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Development of hammerhead ribozymes for HIV-1 gene therapy: principles and progress

Review Article

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Abbreviations: **HIV-1**, human immunodeficiency virus type-1; **FCRs**, flanking complementary regions; **nts**, nucleotides; **TAR**, *trans*-activation response; **LTR**, long terminal repeat; **Ψ**, retroviral RNA packaging signal

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Summary

Hammerhead ribozymes are small RNA molecules that can be designed to specifically recognize and cleave a target RNA. A single ribozyme can theoretically act in a catalytic manner thus cleaving more than one molecule of its target RNA. Because of their sequence specificity, ribozymes are being developed as therapeutic agents to eliminate unwanted cellular and viral RNAs. Ribozymes are being used to inhibit human immunodeficiency virus type-1 (HIV-1) replication. Promising results have been obtained by several groups using ribozymes targeted against various sites within the HIV-1 genome. This article reviews some of the factors relevant to the design of hammerhead ribozymes with a particular focus on their application in HIV-1 gene therapy.

I. Introduction

Ribozymes are small RNA molecules with endoribonuclease activity. They can be designed to cleave RNAs in a sequence-specific manner. Ribozymes base-pair with nucleotides around the cleavage site of the target RNA and catalyze the hydrolysis of a specific phosphodiester bond. There are several different types of ribozymes: the self-splicing group I and II introns (Zaug *et al*., 1986), RNase P (Guerrier-Takada *et al*., 1983), an RNA transcript from *Neurospora* mitochondrial DNA (Saville and Collins, 1990), the hepatitis delta virus (Wu *et al*., 1989), the hammerhead ribozyme (Foster and Symons, 1987), and the hairpin ribozyme (Haseloff and Gerlach, 1989).

The hammerhead ribozyme is the smallest of the above ribozymes. It was first discovered in small circular pathogenic RNAs such as the satellite RNA of the Tobacco ringspot virus (Buzayan *et al*., 1986) and the Avocado sunblotch viroid (Hutchins *et al*., 1986). In its natural setting, the ribozyme motif has evolved to mediate a single turnover reaction *in cis*. However, to have therapeutic applications, the ribozyme must cleave its target RNA *in trans* and in a catalytic manner. The hammerhead ribozyme could be adopted to cleave *in trans* by separating the ribozyme and substrate motifs (Feder and Uhlenbeck, 1992; Hasseloff and Gerlach, 1988; Uhlenbeck, 1987). The *trans*-cleaving hammerhead ribozyme (**Fig. 1**) contains a catalytic domain consisting of 11 conserved nts and two flanking complementary regions (FCRs). The catalytic domain consists of 11 nts that are highly conserved. This region also contains a stem-loop. The sequence and length of this stem-loop can vary except for the innermost G-C base pair, which is conserved. Target RNA specificity is provided by the FCRs on either side of the catalytic domain. FCRs are designed to contain sequences that are complementary to those surrounding the cleavage site within the target RNA. These FCRs allow the ribozyme to recognize and hybridize with the target RNA. Cleavage of the target RNA occurs 3' to the NUH sequence (where N is any nucleotide, and H is any nucleotide but G), although not all NUH sites are cleaved equally well (Foster and Symons, 1987).

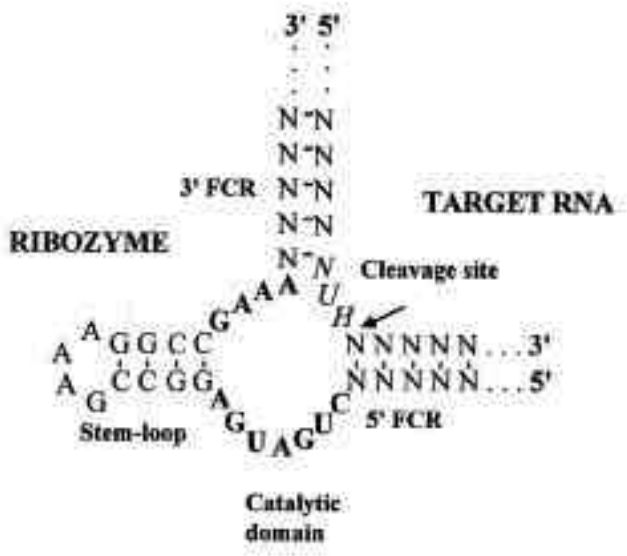

Fig. 1. Schematic representation of a *trans*-cleaving hammerhead ribozyme. The conserved nucleotides within the catalytic domain are indicated in boldface. The cleavage site within the target RNA is italicized. N represents non-conserved nucleotides within the 5'/3' FCRs. The arrow points to the site of cleavage.

Structural and functional characteristics have led to the development of hammerhead ribozymes that can cleave *in trans*. Factors which affect ribozyme-mediated cleavage of cellular or viral RNA have also been investigated. This review discusses factors affecting hammerhead ribozyme activity and provides the progress to date for ribozyme-mediated inhibition of HIV-1 replication.

II. Ribozyme design

A. Flanking complementary regions (FCRs)

In vitro studies suggest that the catalytic activity of hammerhead ribozymes with symmetrical FCRs is greatest if the 5' and 3' FCRs contain 6 to 8 nts (Fedor and Uhlenbeck, 1990; Goodchild and Kohli, 1991). However, short FCRs may not allow the ribozyme to specifically associate with the target RNA *in vivo*.

The effect of the length of the 5' and 3' FCRs on ribozyme activity have been studied and compared both *in vitro* and *in vivo*. Crisell *et al.* (1993) designed and tested a set of ribozymes targeted against the first coding exon of HIV-1 *tat*. These ribozymes were designed to contain either symmetrical 5'/3' FCRs containing 9/9, 12/12, 15/15, 18/18, 21/21, 24/24, 27/27, 30/30, and 33/33 nts or asymmetrical 5'/3' FCRs containing 45/70 and 45/564 nts. Optimum activity *in vitro* was observed with ribozymes containing 9/9 nt-long 5'/3' FCRs, whereas the inhibition of HIV-1 replication was greatest with ribozymes containing ≥ 33/33 nt-long FCRs. Increasing the length of FCRs may have enhanced the ability of the ribozyme to "melt" the secondary structure of the target RNA *in vivo*. However, long FCRs could decrease the rate of dissociation of the ribozyme from the cleaved target RNA and hence reduce the catalytic activity of the ribozyme.

Tabler *et al.* (1994) constructed ribozymes with asymmetric 5'/3' FCRs against the leader-*gag* region of HIV-1. These ribozymes contained a long 3' FCR (289 nts) and a short 5' FCR which varied in length (0, 1, 2, 3, 5, 8, and 13 nts). In this study, as few as 3 nts in the 5' FCR were found to be sufficient for effective cleavage of target RNA *in vitro*. Ribozymes with 3 and 5 nt-long 5' FCRs were also shown to be effective at inhibiting HIV-1 replication *in vivo*. Although the 3' cleavage product was shown to be released *in vitro*, the 5' cleavage product could not be dissociated. Therefore, the design of asymetric ribozymes with long FCRs may decrease ribozyme catalytic activity.

The optimal length of the 3' FCR to be used with ribozymes containing the 3 nt-long 5' FCR has been investigated using a set of ribozymes against the HIV-1 *tat* coding region (Hormes *et al*., 1997). These ribozymes contained a 3 nt-long 5' FCR and variable length 3' FCRs (20, 25, 31, 35, 41, 45, 51, 60, and 195 nts). All ribozymes were shown to be active *in vitro*. A minimum 51 nt-long 3' FCR was found to be necessary to inhibit virus replication in a microinjection experiment where HIV-1 provirus DNA and ribozyme RNA were injected into the nucleus of human cells.

B. The catalytic domain and the stem-loop region

Since most nucleotides in the single-stranded regions of the catalytic domain are highly conserved, efforts have been

made to modify the stem-loop region and determine its effect on cleavage. While decreasing the length of this stem from the conventional 4 bps to 2 bps did not alter the cleavage activity, its further reduction significantly decreased the cleavage activity *in vitro* (Tuschl and Eckstein, 1993). Elongating the stem from 4 bps to 6, 10, 21 or 22 bps seemed to have no effect on *in vitro* cleavage activity of ribozymes, although this resulted in a reduction in their ability to inhibit HIV-1 inhibition (Homann *et al.*, 1994). Therefore, except for the innermost G-C bp that must be conserved, the length or the composition of remaining bps in the stem-loop region does not seem to be critical for ribozyme activity (Tuschl and Eckstein, 1993).

III. Target site selection

The choice of HIV-1 RNA that must be targeted and cleaved is critical as inactivation of not all HIV-1 RNAs would have the same impact on virus replication. Furthermore, within any given HIV-1 RNA only conserved and accessible regions should be targeted. Finally, although hammerhead ribozymes could be designed to cleave HIV-1 RNA at any NUH cleavage site, the most efficiently cleaved sites should be targeted.

A. Target RNA

HIV-1 provirus DNA transcription gives rise to over 20 distinct mRNA species in an infected host cell (**Fig. 2**). Although it has been reported that the unspliced HIV-1 RNA in the nucleus is the primary target of ribozymes (Paik *et al.*, 1997), ribozyme activity may not be exclusively limited to the nucleus and may also occur within the cytoplasm. Ribozymes were also shown to be very effective at cleaving RNA within the progeny virus (Sullenger and Cech, 1993; Westaway *et al.*, 1998). The choice of the target site within HIV-1 RNA only matters for cleavage of spliced mRNAs, as all sites are present within the unspliced HIV-1 RNA. Thus, to allow cleavage of all HIV-1 RNAs within the cell and within the progeny virus, ribozymes may be targeted against regions that are common to all HIV-1 RNAs (Joshi and Joshi, 1996).

B. Target site

The most important factor that must be considered while selecting a ribozyme target site within HIV-1 RNA is the genetic variability. Fortunately, there are numerous regions throughout the viral genome that are highly conserved in all HIV-1 isolates within a given subtype. Some of these sites are also conserved within various subtypes of HIV-1. The ribozyme target sites should be selected from these highly conserved regions. This would decrease the emergence of escape mutants since mutations within these conserved regions are likely to be genetically attenuated and/or replication defective. However, escape mutants may still arise from mutations within non-conserved regions adjacent to the cleavage site. Such mutations may alter target RNA structure and thereby prevent its accessibility to the ribozyme.

To cleave target RNA, a ribozyme must also be able to properly associate with it. However, long RNAs contain complex secondary and tertiary structures involving significant intramolecular base pairing that may reduce the accessibility of a particular target site (Fedor and Uhlenbeck, 1990; Uhlenbeck *et al.*, 1997). Single-stranded regions containing ribozyme target sites should therefore be mapped by enzymatic digestions or chemical modifications (Ehresmann *et al.*, 1987). For example, HIV-1 RNA incubation with oligodeoxyribonucleotides containing potential ribozyme FCRs followed by RNase H digestion could locate target sites within the conserved regions that are likely to be accessible for ribozyme binding (Ho *et al*., 1996; Scherr and Rossi, 1998). It may be desirable to perform these analyses on the full-length target RNA similar to what is to be cleaved *in vivo*. Computer programs may also be used to predict the most probable RNA secondary structures with minimum free energy parameters (Jaeger *et al.*, 1989), although the reliability of computer prediction for very large RNAs is still not high. Once the information on the target RNA secondary structure is available, target sites should be selected within the single-stranded regions that are accessible and highly conserved (Zhao and Lemake, 1998).

C. Cleavage site

With the exception of the AUA-cleaving satellite RNA of the barley yellow dwarf virus (Miller *et al*., 1991) and the GUA-cleaving lucerne transient streak virus (Foster and Symons, 1987), all naturally occurring hammerhead ribozymes cleave their target RNA at a GUC site (Bruening, 1990). Mutagenesis studies have been performed to determine cleavage sites that are best cleaved by the hammerhead ribozyme (Sheldon and Symons, 1989; Ruffner *et al.*, 1990; Perriman *et al*., 1992). Although initial studies led to the development of the general NUH rule (Koizumi *et al*., 1989), detailed kinetic analyses of a target RNA with all possible mutations at the cleavage site (Zoumadakis and Tabler, 1995) identified GUC as the most efficiently cleaved site. The influence of bases surrounding the cleavage triplet was demonstrated by Clouet-d'Orval and Uhlenbeck (1997) who analyzed a hammerhead ribozyme with a 10-fold higher cleavage rate than what was previously reported for hammerhead ribozymes. Mutational analyses demonstrated that the increased cleavage rate was due to the presence of an AU immediately after the GUC cleavage site within the target RNA.

Many attempts have been made to compare and identify ribozyme target sites within HIV-1 RNA that are best cleaved *in vitro* or that inhibit HIV-1 replication most

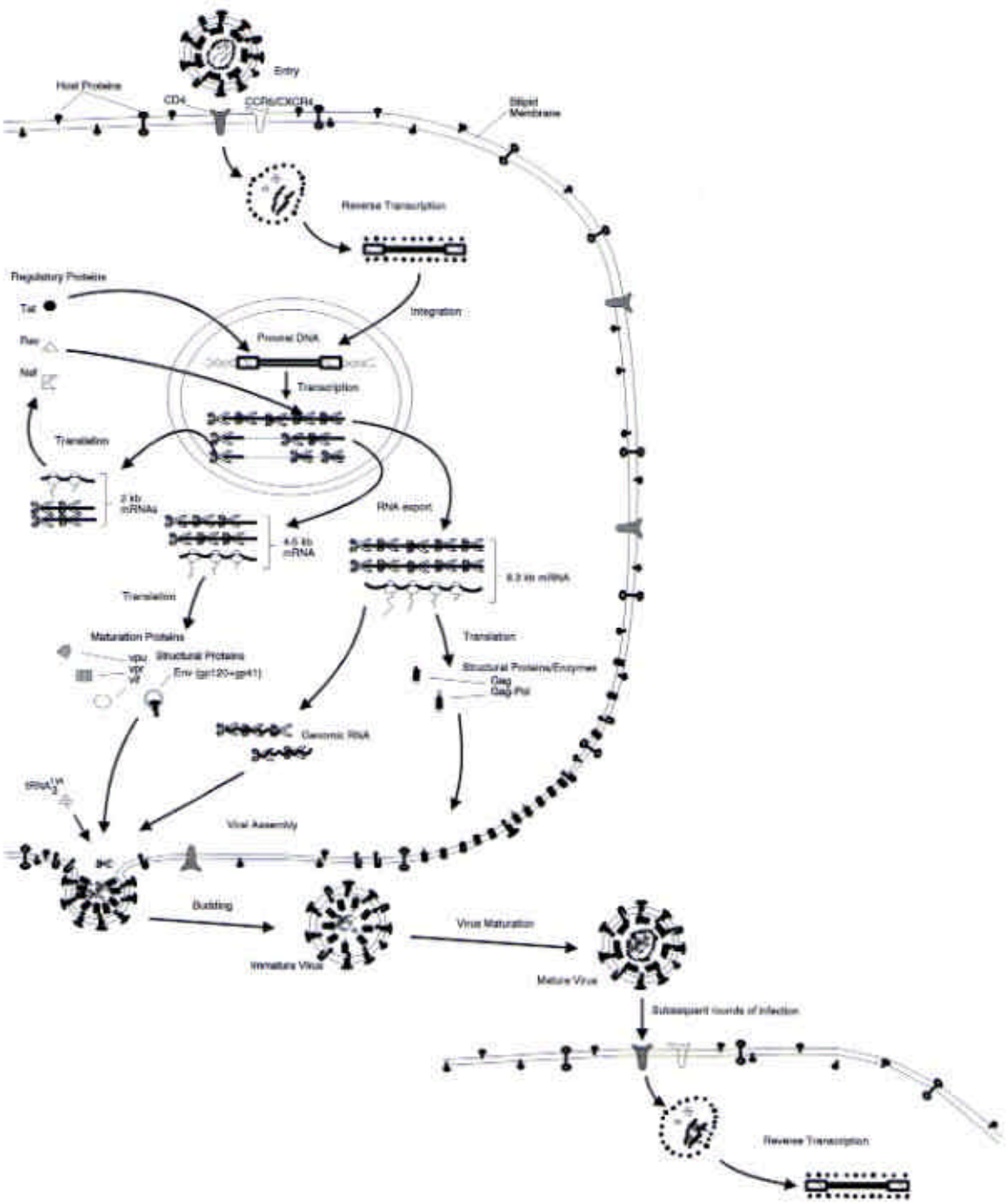

Fig. 2. Ribozyme interference sites within the HIV-1 life cycle. Following entry inside the cell, HIV-1 RNA reverse transcribes and integrates within the cellular genome. Upon transcription, the full length 9.3 kb viral RNA is produced, which is differentially spliced to give rise to various HIV-1 mRNAs. The 2 kb RNAs then give rise to Tat which enhances gene expression and Rev which allows export of 4-5 and 9.3 kb HIV-1 RNAs. Translation of these RNAs then gives rise to various structural and maturation proteins. Virus assembly then takes place and recruits 2 copies of full length HIV RNA and cellular $tRNA_3^{Lys}$. Some of the steps taking place during the subsequent round of infection are also shown. Ribozymes (✿) may cleave HIV-1 RNA in the nucleus, cytoplasm, or progeny virus such that either no virus will be produced or virus produced will be non-infectious.

Table 1 Summary of HIV-1 inhibition results obtained using monomeric hammerhead ribozymes.

Target site	Target cell (pool/clone)	Expression	HIV-1 replication (compared to controls)	Reference
R	**HeLa CD4/pool**	**Transient**	**Suppressed for 9 days**	**Dropulic and Jeang, 1994**
U5	**T-cell line/pool**	**Stable**	**Delayed for 18 days**	**Weerasinghe *et al*., 1991**
	T-cell line/pool	**Stable**	**Suppressed for 5-7 days**	**Dropulic *et al*., 1992**
	HeLa CD4/pool	**Transient**	**Suppressed for 10 days**	**Dropulic *et al*., 1992**
Ψ	**T-cell line/clone**	**Stable**	**Suppressed for 12 days**	**Sun *et al*., 1994**
RRE	**HeLa CD4/pool**	**Transient**	**Suppressed for 6-9 days**	**Dropulic and Jeang, 1994**
gag	**HeLa CD4/pool**	**Transient**	**Suppressed for 7 days**	**Sarver *et al*., 1990**
	T-cell line/pool	**Stable**	**Delayed for 9 days**	**Ramezani and Joshi, 1996**
pro	**T-cell line/pool**	**Stable**	**Delayed for 15 days**	**Ramezani and Joshi, 1996**
RT	**T-cell line/pool**	**Stable**	**Delayed for 9 days**	**Ramezani and Joshi, 1996**
tat	**T-cell line/clone**	**Stable**	**delayed for 8 days**	**Lo *et al*., 1992**
	T-cell line/pool	**Stable**	**Delayed for 6 days**	**Crisell *et al*., 1993**
	T-cell line/pool	**Stable**	**Delayed for 6-8 days**	**Zhou *et al*., 1994**
	T-cell line/pool	**Stable**	**Suppressed for 12 days**	**Sun *et al*., 1995**
	T-cell line/pool	**Stable**	**Suppressed for 9 days**	**Wang *et al*., 1998**
rev	**T-cell line/clone**	**Stable**	**Suppressed for 18 days**	**Michienzi *et al*., 1998**
	T-cell line/pool	**Stable**	**Delayed for 6-8 days**	**Zhou *et al*., 1994**
tat and rev	**LTBMC/pool**	**Stable**	**Suppressed**	**Bauer *et al*., 1997**
	T-cell line/pool	**Stable**	**Delayed for 6-8 days**	**Zhou *et al*., 1994**
env	**T-cell line/pool**	**Stable**	**Delayed for 18 days**	**Ramezani *et al*., 1996**
nef	**T-cell line/clone**	**Stable**	**Delayed up to 14 days**	**Larsson *et al*., 1996**

LTBMC: long-term bone marrow culture.

efficiently *in vivo* (**Table 1**). While targeting some sites has been found to be more effective at inhibiting virus replication than others, the *in vitro* cleavage results could not always be correlated with the results obtained *in vivo* (Dropulic and Jeang, 1994; Ramezani and Joshi, 1996). Even ribozymes with poor *in vitro* cleavage activities have been shown to significantly inhibit HIV-1 replication (Crisell *et al.*, 1993; Ramezani and Joshi, 1996).

IV. Ribozyme delivery, expression, and localization

A. Ribozyme delivery and expression vectors

Retroviral vectors are commonly used for the delivery and expression of genes (Friedman, 1989). Since these vectors are unable to transduce non-dividing cells such as hematopoietic stem cells, lentiviral vectors have recently been developed and successfully used to deliver genes into non-dividing cells (Naldini *et al*., 1996; Uchida *et al.*, 1998). Among the cells transduced with HIV-1 based vectors are the human hematopoietic stem cells (Uchida *et al.*, 1998), macrophages (Corbeau *et al.*, 1998), and terminally differentiated neurons (Naldini *et al*., 1996). The restricted host range of HIV-1, which is limited to $CD4^+$ cells, could be extended using the amphotropic envelope protein from the Moloney murine leukemia virus (MoMuLV) or the G protein from vesicular stomatitis virus (Naldini *et al*., 1996). The later envelope is also more stable and allows ultracentrifugal concentration of virions to high titers (Naldini *et al*., 1996; Reiser *et al*., 1996).

Table 2 *In vitro* selection studies aimed at improving/altering the cleavage activity of various ribozymes.

Ribozyme	# of nts mutated/ mutation rate	Results (approximate improvement compared to unselected RNA)	Reference
***Tetrahymena* group I intron**	**140/5% per position**	**100-fold improved DNA-cleaving activity**	**Beaudry and Joyce, 1992**
***Tetrahymena* group I intron**	**140/5% per position**	**170-fold improved catalytic activity utilizing an altered metal cation**	**Lehman and Joyce, 1993**
RNase P	**9/random**	**30-fold improved catalytic activity**	**Yuan and Altman, 1994**
Hairpin	**50/3 mutations per molecule**	**20-fold improved *trans*-cleavage activity**	**Joseph and Burket, 1993**
Hammerhead	**14/random**	**Consensus activity**	**Ishizaka *et al.*, 1995**
Hammerhead	**4/random**	**Less efficient than consensus**	**Thomson *et al.*, 1996**
Hammerhead	**10/random**	**Less efficient than consensus**	**Vaish *et al.*, 1997**

Ribozyme genes are expressed form the retroviral long terminal repeat (LTR) promoter and/or from internal promoters. However, internal promoters often function poorly when inserted downstream of LTR promoters due to transcriptional interference between promoters (Emerman and Temin, 1984). Optimum functional expression of ribozyme genes has been shown to occur when ribozymes are expressed as part of the long viral RNAs transcribed form the 5' LTR promoter by RNA polymerase (pol) II, rather than as part of transcripts produced form internal pol II (CMV, U1 snRNA) or pol III (tRNA, U6 snRNA) promoters (Zhou *et al.*, 1996; Bertrand *et al*., 1997). High level expression was obtained from pol III promoters when cloned within the 3' LTR (Ilves *et al*., 1996). Upon reverse transcription, this design also resulted in gene duplication within both the 5' and 3' LTRs.

B. Ribozyme localization

Proper co-localization of the ribozyme with its target RNA is essential for ribozyme activity. Pol III-driven human tRNA or U6 snRNA as well as pol II-driven U1 snRNA have been shown to allow nuclear localization of ribozymes, while pol II promoters such as the Rous sarcoma virus LTR promoter mainly localized the ribozyme to the cytoplasm (Bertrand *et al*., 1997).

Anti-HIV ribozymes may be localized to the cytoplasm to inactivate the incoming virion RNA before it is reverse-transcribed. However, hammerhead ribozymes expressed under the control of the pol II promoter were not found to inhibit the incoming HIV-1 RNA (Ramezani *et al.*, 1997; Paik *et al.*, 1997). In contrast, hammerhead ribozymes have been shown to cleave HIV-1 transcripts in the nucleus (Paik *et al*., 1997). Enhanced ribozyme activity in the nucleus may be due to certain RNA-protein interactions which increase the ability of the ribozyme to better associate with the target RNA. Nuclear extracts have been shown to improve the hybridization of complementary RNAs (Portman and Dreyfuss, 1994) and ribozyme activity *in vitro* (Bertrand and Rossi, 1994; Heidenreich *et al*., 1995).

Alternatively, ribozymes could be designed so that they would be co-packaged with HIV-1 virion RNA. Cleavage of HIV-1 RNA within the progeny virus should prevent subsequent viral spread. Sullenger and Cech (1993) used the retroviral RNA packaging signal (Ψ) to develop a packagable ribozyme and demonstrated the feasibility of this approach in a MoMuLV-based system. A chimeric $tRNA_3^{Lys}$-ribozyme was also developed and shown to be packaged by HIV-1 (Westaway *et al*., 1995; 1998). As expected, infectivity of the progeny virus was shown to be significantly decreased.

V. *In vitro* selection of ribozymes

In vitro selection involves isolation of molecules with a desired phenotype (*i.e.* altered or improved catalytic activity) from a pool of partially or randomly mutated molecules. This strategy has been successfully applied (**Table 2**) to RNase P (Yuan and Altman, 1994), the hairpin ribozyme (Joseph and Burke, 1993), and the *Tetrahymena* group I ribozyme (Lehman and Joyce, 1993; Beaudry and Joyce, 1992; Tsang and Joyce, 1994). Larger ribozymes, such as the *Tetrahymena* group I intron, appear to have a very high potential for improvement. In contrast, it seems difficult to further improve upon the catalytic activity of smaller ribozymes, like the hammerhead ribozyme. Many unsuccessful attempts have been reported for selecting hammerhead ribozymes with improved catalytic activities (Nakamaye and Eckstein, 1994; Long and Uhlenbeck, 1994;

Ishizaka *et al.*, 1995; Vaish *et al.*, 1997; Thomson *et al.*, 1996; Ramezani and Joshi, unpublished results).

Long and Uhlenbeck (1994) replaced the stem-loop region within the catalytic domain of a hammerhead ribozyme with 4 or 6 random nts and applied 3 rounds of *in vitro* selection to isolate active ribozymes. However, all selected ribozymes displayed poor catalytic activity. The most effective of these ribozymes contained 6 nts with a tetranucleotide loop and the consensus G-C base pair next to the single stranded regions of the catalytic domain.

Thomson *et al*. (1996) replaced the consensus GAA sequence within the catalytic domain with 4 random nts and used 2 rounds of *in vitro* selection to isolate ribozymes with an improved catalytic activity. However, the most active ribozymes that could be selected contained the sequence HGAA (where H is A, C, or U) instead of GAA and possessed a reduced catalytic activity.

Ishizaka *et al*. (1995) randomized all of the conserved nucleotides in the catalytic domain of a minizyme lacking the stem-loop region, and conducted *in vitro* selection to isolate active ribozymes. However, the ribozymes selected after 7 to 8 rounds of selection contained consensus sequences. Similar results were also reported by Vaish *et al*. (1997), suggesting that the naturally occurring ribozymes might have already evolved to optimum or near optimum levels.

VI. The efficacy of anti-HIV-1 ribozymes

A. Monomeric ribozymes

A variety of sites within HIV-1 RNA have been targeted by monomeric ribozymes (**Table 1**). These include coding regions such as *gag* (Sarver *et al.*, 1990; Ramezani and Joshi, 1996), *pol* (Dropuic and Jeang, 1994), *pro* (Ramezani and Joshi, 1996), *RT* (Ramezani and Joshi, 1996), *tat* (Lo *et al*. 1992; Crisell *et al*., 1993; Zhou *et al*., 1994; Sun *et al*., 1995; Ramezani and Joshi, 1996, Wang *et al*. 1998), *tat/rev* (Zhou *et al*., 1994), *env* (Ramezani and Joshi, 1996), and *nef* (Larsson *et al*., 1996), as well as non-coding regions such as the repeat (R) region (Dropulic and Jeang, 1994), the *trans*-activation response (TAR) element (Ventura *et al*., 1994), the unique 5' (U5) region (Weeraninghe *et al*., 1991; Dropulic *et al*., 1992, Westaway *et al*., 1995), the Ψ region (Sun *et al*., 1994), and the Rev response element (RRE) (Dropulic and Jeang, 1994).

While the above studies have all demonstrated varying degrees of virus inhibition, there has not yet been any report of complete inhibition of virus replication using monomeric ribozymes. Virus replication eventually resumed despite the use of improved expression vectors or co-localization strategies. Partial inhibition was observed even in an extremely exaggerated experimental setting where a ribozyme was expressed as part of the HIV-1 RNA (Dropulic *et al*., 1992; Dropuic and Jeang, 1994). Interestingly, the break-through of virus production was not due to escape virus production, demonstrating the inability of monomeric ribozymes to completely inhibit virus replication.

B. Multimeric ribozymes

The limited success at inhibiting HIV-1 replication using the existing monomeric ribozymes calls for new strategies to further improve on the intracellular activity of ribozymes. The efficiency of the ribozyme is largely dictated by the accessibility of the target site. However, it is unlikely that any given site will be available for cleavage at all times as RNA constantly folds and unfolds and is often masked by various cellular or viral proteins. Targeting various sites within a given RNA would significantly increase the possibility of having at least one site cleaved, which is sufficient for inactivation of the target RNA. Targeting multiple sites within HIV-1 RNA should also reduce the chance of developing escape mutants in clinical settings.

Multimeric ribozymes may be designed to contain ribozymes targeted against various sites within the same or different HIV RNA molecules. Multitarget ribozymes could even be designed to target various subtypes of HIV-1 and/or HIV-2, which would potentially confer simultaneous resistance.

Multimeric ribozymes may be expressed in tandem as part of a single RNA molecule or could be flanked by *cis*-acting ribozymes such that individual ribozymes will be liberated (Chowrira *et al.*, 1994; He *et al.*, 1993; Ventura *et al*., 1993; Yuyama *et al*., 1992, 1994). Self-liberating multimeric ribozymes have been shown to be very effective at cleaving their target RNA *in vitro*, although the efficacy of these ribozymes is yet to be demonstrated *in vivo* (Ohkawa *et al.*, 1993). These ribozymes may be more effective at inhibiting virus replication because of their smaller size and their ability to act simultaneously. However, issues related to the stability and compartmentalization of individual ribozymes remain to be addressed. Furthermore, the use of *cis*-acting ribozymes limits the application of this strategy as it can not make use of retroviral vectors for efficient gene delivery.

Chen *et al.* (1992) constructed several multimeric ribozymes (mono-, di-, tetra-, penta-, and nonameric ribozymes) targeted against various sites within the *env* coding region of HIV-1 RNA. In co-transfection experiments, these ribozymes were able to confer significant inhibition of virus replication. Paik *et al*. (1997) used a defective HIV-1 DNA to express the nonameric ribozyme in HeLa T4 cells. Compared to control cells, which were highly permissive for HIV-1 and HIV-2 replication, only HIV-1 replication was inhibited in multimeric ribozyme-expressing cells.

We developed retroviral vectors expressing mono- or nonameric ribozymes against conserved sites within the HIV-1 Env RNA and compared their anti-HIV-1 efficacy in

a stably transduced T-cell line (Ramezani *et al.,* 1997). The monomeric ribozymes could only delay virus replication, whereas almost no virus production could be detected from nonameric ribozyme-expressing cells for up to sixty days (the length of the experiment). We have also tested the mono- and nonameric ribozyme expression vectors in human peripheral blood lymphocytes (PBLs). When challenged with a primary, patient-derived isolate of HIV-1 (Ramezani and Joshi, 1998), the nonameric ribozyme-expressing PBLs were shown to delay virus replication more than the monomeric ribozyme-expressing PBLs.

VII. Prospects

The hammerhead ribozyme offers a potentially effective means of inhibiting HIV-1 replication. Although the catalytic activity of the hammerhead ribozyme might already be close to optimal, a number of other factors affecting ribozyme design and activity could improve ribozyme activity *in vivo*. Promising results have been obtained using multimeric ribozymes. Combination strategies could be used to achieve even better inhibition of HIV-1 replication.

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