V Discussions
V.1. Ribozyme-Mediated Cleavage in vitro

V.1.1. Design of Ribozymes against Substrates which Assume High Secondary Structures

RNA molecules are known to assume elaborate secondary structures (Zuker et al., 1989) which forms the basis for the higher order structure. The tertiary conformations help in regulation of expression by interaction with a variety of proteins in vivo (Han et al., 1990). Human TNF-α RNA assumes a very high secondary structure which may be involved in regulation of TNF-α gene expression. It is difficult to design ribozymes against TNF-α mRNA, based on secondary structure considerations, primarily because the RNA interacts with a variety of specific (e.g. AUUUA-binding protein) as well as non-specific (nuclear) proteins (Kim et al., 1996). Binding of the TNF-α mRNA in vivo with these proteins might enhance or inhibit ribozyme-substrate interactions (Caput et al., 1986). Thus it is really a matter of chance that some ribozymes work efficiently on such substrates and some do not (Symons, 1991; Ventura et al., 1993; Ferbeyr, 1997). Design of ribozymes based on secondary structure considerations thus could be a very preliminary step towards identification of a suitable target site. Since, in vivo TNF-α mRNA is sequestered by cellular proteins (biologically serving the purpose of maintaining a very low pool of mRNA for rapid protein synthesis upon induction, Grafi et al., 1993), it becomes all the more difficult to achieve complete destruction of the target, unless of course the ribozyme is expressed at very high levels. Thus secondary structure analysis of ribozyme-target interaction should ideally be supported by testing for RNA-protein interactions or substrate-ribozyme interactions in presence of proteins. Interestingly, certain exogenous sequences confer stability to the ribozymes in vivo (Sioud et al., 1992). Such exogenous sequences could be very helpful in maintaining a pool of ribozymes in vivo for effective control of gene expression. Also, they may help in dissociation of ribozyme from the substrate, thereby increasing the catalytic rate.
Our secondary structure analysis of TNF-α RNA indicate that despite its complex structure, ribozymes could be successfully designed based on careful considerations of optimal arm length, proper binding and formation of hammerhead structure of the ribozyme at its cleavage site. Computer simulation of secondary structures indicated that ribozymes having shorter arm lengths (6, 8 & 10) could not achieve the required level of interaction with the substrate (data not shown). Ribozymes with longer than 11 nt arm lengths, however, exhibited proper binding and attainment of active conformation, but were not considered since $K_{cat}$ for such ribozymes could be sub-optimal.

V.1.2. Specificity of Hammerhead Ribozyme Cleavage
To be effective in gene inactivation, the hammerhead ribozyme must cleave the complementary RNA target without deleterious effects from cleaving non-target RNAs that contain mismatches and short stretches of complementarity. The length of the ribozyme-target helices is predicted to be critical for the specificity of cleavage (Hershlag, 1991). If the helices are too short, identical sequences present on non-target regions may be cleaved as efficiently as the target RNA. If the helices are too long, initial binding will be too strong and the ribozyme may also cleave the incorrect targets while requiring a longer time to dissociate from the substrate (Hertel et al., 1996). In order to differentiate optimally between the correct and incorrect targets, a ribozyme requires a helix length that permits substrate dissociation to be faster than the rate of cleavage (Haseloff and Gerlach, 1988). Because of faster dissociation the incorrect sequences may be stopped from cleavage.

Kinetic models for hammerhead ribozyme actions and thermodynamic parameters predict that ribozymes with short antisense arms have a high turnover when compared to their counterparts with long arms (Bertrand et al., 1994; Hertel et al., 1994). However, ribozymes with long antisense arms have been found to be more active in vivo, at least in one case (Jaeger et al., 1989). In this connection, ribozyme directed against IL-2, which contained 16nt antisense arm was found to be significantly less active than those with 21 or 27nt arms (Sioud and Jaspersen, 1996).
In our study, ribozymes designed against TNF-α contained 11nt+11nt antisense arms. Thus, it was necessary to evaluate its specificity in recognizing the target sequence prior to its use in a cellular environment, where it encounters a wide variety of polynucleotide sequences. It is known that the hammerhead ribozymes cleave the substrates preferentially at GUC in trans configuration (Koizumi et al., 1988; Sheldon et al., 1989). However, these studies employed short substrates (typically 17mer-50mer). Thus, it was of interest to map the cleavage site on longer substrates where the structure and sequence content of the substrate could play a dominant role. The evaluation of the specificity of the ribozyme cleavage reaction is important for assessing the safety of ribozyme directed destruction of target RNA, in vivo.

Our results, employing primer extension and sequencing to map the ribozyme cleavage site on a 454nt TNF-α RNA substrate, indicate that the ribozyme cleaves exactly at the predicted nucleotide, confirming the specificity of the reaction.

V.1.3. Effect of Substrate Structure on Ribozyme Cleavage Efficiency
Seventy fold differences in cleavage rates have been reported among hammerhead sequences that contain identical core catalytic sequence but differ in stem I and stem III (Ruffner et al., 1989). The variation in Km values among hammerhead ribozymes appears to result from the propensity of some substrates to form structures that are incompatible with hammerhead assembly. If these structures can equilibrate with cleavable structures during the course of the reaction, high concentrations of RNA will be required to drive the complex formation, resulting in a correspondingly high K_m for the reaction (Koizumi et al., 1988). Some structures of the substrate are so stable that they fail to exchange into cleavable substrates. The ability of RNA to adopt stable conformations incompatible with hammerhead assembly probably accounts for the low extents of cleavage (Sheldon et al., 1989).

Our results suggest that substrate conformation is altered upon binding of the ribozyme at its target site, which led to inhibition of cleavage by one of
the ribozymes, in a multi-ribozyme reaction. Thus, the final outcome of a multi ribozyme reaction could be greatly influenced by substrate structure prior to and after ribozyme binding.

V.1.4. Protein-Mediated Dimerization of Rz762

The observed phenomenon of dimerization has no parallel study for comparison. In the present study, it was observed that dimerization of Rz762 occurs at 5ng/μl concentrations of adenosine deaminase, glucose oxidase, glucose-6-phosphatase and lactate dehydrogenase. It is surprising that such common cytosolic enzymes with specific functions should interact with the ribozyme. Analysis of crystal structures of adenosine deaminase, glucose oxidase and lactate dehydrogenase indicated no common structural motif, other than the fact that all these proteins contain a TIM barrel, initially identified in triose isomerase.

The secondary structure of the ribozymes differed from each other substantially. Rz762 had a more open structure compared to the tight stem structure of Rz316 (Figures.20 & 21). It is interesting to note that the two ribozymes differ from each other only in the substrate binding arms (stem I and stem III). Thus it is evident such interactions could be influenced by the structure of RNA. The aminoacid sequences of these proteins showed no obvious homology with known RNA binding motifs, indicating that the phenomenon involves participation of some novel structural elements. Interestingly, our study indicates that partially trypsinized proteins retained the capacity to promote dimerization, suggesting that the structural element within the protein, required for dimerization, is well protected from partial degradation and thus could be either deep seated and/or involves a very small number of amino acids. In the latter case it would not be surprising if there was no common structural motif found among these proteins, which was the case when 3D structures of adenosine deaminase, glucose oxidase and lactate dehydrogenase were analysed.

The intriguing question of the biological consequence of this type of
interaction remains unanswered. However, the existence of lactate dehydrogenase enhancing virus (LDV), lends suspicion that these common proteins could be exploited for successful establishment of viral infection. Dimerization (or other forms of isomerization) of viral RNA could be caused by enhanced levels of lactate dehydrogenase, since dimerization may not be possible at normal levels of the protein. It is interesting to note that dimerization of viral RNA is crucial in the life cycle of many RNA viruses (Plagemann et al., 1995; Goto et. al., 1998). Our experience with gel retardation and UV crosslinking of the ribozyme-protein complex indicate that it is difficult to catch the complexes at protein concentrations lower than 125ng/μl. Gel retardation experiments could not detect protein-ribozyme complexes with certainty. This indicates that the interaction is rapid and highly transient. This is also in consonance with a relatively small number of residues being involved in protein-ribozyme interaction. This could be the reason for the inability to detect dimerization in vivo, even if it occurred, at least in case of LDV. Thus common cellular proteins could act as ‘RNA Chaperones’ which can resolve certain RNA structures and make efficient trans-interactions possible.

In the present study, UV cross linking experiments indicate that multiple protein monomers might be involved in ribozyme dimerization, as the mobility of the crosslinked product was not matching the expected size of the monomeric proteins. It has been earlier observed that the NCp7 protein of HIV can melt 7-8 bases of the stem to allow RNA-RNA interactions to take place (Bertrand and Rossi, 1994) and in this process more than a single monomer is believed to be involved.

It has been observed recently that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binds tightly to TNFα ribozyme compared to a variety of other ribozymes and RNAs (Sioud and Jasperson, 1996). This activity was shown to be true in vitro and in vivo and that both commercial preparations and HPLC purified GAPDH exhibited this activity.
V.1.5. Effect of Proteins on Ribozyme Cleavage Efficiency

Specific interactions between proteins and RNA are important in many biological processes including protein translation, pre-mRNA splicing, and translational and transcriptional control. Eventhough group I and group II introns can be self-splicing in vitro, there is evidence for assistance by specific proteins in vivo (Lambowitz and Perlman, 1990; Guo et al., 1997). Similarly RNA component of RNaseP, which alone is catalytic, is found complexed with a specific protein in vitro and this provides catalytic enhancement in vitro (Reich et al., 1988). The hnRNP proteins, which coat the nascent RNA, are the most abundant class of non-specific RNA binding proteins (Dryfuss et al., 1993). Here it is relevent to term the non-specificity as rather a broad specificity because of the fact that these cell functions are carried out in a specified manner.

It has been recently shown that a retroviral nucleocapsid (NC) protein binds RNA with broad specificity (Meric et al., 1987; Khan and Giedroc, 1992; Lapadat-Tapolsky et al., 1993; You and McHenry 1993). Also, it has been shown that hammerhead ribozyme activity is enhanced by NC (Tsuchihashi et al., 1993). The increase in turnover has been attributed to effects from its strand annealing and strand dissociation activity. E.coli S12 ribosomal protein appears to aid the activities of group I self-splicing intron and the hammerhead ribozyme (Coetzee et al., 1994). T4 gene 32 protein and HIV Ncp7 bind cooperatively to nucleic acids, in a sequence independent manner through stretches of basic residues (Rocquigny et al., 1992; Surovoy et al., 1993). They bind preferentially and stably to single stranded RNA, and are thought to have helix destabilizing activity (Khan and Giedroc, 1992). Their unwinding activity can directly lead to RNA-RNA annealing, since denaturation of intramolecular structures promotes intermolecular renaturation (Karpel et al., 1982). Therefore, the annealing properties are dependent on the RNA structure and require to completely unwind and coat the RNA prior to renaturation. This annealing activity is very efficient and involves protein-protein interactions which stabilize the transition state of the annealing reaction, allowing more
RNA-RNA encounters to produce double stranded RNA (Kumar and Wilson, 1990; Casas-Finet et al., 1993).

The time interval used for measurement of protein-enhanced ribozyme activities ranged from 7 min to 45 min, although at longer incubation times there was no detectable improvements (Tsuchhaschi et al., 1993; Herschlag et al., 1994; Bertrand and Rossi et al., 1994). In the present study, since dimerization of Rz762 was observed, we expected that if these proteins aided intermolecular interaction then we would be able to detect enhancement in cleavage rate. However, we could not detect any increase in ribozyme activity at 15 min of incubation. A recent report (Sioud and Jasperson, 1996) indicates over 50% enhancement of hammerhead ribozyme (directed against mouse TNFα) catalysis by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In the same study, lactate dehydrogenase was shown to have no effect on the rate of cleavage.

In the present study, since the RNA-protein interactions have been found to be inherently quite rapid, the enhancement, if any should have been detected within 15 minutes. Sioud and Jasperson (1996) observed a 10% increase in the catalytic activity of ribozymes in presence of GAPDH, within 15 min of incubation at 37°C. It will be necessary to perform cleavage reactions over longer periods of time to decipher whether the dimeric form of the Rz762 retains catalytic activity. This would require further purification of the proteins to remove contaminating RNases.

In vivo experiments were, however, carried out with Rz762 because even if the proteins did inhibit the ribozyme activity in vitro, as a consequence of dimerization, the cellular levels of these proteins are not as high as those used in vitro. Also, since ribozyme dimerization was found to be highly dependent on the concentration of the protein, it was not as likely that it occurred in vivo.
V.2. Ribozyme-Mediated Cleavage *in vivo*

Cellular localization, stability and accessibility to the substrate are criteria for assessing the fitness of ribozymes. Ribozymes could be expressed *in vivo* from RNA pol II, RNA pol III, Viral, or other strong/constitutive (eg. CMV, T7 phage) promoters (Cameron and Jennings, 1989; Cotten and Birnsteiel, 1989; L'Huillier et al., 1992). The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. Cassettes containing promoter sequences from human U1 snRNA, U6 snRNA or tRNA genes fused to various processing/stabilizing sequences have been developed and tested (Bertrand et al., 1997). On the other hand, ribozyme expressed as part of the CAT gene, showed 70-80% inhibition of CAT activity *in vivo* in monkey cells (Cameron and Jennings, 1989).

Our results indicate that polyadenylated ribozymes can achieve substantial control of gene expression, in *trans, in vivo*. However, the efficiency of the control of gene expression is much lower that other expression strategies. Polyadenylated ribozyme could achieve 50% reduction on the TNF-α transcript. This could be due to the reason that the poly A messages, containing 5’-cap and 3’ poly A tail, are subject to rapid degradation in the cytosol, by cellular nucleases (Ford et al., 1997). In contrast to our observation, polyadenylated ribozyme RNA against HIV-1 and SIV, transcribed from pol II cassette, was found to be more efficient than when expressed as a part of tRNA, U1 snRNA or U6 snRNA (Bertrand et al., 1997). However, previous studies with other ribozymes embedded in tRNA or snRNA indicate that polyadenylated ribozyme RNA is subject to rapid cellular degradation.

Another strategy which was suggested (Ferbeyr et al., 1996) but so far not employed, is to express the ribozyme as part of the intron so that the ribozyme remains localized in the nucleus. However in the nucleus, it is expected to interact with a variety of proteins which possess broad RNA-binding specificities. The resultant interaction with these proteins could either
enhance or inhibit ribozyme ability to cleave the substrate (Tsuchihaschi et al., 1993; Bertrand and Rossi, 1994) in the cell nucleus. The present study is the first of its kind to express ribozyme embedded in the intron. The results indicate that both Rz316 and Rz762 were highly efficient in cleaving the substrate and achieved 79% cleavage (as assessed by densitometry of northern blotting results) of the target in vivo. Related studies in which nuclear localization was achieved by embedding the ribozyme in tRNA or snRNA (Young et al., 1994) indicate that the ribozyme efficiently cleaved its substrate even in presence of a plethora of RNA-binding proteins with broad specificities.

T7 based expression has been earlier used in many studies to achieve high levels of expression of ribozymes. Almost 100% inactivation of the highly expressed human growth hormone (expressed at 5000 copies/cell) was achieved by T7 dependent transcription in HeLa cells (Leiber ans Strauss, 1995). Expression of T7 directed RNA requires a T7 terminator sequence for efficient termination of the transcripts in vivo. Also, the exogenous T7 terminator sequence has been shown to confer high degree of stability to the ribozyme in vivo. Upto 60% of the ribozymes survived until 72 hrs post transfection with in vitro synthesized, terminator-containing ribozymes, in human peripheral blood lymphocytes (Sioud et al., 1992). Our study confirms the earlier observation that the T7-based expression is highly efficient in destroying the target mRNA, in vivo.

In the present study, expression of the ribozyme alongwith T7 terminator sequences led to 87% decrease in TNF-α transcript, as indicated by densitometry of northern blotting results. Elaborate comparison of relative efficiencies between ribozymes, expressed using different strategies, is limited by lack of parallel studies employing the same set of ribozymes.

V.3. Biological Significance of Control of TNF-α in vivo
TNF-α mediates a variety of cellular functions. It is thought to play a crucial role in normal immune responses as well as during infections. Of the well known functions of TNF-α, its capacity to induce apoptosis in target cells is
thought to be the most important biological function which maintains the cell numbers and deletes unwanted cells (Grell and Scheurich, 1996). TNF-α is a highly potent inducer of apoptosis in a variety of tumor cell lines in vitro (e.g. L929, WEHI164 -mouse fibrosarcoma). The biological importance of TNF-α induced apoptosis can be estimated from the fact that even picomolar concentrations of the cytokine can induce massive cell death in cell cultures (Hannun and Obeid, 1995). Success of the ribozymes, in achieving substantial destruction of TNF-α mRNA, must be assessed using highly sensitive methods which depend on TNF-mediated functions.

In our study, apoptosis was chosen as a biological marker to test the efficacy of the designed ribozymes. Since, even low concentrations of TNF-α cause detectable cell death in a highly TNF-sensitive cell line (WEHI164), it was thought to be the strongest indicator for successful ribozyme mediated depletion of TNF-α mRNA in producer cells. The combined results of northern blot, RNase protection assay, assay of cell viability and flow cytometry indicate that indeed the ribozymes are active in vivo and could diminish TNF-α in terms of apoptosis-inducing units. Expression of ribozyme was found to successfully reduce TNF-α induced apoptosis of WEHI164 cells, indicating that the chosen modes of ribozyme expression were competent to achieve substantial control of TNF-α expression in vivo. This is the first report of its kind to successfully demonstrate the reduction of TNF-mediated apoptosis by expression of TNF-specific ribozymes in vivo. Trypan blue exclusion studies in confirmation with flow cytometry results, indicate that there was an increased survival of the target cells (reaching up to near normal levels) upon ribozyme expression in TNFα secreting cell line.

V.4. Future Prospects
• The designed ribozymes promise application in diminishing TNF-α expression in conditions like rheumatoid arthritis, that are associated with elevated levels of TNF-α.
• Dimerization, as a phenomenon, need to be further studied and the
structural motifs of the proteins need to be identified.

- It remains to be tested if the dimerized ribozyme retains catalytic activity.
- His-tag purification of human TNF-α expressed in *E. coli* provides the means of large scale isolation of this important cytokine.
- The bioactivity of human TNF-α, expressed in *E. coli*, need to be tested prior its use.
- Purified TNF-α can be used to study the role of TNF-α in a variety of cellular processess.
- TNF-α fusion constructs could be generated for targeted delivery and triggering of cell death in chosen subsets of cells.