IV Results
IV.1. Cloning of cDNA for Human TNF-α

TNF-α is a major cytokine produced during inflammatory responses (Beutler and Cerami, 1986). TNF-α is primarily secreted by cells of monocyte-macrophage lineage. Although this cytokine has been shown to be cytostatic or cytotoxic to some solid tumors in vivo and several transformed cell lines in vitro, it has not been possible to delineate cellular responses to TNF-α from the combined influence of the cytokines and lymphokines that seem to be regulated by TNF-α (Reid et al., 1989). The difficulty arises primarily due to the fact that TNF-α influences the expression of various cytokines and lymphokines and so, delineation of effects of TNF-α form the combined influence of all these factors has not been possible. In vitro actions of TNF-α include cytolysis or cytostasis of tumor cell lines, inhibition of lipoprotein lipase in adipocytes, induction of acute phase proteins by hepatocytes and induction of cytokine production by T-cells. TNF-α has been implicated in several clinical syndromes, most notably septic shock and rheumatoid arthritis.

Expression of TNF-α can be induced by a variety of molecules, the most potent being bacterial lipopolysaccharide (LPS). Monocyte-macrophage cells, induced by 100 ng/ml LPS, secrete copious amounts of TNF-α within 6-10 hrs. For the purpose of cloning of cDNA for human TNF-α, peripheral blood lymphocytes were induced for 6 hrs with 100ng/ml LPS, the cells harvested and processed for total RNA isolation as described (Section III.6.). The total RNA was directly used for RT-PCR, performed according to manufacturer’s protocol (Access RT-PCR system, Promega), with minor modifications in the PCR cycling conditions (Section III.7.). Primers were designed to amplify the full length TNF-α cDNA (forward primer primer- 5’TTCTCTCCTCTCACAATGACCCACG 3’, reverse primer- 5’TTTCTCGCCACTGAATAGTAGGCCG 3’) and analysed for their PCR competence, using PCRplan (PCgene software programme, for primer design).

The RT-PCR product was purified using Wizard PCR-preps mini column (Promega) and its size was compared (Figure 1.), on a 1% agarose gel, with a standard DNA size marker (λ DNA, EcoRI-HindIII digested). The
Figure 1. RT-PCR product of human TNF-α cDNA. The RT-PCR product was purified using Wizard PCR-preps mini column and digested with Dral. The size of the product shown is 1.44kb. Lane M- Lambda EcoRI-HindIII marker; Lane 1- Bluescript vector digested with Smal(3.0kb); Lane 2- purified RT-PCR product of human TNF-α.
identity of RT-PCR product was initially confirmed by restriction analysis with PvuII enzyme before ligating in SmaI site of pStuI vector. The multiple cloning region of pStuI vector (modified pBluescript, Ramesh.S.Yadava, 1997) is flanked by T7 and T3 promoters, allowing transcription of cloned inserts.

The ligatón product was transformed into XL-1 blue competent cells and the transfectants were blue/white screened based on the colony color in the presence of IPTG and X-gal. Positive clones were screened with PvuII restriction digestion and sequence of the insert was confirmed by Sanger’s Dideoxy chain termination sequencing (fmol, Promega, USA) (Figure.2.).

The orientation of the insert allowed synthesis of TNF-α RNA using T3 promoter. This construct (named pStuI-hTNF, Figure.3.) was used for in vitro run-off transcription to synthesise TNF-α sense/antisense RNA using T3 /T7 promoters, respectively. The sense RNA was used as a substrate for ribozyme-mediated cleavage analysis and the antisense RNA was used as a full length riboprobe for Northern blotting and RNase protection assays. The complete nucleotide and protein sequence of human TNF-α is shown below:

**IV.1.1. cDNA and Protein Sequence of Human TNF-α**

Ribozyme cleavage sites are indicated by an *arrow*.

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<th>ATG AGC ACT GAA AGC ATG</th>
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<tr>
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<td>Cys Leu Leu His Phe Gly Val Ile Gly Pro Gln Arg Glu Glu</td>
<td></td>
</tr>
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</table>
Figure 2. Sequencing of clones containing human TNF-α cDNA in pStuI. The sequenced portions from these and other constructs exactly matched the published sequence (Wang et al., 1985) given in section 4.1.1. Reaction I and II are from a deletion clone (1-1107 TNF-α cDNA), I-clone 1, II-clone 2, the reaction was done with T7 promoter primer so the sequence read is the complement of TNF-α(1014-1107); reaction III is from the clone containing 1-1440 of TNF-α cDNA, the sequence was read with T3 promoter primer so the sequence appears as the sense strand of TNF-α cDNA (182-340).
Figure 3A. Construction of vectors for generation of ribozymes and TNF-α transcript in vitro.
TCCCAGG GAC CTC TCT CTA ATC AGC CCT CTG GCC CAG GCA
Ser Pro Arg Asp Leu Ser Leu Ile Ser Pro Leu Ala Gln Ala

GTC AGAT CAT CTTCTCT CT CGA ACC CCG AGT GAC AAG CCT GTA GCC
Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala

CATGTTGTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG
His Val Val Ala Asn Pro Gln Ala Gln Gly Gln Leu Gln Trp

CTGA ACC CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG
Leu Asn Arg Arg Ala Ala Leu Leu Ala Asn Gly Val Glu

CTGAGAGATAAAC CAG CTG GTG GTG CCA TCA GAG GGC CTG TAC
Leu Arg Asn Asn Gln Leu Val Val Pro Ser Gly Leu Tyr

CATGTTGTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG
His Val Val Ala Asn Pro Gln Ala Gln Gly Gln Leu Gln Trp

Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro

TCC ACC CAT GTG CTC ACC CAC ACC ATC AGC CGC ATC GCC
Ser Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala

GTC TACCAG ACC AAG GTC AAC CTC CTC TCT GCC ATC AAG
Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys

AGC CCC TGC CAG AGGGAGACC CAGAGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG
Leu Asn Arg Arg Ala Asn Gly Val Glu

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu

GAG AAGG GTG ACCG AACT CTG GCT GAG ATC AAT CGG CCC GAC
Glu Lys Gly Asp Arg Leu Ser Ala Gln Asn Arg Pro Asp

TAGAGCTGTTT TTC GCC GAG TCT GGT GCC TAC TTT GGG ATC
Tyr Leu Asp Phe Ala Glu Ser Gly Glu Val Tyr Phe Gly Ile

ATTGCC CTG TGAGGAGG AGCAAC ATCCAACCTTCCCA AACGC CT
Ile Ala Leu STOP
Figure 3B. (a) *In vitro* run-off transcription of TNF-α RNA. Two independent *in vitro* reactions are shown (I and II) using same amount of independently isolated template DNA. (b) Peak fractions of the *in vitro* transcription product of ribozymes Rz316 & Rz762.
During the course of this study, several portions of TNF-α insert sequence were confirmed using universal primers (T₇ and T₃ promoter primers) on DNA templates which contained either full-length TNF-α cDNA (pStul-hTNF) or deletion clone used for primer extension analysis (pStul-hTNF-del I). The sequenced portions exactly matched the published TNF-α sequence (Wang et al., 1985). Thus, the full-length construct was found suitable for in vivo expression studies.
IV.2. Design of Cognate Ribozymes for TNF-α mRNA

Ribozymes can be engineered to achieve substantial control of gene expression, by effectively destroying the mRNA. One of the advantages in using hammerhead ribozyme is its minimal requirement of recognition sequences for cleavage of the substrate. For a hammerhead ribozyme, the recognition sequence is just a tri-nucleotide stretch of GUX (X= C, A), the invariant nucleotides in the substrate being GU. Statistically, such a sequence would occur once in every 16 nucleotide length, in a random sequence. Thus, hammerhead ribozymes could be designed to target any RNA molecule, provided the latter’s substrate nucleotide sequence is known. The sequences that flank the cleavage site (in the substrate molecule) could be used to direct the ribozyme to a specific GUX site on the target. This can be achieved by including the sequence complement of the flanking bases on either side of the ribozyme catalytic core sequence. This sequence complement would form the two arms (stem I and stem III) of the ribozyme which binds (based on nucleotide sequence complementarity) to the specific site on the substrate. The catalytic core can perform the function of cleavage at this specific sequence and the ribozyme is now said to be targeted to one specific GUC site, from among the many such sequences in a substrate molecule.

Accessibility of the chosen GUX is an important requirement for the ribozymes, to effectively cleave its substrate in a cellular environment, following an intermolecular (trans-) interaction. Since the tertiary structure of the substrate is primarily the result of self-folding, driven by base pairing and energy minimizations, the GUX sequences could also be involved in pairing or could be made unavailable for interactions with ribozyme because of structural constraints. It has been found that the GUX sequence which is not involved in stem regions are exposed to trans-interactions (Denman, 1993). Ribozymes are known to gain access to these sequences in the loop/ bulge regions and cleave their substrates efficiently.
In the present study, ribozymes were directed to cleave GUC sequences. It was, thus, of interest to find out the availability of GUC sites on TNF-α. TNF-α mRNA has 12 GUC sites, out of which 8 occur within the protein coding region. The location of these sites within the coding region (87-784 nt are translated into TNF-α precursor protein) are -273, 316, 499, 568, 586, 682, 752, 762. The other 4 GUC sites occur in the 3’ untranslated region of TNF-α mRNA.

A computer-based (FOLDRNA, GCG, Wisconsin) prediction of secondary structure, for human TNF-α mRNA, was done to analyse the occurrence of these GUC sites in single stranded regions. Analysis of the secondary structure of TNFα mRNA (Figure.5.) indicated that it is a highly structured molecule and that most of the available GUC sites were occupied in stem regions, where base-pairing is expected to hinder binding and optimal folding of the ribozyme on the substrate.

Ribozyme cleavage efficiency depends both on its ability to find the target sequence and the formation of an optimal hammerhead structure upon binding to the substrate. It was, thus, necessary to simulate a trans-configuration of the ribozyme-substrate interaction to choose an optimal target GUC site. The possibility of alternative configurations being assumed by the ribozyme, when annealed with its target, was visualized by folding the RNA sequence consisting of the target RNA linked to the ribozyme sequence via an oligoU linker. Only those sites allowing the hammerhead configuration, in folded state, were chosen as a target site for designing the ribozymes.

Two different GUC sites, at positions 316 and 762, were found to be best suited for ribozyme targeting. Secondary structure predictions showed proper binding and optimal folding of the ribozyme. The FOLDRNA analysis of TNF-α and the evaluation of the two ribozymes targeted at 316 and 762 GUC are presented in Figure.6 (a and b).
**Figure 5.** FOLDRNA predicted secondary structure of full-length TNF-α mRNA. GUC ribozyme cleavage sites within TNF-α protein-coding region are indicated by arrows.
Figure 6. FOLDRNA prediction of secondary structure of TNF-α RNA complexed with the designed ribozymes, Rz\textsubscript{316} and Rz\textsubscript{762}. (a) Rz\textsubscript{316} bound at its target site (b) Rz\textsubscript{762} bound at its target site. Ribozymes bound at their respective target sites are indicated by arrows.
IV.2.1. Synthesis and Cloning of Ribozymes in a Suitable *In Vitro* Expression Vector

Ribozyme oligonucleotides, containing partial complementarity towards their 3’ ends (See below), were analysed for their secondary structure using mFOLD (GCG, Wisconsin). To avoid hairpins and self-copying, the length of the oligonucleotides were varied until predicted secondary structure showed free 3’ ends (data not shown). These 3’ ends contained a 9 base complementary region which was designed to aid formation of partial duplex. Also, the sense-oligonucleotide of the ribozyme was degenerate at one nucleotide position (A/C) so that molecules containing the C residue will effectively code for a mutant ribozyme. This nucleotide position is crucial and mutation from A to C, at this position, completely abolishes ribozyme activity.

The oligonucleotide sequences and their final conformation are indicated below.

*Catalytic Core Sequence of the Hammerhead Ribozyme* :

5’ CTG ATG AGT CCG TGA GGA CGA AA 3’

*Sequence of Ribozyme Oligonucleotides.*

316RBR - 5’ GATCTGAAGATGATC TCT GATGAG TCC GTG A 3’
316RBC- 5’ GATCGCCCAGGCAG(T/G)TT GTCCTCACGGAC T 3’
762RBR- 5’ GATCATCCCAAAGTACTG ATG AGT CCG TGA 3’
762RBC- 5’ GATCTCTGGGCAGG(T/G) TTC GTCCTCACGGACT 3’

*Final sequence of the Ribozyme Duplex Molecules:*

Rz316 complete duplex (5’- 3’)
GATCTGAAGATGATC TCT GATGAG TCC GTG A (A/C)CCGGCACGT
CCTGCCCTTCTACTACACTACTCAGGACT
CCTGCTT(T/G)GACCGAACCCTGCTAG

Rz762 complete duplex (5’-3’)
GATCATCCCAAAGTACTGATGAGTCCGTAGGACGAA(A/C)CTGCTGGGCGAT
CCTGACTTCTTCTACTACACTACTCAGGACT
CCTGCTT(T/G)GGACGGGTCTCTAG
(Note: The partial complementarity of the sense and antisense oligonucleotide pair is indicated in **bold**. The nucleotides in parenthesis indicate that the oligo was synthesised as degenerate at this position.)

The partial duplexes were then extended with T₄ DNA polymerase to form a complete duplex (**Figure.7.**). The duplex DNA was eluted, following electrophoresis in 12% nondenaturing polyacrylamide gel, and blunt-end ligated to StuI digested, CIP-treated pStuI vector. StuI restriction site occurs immediately downstream T₇ promoter, which enables *in vitro* generation of precise 5’ and 3’ end terminated transcript of the insert.

**IV.2.2. Screening of Clones for Ribozyme Inserts**

The product of ribozyme-pStuI ligation was transformed into XL-1 blue competent cells. The putative transformants were picked from IPTG-Xgal plates containing appropriate antibiotic. The white colonies indicated presence of an insert and plasmid DNA of such colonies were isolated, digested with PvuII enzyme and electrophorased on 1.6 % agarose gel to compare the size of the insert with λ EcoRI-Hind III DNA size marker. The cloned ribozyme insert is 53 bp in size and can be conveniently identified using PvuII restriction endonuclease. The insertless vector generates 2.5kb and 447bp fragments, while the ribozyme-containing clones give 2.5kb and 500bp fragments (**Figure.8.**).

Use of oligonucleotides degenerate at a critical position ensured cloning of the wild type as well as the mutant ribozymes from the same pool. The ribozyme-containing clones were sequenced to confirm the nature of the insert (wild/ mutant ribozyme and +ve/-ve orientations). The sequence confirmation of ribozyme containing clones are presented in (**Figure.9.**). The confirmed constructs were scaled up and used for further experimentation.

The ribozyme constructs for *in vitro* expression are presented in **Figure.3**.

**IV.3. *In Vitro* Transcription**

*In vitro* transcription of DNA templates, containing TNF-α and ribozyme
Figure 7. Generation of ribozyme duplex molecules from oligonucleotides with complementarity at their 3' ends. Lane 1 - RBR$_{316}$ + RBC$_{316}$; Lane 2 - RBR$_{762}$ + RBC$_{762}$; Lane 3 - T4 DNA polymerase filled complete Rz$_{316}$ duplex; Lane 4 - T4 DNA polymerase filled complete Rz$_{762}$. The duplex molecules are indicated by an arrow.
Figure 8. Screening of colonies for identification of clones with ribozyme insert. M- λ EcoRI-HindIII DNA size marker; Lanes 1-17- plasmid DNA from various colonies which were restriction digested with PvuII endonuclease. Lanes 2, 4, 6, 8, 11, 12, 15, 16 & 17 show the expected insert of 53bp.
Confirmation of mutant and wild type ribozyme inserts and their orientation by sequencing. (a) Sequencing of Rz762 clones with T7 promoter primer. The sequence indicated is of the sense strand, (b) Sequencing of the Rz316 and Rz762 using T3 promoter primer. The sequence is read as the complement of the ribozyme insert. This was done because the fragment terminating at the third A nucleotide of the ribozyme catalytic core (of GAAA) has aberrant mobility when read with T7 promoter primer. Sequence of Rz762-ve (in panel a) appears the same as Rz762 sequenced with T3 promoter primer.
inserts, were performed as described earlier (Section III.9.). The Sephadex G-50 fractions containing the transcripts are indicated in (Figure. 3 b.)

IV.4 *In Vitro* Characterization of Ribozymes Targeted against TNF-α

IV.4.1. Influence of Length of the Substrate on Ribozyme Cleavage Efficiency

Ribozyme cleavage efficiency depends on the length of the substrate RNA. Since longer targets have high degree of secondary structures, it is rather difficult for the ribozyme to gain access to the target sequence, on a substrate which is in constant flux between alternative conformations, in solution. Usually, *in vitro* cleavage reactions employ smaller target molecules (preferably < 300nt long), where the kinetics of the reaction could be studied reliably. However, since our purpose was to achieve substantial cleavage of TNF-α mRNA *in vivo*, it was thought best to test it on longer substrate.

*In vitro* run-off transcription was performed to synthesize 951nt long TNF-α RNA. 15 pmol substrate was mixed with 30 pmol of each ribozyme and the reaction carried out in 50mM Tris-HCl, 20mM MgCl₂. Analysis of cleavage products indicated that both the ribozymes specifically cleave at their designated target sites to generate fragments of expected size. Cleavage of TNF-α substrate by Rz₃₁₆ generates fragments of 789nt and 160nt. Similarly, cleavage by the Rz₇₆₂ yields fragments of 606nt and 345nt. Thus, *in vitro* cleavage of TNF-α RNA indicate that both ribozymes efficiently cleave longer substrates and are specific to their targeted sites (Figure. 10., Lanes 4-12). Also, the efficiency of the reaction remained unaffected at pH range 7.0-8.0.

To compare influence of length of substrate cleavage efficiency, a smaller (308 nt) substrate generated from PvuII run-off of pStu1-hTNF was used. *In vitro* cleavage of the 308nt RNA using Rz₃₁₆ indicated that the ribozyme is more efficient on smaller substrates (Figure. 10., Lanes 1-3), with over 90% of the substrate being converted to cleaved products, at 37°C within 2hrs of incubation. Thus, although the designed ribozymes were highly efficient
Figure 10. Cleavage reactions of Rz316 and Rz762 at varying pH. Substrate A (308nt) - lanes 2-4; Substrate B (951nt) - lanes 5-13. The cleavage products and their sizes are indicated by arrows. Lane C - control, 951nt TNF-α substrate+308nt TNF-α substrate.
in cleaving its substrate, the progress of cleavage reaction is greatly influenced by the length of the substrate. On longer substrates, ribozyme seems to cleave its target RNA less efficiently as compared to smaller substrates.

IV.4.2. Influence of Temperature on Ribozyme Cleavage Activity

The smaller substrate (308 nt) was observed to be cleaved more efficiently as compared to the full-length TNF-α RNA. It is generally accepted that longer substrates fold into highly structured conformations, which may hinder ribozyme binding at its target site. If this is the case, elevation of the temperature of ribozyme cleavage reaction could relax these structural constraints and possibly improve the cleavage efficiency.

*In vitro* cleavage reactions, on longer substrates, indicated that the ribozyme efficiently cleaves at temperatures of 37°C and below (Figure.11.). At higher temperatures of 42°C and 50°C, the rate of cleavage is drastically lowered (Figure.12.). To understand the influence of temperature, we tested whether the ribozyme behaved in a similar manner on smaller templates. It was observed that the rate of the reaction increased with increasing temperatures (42 and 50°C). Thus, the decrease in ribozyme activity on longer substrate, at higher temperatures, is not due to heat-denaturation of the unbound ribozyme structure nor due to decrease in ribozyme binding efficiency, as evident from the fact that at elevated temperatures the ribozyme cleaved smaller substrates with increased efficiency (Figure.13.). The longer RNA molecules might assume alternative structures involving the target sequence at elevated temperatures.

Alternatively, elevated temperature may result in conformational changes in the ribozyme bound at the target sequence. Temperature-dependent conformational changes in the target molecule result in energy gains which leads to slight relaxations in the structure, at higher temperatures. In longer substrate molecules, these relaxations are localized phenomenon because of the negating effect of relaxations in some other regions. This local relaxation could stretch the bound ribozyme and prevent the formation of a proper hammerhead
### Table

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<td>+</td>
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<tr>
<td>Rz762</td>
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**Figure 11.** Cleavage reactions of Rz$_{316}$ and Rz$_{762}$ at 32 and 37°C. Lanes C3, 1 & 5 contain a 308nt TNF-α substrate; Lanes C4, 2, 3, 4, 6, 7 & 8 contain a 951nt TNF-α substrate.
Figure 12. $R_{762}$-mediated cleavage of 951 nt TNF-α substrate at elevated temperatures. C1 - Negative control; C2 - positive cleavage control, cleavage at 37°C. Lanes 1 & 4 - 1:2 substrate : ribozyme ratio; Lanes 2 & 5 - 1:3 substrate : ribozyme ratio; Lanes 3 & 6 - 1:5 substrate : ribozyme ratio.
Figure 13. Influence of temperature on ribozyme cleavage efficiency of a 308nt TNF-α RNA. Lane C - 308nt TNF-α RNA; Lane 1, 2 & 3 - Rz_{316} + 308nt TNF-α RNA.
structure, which is crucial for its activity. On smaller templates, the relaxations are more global and result in increased exposure of the target bases for intermolecular interactions. Thus, it can be inferred that the ribozyme retains its ability to gain access to the target site at elevated temperatures, but the disruption of the ribozyme structure and subsequent decrease/loss of activity, is a result of the temperature-related conformational changes in the substrate.

IV.4.3. Multiple Ribozyme-Mediated Cleavage of TNF-α Substrate

The use of multiple ribozymes against a substrate has gained popularity recently as it provides possibility of achieving greater control of gene expression. In a cellular environment, the ribozymes could be sequestered by protein-RNA interactions and thus, made unavailable for efficient substrate cleavage. The protein-RNA interactions are influenced, to a great extent, by the conformation of the ribozyme. It is not yet known what structural requirements are fulfilled by the ribozyme (or any small RNA) to contribute towards a profitable interaction with proteins. When multiple ribozymes are used, there is a possibility that a few of them would escape protein-mediated conformational changes and thus would be available for cleavage of the target RNA. Thus, multiple ribozymes have been employed, for in vivo studies, hoping to override the difficulties encountered in a cellular milieu.

In the present study, multiple ribozyme cleavage reactions, in which both the ribozymes were included, produced cleavage products 160nt, 345nt, 606nt and 635nt (Figure.10.-Lanes 6, 9 and 12; Figure.11.-Lanes 4 and 8). It was observed that the two ribozymes, in combination, do not give expected cleavage products. If both the ribozymes were active on the same substrate molecule, a product of 446 nt in length is expected. This product was not detected in multi-ribozyme reactions. The cleavage products seen in multi-ribozyme cleavage reaction are infact the products generated by the two ribozymes on independent substrate molecules. This indicates that the binding of one of the ribozymes to its target site becomes prohibitive to binding of the second ribozyme at the same time. This would also imply that the dissociation of the ribozyme from cleaved substrate and restoration of secondary structure
is a rather slow process.

It is known that the ribozyme cleavage activity requires Mg$^{2+}$ ions. However, in absence of Mg$^{2+}$, binding of the ribozyme to the target site is not affected, which was reaffirmed by using Rz$_{316}$ on a 308 nt substrate in absence of MgCl$_2$ (Figure.18., Lane6.). To study the above aspect, ribozyme cleavage reactions were set up in 50mM Tris-HCl, in absence of MgCl$_2$. Substrate RNA was mixed with either of the ribozymes and heat denatured to disrupt the secondary structures. The two ribozymes were independently bound to the substrate, in absence of MgCl$_2$ for 2hrs, at 37°C. At the end of 2hrs, the corresponding second ribozyme was introduced and MgCl$_2$ was added to a final concentration of 20mM. The reaction was incubated for further 2 hrs at 37°C. The results (Figure.14.) indicated that when Rz$_{316}$ was bound, the conformational change induced becomes prohibitive for either binding or formation of optimal hammerhead structure of Rz$_{762}$. However, when Rz$_{762}$ was bound, it did not inhibit subsequent binding and activity of Rz$_{316}$. It can be observed from the earlier secondary structural analysis data (Figure.6., a and b), that binding of Rz$_{762}$ to the substrate, in fact, opens Rz$_{316}$ cleavage site close to a clear loop region, which allows binding of the arms. On the other hand, binding of Rz$_{316}$ changes the substrate conformation in such a way as to push the Rz$_{762}$ cleavage site into a small bulge with strong neighbouring structures. Thus, binding of ribozyme may result in conformational changes in the substrate and this in-turn, may influence interaction of the ribozymes targeted against other sites on the RNA molecule.

The data, presented here, indicate that it is important to consider the conformational changes induced in the substrate, upon ribozyme binding. Ribozymes could be designed, based on computer aided secondary structure predictions, which take into consideration the altered conformations of the target RNA. The target site and the combination of ribozymes to be used, could then be decided based on the altered conformations of the substrate.

As mentioned earlier, the GUC sites for both the ribozymes (Rz$_{316}$ and
Figure 14. Cleavage activity of individual ribozymes in a multi-ribozyme reaction. C- control, 951nt *in vitro* transcribed TNF-α RNA; Lane 1- Rz316 +TNF-α RNA; Lane 2- Rz316 was mixed with TNF-α RNA in absence of Mg²⁺ to allow binding for 2 hrs at 37°C followed by replenishment of Mg²⁺ along with addition of Rz762. Lane 3- represents a reciprocal sequence of lane 2 treatments. Lane 4- represents binding of both the ribozymes in absence of Mg²⁺ and its replenishment after 2 hrs at 37°C.
RZ762) originally occur in bulge/loop regions. It could be reasoned that since the designed ribozymes cleave the substrate efficiently, the bulge regions are disrupted/opened up to aid binding of the ribozymes at their target sites. This disruption could then result in overall structural rearrangement of the substrate RNA. At this stage, we cannot state if the observed phenomenon is limited to those substrates which are highly structured and in which the GUC sites occur in bulge regions. Substrate RNA containing GUC sites in large loop regions may not be structurally altered in this manner. The observation reaffirms the significance of the location of the target GUC sites for efficient cleavage. It also exemplifies probable alterations in the substrate conformation upon ribozyme binding.

IV.4.4. Specificity of Ribozyme Cleavage Reactions

IV.4.4.1. Mapping of RZ316 Cleavage Site on TNF-α RNA

Ribozymes are known to be highly specific for their target site. The specificity is conferred by stem I and stem III sequence elements which form the two arms of the ribozyme. These two arms are involved in base pairing with the target site, while the catalytic core performs the cleavage function.

The specificity of the designed ribozymes were confirmed by mapping the ribozyme cleavage site using a combination of primer extension and sequencing. For this purpose, 3' deletion of the TNF-α clone (pStuI-hTNF) was done to bring the RZ316 cleavage site closer to the T7 promoter. The construct retained TNF-α 5' ApaI-PvuII fragment (156-488 nt) which contained the RZ316 cleavage site. The PvuII end of this fragment was ligated to the vector- Smal end so that the PvuII recognition sequence is lost. This enabled synthesis of in vitro transcript with PvuII as run-off, beyond the T7 promoter. The T7 promoter sequence complement would also be transcribed along with the target sequence in the synthesized RNA transcript. Primer extension of such RNA was done using T7 promoter primer. Primer extension results in the generation of negative single stranded DNA whose sequence would be the same as the sequence read if T7 promoter primer was used. The
Figure 15. Construction of a deletion clone of TNF-α for primer extension analysis of Rz316 cleavage product.
3' end of primer extension product corresponds to that fragment in a sequencing reaction (performed using the same T7 promoter primer) which was terminated, by dideoxy incorporation, at 3' terminal base of the primer extension product.

In case where ribozyme-mediated cleavage occurs, the T7 promoter complement sequence would be present in the 3' cleavage product and primer extension of this product would generate a DNA fragment corresponding to the precise ribozyme-cleavage point. This primer extension product, when run along with a dideoxy sequencing reaction of the same template DNA (pStuI-hTNF-del I, Figure.15.), would correspond to the sequence fragment terminated at the cleavage site. Thus, the ribozyme cleavage site could be precisely mapped using a combination of primer extension and sequencing. A schematic representation of the procedure is presented in Figure.16. (see below). The result of mapping of ribozyme cleavage site, presented in Figure.17., indicated that Rz316 specifically recognizes and cleaves its designated target site.

Figure.16. Schematic representation of mapping Rz316 cleavage site on TNF-α RNA.
Figure 17. Mapping of ribozyme cleavage site of Rz \(_{316}\) on TNF-\(\alpha\) RNA using a combination of primer extension and sequencing. The sequence read is presented in the inside panel. The outside panel represents the complement sequence (+ strand of TNF-\(\alpha\) RNA) and the ribozyme cleavage site is indicated by an arrow.
IV.4.4.2. Evaluation of Rz316 on Shorter TNF-α Substrate

In addition to the mapping of ribozyme cleavage site on TNF-α RNA, evaluation of the specificity of reaction was done on a 308nt TNF-α substrate. Rz316 was used for this purpose as the smaller substrate contains only Rz316 cleavage site. The ribozyme activity was evaluated on parameters of time of incubation, absence of MgCl₂ and effect of a critical mutation on cleavage of the substrate. The results indicated that Rz316 did not cleave its substrate in absence of MgCl₂ and the designed mutation was found to successfully abolish ribozyme activity. Ribozyme complement RNA (-ve orientation with respect to T₇ promoter) could not perform cleavage of TNF-α transcript. In addition, short intervals of increments in the time of incubation did not affect the extent of cleavage upto 30min duration (Figure.18.).

IV.5. Interaction of the Ribozymes with Purified Cytosolic Proteins

Proteins influence ribozyme structure, target binding, rate of cleavage and rate of degradation. A major factor for the successful use of ribozymes in vivo, is their interactions with cytosolic proteins. Thus, it was necessary to test the ribozymes, in vitro, with a few purified cytosolic enzymes to study their interactions. We chose adenosine deaminase, glucose-6-phosphatase, glucose oxidase, mutarotase and lactate dehydrogenase due to their ubiquitous nature and their highly hydrophilic activities. Such proteins could be candidates for potential interactions with the ribozyme in the cellular environment. Also, a previous report (Sioud and Jasperson, 1996) demonstrated that glyceraldehyde-3-phosphate dehydrogenase binds to one of the ribozymes directed against TNF-α. In the same report, commercial preparation of the enzyme was also found to exhibit the binding activity.

IV.5.1. Dimerization of the Rz762 by Cellular Proteins

Gel retardation experiments were performed, as indicated earlier, with 100ng of each protein and 15pmol of the ribozyme. It was expected that a few of these proteins would bind and retard the mobility of ribozyme. However, it was
Figure 18. Specificity and Mg⁺ dependence of wild type Rz316 (+) strand for catalytic cleavage of 308 nt TNF-α RNA. As indicated, the point mutation (A→C) or antisense transcript did not cleave the target RNA under identical conditions.
observed that the Rz762 was being dimerized (as assessed by the position of the retarded band in a 4\% non-denaturing PAGE) in presence of these proteins, by a hitherto unknown mechanism. Since all the proteins caused identical shifts, it was inferred that the slow band is either a novel structural form of Rz762 or may be a dimer. Dimerization was observed in the case of adenosine deaminase, glucose-6-phosphatase, glucose oxidase and lactate dehydrogenase, the exception being mutarotase, as indicated in Figure.19. Also, dimerization was observed only in the case of Rz762. Thus, some structural requirement is being fulfilled by Rz762 for a meaningful interaction with proteins. The fact that the Rz316 does not interact with any of the cellular proteins, indicates that the tendency to interact with proteins and subsequent dimerization is contributed, in part, by difference in ribozyme nucleotide sequence. The sequences of Rz316 and Rz762 differ from each other at stem I and III (the ribozyme arms). These two ribozymes fold into entirely different conformations, based on nucleotide sequence content (Figures.20. and .21.).

Interestingly, ribozyme-protein complexes, with varied mobility, were not detected, which indicated that the interaction is rapid and transient.

**IV.5.2. Concentration Dependent Influence of Adenosine Deaminase on Dimerization of Rz762**

Cursory study of the available crystal structures of the proteins revealed a common TIM barrel motif (originally defined in triose isomerase). Adenosine deaminase has a classical TIM barrel structure and thus it is a representative molecule for such class of proteins. We investigated whether the observed phenomenon is influenced by protein concentration. Our results indicate a concentration-dependent formation of dimers of Rz762 (15 pmol) in presence of adenosine deaminase. There was no substantial dimerized product at 5, 10, 15 and 20 ng of the protein. However, at these concentrations, the pattern of migration of the ribozyme bands indicated that the molecule was being stretched and undergoing gross structural changes. At 30ng protein concentration, the ribozyme dimers appeared (Figure.22.). At 100ng of protein, we could observe substantial dimerization of Rz762.
Figure 19. Dimerization of Rz_{762} in presence of purified cytosolic proteins. Lanes 1, 7 - Ribozymes in presence of RNasin; A-adenosine deaminase; B-glucose oxidase; C-glucose-6-phosphatase; D-mutarotase and E- lactate dehydrogenase. Lanes 1-6 show that Rz_{316} did not form any novel mobility class as against an apparent dimerization of Rz_{762} in the presence of added proteins (Lanes 7-12).
Figure 20. FOLDRNA predicted secondary structure of Rz_{316}. Please note that the ribozyme assumes a hammerhead structure when appended in *cis* with the target (Figure 6a.).
Figure 21. FOLDRNA-predicted secondary structure of $Rz_{762}$. (Please note that the ribozyme core assumes an active hammerhead structure when appended in *cis* with the target, Figure 6 b).
Figure 22. Influence of increasing concentrations of adenosine deaminase on dimerization of Rz762. Lane 1 - 100ng adenosine deaminase control; Lane 2 - Rz762 control; Lane 3 - 5ng adenosine deaminase + Rz762; Lane 4 - 10ng adenosine deaminase + Rz762; Lane 5 - 15ng adenosine deaminase + Rz762; Lane 6 - 20ng adenosine deaminase + Rz762; Lane 7 - 30ng adenosine deaminase + Rz762.
IV.5.3. UV.-Cross Linking of the Protein-Rz762 Complexes

The cross-linked product was not clearly observed, even after repeated trials, when protein concentrations of 100ng were used (data not shown). The ribozyme concentrations were maintained at 15pmole. Increasing the protein concentration to 400ng-1000ng showed a substantial cross-linked product and the shifting of the radiolabelled ribozyme molecules led to corresponding decrease in the free ribozyme concentrations (Figure 23.). However, the crosslinked products had a mobility shift greater than that with the corresponding monomer proteins, which may result if two monomers of the protein are involved in dimerization of the ribozyme. Also, the procedure could inherently crosslink protein-protein monomers resulting in higher mobility classes. In our understanding, it is safe to call these as 'probable' protein-Rz762 complexes since it does not correspond to multimer RNA mobility.

IV.5.4. RNAse Mapping of the Protein-Rz762 Complex

Attempts to map the protein binding sites on the ribozyme, with a mixture of RNAse A and RNase T1 were not successful (data not shown). This could be due to the fact that the ribozyme-protein interactions are shortlived (as indicated by other experiments). This makes it difficult to catch enough molecules in a bound state. Also, the dimerization phenomenon could involve only a few bases of the ribozyme and mapping these few bases would be extremely difficult, if not impossible. It has been shown, in case of Ncp7, that the protein has the capability to unwind 7 base stems to allow efficient binding to the RNA (Bertrand and Rossi, 1994). If this is true in our case also, then it would be difficult to locate the protein binding site on Rz762, especially given the fact that the interaction seems to be rapid and transient.

IV.5.5. Influence of Proteins on Rz762 Cleavage Activity

Rz762 was found to interact in vitro with cellular proteins. Such ribozyme-protein interactions could lead to either enhancement or decrease in ribozyme activity. Since these proteins caused dimerization within 15 min, enhancement of activity by increasing substrate-ribozyme interactions should be detected
Figure 23. UV cross-linking of probable complexes of Rz_{762} in presence of varied concentrations of the proteins. Lane C1-Rz_{762} control; Lane C2-Rz_{762} + 1000ng BSA; Lanes 1, 2 & 3 - Rz_{762} + 400ng, 600ng & 1000ng adenosine deaminase respectively; Lanes 4, 5 & 6 - Rz_{762} + 400, 600 & 1000 ng lactate dehydrogenase; Lanes 7, 8 & 9 - Rz_{762} + 400, 600 & 1000ng glucose oxidase.
within this period. Although the ribozyme activity in absence of proteins would be negligible within 15 min, any dramatic enhancement, if mediated by proteins, would be due to increase in substrate ribozyme interactions, resolving/unwinding of substrate-ribozyme complex to aid faster cleavage or increasing the turnover by aiding association as well as dissociation of the ribozyme.

Our results indicate that the ribozyme activity is not dramatically affected by these proteins within this time period (Figure.24.). Alternatively, inhibition of ribozyme activity could also occur, which could be tested at longer incubation times. Purification of these proteins is necessary to avoid contaminating RNases which would degrade the transcript upon longer incubation times.

**IV.5.6. Effect of Partial-Trypsinization of Proteins on Dimerization of Rz762**

Dimerization of the ribozyme could be mediated by either gross structural elements of the protein or a stretch of a few amino acids forming an active domain. If any gross structural motif was involved, disruption of such structures should abolish the ability to dimerize the ribozyme. We studied this aspect by using partially -trypsinized proteins and tested their capacity to cause dimerization of the ribozyme. Our results indicate that partially trypsinized proteins retain the dimerization activity (Figure.25.) although the extent of dimerization showed marked decrease. This indicates that the structural motif(s) involved is/are small and resistant to partial trypsin digestion. BSA and trypsin alone could not dimerize Rz762.

**IV.5.7. Ribozyme-Protein Interactions in Human Cytoplasmic Extracts**

Of interest to the problem of ribozyme expression in cell, is the binding and possible sequestering of the ribozymes by cellular proteins. Cytoplasmic extracts were prepared from human THP1 monocyte-macrophage cell line and tested in vitro for their ribozyme binding ability. Gel retardation experiments with 10 µg cytosolic extract indicated that the in vitro synthesised,
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**Figure. 24.** Effect of proteins on Rz\textsubscript{762} mediated cleavage of TNF-\(\alpha\) RNA. C1- control, 951nt TNF-\(\alpha\) substrate; C2- control, 951nt TNF-\(\alpha\) substrate + 100ng BSA; C3- control, 951nt TNF-\(\alpha\) substrate + 0.25% Trypsin; Lanes 4 and 5- 951nt TNF-\(\alpha\) + Rz\textsubscript{762} + adenosine deaminase; Lanes 6 and 7- 951 nt TNF-\(\alpha\)+Rz\textsubscript{762} + glucose oxidase; Lanes 8 and 9- 951nt TNF-\(\alpha\) + Rz\textsubscript{762} + glucose-6-phosphatase; Lanes 10 and 11- 951nt TNF-\(\alpha\) + Rz\textsubscript{762} + mutarotase; Lanes 12 and 13- 951nt TNF-\(\alpha\) + Rz\textsubscript{762} + lactate dehydrogenase. The reactions were electrophorased in 4% denaturing PAGE.
Figure 25. Effect of partially trypsinized proteins on dimerization of Rz762. Lane 1 - 100 ng glucose oxidase + Rz762; Lane 2 - 100 ng partially trypsinized glucose oxidase + Rz762; Lane 3 - 100 ng adenosine deaminase + Rz762; Lane 4 - 100 ng partially trypsinized adenosine deaminase + Rz762; Lane C1 - Rz762 control; Lane 5 - 100 ng glucose-6-phosphatase + Rz762; Lane 6 - 100 ng partially trypsinized glucose-6-phosphatase; Lane C2 - Rz762 + Trypsin, control; Lane 7 - 100 ng lactate dehydrogenase + Rz762; Lane 8 - 100 ng partially trypsinized lactate dehydrogenase + Rz762.
radiolabelled ribozyme was binding to some of the cellular proteins. The two ribozymes appear to bind to distinctly different proteins as indicated by the mobility differences of their bound forms (Figure 26). Silver staining of the gel could not detect a distinct corresponding protein band, as these binding proteins could be present at low amounts. The amount of cytosolic extract used for gel retardation assays was deliberately kept low, in order to rule out non-specific RNA-protein interactions.

IV.6. Ribozyme Expression Strategies in Human Cell Line

IV.6.1. Construction of Vectors for Expression of Ribozymes In Vivo

The designed ribozymes efficiently and specifically cleaved TNF-α RNA in vitro and promised application in controlling TNF-α gene expression in human cell lines. For this purpose, the ribozymes were cloned in mammalian expression vectors (pCI-neo and pVR1012-neo) under the control of human cytomegalovirus (CMV) early promoter/enhancer. Three different strategies were used for expression of the ribozymes in vivo.

IV.6.1.1. Pol II Based Expression

IV.6.1.1.1. Expression of Ribozymes as Polyadenylated RNA

Essential features of pCI-neo mammalian expression vector include the following:

- Human cytomegalovirus (CMV) immediate-early enhancer/promoter region for strong and constitutive expression.
- Neomycin phosphotransferase gene conferring G418 resistance.
- SV40 late polyadenylation signal for efficient RNA processing.
- Chimeric intron composed of 5’ splice site from β-globin intron and the 3’ splice site from an IgG intron, for increased expression levels.
- SV40 origin of replication which provides transient, episomal replication.
- Polycloning region.
- T7 and T3 RNA polymerase promoters for synthesis of in vitro transcripts.
- β-lactamase gene conferring ampicillin resistance.

Rz316 and Rz762 were cloned in NheI site and the constructs were
Figure 26. Detection of ribozyme-binding proteins in THP1 cytoplasmic extracts. (a) Silver staining—Lane 1- THP1 cytoplasmic extract; Lane 2- Rz$_{316}$ + cytoplasmic extract; Lane 3- Rz$_{762}$ + cytoplasmic extract; (b) Autoradiography—Lane 1- Rz$_{316}$; Lane 2- Rz$_{762}$; Lane 3- Rz$_{316}$ + cytoplasmic extract; Lane 4- Rz$_{762}$ + cytoplasmic extract.
initially confirmed by restriction digestions to identify presence of the insert. The positive clones (pClneo-Rz316 and pClneo-Rz762) were sequenced for final confirmation (Figure.27b). The construction route is presented in Figure.27a.

IV.6.1.1.2. Expression of Intron-Embedded Ribozymes

The pVR1012-neo mammalian expression vector is particularly suited for nuclear localization of transcripts of inserts located in the intron. The vector offers the following possibilities:

- Human cytomegalovirus (CMV) enhancer/promoter.
- CMV immediate early 5’ untranslated region.
- CMV immediate early intron.
- Polycloning region.
- TbGH polyadenylation signal.
- Neomycin phosphotransferase gene conferring G418 resistance
- Kanamycin resistance gene.

Within the CMV intron, the unique PvuII restriction site was used for the purpose of cloning the ribozymes. The vector was digested with PvuII enzyme and dephosphorylated with CIP, electrophoresed and eluted from 1% low-melting agarose gel. The PvuII fragment of ribozyme containing clones (pStuI-Rz316 and pStuI-Rz762) was ligated to the vector prepared as above.

The clones were screened by restriction digestion with PvuII enzyme which releases a 500bp insert fragment from the vector. The inserts were confirmed for their orientation relative to the CMV promoter by Sangers dideoxy sequencing (Figure.29.). The clones (pVR1012-Rz316int, pVR1012-Rz762int, Figure.28.) contained ribozymes in the intron, in positive orientation with CMV promoter.

IV.6.1.2. Expression in Mammalian Cells from Bacteriophage Promoter

The U293-3-46 cell line is used to achieve expression in mammalian cells using bacteriophage T7 promoter. This cell line has the following features:

- Subclone of Human embryonic kidney cells.
Figure.27a. Construction of vectors for in vivo expression of ribozymes as polyadenylated RNA
**Figure 27(b).** Sequence of constructs used for expression of ribozymes in mammalian cells as polyadenylated RNA. The results presented here are representative of the ribozymes cloned in mammalian expression vector, pCI-neo. The underlined portion indicates the ribozyme sequence.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Wild Type</th>
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<tr>
<td>Rz762</td>
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<tr>
<td>GATC</td>
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<td>Rz316</td>
<td>Rz316</td>
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Figure 28. Construction of vector for expression of ribozymes embedded in the intron
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<th>Wild Mut.</th>
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<td>Rz316</td>
<td>Rz762</td>
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Figure 29. Nucleotide sequence of ribozymes cloned in the intron of mammalian expression vector, pVR1012-neo. The cloning site is enclosed in a box.
• Contains stably integrated T7 RNA polymerase gene.
• The T7 RNA polymerase gene is faithfully maintained by the cell under neomycin (G418) selection pressure.
• The T7 RNA polymerase gene is under the control of human cytomegalovirus enhancer/promoter for constitutive and high expression levels.

A subclone (U293-3-46, kindly provided by Professor. Martin Billeter, University of Zurich, Switzerland) of human embryonic kidney cell line, contained T7 RNA polymerase gene stably integrated in the genome. Plasmids containing T7 promoter can be transfected into U293-3-46 cells for in vivo expression. The constitutively expressed T7 RNA polymerase gene would transcribe the transfected plasmids by utilizing T7 promoter. Precise in vivo termination of transcription can be achieved by cloning T7 terminator sequence downstream of the inserted sequence.

The ribozyme clones, pStuI-Rz316 and pStuI-Rz762, which were earlier used for in vitro transcription, do not contain terminator sequence, which is necessary for in vivo termination of RNA synthesis. A T7 terminator fragment (BamHI-EcoRV T7 terminator fragment from pET3a, 308 bp) was cloned in BamHI-EcoRV digested pStuI-Rz316 and pStuI-Rz762. This places the terminator sequence downstream to the ribozyme insert and thus enables ribozymes to be expressed in vivo in U293-3-46 cell lines using T7 promoter.

The positive clones (pStuI-Rz316T, pStuI-Rz762T, Figure.30.) were identified by restriction digestion with PvuII enzyme and finally confirmed by sequencing. (Figure.31.)

IV.6.2. Sub-Cloning TNF-α in Mammalian Expression Vector, pCI-neo
Since U293-3-46 cells do not produce TNF-α, TNF-α cDNA was transfected along with ribozyme-containing plasmids to study cleavage of the target, in vivo. pStuI-hTNF contains cDNA for human TNF-α and entire human TNF-α cDNA (1.44Kb) could be released from the vector by digesting at KpnI-Smal restriction sites.
Figure 30. Construction of vectors for T7-based \textit{in vivo} expression of Ribozymes in human U293 cell lines
Figure 31. Sequence of the ribozyme clones containing T7 terminator cloned downstream of ribozyme insert in pStuI-Rz316-T762-\(\phi\).
Mammalian expression vector, pCI-neo, contained two restriction sites for KpnI enzyme, one site occurring in the polycloning region and the other in the polyA+ signal coding sequence, 700bp away. Partial digestion of the vector with KpnI and subsequent sequential digestion with SmaI (occurring in the polycloning site) generated vector fragments which included single restriction cuts at KpnI and SmaI site of the polycloning region. Vector DNA, which received restriction cut at the KpnI site of the polycloning region, could be distinguished from those molecules which received cuts at the second KpnI site based on their mobility in 1% agarose gel. The required band was eluted from 1% low melting agarose and ligated to KpnI-SmaI digested human TNF-α fragment (1-1440nt). The positive clones were identified by restriction digestion and confirmed by sequencing (Figures. 32. And 33.).

IV.7. Expression of Ribozymes in Human THP1 Cells:
Supercoiled plasmid DNA (~5μg) containing ribozyme against human TNF-α, were transfected into human THP1 cell lines at 70% confluence. The growth of the cells, post-transfection, was carefully monitored and neomycin (G418) selection was applied 24 hrs after change to fresh medium. Prior to LPS induction, the cells were harvested and counted to make sure of the density. Induction of TNF-α synthesis was done by addition of bacterial lipopolysaccharide (LPS) at a final concentration 100 ng/ml. Cleared supernatants of these cultures were tested for their apoptosis-inducing ability in TNF-α sensitive mouse cell line (WEHI 164). Simultaneously, the producer cell line (THP1) was harvested and processed for RNA isolation. RNA analysis was done by Northern blotting (for TNF-α transcript levels) and RNase protection assay (for analysis of ribozyme cleavage products). The apoptosis induced in the target cell line, upon challenge with culture supernatants from ribozyme transfected cells, was detected by flow cytometry. Cell viability was assayed by trypan blue staining.
Figure 32. Sequence confirmation of pCI-neo-hTNF, mammalian expression vector containing TNF-α insert.
Figure 33. Construction of vector for expression of human TNF-α in U293 cells
IV.7.1. Detection of Ribozyme Cleavage Activity in Producer Cell Line

IV.7.1.1. Northern Blotting Studies for Background Information

It was necessary to estimate the TNF-α transcript levels, in untransfected THP1 cells, to choose appropriate time point for assay of apoptosis. For this purpose, the THP1 cells were induced with 100ng/ml of bacterial lipopolysaccharide for 24 hrs and the transcript level of TNF-α was studied by Northern blotting at different time points. The results indicate that after a definite lag phase of about 1hr, post-induction, TNF-α transcript level gradually builds up (Figure.34.). TNF-α also acts in an autocrine manner, but in presence of a strong inducer like LPS, overproduction of TNF-α occurs, which is evident in the results presented. Induction time point of 6hrs was chosen for subsequent assays since the levels of TNF-α mRNA builds up sufficiently to be analysed for in vivo ribozyme-cleavage assays. Also, to rule out influence of other interfering factors, which might be induced at increased levels of TNF-α, 6 hrs of induction time point was strictly maintained throughout the study.

IV.7.1.2. TNF-α Transcript Analysis in Ribozyme Transfected Cell Line

Northern blotting was employed to analyse the transcript levels of TNF-α in ribozyme transfected and LPS-stimulated THP1 cells. The producer cell line was transfected with the two ribozymes individually and in combination. Since, a certain pool of ribozyme would already be present, upon transfection of the ribozymes, it was expected that there would be detectable differences in the TNF-α levels, when subsequently induced with LPS.

The results indicate that the transcript levels clearly decreased upon expression of ribozymes in the cells (Figure.35.). Transfection of plasmid DNA which did not contain ribozymes did not lead to decrease in the TNF-α transcript, which indicated that the observed decrease is due to specific destruction of the transcript by the ribozymes. A comparison of the levels of TNF-α among different expression strategies reveal that the T7 based expression of ribozyme was very efficient, especially in the light of the fact that TNF-α construct was cotransfected along with the ribozyme constructs and
Figure 34. (a) Northern blot analysis of TNF-α transcript levels estimated at the indicated time points within 24 hrs of induction with 100ng/ml bacterial lipopolysaccharide (LPS). (b) The equivalent amount of RNA stained with ethidium bromide serving as reference of equal loading of RNA for Northern blot assay.
Figure 35. (a) Northern blot analysis of TNF-\(\alpha\) transcript levels in THP1 cells upon ribozyme expression. C1 - control, LPS induced THP1 cells; C2 - LPS uninduced THP1 cells; C3 - U293 cells transfected with human TNF-\(\alpha\); Lanes 1, 4 & 7 - Rz\(_{316}\) transfection; Lanes 2, 5 & 8 - Rz\(_{762}\) transfection; Lanes 3, 6 & 9 - Rz\(_{316}\) + Rz\(_{762}\) transfection; Lane 10 - control U293, 100 ng/ml LPS; Lane 11 - U293 control, TNF-\(\alpha\) cotransfected with pStul; Lane 12 - U293 control, TNF-\(\alpha\) cotransfected with pET3a. (b) The equivalent amount of RNA stained with ethidium bromide serving as reference of equal loading of RNA for Northern blot assay.
thus the levels of TNF-α would be much higher when compared to the transcript levels in LPS-stimulated THP1 cells, which would be subject to tight cellular regulation.

IV.7.1.3. RNase Protection Assay (RPA) for Detection of Ribozyme Cleavage Products In Vivo.

Ribonuclease protection assays have been extensively used for the purpose of detection of low abundance mRNA. Its sensitivity level is 20 times greater than that of Northern blotting. The antisense RNA, used for hybridization, was internally radiolabelled to assist detection of the protected duplex RNA by autoradiography.

RPA was employed since Northern blotting studies failed to detect in vivo ribozyme cleavage products. The results indicated that the designed ribozymes were active, in vivo. Cleavage products generated by Rz316 could be detected in all the three in vivo expression strategies (Figure.36.). However, cleavage products of Rz762 could not be detected. This could be due to the fact that cleavage products are subject to rapid degradation by cellular RNases (Sioud et al., 1992). Longer RNAs are degraded more efficiently and rapidly compared to the smaller RNAs, which explains the reason for our detection of only the smaller (~300nt) 5'-cleavage product.

IV.8. Ribozyme-Mediated Protection against TNF-α Induced Apoptosis

TNF-α is a potent inducer of apoptosis in a variety of human and mouse tumor cell lines. TNF-α induced apoptosis is mediated by p55 receptor. The mouse receptor recognizes human TNF-α and triggers apoptosis. Culture supernatants of LPS-induced producer cells contains secreted TNF-α, which can be functionally assayed for TNF-α activity in sensitive cell lines. WEHI 164 is a mouse fibrosarcoma cell line, which is highly sensitive to TNF-α at even 10 pmol/ml concentrations. Small amounts of TNF-α in the supernatants can be detected using these cells on the basis of induction of the cell death pathway. The biological assay is highly sensitive, as compared to more conventional
Figure 36. RNase protection assay for detection of ribozyme cleavage products in vivo. (a) P1, P2 - riboprobe 1 and riboprobe 2; Lanes 1, 2 & 3 - Polyadenylated ribozyme expression; Lanes 4, 5 & 6 - Ribozyme embedded in intron. (b) P - riboprobe; Lanes 1, 2 & 3 - T7 based expression of ribozymes in U293 cells.
techniques like ELISA.

Northen blot and RNase protection assays indicated that both the ribozymes efficiently destroyed TNF-α transcript, *in vivo*. However, the present study particularly concerns ribozyme mediated control of TNF-α gene expression at biologically significant levels. It was thus of interest to test the use of these ribozymes in reversing TNF-α induced apoptosis in WEHI 164 cells.

To study this aspect, culture supernatants from LPS-induced, ribozyme transfected cell lines were added to WEHI 164 target cells. After incubation for 10 hrs, the target cells were harvested and processed for flow cytometry for assay of DNA content. Apoptotic cells undergo DNA fragmentation which results in decrease of total DNA content of the cell. Thus, apoptotic cells can be detected, by flow cytometry, as hypodiploid cells with decreased fluorescence when stained with DNA-staining dye like Propidium Iodide. The hypodiploid cells peak earlier than the diploid (Go-G1) cells (*Figure.38a.*).

**IV.8.1. Total Cell Numbers and Trypan Blue Exclusion Studies**

As a confirmatory test to the flow cytometric data, the total cell counts and trypan blue exclusion studies were performed to estimate the fraction of apoptotic cells. The results indicate that ribozyme-mediated cleavage of TNF-α in producer cells, could prevent 80 % of the target cells from undergoing cell death, based on peak area. The total cell count of WEHI164 recovered back to the normal control levels in all the expression strategies used.

Trypan blue exclusion studies also indicated prevention of TNF-α induced apoptosis. Around 90-95% of the cells exhibited active exclusion of the dye which demonstrates competence of ribozymes in achieving the desired level of control of gene expression. The results of total cell count and trypan blue exclusion are presented in *Figure.37.(a, b).*

**IV.8.2. Flow Cytometric Analysis for Detection of Apoptotic Cells**

Our results demonstrate that supernatants from ribozyme transfected TNF-
Figure 37. Assay of relative cell viability of WEHI 164 treated with cleared culture supernatants from TNF-α synthesizing cells. (a) Total cell counts of WEHI 164 cells treated with culture supernatants of ribozyme transfected and untransfected TNF-α synthesizing cell lines. (b) Percentage of live WEHI 164 cells, as assessed by trypan blue staining, upon treatment with culture supernatants of ribozyme transfected and untransfected TNF-α synthesising cells. The data is presented as an average of three independent recordings of samples which were also used for flow cytometric analysis. The histogram shading (indicated in the box) applies to both (a) and (b). The corresponding strategies for ribozyme expression are also indicated in the box.
Figure 38a. Detection of hypodiploid apoptotic cells using flow cytometry. WEHI164 cells were stained with propidium iodide which allowed distinction between diploid (G0/G1) and hypodiploid (apoptotic) cells based on total DNA content of the cell. The above panel represents the overlaid DNA histograms for comparison of apoptotic hypodiploid peak and diploid G0/G1 peak, presented in logarithmic scale for clarity. (1) Histogram of WEHI 164 cells exposed to culture supernatants of LPS uninduced THP1 cells overlayed with the cells exposed to supernatants of U293-3-46 control cells. (2) Histogram of WEHI 164 cells exposed to culture supernatants from LPS induced THP1 cells overlayed with the cells exposed to supernatants of TNF-a transfected U293-4-46 cells. (3) Histogram of WEHI 164 cells exposed to culture supernatants of LPS uninduced THP1 cells overlayed with histogram of the cells exposed to supernatants of LPS induced THP1 cells. (4) Histogram of WEHI 164 cells exposed to supernatants of U293-3-46 overlayed with the cells exposed to supernatants of TNF-a transfected U293-3-46 cells.
Figure 38b. Detection of apoptotic cells using flow cytometry. (1) WEHI164 cells treated with supernatants from LPS uninduced THP1 cells. (2) WEHI 164 cells treated with supernatants from LPS induced THP1 cells. (3) WEHI 164 cells treated with supernatants from LPS induced -R_{z_{16}} transfected THP1 cells. (4) WEHI164 cells treated with supernatants from LPS induced-R_{z_{16}} transfected cells. (5) WEHI164 cells treated with supernatants from LPS induced-R_{z_{16}}+R_{z_{16}} transfected THP1 cells. The transfected ribozymes in this panel correspond to the polyadenylated ribozyme expression strategy.
Figure 38c. Detection of apoptotic cells using flow cytometry. (1) WEHI164 cells treated with supernatants from LPS uninduced THP1 cells. (2) WEHI164 cells treated with supernatants from LPS induced THP1 cells. (3) WEHI 164 cells treated with supernatants from LPS induced -Rz16 transfected THP1 cells. (4) WEHI164 cells treated with supernatants from LPS induced -Rz16 transfected cells. (5) WEHI164 cells treated with supernatants from LPS induced -Rz16 + Rz62 transfected THP1 cells. The transfected ribozymes in this panel correspond to the intron-embedded in vivo ribozyme expression strategy.
Figure 38d. Detection of apoptotic cells using flow cytometry. (1) WEHI164 cells treated with supernatants from U293 cells. (2) WEHI 164 cells treated with supernatants from TNF-α transfected U923 cells. (3) WEHI 164 cells treated with supernatants from TNF-α and -Rz16 cotransfected U293 cells. (4) WEHI164 cells treated with supernatants from TNF-α and -Rz49 cotransfected cells. (5) WEHI164 cells treated with supernatants from TNFα and -RZ16+RZ49 transfected THP1 cells. The transfected ribozymes in this panel correspond to the T7-based \textit{in vivo} ribozyme.
α producer cells have a markedly reduced capacity to induce apoptosis in TNF-α sensitive WEHI164 cells. We observed a complete disappearance of the apoptotic peaks corresponding to all the ribozyme expression strategies, suggesting that each one of them was competent in effectively controlling TNF-α mRNA. Both Rz316 and Rz362 could reverse TNF-α induced apoptosis in WEHI164 cell line. This indicates that control of TNF-α can be achieved to a great extent at the level of mRNA, using ribozymes as mediators for cellular destruction of RNA. The results of flow cytometry are presented in a series of DNA content histograms indicating the various expression strategies (Figure.38a-d.). The apoptotic positive controls were overlaid onto the apoptotic negative controls for comparison of apoptotic peaks (Figure.38a.).

The cell counts and flow cytometric data put together indicate that there is almost complete reversal of apoptosis mediated by TNF-α upon expression of anti-TNF-α ribozymes in vivo.

IV.9. Expression of Human TNF-α in E.Coli

TNF-α is an important cytokine which mediates a variety of immune responses. Although overexpression of this cytokine is unwelcome in pathological states, it is beneficial to express good quantities of this cytokine for use in research and TNF-α based cancer therapy. High levels of expression can be achieved in E.coli and protein purification is possible by expressing TNF-α as a His-tagged protein. His-tagged proteins have a very high affinity towards Ni-NTA matrices (Qiagen) and thus the expressed protein could be purified in large scale.

Human TNF-α was cloned in pQE30 expression vector (Qiagen) (Figure.39.) and expressed in E.coli (SG13009). Preliminary studies indicate the feasibility of expressing TNF-α in a bacterial system. Small-scale isolation (200ml cultures) of the expressed protein demonstrated purification of TNF-α in the imidazole eluate (Figure.40). Further studies are being pursued to isolate the protein in large scale and also to test the bioactivity of the cytokine expressed in bacterial systems.
Figure 39. Construction of Vector for Expression and Purification of Human TNF-α from in E. Coli based on 6x Histidine Tag
**Figure 40.** SDS-PAGE profile of proteins isolated from *E.coli* expressing human TNF-α. 6x-His-tagged TNF-α protein, purified using Ni-NTA matrix, is indicated by arrow. Lane M-Marker; Lane 1- Cleared cell lysate; Lane 2-Flow-through; Lane 3 - 8mM Imidazole wash; Lane 4 -20mM Imidazole wash; Lane 5 -100mM Imidazole elution; Lane 6 - 250mM Imidazole elution; Lane 7 - Control cell lysate; Lane 8- Control flow-through; Lane 9 - Control, 8mM Imidazole wash; Lane 10- Control, 20mM Imidazole wash; Lane 11- Control, elution 100mM Imidazole; Lane 12- Control, 250mM Imidazole elution.