Inhibitory RNA Ligand to Reverse Transcriptase from Feline Immunodeficiency Virus†

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Received January 2, 1996; Revised Manuscript Received March 14, 1996⊗

ABSTRACT: High-affinity, high-specificity RNA ligands for reverse transcriptase from feline immunodeficiency virus (FIV) were isolated from an RNA library by the SELEX (**S**ystematic **E**volution of **L**igands by **EX**ponential enrichment) procedure. The selected RNA ligands bound to FIV reverse transcriptase with dissociation constants in the nanomolar range. One of the ligands was a potent inhibitor of the RNA-dependent DNA polymerase activity of both the recombinant and the virion-derived FIV reverse transcriptase. It also inhibited the reverse transcriptase from an FIV mutant that is resistant to 3′-azido-3′-deoxythymidine (AZT). The inhibition of FIV reverse transcriptase was competitive with respect to template−primer and noncompetitive with respect to deoxyribonucleoside 5′-triphosphates. This ligand was specific for the FIV enzyme and did not inhibit other reverse transcriptases tested (avian myeloblastosis virus, Moloney murine leukemia virus, and human immunodeficiency virus type 1).

Feline immunodeficiency virus (FIV)[1] is a lentivirus that causes a naturally occurring immune deficiency in domestic cats that is similar to the acquired immune deficiency syndrome (AIDS) in humans (Pedersen et al., 1987; Pedersen, 1993). FIV-infected cats develop an AIDS-related complex-like stage, and progress ultimately to an AIDS-like disease (Ishida & Tomada, 1990; Torten et al, 1991). FIV is similar to the human immunodeficiency virus type 1 (HIV-1) in morphology, genome arrangement, and cell tropism (Pedersen, 1993). The FIV reverse transcriptase (RT) has been purified and shown to be nearly identical to the RT from HIV-1 in physical properties, catalytic activities, and sensitivity to antiviral nucleotides (North et al., 1989, 1990a,b, 1994; Cronn et al., 1992). Both of these RTs are heterodimers with subunits of M_r 66 000 (p66) and 51 000 (p51) (North et al., 1994) which have similar Mg^{2+} requirements for catalytic activity and similar template specificities (North et al., 1990b). The FIV and HIV RTs are also nearly identical in susceptibility to inhibition by the active forms of several antiviral nucleosides that display anti-HIV-1 activity, including the 5′-triphosphates of 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T), and the 2′,3′-dideoxynucleotides (ddATP, ddCTP, ddGTP, and ddTTP) (North et al., 1989, 1990a; Cronn et al., 1992). These features make FIV a valuable animal model for studies of RT-targeted therapy for AIDS.

Most of the anti-HIV-1 drugs currently approved for AIDS therapy are nucleoside analogs whose active forms function as inhibitors of RT. These include AZT, ddI, ddC, d4T, and 2′,3′-dideoxy-3′-thiacytidine (3TC). Although these drugs provide some beneficial effects, their use is limited by toxicity (Yarchoan et al., 1989) and the emergence of drug-resistant mutants (Larder et al., 1989; Richmann, 1993). There is a clear need for more specific inhibitors and strategies to combat resistance. The model systems we have developed with FIV have been particularly useful for studies of RT-targeted AIDS therapy and the emergence of FIV mutants resistant to these drugs (Remington et al., 1991, 1994; Gobert et al., 1994). These versatile systems are well-suited for evaluation of new therapeutic strategies.

As an approach to development of more selective RT inhibitors we have used the SELEX (**S**ystematic **E**volution of **L**igands by **EX**ponential enrichment) procedure (Tuerk & Gold, 1990) to isolate high-affinity RNA ligands directed against recombinant FIV RT. We report here the selection of RNA ligands that inhibit the FIV RT in a manner that is competitive with respect to the template−primer complex. Moreover, at least one of the inhibitory RNA ligands is also active against RTs from both wild-type and AZT-resistant FIV.

EXPERIMENTAL PROCEDURES

Materials. Recombinant FIV RT was purified from *Escherichia coli* containing the clone pRTF14 as previously described (North et al., 1994). Virion FIV RTs from wild-type FIV or from the AZT-resistant mutant AZR-17c were purified as previously described (North et al., 1990b; Remington et al., 1994). The latter enzyme has decreased susceptibility to the 5′-triphosphate of AZT (AZTTP). Avian myeloblastosis virus (AMV) RT was purchased from Life

† This research was supported by research grants from National Institutes of Health to L.G. (GM28685 and GM19963) and to T.N. (AI28189) and by funds from NeXstar Pharmaceuticals, Inc., Boulder, CO.

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] Abbreviations: AIDS, acquired immune deficiency syndrome; AMV, avian myeloblastosis virus; AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; dNTPs, deoxyribonucleoside 5′-triphosphates (dATP, dCTP, dGTP, and dTTP); FIV, feline immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; M-MLV, Moloney murine leukemia virus; PCR, polymerase chain reaction; RT, reverse transcriptase; SELEX, **S**ystematic **E**volution of **L**igands by **EX**ponential enrichment.

S0006-2960(96)00010-4 CCC: $12.00

Sciences, Inc., Moloney murine leukemia virus (M-MLV) RT was purchased from Gibco-BRL, and HIV-1 RT was generously provided by Agouron Pharmaceutical Inc. Taq DNA polymerase was purchased from Perkin-Elmer Cetus, T4 polynucleotide kinase from New England Biolabs, and T7 RNA polymerase from U.S. Biochemical Corp. DNA and RNA oligonucleotides were synthesized on an Applied Biosystems Model 394 DNA/RNA synthesizer.

Selection Procedure. High-affinity RNA ligands were selected from an RNA repertoire with a randomized region of 40 nucleotides, containing approximately 10^{14} unique species, as described previously (Tuerk & Gold, 1990; Tuerk et al., 1992; Chen & Gold, 1994). The first 10 rounds of selection were performed by nitrocellulose filter partitioning in 1 mL of binding buffer (50 mM Tris-HCl, pH 7.7, 200 mM potassium acetate, and 10 mM dithiothreitol) containing RNA and target protein. For these selections the RNA concentration was 1520 nM in initial rounds, decreasing to 400 nM in the final rounds of selection; protein concentration ranged from 75 down to 20 nM. Mixtures were incubated at 37 °C for 10 min, and bound RNA was partitioned by nitrocellulose filtration. The recovery of bound RNA was as described previously (Tuerk & Gold, 1990). The last 8 rounds of selections were performed by native gel shift assays (Lim et al., 1991) in 52 μL of binding buffer containing 7% glycerol with appropriate RNA (1240 down to 30 nM) and protein (62 down to 1.5 nM) concentrations. Reaction mixtures were incubated at 37 °C for 10 min, after which samples were loaded onto an 8% native polyacrylamide gel (acrylamide:bisacrylamide 80:1) using 25 mM Tris-HCl, 192 mM glycine, and 1 mM EDTA, pH 8.3, as running buffer. Electrophoresis was carried out at 4 °C for 2 h at 200 V. After autoradiography, RNA in the RNA−RT complexes was purified and cDNA was synthesized as previously described (Tuerk & Gold, 1990; Tuerk et al., 1992; Chen & Gold, 1994). The cDNA product was amplified by PCR and transcribed with T7 RNA polymerase to generate the RNA library for the next round of selection (Tuerk & Gold, 1990; Chen & Gold, 1994).

Determination of Equilibrium Dissociation Constants. Dissociation constants (K_d) were measured as described by Tuerk et al. (1990). Reaction mixtures containing 7.0×10^{-11} M ^{32}P-labeled RNA and 2.3×10^{-10}−3.0×10^{-8} M protein were incubated in 21 μL of binding buffer. After 10 min at 37 °C, 15 μL of each mixture was filtered through nitrocellulose. Filters were dried and placed in vials containing Ecolume (ICN, Inc.) for quantitation of bound radioactivity by liquid scintillation counting in a Beckman LS-133 scintillation counter. K_ds were determined by plotting the percentage of RNA bound versus the log protein concentration (Tuerk et al., 1990).

Boundary Analysis. Determinations of the 5′- and 3′-boundaries required for binding of ligands to FIV RT were performed as described previously (Tuerk & Gold, 1990; Tuerk et al., 1990). For 3′-boundary analyses the RNA was ^{32}P-labeled at the 5′-end with T4 polynucleotide kinase. For 5′-boundary analyses the RNA was labeled at the 3′-end with ^{32}pCp using T4 RNA ligase. Briefly, RNA was partially hydrolyzed by incubation in 50 μL of 50 mM sodium carbonate, pH 9.0, 1 mM EDTA, and 0.5 mg/mL yeast tRNA at 90 °C for 15 min. The reaction was neutralized by addition of 6 μL 3 M sodium acetate, pH 5.2, and the resulting RNA was precipitated with ethanol. The 3′-boundary was determined by incubating 30 pmol of partially alkaline hydrolyzed 5′-^{32}P-labeled RNA with 4.2 pmol of FIV RT in 1 mL of binding buffer as described previously (Tuerk et al., 1990; Chen & Gold, 1994). The 5′-boundary was determined by incubating 30 pmol partially alkaline-hydrolyzed 3′-^{32}pCp-labeled RNA with 4.2 pmol of FIV RT in 1 mL of buffer under similar conditions. Bound fragments were recovered by nitrocellulose filtration and analyzed by denaturing gel electrophoresis.

Inhibition of RT Polymerization Activity. For initial screening and characterization, the ability of RNA ligands to inhibit RT was assayed as described previously (Tuerk et al., 1992; Chen & Gold, 1994). Reactions contained 17.4 nM RT and 10 nM template−primer complex with 5′-^{32}P-end-labeled primer. The RNA template was a fragment of plasmid pT7-1 transcribed by T7 RNA polymerase (RNA template sequence: 5′-GGGAAUUCGAGCUCGGUAC-CCGGGAUCCUCUAGAGUCGACCUGCAG GCAUGC-UAGCUUGGCACUGGGCGUCGUUUUACAACG-UCGUGACGUGG-3′ and primer sequence 5′-CCCACGT-CACGACGTTGTAAAACGACGCCC-3′). Concentrations of RNA ligands used are indicated in individual experiments. Reactions were incubated at 37 °C for 10 min in 20 μL of reaction buffer which consisted of 50 mM Tris-HCl, pH 7.7, 200 mM potassium acetate, 6 mM $MgCl_2$, 10 mM dithiothreitol, 25 μg/mL bovine serum albumin, and 0.4 mM each dNTP (dATP, dCTP, dGTP, and dTTP). Reaction products were analyzed by electrophoresis on a 7 M urea−10% polyacrylamide gel.

Kinetics of Inhibition of RT. In reactions with homopolymeric RNA templates, FIV RT activity was assayed as described previously (North et al., 1989, 1990a, 1994). Reactions were carried out in a volume of 50 μL and under standard conditions contained 50 mM Tris-HCl, pH 8.5, 10 mM dithiothreitol, 0.05% Triton X-100, 250 μg/mL bovine serum albumin (nuclease-free), 6 mM $MgCl_2$, 0.5 OD_{260} unit of poly(rA)−oligo(dT), [*methyl*-^{3}H]dTTP (33 μCi/mL, 20 μM), and 1−5 units (North et al., 1990b) of RT. For experiments to determine kinetic parameters, the concentration of dTTP or of template−primer was varied as indicated. Reaction mixtures were incubated at 37 °C for 30 min, and the radioactivity incorporated into DNA was quantified as previously described (North et al., 1990b). Kinetic parameters (K_m and K_i) were determined as previously reported (North et al., 1990a) using intercept values calculated from double-reciprocal plots.

For experiments using a heteropolymeric RNA we prepared a template corresponding to nucleotides 2979−3295 of the FIV genome (Talbott et al., 1989). This was prepared by PCR amplification of DNA from the 34TF10 clone of FIV (Remington et al., 1994), and then the PCR product was transcribed with T7 RNA polymerase. One of the primers used for PCR had a T7 promoter sequence 5′ to the sequence complementary to FIV cDNA. This primer (designated primer 24′) had the following sequence: 5′-CCCAAGCT-TAATACGACTCACTATAGGGAGCTCCAGAA-GATAAATTACAGG-3′ (T7 promoter sequence underlined). The other primer was 5′-GGGGTCATAGTATCCTAGT-TG-3′ (designated primer 14). PCR reactions, DNA product purification, T7 transcription, and RNA purification were all performed as previously described (Tuerk & Gold, 1990; Chen & Gold, 1994). For RT assays the heteropolymeric template was primed with primer 14. Reactions were carried

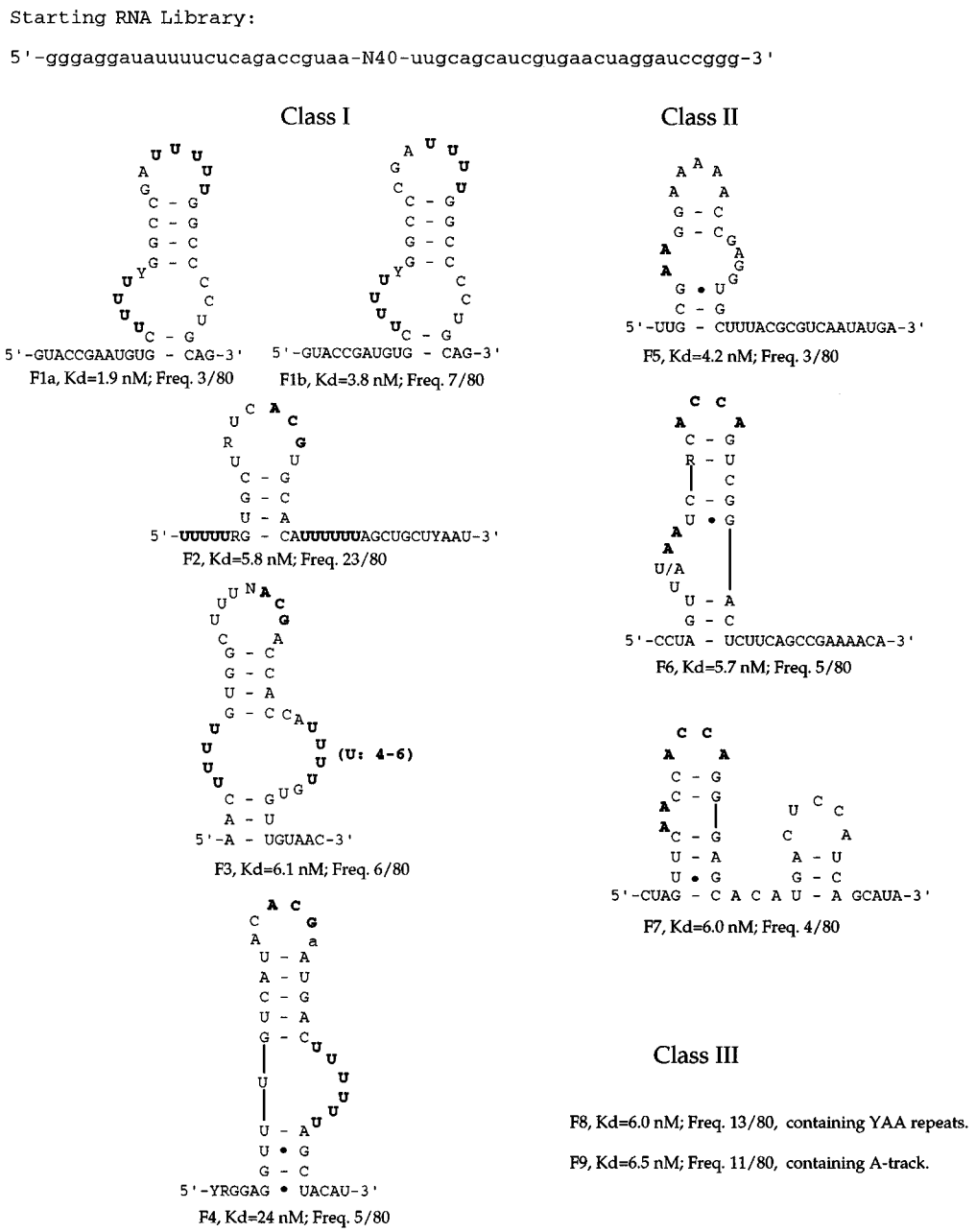

FIGURE 1: Predicted secondary structures of selected RNA ligands. The cDNA from the selected pool of round 18 was cloned into pUC 18 at *Hin*dIII and *Bam*HI sites and sequenced. Each subset was analyzed by an RNA folding program. The K_d and frequency of each ligand are indicated. Bold uppercase letters indicate the consensus sequences. Lowercase letter indicates that the nucleotide is not present in all members of the subset. Y represents pyrimidine; R represents purine; N can be any of the four nucleotides. The predicted structure of the fixed region is indicated in Figure 5B.

out as described above except that the other three dNTPs were present (20 μM each) in addition to [*methyl*-^{3}H]dTTP.

RESULTS

Selection of High-Affinity Oligonucleotide Ligands to FIV RT. The starting RNA repertoire was randomized over a 40-nucleotide stretch flanked by a 25 nucleotide fixed region at the 5′-end and a 27-nucleotide fixed region at the 3′-end (Chen & Gold, 1994). This starting repertoire contained approximately 10^{14} unique RNA species. Recombinant FIV RT was used as the target for selections to evolve high-affinity RNAs that bind to RT. The first 10 rounds of selection were performed by nitrocellulose filtration (Tuerk & Gold, 1990). The final rounds of selection were performed using a native gel mobility retardation method (Lim et al., 1991) as the partitioning strategy. Two major shifted bands appeared on the native gel (presumably the dimeric and monomeric protein−oligo nucleotide complexes, data not shown), and both complexes were collected for further selection. After 8 rounds of selection using the gel retardation assay, the binding affinity of the RNA pool could not be further improved by continued selection. The binding affinity to FIV RT of the round 18 pool (K_d 7.7 nM) was at least 10^3-fold higher than that of the starting repertoire. The PCR-derived molecules from round 18 were subcloned.

Eighty RNA sequences from the round 18 RNA pool were obtained and analyzed by an RNA folding algorithm (Zuker, 1989; Jaeger et al., 1989). The selected RNA molecules fell into three major classes (Figure 1). Class I molecules contain four subsets (F1, F2, F3, and F4), which form a stem−loop

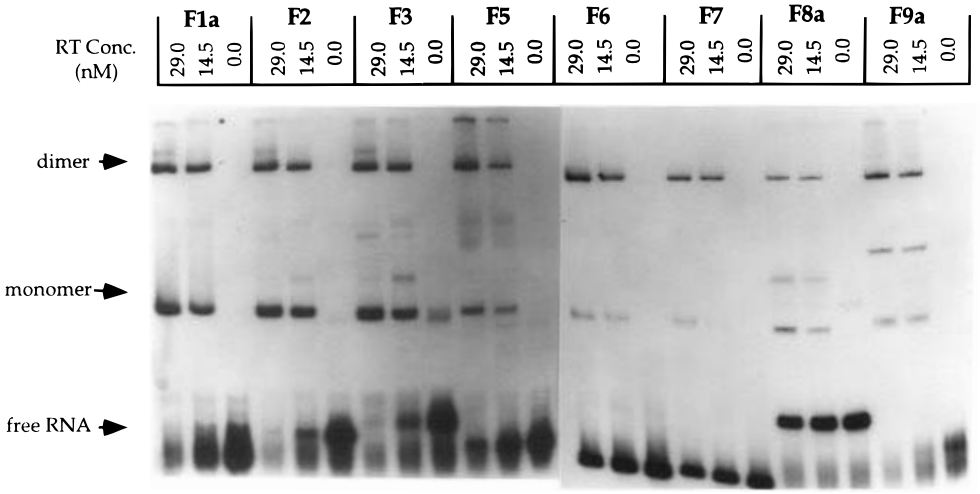

FIGURE 2: Native gel mobility retardation analysis of RT−RNA ligand complexes. For these experiments the indicated ^{32}P end-labeled RNA ligands (approximately 5×10^4 cpm) were each incubated at 37 °C for 10 min with FIV RT (at indicated concentrations) in 21 μL of binding buffer containing 7% glycerol. Samples were analyzed on 8% polyacrylamide gels. Positions of RNA bound to monomer and dimer of FIV RT, and of free RNA, are indicated with arrows.

or a stem−loop with an internal bulge and contain one or two U-tracts in the region predicted to be single-stranded. Three subsets (F2, F3, and F4) of class I ligands also have an ACG consensus in the loop. Class II consists of three subsets of species (F5, F6, and F7) that are able to form stem−loops with internal bulge structures. All of the class II ligands have a consensus AA dinucleotide in the bulge, and two subsets (F6 and F7) of this class contain an ACCA consensus within a loop. Class III consists of two subsets (F8 and F9, sequences not shown). Members of the F8 subset contain YAA repeats, and members of the F9 subset have an A-tract sequence. YAA repeats and A-tract sequences appear to be located in unstructured regions, as predicted by the RNA folding algorithm. Nine orphan sequences were also obtained among the cDNA clones (not shown). The binding affinities of each subset of RNA molecules to FIV RT were measured by filter binding. As shown in Figure 1, the dissociation constants were in the range of 1.9−24 nM.

Native Gel Mobility Retardation Analysis of the Interaction between Selected RNA Ligands and FIV RT. The recombinant, p66/p66 homodimeric FIV RT can dissociate to form p66 monomers. In order to determine whether the selected RNA molecules bind to the protein dimer, monomer, or both forms, gel mobility retardation assays were performed to analyze the RNA−protein interaction with each subset (Figure 2). We presume that the bands observed in Figure 2 represent monomer and dimer forms of RT because larger aggregates of FIV or HIV-1 RT have not been observed. The RNA molecules of class I bound equally to the FIV RT dimer and monomer. The class II RNA ligands bound mainly to dimeric RT and with less affinity to monomeric RT (with the exception of ligand F5, which could bind to both forms but binds preferentially to the dimeric form). The RNA ligands of class III bound to both forms of RT with approximately equal affinity. However, an extra complex with an intermediate mobility was also present. These extra complexes have not yet been identified. They may arise from different conformations of RNA interacting with the monomer protein, from binding of two RNA molecules with one monomer protein, or from an impurity of the recombinant FIV RT. These results indicate that the RNA ligands of the three different classes bind to FIV RT with different patterns.

Inhibition of RT Polymerization Activity. Some of these RNA ligands were expected to interact with FIV RT at the active site and function as inhibitors. Therefore, the ligands were screened with assays previously described (Tuerk et al., 1992; Chen & Gold, 1994) to identify inhibitors of recombinant FIV RT. The most potent inhibitor was RNA ligand F5 of class II (Figure 3) which has a K_d of 4.2 nM (Figure 1). Another ligand, F1a of class I, was less inhibitory to FIV RT even though it had a lower K_d (1.9 nM). Other RNA ligands were much less inhibitory (not shown) than ligand F5. The starting RNA pool did not inhibit FIV RT polymerization activity, even up to a concentration of 1 μM (not shown). The activity of ligand F5 was specific for FIV RT. It did not inhibit RT from AMV, M-MLV or HIV-1 (data not shown).

We also tested the ability of RNA ligand F5 to inhibit the virion-derived (p66/p51 heterodimeric) FIV RTs obtained from wild-type FIV or from an AZT-resistant mutant of FIV, AZR-17c. The RT from AZR-17c has a single mutation at position 202 resulting in substitution of a lysine for glutamate, and this enzyme is 5−6-fold resistant to AZTTP (Remington et al., 1994). Ligand F5 was active against both of these virion-derived FIV RTs (Figure 3). These results demonstrate that the RNA ligand F5, which was selected against recombinant FIV RT, also inhibits virion enzymes derived from both wild-type FIV and an AZT-resistant mutant of FIV.

Minimum RNA Sequences Required for Binding to FIV RT. Because RNA ligand F5 had the greatest inhibitory activity, it was further analyzed to determine the boundaries of the minimal binding domain. Results of these experiments demonstrated that the minimum sequence of RNA ligand F5 required for interaction with FIV RT consisted of a 28-nucleotide region (position U26−C53) (Figures 1 and 4). This 28-nucleotide sequence is predicted to form a stem−loop with an internal bulge structure (Figure 1). A truncated version of the F5 ligand (designated dF5) was designed on the basis of these boundary determinations. The truncated ligand bound to FIV RT with a K_d of about 7.8 nM (which is slightly lower than the K_d of 4.2 nM determined for ligand F5) and was able to inhibit FIV RT, although its activity

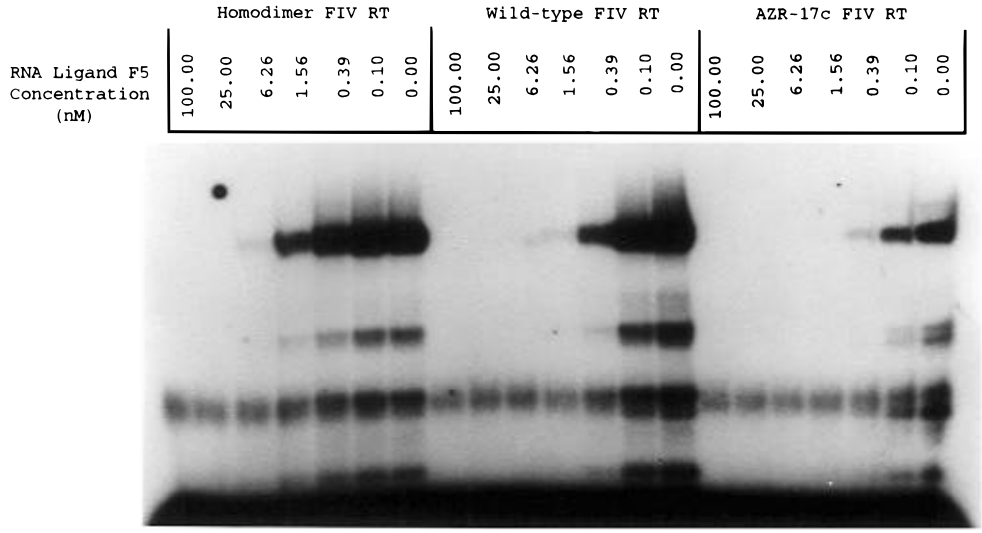

FIGURE 3: Inhibition of FIV RT polymerization activity by RNA ligand F5 . The assay for RT and analysis by gel electrophoresis were performed as described in Experimental Procedures. Bands nearest the top of the gel represent full-length product; bands at the bottom represent free primer; intermedate bands probably represent pause sites.

was slightly lower than that of the intact ligand F5. As expected, dF5 also inhibited virion-derived RT from both wild-type FIV and the AZT-resistant mutant (not shown). A native gel retardation experiment indicated that the truncated ligand dF5 bound to the FIV RT dimer (p66/p66) but not to the monomer (Figure 5). Thus, the truncated ligand appears to be more specific for the active form of FIV RT even though it has a slightly lower binding affinity. These data suggest that sequences flanking the 28-nucleotide region contribute to enhancement of binding and inhibition of RT activity.

Kinetics of RT Inhibition. Kinetic constants were determined for the inhibition of FIV RT by RNA ligands F1a and F5 using poly(rA)−oligo(dT) as the template−primer complex (summarized in Table 1). Both of these ligands inhibited the recombinant FIV RT in a manner that was competitive with respect to template−primer complex and noncompetitive with respect to dTTP (Figure 6). Ligand F5 also inhibited FIV RT in the reaction with poly(rI)−oligo(dC) with a K_i of 57 nM (data not shown). Interestingly, the virion-derived RTs were 3-fold more sensitive to inhibition by ligand F5 than the recombinant FIV RT (Table 1). This is surprising because the ligand was selected from the starting pool with recombinant FIV RT. The K_i values obtained for inhibition of virion-derived RTs from wild-type FIV and from AZR-17c were 31 and 26 nM, respectively (Table 1). Thus, this inhibitor is equally active against the wild-type RT and the AZTTP-resistant form. In contrast, ligand F1a was less inhibitory to the virion FIV RT than to the recombinant FIV RT (Table 1).

Since the inhibition of FIV RT by ligand F5 was competitive with respect to template−primer, we performed additional kinetic analyses with a more biologically relevant, heteropolymeric RNA template. The template used was a 316-nucleotide fragment from the RT-encoding region of the FIV *pol* gene, corresponding to nucleotides 2979−3295 of FIV (Talbott et al., 1989). For RT reactions, this template was primed with a 21-nucleotide DNA primer complementary to the 3′-end of the template, as described in Experimental Procedures. Maximal RT activity was obtained with a primer:template ratio of 2:1, and the velocity was approximately equal to that achieved in reactions with poly(rA)−oligo(dT) (data not shown). In reactions with this heteropolymeric RNA template, ligand F5 inhibited recombinant FIV RT in a manner that was competitive with respective to template−primer and noncompetitive with respect to dNTPs. The K_i value for this inhibition was 45 nM. These data demonstrate that ligand F5 is more potent as an inhibitor of recombinant FIV RT in reactions with the heteropolymeric template than in reactions with poly(rA)−oligo(dT).

We also analyzed the ability of ligand F5 to inhibit the virion-derived FIV RT in reactions with the heteropolymeric RNA template. The K_i determined for this reaction was 12 nM (Table 1). Thus, despite the fact that ligand F5 was selected with recombinant FIV RT, its most potent activity was with the virion-derived enzyme in reactions with a heteropolymeric RNA template.

DISCUSSION

We have used the SELEX method to select potent and specific inhibitors of the FIV RT. The most potent inhibitor, ligand F5, is predicted to form a stem−loop structure with a bulge in the stem. This molecule is a competitive inhibitor of FIV RT with respect to template−primer and a noncompetitive inhibitor with respect to dNTP substrate. Ligand F5 probably blocks accessibility of the active site of RT to the template−primer complex. This ligand was highly specific for the target RT and did not inhibit other RTs tested (AMV, HIV-1, and M-MLV), even at high concentration (1000 nM).

Selections were carried out with recombinant FIV RT, which is a p66 homodimer. Interestingly, ligand F5 was a more potent inhibitor of the FIV RT that was purified from virions, which is a p66/p51 heterodimer. It was also a more potent inhibitor of FIV RT in reactions with an endogenous RNA template than in reactions with homopolymeric templates. Thus, it appears that this ligand is more potent under the more biologically relevant conditions we have examined, and this may be important in its use as an inhibitor of FIV replication. The other ligand (F1a) that was able to inhibit recombinant FIV RT was much less inhibitory to the virion FIV RT.

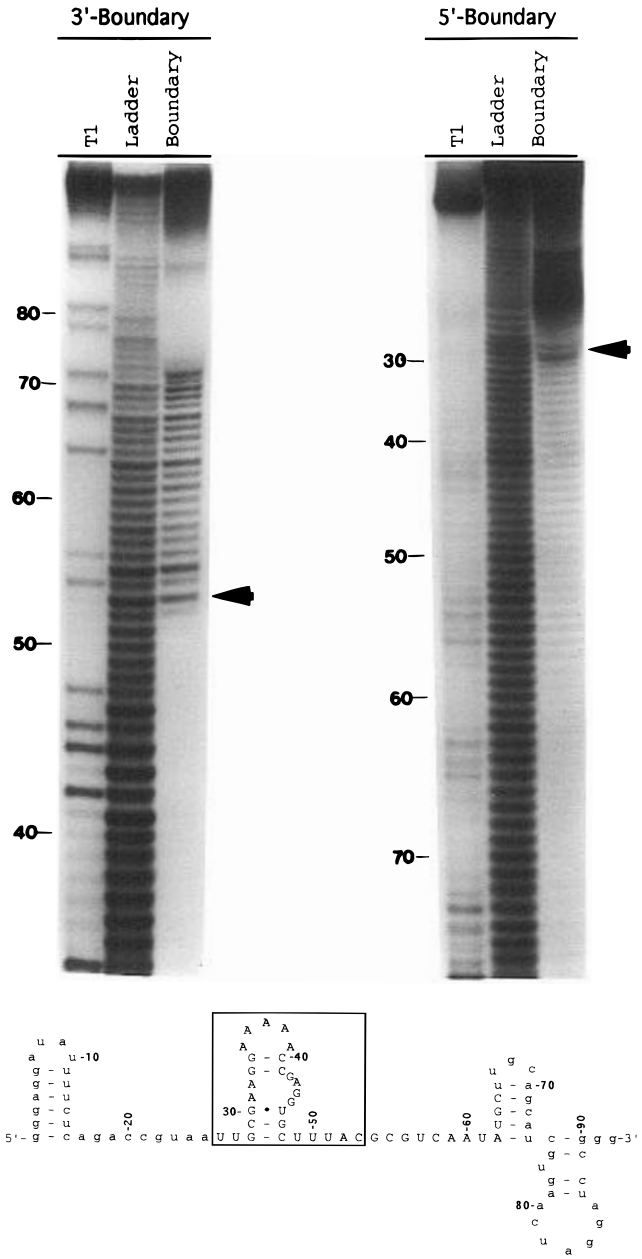

FIGURE 4: Boundary of the binding domain of ligand F5 for FIV RT. (Top) Experiments for determination of the 5′-boundary and the 3′-boundary were as described in Experimental Procedures. Lanes labeled T1 represent RNase T1 digestion of end-labeled RNAs; lanes labeled Ladder depict partial alkaline hydrolysis ladders; lanes labeled Boundary represent analyses of the ability of the partially hydrolyzed RNA fragments to bind to FIV RT on nitrocellulose filters. Arrows indicate the boundaries. (Bottom) Summary of the FIV RT binding domain of ligand F5. The boxed region shows boundaries of the FIV RT binding domain. Capital letters indicate the sequence of F5 for the region that was randomized in the starting pool of RNA. Lowercase letters indicate the fixed sequences. The secondary structure was obtained by a computer RNA folding algorithm.

Ligand F5 was as potent an inhibitor of the RT from an AZT-resistant mutant of FIV as it was against RT from wild-type virus. The enzyme from this mutant has a single amino acid change at position 202 (Glu in wild type to Lys in the mutant) which results in an RT that has a 5−6-fold higher K_i for AZTTP (Remington et al., 1994). On the basis of the crystal structure of HIV-1 RT (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993) and the homology of FIV and HIV RTs (49% identity and 68% similarity of amino acids)

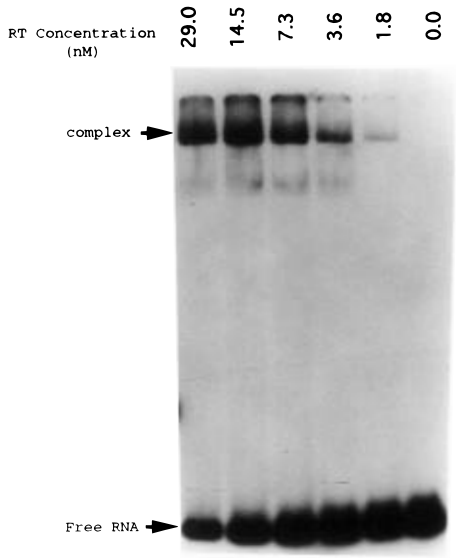

FIGURE 5: Native gel mobility retardation analysis of truncated ligand dF5. Procedures are as described in Figure 1. Arrows indicate positions of RNA bound to the FIV RT dimer (complex) and of free RNA.

Table 1: Inhibition of FIV RT by SELEX Ligands

enzyme[a]	template	variable	K_i[b] (nM)	mode[c]
		(A) Ligand F5		
rRT	poly(rA)−oligo(dT)	dTTP	89 ± 18	NC
rRT	poly(rA)−oligo(dT)	poly(rA)−oligo(dT)	95 ± 7	C
rRT	Endogenous RNA	dTTP	55 ± 5	NC
rRT	Endogenous RNA	Endogenous RNA	45 ± 10	C
vRT	poly(rA)−oligo(dT)	dTTP	37 ± 4	NC
vRT	poly(rA)−oligo(dT)	poly(rA)−oligo(dT)	31 ± 2	C
vRT	endogenous RNA	dTTP	12 ± 1	NC
AZR-RT	poly(rA)−oligo(dT)	dTTP	44 ± 4	NC
AZR-RT	poly(rA)−oligo(dT)	poly(rA)−oligo(dT)	26 ± 7	C
		(B) Ligand F1a		
rRT	poly(rA)−oligo(dT)	dTTP	107 ± 18	NC
rRT	poly(rA)−oligo(dT)	poly(rA)−oligo(dT)	120 ± 7	C
vRT	poly(rA)−oligo(dT)	dTTP	334 ± 4	C

[a] rRT represents recombinant FIV RT, vRT represents virion-wild-type FIV RT, AZR-RT represents virion-AZR-17c RT. [b] Each value represents a mean K_i ± SEM from 6 determinations. [c] NC represents noncompetitive inhibition; C represents competitive inhibition.

(Talbott et al., 1989), we predict that this mutation lies in a helix below the "palm domain" of the RT structure. The fact that this mutation does not alter the sensitivity of the enzyme to ligand F5, despite the large change in charge at position 202, is consistent with our hypothesis that the ligand interacts with the template−primer binding site.

So far, four different viral RTs have been used as targets to isolate high-affinity RNA ligands with K_d values in the nanomolar range. Comparison of *in vitro*-selected RNA molecules against HIV-1 RT (Tuerk et al., 1992), AMV RT, M-MLV RT (Chen & Gold, 1994), and FIV RT showed no obvious conservation of the primary RNA sequences. The only obvious feature these molecules have in common is the presence of two or more consecutive A nucleotides in single-stranded regions (either in the loop or in the bulge) (Figure 7). For instance, the HIV-1-specific ligand has two consecutive As in loop 1 and four consecutive As in loop 2 of a pseudoknot structure. The stacking interaction between stem 1 and stem 2 in the pseudoknot will align these As all on one side of the helix. The RNA ligand F5 for FIV RT contains two consecutive As in the bulge and five consecutive

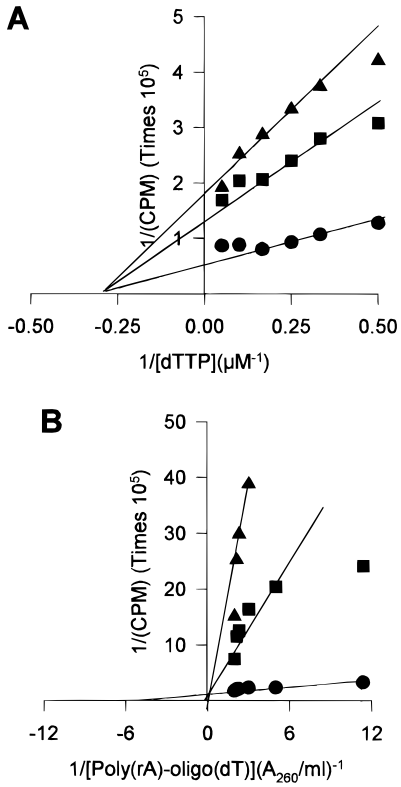

FIGURE 6: Kinetics of inhibition of FIV RT by RNA ligand F5. Lineweaver−Burk plots are from reactions of recombinant FIV RT with variable dTTP (A) and with variable template−primer complex (B). Reactions were run with 200 nM ligand F5 (▲), 50 nM ligand F5 (■), and no ligand F5 (●).

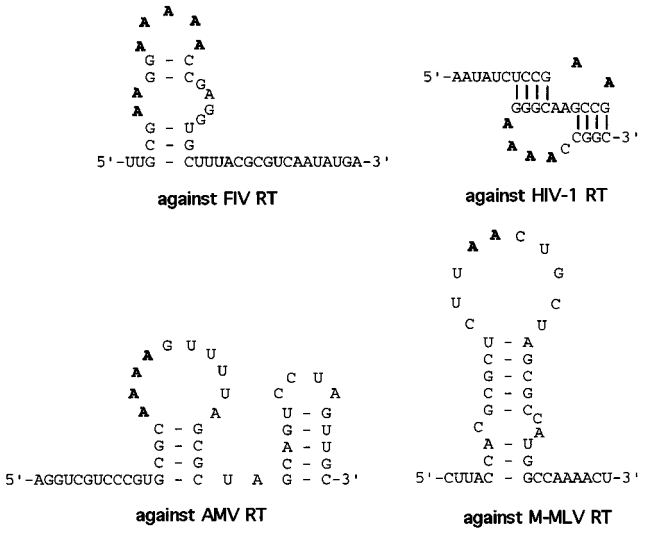

FIGURE 7: Predicted secondary structures of RNA ligands that inhibit HIV-1 RT, AMV RT, M-MLV RT and FIV RT. Bold letters indicate conserved As in the single-stranded region, which are hypothesized to be important for inhibition of RT activity.

As in the loop. There were several other ligands belonging to class I and class II from the FIV selection that do not contain these consecutive As in single-stranded regions; those ligands bind tightly to FIV RT but are poor inhibitors (K_i values greater than 1000 nM). We speculate that the inhibitory RNA ligands fold into tertiary structures that fit into the deep cleft of the nucleic acid binding site in RT and that the stretches of A residues are important for this interaction with the active site. However, the detailed topology of nucleic acid binding sites of different RTs are expected to be different, leading to the diversity of specific ligands obtained with different target RTs. The high specificity of selected RNA ligands to their cognate RTs demonstrates that selected RNA ligands are able to discriminate between evolutionarily closely related proteins.

The successful *in vitro* selection of high-affinity RNA ligands that are potent inhibitors of RT may provide an alternative approach to antiretroviral therapy (Gold, 1995; Gold et al., 1995). However, there are numerous questions pertaining to delivery and intracellular activity that must be addressed if nucleic acids are to be considered as drug candidates. A major problem with current drugs used for AIDS therapy is the rapid emergence of drug-resistant mutants (Richmann, 1993). We do not know whether virus mutants resistant to the SELEX-derived ligands will arise. However, the FIV system has proven particularly versatile for studies of resistance to AIDS therapy (Remington et al., 1991, 1994; Gobert et al., 1994; North & LaCasse, 1995), and this should facilitate studies on the emergence of mutants resistant to these ligands. If FIV mutants resistant to the SELEX ligand are obtained, it will be important to determine whether we can re-evolve the ligand to a form that inhibits the mutant RT. As an initial approach we have expressed ligand F5 in feline cells and demonstrated that FIV replication is inhibited by greater than 99% in some of these cells (manuscript in preparation). This system will provide an excellent opportunity to address resistance issues. FIV also provides an attractive animal model for *in vivo* experimentation to test therapeutic strategies employing these inhibitors (Pedersen, 1993; Barlough et al., 1993).

ACKNOWLEDGMENT

We thank Dr. Steve Ringquist, Dr. Kirk Jensen, and Dr. Mike Lochrie for critical review of the manuscript and for helpful scientific discussion and Joan Strange of the Murdock Molecular Biology Facility, University of Montana, for excellent technical assistance. We also thank the W. M. Keck Foundation for their generous support of RNA science on the Boulder campus.

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BI9600106