Enhancement of anti-HIV-1 Ribozyme Activities by Rev Binding and Multimerization

Yuksel Yildiz

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Enhancement of anti-HIV-1 Ribozyme Activities
by Rev Binding and Multimerization

By

Yuksel Yildiz

A Dissertation submitted in partial satisfaction of
the requirements for the degree of Doctor of Philosophy in Physiology

March 2002
Each person whose signature appears below certifies that this dissertation, in their opinion, is adequate in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HIV NL4-3</td>
<td>an HIV-1 isolate or strain</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>ODNs</td>
<td>oligodeoxyribonucleotide</td>
</tr>
<tr>
<td>AS</td>
<td>antisense</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>env</td>
<td>envelop RNA of HIV genome</td>
</tr>
<tr>
<td>Rev</td>
<td>HIV-1 Rev protein</td>
</tr>
<tr>
<td>Tat</td>
<td>HIV-1 trans-activator protein</td>
</tr>
<tr>
<td>gag</td>
<td>group associated antigen</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>Rz and Rbz</td>
<td>Ribozyme</td>
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<tr>
<td>RBE</td>
<td>Rev binding element</td>
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<tr>
<td>RRE</td>
<td>Rev response element</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>pol III</td>
<td>RNA polymerase III</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>snRNA</td>
<td>small nuclear RNA</td>
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<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HDV</td>
<td>hepatitis delta virus</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>ARM</td>
<td>arginine rich motif</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export sequence or signal</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence or signal</td>
</tr>
<tr>
<td>AD</td>
<td>activation domain</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>wt and mt</td>
<td>wild type and mutant</td>
</tr>
<tr>
<td>FM</td>
<td>fusion molecule</td>
</tr>
<tr>
<td>wtFM</td>
<td>wild type ribozyme-RBE fusion molecule</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA buffer</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
</tbody>
</table>
ABSTRACT

Enhancement of anti-HIV-1 Ribozyme Activities by Rev Binding and Multimerization

By

Yuksel Yildiz

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, March 2002
Dr. John J.Rossi, Chairperson

To effectively apply hammerhead ribozymes as therapeutic agents it is necessary to co-localize them with the desired target. Human immunodeficiency virus type1 (HIV-1) infectivity is dependent on env gene expression. HIV-1 Rev protein binds to a higher ordered RNA structure within the env transcript termed the Rev Binding Element (RBE). In anti-HIV gene therapy employing ribozymes to increase the co-localization of anti-HIV ribozymes with target HIV mRNAs, it has been proposed that when the native HIV-1 RBE is appended to a ribozyme as a decoy molecule, simultaneous binding of Rev monomers to the RBE sequences in both HIV-1 genome and in the ribozyme-RBE fusion molecule and their subsequent multimerization may serve to increase the co-localization of ribozyme with HIV-1 mRNA. In this respect, Yamada et al. (1996) have combined the native HIV-1 RBE sequence with a hairpin ribozyme targeted to the U5 region of HIV-1. Their data have demonstrated a substantial enhancement of antiviral activity in vivo when both RBE and ribozyme were present in comparison to either one alone. But their studies never demonstrated co-localization in vitro. In this study we have tested the concept of Rev mediated co-localization in vitro. First of all, we have detected the most accessible sites for hammerhead ribozymes targeting a region of the HIV-1 env gene encoding
gp120 and gp41 proteins using antisense & RNAsesH mapping in cell extracts prepared from the HIV-1 infected CEM cells. We have next designed anti-env hammerhead ribozymes against the best sites and fused them with the native HIV-1 RBE sequences. Using Rev binding and gel shift retardation assays we have tested whether or not RNP complexes which include Rev, the RBE and the HIV-1 mRNA are formed as a result of Rev multimerization. Our results here demonstrate simultaneous binding of Rev monomers to the RBE sequences in both HIV-1 genome and in the fusion molecule and their subsequent multimerization can co-localize ribozyme and target RNAs.
I. INTRODUCTION

A. Anti-HIV Gene Therapy

Anti HIV-gene therapy is based on the idea that transfer of therapeutic genes into HIV-infected target cells will render them resistant to HIV replication. Re-infusion of the protected cells to the patient may limit virus spread and delay disease progression. While current studies involve gene transfer into mature CD4+ T cells, efforts are underway to deliver antiviral genes to pluripotent hematopoietic stem cells to ensure a renewable supply of HIV-protected cells for the life of the patient (Bridges and Sarver, 1995). To date, various anti-HIV-1 gene therapies have been developed and shown to inhibit HIV replication in cell cultures. These include (i) RNA-targeted antivirals, such as Antisense (DNA and RNA), Ribozymes and RNA Decoys, (ii) transdominant incomplete HIV proteins that compete or interfere with viral proteins such as Rev, Tat, Gag and Env, and (iii) inducible toxins that kill host cells infected by the virus (Yu et al., 1994).

In all applications of gene therapy, the therapeutic benefit will be determined by the intrinsic activity of the chosen trans-gene as well as its intracellular expression, stability and localization. The delivery system and the biology of target cells will also influence the success of a gene therapy strategy.

Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) are still a scientific challenge as to their in vivo eradication. Since continuous variability of the HIV genome causes the present therapies to lose efficiency over the course of time and prevents the successful development of a vaccine and since all genes are expressed through RNA intermediates, gene therapy employing ribozymes has gained important consideration as a possible treatment for AIDS.
B. Hammerhead Ribozyme

The hammerhead ribozyme is a very small RNA motif that may cleave at a specific phosphodiester bond to produce 2',3' cyclic phosphate and 5' hydroxyl termini (Hutchins et al., 1986; Forster & Symons, 1987). Cleavage by hammerhead ribozymes is magnesium dependent and is site specific. The hammerhead consists of three helices connected by three single-stranded regions.

In nature, hammerhead ribozymes are utilized by a number of small pathogenic plant viroid and satellite RNAs for processing during rolling-circle replication (Symons, 1992) and they act “in cis” (on the same strand of RNA). Later, the hammerhead ribozyme was designed to work “in trans” [work against other RNA molecules (Uhlenbeck, 1987)]. The trans-acting hammerhead ribozyme has two antisense arms (stem I and III) and a catalytic domain with a flanking stem II as well as a loop section. In the hammerhead motif, a specific set of three nucleotides is required at the cleavage site. The triplet is defined by NUH (where N is U, G, A, or C and H is U, C, or A), with cleavage occurring 3’ to H. The GUC triplet is the most commonly used site in nature (Frostier & Simons, 1987) and is cleaved most efficiently (Refiner et al., 1990). This is the target site of choice in designing a new ribozyme. However, the optimal triplet depends also on the composition and nucleotide lengths of stem I and III. Because -UH- occurs very frequently in RNA (once in every six nucleotides on average), the hammerhead ribozyme can be designed to target any RNA. The ribozyme hybridizes to the complementary sequences flanking the-UH- cleavage site within the target RNA. The complementary sequences form stem I and III. If these two stems are short enough, the cleavage products can dissociate from the ribozyme easily, allowing the ribozyme to bind
and cleave another molecule. By repeating the cycles, one ribozyme molecule can be recycled to cleave many substrate molecules. The length and sequence of stems I and III affect the dissociation of the hammerhead ribozyme from the substrate and affect the catalytic properties of the ribozyme. The optimal lengths of each section of stems I and III are 9-12 nucleotides (Herschlag, 1991). Other factors that should be considered in optimizing the efficiency of hammerhead ribozymes include substrate secondary structure, creation of A-rich sequences in the hammerhead ribozyme that minimize U-G wobble pairs, and increased discrimination against non-targeted mRNAs (Herschlag, 1991; Bertrand et al. 1994).

Forster and Symons (1987) defined a consensus as the catalytic core of hammerhead ribozymes. Single base changes in each of the 14 conserved sites show some effect on catalytic efficiency (Ruffner et al., 1990). Mild to significant loss of catalytic activity can result from any change in the consensus sequence of the ribozyme catalytic core (Ruffner et al., 1990).

Magnesium is needed to stabilize the 5' oxygen leaving group (Taira et al., 1990) probably due to its ability to stabilize the tertiary structure of the hammerhead complex. Spermidine has been found to lower the concentration of magnesium required for hammerhead ribozyme cleavage, suggesting that spermidine can replace magnesium in stabilizing the active ribozyme structure (Dahm et al., 1991)
Figure 1. Hammerhead Ribozyme

The boxed NUH is the consensus target site where N is A,C,G,U and H is A,C,U). The Phosphodiester bond between H and downstream nucleotide indicated by an arrow is cleaved by the catalytic core, which is formed by 11 conserved nucleotides and stem loop II, in presence of Mg$^{+2}$. The Asterisk indicates the nucleotide, changed to make the mutant form of the ribozyme. (Usually a G to A mutation is used.) The sequence in the ribozyme, which forms stem I with the target RNA, is called arm I; and the sequence, which forms stem II with target, is called arm II.
1. Factors govern ribozyme activity

Developments in the following areas are prerequisites for successful ribozyme-based strategies: 1) persistent high-level expression in transduced cells; 2) targeting of the ribozyme into the correct subcellular compartment and colocalization of the ribozyme with its substrate; 3) appropriate folding of the ribozyme for catalytic activity; and 4) stable intracellular maintenance of the ribozyme.

2. Potential therapeutic applications of hammerhead ribozymes

Ribozymes can target specific RNA molecules and each molecule has the potential to cleave multiple copies of a given RNA. The hammerhead ribozyme is one of the smallest ribozymes known and can have great potential in gene therapy. The effects of hammerhead ribozymes have been shown to be catalytic rather than behaving as a simple antisense effector.

Traditional pharmaceuticals inhibit protein function. However, ribozymes either disrupt the flow of genetic information from DNA to protein or may even directly disrupt RNA genomes, as in the case of RNA viruses. Diseases that result from undesirable expression of RNA, such as neoplastic disorders and viral illnesses, are amenable to such a therapeutic approach. Hammerhead ribozymes have been studied as a therapeutic agent for many viral infections as well as malignancies and genetic diseases.

Rossi and colleagues described the first use of a hammerhead ribozyme to inhibit HIV-1 replication in tissue culture cells (Sarver et al., 1990). In this study, a ribozyme targeting the gag sequence was stably expressed in HeLa-CD4+ cells and the p24 levels were reduced compared with the non-ribozyme expressing cells upon infection with HIV.
Hammerhead ribozymes developed against the HIV-1 tat and tat/rev genes (Zhou et al. 1994) have been in clinical trials for several years. There have been reports of hammerhead ribozymes targeting other HIV genes including vif (Lorentzen et al., 1991), int (integrase) (Sioud et al., 1991) and the 5’UTR leader sequences (Weerasinghe et al., 1991). Each of these ribozymes resulted in delays or reduction in virus expression. Hammerhead ribozymes were used for other viral infections such as chronic management of chronic hepatitis B (HBV) infections (Kim et al., 1999; Hsieh ad Taylor, 1992; Netter et al., 1993).

Since ribozymes can distinguish RNA transcripts that differ from the wild-type transcripts by only one nucleotide, they can be designed as a therapeutic to eliminate mutated transcripts while leaving wild type transcripts intact. One example is the mutant H-ras gene implicated in bladder carcinoma targeted by a hammerhead ribozyme (Tone et al., 1993; Kashani et al., 1992). Another example is the bcr-abl transcript caused by the t(9;22) translocation in chronic myelogenous leukemia (CML) (Wright et al., 1993; Shore et al., 1993). Other potential targets for ribozyme-based cancer therapeutics include the MDR-1 (multidrug resistance gene) transcripts, topoisomerase α, methylguanine methyltransferase, dihydrofolate reductase, bcl-2, bcl-x and certain cytokines such as interleukin 6 and 9.
Figure 2. HIV-1 Map.

The upper panel of the figure was adapted from Human Retroviruses and AIDS 1990 database of NIH. Brief information about the coding regions are as follows:
- **LTR**: long terminal repeat, includes binding sites for cellular transcription factors
- **TAR**: target sequence for transactivation, stretching from the mRNA start site to approximately 50 nucleotides downstream in HIV-1
- **Gag**: gene encoding nucleocapsid, core and matrix proteins
- **Pol**: gene encoding a polymerase polyprotein, which upon cleavage yields protease, reverse transcriptase, and endonuclease/integrase proteins
- **Vif**: viral maturation and infectivity factor, typically a 23kD protein (localized in the cytoplasm/cell membrane) that determines rate of viral spread
- **Vpr**: gene product of about 15 kD localized in the nucleus; plays role in viral maturation
- **Tat**: gene encoding the transactivating regulatory protein of typically 14 kD, localized in the nucleus or nucleolus; tat-minus mutants do not replicate; tat protein has been found in the extracellular milieu
- **Rev**: gene product of 20kD (typically) localized in the nucleus or nucleolus; facilitates structural protein synthesis through transport of unspliced messages; interacts with RRE, the "Rev responsive element" found in the envelope coding region of transcripts
- **Vpu**: gene encoding a 15-20 kD cytoplasmic protein that functions in viral maturation and down regulation of CD4
- **Env**: gene product is the envelope or coat protein consisting of at least an extracellular glycosylated protein of about 120 kD and a transmembrane protein, also glycosylated of about 41kD. Viral envelope glycoproteins play role in CD4 binding and membrane fusion
- **Nef**: gene encoding a potentially myristoylated protein of about 27 kD; the function (or functions) is controversial, but it is related to down regulation of CD4 and unknown functions associated with in vivo pathogenesis; localized in the cytoplasm, the protein is the most immunogenic of the accessory proteins

Lower panel was obtained from Los Alamos National Lab’s HIV Database (http://hiv-web.lanl.gov/seq-db.html). This database allows the user to request particular coding regions, for example only protease, out of partial or full-length genomes. HIV-MAP also provides translations of the gene fragments that are retrieved.
HIV-1 Exons
C. HIV-1 envelope gene and envelope protein

The HIV-1 envelope gene is one of the most variable regions in the HIV genome, which is a reason why development of an effective vaccine has been very difficult. Why should we target the env gene with ribozymes then? First, HIV-1 infectivity is dependent on envelope gene expression. The gp 120 and gp 41 glycoproteins of the HIV envelope are absolutely necessary for viral fusion to target cell surface receptors. Thus, by selectively destroying the env mRNA with ribozymes env protein expression and hence HIV infectivity can be inhibited. In this respect, it has been shown that hammerhead ribozyme targeting of the conserved sequences in the envelope-coding region inhibited HIV-1 replication (Chen et al., 1992) and conferred longer protection (15 days) against HIV infection than ribozymes targeting other sites (Gag, protease, reverse transcriptase, or the Tat-coding regions) of HIV-1 (Ramezani and Joshi, 1996). Second, the HIV Tat and Rev proteins are essential for early steps in the HIV life cycle. Tat and Rev precursor mRNAs contain introns that are fully spliced out in the early stages of viral replication. Portions of these introns, when unspliced in the later stages of infection, comprise the coding sequence for the env protein. If a ribozyme should target a region in the env gene corresponding to a sequence important in correct early splicing to produce mature Tat and Rev mRNA, the expression of these proteins will also be adversely affected. Third, in order to co-localize ribozyme and substrates via Rev multimerization, use of the RRE and surrounding sequences is a logical region for ribozyme targeting.

Therefore, we have decided to select a region between nucleotides 7250 and 8131 in the env gene of HIV-1 NL4-3 which includes the RRE and also encodes gp120 and gp41, which play very important roles in viral fusion to CD4+ T lymphocytes.
D. The HIV-1 Rev Response and Rev Binding Elements (RRE &RBE)

The HIV-1 RRE is a 234 nt stem-loop structure located in the env coding region, present in all incompletely spliced HIV-1 mRNAs. This structure contains one major stem (stem I) and four stem-loops branching from the main stem (stem-loops II-V). Stem loop II can further be subcategorized into 3 regions: IIA, IIB and IIC (Bartel et al., 1991). Using deletion analyses Olsen et al. (1990b) and Huang et al. (1991) determined the regions of the RRE important for Rev function. Deletion of stem-loop II or IIB ablated Rev function and in vitro Rev binding. Therefore, in the context of the RRE, stem-loop IIB was considered the primary determinant of Rev interaction and Rev responsiveness in vivo.

The Rev binding element (RBE) is located between nucleotides 45 and 75 of the RRE (Kjem et al., 1992). It has been determined that the RBE forms a stem-purine rich asymmetric bulge-stem structure (Bartel et al., 1991; Heaphy et al., 1991; Kjems et al., 1992). RBE deletion or substitution mutants inhibit Rev-RRE interaction in vitro and cause RRE containing mRNAs to become unresponsive to Rev in vivo (Holland et al., 1990; Malim et al., 1990; Olsen et al., 1990b; Huang et al., 1991).
Figure 3. The HIV-1 Rev Response and Rev Binding Elements

The RRE is drawn as a 234 nt structure using the generally accepted nomenclature for stems and loops. The primary Rev binding site (RBE) is boxed and shown in greater detail in the inset. Watson-Crick base pairs are represented with the bases in close proximity (i.e., C49-G70). The two noncanonical purine–purine base pairs (G47-A73 and G48-G71) are drawn with lines between the bases; these lines also reflect the widening of the major groove of the helix in this region. The bases are circled were invariant in iterative in vitro genetic selection experiments. Chemical modification of the purines marked with dots resulted in severely reduced Rev binding in vitro, as did modification of the sugar-phosphate backbone at the positions indicated (with arrows) (Adapted from Pollard, V.W. and Malim, M.H. 1998).
E. HIV-1 Rev Protein

Rev is a regulatory protein synthesized during the early life cycle of HIV. It controls the expression of the HIV-1 structural genes through binding to a Rev responsive element (RRE) present only in the unspliced (9 kb) or singly spliced (4.3 kb) viral mRNA. Binding facilitates the export of the unspliced viral transcripts from the nucleus into the cytoplasm for translation of the viral structural proteins and assembly into viral particles.

Based on compensatory mutation studies it was shown that Rev binding to stem-loop 2B was dependent on the secondary structure and not the sequence (Olsen et. al, 1990; Huang et al., 1991). Bartel et al. (1991) however, reported that some sequence was necessary for Rev binding. Rev specifically recognizes a non-canonical Watson-Crick base pair in a bulged region directly adjacent to stem-loop 2B. This G:G base pair, is essential for Rev binding and can be replaced only with A:A, if binding is to occur. They proposed that the G:G base pair distorts the sugar-phosphate backbone of viral RNA and that this distortion is critical for recognition by Rev (Green, 1991).

Rev is a 13kDa and 116 amino acid protein. It localizes predominantly to the nucleolus of HIV-1 infected cells (Hauber et al., 1988; Cullen et al., 1988; Cochrane et al., 1989; cochrane et al., 1990) and is able to shuttle between the nucleus and the cytoplasm (Meyer B.E and Malim MH., 1994; Richard et al., 1994). The functional regions within the protein have been defined by mutagenesis studies combined with in vitro and in vivo functional assays. Within the N-terminal half of the protein, an arginine-rich motif spanning residues 35-50 mediates the sequence specific binding of Rev to the high affinity binding site within the RBE (Kjems et al., 1992; Daly et al., 1989; Heaphy et al., 1990). This motif also acts as a nuclear localization signal (Hope et al., 1990; Hammerschmid et al., 1994; Cochrane et al., 1990). Between residues 75 and 84 in the C-
Figure 4. Early and late phases of HIV-1 mRNA expression

Full-length unspliced ~9-kb mRNA, partially spliced ~4-kb mRNA, and fully spliced ~2-kb mRNA species are constitutively expressed in the nucleus. In the absence of Rev (upper panel), or when the concentration of Rev is below the threshold necessary for function, the ~9-kb and ~4-kb transcripts are excluded from the cytoplasm and either spliced or degraded. In contrast, the fully processed ~2-kb mRNAs are constitutively exported to the cytoplasm and used to express Rev, Tat and Nef. When the levels of Rev in the nucleus are sufficiently high (lower panel), the nuclear export of ~9-kb and ~4-kb RNAs is activated and the translation of all viral proteins ensues (Adapted from Pollard, V.W. and Malim, M.H. 1998).
Early Phase (no/low Rev)

nucleus  cytoplasm

-9 kb
-4 kb
-2 kb

splicing/degradation

Rev, Tat, Nef

Late Phase (high Rev)

nucleus  cytoplasm

-9 kb
-4 kb
-2 kb

splicing

Gag, Pol

Env, Vif, Vpr, Vpu

Rev, Tat, Nef
terminal portion of Rev lies a leucine-rich sequence termed the activation domain (Mermer et al., 1990; Hope et al., 1991; Malim et al., 1991); this sequence is a nuclear export signal which is able to bind the conserved nuclear export factor Crm1 (Fornerod, et al., 1997; Fukuda et al., 1997). This interaction with Crm1 and other nucleoproteins, probably including RIP/Rab (Bogerd et al., 1995; Fritz et al., 1995), allows the activation domain to direct the nuclear export of Rev proteins and the mRNAs to which they are bound. The multimerization, which is essential for \textit{in vivo} Rev activity, involves two loci (between residues 18-26 and 54-56) flanking the arginine-rich motif.

Mutant Rev proteins that are deficient in multimerization are still able to occupy the high affinity site and form a binary complex but are unable to assemble into higher ordered complexes (Madore et al., 1994; Malim MH and Cullen BR., 1991; Tiley et al., 1990). It has also been shown that multimerization can occur in the absence of the RRE both \textit{in vitro} (Cole et al., 1993; Flugel et al., 1993) and \textit{in vivo} (Bogerd et al., 1993; Hope et al., 1992; Olsen et al., 1990). The possible contribution of bridging factors (unidentified cellular proteins or factors) to multimerization \textit{in vivo} should not be overlooked, however.

Mutagenesis experiments demonstrated that disruption of leucine-rich domain (residues 75 to 83) yields proteins (for example, Rev M10) that localize to the nucleus, bind RRE-containing RNA, multimerize, and yet do not facilitate the export of those RNAs from nucleus (Daly et al., 1989; Hope et al., 1990; Malim et al., 1989; Malim and Cullen 1991; Olsen et al., 1990; Tiley et al., 1992; Venkatesh et al., 1990; Zapp et al., 1991). Because such mutants also exhibit a dominant negative (trans-dominant) phenotype (Malim et al., 1989; Mermer et al., 1990; Venkatesh et al., 1990) and because
Figure 5. The HIV-1 Rev protein domains

The 116 amino acid Rev protein harbors regions that mediate RNA (RRE) binding and nuclear localization (gray box), are required for protein multimerization (hatched boxes) and function as the NES/activation domain (solid box). The amino acid sequences of the arginine-rich and leucine-rich domains (residues 34 to 50 and 75 to 83, respectively) are indicated below, together with the changes at positions 78 and 79 that are present in the Rev M10 mutant. (Adapted from Pollard, V.W. and Malim, M.H. 1998).
multimerization

NLS & RRE binding

activation domain/NES

\[ \text{TRQARRNRRWRERQR} \]

\[ \text{LPPLERLTL} \]

DL (M10)
repeats of similar leucine-rich motifs are involved in protein-protein interactions between diverse proteins (Kobe et al., 1994), it was proposed that this domain interacts with cellular proteins required for Rev function (Malim et al., 1991).

Chemical cross-linking and gel filtration data indicate that Rev exists predominantly as a tetramer (Nalin, C.M. et al. 1990) and Zapp, M.L., et. Al 1991) and there is evidence that higher-order multimers can form in vitro even in the absence of RNA. (Heaphy et al. 1991).

Mutations that prevent oligomerization eliminate Rev activity, but the basis of this loss of activity remains controversial. While some studies indicate that oligomerization may be a prerequisite for RRE binding (Olsen H.S., et al. 1990 and Zapp, M.L., et al. 1991), others suggest that monomers of Rev can bind the RRE and subsequently oligomerize on the RNA, and that it is this substrate-dependent oligomerization which is essential for function (Cook, K.S., et al. 1991 and Malim, M.H., 1990). Either of these mechanisms (or a combination of the two) could account for the ability of a single RRE to bind up to eight Rev monomers simultaneously (Cook, K.S., et al. 1991 and Heaphy, S.C., et al. 1990). Significantly, both RRE-dependent and RRE-independent oligomerization appear to be mediated by the same sequences in Rev, implying that two phenomena are closely related: both can be blocked by N-terminal mutations (particularly at residues 14 to 26), but neither is affected by mutations in the C-terminal domain (Malim, M.H., 1990; Olsen, H.S., et al. 1990 and Zapp, M.L., et al. 1991, and Hope et al., 1992).
F. Goals, Hypothesis and Objectives

The present study here utilizes the idea that simultaneous binding of Rev monomers to the RBE sequences in both the HIV-1 genome and in the fusion molecule and their subsequent multimerization may co-localize ribozyme and target RNAs and hence may increase the functional activity of ribozymes.

To increase the antiviral potency of the ribozyme vector, as well as reduce the chance of viral resistance, the possibility of adding other antiviral genes to ribozyme constructs has been explored. Yamada et al. (1996) have combined the antiviral effects of a hairpin ribozyme targeted to the U5 region of HIV with the native RBE.

As Yamada et al. (1996) pointed out that not only would this molecule be bifunctional (ribozyme and decoy effect), but ribozyme activity should also be facilitated by linking to RBE for several reasons: The RBE would further stabilize the ribozyme molecule; the binding of Rev to the ribozyme RBE fusion molecule would allow it to traffic the same nuclear-cytoplasmic pathway as HIV mRNA does, thereby increasing the opportunity of interaction between enzyme and substrate; and the binding of Rev to the ribozyme-substrate complex is expected to increase ribozyme turnover, resulting in increased catalytic activity.
Figure 6. The Ribozyme-RBE Fusion RNA Molecule

The box indicates ribozyme cleavage target site; cleavage occurs after “A” nucleotide. The length of ribozyme used in this study was 80 nt and the RBE was 31nt. With added restriction site sequences at both ends the fusion RNA molecules’ length was 119 nt whereas target HIV-1 RNA’ length was 933 nt.
Fusion molecule

5'-AAUAUAAGUA GUAAAAAUUG-3'
3'-UCGUUAUAUUUCA CAUUUUUAACGCAGAU CC GCA-U A U C U
II. MATERIALS AND METHODS

A. Materials

1. Cells

Human CEM T lymphocytes were obtained from the American Type Culture Collection (Rockville, MD).

2. Reagents

RPMI 1640, Fetal calf serum was purchased from Irvine Scientific, Inc. (Irvine, CA). Oligonucleotides were synthesized by the DNA/Peptide Core Facility of City of Hope Medical Center (Duarte, CA) and Integrated DNA Technologies (IDT), Inc. (Coralville, IA)

3. Solutions

DEPC water: 0.1% diethyl-pyrocarbonate was added to double-distilled water overnight. The treated water was then autoclaved.

RNA loading buffer: 80% (vol/vol) formamide, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol.

20X SSC: 3.0 M NaCl, and 3.0 M sodium citrate, pH 7.0

10X TBE: 0.4 M Tris-HCl, pH 8.0, 0.4 M boric acid and 0.012 M EDTA, pH 8.3

TE buffer: 0.01 M NaOH, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA and 0.1% SDS

4. Chemicals:

[α-32P]UTP and [γ-32P]ATP were obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). tRNA was obtained from Boehringer Mannheim (Indianapolis, IN). All other
chemicals were obtained from Fisher Scientific Co. (Irvine, CA) or Sigma Chemical Co. (St.Louis, MO).

5. Enzymes:
DNase I was obtained from Ambion Inc. (Austin, TX) and Boehringer Mannheim (Indianapolis, IN). RNase inhibitor was obtained from Promega Corp. (Madison, WI). T4 polynucleotide kinase and T7 RNA polymerase were obtained from New England Biolabs Inc. (Beverly, MA). T4 DNA ligase was obtained from Promega Corp. (Madison, WI). Restriction enzymes were obtained from New England Biolabs (Beverly, MA), Boehringer Mannheim (Indianapolis, IN) and Promega Corp. (Madison, WI).

6. Proteins:
HIV-1 Rev wild type protein was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr. David Rekosh, Dr. Marie-Louise Hammarskjold and Dr. Michael Orsini.

7. Miscellaneous Materials
The dNTPs and NTPs were purchased from Pharmacia (Piscataway, NJ) and Boehringer Mannheim (Indianapolis, IN). QIAprep Miniprep Kit, QIAprep Maxiprep Kit, QIAquick PCR Purification Kit and QIAquickNucleotide Removal Kit were purchased from Qiagen Inc. (Chatsworth,CA). The pBluescript II SK and KS (+) plasmids were purchase from Stratagene (SanDiego,CA).
B. Methods

1. RNA Accessibility Determination

   a. HIV-1 Infection and Preparation of Cell extracts from HIV-1 Infected CEM Cells.

      CEM cells were infected with supernatants of HIV-1 pNL4-3: 1x10^5 cells in 200 μl of RPMI1640 +10%FBS were transferred into a 12x75 tube, and 100 μl of virus containing supernatant was added. The cells were incubated overnight at 37°C and 5%CO₂. Next day the cells were washed 4 times with HBSS (Hank’s Balanced Salt Solution, Bio Whitacker) and transferred into one well of a 24 well plate, 1ml total volume. Culture medium was the same as above (RPMI 1640+10% FBS) through the entire procedure. After 5 days, the cell density was high enough to transfer the cells into a T25 flask, into a total volume of 10 ml of culture medium.

      After approximately 10 days the cell density was high enough to transfer the cells into a T75 flask into a total volume of 15 ml of culture medium. For approximately 3-5 days culture supernatant samples were collected and measured in a p24 or RT assay. If the p24 output or the RT activity were high enough (compared to a standard HIV supernatant) the cells were harvested. Approximately 2x 10^7 cells were washed 2x with cold PBS. The cells were pelleted in an autoclaved microcentrifuge tube, the volume of the pellet was approximately 50 μl. 100 μl Buffer A (7mM Tris-HCl, pH7.5, 7 mM KCl, 1mM MgCl₂, 1mM β-mercaptoethanol) was added, cells were allowed to swell on ice for 10 minutes. After the incubation time on ice, the cells were transferred into an autoclaved dounce on ice and dounded (20 strokes).

      The total recovered volume (approximately 100 μl) was transferred into a new autoclaved microcentrifuge tube. 10 μl of Buffer B (neutralizing buffer: 21 mM Tris-HCl, pH 7.5, 116 mM KCl, 3.6 mM MgCl₂, 6 mM β-mercaptopoethanol) was added, the
tube was spun at 16,000x g at 4°C in a microcentrifuge for 10 minutes. After the spin the supernatant containing the cell extracts were collected.

b. **Antisense Directed Cleavage of 933 nt Labeled HIV-1 RNA by *Escherichia coli* RNaseH Enzyme**

A 7.5μl and 1:10 antisense oligo excess reaction was setup as follows: 75 nM 933 nt P$^{32}$ labeled substrate and 750 nM AS ODN heated to 90°C for 2 min and cooled on ice for 3 minutes. Both RNAs were transferred to 7.5X buffer (50mM Tris-HCl, pH 7.5 and 10mM MgCl$_2$) and DEPC water. 3 units (1/10 dilution of the original) *E. coli* RNaseH (Gibco/BRL) were added to the reaction mix. After 1 hour incubation at 37°C, 7.5μl 2X formamide loading buffer was added and the samples were electrophoresed on a 5% denaturing polyacrylamide gel. The reactions were carried out separately for all 8 sites using the corresponding antisense oligo.

c. **Antisense Directed Cleavage of 933 nt Labeled HIV-1 RNA in non-infected CEM Cell Extracts by Cell Extracts’ RNaseH Activity**

Cell extracts from non-infected CEM cells were prepared as in #1 of Methods. A 10 μl reaction was setup as follows: 2μl cell extract, 1μl (20nM) 933nt transcribed hot substrate, 0.5μl (20units) RNasin, 1μl 10Xbuffer (40mMTris-HCl pH7.5; 10mM MgCl$_2$; 1mMDTT) and DEPC water (until 10μl) were all mixed. 1500nM (in 1μl) antisense oligo were added as final reagent and mixed gently. After 5 minutes of incubation at 37°C, each sample was treated with 5 μl 3X proteinase K buffer (0.6mg/ml proteinase K, 30 mM Tris-HCl pH 7.5, 60 mM EDTA, 1% SDS and 0.02mM CaCl$_2$) at 37°C for 15 minutes. Then samples were subjected to phenol chloroform extraction and alcohol precipitation and electrophoresed in a 5% denaturing polyacrylamide gel.
d. Antisense Directed RNaseH Cleavage of HIV-1Env RNA in HIV-1 infected CEM Cell Extracts and RT-PCR Analyses

Antisense oligodeoxyribonucleotides used in these studies were as follows: Each antisense oligodeoxyribonucleotide was named according to its prospective ribozyme cleavage site triplet location. For instance, AS 7386 refers to the ribozyme cleavage site triplet in the middle portion of this oligo that is located in nucleotide 7386 of the HIV-1 NL-43 RNA.

\[ \text{AS1} = \text{AS7386}, \ 5'\text{-AGTACTATTAAACAGTTGTGTTG-3'}; \]
\[ \text{AS2} = \text{AS7462}, \ 5'\text{-TTCTGCATGGGAGTGTGATTGTG-3'}; \]
\[ \text{AS3} = \text{AS7518}, \ 5'\text{-GGGAGGGGCATAACATTGCTTTTC-3'}; \]
\[ \text{AS4} = \text{AS7637}, \ 5'\text{-TTGTCCCTCATATCGCCTCCCTC-3'}; \]
\[ \text{AS5} = \text{AS7678}, \ 5'\text{-CAATTTTTACTACTTTATATTTA-3'}; \]
\[ \text{AS6} = \text{AS7858}, \ 5'\text{-GCTGCTGCATATACAGACAAT-3'}; \]
\[ \text{AS7} = \text{AS7918}, \ 5'\text{-CCCAGACTGTGAGTTGCAACAGA-3'}; \]
\[ \text{AS8} = \text{AS8010}, \ 5'\text{-GAATTTCGCCAGAGCAAACCAACAAA-3'}; \]

Control Sense oligo (7278-7298), 5'\text{-AATAATAAAACACAATAATCTT-3'}.

(Underlined triplet nucleotides refer to prospective ribozyme cleavage sites in the target RNA.)

HIV-1 5'-priming oligo for PCR (7228-7251) (24 nt):
5'\text{-GAATGCCACTTTAAAACAGATAGC-3'}

HIV-1 3'-priming oligo for PCR (8049-8030) (20nt):
5'\text{-GACTTCTGGAGGAGGTGCAACAGA-3'}

(5' labeled with TET fluorescent dye).

Human β-Actin 5'-priming oligo for PCR (26nt):
5'-GCCCCCCTGAACCCCAAGGCCAACC-3'  
(5' labeled with Rhodamine fluorescent dye).

It is located in exon 3. This sequence is complementary to nucleotides 1372 to 1397

**Human β Actin 3'-priming oligo for PCR (24nt):**

5'-GAAGTCCAGGGCGACGTAGCAG-3'  
It is located in exon 4. This sequence is complementary to nucleotides 2160 to 2137  
and the PCR product is 348 nt for the spliced form and 789 nt for the unspliced form.

i) Antisense & RNaseH Cleavage Reaction

A 20μl reaction was setup as follows: 10μl cell extracts, 2μl 10X buffer  
(40mMTris-HCl pH7.5; 10mM MgCl₂; 1mMDTT), 1μl RNasin (40 U/μl) (Promega),  
1500 nM antisense oligo and DEPC water were mixed gently and incubated at 37°C for 5  
minutes. Then 10 μl 3X Proteinase K buffer (0.6mg/ml proteinase K, 30 mM Tris-HCl  
pH 7.5, 60 mM EDTA, 1% SDS and 0.02mM CaCl₂) were added and incubated for 15  
minutes at 37°C. After phenol-chloroform extraction and alcohol precipitation, the  
samples were treated with DNAse I using 1μl DNAse I (Roche). After a second phenol-  
chloroform extraction and alcohol precipitation, Total RNA was measured by  
spectrophotometry.

ii) RT-PCR reaction

Using 500-1000 ng of total RNA as a template, a 20 μl RT reaction was carried  
out as follows: 4 μl 5X RT buffer, 1.6 μl 0.1 M DTT, 0.4 μl RNasin, 1μl Hexamer  
random primer (50 ng/μl), 8 μl dNTP and 1 μl MMLV (Moloney murine Leukemia  
Virus) Reverse Transcriptase (200U/μl) (Gibco BRL) enzyme and an appropriate volume
of DEPC water were mixed and incubated at 42°C for 1 hour. A 50 µl PCR reaction was setup as follows: 20 µl RT reaction mix, 5 µl 10X Buffer, 10 pmols of HIV 5’ and 3’ primers and 10 pmols of human β-actin 5’ and 3’ primers and DEPC water until 48.5 µl were mixed and placed at 94°C for 5 minutes. The samples were transferred to ice for 2 minutes. After addition of 2 µl dNTP and 0.5 µl Taq DNA polymerase (Boehringer Mannheim), the PCR reaction was carried out for 26 cycles at 94°C 1 min; 62°C 1 min; 72°C 1 min and an extension was carried out at 72°C for 10 minutes.

e. Radioactive oligonucleotide probe labeling

A 10 µl reaction was setup as follows: 1 µl (10 pmol) of oligonucleotide, 1 µl 10X T4 PNK buffer, 6.5 µl of water, 1 µl of [γ-32P]ATP and 0.5 µl of T4 PNK kinase were mixed. The reaction was then incubated at 37°C for 30 min.

f. Southern Hybridization

10 µl of RT-PCR products were fractioned on a 0.8% agarose gel and transferred to a Hybond™-N nylon membrane (Amersham) by capillary blotting. The RT-PCR products were then crosslinked to the membrane with a Stratagene UV Stratalinker. The membrane was prehybridized in 6X SSC, 7% SDS at 55°C for 2 hours. 10 pmol of oligonucleotide was labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs). The probe was added to the prehybridization solution and hybridization was carried out overnight at 55°C. Membranes were washed and exposed to both X-ray film and a Phosphoimager screen and subsequently analyzed with ImageQuant Tools software (Molecular Dynamics). The HIV probe was one of the
antisense oligonucleotides used in the RNaseH Cleavage and RT-PCR analyses in the cell extracts. The human β actin probe, which was used as an internal control, was the 3’ primer of the PCR reaction. The quantitations of the HIV signals obtained by the Phosphoimager were normalized with to actin signal intensity.

g. *In vitro* transcription

The HIV-1 sequences between 7250 nt (Nhe I) and 8131 nt (Hind III) in the *env* gene of HIV-1 NL4-3 which include the RRE were cloned into the pBS-KS II+ vector (Stratagene) between the Sma I and Hind III sites. After linearization with Hind III digestion, the plasmid DNA was purified with a Qiaquick PCR purification kit.

A 20 μl reaction was prepared using a minimum of 500-1000 ng DNA template. Reaction samples contained an appropriate amount of DNA template, 2 μl 10X NEB RNA polymerase buffer, 1 μl of NTP mix (10mM each of ATP, CTP, GTP and UTP), 1 μl 0.1M DTT, 1μl Rnasin (40U/μl) and 1 μl of T7 or T3 RNA polymerase (NEB,50u/μl) and water to make the total volume 20 μl. The reaction was incubated at 37°C for 2hr.

h. *In vitro* transcription for preparing radioactive RNA transcripts

In order to transcribe the target region between 7250 nt (Nhe I) and 8131 nt (Hind III) in the *env* gene of HIV-1 NL4-3, which includes the RRE, these sequences were cloned into pBS-KS II+ vector (Stratagene) between the SmaI and HindIII sites. After linearization with Hind III digestion, the plasmid DNA was purified with a Qiaquick PCR purification kit.
a) \textit{In vitro transcription reaction:} Reactions were prepared in a 20 µl reaction volume and included a minimum of 500-1000 ng of DNA template, 2 µl 10X NEB RNA polymerase buffer (400mM Tris-HCl (pH7.9), 60 mM MgCl₂, 20 mM spermidine, 100 mM dithiothreitol), 2 µl 100 mM DTT, 2 µl AGC mix (10 mM each of ATP,GTP,CTP), 3.125 µl 0.8mM cold UTP, 2 µl α³²P-UTP (3000 Ci/mmol), 1.5 µl T7 RNA Polymerase enzyme and an appropriate amount of DEPC water to a final volume of 20 µl. Incubation was carried out at 37°C for 2 hours. 1µl DNase I was then added and incubated further for 15 min at 37°C.

b) \textit{Purification:} The \textit{in vitro} transcribed RNA was electrophoresed in a 5% denaturing polyacrylamide gel and then exposed on an X ray film for 2 minutes to determine the position of the desired transcripts. The corresponding gel pieces were cut out and minced in an Eppendorf tube and eluted in 350µl elution buffer. (0.5M NH₄OAc, 1mMEDTA, 0.2% SDS) at room temperature overnight (12-16 hrs). The eluted contents were transferred into a Costar Spin-X centrifuge filter unit and centrifuged for 3 minutes at maximum speed using a microcentrifuge. One µl of glycogen (Boehringer) was added followed by 1/10 volume of 3M NH₄OAc and 2.5 volume ethanol to the supernatant. The RNA was pelleted and washed with 70% ethanol. The final sample was resuspended in 22 µl RNase free water or TE buffer. Aliquots were measured for cpm and OD to estimate the specific activity and concentration of the transcripts.
2. Ribozyme Preparation

Design and Construction of Ribozyme and Chimeric Ribozyme-RBE Fusion RNAs.

Ribozymes targeting three different sites (7637, 7678 and 7918) were designed in wild type (wt) and mutant (mt) forms and combined with the native HIV-1 RBE sequence. The fusion molecules have been cloned into the pBS-SK II expression plasmid vector. In order to investigate the expression level of the ribozymes and the RBE genes using pol III expression cassettes or retroviral vector in transfected and/or transduced cells, two restriction sites (Xho I and Mlu I) have been included in both ends of the fusion molecules. Using these and all other appropriate sites they can be cloned into a pol III or retroviral vector.

The preparation scheme is shown in Figure 7.

7637 Ribozyme (Rbz) constructs:

Oligo A for 7637 rbz constructs (7637-5’ oligo) (38nt):

BamH I
5’-CGGGATCCCGAGGAGGCGATTTCGTCCTCACGGACTCA-3’

(common for 7637 wt and mt rbz constructs)

Oligo B for 7637 wt Rbz (7637wt-3’ oligo) (38nt):

EcoR I
5’-CGGAATTCCGTTGTCCCTCACTGATGAGTCCGTGAGGA-3’

Oligo B for 7637 mt Rbz (7637mt-3’ oligo) (38nt):

EcoR I
5’-CGGAATTCCGTTGTCCCTCACTAATGAGTCCGTGAGGA-3’
7678 Rbz constructs:

Oligo A for 7678 rbz constructs (7678-5’ oligo) (38nt):

BamH I
5’-CGGGATCCCCGAATATAAAGTTTCGTCCTCACGGACTCA-3’
(common for 7678 wt and mt rbz constructs)

Oligo B for 7678 wt Rbz (7678wt-3’ oligo) (38nt):

EcoR I
5’-CGGAATTTCGCCAATTTTTACCTQTGATGAGTCCGTGAGGA-3’

Oligo B for 7678 mt Rbz (7678mt-3’ oligo) (38nt):

EcoR I
5’-CGGAATTTCGCACAATTTTTACCTAATGAGTCCGTGAGGA-3’

7918 Rbz constructs:

Oligo A for 7918 rbz constructs (7918-5’ oligo) (38nt):

BamH I
5’-CGGGATCCCCGTGGTATGCAACTTTCGTCCTCACGGACTCA-3’
(common for 7918 wt and mt rbz constructs)

Oligo B for 7918 wt Rbz (7918wt-3’ oligo) (38nt):

EcoR I
5’-CGGAATTTCGCACAGCTGTCTCAGTATGAGTCCGTGAGGA-3’

Oligo B for 7918 mt Rbz (7918mt-3’ oligo) (38nt):

EcoR I
5’-CGGAATTTCGCAGGAGCTGATCTAATGAGTCCGTGAGGA-3’

7637, 7678 and 7918 wt/mt Rbz-RBE Fusion RNA Constructs:

Oligo C (5’RBE oligo) (57nt): (common for all wt and mt constructs)

Xba I Xho I wt RBE
5’-GCTCTAGAGCCTCGAGTGGGCAGCGACTGCAATGACGGTGACGGTACACG
GGATCCCG-3'
BamH I

Oligo D (3’ RBE oligo) (26nt): (common for all wt and mt constructs)
5’-GGGGTACCCACGCGTCGGAATTCCG-3’
        Kpn I   Mlu I   EcoR I
Figure 7. Design scheme for ribozyme and ribozyme-RBE fusion molecules

To create the fusion molecules, overlapping primers A and B of each ribozyme were extended first in a 50 μl reaction using 100 pmol A and B oligos, 1μl Vent DNA polymerase. After gel purification of desired size products, a PCR reaction was carried out to create fusion RNA molecules using 100 pmols oligo C and D and 1-2 μl of first PCR products.
To create the fusion molecules, A and B oligos of each ribozyme were amplified in 50 μl PCR reaction as follows: each sample contained 100 pmol A and B oligos, 5 μl 10X Buffer (NEB) (100 mM KCl, 200mM Tris-HCl (pH 8.8), 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 0.1% Triton X-100) 4μl dNTP (2.5mM) and 1μl Vent DNA polymerase (200U/μl) (NEB) and DEPC water until 50 μl. Using the hot start technique and conditions of 94°C 3 min; 94°C 1min, 55°C 1min, 72°C 1min; 72°C 10min the PCR reactions were carried out for 5 cycles. After gel purification of the desired size PCR products, a second PCR reaction was carried out to create fusion molecules as follows: 1-2μl of first PCR products (volume may increase / decrease, depends on the concentration), 100 pmols oligo C and D, 5 μl 10X Buffer, 4μl dNTP (2.5mM) and 1μl Vent DNA polymerase (200U/μl) (NEB) and DEPC water until 50 μl. The PCR conditions were the same as above and at least 20 cycles were used. After the reaction, the PCR products were gel purified and digested with Xba I and Kpn I and inserted into the pBS-SKII plasmid, already cleaved with the same enzymes. A 20 μl ligation reaction with 1:10 vector-to-insert ratio using T4 DNA ligase (Promega) was carried out at 16°C overnight.
Figure 8. Structure of Ribozymes and Fusion Molecules Created by *In vitro* Transcription
7637wtRbz+RBE (AS)  
\[ \text{armII catalytic core armI} \]  
5'gcuuacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuaacgacgacuggccagggugacgccuucuugacuguaccccuccuaacgacgacgucuucucaggaac gccuuaacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuaacgacgacgacguggucuacgccuuaacgacgacgacgucuucucaggaagcccuccuag 3'  
wtRBE

7637mtRbz+RBE (AS)  
\[ \text{armII catalytic core armI} \]  
5'gcuuacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuaacgacgacgacgucuacgccuuaacgacgacgacgucuucucaggaagcccuccuag 3'  
wtRBE

7637wtRbz (AS)  
\[ \text{armII catalytic core armI} \]  
5'gcuuacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuaacgacgacgacgucuacgccuuaacgacgacgacgucuucucaggaagcccuccuag 3'  
wtRBE

7678wtRbz+RBE (AS)  
\[ \text{armII catalytic core armI} \]  
5'gcuuacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuaacgacgacgacgucuacgccuuaacgacgacgacgucuucucaggaagcccuccuag 3'  
wtRBE

7678wtRbz (AS)  
\[ \text{armII catalytic core armI} \]  
5'gcuuacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuaacgacgacgacgucuacgccuuaacgacgacgacgucuucucaggaagcccuccuag 3'  
wtRBE

7918 wtRbz+RBE (AS)  
\[ \text{armII catalytic core armI} \]  
5'gcuuacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuaacgacgacgacgucuacgccuuaacgacgacgacgucuucucaggaagcccuccuag 3'  
wtRBE

7918wt Rbz (AS)  
\[ \text{armII catalytic core armI} \]  
5'gcuuacccaugggguggcagccuuaaggcaacacaggagugacuacucgcuuuaacgaggagcccuccuaacgacgacgacgucuacgccuuaacgacgacgacgucuucucaggaagcccuccuag 3'
To obtain the Rbz alone (no RBE) and fusion molecules, constructs were digested with BamH I and Xho I restriction enzymes respectively. After purification with a Qiaquick PCR purification kit, 500-1000ng linearized template DNA was used for cold or hot in vitro transcription reaction as explained in methods 1g and 1h.

3. RNA/Protein Binding Studies

Rev Binding Assay with Ribozyme, Ribozyme-RBE Fusion RNAs and/or Labeled/Unlabeled Target RNAs (Gel Retardation Assays)

Mobility shift assays were performed at 37°C using a protocol described by Brice et al (1999) with some modifications. Binding reactions (total volume of 20 µl) were set up in siliconized Eppendorf tubes at room temperature. Each sample contained: 8 µl 2.5% Triton-100X (SIGMA); the desired concentration of RNAs [Rbz; Rbz+RBE (hot and/or cold); 933nt HIV RNA(hot and/or cold); tRNA] in 1- 4 µl; 0.5µl (20 units) RNasin (Promeaga); Rev protein (desired amount in desired volume) (usually 50ng/µl) and binding buffer (50 mM NaCl, 10mM Tris-HCl (pH 7.5), 1mM DTT, 2mg/ml BSA) until 20 µl (but minimum volume for the binding buffer should be 5.5µl). The original Rev protein concentration was 2µg/µl, this was diluted to the desired concentration using dilution buffer (500 mM NaCl, 10mM Tris-HCl (pH 7.5), 1mM DTT, 2mg/ml BSA).

a. Single binding reaction: Every reagent except the RNAs and Rev were mixed in a siliconized Eppendorf tube. RNAs were denatured and renatured by heating at 85°C for 4 minutes and cooled in ice for 5 minutes. Following a quick spin, the corresponding volume of RNAs was added to the buffer. Rev protein was added as the last reagent and mixed gently. After a 20 minute incubation at 37°C, 5µl loading buffer (25% glycerol, 0.25% bromophenol blue, 0.25%xylene cyanol FF) were added to each sample. The samples were loaded onto a 3.5% 10x15 cm nondenaturing polyacrylamide gel (29:1;
1.5mm) and electrophoresed at $4^0\text{C}$ in 0.5X TBE buffer and at a 20 mA constant current. Gels were always prerun for 1 hour at the same current.

b. Mixing of two independent binding reactions: Each 20 $\mu$l binding reaction was performed independently as above. After 20 minutes incubation at $37^0\text{C}$, the reactions were mixed in one tube (total volume of 40$\mu$l) and incubated 30 minutes at $37^0\text{C}$. Ten $\mu$l loading buffer were added and half volume of mix (25$\mu$l) was loaded onto a 3.5% nondenaturing polyacrylamide gel (29:1, 1.5mm) and run at $4^0\text{C}$ in 0.5X TBE buffer at 15 mAmp constant current.
III. RESULTS

A. Selection of the most accessible sites in the HIV-1 env RNA

One of the major parameters determining efficacy of ribozyme strategies is the identification of an accessible target site in the chosen target RNA. For trans-acting hammerhead ribozymes, accessibility to target RNAs is the rate-limiting step in vivo. As of now, since information about the relationship between accessibility and RNA structure is very limited, and since it is a function of many unknown factors, accessibility is currently beyond our ability to predict.

Despite that, currently, there are several in vitro techniques available to determine the accessibility of ribozymes or antisense agents (DNA/RNA) to the target mRNA. They are (i) computer based RNA folding programs such as Mfold (Zuker M, 2000) (ii) probing secondary structure through the use of single-strand specific probes such as nuclease S1, lead acetate, imidazole, DMS (dimethyl sulfate) and CMCT (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate) (Campbell et al, 1997), (iii) the use of antisense oligodeoxynucleotides (AS ODNs) or AS ODN libraries & RNase H cleavage (Ho et al, 1998; Scherr and Rossi, 1998) and (iv) the use of ribozyme libraries (Pierce and Ruffner, 1998; Lieber and Strauss, 1995).

Ideal screening should use intact cells or cell extracts harboring target RNAs of interest. Since random approaches (AS ODN and ribozyme libraries) provide the greatest range of possibilities, they are preferred over more directed approaches. However, again, methods need to be developed that allow rapid, unambiguous identification of the most accessible ribozyme target sequences. Once such sites have been identified, specific ribozymes must be tested efficiently in the appropriate cell culture or an animal model (Rossi 1999).
Figure 9. Target Sites

All eight sites were chosen from regions highly conserved between different HIV isolates. There were three CUC, two GUA, two AUA and one GUU sites. Five sites among them were upstream region of RRE, two sites were in the RRE and one site was in the downstream of RRE.
Target Sites

6221

env

7385
(GUU)

7461
(GUA)

7518
(GUA)

7657
(GUA)

7678
(GUA)

7759

7857
(AUA)

7917
(CUC)

7992

6908

(CUC)

8785

tat

rev
Figure 10. Computer predicted folding of 933nt HIV-1 target RNA

Computer predicted folding of the 933 nt HIV-1 target RNA was obtained from the Mfold program (3.1 version). Because the program gives the folding results for bigger transcripts (more than 800 nt) as just an "outline" instead of showing each nucleotide position, the exact position of the cleavage sites could not be shown.
Figure 11. *In vitro* cleavage reaction using AS ODNs, labeled substrate transcript and *E. coli* RNaseH enzyme

Antisense directed cleavage of the 933 nt labeled HIV-1 RNA by *E. coli* RNaseH enzyme to determine the most accessible target site(s). Bands in mid portion of gel refer to cleavage products. As seen in 7678 and 7637 lanes, *in vitro* transcribed 933 nt HIV-1 target RNA was cleaved from these two sites very effectively. In this *in vitro* cleavage experiment, there was no additional protein. Numerical labels refer to each target site position, S is substrate, AS oligo is Antisense oligo, buffer is reaction buffer.
Figure 12. *In vitro* cleavage reaction in non-infected CEM cell extracts using Antisense ODNs, labeled substrate and cell extract RNaseH activity.

Consistent with previous experiments (Fig.11) this experiment was designed to determine the most accessible target sites in a more *in vivo*-like context. Cell extract provide both proteins and endogenous RNaseH enzyme, The bands are cleavage products. 7678 and 7637 sites appear to be more accessible.
We have chosen eight conserved sequences in the target region (between nucleotides 7250 and 8131 of the *env* gene of HIV-1 NL4-3 as potential hammerhead ribozyme target sites. We have used four different *in vitro* techniques to detect the most accessible sites in this target region. Techniques used are as follows:

1. **Computer predicted RNA folding**
   Computer predicted folding of the 933 nt HIV-1 target RNA and the ribozyme and fusion molecule RNAs were obtained from the Mfold program (3.1 version), which is an RNA and DNA folding package developed by Dr. Michael Zuker at Rensselaer Polytechnic Institute, Troy, NY (http://bioinfo.math.rpi.edu/~zukerm/rna/)

   Because the program gives the folding results for bigger transcripts (more than 800 nt) as just an "outline" instead of showing also each nucleotide position, I could not show the exact position of the cleavage sites in figure 10.

   The predictions from mfold for small size RNA (until 200nt) is really consistent and correlated with the experimental data. (Jain et al., 2001; Charpentier et al., 1997; Mann et al., 1994; Heaphy et al., 1991).

2. **In vitro cleavage reaction using antisense ODNs, labeled substrates and *E. coli* RNaseH enzyme**

   Reactions were carried out as described in Methods 1b. Because the fragments were at expected size, the reductions in the target RNA were most likely due to the specific target annealing by corresponding antisense oligonucleotides and cleavage by *E. coli* RNaseH. Consequently, the site 7678 was best followed by 7637 (Figure 11).
3. *In vitro* cleavage reaction in non-infected CEM cell extracts using antisense ODNs, labeled substrate and Cell extracts’ RNaseH activity

Reactions were carried out as explained in Methods 1c. As a difference from previous technique, we have used non-infected CEM cell extracts in this method to provide the protein environment to labeled target for folding purpose. Also there was no need for exogenous RNaseH in the reaction because of cell extracts’ endogenous having. However, there were no significant difference in the results from technique 2 and 3. Again site 7678 was best followed by site 7637 (Figure 12).
Figure 13. Scheme of accessibility studies in the HIV-1 infected CEM cell extracts

This figure summarizes the all steps followed throughout entire technique. Briefly, it is expected that antisense DNA oligos can hybridize only the accessible target sites; RNaseH degrades the RNA only in the resulting RNA/DNA hybrids. RT-PCR performed with flanking primers will show reduced product formation when target site is accessible.
Accessibility of Antisense ODNs to target sites

Cell extracts from HIV-1 infected CEM cells

5 min at 37 C

Sense and Antisense ODNs

Inaccessible

Accessible

HIV-1 mRNA

ODN

DNaseI treatment

RT

PCR

Quantification

RNaseH

HIV-1 mRNA

ODN

No Product

55
Figure 14. RT-PCR determination of target site accessibility

The figure shows agarose gel picture of RT-PCR reactions in triplicate. The upper band (821 bp) is the expected HIV-1 product and lower band (348 bp) is derived from actin as an internal control. The greater the accessibility of the given target to the corresponding antisense oligo (or by extension, ribozyme) the greater the reduction in the PCR product, relative to the internal control.
4. Antisense directed RNaseH Cleavage of HIV-1 Env RNA in HIV-1 infected CEM cell extracts and RT-PCR analyses

Reactions were carried out as in Methods 1d. Figure 13 outlines the scheme of the accessibility studies. Experiments were carried out in the HIV-1 infected CEM cell extracts. The numbers in Table 1 were derived from nine separate experiments using cell extracts, which were prepared at two different times. One experiment was performed in triplicate (Figure 14). Following southern hybridization of RT-PCR products, radioactive bands were visualized and quantitated using a Molecular Dynamics PhosphoImager.

Although we have only used 881nt transcript of the env gene of the HIV-1 NL4-3 in techniques 2 and 3, the results of both methods were similar to the results of technique 4, which allowed screening of the entire the HIV-1 NL4-3 genome (9.4 kb) in the CEM cell extracts (Figure 14 and 15).

Actually, previous two experimental techniques (2 & 3) was showing that the site 7678 was best followed by 7637, since method 1d and technique 4 (Antisense directed RNaseH Cleavage of HIV-1 Env RNA in HIV-1 infected CEM cell extracts and RT-PCR analyses) was closer to in vivo conditions, we decided to carry out the site selection using this procedure. In conclusion, we have chosen sequences at position 7637, 7678 and 7918 as the most accessible, intermediate accessible and inaccessible sites (as a negative control) respectively. (Fig. 14, 15, 16 and Table 1)
Figure 15. RT-PCR and Southern Hybridization

Figure shows +RT and −RT agarose gel pictures of one of nine RT-PCR experiments and the corresponding southern hybridized with anti-HIV and anti-actin probes. +RT refers to presence of reverse transcriptase enzyme during RT (Reverse transcription) reaction. −RT means no reverse transcriptase enzyme available in the reaction. Southern hybridization was done for quantitation purpose. 7637 site appeared to be best site in this gel picture.
RT-PCR

+RT panel

-RT panel

821nt HIV-1 bands

348nt Actin bands
Figure 16. Efficiency of RNaseH Mediated Cleavages

Table 1. Quantitative analysis of RT-PCR products
   (After normalization)

Figure 16 and Table 1 show statistical analyses of RNaseH mediated reduction of HIV-1 RNA in the presence of antisense oligos. Two-tailed t set analysis indicates that the reductions of HIV-1 mRNA with AS4+AS5 oligos were significant. For both 7637 and 7678 site P value was less than 0.001 (P<0.001). In agreement with figure 16, AS4 site (7637) had the highest significance.
Efficiency of RNaseH Mediated Cleavages

<table>
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<tr>
<th>Cleavage Sites</th>
<th>Mean</th>
<th>St.Dev.</th>
</tr>
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<td>0.000</td>
</tr>
<tr>
<td>S</td>
<td>0.7259</td>
<td>0.4928</td>
</tr>
<tr>
<td>AS1</td>
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<td>0.1532</td>
</tr>
<tr>
<td>AS2</td>
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<td>AS4</td>
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<td>AS5</td>
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<td>AS8</td>
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</table>
B. Determination of in vitro co-localization of ribozyme-RBE fusion molecules with substrate.

1. Rev protein binding to the 933 nt labeled HIV-1 target RNA

The Rev binding assay was performed at 37°C using a protocol described by Brice et al (1999) with some modifications. The observed gel shifts were dependent on the concentration of Rev protein. To demonstrate if the binding of Rev to target was specific, competition assays with a 100X excess (3 μM) of unlabeled 933 nt HIV-1 RNA and E. coli tRNA were carried out. Unlabeled 933 nt HIV-1 RNA prepared by in vitro transcription was used as a specific competitor and negative control, and E. coli tRNA (Roche) was used as a nonspecific competitor. As seen in the last two lanes of Figure 18 Panel B, Rev binding to the target is specific.

2. Rev binding to the 7637 Ribozyme-RBE fusion molecule

Rev Binding assays for the 7637 chimeric molecule were performed under the same conditions of Rev binding to target (Figure 19 Panel A and B). The labeled fusion RNA concentration was 40 nM for the binding assay in Panel A and was 500 nM in Panel B. Concentrations of E. coli tRNA used as nonspecific competitor were 4 μM (100X) in Panel A and 12.5 μM (25X); 25 μM (50X) and 50 μM (100X) (lines 3, 4 and 5) in Panel B. The concentration of unlabeled 7637 fusion RNA, used as a specific competitor in Panel A, was 800nM (20X).

Since Rev binding to the 7637 fusion RNA was inhibited by all different concentrations of nonspecific competitor E. coli tRNA (lanes 3, 4 and 5 in Figure 19 Panel B), Rev binding to the chimeric transcript was not specific. After the denaturation and refolding steps, all competitors were added to the binding buffer immediately after
addition of labeled fusion molecule. Rev proteins were always the last reagent added to the reaction.
As mentioned in the text, the exact mechanism of rev binding to the RBE molecule is currently incomplete. One hypothesis is that a single monomer first binds to RBE, then others bind to the first cooperatively. A second proposal suggests that Rev binds to RBE as an oligomer. This figure’s representation resembles mostly cooperative action, and shows how co-localization of HIV-1 RNA and Fusion RNAs might occur via Rev multimer bridge.
Figure 18. Rev binding and competition assays to 933nt labeled HIV-1 RNA with unlabeled 933nt HIV-1 RNA and *E. coli* tRNA

The observed gel shifts were dependent on the concentration of Rev protein. Unlabeled 933 nt HIV-1 RNA was used as a specific competitor and negative control, and *E. coli* tRNA was used as a nonspecific competitor. As seen in the last two lanes of Panel B, Rev binding to the target is specific.
Panel A

Panel B
Figure 19. Rev binding and competition assays to 7637Rbz-RBE fusion molecule with unlabeled 7637 rbz-RBE fusion molecule and E. coli tRNA

The labeled fusion RNA concentration was 40 nM for the binding assay in Panel A and was 500 nM in Panel B. Concentrations of E. coli tRNA used as nonspecific competitor were 4 μM (100X) in Panel A and 12.5 μM (25X); 25 μM (50X) and 50 μM (100X) (lines 3, 4 and 5) in Panel B. The concentration of unlabeled 7637 fusion RNA, used as a specific competitor in Panel A, was 800nM (20X). Since Rev binding to the 7637 fusion RNA was inhibited by even 25X excess nonspecific competitor E. coli tRNA (lanes 3), Rev binding to the chimeric transcript was not specific.
3) Rev binding to three different ribozymes and fusion molecules

Figure 20 shows that Rev binds to all the fusion RNA molecules as expected. However, although there is no RBE sequence in 7637 and 7678 ribozyme molecules, interestingly, Rev also binds to these ribozymes. There must be some putative binding sequence in these molecules.

4) Co-localization of the 7637, 7918 and 7678 fusion RNA molecules with the 933nt HIV-1 target RNA via Rev multimerization

To demonstrate the co-localization of the fusion molecule with the HIV-1 target RNA via Rev multimerization, two independent Rev binding reactions using a labeled fusion RNA and an unlabeled 933nt HIV-1 target RNA were carried out as explained in Methods3b (mixing of two independent binding reactions). In Panel A, 790 nM of unlabeled 933 nt HIV RNA and 1380 nM of labeled 7637 fusion molecule (1:1,7 ratio) were used. Assays were also performed in the absence of protein and in the presence of Rev and BSA (bovine serum albumin) proteins. In each independent binding reaction, the concentrations of Rev and BSA were 100 ng. After mixing, the total amounts were 200 ng for each protein.

Lines 3 and 4 in Figure 21 demonstrate our hypothetical complex formation, which includes HIV-1 RNA, the fusion RNA with the RBE sequence and the Rev protein. The stronger band for the 7637 ribozyme could be due to the fact that (i) the affinity of the 7637 ribozyme for Rev binding is higher than that of 7637 fusion molecule; and (ii) the ribozyme molecule (80nt) is shorter than the fusion molecule (119 nt.) Thus, more ribozyme molecules can be shifted compared to the RNA-RBE fusion molecule. The absence of these shifted bands in the other lanes (no protein, BSA and other control lanes) (lanes 1, 2, 5, 6, 7 and 8) indicates that the bands formed as a result of specific binding of Rev to both molecules.
Panel B in Figure 21 was carried out exactly as in Panel A. The only difference is that the HIV-1 RNA-to-fusion molecule ratio was 1:1 (870 nM : 870 nM).
Figure 20. Rev Binding assays with labeled 7918, 7637, and 7678 ribozymes and fusion molecules.

This figure shows that addition of RBE sequence to the Ribozyme molecule does not significantly change the Rev binding pattern. Although we used the RBE sequence, which is critical for Rev binding, additional sequences from the RRE may be required to obtain the correct RBE secondary structure in this context. 18 is 7918 rbz and fusion molecule; 37 is 7637 rbz and fusion molecule; and 78 is 7678 rbz and fusion molecule.
<table>
<thead>
<tr>
<th>Ribozymes</th>
<th>Fusion Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Rev</td>
<td>-Rev</td>
</tr>
<tr>
<td>18 37 78</td>
<td>18 37 78</td>
</tr>
<tr>
<td>+Rev</td>
<td>+Rev</td>
</tr>
<tr>
<td>18 37 78</td>
<td>18 37 78</td>
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</table>
Figure 21. Rev binding assays with labeled 7637Rz, labeled 7637 fusion molecule and unlabeled 933 nt HIV-1 RNA.

Lanes 3 and 4 demonstrate that co-localization of 7637 ribozyme and 7637 fusion molecules with unlabeled 933 nt HIV-1 RNA takes place. In Panel B the hypothetical complex formation appears much strongly. Rz+R is ribozyme plus Rev protein; S+R is substrate (unlabeled 933nt HIV-1 RNA) plus Rev protein; 37 Rz is 7637 ribozyme; 37 FM is 7637 fusion molecule, marker is labeled 933 nt HIV RNA.
Co-localization experiments via Rev multimerization were also performed with labeled 7637 ribozyme, labeled 7637 RNA-RBE fusion molecule and labeled HIV-1 target RNA (all-hot method). After the mixing and incubation at 37°C for 30 minutes, the samples were electrophoresed in 3.5% native polyacrylamide gel. Gel pieces, which represent complex formation, were cut out (data not shown) and recovered from the gel in elution buffer by shaking overnight. After phenol chloroform extraction and alcohol precipitation, isolated RNA was electrophoresed in 5% denaturing polyacrylamide gel. Figure 22, Panel A and B, demonstrates co-localization of HIV-1 RNA with the 7637 ribozyme and 7637 fusion molecule. Although there were 10:1 and 20:1 ratios of fusion molecule to HIV-1 in the binding assays, no increases in the co-localization were observed (Panel A). Thus, there appears to be saturation of ribozyme binding to the target RNA at these ratios. However, there was an increase in the co-localization of the 7637 fusion molecule when the ratio was increased from 2:1 to 10:1 (Figure 22 Panel B). Since the 7637 ribozyme had a higher cpm than the 7637 fusion molecule, co-localization of the 7637 ribozyme with HIV RNA is higher than the fusion molecules.

Since 7918 and 7678 ribozyme and fusion molecule did not show a clear co-localization with “cold-hot” method (Figure 23), this “all-hot” experiment was not performed for the 7918 and 7678 ribozymes and fusion molecules. Even if there were a positive result in the “all-hot” method for 7918 and 7678 ribozyme and fusion molecules, there would be no scientific reason for this (which means that it is working in one way but not working in the other way).

The results of “all-hot” method (Figure 22) confirm the results (co-localization) of the “cold-hot method” seen in Figure 21.
Figure 22. Rev Binding assay with labeled HIV-1 RNA, labeled 7637 Rz and labeled 7637wt and mt fusion molecule

Co-localization experiments via Rev multimerization were also performed with labeled 7637 ribozyme, labeled 7637 RNA-RBE fusion molecule and labeled HIV-1 target RNA (all-hot method). After the mixing and incubation at 37°C for 30 minutes, the samples were electrophoresed in 3.5% native polyacrylamide gel. The band corresponding to complex was eluted from the gel, and re-electrophoresed by 5% denaturing Polyacrylamide gel to visualize components of the complex. S is 933nt labeled substrate; R is Rev protein; wtFM is wt7637 ribozyme plus RBE; mtFM is mt7637 ribozyme plus RBE; RBE control is 119 nt 7637 ribozyme-RBE fusion RNA; Rbz control is 80 nt rbz alone RNA.
Panel A

Rbz control
RBE control
S Control
S+R
(Rz+R)+(S+R)
(wtFM+R)+(S+R)
(wtFM+R)+(S+R)
(mtFM+R)+(S+R)
(mtFM+R)+(S+R)

Panel B

S+R
(Rz+R)+(S+R)
(wtFM+R)+(S+R)
(wtFM+R)+(S+R)
(mtFM+R)+(S+R)
(mtFM+R)+(S+R)
S control
Since we have obtained positive results for the 7637 ribozyme and 7637 RNA-RBE fusion molecule in co-localization experiments with HIV-1 RNA (Figure 21), and since the 7918 and 7678 fusion RNA molecules bind to Rev (Figure 20), we also performed the Rev binding assay for co-localization of the 7918 and 7678 fusion molecules with HIV-1 RNA.

The 7918 fusion RNA molecule demonstrates gel shifts with Rev (Figure 23, lane 3). The absence of gel shifts with unlabeled 933 nt target HIV-1 RNA at the 933 nt level, and the presence of strong bands at the lower molecular weights indicates that some co-localization occurs with HIV-1 RNA, but it most probably happens with some of its truncated forms. We have not obtained any gel shift bands at the 933 nt level and even at the lower level with 7678 fusion RNA molecule (Figure 23, lane 5).
Figure 23. Rev Binding assays with 7918 Rz, 7918 fusion molecule, 7678 Rz, 7678 fusion molecule and unlabeled 933 nt HIV-1 RNA.

Co-localization experiments with HIV-1 RNA, 7918 and 7678 fusion RNA molecules (two exposures). While no shifts are seen with the full-length 933 nt RNA, some shifts are observed in lane 3, probably with truncated forms of the RNA (To able to see the top of the hot marker much clearly, lesser exposure form of gel picture was placed next to higher exposure form).
IV. DISCUSSION

The HIV-1 still plays a central role in AIDS progression and thus a strong rationale for the development of effective, long-term antiviral therapy has been reaffirmed. Since all genes are expressed through RNA intermediates, anti-HIV-1 gene therapies employing ribozymes have gained important consideration as a possible treatment for AIDS. Previous studies have demonstrated that ribozymes can be effective in inhibiting virus replication in human T cell lines (Michienzi et al., 2000; Westaway et al. 1998; Yamada et al. 1994; Yu et al; 1993; Sarver et al., 1990). However, because of the high rate of viral replication, the possibility of escape mutants is still a major issue with anti-HIV gene therapy. To increase further the antiviral potency of the ribozyme vector, as well as reduce the chance of viral escape mutants, Yamada et al., (1996) has explored the possibility of adding other antiviral genes to ribozyme constructs.

Rev, an early gene product of HIV, controls the expression of the HIV-1 structural genes through binding to an RRE present in unspliced or partially spliced viral transcripts and facilitates the nuclear export and utilization of such transcripts in the cytoplasm (Felber et al., 1989; Kjems et al., 1991; Malim et al., 1989). Previous studies have shown that ribozyme/target co-localization increases the efficiency of target down-regulation in vivo (Sullenger and Cech, 1993; Samarsky et al.,1999 ). Like Yamada et al. (1996) we reasoned that Rev-binding sequences attached to a ribozyme could co-localize with a target containing the RRE, via a Rev multimer “bridge”. Adding RRE-derived sequences to the ribozyme would have an independent anti-HIV effect via a decoy mechanism as well. We wished to test the feasibility of this combined approach, in which ribozymes are tethered to RRE-derived sequences in vitro.
We chose the HIV-1 RBE (stem loop IIB) of the RRE to append to the hammerhead ribozyme, based on the following experiments. Yamada et al (1996) used the entire RRE sequence as a decoy in their studies of HIV inhibition and they obtained inhibition of expression by their fusion molecule (hairpin ribozyme + the RRE) at week 15 after transfection. One potential problem is that the RRE RNA has been reported to bind one or more mammalian cellular proteins (Vaishnav et al., 1991), and such binding may induce cellular toxicity and provide a negative selection for cells expressing this fusion molecule. However, a minimal sequence consisting of the stem loop II binds Rev but no known cellular factors; consequently Yamada et al. (1996) finally used only stem loop II as a decoy molecule in their study. Based on the idea that the RBE should be good enough for the in vitro Rev binding assays and to avoid other cellular factor binding, we decided on a similar approach; three ribozymes, targeted against the HIV 7637, 7678, and 7918 sites, were tethered to the RBE to form the corresponding chimeric RNAs, to allow comparison of the relative effects of target accessibility and protein facilitation. We have tested the feasibility of this approach in vitro. Our results show that RNA-protein-RNA complex formation between two different RNA molecules containing the HIV-1 RBE molecules via Rev multimerization can take place, thus co-localizing ribozymes with the HIV target RNA in vitro.

As a first step, we have determined the most accessible sites for hammerhead ribozyme targeting in a region of the HIV-1 envelope gene, which encodes gp41 and gp120 envelope proteins and harbors the RRE of HIV-1. Currently there are some in vitro techniques for accessible site screening. However, as pointed out in the Introduction, ideal screening will be in intact cells or cell extracts harboring target RNAs of interest. Since random approaches (AS ODN and ribozyme libraries) provide the greatest range of
possibilities, they are preferred over more directed approaches. However, they take longer to perform and are much more costly, without any great gains in sensitivity. Therefore, methods need to be developed that allow rapid, unambiguous identification of ribozyme target sequences. Once such sites have been identified, specific ribozymes must be tested efficiently in the appropriate cell culture or an animal model (Rossi 1999). By using a defined antisense oligonucleotide and RNaseH mapping technique we have investigated the region of the HIV-1 RRE between 7250 nt and 8131 nt of HIV-1 NL4-3. This study is the first investigation in this region using antisense probing on HIV RNA to find the most accessible sites for ribozyme targeting. Our results demonstrate that the region surrounding position 7637 site is the best candidate for ribozyme targeting.

As a second step, we measured Rev binding to both the 933 nt RRE-containing substrate and the ribozyme-RBE fusion molecule. We have demonstrated that the HIV-1 Rev protein binds to the 933 nt transcribed substrate very specifically; however, Rev binding to the 119 nt ribozyme-RBE fusion molecule is not as specific. As seen in Figure 19, tRNA competes effectively with the chimeric fusion molecule for Rev binding. Daly et al., (1993) also observed that Rev binds to antisense RNA with high affinity in the absence of excess unlabeled RNA. Our results support their explanation that common, nonspecific recognition motifs may exist on many folded RNA molecules including antisense RNA and tRNA. In addition, Hemmerich et al. (1997) also reported that human ribosomal protein L7 demonstrates specific binding to its labeled and unlabeled L7 mRNA as well as ribosomal RNA (rRNA) and poly(A)-rich mRNA. These data demonstrate that sometimes tRNAs, rRNAs or some RNA homopolymers that are mostly used as nonspecific competitors, can be specific competitors as well.
Interestingly, the A Box of the tRNA molecule has a striking similarity to the GUA—GG loop region of the HIV-1 RBE (Figure 24 and Figure 31). Such binding to tRNAs may not be significant *in vivo*, especially in light of our observation that tRNA is a poor competitor of the 933 nt substrate. Interestingly, Lee et al. (1992) reported that when a chimeric tRNA-RRE was expressed in CEM SS cells, viral replication was inhibited by more than 90%.

Rev is a member of a class of RNA binding proteins characterized by the presence of an arginine-rich motif (Lazinski et al., 1989 and Zamore et al., 1990). Based upon their results Zapp et al. (1991) and Olsen et al. (1990) concluded that Rev is an oligomer in the absence of the RRE. Two other proteins containing the arginine-rich motif, Tat (Frankel et al., 1988) and Gag (Trono et al., 1989) also appear to be oligomers. Oligomerization may thus be a general feature of this class of RNA binding proteins.

On the basis of their results as well as previous studies, Daly et al., (1993) suggest a mechanism of action for Rev protein as follows: The arginine-rich nucleolar localization domain provides nearly all of the free energy of binding of Rev to the RRE through a number of contacts. Arginines, however, have previously been shown to interact with high affinity with guanine residues, an observation that explains why Rev interacts with high affinity with many RNA fragments. (Helene 1977; Porschke, 1978; Seeman et al., 1976). Arginines have also been shown to interact with phosphate groups on bulged or looped regions of RNA (Calnan et al., 1991). The fundamental discrepancy of a how a protein with equivalent affinities can discriminate the RRE from large excesses of nonspecific binding sites *in vivo* may be explained through this interaction. This could also explain why Rev binds to RRE, antisense RNA and tRNA with high affinity *in vitro*, each which possess guanine-rich sequences. The high affinity of the arginines for guanine
residues may play a role in searching for the correct target binding sequence on the nascent mRNA.

In this regard, we examined the guanine content of our constructs, (expressed as # of guanine / total # of nucleotides) which are: 7637 fusion molecule, 40/119; 7678 fusion molecule, 34/119; 7918 fusion molecule, 37/119; 7637 ribozyme, 25/80; 7678 ribozyme, 19/80 and 7918 ribozyme, 22/80. When we looked at the folding picture of each RNA construct, some of the salient features are the following:

In the folding prediction of the 7637 ribozyme and fusion molecule (Figures 25 and 26), we see a purine-rich loop containing 5 Guanines and 3 Adenines. Interestingly, comparison of the predicted folding of the 7637 ribozyme and 7637 fusion molecule shows that the ribozyme motif of the 7637 fusion molecule remains the same when the RBE sequence is added. This shows that the secondary structure of the 7637 ribozyme is very stable. In addition, two G nucleotides (Figure 24) that form the high affinity- bubble region of the fused RBE molecule in the 7637 fusion molecule disappear. In this regard, Heaphy et al., (1991) reported that deletion of these two guanines or replacement with adenine residues abolished specific Rev binding. These observations may serve to explain why we also obtained gel shifts with the 7637 ribozyme alone, and also why this binding was better than that observed with the 7637 fusion molecule. Because the 7637 ribozyme is shorter than the 7637 fusion molecule, and because no additional high affinity site can form in the predicted secondary structure of the 7637 fusion molecule, gel-shift bands with the 7637 ribozyme are stronger than those formed with the 7637 fusion molecule (Figure 21).

When we investigated the potential folding patterns of the 7918 ribozyme and its fusion molecules (Figure 27 and 28), there were no purine-rich loops in the predicted
folding of the 7918 ribozyme (the top hairpin loop could be candidate but it contains only one guanine nucleotide and purine and pyrimidine nucleotide numbers are equal). However, the small central loop of the 7918 fusion molecule could be a candidate. Because it contains two guanines and three adenines in the loop region, and while the total purines number five, the pyrimidines number four in this region. This loop could explain the weak shifts at the higher molecular weight (933 nt) and the strong shifts at the lower molecular weights in the co-localization assays with the 7918 fusion molecule (Figure 23).

The predicted folding of the 7678 ribozyme and fusion molecule generates one hairpin loop, which contains one guanine and four adenines (Figure 29 at right bottom) in the 7678 ribozyme. This loop could bind Rev and thus could account for the binding of Rev to the 7678 ribozyme observed in Figure 20. The absence of gel shift bands corresponding to 933 nt fragment with the 7678 fusion molecule in Figure 23 could be explained by the disappearance of the guanine nucleotide from the loop after RBE addition to the 7678 ribozyme (Figure 30).

Consequently, the predicted folding profiles give a reasonable hypothesis for our in vitro results. However, even though there are many studies (Jain et al., 2001; Charpentier et al., 1997; Mann et al., 1994; Heaphy et al., 1991) which use the folding of the RRE and have high correlation between predicted folding and experimental results, without mutational analyses it cannot be convincingly stated that the differential binding of Rev to the 7637 ribozyme and RNA-RBE fusion molecules as well as for other constructs is due to the proposed structural determinants, which are purely based upon computer assisted folding.
As pointed out in the Introduction, multimerization, while clearly essential for Rev function, remains poorly understood at the molecular level. While some studies indicate that oligomerization may be a prerequisite for RRE binding (Olsen H.S., et al. 1990 and Zapp, M.L., et al. 1991), others suggest that monomers of Rev can bind the RRE and subsequently oligomerize on the RNA, and that it is this substrate-dependent oligomerization which is essential for function (Cook, K.S., et al. 1991 and Malim, M.H., 1990). Either of these mechanisms (or a combination of the two) could serve for the co-localization of ribozymes with HIV target RNAs as Rev monomers oligomerize and bind to the RBE molecules simultaneously in the fusion molecule and HIV-1 genome.

*In vivo* formation of the multimeric Rev:RRE ribonucleoprotein complex is facilitated by bridging of a cellular cofactor for Rev that likely interacts with multiple Rev activation domains (Bogerd and Greene, 1993; Madore et al., 1994). Thus, the possible contribution of bridging factors (unidentified cellular proteins) in the multimerization may also play an additive role in co-localization *in vivo*.

The nucleolar localization properties of Rev and Tat proteins have been demonstrated (Cullen et al., 1988; Luznik et al., 1995; Dundr et al., 1995 Endo et al., 1989; Siomi et al., 1990; Stauber et al., 1998). Michenzi et al (2000) demonstrated accumulation of an anti-HIV ribozyme in the nucleolus and potent inhibition of HIV-1 replication using a hammerhead ribozyme. On the basis of these results, they speculate that the assembly of a ribonucleoprotein particle containing the HIV-1 RNA and the two HIV-1 regulatory proteins along with other cellular factors may take place in the nucleolus. This ribonucleoprotein could be involved in the export and subsequent translation or packaging of HIV-1 RNA in the cytoplasm. In the light of this, we can speculate that this ribonucleoprotein complex formation could originate directly from
oligomerization of Rev and Tat proteins or from facilitation by oligomerization of Rev and Tat proteins along with other cellular factors during trafficking through the nucleolus. Thus, the weak ribonucleoprotein complex formation observed in our \textit{in vitro} experiments could be much stronger during cotrafficking of HIV-1 RNA and ribozyme-RBE fusion molecule RNAs through the nucleolus. Formal proof of the co-localization of the ribozymes with HIV-1 RNA via Rev multimerization will require determinations of the cellular localizations of these fusion RNA molecules and Rev-bound ribonucleoprotein complexes by in situ hybridization or in situ RT-PCR coupled with immunofluorescence assays.

In conclusion, our study has demonstrated that multimerization of the HIV Rev monomers can co-localize ribozyme and HIV RRE containing target transcripts. Based on these \textit{in vitro} data it can be argued that multimerization of HIV Rev is a potential contributing factor for co-localization of ribozyme and HIV RNAs. This multimerization most likely takes place simultaneously with trafficking of these RNAs from the nucleus to the cytoplasm. \textit{In vivo}, the extent of multimerization is unknown. Judging from our \textit{in vitro} data, it appears to be weak. Together with co-trafficking, this weak oligomerization could prove to be significant. In terms of bringing two molecules together through the Rev multimerization, there is no study to date, which directly addresses this point other than the present study.
Future Directions

Our results from accessibility and Rev binding assays demonstrated that just as the 7637 site is the most accessible site for ribozyme targeting in HIV, so ribozyme and fusion molecules targeting the 7637 site are also the best constructs for co-localization with HIV RNAs *in vivo*. Thus, the 7637 ribozyme and fusion molecule constructs are the best candidates for inhibiting HIV replication. Using a retroviral such as pMND or pol III vector such as U6+27 to express the fusion molecules, the anti-HIV capacity of these constructs should be tested in the appropriate cells.

By using antisense oligos to identify additional ribozyme cleavage sites, along with RNA molecules that contain either the ribozyme alone or a ribozyme+RBE sequence, it should be possible to identify additional optimal combinations of ribozymes and a Rev decoy for testing in gene therapy settings.

Daefler et al. (1990) have shown that the first 77 nucleotides of the RRE (LT-D1), which produce the first (or upstream) complementary half of the stem loop I and the first (or upstream) complementary half of the stem loop IIA and entire stem loop IIB, are sufficient to bind the Rev protein *in vitro*. By using the same construct, Vainshnav et al. (1991) checked whether the first 77 nucleotides could functionally substitute for RRE in a Rev dependent heterologous gene expression assay. LT-D1 did not confer Rev responsiveness to the CAT reporter gene even at Rev concentrations that could maximally stimulate RRE-linked CAT gene expression in HeLa cells, suggesting that RRE sequences 3’ to LT-D1 are also important for trans-activation. After these two studies, Yamada et al. (1996) used entire stem loop II of the HIV-1 RRE and Lee et al. (1992) used stem loop IIA and IIB except IIC in their *in vivo* studies using the same combined approach and their constructs were able to inhibit HIV replication. The
inhibition observed in the Lee et al. (1992) study was purely due to a decoy mechanism whereas in the Yamada et al. (1996) study it could be also due to enhancement of ribozyme activity by the co-localization via Rev multimerization. The critical point is that both studies used additional sequences adjacent to stem loop IIB to obtain specific Rev binding and inhibition. Consequently, by including additional sequences in our decoy molecule, either from stem loop II A, stem loop I, or the entire stem loop II as in Yamada et al (1996), the weak co-localization with multimerization observed in our study may be improved.

Yamada et al. (1996) used stem loop II of the RRE as a decoy molecule downstream of the hairpin ribozyme. However, based on Bertrand and Rossi et al (1994), they proposed to use of stem loop II of the RRE upstream of the hairpin ribozyme because i) the 5' half of the hairpin ribozyme does not have any intrinsic secondary structure due to its short size and reduced stability ii) stem loop II could conceivably stabilize the ribozyme molecule by adding a highly folded structure to the 5' end of the molecule. The hammerhead ribozyme used in my study is expected to have a more symmetric structure than a hairpin ribozyme, in terms of the position of the arms with respect to the catalytic core. Although RNA molecules are usually degraded first from their 3' ends by exonucleases in vivo, the use of a decoy molecule in the upstream region of a hammerhead ribozyme molecule could make a difference in terms of the secondary and tertiary structures, thereby stabilizing the transcripts.

Use of different ribozyme-substrate hybridizing arm lengths could also improve efficacy of ribozyme. In the present study our ribozymes’ arm lengths were 10 nucleotides on each side of the catalytic core. Using eight or nine base pairs in the arms
could possibly produce higher cleavage turnover rates, or affect Rev multimerization. This is a worthwhile alteration for investigation.

Simultaneous multitargeting of HIV-1 RNA from different accessible sites with ribozymes using a combined approach could also be a very potent therapy. By using just hammerhead ribozyme sequences, Chen et al. (1992) showed that the multitarget-ribozymes could potentially be effective against many HIV-1 isolates, and could slow the selection of viral escape mutants, thereby prolonging their effectiveness. Multitarget-ribozymes could possibly be much more effective with the fused decoy molecules.

Because no appropriate Rev multimerization mutant is currently available, proving enhancement of ribozyme activity with a combined approach of Rev multimerization and Ribozyme action would be very difficult in vivo. However, the following experiments could prove the hypothesis in vivo: 1) Cell culture experiments that compare the efficacy of 7637 Rbz-RBE fusion molecule (ribozymes should be both wild type and mutant forms to clarify the ribozyme effect), placebo sequence-RBE fusion molecule (placebo sequence should replace 7637 ribozyme with same length and should not have any ribozyme and Rev binding activity), 7637 Rbz alone and the RBE alone. We suggested the use of rbz-RBE (both wild type and mutant forms), placebo-RBE, the RBE alone and the ribozyme alone to better compare the inhibition effects of each of these constructs. We would expect that 7637 rbz-RBE fusion molecule would have the greatest anti-HIV effect. 2) in situ hybridization would demonstrate more rev-bound ribonucleoprotein complex formation with the 7637 Rbz-RBE fusion molecule inside the cells and 3) parallel in vitro cleavage assays would indicate significant enhancement of ribozyme cleavage.
In order to prove the *in vitro* enhancement of ribozyme cleavage of the ribozyme - RBE fusion molecule via Rev multimerization, it should be demonstrated first that ribozyme-substrate co-localization (annealing) occurs more strongly with the fusion molecule than that with ribozyme alone, followed by a clear demonstration of significant *in vitro* cleavage activity. The present study has proved that co-localization happens via Rev multimerization. As pointed out above, by using additional sequences to the RBE, co-localization could be significantly increased. The next step is to show the enhancement of the ribozyme cleavage activity via Rev multimerization. In this regard, preliminary *in vitro* ribozyme cleavage assays were actually carried out in our study (data not shown). However, conditions of *in vitro* cleavage assays need to be optimized.

In conclusion, by co-expressing a combination of different small therapeutic RNAs (ribozymes and RNA decoys) that function additively or synergistically to target multiple conserved sites of the HIV-1 genome, the possibility of the emergence of escape virus mutants can be reduced in long-term gene therapy.

Gene therapy approaches have the potential to extend the effective treatment time of HAARTs (highly active antiretroviral therapies), or allow lower dosages of standard drug treatments to ameliorate side effects and improve patient quality of life.
Figure 24. Computer predicted folding of the wild type HIV-1 RBE (obtained from Mfold 3.1)
\[ dG = -14.3 \ [\text{initially} \ -14.3] \]
Figure 25. Computer predicted folding picture of 7637 ribozyme RNA (obtained from Mfold 3.1)
Figure 26. Computer predicted folding picture of 7637 fusion molecule RNA (obtained from Mfold 3.1)
Figure 27. Computer predicted folding picture of 7918 ribozyme RNA (obtained from Mfold 3.1)
Figure 28. Computer predicted folding picture of 7918 fusion molecule RNA (obtained from Mfold 3.1)
Figure 29. Computer predicted folding picture of 7678 ribozyme RNA (obtained from Mfold 3.1)
\[ \Delta G = -17.12 \text{ [initially -19.1] } 78Rz \]
Figure 30. Computer predicted folding picture of 7678 fusion molecule RNA (obtained from Mfold 3.1)
$\Delta G = -39.32$ (initially -44.5) 78uFM
Figure 31. Nucleotide sequence of tRNA Lys3 presented in cloverleaf form
D refers to dihydrouridine (Adapted from Wilson and Abbots, 1992)
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