III. RESULTS
III.1. Selection of Target Sites in \textit{lexA} RNA

For designing a targeted ribozyme, the site for cleavage must be chosen within the target RNA. In naturally occurring self-cleavage (e.g., RNA which self-cleaves via hammerhead structure), the sequence \textit{GUC} usually serves as the preferred site of cleavage. It is now known that the sequence \textit{GUX} (\(X = A, U\) or \(C\)) in the target RNA, is susceptible to such cleavage reactions mediated by ribozymes.

The complete nucleotide sequence of \textit{lexA} RNA was scanned for the occurrence of \textit{GUC} target site. A total of sixteen such sites were identified (Fig. 9A). The experimental method for evaluation of target site accessibility is, RNase H-directed cleavage using oligonucleotides complementary to the target site (Young et al., 1994). The results indicate that certain sites are more accessible than other. But in this study, we used a computer-assisted program \textit{RNAFOLD} (University of Wisconsin, Genetics Computer Group) to evaluate the accessibility of target site. Computer-assisted prediction of possible secondary structures which are thermodynamically stable may be helpful in the search for loop regions remaining single stranded. Full length (952 nt) and partial length (467 nt) \textit{lexA} RNA were folded using \textit{RNAFOLD} computer program (Fig. 10A, B). All the potential ribozyme target sites were scanned for their position in predicted secondary structure. The region flanking target site 343 seems to assume very stable structure with the \textit{GUC} always on the loop region, irrespective of whether full- or partial-length \textit{lexA} RNA is folded (Fig. 13). Dot plot analysis of full- and partial-length \textit{lexA} RNA showed no additional structures with significant stability of folding around the target sites (Fig. 11).

Several different ribozymes were modelled by computer program for different \textit{GUC} sites along the RNA with ribozyme sequence attached to the 3' of the substrate RNA by five U nucleotides in \textit{cis} and their minimum energies were compared. Ribozyme against 343 position showed most stable structure according to \textit{RNAFOLD} (minimum potential energy were taken as a criterion to depict the most stable structure). The secondary structure of the ribozymes with 10 nucleotide long arms complementary to the target sites on RNA at position 303, 343, 390 and 528 in \textit{cis}-form are shown in Fig. 12. However, in all cases appropriate structures were predicted when ribozymes were modelled in the presence of the substrate except in one
Fig. 9. (A) Location of GUC sites on lexA RNA. The black region indicate the polylinker of pTZ18U; the hatched region , the 5' and 3' untranslated region; and the open region, the lexA coding region. +1 indicates the transcription start site. Arrow below the map indicates the position which was chosen as a target site. EcoRV and BamHI sites were used for run-off transcription.

(B) Structure of the hammerhead ribozyme and substrate used in this study. Cleavage of substrate occurs 3' of the GUC sequence.
Fig. 10. Model of the secondary structure of *lexA* RNA generated by the programme RNAfold. The graphic representation was obtained by Squiggles (University of Wisconsin, Genetics Computer Group). (A) full length (952 nt) transcript.
Fig. 10. Model of the secondary structure of *lexA* RNA generated by the programme RNAfold. The graphic representation was obtained by Squiggles (University of Wisconsin, Genetics Computer Group). (B) partial length transcript (467 nt)
Fig.11. Dot plot analysis of *lexA* RNA (A) full-length transcript (952 nt)
Fig.11. Dot plot analysis of *lexA* RNA. (B) partial-length transcript (467 nt)
Fig. 12. Ribozymes structure at different GUC sites. The secondary structure of the ribozyme with 10 nt long arms complementary to different GUC sites in cis form by computer program RNAfold. Please note that the GUCs are accessible excepting in the position 390.
Fig. 13. A portion of secondary structure of $lexA$ showing GUC cleavage site. Boxed portion in Fig. 10 in secondary structure of $lexA$ RNA generated by the program RNAfold is shown enlarged. Arrow indicates the cleavage site.
Fig. 14. Secondary structure of RNA by MFOLD. A minimum free energy structure of lacI RNA which was connected by ribozyme sequence in cis form. (A) lacI RNA + 3.1 ribozyme.
Fig. 14. Secondary structure of RNA by MFOLD. A minimum free energy structure of
lexA RNA which was connected by ribozyme sequence in cis form. (B) lexA RNA+
4.11 ribozyme.
Fig. 15. A portion of secondary structure of substrate when folded with ribozyme sequence. (A) lexA RNA + 3.1 ribozyme; (B) lexA RNA + 4.11 ribozyme and (C) sequence of the one of the predicted structure near target site.
Fig. 16. Screening of ribozyme clones (PvuII digestion pattern). Lane 1 contains pBlOT7 digested with Pvu II and lane 4 contains 1 kb ladder from GIBCO-BRL. Lane 2, 3, 5, 6, contain different ribozyme clones digested with PvuII. Please note that new Pvu II site is created at 3' end of the insert. (see Fig. 8 for restriction map of these clones)
Oligonucleotide duplexes (lane 2, Fig. 5) corresponding to the ribozyme sequence (degenerate at selected sites for isolating mutants) were cloned in Nsi I site of pBlot7 vector near T7 promoter and recombinant clones were characterized by Pvu II restriction digestion. PBlot7 was digested with different restriction enzymes to confirm the site available in the vector and these restriction sites were used for cloning and characterization of ribozyme clones (Fig. 5). Figure 16 shows Pvu II restriction pattern of different clones having ribozyme sequence. Lane 1 is pBlot7 digested with Pvu II whereas lanes 2, 3, 5 and 6 contain clones having insert. The clones having an insert in positive orientation with respect to T7 RNA polymerase promoter gave two fragments of 333 bp and 155 bp whereas clones having insert in negative orientation with respect to T7 RNA polymerase gave rise to 370 and 118 bp fragments. These clones were named as pRZ4.9, pRZ4.1, pRZ3.1 and pRZ3.7. These four constructs are depicted in Fig. 17. The nucleotide sequence analysis of these clones are shown in Fig. 18. Arrows indicate 'A' to 'C' mutation in the catalytic domain used to inactivate ribozyme activity.

III.4. Subcloning of Target gene in pBlot7 vector

The lexA gene as obtained from Prof. D.W. Mount was originally in pJL40 which is a derivative of pBR322. To generate an in vitro transcript of lexA gene, it was necessary to subclone it under promoter which can be used to transcribe in vitro using phage RNA polymerase. The lexA coding region was PCR amplified using lexA-specific primers (lane 6, Fig. 5) and was subcloned into the Sma I site of pBlot7 vector which is a derivative of pBluescript. Figure 17 shows a linear map of the resulting lexA clones Sma I site of pBlot7 vector. Plasmid pBlx4 contains the sequence in positive orientation while pBlx6 has the same insert in negative orientation with respect to the T7 promoter (maps are not to the scale). The clones were characterized by EcoRI restriction digestion which releases an insert of 952 bp. Figure 19 shows the release of 952 bp lexA insert following EcoRI restriction digestion. Orientation of these clones were confirmed by Hinc II restriction digestion. Cloning junctions were confirmed by sequencing.
Fig. 17. Maps of the plasmid templates for lexA RNA and ribozyme synthesis by in vitro transcription with T7 or T3 RNA polymerase.
Fig. 18. Sequence analysis of ribozyme clones in positive (A) and negative (B) orientation with reference to T7 promoter. G-A-T-C refers to the order of loading the dideoxy reactions. The sequence around the mutation site is indicated on the right. Arrow shows nucleotide mutated to inactivate ribozyme catalysis. Sequences from a few representative clones are shown. Sequence ladders were separated on a 6% polyacrylamide gel containing 7M urea.
Fig. 19. Screening of lexA clones. Miniprep DNA was digested by EcoRI restriction enzyme. Lane 6 has EcoRI-Hind III cut λ DNA as size marker. Lanes 2, 4, 5, 8 and 9 show the expected 952 bp lexA fragment (indicated by the arrow at the right hand side).
III.5. Limitation of pBloT7 Vector

pBloT7 vector differs from pBluescript M13+(KS) in possessing an Nsi I site partially overlapping with the transcription initiation site downstream of T7 RNA polymerase promoter (Fig. 4). Digestion of pBloT7 vector with Nsi I and direct ligation of complementary inserts give rise to the vectors having ‘T’ nucleotide at +1 position of T7 promoter. In vitro transcription of these resulting vectors gave very poor yield of transcript (8 copies/template under defined conditions, Fig. 20, lane 2). We could not detect any transcription product with these clones. This was further substantiated by changing +1 position from T to G resulting in efficient transcription (88 copies/template) of constructs using T7 RNA polymerase (Fig. 20, lanes 7 & 8).

III.6. Transcription Efficiency of T7 Promoter having Different Nucleotides at +1 Position

Vectors containing different nucleotides at +1 position of T7 promoter were constructed. In order to test the efficiency of the initiation of transcripts by T7 RNA polymerase on various constructs having different nucleotides at +1 position, these constructs were transcribed in vitro. Transcription yields in each case were quantitated (Table 2).

For qualitative analysis of the RNA, 1/10th of the synthesis product was electrophoresed on a 1.2% agarose gel and products were detected by autoradiography of dried agarose gel (Fig. 20). In vitro transcription of pBloT7 vector linearized with Sca I, gave very low yield of RNA (8 copies/template) while newly constructed vector pStu I which has G as −1 nucleotide yielded very good quantity of RNA (88 copies/template). In other cases, transcription efficiency was also very low (Table 2).
Fig. 20. \textit{In vitro} transcription with T7 RNA polymerase. (A) Photograph showing an aliquot (1/10th) of the transcription products electrophoresed through 1.2% agarose gel produced from templates having different nucleotides at +1 position of T7 promoter. The template DNA, site of run-off and base at +1 were as follows: Lane 1. pStu/Sca I (G); Lane 2. pBloT7/Sca I (T); Lane 3. pBlx6/Hind III (T); Lane 4. pBlx42/EcoRV (T); Lane 5. pBlx423/EcoRV (A); Lane 6. pID4.3/Sca I (C); Lane 7. pR15/BamH I (G) and Lane 8. pR18/BamH I (G). (B) Autoradiograph of the transcripts as in 'A' above in dried agarose gel. Please see table 4 for quantitation of the yield.
Table 2. Yield of transcription reactions from template which are variants in the +1 position

<table>
<thead>
<tr>
<th>SEQUENCE</th>
<th>LENGTH (nt)</th>
<th>PLASMIDS</th>
<th>No. of transcripts / template</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'...TATATG</td>
<td>pBloT7 / Pvu II</td>
<td>330</td>
<td>8</td>
</tr>
<tr>
<td>5'...TATAGN</td>
<td>pR15 / EcoRV</td>
<td>460</td>
<td>88</td>
</tr>
<tr>
<td>5'...TATACN</td>
<td>pID4.3 / Pvu II</td>
<td>414</td>
<td>10</td>
</tr>
<tr>
<td>5'...TATAAN</td>
<td>pR423 / EcoRV</td>
<td>400</td>
<td>6</td>
</tr>
</tbody>
</table>

III.7. Construction of In Vitro Transcription Vector Allowing Precise Initiation of Transcript

The pBluescript derivative pBloT7 contains Nsi I restriction site immediately adjacent to the T7 promoter sequence. Direct ligation in Nsi I site or ligation of the blunt ended fragments with a 'T' at their 5' end in the Nsi I cut and overhang-resected vector that result in 'T' occupying +1 position, exhibit extremely low efficiency of the transcription from the T7 promoter in vitro. In order to overcome this problem, we constructed two new vectors having Bgl II and Stu I sites respectively in place of Nsi I site (Fig. 7). Advantage with these vectors are that they have G at +1 position and they show a very high efficiency of transcription. Cloning of any gene in Bgl II site of this vector (pBglII) gives rise to a construct offering transcription initiation at +1 of the insert. Similarly, transcription of pStuI gives two nucleotides (GG) at the 5' end. Blue-white screening is retained in the vectors. All other properties are similar to the pBluescript vectors (Fig. 7B).
III.8. Primer Extension Analysis

Based on the construction strategy, it could be assumed that transcription initiation site would respond to the first base of the inserted sequence. Primer extension analysis was used to confirm location of initiation of transcription in transcripts which were made in vitro using phage RNA polymerase. In this method, [$\gamma^{32}$p] ATP kinased T3 primer placed near 3' end of the transcript produced after run-off transcription was used. Figure 21.B shows extension of the annealed primer with superscript RT II (GIBCO-BRL) in the presence of dNTP. In parallel to the primer extension reaction, a sequencing reaction with the same primer of the plasmid was run to match the extended band with sequencing reaction products. The size of the extended product matched precisely with +1 G of the T7 promoter sequence (Fig.21B), which means transcription actually starts from this position. The result of this experiment also shows the precise size of in vitro transcription product which is being made from T7 promoter with T7 RNA polymerase.

III.9. Construction of Ribozyme Clones in pBII Vector

Ribozyme sequences cloned in pBloT7 vector were ineffective as discussed under 'Limitation of pBloT7 Vector'. These ribozyme clones were transferred in pBII vector following a cloning strategy as given in Fig. 8. pBII vector was constructed by ligation of small fragment from TKS(-) into large fragment of pBloT7 vector. The resulting vector pBII has one BssHII site upstream of T7 promoter, whereas pBloT7 does not have any BssHII site and TKS(-) has two BssHII sites.

Ribozyme clones (pRZ4.9/ pRZ4.11) were PCR amplified with pK7, which has BssHII site upstream of T7 promoter and mutation at +1 position from T to G, and reverse primers. The fragment following restriction digestion with BssHII and XhoI was cloned in pBII vector which was digested by the same restriction enzymes as PCR products. The clones were characterized by the presence of BssHII restriction site and confirmed by PvuII restriction digestion (Fig. 22.A).
Fig. 21. (A) Screening of pBgl II / pStu I vectors digested by restriction enzyme Bgl II (a) and Stu I (b). Plasmids having respective restriction site were linearized after digestion with the restriction enzyme.

(B) Primer extension analysis. Reverse transcription was performed with the transcript produced from pStu I vector (lane 1 and 2). Lane 3-6 labeled G-A-T-C display dideoxy sequence reaction employing same primer to locate the exact base position of the extension product. The base corresponding to the extension product represent the transcription initiation site.
Fig. 22. (A) Restriction analysis of pB II vector. pB II vector was constructed as described in Methods. Restriction digestion of pB II vector with BssH II (Lane 3), BssH II + Xho I (Lane 4) and Nsi I (Lane 6). Lane 1 was λ DNA digested with EcoRI + Hind III, Uncut pB II (Lane 2) and TKS (-) vector digested with BssH II (Lane 5). Also see fig. 7 for the map of the pB II vector.

(B) Restriction analysis of vectors used for in vivo studies. 1. uncut pRZ4.11B; 2. uncut pRZ4.11BT; 3, 4, 5 and 6 show BssH II digest of pRZ4.11B, pRZ4.11BT, pRZ4.9B and pRZ4.9BT respectively. 7, λ DNA / BstE II + pBluescript / Msp I; Lane 8-11 show Pvu II digest of pRZ4.9B, pRZ4.9BT, pRZ4.11B and pRZ4.11BT respectively. Lane 7 was BstE II fragments of λ DNA along with Msp I fragments of pBluescript KS(+). (cf. fig. 7 for details of construction)
III.10. Construction of Vectors for In Vivo Studies

For in vivo studies, the vector should have proper signal to initiate and terminate transcription of any gene at defined site. Otherwise, ribozyme sequence transcribed in vivo having 5'- or 3'- additional sequence can fold into such secondary structures which may not be catalytically active. Plasmids pRZ4.9B and pRZ4.11B are naturally transcribed from +1 of insert, but they do have T7 terminator sequence to terminate at particular site. Plasmid pET-3a containing T7 terminator sequence (Rosenberg et al., 1987) was digested by BamHI and EcoRV restriction enzymes and a fragment of 306 bp was isolated, and cloned into BamHI and HincII sites of pRZ4.9B and pRZ4.11B. Recombinant clones having T7 terminator sequence were confirmed by release of insert with BamHI and XhoI restriction digestion, respectively (Fig. 22.8).

III.11. Subcloning of lexA gene in pTZ18U Vector

Initially lexA gene was subcloned in pBlOT7 vector in which T7 promoter was not efficient. The reason for this is discussed under the subtitle 'limitation of pBlOT7 vector'. So it was necessary to subclone lexA gene in some other vector or to modify this vector so as to obtain very high yield of in vitro transcript with phage RNA polymerase. The lexA insert was released from pBlx4 as an EcoRI fragment of 952 bp and purified from agarose gel. This insert was subcloned in EcoRI site of pTZ18U vector (map of pTZ18U is given in Fig. 4). In order to confirm the insert and its orientation, restriction analysis of clones having lexA gene in pTZ18U was done by EcoRI and HincII respectively (Fig. 23B). The orientation of the clones having insert was also confirmed by polymerase chain reaction using T7 promoter primer and lexA-specific primers (PR2). Clones having insert in positive orientation with respect to T7 promoter gave rise to a ~952 bp band (lane no 1, 3, 5) and clones having insert in negative orientation did not give rise to any band as expected (Fig. 23.A).
Fig. 23. (A) Colony PCR screening of *lexA* clones. Different clones were PCR amplified directly from colonies using T7 promoter primer and *lexA* specific primer (PR2). Clones having insert in positive orientation with respect to T7 promoter give a fragment of expected size whereas other clones did not give any PCR amplified product. Lane 5 contains EcoRI-HindIII fragments of λ DNA.

III.12. Subcloning of recA Gene in pBgl II Vector

DNA fragment containing recA gene as in JAM24 (pBR322 based plasmid obtained from Prof. D. W. Mount) was isolated with SacII and EcoRI restriction enzyme, respectively. This fragment was subcloned in corresponding sites of pBgl II vector. Recombinant clones were confirmed by release of insert with SacII and EcoRI restriction digestion (Fig. 24). This plasmid was linearized with Bgl II and antisense recA probe was generated with T3 RNA polymerase to study recA gene expression pattern in E.coli.

III.13. Ribozyme-mediated Cleavage of lexA RNA In Vitro

Fig. 9A shows the location of GUC on lexA RNA, with the cleavage site occurring at position 343. Fig. 9B depicts the sequence of the lexA-ribozyme including its structure and complementary sequence of lexA RNA containing the target GUC site. In vitro cleavage of lexA RNA was directly demonstrated using (α-32P) UTP labelled lexA RNA substrate as described earlier in materials and methods. The lexA RNA target that spanned cleavage site 343 was prepared by transcription of plasmid pR15 after linearization with EcoRV using T7 RNA polymerase. Likewise, labelled ribozyme was synthesized in vitro by utilizing T3 RNA polymerase promoter of plasmid pRZ3.1.

We have shown the optimal conditions for lexA-ribozyme mediated cleavage to be 50 mM Tris-HCl (pH 7.5) with 10 mM MgCl₂ at 37°C. The cleavage reaction was dependent on time, pH, magnesium concentration and molar ratio of substrate to ribozyme (Fig. 25). The cleavage reaction seems to have an absolute requirement for divalent cations. When substrate RNA was incubated in 50 mM Tris-HCl, 1 mM MgCl₂, 1 mM EDTA, pH7.5, no detectable cleavage occurs upto 3h (Fig.25C, lane 2). The reaction rate was found to increase steadily with MgCl₂ concentration until a maximum (80%) is reached at ~20 mM MgCl₂. At low pH (6.2), cleavage rate is slow and the rate increases slowly with increase in pH. Very high pH (>10), causes non specific degradation of RNA. The reaction rate is time dependent and it reached a maximum of 75% cleavage in 3h. There is no further increase in cleavage product with
Fig. 24. Restriction analysis of recA gene. 1. BstE II fragments of λ DNA; 2. Uncut pBglRI; 3. pBglRI cut with EcoRI; 4. pBglRI cut with Sac II + EcoRI; 5. HinflI digested pBR322 was used as size marker; 6, 7, 8, is in same order as 2, 3, 4.
Fig. 25. *In vitro* characterization of ribozyme cleavage reactions. An autoradiograph showing *lexA* cleavage in different reaction conditions. (A) Time course of cleavage reaction. The lanes correspond from L to R to 0, 30, 60, 90, 120, 150 and 180 min of incubation. (B) pH dependence of ribozyme cleavage. The lanes correspond from L to R to pH (6.2, 6.8, 7.1, 7.5, 7.8, 8.2 and 8.6). (C) Mg$^{2+}$ dependence of cleavage reaction. The lanes correspond from L to R to 0, 1, 5, 10, 15 and 20 mM Mg$^{2+}$ and (D) Ribozyme concentration dependence of cleavage reaction. The lanes correspond from L to R to 0, 10, 20, 40, 80 and 120 nM of ribozyme.
Fig. 26. A densitometric quantitation of the reactions displayed in Fig. 25. The fraction of *lexA* RNA (FracS) remaining was plotted versus different Mg2+ (A), pH (B), time (C) and (D) ribozyme concentration. The dotted line represents fraction of substrate converted to products in various conditions.
increase in incubation time. In the above experiment, substrate to ribozyme ratio was kept at 1:3. The cleavage rate increases with increase in ribozyme concentration. Under optimal conditions, the maximum cleavage obtained was ~90% at a substrate to ribozyme ratio 1:10 (Fig. 26).

The reaction rate at different temperature was also examined and it shows that product formation increases as temperature was increased up to 55 °C. Beyond 55 °C, non-specific degradation occurs (Fig. 27A, lanes 6, 7, 13, 14 and 20). After denaturation of samples and addition of Mg²⁺ to a final concentration of 10 mM, one aliquot was kept on ice, while other were placed at indicated temperature with different ribozyme:substrate ratio. 96% cleavage occurred at 55°C as compared to 70% at 37°C with ratio 1:3 (Fig. 27B).

III.14. Specificity of Cleavage of lexA RNA by Wild Type and Mutant Ribozyme

A lexA ribozyme with a mutation A15.1C in the catalytic core failed to cleave lexA RNA over 3h. In a previous study, mutation A15.1G was shown to have <5% of the cleavage activity of the normal ribozyme (Scanlon et al., 1991). To check the specificity of the ribozyme against lexA RNA, antisense lexA RNA was also used as substrate for ribozyme 3.1 and ribozyme 4.11. No detectable cleavage was observed. This shows specificity of ribozyme to its substrate RNA (Fig. 28.2). Figure 30 shows mutant ribozyme incubated with lexA RNA with elevated concentration of magnesium (upto 700 mM) to check whether increase in ion concentration can compensate for mutation in the catalytic core region, but we could not detect any cleavage activity.

III.15. Cleavage of Full- and Partial-length lexA RNA by ribozyme

Transcription of the plasmid pR15 after linearization with EcoRV yielded a 467 bases-long fragment of lexA RNA, whereas BamHI-linearized plasmid gave rise to a 973 bases-long full-length lexA RNA (Fig. 29A). Ribozyme which was designed to cleave at 343 position of lexA RNA, would yield two fragments in either case. The ribozyme was examined for RNA
Fig. 27. Cleavage of *lexA* transcript by ribozyme at varied substrate ribozyme ratio and different temperature. (A) Ribozyme-mediated cleavage reaction were performed 0°C (lanes 1, 8 and 15); 22°C (lanes 2, 9 and 16); 37°C (lanes 3, 10 and 17); 45°C (lanes 4, 11 and 18); 55°C (lanes 5, 12 and 19); 65°C (lanes 6, 13 and 20) and 75°C (lanes 7 and 14) employing substrate : ribozyme of 1:1 (lanes 1-7), 1:3 (lanes 8-14) and 1:10 (lanes 15-20). (B) A densitometric scanning of the above data. (*Filled triangles, 1:1; Filled circles, 1:3*)
Substrates:

S467 5' GG-325 bases-TGCCGCTGGTAGGTC GTGTGGCTGCCGGTG-110 bases-3'
S973 5' GG-325 bases-TGCCGCTGGTAGGTC GTGTGGCTGCCGGTG-616 bases

Ribozymes:

3' GACCAUCCR CACACCGACGG 5'

A C
A U
G A G
C-G G A
R = A for 4.11
A-U U
R = C for 4.9
G-C
G-C
A G
G U

3' GACCAUCCR CACACCGACGU-126 bases-GGG 5'

A C
A U
G A G
C-G G A
A-U U
R = A for 3.1
G-C
R = C for 3.7
A G
G U

Fig. 28.1. Schematic representation of the substrates and ribozymes used in this study. The ribozyme cleavage site in the RNAs is indicated by an arrow. The base-pairing between the substrates and ribozyme is underlined in the substrates sequence.
Fig. 28.2. (A) Cleavage of sense/antisense *lexA* RNA by wild type (4.11) and mutant (4.9). Substrate RNA was incubated in cleavage buffer (50 mM Tris-HCl, pH-7.5, 10 mM Mg$^{2+}$) with wild type and mutated ribozyme at 37°C for 0 h and 1h. Lane 1-2, sense RNA + mutant ribozyme; lane 3-4, sense RNA + ribozyme; lane 5-6, antisense RNA + mutant ribozyme; lane 7-8, antisense + ribozyme.

(B) Specificity of targeted ribozyme for sense RNA. Substrate RNA was incubated in cleavage buffer (50 mM Tris-HCl, pH-7.5, 10 mM Mg$^{2+}$) with wild type (3.1 and 4.11) and mutated ribozyme (3.7 and 4.9) at 37°C for 2 h. Cleavage products were separated on 6% polyacrylamide containing 7M urea. Lane 1, sense RNA + 3.1 ribozyme; lane 2, antisense RNA + 3.1 ribozyme; lane 3, antisense RNA + 3.1 mutant ribozyme; lane 4, sense RNA + 4.11 ribozyme; lane 5, sense RNA + 4.11 mutant ribozyme; lane 6, antisense RNA + 4.11 ribozyme; lane 7, antisense RNA + 4.11 mutant ribozyme. Ribozymes 3.1 and 3.7 have extra nucleotides corresponding to vector sequence at 5' end.
Fig. 29. (A) Autoradiograph of the dried agarose gel showing partial length (Lanes 2-4) and full length (Lanes 5-8) transcripts produced \textit{in vitro} and electrophoresed through 1.2% formaldehyde 1.2% agarose gel. Lanes 1 and 5 contain vector only. Lanes 2 and 5 contain \textit{in vitro} transcription reaction while 3 and 6 contain transcripts after heat denaturation. Lanes 4 and 6 contain sample treated with DNasel (RNase-free). (B) Ribozyme-mediated cleavage of full- (973 nt) and partial-length (467 nt) \textit{lexA} transcripts. Ribozyme and substrate synthesized in the presence of radioactive tracer and quantitated. Cleavage reactions were done at 37°C for different time with 10 mM Mg²⁺. Cleavage products were electrophoresed on urea-polyacrylamide gel. Lane 1, truncated substrate RNA + 3.1 ribozyme (15 min); Lanes 2 and 3 have products of this reaction after 60 and 120 min, respectively; Lane 4, full-length substrate RNA only; Lanes 5, 6 and 7 show cleavage products of this reaction following 15, 60 and 120 min incubation with ribozyme.
cleavage activity by using both the 467-bases-long as well as 973-bases-long transcript of the \textit{lexA} sequences as a substrate. Schematic representation of the substrates and ribozymes used is shown in Fig.28.1. Under experimental conditions, ribozyme produced two main cleavage products of expected size from 467-bases-long (lane no 1, 2, 3) and 973-bases-long full-length transcript (lane no 5, 6, 7). Lane no 4 shows that no cleavage product was seen when the 467 base long substrate was incubated without ribozyme. Arrow at right of the photograph shows the band which is common in both full- and partial-length \textit{lexA} RNA transcript and arrow at left of photograph points towards uncleaved transcript (Fig.29.B). Cleavage products showed no heterogeneity, and in fact the long and short substrates gave the same 343 bases product thus confirming the ribozyme mediated cleavage at the site as designed. It was noted that both the substrates were cleaved with equal rate. These findings indicate that GCC sequence chosen is accessible to ribozyme-mediated cleavage under the defined conditions \textit{in vitro}. Cleavage was specific because the resulting products were of the expected size.

\textbf{III.16. Ribozyme-substrate Binding Studies}

To assess the structural homogeneity of the substrate RNAs, their electrophoretic behavior was examined under conditions similar to those used for the cleavage reaction. The ribozyme mediated reaction involves complex formation between substrate and ribozyme, cleavage and cleaved fragments dissociate from the ribozyme ($K_{diss}$) and the liberated ribozyme is now available for a new series of the catalytic events (Fig. 31). Gel shift experiments were performed with radiolabeled substrate RNA and ribozyme to show that ribozyme does form a complex with target RNA and it gets dissociated, but the same is not true with mutant ribozyme (Fig. 32.A). Two predominant conformational species of the \textit{lexA} RNA are seen under non-denaturing assay condition (Fig. 32A). Mutant ribozyme, intended to bind, but to be inactive, were also examined for the catalytic activity. As expected, no amounts of substrate cleavage were observed after incubation of an excess of ribozyme over substrate for 3 hrs at 37°C. To verify that ribozyme indeed binds substrate, varied concentration of ribozyme was incubated with substrate under same condition, and hybridization was evaluated by non-denaturing electrophoresis (Fig.32B). In case of mutant ribozyme, we could see a retardation in substrate RNA compared to substrate alone or substrate with wild type ribozyme. In case
Fig. 30. (A) Autoradiograph showing peak gel filtration fraction of *lexA* (lanes 1, 2 and 3), ribozyme 3.1 (lanes 4, 5 and 6), ribozyme 4.11 (lanes 7, 8 and 9) and ribozyme 4.9 (lanes 10, 11 and 12) produced by *in vitro* transcription. (B) Autoradiograph showing cleavage of *lexA* transcript at higher concentration of Mg$^{2+}$. Substrate RNA was incubated with (a) wild type ribozyme and (b) mutant ribozyme with increasing concentration of Mg$^{2+}$ ions (up to 700 mM).
Fig. 31. A schematic representation of the kinetics of the ribozyme-catalyzed reaction. The reaction catalyzed by the hammerhead ribozyme consists of at least three steps. The substrate first binds to the ribozyme ($k_{\text{assoc}}$). The phosphodiester bond of the bound substrate is cleaved by the action of Mg$^{2+}$ ions ($k_{\text{cleave}}$). The cleaved fragments dissociate from the ribozyme ($k_{\text{diss}}$) and liberated ribozyme is now available for the new series of the catalytic events (Shimayama et al., 1995).
Fig. 32. Analysis of ribozyme-substrate complex. (A) Substrate RNA labeled during transcription with [α-32P]UTP, were combined with labeled wild type and mutant ribozyme in cleavage buffer for different time intervals and loaded on 6% polyacrylamide gel containing 10 mM Mg²⁺. Lane 1, substrate in 50 mM Tris-HCl, pH 7.5; lane 2, substrate + 1x CB; lane 3, substrate + 1x CB+ mutant ribozyme (0 h), lanes 4 and 5 represent complex formed after 1 h and 2 h of incubation with above conditions respectively. lanes 6 and 7 contain ribozyme incubated in 1xCB for 1 h and 2 h respectively. (B) Different concentration of mutant ribozyme was mixed with substrate RNA in cleavage buffer and analyzed on nondenaturing polyacrylamide gel. Lane 1, substrate only; lane2, mutant ribozyme only; lanes 3-7 contain 0-120 nM ribozyme with 40 nM substrate RNA incubated in 1x CB for 30 min. (1x CB, 50 mM Tris-HCl, pH 7.5, 10 mM Mg²⁺).
of the active ribozyme, the complexed state could not be visualized as a single discrete species perhaps because of these complexes being in a state of flux and assuming different conformational states during the course of reaction. The cleavage products may also offer potential competitors for the ribozyme binding since they retain the cognate sequence for one of the arms of the ribozyme.

III.17. Kinetic Characterization of Ribozymes 3.1 and 4.11

To find out if the 5' additional vector sequences in the ribozyme had any effect on the rate of cleavage, ribozymes with additional sequences were generated. Secondary structure prediction of these ribozymes when attached to 3' end of the substrate by five U nucleotides have shown that they fold properly with their target site (Fig. 14). Plasmid pRZ4.11B upon digestion with PvuII restriction enzyme gives precise transcript having no additional vector nucleotides at 5' and 3' end of the ribozyme with T7 RNA polymerase whereas in vitro transcription of plasmid pRZ3.1 linearized with PvuII restriction enzyme with T3 RNA polymerase gave rise to 127 nucleotides from vector at 5' end of the ribozyme, but 3' end of the ribozyme does not have any additional vector nucleotide. Steady-state cleavage velocities were measured for each ribozyme at several substrate concentration that were at least 10-fold greater than ribozyme concentration (Fig. 33). Michaelis-Menten parameter for these hammerhead ribozyme are shown in Table.3. Values of $K_{cat}$ are similar, but $K_m$ values differ, being particularly high for ribozyme 3.1. Dependence of the $lexA$ RNA cleavage on different ribozyme concentration were carried out (Fig. 34 A) and the slope obtained by plotting the observed reaction rate against the ribozyme concentration used for the calculation of $k_{cat}$ / $K_m$ values for the ribozyme (Table. 3). When the reaction was allowed to proceed for different time interval, after 3.5h, 58% of the substrate converted to the product in presence of ribozyme 3.1 whereas 50% cleavage occurs in case of ribozyme 4.11 (Fig. 35). Both the ribozymes cleaved with different rate at different temperature. Above 60% of the substrate was converted into product in case of ribozyme 3.1 as compared to 15% cleavage by the ribozyme 4.11 at 45°C when the reaction was carried out for 1h (Fig. 36). $lexA$ RNA cleavage reactions are pH and Mg$^{2+}$ dependent. Almost no detectable cleavage product were seen at pH<7 when the reaction was incubated
Fig. 33A Comparison of Ribozyme 3.1 and 4.11. Dependence of ribozyme-mediated cleavage on substrate concentration.
Fig. 33B. Steady-state kinetics of hammerhead cleavage reactions. (a) The steady-state rate of cleavage ($V$, pmoles min$^{-1}$ x 10$^{-1}$) normalized to ribozyme (R) concentration (45 nM) is plotted versus substrate (S) concentration for hammerhead ribozyme 3.1. (b) Eadie-Hofstee plots of these data used for the calculation of $K_m$. 
Fig. 33C. Steady-state kinetics of hammerhead cleavage reactions. (a) The steady-state rate of cleavage \( V \) (pmoles min\(^{-1} \times 10^{-2} \)) normalized to ribozyme \( R \) concentration (45 nM) is plotted versus substrate \( S \) concentration for hammerhead ribozyme 4.11. (b) Eadie-Hofstee plots of these data used for the calculation of \( K_m \).
Fig. 34. (A) Dependence of the *lexA* RNA cleavage on ribozyme concentration. (B) The graph is a representation of the above data. The slopes obtained by plotting the observed reaction rate $k$ against the ribozyme concentration represent the $k_{cat}/K_m$ values for the different ribozymes. *Filled circles*, 3.1 ribozyme; *Open circles*, 4.11 ribozyme.
**Fig. 35.** Time course of cleavage. (A) Substrate RNA and ribozymes 3.1, 4.11 were incubated in 50 mM Tris-HCl (7.5), 20 mM Mg2+ at different time. (B) The fraction of substrate converted to product at various times is shown for hammerhead 3.1 (Filled circles) and for hammerhead 4.11 (Open circles) in a reaction containing substrate to ribozyme ratio is 1:1.
Fig. 36. Dependence of cleavage reaction on different temperature. (A) Substrate RNA and ribozymes (ratio 1:1) were incubated at temperature 0°C, 22°C, 37°C, 45°C, 55°C and 65°C (Lane 1-6). (Filled circles, ribozyme 3.1; open circles 4.11). (B) A densitometric scanning of the above gel.
for 1h. but the cleavage pattern are same in both ribozyme (Fig. 37). In the above experiment, substrate to ribozyme ratio was kept 1:1.

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<th>Hammerhead</th>
<th>$K_m$ (nM)</th>
<th>$K_{cat}$ (min$^{-1}$)</th>
<th>$K_{cat} / K_m$ (nM$^{-1}$ min$^{-1}$)</th>
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<td>3.1</td>
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<td>0.0028</td>
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<td>4.11</td>
<td>64</td>
<td>0.0016</td>
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* indicates average values of three separate experiment.

III.18. Effect of Factors Interacting with Nucleic Acids on Ribozyme Activity

The ribozyme cleavage reaction was studied under different conditions known to influence stability of nucleic acid using labelled substrate and ribozymes. The cleavage reaction seems to have an absolute requirement for divalent cations. No cleavage was observed in NaCl which is generally equally effective in stabilizing RNA structure. Spermine is not able to replace magnesium ion in cleavage reaction. Other metal ions (Mn$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Al$^{3+}$, Zn$^{2+}$, Hg$^{2+}$, Fe$^{2+}$, Ni, Va and Zr) were also tested in cleavage conditions. Substrate RNA was incubated with ribozyme in 50 mM Tris-HCl, pH 7.5 containing 10 mM different metal ions. The observed cleavage of RNA was found to be efficient in the presence of Mn$^{2+}$, Co$^{2+}$, slower with Ca$^{2+}$. Al$^{3+}$ and it was very slow with the other ions with respect to cleavage observed in presence of Mg$^{2+}$ (Fig. 38).

The ribozyme cleavage reaction was also carried out in denaturants like formamide, urea and ethanol. As the percentage of formamide, ethanol or urea was increased in the reaction mix, the rate of conversion of substrate to product decreased. Cleavage products could be detected in reactions incorporating upto 2M urea but at concentration exceeding 4M of this chaotropic agent, the reaction was apparently completely inhibited (Fig. 39).
Fig. 37. Dependence of cleavage reaction on Mg$^{2+}$ (a) and pH (b). Substrate RNA and ribozyme 3.1 (Lane 1-5), ribozyme 4.11 (Lane 6-10) were incubated in 50 mM Tris-HCl (7.5) containing 0-20 mM Mg$^{2+}$ (a). Substrate RNA and ribozyme 3.1 (Lane 1-5), ribozyme 4.11 (Lane 6-10) were incubated in presence of 20 mM Mg$^{2+}$ containing different pH 6.2, 6.8, 7.5, 7.8, 8.2 and 8.6 of 50 mM Tris-HCl (b).
Fig. 38. Effect of metal ions on ribozyme activity. Substrate RNA was incubated with 3.1 ribozyme in 50 mM Tris-HCl, pH-7.5 containing 10 mM different metal ions. Reactions were carried out for 0, 1, 2 and 3h. Cleavage products were analyzed on 6% polyacrylamide gel containing 7M urea. (A) Lanes 1-4, Al^{3+}; lanes 5-8, Ca^{2+}; lanes 9-12, Cd^{2+}; lanes 13-16, Co^{2+}; lane 17, Mg^{2+}; lanes 18-20, Hg^{2+}. (B) Lanes 1-4, Co^{2+} and lanes 5-8, Mn^{2+}. 
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Fig. 39. Effect of factors affecting nucleic acid interaction on ribozyme activity. Substrate RNA and 3.1 ribozyme were incubated in cleavage buffer (50 mM Tris-HCl, pH-7.5, 10 mM Mg$^{2+}$) containing formamide, ethanol or urea. (A) Lanes 1-5: 0, 10, 20, 30 and 40% formamide (B) Lanes 6-10: 0, 5, 10, 20 and 30% ethanol and (C) Lanes 11-15: 0, 2, 4, 6 and 8 M urea.
Fig. 40. (A) Isolation of total RNA. Total cellular RNA was isolated after IPTG addition and electrophoresed on 1.2% denaturing agarose gel. Lanes 1-6 contain RNA samples at 0, 15, 30, 45, 60, 90 min following induction.

(B) RNase protection assay for lexA-ribozyme. 5 μg total cellular RNA isolated were hybridised with 32P-labeled antisense ribozyme riboprobe, followed by RNaseA/T1 digestion. Resulting digest was electrophoresed in denaturing 6% polyacrylamide gel and visualized by autoradiography. Arrow shows a protected band. Lane no. 1 contain riboprobe used for hybridization.
Fig. 41. Cellular ribozyme activity. Total cellular RNA was incubated with *in vitro* made *lexA* transcript for 90 min. Lane 1 contains transcript used for assay while lanes 2 and 3 contain ribozyme *in vitro* made instead of total cellular RNA to serve as a control. Lanes 4, 5 and 6 shows labeled substrate incubated with total cellular RNA prepared at 15, 30 and 45 min following IPTG induction.
the ribozyme accumulated per cell, 45 minutes after induction by IPTG of the lacZ promoter driven T7 RNA polymerase gene (This estimation is based on the assumption that each cell of E. coli contains ca. 100 femtograms of total RNA).

III.19.3. Effect of Ribozyme on Expression Pattern of lexA and recA Genes

E. coli strain BL21(DE3) was transformed with plasmids containing ribozyme sequence. These transformants were grown in LB medium containing chloroamphenicol, till the O.D$_{600}$ reaches 0.3-0.5, and IPTG was added to growth medium at final concentration of 1mM. Cultures were collected at different time intervals (0, 15, 30, 45, 60, 90 min) and total cellular RNA were isolated. These samples were analyzed for lexA and recA expression by RNase protection assay. Plasmids pR18 digested with BamHI was used to generate full-length antisense lexA riboprobe and pBgR I was used to generate antisense recA riboprobe. Total RNA was heated and allowed to anneal with antisense lexA or antisense recA riboprobe, followed by digestion with a single strand-specific RNase A/T1. Digestion of pre-annealed RNA produced a protected species of the expected size (Fig.42B, protected band marked by arrow).

Expression of lexA was increased compared with control, reaching a maximum at 45 min after the induction of ribozyme, followed by a gradual decline but the pool remained higher than its basal level (Fig.42B). Expression of recA gene is induced and attains a maximum at 60 min after the IPTG induction of ribozyme transcription(Fig.43). Figure 42A shows derepression of lexA gene in cells containing wild-type (lanes 4, 5, 6 and 7) and mutant ribozymes (lanes 2 and 3). Total RNA was isolated after 45 min of the IPTG addition (lanes 3, 5 and 7) and before IPTG addition (lanes 2, 4 and 6).
Fig. 42A. RNase protection assay for lexA transcript. Total cellular RNA were hybridised with antisense lexA riboprobe (produced from run-off transcription on template pR18 with T7 RNA polymerase), followed by RNaseA/T1 treatment. Samples were electrophoresed in polyacrylamide gel under denaturing condition. (A) Samples taken at 0 and 45 min. Lanes 1 and 8, control; lane 2, pRZ4.9BT (0 min); lane 3, pRZ4.9BT (45 min); lane 4, pRZ4.11BT (0 min); lane 5, pRZ4.11BT (45 min); lanes 6-7 is same as lanes 4-5; lane 9-10, riboprobe used for hybridization.
**Fig. 42B.** RNase protection assay for *lexA* transcript. Total cellular RNA were hybridised with antisense *lexA* riboprobe (produced from run-off transcription on template pR18 with T7 RNA polymerase), followed by RNaseA/T1 treatment. Samples were electrophoresed in polyacrylamide gel under denaturing condition. (B) Samples taken at different time intervals. Lane 1, riboprobe (truncated *lexA* transcript); lane 2, antisense riboprobe (~950 nt); lane 3, tRNA+ antisense riboprobe+RNaseA/T1; lane 4, full-length antisense transcript + truncated transcript + RNaseA/T1; lanes 5-10, RNA samples prepared at 0, 15, 30, 45, 60 and 90 min after induction + full-length riboprobe + RNaseA/T1; lanes 11-16 is same as lanes 5-10 except that hybridised samples were treated by RNase ONE (Promega). Arrow indicates the protected band. Cleavage products were marked *.
Fig. 43. RNase protection assay for recA transcript. Total cellular RNA was hybridised with antisense recA riboprobe, followed by RNaseA/T1 digestion. The resulting digest was electrophoresed in denaturing 6% polyacrylamide gel and visualized by autoradiography. Lane no.1 contains probe hybridised with tRNA as a control. Lanes 2-7 contain protected band corresponding to cellular RNA prepared at 0, 15, 30, 45, 60 and 90 min after induction. Arrow indicates protected band.
Fig. 44. Survival of *E. coli* BL21 cells induced for ribozyme expression (1 mM IPTG for 45 min) (filled circles) and the same strain carrying the ribozyme-less vector (open circles), treated under otherwise identical conditions, under varied doses of ultraviolet radiation.
III.19.4. UV-Sensitivity of Cells after IPTG Induction of Ribozyme

*E. coli* strain BL21(DE3) cells transformed with plasmid containing ribozyme sequence and grown in LB media until the $A_{600}$ of the culture was $\sim 0.3$. IPTG (Isopropyl thiogalactosid) was added to the final concentration 1.0 mM to induce the expression of the endogenous T7 RNA polymerase gene. Cells were grown for another 45 min with shaking at 37°C, and were tested for their sensitivity to the UV light source of 312 nm wavelength. Cells containing pBloT7 plasmid vector without ribozyme insert were taken as control. Figure 44 shows that cells induced for ribozyme expression were more sensitive to UV light than normal cells lacking ribozyme. There are signs of inactivation being biphasic in transfectant expressing ribozyme against *lexA*. 