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# Endogenous expression of a high-affinity pseudoknot RNA aptamer suppresses replication of HIV-1

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## ABSTRACT

**Aptamers, small oligonucleotides derived from an *in vitro* evolution process called SELEX, are promising therapeutic and diagnostic agents. Although very effective *in vitro*, only a few examples are available showing their potential *in vivo*. We have analyzed the effect of a well characterized pseudoknot RNA aptamer selected for tight binding to human immunodeficiency virus (HIV) type 1 reverse transcriptase on HIV replication. Transient intracellular expression of a chimeric RNA consisting of the human initiator $tRNA^{Met}$ ($tRNA^{Meti}$)/aptamer sequence in human 293T cells showed inhibition of HIV particle release by >75% when the cells were co-transfected with proviral HIV-1 DNA. Subsequent virus production of human T-lymphoid C8166 cells, infected with viral particles derived from co-transfected 293T cells, was again reduced by >75% as compared with the control. As the observed effects are additive, in this model for virus spread, the total reduction of HIV particle formation by transient intracellular expression of the pseudoknot RNA aptamer amounts to >95%. Low-dose HIV infection of human T cells stably expressing the aptamer did not show any virus replication over a period of 35 days. This is the first example of an RNA aptamer selected against a viral enzyme target to show powerful antiviral activity in HIV-1-permissive human T-lymphoid cell lines.**

## INTRODUCTION

About a decade ago, a method for *in vitro* selection of combinatorial oligonucleotide libraries was described (1–3). This technique, generally termed 'Systematic Evolution of Ligands by EXponential enrichment' (SELEX), has in recent years led to an explosion in the discovery of potential nucleic acid ligands (aptamers) for target proteins. Important properties of aptamers are tight binding to proteins ($K_D$ range from low picomolar to nanomolar) combined with high specificity and virtually no immunogenity. Often, aptamers block the enzymatic activity of their target proteins. Consequently, several concepts of using these molecules as therapeutics and/or diagnostics have been proposed (for a review see 4–6). However, despite the increasing number of aptamers described, there are still few examples of their effectiveness in living cells and *in vivo*. Obviously, turning an *in vitro* selected oligonucleotide into a therapeutic agent is no easy task (7). Nevertheless, there are a few promising examples of aptamers selected against extracellular protein targets. One of these aptamers is currently being assessed in clinical trials (8).

However, a far greater number of protein targets are localized within the cell resulting in the need for different strategies of drug delivery (for example 9–11, for a review see 12). One clinically relevant example of interesting intracellular targets is viral proteins. A major challenge in the treatment of AIDS is the fast emergence of resistant human immunodeficiency virus (HIV) mutants (for a review see 13). A second problem is the severe side effects encountered by AIDS patients treated with the available anti-HIV drugs. Theoretically, aptamers are promising candidates to overcome the limitations of current HIV therapy. Because of their high specificity towards the intended target there should be fewer side effects. Consequently, the effect of anti-HIV-1 Rev and anti-HIV-1 Tat decoys/aptamers have been investigated in a cellular environment (14,15). Furthermore, several groups could show inhibition of viral replication in cell culture by anti-HIV-1 Rev decoys/aptamers (16–20). Because of the pivotal role of the reverse transcriptase (RT) in the retroviral life cycle (for a review see 21) it represents an even more attractive target for developing such innovative antiviral strategies. Being an integral component of the released viruses, there is a good chance that an aptamer against this viral enzyme would also be packed into the particles, thus leading to non-infectious viruses. Thus, not only cells expressing the aptamer would be protected.

Applying the SELEX procedure a pool of about 20 RNA aptamers selected against HIV-1 RT could be identified (22).

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The analysis of the isolated aptamers revealed a consensus sequence that resulted in the formation of an RNA pseudoknot (23,24). Recently, we determined the X-ray structure of one of these aptamers complexed with its target (25). The pseudoknot RNA partially overlaps with the binding site for the natural substrate consistent with the aptamer acting as potent competitive inhibitor. Considering the aptamer covers a much larger area of the target protein (25) than the small drugs currently used in antiviral therapy (for a review see 26), the likelihood of viral escape via single point mutations should be drastically reduced (27). Further, thorough biochemical characterization of this protein/RNA interaction *in vitro* revealed an extraordinarily tight binding with a $K_D$ of ~25 pM for the complex (28). This is at least 100 times lower than for the natural substrate. Additionally, the inhibitory aptamer is highly specific for HIV-1 RT, with the closely related HIV-2 enzyme showing a binding affinity close to four orders of magnitude lower.

To explore the effect of this RNA aptamer on HIV replication, we transiently expressed the molecule in eukaryotic cells as a polymerase III-driven chimeric gene consisting of the human initiator $tRNA^{Met}$ ($tRNA^{Meti}$) sequence and the 33 nt pseudoknot sequence. Transient co-transfection of 293T cells with the pseudoknot expressing vector and a proviral HIV-1 DNA resulted in a >75% reduction of viral particle release from these cells. Furthermore, such viral particles showed a strongly reduced intrinsic RT activity, indicating package of the aptamer into the virions. Subsequent infection of T-lymphoid cells with viruses produced in the presence of chimeric $tRNA^{Meti}$/pseudoknot RNA showed a >75% decline in viral replication as compared with the control. Moreover, a T-lymphoid cell line stably transfected with the aptamer construct did not show any virus replication after low-dose HIV-1 infection. This demonstrates that the aptamer functions as a powerful inhibitor of HIV replication in cell tissue culture (proof of principle).

## MATERIALS AND METHODS

### *In vitro* RNA preparation

The 33 nt pseudoknot RNA aptamer (sequence: 5′-GGGAGAUUCCGUUUUCAGUCGGGAAAAACUGAA) and the $tRNA^{Meti}$/pseudoknot RNA chimera (see Fig. 1B) were prepared in a standard 10 ml T7 reaction mixture for *in vitro* transcription and purified by gel electrophoresis as described previously (25,29). The RNAs were refolded at a concentration of 200–300 µM at 65°C for 5 min followed by slow cooling to room temperature in 20 mM cacodylate buffer pH 6.5, 25 mM NaCl and 5 mM $MgCl_2$. 5′-End-labeling of the RNA with T4 polynucleotide kinase (New England Biolabs) was performed as described previously (30). Dephosphorylation of the *in vitro* transcribed RNA prior to end-labeling was carried out according to standard procedures (31).

### RNA secondary structure prediction

The secondary structures of the chimeric RNA transcripts were predicted by using the program PKNOTS (32). Secondary structure models were drawn using the program RnaViz (33).

### Protein purification

Recombinant heterodimeric wild type HIV-1 and HIV-2 RT were expressed in *Escherichia coli* and purified as described before (34,35). Enzyme concentrations were routinely determined using an extinction coefficient at 280 nm of 260 450 $M^{-1}$ $cm^{-1}$ (HIV-1 RT) and 238 150 $M^{-1}$ $cm^{-1}$ (HIV-2 RT). The purified RTs were free of nuclease contamination.

### *In vitro* binding assay

Protein and 5′-$^{32}$P-labeled RNA were mixed in standard buffer (50 mM Tris–HCl pH 8.0, 50 mM KCl, 1 mM DTT and 10 mM $MgCl_2$) and incubated at 25°C for 10 min. An aliquot of this mixture was filtered under suction through a prewet (standard buffer) nitro-cellulose filter (Schleicher & Schuell BA85) and rinsed with 4 ml of standard buffer. Radioactivity retained on the filters was measured by scintillation counting.

### Polymerase activity determination

RNA-dependent DNA polymerase activity on poly(rA)/oligo$(dT)_{12–18}$ substrates was measured by a standard assay described previously (36,37) with 2.8 nM of RT for 10 min at 37°C in a buffer containing 50 mM Tris–HCl pH 8.0, 80 mM KCl, 5 mM DTT, 6 mM $MgCl_2$ and 0.05% (v/v) Triton X-100.

### Cell lines and cell tissue culture

HS 68 (human newborn foreskin fibroblast) and 293T (human embryonic kidney cells) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Karlsruhe, Germany), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% $CO_2$. Jurkat and C8166 cells (both human CD4 positive T-lymphoid cells) were cultured under the same conditions but with RPMI medium instead of DMEM. For transfection and infection experiments the cells were seeded in 48-well culture plates at a density of $10^5$ cells/well.

### Northern blot analysis

Nuclear and cytoplasmic RNAs were isolated from HS 68, 293T and Jurkat cell lines using the Trizol reagent (Life Technologies) according to the manufacturers protocol with a few modifications. Briefly, $5 \times 10^6$–$10^7$ cells were transfected with different double-copy (vector) (DC)T5T constructs using either the LipofectAMINE PLUS™ reagent (Life Technologies) or the calcium phosphate transfection procedure (38). Forty-eight hours post-transfection, cells were incubated in a buffer containing 50 mM Tris–HCl pH 8.0, 140 mM NaCl, 1 mM DTT, 1.5 mM $MgCl_2$ and 0.5 % (v/v) NP-40 for 5 min on ice. Cytoplasmic RNA was separated from nuclear RNA by centrifugation (2 min at 300 *g* and 4°C). Supernatant containing the cytoplasmic fraction and pellets containing the nuclear fraction were then incubated with the Trizol reagent and RNA purification was performed as described by the manufacturer. The amount of RNA in each sample was determined by $OD_{260}$ measurements. RNA samples were subjected to electrophoresis in formaldehyde agarose gels (1.2%), transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) and hybridized with either a $^{32}$P-labeled pseudoknot, $tRNA^{Lys3}$ or U6 snRNA probe. After extensive washing of the membrane, data were

quantified by phosphorimaging (Fuji FLA 3000 radioluminograph scanner).

### Transfection and infection experiments

Human 293T kidney cells were transiently co-transfected with proviral HIV-1 DNA [pNL4-3 (39), 150 ng/well], and different DCT5T constructs (250 ng/well): the parental vector (DCT5T), an antisense RNA vector (DCT5T-αrev) and the $tRNA^{Meti}$-pseudoknot vector (DCT5T-pseudoknot). Transfection was performed according to the calcium phosphate procedure (38). Transfection efficiency was determined using the pEGFP-C1 vector (Clontech Laboratories, Inc., Palo Alto, CA). Supernatants were collected 36 h post-transfection and virus production was measured by a commercial p24 ELISA (Innotest™, Innogenetics, Heiden, Germany). In a subsequent step, human CD4-positive T-lymphoid cells (C8166) were infected with these supernatants. Virus concentration, determined via p24 ELISA, was held constant in all infection experiments. Forty-eight hours post-infection, virus production was measured by ELISA.

For the generation of stably transfected clones, Jurkat cells were electroporated in the presence of different DCT5T constructs and grown in the presence of G418 for selection as described previously (40). Stable clones were infected with HIV-1 particle containing cell-free supernatants of transiently transfected 293T cells as described above. At given time points virus production was determined via p24 ELISA.

## RESULTS

### Vector construction

The vector DCT5T originates from a Moloney murine leukemia virus-based, polylinker-modified vector designated N2A (41). This vector contains a Δ3′-5 mutant $tRNA^{Meti}$ gene (42) and a polymerase III termination signal in the U3 region of the 3′ long terminal repeat (43). The deletion within the tRNA gene (18 bp at the 3′-end of the gene and 11 bp of the mature tRNA product) abolishes processing of the primary tRNA transcript. Insertion of a foreign sequence between the Δ3′-5 tRNA gene and a transcription signal generates a chimeric transcript in which the foreign sequence is fused to the 3′-end of the tRNA (42). An oligonucleotide containing the pseudoknot sequence was cloned into the unique *Bam*HI/*Mlu*I restriction sites downstream of the tRNA gene and upstream of the polymerase III termination signal, generating the retroviral vector DCT5T-pseudoknot (Fig. 1A). Figure 1B shows a secondary structure prediction of the 117 nt chimeric RNA transcript using the program PKNOTS (32). According to this prediction the pseudoknot motif folds as expected. As described in the next section, these computer-based results were confirmed by biochemical analysis.

### Functional evidence for proper folding of chimeric RNA *in vitro*

As it cannot be predicted for certain whether or not the pseudoknot motif folds properly in the sequence context of the tRNA based on computer models, we performed *in vitro* binding studies with this RNA chimera. For this purpose, the corresponding $tRNA^{Meti}$/pseudoknot transcription unit (see Fig. 1A) was cloned into the T7 RNA transcription vector

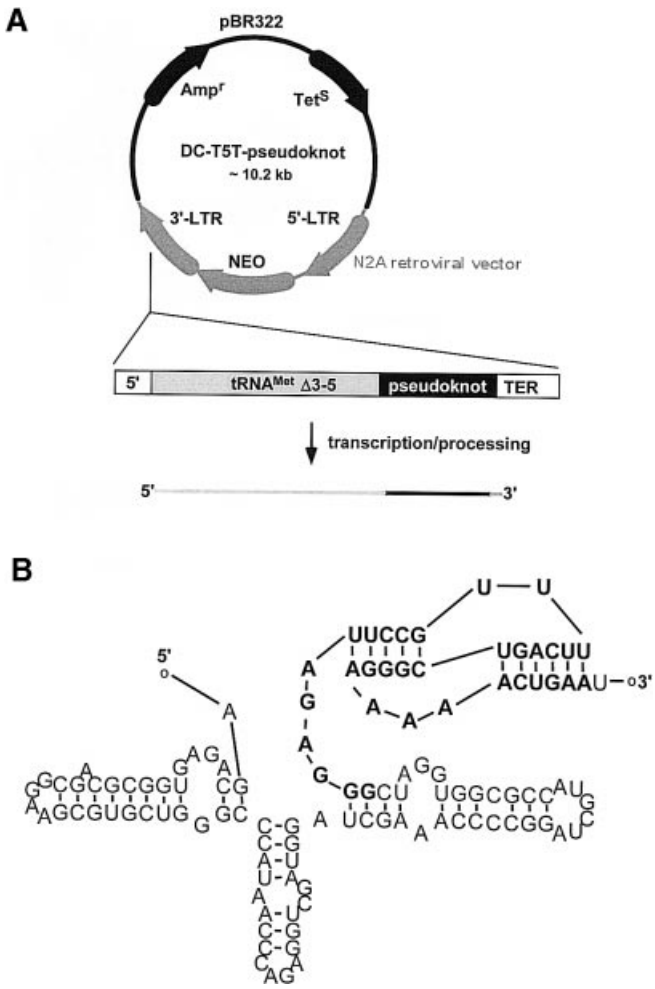


**Figure 1.** Vector construction. (**A**) The original retroviral vector DCN2A contains a Δ3′-5 mutant $tRNA^{Meti}$ gene and a polymerase III termination signal (TER) in the U3 region of the 3′ long terminal repeat. An oligonucleotide containing the pseudoknot sequence was cloned downstream of the tRNA gene and upstream of the polymerase III termination signal generating the DCT5T-pseudoknot vector. (**B**) Secondary structure prediction of the pseudoknot motif within the chimeric tRNA-pseudoknot RNA transcript. The prediction was calculated using the pseudoknot folding software PKNOTS. The pseudoknot part is shown in bold letters. The additional base at the 3′-end of the transcript arises from the termination signal.

pBluescript SK (Stratagene, La Jolla, CA) via the *Kpn*I/*Xba*I restriction sites. Transcription and PAGE purification of the chimeric RNA (see Fig. 1B) was performed as described in Materials and Methods. Because of technical reasons the *in vitro* transcribed RNA is 137 nt long as compared with the *in vivo* transcribed RNA with 117 nt. The additional bases are located at the 5′-end of the transcript and originate from the transcription vector pBluescript SK. Figure 2 shows a typical filter binding experiment using radiolabeled RNA. As compared with the aptamer alone the $tRNA^{Meti}$/aptamer construct shows a slight reduction in binding affinity for HIV-1 RT dropping from 37 to 86 pM. Similar results were obtained applying fluorescence measurements as described recently by Kensch *et al.* (28) to determine the binding affinity (data not shown). Binding affinities of a chimera consisting of $tRNA^{Meti}$ and the complementary pseudoknot sequence could not be

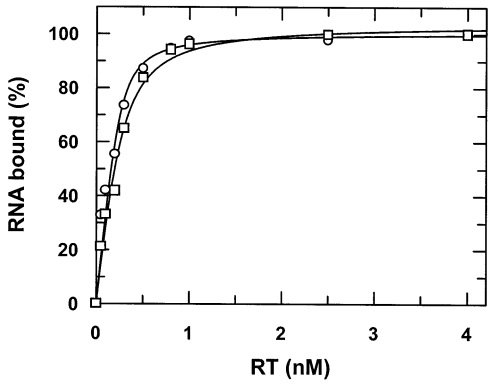


**Figure 2.** Binding studies. Filter binding assay of radiolabeled pseudoknot RNA and tRNA-pseudoknot RNA with RT. 5′-$^{32}$P-labeled RNAs (200 pM) were titrated with increasing amounts of RT. The curves show best fits to a quadratic equation yielding a $K_D$ of 37 (±14) and 86 (±17) pM for the pseudoknot (circles) and the tRNA-pseudoknot (squares), respectively.

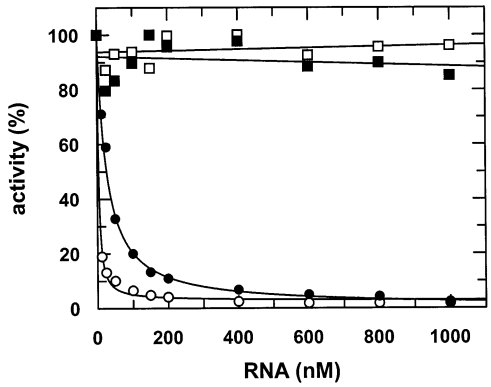


**Figure 3.** Inhibition of RT activity *in vitro.* RNA-dependent DNA polymerase activity on poly(rA)/oligo(dT)$_{12–18}$ substrates was measured for 10 min at 37°C. 2.88 nM HIV-1 (circles) or HIV-2 (squares). RT was preincubated with increasing amounts of pseudoknot (open symbols) or tRNA-pseudoknot RNA (filled symbols).

determined accurately. However, the binding is at least three orders of magnitude weaker than for a construct containing the pseudoknot sequence (data not shown). These results clearly indicate proper folding of the pseudoknot motif within the RNA chimera yielding a highly specific interaction with the target protein.

### Effect of the tRNA/pseudoknot chimera on RT polymerase activity

To test the inhibition of the enzymatic activity of the target protein by the chimeric RNA we performed a standard RT assay using poly(rA)/oligo(dT) as substrate. The reactions were started with preincubated RT/RNA complexes. Figure 3 shows strong inhibition of the HIV-1 RT polymerase activity by the pseudoknot RNA alone as compared with the closely related enzyme of HIV-2 (28). The chimeric transcript is somewhat less efficient in blocking the polymerase activity of HIV-1 RT and as found for the aptamer alone shows no effect on HIV-2 RT. Inhibition constants for the pseudoknot and the tRNA-pseudoknot were determined by fitting the experimental data to a hyperbolic equation yielding $K_i$ values (defined as

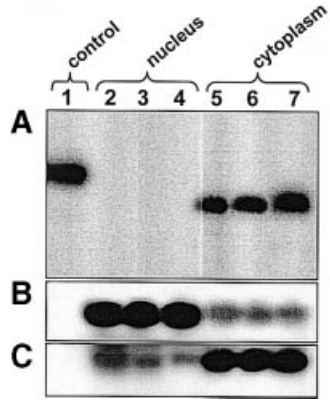


**Figure 4.** Subcellular distribution of the tRNA-pseudoknot chimera. Total nuclear and cytoplasmic RNAs were prepared from $10^7$ cells (HS 68, 293T and Jurkat) transfected with the DCT5T-pseudoknot vector. Each lane was loaded with the same amount of RNA (20 µg) as determined by $OD_{260}$ measurements. (**A**) Pseudoknot RNA, (**B**) U6snRNA and (**C**) tRNA$^{Lys3}$. Lane 1, control (137 nt RNA chimera prepared by *in vitro* transcription). Lanes 2–4, nuclear RNA. Lanes 5–7, cytoplasmic RNA from transfected HS 68 (2,5), 293T (3,6) and Jurkat (4,7) cells.

RNA concentration that inhibited polymerase activity by 50%) for HIV-1 RT of 2.8 (±0.24) and 25.5 (±0.03) nM, respectively. No inhibition of polymerase activity was observed with a chimera consisting of tRNA$^{Meti}$ alone or the complementary pseudoknot sequence at concentrations up to 1 µM (data not shown).

### Intracellular distribution of endogenously expressed aptamer

The polymerase III-driven chimeric tRNA gene for *in vivo* transcription of the aptamer was primarily chosen for two reasons: transcription efficacy and cytoplasmic distribution of the transcript. The latter being a perquisite for effective inhibition of the target protein that is localized in the cytoplasm. In order to investigate the intracellular localization of the aptamer, we performed northern blot analysis. As shown in Figure 4 the chimeric RNA is essentially found in the cytoplasmic fraction when transiently expressed in different human cell lines. In order to exclude leakage form one compartment to the other during sample preparation, probes against tRNA$^{Lys3}$ and U6 snRNA were chosen as cytoplasmic and nuclear controls, respectively.

### Transient suppression of the production of infective HIV-1 particles by pol III-driven pseudoknot aptamers

Human 293T kidney cells were transiently co-transfected with infectious proviral HIV DNA and different DCT5T constructs: the parental vector (DCT5T), an antisense RNA vector (DCT5T-αrev) and the tRNA$^{Meti}$-pseudoknot vector (DCT5T-pseudoknot). The transfection efficacy was monitored by expression of a plasmid-encoded green fluorescence protein and was found to be ~80% (data not shown). The amount of virus produced and released by these cells was measured 36 h post-transfection via a p24 antigen ELISA (Fig. 5A). Each experiment was performed in triplicate and repeated twice. The parental vector (DCT5T) served as a negative control and the amount of virus produced in the presence of this construct was set to 100%. As a positive control for inhibition, the well characterized antisense

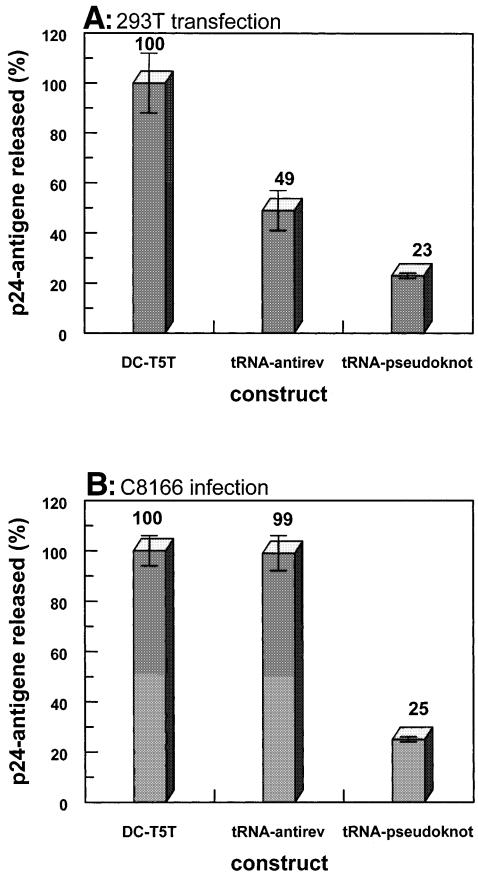


**Figure 5.** Transient transfection and infection experiments. Each experiment was performed in triplicate and repeated twice. The error bars indicate the standard deviation error from the mean. (**A**) 293T cells were co-transfected with a HIV-1 proviral DNA and different retroviral constructs: the parental vector (DCT5T), an antisense RNA vector (DCT5T-αrev) and the $tRNA^{Meti}$-pseudoknot vector (DCT5T-pseudoknot). Virus production was measured 36 h post-transfection via ELISA analysis. (**B**) Infection of human T-lymphoid cell line (C8166) with viruses produced by the 293T cell line (see Fig. 7A). In each experiment the same amount of virus was applied. HIV-1 replication was measured 48 h post-infection by ELISA.

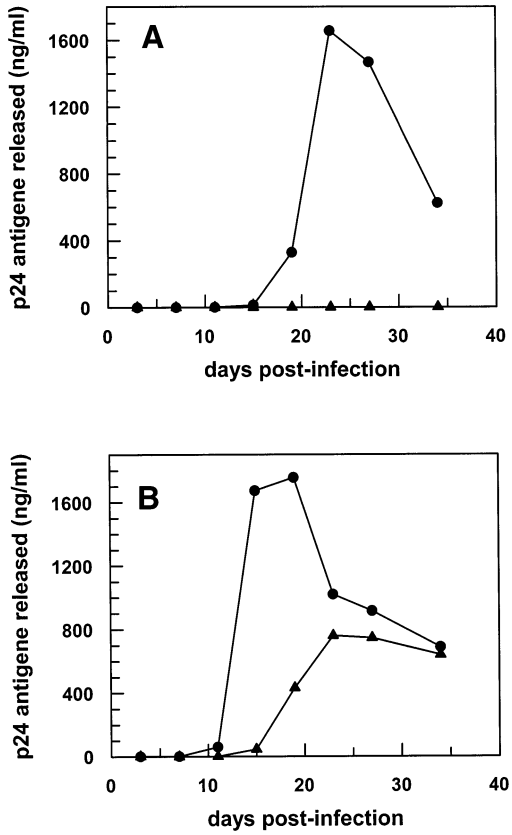


**Figure 6.** Infection of stably transfected Jurkat cells. Jurkat cell clones either stably transfected with the parental vector DCT5T (circles) or the DCT5T-pseudoknot vector (triangles) were infected with different amounts HIV-1 particles. (**A**) 190 pg p24 per $10^5$ cells and (**B**) 1700 pg p24 per $10^5$ cells. At the time points given, viral replication was measured by p24 ELISA.

construct DCT5T-αrev was used (44). This anti-rev RNA has been shown previously to be a potent inhibitor of HIV-1 replication in transient quantitative co-transfection assays as well as in the long-term experiments after retroviral transduction of HIV-1-susceptible cells and challenge with HIV-1 (40).

Thus, this DCT5T-αrev construct was used as internal positive control to calibrate the system. Consistent with earlier measurements, the anti-rev RNA showed an inhibition of virus production under the specific experimental conditions used here by ~50%. Upon co-transfection with the DCT5T-pseudoknot vector virus production was reduced by ~77%. Analyzing the RT activity of viruses released by the transient transfected 293T cells, we found ~70% reduction after normalizing for p24 in case of the aptamer co-expressed (data not shown).

### Reduced infectivity of HIV-1 particles generated in the presence of the pseudoknot aptamer

To measure the influence of the RT-binding aptamer on the specific infectivity of HIV-1 particles, human T-lymphoid cells C8166 were infected with constant amounts of HIV produced by co-transfection of 293T cells with proviral HIV DNA and different constructs of the DCT5T vector (see Fig. 5A and previous section). The new cycle of virus replication in C8166 cells was monitored 48 h post-infection via a p24 antigen ELISA (Fig. 5B). When standardized to the DCT5T control, no inhibition of viral replication was observed in the case of the DCT5T-αrev construct. However, viruses assembled in the presence of the $tRNA^{Meti}$/pseudoknot RNA showed a substantial reduction (~75%) of their ability to infect and/or replicate in newly infected T-lymphoid cells.

### Complete inhibition of viral replication in stably transfected Jurkat cells after low-dose HIV infection

In order to overcome the intrinsic problems of transient transfection assays, we generated stably transfected T-lymphoid cells. Figure 6 shows HIV-1 infection of Jurkat cells either stably transfected with the parental vector DCT5T or the DCT5T-pseudoknot vector. Low-dose HIV infection (190 pg p24/$10^5$ cells) of Jurkat cells stably expressing the aptamer did not show any virus replication over a period of 35 days (Fig. 6A). Increasing the initial virus titer by 10-fold, HIV-1 particle production can be observed in the presence of the $tRNA^{Meti}$/pseudoknot RNA chimera. However, there is a

significant reduction of virus production and a delay of virus release of ~1 week as compared with the control.

## DISCUSSION

In order to investigate the effect of an inhibitory RNA aptamer, selected for tight binding to HIV-1 RT, on viral replication *in vivo*, we constructed a retroviral vector for intracellular expression of this aptamer. The pseudoknot RNA sequence was cloned into the DCT5T vector downstream of a polymerase III-driven Δ3′-5 mutant $tRNA^{Meti}$ gene and upstream of a polymerase termination site generating a $tRNA^{Meti}$/pseudoknot RNA chimera.

Northern blot analysis of 293T cells transiently transfected with this construct revealed the transcript to be predominantly localized in the cytoplasm. This is in agreement with findings reported by others using such a vector for intracellular expression of RNA molecules (45,46). However, it should also be mentioned here that other studies reported opposite results (17,47). The reason for this obvious discrepancy remains unclear. It could be speculated that the overall folding of the transcript influences the interaction with export carriers and thus determines whether or not the tRNA chimera is actually transported into the cytoplasm.

First, we confirmed that the aptamer within the chimeric RNA still specifically binds the target protein. This was investigated by binding studies with *in vitro* transcribed RNA showing similar affinities for HIV-1 RT compared with the aptamer alone indicating that the pseudoknot motif is properly folded in the sequence context of the $tRNA^{Meti}$ and the extra RNA part at the 5′-end is not interfering with binding to its target. The somewhat lower binding affinity observed for the chimera could be in part explained by the additional base at the 3′-end of the pseudoknot sequence generated *in vivo* due to the polymerase termination signal. According to the X-ray structure there should not be sufficient space for an additional nucleotide at that position. The binding studies were further supported by inhibition experiments. Here we tested the effects of the chimera on the polymerase activity of RT. As already shown (28) for the pseudoknot alone, the chimera is highly specific for the HIV-1 enzyme compared with HIV-2. This clearly indicates that the strong inhibition of the polymerase activity is due to specific interactions with the pseudoknot part of the chimera. The reduced $K_i$ value in the case of the $tRNA^{Meti}$/pseudoknot RNA by a factor of about 9 as compared with the pseudoknot alone is most likely caused by the difference in binding affinities as minor changes here have a more pronounced effect in this kind of assay. Here the poly(rA)/oligo$(dT)_{12–18}$ substrate is in large excess over inhibitor RNA. Therefore, inhibition is due to enzyme trapped by the aptamer as the reaction was started by adding RT/aptamer complexes. Differences of a factor of 2 in the $k_{off}$ values for the enzyme/inhibitor complex as indicated by the $K_D$ values could easily cause a difference of 5–10 for the $K_i$ values in the inhibition assay.

To analyze effects of the aptamer in HIV-infected cells we first investigated the production/release of HIV in cells co-transfected with the vector coding for the aptamer chimera and infectious proviral HIV-1 DNA. In this situation no reverse transcription takes place. However, the production of viral particles was reduced by >75% and we cannot be certain at which stage of the replication cycle this aptamer interferes. It is conceivable that the RNA disturbs the viral packaging by binding to the RT within the premature Gag-Pol-polyprotein or the aptamer might even affect the maturation of the gag-pol precursor. There are examples where mutations within the RT gene caused similar effects (48,49). This was attributed to problems during packaging events. A second scenario could be that the binding of the aptamer to the Gag-Pol-polyprotein interferes with the selective packaging of the primer $tRNA^{Lys3}$ into viral particles (50).

In a second set of experiments, we infected human T-lymphoid cells with viral particles generated in the presence of the aptamer. In this case reverse transcription of the viral RNA genome has to take place before new viral particles can be released. The replication capability of these viruses was reduced by ~75%. This is most likely due to an aptamer bound to RT and packed into the virus causing inhibition of reverse transcription during the next replication cycle. This argument is strongly supported by the finding that HIV particles produced by 293T cells in the presence of the aptamer show dramatically reduced polymerase activity. If normalized for p24, those particles show ~70% lower RT activity as compared with particles produced in the presence of the parental vector (data not shown). This is in perfect agreement with the 75% inhibition observed for the infection experiment. However, currently we cannot entirely rule out that these viruses are also impaired in infecting T-lymphoid cells.

The described effects are additive causing an inhibition of the viral replication rate by >95% in total. It should be noted here that the transfections were transient. This could be important in respect to the maximal inhibitory potential of the aptamer since the transfection efficiency was determined to be in the range of ~80% in the chosen set up. Therefore, it is conceivable that in the first round where cells were co-transfected with two plasmids they might have taken up only one. If this happens to be the proviral DNA no inhibition would be observed. Consequently, we believe the detected rate of inhibition in this kind of experimental set up to be at the lower limit.

In order to study the effect of the aptamer in a cell culture system closer to the *in vivo* situation, we performed infection experiments with stably transfected T-lymphoid cells. As already expected from the transient transfections, here we observed complete inhibition of viral replication in the presence of the aptamer after low-dose HIV-1 infection. As anticipated, increasing the tissue culture infectious dose $(TCID)_{50}$ above a certain threshold level, the amount of aptamer produced by the cells is not sufficient anymore to fully block viral replication. Still, the number of particles released is significantly lower and delayed by ~1 week as compared with control. Further fine-tuning of the experimental system should enable us to gain insight into the delicate balance between full inhibition versus viral breakthrough.

So far we could not observe any toxic effects caused by the RNA. Using a carrier peptide system to transport *in vitro* transcribed pseudoknot RNA into human fibroblasts no toxic effects were observed at concentration up to 100 μM (data not shown; T. Restle, manuscript in preparation).

Aptamers are fascinating new reagents to be used as diagnostic tools as well as therapeutics to battle a variety of diseases. However, the main obstacle remains their delivery.

This is especially problematic when the target is localized inside the cell. Here we show for the first time that an RNA aptamer selected for tight binding to a viral enzyme is capable of blocking HIV replication. Consequently, we conclude that the application of this aptamer by gene transfer techniques represents a very promising and innovative approach to treat infections with HIV-1. Comparing our results to results obtained by others using anti-HIV-1 Rev aptamers (16–20) is difficult due to the differences in the methodologies applied. It remains to be seen which approach turns out to be more effective. In terms of mechanistic aspects, targeting a viral enzyme could have the advantage of the aptamer being packed into the viral particles and thus rendering them inactive, i.e. decreasing the apparent infectivity.

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