Inhibition of the Human Immunodeficiency Virus Type 1 Integrase by Guanosine Quartet Structures[†]

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ABSTRACT: An oligonucleotide (T30177) composed entirely of deoxyguanosine and thymidine has previously been shown to fold upon itself in the presence of potassium into a highly stable four-stranded DNA structure containing two stacked deoxyguanosine quartets (G4s). T30177 also protects host cells from the cytopathic effects of human immunodeficiency virus type 1 (HIV-1). We report that this G4 oligonucleotide is the most potent inhibitor of HIV-1 integrase identified to date, with IC_{50} values in the nanomolar range. Both the number of quartets formed and the sequence of the loops between the quartets are important for optimal activity. T30177 binds to HIV-1 integrase without being processed and blocks the binding of the normal viral DNA substrate to the enzyme. The normal DNA substrate was not able to compete off T30177 binding to HIV-1 integrase, indicating a tight binding of G4s to the enzyme. Experiments with truncated HIV-1 integrases indicate that the N-terminal region containing a putative zinc finger is required for inhibition by T30177 and that T30177 binds better to full-length or deletion mutant integrases containing the zinc finger region than to a deletion mutant consisting of only the central catalytic domain. The N-terminal region of integrase alone is able to bind efficiently to T30177, but not the linear viral DNA substrate, in the presence of zinc. Hence, G4s represent the first class of compounds that inhibit HIV-1 integrase by interacting with the enzyme N-terminal domain. The greater inhibitory potency of T30177 in buffer containing magnesium versus manganese suggests that divalent metal ion coordination along the phosphodiester backbone may play a role in the inhibitory activity. T30177 inhibited HIV-2 integrase with similar potency as HIV-1 but inhibited feline and simian immunodeficiency virus integrases at higher concentrations, suggesting selectivity can be achieved. We propose that novel AIDS therapies could be based upon guanosine quartets as inhibitors of HIV-1 integrase.

Integration, whereby the double-stranded viral DNA generated by reverse transcriptase is inserted into a chromosome of the host cell, establishes the proviral state. Integration is catalyzed by the retroviral enzyme integrase which is encoded at the 3′-end of the *pol* gene (Varmus & Brown, 1989). Integrase first catalyzes the excision of the last two nucleotides from each 3′-end of the linear viral DNA, leaving the terminal conserved dinucleotide CA-3′-OH at these recessed 3′-ends (Figure 1A). This activity is referred to as the 3′-processing or dinucleotide cleavage. After transport to the nucleus as a nucleoprotein complex (Varmus & Brown, 1989), integrase catalyzes a concerted DNA strand transfer reaction by nucleophilic attack of the two viral ends onto a host chromosome. This reaction generates a recombination intermediate resembling an X structure, analogous to a Holliday junction intermediate [for recent reviews, see Katz and Skalka (1994) and Vink and Plasterk (1993)]. Mutation analyses of the viral integrase gene demonstrate that integration is required for retroviral replication and that it is a legitimate target for the design of antiretroviral drugs (Engelman et al., 1995; Englund et al., 1995; Leavitt et al., 1996).

We have previously reported that AZT nucleotides can inhibit HIV-1 integrase (Mazumder et al., 1994) and that substitution or unsaturation at the 3′-position of the deoxyribose confers potency against HIV-1 integrase (Mazumder et al., 1996). These results suggested that the enzyme's nucleotide binding site could serve as a potential drug target. Further studies using dinucleotides have shown that the potential stacking interactions gained from the heterocyclic rings can further enhance potency against HIV-1 integrase (A. Mazumder and Y. Pommier, submitted for publication).

Recently, oligonucleotides composed of deoxyguanosine and thymidine have been reported to inhibit HIV-1 replication (Rando et al., 1995; Wyatt et al., 1994). Such oligonucleotides can form deoxyguanosine quartets (G4s) (Gellert et al., 1962; Rando et al., 1995; Wyatt et al., 1994). Oligonucleotides forming intramolecular G4s did not block virus adsorption but rather inhibited viral-specific transcripts (Rando et al., 1995). In the present report, we demonstrate the potent inhibition of HIV-1 integrase by oligonucleotides containing intramolecular G4s and report on the structure−activity results from a series of these structures and the site of molecular interactions with HIV-1 integrase. The relevance of these findings with respect to HIV-1 integrase binding to its DNA substrate and to dimerization of the retroviral genome is also discussed.

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MATERIALS AND METHODS

Preparation of Oligonucleotide Substrates and Inhibitors. The following HPLC-purified oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, TX): AE117, 5′-ACTGCTAGAGATTTTCCACAC-3′; AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′; AE157, 5′-GAAAGCGACCGCGCC-3′; AE146, 5′-GGACGCCATAGCCCCGGCGCGGTCGCTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAGCAGGGGCTATGGCGTCC-3′; AE118S, 5′-GTGTGGAAAATCTCTAGCA-3′; RM22M, 5′-TACTGCTAGAGATTTTCCACAC-3′. AE117, AE118, and the first 19 nucleotides of AE156 correspond to the U5 end of the HIV-1 long terminal repeat (LTR).

To analyze the extents of 3′-processing and strand transfer using 5′-end-labeled substrates, AE118 was 5′-end labeled using T_4 polynucleotide kinase (Gibco BRL) and [γ-^{32}P]ATP (Dupont-NEN). The kinase was heat-inactivated, and AE117 was added to the same final concentration. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and run on a G-25 Sephadex quick spin column (Boehringer Mannheim) to separate annealed double-stranded oligonucleotide from unincorporated label.

To analyze the extent of strand transfer using the "precleaved" substrate, AE118S was 5′-end-labeled, annealed to AE117, and column-purified as above.

To analyze the choice of nucleophile for the 3′-processing reaction, AE118 was 3′-end-labeled using [α-^{32}P]cordycepin triphosphate (Dupont-NEN) and terminal transferase (Boehringer Mannheim) (Engelman et al., 1991; Vink et al., 1991). The transferase was heat-inactivated, and RM22M was added to the same final concentration. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and run on a G-25 spin column as before.

To determine the extent of 30-mer target strand generation during disintegration (Chow et al., 1992), AE157 was 5′-end-labeled, annealed to AE156, AE146, and AE117, and column-purified as above.

Oligonucleotides composed of deoxyguanosine and thymidine were synthesized, purified, and incubated with potassium ion to generate the G4s. The deoxyguanosine quartet (G4)-forming stuctures were then purified as previously described (Rando et al., 1995). Radiolabeling at the 5′-end was accomplished as described for the AE118 oligonucleotide (see above).

Integrase Proteins and Assays. Purified recombinant wild-type HIV-1 integrase, deletion mutants IN^{1-212}, IN^{50-288}, IN^{50-212} (Bushman et al., 1993), and IN^{1-55}, and site-directed mutants $IN^{F185K/C280S}$ and $IN^{F185K/C280S/H12N/H16N}$ were generous gifts of Drs. T. Jenkins and R. Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. Dr. Craigie also provided the expression system for the wild-type HIV-1 integrase. A plasmid encoding the HIV-2 integrase was generously provided by Dr. R. H. A. Plasterk (Netherlands Cancer Institute). Purified recombinant wild-type FIV and SIV integrases were generous gifts of Drs. S. Chow (UCLA) and R. Craigie (NIDDK), respectively.

Integrase was preincubated at a final concentration of 200 nM (for HIV-1 and HIV-2) or 600 nM (for FIV and SIV) with inhibitor in reaction buffer [50 mM NaCl, 1 mM HEPES, pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$ or $MgCl_2$ (when specified), 0.1 mg/mL bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, and 25 mM MOPS, pH 7.2] at 30 °C for 30 min. When magnesium was used as the divalent metal ion, poly(ethylene glycol) was added at a final concentration of 5% to increase the activity as previously described (Engelman & Craigie, 1995). Preincubation for 30 min of the enzyme with inhibitor was performed to optimize the inhibitory activity in the 3′-processing reaction (Fesen et al., 1994). Then, 20 nM of the 5′-end ^{32}P-labeled linear oligonucleotide substrate was added, and incubation was continued for an additional 1 h. The final reaction volume was 16 μL.

Disintegration reactions (Chow et al., 1992) were performed as above with a Y oligonucleotide (i.e., the branched substrate in which the U5 end was "integrated" into target DNA was used).

Electrophoresis and Quantitation. Reactions were quenched by the addition of an equal volume (16 μL) of loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue). An aliquot (5 μL) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M Tris−borate, pH 8.3, 2 mM EDTA, 20% acrylamide, and 8 M urea). Gels were dried, exposed in a Molecular Dynamics Phosphorimager cassette, and analyzed using a Molecular Dynamics phosphorimager (Sunnyvale, CA). Percent inhibition was calculated using the equation:

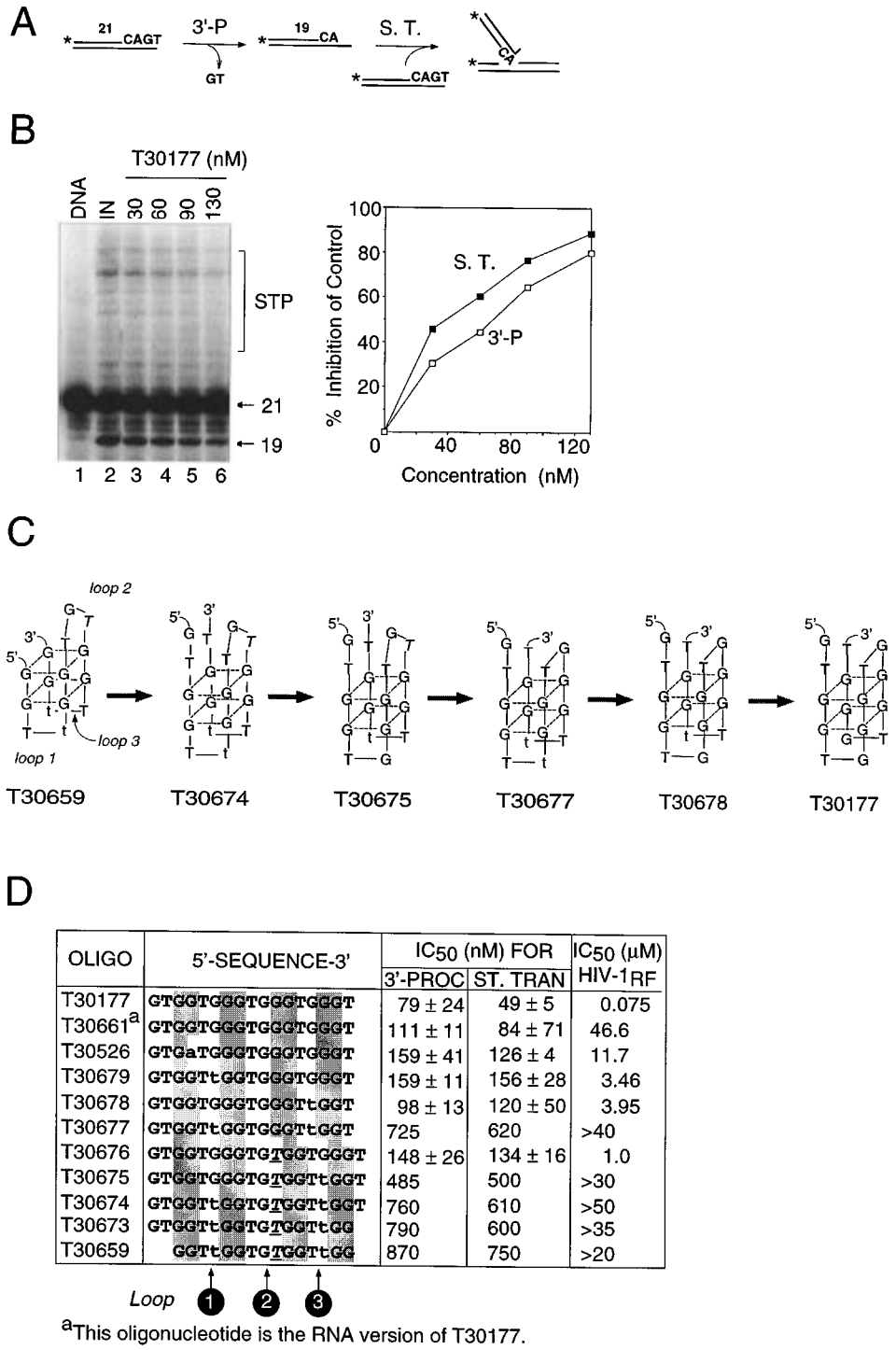
$$100 \times [1 - (D - C)/(N - C)]$$

where *C*, *N*, and *D* are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus integrase, and integrase plus drug, respectively. IC_{50} was determined by plotting the drug concentration versus percent inhibition and determining the concentration which produced 50% inhibition.

UV Cross-Linking Experiments. The method used has been described by Engelman et al. (1994). Briefly, integrase (at the indicated concentration) was incubated with substrate in reaction buffer as above for 5 min at 30 °C. Reactions were then irradiated with a UV transilluminator (254 nm wavelength) from 3 cm above (2.4 mW/cm^2) at room temperature for 10 min. An equal volume (16 μL) of 2× SDS−PAGE buffer (100 mM Tris, pH 6.8, 4% 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) was added to each reaction. Twenty microliter aliquots were heated at 95 °C for 3 min prior to loading on a 12% or 18% SDS−polyacrylamide gel. The gel was run at 120 V for 1.5 h, dried, and exposed in a PhosphorImager cassette. For inhibition of DNA binding experiments (Figure 3), integrase (200 nM) was preincubated with the deoxyguanosine quartet (at the indicated concentration) for 30 min at 30 °C prior to the subsequent addition of the radiolabeled viral DNA substrate (20 nM). For the competition experiments (Figure 7), integrase (200 nM) was preincubated with either the radiolabeled viral DNA substrate (20 nM) or T30177 (20 nM) for 5 min at 30 °C prior to the addition of competitor DNA at the indicated concentration.

RESULTS

Guanosine Quartet Oligonucleotides Inhibit HIV-1 Integrase. The inhibition of HIV-1 integrase by a series of oligonucleotides which can form G4s is shown in Figure 1.



OLIGO	5'-SEQUENCE-3'	IC$_{50}$ (nM) FOR 3'-PROC	IC$_{50}$ (nM) FOR ST. TRAN	IC$_{50}$ (μM) HIV-1$_{RF}$
T30177	GTGGTGGGTGGGTGGGT	79 ± 24	49 ± 5	0.075
T30661[a]	GTGGTGGGTGGGTGGGT	111 ± 11	84 ± 71	46.6
T30526	GTGaTGGGTGGGTGGGT	159 ± 41	126 ± 4	11.7
T30679	GTGGTtGGTGGGTGGGT	159 ± 11	156 ± 28	3.46
T30678	GTGGTGGGTGGGTtGGT	98 ± 13	120 ± 50	3.95
T30677	GTGGTtGGTGGGTtGGT	725	620	>40
T30676	GTGGTGGGTG*T*GGTGGGT	148 ± 26	134 ± 16	1.0
T30675	GTGGTGGGTG*T*GGTtGGT	485	500	>30
T30674	GTGGTtGGTG*T*GGTtGGT	760	610	>50
T30673	GTGGTtGGTG*T*GGTtGG	790	600	>35
T30659	GGTtGGTG*T*GGTtGG	870	750	>20

Loop ❶ ❷ ❸

[a]This oligonucleotide is the RNA version of T30177.

FIGURE 1: Inhibition of HIV-1 integrase 3′-processing and strand transfer and HIV-1$_{RF}$ cytopathicity by guanosine quartets. (A) Schematic diagram showing 3′-processing (3′-P, which liberates a GT dinucleotide) and strand transfer (S. T., which results in the insertion of one 3′-processed oligonucleotide into another target DNA), with 5′-end-labeled (asterisk), blunt-ended oligonucleotide. (B) Left panel, concentration response obtained from a typical experiment. The DNA substrate (21-mer), 3′-processing product (19-mer), and strand transfer products (STP) are shown. Lane 1, DNA alone; lane 2, with integrase; lanes 3−6, with integrase in the presence of the indicated concentrations of T30177. Right panel, graph derived from quantitation (see Materials and Methods) of the dose response in the left panel showing inhibition of integrase-catalyzed 3′-processing (open squares) and strand transfer (filled squares). (C) Structures of guanosine quartet oligonucleotides. All of the oligonucleotides shown have a phosphorothiodiester linkage at both the 5′- and 3′-ends. (D) IC$_{50}$ values for several G4 oligonucleotides against both activities of HIV integrase and HIV-1$_{RF}$ in cell culture. Insertions into the parent compound T30177 are shown by an italicized and underlined nucleotide while mutations are designated by a lower case nucleotide. The guanosines involved in the quartets are shaded, and the loops are designated by the corresponding numbers (see panel C, left).

Oligonucleotides T30177 and T30659 (Ojwang et al., 1995) fold upon themselves into structures stabilized by two G4s stacked upon each other to form a deoxyguanosine octet (Rando et al., 1995; Schultze et al., 1994). Interestingly, T30177 is active against HIV-1 in cell culture and against purified HIV-1 integrase *in vitro* (Ojwang et al., 1995) while T30659 is not. Inhibition of both the 3′-processing and strand transfer activities of HIV-1 integrase (Figure 1A) by T30177 was observed in the nanomolar range (see Figure 1B).

In order to ascertain why T30177 was effective and T30659 was not, we made a series of oligonucleotides to incrementally change one compound into the other. The structures are shown in panels C and D of Figure 1. All of the oligonucleotides shown have a phosphorothiodiester linkage at both the 5′- and 3′-ends. The differences between T30177 and T30659 (i.e., the presence of additional bases at both ends, different sequences in all three loops, and extension of loop 2) manifest themselves in dramatic increases in the IC_{50} values (Figure 1D). To distinguish the contributions of each of these changes, we first added the same 5′- and 3′-nucleotides to T30659 as are present on T30177, yielding T30674 (Figure 1C). These changes did not confer potency (Figure 1D). Then, we changed either loop 1 to obtain T30675 (Figure 1C) or the three bases in loop 2 into those found in T30177, yielding compound T30677 (Figure 1C). Neither change by itself conferred potency (Figure 1D). However, when we changed two of the loops to resemble T30177, yielding T30676 or T30678 (Figure 1C), we were able to significantly improve the activity over that of T30659. We are in the process of substituting other bases for the Gs in the loops to determine what effect(s) such substitutions may have. The importance of having two deoxyguanosine quartets was investigated by mutation of one of the deoxyguanosines involved in the formation of the second quartet to a deoxyadenosine. This oligonucleotide, T30536, exhibited a 2−3-fold decrease in potency (Figure 1D). These data suggest not only that the octet structure is critical but that the loops are important for interaction with HIV-1 integrase.

The activities of the oligonucleotides in the cellular assays did not strictly correlate with the *in vitro* anti-integrase activity (Figure 1D). The correlation is complicated by the differential stabilities and susceptibilities to nuclease digestion of the oligonucleotides *in vivo* (Bishop et al., 1996).

In Figure 1, G4 oligonucleotides were tested in a dual assay which measures both 3′-processing and strand transfer (Craigie et al., 1990; Katz et al., 1990). A strand transfer assay using a "preprocessed" (3′-recessed) substrate (19-mer in Figure 2A, left panel) was also performed to determine whether the strand transfer reaction was truly being inhibited or whether the inhibition of the 3′-processing reaction caused the decrease in the subsequent strand transfer products. Inhibition of strand transfer using this preprocessed substrate was observed in the same concentration range (Figure 2A, right panel) as that seen with the blunt-ended, duplex oligonucleotide substrate. Therefore, G4 oligonucleotides inhibit both steps of the integration reaction: 3′-processing and strand transfer.

Inhibition of 3′-processing was confirmed using DNA substrates labeled at the 3′-end (Engelman et al., 1991; Vink et al., 1991) (Figure 2B, left panel). All of the G4s tested inhibited glycerolysis, hydrolysis, and circular nucleotide formation to the same extent (Figure 2B, right panel). Thus, G4 oligonucleotides exert a global inhibition of the three nucleophiles in the 3′-processing reaction (glycerol, water, or the hydroxyl group of the viral DNA terminus).

Having demonstrated that the catalytic activities of integrase could be inhibited by G4 oligonucleotides, we next examined whether DNA binding was also affected. We performed UV cross-linking of integrase−DNA reactions to address this question. Cross-linking of substrate DNA to

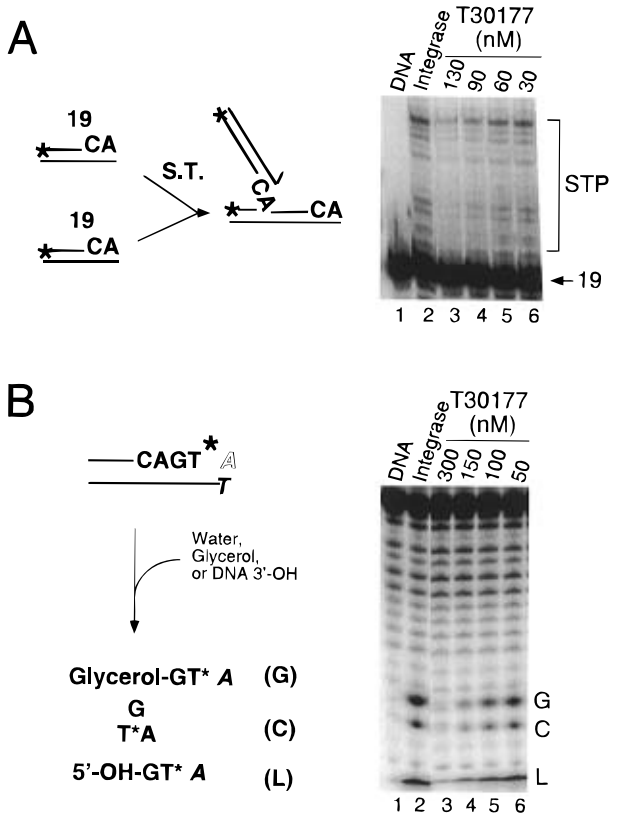


FIGURE 2: Inhibition of strand transfer and 3′-processing activities of HIV-1 integrase by the deoxyguanosine quartet T30177. (A) Left, schematic diagram depicting the strand transfer assay using the precleaved oligonucleotide (19-mer substrate). Right, Phosphorimager picture showing inhibition of strand transfer with T30177. The DNA substrate (19-mer) and strand transfer products (STP) are shown. Lane 1, DNA alone; lane 2, plus integrase; lanes 3−6, plus integrase in the presence of the indicated concentrations of T30177. (B) Left, schematic diagram depicting the 3′-processing assay using the oligonucleotide labeled at the 3′-end with [^{32}P]cordycepin (*A) (22-mer substrate). Right, Phosphorimager picture showing inhibition of HIV-1 integrase-catalyzed 3′-processing with T30177. Lane 1, DNA alone; lane 2, with integrase; lanes 3−6, in the presence of the indicated concentrations of T30177.

integrase followed by electrophoresis results in a product having a molecular mass of approximately 39 kDa (Engelman et al., 1994; Yoshinaga et al., 1994). As seen in Figure 3, binding of HIV-1 integrase to radiolabeled U5 DNA substrate was inhibited by preincubation of the enzyme with T30177 in the same concentration range as its IC_{50} value for strand transfer (lanes 3−7). In contrast, preincubation of the enzyme with T30659, which was poorly active in the 3′-processing/strand transfer assay (Figure 1D), resulted in only modest inhibition of DNA binding even at a T30659 concentration of 500 nM (Figure 3A, lanes 9−13).

Importance of the HIV-1 Integrase Zinc Finger Region for Deoxyguanosine Quartet Oligonucleotide Inhibition. Integrase can catalyze *in vitro* an apparent reversal of the DNA strand transfer reaction called disintegration (Chow et al., 1992). In contrast to the 3′-processing and strand transfer reactions, disintegration requires neither the N-terminal zinc finger region nor the C-terminal DNA binding domain of integrase (Bushman et al., 1993). For this reason, the HIV-1 integrase catalytic core domain, IN^{50-212} (Figure 4A), can be used in the intramolecular disintegration assay for testing the site of action of inhibitors (Mazumder et al., 1994, 1995b).

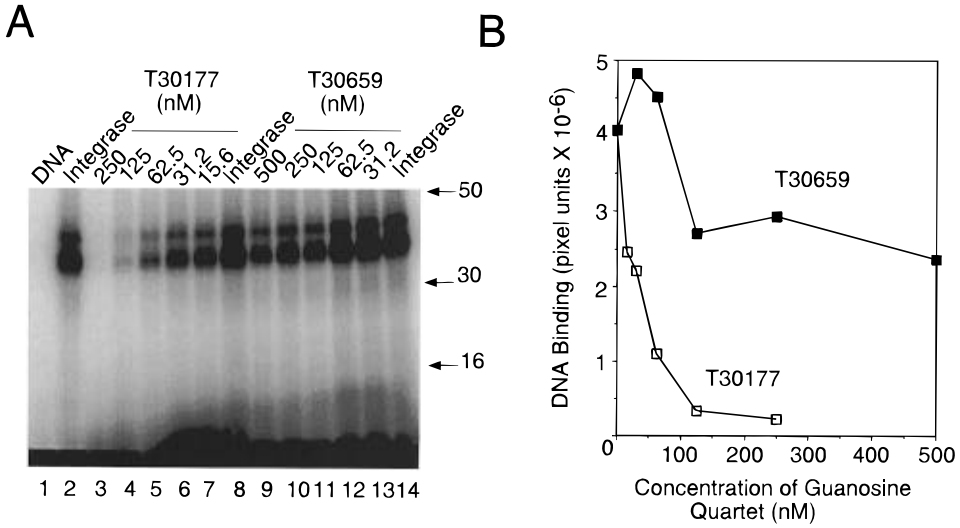


FIGURE 3: Inhibition of the DNA binding activity of HIV-1 integrase by deoxyguanosine quartets. DNA binding was measured after UV cross-linking of reactions in which integrase was preincubated for 30 min at 30 °C with the deoxyguanosine quartet prior to addition of the DNA substrate. (A) Phosphorimager picture showing differential inhibition of DNA binding with T30177 and T30659. Lane 1, DNA alone (20 nM); lanes 2, 8, and 14, with integrase (200 nM); lanes 3−7, in the presence of the indicated concentrations of T30177; lanes 9−13, in the presence of the indicated concentrations of T30659. The migrations of proteins of known molecular weight are shown to the right of the gel. (B) Graph derived from quantitation of the dose response in (A) showing inhibition of integrase binding by T30177 (open squares) but not by T30659 (filled squares).

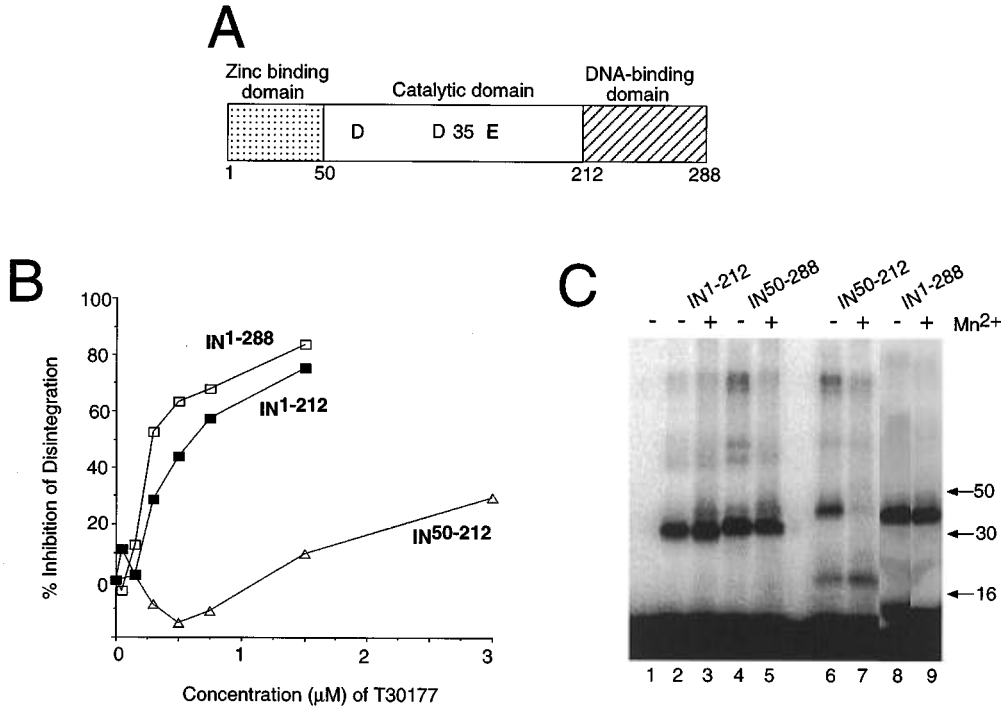


FIGURE 4: Differential activities of T30177 on wild-type and deletion mutants of HIV-1 integrase. (A) Schematic diagram showing the three domains of HIV-1 integrase. (B) Inhibition of wild-type IN^{1-288} (open squares), IN^{1-212} (closed squares), and IN^{50-212} (open triangles) in the disintegration assay. (C) Binding of HIV-1 integrase wild-type (IN^{1-288}) and deletion mutants at a final concentration of 1 μM to ^{32}P-end-labeled deoxyguanosine quartet T30177 at a final concentration of 250 nM. The mobilities of proteins of known molecular mass (in kDa) are shown to the right of each figure. Lane 1, T30177 alone; lanes 8−9, binding to wild-type, full-length HIV-1 integrase (IN^{1-288}) in the absence or presence of Mn^{2+}; lanes 2−3, binding to IN^{1-212} in the absence or presence of Mn^{2+}; lanes 4−5, binding to IN^{50-288} in the absence or presence of Mn^{2+}; lanes 6−7, binding to IN^{50-212} in the absence or presence of Mn^{2+}.

In the disintegration assay, only the IN^{1-288} and IN^{1-212} proteins (Figure 4B) were inhibited by T30177 (with IC_{50}s of 270 and 600 nM, respectively) while neither IN^{50-212} (Figure 4B) nor IN^{50-288} (data not shown) showed more than 30% inhibition at a 3 μM concentration of T30177. The concentration of T30177 required for inhibition of disintegration was higher than that required for inhibition of either 3′-processing or strand transfer. These results are consistent with those observed with other molecules (Fesen et al., 1994; Mazumder et al., 1994). This observation suggests that the active site of HIV-1 integrase may tolerate drug-induced interference of DNA−protein interactions during the disintegration reaction. These results are consistent with the relative tolerance of this reaction to mutagenesis of either DNA substrate features (Chow & Brown, 1994) or protein structural domains (Bushman et al., 1993). To our knowl-

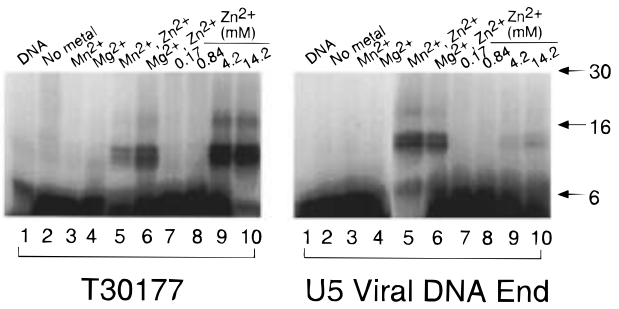


FIGURE 5: DNA binding activity of the zinc finger domain of HIV-1 integrase. Binding of IN^{1-55} to T30177 or the viral DNA substrate (see Figure 1A, 21-mer). Lanes 1, DNA alone (50 nM); lanes 2, IN^{1-55} (2 μM) with no metal; lanes 3, IN^{1-55} with manganese (7.5 mM); lanes 4, IN^{1-55} with magnesium (7.5 mM); lanes 5, IN^{1-55} with manganese (7.5 mM) and zinc (4.2 mM); lanes 6, IN^{1-55} with magnesium (7.5 mM) and zinc (4.2 mM); lanes 7−10, IN^{1-55} in the presence of the indicated concentration of zinc alone.

edge, T30177 is the first example of an HIV-1 integrase inhibitor requiring the enzyme zinc finger region for inhibitory activity. These results suggest that the zinc finger may assist in stabilizing binding to T30177.

This hypothesis was investigated further by monitoring binding of wild-type, full-length integrase (IN^{1-288}) and of deletion mutants to radiolabeled T30177. The concentration of T30177 required for DNA−protein complex formation was the same as that required for complex formation using the viral U5 DNA substrate (i.e., in the 20 nM range). UV cross-linking assays (Engelman et al., 1994) showed that IN^{1-288} formed a DNA−protein complex of the expected molecular weight in the absence or presence of added manganese (Figure 4C, lanes 8 and 9). The IN^{1-212} protein, which has previously been shown to bind to linear viral DNA only at high concentrations (approximately 2.56 μM) and only in the presence of divalent metal ion (Engelman et al., 1994), was able to cross-link to T30177 with the same efficiency as wild-type integrase in the absence or presence of added manganese (lanes 2 and 3). The IN^{50-288} protein, which contains a nonspecific DNA binding domain, was also able to cross-link to T30177 with the same efficiency as wild-type integrase in the absence or presence of added manganese (lanes 4 and 5), consistent with its ability to bind to viral U5 DNA (Engelman et al., 1994). The extent of cross-linking was significantly diminished in the case of the core mutant IN^{50-212} compared to IN^{1-212} in the absence or presence of manganese (compare lanes 2 and 3 with 6 and 7, faster migrating complex). The higher molecular weight species in lane 6, having the expected molecular weight of a dimer, has been reproducibly observed, but its identity has not been confirmed. These data support the notion that the N-terminus of HIV-1 integrase assists in the formation or stabilization of an HIV-1 integrase−T30177 complex, perhaps by binding the oligonucleotide.

DNA Binding Activities of the HIV-1 Integrase Zinc Finger Domain. To further analyze the binding of the N-terminal zinc finger region to T30177 and compare these results to the viral U5 substrate, UV cross-linking was performed with an IN^{1-55} deletion mutant (see Figure 4A) containing only this domain. As seen in Figure 5, this mutant could not bind either the T30177 oligonucleotide or the viral DNA substrate when only manganese or magnesium was present (left and right panels, lanes 3 and 4). However, the IN^{1-55} protein could bind to both DNAs in the presence of zinc and either manganese or magnesium (left and right panels, lanes 5 and 6). Significantly, the IN^{1-55} protein was able to efficiently bind to the T30177 G4 oligonucleotide, but not the viral DNA substrate, in the presence of zinc alone (left and right panels, lanes 9 and 10). These results are in accord with the known zinc binding ability of this domain (Burke et al., 1992; Bushman et al., 1993). Possible interactions between the zinc ion and the sulfur atom in the internucleotidic phosphorothiodiester linkages of T30177 could account for the efficient binding of IN^{1-55} to this substrate, whereas such interactions could not occur with the viral DNA substrate which is composed entirely of phosphodiester linkages. However, efficient binding of IN^{1-55} to T30177 analogs which do not contain phosphorothiodiester linkages (e.g., T30175; see Figure 6B) suggests that such zinc−sulfur interactions may not play a key role in the observed efficient binding of IN^{1-55} to T30177. The data in Figure 5 also suggest that the N-terminal domain of integrase has DNA binding capabilities on its own. Finally, these experiments demonstrate comparable binding of the HIV-1 integrase zinc finger domain to an oligonucleotide containing G4s or to a double-stranded, linear, viral DNA oligonucleotide when both manganese (or magnesium) and zinc are present but more efficient binding to the G4 oligonucleotide under nonphysiological conditions (zinc alone). We also found that the nucleocapsid protein of HIV-1, a nucleic acid annealing protein which contains two CCHC zinc fingers and which is essential for dimerization of the retroviral RNA genome (Tsuchihashi & Brown, 1994), also binds efficiently to T30177 (data not shown). The ability of zinc to confer DNA binding ability on the IN^{1-55} protein was examined by replacement of this ion with other transition metals. Consistent with spectroscopic data (Burke et al., 1992), only zinc was able to induce detectable DNA binding to the G4 oligonucleotide (data not shown).

Increased Potency of Deoxyguanosine Quartets in Magnesium. In contrast to IN^{1-55}, the extent of cross-linking (and presumably binding) of wild-type integrase to radiolabeled deoxyguanosine quartet was increased in the presence of magnesium relative to manganese at several concentrations of the deoxyguanosine quartet (Figure 6A). This observation led us to examine whether the inhibitory activity of T30177 and analogs could also be enhanced by buffer containing magnesium. In order to address this question, we tested three versions of T30177 as shown in Figure 6B. T30175 has the same base sequence as T30177 but is composed entirely of phosphodiester internucleotidic linkages. T30177 has phosphorothiodiester linkages at both the 5′- and 3′-ends. T30038 also has the same base sequence as T30177 but is composed entirely of phosphorothiodiester internucleotidic linkages. The inhibition of 3′-processing catalyzed by HIV-1 integrase by these deoxyguanosine quartets is shown in Figure 6C. Both T30175 and T30177 showed 4−5-fold increases in potency when magnesium was used as the divalent metal instead of manganese. In contrast, T30038 showed no significant increase in potency when magnesium was used as the ion (Figure 6D). These data are in accord with the increased stability constants for magnesium−nucleotide complexes when oxygen replaces sulfur (Pecoraro et al., 1984). The opposite is true for manganese. Therefore, the greater inhibitory potency of T30177 in buffer containing magnesium versus manganese may reflect a requirement for magnesium ion coordination along the phosphodiester back-

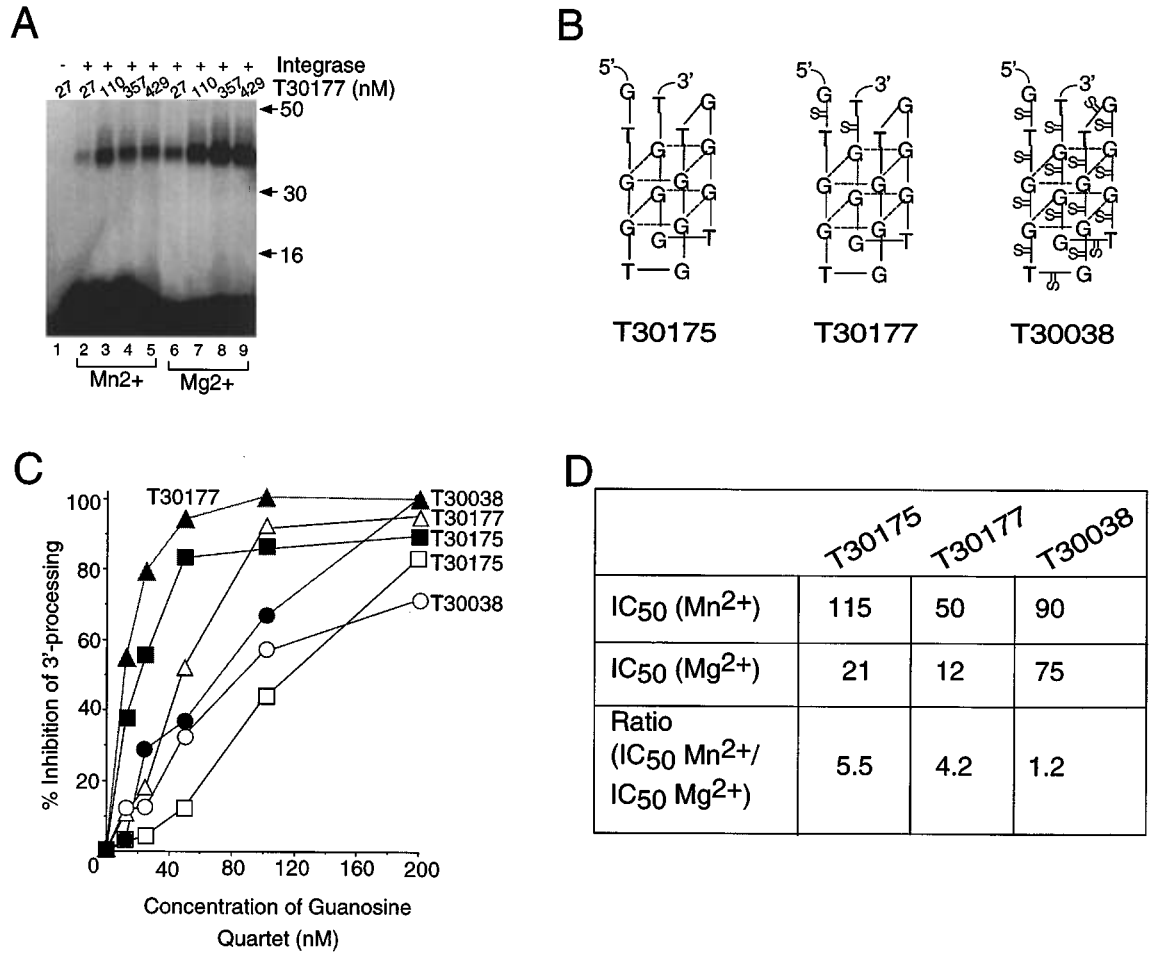


FIGURE 6: Increased binding to and inhibition by deoxyguanosine quartets in magnesium versus manganese. (A) Phosphorimager picture showing DNA binding of wild-type integrase to radiolabeled T30177. Lane 1, DNA alone (27 nM); lanes 2−5, binding of integrase (200 nM) in manganese buffer to the indicated concentration of T30177; lanes 6−9, binding of integrase (200 nM) in magnesium buffer to the indicated concentration of T30177. The migrations of proteins of known molecular weight are shown to the right of the gel. (B) Structures of T30177 and two analogs in which the internucleotidic linkages have been changed. (C) Graph derived from quantitation (see Materials and Methods) of the inhibition of integrase-catalyzed 3′-processing in the presence of T30177 and analogs in either magnesium or manganese. Inhibition by T30177 (triangles), T30175 (squares), and T30038 (circles) is shown using buffer containing magnesium (filled symbols) or manganese (open symbols). (D) Table showing IC_{50} values for 3′-processing for the deoxyguanosine quartets in buffer containing manganese and magnesium and the ratio of these values.

bone of T30177 in order to confer inhibitory activity and optimum interaction of T30177 with HIV-1 integrase. This coordination can occur with more stability when either T30177 or T30175 are assayed in buffer containing magnesium rather than manganese and is manifested in a greater potency against 3′-processing.

DNA Competition Experiments. The relative affinity for the G4 oligonucleotide was probed by attempting to compete off the integrase bound to radiolabeled HIV-1 viral U5 DNA with increasing concentrations of unlabeled T30177 (Figure 7A). The converse experiment, where binding of integrase to radiolabeled G4 oligonucleotide was carried out prior to the addition of increasing concentrations of unlabeled HIV-1 viral U5 DNA, was also performed (Figure 7B). In each case, a band having the apparent mobility of an integrase−DNA complex was evident. In Figure 7A, the viral DNA-integrase complex has a molecular weight of 38 500 while in Figure 7B, the T30177−integrase complex has a molecular weight of 37 000. Neither complex could be competed off by either competitor DNA even at concentrations where the competitor was in 500-fold excess (Figure 7A, lane 6). Similar results were seen when the IN^{1-212} and IN^{50-212} proteins were used in competition experiments (data not shown). Therefore, the stability of the G4 oligonucleotide DNA−integrase complex is comparable to that of the viral DNA−integrase complex (Ellison & Brown, 1994; Vink et al., 1994a).

Inhibition of Related Lentiviral Integrases. T30177 was tested for inhibition of the related retroviral integrases from HIV-2 (van Gent et al., 1992), simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV) (Vink et al., 1994b). As seen in Figure 8, T30177 inhibited 3′-processing catalyzed by HIV-1 integrase in the expected concentration range (Figure 8A, lanes 2−8; IC_{50} = 55 nM). Inhibition of HIV-2 integrase (using HIV-1 DNA) was also observed in the same range (lanes 9−15; IC_{50} = 90 nM). However, FIV integrase was inhibited at 3-fold higher concentrations of T30177 (lanes 16−22; IC_{50} = 175 nM), and SIV integrase was inhibited at 7-fold higher concentrations of T30177 (lanes 23−29; IC_{50} = 420 nM). Therefore, the T30177 G4 oligonucleotide displayed some selectivity among the lentiviral integrases.

DISCUSSION

Guanosine Quartet Oligonucleotides Are Novel and Potent Inhibitors of HIV-1 Integrase. Oligonucleotides composed

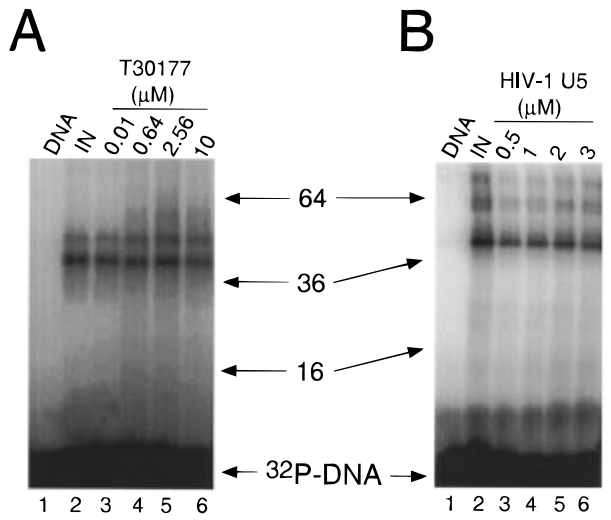


FIGURE 7: Competition of binding to either U5 viral oligonucleotide (see Figure 1A, 21-mer) (A) or deoxyguanosine quartet T30177 (B). Lanes 1, DNA alone; lanes 2, with wild-type, full-length HIV-1 integrase. Lanes 3−6 in panel A, with integrase in the presence of the indicated concentrations of T30177 added after a 5 min preincubation with the U5 viral DNA oligonucleotide. Lanes 3−6 in panel B, with integrase in the presence of the indicated concentrations of viral U5 DNA oligonucleotide added after a 5 min preincubation with the deoxyguanosine quartet T30177.

of deoxyguanosine and thymidine and forming G4 structures have previously been shown to inhibit HIV replication (Rando et al., 1995; Wyatt et al., 1994). Two mechanisms have been invoked. First, some oligonucleotides have been shown to bind to the V3 loop of the envelope protein gp120 and subsequently inhibit virus adsorption and cell fusion (Wyatt et al., 1994). Second, oligonucleotides such as those described in the present study also inhibit viral-specific transcripts (Rando et al., 1995) presumably by inhibiting viral integration (Ojwang et al., 1995). The present finding that inhibition of the HIV-1_{RF} strain in cell culture parallels that of purified integrase *in vitro* in the series of G4 oligonucleotides tested (Figure 1D) further demonstrates the possibility that HIV-1 integrase can be targeted by some G4 oligonucleotides.

G4 oligonucleotides differ from previously published HIV-1 integrase inhibitors in several ways. First, they are among the most potent inhibitors to date with IC_{50}'s in the nanomolar range. Their potency range is comparable to flavone (Fesen et al., 1994) and tyrphostin derivatives (Mazumder et al., 1995a), which, however, generally fail to show antiviral activity. Second, the zinc finger domain of HIV-1 integrase contributes to the inhibition by G4 oligonucleotides, as truncation mutant enzymes lacking this domain are resistant to the G4 oligonucleotides. This property is unique, as all the other inhibitors to date are active against the HIV integrase catalytic core domain (Fesen et al., 1994; Mazumder et al., 1994, 1995a, 1996). Finally, G4 oligonucleotides form stable enzyme complexes that cannot be displaced by excess viral DNA oligonucleotide.

Role of the HIV-1 Integrase Zinc Finger Region. Mutation and deletion analyses show that the zinc finger motif (H-H-C-C) of retroviral integrases is required for integration (3′-processing and strand transfer) activity (Engelman & Craigie, 1992). However, the structural role of this region has not been elucidated. It has been postulated to provide DNA sequence-specificity (Bushman et al., 1993) and stabilize DNA−enzyme (Vink et al., 1994a) and enzyme−multimer complexes (Ellison et al., 1995). Our data provide the first direct evidence that the HIV-1 integrase N-terminus region [amino acids 1−55 (Figure 4A)] can interact directly with viral DNA in the presence of both zinc and magnesium (or maganese). The fact that the IN^{1-55} protein binds to the G4 oligonucleotides in the presence but not in the absence of zinc is consistent with the formation of a zinc finger in this region (Burke et al., 1992). Hence, it is possible that the zinc finger region can selectively bind to non-B DNA structures. It is noteworthy that the recently solved structure of HIV-1 integrase (Dyda et al., 1994) resembles that of the Ruv C Holliday junction-resolving enzyme (Ariyoshi et al., 1994) and that of the bacteriophage Mu transposase core (Rice & Mizuuchi, 1995). These structurally related proteins also bind multiple double helices, generating X structure intermediates.

Although the zinc finger region of integrase is required for inhibition by the G4s, the IN^{50-212} protein, which contains only the central catalytic domain, was capable of binding to T30177 (Figure 4C). These results suggest that the binding site of T30177 may reside in the catalytic domain but that the zinc finger domain is necessary for stabilization of the enzyme−inhibitor complex and for the subsequent inhibition by the T30177 oligonucleotide. These conclusions are also consistent with the finding that a site-directed HIV-1 integrase mutant containing both the C-terminal DNA binding domain and the zinc finger domain in which the two zinc finger histidines were mutated to asparagines was able to bind to G4 oligonucleotides (data not shown). These data suggest that when the nonspecific DNA binding domain is present, a second binding site for the T30177 oligonucleotide may become available. Therefore, HIV-1 integrase may have two separate DNA binding sites: one for viral DNA and one for target or "host" DNA. This scenario would be expected if integrase were to bind both the viral and host DNA at sites which were distinct but in close proximity *in vivo* (Vincent et al., 1993). It should be noted that the existence of a single binding site on HIV integrase for both viral and target DNA has been proposed by others (Vink et al., 1993).

Biological Relevance of G4 Structures. Several similarities exist between retroviral genomes and telomeric regions of eukaryotic chromosomes. The two RNA strands comprising the HIV-1 genome can potentially dimerize and form intermolecular G4s *in vitro* (Awang & Sen, 1993; Sundquist & Heaphy, 1993), as does telomeric DNA (Sundquist & Klug, 1989). In addition, the β subunit of the Oxytrichia telomere binding protein has been proposed as a molecular chaperone for the formation of G4s at the ends of chromosomes by enhancing the rate of a thermodynamically favored transition (Fang & Cech, 1993). In retroviruses, the nucleocapsid protein may also act as a molecular chaperone to enhance dimer formation (Sundquist & Heaphy, 1993). In this manner, it may facilitate the formation of and bind to the G4. We also found that G4s can bind to purified nucleocapsid protein (data not shown). Thus, G4s may be structurally important as molecular scaffolds in both retroviral preintegration complexes and telomeres, and these structures may have associated chaperones in both cases. Finally, a G4-containing structure may act as a negative regulator of telomere elongation *in vivo* due to its ability to inhibit telomerase *in vitro* (Zahler et al., 1991). Analogously, G4

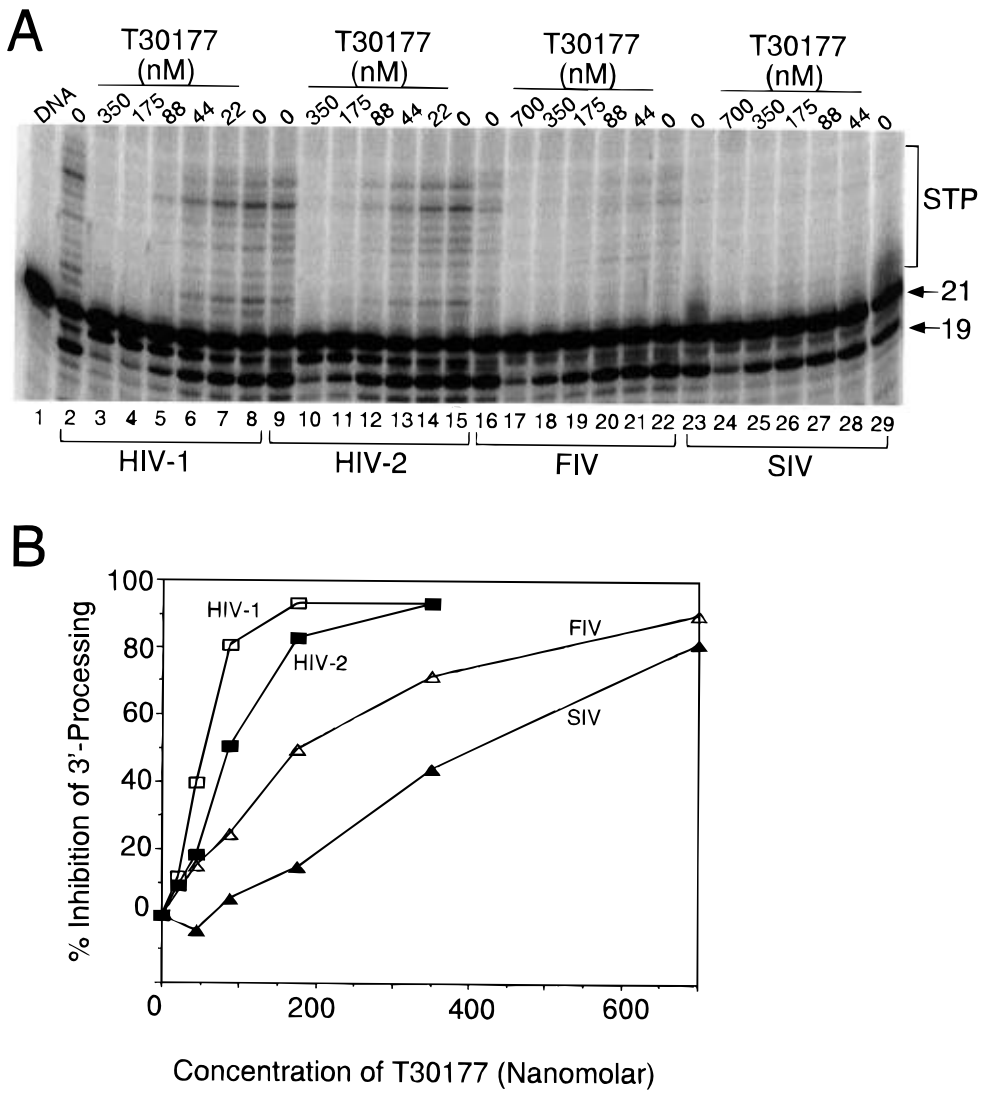


FIGURE 8: Inhibition of the related retroviral integrases. (A) Inhibition of 3′-processing and strand transfer catalyzed by HIV-1 (lanes 2−8), HIV-2 (lanes 9−15), FIV (lanes 16−22), and SIV (lanes 23−29) integrases in the presence of T30177. Lane 1, DNA alone; lanes 2, 8, 9, 15, 16, 22, 23, and 29, with integrase; lanes 3−7, 10−14, 17−21, and 24−28, with integrase in the presence of the indicated concentrations of T30177. (B) Graph derived from quantitation (see Materials and Methods) of the dose responses in (A) showing inhibition of HIV-1 (open rectangles), HIV-2 (filled rectangles), FIV (open triangles), or SIV (filled triangles) integrase-catalyzed 3′-processing.

structures may act to inhibit integrase (Figure 1) and thereby act as a block to autointegration or digestion of the viral DNA prior to insertion into the host chromosome.

The existence of G4s *in vivo* has not been demonstrated. However, they have been shown to form *in vitro* in telomeric sequences (Kang et al., 1992; Smith & Feigon, 1992; Sundquist & Klug, 1989), HIV-1 RNA sequences (Awang & Sen, 1993; Sundquist & Heaphy, 1993), fragile X syndrome nucleotide repeats (Fry & Loeb, 1994), the retinoblastoma susceptibility gene (Murchie & Lilley, 1992), immunoglobulin switch region sequences (Sen & Gilbert, 1988), and possibly during meiotic recombination (Liu & Gilbert, 1994). Given these results, it is not surprising that proteins such as thrombin (Bock et al., 1992) (not normally known to bind nucleic acids), chick topoisomerase II (Chung et al., 1992), MyoD (a transcription factor that regulates myogenesis) (Walsh & Gualberto, 1992), a hepatocyte chromatin protein (Weisman-Shomer & Fry, 1993), macrophage scavenger receptors (Pearson et al., 1993), and a protein from *Tetrahymena thermophila* (Schierer & Henderson, 1994) have been found to bind G4-containing nucleic acids. Another G4 binding protein, KEM1, has been isolated and implicated in recombination-type reactions *in vivo* (Liu & Gilbert, 1994). The catalytic activities of this protein and of the integrase protein are DNA endonucleolytic cleavage and strand transfer. However, unlike KEM1, integrase does not catalyze endonucleolytic cleavage reactions on G4s (data not shown). Thus, G4s may be mechanistically relevant in a diverse set of biological processes involving enzymes with similar activities.

In summary, we have demonstrated that oligonucleotides containing intramolecular G4s are potent inhibitors of HIV-1 integrase. Inhibition is dependent on the zinc finger region of integrase and on the structure and sequence of the G4s.

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