**lexA** mRNA was chosen as a model target for ribozyme mediated inactivation

Ribozymes are RNA molecules which by assuming specialized structures acquire the capability to catalyze modification of biomolecules. One class of such catalytic RNA molecules cleave RNA in a targeted manner and are potentially useful for modulation of intracellular gene expression. It is of interest to determine whether ribozymes can really be used in living cells to modulate gene expression. Towards this end, we examined the ability of a ribozyme to cleave *lexA* transcript, and hence to induce the SOS response in *E. coli*. LexA is a repressor protein and binds to similar operator elements (SOS box) of most of SOS genes, including *recA* and *lexA*. It was our interest to determine whether the modulation of *lexA* gene by ribozymes affects the SOS response.

**GUC of the *lexA* RNA sequences were scanned for the accessibility with the help of the computer program RNAFOLD**

The only sequence requirement for the cleavage site of the target RNA by a ribozyme is that it contains the trinucleotide GUC. So, before designing a ribozyme against *lexA* RNA, the complete RNA sequence was scanned for the presence of GUC target sites. Secondary structure prediction of *lexA* RNA was carried out using RNAFOLD computer program (GCG, University of Wisconsin). Since prediction of accessibility of target sites is very important while designing a ribozyme against any gene, the accessibility of different target sites were also examined by employing RNAFOLD. Secondary structure prediction showed that GUC at position 343 resides in the loop region. Position of this loop remain unaltered when different lengths of *lexA* RNA were folded. This showed that hairpin-loop structure which spans this GUC site is more stable than the alternative structures the sequence could assemble. Different GUC sites along the RNA were also tested for their availability to form complex with ribozyme with 10 nucleotides stem I and stem III. Ribozyme targeting at position 343 gave minimum free energy. According to the program RNAfold, the best structure within 10% of the lowest free energy contained roughly 90% of phylogenetically related helices.

**Annealing of ribozyme with chosen target was visualized by RNAFOLD**

When ribozyme sequence which was designed for GUC at the position 343 connected by five U's at the 3' end to *lexA* RNA was folded, the ribozyme could form complex with its target site as predicted. Accessibility of target site selected in *lexA* RNA, located within the loop region as predicted by the computer program was substantiated by experimental results.
A hammerhead ribozyme was designed and cloned in pBLoT7 vector
Based on the above observations, a hammerhead ribozyme as refined by Haseloff and Gerlach, was designed to cleave lexA RNA having 11 and 9 nucleotides stem I and stem III respectively. Oligonucleotides corresponding to lexA-ribozyme were cloned in Nsi I site of pBLoT7 (a derivative of pBluescript) under phage T7 promoter. This plasmid was named as pRZ4.11. We also constructed a plasmid that directs the in vitro synthesis of mutant ribozyme named, pRZ4.9, which contains a point mutation A15.1C in the catalytic domain. Numbering of hammerhead was done according to Hertel et al., 1992. Other Plasmids pRZ3.1 and pRZ3.7 with ribozyme sequence in negative orientation with respect to T7 RNA polymerase promoter were also constructed.

Wild type as well as critical point mutant forms of the ribozyme were prepared
Ribozymes directed against GUC located at position corresponding to nucleotide 343 of lexA RNA were prepared. Ribozymes 3.1 and 3.7 transcripts were synthesized in vitro by transcription of Pvu II linearized plasmid pRZ3.1 with the help of phage T3 RNA polymerase. Ribozyme 3.7 which is identical to ribozyme 3.1 except that it possessed a mutation at A15.1C. Wild type (4.11) and mutant (4.9) ribozyme were synthesized by in vitro transcription of Pvu II linearized pRZ4.11 and pRZ4.9 respectively, with the help of phage RNA polymerase. Ribozyme 3.1 differs from ribozyme 4.11 in having 126 extra vector nucleotides at the 5' end whereas the 3' end is same in both the cases.

The in vitro transcription vector was suitably modified for optimal yield of the transcripts from T7 promoter
Upon carrying out the in vitro transcriptions of the constructs having ribozymes and lexA sequence, it was found that T7 RNA polymerase did not yield any transcripts whereas T3 RNA polymerase was very efficient. When the sequence of the T7 promoter was analyzed, it was found that T7 promoter has T as +1 nucleotide which is not being transcribed. It is known that G as +1 nucleotide is transcribed efficiently. It was again confirmed by changing T to G at +1 position of T7 promoter in these clones by site directed mutagenesis which led to efficient synthesis of transcripts. These new constructs having G at +1 position were named as pRZ4.11B and pRZ4.9B, which were obtained by PCR amplification of pRZ4.11 and pRZ4.9 with modified T7 promoter (Rb) and reverse primer (RevP), and subsequently cloning the product in pBlI vector.

The 5' end of the transcripts was mapped by primer extension
Two new vectors were constructed having Bgl II and Stu I sites respectively in place of Nsi I site of pBLoT7. The advantage with these vectors is that they have G at +1
position. In addition they show a very high efficiency of transcription with T7 RNA polymerase. The transcription initiation site of these vectors was mapped by primer extension on the transcripts produced.

Relative efficiency of the transcription of the templates offering varied bases at +1 was estimated

Vectors containing G, A, T and C nucleotides at +1 position of the T7 promoter were constructed. In order to test the efficiency of the initiation of transcripts by T7 RNA polymerase on various variants, the four constructs were transcribed in vitro. Cherenkov measurements of offered and incorporated [$\alpha^{32}$P]-UTP was used to estimate the amount of RNA synthesized. Transcription with T7 RNA polymerase was most efficient in pR15, pR18 and pStuI each having a G nucleotide at +1 of the transcript. Initiation on other constructs, with non ‘G’ nucleotides at +1 was very poor or no detectable transcripts were produced.

Transcripts lacking any additional sequences at 5' or 3' end were produced

The transcripts produced by in vitro transcription of PvuII linearized plasmids carrying ribozyme sequence (pRZ4.9B and pRZ4.11B), with the help of phage T7 RNA polymerase contains no additional vector nucleotides at their 5' or 3' end. The advantages of cloning of ribozyme in this system are the high level of expression achieved with viral RNA polymerases and the ease with which T7 or T3 constructs can be tested in vitro.

Ribozyme mediated cleavage reactions was carried out in vitro for establishing optimal conditions

Transcription from the plasmid template that contained the lexA gene and the ribozyme sequence generated lexA RNA substrate and the ribozyme. These transcripts were used to determine the specificity of the ribozyme cleavage and to optimize cleavage conditions in vitro. The cleavage product showed no heterogeneity, and the fact that the long and short substrates gave the same 343 nucleotide product proved that the cleavage was occurring at the expected site. No such cleavage was seen during incubation without ribozyme, or with anti-ribozyme RNA produced by transcription of the ribozyme gene in opposite orientation. It was noted that both substrates were cleaved at the same rate. This finding indicates that the GUC sequence chosen is accessible to ribozyme mediated cleavage in vitro.

The mutation A15.1C inactivates the lexA-ribozyme

Activities of wild and mutant ribozymes were examined in vitro using labelled transcript of lexA which is 467 nucleotides in length. Specificity of cleavage was demonstrated as
the actual size of the cleaved fragments corresponded to the expected size, if cleavage were to occur immediately 3' to the GUC sequence. A mutant ribozyme with a mutation in the catalytic domain (A15.1C) was shown to be inactive even if the incubation was carried out for an extended period of time, whereas in an earlier study a mutation A15.1G was shown to have <5% of the cleavage activity as compared to wild type ribozyme.

Hammerhead ribozyme mediated cleavage is Mg²⁺, pH, time and ribozyme concentration dependent
Increase in the amounts of the ribozyme relative to the substrate resulted in a progressive increase in the amount of the cleavage products. The cleavage reaction was also found to be time-dependent and had an absolute requirement of Mg²⁺. At low pH, no cleavage products were seen and very high pH caused non-specific degradation of RNA. This showed that reactions were pH-dependent. When the ribozyme was incubated with the substrate in pH 7.5, no cleavage occurs. Spermidine could not replace magnesium ion in the reaction and no cleavage was observed in 1M NaCl which is generally equally effective in stabilizing RNA secondary structure.

Ribozyme 3.1 and ribozyme 4.11 were compared in terms of cleavage kinetics
Ribozyme having 5' additional vector nucleotides with ribozyme having no vector nucleotides at both the ends were compared, with respect to time, Mg²⁺, pH and temperature requirement for cleavage reactions. Kinetic characterization of ribozyme having 5' additional vector sequences along with ribozyme having no additional sequence at either ends were carried out. The ribozyme 3.1 having 126 extra bases from the vector has higher Km value than 4.11 ribozyme which does not have any additional nucleotide. The two ribozyme have similar $k_{cat}/K_m$ values.

Co²⁺ and Mn²⁺ can partially support the ribozyme reactions
Since ribozymes appear to be metalloenzymes, the effects of various metal ions, Co²⁺, Ca²⁺, Mn²⁺, Zn²⁺, Cd²⁺, Ni²⁺, Hg²⁺, Al³⁺, Fe³⁺, Zr and Vanadium on hammerhead ribozyme were examined.

Denaturing and precipitating conditions inactivate the ribozyme activity
The ribozyme cleavage reactions were also carried out in formamide, urea and ethanol. As the concentration of formamide, urea or ethanol increased, rate of product formation decreased.
Formation of substrate-ribozyme complex could be visualized by mobility shift. Gel shift experiments showed a retardation in substrate RNA complex as compared to substrate alone (without the ribozyme).

The ribozyme constructs to study expression of ribozyme in cells
To study the effect of ribozyme-mediated cleavage of lexA mRNA within bacterial cells, plasmids containing wild type (pRZ4.11BT) and mutant ribozyme (pRZ4.9BT) sequences flanked by the T7 RNA polymerase promoter and T7 terminator were constructed. Escherichia coli BL21 (DE3), a λ lysogen containing T7 RNA polymerase gene under lacZ promoter was transformed with plasmids pRZ4.11BT and pRZ4.9BT. These plasmids have a terminator sequence and produce a transcript of limited and defined size, and because of the transcript's likely stem-loop structure, ribozyme stability is improved probably by limiting the action of RNases.

We have for the first time been able to show ribozyme activity in cell extracts
Induction of ribozyme was monitored by RNase protection assay after addition of IPTG to BL21(DE3) cells. Induction of lexA transcript was observed in cells carrying the ribozyme compared to the mutant ribozyme where a little induction was apparent perhaps because of antisense effect of the arms of the ribozyme. Derepression of lexA occurred up to 45 min following induction of ribozymes. Prolonged incubation after induction showed a gradual decrease in lexA RNA concentration. Also for the first time we have demonstrated the presence of cleavage products of expected size in Escherichia coli. This was difficult to detect in vivo as reported earlier. Cleavage of lexA transcript also affects the expression of recA gene. Induction of recA gene is also a good indicator of SOS response in E. coli. Taking the above parameters as indicators of SOS alarm, the present study would be the first record of the induction of SOS function under non DNA damaging conditions.

Prolonged derepressed state of lexA results in increased UV-sensitivity in bacteria
Cells taken after 45 min of the IPTG induction for ribozyme expression showed increased sensitivity to UV light. It must be mentioned in this context that LexA is an autoregulatory protein and the time required for capturing bacterial cells in the transient states of SOS expression is shorter than that required to reach equilibrium of expression based on the lacZ promoter used for derepression of the ribozyme in the present experiments. It is quite likely that at the time of aliquoting the bacteria for test of UV-sensitivity, they would have already passed the initial expression of SOS functions and have reached the second equilibrium with respect to LexA pool and, therefore, have most of the repair related SOS functions repressed.
The effect of the expression of ribozyme *in vivo* on the cells carrying the ribozyme expression vector on the level of transcripts of genes regulated by LexA protein was also studied. The result of the above experiments suggest that SOS regulon of *E. coli* may be subjected to regulation at a point downward of DNA damage. This also mean that damaged DNA is not directly involved in derepression of *lexA* and other SOS genes even though it is the common product of conditions causing SOS alarm in *Escherichia coli*.

These conclusions however, remain to be confirmed by an assay of the LexA autoprotease activity, SOS repair reactions, level of expression of other SOS-related genes and genetic consequences, if any, of ribozyme mediated cleavage of *lexA* transcript.

A model ribozyme in *E. coli* has been developed. However, this approach can also be adopted for application in higher organisms. Ribozyme chosen against any gene can be constructed and expressed *in vivo*. In situation where hyperexpression of a gene results in a pathological state, inactivation of gene function may help in managing the disease e.g., inactivation of oncogene transcript and/or telomerase transcript (which are known to be associated with transformed state) promise to offer a molecular approach to control cancer. Ribozyme targeted against HIV-1 have shown promise to restrict virus replication in cultured cells. We are currently developing ribozymes targeted against mammalian TNFα and the RNA component of telomerase in our laboratory.