MATERIALS AND METHODS
V. MATERIALS AND METHODS

V.1. Materials

V.1.1. Reagents

Acetic acid:- Acetic acid glacial. (Merck-GR, Qualigens-AR). Concentration 99.7%. Stored at RT.

Acrylamide 30%:- 29 g Acrylamide (Sigma, A-9909) + 1 g N,N’-methelyene-bis-acrylamide (Sigma, M-7256) dissolved in 60 ml H₂O. Warmed on the magnetic stirrer to dissolve and made final volume to 100 ml, added 1 g activated charcoal/100ml acrylamide, stirred for an hour, filtered through Whatman 1 MM filter paper, stored at 4°C in dark brown bottles. Bench life of 1 month.

Agar plates:- LB agar: To 800 ml H₂O added 10 g Bacto Tryptone (Hi Media RM-014; Difco laboratories), 5 g Yeast Extract (Hi media RM-027), 10 g NaCl (Qualigens-AR). Adjusted the pH to 7.0 with 5 N NaOH and made the final volume to 1L. Added 15 g agar/L (Hi media RM-301), 1.5%, autoclaved and cooled down to 45-50°C. The antibiotics were added from sterile stocks and were poured into 90-mm bacteriological plates. Allowed the plates to polymerise for 1 hour in the laminar flow or on the bench overnight.

Agarose gel:- Type V High melt (Sigma, A-3768) or Type VII Low melt (Sigma, A-40180) or Agarose, LMP (Promega, V283A). Typically 0.8 - 2% agarose gels in 1 X TAE buffer were used with a final concentration of 0.5 μg/ml Ethidium bromide.

Antibiotics:- All antibiotic stock solutions were sterile filtered
through 0.45 µM or 0.22 µM filters

**Ampicillin**: Ampicillin-sodium salt injection - Biocillin 500mg. Injection (Biochem Pharmaceuticals Industries) was dissolved in the 5ml sterile H₂O provided with the antibiotic. Kept frozen as 500 µl aliquots at -20°C. Working concentration: 100 µg/ml.

**Chloramphenicol**: Chloramphenicol (Sigma, C-7795). Dissolved in absolute ethanol for a stock concentration of 10 mg/ml and stored at -20°C. Working concentration 34 µg/ml.

**Nalidixic acid**: Nalidixic acid-sodium salt (Sigma, N-3143) was dissolved in H₂O at 5 mg/ml as stock concentration and stored in 500 µl aliquots at -20°C. Working concentration: 15 µg/ml.

**Penicillin G**: Sodium salt of benzylpenicillin, (Sigma, P-3032). Stock solution 500 mg/ml. Stored at 4°C. Added to a final concentration of 100 U/ml. Used for cell culture.

**Streptomycin**: (Sigma, S-9137) added dry powder to the medium to a final concentration of 100 mg/ml.

**Tetracycline**: Tetracycline hydrochloride (Sigma, T-8032). Stock concentration was 10 mg/ml in 50% ethanol. Stored in aliquots at -20°C. Working concentration: 15µg/ml.

**Protease inhibitor**: (Sigma, A-6279). Prepared in H₂O to a final concentration of 1 mg/ml and stored at -20°C. Used at a final concentration of 1-2 µg/ml.

**Ammonium per sulphate**: (Sigma, A-9164) 0.10 g APS was dissolved in 1 ml H₂O to get a 10% solution. Prepared fresh just before use.
Materials and Methods

ATP 0.1 M:- Adenosine triphosphate (Sigma, A-6419). Dissolved 60 mg ATP in 0.8 ml H₂O and adjusted the pH to 7 with 0.1 N NaOH. Adjusted the volume to 1 ml with sterile distilled H₂O. Diluted a 1 μl aliquot 1000 times and read the wavelength at 259 nm and calculated the actual concentration of ATP. \[ \varepsilon_{\text{ATP}} = 1.54 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \]. Stored as 20 μl aliquots at -20°C.

Bacterial Cells:- Stored in 15% glycerol as 1 ml aliquots at -80°C and stab cultures at 4°C.

- *E. coli* DH5α: φ80dλacZΔM15, recA1, gyrA96(Nal'), thi-1, hsdR17 (rK-, mK+), sup E44, relA1, deoR, Δ(lacZYA-argF)U169
- *E. coli* XL-1 Blue: recA' endA1 gryA96(Nal') thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZ~M15 Tn10(Tet')]
- *E. coli* BL21(DE3)pLysS: F'ompT, hsdSbr, (rB·mB'), dcm. gal, λ (DE3), pLysS (Cam')
- *E. coli* BL21(DE3)pLysE: F'ompT, hsdSbr, (rB·mB'), dcm. gal, λ (DE3), pLysE (Cam')

Benzamidine:- Hydrochloride: Hydrate (Sigma, B-6506) Peptidase inhibitor. Prepared in H₂O to a final concentration of 250 mg/ml stored at -20°C. Used at a final concentration of 0.5 mg/ml.

(250 mg/ml)

Binding Buffer (10X):- 200 mM HEPES, pH 7.9., 4 mM EDTA, pH 8.0., 100% glycerol. Added 4 mM DTT just prior to use.

(EMS A)

Bradford’s Reagent:- Commassie Brilliant Blue G-250 (Merck-GR) 10 mg dissolved in 5 ml Ethanol + 10 ml conc. H₃PO₄ mix), make volume to 100 ml with distilled H₂O.
<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
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<tbody>
<tr>
<td>BSA:-(10 mg/ml)</td>
<td>Bovine Serum Albumin. (Fraction V, Sigma, A9647). Dissolved 10 mg BSA in H$_2$O and stored as 1 ml aliquots at $-20^\circ$C.</td>
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<tr>
<td>Buffer A (10X):-</td>
<td>150 mM Tris.Cl, 150 mM NaCl and 250 mM KCl in H$_2$O, the pH adjusted to 7.4 with conc. HCl, autoclaved and stored at 4$^\circ$C. Just before use added to final concentrations of 25mM $\beta$-Mercaptoethanol, 0.15 mM Spermine, 0.5 mM Spermidine and 0.2 mM PMSF from stock solutions (see same section and preparation of the respective stock solutions and their concentrations).</td>
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<tr>
<td>CaCl$_2$ 1M:-</td>
<td>Dissolved 14.7 g of CaCl$_2$.2H$_2$O (Sigma, C-3306) in 100 ml H$_2$O and sterile filtered through a 0.22-micron filter. Stored at $-20^\circ$C.</td>
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<tr>
<td>Carrier DNA:-(10 mg/ml)</td>
<td>Salmon sperm DNA, sodium salt (Sigma, D-1626) 10 mg/ml in H$_2$O, added NaCl to a final concentration of 0.1 M, extracted with phenol/chloroform (1:1) once, sonicated and ethanol precipitated. Dissolved the sheared DNA in H$_2$O to a final concentration of 10 mg/ml. Stored as 250 $\mu$l aliquots at $-20^\circ$C.</td>
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<tr>
<td>Carrier RNA:-(10 mg/ml)</td>
<td>Ribonucleic acid Type VI from Torula Yeast (Sigma R-6625) dissolved to a final concentration of 10 mg/ml in TE pH 7.5, 0.1 M NaCl, vortexed vigorously to dissolve, extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Ethanol precipitated and was re-dissolved in TE, pH 7.5 at a concentration of 10 mg/ml. Stored as 100 $\mu$l aliquots at $-20^\circ$C.</td>
</tr>
</tbody>
</table>
Materials and Methods

Cell lines:-
Daudi cells National Centre for Cell Science, (DBT, GOI), University of Pune Campus, Ganeshkhind, Pune.
HeLa cells S3 American Type Culture Collection, ATCC No. CCL-2.2.

Chloroform:-
(E.Merck, Germany), Cat. No. 2445 Stored at RT.

CIAP buffer (10X):-
1 M NaCl, 500 mM Tris.Cl, pH 8.2, 100 mM MgCl₂ and 10 mM DTT, pH 7.9. Stored at -20° C.

CIAP:-

Cisplatin:-
Kemoplat; Cisplatin Injection, 1 mg/ml; Dabur Pharmaceuticals. Made serial dilutions in 1 X PBS. Stored at 4° C.

Coomassie Brilliant Blue R-250 dye:-
Dissolved 0.25 gm Coomassie Brilliant Blue-R250 (SRL) in MeOH:H₂O:AcOH::45:45:10. Filtered through Whatman No. 1 filter to remove any particulate matter. Stored in dark bottles and re-used.

Cracking Buffer:-
Added 5 N NaOH and 20% SDS to a final concentration of 2 N NaOH and 0.2% SDS in 6 X DNA loading Buffer. Store at RT.

Cycloheximide:-
(Sigma, C-6255). Made a stock solution of 10 mg/ml in sterile H₂O, sterile filtered and stored at -20° C.

Denaturing Buffer:-
0.5 N NaOH, 1.5 M NaCl for denaturing DNA in
Materials and Methods

the agarose gel for Southern blotting.

Denhardts' reagent (50X):-

1 g Ficoll (Sigma F-2637), 1 g Polyvinylpyrrolidine (Sigma, P-5288) and 5 g Bovine serum albumin (Fraction V, Sigma, A-9647) in 100 ml H₂O. Sterile filtered and stored in 20 ml aliquots at -20°C.

DEPC:-

Diethyl pyrocarbonate. (Sigma, D-5758). Stored at 4°C in a dark bottle.

Depurination Buffer:-

0.25 N HCl to depurinate DNA in the agarose gel for Southern blotting.

Developer:-

(Silver staining)

6 g Na₂CO₃ + 4 g sodium thiosulphate/100ml H₂O.

Developer:-

(autoradiography)

Mixed 200 ml of the 5 X GBX Developer (Sigma, P-7042) in 800 ml H₂O and stored at RT in dark glass bottles in the dark.

Dextran sulphate:-

Sodium salt of dextran sulphate (Sigma, D-8906). Made stock solution of 50% in H₂O and used at a final concentration of 10% in pre-hybridization and hybridization buffers.

Dimethylformamide:-

N,N-Dimethylformamide (E. Merck-GR. India). Cat No. 17754. Prepared 50% in sterile H₂O for tissue culture work.

Dissolving Buffer:-

(E.coli RNA)

200 mM sodium acetate, pH 5.0., 0.2% SDS., 1mM EDTA, pH 7.6.
DMEM: Dulbecco’s Modified Eagle Medium. (Sigma, D-5523). To prepare 500 ml of Dulbecco’s Modified Eagle Medium added 5.015g DMEM and 1.85g sodium bicarbonate to 400 ml H₂O and dissolved with stirring. Added streptomycin and penicillin to the final concentrations of 100 µg/ml and 100 U/ml respectively. Adjusted the pH to 7.5 with 1 N HCl. Added 50 ml heat inactivated foetal calf serum to the medium and made up the volume to 500 ml with H₂O. Moved the medium to the laminar flow and filter sterilised into an autoclaved bottle. Stored the medium at 4°C under sterile conditions. 5 ml of the medium was kept overnight in the CO₂ incubator and 1 ml plated on LB agar plates overnight to check for bacterial contamination.

DMSO: Dimethylsulphoxide. (Sigma, D-2650; Merck-GR, 1.02952.1000). Used at a final concentration of 10% in FCS to freeze cells in liquid N₂.

DNA loading dye (6X): 25 mg Xylene cyanol FF., 25 mg bromophenol blue, 7 ml H₂O, 3 ml sterile glycerol. Mixed well and stored at 4°C in 1 ml aliquots.

Doxorubicin: Cadria; Doxorubicin hydrochloride injection, 2 mg/ml, Cadila Pharmaceuticals. Made serial dilutions in 1 X PBS and stored at 4°C. Used at a final concentration of 5 µM.

DTT 1M: D-L-Dithiothreitol (Sigma, D-9779) dissolved in deionised H₂O and kept frozen as 100 µl aliquots at -20°C.

EDTA, 0.5 M: Added 93.05g of di-sodium ethylenediaminetetra-
Materials and Methods

pH 8.0. Acetate. 2H₂O (Qualigens-AR; Sigma, E-5134) to 400 ml H₂O and adjusted pH to 8.0 with 40 g NaOH pellets and 5 M NaOH, autoclaved and stored at RT.

EGTA, 0.5 M:-

pH 7.0. Ethylene glycol-bis-[B-amino ethyl ether]-N,N,N',N', tetraacetic acid. Sigma, E-3889. Added 1.9092 g EGTA to 35 ml H₂O and adjusted the pH to 7.0 by adding 10 M NaOH drop-wise while stirring. Made up the volume to 50 ml with H₂O, sterilised by autoclaving and stored at RT.

EMSA Buffer (5X):-

Dissolved 151.425 g Trizma (Sigma, T-1503) and 750 g Glycine (Merck-GR) in 4.5 litre H₂O. Added 100 ml 0.5 M EDTA, pH 8.0 and made up the volume to 5 litre. Checked the pH - should be 8.5. Sterilised by autoclaving. Stored at RT.

Ethanol:-

(Merck-GR 1.00983.0511), Bengal Chemicals and Pharmaceuticals, Ltd. Stored at -20°C.

Ethidium bromide:- (10 mg/ml)

Dissolved 100 mg ethidium bromide (Sigma, E-8751) to 10 mg/ml in 10 ml sterile H₂O, stored as 1ml aliquots at 4°C in dark coloured Eppendorf tubes or in Eppendorf tubes covered in aluminium foli. Working concentration = 0.5 μg/ml.

FCS:-

Foetal Calf Serum. Biological Industries; Kibbutz Beit Haemek, Israel 04-001-1B; 0.07μM membrane filtered. Purchased from Centre for Biochemical Technology (CBT), C.S.I.R., New Delhi. Incubated at 55°C for 20 min to heat inactivate, aliquots of 50 ml stored at -20°C under sterile conditions.
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Fixer: Dissolved 238 g of X-ray acid fixing salts (Kodak F 9000720) in 900 ml H₂O. Filtered through Whatman 1 MM filter paper and stored at RT in dark glass bottles. Took care never to use copper, tined or galvanised ware to prepare or store the fixer.

Fixer: 40% (v/v) ethanol, 12% (v/v) acetic acid, and 25 μl/100ml formaldehyde. (Silver staining)

Formaldehyde:- Formaldehyde solution (37%). (Qualigens-AR). Stored at RT.

Formamide:- (Sigma, F-7508., Merck, 1.12027.0100). Deionised by DOWEX MR-3 mixed bed resin (Sigma, m1-005), filtered through Whatman No.1 and stored at -20°C.

Geimsa Stain:- Qualigens stains. Stored at RT.

Glucose:- (Sigma, G-6136). Stored at 4°C.

Glycerol:- (Sigma, G-5150; Qualigens AR). Stored at RT, autoclaved and stored at 4°C when needed.

Glycine:- (Merck-GR). Stored at RT.

H₂O₂:- Hydrogen peroxide (Merck. 17544). 30% purified solution. Kept in dark bottle in dark at RT. Made fresh dilutions in H₂O and discarded after use.

HCl 1N:- Hydrochloric Acid (Qualigens-AR). Added 8.25 ml of concentrated HCl (Sp. gravity 1.19; ~12.1N) to H₂O and made the volume to 100 ml.
Heparin:- Heparin sodium salt, injection (25,000 IU/5 ml) Biological E. Ltd. Stored at -20°C. Used at a final concentration of 200 μg/ml.

HEPES 1M:- (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), a zwitterionic buffer. (Sigma, H-1016). Dissolved 23.83 g in 75 ml H₂O and adjusted the pH to 7.9 with NaOH, made up the volume to 100 ml and stored at RT.

HEPES Buffer (2X):- Dissolved 1.19 g HEPES, 1.64 g NaCl in 80 ml H₂O, added 1 ml 100X Phosphate Buffer and adjusted the pH to 7.05 with 1N NaOH. Made the volume to 100 ml and rechecked the pH. Sterile filtered, and made aliquots of 5 ml Stored at -20°C.

Hybridization Buffer:- 50% formamide, 10% dextran sulphate, 2 X SSC, 1% SDS,. 200μg/ml denatured salmon sperm DNA, 1 X 10⁶ cpm/ml denatured α-[³²P]-labelled DNA probe.

Immidazole:- (Sigma, I-0125). Stored at RT.

Impregnation Solution:- 0.2 g silver nitrate/100ml H₂O. (Silver staining)

IPTG:- Isopropyl-β-D-thiogalactopyranoside (MBI-Fermentas, #R0392). 100 mM stock solution was made in sterile H₂O and was stored as 1 ml aliquots at -20°C. While using, thawed on ice and replaced immediately in -20°C after use.

Isopropanol:- Isopropanol (Spectrochem, HPLC grade). Stored at RT.
Materials and Methods

**KCl 1M:-**
Potassium chloride (Qualigens-AR). Dissolved 7.45 g KCl in 100 ml H₂O and autoclaved. Stored at RT.

**KCl 2M:-**
Potassium chloride. (Qualigens AR). Prepared a stock solution of 2M by dissolving 14.91 g in 60 ml H₂O and making the volume to 100ml. Autoclaved and stored at RT.

**Leupeptin:-**
Hydrochloride (Sigma, L-0649). Serine and cysteine protease inhibitor made in H₂O at a concentration of 1 mg/ml and stored at -20°C. used at a final concentration of 2 μg/ml.

**LiCl:-**
Lithium chloride (Sigma L-0505). Anhydrous salt. Stored at RT.

**Ligase:-**
T₄ DNA Ligase (Gibco Life Technologies, 15224-025), concentration: 1 Weiss unit/μl. Stored at -20°C in supplier's buffer.

**Ligation Buffer:-**
50 mM Tris.Cl (pH 7.6), 10 mM MgCl₂, 1mM DTT, 1 mM ATP, 5% PEG-8000. Supplied by Gibco Life Technologies. Stored at -20°C in supplier's buffer.

**Liquid Nitrogen:-**
Central Instruments Facility, School of Life Sciences, J.N.U., New Delhi.

**Loading Buffer (6X):-**
0.25% Bromophenol blue (Sigma, B-7021), 0.25% Xylene cyanol FF (Sigma, X-4126) in 30% glycerol, stored at 4°C as 1 ml aliquots.

**Loading dye (2X):-**
1% low melting point agarose, 10 mM EDTA, pH
### Materials and Methods

| Lysis Buffer: |- (DNA laddering) | 8.0, 40% sucrose. Reagents were mixed and incubated at 70°C to mix. |
| Lysis Buffer: |- (plasmid DNA) | 20 μl H2O, 50 μl 20% SDS, 10 μl 0.5 M EDTA, pH 8.0, 10 μl 10 N NaOH. |
| Lysis Buffer: |- (MTT assay) | 20% SDS (Sigma, L-4390) in 50% dimethylformamide. |
| Lysis Buffer: |- (rRNA isolation) | 6M Urea, 3M LiCl, 50 mM Sodium acetate, 1% SDS, 200 μg/ml heparin. Stored at 4°C. Added urea to DEPC-treated H2O and mixed on magnetic stirrer. After urea had completely dissolved added LiCl and mixed on magnetic stirrer. Added 3M sodium acetate, pH 5.0. and filter-sterilised the solution. Added heparin and SDS just prior to use. |
| Lysis Buffer: |- (whole cell lysate) | 1 X PBS with 1% Triton X-100. Just prior to use added the protease inhibitors: 1 mM DTT, 0.5 mM PMSF, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin, 0.5 mg/ml Benzamidine, and the phosphatase inhibitor : 1 mM Sodium ortho-vanadate. |
| Lysis Buffer: |- (EMSA) | 10 mM HEPES, pH 7.9., 10 mM KCl., 0.1 mM EDTA, pH 8.0, 0.1 M EGTA, pH 7.0 Stored at -20°C. Protease inhibitors added just prior to use to a final concentration of 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine |
| Lysozyme: |- (10 mg/ml) | (SRL, 1240123) 10 mg/ml Lysozyme in 50 mM Tris.Cl (pH 8.0). Stored at -20°C as 100 ul aliquots. |
### Materials and Methods

<table>
<thead>
<tr>
<th>Component</th>
<th>Details</th>
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<tbody>
<tr>
<td><strong>β-Mercaptoethanol:</strong></td>
<td>β-Mercaptoethanol (14.4 M <em>Merck</em>-GR). Stored at 4°C.</td>
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<tr>
<td><strong>M9 Minimal Media (5X):</strong></td>
<td>Dissolved in a final volume of 1 L the following:</td>
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<td>64 g Na₂HPO₄·2H₂O, 15 g KH₂PO₄, 2.5 g NaCl, 5 g NH₄Cl. The solution</td>
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<td></td>
<td>was divided into 200 ml aliquots and sterilised by autoclaving for 15</td>
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<td>minutes at 15 lb/sq.inch pressure.</td>
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<td><strong>Media:</strong> (Bacteriological)</td>
<td>LB medium: 10 g Bacto-tryptone (Hi-media, RM-014), 5 g Bacto-Yeast</td>
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<td></td>
<td>Extract (Hi-media RM-027), 10 g NaCl (Qualigens-AR) in 1 L H₂O and</td>
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<tr>
<td></td>
<td>adjusted the pH to 7.0 with 5 M NaOH. Autoclaved at 15 lb/sq.inch</td>
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<tr>
<td></td>
<td>pressure for 15 minutes and stored at 4°C.</td>
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<td></td>
<td>YT medium: 16 g Bacto-tryptone (Hi-media RM-014), 10 g Bacto Yeast</td>
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<td></td>
<td>Extract (Hi-media RM-027), 5 g NaCl (Qualigens-AR) in 1 litre H₂O,</td>
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<tr>
<td></td>
<td>adjusted the pH to 7.0 with 5 M NaOH, autoclaved at 15 lb/sq.inch</td>
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<td>pressure for 15 minutes, stored at 4°C.</td>
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<td></td>
<td>M9 Minimal Media: To 750 ml autoclaved H₂O added 200 ml 5X M9 Minimal</td>
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<td>Media and 20 ml sterile 20% glucose. Added the required antibiotics</td>
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<td></td>
<td>and used immediately.</td>
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<td><strong>Membranes:</strong></td>
<td>Biodyne A: (Gibco-Life Technology, 14866-016). Neutrally charged nylon</td>
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<td></td>
<td>membrane. Stored in a clean dry place at RT.</td>
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<td></td>
<td>Millipore GS WP-02400: 0.22 µm pore size filters for sterilisation.</td>
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<tr>
<td></td>
<td>Millipore Corporation, USA. Stored in a clean dry place at RT.</td>
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<tr>
<td></td>
<td>Trans-Blot Transfer Medium: (BIO-RAD, Cat. No. 162-0115). Pure Nitrocellulose Membrane 0.45 µm. Stored in a clean dry place.</td>
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</tbody>
</table>
Methanol:-(Qualigens-AR; Merck-GR), stored at RT in a dark bottle.

MgCl₂ 1M:- Magnesium chloride, hexahydrate (Qualigens-AR). Dissolved 20.33 g of MgCl₂·6H₂O in 80 ml of H₂O and adjusted the volume to 100 ml. Sterilised by autoclaving.

MgSO₄ 1M:- Magnesium sulphate, heptahydrate. Dissolved 24.65 g of MgSO₄·7H₂O (Qualigens-AR) in 100 ml H₂O and sterile filtered through 0.22 µM filter and stored at 4°C.

Milk (non-fat):- Nestle CARNATION Nonfat dry milk. Stored at 4°C. Used at a final concentration of 5% in 1X PBST.

MnCl₂:- Manganese chloride. (Qualigens-AR). Stored at RT.

MOPS 1M:- (3-[N-Morpholino]propanesulphonic acid), MOPS free acid (Sigma, M-8899). Dissolved 20.93 g in 100 ml H₂O, adjusted pH to 7 with NaOH. Filter sterilised and stored at 4°C.

MTT:- [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide]. (Sigma, M-2128). Made fresh at a final concentration of 5 mg/ml in 1 X PBS. Stored at 4°C. Used at a final concentration of 25 µg/ml.

Na₂CO₃:- Sodium carbonate (Qualigens-AR). Stored at RT.

NaCl 5M:- Sodium chloride (Qualigens-AR). Dissolved 29.2 g
Materials and Methods

NaCl in 100 ml H₂O, autoclaved, stored at RT.

**NaHCO₃:**
Sodium hydrogen carbonate
*(E.Merck [India] Cat. No. 17520). Stored in a cool, dark place.*
*(Sigma, S-4019). Stored at 4°C.*

**Ni-NTA agarose:**
Qiagen. Stored as slurry at 4°C.

**Normal saline:**
0.98% NaCl solution in sterile H₂O. Autoclaved and used chilled.

**NP-40 10%:**
IGEPAL CA-630. *(Sigma, I-3021). Nonionic detergent. (Octylphenoxypolyethoxyethanol, chemically indistinguishable from Nonidet P-40. Dissolved 1 ml 100% Nonidet P-40 in 9 ml H₂O and mixed gently by inverting the tube. Stored at -20°C.*

**Nuclear Extraction Buffer:**
20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 7.0. Stored at -20°C. Protease inhibitors added just prior to use to a final concentration of 1 mM DTT, 0.5 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 0.5 µg/ml benzamidine.

**Nucleosides:**
**Deoxyribonucleotides:**
dATP (Gibco Life technology, 10216-018)
dCTP (Gibco Life technology, 10217-016)
dGTP (Gibco Life technology, 10218-014)
dTTP (Gibco Life technology, 10218-012)

**Radioactive nucleotides:**
[α³²P] dATP: Amersham, Cat. No. AA0074, 6000 Ci/mmmole, 10 mCi/ml Stored at -20°C in lead vials.
### Materials and Methods

[\(^{32}\)P] ATP: Amersham, Cat. No. AA0018, 6000 Ci/mmole, 10 mCi/ml. Stored at \(-20^\circ\)C protected in vials.

**PAGE:**
Polyacrylamide gel electrophoresis:  
**Stacking gel:** 5% Polyacrylamide in Tris.Cl pH 6.8.  
**Resolving gel:** 8 to 10% Polyacrylamide in Tris.Cl pH 8.8

**PBS (10X):**
1.3 M NaCl, 20 mM KCl, 78 mM Na\(_2\)HPO\(_4\).2H\(_2\)O, 14 mM KH\(_2\)PO\(_4\), autoclaved and stored at 4\(^\circ\)C.

**PBS/EDTA:**
1 X PBS, 2 mM EDTA, pH 8.0. Autoclaved and stored at \(-20^\circ\)C.

**PBST:**
1 x PBS, pH 7.6 containing 0.1% Tween-20.

**PEG 30%:**
Polyethylene glycol-8000 (Sigma, P-5413). 30% made in H\(_2\)O, autoclaved and stored at RT.

**Phenol:**
(Ranbaxy-AR) Double-distilled, added hydroxyquinoline to a final concentration of 0.1%, equilibrated with 0.5 M Tris.Cl pH 8.0, followed by equilibration in Tris.Cl pH 8.0 and stored in 200 ml aliquots at \(-20^\circ\)C. Working solution was stored at 4\(^\circ\)C.

**Phosphate Buffer (100X):**
Added 2.67 g Na\(_2\)HPO\(_4\).2H\(_2\)O to sterile H\(_2\)O and made up the volume to 100 ml. The final 100X solution is 150 mM. Sterile filtered and stored at RT.

**PMSF (10 mM):**
Phenylmethylsulfonyl fluoride. (Sigma, P-7626). Dissolved 1.74 mg in 1 ml isopropanol to get a final concentration of 10 mM. Stored the aliquots
Materials and Methods

PMSF (100 mM):-
(Sigma, P-7626). Stored at 4°C in a dark brown bottle. Prepared fresh in isopropanol by adding 17.2 mg per ml isopropanol.

Poly[I]: poly[C]:-
Sodium salt of polyinosinic-polycytidylic acid. (Sigma, P-1530). High molecular weight, double-stranded homopolymer. Prepared in 1 X PBS at a concentration of 1 mg/ml and stored at -20°C. Used at a final concentration of 25 μg/ml.

Polymerases: -
Klenow fragment: E. coli DNA Polymerase I, Large (Klenow) Fragment, New England Biolabs. 210L, 5 U/μl, stored at -20°C.
1 X E. coli Polymerase/Klenow Buffer: 10 mM Tris.Cl (pH 7.5), 5 mM MgCl₂, 7.5 mM DTT. Buffer to be supplemented with dNTPs. Stored at -20°C.

Taq DNA polymerase, recombinant: Gibco Life technology, Cat. No. 10342-020, 5U/μl, stored -20°C.
10X buffer: 200 mM Tris.Cl (pH 8.4), 500 mM KCl. Stored at -20°C.

T₄ DNA Polymerase: New England Biolabs, stored at -20°C.

Potassium acetate 3/5 M: -
30 ml 5 M Potassium acetate (Qualigens-SQ) and 5.75 ml Glacial acetic acid were mixed to a final vol of 50 ml, autoclaved and was stored at RT.

Pretreatment Solution: -
(Sliver staining)
0.2 g sodium thiosulphate/100 ml H₂O.

Pronase: -
(Sigma, P-5147). Dissolved to a final
Proteinase K:—
(20 mg/ml)

(Promega, V3021). Made at a final concentration of 20 mg/ml in 50 mM Tris.Cl pH 8.0, 10 mM CaCl₂. No pre-treatment required. Stored at -20°C.

Restriction Enzymes:—
(a) New England Biolabs, stored at -20°C.

- **Bam HI**: 20 U/μl. Cat. No. 136S.
- **Bgl II**: 10 U/μl. Cat. No. 144S.
- **Bss HII**: 4 U/μl. Cat. No. 199S.
- **Hinc II**: 10 U/μl. Cat No. 103S.
- **Kpn I**: 10 U/μl. Cat. No. 142S.
- **Nco I**: 10 U/μl. Cat No. 193S.
- **Not I**: 10 U/μl. Cat. No. 189S.
- **Pvu II**: 10 U/μl. Cat. No. 151S.
- **Sac I**: 20 U/μl. Cat No. 156S.

1 X NEBuffer Bam HI + BSA. for Bam HI (150 mM NaCl, 10 mM Tris.Cl, 10 mM MgCl₂, 1 mM DTT [pH 7.9]).

1 X NeBuffer Bss HII for Bss HII [100 mM NaCl, 10 mM Bis Tris Propane.Cl, 10 mM MgCl₂, 1 mM DTT (pH 7.0)].

1 X NEBuffer 1 for Kpn I (+BSA), Sac I (+ BSA), [10 mM Bis Propane, 10 mM MgCl₂, 1 mM DTT (pH 7.0)].

1 X NeuroBuffer 2 for Pvu II [10 mM Tris.Cl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT (pH 7.9)].

1 X NEBuffer 3 for Bgl II, Bst XI, Hinc II (+BSA), Not I (+BSA), [50 mM Tris.Cl, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT (pH 7.9)].

1 X NEBuffer 4 for Nco I [20 mM Tris-acetate, 10 mM Mg-acetate, 50 mM potassium acetate,
Materials and Methods

1 mM DTT (pH 7.9)].

(b) Gibco Life technology:

Eco RI: (10 U/µl), Cat. No.15202-021.
Hind III: (10 U/µl), Cat. No. 15207-020.
Pst I: (10U/µl), Cat. No. 15215-049.
Xba I: (10U/µl), Cat. No. 15226-012.

700 µl deionised formamide, 160 µl 10X MOPS,
260 µl formaldehyde, 18 µl H2O, 100 µl glycerol,
80 µl bromophenol blue.

RNA loading dye (2X):-

Pancreatic RNase A, Ribonuclease Type IA.
(Sigma, R-4875). Made at a final concentration of
10 mg/ml in 10 mM Tris.Cl (pH 8.0), 15 mM NaCl.
Incubated for 15 minutes at 85°C to inactivate
any contaminating DNases. Stored at -20°C.

Roeferon A:-
(recombinant human IFN-α2a)

Roche U.S. Pharmaceuticals; 2.7 X 10^8 IU/mg
protein. Made dilutions in 1 X PBS and stored at
4°C. Used at a final concentration of 0 - 1000 U
for treatment of cells in 1 ml.

RPMI-1640 Medium:-

Roswell Park Memorial Institute-1640 Medium.
(Sigma, R-6504). Dissolved 2.08 g RPMI-1640 in
150 ml H2O. Added 900 mg glucose (Sigma, G-6138) and adjusted the pH to 4.0 with 1 N HCl
when RPMI-1640 medium dissolved. Raised the
pH to 7.0 with 1 N NaOH. Added 0.4 g sodium bicarbonate (Sigma S-4019) and checked the pH.
If the pH overshot 7.2 readjusted the pH to 7.2.
Materials and Methods

with 1 N HCl. In the hood added 20 ml heat inactivated foetal calf serum to make a final concentration of 10%. Added 20 mg Streptomycin (Sigma S-9137, final concentration 100 mg/ml) and 24 μl Penicillin (final concentration 100 U/ml, Sigma P-3032). Sterile filtered the medium and stored at 4°C. 5 ml of the medium was kept overnight in the CO₂ incubator and 1 ml plated on LB agar plates overnight to check for bacterial contamination.

Sample Buffer (2X):
(SDS-sample buffer)
100 mM Tris.Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% glycerol, 200 mM DTT in H₂O DTT is added just prior to use. Stored at RT (otherwise the SDS will precipitate). Shelf life of 3 months.

Sample Buffer (2X):
(Discontinuous Native PAGE)
0.5 M Tris.Cl pH 6.8, 30% glycerol, 0.5% (w/v) bromophenol blue in H₂O.

SDS 10%:
Lauryl sulphate-Sodium salt (Sigma, L-4390), 10% (w/v) SDS dissolved in 50 mM Tris.Cl, pH 8.0. Stored at RT.

Sephadex G-50:
Sephadex G-50 (Sigma, G-50-80), 10 g was soaked and washed several times in excess of H₂O to remove all traces of dextran, re-suspended in 100 ml 1 X TNE, autoclaved and was stored at 4°C.

Silver nitrate:
(Qualigens-AR), stored in dark in a sealed bottle.

Sodium acetate 3M:
(pH 5.2)
Dissolved 40.8 g sodium acetate.3H₂O (Qualigens-AR), in 80 ml H₂O and adjust the pH to 5.2 with glacial acetic acid. Made the volume
Materials and Methods

100 ml and made 20 ml aliquots, autoclaved and store at RT.

Sodium acetate 3M:-(pH 5.0)
Added 20.41 g sodium acetate (Qualigens AR) to 20 ml DEPC-treated H2O. On the magnetic stirrer adjusted the pH to 5.0 with glacial acetic acid. Made final volume to 50 ml with DEPC-treated H2O. Stored at RT.

Sodium thiosulphate:- Qualigens-SQ. Store at RT.

Solution I:- (DNA fragmentation)
50 mM Tris.Cl, pH 8.0, 10 mM EDTA, pH 8.0, 0.5% SDS. Added Proteinase K to a final concentration of 0.5 mg/ml just prior to use.

Solution II:- (DNA fragmentation)
RNase A solution in H2O at a concentration of 1 mg/ml.

Solution I:- (genomic DNA isolation)
0.34 M sucrose in 1 X Buffer A.

Solution II:- (genomic DNA isolation)
1.8 M sucrose in 1 X Buffer A.

Spermidine, 1M: - Dissolved 0.1452 g Spermidine (Sigma, S-0266) to a final vol of 1 ml in 10 mM Tris.Cl, pH 9.5, 0.1 mM EDTA. Stored as 500 µl aliquots at -20°C.

Spermine, 1M: - Dissolved 0.3482 g Spermine tetrahydrochloride (Sigma, S-1141) to a final vol of 1 ml in 10 mM Tris.Cl, pH 9.5, 0.1 mM EDTA. Stored as 500 µl aliquots at -20°C.

SSC (20X):- 175.3 g NaCl (Qualigens-AR) + 88.2 g Sodium citrate (Qualigens-AR) dissolved in 800 ml H2O,
adjusted pH to 7.0 with 1 N HCl, made the vol to 1 L, autoclaved, stored at RT.

SSPE (20X):-

40.175.3 g NaCl (Qualigens-AR), 27.6 g of Sodium dihydrogen phosphate di-hydrate (SRL 1944113) and 7.4 g EDTA (Qualigens-AR) were dissolved in 800 ml H2O. adjusted pH to 7.4 with 5 M NaOH and made up the vol to 1 L with H2O, autoclaved and stored at RT.

Studier's RNA Isolation Buffer:-

50 mM Tris. Cl, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 19% glycerol, 0.025% bromophenol blue in DEPC-treated H2O.

Sucrose:-

(Sigma, S-0389). Stored at RT.

T10N10:-

10 mM Tris.Cl, pH 8.0, 10 mM NaCl.

TAE Buffer (50X):-

Mixed 242 g Tris base (Qualigens-AR), 57.1 ml glacial acetic acid (Qualigens-AR), 100 ml 0.5 M EDTA pH 8.0 (Qualigens-AR) and made to 1 L with H2O, autoclaved and stored at RT.

TBE Buffer (5X):-

54 g Tris base, 27.5 g Boric acid (Qualigens-AR) and 20 ml 0.5 M EDTA pH 8.0, to 1 L H2O, autoclaved and stored at RT. If on long storage a precipitate was formed the solution was discarded.

TCM buffer:-

0.3 M CaCl2, 0.3 M MgCl2 and 0.1 M Tris.Cl pH 7.5 in H2O, autoclaved and stored at RT. For all reagents, stock solutions were used.

TE buffer:-

10 mM Tris.Cl, pH 7.5 or 8.0 and 1 mM EDTA in H2O, autoclaved and stored at RT.
### Materials and Methods

**TEL T buffer:**

50 mM Tris.Cl, pH 7.5, 62.5 mM EDTA, 0.4% Triton X-100 and 2.5 M LiCl in H$_2$O, autoclaved and stored at RT. (For all the reagents except LiCl, stock solutions were used. LiCl was added as a dry powder).

**TEMED:**

N,N,N',N', Tetra methyl ethylene diamine. (Sigma, T-7024), stored at 4°C in a dark bottle.

**TNE (10X):**

0.5 M Tris.Cl, pH 7.5, 1 M NaCl and 0.05 M EDTA in H$_2$O, pH was 7.4 - 7.5 in a 1/10 dilution, autoclaved and stored at RT.

**TNF-α:**

(recombinant human tumour necrosis factor-α)

Genentech Inc., South San Francisco. Specific activity 5 $\times$ 10$^7$ Units/mg protein. Made dilutions in 1 X PBS from a concentration 10 $\mu$g/0.1ml and stored at 4°C. Used 29 µl from a stock solution of 34.58 nM to treat cells in 1 ml medium to get a final concentration of 1 nM.

**Transfer Buffer :-**

(Western Blotting)

For every 1 L of the buffer added 200 ml 5X Tris-Glycine Buffer (Western), 200 ml MeOH and made up the vol to 1 L with H$_2$O.

(Final concentration: 25 mM Tris base, 0.2 M glycine, 20% MeOH, pH 8.5).

**Transformation Buffer I:-**

To sterile H$_2$O added 15% glycerol and autoclaved. Added to a final concentration 30 mM potassium acetate, 10 mM CaCl$_2$, 50 mM MnCl$_2$, 100 mM KCl, and adjusted the pH to 5.8 with 0.1 N acetic acid. Sterile filtered through 0.2 µM membrane and stored at 4°C. For every 100 ml culture required 40 ml of the buffer.
### Materials and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformation Buffer II</td>
<td>To sterile H₂O added 15% glycerol and autoclaved. Added to a final concentration 10 mM MOPS pH 7.0, 75 mM CaCl₂, 10 mM KCl, and sterile filtered through 0.22 μm membrane and stored at 4°C. For every 100 ml of starting culture required 4 ml of transformation buffer II.</td>
</tr>
<tr>
<td>Tris-Glycine Buffer (10X)</td>
<td>Added 30.3 g Tris base, 144.0 g glycine and made up the volume to 1 L with H₂O. Do not adjust the pH, which becomes 8.3 when the salts dissolve.</td>
</tr>
<tr>
<td>Tris.Cl</td>
<td>(Qualigens-AR, Sigma, T-1503). Tris or Trizma base 1 M in H₂O; pH was adjusted to 7.5 or 8.0 with concentrated HCl, autoclaved and stored at RT.</td>
</tr>
<tr>
<td>Tris-glycine SDS Buffer (5X)</td>
<td>15.1 g Tris base (Qualigens-AR), 94 g Glycine (Merck-GR), 50 ml 10% SDS (Qualigens-AR). If the buffer had to be used for native PAGE then the SDS was not added.</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>(Sigma, T-8787; SRL- 2020130), diluted to 25% in H₂O and stored at RT.</td>
</tr>
<tr>
<td>Trypan Blue Stain</td>
<td>(Sigma, T-6146). Made in 1 X PBS at a final concentration of 0.4%. Stored at RT.</td>
</tr>
<tr>
<td>Trypsin</td>
<td>(Sigma, T-2271). Prepared a working solution of 0.03% in 1 X PBS/EDTA and stored at -20°C.</td>
</tr>
<tr>
<td>Tween-20</td>
<td>(E. Merck- India, Prod 437082Q). Store at RT.</td>
</tr>
<tr>
<td>Urea</td>
<td>(Sigma, U-5378, SRL, 214321), stored at RT.</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vinblastin</strong></td>
<td>Cytoblastin; Vinblastin sulphate injection, 10 mg/ml; Cipla Ltd. Made serial dilutions in 1 X PBS and stored at -20°C.</td>
</tr>
<tr>
<td>(1mM)</td>
<td></td>
</tr>
<tr>
<td><strong>Vincristin</strong></td>
<td>Cytocristin; Vincristin sulphate injection, 1 mg/ml; Cipla Ltd. Made dilutions in 1 X PBS and stored at -20°C.</td>
</tr>
<tr>
<td>(1mM)</td>
<td></td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td>Milli Q Water (Millipore deionizer) or quartz double-distilled, autoclaved H₂O.</td>
</tr>
<tr>
<td><strong>Whatman paper</strong></td>
<td>Whatman 3 MM Paper (3030917), stored in a clean dry place.</td>
</tr>
<tr>
<td><strong>X-gal</strong></td>
<td>5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma, B-4252). Stock solution of 20 mg/ml in dimethylformamide made or polypropylene tubes. The aliquots were stored at -20°C, in Eppendorf tubes that were wrapped in Aluminium foil to prevent damage by light.</td>
</tr>
<tr>
<td>(20 mg/ml)</td>
<td></td>
</tr>
<tr>
<td><strong>X-ray films</strong></td>
<td>XAR5, Kodak, (Sigma, F-5388), Hyperfilm™ MP (Amersham). Stored dry at 4°C.</td>
</tr>
<tr>
<td><strong>ZnSO₄ 1M</strong></td>
<td>Zinc sulphate (Qualigens-AR). Prepared in H₂O; autoclaved and was stored at RT.</td>
</tr>
</tbody>
</table>

### V1.2. Plasmids, Oligonucleotides, Antibodies, Cofactors, Kits and Animals

#### V1.2.1. Plasmids

**pBluescript KS II (+) and pBluescript SK II(-):** 2961 bp phagemid from Stratagene, USA (Short et al., 1988; Alting-Mees et al., 1989). The vector possesses a f1 origin and CoIE1 origin with T₇ and T₃ promoters flanking the polylinker (bases 657-759) that contains 21 restriction enzyme sites. The vector contains the lacZ gene under lac promoter for blue/white selection after induction with IPTG. KS
indicates that the polylinker is oriented such that β-galactosidase (bases 816-938) transcription proceeds through the Kpn I site first and the Sac I site last. SK indicates that the polylinker is oriented such that β-galactosidase (bases 816-938) transcription proceeds through the Sac I site first and the Kpn I site last. II indicates the presence of two Bss HII sites that allow the entire polylinker and the T7 and T3 promoter sequences to be excised from the vector. bla gene (1975-2832) confers ampicillin resistance and allows for selection in E. coli.

**pRSETA**: 2897 bp high-level prokaryotic expression vector from Invitrogen. Expression is controlled by bacteriophage T7 promoter (bases 20-39) and induced by the production of T7 RNA polymerase in BL21(DE3)pLys S/E strain of E. coli where the T7 RNA polymerase gene is under the control of the lacUV5 promoter that is inducible by IPTG. The T7 gene 10 sequence (bases 133-162) provides protein stability, whereas the N-terminal poly-histidine tag (6XHis: bases 112 - 129) provides for rapid purification through Ni-columns. There is an N-terminal Xpress epitope (bases 169-192) for protein detection with the anti-Xpress antibody and an Enterokinase cleavage site for removal of the fusion tag. F1 origin (bases 456-911) for ssDNA allows easy sequencing and mutagenesis. The term A signifies the N-terminal coding sequence is in a frame relative to the multiple cloning sites (bases 202-248) such that it allows in-frame cloning of the gene of interest. The pUC origin (bases 916-2852) and ampicillin resistance gene bla, allows for replication and selection in E. coli. A kind gift from Dr. R. Madhubala, from the School of Life Sciences, JNU, New Delhi.

**pRSETB**: 2897 bp high-level prokaryotic expression vector from Invitrogen. Expression is controlled by bacteriophage T7 promoter (bases 20-39) and induced by the production of T7 RNA polymerase in BL21(DE3)pLys S/E strain of E. coli in which the T7 RNA polymerase gene is under the control of the lacUV5 promoter that is inducible by IPTG. The T7 gene 10 sequence (bases 133-162) provides protein stability, whereas the N-terminal poly-histidine tag (6XHis: bases 112 - 129) provides for rapid purification through Ni-columns. There is an N-terminal Xpress epitope (bases 169-192) for protein detection with the anti-Xpress antibody and an Enterokinase cleavage site for removal of the fusion tag. F1 origin (bases 456-911) for ssDNA allows easy sequencing and mutagenesis. The term B signifies the N-terminal coding sequence is in a frame relative to the multiple cloning site (bases 94-248).
202-248) that allows in-frame cloning of the gene of interest. The pUC origin (bases 916-2852) and ampicillin resistance gene *bla*, allow for replication and selection in *E. coli*. A kind gift from Dr. R. Madhubala, from the School of Life Sciences, JNU, New Delhi.

**pZB1:** 5128 bp plasmid containing the murine RNase L partial 5' cDNA cloned in pBluescript SKII(-) within Eco RI-Xho I sites (Zhou *et al.*, 1993). The cDNA extends over exon 1 till exon 4. A kind gift from Prof. R. H. Silverman, Cleveland Clinic Foundation, Ohio, USA. Genbank accession No. L10382.

**pZC5:** 5466 bp plasmid containing the complete cDNA of the human RNase L gene cloned in the Hind III site of pBluescript KS(+) downstream of the T7 promoter sequence (Zhou *et al.*, 1993). A kind gift from Prof. R. H. Silverman, Cleveland Clinic Foundation, Ohio, USA. Genbank accession No. L10381.

### V.1.2.2. Primers and Oligonucleotides

Primers were either obtained from Genmed Synthesis, 1129 Grandview Drive, South San Francisco, USA (Primers No. 1 & 2) or from Macromolecular Resources, Boulder, Colorado, U.S.A (Primers No. 3 & 4). Primers were checked on 12% denaturing polyacrylamide + 7M urea gel to check for purity and integrity. Primers were dissolved at a concentration of 1 nmole/µl in deionised H₂O, Absorbance at A₂₆₀ was measured and serially diluted stock to 25 pmole/µl. Primers were stored at -20°C and thawed only on ice.

1. **PvuHrn5’85-106:** 5'-GCAGCTGGCAATTTACCGTCA-3'
2. **hindHrnNC5’2531:** 5'-TTAAGCTTATGGACTAGTGTAGTCTGGG-3'
3. **5’-NFκB:** 5'-TTGTTACAGGGACTTTCGCTGGGACTTTCACGGGAGGTGGG-3'
4. **3’-NF-κB:** 5'-CCACGCTCCCTGGAAAGTCCCACCGGAAGTCCCTTGGTAACAA-3'

(Primers and oligonucleotides have been described more extensively in Table 1 of Results section).
V.1.2.3. Antibodies

**Mouse anti-human RNase L antibody (monoclonal):** Monoclonal antibody against the C-terminal portion of human RNase L protein (Dong and Silverman, 1995) was a kind gift from Prof. R. H. Silverman, Cleveland Clinic Foundation, Ohio, USA. The antibody was used at a dilution of 1:5000 in 1 X PBST and stored at -20°C. Thawed only on ice. Used antibody solution was stored at 4°C.

**Rabbit antihuman-IkB antibody (polyclonal):** polyclonal antibody raised in rabbit against human IxB-α (C210, Santa Cruz Biotechnology, CA.

**Rabbit anti-mouse IgG-HRP conjugated antibody (polyclonal):** polyclonal antibody raised in rabbit against mouse IgG and conjugated to horse-radish peroxidase (HRP) was kindly provided by Dr. Rathod, Immunochemistry Laboratory, National Institute of Immunology, New Delhi.

**Goat anti-rabbit IgG polyclonal-HRP conjugated antibody:** polyclonal antibodies raised in goat against rabbit IgG and conjugated to horse raddish peroxidase (HRP) were from Santa Cruz Biotechnology, CA.

V.1.2.4. Cofactor

The 2',5'-oligoadenylate (2-5A) cofactor was a kind gift from Prof. R. H. Silverman, Cleveland Clinic Foundation, Ohio, USA. Stored at -20°C. Thawed only on ice. The 500 nM stock solution of 2-5A was diluted in H2O and used at a concentration of 100 nM.

V.1.2.5. Kits

1. **QIAquick Gel Extraction Kit.** Qiagen. Cat. No. 28704. DNA size range : 70 bp-10 kb

V.1.2.6. Animals

Healthy female Swiss albino mice 12-14 weeks of age were obtained from the animal house facility of the School of Life Sciences, JNU, New Delhi. Mice were sacrificed by cervical dislocation and the liver dissected quickly and washed in cold normal saline and briefly kept on ice. All experiments were conducted with fresh tissue.
V.2. GENERAL METHODS

V.2.1. Selection of *E. coli* strain

The *E. coli* strains to be used were streaked on LB agar plates containing the appropriate antibiotics. The cells were grown overnight at 37°C. Colonies were picked and glycerol stocks made for the bacterial strain.

**Table VI** Strains of *E. coli* used along with the antibiotics used to select them on LB agar plates

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Strain of <em>E. coli</em></th>
<th>Antibiotic (µg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.</td>
<td>DH5α</td>
<td>Sodium nalidixate (15 µg/ml)</td>
<td>Hanahan, 1983.</td>
</tr>
</tbody>
</table>

V.2.2. Glycerol stocks of *E. coli* cells

A colony scraped from a plate containing cells grown overnight was resuspended in 500 µl of LB medium contained in an Eppendorf tube. To the cell suspension added an equal volume of LB containing 30% glycerol. The Eppendorf tube was vortexed to ensure that the glycerol was thoroughly dispersed and the culture transferred to -70°C for long term storage. Alternatively, inoculated a single bacterial colony in 850 µl LB medium with the appropriate antibiotics, in an Eppendorf tube. The culture was allowed to grow overnight at 37°C with shaking. To the overnight grown culture added 150 ul sterile glycerol. Stored the culture at -70°C.
V.2.3. Stab cultures of E. coli cells

2-3 ml glass vials were filled $\frac{2}{3}\text{rd}$ with molten LB agar and the vials autoclaved for 20 minutes at 15 lbs/sq.inch on liquid cycle. Allowed the vials to cool and then screwed their caps tightly and stored at RT till needed. To store the bacteria picked a single well isolated colony with a sterile needle and stabbed the needle several times through the agar to the bottom of the vial. Replaced and tightened the vial and stored in the dark at 4°C. To revive the cells pricked the surface of the stab culture and streaked colonies on agar plates containing the appropriate antibiotics.

V.2.4. Preparation of competent E. coli cells

Prepared competent cells by a modification of the method of Hanahan (1985). Streaked the required bacterial strain of E. coli on an agar plate containing the appropriate antibiotics. The next day a single isolated colony was inoculated into 5ml LB containing appropriate antibiotics and allowed to grow overnight with shaking at 200 rpm at 37°C. The next morning inoculated 1 ml of the overnight culture/100 ml LB containing 20 mM MgCl$_2$. The culture was allowed to grow with shaking at 37°C till an O.D.$_{550}$ of 0.5 - 0.6 was attained. (This typically took 3-4 hours for all the strains of E. coli that were used). All subsequent steps were carried out at 4°C or on ice. At no point were the cells exposed to any variation in temperature.) The cells were immediately put into pre-chilled 250 ml GSA bottles and spun at 2.5K rpm for 5 minutes at 4°C in Sorval RC-5B refrigerated centrifuge. The supernatant was discarded and ice-cold 40ml Tranformation Buffer I/100ml starting culture was added. (Added about 10ml Buffer initially and resuspended the cell pellet with a sterile Pasteur pipette. When no cell clumps were visible added the rest of the buffer). After the cell pellet had been completely resuspended allowed the cells to incubate on ice for 10 minutes. Centrifuged the cells at 2K rpm for 5 minutes at 4°C. Discarded the supernatant and added ice-cold 4ml Transformation Buffer II/100ml starting culture. Resuspended the cell pellet with a sterile Pasteur pipette. Made 200 μl aliquots in pre-chilled Eppendorf tubes and stored the cells at -70°C for further use.
V.2.5. Transformation of competent *E. coli* cells

Thawed competent *E. coli* cells on ice for 10 minutes. Took 1 ng/2 μl of plasmid DNA or 4 μl ligation mixture and added double the volume of TCM Buffer. Incubated on ice for 5 minutes. Added 200 μl competent cells. Left on ice for 20 minutes. Gave heat-shock at 42°C for 90 seconds. Incubated on ice for 5 minutes. Added 800 μl LB medium and incubated at 37°C for 1 hour with shaking at 200 rpm. Plated 200 μl of the transformation mixture per agar plate containing the appropriate antibiotics. The plates were allowed to dry in the hood for 5 minutes and incubated overnight at 37°C. The next day the total number of colonies were counted and the transformation efficiency measured by the formula:

\[
\text{Transformation efficiency} = \frac{\text{Total no. of colonies}}{\text{μg DNA X100}}.
\]

V.2.6. Isolation of plasmid DNA

V.2.6.A Rapid isolation of plasmid DNA

The transformed cells were streaked on a grid prepared on agar plates containing the appropriate antibiotics and allowed to grow at 37°C. A small amount of the streak was taken into a 500 μl Eppendorf tube and resuspended in 10 μl T10. Added 10 μl Cracking buffer and allowed to stay on the bench for 2 minutes. Loaded to an agarose gel that was not submerged in TAE Buffer. After all the samples were loaded, the gel was immersed into the tank containing TAE electrophoresis buffer and EtBr at a final concentration of 0.5 μg/ml with the current switched on. Visualised and selected the clones after EtBr staining from the relative migration of the supercoiled DNA of the recombinant clones as compared to the vector.

V.2.6.B LiCl rapid miniprep for isolation of plasmid DNA

Mini preparation of plasmid DNA was carried out by a modification of the LiCl method of Homes and Quigley, 1981. Inoculated 5 ml medium with a single isolated bacterial colony and grew overnight with shaking at 37°C. Centrifuged 1.5 ml of the overnight grown culture at maximum speed for 10 seconds, at room temperature. Discarded the supernatant, added 100 μl TELT Buffer and vortexed briefly. Added 10 μl freshly prepared lysozyme (10 mg/ml) and immediately put the Eppendorf tubes into boiling water bath for 1 minute (for optimal yield of plasmid DNA never handled more than 12 tubes at a time).
Chilled the tubes on ice for 5 minutes. Centrifuged the tubes for 8 minutes at maximum speed, at room temperature. The pellet of cell debris formed at the bottom of the Eppendorf tube was carefully removed with a sterile toothpick. Added 11 μl sodium acetate, pH 5.2 to the tubes and vortexed well. Added 100 μl isopropanol and vortexed well. Kept at -20°C for at least 30 minutes. (If handling more than 12 samples left the DNA to precipitate in isopropanol and processed the next 12 samples). Centrifuged the Eppendorf tube at 15K rpm for 15 minutes at 4°C. The pellet was washed in 200 μl 70% ethanol and centrifuged at 12K rpm for 10 minutes. The pellet was dried at 37°C and resuspended in 10mM Tris.Cl, pH 8.0. The DNA was loaded to an agarose gel and electrophoresed in 1 X TAE Buffer containing 0.5 μg/ml ethidium bromide and visualised on a UV-transilluminator at 300 nm.

V.2.6.C Rapid isolation of plasmid DNA using MgCl₂
Inoculated a single isolated colony in 5 ml medium containing the required antibiotics. The culture was grown overnight at 37°C with shaking. The next day 3 ml of the culture was centrifuged at maximum speed for 10 seconds. After discarding the media the cell pellet was resuspended in 100 μl of H₂O followed by 100μl of freshly prepared Lysis Buffer. Placed the Eppendorf tube in boiling water-bath for exactly 2 minutes. Added 50 μl of 1 M MgCl₂ and kept on ice for exactly 2 minutes. Centrifuged at 4°C at 2K rpm for 2 minutes. Added 50 μl of 5 M Potassium acetate and kept on ice for exactly 2 minutes. Centrifuged at 12K rpm for 5 minutes. Transferred the supernatant to a fresh Eppendorf tube containing 600 μl isopropanol at room temperature and left on ice for exactly 5 minutes. Centrifuged at 12K rpm for 5 minutes at 4°C and discarded the supernatant by inverting. Added 250 μl 75% EtOH and left on the bench for 5 minutes followed by centrifuging at 12K rpm for 5 minutes at 4°C. The pellet was air-dried and dissolved in 20 μl Tₐ₀, pH 8.0. Loaded 2 μl to agarose gel and electrophoresed it at 100 mA in 1 X TAE buffer containing 0.5 μg/ml EtBr and visualised the DNA in an UV trans-illuminator.

V.2.6.D Large scale isolation of plasmid DNA
Inoculated 250 ml LB medium with 1 ml of the glycerol stock or 1 ml of an overnight grown culture. Grew the culture overnight at 37°C with shaking at 200 rpm. The next day transferred the culture to 250 ml GSA bottles,
centrifuged the culture at 6K rpm at 4°C for 20 minutes and resuspended the pellet in 12 ml 50 mM Tris.Cl, pH 8.0. Transferred to an Oakridge tube. While vortexing added 3 ml freshly prepared 10 mg/ml lysozyme (prepared fresh in 50 mM Tris.Cl, pH 8.0) and left on ice for 10 minutes. With vortexing added 1.8 ml 0.5 M EDTA, pH 8.0 and left on ice for 10 minutes. With vortexing added 0.75 ml 2% Triton-X 100 and left on ice for 1 hour after which it was centrifuged in a SS-34 rotor for 30 minutes at 4°C and 15K rpm. The supernatant was measured and transferred to a 50 ml beaker. The volume is generally 18 ml and the subsequent calculations are based on this. While stirring with a magnetic bead, added 1.8 ml freshly prepared 1 N NaOH (diluted from a 5 N NaOH stock) and left on the bench for 10 minutes. Added 8 ml of Tris. Cl, pH 7.5 and left for 3 minutes after which 3 ml of 5 M NaCl and an equal volume of phenol was added. Stirred for 3 minutes and divided into two 30 ml Oakridge tubes. Centrifuged for 5 minutes at 4°C at 9K rpm and transferred the aqueous phase to a conical flask. Added an equal volume of chloroform and stirred for 3 minutes. Allowed the phases to separate and removed the lower organic phase. Added an equal volume of chloroform and stirred for 3 minutes. After the phases had separated removed the lower organic phase and transferred the upper aqueous phase to a 30 ml Oakridge tube and centrifuged at maximum speed at 4°C for 5 minutes. Transferred the upper aqueous phase to fresh Oakridge tubes and added 60 µl of 10 mg/ml RNase A to each tube. Incubated each tube at 37°C for 1/2 an hour. Added 3 ml of 5 M NaCl and 5 ml of 30% PEG-8000 to each tube and left on ice at 4°C. Centrifuged the tubes in HB-4 swing bucket rotor at 9K rpm for 20 minutes at 4°C and resuspended each pellet in 500 µl 1 X TNE, left on bench to dissolve. Pooled the DNA into Eppendorf tubes. To each Eppendorf tube containing pooled DNA added 12.5 µl 20% SDS and 10 µl 10 mg/ml pronase and incubated at 37°C for 30 minutes. Phenol/chloroform treatment (1/2 volume phenol, vortexed; 1/2 volume chloroform vortexed) was carried out, centrifuged for 15 minutes at RT and removed aqueous phase to fresh Eppendorf tubes, added 3/4th volume phenol, vortexed; 1/4th volume chloroform, vortexed. Centrifuged for 15 minutes and removed the aqueous phase to fresh Eppendorf tubes. Added 1/10th volume of 3 M sodium acetate, pH 5.2, vortexed and added 0.7 volume of isopropanol and left at -20°C for 1 hour. Centrifuged for 30 minutes at 4°C at 15K rpm. Washed the pellet in 200 µl 80% EtOH and centrifuged at 4°C for 15 minutes at 12K
rpm. Air dried the pellet and resuspended in 200\mu l T_{10}. Made a 1:10 and 1:20 dilution of the DNA to estimate its concentration.Measured O.D. at 260 nm and 280 nm and took the spectrum from 200 nm to 300 nm. In a 1-cm-wide cuvette 50 \mu g/ml dsDNA has an absorbance at 260 nm of 1.00. The concentration of the DNA was estimated from the formula:

\[
\text{Concentration of DNA} = A_{260} \times \frac{50 \mu g/ml}{1 \text{ absorbance unit}} \times \text{dilution factor}
\]

The O.D. $260/280$ ratio of pure DNA (free of proteins) is 1.7 - 2.0. Alternatively, the concentration of plasmid DNA can be estimated from the intensity of the DNA bands in an ethidium bromide stained agarose gel.

**V.2.7. Sephadex-G50 column purification of DNA**

Filled Sephadex-G50 into a 1 ml sterile syringe whose tip had been blocked with sterile glass-wool and washed the column 5 times with 500 \mu l 1 X TNE. To the column added 100 \mu l plasmid DNA at a concentration 1 mg/ml and eluted with 200\mu l TNE into Eppendorf tubes. Checked 6 \mu l of each eluent on an agarose gel and pooled fractions containing the plasmid DNA. Precipitated the DNA after adding $\frac{1}{10}$th volume 3 M sodium acetate, pH 5.2 and 0.7 volume isopropanol. Dissolved the DNA in 200 \mu l T_{10}. This plasmid DNA was used for all experiments and remained stable after prolonged storage at -20°C.

**V.2.8. Isolation of murine genomic DNA**

Murine genomic DNA from the nuclei purified from mouse the livers of Swiss Albino mice, extracted as per the method of Hewish and Burgoyne (1973) modified by Chaturvedi and Kanungo (1985). Five female Swiss Albino mice were sacrificed by cervical dislocation. All subsequent steps were carried out quickly at 0°C - 4°C. The livers were quickly removed and kept in chilled normal saline. The liver was minced well and every 3 gm of minced liver was mixed with 30 ml of chilled Solution I (final concentration of 0.1 mg tissue/ml). A 10% homogenate of the tissue was prepared with four up and down strokes in a glass homogeniser, fitted with a teflon pestle at 8000 - 10,000 rpm. The homogenate was filtered through four layers of cheesecloth into a beaker kept on ice and subsequently the filtrate was transferred to a cushion of solution II in 35 ml SW-28 rotor tubes and centrifuged at 27,000 rpm for 1 hour at 4°C in a Beckman L5-50 B Ultracentrifuge in a SW-28 rotor. At the bottom of the cushion is present the nuclear pellet. Very carefully, decanted the supernatant,
which may have a top layer of lippopolysaccharide. The nuclear pellet was allowed to swell in 1.6 ml solution II on ice with gently resuspended with a sterile Pasteur pipette. The nuclei were visualised under phase contrast microscope under 10 - 100 X magnification to ensure integrity. The nuclear pellet was checked for yield and purity by measuring the O.D.260 in UV 160A Shimadzu spectrophotometer, of a 1:1000 dilution of the nuclear pellet thoroughly disrupted in 2M NaCl/5 M urea. A scan from 200 to 300 nm of the disrupted nuclei gives a characteristic peak at 260 nm. The nuclear pellet was resuspended in solution II at a final concentration of 1 mg DNA/ml. Genomic DNA was extracted from the nuclei by a modification of the method of Marmur (1961) and Sambrook et al. (1989). The nuclear suspension (500 µg DNA/ml) in solution II was lysed in presence of 1% SDS and 1 N NaCl with gentle shaking for 1 hour. An equal volume of phenol was added to the tubes and rotated by hand for 10 minutes and left at slow shaking overnight. A turbid aqueous layer became visible, to which an equal volume of chloroform, mixed for 15 minutes and centrifuged at 10K rpm in a SS-34 rotor for 15 minutes. The aqueous phase was similarly treated twice with chloroform (1:1) and 3 M sodium acetate added to the final aqueous phase up to 0.3 M and the genomic DNA was precipitated in isopropanol on bench for 1 hour. On addition of isopropanol, a gelatinous white precipitate was formed, spooled the genomic DNA into a sterile Eppendorf tube and it was washed with 80% ethanol, briefly dried at RT and dissolved in T_{10}N_{10}. Genomic DNA takes a while to go into solution and was left at 4°C. Loaded 6 µl to a 0.8% agarose gel and electrophoresed in 1 X TAE buffer at 50 mA to estimate the integrity of the DNA isolated. Made a 1:1000 dilution and estimated concentration of the genomic DNA and its purity spectrophotometrically. For dsDNA an O.D.260 of 1 is equivalent to 50 µg dsDNA/ml and a ratio of O.D.260/280 of 1.8 - 2.0 is indicative of DNA free of protein contamination. A scan of the genomic DNA from 200 to 300 nm, revealed the characteristic peak at 260 nm. The DNA was stored at 4°C.

V.2.9. Restriction digestion of DNA

V.2.9.A Restriction digestion of plasmid DNA

The plasmid DNA was dissolved in appropriate 1 X Restriction Enzyme Buffer (as recommended by the manufactureres) to get a final concentration of
50 ng DNA/μl in the reaction mixture. If required BSA was added to a final concentration of 0.1 mg/ml. The restriction enzyme was added to a final concentration of 1 U/μg DNA and the Eppendorf tube left at the appropriate incubation temperature overnight.

**V.2.9.B Restriction digestion of murine genomic DNA**

The genomic DNA was dissolved in appropriate 1 X restriction enzyme buffer containing BSA (final concentration of 0.1 mg/ml) at a final concentration of 50 ng DNA/ml. Added the restriction enzyme to a final concentration of 5 U/μg DNA. Incubated overnight at the appropriate temperature.

**V.2.10. Purification of DNA fragments through low melt agarose**

DNA fragments were purified from agarose gels as per the method of Ausubel et al., 1995). Electrophoresed the digested DNA fragments through the required percentage of high melting agarose in 1 X TAE buffer containing 0.5 μg/ml ethidium bromide at 100 mA. When the fragment had resolved sufficiently cut a window below the target fragment and poured 1% low melting agarose in same buffer into it. Polymerised the agarose at 4°C, allowed the gel to come to room temperature by submerging it in the buffer tank for 15 min and electrophoresed it again till the target fragment completely entered the window. Cut the fragment and trimmed off any extra pieces of agarose on the transilluminator. The minced piece of agarose containing the target fragment was kept at 65°C to melt the agarose and ascertain the volume, mixed with equal volume of TE buffer, pH 8.0. (to a final concentration of ≤ 0.4%), vortexed and kept at 37°C for 10 minutes to mix well. To remove the agarose, added an equal volume of buffered-phenol, centrifuged for 5 min at 15,300 X g at RT. Collected the aqueous phase. Reextracted the phenol phase and the interphase with an equal volume of TE buffer, pH 8.0, centrifuged at 15,000 X g, collected the aqueous phase and ethanol precipitated the DNA from the pooled aqueous phase in the presence of 0.1 vol of 3 M sodium acetate, pH 5.2. The pellet was dissolved in 20 μl Tris.Cl, pH 8.0. The DNA concentration was estimated from an ethidium bromide-stained agarose gel after electrophoresing the gel-purified DNA fragment. The gel purified fragments were used to prepare probes for Southern hybridization and ligation reactions.
V.2.11. Preparation and purification of probes for Southern Hybridization

V.2.11.A Preparation of the probe for Southern Hybridization

The full length mouse RNase L cDNA probe (pZB1/Kpn I - Sac I, 2269 bp) and the 5' probe (pZB1/Eco RI - Eco RI, 717bp) probe were prepared using the MBI Fermentas Labelling Kit as per the manufacturer’s instructions. The method involved the in vitro synthesis of random primed $^{32}$P-labelled DNA probes. 25 ng of the gel purified probe DNA was boiled in a final volume of 41 µl in 1 X Hex Buffer (contains the hexamer primers) for 10 minutes and immediately chilled on ice for 3 minutes. After a pulse spin at RT, added 3 µl Mix A (-dATP), 2.5 µl α-$^{32}$P-dATP (10 µCi/µl, Sp. Activity = 6000 Ci/nmole), and 1 µl DNA polymerase Klenow Fragment (5 U/µl). gave pulse spin at RT and incubated for 2 hours at 37°C. Stopped the reaction by adding 4 µl dNTP, incubating for 5 minutes at 37°C and adding 1 µl 0.5 M EDTA, pH 8.0. The random primed probe was either processed for purification or stored at -20°C.

V.2.11.B Purification of the random primed $^{32}$P-labelled probe

Probe purification was done through Sephadex G50 spin columns. The tip of a 1 ml sterile syringe was plugged with autoclaved and siliconised glass-wool. The column was packed with Sephadex G50, taking care that no air-bubbles or cracks were present in the column. Column was then centrifuged in a clinical centrifuge with a swinging bucket rotor at 3K rpm for 1 minute at RT. The column was washed thrice with 100µl TE, pH 8.0, by centrifugation at 3K rpm for 1 minute at RT. Added 50 µl TE, pH 8.0, to the random primed probe to make the volume to 100 µl and gave a pulse spin to the Eppendorf tube. Carefully loaded the reaction to the column, which was lodged in an improvised Falcon tube (15 ml) with a hole in its cap and held within a 1.5 ml Eppendorf tube placed on a cotton-wool cushion inside, to collect the eluent. Centrifugation at 3K rpm for 3 minutes at RT eluted the purified random primed probe into the Eppendorf tube. 1 µl of the reaction was used to take Cerenkov counts in a LKB-β counter. Specific activity of the probe was 0.5 - 1 X 10⁹ cpm/µg.
V.2.12. Southern Hybridization

Digested 30 μg murine genomic DNA with Bam HI, Bgl II, Eco RI, Hinc II, Hind III, Nco I, Pst I, Sac I and Xba I. 10 μl of the digestion reaction was checked on 1% agarose gel to ensure that the genomic DNA was completely digested. After heat inactivation of the enzyme, the DNA was precipitated by the addition of 1/10th volume (60 μl) of sodium acetate, pH 5.2 and 0.6 volumes of Isopropanol, DNA was dissolved in 48 μl TE Buffer and stored at -20°C. The DNA was loaded in a 0.8%, 20 x 25 cm² agarose gel in 1 X TAE Buffer, which had been allowed to polymerise overnight. The gel was electrophoresed at 100 mA in 1 X TAE Buffer containing 0.5 μg/ml ethidium bromide till the bromophenol blue had migrated to 2/3rd the length of the gel. Photograph of the gel was taken when necessary.

Depurination: The gel was depurinated in two volumes (500 ml) of 0.25 N HCl till the bromophenol blue became yellow in colour (~ 10 minutes). The gel was rinsed twice in sterile distilled H₂O to remove all traces of HCl, which otherwise would have interfered in the subsequent denaturation step.

Denaturation: The gel was denatured in 500 ml Denaturation Solution for 15 minutes with slow shaking. The Denaturation Solution was replaced after 15 minutes and it was denatured for another 15 minutes with shaking. During this denaturation step the bromophenol blue indicator dye returned to the original blue colour.

Southern transfer: The denatured gel was transferred directly from the Denaturation Solution to a buffer reservoir containing a supporting wick of two sheets of 3 MM Whatman paper and 10 X SSC. A dry piece of Biodyne A membrane was placed directly over the gel. This was covered with three sheets of 3 MM Whatman 1 paper, blotting pads, a top glass cover and a small weight from bottom to top sequence. The transfer was allowed to proceed for 18 hours by capillary blotting method to ensure complete transfer of all the fragments. To ensure that the transfer was complete the agarose gel was restained with 0.5 μg/ml ethidium bromide for an hour and visualised under the UV-transilluminator.

Fixation: The membrane was removed from the gel surface, put on a sheet of 3 MM Whatman paper soaked in 2 X SSC and fixed by exposure to 12000 μJ of UV radiation. The membrane was washed in 2 X SSC for 5 minutes.
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After this the membrane was dried at 37°C and stored in a sealed plastic bag at 4°C or prehybridized immediately.

Prehybridization: The membrane was put in a hybridization bag and 0.1 ml/cm² (40 ml) prehybridization solution added, and the bag sealed. The membrane was prehybridized overnight and incubated with slow agitation at 42°C.

Hybridization: Prehybridization solution was removed and fresh 0.1 ml/cm² (40 ml) prehybridization solution was added. To this added 1 X 10⁶ cpm/ml of denatured probe along with 200 μg/ml denatured salmon sperm DNA, incubated overnight at 42°C with slow agitation. The ³²P-DNA probe containing 200 μg/ml salmon sperm DNA was mixed together in an Eppendorf tube and was denatured in boiling water bath for 5 minutes, chilled on ice for 5 minutes and a pulse spin was given.

Washes: The Southern blots were successively washed in wash solution I at 42°C - 65°C. The blots were then washed more stringently in wash solution II (42°C - 65°C) till there was no background.

Autoradiogram: The blot was exposed to Kodak X-AR5 film overnight and developed next day. Sizes of the different bands were calculated from DNA molecular size marker.

V.3. EXPRESSION of RNase L PROTEIN IN E. coli

Strategy:

To express murine (pZB1) and human (pZC5) RNase L cDNAs in E. coli, the cDNAs were subcloned into pRSET vectors. pRSET vectors are expression vectors where the multiple cloning site (MCS) is downstream of the T₇ RNA polymerase promoter. Initially, the ligation mixture was transformed into E. coli DH5α. DH5α is a rec strain of E. coli, prevents integration of the recombinant plasmid into bacterial genome. In addition, it is lac⁰ that is, excess amount of the lac repressor is made to prevent leaky expression by E. coli RNA polymerase in the absence of IPTG. The plasmid DNA from the selected recombinant clones was introduced to E. coli strain BL21(DE3)pLysS or BL21(DE3)pLysE, where the T₇ RNA polymerase is chromosomally integrated into the bacterial genome as the DE3 lysogen under the control of lac UV5 promoter. In order to prevent leaky expression this bacterial strain has a
second compatible plasmid pLysS that carries the gene for the T7 lysozyme and confers chloramphenicol resistance. In addition this strain of *E. coli* also lacks the *ompT* outer membrane protease and *lon* protease. This helps in stabilising the foreign protein being expressed which maybe sensitive to endogenous protease activity.

V.3.1. Expression of recombinant murine RNase L

V.3.1.A Preparation of the vector DNA

Digested 20 µg pRSETB plasmid DNA with Hind III. The 3'-end of the Hind III digest was made blunt-end by Klenow treatment. The fragment was isopropanol precipitated in the presence of 0.3 M sodium acetate, pH 5.2, dissolved in 10 mM Tris.Cl pH 8.0 and digested with Nco I. The digest was isopropanol precipitated in the presence of 0.3 M sodium acetate, pH 5.2, dissolved in 20 µl 10 mM Tris.Cl, pH 8.0 and given Calf Intestinal Alkaline Phosphatase (CIAP) treatment. After isopropanol precipitation, the 2885 bp fragment was gel-purified, dissolved in 10 mM Tris.Cl and the DNA concentration was estimated from an agarose gel.

V.3.1.B Preparation of the murine RNase L cDNA insert

The mRNase L cDNA (pZB1) was digested with Apa I, blunt-ended with T₄ DNA polymerase and Klenow. After isopropanol precipitation, the fragment was digested with Nco I and precipitated in 0.1 vol of 3 M Na-acetate, pH 5.2 and an equal vol of isopropanol and dissolved in 20 µl 10 mM Tris.Cl, pH 8.0 and gel-purified. The DNA was checked on an agarose gel to estimate the concentration and the integrity of the 2044 bp fragment.

V.3.1.C Ligation of pRSETB and mRNase L cDNA to get expression clones for recombinant murine RNase L

30 fmoles of the dephosphorylated vector (2885 bp) and 90 fmoles of the mRNase L insert (2044 bp), 1 Weiss unit T₄ DNA Ligase was used for 20 µl ligation reaction set at 14°C for 16 hrs. 4 µl of the ligation mixture was transformed into competent *E. coli* DH5α and selected on LB agar plates supplemented with 100 µg/ml ampicillin. The recombinant colonies were randomly selected, streaked on a LB plated supplemented with 100 µg/ml ampicillin and used to inoculate 1.5 ml LB medium + 100 µg/ml Ampicillin in a
2.0 ml Eppendorf tube and grown at 37°C with shaking overnight. Plasmid DNA was isolated by the LiCl miniprep method (see section V.2.6.B of Materials and Methods) and the recombinant clones (4929 bp supercoiled plasmid) were selected on the basis of the relative migration as compared to the vector (pRSETB, 2897 bp supercoiled plasmid) in 1% agarose gel. Eight clones were selected that migrated slower than the vector. Each of these plasmid DNA was digested with Nco I to check for the presence of the 4929 bp linear plasmid. From here seven clones were selected namely, pRSETB-mRNase L # 4, 6, 8, 10, 13, 14, 15. Plasmid DNA from these clones were isolated and used to transform competent E. coli BL21(DE3)pLysS cells and selected colonies on LB agar plates supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol.

V.3.2. Expression of recombinant human RNase L

V.3.2.A Preparation of vector pRSETA

Digested 10 μg pRSETA plasmid DNA with Hind III, precipitated it in 0.1 vol Na-acetate, pH 5.2 and an equal vol of isopropanol, dissolved in 10 mM Tris.Cl, pH 8.0 and digested with Pvu II. The double-digested 2878 bp fragment was treated with 1U CIAP/pmole end, and precipitated in the presence of 0.1 vol 3 M Na-acetate, pH 5.2 and an equal vol of isopropanol. The DNA fragment was gel-purified, dissolved in 20 μl 10 mM Tris.Cl, pH 8.0 and integrity and concentration of the DNA estimated from gel.

V.3.2.B Preparation of the insert

The human RNase L cDNA has six stop codons before its ATG translational start codon. To circumvent this problem, the cloning strategy involved creating a Pvu II site immediately upstream of the hRNase L coding sequence. This was done through PCR-mediated site directed mutagenesis and oligonucleotide primers were designed such that a 'C' residue was introduced instead of a 'G' residue and thus create a Pvu II restriction motif (CAGCTG) and a 'A' residue was introduced so as to stay within the translational reading frame after the ligation in pRSETA (see flow chart).
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Fig. IX Flow chart depicting the scheme used to introduce a 5' upstream Pvu II site in the human RNase L cDNA, pZC. (All nucleotides in italics represent the primer sequence, \textit{CAGCTG} = Pvu II restriction site, \textit{C} and \textit{A} represent the sites mutated and introduced through PCR respectively. The cDNA sequence is not in italics and codons in bold represent the stop codons.)

The natural Hind III sites present in the 3' end of the pZC5 was used in the oligonucleotide primer at the 3'-terminus. 200 pg DNA/4 \( \mu l \) linearised pZC5 digested with Cia I was used as the template for a 35 cycle PCR reaction: 94°C X 5', (94°C X 30'', 54°C X 30'', 72°C X 3') X 34 cycles, 72°C X 10 minutes. The PCR amplicon was precipitated in 0.1 vol 3 M Na-acetate, pH 5.2 and an equal vol of isopropanol and dissolved in 10 mM Tris.Cl, pH 8.0. The 2444 bp amplicon was sequentially digested with Hind III and Pvu II to generate a 2440 bp fragment and gel-purified. The DNA was checked on gel to estimate the fragment integrity and concentration.

V.3.2.C Ligation of pRSETA and hRNase L PCR amplicon from pZC5 to obtain the expression clone for human RNase L

Ligation was carried out with 60 fmole of the dephosphorylated (pRSETA) vector (2878 bp) and 180 fmole of the insert (2440 bp) in a 20 \( \mu l \) reaction at 14°C for 16 hrs with 1 Weiss Unit T4 DNA Ligase. 4 \( \mu l \) of the ligation reaction was used to transform 200 \( \mu l \) competent \textit{E. coli} DH5\( \alpha \), transformants selected on LB plates supplemented with 100 \( \mu g/ml \) ampicillin. Plasmid DNA was isolated using the LiCl-rapid miniprep method (see V.2.6.B of Materials and Methods) and 4 clones were selected on the basis of their relative migration (5318 bp supercoiled DNA) as compared to the uncut pRSETA vector 92897 bp
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supercoiled plasmid DNA). The clones selected were sequentially digested with Pvu II and Hind III to check for the insert DNA and its orientation and recombinant clones selected. Four clones pRSETA-hRNase L #51, #53, #58 and #59 were selected.

Once the pRSET fusion constructs of RNase L were obtained and confirmed, the plasmid DNA was transformed into E. coli BL21(DE3)pLysE cells grown on LB agar plates supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. This pRSETA-hRNase L clone was found to be toxic to E. coli so every set of experiment was carried out with fresh transformants.

V.3.3. Small scale induction of recombinant RNase L protein expression

V.3.3.A Induction of murine RNase L

Protein induction of the expression clones of RNase L was carried out as per the method of Williams et al., 1995. The pRSETB-mRNase L containing BL21(DE3)pLysS cells were grown overnight at 30°C, in LB medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The O.D.₆₀₀ was taken and inoculated 20 ml LB medium with cells equivalent to an O.D.₆₀₀ ~ 0.08. The cells were allowed to grow at 30°C till they attained an O.D₆₀₀ ~ 0.3. Three aliquots of cells equivalent O.D.₆₀₀ ~ 0.3 were taken for each clone. This can be calculated by the general formula:

\[ \text{µl of cells to be taken} = \frac{0.3}{\text{O.D.}_600} \times 1000. \]

The aliquots were centrifuged for 1 minute and the supernatant removed. These sets were later used to isolate the total RNA, plasmid DNA and the recombinant RNase L protein. The sets used to isolate the RNA and DNA were stored at -20°C without any treatment. For protein estimation 80 µl of 2 X SDS sample buffer was added to the pellet, vortexed well and stored at -20°C. This was used as the uninduced protein extract. Immediately, added IPTG to the remaining of the culture to a final concentration of 1mM and resumed incubation at 30°C. Collected O.D.₆₀₀ equivalent to 0.3 cells after 3 hours.

After every 1 hour two sets of samples with the O.D.₆₀₀ = 0.3 were collected and processed as before. (One for RNA isolation and the other for protein estimation).
V.3.3.B Induction of human RNase L

Transformed competent BL21(DE3)pLysE cells with recombinant DNA clones of pRSETA/hRNase L and plated the transformants on LB agar plates supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. It was observed that the E. coli cells containing the pRSET-hRNase L plasmid DNA grew into much smaller colonies on LB-agar 100 μg/ml ampicillin-34 μg/ml chloramphenicol plates, when compared to that of the pRSETA vector DNA. This occurred in the absence of IPTG. Streaked some colonies to fresh LB agar plates the next day and allowed the colonies to grow overnight. Always streaked at least 6 colonies on the LB-agar plates supplemented with antibiotics mentioned about. The next day, inoculated only those recombinant clones that were still growing slower than the vector (pRSETA) into 5 ml LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Grew with shaking at 37°C till an O.D.₆₀₀ ~ 0.3 was obtained. Took cells equivalent to O.D.₆₀₀ ~ 0.3 and prepared the lysate for SDS-PAGE (see section V.3.3.C). Added IPTG to a final concentration of 0.5 mM IPTG to the cultures of recombinant human RNase L clones and allowed them to grow for 3 hours with shaking at 37°C. Collected cells equivalent to O.D.₆₀₀ ~ 0.3 and prepared lysate for SDS-PAGE. Loaded to a 8% polyacrylamide-SDS gel and electrophoresed in 1 X Tris-Glycine-SDS buffer at 60 V for 1 1/2 at RT. Fixed and stained the SDS-PAGE gels with Coomassie Brilliant Blue R-250 (see section V.3.3.F).

V.3.3.C Preparation of lysates for SDS-PAGE

After all the aliquots at different time points were collected, the samples were stored at -20°C after extraction in 2 X SDS loading Buffer, were then thawed to room temperature and placed in a boiling water bath for 5 minutes and clarified by centrifugation for 2 minutes at 12K rpm. Loaded the entire sample (equivalent to O.D.₆₀₀ ~ 0.3 cells) to a polyacrylamide/SDS gel and electrophoresed at 60 Volts in 1 X Tris-glycine SDS Buffer at RT. The gel was fixed in and stained with Coomassie Brilliant Blue R-250 dye. The induced protein bands were identified by comparison with the uninduced protein control lane. The gels were stored in 20% glycerol and photographed.
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V.3.3.3. D Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared as per Laemeli’s method (Laemali, 1970). 30% stock solution of degassed acrylamide (29:1 acrylamide:bis-acrylamide) was mixed with 1.5 M Tris.Cl, pH 8.8, water and 10% SDS. The solution was swirled gently to avoid formation of bubbles and quickly added freshly prepared 10% APS and TEMED. The solution was poured into sealed glass plates. A layer of water was poured on the resolving gel and the gel allowed to polymerise at room temperature for one hour. Removed the upper layer of water completely and poured the 5% stacking gel which should be at least 1 cm below the bottom of the position of the comb. Allowed the resolving gel to polymerise for an hour. Removed the comb carefully and washed the wells thoroughly to remove any traces of unpolymerised acrylamide. Placed the gel in the vertical gel apparatus, ensuring that there were no air-bubbles trapped between the agarose and the buffer at the bottom of the gel. Loaded the entire 80 μl of the protein sample into the wells and electrophoresed in 1 X Tris-glycine-SDS Buffer at 60 V overnight.

V.3.3.3. E Fixing and staining of SDS-PAGE gels using Coomassie Brilliant Blue R-250

Polypeptides resolved in SDS-PAGE gels can be simultaneously fixed with 50% (v/v) methanol, 10% (v/v) acetic acid and stained with Coomassie Brilliant Blue R-2501 (Merril et al., 1979; Switzer et al., 1979). The gel was immersed overnight in 5 times its vol of Coomassie Brilliant Blue R-250 with slow agitation. The stain was removed and kept for further use. The gel was destained by soaking it in 50% (v/v) methanol, 10% (v/v) acetic acid, changing the solution once. The next day the gel was stored in 20% glycerol.

V.3.3.3. F Silver staining of protein gels

The proteins resolved by SDS-PAGE were silver stained using a protocol for the nondiamine silver staining of protein gels, with some modifications. The 1Coomassie Brilliant Blue: Originally developed as acid dyes, they were named "Coomassie Dyes" to commemorate the 1896 British occupation of the Ashanti capital, Kumasi or "Coomassie", now in Ghana. The "R" stands for the reddish hue while the number "250" is an indicator of the dye strength. The "G" in Coomassie Brilliant Blue G-250 stands for the greenish blue hue. 1 mg of protein will bind to 1.2 mg of Coomassie Brilliant Blue R-250 and 1.4 mg of Coomassie Brilliant Blue G-250.

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The non-diamine method of silver staining relies on the ability of silver nitrate to bind to proteins under acid condition, followed by selective reduction of silver ion to metallic silver by formaldehyde under alkaline conditions. Sodium carbonate is used to maintain an alkaline pH during development of the colour of the proteins. Formic acid produced by the oxidation of formaldehyde is buffered by sodium carbonate.

The gels were fixed for 2 hours (minimum time is 2 hours. The gels can be left overnight in the fixer solution) in the fixer for silver staining. It is imperative that in each of the subsequent steps the exact time is adhered to, the gels are completely submerged in the solutions and are accompanied by brisk shaking. The gels were washed thrice in 50% ethanol exactly for 10 minutes, followed by a 1 minute wash in Pre-treatment Solution. The gels were washed in water for 1 minute, agitated in the Impregnation Solution for 20 minutes, followed by a 1 minute rinse in water. The gels were developed [6% sodium carbonate, 0.4% sodium thiosulphate solution, 200 μl. formaldehyde] till the desired band intensity was obtained. The gel was rinsed in water and the reaction stopped with addition of the Stop Solution [40% ethanol, 12% acetic acid]. The gel was given a final treatment in 50% MeOH and stored in 20% glycerol. Gels can be washed, stored and photographed in 20% glycerol.

V.3.4. E. coli growth assay
V.3.4.A Growth assay for murine expression clones

Inoculated 20 ml LB medium supplemented with 100 ampicillin and 34 μg/ml chloramphenicol with O.D.₆₀₀ ~ 0.08 and allowed to grow at 30°C till O.D.₆₀₀ reached 0.3, when cells equivalent to O.D.₆₀₀ ~ 0.3 were removed as uninduced protein fraction. Immediately induced the rest of the culture with 1 mM IPTG and grew cells for 4 hours at 30°C with shaking at 200 rpm. After intervals of 1 hour removed cells equivalent to O.D.₆₀₀ ~ 0.3 (to ensure that equal volume of protein was checked) and processed for sample preparation for SDS-PAGE and stored at -20°C till all the samples were ready. Loaded the samples to a 10% polyacrylamide gel and electrophoresed overnight at 20 V in 1 X Tris-Glycine-SDS Buffer. Proteins were visualised after staining with Coomassie Brilliant Blue-R250.
V.3.4.B Growth assay for human expression clones

Freshly transformed pRSETA-hRNase L clones in *E. coli* BL21(DE3)pLysE cells were streaked on LB-agar plates supplemented with 100 $\mu$g/ml and 34 $\mu$g/ml ampicillin and chloramphenicol, respectively. The next day inoculated 5 ml LB-medium containing the above mentioned antibiotics with inoculum from those streaks which were still small as compared to the vector and had not become bigger than the vector pRSETA containing BL21(DE3)pLysE cells. Grew the cells with shaking at 37°C till O.D$_{600}$ $\sim$ 0.3 was attained when the uninduced protein fraction was removed (cells equivalent to O.D$_{600}$ $\sim$ 0.3). Immediately induced the cell culture with 0.5 mM IPTG and resumed the growth conditions. Collected aliquots of cells equivalent to O.D$_{600}$ $\sim$ 0.3 after every 1 hour and prepared samples for SDS-PAGE and stored at -20°C. When all the samples had been collected, loaded to a 8% polyacrylamide gel and electrophoresed at 60 V in 1 X Tris-glycine-SDS Buffer. Visualised gels after staining with Coomassie Brilliant Blue R-250.

V.3.5. Isolation of total RNA from *E. coli*

Total RNA was isolated from *E. coli* by a modification of Studier's method (Studier *et al.*, 1986). Titres of BL21(DE3)pLysS cell cultures equivalent to an O.D$_{600}$ of 0.3, containing either the pRSET vector or the recombinant RNase L clone were collected immediately before and at different time intervals following IPTG induction. Cells were collected by centrifugation at 10k rpm for 1 minute 200 $\mu$l of Studier's RNA Isolation Buffer was added to each sample and mixed thoroughly by tapping by hand. The samples were placed in a boiling water bath for 2 minutes. Allowed the samples to come to room temperature and precipitated the RNA by the addition of 3 M Na-acetate, pH 5.2, to a final concentration of 0.2 M. Added an equal vol of isopropanol and allowed precipitation either overnight at -20°C or at -80°C for 1 hour. Centrifuged at 4°C for 30 minutes at 10K x g. Washed each sample with 50 $\mu$l. of 70% ethanol and centrifuged at 4°C for 30 minutes at 10K x g. The RNA samples were dried at 37°C and dissolved in 20 $\mu$l deionised formamide. Stored the samples at -20°C till all the samples were ready. The RNA was visualised by Ethidium bromide staining after electrophoresis at 40 Volts through a 1.4% agarose gel. Electrophoreses was carried out in 1 X TAE containing 0.5 $\mu$g/ml ethidium
bromide (the agarose gel was first prepared in water and autoclaved, after which autoclaved 50 X TAE buffer was added to a final concentration of 1 X).

V.3.6. Assay for solubility of recombinant murine RNase L protein

To assay for the solubility of the recombinant mouse RNase L protein, inoculated 5 ml LB medium with 20 µl of overnight grown culture of mRNase L. Allowed the culture to grow at 30°C till an O.D.₆₀₀ ~ 0.3 was attained. Removed 1 ml cell culture, prepares SDS-PAGE lysates with it (uninduced protein fraction) and induced the culture with 1 mM IPTG and allowed the culture to grow for 3½ hours. Removed O.D.₆₀₀ ~ 0.3 worth cells and processed for preparation of lysates for SDS-PAGE. This is the total induced protein sample. Centrifuged 1 ml induced culture at RT at maximal speed for 20 seconds and resuspended in 150 µl of lysis buffer (for protein solubility). Incubated at 30°C for 15 minutes. Sonicated twice with a microtip at amplitude of 18 for 10 seconds on ice followed by centrifugation for 2 minutes at maximum speed at RT and removed the supernatant. This is the soluble protein fraction, while the pellet contains the insoluble protein fraction. To the soluble protein fraction Added 150 ul 2 X SDS sample buffer to both the fractions and loaded 10 µl from each along with the uninduced and induced protein to a 10% acrylamide-SDS gel. Electrophoresed in 1 X Tris-glycine-SDS Buffer and visualised proteins after staining with Coomassie Brilliant Blue R-250.

V.3.7. Purification of murine RNase L protein using Ni-NTA columns

Growth and induction: Inoculated 5 ml LB with a clone of mRNase L (clone #4) and allowed the culture to grow overnight at 30°C with shaking. The next morning the entire culture was transferred to 1 L LB medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. Allowed the culture to grow at 30°C till the O.D.₆₀₀ of 0.3 was obtained. Took cells equivalent to O.D.₆₀₀ ~ 0.3 and prepared lysate for SDS-PAGE. The rest of the culture was induced with 1 mM IPTG and allowed to grow till 3½ hours.

Purification on Ni-NTA agarose columns: The recombinant mouse protein was extracted under denaturing conditions using 8M urea. Once denatured the protein slurry was passed through the Ni-NTA column and the recombinant protein allowed to bind to the column via the histidine tag. The recombinant protein was renatured while still on the column by a gradient wash of urea.
(from 8M to 0M urea) in the phosphate buffer. The recombinant protein was then eluted out with imidazole. After collecting the induced protein fraction, 3½ hours after induction, culture was centrifuged at 4K X g at 4°C for 20 minutes. The cells were resuspended in 1 X sodium phosphate buffer, pH 8.0 and centrifuged again at the same rpm for 10 minutes. Added 5 ml sodium phosphate buffer, pH 8.0 containing 8M urea and left on a shaker for ½ an hour at RT. The suspension of the cells was centrifuged at 13k rpm in a SS-34 rotor at 4°C. Meanwhile a column was packed with fresh Ni-NTA slurry and given repeated washes (at least 5 washes) with 8M urea in phosphate buffer, pH 8.0. The supernatant after centrifuging the recombinant protein was passed through the column thrice to ensure that all the recombinant protein bound to the column. The flow through was collected and stored at -20°C. To renature the recombinant protein while bound to the column, the column was washed in a gradient of 10 ml 8M urea to 0M urea in phosphate buffer, pH 8.0. The eluants from the gradient washes were collected and stored at -20°C. Elution of the bound recombinant mouse protein was done with 5 ml of 250 mM imidazole in phosphate buffer, pH 8.0. Collected the eluants in 1 ml fractions and stored at -20°C.

To check for the fractions containing the recombinant mouse protein, loaded uninduced protein, induced protein, flow through, gradient washes, and eluant 1-5 after elution of the protein was loaded to a 10% polyacrylamide/SDS gel and electrophoresed in 1 X Tris-glycine-SDS buffer. After staining with Coomassie Brilliant Blue-R250, it was determined that eluants 2 - 5 contain the recombinant mouse protein. Fractions 2 - 5 of the eluted protein were pooled and dialysed to remove the divalent ions. Dialysis was carried out at 4°C in 1 L sodium phosphate buffer, pH 8.0 containing 1 mM PMSF with changes after every ½ an hour. Dialysis was carried out for 4 hours. The dialysed protein was stored at -20°C in 500 μl aliquots. The protein continuing aliquots were always thawed only on ice just prior to use.

V.3.8. 2-5A binding and dimerization

V.3.8.A Reaction for 2-5A binding

The entire reaction was carried out on ice and electrophoresis in the native PAGE was carried out at 4°C in the cold room. 5 μg BSA was used as control. 10 μl of purified murine RNase L protein was used for the binding and
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dimerization reaction to 2-5A. 2-5A, ATP and Mg\(^{2+}\) ions were used at a final concentration of 100 nM, 50 µM and 2.5 mM respectively in a 15 µl reaction. Set reactions with BSA (5 µg/5ml) and mRNase L (10 µl) as per the table given below:

**Table VII Reaction conditions and volumes of various reagents used for the 2-5A binding and subsequent dimerization of the NiNTA column-purified recombinant murine RNase L**

<table>
<thead>
<tr>
<th>Reaction tube #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
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<tbody>
<tr>
<td>2-5A</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>MgCl(_2)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>ATP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>500nM 2-5A</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(final conc. 100nM)</td>
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<tr>
<td>25mM MgCl(_2)</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>(final conc. 2.5 mM)</td>
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<tr>
<td>500µM ATP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>(final conc. 50µM)</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>H(_2)O</td>
<td>10</td>
<td>7</td>
<td>8.5</td>
<td>5.5</td>
<td>5.5</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>3.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>BSA (lanes 1-6)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>mRNase L (lanes 7-12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The reaction was allowed to incubate on ice for 30 minute and mixed with 15 µl 2 X native PAGE loading buffer and loaded to a 8% native polyacrylamide gel pre-run for 30 minutes at 4°C and electrophoresed in 1 X Tris-glycine buffer at 140 volts till 1/2 hour after the bromophenol blue had run out of the gel. Cut the gel so that the lanes containing BSA could be stained overnight in Coomassie Brilliant Blue R-250 (see section V.3.3.E of Materials and Methods), while the lanes containing the mRNase L were left overnight in the fixative for silver staining (see section V.3.3.F of Materials and Methods).

**V.3.8.B Native Polyacrylamide gel electrophoresis (Native PAGE)**

Native PAGE was carried out as per the method of Ornstein and Davis, 1964. Autoclaved water was used to polymerise the gel and to make the electrode buffer. Polymerised degassed acrylamide, 1.5 M Tris.Cl, pH 8.8 in H\(_2\)O with fresh 10% APS and TEMED for 1 hour for resolving gel. Poured a thin layer
of 5% stacking gel made of degassed acrylamide, 0.5 M Tris.Cl, pH 6.8 and H₂O with fresh 10% APS and TEMED and polymerised for 1 hour. Loaded the gel with 10 μl 2 X Native PAGE loading buffer and the gel was pre-run in the cold-room at 4°C at 140 V in 1 X Tris-Glycine buffer. A ½ an hour to 1 hour pre-run is sufficient, after which samples were loaded. Loaded 30 μl sample/well and electrophoresed at 140 V in the cold room until ½ hour after the bromophenol blue had run out of the gel. Protein bands were visualised after silver staining.

V.3.9. Bradford’s Method of Protein Estimation

A standard curve of BSA was made by using a serial dilution of BSA (1 μg/ml) from 0, 2, 4, 6, 8, 10, 12, 14 μg in a final volume of 20 μl in a 96 well plate. Each concentration was taken in triplicates. Made the volume to 20 μl with H₂O. Added 180 μl Bradford’S Reagent (Bradford, 1976) to each well and took the reading in an ELISA reader at 655 nm. Plotted a standard curve of the mean values of the different concentrations against the O.D.₆₅₅.

To estimate the concentration of the protein sample took 2 μl of the protein extract, made the volume to 20 μl with H₂O and added 180 μl Bradford reagent. Took the O.D.₆₅₅ in an ELISA Reader and estimated the concentration of the protein from the standard curve.

V.3.10. Western Blotting

Electrophoresis: After estimating the total protein content in the E. coli extracts using Bradford’s reagent, loaded to a 8% SDS-PAGE gel 100 μg protein per lane (or O.D.₆₀₀ ~ 0.3 equivalent of cell extract) along with SDS-prestained marker. The gel was electrophoresed in Tris-Glycine-SDS buffer at 100 V till the bromophenol blue dye migrated out of the gel.

Blotting: Trimmed the gel to remove the stacking gel. On top of the gel was placed nitrocellulose membrane. The gel and membrane were sandwiched between two sheets of Whatman 1MM sheets and packed into the Western Blotting apparatus (Bio-Rad). Transfer was carried out at 40 V/gel in Tris-Glycine-Methanol Transfer Buffer overnight at 4°C in the cold room with constant mixing of the buffer. Removed the gel - the prestained marker should always be completely transferred to the nitrocellulose membrane and be visible. The membrane can either be stored in the moist condition at 4°C in 1 ml 1 X PBS or processed directly for antibody incubation.
Western blotting: All steps were carried out with shaking at RT and for each blot 10 ml of the reagent was used. When processing 2 blots simultaneously only 15 ml reagent was used for every 2 blots. The blot was given 3 washes of 10 minutes each in 5% non-fat milk in 1 X PBST (Baetteiger et al., 1982). This serves to block all the non-protein bound regions in the blot that might interfere with antibody binding. Incubation with the primary antibody at 1:3000 dilution in 1 X PBST was done for 1\frac{1}{2} hours. The blot was given 5 washes of 10 minutes each in 1 X PBST to remove all non-specifically bound primary antibody. The blot was incubated with the secondary antibody at a 1:3000 dilution in 5% non-fat milk in 1 X PBST for 1\frac{1}{2} hours. The blot was given 5 washes in 1 X PBST for 10 minutes each to remove all non-specifically bound secondary antibody.

Developing: All the subsequent steps have to carried out in minimum time. In a clean container added 1 ml ECL solution I and 1 ml ECL solution 2 and swirled to mix. Placed the blot in the ECL I and II solution mixture and swirled for 1 minute taking care to ensure that the entire blot is constantly in contact with the solution. Blotted all extra ECL solution on tissue paper and wrapped the blot in Saran wrap. Attached the blot in the same orientation on the bottom left corner of the intensifying screen with scotch-tape. In dark, exposed the blot to Amersham hyperfilm for varying time - flash, 30 seconds, 1 minute or more and developed the film with X-Ray developing and fixing solutions with intermediate wash in H2O. After the film had dried aligned it with the blot and marked the position of the lanes as pre-stained markers on the film. The blot was restored at 4°C.

V.4. Tissue Culture Methods

V.4.1. Seeding of human lymphoblastoma Daudi cells

Quickly thawed a vial of frozen human Burkitt lymphoma Daudi cells (Moore and Kitamura, 1968) stored in liquid nitrogen by putting 500μl RPMI-1640 medium that was at 4°C. Added the contents of the cryovial to 4 ml of RPMI-1640 medium contained in a 15 ml Falcon tube and resuspended the thawed cells with a sterile Pasteur pipette. Kept at RT for 5 minutes to allow the DMSO to diffuse out of the cells. Centrifuged the cells at 1.5K rpm for 3 minutes at RT. Discarded all the RPMI medium leaving no traces of the DMSO used to store the cells, taking care not to disturb the cell pellet. Resuspended the cell
pellet in 1 ml RPMI-1640 medium and made up the volume with the addition of
4 ml RPMI-1640 medium. Added the resuspended cells to a fresh T25 flask and
placed in the CO2 incubator. Usually Daudi cells were seeded at 0.3 - 0.5 X 10^6
cells/ml density at the time of harvesting. Cells were checked for growth after
intervals of 24, 48 and 72 hours under a phase contrast microscope.

V.4.2. Trypsinisation and Seeding of human cervical adenocarcinoma
HeLa cells

Human cervical adenocarcinoma cells were thawed from liquid nitrogen
as mentioned above. In a T25 flask grew HeLa cells to 80% confluency.
Discarded the media and added 1 ml 0.03% trypsin made in 1 X PBS/2 mM
EDTA, pH 8.0. Left the flask at 37°C in the CO2 incubator for 3 minutes. Added 2
ml complete medium and collected the cells in a 15 ml Falcon tube. Pelleted the
cells by centrifugation at 1.5K rpm for 3 minutes at RT. Discarded the medium
and resuspended cells in fresh 5 ml DMEM. This brings the cell number to 1 X
10^6 HeLa cells per T25 flask.

V.4.3. Freezing cells

A growing culture of cells (Daudi or HeLa) was harvested as mentioned
earlier, centrifuged at 1.5K rpm for 3 minutes and the cell pellet resuspended at
a concentration of 2 - 5 X 10^6 cells/ml in 90% fetal calf serum and 10% DMSO
in labelled cryovials. Cells were put on ice and then transferred to -80°C
overnight. Cells were transferred to liquid nitrogen for long term storage.

V.4.4. Determination of viable cells by Trypan Blue Staining

Trypan Blue Stain is used in dye exclusion procedures for viable cell
counting. This method is based on the principle that live (viable) cells do not
take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the
visualisation of cell morphology. 20 µl of cell suspension were resuspended in
30µl 1 X PBS and added 50µl of 0.4% Trypan Blue Stain. Cells were allowed to
stand maximally for not more than 15 minutes as viable cells in addition to non-
viable cells could also take up the dye upon prolonged staining. With the cover
slip in place, a drop of the Trypan Blue cell-suspension was transferred to both
the chambers of the haemocytometer. Starting from chamber I of the
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haemocytometer, counted the cells in the 1 mm centre square and four 1 mm corner squares. The non-viable cells stained blue and the non-stained viable cells were counted. Each square of the haemocytometer with the cover slip in place represents a total volume of 0.1 mm$^3$ or 10$^{-4}$cm$^3$. Since 1 cm$^3$ is equivalent to approximately 1 ml the subsequent cell concentration per ml. was determined using the calculation:

$$\text{Cells per ml} = \text{average number of cells per square} \times \text{dilution factor} \times 10^4$$

V.4.5. MTT Assay for Cytotoxicity

The MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] assay provides an indirect measurement of cell growth through the measurement of the mitochondrial enzyme system to convert the tetrazolium salt, MTT to a coloured formazan complex that can be measured spectrophotometrically (Mahy and Kangro, 1996). Live, growing cells have respiratory-competent mitochondria and actively convert MTT to formazan. Conversely dead or non-viable cells have a compromised mitochondrial system and have a lower conversion rate of MTT to formazan. To 100 µl. cells contained in a 96 well plate in triplicates added 25 µl MTT (5 mg/ml.) and incubated the cells in the CO$_2$ incubator for 2 hours. During this time the MTT gets converted to formazan. Blue formazan crystals are usually observed inside cells under the microscope at this stage. Added 100 µl of Lysis Buffer for MTT assay (20% SDS in DMF) to solubilise the water insoluble formazan, incubated overnight at 37°C under humid conditions and measured the absorbance at 570 nm. Calculated the mean and standard deviation of the absorbance.

V.4.6. Treatment of Daudi cells with cytokines

For MTT assay 0.5 X 10$^6$ Daudi cells/ml were grown in presence of 0, 1, 10, 100 and 1000 U/ml IFN-α (Roferon A) to measure cytotoxicity by MTT assay.

For NF-κB EMSA assay 1 X 10$^6$/ml Daudi cells were grown in 24 well plate in presence of 0, 1, 10, 100, 1000 U/ml of IFN-α (Roferon A) for 24 hours
then treated with 0 - 1 nM TNF for 30 minutes or 2 X 10^6 Daudi cells /ml were treated with IFN-α or IFN-β or 1 nM TRAIL for 0, 5, 10, 15, 30 and 60 minutes. The varying dose of cytokines and the duration of treatment and pretreatment were selected to find out low-dose, high-dose and short and long duration effects of the cytokines used. The treatments were done in triplicates for MTT or 3H-thymidine uptake assay and in duplicates for NF-κB EMSA assay.

**V.4.7. Treatment of HeLa cells with different agents**

HeLa cells were plated at a density of 0.5 X 10^6 cells per well in 6 well plate in 1.5 ml DMEM and allowed to grow overnight. After 23 hours the medium was replaced with 1 ml fresh DMEM and the cells were allowed to grow for 1 hour. Cell were treated with various agents as per Table VIII and allowed to grow for 24 hours. Cells were then harvested by scraping the plate on ice and washing each well with 0.35 ml 1 X PBS to collect the remaining cells. Cells were collected by briefly centrifuging at 4°C. The cell pellet was washed once with 1 X PBS and the pellet was processed for various experiments.

**Table VIII** Various treatments given to HeLa cells with the amount added per 0.5 X 10^6 cells contained in 1 ml DMEM, with concentrations of the stock solutions and final concentration of each treatment

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>Stock solution</th>
<th>Volume added per plate</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>No treatment</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Cycloheximide</td>
<td>5 mg/ml</td>
<td>10 µl</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>3.</td>
<td>Poly(I:C)</td>
<td>2.5 mg/ml</td>
<td>10 µl</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>4.</td>
<td>Cycloheximde + poly(I:C)</td>
<td>5 mg/ml</td>
<td>10 µl + 10 µl</td>
<td>50 µg/ml + 25 µg/ml</td>
</tr>
<tr>
<td>5.</td>
<td>Cisplatin</td>
<td>1 mM</td>
<td>5 µl</td>
<td>5 µM</td>
</tr>
<tr>
<td>6.</td>
<td>Doxorubicin</td>
<td>1 mM</td>
<td>5 µl</td>
<td>5 µM</td>
</tr>
<tr>
<td>7.</td>
<td>Vinblastin</td>
<td>1 mM</td>
<td>5 µl</td>
<td>5 µM</td>
</tr>
<tr>
<td>8.</td>
<td>Vincristin</td>
<td>1 mM</td>
<td>5 µl</td>
<td>5 µM</td>
</tr>
<tr>
<td>9.</td>
<td>H2O2</td>
<td>1 mM 100 mM</td>
<td>5 µl 10 µl</td>
<td>5 µM 1 mM</td>
</tr>
<tr>
<td>10.</td>
<td>CaCl2</td>
<td>10 µM</td>
<td>5 µl 10 µl</td>
<td>50 nM 100 nM</td>
</tr>
</tbody>
</table>
V.4.8. Preparation of whole cell lysate for Western Blotting

Cell lysates were prepared by a modification of the method of Walczak et al., 2000. Cells (0.5 X 10^6 per sample) were harvested by centrifugation at 600 X g for 10 minutes at 4°C and washed twice in 1 X PBS. The lysate prepared by resuspending the cell pellet in 20 μl of Lysis Buffer (for whole cell lysate). After a 30 minute incubation on ice, the lysates were centrifuged at 15,000 X g at 4°C for 5 minutes to remove the nuclei. The cell lysate was either stored at -20°C or 2 μl per sample was processed for estimation of protein concentration by Bradford's method. 50 μg/lane of the total cell lysates were used for Western blotting. Equal volume of each lysate was loaded per well for SDS-PAGE by making up the volume with Lysis Buffer.

V.4.9. Isolation of total RNA from HeLa cells

Total cellular RNA was extracted from the HeLa cells by a modification of the method of Auffray and Rougeon (1980). Certain precautions were taken throughout the experiment. H2O used was DEPEC-treated, autoclaved all glassware used was baked at 180°C overnight, all gel apparatus and other plastic wares were treated with H2O2 overnight and unless otherwise mentioned all steps were carried out 4°C in RNase-free conditions in front of a flame. Hela cells were harvested 24 hours after treatment by scraping the plates followed by centrifugation at 12K rpm at 4°C for 10 minutes. The cell pellet was washed once in 1 X PBS (ice-cold) and centrifuged at 12K rpm at 4°C to collect the cell pellet. After aspirating the PBS, the cell pellet was lysed in 200 μl Lysis Buffer (for RNA isolation) by resuspending the pellet with a 200 μl micropipette. Added another 300 μl lysis buffer and sonicated the sample thrice on ice at an amplitude of 12 micron for 30 seconds with a 1 minute break. Left the samples overnight on ice in the cold-room. Next day centrifuged the samples at 13K rpm at 4°C for 20 minutes and removed the supernatant in front of a flame. Added 300 μl Dissolving Buffer to each sample and tapped the Eppendorf tube. Added equal amount of Tris. Cl, pH 8.0 saturated phenol and vortexed. Centrifuged at 12K rpm at 4°C for 10 minutes. Added equal volume of chloroform to the aqueous phase, vortexed and centrifuged at 12K rpm for 10 minutes at 4°C.
Materials and Methods

rRNA from the aqueous phase was precipitated by adding 30μl 3M sodium acetate, pH 5.2, and 300 μl. isopropanol, vortexed and stored at −20°C overnight. Centrifuged the samples at 13K rpm at 4°C for 30 minutes, and washed the rRNA pellet in 200 μl 80% Ethanol made in DEPC-treated H₂O. Centrifuged for 5 minutes at 4°C at 13K rpm and aspirated the ethanol. Air-dried the pellet and dissolved it in 10 μl DEPC-treated H₂O, by tapping and keeping at 4°C for 10 minutes. Added 10 μl 2 X RNA loading dye, resuspended with a micropipette and loaded to a 1.2% agarose gel and electrophoresed at 50 V in 1 X TAE Buffer. rRNA was visualised by UV-transilluminator and the gel was photographed.

V.4.10. DNA fragmentation assay for HeLa cells

DNA laddering, as an indicator of apoptosis, was assayed by extracting genomic DNA from treated HeLa cells (1 X 10⁶ cells per sample) by a modification of the method of Loweth and Morgan, 1998. Briefly, cells were harvested by centrifuging at 2K rpm for 5 minutes at 4°C. The cell pellet was washed once in 1 X PBS and resuspended in 20 μl Solution I (for DNA laddering) containing freshly added Proteinase K to a final concentration of 0.5 mg/ml. Samples were incubated at 50°C for 1 hour before adding 10 μl 0.5 mg/ml RNase A and incubating at 50°C for a further 1 hour. Samples can now be stored at −20°C, if necessary. Heated the lysate at 70°C for 5 minutes and mixed with 10 μl of pre-heated loading buffer (for DNA laddering) and immediately loaded to a 1.7% agarose gel containing 0.1 μg/ml ethidium bromide and the gel cooled at 4°C for 10 minutes to allow the low-melt agarose to polymerise. This prevents the small sized DNA molecules from floating out of the well when the gel is immersed into the electrophoresis buffer. The DNA was electrophoresed in 1 X TAE buffer at 40 V till the dye front had migrated 4 - 5 cm. (Do not allow the DNA to run more than 5 cm as the bands get diffused and it is difficult to photograph). DNA was observed using UV transillumination and was photographed.

V.4.11. Electrophoretic mobility shift assay for NF-κB

EMSA for NF-κB was performed as per the method described by Schreiber et al., 1989 and Chaturvedi et al., 1994). The entire experiment was divided into 3 parts: A. preparation of nuclear extract, protein estimation, B.
preparation of the labelled oligo, column purification and C. running of the gel, exposure to X-ray film and analysis of the results.

**V.4.11.A Preparation of nuclear extract for NF-κB EMSA**

All steps were carried out at 4°C, unless otherwise mentioned. Collected treated HeLa cells (0.5 × 10^6 per sample) 24 hours after treatment, by scraping the wells on ice and collecting the cells in a 1.5 ml Eppendorf tube. 1 well was treated with 1 nM TNF just 1 hour prior to harvesting the cells. For time course experiments 0.5 ml chilled 1 X PBS was added to the cells in 1 ml medium in 6 well plate immediately after the indicated time. Washed the wells with 350 µl ice cold 1 X PBS and to collect remaining cells by centrifugation at 12K rpm at 4°C for 1 minute. The cell pellet was washed once in 500 µl 1 X PBS by resuspending with a 200 µl micropipette and cells collected after centrifugation at 12K rpm at 4°C for 1 minute. Added 200 µl Lysis Buffer (for NF-κB) and resuspended the pellet with a 200 µl micropipette. Allowed the cells to swell on ice for 15 minutes. Added 6 µl 10% NP-40 and vortexed vigorously to lyse the cells. Incubated on ice for 1 minute. Centrifuged for 5 minute at 12K rpm at 4°C and collected the cytoplasmic extract in pre-chilled 1.5 ml labelled Eppendorf tubes and stored at -80°C. Protein concentration was estimated by Bradford's method prior to use.

To the dirty looking pellet added 25 µl nuclear extraction buffer and resuspended with a 200 µl micropipette, incubated on ice for 30 minutes with intermittent vortexing. The nuclear suspension was centrifuged at 4°C for 8 minutes at 12K rpm can be stored at -80°C and the protein concentration be estimated by Bradford’s method, the supernatant was transferred to a pre-chilled labelled 1.5 ml Eppendorf tube. Nuclear fraction is always kept on ice while outside -80°C.

**V.4.11.B.i. Preparation of the ³²P-labelled NF-κB oligonucleotide**

The wild- type 45 mer oligonucleotide (see Results: Table 1) derived from the HIV-LTR that contains two NF-κB binding sites was used in the EMSA for detection of NF-κB activation in nuclear extracts from the HeLa cells. Since these oligos have a 5’-OH, they were labelled with γ-³²P-ATP and T₄ polynucleotide kinase. 2 pmole of the 5’-NF-κB oligo was labelled and annealed with 100 times excess (200 pmole) of the unlabelled 3’-NF-κB complementary
strand to make it double-stranded for EMSA. Thawed the oligonucleotides, T₄ Polynucleotide buffer, γ₃₂P-ATP on ice and added the following components in a 1.5 ml Eppendorf tube.

Mixed the reaction by tapping, gave a pulse spin and allowed the kinase reaction to continue at 37°C for 30 minutes and stopped the reaction with the addition of 2 μl 0.5 M EDTA, pH 8.0, left on ice for 5 minutes and made up the volume to 100 μl with 10 mM Tris.Cl, pH 8.0.

### Table IX  Kinase reaction to prepare NF-κB probe for EMSA

<table>
<thead>
<tr>
<th>Order of adding</th>
<th>Reagent</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sterile H₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>10X T₄ Polynucleotide Kinase Buffer</td>
<td>5 μl</td>
<td>1X</td>
</tr>
<tr>
<td>3.</td>
<td>0.02 M DTT</td>
<td>2.5 μl</td>
<td>1 mM</td>
</tr>
<tr>
<td>4.</td>
<td>5' NF-κB oligo (1 pmole/μl)</td>
<td>2 μl</td>
<td>200 pmole</td>
</tr>
<tr>
<td>5.</td>
<td>γ₃₂P-ATP (6000 Ci/mole, 10 μCi/μl)</td>
<td>2.0 μl</td>
<td>20 μCi</td>
</tr>
<tr>
<td>6.</td>
<td>T₄ Polynucleotide Kinase (5 U/μl)</td>
<td>1.0 μl</td>
<td>1 U/50 μl</td>
</tr>
</tbody>
</table>

V.4.11.B.ii.  **Column purification of the NF-κB oligo**

Prepared a spin column in a 1 ml syringe by packing with sterile Sephadex-G50 slurry. Washed the column thrice with 200 μl T₁₀ and gave a spin at RT for 2 minutes at 2K rpm. Added 100 μl of the 5'-NF-κB-labelled probe mixture to the top of the column, centrifuged at 2K rpm for 2 minutes, RT and collected the eluent in a sterile Eppendorf tube. Took 1 μl purified probe in a 0.5 ml Eppendorf tube and made the volume to 10 μl with 9 μl T₁₀. Similarly, this dilution was used to make another 1:10 dilution. Took Cerenkov counts for each vial and estimated the labelling efficiency (~ 1.8 X 10⁵ cpm/pmole). Usually the eluant volume was 200 μl. To the eluent added 2 μl of 50 pmole/μl 3'-NF-κB oligo. Generally 50-fold molar excess of the unlabelled complementary strand is good to ensure the complete conversion of labelled single-stranded to double-stranded oligonucleotides.
Denaturing: Kept the oligo mix in boiling H₂O for 3 minutes and gave a pulse spin to collect the reaction at the bottom of the tube.

Annealing: Left at RT for 1 hour to allow the strands to anneal completely. Made the volume to 500 µl with the addition of 200µl T₁₀ and transferred the reaction to ice. By making the volume up to 500 µl the final concentration of the 5³²P-dsNF-κB probe becomes 4 fmole/µl. Made 100 µl aliquots and stored the reaction at -20°C.

V.4.11.C  Electrophoretic mobility shift assay
V.4.11.C.i.  Casting the acrylamide gel

The 7.5% polyacrylamide gel is cast prior to setting up the binding reaction. The gel was prepared by mixing the following solutions in the order mentioned below:

<table>
<thead>
<tr>
<th>Order of addition of the reagents</th>
<th>Stock solutions</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>H₂O</td>
<td>27.06</td>
</tr>
<tr>
<td>2.</td>
<td>5 X EMSA Buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>3.</td>
<td>30% acrylamide</td>
<td>12.5</td>
</tr>
<tr>
<td>4.</td>
<td>10% APS</td>
<td>0.40</td>
</tr>
<tr>
<td>5.</td>
<td>TEMED</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Total volume</td>
<td>50.0</td>
</tr>
</tbody>
</table>

The gel was allowed to polymerise for 45 minutes at RT. After polymerisation the comb was removed, the wells were thoroughly rinsed with buffer and the gel was fixed to the chamber. Added 1 X EMSA buffer and flushed the wells with the buffer with a 10 ml hypodermic needle and syringe. Started the pre-run at 150 V (~40 mA) at RT for at least 30 minutes immediately after setting the binding reaction (see V.4.11.C.ii)

V.4.11.C.ii. Binding reaction for NF-κB EMSA

The nuclear extracts were thawed on ice. For the binding reaction protein from nuclear extract (4 µg for Daudi cells, 8µg for HeLa cells) was used per binding reaction. Made the volume of the protein extract to 9.6 µl with sterile H₂O. Added 6.4 µl master mix to the nuclear extract, added 4 µl ³²P-
labelled dsNF-κB oligo, gave a pulse spin in a refrigerated microfuge and incubated at 37°C for 30 minutes. Transferred the tubes to ice and added 4 μl 6 X Loading dye. Stopped pre-run of the gel and washed the wells of the gel. Loaded the samples and electrophoresed at 150 V (~40 mA) for 3 hours.

### Table XI Reagents for EMSA binding reaction

<table>
<thead>
<tr>
<th>Sequence of addition of the reagents</th>
<th>Stock solution</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10 X Binding Buffer</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>0.2 M DTT</td>
<td>0.4</td>
</tr>
<tr>
<td>3.</td>
<td>1 μg/ml calf thymus DNA</td>
<td>2</td>
</tr>
<tr>
<td>4.</td>
<td>10% NP-40</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total volume (6.4 μl per binding reaction)</td>
<td>6.4</td>
</tr>
</tbody>
</table>

After the run was complete removed the notched glass plate and placed a 3 MM Whatman sheet (slightly larger than the gel) on the gel. Pressed the sheet uniformly and gently so that the gel was firmly stuck to the Whatman sheet. Covered the gel with Saran wrap and dried at 80°C for 2 hours under vacuum suction. The gel was then exposed to Hyperfilm™.

**V.4.12. Geimsa staining of HeLa cells**

HeLa cells after 24 hours of treatment with various agents were stained with Geimsa stain as per the method described by Freshney. Seeded 0.5 X 10⁶ cells per well in 6 well plates and 24 hours after treatment with the various agents removed the media. Added 2 ml 1 X PBS per well and washed the cells. Added 1 ml Methanol:PBS::1:1 per well and left at RT for 10 minutes. This was repeated thrice. Added 1 ml Methanol per plate and left at RT for 1 minute. (At this point the cells can be stored at 4°C). Added 2 ml/well of Geimsa stain and left for 3 minutes at RT. Added 8 ml autoclaved H₂O/well and swirled gently for 3 minutes. This was repeated twice. Finally, immersed the plate into a tray of water and allowed all the extra stain to wash out and the background appeared clear. Checked the plate under a microscope to ensure that the cells were completely destained. The plate was left inverted to allow it to dry completely. Added 1 ml 10% sterile glycerol to every well and stored the plate at 4°C. The plate was observed under microscope (Zeiss). Photographs of the apoptotic
cells fixed in the plates were taken at 320 X magnification on Kodak Gold Plus 100 colour film.