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HIV-1 inactivation by nucleic acid aptamers

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TABLE OF CONTENTS

1. ABSTRACT

Although developments in small-molecule therapeutics for HIV-1 have been dramatic in recent years, the rapid selection of drug-resistant viral strains and the adverse side effects associated with long-term exposure to current treatments propel continued exploration of alternative anti-HIV-1 agents. Non-coding nucleic acids have emerged as potent inhibitors that dramatically suppress viral function both *in vitro* and in cell culture. In particular, RNA and DNA aptamers inhibit HIV-1 function by directly interfering with essential proteins at critical stages in the viral replication cycle (Figure 1). Their antiviral efficacy is expected to be a function, in part, of the biochemical properties of the aptamer-target interaction. Accordingly, we present an overview of biochemical and cell culture analyses of the expanding list of aptamers targeting HIV-1. Our discussion focuses on the inhibition of viral enzymes (reverse transcription, proteolytic processing, and chromosomal integration), viral expression (Rev/RRE and Tat/TAR), viral packaging (p55^{Gag}, matrix and nucleocapsid), and viral entry (gp120) (Table 1). Additional nucleic acid-based strategies for inactivation of HIV-1 function (including RNAi, antisense, and ribozymes) have also demonstrated their utility. These approaches are reviewed in other chapters of this volume and elsewhere (1-5).

Table 1. Anti-HIV-1 aptamers

Target	Aptamer	K_d (nM)	IC_{50} (nM)	Viral Inhibition	References
RT	RNA	0.025-20	1-10	10 to 100-fold[a,d]	14, 15, 31, 49, 68, 80
	DNA	1-11	0.3-62	10-fold[b,e]	70, 71
PR[1]	RNA	10	NA	NA	17-19
IN	RNA	2	NA	NA	106
	DNA	NA	6-166	10-fold[a,d,e]	78, 108, 109, 191
Rev	RNA	1.3	NA	10-fold[a,c,d]	24, 111, 112, 134, 192
Tat	RNA	0.12-120	NA	2 to 10-fold[a,e] 100-fold[c,d]	111, 123-125, 139-141, 145, 146
NC	RNA	0.5-50	0.5-2	2-fold[c, d]	172-177
gp120	2'F-RNA	5-100	NA	10,000-fold[b,d]	182, 183
	5-Br-DNA	83	NA	NA	184

Aptamers to HIV-1 target proteins are listed according to nucleic acid type, [1], Protease (PR) aptamers were selected and assayed against Hepatitis C Virus NS3 protease, NA, not assessed, a, Aptamers expressed in cultured cells, b, Aptamers added exogenously to cultured cells, c, Decoy RNA expressed in cultured cells, d, Inhibition of viral replication in cultured cells, as assessed by viral titres, e, Inhibition of protein function in cultured cells, as assessed by Tat-driven reporter expression.

2. NUCLEIC ACID APTAMERS

Aptamers are small single-stranded nucleic acid molecules that assume specific three-dimensional folded conformations to achieve high affinity binding interactions with defined molecular targets. Individual aptamers are generated from libraries of random-sequence RNA or single-stranded DNA through an iterative process of selection and amplification known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) (6, 7) (see chapter by A. C. Yan *et al.* in this volume). The affinity and specificity of aptamers for their targets can often surpass that achieved with monoclonal antibodies (8). The breadth of molecular targets successfully selected for aptamer binding includes amino acids, nucleotide cofactors, sugars, antibiotics, lipid micelles, small peptides, proteins, viral particles, and even whole cells (9-13). Naïve aptamers often include many more nucleotides than are required for target recognition, and most of the aptamer studies described below include efforts (in some cases quite exhaustive) to identify the core RNA or DNA elements involved in target recognition. For example, anti-RT pseudoknot aptamers of 30 to 45 nt have been selected from libraries in which transcripts were 93, 97, 128, or 134 nt in length (14, 15). Aptamer-protein interactions often antagonize native protein functions, even for proteins which normally do not interact with nucleic acids. As a consequence, considerable effort has been devoted to developing aptamers as therapeutic and diagnostic agents. An anti-VEGF aptamer known as Macugen (pegaptanib sodium injection) was approved by the U.S. Food and Drug Administration in December of 2004 for the treatment of age-related macular degeneration. Similarly, an anti-thrombin aptamer entered phase I clinical trials in 2004, and numerous other aptamers are currently in pre-clinical development (16).

Although this review focuses on aptamers directed against HIV-1 targets, anti-viral aptamers have also been described for targets derived from hepatitis C virus (HCV) (17-20), influenza virus (21), and cytomegalovirus (12). Aptamers can aid efforts to dissect the molecular steps of viral pathogenesis and to establish the validity of targeting specific viral proteins for future small molecule drug design. This is in contrast with anti-mRNA-based methods such as RNAi, antisense and ribozymes, which are much less precise in their viral action. Rather than antagonizing individual proteins, these other methods can lead to degradation of polycistronic viral mRNAs and entire viral genomes, precluding the validation of individual target proteins. Unlike the lead compounds that arise from screens of small molecule libraries, aptamers recovered from *in vitro* selection require relatively little structural modification to be adapted for gene therapy applications. Finally, detailed biochemical understanding of the aptamer-target interaction interface can help identify neutralizing epitopes on the viral proteins and aid in the design of new small molecule drugs.

It is anticipated that therapeutic aptamers could be delivered as either intracellular or extracellular agents. Intracellularly expressed aptamers have proven their efficacy for protein antagonism in both eukaryotic and bacterial systems (reviewed in (22, 23)). When expressing aptamers inside cells, consideration of target protein location is essential to the design of the expression cassette. For example, RNA polymerase II (pol II) promoters generally direct cytoplasmic accumulation of transcripts, while pol I or pol III promoters direct nuclear or nucleolar accumulation (24, 25). Addition of specific RNA structural elements such as the "S35 element" (26) or fusion to a tRNA can augment cytoplasmic accumulation and stability, and expression of tandem aptamer arrays to generate polyvalent transcripts can increase target antagonism (27-30). The possibility of forming alternative, inactive secondary structures in aptamer transcripts can be minimized by including self-cleaving ribozymes within expressed RNA adjacent to the core target-binding structural element. In this strategy, self-cleavage of the ribozyme releases the aptamer from the nascent transcript (31). Intracellular expression of aptamers obliges the use of unmodified "biological" RNA that includes only the four standard nucleotides (A, C, G and U). In contrast, aptamers delivered as extracellular agents may contain "nonbiological" covalent modifications. For example, 2' fluoro- and other substitutions on the ribose sugar, "locked nucleic acids," phosphate backbone modifications, 5' inverted abasic residues and enantiomeric RNA and DNA

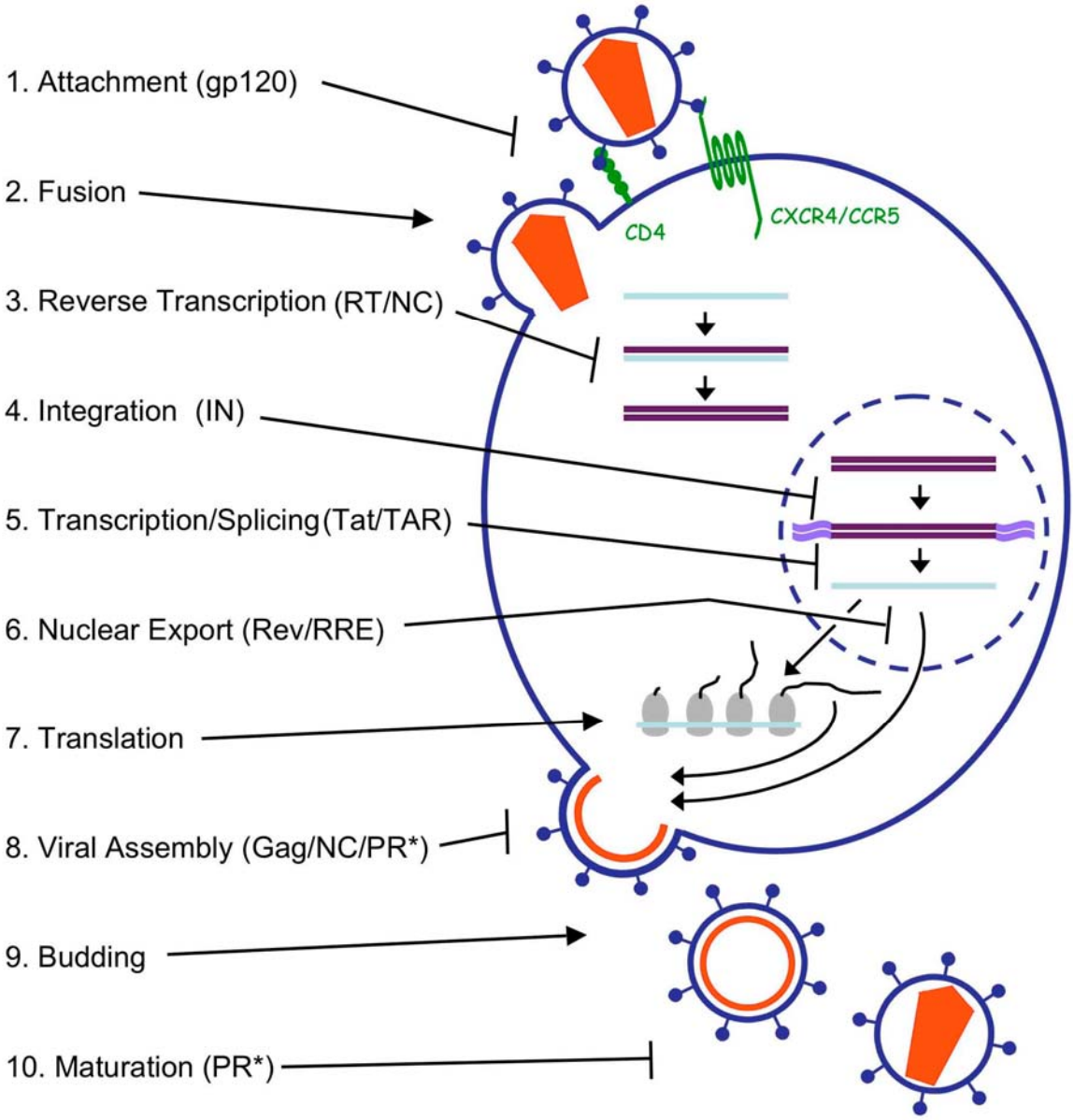

Figure 1. Inhibition of HIV-1 replication by nucleic acid aptamers. Key steps in the viral life cycle are numbered sequentially. Viral proteins for which aptamers have been selected are indicated in parentheses, and the corresponding points at which viral replication is disrupted are indicated by flat-ended bars. Aptamers to the HIV protease (*) are not yet available, although inhibitory aptamers have been selected against the hepatitis C virus (HCV) NS3 protease.

all stabilize nucleic acids against degradation by serum nucleases, while tethering a polyethylene glycol or cholesterol moiety to aptamers reduces uptake by the kidney and liver, greatly improving circulating half-life (32-43)

3. INHIBITION OF HIV-1 ENZYMATIC FUNCTION

The HIV-1 *pol* gene encodes the protease (PR), reverse transcriptase (RT), and integrase (IN). These three enzymes are translated as part of the Gag-Pol fusion protein resulting from a ribosomal frame shift near the 3' end of the *gag* gene. Processing of the Gag-Pol polyprotein by PR yields the mature, active forms of PR, RT, and IN, along with all of the mature Gag protein products. Eighteen of the nineteen anti-HIV-1 drugs currently approved for use by the FDA inhibit the function of either RT or PR, making these viral enzymes the primary targets of current therapeutic approaches (44-46).

3.1. Reverse Transcriptase.

Retroviruses carry an RNA genome that is copied into double-stranded cDNA by the viral RT prior to integration into the host genome. HIV-1 RT is released

from the Pol polyprotein as a 66kDa protein product that assembles into a p66/p66 homodimer. Protease removes the C-terminal 120 amino acids from one of the p66 subunits to generate the mature p66/p51 heterodimer (47). Mature RT has two essential enzymatic activities: DNA polymerization (RNA-dependent for minus strand synthesis and DNA-dependent for plus strand synthesis) and cleavage of the RNA strand in RNA/DNA hybrids (RNase H activity). Both active sites are located within the p66 subunit. The p51 subunit serves as a scaffold that stabilizes the active conformation of p66 and provides essential contacts to the primer/template substrate complex.

The p66/p51 heterodimer has been crystallized in a variety of conformations that group into two categories loosely defined as "fingers-touching-thumb" or "fingers-and-thumb-separated" (reviewed in (48)). (We choose the "touching" and "separated" nomenclature rather than "closed" and "open," to avoid confusion with the "closed" and "open" conformations commonly referenced in the polymerase literature. The latter nomenclature denotes a conformational change in the ternary complex—polymerase, template/primer and the dNTP—when the fingers region close on the dNTP.) In the "touching" conformation of RT, which is observed for unliganded RT and for RT bound to an RNA aptamer pseudoknot, the fingers and thumb domains of the protein contact each other. In the "separated" conformation, which is observed in co-crystals of RT bound to double-stranded DNA (dsDNA), to an RNA-DNA hybrid or to non-nucleoside drugs, a rigid-body rotation of the thumb domain by 30-40° relative to the palm produces a gap of approximately 13 Å between the tips of the fingers and thumb. The two conformations are not artifacts of the crystallization; electron pair resonance (EPR) measurements of spin-labeled RT show the two conformations in equilibrium in solution. The "touching" conformation is strongly favored at physiological temperature (95%), with increasing amounts of the "separated" conformation at low temperature (65% "separated" at 0°C) (49). Thus, the "touching" conformation is the target that would be most frequently encountered by an evolving aptamer library *in vitro* and by an expressed aptamer that encounters unliganded RT within a cell. Furthermore, the EPR signature of a spin-labeled RT in complex with aptamer is very similar to that of the free protein, and dissimilar to that of an RT-dsDNA complex (49).

3.1.1. Anti-RT drugs, side effects and resistance

The eleven FDA-approved RT inhibitory drugs fall into two classes, both of which target polymerization activity. Eight of these drugs are nucleoside analog RT inhibitors (NRTI): AZT (azidothymidine or zidovudine), ddI (dideoxyinosine or didanosine), ddC (dideoxycytidine or zalcitabine), abacavir, tenofovir, d4T (stavudine), FTC (emtricitabine), and 3TC (lamivudine). The NRTI drugs all lack 3'-OH and therefore terminate DNA chain elongation upon incorporation into replicating strands. The non-nucleoside RT inhibitors (NNRTI)— delavirdine, nevirapine, and efavirenz— bind near the nucleotide-binding site and inhibit RT function through allosteric means (50, 51). High-activity anti-retroviral therapies (HAART) against HIV-1 usually involve the combination of two NRTIs and one NNRTI, or two NRTIs and a PR inhibitor (52). Small molecule inhibitors of HIV-1 RNase H are being developed (53), but have not yet reached clinical trials.

While NRTI and NNRTI drugs are effective antiviral agents, they can produce significant adverse side effects including mitochondrial toxicity, lipodistrophy, liver disfunction, nausea, diarrhea, headaches, insomnia, and hypersensitivity (54). Resistance to these drugs can arise rapidly during infection, in part because only one or a few amino acid changes at specific locations in RT decrease viral susceptibility to inhibition (55). Between 1999 and 2000, drug resistant strains made up 12.4 % of new HIV-1 infections in ten North American cities, and multi-drug resistance reached as high as 6.2 % (56). Other estimates put the prevalence of drug resistant strains as high as a quarter of all circulating virus (57-59). Mathematical modeling suggests that the prevalence of drug-resistant HIV-1 strains in San Francisco may reach 40% or higher within the next few years (60).

At least 39 of the first 300 codons of HIV-1 RT are associated with drug resistance, and cross-resistance between other approved anti-RT drugs is often observed (61-63). This can be understood from an examination of crystal structures of RT-drug and RT-substrate complexes wherein resistance mutations map closely together in the three-dimensional structure (50, 64-67). Thus, adverse side effects and facile resistance limit the ultimate effectiveness of NRTI and NNRTI drugs. Nevertheless, the proven therapeutic effectiveness of targeting RT and the need to overcome the limitations of existing drugs has inspired intense analysis of RT structure and function and a correspondingly intense search for new classes of anti-RT drugs. Polymeric compounds, such as nucleic acid aptamers, which make different and broader atomic-level interactions with RT, may limit viral evasion by the known spectrum of resistance mutations. They may also offer an opportunity to overcome drug resistance altogether through combined use with members of the current suite of anti-RT drugs.

3.1.2. RNA aptamers *in vitro*

The highly positively charged template-binding cleft of RT is well suited for binding not only to nucleic acid duplexes, but to aptamers as well. At least 300 different RNA and single-stranded DNA (ssDNA) aptamers against HIV-1 RT and related reverse transcriptases have been isolated by us (14) and by others (15, 68-71). Nearly all RNA aptamers selected to bind HIV-1 RT are predicted to fold into pseudoknots. About half of these RNAs form the so-called "Tuerk-type pseudoknots" (TPK), characterized by an invariant sequence in stem 1, and highly conserved sizes and compositions of loops 1 and 2 (Figure 2, top left) (14, 15, 72). Most of the remaining RNA aptamers to HIV-1 RT are predicted to fold into pseudoknot structures that differ moderately or radically from the canonical TPK pseudoknot, with elongated stems or loops or with sequences that do not match the TPK consensus (Figure 2, bottom left) (14). A few RNA

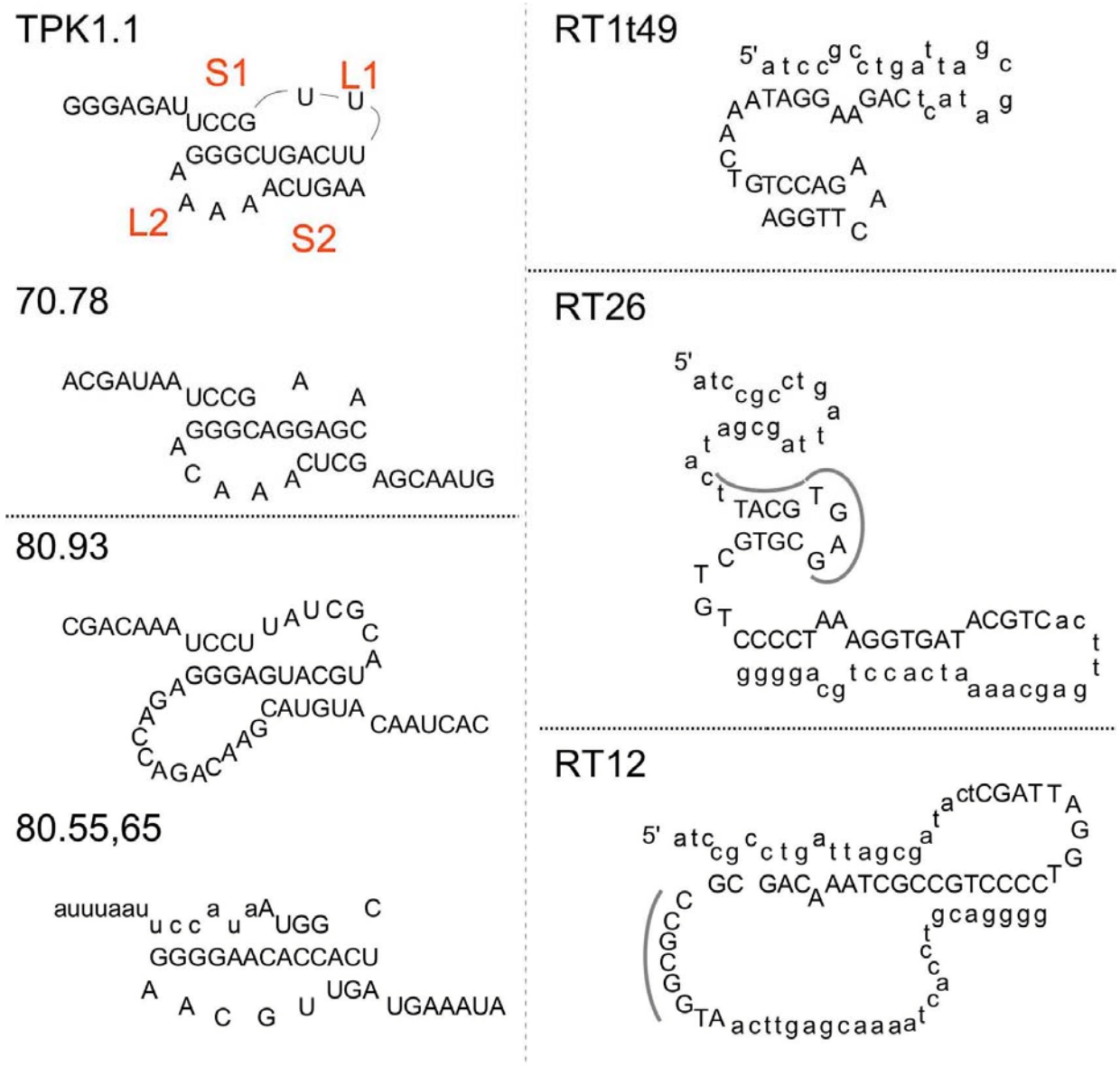

Figure 2. Aptamers against HIV-1 reverse transcriptase. A. Comparison of two representatives each from the "TPK" (top) and "non-TPK" (bottom) classes of pseudoknot aptamers. Only the pseudoknot portions are shown; the original aptamers in each case contain up to 100 additional nucleotides of flanking sequence. Stems (S) and loops (L) are labeled for TPK1.1. Secondary structures in this review follow the convention that lower case letters indicate nucleotides derived from original primer binding sequence and upper case letters indicate selected nucleotides. B. ssDNA aptamers to HIV-1 RT (70). Segments that could permit alternative secondary structures or dimerization are indicated with arcs.

aptamers to HIV-1 RT appear to adopt unrelated structures, including a set of small RNA hairpins reported to inhibit RNase H function selectively at micromolar concentrations (73). ssDNA aptamers to HIV-1 RT (Figure 2, right) (70) and RNA aptamers to RTs from other retroviruses (AMV, FIV and MMuLV) (68, 69) show greater structural variability, including stem-loop structures, double-stem-loops and recessed 3' ends in addition to pseudoknots.

Many of the RNA aptamers from different structural classes bind RT with dissociation constants in the low nanomolar range when assayed using gel-shift and nitrocellulose binding (14, 15). Biochemical and biophysical studies of aptamer-RT interactions have focused on TPK aptamer "1.1" (Figure 2) (49, 72, 74), which was one of the first RT aptamers to be identified (15). This aptamer and several related RNAs bind RT with Kd values in the 0.5 to 10 nM range when measured by nitrocellulose filter binding or gel mobility shift methods (14, 15, 31, 72). TPK1.1 yielded a remarkably low K_d of 25pM when measured using fluorescent aptamer displacement or electron paramagnetic resonance

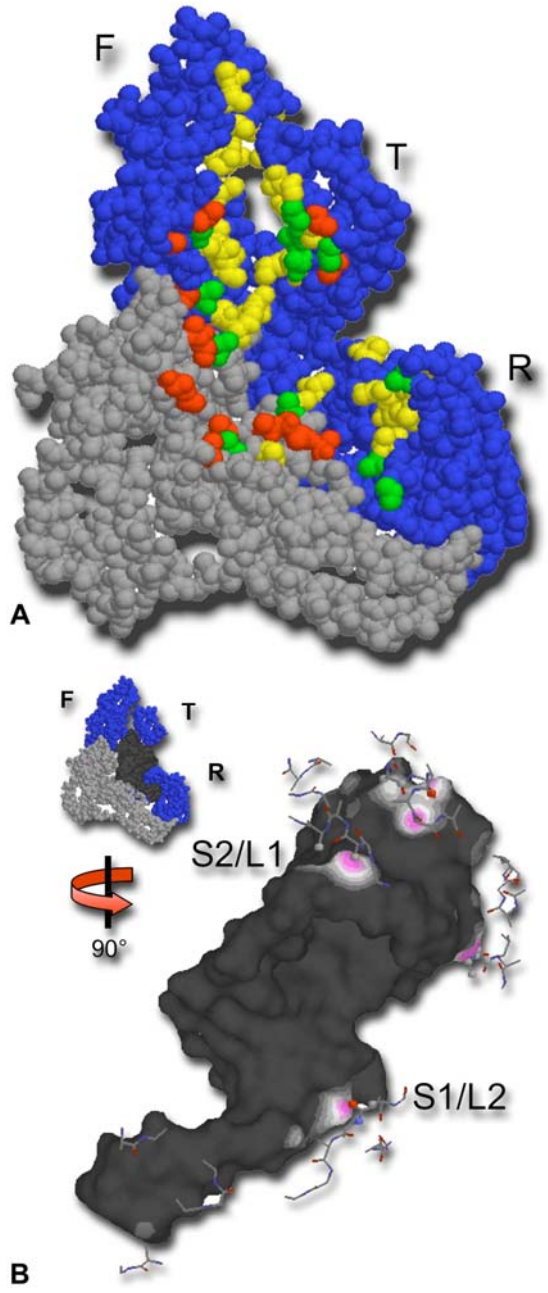

Figure 3. RT-nucleic acid interactions. A. Amino acid positions contacting various nucleic acids (Table 1) are mapped onto the surface of HIV-1 reverse transcriptase structure 1HVU. Yellow, contacts with primer-template complexes (64, 75, 76); green, contacts with pseudoknot aptamer (74); red, contacts with both targets. RT subunits p66 (blue) and p51 (gray), and fingers (F), thumb (T), and RNase H (R) structural domains are indicated. B. Protein environment of pseudoknot aptamer bound to RT (74). Aptamer surface (dark gray) is shown with protein removed except for residues within 7Å of aptamer surface. Pink regions of aptamer surface are within hydrogen bonding distance of RT residues. Orientation is rotated 90° relative to that shown in Figure 3A and in inset. Image was created using Protein Explorer Contacts tool.

spectroscopy (49). Enzyme inhibition has been measured in primer-extension assays of DNA polymerization using RNA or DNA templates. When RT was included in the reaction in low nanomolar concentrations, RNA aptamers from different structural groups inhibited DNA polymerase activity with IC50 values that were similar to the total RT concentration (14, 15, 72), while related polymerases were unaffected (14). We recently found that many previously uncharacterized aptamers, including a number of structurally diverse non-TPK pseudoknots, inhibit RT polymerization activity at least as well as TPK1.1 (D. Saran & DHB, unpublished). Although it seems likely that the molecular interactions vary considerably among the non-TPK pseudoknots, they appear to target a common surface, given that several structurally unrelated RNA aptamers mutually compete for binding to HIV-1 RT (14).

RT is the only HIV-1 protein for which a crystal structure is available for the aptamer-protein complex. Binding of RNA aptamer TPK 1.1 buries more than 2600Å^2 of RT surface area (74) in the region closely overlapping the dsDNA-binding surface (75). Although this structure is of low resolution (4.8Å), Jaeger et al. noted 23 amino acids as lying within 5Å of the aptamer (Figure 3A), and we note here five additional residues that appear from the crystallographic data to make similarly close contact (K261, G262, and N265 in p66; K20 and V21 in p51) (Table 2). Unexpectedly, close approaches between the RNA and RT appear to be especially extensive near the 3' end of the pseudoknot in stem 2 and loop 1 (Figure 3B). Assuming that the RNA structural elements were accurately assigned in the low-resolution structure, this observation would appear to contradict solution biochemical analysis. Green et al. demonstrated that the opposite end (stem 1 and loop 2) of a closely related 28 nucleotide aptamer is highly sensitive to chemical modification of phosphates and bases, to substitution with 2' O-methyl and 2'-deoxynucleotides, and to nucleotide substitutions and deletions. Stem 2 and loop 1 of this same aptamer, which appear in the crystal structure to make the most contacts with RT, were largely insensitive to these changes (72). These data would appear to suggest that the aptamer 5' end (stem 1 and loop 2) , and not its 3' end (stem 2 and loop 1), makes most of the essential contacts with RT.

Many residues identified as contacting the pseudoknot RNA aptamer are also contacted by nucleic acids in co-crystallized complexes with double-stranded DNA (75), with a covalently tethered dsDNA (64), or with an RNA-DNA hybrid analog of the poly-purine tract that primes plus-strand synthesis (76) (Table 2 and Figure 3A). Of the 80 total contacts between RT and these various nucleic acids, 15 are with p51 and 10 lie within the RNase H domain of p66. Thus, the structural data are consistent with a model in which RNA aptamers inhibit both polymerase and RNase H activities by competing with the primer-template complex for access to RT. Because of this presumptive mechanism of inhibition, aptamer inhibitors of RT are sometimes referred to as Template Analog RT Inhibitors (TRTI).

3.1.3. Anti-RT ssDNA aptamers *in vitro*

Two very different sets of ssDNA aptamers have also been identified. The first of these was isolated against

Table 2. Amino acid contacts in four RT-nucleic acid co-crystals

COMPLEX[a]	A	B	C	D
p66 contacts[b]				
Lys13				X
Trp24	Y			
Pro25	Z			
Phe61	Y			
Lys65	Y			
Arg72	Y			
Leu74	Y			
Val75	Z	Y		
Asp76	Y			
Arg78	Y	Y		
Asn81	X	X		
Lys82				X
Trp88				X
Glu89	Y	Y	Y	X
Gln91	Y	Y		
Leu92	Y	Z		
Gly93	Y	Y	Y	
Ile94	Y	Y		
Asp113	X			
Ala114	X			
Tyr115	X		Y	
Gln151	Y		Y	
Gly152	Y	Y	Y	
Trp153	Z	Y		
Lys154	Y	Z	Y	X
Pro157	Y	Y	Y	
Tyr183	Y	Y	Y	
Met184	Y	Z	Y	
Asp185	X	Y	Y	
Asp186	Y	Y		
Met230	Y	Y	Y	
Gly231	Y	Y	Y	
Gln255	X	Y		
Gln258	Y[c]	Y	Y	
Lys259	Y	Y	Y	
Val261	Z			Z
Gly262	Y	Y	Y	Z
Lys263	Y	Y	Y	
Asn265	Z	Y	Y	Z
Trp266	Y	Y	Y	
Lys275				Y

COMPLEX[a]	A	B	C	D
p66 contacts[b]				
Arg277		Z		Y
Ser280	Y	Y	Y	
Lys281				X
Leu283	Z	Z		
Arg284	Y	Y	Y	X
Gly285	Y	X	Y	
Thr286	Y			
Leu289	Y			
Lys353	Z	Y	Y	
Ala355	X	X		
Gly359	X	X		
Ala360		X		
His361	Y	Z		
Lys374	Y	Y		
Arg448	Y	Y		X
Lys451	Y			
Thr473	Y	Y	Y	
Asn474	X	Y		
Gln475	Z	Y	Y	
Lys476	X	Y		
Gln500	Y	Y		X
Tyr501	Y	Y	Y	
Ile505		X		
His539	X	X		X
p51 contacts				
Lys20(p51)				
Val21(p51)				
Lys22(p51)				
Arg78(p51)				
Lys82(p51)				
Glu324(p51)				X
Lys388(p51)				X
Lys390(p51)		X		
Lys395(p51)	Y	Y		X
Glu396(p51)		X	Y	
Glu413(p51)				X
Glu415(p51)				Y
Phe416(p51)				Y
Val417(p51)				Y
Asn418(p51)				X

Yellow squares, RT residues that contact dsDNA or RNA-DNA hybrid; green squares, RT residues that contact pseudoknot aptamer; red squares, RT residues that contact aptamer and at least one additional nucleic acid (same color scheme as in Figure 3A). [a] Complexes: **A** = RT covalently linked with ds DNA (64); **B** = RT bound polypurine track RNA-DNA primer-template (76); **C** = RT bound to dsDNA (75); **D** = RT bound to RNA pseudoknot aptamer TPK1.1 (74). [b] Contacts:**X** = Identified in the original publications but not evident to the authors of this review using Protein Explorer; **Y** = Contacts identified the original publications and confirmed by the authors using Protein Explorer. Protein Explorer contacts defined as being close enough to be putatively noncovalently bonded; **Z** = Contacts identified by the authours using Protein Explorer but not mentioned in the original publications. Structures (Protein Data Bank ID)1RTD (64), 1HYS (76), 2HMI (75), 1HVU (74) used for Protein Explorer analysis. [c] Gln258 is replaced with Cys for cross-linking RT to dsDNA in this complex.

the p66/p51 holoprotein, and includes many DNAs that have the capacity to form complex secondary structures either as monomers or as dimers (Figure 2, right). Interestingly, Schneider *et al.* showed that aptamers with recessed 3' ends could be partially extended by RT. The aptamers may make stronger or more extensive contacts with the RT than would be observed for dsDNA, given that the partially extended ssDNA aptamers bound much more

weakly and were poor inhibitors (70). The second set of ssDNA aptamers was targeted to bind the RNase H domain by alternating cycles of positive selection for binding to the p66/p51 heterodimer with cycles of negative selection to remove aptamers binding the p51/p51 homodimer (71). Each of the dominant species from this selection can form a guanosine tetrad structure ("G-quartet"). Andreola and co-workers found that these ssDNAs inhibit both DNA polymerization and RNase H activities *in vitro* with IC_{50} values of 500nM. When added to cell culture simultaneously with virus, these same DNAs interfered with viral infectivity as assessed by monitoring Tat-driven β-galactosidase activity. Enzymatic inhibition appears to be restricted to HIV-1 proteins, in that the anti-HIV-1 RNase H aptamer ODN93 showed poor affinity for human RNase H (71, 77). Furthermore, a separate set of aptamers generated against human RNase H were ineffective against *Escherichia coli* and HIV-1 RNase H (77). However, the anti-HIV-1 RNase H aptamers also cross-react with HIV-1 integrase, which is in the same structural superfamily, blocking both "end-processing" and strand-insertion activities by that enzyme (78).

3.1.4. Anti-RT RNA aptamers in cells

RNA aptamers retain their RT-inhibitory effect *in vivo*, severely attenuating viral replication in cultured human T-lymphoid cells. When Joshi and Prasad (31) expressed six different pseudoknot RNA aptamers in human 293T kidney cells co-transfected with proviral HIV DNA, virions harvested from these cells demonstrated a 90-99% reduced capacity for subsequent infection (1-2 logs of reduced infectivity). Removal of extra nucleotides 5' and 3' of the aptamer core was accomplished by including within the expression cassette self-cleaving hammerhead ribozymes designed to release the aptamers from the nascent transcripts (79). Dot blot hybridization analysis of RNA isolated from these viral particles revealed that the expressed aptamers are packaged into mature virions, where they may already be bound to RT in position to inhibit the next round of replication. These aptamers also inhibited replication of HIV-1 strains resistant to AZT, 3TC, ddI, ddC and nevirapine (79). This result might be expected given that the sites of drug resistance do not overlap with the aptamer-binding surface. Chaloin *et al.* obtained similar results when they expressed a 33nt RNA aptamer TPK1.1 fused to the 3' end of $tRNA^{met}$ in 293T cells infected with HIV-1 strain NL4-3 (80). Viral production was diminished by >70% as measured by endogenous RT assays in 293T cells co-transfected with aptamer and proviral DNA. When these viral particles were used to infect cultured human T-lymphoid C8166 cells, infectivity was diminished by ~75% as monitored by p24 ELISA 48hrs after infection. Additionally, stably-transfected aptamer-expressing Jurkat cells infected with with a low viral dose (190 pg $p24/10^5$ cells) were resistant to viral infection and replication out to 35 days (80). Thus, intracellularly expressed aptamers have the potential to complement the current suite of small molecule RT-inhibitors as a new class of highly-specific anti-retroviral therapeutics.

In anticipation of the potential clinical application of antiviral aptamers, it is necessary to assess the capacity of HIV-1 to evolve resistance to inhibition by anti-RT aptamers. We have speculated that viable aptamer-resistant mutants will be rare (14, 23), and others have advanced similar viewpoints (4). As part of its normal function in viral replication, RT must bind non-specifically with both RNA and single-stranded DNA. Mutations that disrupt RT-aptamer interactions might therefore be expected to interfere with generic nucleic acid binding and render the polymerase inactive. The few data available in this regard are consistent with this speculation. Fisher *et al.* screened RT mutants for variants that resisted aptamer inhibition (ssDNA aptamer RT1t49, Figure 2) in an enzymatic primer extension assay. Two point mutations were identified in helix H (N255D/N265D in the primer-template minor groove-binding tract) that diminished aptamer binding. However, purified mutant RT's displayed severe processivity defects that were especially evident on DNA templates (81), and viruses carrying these mutations were inviable (82). It remains to be seen whether aptamer-resistant mutants can arise in cell culture with frequencies approaching those observed for resistance to small molecule drugs.

In pursuit of a functional selection of aptamer-resistant RT mutants, we adapted a bacterial complementation system originally designed for testing heterologous polymerase activity. This system utilizes an *E. coli* strain (BK148) that carries a temperature sensitive mutation in its DNA polymerase I (*polA12*) such that isolated colonies form at 30°C but not at 37°C (83). Expression of HIV-1 RT in BK148 complements the Pol I^{ts} mutant, restoring DNA replication and enabling normal growth at 37°C. Small molecule nucleoside prodrug inhibitors of HIV-1 RT reestablish the temperature sensitivity of the Pol I^{ts}/HIV-1 RT strain (84, 85), validating this system for use in screening novel potential RT inhibitors. We showed that expression of several different aptamers in BK148 prevented complementation of the Pol I^{ts} defect by HIV-1 RT (23). In principle, this system could be adapted to screen RT variants for aptamer resistance, or to screen aptamer variants for enhanced anti-RT efficacy. However, we have recently found that selective pressures in the bacterial context are much less stringent than during viral replication. Specifically, when we tested RT variants carrying the N255D and N265D point mutations described above, both the single mutants and the double mutant were able to complement DNA pol I activity at the restrictive temperature, even though these same mutations produce viral replication defects (J. D. Kissel and D. H. Burke, unpublished). Intact viruses or conditionally replicating viruses thus seem to be more suitable systems with which to monitor interactions between aptamers and mutant RTs.

3.2. Protease

The HIV-1 protease (PR) plays a critical role in viral replication by processing the Gag and Pol polyproteins into their mature structural proteins and enzymes (matrix, capsid, nucleocapsid, PR, RT, and IN) (86). A member of the aspartyl protease family, PR functions as a homodimer in which each monomer contributes one of the two catalytic Asp residues to the

active site at the dimer interface. Viruses that encode defective PR are able to package and bud normally, but the resulting viral particles fail to mature and are rendered non-infectious (87-89). The absolute requirement for active PR, coupled with its high substrate specificity, has made the HIV-1 protease an attractive target for anti-HIV-1 therapies. Clinically available PR inhibitors developed during the last decade are categorized either as substrate-based peptidomimetic inhibitors or as non-substrate small molecule inhibitors (reviewed in (90)). Unfortunately, anti-PR drugs are also associated with toxic side effects including lypodistrophy and hyperlipidemia due to cross-reactivity with cellular proteases (91, 92). Additionally, point mutations in the PR substrate-binding site and/or in the cleavage sites within Gag and Pol can produce resistant viral strains (44, 55, 90). As with anti-RT drugs, the combination of toxicity and drug resistance drives the continued development of alternative therapeutics.

3.2.1. Protease aptamers

Nucleic acid aptamers targeted to HIV-1 PR have not yet been reported.Nevertheless, there is precedence for their potential utility based on studies of aptamers selected to bind the NS3 protease of the hepatitis C virus (HCV) (18). NS3 is a serine protease required for the maturation of the HCV polyproteins, analogous to HIV-1 PR. One of the selected RNA aptamers (74nt in length) binds NS3 with a K_d of ~10nM and inhibits approximately 90% of protease activity *in vitro* when enzymatic assays were performed with low micromolar concentrations of aptamer in 5-fold excess over PR concentrations (17). Inhibition of NS3 protease function inside cells was recently demonstrated when Nishikawa *et al.* co-expressed the aptamer, the protease, and a protease substrate in HeLa cells. Substrate cleavage was monitored by immunoblot analysis of cell lysates (19). The aptamer was expressed in four tandem repeats flanked by a self-cleaving ribozyme (from the hepatitis delta virus) to release individual aptamers from the transcript. Aptamer expression inhibited HCV protease activity by roughly 50% (19). HCV has traditionally been difficult to propagate in cultured cells, but recent breakthroughs (93) have resulted in systems more amenable to drug development (94). Therefore, although these aptamers have yet to be used to disrupt HCV replication in cell culture, the preliminary *in vitro* and *in vivo* enzyme inhibition studies suggest promise for the development of anti-HIV-1 PR aptamers.

3.3. Integrase

HIV-1 integrase (IN), the third of the *pol*-encoded enzymes, catalyzes the insertion of a double-stranded DNA copy of the viral genome into preferred locations within actively transcribed genes in the host chromosome (95, 96). IN contains an N-terminal HHCC zinc finger domain, a central catalytic core (characterized by a 'DD23E' motif), and a C-terminal DNA-binding domain. The protein belongs to a family of polynucleotidyltransferase enzymes that includes the eukaryotic Rag1 and Rag2 proteins and both prokaryotic and eukaryotic retrotransposon integrases. The RNase H of HIV-1 also shares structural similarity with members of this family, although it is mechanistically unrelated. (97, 98). Mutational analyses of IN established that its function is essential to viral replication in cell culture (99-103). The apparent lack of a functionally equivalent human enzyme raises the possibility that anti-IN agents may display reduced cytotoxicity arising from off-target inhibition relative to currently available therapeutics. A number of anti-IN compounds have been reported in the literature in recent years (44, 46, 104). A diketo acid known as S-1360 has completed phase II clinical trials (44, 105), and Merk and Gillead each have compounds in Phase I trials (L870810 and JTK 303, respectively).

3.3.1. Integrase aptamers *in vitro*

Aptamers to IN include several RNA and ssDNA species (Figure 4) (78, 106-109). Allen *et al.* (106) obtained high-affinity IN-binding RNA aptamers, the best of which displayed a K_d for IN of 2nM when assayed in the presence of 50mM NaCl. Although the binding affinity of IN for the aptamer is one thousand-fold better than its affinity for random RNA at 250mM NaCl (K_d of 12nM), this difference diminishes to only ten-fold when assayed in 50mM NaCl, probably due to increased non-specific nucleic acid binding by the cationic IN at low ionic strength. While the anti-viral efficacy of these RNA aptamers has not yet been ascertained in cell-based assays, ssDNA aptamers inhibit Tat-driven reporter gene expression in cell culture (78). Derived from longer aptamer sequences originally selected as inhibitors of HIV-1 RNase H (71), two of the most effective of these G-quartet-forming DNAs inhibited viral infectivity with IC_{50} values of ~6nM as measured by monitoring expression of a β-galactosidase reporter, and they were over 90% inhibitory when included in the medium at concentrations of ~300nM. These two aptamers displayed IC_{50} values of 108nM and 166nM in assays for disruption of the strand transfer ability of IN. The 3'-end processing activity of IN was even more severely inhibited by these aptamers, with IC_{50} values measured at 9nM and 42nM.

Another set of short, G-quartet-forming single-stranded DNAs has demonstrated promising anti-viral effects (109). The most potent inhibitory sequence (referred to in some publications as T30177 and in others as zintevir, Figure 4) demonstrated IC_{50} values of 50nM and 80nM for *in vitro* disruption of HIV-1 IN-catalyzed strand transfer and 3'-end processing activities, respectively (108). These experiments also revealed that the N-terminal 50 amino acids of IN are required for inhibition by zintevir, suggesting a direct interaction between the ssDNA aptamer and the zinc-finger domain of the enzyme. The antiviral efficacy of zintevir has been measured in assays of primary human cell types (including PBMCs and macrophages) and cultured cell lines (including the $CD4^+$ T-lymphoid line CEM-SS and the HTLV-1 infected T-cell line MT2). In both contexts, addition of zintevir to the growth medium resulted in IC_{50} values from mid-nanomolar to low-micromolar for inhibition of replication of multiple HIV-1 strains (109). Impressively, when cultured MT4 cells (a $CD4^+$ lymphocyte line) were infected with HIV-1 in the presence of zintevir and treatment was maintained for four days, HIV-1 replication over the 27 days following removal of drug was suppressed ten-fold relative to cells treated

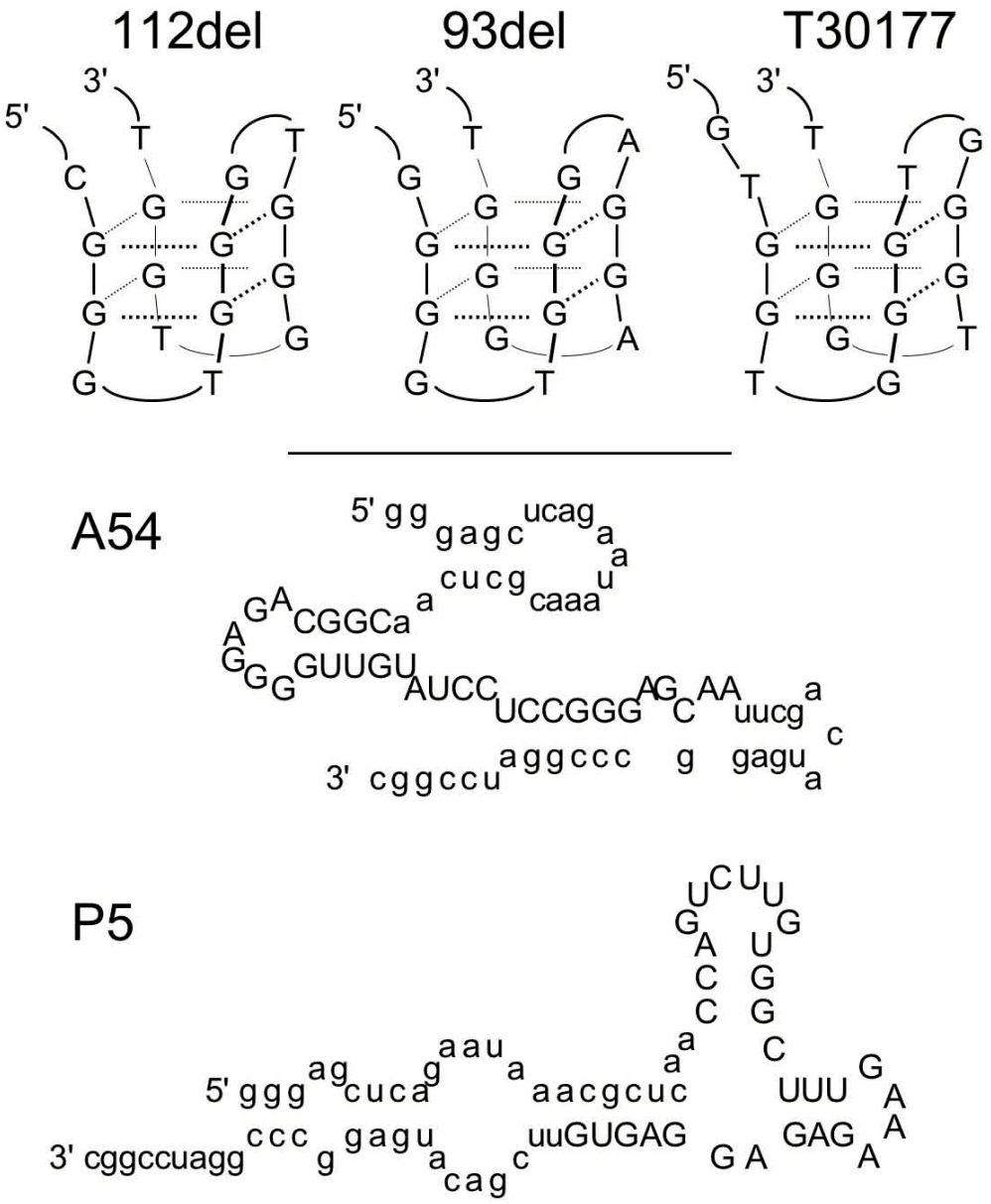

Figure 4. Integrase aptamers. ssDNA (78, 109)(top) and RNA (106) (bottom) aptamers to IN. Aptamer T30177 is also known as zintevir.

with AZT as measured by p24 levels. The mode of cellular uptake of these DNA aptamers is not yet well understood, although the fact that inhibition is observed suggests that the aptamers must penetrate the cells, the free virus, or both.

4. INHIBITION OF HIV-1 GENE EXPRESSION: TRANSCRIPTIONAL REGULATION AND RNA TRANSPORT

4.1. Rev

The HIV-1 genome encodes two proteins that serve to regulate viral gene expression: the *Re*gulator of *V*iral Gene Expression (Rev), and the *T*rans*A*ctivator of *T*ranscription (Tat). Rev is a 13-kDa protein that directs the export of partially spliced (~4kb) and unspliced (~9kb) viral mRNA from the nucleus to the cytoplasm. Rev-dependent export appears to share the same pathway as 5S rRNA and capped U1 snRNA. Expression of Rev's leucine-rich nuclear export signal (NES) coupled to BSA blocks cap-dependent export of 5S rRNA and capped U1snRNA, as well as Rev-dependent export of a reporter RNA, though it does not inhibit the export of mRNA, tRNA or ribosomal subunits (110). Rev's role as an essential RNA-binding viral protein made it a natural choice as one of the earliest protein targets for RNA aptamer selections (111, 112). Rev

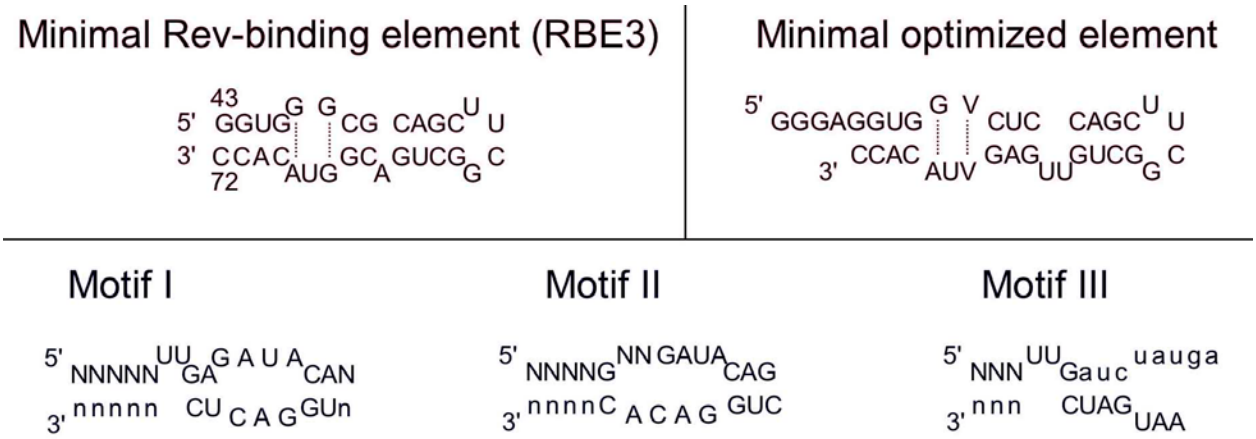

Figure 5. Rev aptamers. Secondary structures are shown for natural Rev-binding element (top left), and for RNA aptamers described by Giver *et al.* (112) (top right) and by Tuerk and MacDougal-Waugh (111) (bottom). V = A, C or G; N = any nucleotide; n = Watson-Crick partner for N.

contains an arginine-rich motif that serves as a nuclear localization signal as well as the site for specific interactions with the *R*ev-*R*esponsive *E*lement (RRE). The RRE is a highly structured 234-nucleotide RNA stem loop located within the envelope coding region of HIV-1 mRNA (113, 114). Rev initially binds within stem-loop IIB of the RRE in a 13 nucleotide region called the Rev binding element (RBE), after which it oligomerizes along the RNA (reviewed in (22, 114)). Within the RBE, two non-canonical base pairs at G47:A73 and G48:G71 (separated by U72) widen the major groove of the double-stranded RNA stem, creating a structural perturbation that is recognized by the arginine-rich motif of Rev (115, 116). The resulting protein-RNA complex prevents nuclear splicing (117). Instead, the leucine-rich NES binds to CRM1 (also known as exportin 1) and directs unspliced and singly-spliced viral mRNAs for export from the nucleus to the cytoplasm for translation and/or viral packaging.

4.1.1. Rev aptamers *in vitro*

When the Szostak group isolated Rev-binding aptamers from a library of natural RBE variants, covariation patterns among the selected isolates suggested non-canonical base pairings within the core binding element, including a G^{syn}-G^{anti} base pair, a G^{anti}-A^{anti} base pair, and a looped out U (Figure 5) (115). Each of these structural features was later confirmed by high-resolution NMR analysis (118). The structure of a 35 nt Rev aptamer bound to a 17 amino acid, arginine-rich peptide from Rev shows some similar interactions, in which the major groove of the RNA is widened near two adjacent G-A and A-A mismatches to accommodate the peptide, and a U-AU triplet is sandwiched between two arginines of the peptide (119). An additional set of Rev aptamers isolated from a random-sequence library contained some similarity to the native RBE (Figure 5) but has not been characterized biochemically (111). In related work, Baskerville, Ellington and co-workers identified RNA aptamers to the Rev homolog Rex found in type I human T-cell leukemia virus (HTLV-1). These aptamers directed Rex-dependent mRNA export from the nucleus to the cytoplasm (120, 121). Deletion and mutational analysis of the selected elements led to a model in which the Rex-binding element (XBE) is thought to contain two Rex-binding sites, each of which is remarkably similar to the HIV-1 TAR element (although cross-reactivity with Tat was not assessed). Although the XBE and selected aptamers bind to an arginine-rich alpha helical element in Rex, the interaction is specific in that they do not bind the analogous motif in HIV-1 Rev.

4.1.2. Non-aptamer Rev decoys *in vivo*

A number of groups have investigated anti-Rev inhibitors, including small molecule compounds (114, 122) and RNA decoys (30, 123-129). When Sullenger *et al.* expressed a 45-nt RNA transcript encompassing RRE stem-loops IIA and IIB in CEM cells, HIV-1 replication was reduced by 90% (123). However, the intact RRE (234 nt) appears to interact with a number of endogenous cellular proteins, including splicing factors (130-132), raising the possibility that the use of RRE itself as a therapeutic agent could lead to unwanted side effects due to sequestration of these or other host proteins. To circumvent this potential problem, Sullenger and colleagues expressed a tRNA fusion with the 13-nt RBE, for which there is no known interaction with any cellular factors (124). When expressed in reporter cell lines, the minimal decoy more effectively inhibited HIV-1 replication than the larger 45-nt decoy (123, 124). There was no measurable difference in CEM cell growth rates when this minimal decoy was expressed, consistent with a model in which decoy expression may be both effective and relatively free of cytotoxic effects.

4.1.3. Rev aptamers *in vivo*

While decoys exhibit therapeutic potential, affinity for their targets may be limited due to overlapping functional sequence constraints; i.e., RBE may be sub-optimally suited for Rev binding as it is also constrained to encoding a segment of gp120. In contrast, aptamers are unencumbered by such genetic constraints. Several aptamers have been generated with affinities for Rev that are 10-times greater than that of the wild type RRE (K_d =

1.3nM for the best Rev aptamer) (111, 112). Symensma *et al.* found that several different Rev-aptamers could direct the export of unspliced mRNA in a Rev-dependent *in vivo* reporter system. These results suggest that the aptamers can functionally substitute for the RBE, and that they may compete with the RRE for binding to Rev (133).

Expression of Rev aptamers and decoy RNAs in cultured cells has demonstrated viral inhibition, and has illustrated the insight that can be gained from targeting transcripts to specific subcellular locations through strategic promoter choice. In one study, expression of an RBE decoy from a U6 snRNA *pol III* promoter resulted in nuclear localization and a corresponding 90% reduction in HIV-1 gene expression as assessed by monitoring endogenous RT activity. In the same report, anti-sense RNA and two ribozymes targeting the U5 region of the viral LTR (a hammerhead ribozyme and a hairpin ribozyme) were less effective viral inhibitors (24). In another study, co-expression of a Rev aptamer and an anti-envelope ribozyme driven by the RSV *pol II* promoter (targeting transcripts to the cytoplasm) also decreased viral production by about one log (88% reduction) (134). Expression of the ribozyme segment without the aptamer did not inhibit viral production to nearly the same degree, suggesting that cytoplasmic accumulation of the aptamer, rather than the ribozyme, may have been responsible for blocking the virus (134). Together, these results demonstrate the effective disruption of Rev function by expressed aptamers localized to either the nucleus or cytoplasm.

4.2. Tat

The Tat protein enhances expression of the viral genome by the host RNA polymerase II. Tat binds directly to a stem-loop structure near the 5' end of the viral RNA known as the *T*rans*A*ctivation *R*esponse element (TAR). Similar to Rev, Tat contains a basic residue-rich alpha helix that recognizes a UCU bulge and the flanking four base pairs in the helical stem of TAR (135). Transcriptional activation results from hyperphosphorylation of the carboxyl-terminal domain (CTD) of RNA pol II by cyclin-dependent kinase 9 (CDK9), which is recruited to the transcription complex by Tat-bound cyclin T1 (136). Tat interacts with a number of other host proteins in TAR-independent complexes, including the transcription factor NF-κB, several histone acyltransferases, and importin β. Tat even appears to function as an autocrine/paracrine factor for a number of cell types when secreted by infected cells (137). Although its role as a transcriptional upregulator makes it an ideal target for anti-HIV-1 therapeutics, the number of Tat-inhibitory compounds to reach clinical trials has been limited, and none has yet been approved for use in patients (138). The need for continued development of novel Tat inhibitors remains urgent, suggesting a utility for Tat aptamers in that role.

4.2.1. Tat aptamers *in vitro*

Tat aptamers have been isolated independently on several occasions from random sequence pools (111, 139-141). Tuerk and MacDougal-Waugh describe three sequence families of aptamers arising from a selection to HIV-1 Tat protein (111), although these have not been characterized biochemically. Subsequently, Yamamoto *et al.* described aptamers that bind Tat with an affinity (K_d = 0.12nM) over two orders of magnitude greater than that of the Tat-TAR interaction. These aptamers contain a tandem repeat of a TAR-like motif (Figure 6), although the binding stoichiometry appears to be approximately 1:1 (139, 140). Variants of this motif were later tagged with fluorescent dyes for detection of Tat protein using molecular beacon technology (142). Another set of aptamers selected in the presence of 2mM Zn^{2+} showed no similarity to the TAR element. These aptamers demonstrated a slight preference for Zn^{2+} vs. Mg^{2+} (2- to 7-fold difference in Tat-binding affinity), and their affinity for Tat is approximately equivalent to the native Tat-TAR element (K_d = 12 to 120nM) (141).

4.2.2. Tat aptamers and TAR decoys *in vivo*

Inhibition of viral replication in cell culture by TAR RNA decoys suggests the promise of expressed Tat aptamers. Sullenger and colleagues (143) achieved a 99% decrease (2 logs) in HIV-1 replication in cultured human T-lymphoid cell line expressing a $tRNA^{met}$-TAR fusion transcript. At least four additional studies demonstrate the efficacy of TAR RNA decoy for inhibition of viral replication in cultured cells. Two of these used constitutive promoters to express HIV-2 TAR (144) or a nucleolus-targeted U16 snoRNA-TAR chimera (145, 146). The other two systems used Tat-regulated promoters to express the decoys. The transcript contained polymeric TAR motifs of up to 50 tandem repeats in one case (29), and an HIV-2 TAR/HIV-1 RRE hybrid transcript in the other (129).

Both the TAR and RRE RNA elements interact with several essential cellular proteins, including nuclear protein p140, cellular factor TRP-185, interferon-induced double-stranded RNA-activated protein kinase, the single-stranded DNA binding protein, Pur-alpha and others (147-154). While this circumstance raises the possibility of cytotoxicity resulting from expressed decoys, no detrimental effects on cell viability were observed during continuous expression of wild type and mutant TAR sequences in cultured T-lymphoid cells (155). Because TAR and RRE decoy systems utilize viral RNA sequences, the probability of HIV-1 evolving escape mutations that evade this mode of inhibition would appear to be extremely low, as such an event would require simultaneous mutations in both the protein (Tat or Rev) and RNA (TAR or RRE) components. Similar to the situation for Rev aptamers and RRE decoys discussed above, expression of non-TAR-like RNA aptamers may lessen fears of cytotoxicity resulting from unintended sequestering of cellular factors. No reports have been published to date describing inhibition of viral replication in cell culture through the use of expressed Tat aptamers. However, Yamamoto and colleagues expressed Tat protein in a HeLa cell line transduced with an HIV-1 LTR-luciferase fusion to monitor Tat-driven reporter expression (140). Co-expression of either TAR decoys or a Tat aptamer decreased Tat-dependent luciferase activity by 50-70% as compared with a Tat only expression control, demonstrating that an expressed aptamer can significantly inhibit Tat function in cells for a reporter system.

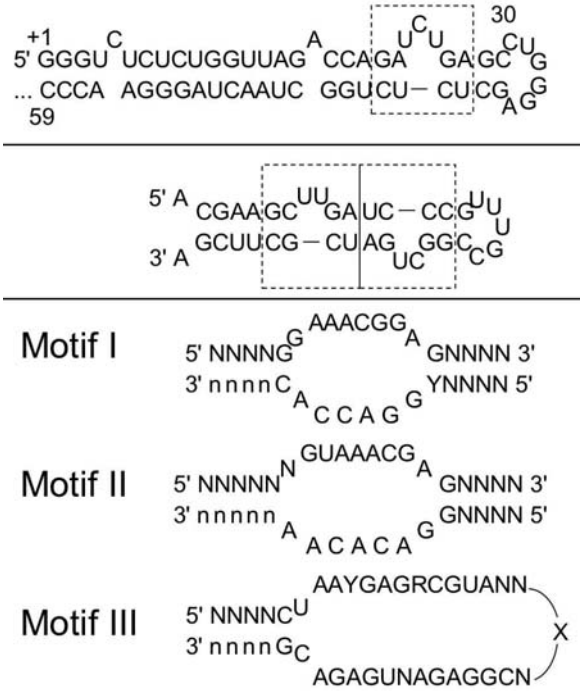

Figure 6. Tat aptamers. Secondary structures of Tat-binding RNAs. Top, native 5' end of HIV-1 genomic transcript, including the TAR element (boxed). Middle, aptamer containing a tandem repeat of a TAR-like motif identified by (140). Bottom, three additional motifs identified by Tuerk and MacDougal-Waugh (111). X = variable number of unspecified nucleotides; N = any nucleotide; n = Watson-Crick partner for N; R = purine; Y = pyrimidine.

4.3. TAR, RRE and other structured viral RNAs

Aptamers can be selected to recognize structured RNA through non-base-pairing quaternary interactions (156-158). Indeed, both RNA and DNA aptamers have been generated that interact specifically with the TAR element at the 5' end of the viral RNA (156, 157). Unlike antisense approaches, these aptamers appear to interact with their target transcripts through quaternary interactions which depend on structural context rather than simple base pairing between two linear strands. Although their anti-viral efficacy has not been measured, these aptamers reduce the transcription of partial viral message *in vitro* (325 and 525 nt initiated from native +1 site). Inhibition was approximately 50% when aptamer was used at 0.4 micromolar, and 80-90% when used at 4 micromolar (159, 160). It is unclear whether TAR mutations could overcome the inhibitory effects of these aptamers without simultaneously incapacitating the virus, or whether sequence constraints on the Tat-TAR interaction may prevent significant mutation of the TAR element. Other viral RNA sequences, such as the RRE, the psi element (ψ) and the genome dimerization initiation site (DIS) form discrete structures and could also serve as targets of future selection experiments.

5. INHIBITION OF VIRAL PACKAGING

The Gag gene products are initially translated as a p55 polyproteinthat is processed by PR into the mature proteins. The matrix (MA) portion of the polyprotein is myristoylated and assembles at the cell membrane to form the outer shell of the virion, where it is processed into the mature p17 form during budding. p7 nucleocapsid (NC) is a 55 amino acid protein that binds tightly to the RNA genome. p24 capsid (CA) forms an inner shell around the NC-bound genomic RNA. The C-terminal p6 fragment of Gag ($p6^{Gag}$) is important for virion release and for incorporation of Vpr into the virus. Finally, p1 and p2 are two small spacer peptides of unknown function that are released from either side of NC (161). The Gag polyprotein is also believed to recruit the genomic replication primer $tRNA_3^{Lys}$ through its interactions with the lysyl tRNA synthetase (162, 163). Of these products, only the $p55^{Gag}$ polyprotein and two versions of nucleocapsid (mature NCp7 and a partially processed NC precursor NCp9) have been used to generate aptamers.

5.1. Nucleocapsid and $p55^{Gag}$ polyprotein

NC is a highly positively charged protein characterized by the presence of two CCHC "zinc finger" motifs. In the context of the Gag precursor polyprotein, the two zinc fingers facilitate binding of NC to the ψ sequence of the viral RNA genome during packaging of new viral particles (161). NC remains tightly associated with the viral RNA in the mature viral particle following processing of Gag by PR. In addition to its role in viral particle encapsidation and assembly, NC promotes annealing of the cellular $tRNA^{Lys}$ primer to the viral genomic RNA during initiation of reverse transcription (164-166), as well as strand transfer following synthesis of minus-strand-strong-stop DNA (167-169). The involvement of NC in multiple steps of the viral life cycle, along with an apparent intolerance for mutations at the two zinc finger motifs (170, 171) make it an attractive target for inactivation by anti-HIV-1 therapeutic agents.

5.1.1. NC and $p55^{Gag}$ aptamers *in vitro*

The first *in vitro* selection for nucleic acid aptamers against NCp7 produced a family of RNA sequences with affinity for the protein in the 2nM to 6nM range (172). Among 55 sequenced clones from the sixteenth round of the selection by Allen *et al.*, all shared a conserved 14 nucleotide sequence motif of considerable sequence similarity to three known NC binding regions within the viral genome (Figure 7A and 7B). *In vitro* assays for the inhibition of strand annealing by two of the selected clones revealed IC_{50} values of 0.5nM and 2nM. In separate work by Berglund *et al.*, an RNA aptamer with an unrelated sequence (designated SelPsi), derived from a selection against a partially processed seventy-one amino acid NCp9, binds its target with K_d~2.3nM. Berglund's aptamers share sequence similarity with a site within the viral ψ element (Figure 7C) (173). Although the site of similarity overlaps one of the sites identified by Allen *et al.*, the matching nucleotides are distinct and the two sets of aptamers form different secondary structures, each of which is consistent with the segment of viral RNA to which it is compared. An unexplored possible explanation for this difference is that the viral genomic RNA may contain multiple overlapping NC-binding sites within the ψ element, each with distinct structural features, or that the two sets of aptamers may

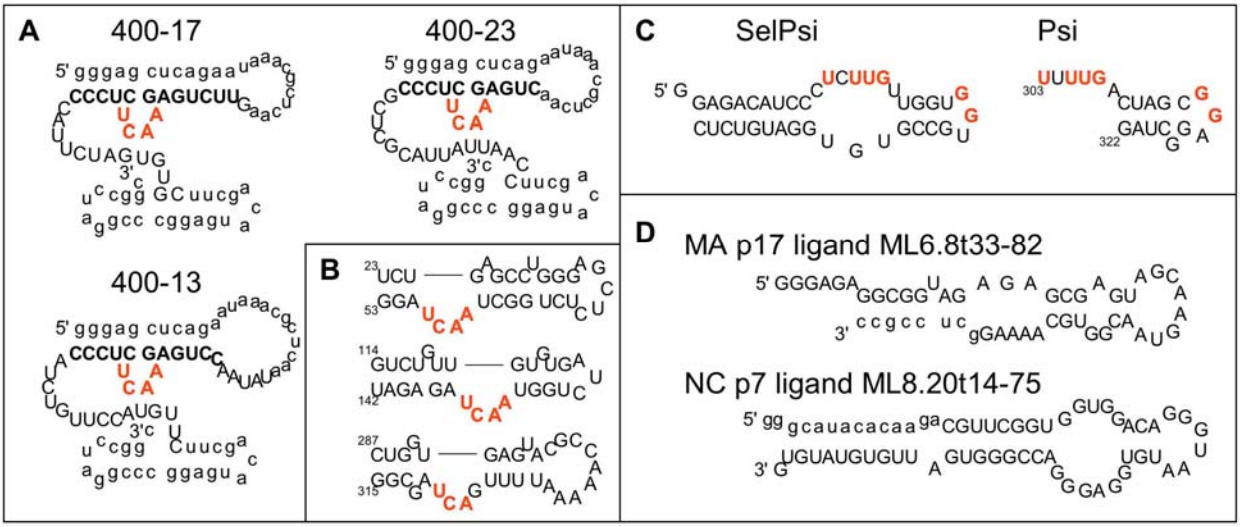

Figure 7. RNA aptamers targeted to matrix, nucleocapsid, or $p55^{Gag}$. A. Representatives of the aptamer set isolated by Allen *et al.* (172). Conserved motif found in these and several other aptamers from this set is bolded. Nucleotides shared between aptamers and genomic RNA are shown in red. B. Sites in HXB2 genome with similarities to Allen aptamers. C. SelPsi aptamer identified by Berglund *et al.* and corresponding segment of viral ψ element (173). D. Lochrie aptamers to MA (above) and to NC (below) segments of $p55^{Gag}$ polyprotein (174).

contact different surfaces of NC. Lochrie *et al.* selected aptamers to the full $p55^{Gag}$ polyprotein expressed as a GST fusion (174). At least 16 aptamers were identified that bound to MA, NC, or the $p55^{Gag}$-GST fusion with K_d values of 13 nM or better (Figure 7D). Unlike the Allen and Berglund aptamers, none of the Lochrie aptamers to NC demonstrated significant sequence similarity to HIV-1 viral RNA. This result might be expected given that the Allen aptamers incorporated the experimenter-imposed 5' constant region into the active structure, and the Lochrie selection utilized a different constant sequence. Several high-affinity aptamers bind in the NC segment of $p55^{Gag}$ (K_d of 1nM), while others appear to bind in the matrix MAp17 region (K_d of 3 nM). Finally, Kim *et al.* (175) have reported RNA aptamers selected against NCp7 with K_d values of 0.5nM to 3nM. The various forms of NC bind up to 100-fold more tightly to individual isolates from all four sets of NC aptamers than to the natural viral ψ element (reported K_d of 58nM (173)). This strong preference for the selected RNA over the natural binding target parallels the preference noted above for Tat binding to selected aptamers versus to the natural TAR element.

5.1.2. NC aptamers and decoys *in vivo*

Dorman and Lever recently expressed a 109nt portion of the HIV-1 ψ decoy RNA element (containing the core packaging sequence) in cultured Jurkat T lymphocytes (176). Following viral challenge at a low multiplicity of infection (MOI = 0.008), cell cultures were monitored for viral RT activity for seven weeks. Cells expressing the ψ decoy construct prevented viral replication for the first four weeks, after which time viral RT levels rose to half that of the empty vector control. The reason for the loss of viral suppression is not clear, although one possibility is that decoy expression may not have been maintained through such an extended assay. Surprisingly, the investigators were unable to confirm a disruption of viral packaging by the decoy in the transfected cells. Although there have been reports of antiviral assays using anti-NC aptamers expressed in human cells, the anti-viral effectiveness is modest (up to 60% reduction as monitored by viral RNA quantitation three days after transfection) (177). However, because the aptamers in that study are expressed within very large transcripts that could prevent either their proper folding or their incorporation within viral particles, an alternative design may yield more convincing anti-viral effects. Nevertheless, the multi-week viral suppression observed for expression of the ψ element decoy and the abundance of high-affinity NC-binding RNA aptamers warrant continued exploration of expressed NC aptamers.

6. INHIBITION OF VIRAL ENTRY

6.1. Glycoproteins gp120 and gp41

Targeting the HIV-1 envelope glycoproteins or host cell receptors provides an opportunity for aptamer therapies without the need for intracellular delivery or expression from gene therapy vectors. As such, it is conceivable that aptamers to viral glycoproteins could proceed more rapidly through testing in animal models (and reach clinical trials sooner) than expressed aptamers. Cellular invasion by HIV-1 is a two step process mediated by the actions of the viral envelope glycoproteins gp120 and gp41, both derived from proteolytic processing of the gp160 *env* gene product. gp120 mediates viral attachment via interactions with host cell surface protein CD4 . Prior to cell attachment, two helical regions of gp41 known as HR1 and HR2 form three-helix coiled-coil interactions at the trimer interface. Following attachment and binding to chemokine receptor CCR5 or CXCR4, HR1 and HR2 fold back and interact with each other to form a six-helix bundle. This movement pulls the viral and cell membranes

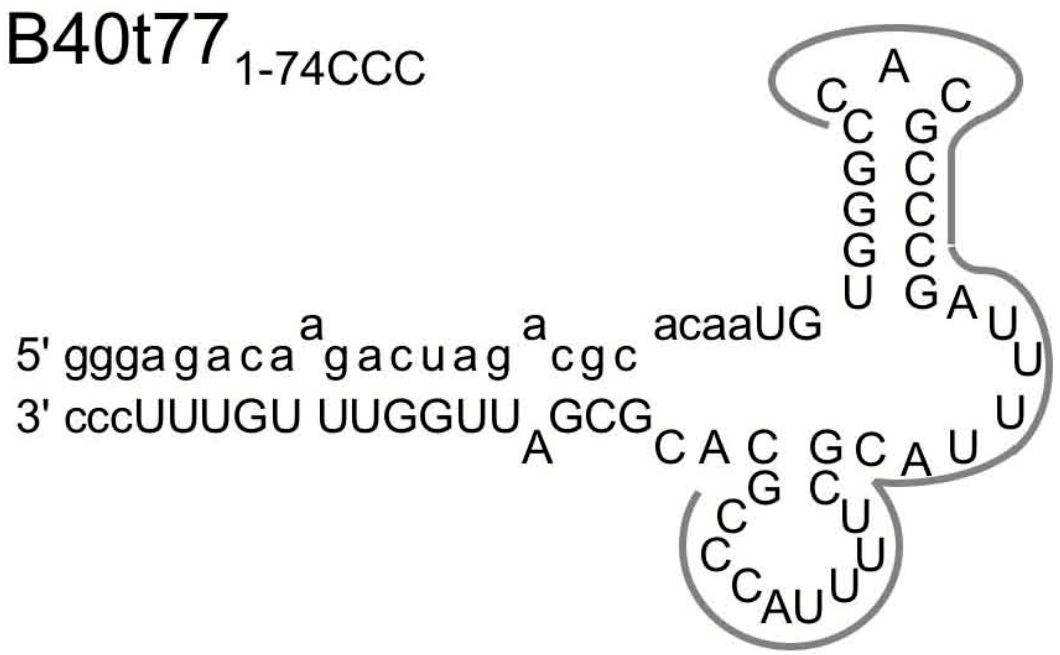

Figure 8. gp120 aptamer. A nuclease-stabilized RNA aptamer targeted to gp120 (182). All C and U residues were transcribed as 2'-fluoro analogs in original selection. Nucleotides suggested by footprinting data to be in binding core are indicated by grey line.

into proximity, helping to initiate their fusion, and resulting in viral entry into the host cell. The first FDA-approved HIV-1 inhibitor directed at a target other than reverse transcriptase or protease was enfuvirtide (178, 179). As a 36 amino acid peptide corresponding to residues 127-162 of the HR2 region of gp41, the biopolymeric enfuvirtide has more in common with nucleic acid aptamers than with the small molecule drugs directed against RT and PR. Like an aptamer, enfuvirtide appears to inhibit viral function through a competitive binding event by interacting with and disrupting the native HR1-HR2 interaction (180, 181).

6.1.1. gp120 aptamers *in vitro* and in antiviral assay

Aptamers to gp120 have been generated in several labs in recent years (182-184). The selections differ both in the HIV-1 strain from which the gp120 was derived and in the chemical composition of the aptamer polynucleotide backbones. Two separate selections from the James lab targeted gp120 from subtype B strains of HIV-1, with strain HXB2 being used in one case (183) and Ba-L in the other (182). Both of the James lab RNA aptamer libraries were stabilized against serum nucleases by substituting fluorine in place of OH at the 2' position of the pyrimidine nucleotides. These selections yielded individual sequences with K_d values for gp120 ranging from 5nM to 100nM (Figure 8). The 2' fluoro-pyrimidine-substitued RNA aptamers isolated by Khati *et al.* show the most potent antiviral efficacy of all aptamers described in this chapter, suppressing viral replication in cultured PBMC's by up to 10,000-fold relative to untreated cells (182). The magnitude of this inhibition is at least 100-fold greater than that of any of the studies described above. Importantly, when Khati and colleagues applied several of their gp120 aptamers to cell culture assays for inhibition of replication by six HIV-1 group M non-B subtype clinical isolates and one group O isolate, they found them all to be equally or more effective than a handful of neutralizing antibodies assayed in parallel (182). This is a promising result in the face of HIV-1's ability to evade antibody inhibition *in vivo*. Time will tell whether the virus will be able to generate escape mutations to gp120 aptamers. The James group speculates that their aptamers may be able to penetrate the highly variable exterior surfaces of gp120 to gain access to the conserved core. It is not know whether these aptamers bock HIV-1 entry by preventing gp120 from docking with the cell surface, or by disrupting the gp120-gp41 interaction. In another work, Smith and colleagues isolated modified aptamers to gp120 using 5-Bromo-2'-deoxyuridine in place of thymidine in their DNA aptamer pool. The 5-bromo modification permits covalent attachment of their aptamers to the glycoprotein through photocrosslinking, making them irreversible inhibitors (184). Although photoselected DNA aptamers were designed as proteomic diagnostic reagents (185), it is possible they will find additional use in other applications.

7. FURTHER CONSIDERATIONS

The results presented above highlight the advances in developing aptamers as anti-HIV-1 reagents. In addition to the dramatic antagonism of viral replication by aptamers selected to the extracellular target gp120, especially promising aptamers are also available that disrupt the function of the intracellular viral targets RT, IN, Tat and Rev. Anti-NC aptamers have yet to exhibit significant anti-viral activity. Among the list of largely unexplored targets are host proteins that facilitate viral pathogenesis and several viral proteins (MAp17, $p6^{Gag}$, the

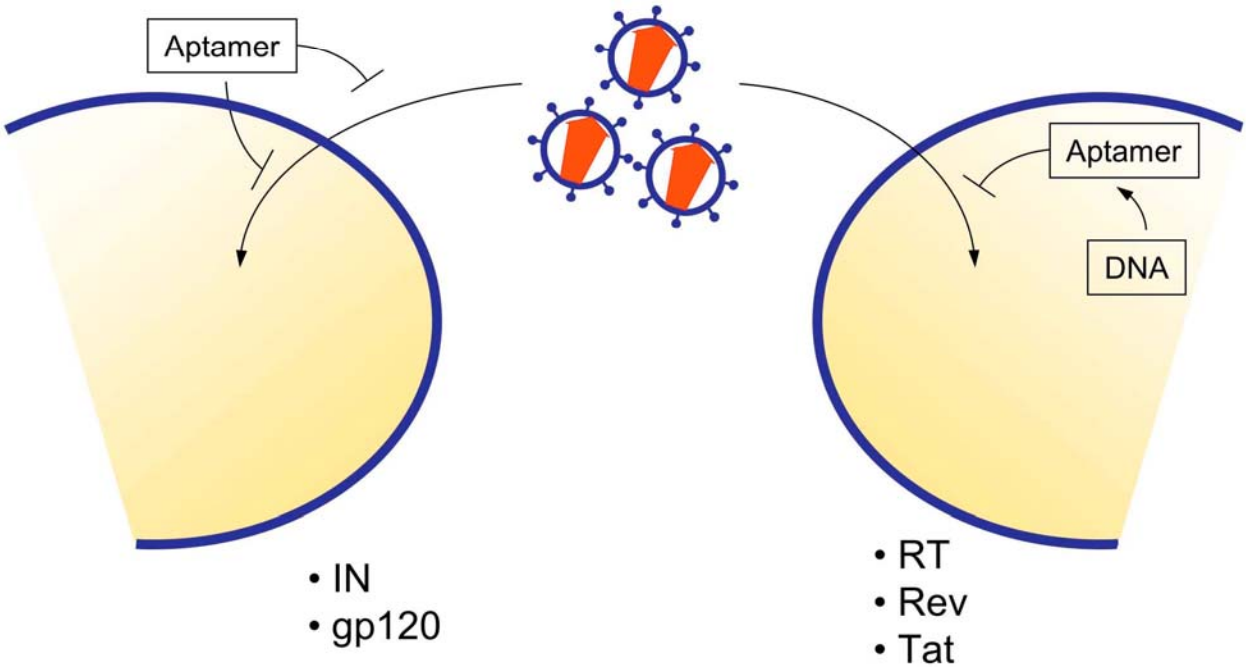

Figure 9. Neutralization of HIV-1 replication by nucleic acid aptamers can be accomplished either through exogenous delivery (left) or through intracellular expression (right). Targets for which viral neutralization has been best demonstrated are noted below the corresponding pathway.

Gag linker peptides p1 and p2, PR, gp41, Nef, Vif, Vpr and Vpu). Future developments can be expected to improve aptamer efficacy and to exploit targeted antagonism to gain greater understanding of viruses. Advances in the SELEX methodology, coupled with our ever deepening understanding of the molecular mechanisms of HIV-1 pathogenesis, should enable the development of more effective second- and third-generation aptamers. A better understanding of the biochemical properties of aptamers and of aptamer-target interactions will yield aptamers with greater affinity for the intended targets, reduced off-target reactivity, increased ability to localize temporally and spatially onto the intended target, and minimized susceptibility to the appearance of aptamer-resistant mutations. Added potency may be achievable through expressing polycistronic aptamers targeted against one or multiple HIV-1 proteins. Like currently available HIV-1 inhibitors, aptamers may find their greatest utility as adjuvants to other therapies, serving as components of a multi-pronged "cocktail" of different classes of inhibitors targeting multiple aspects of HIV-1 function.

Several themes emerge in this overview. First, HIV-1 can be neutralized by nucleic acid aptamers that are delivered either exogenously or via intracellular expression (Figure 9). Second, in their current formulations, most of the aptamers described above reduce viral infectivity by one to two logs (10- to 100-fold) (Table 1). The gp120 aptamers are exceptional in providing over four logs of viral inhibition (182). Third, some aptamers arise with sequence features that resemble HIV-1 genomic RNA. Comparative structural analyses of how the aptamers and the natural RNAs bind their targets may be especially valuable for establishing how inhibitory interactions differ from productive interactions. Fourth, aptamers to nucleic acid-binding proteins tend to bind these proteins 10- to 100-fold more tightly than these proteins bind their native RNA targets. This trend likely reflects both the fact that natural macromolecular interactions are nearly always dynamic rather than optimized for binding and the fact that biological selection occurs simultaneously for multiple functions in addition to binding, such as encoding for proteins. As a corollary, we speculate that aptamers that strongly mimic viral RNA sequences may offer little inhibitory advantage over expression of the viral RNA segment as a decoy. Fifth, some aptamers appear to exert their effects by getting packaged within the nascent virions and preventing subsequent infection. This is best demonstrated for RNA aptamers targeted to RT (31). In this respect they operate similarly to the host restriction factor APOBEC3G, which is packaged into virions and prevents productive infection by deaminating the dC residues in the newly synthesized ssDNA of the replicating viral genome (186, 187).

A sixth theme relates to the extensive natural sequence diversity encompassed by the HIV-1 global pandemic. It is not yet known whether aptamers will demonstrate broad neutralizing capabilities, although the effective cross-clade inhibition by gp120 aptamers (182), and to a lesser extent by RT aptamers (31) is encouraging in this respect. Both natural sequence variation and the treatment-driven evolution of resistant strains have proven problematic for small molecule inhibitors. Aptamer resistance may be less of a problem than small molecule resistance due to the large area buried at the protein-

aptamer interface and to the requirement in several cases (RT, IN, Tat, Rev and NC) that any mutations in the protein not disrupt generic RNA-protein interactions. All of the currently available aptamers were selected against protein targets from HIV-1 subtype B, the dominant clade in North America and Europe (188). When a small sampling of pseudoknot RT aptamers was assayed against a panel of non-B HIV-1 strains, they proved to be effective inhibitors of isolates from subtypes A, B, D, E and F, but they were less effective against a subtype C isolates, a recombinant A/D strain and an isolate from group O (31). An approach being pursued in our lab is to generate broad-spectrum inhibitors using "toggle SELEX" (189), wherein aptamers are selected to recognize proteins from several clades through alternate exposure of the RNA pool to each target during successive rounds of *in vitro* selection.

Finally, as the majority of HIV-1 protein targets function intracellularly, inhibitory aptamers must encounter their targets inside cells. The use of RNA aptamers to disrupt their function therefore essentially describes a gene therapy approach. Accordingly, as with all potential gene therapies, an efficient delivery technology is needed before advance to the clinic can be considered. Long-term, stable expression of the aptamer is another requirement for gene therapies, especially since latent viral reservoirs could potentially be reactivated many years after initial treatment. One delivery system that has been explored is a conditionally replicating virus carrying three components: a gene encoding the aptamer, a ψ element for packaging, and two LTRs. This RNA would be packaged into viral particles along with the normal complement of HIV-1 proteins, including the products of the HIV-1 env genes (190). Upon infection of target cells, which would include the same range of cell types as that of infectious HIV-1, the aptamer-encoding construct would be integrated into the host genome, where it would be competent to direct the expression of the protective aptamer. Although gene therapy has yet to become a generally applicable, safe, and reliable technology, it is a highly sought after treatment paradigm for myriad genetic and infectious diseases. The widely shared desire to make gene therapy a reality will keep many laboratories busy at the task, and the continued development and refinement of HIV-1 aptamers assures that there will be an increasingly potent antiviral arsenal in waiting.

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