2 materials and methods

2.1 Materials

2.1.1 Source of chemicals used

All media for tissue culture (DMEM), fetal calf serum and sodium bicarbonate were obtained from Sigma chemical company. Agarose, β-mercaptoethanol, BSA (fraction V), calcium chloride, DTT, ethidium bromide, lysozyme, magnesium chloride, SDS, TEMED, IPTG (isopropyl thio-β-D-galactoside), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), Tween20, leupeptin, antipain, aprotinin and tetracycline were from Sigma. Acrylamide and bis-acrylamide were from Serva chemical company. Sephadex G-50, PEG 8000, low melting point agarose, RNaseA, low molecular weight protein markers for SDS-PAGE and formamide were obtained from Pharmacia. BCIP (4-chloro-3-indolylphosphate), NBT (Nitro Blue Tetrazolium), BromodeoxyUridine (BrdU) labeling reagent and enhanced chemiluminescence detection reagents were from Roche. Nitrocellulose membrane, Hybond N and N+ nylon membranes were purchased from Amersham. Molecular biological enzymes were purchased from New England BioLabs, Pharmacia and Bangalore Genei. Whatman filter papers were purchased from Whatman International Ltd. X-ray films were obtained from Konica Corporation or Kodak; intensifying screens were from Fuji. All radiolabeled nucleotides were from BRIT, Bombay. Tryptone, agar, and yeast-extract were purchased from Difco Laboratory. PCR and RT-PCR kits were purchased from Perkin Elmer-Cetus Corporation. The sequencing kits were obtained from Promega. Trizol reagent for single step isolation of total RNA, Geneticin, Hygromycin, and Lipofectamine plus reagent were from GIBCO BRL. All other chemicals were purchased from local manufacturers and were of analytical grade.

Antibodies

FITC-conjugated and alkaline phosphatase conjugated anti-mouse secondary antibodies were from Bangalore Genei. Anti-BrdU antibody was from Roche. Rabbit polyclonal anti-CDK2, mouse monoclonal anti-PCNA and anti-phosphotyrosine antibody, Py20 were purchased from
Santa Cruz Biotechnology, Inc. Anti-c-Myc antibody (9E10) was from Developmental studies Hybridoma bank, Department of Biological Sciences, University of Iowa. Anti-CyclinE antibody was purchased from Oncogene Research Products.

### 2.1.2 Bacterial strains

**E. coli DH5α:** *F. recA1 endA1 hsdR17 (rK-mK) (lacZYA-argF) U169 (φ 80 lacZΔM15) supE44 thi-1 gyrA96 relA*

This strain was used for all routine transformations, plasmid isolations, selection of recombinant plasmids, etc.

**E. coli BL21(DE3) pLys S:** *F ompT hsdSB (rB.mB.) gal dcm (DE3) pLysS (CmR)*

This strain was used for expression of recombinant proteins cloned in the vector pET3a.

The bacterial strains were stored on LB agar plates (containing the appropriate antibiotic) at 4°C or in LB having 15% glycerol at −70°C.

### 2.1.3 Cell lines

a. **COS1:** A monkey kidney cell line, transformed with SV40 having a defective origin of replication.

b. **NIH3T3:** A mouse embryo fibroblast cell line.

c. **C3H, 10T1/2:** A mouse embryonic cell line.

d. **HeLa-tTA Tet-Off inducible cell line:** A human epithelial-like cell line, derived from cervical carcinoma. It has been stably transfected with the Tet-Off regulator plasmid containing a G418 (Geneticin) resistance cassette. Pre-made HeLa-Tet-Off inducible cell line was a kind gift from Dr. Helen M. Blau, Stanford University, U S A.

e. **HeLa-PTP-S2:** It is the HeLa-tTA Tet-Off inducible clone, stably transfected with the pTRE-PTP-S2 response plasmid encoding the protein tyrosine phosphatase gene PTP-S2. The response plasmid was co-transfected with pTK-hyg that confers resistance to hygromycin. Two of the clones C14 and C5, which were used in this study, gave inducible expression of PTP-S2, on removal of tetracycline. These clones were prepared by Sanjeev Gupta in our laboratory and kindly provided by him for studies reported in this thesis.

### 2.1.4 Plasmids

**pUC18/pUC19:** These two vectors are identical except that they contain multiple cloning sites arranged in opposite orientations. They are high copy number plasmids derived from pBR322
with ampicillin resistance marker. They display α-complementation in appropriate host and hence the recombinants can be identified by blue/white color selection (Messing, 1983; Yanisch-Perron et al., 1985).

**Pbluescript II KS+**: It is a 2958 base pair long high copy number ColE1 replication origin based phagemid, derived from pUC19 and carries ampicillin resistance marker. The "KS" designation indicates the multiple cloning site is oriented such that the lacZ′ transcription proceeds from KpnI to SacI. The lacZ′ expresses the N-terminal part of β-galactosidase. An inducible lac promoter upstream from the lacZ′ gene permits the expression of N-terminal peptide which complements the rest of the protein produced from the chromosome to produce functional β-galactosidase, enabling blue/white color selection in the presence of chromogenic substrate like X-gal. (Short et al., 1988; Alting-Mees, M.A., and Short, J.M., 1989).

**pET3a**: A prokaryotic expression vector with ampicillin resistance marker that allows cloning of target DNAs at sites where they will be minimally transcribed by *E. coli* RNA polymerase but selectively and actively transcribed by T7 RNA polymerase. Translation is controlled by the strong ϕ10 promoter for T7 RNA polymerase, gene10 translation start site (s10) and transcription termination (T). These vectors are capable of directing the production of large amounts of target RNAs and protein in *E. coli* BL-21pLys S, which carries the gene for T7 RNA polymerase in the chromosome under the control of lacUV5 promoter (Rosenberg et al., 1987).

**pCB6**: It is a mammalian expression vector. Target DNAs can be cloned in this vector under the cytomegalovirus (CMV) promoter, which is followed by a segment of DNA containing transcription terminator and polyadenylation signals from the human growth hormone (hGH) gene. This vector also contains the SV40 origin of replication and neomycin resistance gene, followed by the SV40 poly (A) addition sequence (Brewer, 1994).

**Tet-Off regulator/response plasmids, pTKHyg**: The Tet-Off and Tet-On gene expression systems are based on two regulatory elements derived from the *E. coli* tetracycline-resistance operon: the Tet repressor protein (TetR) and the Tet operator DNA sequence (tetO). The regulator and response plasmids deliver these elements into mammalian cells where they are integrated into the host genome to establish a double-stable cell line. Once established, this cell line responds to tetracycline or doxycycline in a dose dependent manner, allowing precise control of expression of the target gene. The Tet-Off system uses the pTet-off regulator plasmid. This plasmid
expresses a fusion protein known as the tetracycline-controlled transactivator (tTA), which is composed of TetR and the VP16 activation domain (AD). tTA activates transcription in the absence of Tet/Dox. pTRE2 is the response plasmid that encodes the tetracycline-response element (TRE), which contains seven repeats of the tetO sequence, and the target gene. tTA binds to the TRE, activating transcription of the target gene. The Tet regulator plasmid has a neomycin resistance cassette and allows selection of stable clones. pTK-Hyg is a selection vector using hygromycin. When co-transfected with pTRE2, it is useful for selecting double-stable Tet-Off or Tet-On cell lines.

**2.1.5 Bacterial media, antibiotics and chemicals**

1. **Ampicillin**: 100μg/ml final concentration, Stock solution (1000X) was made in sterile double distilled water.
2. **LB**: 1% bacto-tryptone, 1% sodium chloride, 0.5% yeast-extract, pH adjusted to 7.0 with 0.1N sodium hydroxide.
3. **LB agar**: LB containing 1.5% agar.
4. **IPTG**: 40μl/plate, 0.5M stock of isopropyl thio β-D-galactoside in sterile double distilled water.
5. **X-Gal**: 100μl/plate, 5mg/ml of 5- bromo- 4- chloro- 3- indolyi- β D- galactoside in dimethyl formamide.
6. **TE**: 10mM Tris-HCl (pH 8.0), 1mM EDTA.
7. **1XTBE**: 89mM Tris base, 89mM boric acid and 1mM EDTA.
8. **1X SSPE**: 0.15 M NaCl, 10mM sodium phosphate (pH 7.4) and 1mM EDTA.
9. **1X 2-(N-morpholino) propanesulphonic acid (MOPS)**: 20mM 2-(N-morpholino) propanesulphonic acid (pH 7.0), 8mM sodium acetate and 1mM EDTA.
10. **1X PBS**: 137mM sodium chloride, 2.7mM potassium chloride, 4.3mM di-sodium hydrogen phosphate and 1.4mM potassium di-hydrogen phosphate. 10X stocks were made by dissolving required amounts in milli-Q water and the pH was adjusted to 7.4 with HCl.
11. **Water-saturated mineral oil for PCR**: Equal volumes of mineral oil and autoclaved double distilled water are vigorously mixed and allowed to settle and sterilized by autoclaving.
12. **Buffer-saturated phenol**: Phenol needs to be equilibrated to >pH 7.8 for DNA purification since DNA partitions into the organic phase at acid pH. Melted phenol was mixed with equal volumes of 0.5M Tris-HCl pH 8.0 and 0.1% hydroxyquinoline, stirred and allowed to phase separate at room temperature. Following this, the upper aqueous phase was removed and equal volumes of 0.1M Tris-HCL pH 8.0 was mixed with the phenolic layer and again left for
phase separation. This step was repeated until pH of the aqueous solution reached 8.0. Once the phenol was equilibrated and the final aqueous phase had been removed, it was stored in a light-tight bottle at 4°C after mixing with 0.1 volume of 0.1M Tris-HCl pH 8.0.


14. De-ionized Formamide: Formamide was mixed with mixed bed resin, stirred for an hour, filtered, aliquoted and stored at −20°C.

15. DEPC water: Water with 1% DEPC left overnight for equilibration and autoclaved.

16. Hybridization buffer: 1:1 v/v of 1N sodium di-hydrogen phosphate pH 7.2 (adjusted with 85% ortho phosphoric acid) and 14% SDS with 1mM EDTA.

17. SDS-PAGE Sample buffer (1X): 50mM Tris-HCl pH 7.5, 3% SDS, 10% glycerol, 4mM EDTA, 10% β-mercaptoethanol and 0.01% bromophenol blue. Usually 3X or 2X stocks were made and stored frozen at −20°C.

2.1.6 Tissue culture media, antibiotics and chemicals

1. Dulbecco’s modified eagles’ medium (DMEM): 13.4gm of DMEM (Sigma) and 3.7gm of sodium bicarbonate were dissolved in one liter of milliQ water. The pH was adjusted to 7.3, then filter sterilized and stored at 4°C.

2. Penicillin, Streptomycin, Kanamycin: Penicillin, 60µg/ml final concentration. Streptomycin and kanamycin, each 50µg/ml final concentration. A stock solution (100X) was made in sterile 1X PBS (Phosphate buffered saline), filtered and stored at −20°C.

3. Tetracycline: 2µg/ml final concentration. Stock solution (1000X) was made in absolute ethanol and stored in aliquots at −20°C.

4. Trypsin-EDTA (1X): 0.125% Trypsin and 0.125% EDTA in milliQ water, filter-sterilized and stored at −20°C.

5. Complete DMEM: DMEM containing 10% fetal calf serum and 1X antibiotics. The solution was filter-sterilized and stored at 4°C.

6. Complete selection medium: DMEM containing 10% fetal calf serum, 200µg/ml of G418 (geneticin) and 100µg/ml of Hygromycin. The medium was filter-sterilized and stored at 4°C.
2.2 Methods

2.2.1 Sterilization

All glassware and plasticware were sterilized by autoclaving at 15lb pressure for 20 minutes or by baking at 180°C for 12 hours, before using for the experiments. All solutions were prepared in double-distilled water or milliQ water. Sterilization of the solutions was generally done by autoclaving. Bacterial growth media were autoclaved at 15lb pressure for 20 minutes.

2.2.2 Isolation of plasmid DNA

Small scale isolation

Plasmid DNA, on a small scale, was prepared by the boiling-method as described by Sambrook et al., (1982), with some modifications. 5ml of LB medium containing 100μg/ml ampicillin was inoculated with a single colony of E. coli DH5α containing plasmid. Bacterial cell pellet from 3ml overnight culture was suspended in 0.5ml of solution A (8% sucrose, 0.5% Triton X-100, 50mM EDTA pH 8.0, 10mM Tris pH 8.0) by vortexing. 25μl of freshly prepared lysozyme solution (10mg/ml in TE₅) was added and vortexed vigorously. The tube was kept in a boiling water bath for 1 minute and then centrifuged for 20 minutes at room temperature in a microfuge. 15μl of 3M sodium acetate, pH 5.2 and 0.6 volume of isopropanol (300μl) were added to the supernatant, and kept at room temperature for 15 minutes. The mixture was centrifuged at room temperature for 15 minutes. The pellet was dried under vacuum and dissolved in 200μl TE₅ containing 20μg/ml Dnase-free RNase. The plasmid solution was then extracted with phenol-chloroform, precipitated and dissolved in TE₅ and checked on a 1% agarose gel as described below.

Large scale isolation

For transfection quality DNA, the Qiagen Midi protocol was used for isolating plasmid. A single colony from a freshly streaked selective plate was used to inoculate a starter culture of 2-5ml LB medium containing the appropriate selective antibiotic. This was incubated for ~8 hours at 37°C with vigorous shaking (~300 rpm). The starter culture was diluted to 1/1000 into 25ml selective LB medium and grown at 37°C for 12-16 hours with vigorous shaking. The culture was allowed to reach a cell density of approximately 3-4×10⁹ cells/ml, which typically corresponds to a pellet net weight of approximately 3g/liter medium. The bacterial cells were harvested by centrifuging at 6000xg for 15 minutes at 4°C in a Sorvall GSA rotor. All traces of supernatant were removed by inverting the open centrifuge tube until all medium had been drained. The
pellet was resuspended in 4ml of buffer P1. Care was taken to ensure that no cell clumps remained. 4ml of buffer P2 was added to this and mixed gently by thoroughly inverting 4-6 times and incubating at room temperature for 5 minutes. For precipitation, 4ml of chilled buffer P3 was added, mixed by inversion and incubated on ice for 15 minutes. The lysate was mixed thoroughly to avoid localized potassium dodecyl sulfate precipitation. The lysate was centrifuged at 20,000xg for 30 minutes at 4°C. The supernatant containing plasmid DNA was removed and loaded onto a pre-equilibrated Qiagen-tip 100 (using 4ml buffer QBT and allowing the column to empty by gravity flow). The Qiagen-tip was washed twice with 10ml of buffer QC and the DNA was eluted with 5ml of buffer QF. Adding 3.5ml of isopropanol at room temperature to the eluate and centrifuging at 15,000xg for 30 minutes precipitated DNA. The DNA pellet was washed with 2ml of room temperature 70% ethanol and centrifuged at 15,000xg for 10 minutes. The pellet was air-dried for 5-10 minutes, re-dissolved in a suitable volume of TEa. The plasmid was checked on a 1% agarose gel and quantitated by a UV spectrophotometer.

2.2.3 RNA isolation

Usual precautions to minimize RNase contamination were taken. Glassware was washed and treated with 0.1% diethyl polycarbonate (DEPC) overnight at room temperature and baked at 180°C for 24 hours.

Preparation of total RNA

TRIZOL reagent (Gibco BRL) was used to prepare total RNA from tissue culture cell lines. Trizol reagent (1ml) was added to a 75cm² flask of cells (approximately 80% confluent) and kept for 2-3 minutes. The resulting slurry was passed through a pipette 4-5 times, and then transferred to 1.5ml eppendorf tubes and vortexed briefly. It was allowed to stand at room temperature for 2 minutes, following which 200µl of chloroform was added and vortexed. The suspension was spun at not more than 12,000g for 15 minutes between 2-8°C for phase separation. The aqueous phase was collected and divided into two tubes. 500µl of isopropanol was added per 1ml of Trizol reagent, mixed well and left at room temperature for 10 minutes. Centrifuging at not more than 12,000g for 10 minutes between 2-8°C pelleted the RNA. The total RNA pellet obtained after the final centrifugation was washed with 1ml of 70% ethanol (in DEPC water), spun at 7,500g for 5 minutes between 2-8°C. The RNA pellet was stored in absolute ethanol at -70°C. Prior to checking the RNA preparation, absolute ethanol was removed, the pellet was dried briefly under vacuum, dissolved in approximately 30-50µl of DEPC water by
heating at 56°C for 15 minutes and checked on a 1% agarose gel containing 0.5μg/ml ethidium bromide.

**2.2.4 Estimation of nucleic acid concentration**

Nucleic acid concentration in the samples was estimated by measuring O.D. at 260 nm (Sambrook et al., 1982). An empirical relationship used for this purpose was that, an O.D. of 1 was equivalent to 50μg/ml of double stranded DNA or 40μg/ml of RNA or 33μg/ml of single stranded oligonucleotides.

**2.2.5 Phenol:Chloroform extraction**

Deproteinization of DNA by phenol:chloroform extraction was generally done as follows unless otherwise mentioned. DNA samples were mixed with an equal volume of 1:1 mixture of phenol (saturated with 0.1 M Tris-HCl, pH 8.0) and chloroform:isoamyl alcohol (24:1, v/v) by vortexing, followed by centrifugation at 12,000rpm for 5 minutes. The aqueous layer was separated and the above step was repeated.

**2.2.6 Precipitation and desalting of nucleic acids**

Nucleic acids (DNA and RNA) were generally precipitated from aqueous solution by the following procedure unless otherwise mentioned. To the aqueous solution containing DNA or RNA, 1/10 volume of 3M sodium acetate, pH 5.2 and 2.5 volumes of 95% ethanol were added, mixed well and precipitated at −20°C for 1 hour to overnight (when overall concentration of the nucleic acid was very low). The nucleic acid was then recovered by centrifugation at 12,000rpm for 10-15 minutes at 4°C. Salt from the pelleted nucleic acids was removed by washing twice with ice-cold 70% ethanol, followed by centrifugation at 12,000rpm for 5 minutes at 4°C. The supernatant was removed, after which the pellet was vacuum-dried and dissolved in TEa or water.

**2.2.7 PEG precipitation of DNA**

Purified DNA was further precipitated by the following procedure for removing small molecules of nucleic acids. The purified DNA was dissolved in a volume of 20μl of TEa, to which 5μl of 5M NaCl and 25μl of 20% PEG 8000 were added. This mixture was placed on ice for 15-
20 minutes and then centrifuged at room temperature at 10,000rpm for 15 minutes. The pellet was washed twice with 70% ethanol, vacuum dried and dissolved in 20μl TE₈.

2.2.8 Agarose gel electrophoresis

The DNA/RNA samples were mixed with an appropriate volume of 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) and electrophoresed through 0.5%-2% agarose gels, in TBE depending on the size of the fragments to be separated. Electrophoresis was carried out at 5V/cm. The DNA/RNA was visualized by adding 0.5μg/ml ethidium bromide to the gel.

2.2.9 Restriction endonuclease digestion

The necessary amount of plasmid DNA (1-2 μg) was mixed with 2-4U enzyme/μg of DNA, in a final volume of 20μl using the buffers and incubation conditions recommended by the manufacturers. The reaction was terminated by freezing the tube to -20°C. The fragments were visualized by electrophoresis on 1%-2% agarose gels stained with ethidium bromide (0.5μg/ml).

2.2.10 Purification of insert DNA from low melting agarose gels by freeze squeeze method

DNA inserts used for cloning were prepared from low melting agarose gels using the following protocol. The cloned DNA in vectors was digested with appropriate restriction enzymes and the digested samples were electrophoresed in 1%-1.5% low melting agarose gel in 1X TBE. The gel was stained in ethidium bromide and viewed using a UV-transilluminator and the appropriate DNA band was cut out. The piece of gel containing the band of interest was put into a 0.5ml eppendorf tube containing autoclaved glass wool and a hole was pierced through the bottom. This whole set up was placed in a 1.5ml eppendorf tube and kept at -70°C for 15-20 minutes. The frozen piece of agarose containing the DNA fragment was spun at 4°C at 5,000rpm for 5 minutes and the supernatant was collected in the 1.5ml tube. In order to get a higher percentage of recovery, a small volume of TE₈ buffer was added to the agarose piece and the spin was repeated. All the solutions were collected and were subject to a butanol extraction to remove ethidium bromide. This was followed by a phenol:chloroform:isoamyl alcohol extraction as described earlier and finally ethanol precipitated. The resultant DNA pellet was washed twice in 70% ethanol, vacuum dried, dissolved in TE₈ and used for all subsequent reactions.
2.2.11 Ligation

Ligations were carried out at 15°C for 16-20 hours in 10µl reaction volume containing 1µl 10X ligation buffer (1X: 50mM tris HCl pH 7.6, 10mM MgCl₂, 10mM DTT and 1mM ATP), 50-100 ng of the desired vector, three molar excess of the DNA insert of choice and 1 unit of T4 DNA ligase.

2.2.12 Preparation of competent cells

Competent cells were prepared according to the method described by Hanahan (1985) with some modifications. A single colony of DH5α was inoculated from an overnight culture of the bacteria grown on LB agar plate into 5ml LB medium and incubated at 37°C for 12 hours. 1ml from this was inoculated into 35ml of LB medium in a conical flask, and incubated at 37°C till the cell density was such that its absorbance at 600nm was between 0.45 and 0.55. The cells were collected by centrifuging at 3,000rpm for 15 minutes at 4°C in a HB-4 rotor in Sorvall RC5B centrifuge. The supernatant was completely drained and the pellet was suspended in 10ml of RF1 buffer (100mM RbCl, 50mM MnCl₂·4H₂O, 10mM CaCl₂·2H₂O, 30mM Potassium acetate, 15% glycerol, pH 5.8). The cell suspension was incubated on ice for 15 minutes and the cells were centrifuged as above. The pellet was re-suspended in 2.4ml of RF2 (10mM MOPS, 10mM RbCl, 75mM CaCl₂·2H₂O, 15% glycerol, pH 6.8). The cell suspension was again incubated on ice for 15 minutes and distributed into pre-chilled microfuge tubes in 100µl aliquots. The aliquots were flash frozen in liquid nitrogen and stored at -70°C till further use.

2.2.13 Transformation of competent cells

The competent cells were removed from -70°C and allowed to thaw on ice. 5-10µl of ligated sample was added to 100µl of competent cells and mixed well. The transformation mixture was then incubated on ice for 30-45 minutes, followed by a heat shock at 42°C for 90 seconds. The tubes were chilled by returning them immediately to ice. 400µl of LB medium was added and the transformed cells were incubated at 37°C for 60 minutes before plating them.

The transformed bacteria were plated on 90mM LB agar plates containing 100µg/ml ampicillin, 1mM IPTG and 25µg/ml X-gal. The plates were incubated at 37°C for 12-16 hours to allow the bacteria to grow. The white colonies were either used for isolating plasmids or for screening to pick up recombinant clones.
2.2.14 Selection of recombinant clones

The recombinant white colonies were subjected to colony hybridization using the method of Buluwela et al., (1989) to check for the presence of insert DNA. The recombinant white colonies (as well as a few blue colonies that served as a negative control) were patched on LB agar plates containing ampicillin. The plates were incubated overnight at 37°C. The plates were layered with a Hybond N nylon membrane, and the filter was marked asymmetrically with a needle dipped in Indian ink. When the filter was thoroughly wet with the colonies sticking to it, it was peeled off from the plate, inverted and placed on a Whatman 1M filter paper soaked in 2X SSPE/5% SDS for 2 minutes in a glass tray. The tray with the filters was shifted to a microwave oven and heated at 650W for 2.5 minutes, thereby lysing the cells, denaturing and fixing the bacterial DNA to the membrane. Filters were then wetted in 5X SSPE/0.1% SDS for 5 minutes. They were kept for pre-hybridization in hybridization solution containing 7% SDS, w/v (1:1 v/v 1N Na₂HPO₄: 14% SDS with 1mM EDTA) for 1-1½ hours at 65°C (in polythene bags). The radioactive probe was allowed to thaw to room temperature, denatured by adding 1/10th the volume of 3N NaOH and heated at 37°C for 5 minutes. The denatured probe was immediately added to the prehybridized membrane containing the same buffer and hybridized overnight at 65°C for 14-16 hours. After hybridization, the membrane was washed with a large volume of 2X SSPE/0.1% SDS for half an hour at room temperature, followed by a second wash with 0.5X SSPE/0.1% SDS for half an hour at room temperature. The third and final wash was given with 0.5X SSPE/0.1% SDS for 45-60 minutes at 65°C. The radioactive counts were checked after each wash. The filter was dried by placing on a Whatman paper and kept inside a thin polythene sheet and exposed to an X-ray film either at room temperature or at -70°C.

2.2.15 Northern transfer and hybridization

RNA samples were electrophoresed through a 1% agarose/2.2M formaldehyde gel made in formaldehyde gel-running buffer (20mM MOPS pH 7.0, 5mM sodium acetate and 0.5mM EDTA). The samples were prepared by mixing together 10μg RNA (in 5μl), 1μl of 10X formaldehyde gel-running buffer, 3.5μl formaldehyde and 10μl of formamide in a sterile microfuge tube. The samples were heated at 56°C for 15 minutes and chilled on ice. 2μl of sterile formaldehyde gel-loading buffer (50% glycerol, 1mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol) was added. The gel run was performed in formaldehyde gel-running buffer. After electrophoresis the gel was washed in 3-4 changes of sterile water for 30 minutes each, to remove formaldehyde and then stained with ethidium bromide (0.5μg/ml) The gel was
photographed and then vacuum-transferred to Hybond N+ nylon membrane using 50mM NaOH for 2 hours. The filter was washed once in 2X SSPE and hybridized to a labeled probe, the specific activity of which was approximately 2.5x10^8 cpm/μg. Hybridizations and washings were carried out essentially as described for colony hybridization. Blots were autoradiographed by exposing to X-ray film along with intensifying screen at -70°C.

### 2.2.16 Densitometric scanning

Phosphor imager (FUJIFILM, BAS 1800, Japan) was used for scanning and quantitation of autoradiograms of northern blots and colony hybridization.

### 2.2.17 DNA Sequencing

DNA was sequenced using the dideoxy-termination method of Sanger et al., (1977), as modified by Chen and Seeburg (1985) for sequencing double stranded plasmid DNA. T7 DNA polymerase and Taq DNA polymerase based sequencing kits (Promega Corporation) were used depending on the availability and requirement.

#### Preparation of plasmids for sequencing

The plasmids used for sequencing, isolated as described earlier in section 2.2.2 (small-scale isolation) were further purified by PEG precipitation as described in section 2.2.7.

#### Denaturing the DNA for sequencing

In order to denature the DNA, 1-2μg of DNA in 20μl TE3 was mixed with 2μl of 2M NaOH and left for 5-10 minutes at room temperature. The reaction was neutralized with 3μl of 3M sodium acetate (pH 5.2). The DNA was precipitated using ethanol at -20°C for 20 minutes, pelleted by spinning in a refrigerated microfuge at 12,000rpm for 15 minutes at 4°C. The pellet was washed with 70% ethanol, dried and dissolved in appropriate volume of de-ionized water.

#### Sequencing reaction

**A. With Taq DNA polymerase**

For sequencing with Taq DNA polymerase Taq-Track sequencing kit from Promega was used. The denatured DNA was dissolved in 18μl of water to which 5μl of 5X Taq polymerase buffer and 2μl of labeled primer were added. The primer was annealed by incubation at 37°C for 30 minutes. To this mix 1.6μl (7.5 units) of Taq DNA polymerase (sequencing grade) was added
and 6μl aliquots of this reaction mixture was added to four different siliconized microfuge tubes containing 1μl each of A, C, G, or T mixes provided in the kit. The tubes were incubated at 72°C for 20 minutes. The reactions were finally stopped by the addition of 4μl of the stop solution provided in the kit.

The sequencing primers were labeled as follows. In a 10μl reaction, 5μl (10pmol) of 10ng/μl primer, 3μl (10pmol) of [γ-32P] dATP and 5 units of T4 polynucleotide kinase were mixed and incubated at 37°C for 10 minutes. The reaction was stopped by heating for 2 minutes at 90°C.

B. With T7 DNA polymerase

For sequencing with T7 DNA polymerase a kit from BRIT was used. The denatured and dried DNA was mixed with 0.5pmol (approximately 10ng of a 24mer primer), 2μl of 5X sequencing buffer and the volume was made up to 10μl with water. Annealing was done at 65°C for 5 minutes and cooled to room temperature slowly over 30 minutes. It was then centrifuged and cooled on an ice bath. To this annealed template-primer mixture, 1μl of 0.1 M DTT, 2μl of diluted (1:5) stock labeling mix, 1μl of [γ-32P] dATP (10μCi) and 2.5μl (4 units) of T7 DNA polymerase were added and incubated at room temperature for 5 minutes. 3.5μl of this reaction was added to each of the four different microfuge tubes containing 2.5μl of the respective termination mixture A, C, G and T. The tubes were incubated at 37°C for 5 minutes and the reactions were stopped by adding 4μl of stop solution to each of the termination reactions.

Electrophoresis of sequencing reactions

The reactions were heated to 70-75°C for 3-5 minutes and 1-3μl of each reaction was loaded on an 8% denaturing urea-polyacrylamide gel of 0.35mM thickness assembled into the apparatus of Atto Corporation, Japan. Electrophoresis was done in 1X TBE at a constant power (100 watts) at 50-60°C. The gel was pre-run for 30-60 minutes before the samples were loaded. In general, two loadings of the samples were done. After the run, the gel was fixed in a solution of 10% methanol and 10% acetic acid for about 45 minutes; dried under vacuum and autoradiographed. After developing the autoradiogram, the sequencing ladders were read manually.
2.2.18 Oligonucleotide synthesis

The list of oligonucleotide primers used in this study is given in table 2.1. The oligonucleotides were synthesized using phosphoramidite chemistry in a Pharmacia gene assembler provided by the central facility at CCMB.

2.2.19 Polymerase chain reaction

DNA was amplified by PCR according to Saiki et al., (1987) using the GeneAmp kit (Perkin-Elmer-Cetus Corporation) or by using reagents from Bangalore Genei following the manufacturers' instructions. About 10ng template DNA was used in a reaction mix containing 1X PCR buffer (10mM Tris-HCl pH 8.3 at 25°C, 50mM potassium chloride, 1.5mM magnesium chloride, 0.001% (w/v) gelatin), 0.2mM dNTPs, 250ng of primers in a volume of 50μl. To this mix, 1.25 units of Taq DNA polymerase/50μl reaction was added and the reaction mix was overlaid with 50μl of sterile, water saturated mineral oil to prevent evaporation during the PCR reaction cycles. The samples were subject to 30-35 cycles of amplification, using denaturation at 94°C, annealing at appropriate temperature depending on the T_m of primer and chain extension at 72°C. The last cycle was followed by an extension at 72°C for 5 minutes. At the end of the specified number of cycles, 5-10μl of the reaction mix was subjected to agarose gel electrophoresis.

2.2.20 Amplification of RNA

Amplification of RNA by subjecting it to reverse transcription followed by PCR amplification of resultant cDNA was performed as described by Innis et al., (1990) using reagents from GIBCO BRL.1μg RNA was made up to a volume of 11μl, to which was added 1μl oligo-dT (2.5μM final concentration) and the mix was incubated at 70°C for 10 minutes. It was then spun and chilled on ice. RT reactions were carried out by adding the following reagents to the annealed RNA and oligo-dT; 50mM KCl, 20mM Tris-HCl, pH 8.4, 2.5mM MgCl2, 0.5mM dNTPs, 10mM DTT and 25 units of Superscript II reverse transcriptase, which was added after incubating this mix at 42°C for 2-5 minutes. This was then subjected to a cycle of RT reaction at 42°C for 50 minutes. The RT reaction was stopped by incubation at 70°C for 15 minutes, which inactivates the enzyme and finally an incubation at 4°C for 5 minutes. The reaction products were frozen immediately at -20°C. The first strand cDNA thus synthesized was divided and each part (2μl) was amplified using a specific set of primers in a 50μl PCR reaction containing 2mM
Table 2.1 Oligonucleotide Primers used for RT-PCR analysis and Probe Labeling

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>NUCLEOTIDE POSITION</th>
<th>cDNA</th>
<th>SEQUENCE (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK7</td>
<td>648-670 sense</td>
<td>PTP-S (rat)</td>
<td>GGAATTCCATGGGCCCTGCAGTGATCCATTCC</td>
</tr>
<tr>
<td>RR12</td>
<td>1098-1120 antisense</td>
<td>PTP-S (rat)</td>
<td>CGGGATCCATTAGGTGTCTGTCAATCTTG</td>
</tr>
<tr>
<td>RR14</td>
<td>828-854 sense</td>
<td>PTP-S (rat)</td>
<td>GGATTCTACATGGCCATATAAGAAGGACAAA</td>
</tr>
<tr>
<td>CCNA1</td>
<td>670-694 sense</td>
<td>cyclinA (human)</td>
<td>GTTTAATGATACCTAAAGTGTT</td>
</tr>
<tr>
<td>CCNA2</td>
<td>915-939 antisense</td>
<td>cyclinA (human)</td>
<td>CCCCAGAAATGAGAGGTTTGTG</td>
</tr>
<tr>
<td>M1</td>
<td>924-948 sense</td>
<td>c-Myc (human)</td>
<td>GACCTTCATCAAAAAACATCATCAT</td>
</tr>
<tr>
<td>M3</td>
<td>1345-1368 antisense</td>
<td>c-Myc (human)</td>
<td>CCTTCTCCAGAAACACAT</td>
</tr>
<tr>
<td>RR6</td>
<td>829-855 sense</td>
<td>PTP-S (rat)</td>
<td>TACATGCC[CT]ATAATAGGAGGACAA</td>
</tr>
<tr>
<td>RR7</td>
<td>1097-1119 antisense</td>
<td>PTP-S (rat)</td>
<td>ATTTAGGTGTCTGTCAATCTTG</td>
</tr>
<tr>
<td>RR2A1</td>
<td>518-537 sense</td>
<td>RR2 (human)</td>
<td>CCTCTCAAGGACATTCAG</td>
</tr>
<tr>
<td>RR2A2</td>
<td>803-822 antisense</td>
<td>RR2 (human)</td>
<td>CCGCGTCTGGCCTCTTCTT</td>
</tr>
<tr>
<td>E1</td>
<td>841-860 sense</td>
<td>cyclinE (human)</td>
<td>CAGATTGCAGAGCTTG</td>
</tr>
<tr>
<td>E2</td>
<td>1020-1039 antisense</td>
<td>cyclinE (human)</td>
<td>TCCCCGTCTCCCTAT</td>
</tr>
</tbody>
</table>

MgCl$_2$, 50mM KCl, 10mM Tris-HCl, pH 8.3, 150µM dNTPs, 0.2µM of each primer and 1.25 units of Taq DNA polymerase. The polymerase chain reaction conditions for cdc25 were; denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute (or annealing and extension at 64°C for 90 seconds for GAPDH primers) for 35 cycles in an automated Perkin-Elmer Cetus thermal cycler. The PCR cycling conditions for cyclinE were; denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds for 35 cycles. After the last cycle, an extension was given at 72°C for 5 minutes. An aliquot (10µl) from each reaction was analyzed on a 1-1.5% agarose gel depending on the size of the fragment.

2.2.21 Vector construction

A. Construction of mutant PTP-S2

Strategy

The strategy employed to construct a catalytically inactive form of PTP-S2 made use of PCR to introduce the cysteine to serine mutation at amino acid position 216 by utilizing a unique Ncol site upstream of Cys216. This was done by a single base change from G to C at position 647 (of the full length PTP-S2) introduced into one of the primers used for PCR amplification. Construction of this vector was carried out in two steps. In the first step, the 3' fragment into which the mutation was introduced by PCR mutagenesis was cloned into pBluescript II KS+. This fragment was later ligated to the 5' fragment to obtain the full-length mutant PTP-S2.

Description

Full length PTP-S2 in pUC18 was used as a template to amplify by PCR a 530 base pair 3' fragment using SK7 and RR12 as forward and reverse primers respectively. The SK7 primer introduced the base change at nucleotide position 647 to cause the cysteine at position 216 to serine (TGC to TCC) mutation. The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI-BamHI site of pBluescript II KS+. The mutation was confirmed by sequencing. Digestion of this pBluescript II KS+ clone with Ncol and HindIII linearized the plasmid (and in the process lost the EcoRI site), which was ligated with the 5' Ncol-HindIII fragment released from full length PTP-S2. The clones were screened for positives by colony hybridization. The full-length mutant PTP-S2 (mPTP-S2 cDNA released by BamHI digestion) was then cloned in BamHI site of pET3a (bacterial expression vector) and BgIII site of pCB6 (mammalian expression vector).
B. Construction of vector expressing non-catalytic domain of PTP-S2

The non-catalytic domain of PTP-S2 is encoded by the C-terminal 112 amino acids from position 270 to 382, the amino acid at position 270 being methionine. The desired region was amplified by PCR using the RR12 and RR14 primers and full length PTP-S2 in pUC18 as the template. The 360 base pair PCR fragment was digested with BamHI and cloned in the BamHI site of pBluescript II KS+. The fragment was confirmed by sequencing and then cloned in the BglII site of the mammalian expression vector pCB6.

2.2.22 Extraction and partial purification of mutant PTP-S2 protein expressed in bacteria

Extraction

The full-length mutant PTP-S2 clone encoded in pET3a was transformed into BL21DE(3) cells and selected on LB-ampicillin (100μg/ml) and chloramphenicol (30μg/ml). A single colony was inoculated into 5ml culture and grown overnight. The overnight culture was subcultured at a ratio of 1:10 and grown till the O.D.600 reached between 0.45-0.55. The culture was then induced with 1mM IPTG for 4 hours. 1.5ml of the culture was spun down and the pellet resuspended in 0.5ml extraction buffer (pH 8.0 with 200mM NaCl). This was incubated in a water bath at 25°C for 30 minutes. The suspension was spun at 10,000rpm for 20 minutes at 4°C. A fraction of the supernatant and the pellet were boiled in SDS-sample buffer and fractionated on SDS-PAGE for analysis by coomassie blue staining, as well as immunostaining.

Purification (Sephadex G-75 column chromatography)

A sephadex G-75 superfine column (1.6 x 65cm) was equilibrated using column buffer (25mM Tris-HCl, pH 7.1, 1mM EDTA, 0.25% β-mercaptoethanol, 200mM NaCl and 10% glycerol). The extracted supernatant was applied on the G-75 column. Twelve 2.5ml fractions were collected after allowing the flow-through (40ml) to pass. Small aliquots from each fraction were used for estimating phosphatase activity (10μl); immunoblotting (10μl) and analyzing the protein profile by coomassie blue staining (40μl).

2.2.23 Assay for protein tyrosine phosphatase activity using para Nitro Phenyl Phosphate (pNPP) as substrate

PTP activity was measured essentially as described by Swarup and Subramaniam (1989). The enzyme was incubated at 30°C for 5-10 minutes in 50mM Tris-HCl (pH 7.1)
containing 5mM pNPP substrate in a volume of 100μl. The reaction was stopped by adding 1ml of 2M sodium carbonate. The O.D. was measured at 430nm.

2.2.24 Culture of cell lines and harvesting

Cos1 cells, NIH 3T3 cells and HeLa Tet-inducible clone expressing PTP-S2 were cultured as monolayers in Dulbecco’s modified eagle’s medium (DMEM), supplemented with 10% fetal calf serum and antibiotics at 37°C in a humidified CO2 atmosphere.

For harvesting cells, medium was aspirated and the cells were washed once with sterile 1X PBS. They were then treated briefly with a small volume of 0.125% trypsin-EDTA for detaching them from the substratum. Neutralizing with complete medium stopped further trypsinization. The cells were pelleted by centrifuging at 1000g for 3 minutes and washed once with 1X PBS. The pellet was either boiled directly in SDS-sample buffer to make whole cell lysates or suspended in 1ml of Trizol solution for isolation of total cellular RNA.

2.2.25 Transient transfection of cell lines

The cell lines of choice (COS1 and HeLa) were transiently transfected with the desired plasmid constructs using either Lipofectamine transfection reagent (GIBCO BRL) or Lipofectamine Plus Reagent (GIBCO BRL) depending on the availability and requirement.

A. With Lipofectamine reagent

The cells (4x10⁴ cells/coverslip) were plated on coverslips 24 hours prior to transfection in DMEM containing 10% serum and 1X antibiotics. The ratio of DNA:lipoferctamine reagent was 1:6. 250ng of DNA was used for each coverslip. Transfection quality sterile DNA (1μg) and 6μl of Lipofectamine reagent were added to 100μl of plain DMEM (without antibiotics and serum) and incubated for about 30-45 minutes, allowing the complexes to form. Meanwhile, the coverslips were washed twice with PBS to remove serum and replaced with plain DMEM. Following incubation, an additional 400μl of plain DMEM was added to the complexes and this was distributed equally among 4 coverslips. 4-5 hours following transfection, medium containing 20% serum was added to the cells, so that the final concentration of serum became 10%. The transfected cells were allowed to remain as such overnight. 24 hours following transfection, the medium was replaced with fresh complete medium. 48 hours after transfection, the cells were fixed and processed for immunofluorescence as described previously (Radha et al., 1994).
some cases where the expression of protein was low, the cells were induced with sodium butyrate (5mM) for a maximum of 16 hours, coinciding with 48 hours after transfection and then processed for immunofluorescence as before.

**B. With Lipofectamine Plus reagent**

The transient transfections were carried out according to the Life Technologies protocol. 5x10^4 cells were plated on each coverslips. The medium did not contain any antibiotics. 24 hours after seeding the cells, the medium was replaced with plain medium. In the meantime, the DNA:lipid complexes were prepared. For each coverslip, 0.8μg DNA in case of Cos1 cells (0.4μg DNA in case of HeLa cells) was added to 17μl DMEM and this was mixed with 8μl Plus reagent (3μl in case of HeLa cells) and incubated at room temperature for 15 minutes. 23μl DMEM was added to 2μl lipofectamine reagent, mixed and combined with the DNA:Plus complex and incubated at room temperature for 15 minutes. For each 50μl reaction volume, another 200μl DMEM was mixed and added to the coverslips. Transfection was allowed to occur for 3-5 hours, following which, medium containing 20% serum was added so that the final concentration of serum became 10%. In some apoptosis experiments, which required to show the effect of certain agents like DMSO and caspase inhibitors on PTP-S2 induced apoptosis, these agents were added to the cells 3-5 hours after transfection, at the time of replenishing with serum and this was continued throughout, till the cells were ready to be processed for immunofluorescence.

### 2.2.26 Stable transfection of cells

**A. Establishment of tetracycline-inducible PTP-S2 clones in HeLa**

A full length PTP-S2 cDNA fragment was cloned in the BamHI site of pTRE response plasmid by Ch. Sudhakar. Isolation of stable, tetracycline-inducible PTP-S2 clones in HeLa cells was done by Sanjeev Gupta (section 2.1.3).

**B. Establishment of stable clones expressing PTP-S2 in C3H, 10T1/2 cell line**

2x10^5 C3H, 10T½ cells were plated in 35mm dishes in duplicates, twenty four hours prior to transfection. The cells were transfected with 1μg DNA (control vector, PTP-S2 and mutant PTP-S2 vector) using lipofectamine reagent. Forty eight hours after transfection, medium containing 400μg/ml active concentration of genetin was added for selection. Two weeks after selection the surviving clones were picked up and maintained in selection medium.
2.2.27 Determination of growth kinetics

Growth kinetics of the HeLa Tet-inducible PTP-S2 clone was measured by plating $5 \times 10^4$ cells in 35mm dishes in duplicates in DMEM with 10% FCS and cell numbers were counted after 24, 48, 72 and 96 hours of growth and induction following removal of Tetracycline. At the same time another set of cells was plated for each of the above time points without removing Tetracycline. These served as the uninduced controls.

2.2.28 Immunofluorescence

In transiently transfected cells, 48 hours after transfection, the cells were processed for immunofluorescence as described by Radha et al., (1994). The cells were thoroughly rinsed thrice with 1X PBS and fixed with 3.7% formaldehyde (in PBS) for 10 minutes at room temperature. They were washed twice with PBS and permeabilized with 1X PBS containing 0.5% TritonX-100 and 0.05% Tween20 for 6 minutes. Following permeabilization, the cells were washed and blocked in PBS containing 2% BSA for 1 hour at room temperature. The coverslips were incubated in first antibody overnight at 4°C, washed thoroughly to remove excess unbound antibody and then incubated in secondary antibody (FITC conjugated) for one hour at room temperature. The coverslips were washed with PBS, mounted on glass slides using in mountant [90% glycerol, 1mg/ml Antifade( PPD-para-phenylene diamine hydrochloride) in PBS] with DAPI (1μg/ml) and observed under the fluorescence microscope.

2.2.29 Quantitative analysis of apoptotic cells

Cos1 cells were transiently transfected with the desired plasmids and processed for immunofluorescence as described in sections 2.2.25 and section 2.2.28. Cells were observed using an Olympus microscope. PTP-S expressing cells, i.e. cells showing FITC staining were counted and those that showed condensed chromatin or fragmented nuclei were scored as apoptotic cells. Cells not showing PTP-S expression in the same coverslips were also scored for the number of apoptotic cells. An average of 300 PTP-S expressing and 500 non-expressing cells were counted in each experiment.

2.2.30 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by the method described by Laemmli (1970) using a discontinuous buffer system. Composition of the buffers used is given below:
Stacking gel: 0.125M Tris-HCl, pH 6.8
Resolving gel: 0.375M Tris-HCl, pH 8.8
Electrophoresis buffer: 0.025M Tris-HCl, 0.192M glycine, 0.1% SDS pH 8.3

A stock solution containing 30% acrylamide (w/v) and 0.8% N, N’ methylene bisacrylamide (w/v) was used. The resolving gel had 10% acrylamide and the stacking gel had 4.5% acrylamide. The gel and the buffers contained 0.1% SDS. The gels were polymerized using TEMED (15µl for 30ml of gel mixture) and freshly prepared ammonium persulphate (200µl of 10% solution). The gels were cast on a vertical gel apparatus (Minikin, India). Prior to loading, the protein samples were boiled in a water bath for 5 minutes in SDS-gel sample buffer (50mM Tris-HCl, pH 7.5, 3% SDS, 10% glycerol, 4mM EDTA, 0.01% bromophenol blue and 10% β-mercaptoethanol). Electrophoresis was carried out in constant current mode at 20mA till the samples crossed the stacking gel and then at 40mA through the resolving gel.

2.2.31 Staining with Coomassie brilliant blue

The gels were stained with 0.05% coomassie brilliant blue R250 in methanol:acetic acid:water (45:10:45 v/v) for at least 1-2 hours. The gels were destained in 10% acetic acid:20% methanol solution in water for 2-4 hours on a rocking platform with 2-3 changes of the solution.

2.2.32 Western blotting

Color reaction and Enhanced Chemiluminescence (ECL)

The protein samples separated on the SDS-PAGE were transferred onto nitrocellulose membrane (Hybond N, Amersham) electrophoretically, using a Nova Blot apparatus (Pharmacia). The membrane was wetted in the transfer buffer (39mM glycine, 48mM Tris base, 0.0375% SDS and 20% methanol). The gel was placed in contact with the membrane and sandwiched between four pieces of buffer-soaked Whatman 3M paper on each side. The sandwich was then placed between graphite plate electrodes, with the membrane facing the anode. The transfer was performed for 2 hours using a current of 0.8mA/cm². After transfer, the molecular weight markers were detected by Ponceau S stain. The membrane was blocked for one hour with 2% BSA in TBSTG (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% Tween 20 and 0.2% gelatin) at a volume of 0.1ml/cm² of filter. The blot was then incubated for 2 hours at room temperature with primary antibody in TBSTG wash buffer. After three washes of 5 minutes each in wash buffer, the membrane was incubated for 1 hour at room temperature with horseradish peroxidase-labeled secondary antibody or alkaline phosphatase conjugated secondary antibody in TBSTG. The
membrane was washed three times in TBSTG and bound antibodies were detected by enhanced chemiluminescence (ECL) detection system. The immunoreactive bands could also be detected by a color reaction (for alkaline phosphatase conjugated secondary antibody) using 100mM Tris- HCl pH 9.0 buffer containing 10mM MgCl₂, 0.005% 4-chloro-3-indolyl phosphate (BCIP) and 0.01% Nitro Blue Tetrazolium (NBT).

2.2.33 BrdU (5-bromo-2'-deoxy-uridine) incorporation and detection

Approximately $10^4$ HeLa PTP-S2 Tet-inducible cells were plated on coverslips and induced for PTP-S2 expression for 72 hours by thorough washing with 1X PBS to remove Tetracycline. Another set of cells were plated and grown for the same length of time without induction of PTP-S2 expression to serve as a control. A pulse of 100μM BrdU (in DMEM containing 10% serum) was given for one hour at 37°C in CO₂ incubator. The cells were washed twice with 1X PBS, prior to fixing with cold 70% ethanol for 30 minutes at 4°C. They were then exposed to 2N HCl for 30 minutes at room temperature to ensure DNA denaturation and neutralized by several washes with sodium tetraborate (1mg/ml) and with PBS. All antibody incubations were done at room temperature. The coverslips were then incubated in blocking solution (1X PBS containing 2% BSA) for 2 hours at room temperature. For BrdU detection, cells were incubated for two hours with anti-Brdu antibody and then incubated for 1 hour with FITC-conjugated secondary antibody. The antibody incubations were always followed by three washes with 1X PBS to remove any excess unbound antibody. The cell nuclei were then labeled with DAPI (1μg/ml) for 10 minutes. Cover slips were mounted in 90% glycerol and observed using a fluorescence microscope.

2.2.34 Cell cycle analysis by flow cytometry

For analysis of DNA content by flow cytometry, exponentially growing culture of cells (approximately 50%-60%) were trypsinized uniformly, avoiding clumping and spun down in a 15ml centrifuge tube. They were washed once with PBS to remove medium. The cells were fixed by resuspending the pellet in 1.25ml PBS and added drop wise to 3.75ml ice-cold absolute ethanol. The fixed cells were kept overnight at 4°C. Prior to analysis, the fixed cells were washed twice with PBS to remove ethanol and re-suspended in PBS containing 20μg/ml propidium iodide, 50μg/ml DNase-free RNase and 1% TritonX-100. Cells were incubated at 37°C for 30 minutes before being analyzed in a FACSCAN flow cytometer. In the double-staining procedure for staining cells with anti-PTP-S2 antibody, prior to propidium iodide staining, the fixed cells were
washed once with PBS and permeabilized using 0.5% TritonX-100, 0.05% Tween20 in PBS for 6 minutes. The permeabilized cells were washed twice to remove detergent and blocked in PBS containing 1% BSA for 1 hour at room temperature. They were incubated in primary antibody overnight at 4°C. Following 3 washes with PBS to remove unbound primary antibody, cells were stained with FITC conjugated secondary antibody for 1 hour at room temperature. After three washes with 1X PBS, they were stained with propidium iodide as described above and analyzed by flow cytometry using the FACSTAR plus equipment and Cell Quest software from Becton Dickinson.

2.2.35 Cell synchronization

Induced and uninduced HeLa clones were synchronized at the G1/S boundary by the double thymidine block protocol (Stein et al., 1998). Thymidine block was initiated in cultures at a cell density that would permit active growth throughout the time course of the synchronization procedure. The first thymidine block was imposed for 14 hours by removing the growth medium by aspiration and providing fresh medium containing 2mM thymidine. Cells were released from the first block for 9 hours by removing the thymidine containing medium by aspiration and washing the monolayers thrice with an equal volume of serum-free medium (at 37°C) prior to replacement with normal growth medium. Following the 9-hour release period, a second thymidine block was imposed for 14 hours by adding thymidine. The cells were released from the second thymidine block and re-plated in normal growth medium and fixed for flow cytometric analysis at different times after release from the block. Samples for FACS analysis were made for both induced and uninduced cells at every two-hour interval starting from the release from second block till 22 hours after release from the block. The fixed cells were processed as described above for cell cycle analysis by flow cytometry.

2.2.36 Immunoprecipitation

Whole cell lysates were prepared by extracting with 2X IP buffer (40mM Tris-HCl, pH 7.4, 2% TritonX-100, 1% Sodium deoxycholate, 300mM sodium chloride, 2mM PMSF, 2µg/ml protease inhibitors like STI, leupeptin and aprotinin) for 15 minutes on ice. The cell debris was removed by spinning at 10,000rpm for 10 minutes at 4°C. The supernatant was diluted 1:1 and incubated with the antibody (30µl of anti-PTP-S2 antibody, G11, or 2µl of anti-Cdk2 antibody) for 1 hour. Following this, Protein A-agarose beads were added and the immunoprecipitation process was carried out for one more hour. The beads with the antibody attached to them were
pelleted by spinning at 5,000 rpm for 3 minutes and washed with 1X IP buffer 3-5 times and either boiled with 1X sample buffer for western blotting or processed for kinase/phosphatase assay.

2.2.37 Kinase assay

Cdk2 activity of the immunoprecipitated proteins attached to the Protein A-agarose beads was checked by phosphorylation of purified Histone H1 (2 μg) in kinase buffer (10 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM DTT, 2 μg/ml protease inhibitors, 0.1 mM orthovanadate and 10 μM [γ-³²P] ATP) in a total volume of 50 μl for 10 minutes at 25°C. The reaction was stopped by adding 25 μl of 3X SDS-PAGE sample buffer and boiled for 5 minutes. The samples were then resolved by SDS-PAGE, transferred to Immobilon-P and exposed to Fuji phosphor imager cassette. This blot was then probed with anti-Cdk2 antibody and Cdk-2 activity (Histone H-1 phosphorylation) was normalized for Cdk2 protein levels.

2.2.38 Preparation of [³²-P] labeled substrate for tyrosine phosphatase assay

The substrate was labeled according to the method of Swarup and Subramanyam (1989). 1 mg of the poly (Glu⁴, Tyr¹) from Sigma was incubated with 0.1 mM [γ-³²P]ATP in TMV buffer (10 mM Tris-HCl pH 7.5, 5 mM MgCl₂) containing 10 μg of TK-1, a tyrosine kinase purified by Swarup et al., (1988). The reaction was carried out overnight at 25°C and was stopped by adding ice-cold 100% Trichloroacetic acid to a final concentration of 10% and kept on ice for 30 minutes. Following incubation on ice, the reaction mix was centrifuged for 10 minutes at 15,000 rpm in a microfuge. The supernatant was discarded and the pellet was washed once with 10% TCA, followed by a quick wash with 1 M Tris-HCl pH 8.0. The pellet was dissolved in 50 μl 1 M Tris-HCl, pH 8.0 by gentle vortexing. The tube was centrifuged for 5 minutes and the supernatant was checked for counts. The labeled substrate was purified by passing the supernatant through a 1 ml column of Sephadex G-50 in TE₈. Six fractions of 200 μl each were collected and 2 μl of each fraction was counted in a scintillation counter. The concentration of the [³²P]-labeled substrate was determined from the specific activity of the [γ-³²P]ATP used for phosphorylating the substrate.
2.2.39 Assay of protein tyrosine phosphatase activity

The phosphatase activity was measured essentially as described by Swarup and Subramanyam (1989). The immunoprecipitate was incubated at 30°C for 5 minutes in 25mM Tris-HCl pH 7.1 containing 1.5-2μM [32-P]-labeled poly (Glu⁴, Tyr¹), 0.02% TritonX-100, 1mM DTT, 0.2mM EDTA in a volume of 50μl. The reaction was stopped by adding 250μl of ice-cold 10% Trichloroacetic acid followed by addition of 20μl of bovine serum albumin (20mg/ml). After incubating the tubes on ice for 15 minutes, they were centrifuged for 2 minutes at 12,000rpm in a microfuge. 200μl of the supernatant, which contained the TCA-soluble material, was transferred to a 0.5ml microfuge tube and its [32P] disintegration was estimated by Cherenkov counting.

2.2.40 Pervanadate treatment

The treatments were done as described by Garton, A.J. et al., 1996 with some modifications. Prior to cell lysis, 70-90% confluent cultures of cells were treated for 30 minutes with 0.1 mM pervanadate at 37°C (20μl of a fresh solution containing 50mM Sodium orthovanadate [Na₃VO₄] and 100mM H₂O₂ was added to 10ml growth medium). Following treatment, the cells were washed thoroughly with 1XPBS and lysed in SDS sample buffer.