likely to be the relevant target in vivo. From this viewpoint, the use of modified bases in oligonucleotidic inhibitors may also provide a way of overcoming the limitations of natural ODNs such as limited cellular delivery and stability.

REFERENCES

1. Palella, F. J. J., Delaney, K. M., Moorman, A. C., Loveless, M. O., Fuhrer, J., Satten, G. A., Aschman, D. J., and Holmberg, S. D. (1998) *N. Engl. J. Med. 338*, 853−860.
2. Sakai, H., Kawamura, M., Sakuragi, J., Sakuragi, S., Shibata, R., Ishimoto, A., Ono, N., Ueda, S., and Adachi, A. (1993) *J. Virol. 67*, 1169−1174.
3. Bushman, F. D., Fujiwara, T., and Craigie, R. (1990) *Science 249*, 1555−1558.
4. Brown, P. O. (1990) *Curr. Top. Microbiol. Immunol. 157*, 19−48.
5. Craigie, R., Mizuuchi, K., Bushman, F. D., and Engelman, A. (1991) *Nucleic Acids Res. 19*, 2729−2734.
6. Brown, P. O. (1997) Integration, in *Retroviruses* (Coffin, J. M., Hughes, S. H., and Varmus, H. E., Eds.) pp 161−203, Cold Spring Harbor Laboratory Press, Plainview, NY.
7. Chow, S. A. (1997) *Methods 12*, 306−317.
8. Chow, S. A., Vincent, K. A., Ellison, V., and Brown, P. O. (1992) *Science 255*, 723−726.
9. Pommier, Y., and Neamati, N. (1999) *Adv. Virus Res. 52*, 427−458.
10. Jing, N., Marchand, C., Liu, J., Mitra, R., Hogan, M. E., and Pommier, Y. (2000) *J. Biol. Chem. 275*, 21460−21467.
11. Ojwang, J., Elbaggari, A., Marshall, H. B., Jayaraman, K., McGrath, M. S., and Rando, R. F. (1994) *J. Acquired Immune Defic. Syndr. 7*, 560−570.
12. Ojwang, J. O., Buckheit, R. W., Pommier, Y., Mazumder, A., De, V. K., Este, J. A., Reymen, D., Pallansch, L. A., Lackman-Smith, C., Wallace, T. L., et al. (1995) *Antimicrob. Agents Chemother. 39*, 2426−2435.
13. Brodin, P., Pinskaya, M., Volkov, E., Romanova, E., Leh, H., Auclair, C., Mouscadet, J. F., and Gottikh, M. (1999) *FEBS Lett. 460*, 270−274.
14. Mouscadet, J. F., Carteau, S., Goulaouic, H., Subra, F., and Auclair, C. (1994) *J. Biol. Chem. 269*, 21635−21638.
15. Mouscadet, J. F., Ketterle, C., Goulaouic, H., Carteau, S., Subra, F., Le Bret, M., and Auclair, C. (1994) *Biochemistry 33*, 4187−4196.
16. Neamati, N., Sunder, S., and Pommier, Y. (1997) *DDT 2*, 487−498.
17. Caumont, A., Jamieson, G., de Soultrait. V., Parissi, V., Fournier, M., Zakharova, O. D., Bayandin, R., Litvak, S., Tarrago-Litvak, L., and Nevinsky, G. A. (1999) *FEBS Lett. 455*, 154−158.
18. Tramontano, E., Colla, P. L., and Cheng, Y. C. (1998) *Biochemistry 37*, 7237−7243.
19. Snasel, J., Rejman, D., Liboska, R., Tocik, Z., Ruml, T., Rosenberg, I., and Pichova, I. (2001) *Eur. J. Biochem. 268*, 980−986.
20. Drake, R. R., Neamati, N., Hong, H., Pilon, A. A., Sunthankar, P., Hume, S. D., Milne, G. W., and Pommier, Y. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 4170−4175.
21. Mazumder, A., Cooney, D., Agbaria, R., Gupta, M., and Pommier, Y. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5771−5775.
22. Mazumder, A., Neamati, N., Sommadossi, J. P., Gosselin, G., Schinazi, R. F., Imbach, J. L., and Pommier, Y. (1996) *Mol. Pharmacol. 49*, 621−628.

23. Taktakishvili, M., Neamati, N., Pommier, Y., and Nair, V. (2000) *Bioorg. Med. Chem. Lett. 10*, 249−251.
24. Brodin, P., Pinskaya, M., Parsch, U., Bischerour, J., Leh, H., Romanova, E., Engels, J., Gottikh, M., and Mouscadet, J. F. (2001) *Nucleosides, Nucleotides Nucleic Acids 20*, 481−486.
25. Berressem, R., and Engels, J. W. (1995) *Nucleic Acids Res. 23*, 3465−3472.
26. Leh, H., Brodin, P., Bischerour, J., Deprez, E., Tauc, P., Brochon, J. C., LeCam, E., Coulaud, D., Auclair, C., and Mouscadet, J. F. (2000) *Biochemistry 39*, 9285−9294.
27. Pemberton, I. K., Buc, H., and Buckle, M. (1998) *Biochemistry 37*, 2682−2690.
28. Hansen, M. S., Smith, G. J., Kafri, T., Molteni, V., Siegel, J. S., and Bushman, F. D. (1999) *Nat. Biotechnol. 17*, 578−582.
29. Dyda, F., Hickman, A. B., Jenkins, T. M., Engelman, A., Craigie, R., and Davies, D. R. (1994) *Science 266*, 1981−1986.
30. Rice, P., Craigie, R., and Davies, D. R. (1996) *Curr. Opin. Struct. Biol. 6*, 76−83.
31. Pemberton, I. K., Buckle, M., and Buc, H. (1996) *J. Biol. Chem. 271*, 1498−1506.
32. Bukrinsky, M. I., Sharova, N., Mcdonald, T. L., Pushkarskaya, T., Tarpley, W. G., and Stevenson, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6125−6129.
33. Farnet, C. M., and Bushman, F. D. (1997) *Cell 88*, 483−492.
34. Carteau, S., Hoffmann, C., and Bushman, F. (1998) *J. Virol. 72*, 4005−4014.
35. Brown, P. O., Bowerman, B., Varmus, H. E., and Bishop, J. M. (1987) *Cell 49*, 347−356.
36. Jenkins, T. M., Esposito, D., Engelman, A., and Craigie, R. (1997) *EMBO J. 16*, 6849−6859.
37. Mazumder, A., Neamati, N., Ojwang, J. O., Sunder, S., Rando, R. F., and Pommier, Y. (1996) *Biochemistry 35*, 13762−13771.
38. Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., and Miller, M. D. (2000) *Science 287*, 646−650.
39. Wai, J. S., Egbertson, M. S., Payne, L. S., Fisher, T. E., Embrey, M. W., Tran, L. O., Melamed, J. Y., Langford, H. M., Guare, J. P., Zhuang, L., Grey, V. E., Vacca, J. P., Holloway, M. K., Naylor-Olsen, A. M., Hazuda, D. J., Felock, P. J., Wolfe, A. L., Stillmock, K. A., Schleif, W. A., Gabryelski, L. J., and Young, S. D. (2000) *J. Med. Chem. 43*, 4923−4926.
40. Asante-Appiah, E., and Skalka, A. M. (1999) *Adv. Virus Res. 52*, 351−369.
41. Cherepanov, P., Este, J. A., Rando, R. F., Ojwang, J. O., Reekmans, G., Steinfeld, R., David, G., De Clercq, E., and Debyser, Z. (1997) *Mol. Pharmacol. 52*, 771−780.
42. Scottoline, B. P., Chow, S., Ellison, V., and Brown, P. O. (1997) *Genes Dev. 11*, 371−382.
43. Katz, R. A., Gravuer, K., and Skalka, A. M. (1998) *J. Biol. Chem. 273*, 24190−24195.

BI015732Y