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.

Agar plates:
1. LB agar: (Hi media RM-301). 15 gm agar (1.5%) in 1 litre medium, autoclaved, cooled down to 45 °C. The antibiotics were added from sterile stocks (Ampicillin 50-100 μg/ml or Tetracycline 10 μg/ml final conc.) and were poured into 90 mm plates.
2. LB top agar: 10 gm Bacto-tryptone, 5 gm Bacto-yeast extract and 5 gm NaCl were dissolved in water to a final volume of 1 litter. pH was adjusted to 7.0 with 5.0 M NaOH and 7.2 gm agar was added. After autoclaving, cooled down to 55 °C, 1 ml of sterile 1 M MgSO₄ was added and poured.

Agarose gel: Type V High melt, (Sigma-A-3768) or Type VII low melt, (Sigma A-4018) or ultra pure grade (Gibco BRL 15510-027). Typically 0.6-1.8% agarose in 1X TAE buffer were used with a final concentration of 0.5 μg/ml EtBr.

Antibiotics:
1. Ampicillin: Ampicillin sodium injection solution (Biocilin, India) 500mg of ampicillin salt was dissolved in 5ml of sterile water, final conc. was 100mg/ml, kept frozen as 500 μl aliquots at -20°C.
2. Nalidixic acid: Nalidixic acid sodium salt, (Sigma N-3143) was dissolved (5 mg/ml) in water and stored as 100 μl aliquots at -20°C.conc. 3. Tetracycline: Tetracycline hydrochloride (Sigma T-7508). Stock conc. was 10 mg/ml in 50% ethanol and was stored as 100-500 μl aliquots at -20°C. Final conc used was 10μg/ml.

Bacterial cells: Stored as 25% glycerol stocks, 250 μl aliquots at -80 °C.
1. E.coli DH5α, (genotype): F'/endA1 hsdR17 (rk- Mk+) sup E44, thi-1, λ, rec. A1 gyr A (Nal r) relA1(lac IZYA-argF)U169 deoR (φ80dlc (lacZ)M15)).
2. E. coli XL-1 Blue: genotype: endA1, hsdR17 (rk-, mk+) sup E44, thi-1, λ, rec. A1 gyr A 96, rel A1, ( lac) [F', pro AB, lacZ, M15, Tn10 (tetK)]]

4. *E. coli* Y1090r*: genotype: araD139, hsdR(rk-, mk+), mcrA-, rpsL, supF, trpC22::Tn10, lacU169, lon, (PMC9).

**BSA**: Bovine serum albumin (Sigma, fraction V, A-9647) 1mg/ml solution in sterile water, stored at -20°C.

**CaCl₂ 1M**: Dissolved 14.7 g of CaCl₂ 2H₂O (Sigma, C-3306) in 100 ml H₂O and sterile filtered through a 0.22 micron filter. Stored at -20°C as 10 ml aliquots.

**CaCl₂ 2.5 M**: 2.5 M CaCl₂ 2 H₂O (Sigma, C7902), prepared in sterile milliQ water. Filter sterilized and stored as aliquots of 0.5 ml at -20°C.

**Carrier DNA**: Salmon sperm DNA (sodium salt, Sigma D-1626) or calf thymus DNA (sodium salt, Sigma D-1501). 1 mg/ml in 10 mM Tris-Cl pH-8.0, 10 mM NaCl (NT-buffer), sonicated, once phenol : chloroform extracted, ethanol precipitated, redissolved and was stored as 250 μl aliquots at -20°C.

**Cell lines**: HeLa S3 cells American Type Culture Collection, ATCC No. CCL-2.2.

**Chloroform**: E. Merck (GR), Cat. No. 2445, stored at room temperature.

**CIAP**: Calf intestinal alkaline phosphatase, (New England Biolabs, 290S), conc. was 10 U/μl, stored at -20°C in supplier's buffer.

**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 as aliquots.

**Denaturing Buffer**: 0.5 N NaOH, 1.5 M NaCl for denaturing DNA in the agarose gel for Southern blotting.

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

**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:**
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.

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

**Dissolving Buffer (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 5.0159 g DMEM and 1.858 g 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 1N 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 100% in FCS to freeze cells in liquid N₂.

**DNase I:**
Deoxy-ribonuclease I (Sigma D-5025), 4.5 U/µl in 0.15 M NaCl. Stored as 20 µl aliquots at -20 °C.

**EDTA:**
Ethylene diamine tetra acetic acid, disodium salt, (Qualigen-AR) was gradually dissolved upto 0.5 M in water as the pH adjusted to 7.5 or 8.0 with NaOH pellets and 5 M NaOH soln., final vol adjusted with H₂O, autoclaved and stored at RT.

**EtBr:**
Ethidium bromide (Sigma, E-8751) was dissolved at 10 mg/ml in sterile water, stored as 100 µl aliquots at 4 °C in dark. Working concentration = 0.5 µg/ml.

**Ethanol:**
Merck-GR 1.00983.0511, Bengal Chemicals and Pharmaceuticals, Ltd. Stored at -20°C.
FCS : Fetal Calf Serum, (Biological Industries, Israel, cat #04-001-1B). The serum was heat-inactivated at 56°C for 20 min., stored as 50 ml aliquots at -20°C, used at a final concentration of 10% in DMEM medium.

Fixer : Fixer (Kodak, F-9000720). 2.38 Kg was dissolved in 9-litre water, filtered through Whatman 1 MM paper and stored at RT in brown bottle in dark.

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

Formamide : (Sigma, F-7508). Deionised by DOWEX MR-3 mixed bed resin (Sigma 1-9005), stored as 1 ml aliquots at -20°C.

Formamide dye : 95% deionised Formamide, 20 mM EDTA, stop solution: 0.05% Bromophenol blue and 0.05% Xylene Cyanol FF. Stored at -20°C as 1 ml aliquots.

Geimsa Stain : Qualigens stains. Stored at RT.

Gelatin (2%) : (Merck-GR grade). 2 gm Gelatin in 100 ml water, autoclaved and stored at 4°C.

Geneticin G418 sulphate: (Gibco-BRL 11811-049, 1g) Geneticin selective antibiotic was used for selection of pCDNA 3.1 stably transfected HeLa cells. Dissolved as per manufacturers instructions: G418, supplied as a powder, labelled potency was 735μg/mg of powder. To prepare a stock of 100 mg/ml, 142 mg of G418 powder was taken per ml of autoclaved milliQ water (7 ml total solution was prepared), filter sterilized through 0.22 μm filter, and stored at 4°C as 500μl aliquots. Final concentration used was 0.6 mg/ml.

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

Glycerol : (Sigma, G-5150; Qualigens AR). Stored at RT.

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.

HeBS Buffer (2X) : 50 mM HEPES (free acid, crystalline SIGMA H3375), 280 mM NaCl, 1X PO₄⁻³ buffer pH adjusted to 7.05 with 1N NaOH. Sterile filtered through 0.22 μm filter, stored as 5 ml aliquots at -20°C.
Heparin: Commercially available Heparin sodium injection (25,000 I.U in 5 ml) obtained from (Trade name Beparin) Biological E. limited. India, stored at -20 °C. Used at a final concentration of 200 μg/ml, for RNA isolation.

HEPES: (Sigma, H-3375) for 1 M. 23.83 gm was dissolved in 70-80 ml of distilled water, adjusted pH 7.9 with NaOH, vol. made upto 100 ml, autoclaved and stored at room temperature.

I.P.T.G: Isopropyl β-D-thio galactopyranoside. (Bangalore Genie FC-1). 100 mM stock solution was made in sterile water and stored as 0.5 ml aliquots at -20 °C.

Isopropanol: Isopropanol (Spectrochem, HPLC grade). Stored at RT.

KCl: Potassium Chloride (Qualigens-AR grade, MW= 74.55)

LiCl: Lithium chloride anhydrous, (Sigma, L-0505), stored at RT.

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

Ligation Buffer (1X):- 50 mM Tris-Cl (pH 7.6), 10 mM MgCl2, 1mM DTT, 1 mM ATP, 5% PEG-8000. Supplied by Gibco-BRL. Stored at -20 °C.

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.

Lysis buffer (genomic DNA isolation): 5 mM MgCl2, 10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 320 mM sucrose. Filter sterilized and stored at 4 °C.

Lysis buffer (plasmid midiprep): 15% sucrose w/v (Qualigens-AR grade), 25 mM Tris-Cl (pH 8.0), 10 mM EDTA. Stored at 4 °C, added 2mg/ml Lysozyme, freshly prepared before use.

Lysis Buffer (RNA 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.
Lysozyme: (SRL, 1240123) 10 mg/ml Lysozyme in 50 mM Tris.Cl (pH 8.0). Stored at -20°C as 100 ul aliquots.

Maltose: (Sigma M 5895). 20 g was dissolved in 100 ml water final volume, filter sterilized through 0.22 μM membrane and stored as 15 ml aliquots at -20°C.

Media:
1. LB medium: 10 g Bacto-tryptone (Hi-media, RM-014), 5 gm Bacto yeast extract (Hi-media RM-027), 10 gm NaCl (Qualigens-AR) in 1 litre water. pH was adjusted to 7.0 with 5 M NaOH, autoclaved and stored at 4°C.
2. NZCYM broth: 22 g was dissolved in 1 litre water. pH was adjusted to 7.0 with 5 M NaOH, autoclaved and stored at 4°C.

Membranes:
1. Millipore: (GS, WP-02400), pore size 0.22 μM filters for filter-sterilization from Millipore Corporation, USA.
2. Biodyne A: (Gibco-Life Technology, 14866-016). Neutrally charged nylon membrane. Stored in a clean dry place at RT.

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.

M-MLV reverse transcriptase: 200 U/μl (Promega, M1701) stored at -20°C. supplied with 5 X reaction buffer (M531A) 250mMTris-HCL, pH 8.3; 375mM KCl; 15mM MgCl₂; 50mM DTT.

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

NaCl 5M: Sodium chloride (Qualigens-AR). Dissolved 29.2 g NaCl in 100 ml H₂O, autoclaved, stored at RT.

NEBlot Kit: (NEB, 1550-50) Random primer Klenow based labeling kit from New England Biolabs, U.S.A. stored at -20 \degree C

Normal saline: 0.98% NaCl solution in sterile H_2 O. Autoclaved and used chilled.

NP-40: Nonidet P-40 (Sigma, N-0896)

Nucleotides:

1. Deoxynucleotides: (dATP, dGTP, dCTP, dTTP), 100 mM stocks were from Promega Corporation, USA and NEB, USA and diluted stocks were 25 mM, both were stored at -20 \degree C.
2. Radio active nucleotide triphosphates: (i) [\alpha^{32}P] dATP: BRIT, India, (LCP-103, 3000 Ci/m mole, 10 \mu Ci/\mu l) or Amersham, Cat no. AA0074 (6000 Ci/m mole, 10 \mu Ci/\mu l), stored at -20 \degree C.

PBS (10X): 1.3 M NaCl, 20 mM KCl, 78 mM Na_2 HPO_4 .2H_2 O, 14 mM KH_2 P O_4, autoclaved and stored at 4\degree C.

PBS (1X): 130 mM NaCl, 2 mM KCl, 7.8 mM Na_2 PHO_4 .2H_2 O, 1.4 mM KH_2PO_4 .2H_2 O made in water, pH 7.4 with HCl, autoclaved and stored at RT.

PEG: Polyethylene glycol 8000 (Sigma, P-5413), 30% solution made in water, autoclaved and stored at RT.

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\degree C. Working solution was stored at 4\degree C.

Phosphate buffer: 0.5 M Na_2 HPO_4 (Qualigens-AR grade) in water, pH was adjusted to 7.2 with phosphoric acid, autoclaved and was stored at RT.

PO_4^{3-} Buffer (100X): 150 mM Na_2 HPO_4 .2 H_2 O (Qualigens, India) autoclaved.

Polymerases:

1. Klenow fragment: From *E. coli* DNA polymerase I, (New England Biolabs, 210L), 5 U/\mu l, stored at -20 \degree C and 10x Klenow buffer: 0.1 M Tris-Cl (pH-7.5), 50 mM MgCl_2, 75 mM DTT, stored at -20 \degree C.
2. Taq DNA polymerase: Genie Bangalore (MME 5J), 3 U/µl or Gibco Life technology (10342-020), 5U/; stored -20 °C. 10X buffer: Genie Bangalore 100 mM TAPS, 15 mM MgCl₂, 500 mM KCl, 0.1% gelatin. Stored at -20 °C. Gibcobrl: 10X buffer: 200mM Tris-HCl (pH 8.4), 500mM KCl.

3. PFU polymerase: Pfu DNA polymerase 2.5 U/µl, MBI fermentas #EP0509.

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.

Pronase: (Sigma, P-5147), 20 mg/ml dissolved in 0.15 M EDTA, self digested at 37°C for 2 hr, stored frozen at -20°C in 200 µl aliquots.

Proteinase K: (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.

Radio active nucleotides: [α³²P] dATP: Amersham, Cat. No. AA0074, 6000 Ci/m mole, 10 mCi/ml Stored at -20°C in lead vials.

Restriction enzymes:
1. Bam HI: (20 U/µl), 101LBam HI buffer (10x): 150 mM NaCl, 10 mM Tris-Cl, 10 mM MgCl₂, 1mM DTT and 1 mg/ml acetylated BSA, stored at -20 °C From Life Technology, U.S.A., stored at -20°C.
2. Eco RI: 10U/µl, 15202-021. Eco RI Buffer (REACT 3, 10X) 500 mM Tris-HCl (pH 8.0 ), 100 mM MgCl₂,1000 mM NaCl, stored at -20° C.
3. Hind III: 10U/µl, 15207-020, Hind III buffer (REACT 2, 10X): 500 mM Tris-HCl (pH 8.0 ), 100 mM MgCl₂, 500 mM NaCl, stored at -20°C.

RNA loading dye (2X):- 700 41 deionised formamide, 160 µl 10X MOPS, 260 µl formaldehyde, 18 µl H₂O, 100 µl glycerol, 80 µl bromophenol blue.

RNase A: Pancreatic RNase A (Sigma, R-9009), 10 mg/ml dissolved in 1x TNE and was incubated at 85°C for 10 min to inactivate contaminating DNase, stored as 100 µl aliquots at -20 °C.

SM (1X): 10 ml 10X SM, 0.5 ml 2% gelatin, water added to a final volume of 100 ml, autoclaved and stored at 4°C.
SDS 20%:— Lauryl sulphate-Sodium salt (Sigma, L-4390), 20% (w/v) SDS dissolved in 50 mM Tris.Cl, pH 8.0. Stored at RT.

Sephadex: G-100, (Sigma, G100-120) and G-50 (Sigma, G50-80). 10 gm was soaked and washed properly with excess water to remove all traces of dextran, re-suspended in 100 ml 1x TNE, autoclaved and was stored at 4 °C.

SM (10X): 1 M NaCl, 0.1 M MgSO₄·7H₂O and 0.35 M Tris-Cl (pH-7.5). Autoclaved and was stored at 4°C.

Sodium acetate: (Qualigens-AR grade), stored at RT.

Sodium acetate 3M: 1. Dissolved 40.8g sodium acetate.3H₂O (Qualigens-AR), in 80 ml H₂O and adjusted the pH to 5.2 with glacial acetic acid. Made the volume 100 ml and made 20 ml aliquots, autoclaved and store at RT.

2. Dissolved 40.8g sodium acetate.3H₂O (Qualigens-AR), in 20 ml H₂O and adjusted the pH to 4.6 with glacial acetic acid. Made the volume 100 ml and made 20 ml aliquots, autoclaved and store at RT.

Sodium chloride: Sodium chloride (NaCl) (Qualigens-AR grade), stored at RT.

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 pl aliquots at -20°C.

Spermine, 1M:— Dissolved 0.3482 g Spermine tetra hydrochloride (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): 3 M NaCl and 0.3 M sodium citrate, 175.3 gm NaCl + 88.2 gm Sodium citrate (Qualigens-AR grade) dissolved in 1 litre water, adjusted pH to 7.0 with Citric acid, autoclaved, stored at RT.

Sucrose: (SRL-1944115) or (BDH-AR grade), stored at RT, solution kept at 4°C for limited period.

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

T₁₀N₁₀: 10 mM Tris.Cl, pH 8.0, 10 mM NaCl.

TAE (50x): 242 gm Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0) and volume was adjusted to 1 litre with water,
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.

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, autoclaved and stored at RT.

TELT buffer : 50 mM Tris-Cl (pH 7.5), 62.5 mM EDTA, 0.4% Triton X-100 and 2.5 M LiCl, autoclaved and stored at RT.

Tfb I : 30 mM Potassium acetate, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂, 150 gm/l glycerol (pH 7.0), stored at 4°C. Prepared fresh.

Tfb II : 10 mM Na-MOPS, 75 mM CaCl₂, 10 mM KCl, 150 gm/l glycerol, stored at 4°C. Prepared fresh.

TNE (10x) : 0.5 M Tris-Cl (pH-7.5), 1 M NaCl and 0.05 M EDTA, pH was 7.4-7.5 in a 1/10 dilution, autoclaved and stored at RT.

Tris-HCl : (Qualigens-AR grade). Tris base 1 M in water, pH was adjusted to 7.5 or 8.0 with HCl, autoclaved and stored at RT.

Triton X-100 : (Sigma, T-8787), diluted to 25% in water and stored at RT.

Trypan Blue Stain : (Sigma, T-6146). Made in 1 X PBS at a final concentration of 0.4%. Stored at RT.

Trypsin : (Sigma, T-2271). Prepared a working solution of 0.03% in 1 X PBS/EDTA and stored at -20°C.

Tween-20 : (E. Merck- India, Product no. 437082Q). Store at RT.

Urea : (Sigma U-5378), (SRL-214321), stored at RT.

Water : Milli Q Water (Millipore deionizer) or Quartz double distilled autoclaved water.

X-gal: 5-Bromo 4-Chloro 3-Indolyl β-D-galactopyranoside. Genie Bangalore, (FC-5). Stock solution was 50 mg/ml in dimethyl formamide. Filter sterilized with 0.22 μM membrane and was stored at -20 °C in a dark.

X-ray films: XAR5, Kodak, (Sigma, F-5388), HyperfilmTM MP (Amersham). Stored dry at 4°C.

V.1.2. Oligonucleotides and plasmids

V.1.2.1 Oligonucleotides:

AG primer: 5' AGA GAG AGA GAG AGA GAG A 3' (PCR probe preparation)
TC primer: 5' TCT CTC TCT CTC TCT CTC T 3' (PCR probe preparation)
gt11 5' primer: 5' TCA ACA GCA ACT GAT GGA AAC CAG 3' (amplification of inserts in λgt11)
gt11 3' primer: 5' TTG ACA CCA GAC CAA CTG GTA ATG 3' (amplification of inserts in λgt11)
M13 rev primer: 5' AAC AGC TAT GAC CAT G 3' (PCR-3.3 DNA, sequencing)
T7 primer: 5' AAT ACG ACT CAC TAT AG 3' (sequencing)
T3 primer: 5' ATT AAC CCT CAC TAA AG 3' (sequencing)
Rb primer: 5' TGA GCG CGC GTA ATA CGA CTC ACT ATA GGC AG 3' (PCR-3.3 DNA)
Random primer: 5' NNN NNN 3' (Random priming probe preparation)

Primer A: 5' TAA TAC GAC TCA CTA TAG GGT TTT TTT TTT TT 3' (First strand synthesis.)
Primer B: 5' AAT TAA CCC TCA CTA AAG GGN NNN NN 3' (Second strand synthesis)
HUM1 fwd: 5' AAG AAT AAG CTT ATG GTT GTG GCC CTC CTT ATT G 3' (HUM1-PCR)
HUM1 reverse: 5' TCA TTG AAG CTT CAT TTC TCG CCA AAA AGT ATC TCC A3' (HUM1-PCR)
G3PDH 5' primer: 5' ACC ACA GTC CAT GCC ATC AC 3' (GAPDH PCR)
G3PDH 3' primer: 5' TCC ACC ACC CTG TTG CTG TA 3' (GAPDH PCR)

Primers were commercially synthesized by Genmed synthesis, USA or GIBCOBRL or at Biobasic, Inc. Canada. Primers were synthesized at 0.2 nm scale, purified by desalting, dissolved in deionised water at a concentration of 2 nM and 25 pmole/μl and were stored at -20 °C. The G3PDH 0.45 kb amplimer set (5' and 3' primers)
was from Clontech Laboratories Inc., USA cat no. 5405-3; a kind gift from Dr. Arvind Gill.

V.1.2.2. Plasmids:

**pBlot7**: pBlot7 is a modified version of pBluescript KS\(^+\) plasmid (Stratagene) 2961 bp, by introducing one Nsi I site in-between the TATA box of the T7 promoter and multiple cloning site of the plasmid pBluescript KS\(^-\) (Karin, 1989). A kind gift from Dr. P. K. Yadava, School of Life Sciences, J.N.U. New Delhi, India.

**pBS II KS\(^+\)**: pBluescript II KS\(^-\), 2961 bp from Stratagene USA. Important sites are ampicillin resistance gene (1975-2832) bp, multiple cloning sites (657-759) bp and lac Z gene (816-938) bp.

**pCDNA3.1 (+)**: Mammalian expression vector for constitutive CMV promoter-enhancer directed, high level expression of cDNA in mammalian cells (Invitrogen V790-20). In addition, the plasmid has the SV-40 origin of mammalian replication, bovine growth hormone polyadenylation signal and termination sequence, and *E. coli* ColE1 origin and Ampicillin resistance gene for selection in bacteria. The selectable marker for mammalian expression is the neomycin resistance gene that confers G418 resistance to the transfected cells.

V.1.3. Human testis cDNA library: Human testis 5'-stretch plus cDNA library in \(\lambda\)gt11 was obtained from Clontech, USA. RL3004b (A kind gift from Dr. Y. K. Jaiswal, Biochemistry Department, Jiwaji university, Gwalior, India) and was stored at \(-70^\circ\)C.

V.1.4. Human multiple tissue northern blot (MTN) and multiple tissue expression array (MTE): The human multiple tissue northern blots (Clontech Laboratories Inc., USA cat no. 7759-1, 7760-1), human multiple tissue expression array (Clontech Laboratories Inc., USA cat no.7775-1) and ExpressHyb solution were kind gifts of Dr. Sanjaya Singh, M. D Anderson Cancer Centre, Houston TX, USA.

V.1.5. Animals: Healthy male swiss albino mice, 12-14 weeks of age were procured from the animal house facility of the School of Life Sciences, J.N.U. Mice were sacrificed by cervical dislocation, the testis were dissected out quickly and used fresh for the RNA isolation.
V.2 METHODS

V.II General methods

V.II.1 Routinely used procedures:
Restriction digestions were done according to manufacturer's instruction (New England Biolabs, GIBCO Life Technology). Phosphatase treatment of DNA, ligation of DNA fragments, phenol : chloroform extraction, ethanol or isopropanol precipitation and extraction of DNA from low melting agarose gels were basically carried out as described in Sambrook, Fritsch and Maniatis, (1989). Optimizations of different PCR reactions for primer annealing, Mg²⁺ concentrations, and optimum specific amplification of DNA were mainly carried out as described in White (1997). DEPC treatment of water and preparation of solutions used for RNA was carried out using 0.1% v/v DEPC. The solutions were autoclaved 15lb, 30 min. Tris based solutions were not treated by DEPC.

V.II.2 Preparation of competent E. coli cells:
E. coli XL-1 blue cells were made competent for transformation as per the method Hanahan et al., (1985). Cells were streaked on LB + 1.5% agar + tetracycline (tet) 10 µg/ml plate and allowed to grow at 37° C overnight to select for pure antibiotic resistant colonies. 5ml LB + tet (10µg/ml) was then inoculated with a single colony from the plate and allowed to grow overnight at 37° C, 200 rpm. Next day 100 ml LB + tet (10µg/ml) medium was inoculated with 1ml of overnight grown culture and allowed to grow at 37° C and 200 rpm for 2 to 3 hours until O.D. at 550 nm of the culture reached 0.4 to 0.6, which corresponded to approximately 5-6 X 10⁷ cells/ml. Cells were pelleted down in 250 ml GSA bottles at 3500 rpm for 5 min. at 4° C in a Sorvall RC5B centrifuge using GSA rotor, the medium was discarded. The pellet was gently resuspended in 40 ml of ice cold Tfb I buffer (30 mM Pot. Acetate, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂, 150 g/L glycerol, pH 5.8) on ice. After being left undisturbed on ice for 15 min, the cells were again pelleted down at 3500 rpm at 4° C for 5 min. The pellet was then gently resuspended in 4ml of Tfb II (10 mM Na-MOPS, 75 mM CaCl₂, 10 mM KCl, 150g/L glycerol, pH 7.0 ) on ice. Aliquots of 100 to 200 µl cells were saved in prechilled 0.5 ml Eppendorf tubes and stored at -80° C. E. coli DH 5α cells were selected on LB + 1.5%agar + nalidixic acid
V.II.3 Transformation of *E. coli* with plasmid DNA:

Transformation of the competent *E. coli* cells with plasmid DNA was carried out according to Sambrook et al., (1989). An aliquot of competent cells was thawed on ice for 15 to 30 min. 0.1, 0.2, 0.5 and 1 ng of plasmid DNA were mixed with 2 μl TCM buffer (0.3 M CaCl₂, 0.3 M MgCl₂, 0.1 M Tris-HCL pH 7.5) and kept on ice for 5 min. 50 μl of competent cells were mixed with the DNA+TCM buffer mixture and left undisturbed on ice for 30 min. The cells were then given a heat shock at 42°C for 90 sec. The cells were then immediately transferred to ice and left for 5 min. 1 ml LB medium was added to each tube and the cells were incubated at 37°C for 1 hr with shaking at 200 rpm. 200 μl of the transformation mixture was spread on LB + 1.5% agar + amp. (100 μg/ml) plates. Plates were incubated at 37°C overnight. The number of colonies was counted and the transformation efficiency was checked with 0.1 ng of plasmid DNA and expressed as number of colonies/μg plasmid DNA. Transformation efficiency of the cells was in the order of $10^7$ to $10^9$ cfu/μg DNA.

V.II.4 Purification of plasmid DNA

V.II.4.A LiCl boiling method for mini - preparation of plasmid DNA:

Mini preparation of the plasmid DNA was carried out by a LiCl boiling method of Holmes and Quigley (1981). 5 ml LB + amp (100 μg/ml) medium was inoculated with a single transformed bacterial colony and allowed to grow at 37°C, 200 rpm overnight. 1.5 ml of the overnight grown culture was pelleted down by spinning at 12000 rpm for 30 sec. in a microfuge at room temperature. The supernatant was aspirated without disturbing the pellet. The bacterial pellet was then resuspended in 100 μl TELT Buffer (50 mM Tris-HCL pH 7.5, 62.5 mM EDTA, 0.4% Triton X-100, 2.5 M LiCl) by vortexing, to which 10 μl of freshly prepared aqueous solution of lysozyme (10 mg/ml) was added. The tubes were immediately transferred to boiling water bath for 1 min, then incubated on ice for 5 min. The tubes were centrifuged at 12000 rpm for 8 min. at room temperature to pellet down cell
debris, which was removed with a sterile toothpick. The supernatant was treated once with equal volume of phenol: chloroform (1:1) mixture. The aqueous layer was collected in a fresh Eppendorf tube and precipitated with 0.6 volume of isopropanol in presence of 0.3 M sodium acetate, kept on ice for 15 min. DNA was pelleted at 15000 rpm for 20 min. at 4°C and washed once with 80% ethanol to remove excess of salt. The pellet was air dried for 30 min. and dissolved in 20 μl TE. This purified plasmid DNA was used to check for recombinant DNA clones by restriction digestion and agarose gel electrophoresis.

V.II.4.B Alkaline lysis method for midi-preparation of plasmid DNA:

Midipreparation of plasmid DNA was carried out according to Sambrook et al., (1989). 50 ml of LB + antibiotic was inoculated with a single transformed colony, allowed to grow overnight at 37°C, 200 rpm. Cells were pelleted down by spinning at 5000 rpm for 5 min. at 4°C in a Sorvall RC 5B centrifuge. The cell pellet was resuspended well by vortexing in 1.5 ml lysis buffer (15% sucrose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA, pH 8.0). A final concentration of 2 mg/ml freshly prepared Lysozyme (40mg/ml in 50 mM Tris-HCl pH 8.0) was added to the cell suspension and left on ice for 10 min. The lysed cell suspension was denatured by adding 3.0 ml of freshly prepared 0.2 N NaOH + 1% SDS and mixed by inverting the tube. After 10 min. on ice, 1.6 ml of 3 M sodium acetate (pH 4.6) was added, mixed by inverting and left on ice for 20 min. The tubes were then centrifuged at 12000 rpm for 15 min at 4°C and to the supernatant collected in a fresh tube, 5 μl of DNase free RNase (10 mg/ml) was added, incubated for one hour at 37°C. The plasmid DNA solution was extracted twice with equal volume of phenol : chloroform (1:1) and once with chloroform. The plasmid DNA was precipitated by adding 0.6 volume of isopropanol in presence of 0.3 M sodium acetate and kept on ice for 15 min. DNA was pelleted by spinning at 15000 rpm for 20 min. at 4°C and the pellet was washed with 80% ethanol to remove excess salt. The pellet was then air dried for 30 min. at RT and dissolved in 100 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 1μl DNA was loaded on 1% agarose-TAE gel to check the quality and quantity of plasmid DNA. The DNA was pure enough to carry out restriction digestion, dot blots and probe preparation.
V.II.4.C Large scale purification of plasmid DNA:

Large scale plasmid DNA purification was carried out by a modified method of Sambrook et. al., (1989). 5ml of LB medium with appropriate antibiotic was inoculated with a single transformed colony and allowed to grow at 37° C, 200 rpm for overnight. 1 ml of overnight grown culture was used to inoculate a 250 ml LB + antibiotic medium and allowed to grow at 37° C, 200 rpm for 14-16 hours. Cells were pelleted down at 6000 rpm for 20 min at 4° C in a Sorvall RC 5B centrifuge with a GSA rotor. The cell pellet was resuspended in 12 ml of 50 mM Tris-HCl, pH 8.0. The cell suspension was transferred from the GSA bottle to a 35 ml tube and 2.0 mg/ml lysozyme (from a freshly prepared stock of 10 mg/ml) was added while vortexing and kept on ice for 10 min. Then 1.8 ml 0.5 M EDTA (pH 8.0) was added and mixed by vortexing. To the mixture 750 μl of 2% Triton X-100 was added and vortexed. The extract was left on ice for 60 min. and then centrifuged at 15,000 rpm for 30 min. at 4° C in a SS34 rotor of Sorvall RC5 B centrifuge. The supernatant was transferred to a fresh 35 ml tube, allowed to reach room temperature. 1.8 ml freshly prepared 1 M NaOH was then added while stirring for denaturing the genomic DNA and left for 10 min. at room temperature. The pH was checked with pH paper and it was around 12.5. The mixture was then neutralized by adding 8 ml 1 M Tris-HCl (pH 7.5) and kept for 3 min, the pH of the mixture came down to 8.3 - 8.5. To the crude mixture, 3 ml 5 M NaCl was added. The mixture was extracted with equal volume of 0.5 M NaCl saturated phenol, centrifuged at 10,000 rpm for 15 min at 4° C. The aqueous layer was extracted twice with equal volume of chloroform. The aqueous phase was collected. The RNA in the aqueous phase was digested with 50 μl DNase free RNase A (10 mg/ml), kept at 37° C for 1 hr. DNA was precipitated by adding 3 ml of 5 M NaCl and 5 ml of 30% PEG 8000. The mixture was mixed by vortexing and kept on ice overnight. The precipitated DNA was pelleted down by centrifuging at 10,000 rpm for 30 min. at 4° C in a HB4 rotor of Sorvall RC5B centrifuge. The DNA pellet was washed with 80% ethanol, air dried, and to the pellet 400 μl TNE buffer was added and kept at 37° C for 1 hr to dissolve the DNA. To the DNA solution 10 μl 20 % SDS (1/40 volume) and 8 μl (1/50 volume) pronase (20 mg/ml) were added and incubated at 37° C for 30 min. The reaction mixture was extracted twice with equal volume of phenol: chloroform (1:1) and once with equal volume of chloroform. DNA was precipitated by 0.7 volume of isopropanol in the presence of 0.3 M Sodium
acetate (pH 5.2), kept at -20° C for 2 hr. or - 80° C for 15 min. DNA precipitate was pelleted down by centrifuging at 15,000 rpm for 20 min. at 4° C. The pellet was washed with 70 % ethanol; air dried for 30 min. and dissolved in 200 µl 10 mM Tris-HCl (pH 8.0). 1 µl DNA solution was checked on 1 % TAE agarose gel. DNA quantity was measured at A_{260/280} nm and the quality by wavelength scan from A 200nm-300nm in a UV-160A Shimadzu spectrophotometer. The yield of the pBluescript plasmid was 1.6 - 1.8 mg from a 250 ml culture. Plasmid DNA isolated by this method was pure enough to be used for restriction digestion and preparation of probe by random priming and other purposes.

V.II.5 Purification of plasmid DNA through Sephadex G-100 column:

Plasmid DNA was purified through Sephadex G-50 as per the method of Sambrook et. al., (1989). Sephadex G-50 was soaked overnight in water and washed 2-3 times to remove the fines, autoclaved and resuspended in TNE buffer. The column was prepared by packing Sephadex G-50 to a bed volume of 1 ml in a 1ml insulin syringe plugged at the bottom with sterile glass wool. 100 µl of DNA solution at a concentration of 0.5-1 µg/µl was loaded and eluted with equal volumes of 1X TNE. 4 µl was checked on a 1% agarose/TAE gel. Fractions with plasmid DNA were pooled from 4-5 fractions and precipitated at -80 °C for 15 min by adding 2.5 volume ethanol in presence of 0.3 M sodium acetate pH 5.2. DNA was collected by centrifugation at 12,000 rpm, 15 min at 4 °C, washed once with 80% ethanol and was finally dissolved in 100 µl 10 mM Tris-HCl (pH 8.0). This plasmid DNA was pure enough to be used for transfection into cultured mammalian cells and all other purposes and it remained good even after prolonged storage at -20 °C. Plasmid DNA to be used for transfections was dissolved in autoclaved milliQ water at a final concentration of 500 ng/µl and stored in -20°C in aliquots of 50 µl till further use.

V.II.6 Purification of DNA through low melting agarose gel:

DNA was purified from agarose gels by the method of Ausubel et. al., (1995). Plasmid DNA, digested with appropriate restriction enzymes was loaded to 1 % high melting agarose-TAE gel, and electrophoresed till the DNA had resolved into distinct bands. A cavity was made ahead of the desired band to be purified into which 1% low melting agarose in 1X TAE was poured and allowed to set at 4°C for 15-30
The gel was electrophoresed till the desired band completely entered into the low melting agarose block. The desired band was cut out with a sterile blade under brief UV visualization and melted at 65°C for 10 min. Then equal volume of 50 mM Tris-HCl (pH 7.5) was added. The mixture was vortexed vigorously and heated at 65°C for 5 min. and immediately transferred to 37°C for 5 min. An equal volume of TNE-saturated phenol (prewarmed to 37°C) was added and mixed properly by vigorous vortexing to extract the DNA. The aqueous layer, obtained after centrifugation at 10,000 rpm at room temperature, was taken in a fresh tube. The DNA in the aqueous phase was precipitated by adding 0.6 volume of isopropanol in presence of 0.3 M sodium acetate (pH 5.2) and was precipitated at -20°C for 1 - 2 hr. The DNA was collected by centrifuging at 15,000 rpm for 10 minutes at 4°C. The DNA pellet was washed with 80% ethanol, air dried and dissolved in 20-50 μl 10 mM Tris-HCl (pH 8.0). 1 μl of the DNA solution was loaded on 1% agarose gel to check the approximate concentration by comparing with DNA of known size and concentration. Usually the concentration was 10-20 ng/μl.

V.II.7 Preparation of radiolabelled probes

V.II.7.A Preparation and purification of 32P labelled random primed DNA probes:

32P labelled DNA probes were prepared by random primed DNA synthesis in vitro as per manufacturer's (New England Biolabs, USA) instructions. Typically, 25 ng of gel purified DNA fragment in water was boiled for 3 min in a boiling water bath and immediately chilled on ice for 5 min. It was 32P labelled using NEBlot™ Kit (NEB, USA, cat. No. 1550-50) as recommended by the manufacturer and mentioned below.

<table>
<thead>
<tr>
<th>Stock</th>
<th>volume</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling buffer (10 X) (containing random primers)</td>
<td>2.5 μl</td>
<td>1 X</td>
</tr>
<tr>
<td>dGTP (100 mM)</td>
<td>1.0 μl</td>
<td>4 mM</td>
</tr>
<tr>
<td>dCTP (100 mM)</td>
<td>1.0 μl</td>
<td>4 mM</td>
</tr>
<tr>
<td>dTTP (100 mM)</td>
<td>1.0 μl</td>
<td>4 mM</td>
</tr>
<tr>
<td>α32P dATP (10 μCi /μl) (Specific activity of 6000 Ci/m mole)</td>
<td>2.0μl</td>
<td>20μCi</td>
</tr>
<tr>
<td>E. coli DNA polymerase I Klenow fragment, 5U/μl</td>
<td>0.5 μl</td>
<td>2.5U</td>
</tr>
</tbody>
</table>
The total reaction volume was 25 µl and it was incubated at 37°C for 1 hour. The reaction was terminated by 2µl of 0.5 M EDTA and processed for purification or stored at -20°C.

V.II.7.B 32P GA/CT PCR probe:

3.3 AG-primer and 3.3 CT-primer (12.5 pmole each) were used as self annealing templates for PCR amplification using 3.5 µl α32P dATP (3000 Ci/m mol, 10 µci/µl) in presence of dGTP, dCTP, dTTP (0.2mM each), 2 mM MgCl2 and 0.9 U Taq DNA polymerase in a final volume of 25 µl using the following program in an MJ research PTC-100 thermal cycler (94 °C for 5min) 1 cycle, (94 °C for 30 sec, 43 °C for 30 sec and 72 °C for 2min) 35 cycle and 72 °C for 10 min. Specific activity of the 32P GA/CT probe was 10^7-10^8 cpm/µg DNA. This probe was used in the Southern hybridization of cDNA clones to check for presence of repeats in cDNA inserts.

V.II.7.C 32P 3.3 PCR probe:

Intact 1 ng p7SKM3.3 plasmid (227 bp 3.3 DNA in bluescript) was PCR amplified by Rb and M13 reverse primers (12.5 pmole of each) using 2.5 µl α32P dATP (3000 Ci/m Mol, 10 µci/µl) in presence of dGTP, dCTP, dTTP (0.2 mM each), 2 mM MgCl2 and 1 U Taq DNA polymerase in a final volume of 25 µl using the following program (94 °C for 5 min) 1 cycle, (94 °C for 1 min, 43 °C for 1 min, 72 °C for 1 min) 35 cycle and 72 °C for 10 min. Amplified 3.3 DNA was digested with Sac I to remove the vector DNA (164 bp). Digested DNA was checked in a 1% agarose-TAE gel. The higher band (236 bp) having 32P labelled 3.3 insert DNA was purified from a preparative 1% low melt agarose gel as described before. Specific activity of the probe was 10^8 cpm/µg DNA. This probe was used to screen the cDNA library and Southern hybridization of the PCR amplified cDNA inserts from cDNA clones.

V.II.7.D PCR amplification of HUM1 ORF:

The HUM1 ORF DNA (603 bp) was amplified using 50 pg of the gel purified HUM1 cDNA insert as the template. The final concentrations of other PCR components were: 1X PCR buffer, 0.5mM dNTPs, 25 pmole/50 µl reaction, 2mM MgCl2, 2.5 units Taq polymerase, in a final reaction volume of 50 µl. The PCR
amplification parameters: denaturation for 4 min at 94°C, 35 cycles of 94°C for 45 sec, 68°C for 45 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. 5 μl of the PCR reaction were loaded on a 1.6% agarose gel and checked for amplification. The PCR amplified band was then resolved on a 1.2% agarose gel, and the band was purified from the gel as described above. 25 ng of the purified fragment was used as a template for random primed \(^{32}\)P labelling as described above.

All probes were purified by Sephadex G-50 spin column. The nozzle of a sterile 1 ml plastic syringe was plugged with autoclaved siliconized glass wool. Sephadex G-50 was swollen in H\(_2\)O, washed several times in sterile water, the resin was resuspended in TNE buffer and was autoclaved. The column was filled by adding sephadex G-50 suspension slowly and allowed to pack uniformly under gravity and then by prespin inside a 15 ml plastic centrifuge tube at 3000 rpm for 1 minute in a clinical centrifuge with swinging bucket rotor. The column was washed twice with 100 μl TE buffer (pH 8.0) by centrifugation at 3000 rpm for 1 minute each. 75 μl of TE (pH 8.0) buffer was added to the 25 μl of labelled probe and this 100 μl mixture was loaded on to the column which was lodged inside an improvised stand made out of a Falcon tube (15 ml) with a hole in the cap and containing an Eppendorf tube on a paper cushion inside to collect the elute. This was done in such a manner that the nozzle was suspended into the collection tube and to prevent leakage of \(^{32}\)P material outside. Centrifugation at 3000 rpm for 3 minutes brought the purified \(^{32}\)P probe into the Eppendorf tube, which was then taken out with a fine forceps, closed with the cap, stored at -20°C. 1 μl from it was aliquoted to check for the specific activity of the in a LKBβ–counter by Cerenkov counting. The specific activity of the probe was found to be about 5-9 X 10^8 cpm/μg.

V.II.8 RNA isolation from cells and tissues:

Total RNA from mouse testis or human tumour samples was isolated by the method of Auffray and Rougeon (1980). All following steps were carried out under RNase-free conditions (as per standard protocol) and on ice at 0-4°C unless otherwise mentioned. Fresh tissues from the mice or flash frozen human tumour samples were washed in normal saline twice at 4°C to remove blood, blotted, weighed and then minced into small pieces on ice. The tissue was then ground in a mortar-pestle under liquid nitrogen. The cell pellet was processed directly. Lysis buffer (6 M urea, 3 M
LiCl, 50 mM NaOAc, 0.1% SDS and 200 μg/ml heparin, last two components added freshly just before use) was added to the minced tissue to make a 10% homogenate and was thoroughly homogenized in a mortar and pestle for the tissue, while cells were vigorously vortexed till lysis was complete. Homogenates were then sonicated on ice in a ultrasonicator for 30 sec each for 6 times with 1 min gap in between and were kept on ice at 4°C for 16-18 hr for precipitation. The extracts were centrifuged in a Sorvall, SS-34 rotor, at 10,000 rpm for 15 min at 4°C. Precipitates were pelleted and resuspended/washed in 4 ml (8 M urea + 4 M LiCl) and centrifuged as before. Final precipitates were dissolved in 3-5ml (200 mM NaOAc pH 5.0, 0.2% SDS and 1 mM EDTA) by vigorous vortexing until it was soluble. The RNA solutions were extracted twice: once with equal volume of TNE-phenol and then with equal volume of phenol: chloroform (1:1). Care was taken to ensure that there was no residual interphase material. Aqueous phases were collected by centrifugation at 12,000 rpm for 8 min, at 4°C. RNA was then precipitated by adding 2.5 volume chilled ethanol in presence of 0.3 M sodium acetate (pH 5.0), kept at -20°C for overnight. RNA was precipitated by centrifugation at 12000 rpm for 15 min, at 4°C. It was thoroughly washed with 80% ethanol, air-dried and finally dissolved in 100 μl sterile distilled water. A260/A280 nm ratio of the RNA and A200-300 nm scan were also measured spectrophotometrically. Both the yield and quality of the RNA were determined from the A260 nm value and the ratio of A260nm/280nm values as well as the A200-300 nm spectrum as per standard recommendations. RNA solutions were also routinely checked in 1% agarose-TAE gels, stained with ethidium bromide to visualise the intactness of the 28S and 18S ribosomal RNAs. RNA solutions were stored at -20°C as 70% ethanol precipitates.

V.II.9.A. Screening of λgt11 cDNA library:

One single E. coli Y1090r– colony was selected from a LB-agar-amp plate. One single isolated colony was picked and inoculated into 100 ml (LB broth + 10 mM MgSO₄) in presence of 0.2% maltose, incubated in a shaker at 37 0°C and 200 rpm overnight till the O.D₆₀₀ of the culture reached 2.0. Rat testis (RT) library was first serially diluted to 1:500, and then to 1:250,000 in 1X SM medium. Different dilutions of the library were diluted with 100 μl of 1X SM mixed with 200 μl overnight Y1090r– culture and were incubated in a 37 0°C water bath for 20 min to adsorb the
phage particle. 5 ml of melted (at 55 °C) LB top agar (0.7%) + 10 mM MgSO₄ was added quickly to each tube, mixed well and poured onto LB agar (1.5%) + 10 mM MgSO₄ plate (90 mm diameter) at 37 °C quickly. Plates were allowed to set at RT, incubated (at inverted position) at 37 °C for ~10-12 hrs until plaques developed. Plates were sealed and were stored at 4 °C. To determine titer (pfu/ml), plaques were counted and titer was determined using the formula: pfu/ml = (# of plaques X dilution factor X 10⁻³ μl/ml) / used.

Titer values were generally between 10¹⁰- 10¹¹ pfu/ml. HT (Human Testis) library plates (132 mm diameter) were then screened (primary) using ³²P 3.3 DNA probe. Positive plaques were picked out by sterile Pasteur pipettes and put into 400 μl of 1X SM, mixed and kept at 4 °C overnight with 1 drop of chloroform to diffuse out the phages. Next day it was centrifuged at 12 Krpm for 5 min and supernatant was stored in a fresh tube with one drop of chloroform at 4 °C. Similarly positive plaques from primary screening were plated again and screened for secondary and subsequently tertiary screening using the ³²P 3.3 DNA probe.

V.II.9.B. PCR amplification of inserts and Southern hybridization:

After tertiary screening, positive clones were PCR amplified using λgt11 specific primers flanking the cloning junction (Eco RI) and PCR products were Southern hybridized using ³²P 3.3 DNA probe and 3.3 GA probe separately. Positive clones having the GA or CT stretches from both the hybridization s were selected. 5 μls of phage particle (1.25X10⁴) in 1XSM were diluted to 6.6 times with dH₂O, boiled for 2 min, quick chilled on ice for 5 min and were centrifuged for 5 min at RT, 12 Krpm. Supernatants were collected and PCR reactions were set as follows: 10 μl of phage supernatant, both λgt11 primers (12.5 pmole each), 2.5 μl of 10X PCR buffer, 1.5 mM Mg²⁺, 0.4 mM dNTPs mix and 0.9 U of Taq DNA polymerase as final conc. 25 μl with one drop of mineral oil on the top and put in a PCR machine using the following cycle:- (a) 95 °C for 5 min, (b) 93 °C for 50 sec, 54.2 °C for 1 min 55 sec, 72 °C for 4 min repeated for 35 cycle, (c) 72 °C for 10 min. All PCR conditions were optimized. 1/3 of the each PCR reaction was loaded in 1% agarose-TAE gel along with a DNA size marker (λ DNA + Eco RI + Hind III). After completion of electrophoresis, gels were denatured, transferred onto nylon membranes and Southern hybridized using either ³²P 3.3 DNA or ³²P (GA)ₙ oligo as probe as mentioned earlier.
DNA in the gel was denatured with 200 ml denaturing solution (1.5 M NaCl + 0.5 M NaOH) for 30 min 3 times, by shaking at RT. DNA was transferred onto a nylon membrane (Nytran-N-) by a improvised vacuum blotting system (for 1hr) at 35-40 mm water height as an indicator for the vacuum and using denaturing solution as transfer buffer. DNA on membrane was then UV cross-linked in moist condition in a Stratalinker 1800 (Stratagene), washed in 6X SSC, dried at RT and stored at 4 °C in a sealed plastic bag. For Southern hybridization (essentially carried out as mentioned in Church and Gilbert), DNA transferred-nylon membrane was sealed in a polypropylene bag with 0.1 ml/ cm² pre-hybridization solution (0.25 M Na₂HPO₄ pH-7.2, 7% w/v SDS, 1% w/v BSA and 1 mM EDTA) was prehybridized at 65 °C temperature for 30 min. ³²P labelled denatured probe (10⁵ cpm/ml) and tRNA (10 μg/ml) were added to prehybridization mix. Hybridization was carried out at 55 °C for 18 hr with slow shaking. After hybridization membranes were sequentially washed in 200 ml soln-I (0.25M Na₂HPO₄, pH 7.2 + 1% SDS + 1 mM EDTA), soln-2 (0.125M Na₂HPO₄, pH 7.2 + 1% SDS + 1 mM EDTA) for 2X15 min first at RT, then at 55 °C. During washing radioactivity on the filter was monitored by a GM counter (Rad-Monitor™, USA) and washing was continued until background was clear. Membranes were then exposed to X-ray films in moist condition in a sealed plastic bag at -70 °C with intensifying screen.

V.II.9.C. Preparation of genomic DNA from λgt11 clones:

One single colony of LE 392 was inoculated into a 15 ml LB + 10 mM MgSO₄ + 0.2 % maltose and was grown at 37 °C, 200 rpm for 16-18 hrs until O.D₅₅₀/ml attained 2.0. 200 μl of cells having 2 OD/ml were infected with 5 μl of phage lysate (~10¹⁰ pfu/ml) and kept at 37 °C for 20 min. It was then transferred into 60 ml NZCYM and allowed to grow at 37 °C, 200 rpm for 5-6 hrs. Lysis generally was complete by this time otherwise 300 μl chloroform was added and kept at 37 °C, 200 rpm for 15 min. Culture was centrifuged for 5 min, at 4 °C, 10 krpm. Supernatant was collected, 5 μl DNase I (4.5 U/μl) and 5μl RNase A (10 mg/ml) were added and allowed to digest E. coli genomic DNA and RNA for 40 min at 37 °C. 5 M NaCl and solid PEG-8000 were added to final 1 M and 10 %, respectively and left on ice at 4 °C for 16 hrs. Next day phage particles were pelleted down at 4 °C, 10 K rpm for 20 min and was resuspended well in 1 ml TE. The phage suspension was then extracted very
gently twice with equal volume of phenol followed once with equal volume of chloroform. 0.1 volume of 3 M sodium acetate (pH-5.2) and 0.7 volume isopropanol were added to the aqueous layer and kept at −20 °C for 16-18 hrs. Phage DNA was collected at 10 K rpm for 15 min at 4 °C, washed with cold 80% ethanol, dried and dissolved in 10 mM Tris-Cl + 10 mM NaCl pH 7.5. Yield and purity of the λ DNA was checked in a 0.6% agarose-TAE gel and by O.D of A_{260}, A_{260/280} and scan at 200-300 nm in a spectrophotometer. Purified genomic DNA was stored at 4 °C with one or two drops of chloroform added to the bottom of the tube for further use.

V.II.9.D. Subcloning of cDNAs to pBluescript (KS+) vector

(i) Vector preparation:

5 μg pBluescript II KS+ plasmid DNA was digested with 20 U of Eco RI in a 50 μl reaction volume at 37 °C for 16 hr. After complete linearization of the plasmid as judged by agarose gel, the enzyme was inactivated by incubating the reaction mixture at 65 °C for 15 min. Subsequently, the vector DNA was dephosphorylated with 1 U of CIAP in 120 μl 1X CIAP buffer at 37 °C for 60 min. The enzyme was inactivated by incubation at 75 °C for 20 min. DNA was then extracted once with equal volume phenol : chloroform (1:1) mixture, precipitated by adding 2.5 volume ethanol in presence of 0.3 M sodium acetate (pH 5.2) at −80 °C for 15 min, collected by centrifugation at 12 Krpm for 15 min at 4 °C, washed once with 80% ethanol, dried and dissolved in TE. 1 or 2 μl of dissolved DNA was mixed with 1/6th of 6X loading dye and was electrophoresed in 1% agarose gel in TAE buffer, purified through low melt agarose as described before, dissolved as 100 ng/μl (50 fmole/μl) i.e. in 40 μl of 10 mM Tris-Cl and stored at −20 °C. 1 μl of the DNA was checked on 1% agarose gel to assess the DNA concentration.

(ii) Preparation of Eco RI cDNA fragment(s) from cDNA clones:

Genomic DNA (50 μg) from the λgt11 clones were digested with 60U Eco RI at 37 °C for 16-18 hrs. Digested DNA was then mixed with 1/6th volume of 6X loading buffer and was loaded on a 1% low melting agarose-TAE gel. Inserted cDNA fragments were then cut out from the gel and was purified as mentioned above. Purified cDNA was redissolved (25 fmole/μl) in 30 μl TE and stored at −20 °C.
(iii) **Ligation of the cDNA fragments to Vector DNA:**

100 fmole of the vector DNA (KS⁺) and 100 fmole of purified cDNAs fragment was ligated in a 20 μl reaction mixture using 1X ligation buffer (Gibco-Life tech) containing 1 mM ATP, 1 Weiss U of T4 DNA ligase at 16 °C for 16-18 hours. The ligation mix was then stored at -20 °C and 5 μl of ligation mix was directly mixed with 2 μl TCM buffer and transformed into competent cells as described above.

(iv) **Identification of recombinant clones by Eco RI digestion:**

Plasmid DNAs were prepared by mini-prep from the recombinant clones and were checked on agarose gels. Colonies were finally selected after digesting by Eco RI. 1 μg of plasmid DNAs was digested by 2.5 U Eco RI in 20 μl reaction volume at 37 °C for 2 hrs. Vector plasmid cut by Eco RI was the reference DNA. Clones with Eco RI inserted cDNAs were selected. The positive clones were picked and grown in 50 ml LB medium containing 100 μg amp/ml to purify larger amount of the plasmid cDNA clones for further use.

V.II.10 **Analysis of cDNA sequences**

V.II.10.A. **Automated sequencing:**

Plasmid DNA from the positive clones was prepared and purified from 50 ml culture as mentioned above. All the cDNA clones were sequenced by the Bigdye primer/terminator chemistry and automated fluorescence based sequencing in the ABI-377 or ABI-3700 (Perkin-Elmer) system (in collaboration with Dr. Sanjaya Singh, Biochemistry and Molecular Biology Department, University of Texas, M. D. Anderson Cancer Center, Houston, Tx, USA and Dr. Lalji Singh, Director, CCMB, Hyderabad).

V.II.10.B. **Computational Analysis of cDNA sequences through homology search:**

cDNA sequences were tested for vector DNA contamination against the VecScreen database at NCBI, extra sequence was trimmed and applied to further analysis (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html).
Sequences of the cDNAs were searched for their homology against nucleotide databases by using the BLASTN program version 2.2.1. The databases searched by BLASN included the 'nr' (all GenBank+EMBL+DDBJ+PDB sequences, excluding EST, STS, GSS ³ sequences) and human EST (Expressed Sequence Tags subset of GenBank+EMBL+DDBJ) databases, and this gave information on matches on genes, genomic matches and expression information respectively. All EST matches that were linked to the unigene database were also followed up for precise annotation of the cDNAs. UniGene system partitions GenBank sequences into a non-redundant set of gene-oriented clusters, each representing a unique gene. Related information such as the tissue types from which the gene expression has been detected and map location. UniGene clusters are compiled from sequences of well-characterized genes as well as expressed sequence tag (EST) sequences.

Coding potential of the sequences was determined by searching for the Open Reading Frames (ORF) with the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). ORFs were then searched for homology to the known protein databases with the BLASTP program (version 2.2.1) at NCBI⁴. cDNA sequences were also searched for protein sequences similar to query at the Baylor College of Medicine (BCM) search launcher WU-BLAST program version 2.0. Sequences were translated into all six possible reading frames and searched for homology by BLASTX+BEAUTY (http://searchlauncher.bcm.tmc.edu/seq-search/nucleic_acid-search.html).

Repeat sequences within the cDNA sequences were also searched for by a combination of three methods. The RepeatMasker program available through the, Baylor College of Medicine WU-BLAST 2.0 was used to identify and mask interspersed repeats known to exist in mammalian genomes as well as low complexity DNA sequences. One of the limitations of the program is RepeatMasker is not reliable for accurate annotation of simple repeat sequences for three reasons. (i)

³ EST: Expressed Sequence Tags. Contains sequence data and other information on "single-pass" cDNA sequences.
STS: Sequence Tagged Sites.
GSS: Genomic Survey Sequence. Includes single-pass genomic data, exon-trapped sequences, and Alu PCR sequences.
⁴ Standard protein-protein BLAST (blastp) is used for both identifying a query amino acid sequence and for finding similar sequences in protein databases.
The interspersed repeats are masked before the simple repeats, thus hiding many simple repeats contained within an interspersed repeat, a commonly occurring phenomenon, before these can be detected. (ii) Only all di- to tetrameric and some pentameric simple repeats are scanned for. (iii) Often regions that are indicated to be simple repeats are highly diverged. These may have had a different unit repeated as currently recognized, and/or constitute plain low-complexity DNA. As an example of the latter, mixed (GGA)n or (GGAA)n etc. simple repeats are often annotated as poly-purine regions, thus hiding the true simple repetitive nature.

Therefore, to identify the cryptic repeat regions more accurately, the SIMPLE algorithm was used. SIMPLE v.3.0 is an algorithm for the identification of simple sequences in proteins, RNA or DNA. It provides a general relative simplicity factor for the sequence, representing the amount of repeats of short motifs (1-4) in respect to random sequences of the same composition. The simplicity is not restricted to tandem repeats but cryptic repeats are also taken into account. The program also provides a list of the short motifs which reiterate significantly and their position within the sequence. The parameters used for running the N-SIMPLE (SIMPLE V 3.0 for the analysis of DNA and RNA sequences) were set at the default apart from the window length that was increased to 100, to facilitate detection over longer stretches of simplicity. The program assigns the sequence an overall simplicity factor based on the cumulated simplicity scores of the different motifs (over the entire sequence) detected. This score is then divided by a simplicity score of 10 randomizations of the same sequence, to test whether the simplicity factor (SF) of the sequence is above the expectation under random distribution of the elements and whether specific short motifs show significant clustering. This is the final simplicity score (SS) assigned to a sequence and it is only considered significant to a level of 95% or 99%, where a sequence with significant simplicity to a significance level of 0.99 is considered to be simple to a level of 99%. Most sequences tested, showed simplicity to a level of 99%.

Visual examination of the sequences was also carried out to pick out any motifs/stretches of simple repeats that may have escaped the RepeatMasker or SIMPLE programs due to their detection parameters, and their stringency in identifying stretches ≥ a minimal repeating unit. In addition there were motifs e.g. (GAAA)n that were found dispersed regularly within some sequences, that were not detected by either program, and where lowering the stringency levels of the programs
to facilitate detection also increased the background noise. Thus visual examination was relied upon to detect certain repeating motifs.

Unigene and OMIM databases were referred to for additional information on each sequence whenever possible. UniGene is a system for automated sequence clustering and partitioning GenBank sequences into a non-redundant set of gene-oriented clusters. In addition to sequences of well-characterized genes, novel expressed sequence tag (EST) sequences are also included and each UniGene cluster contains sequences that represent a unique gene, as well as related information such as the tissue types in which the gene has been expressed and map location. Sequences from *Homo sapiens*, *Rattus*, *Mus musculus*, *Bos Taurus*, *Danio rerio*, *Xenopus* sp., and *Drosophila* are available (http://www.ncbi.nlm.nih.gov/UniGene).

The OMIM or Online Mendelian Inheritance in Man database is a catalog of human genes and genetic disorders with textual information, references and links to MEDLINE and sequence records in the Entrez system, and links to additional related resources at NCBI and elsewhere. OMIM focuses primarily on inherited, or heritable, genetic diseases and provides comprehensive information of cytogenetic locations of genes that are linked to genetic disorders (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=OMIM).

V.II.11. Southern analysis of HUM1-ORF

V.II.11.A Isolation of genomic DNA from human lymphocytes:

Genomic DNA was isolated from peripheral blood drawn from healthy adult human subjects as per the method of Kunkel et. al., (1985). Six to seven ml of blood was resuspended in 50 ml of lysis buffer (5 mM MgCl2, 10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 320 mM sucrose) and mixed gently on ice for 15 minutes. The mixture was centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was discarded. The transparent white pellet obtained was resuspended in 4.5 ml of nuclear buffer (100 mM NaCl, 24 mM EDTA). The nuclei were lysed by adding 250 μl of 10% SDS and 5 μl of Proteinase K (20 mg/ml) and the tubes were incubated at 37°C overnight. The solution was cooled to room temperature, equal volume of TNE-saturated phenol was added and the two phases were gently mixed by slowly turning the tube end to end for 15 minutes until the two phases formed an emulsion. The tubes were centrifuged at 4000 rpm for 15 minutes at 4°C. The aqueous phase was
transferred to a clean 35 ml tube using a thick bore Pasteur pipette. The extraction was repeated once with phenol and twice with chloroform : isoamyl alcohol (24: 1) mixture. To the aqueous phase 0.1 volume 3M sodium acetate (pH 5.2) and 0.7 volume isopropanol were added. The DNA was allowed to precipitate at room temperature and transferred to an Eppendorf tube using a wide mouth tip. The DNA pellet was washed thrice with 80% ethanol at room temperature for 10 minutes each. The DNA was allowed to briefly dry at 37° C and the pellet was resuspended in 250 μl of TE buffer (pH 8.0). It was allowed to dissolve at room temperature and stored at 4° C.

V.II.11.B Restriction digestion of genomic DNA :

50 μg genomic DNA was digested in appropriate buffers with 245 U Eco RI, 250 U Hind III, and 500 U Bam HI in 700 μl reaction volume separately, in an incubator for 16 hr at 37° C. Digestion was checked by electrophoresis in a 1% agarose-TAE gel. Digestion was further continued if it was not completed, either by increasing the reaction volume and adding fresh enzyme or by ethanol precipitation of the DNA and further redissolving in appropriate buffer and adding fresh enzyme. After ethanol precipitation, the pellet was washed by 80% cold ethanol, dissolved in 10 mM Tris-Cl pH 7.5 + 10 mM NaCl and rechecked for complete digestion. Digests were stored at -20° C for further use.

V.II.11.C Gel electrophoresis and Southern hybridization :

Agarose gel electrophoresis of DNA and Southern blot hybridization were carried out as per the modified method of Sambrook et. al., (1989) and Church and Gilbert. 20 μg of human genomic DNA digested by Bam HI, Eco RI, Hind III were electrophoresed in 1% agarose gels in TAE buffer (13 X 6.5 cm²) containing 0.5 μg /ml ethidium bromide at 50 Volts for 4 hr. Bioline Hyperladder (HYPL1200) was used as the marker for the gel electrophoresis. The gel was observed under transilluminated UV light (300 nm) in a IBI-Kodak transilluminator and photographed by a Polaroid camera. The gel was depurinated in 0.25 N HCl by slow shaking at room temperature for 15 min., washed with autoclaved distilled water to remove excess acid. The gel was denatured by keeping in denaturation solution (1.5 M NaCl + 0.5 M NaOH ) twice for 30 min. with gentle shaking at room temperature. Then the gel was neutralized by keeping in neutralization solution (1.5 M NaCl and 1 M Tris –
HCl, pH 7.5) twice for 30 min with gentle shaking. DNA was transferred on to nylon membrane (Biodyne nylon membrane A, GIBCO-Life technology 14866-016) by capillary blotting in 10 x SSC for 16-20 hours on bench. The moist blot was UV crosslinked for 30 sec at 1200 μJoules in a UV cross linker (Amersham). The blot was washed in 6 x SSC, dried at RT and sealed in polypropylene bag and stored at 4 ° C until used.

For Southern hybridisation, DNA blot was put inside hybridization bottle and first rinsed with 5 x SSC at room temperature, 10 ml of prehybridization solution (0.3 M phosphate buffer, pH 7.2, 7% SDS (w/v), and 1mM EDTA) was added into the hybridization bottle and prehybridization was carried out at 65 °C for 30 min to 1 hour with constant rolling of the bottle in a hybridization incubator (Robbins Scientific, USA). \(^{32}\)P labelled HUM1-ORF DNA probe prepared as described earlier, (>10⁵ cpm/ml) and sonicated Salmon sperm carrier DNA (100 μg/ml) was mixed well, denatured by heating in a boiling water bath for 5 min, immediately chilled on ice for 5 min and then added to the hybridization bottle (containing the prehybridization solution). Hybridization was carried out at 65° C for 16-20 hours with constant rolling of the bottle in the hybridization incubator.

Then the hybridised membrane was taken out, first washed in 2 x SSC and 1% SDS for 20 min at room temp, followed by washing at 65°C by following the sequence of low to high stringency as follows: Wash solution I : 1x SSC, 0.5% SDS; Wash solution II : 0.6 x SSC, 0.1% SDS, Wash solution III : 0.2 x SSC + 0.1% SDS, for 20 min each. The radioactivity on the blot was monitored by a hand held radioactive monitor (Rad-Monitor\textsuperscript{TM}, USA) to check the signal : background ratio as well as the positive and negative controls. If required, washing was continued further. The blot was checked for reference and was wrapped in saran wrap and exposed to X-ray film (Amersham Hyper film) with intensifying screens in the dark room. The cassette was kept at -70 °C for 6 days. The X-ray film was developed manually in developer (Sigma GBX developer) for 1-2 min (in dark room), rinsed sufficiently in water for 5 min and fixed in fixer solution (Kodak, cat. No. 900 0720) for 3-4 min., then the film was again washed in running tap water for 15-30 min. to remove fixer and air dried, visualized and stored at room temperature.
V.II.12 RNA expression for HUM1 cDNA

V.II.12.A. Human multiple tissue northern:

The human multiple tissue northern blot (Cat. no 7759-1, Clontech Laboratories Inc., USA) was hybridized to the $^{32}$P labelled HUM1 ORF DNA probe as per manufacturer's instructions. Random primed labelling was carried out on the PCR amplified and gel purified HUM1 ORF fragment. The MTN was sealed in a plastic bag with 5 ml of ExpressHyb solution and suspended in a waterbath set at 68 °C for 60 min for prehybridization. The probe (2.25 x 10$^6$ cpm/ml) was denatured at 95 °C for 5 min., and chilled on ice immediately and then added to prewarmed (68 °C) ExpressHyb that was used to replace the spent prehybridization solution in the bag. Hybridization was allowed to proceed at 68 °C for 1 hour. The blot was then rinsed successively in Wash solution 1 : 2X SSC, 0.05% at room temperature and Wash solution 2 : 0.1% SSC, 0.1% SDS at 50°C -65°C till there was no background detected by the hand held radioactive monitor (Rad-Monitor™, USA). The blot was wrapped and sealed in saranwrap, immediately to avoid drying and exposed to Xray film (HyperFilm, Amersham) with intensifying screens and stored at-70 °C. Short exposure of 4 hours and long exposure of 2 days were carried out.

V.II.12.B. RT-PCR analysis of HUM1 RNA from transfected HeLa cells and mouse testis:

Synthesis of the first strand cDNA was carried out on 1 μg of total RNA isolated as described above, from the transfected HeLa cells or the mouse testis. A ratio of 100 ng of primer A : RNA per reaction was maintained to prime the first strand synthesis with the help of MMLV reverse transcriptase as per manufacturer's instructions (M1701, Promega, USA). The reaction was set up as follows: 1μg of RNA and 100 ng of the Primer A (100 ng/μl working stock) were mixed with DEPC treated water to a final of 15 μl and heated to 70°C for 5 min to open out the RNA secondary structure. The tube was transferred immediately to ice, left for 5 minutes, and then to the tube added 1.25 μl of a 10 mM dNTP mix, 5 μl of the 5X MMLV reaction buffer (250mMTris-HCL, pH 8.3; 375mM KCL; 15mM MgCl$_2$; 50mM DTT)
and 0.5 μl of the MMLV enzyme (200 U/μl) in a final reaction volume of 25 μl made up by nuclease free water. The reaction was allowed to proceed at 37°C for 60 min. Half the reaction i.e. 12.5 μl was then used as a template for second strand synthesis, with the following reaction conditions. The PCR reaction buffer 10X was kept at a final of 1X, 0.2mM dNTPs, MgCl₂ to a final of 2.0 mM, primer concentrations (primers A and B) were 70 pmole per reaction, Taq polymerase was used at 2 units/reaction, in a final reaction volume of 50 μl. The cycle parameters for PCR amplification were denaturation at 95°C for two min, 35 amplification cycles of 94 °C for 40 sec, 42°C for 1 min, 72°C for 2 min. and a final extension of 10 min at 72°C. 1/10th of this reaction 5 μl was used as a template for the amplification of either HUM1 or GAPDH as a positive control for the RT-PCR. The final reaction conditions were: 1X PCR buffer, 0.2 mM dNTPs, MgCl₂ to a final of 2.0 mM, primer concentrations of 25 pmole/ 50 μl reactions, Taq polymerase 2 Units, in a total volume of 50 μl. The PCR parameters for amplification used were: denaturation at 94°C for 4 min, 35 amplification cycles of 94 °C for 45 sec, 68°C (HUM1 primers) for 1 min or 60 °C (G3PDH primers) for 45 sec., 72°C for 1 min. and a final extension of 10 min at 72°C. The Primers used for these reactions were specific: HUM1 fwd and reverse (Biobasic) and G3PDH 5' and 3' primers (Clontech).

V.II.13 RNA expression for the cDNAs in tumours by reverse northern blot:

Total RNA was isolated from three human nervous system tumours. The first strand cDNA was synthesized from 1μg RNA by a 15 mer oligo dT primer with a T7 primer attached to its 5' end (Primer A). The second strand cDNA was synthesized by a 6 mer random primer with a T3 primer attached to its 5' end (Primer B). The ds cDNA was then amplified by PCR using the T7 and T3 primers. The PCR product showed >7 kb to 600 bp size distribution in an 1% agarose gel. From this amplified cDNA population, 5 μl was used to prepare 32P cDNA by PCR using the T7 and T3 primers. The 32P labelled cDNA (specific activity 10⁶ - 10⁷ cpm/μg) was used as the probe to hybridize various cDNA inserts (1ng) spotted onto the nylon membrane as a dot blot. 1μg of pBluescript II KS+ plasmid was used as a negative control, and 1μl of total amplified RNA from the PCR amplification step was the positive control. Hybridization and washing conditions were as described earlier for southern blots.
Autoradiographic exposure of this reverse northern blot showed differential expression of the RNA from the tumours corresponding to the cDNAs.

V.II.14 Maintainence of HeLa and Transfection protocols

V.II.14.A. Tissue culture methods:

The human cervical carcinoma HeLa S3 cells (ATCC CCL-2.2) were maintained in complete DMEM with 10% FCS (with 100 µg/ml streptomycin and 100 U/ml penicillin), grown to 80% confluency in T25 flasks typically for 3 days and then split in a 1:3 ratio for seeding in a fresh T25 flask. Medium was discarded, 1ml of 0.03 % Trypsin in PBS-EDTA was added, and the flask was incubated at 37°C for 3 min. till the cells detached, two ml of complete DMEM was added to stop the action of trypsin, and the cell suspension was collected, centrifuged at 1.5 K rpm, 3 min in a Heraeus clinical centrifuge to collect the cells. The supernatant was discarded, the cells resuspended in 3 ml fresh DMEM and 1 ml each was seeded into a fresh T25 flask containing 4 ml complete DMEM. The cells were then allowed to grow at 37°C, 5.0 % CO2. Cell were observed under a phase contrast inverted microscope.

V.II.14.B. Freezing/ Thawing of the cells:

A healthy growing culture of cells was harvested after trypsinization and the cell pellet was resuspended at a concentration of 2-5x 10^6 cells/ml freezing medium (10% DMSO and 90% FCS). The cryovials were put on ice, wrapped with tissue, transferred to -80°C overnight and then stored for long term in liquid nitrogen. Revival of the frozen cells involved fast thawing (37°C) of the frozen cells and resuspension in 4 ml of complete DMEM. The cells were then harvested by centrifugation, the medium containing the DMSO was discarded, and the cells were resuspended gently in fresh complete DMEM and seeded into a fresh T25 flask. The cells were allowed to grow upto 80% confluency and maintained as described above.

V.II.14.C. Cell counting:

Cells were counted on the Hemocytometer before seeding for transfection to ensure that the constant number of cells was seeded per well of a six well plate. 20 µl of the cell suspension was taken along with 30 µl of 1X PBS and 50µl of 0.4%
Trypan blue stain. A drop of this cell suspension was allowed to spread by capillary action between the cover slip and the counting chamber. The number of cells was counted under the microscope from all 9 subchambers and the average was taken. The number of cells per ml of the original suspension was then calculated as:

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

V.II.14.D. **Transfection of HeLa cells with pCDNA-HUM1:**

Transfection of HeLa cells was carried out by a modified method of the original CaPO₄ method (Wigler et al., 1979). Healthy HeLa cells were seeded at a density of 0.2 x 10⁶ cells/well of a six well plate in a volume of 2 ml complete DMEM. [DMEM medium was chosen as the medium of growth and transfection as the procedure for CaPO₄ transfection requires a growth medium with lower concentrations of calcium.] Cells were allowed to grow for a period of 18 hrs, when the medium was changed to fresh complete DMEM. The cells were allowed to stabilize at 37°C, 5.0% CO₂ for two hours. Meanwhile the CaPO₄ precipitate of the DNA was prepared under sterile conditions in the hood to be added to each well as follows:

A 90μl mixture of the pCDNA vector and pCDNA:HUM1 plasmids were prepared in the concentration ranges given in Fig. 36 A (i & ii). To this plasmid DNA mixture, 10 μl of 2.5 M CaCl₂ was added with a fine tip and with constant mixing to ensure an even suspension of the DNA with CaCl₂. It is important to ensure that the mixing is even and slow. To this tube, 100 μl of 2X HeBS pH 7.05 buffer was added drop wise while holding the tube on the vortex mixer to ensure that the mixing was even and that aeration was provided for an even, small precipitate formation. All steps were carried out under sterile conditions in the hood, and the tubes were left for 30 min to allow the cloudy white precipitate to form. At the end of the half hour that coincided with the end of the two hour incubation of the cells at 37°C, the precipitate was added drop wise to each respective well, the plate was swirled and the precipitate was allowed to settle on the cells for 2 min. The plates were then transferred to the 37°C, 5.0% CO₂ incubator and allowed to take up the precipitate for 16 hours at the end of which the medium was discarded, a wash was given with 1XPBS to remove all last traces of the precipitate and fresh medium (2 ml complete DMEM) was added per well.
In case of the transient transfections, cells were allowed to grow for 24 hours at 37°C, 5.0% CO₂, the medium was discarded, three washes of 1X PBS (5ml, 5 min) were given and the cells were harvested for RNA preparation by scraping. 0.5 ml of RNA lysis buffer was added per cell pellet (typically 1 x 10⁶ cells) and the RNA isolation procedure was then carried out as described earlier.

In case of the stable transfections for the growth suppression assay, the cells were maintained in complete medium containing 0.6 mg/ml G418. Typically, fresh medium with G418 was changed every three days for a period of 21-24 days till the G418 resistant colonies were visible to the naked eye. The colonies were then fixed, stained with geimsa dye (Freshney, 1987) and counted. The staining procedure was as follows: After discarding the medium, each well was given a 2ml 1X PBS wash, next three changes of 1 ml Methanol : PBS (1:1) mixture for 10 min each were given to the cells and the plate was left at room temperate. The fixation was completed with addition of 1 ml of absolute methanol for 1 min. 2 ml /well of geimsa stain was added and washed off with three changes of sterile water. The remaining dye was allowed to wash off by gently immersing the plate in a tray of running water till the background was clear. The plate was inverted and allowed to dry completely. The colonies appeared as large visible purple dots and some faintly stained smudges were also visible that were later confirmed to be loosely formed colonies under the microscope. Colonies were observed under a Zeiss inverted phase contrast microscope and photographs of both types of colonies were taken on a Zeiss stereo microscope at 40X magnification on Konica 100 colour film.