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High affinity interaction of HIV-1 integrase with specific and non-specific single-stranded short oligonucleotides

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Abstract Retroviral integrase (IN) catalyzes the integration of double-stranded viral DNA into the host cell genome. The reaction can be divided in two steps: 3′-end processing and DNA strand transfer. Here we studied the effect of short oligonucleotides (ODNs) on human immunodeficiency virus type 1 (HIV-1) IN. ODNs were either specific, with sequences representing the extreme termini of the viral long terminal repeats, or non-specific. All ODNs were found to competitively inhibit the processing reaction with K_i values in the nM range for the best inhibitors. Our studies on the interaction of IN with ODNs also showed that: (i) besides the 3′-terminal GT, the interaction of IN with the remaining nucleotides of the 21-mer specific sequence was also important for an effective interaction of the enzyme with the substrate; (ii) in the presence of specific ODNs the activity of the enzyme was enhanced, a result which suggests an ODN-induced conformational change of HIV-1 IN.

Key words: HIV-1 integrase; Specific oligodeoxynucleotide; Inhibitor

1. Introduction

Replication of retroviruses depends on the integration of a ds DNA copy of the retroviral genome into the host cell nuclear genome [1–3]. Integration requires a virus-encoded protein, integrase, and DNA sequences located in the U3 and U5 regions at the ends of the viral long terminal repeats (reviewed in [4]). The integration reaction has been analyzed in vivo and in vitro and it has been shown that it proceeds in three steps [5–7]. The first step is 3′-processing: two nucleotides (GT) from the 3′-ends of each strand of linear viral DNA are removed by IN leaving at the viral 3′-ends the conserved CA dinucleotide. A highly conserved feature of all retroviruses is the presence of a CA dinucleotide immediately upstream of the cleavage site in the LTR. This sequence is critical for site-specific cleavage and integration. The second step or strand transfer, is a concerted cleavage ligation reaction during which IN makes staggered cuts in the target DNA and ligates the recessed 3′-OH ends of the viral DNA to the overhanging 5′-phosphate ends of the target DNA at the cleavage site. The product of this reaction is a gapped intermediate. The last step is 5′-end joining, in which the integration process is completed by removal of the two unpaired nucleotides at the 5′-ends of the viral DNA and repair of the gaps between the viral and target DNA sequences.

Since IN is essential for productive retroviral infection, the enzyme is a potential target for antiviral chemotherapeutic intervention. In contrast to HIV-1 protease and reverse transcriptase, for which an extensive number of chemical inhibitors have been described, there are few reports about selective inhibitors of integrase. In the absence of a detailed complete model of how integrase functions, structure-activity analysis and molecular basis of retroviral DNA integration can provide some clues for the design of potent inhibitors against this viral target.

Here we studied the interaction of short oligonucleotides with HIV-1 IN. Our hypothesis was that ODNs having high affinity for the enzyme could offer a unique opportunity for rational design of IN-targeting drugs. To address this question we studied the in vitro processing reaction employing purified recombinant HIV-1 IN. The effect of short ODNs on this reaction was analyzed to assess interactions with IN. ODNs were either single-stranded or double-stranded, with sequences related or not to the natural substrate.

Our results showed that ODNs were able to interact with IN and that enzyme affinity was higher for specific than non-specific ODNs of the same length. All ODNs were competitive inhibitors towards substrate and the K_i values for the best inhibitors were in the nM range. This type of inhibitors which binds strongly to the active site of HIV-1 IN provides another type of potential therapeutic agent against the virus.

2. Materials and methods

All ODNs were synthesized as described in [8] and their concentration was determined according to [9]. Double-stranded DNA substrate for 3′-end processing was prepared by annealing the 19-mer ODN (5′-GTGTGGAAAATCTCTAGCA) with the 21-mer complementary strand (5′-ACTGCTAGAGATTTTCCA) by heating for 2 min at 90°C and slow cooling. This ds DNA was labeled at the 3′-end with the exonuclease-free Klenow fragment of *E. coli* DNA polymerase, [α-^{32}P]dGTP and [α-^{32}P]TTP [10].

HIV-1 IN was purified from the JSC 310 proteases deficient yeast strain transformed with the integrase expression plasmid, pHIV1S-F2IN [11]. Total protein was extracted as described in [12]. Integrase was purified using the protocol described by Engelman and Craigie [13] with some modifications to improve the yield and purity of the enzyme. All buffers used in the purification procedure were as described in [12], except that they contained 1 M ammonium sulfate

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Abbreviations: HIV-1, human immunodeficiency virus type 1; IN, integrase; LTR, long terminal repeat; ODN, oligodeoxynucleotide; ss, single-stranded; ds, double-stranded

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and 7 mM CHAPS (to avoid aggregation of IN). The soluble fraction obtained from yeast extract was applied to a 1 ml Hitrap butyl-Sepharose 4B column (Amersham Pharmacia Biotech) previously washed with 5 volumes LSC buffer and then equilibrated with 5 volumes HSC buffer containing 1 M ammonium sulfate. Proteins were eluted by a step of HSC buffer without ammonium sulfate. Fractions containing IN were pooled, diluted to 1/3 with buffer D (50 mM HEPES pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 7 mM CHAPS) and charged on a 1 ml Hitrap Heparin-Sepharose CL-4B column (Amersham Pharmacia Biotech). After washing with 5 volumes HS buffer, the column was equilibrated with 5 volumes LSC buffer. IN was then eluted by a step (1 M NaCl), pooled and diluted (1/4) with buffer D. The diluted solution was charged on a second Hitrap Heparin-Sepharose CL-4B column and after extensive washing, proteins were eluted with a linear gradient of 100% LSC buffer to 100% HS buffer. Fractions containing IN were pooled, diluted (1/4) with buffer D and applied to a MonoS PC 1.6/5 column (Amersham Pharmacia Biotech) equilibrated with buffer D plus 100 mM NaCl. Proteins were eluted with a linear gradient of 100% buffer D (containing 100 mM NaCl) to 60% HS. CHAPS (7 mM) was added to all fractions containing IN. The pooled fractions were stored at −80°C.

IN activity was determined by measuring the 3′-end processing reaction at 30°C. The standard reaction mixture (20–100 μl) contained: 20 mM HEPES (pH 7.5), 10 mM DTT, 0.1 mM EDTA, 4 mM NaCl, 7.5 mM $MnCl_2$, 0.05% NP 40, 1.5 nM ds [^{32}P]substrate. The reaction mixture was incubated for different times (2–60 min) in the presence of 10–40 nM IN. The reaction products of IN-dependent [^{32}P](GT) dinucleotide removal were estimated using two methods: (a) separation on 12% polyacrylamide gel electrophoresis in the presence of 7 M urea, or (b) measuring the label remaining in the acid-insoluble material by the utilization of dry Whatman 3MM filters (15×15 mm) presoaked in 5% trichloroacetic acid (TCA). Further treatment of the filters was carried out as described in [14]. All measurements were carried out in the linear ranges of time dependence and IN concentration.

Initial rates were measured in kinetic experiments. The type of inhibition, K_i and K_m values were estimated using the Lineweaver-Burk plot $1/v$ (where v is the reaction velocity) *versus* 1/substrate. IC_{50} values were determined at substrate concentrations comparable to K_m values. In these conditions and as theoretically calculated, $IC_{50} = 2K_i$ [15]. Errors in the estimation of K_i and K_m were within 10–30%.

3. Results and discussion

The first step of the integration process, 3′-end processing, involves a sequence-specific endonucleolytic cleavage of two nucleotides. This reaction is commonly assayed with a ds oligonucleotide derived from the HIV-1 U5 end of the LTR, containing the following sequence:

point of cleavage
5′ GTG TGG AAA ATC TCT AGC A↓**GT** 3′
3′ CAC ACC TTT TAG AGA TCG T CA 5′

We used this specific 21-mer duplex DNA containing [^{32}P]-labeled GT nucleotides in the 3′-end as substrate of the 3′-processing reaction catalyzed by HIV-1 IN. Sequence-specific removal from the 3′-end generated a radiolabeled dinucleotide product. The kinetic analysis of IN was done by measuring the label remaining in the acid-insoluble material after dinucleotide cleavage. By determining the initial velocity of the reaction at different substrate concentrations, we calculated the K_m for the specific 21-mer duplex DNA substrate to be 1.5 ± 0.3 nM.

As we were interested in studying the interaction of HIV-1 IN with short ODNs, their effect was measured in the processing reaction. ODNs were single- or double-stranded molecules of different lengths (1 to 21 nucleotides) and either related ('specific') or unrelated ('non-specific') to the specific sequence of the 21-mer substrate.

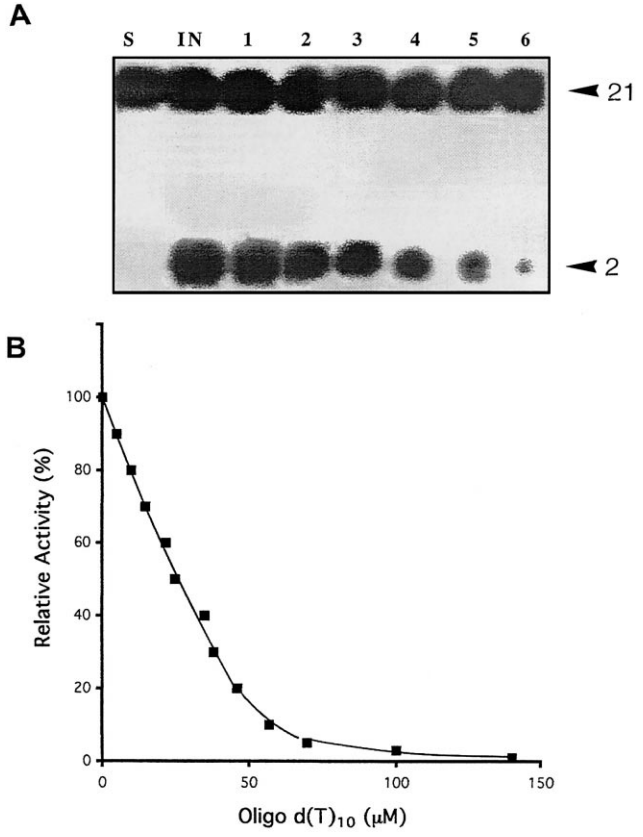

Fig. 1. Inhibition of the IN-catalyzed 3′-processing reaction by oligo $d(T)_{10}$. IN (30 nM) was incubated for 30 min at 30°C with the labeled ds 21-mer substrate as described in Section 2. A: Reaction products were analyzed by 12% polyacrylamide gel electrophoresis in the presence of 7 M urea. Complete reaction mixture containing the labeled substrate incubated in the absence of integrase (lane S) or in the presence of IN (lane IN). Lanes 1 to 6: same as lane IN, but in the presence of 5, 15, 25, 35, 50 and 140 μM of oligo $d(T)_{10}$, respectively. Arrows indicate 21 and 2 nucleotides. B: Reaction products were determined by measuring the TCA-precipitable radioactivity remaining after IN-dependent processing. Results are expressed as relative activity, 100% corresponding to IN activity in the absence of inhibitor.

We first study the effect of non-specific ODNs on the 3′-processing reaction. Fig. 1 illustrates the results obtained with oligo $d(T)_{10}$. As shown in Fig. 1A (lane IN), sequence-specific removal from the 3′-end generated a radiolabeled dinucleotide product detected following electrophoresis. In the presence of increasing concentrations of oligo $d(T)_{10}$ a dose-dependent inhibition of the 3′-end processing reaction was obtained (Fig. 1A, lanes 1–6). In Fig. 1B are presented the results obtained in a parallel experiment in which the reaction products were analyzed by counting the remaining labeled material in the acid-insoluble fraction. Oligo $d(T)_{10}$ inhibited the processing reaction with an IC_{50} value of 25 μM. Similar patterns of inhibition were obtained for all other oligo $d(T)_n$. As the measurements using both determinations matched very well, we used the second method for quantitative kinetic experiments.

The inhibitory effect produced by non-specific ODNs was then analyzed as a function of the chain length. The results

Table 1
Effect of oligonucleotides on the 3′-processing reaction catalyzed by IN

A. Specific or related ODNs	$IC_{50}(1)$ (μM)	K_i	Non-specific ODNs	$IC_{50}(2)$ (μM)	K_i	Ratio[a]
			TMP	23000		–
			dGMP	45000		–
GT	400	130	T_2	1000	330	2.5
AGT	160		T_3	500		3.1
CAGT	70		T_4	220		3.1
GCAGT	40	13	T_5	130		3.3
AGCAGT	20		T_6	100		5
TAGCAGT	10		T_7	80		8
CTCTAGCAGT	1.5		T_{10}	25	8.3	16.7
GGAAAATCTCTAGCAGT	0.1		T_{17}	5		50
GTGTGGAAAATCTCTAGCAGT	0.03		T_{21}	3		100
TTTTGTAAAACCACGGCCAGT	0.5					
T_{19}GT	1.2					
GTGTGGAAAATCTCTAGCAGrU	0.25					
GTGTGGAAAATCTCTAGCAG	0.45					
GTGTGGAAAATCTCTAGCA	0.09					
B. Specific or related duplex ODNs[b]	$IC_{50}(1)$ (μM)		Non-specific duplex ODNs	$IC_{50}(2)$ (μM)		Ratio[a]
GTGTGGAAAATCTCTAGCAGT••d(N)$_{21}$	0.004		T_{21}•A_{21}	0.32		80
T_{19}GT••A_{19}CA	0.11					
GTGTGGAAAATCTCTAGCAGrU••d(N)$_{21}$	0.15					
GTGTGGAAAATCTCTAGCAG••d(N)$_{21}$	0.17					
GTGTGGAAAATCTCTAGCA••d(N)$_{21}$	0.012					

IC_{50} corresponds to the ODN concentration giving 50% inhibition of the 3′-processing reaction.
Standard errors did not exceed 10–30%.
[a]Ratio = $IC_{50}(2)/IC_{50}(1)$.
[b]Sequence of complementary d(N)$_{21}$: 5′-ACT GCT AGA GAT TTT CCA CAC-3′.

reported in Table 1 showed that IC_{50} values decreased with ODN lengthening ($n = 1$ to 21). Kinetic studies of enzyme inhibition were conducted to determine the mechanism by which non-specific ODNs inhibit HIV-1 IN (Fig. 2). The pattern of inhibition showed that oligo d(T)$_{10}$ was a competitive inhibitor of the specific 21-mer DNA substrate with a $K_i = 8.3 \pm 1.3$ μM. The same type of inhibition was obtained for all other ODNs. In all experiments, substrate was used at concentrations comparable to the K_m value; in these conditions and as predicted theoretically, $IC_{50} = 2$–$3K_i$. The K_i values obtained for the inhibition by ODNs were, as expected, 2–3 times lower than the corresponding IC_{50} (Table 1).

Next, we determined the effect of specific ODNs, containing sequences related to the specific 21-mer substrate. The addition of a GT dinucleotide to the processing reaction produced an inhibitory effect similar to that observed with non-specific ODNs (Fig. 3A). However, an unexpected result was obtained when we tested the effect of longer specific single-stranded ODNs on the 3′-processing reaction. In contrast to the inhibition observed with non-specific ODNs and also with the GT dinucleotide, the addition of the AGT trinucleotide or longer ODNs to the 3′-processing reaction led to a detectable increase of the reaction rate at relatively low ODN concentrations, followed by an inhibition at high concentrations (Fig. 3A). To investigate the nature of the activation process we analyzed the behavior of HIV-1 IN when preincubated with different ODNs. As shown in Fig. 3B, preincubation of the enzyme with low concentration of GT-containing ODNs led to an increase of the initial rate of the 3′-processing reaction. It has been already reported, concerning integrase activation, that preincubation of HIV-1 IN with Mn^{2+} increased the specific activity of the enzyme [16]. The role of Mn^{2+} appears to be related to the induction of protein-protein interactions able to stabilize complexes with DNA [17]. The enzyme activation we observed with IN was further studied by assaying the effect of preincubating HIV-1 IN with either Mn^{2+} or specific ODNs. In both cases the enzyme activity was stimulated (Fig. 3B). At initial velocities the activity of IN was increased 2-fold in the presence of AGT, 4-fold with Mn^{2+} and 6-fold with GCAGT. It is important to recall that non-specific ODNs such as oligo d(T)$_{10}$ under these preincubation conditions, did not activate the enzyme, but inhibited HIV-1 IN as described above.

To further evaluate the inhibitory effect produced by specific ODNs in the processing reaction, it was important to eliminate the integrase activation produced by specific ODNs. We thus preincubated IN in the presence of Mn^{2+} in order to obtain the 'activated form' of the enzyme, and then the processing reaction was performed in the presence of specific ODNs. In Fig. 3C are presented the results with AGT or CAGT, where it is shown that, when starting with an Mn^{2+}-activated form of IN, an inhibition similar to that obtained with non-specific ODNs was obtained. In these conditions, specific ODNs were also competitive inhibitors towards the substrate. The IC_{50} values for specific ODNs were determined and they are summarized in Table 1. Although all ODNs were capable of inhibiting the processing reaction, an important inhibition was obtained at nmolar concentrations with the specific 21- and 19-mer ODNs.

Based on these results, we hypothesized that the effect of specific ss ODNs on the processing reaction rate may be the sum of two processes: an ODN-mediated activation of IN, followed by an inhibitory effect produced by the specific ODN, similarly to that produced by non-specific ODNs. Different reasons may explain the activation effect: (i) the influence of specific ligands on enzyme aggregation; (ii) a stimulation of the transition between an inactive IN molecule to an active one due to complex formation with ODNs; (iii) a

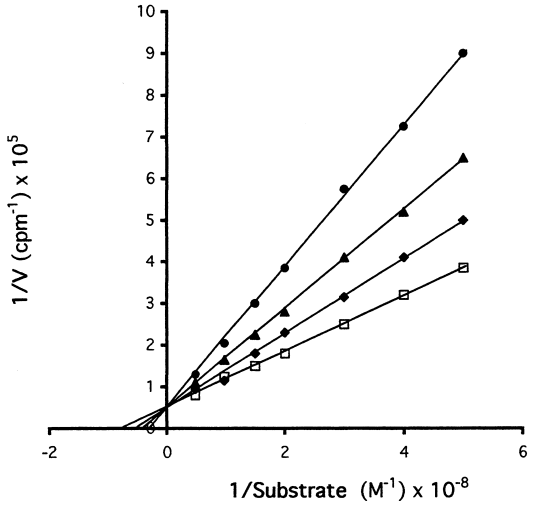

Fig. 2. Inhibition produced by oligo d(T)$_{10}$. Double-reciprocal plot according to Lineweaver and Burk. The 3′-processing reaction was performed as a function of substrate concentration as indicated. Oligo (T)$_{10}$ was added at different concentrations: 0 (□), 3.8 μM (♦), 8.3 μM (▲) and 18.3 μM (●).

change of the enzyme conformation leading to optimal catalysis.

Results of kinetic analysis showed that ODNs are competitive inhibitors that bind to the active site of IN catalyzing the 3′-processing reaction. As the inhibition experiments were done at substrate concentrations comparable to the K_m value where, as predicted theoretically, $IC_{50} = 2K_i$, we used the IC_{50} values as an estimation of the affinity of ODNs for HIV-1 IN. From data in Table 1 it can be concluded that the affinity of HIV-1 IN for ODNs depends on length, sequence and structure of the inhibitory agent. While lengthening of the ODN chain resulted always in higher affinity to HIV-1 IN, the enhancement of affinity was more important for specific than for non-specific ODNs. As a result the ratio between IC_{50} values of non-specific and specific ODNs of the same length, significantly increases with length (Table 1A). For example, the IC_{50} value for the specific 21-mer ODN is about 100 times lower than that for d(T)$_{21}$. In addition, annealing of specific ss ODNs to their complementary strands also led to an increase in affinity. The affinity of IN for $T_{21}xA_{21}$ duplex is about 10 times higher than that for d(T)$_{21}$. Thus, the contribution to the affinity for IN of the two chains of complementary ODNs is far from being simply additive. It seems most likely that one chain of the duplex forms strong specific contacts with the enzyme, while the other form only weak contacts. This is the case for other enzymes interacting with DNA we have previously analyzed, such as DNA polymerases, topoisomerases, repair and restriction enzymes [11,18–24].

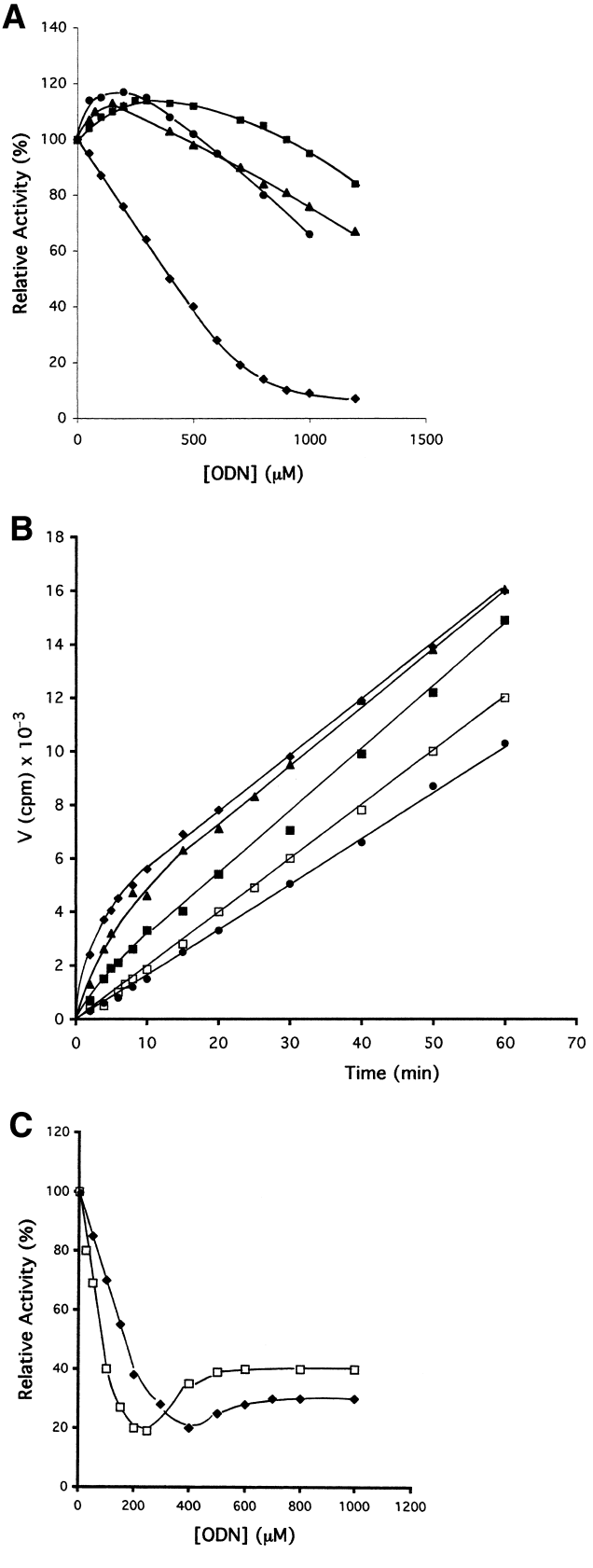

Fig. 3. A: Effect of specific ODNs on the rate of the 3′-processing reaction. Reaction mixture was incubated with IN for 30 min at 30°C in the presence of GT (♦), AGT (■), CAGT (▲) and GCAGT (●). B: Influence of IN preincubation on the 3′-processing activity. IN was preincubated at 30°C in a final volume of 100 μl, using the following conditions: integrase alone preincubated for 30 min (□); in the presence of 15 mM $MnCl_2$ for 30 min (▲); with 250 μM AGT for 2 min (■); with 63 μM GCAGT for 2 min (♦); with 20 μM oligo (T)$_{10}$ for 2 min (●). At different time intervals, aliquots (10 μl) of the preincubated mixture were drawn and the processing reaction mixture was added. C: Effect of specific ODNs on the activity of preincubated IN. The enzyme was preincubated with 15 mM $MnCl_2$ for 30 min at 30°C. Then, ODNs were added and the processing reaction was performed as described above. AGT (♦); CAGT (□).

Concerning the sequence, a higher affinity was observed for GT-containing ODNs compared to non-specific ODNs of the same length. Most interesting, a specific 20-mer ss ODN lacking the 3′-terminal T-nucleotide, or a 21-mer ss ODN in which the 3′-end T has been replaced by a ribo U led to a decrease of affinity of 15- or 8-fold, respectively (Table 1A). Also in the case of double-stranded ODNs, removal of the T in 3′-end or replacement by rU, decreased the affinity around 40 times (Table 1B). For these ds ODNs (20-mer without the 3′-end T or with a ribo U instead of T) the affinity was comparable to that of non-specific ds ODNs of the same length. In contrast, a specific 19-mer ss ODN without the GT at the 3′-end, presented higher affinity than the 20-mer, with IC_{50} values similar to that of the specific 21-mer ss ODN. The high affinity obtained with the 21- and the 19-mer was expected, since these ODNs have sequences representing specific substrates for processing and strand transfer reactions, respectively.

Different studies have been carried out in which viral DNA end sequences were altered and effects on processing were monitored. Some reports have suggested that there is limited sequence specificity required, with only the well conserved CA dinucleotide being needed for processing and strand transfer [25]. Other studies have suggested the importance of 2–7 base pairs upstream of the conserved CA, with varying effects on the reaction [26–30]. Recently, the importance of residues at positions 8–11 distal to the LTR which interact with the C-terminal domain of IN has been reported [31].

All our results taken together show the important role of the 3′-terminal GT dinucleotide in the affinity for HIV-1 IN. Our data also suggest that besides the 3′-terminal GT, and the conserved CA, the interaction of IN with the remaining nucleotides of the 21-mer specific sequence is also important. This was illustrated by the results obtained when the canonical bases situated in the 5′-terminal fragment were changed giving a 17-fold decrease of affinity with respect to the specific 21-mer. After removal of the 3′-terminal GT dinucleotide there still can be formation of additional contacts between IN and the cleaved chain of the substrate.

We have shown here that HIV-1 IN can interact with high affinity with short ODNs. Since the affinity of ODNs may be increased by introducing additional groups able to interact with the enzyme, specific ODNs offer a unique opportunity for rational design of drugs targeted directly towards HIV-1 IN.

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