Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Sources of Chemicals

Acetyl Coenzyme A, Acrylamide, aprotinin, bis-acrylamide, Agarose, β-mercaptoethanol, BSA fraction V, Butyryl Coenzyme A, BCIP, calcium chloride, cycloheximide, DEPC, digitonin, DMSO, doxorubicin, DTT, ethidium bromide, formamide, HEPES, IPTG, kanamycin, leupeptin, lysozyme, NBT, penicillin, PIPES, PMSF, Ponceau S, Sodium dodecyl sulphate (SDS), streptomycin, TEMED, tetracycline, Tris base, Triton X-100, trypsin, Tween-20, X-gal were all obtained from Sigma. Sephadex G-25 and Sephadex G-50 were obtained from Pharmacia. Medium molecular weight marker proteins for SDS-PAGE, DNA markers and PCR reagents were from Bangalore Genei. Lipofectamine 2000, Trizol Reagent, Superscript II first strand cDNA synthesis kit, FBS and DMEM were obtained from Invitrogen. RNase A was from Calbiochem. 5X RLB (reporter lysis buffer) for reporter assays was obtained from Promega. D-threo- (dichloroacetyl-1-14C)-chloramphenicol (CAT assay grade), Hybond C, Hybond N, and Hybond N+ membranes were purchased from Amersham Life Science. Western lighting chemiluminescence reagent was from Perkin Elmer Life Science. Mitotracker Red was obtained from Molecular Probes. Columns for preparing transfection grade DNA were from Qiagen or Life Technologies. Scintillation grade dimethyl-POPOP and PPO were from Loba-chemie. Restriction enzymes, T4 DNA ligase were from New England Biolabs and Bangalore Genei. Poly (dl-dC) was from Roche. Tryptone, agar and yeast extract were from Difco Laboratory and Blotto was from Santacruz. Whatman filter papers were from Whatman International Ltd; X-ray films were from Konica Corporation or Kodak. Intensifying screens were obtained from Dupont. Phosphor-imager screens were from Fuji. All 32P-radiolabelled nucleotides were obtained from BRIT, Mumbai. Other reagents were from local suppliers like Qualigens, SRL, SD fine-chemicals Ltd., and Merck India Ltd. and were of analytical grade.

2.1.2 Antibodies

G11 monoclonal antibody against PTP-S2 was prepared in our laboratory by Dr. V. Radha and the culture supernatant was used as such for immunofluorescence and 1/10 diluted for western blotting.

-Rabbit polyclonal anti-caspase-1, rabbit polyclonal IL-1β antibody and rabbit polyclonal cytochrome c antibody were from Santa Cruz biotechnology.

-Rabbit polyclonal Omi antibody and Sheep polyclonal hsp60 antibody were gift from Dr. Alnemri, Thomas Jefferson University, USA
Mouse monoclonal anti-α tubulin antibody was from Amersham LifeScience.

Mouse monoclonal anti-T7 tag antibody was from Novagen.

Goat polyclonal Bid antibody was from R and D systems.

Rabbit polyclonal Bax antibody was obtained from Upstate.

Secondary antibodies for western blotting and immunofluorescence were from Bangalore Genei, Amersham LifeScience or Vector labs.

2.1.3 Bacterial Strains

_E. coli_ DH5α : F' end A1 hsd R17 (rK-, Mk-) sup E44 thi-1 rec A1 gyr A96 (Nal')
rel A1 Δ (lac ZYA-arg F) u169 (φ80 lac Z8 M15)

The above-described strain of _Escherichia coli_ was used for all transformations, plasmid isolations and for selection of recombinant clones.

2.1.4 Cell Lines

The cell lines used in this study and their tissue types are listed below.

a) MCF-7 - human breast carcinoma
b) HeLa - human cervical adenocarcinoma
c) A549 - human lung carcinoma
d) U2OS - human osteosarcoma
e) HCT116 - human colorectal carcinoma
f) MCF-mp53 - This cell line was derived from MCF-7 cells by transfection with R273H mutant of p53 followed by selection with G418 (Gupta _et al._, 2001)
g) Cos-1 - African green monkey (Cercopithecus aethiops) kidney cell line transformed by SV 40, established from CV-1 Simian cells

2.1.5 Plasmids

a) pcDNA3-T7-Ipaf has the Ipaf cDNA cloned in frame with a N-terminal T7-Tag, the Ipaf insert can be released with Bam HI and Xho I. This plasmid was a gift from Dr. E.S. Alnemri, Thomas Jefferson University, USA and has been described in Poyet _et al._, 2001.

b) pcDNA3-T7-DN-Ipaf was constructed by deleting most of the nucleotide binding domain (∆253-659 amino acids) of full length Ipaf by Ms. Subhashini Sadasivam and has been described in Sadasivam _et al._, 2005.

c) pcDNA3-T7-activated Ipaf was made by deleting the LRR domain of Ipaf and has been described (1-560 amino acids) (Sadasivam _et al._, 2005)
d) The mU6-pro vector contains the mouse U6 snRNA promoter (RNA polymerase III) with a Bbs I cloning site arranged to allow insertion of the shRNA template sequences after the first nucleotide of the U6 snRNA. The vector has a GFP insert that can be released with Bbs I and Xba I and the hairpin oligonucleotide can be subsequently cloned in its place. This vector was a gift to Dr.Jyotsna Dhawan of CCMB from Dr.David L. Turner, University of Michigan and has been described in Yu et al., 2002. Ipafl shRNA cloned in mU6Pro vector (which targets nucleotides 1294 - 1312) has been described previously (Sadasivam et al., 2005).

e) T7-pcDNA-p53DD plasmid has human p53 cDNA encoding amino acids 302-390 cloned in-frame with a N-terminal T7 tag into pcDNA3 vector. This plasmid was a gift from Dr William G. Kaelin Jr, DFCI, Harvard medical school, USA and has been described in Irwin and Kaelin, 1998.

f) pcDNA-HA-p73α and pcDNA-HA-p73β plasmids have cDNAs for the different isoforms of p73 (a homologue of tumor suppressor p53) namely α, β cloned in-frame with a hemagglutinin (HA) tag into pcDNA vector. These plasmids were a gift from Dr Gerry Melino, University of Rome, Italy and have been described in De Laurenzi et al., 1998.

g) pCB6-procaspase-1 plasmid expresses the human caspase-1α isoform under the control of the CMV promoter and has been described in Gupta et al., 2002.

h) pTET-Off and pTRE- plasmids were from clonetech. pTRE-PTP-S2 plasmid has been described in Ganapati et al. 2001.

i) pcDNA3.1-IL-1β expression plasmid was a gift from Dr. Alnemri, Thomas Jefferson University, USA.

j) pCAT-promoter vectors were from Promega. pCAT-Basic vector lacks eukaryotic promoter and enhancer sequences and was used to clone putative promoter sequences upstream of a chloramphenicol acetyl transferase (CAT) reporter gene. pCAT-promoter vector contains the CAT reporter gene under the control of a SV-40 minimal promoter. Putative enhancer elements can be cloned either upstream or downstream of the promoter/CAT transcriptional unit.

k) pCMV-p53 plasmid has the human wild-type p53 cDNA downstream of CMV promoter.

l) pC53-4.2N3 plasmid has the mutant human p53 cDNA from nucleotide position 1 to 1794. In this construct arginine 273 is mutated to histidine (R273H) and was derived from an epidermoid carcinoma cell line. In addition, the polymorphic amino acid residue 72 is proline. This mutant has been described in Harlow et al., 1985.
m) pCMV-SPORT-βGal containing the E.coli β-Galactosidase gene under a CMV promoter was from Invitrogen.

n) pEGFP-C1 vector contains enhanced GFP to which proteins of interest can be expressed as a fusion to the GFP C-terminus. This plasmid was from Clontech.

o) pMOSBlue was used for blunt end cloning of PCR products. This plasmid was part of the pMOSBlue blunt ended cloning kit purchased from Amersham Pharmacia Biotech.

2.1.6 Bacterial Media, Antibiotics and Chemical Stocks

β-Gal assay buffer (2X): A solution of 1.33 mg/ml ONPG, 2 mM MgCl₂ and 100 mM β-mercaptoethanol was made in 200 mM sodium phosphate buffer (pH 7.3), and stored in aliquots at -20°C.

1M DTT: 3.09 g of DTT was dissolved in 20 ml of 0.01 M Sodium acetate (pH 5.2), sterilized by filtration and stored as aliquots in -20°C.

1X PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄. A 10X stock solution was prepared in double distilled water, pH adjusted to 7.4 with HCl and autoclaved.

1X SSPE: 150 mM NaCl, 10 mM Na₂HPO₄ and 1 mM EDTA. 20X stock solution was prepared in double distilled water and pH adjusted to 7.4 with 10N NaOH.

1X TBE: 89 mM Tris base, 89 mM boric acid and 1 mM EDTA dissolved in double distilled water. 10X stock solution prepared was filtered, autoclaved and stored at room temperature.

30% acrylamide solution: 29.2 g of acrylamide and 0.8 g of N, N’-Methylene bis-acrylamide was dissolved in double distilled water, made up to 100 ml and stored at 4°C in an amber-coloured bottle.

6X Agarose gel loading buffer: 0.25% bromophenol blue and 0.25% xylene cyanol in 30% glycerol.

Acetyl Coenzyme A: 3.5mg/ml stock solution was made in water, aliquoted and stored at -80°C.

Amido-black staining solution: 0.1% amidoblight 10B, 25% isopropanol and 10% acetic acid in water.

Ampicillin: 1000X stock solution was made by dissolving 100 mg Ampicillin per ml of sterile double distilled water.

BCIP: 50 mg/ml stock solution was made using di-sodium salt of BCIP in double-distilled water and stored at -20°C.
Buffer saturated phenol: Melted phenol was mixed with equal volume of 0.5M Tris-HCl pH 8.0 and 0.1% 8-hydroxyquinoline and mixed well. The upper aqueous phase was removed and the process repeated with 0.1M Tris-HCl pH 8.0. This step was repeated till the pH of the aqueous solution was equilibrated to 8.0. It was mixed with 0.1 volume of 0.1M Tris-HCl (pH 8.0) and stored in amber-colored bottle at 4°C.

Chloroform:isoamyl alcohol: 24:1 (v/v) chloroform:isoamyl alcohol mixture was prepared.

Deionized formamide: Formamide was mixed with mixed-bed resin, stirred for an hour, filtered, and stored in aliquots at -20°C.

DEPC water: Water for RNA isolation was treated with 1% DEPC, left overnight and subsequently autoclaved to remove excess DEPC.

DMEM (1X): 13.4 g of DMEM and 3.7 g of NaHCO₃ was dissolved in milli-Q water to make 1 litre of the medium. The pH was adjusted to 7.4 using HCl and subsequently filter sterilized.

Doxorubicin: A stock solution of 1mg/ml of doxorubicin in milli-Q water was made and stored at -20°C.

Ethidium Bromide: 10 mg/ml solution in water.

Hybridisation buffer: Equal volumes of 14% SDS and 1N Na₂HPO₄ (pH 7.4) with 1 mM EDTA (pH8.0).

LB Agar: LB media reconstituted with 1.5% Bactoagar was used for pouring LB plates.

Luria-Bertani (LB) broth: 1% Bactotryptone, 1% NaCl and 0.5% Bacto-yeast extract were dissolved in double distilled water. pH was adjusted to 7.4 using 10N NaOH and then autoclaved.

Mountant: 90% glycerol, 10% antifade solution (10 mg/ml para-phenylene diamine hydrochloride in 10X PBS) and 0.5μg/ml DAPI (from 5 mg/ml stock) in 1X PBS.

NBT: 50 mg/ml stock solution was made in 70% Dimethyl formamide and stored at -20°C.

Penicillin, streptomycin, kanamycin: 600 mg penicillin, 1000 mg kanamycin, 500 mg streptomycin were dissolved in 100 ml 1X PBS and filter sterilized to make a 100X stock which was stored frozen at -20°C. 1X antibiotic solution was used in all tissue culture media.

Ponceau S: A 10X stock of Ponceau S was made containing 2% Ponceau S, 30% trichloroacetic acid and 30% sulfosalysilic acid in water. For use with PVDF membranes, 1X solution of 0.1% Ponceau S in 5% acetic acid solution was used.
RNase A: 20 mg/ml stock solution was made in 15 mM NaCl and 10 mM Tris (pH 7.5). It was then boiled for 15 minutes and cooled slowly to room temperature. The stock was then aliquoted and stored at -20°C.

Scintillation Fluid: 5 g/litre PPO and 0.5 g/litre Di-methyl POPOP solution was made in toluene and stored in amber coloured bottle.

SDS-PAGE sample buffer (3X): 180 mM Tris-Cl pH 6.8, 6% SDS, 15% glycerol, 7.5% β-mercaptoethanol and 0.01% bromophenol blue in double-distilled water and stored at -20°C.

Tris EDTA pH 8.0 (TE): 10 mM Tris HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

Trypsin EDTA: 0.125% trypsin (cell culture grade) and 0.1% EDTA was dissolved in 1X PBS, filter sterilized and stored at -20°C.

2.2 Methods

2.2.1 Plasmid Isolation

Plasmid DNA miniprep was carried out by boiling lysis method as described by Sambrook et al., 1989 with certain modifications. Restriction digestion of plasmids for screening purposes was carried out after the Rnase treatment. In order to prepare plasmids for sequencing purposes, an alternate method of plasmid purification from technical manual of the ABI prism 3700 sequencer was used. In this method, 3 ml of overnight grown bacterial culture was pelleted and re-suspended in 100 μl of double-distilled water. 100 μl of lysis buffer (100 μl of 10% SDS, 20 μl of 0.5M EDTA and 10 μl 10N NaOH made up to 1ml with water) was added and the tube was kept in boiling water for 2 minutes. 50 μl of 1M MgCl₂ was added to these, mixed and kept on ice for 2 minutes. The mixture was spun at maximum speed in a microcentrifuge for 2 minutes. 50 μl of 5M potassium acetate was added to the supernatant and kept on ice for 2 minutes. The tube was then spun at maximum speed for 2 minutes and the supernatant transferred to a separate tube. DNA was precipitated by adding 600 μl of Isopropanol followed by 2 minutes incubation on ice. The tube was then spun at maximum speed for 2 minutes to pellet the plasmid DNA. The pellet was washed with 1 ml of 70% ethanol and air-dried. The pellet was subsequently re-suspended in 100 μl TE pH 8.0 containing RNase A.

Plasmids for transfections were prepared using QIAGEN-tip20 (miniprep) and QIAGEN-tip 100 (midiprep) columns or using the GibcoBRL CONCERT High Purity
plasmid columns according to manufacturer’s instructions. The protocol involves alkaline lysis of cells followed by column purification of DNA that yields high purity plasmids with relatively low levels of impurities.

2.2.2 Quantitation of Nucleic Acids

The nucleic acid concentration was determined by measuring the absorbance at 260 nm. Empirical relationship of 50 μg of double stranded DNA, 33 μg of single stranded DNA or 40 μg of single stranded RNA was taken to be equal to 1.0 OD$_{260}$. Purity of the preparation was estimated using ratio of absorbance at 260 nm to 280 nm.

2.2.3 Agarose Gel Electrophoresis

DNA and RNA samples were mixed with 6X loading dye (so as to make it 1X) and were resolved, using 0.8-1.2% agarose gels made in 1X TBE buffer. 0.25 μg/ml ethidium bromide was added to gels during preparation for visualizing DNA.

2.2.4 Restriction Endonuclease Digestion

Plasmid DNA (1-2.5 μg) was digested with 1-2.5 units of restriction enzyme in a compatible buffer in a 20 μl final volume, as per manufacturers instructions. Digested products were visualized by resolving in an agarose gel along with appropriate DNA markers.

2.2.5 Gel Elution of DNA Fragments

The GeneCiean purification kit from Bio101 was used to purify DNA by gel elution. Eluted DNA was used for further protocols such as ligation.

2.2.6 Ligation

DNA fragments obtained after gel purification of PCR products or restriction digests were ligated using T4 DNA ligase at 22°C (for blunt ended cloning) or 16°C for 8 hours to overnight. A molar ratio of vector to insert of 1:3 was generally used. When using the pMOS Blue vectors for cloning PCR products, the protocol suggested in the product literature was followed.

2.2.7 Preparation of Ultracompetent Cells

The method of Inoue et al., 1990 was used for high efficiency ultracompetent cells. Pre-inoculum of E.coli DH-5α strain from a single colony was grown overnight in 10 ml of LB at 37°C. 0.1% inoculum of this culture was added to 250 ml LB medium in a 2-litre flask and kept under vigorous shaking at 18°C till absorbance reached about 0.6. The culture was kept on ice for 10 min and the cells harvested by centrifuging at 2500 g for 10 min at 4°C. The cell pellet was resuspended in 80 ml of ice-cold filter sterilized
PIPES buffer (10 mM PIPES, 15 mM CaCl<sub>2</sub>, 250 mM KCl, 55 mM MnCl<sub>2</sub> pH 6.7) and placed on ice for 10 min. Cells were re-centrifuged and gently resuspended in 10 ml of PIPES buffer with 7% DMSO. The cells were kept on ice for about 10 min, aliquoted and snap frozen using liquid nitrogen for storage at -80°C.

2.2.8 Purification of Radiolabelled Probe

Radiolabelled oligonucleotides generated by PCR for colony hybridisations were purified by Biogel P60 column. Biogel P60 was mixed with TE (pH 8.0) and kept overnight for swelling and autoclaved. The slurry was packed upto 1 ml mark in a syringe and a single wash was given with TE (pH 8.0). The radiolabelled PCR product (50 μl) was loaded on the column followed by 250 μl of TE (pH 8.0). This eluant was collected as first fraction and five additional 200 μl fractions were collected subsequently. 2 μl of each of these fractions was spotted on small Whatman No3 filter paper pieces and radioactivity was measured by scintillation counter. Only the second fraction which had maximum radiolabelled DNA and third fraction were used for hybridization.

2.2.9 Southern Blotting and Hybridisation

DNA separated on agarose gel was vacuum transferred for 1 hour onto Hybond N+ using 0.4 M NaOH. Following transfer, the membrane was rinsed in 2X SSPE and marker positions were marked on it using transilluminator. Pre-hybridisation was carried out at 65°C in hybridisation buffer for 1-2 hours. Alkali-denatured-probe (probe incubated with 0.3N NaOH at 37°C for 5 min) was added to the fresh hybridisation buffer and incubated at 65°C for 4 hours to overnight with shaking. After hybridisations, the blots were washed sequentially with 2X SSPE + 0.1% SDS at room temperature for 30 min, 0.5X SSPE with 0.1% SDS at room temperature for 30 min and finally with 0.5X SSPE having 0.1% SDS at 65°C for 45 min. Autoradiography and/or phosphor-imager analysis was used to develop the blot or hybridisation membrane.

2.2.10 DNA Sequencing

ABI Prism Model 3700 DNA Analyzer or Model 3730 DNA Analyzer were used for all DNA sequencing reactions. 200 ng of plasmid DNA and 2.5-5 pmoles of primer was constituted in a volume of 3.2 μl and mixed with 1.8 μl of the big-dye terminator sequencing kit (Perkin-Elmer). PCR was carried out in the Gene Amp PCR System 9600 Thermal Cycler with denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 50°C for 4 min, for a total of 35 cycles and a final extension was done for 5
minutes. DNA was ethanol precipitated and washed with 70% ethanol. The samples were then resuspended in 10 μl High-dye-formamide (Perkin-Elmer) and 5 μl of this was loaded in the capillaries.

2.2.11 Oligonucleotide Synthesis and Purification

Oligonucleotides used in this study were synthesized in Applied Biosystem 394 DNA/RNA synthesizer and purified by spun-column using Sephadex G50. For gel shift assays oligonucleotides were purified by elution from Urea-PAGE gels. The oligonucleotides to be purified were resolved using denaturing urea-PAGE gel. The bands were visualized using ethidium bromide staining and excised out using a clean scalpel and chopped into fine pieces. These fragments were incubated in 0.3M sodium acetate solution (pH 7.0) overnight on a rototorque. The buffer was then transferred to a 2 ml tube. The sample was then extracted with equal volumes of n-Butanol till the volume reduced to about 500 microlitres. DNA was precipitated using 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol at -20°C for 1 hour. The oligonucleotide was then pelleted by centrifuging at 13,000 rpm for 30 minutes and air-dried. It was dissolved in 100 microlitres of TE or double distilled water. Further desalting was carried out using a Sephadex G25 spun column.

2.2.12 RNA Isolation

RNA was prepared using TRIZOL reagent, monophasic mixture of phenol and guanidium isothiocyanate (GITC), according to the manufacturer’s instructions. Isolated RNA was dissolved in appropriate amounts of DEPC water, quantitated and visualized on gel.

2.2.13 Polymerase Chain Reaction (PCR)

PCR was done in a reaction mix containing 1X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin- Taq buffer 10A from Bangalore Genei), 250 mM each of the dNTPs, 200 ng primers and 1.5 units of Taq DNA polymerase. After an initial denaturation at 94°C for 2 min required number of cycles was carried out. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 4°C below melting temperature of the primer for 1 min and extension at 72°C for 1 min. A final extension for 7 min was given at 72°C for completion of elongation products.

2.2.14 Reverse transcription and polymerase chain reaction (RT-PCR)

RT reaction was carried out using the Superscript II first strand cDNA synthesis kit (Invitrogen) according to the manufacturers recommended protocol. 2 μg of total RNA was used as the template and oligo-dT was used as primers for reverse transcription in
20 μl reaction volume. The RNA was annealed with 500 ng of oligo-dT\textsubscript{(12-18)} at 65°C for 5 min in a 10 μl reaction volume. This was incubated at 42°C for 2 min in a reaction mix containing 1X RT buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 5 mM MgCl\textsubscript{2}, 10 mM DTT, 500 μM of each dNTP and 40 units of RNase inhibitor. The reverse transcription was carried out at 42°C for 50 min using 50 units of Superscript II RT enzyme. The reaction was stopped by incubation at 70°C for 15 mins. The reverse transcription product was used as template for semi-quantitative PCR reactions. The list of primers used for RT-PCRs in the study is listed below.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>Amplicon size (bp); Target gene</th>
<th>Annealing Temp, No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP2</td>
<td>5'-CGAATTCAATGTCCTGGGAAGAGGTGAA-3'</td>
<td>408 bp; human caspase-1</td>
<td>58°C, 40 cycles</td>
</tr>
<tr>
<td>CTP3</td>
<td>5'-CGAATTCAGGACAAACCGAAGGTGATC-3'</td>
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<td></td>
</tr>
<tr>
<td>IP-1</td>
<td>5'-CTCTCATGGTGAAGCCAGTCC-3'</td>
<td>301 bp; human</td>
<td>64°C, 40 cycles</td>
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<tr>
<td>IP-2</td>
<td>5'-GACAGAGACTTGACTGTATCCA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apaf-1</td>
<td>5'-GCTGATGGAACCTAAAGCTTTGGGA-3'</td>
<td>350/221 bp; both forms of human Apaf-1</td>
<td>64°C, 30-35 cycles</td>
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<tr>
<td>Apaf-2</td>
<td>5'-CACCTTTGAACGTGAGTCTGATTTCC-3'</td>
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<td></td>
</tr>
<tr>
<td>RR15</td>
<td>5'-GACCCCTTCATTGACCTCAACTA-3'</td>
<td>557 bp; human and murine GAPDH</td>
<td>64°C, 18-20 cycles</td>
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<td>RR17</td>
<td>5'-ACCTTGCCACAGCTTGGCAG-3'</td>
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<tr>
<td>ASC1</td>
<td>5'-CGAGGAGCTCAAGAATTTGAACGC-3'</td>
<td>387 bp Human ASC</td>
<td>58°C, 35 cycles</td>
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<td>ASC2</td>
<td>5'-TACTGCTATCCGCAGTCACTT-3'</td>
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<td></td>
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<tr>
<td>CTP1</td>
<td>5'-GCTCTGGAGGGGATGTGGAC-3'</td>
<td>355 bp; human caspase-2</td>
<td>60°C, 32 cycles</td>
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<tr>
<td>CTP2</td>
<td>5'-TCAGTGATTCAAAATCCATTAA-3'</td>
<td>723 bp; human caspase-3</td>
<td>56°C, 30 cycles</td>
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<tr>
<td>CTP3</td>
<td>5'-AAATTCTGTTGCCACCCCTTGC-3'</td>
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<tr>
<td>C6P1</td>
<td>5'-TTC AAT CAT GAG AGG TTC TTT TGG-3'</td>
<td>654 bp; human caspase-6</td>
<td>60°C, 35 cycles</td>
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<tr>
<td>C6P2</td>
<td>5'-TGGGTCTTTGCAAAAGTCCAC-3'</td>
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<tr>
<td>Oligonucleotide</td>
<td>Sequence</td>
<td>Length (bp)</td>
<td>Temperature/Repetitions</td>
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<tr>
<td>C7P1</td>
<td>5'- TGTATCCATCAGGATCCAGTCCTG-3'</td>
<td>302 bp</td>
<td>56°C, 30 cycles</td>
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<td>C7P2</td>
<td>5'- GCTCACTCCATCTCAGTCAGTG-3'</td>
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<td>C8P1</td>
<td>5'- CTGGACTACATTCCGCAAAGGA-3'</td>
<td>330 bp</td>
<td>55°C, 32 cycles</td>
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<td>C8P2</td>
<td>5'- TCATCATCCAGTTGCTCATTTGG-3'</td>
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<tr>
<td>C10P1</td>
<td>5'- GTGAGCTTTTCGAGAAGCTTC-3'</td>
<td>382 bp</td>
<td>62°C, 29 cycles</td>
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<tr>
<td>C10P2</td>
<td>5'- CTGAGTCAATGCTTCTGACAG-3'</td>
<td></td>
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</tr>
</tbody>
</table>

2.2.15 Identification of putative p53 binding sites in caspase-3 gene

The genomic location and sequence of human caspase-3 gene was determined using the human genome blast at NCBI. Using the NCBI software Spidey, intron-exon structure of the gene was determined. Presences of p53 binding sites were determined in these sequences using the Matlnspector 2.2 software (Genomatix) with a stringency level of 0.6 for both core and matrix.

2.2.15 End-labelling and Purification of End-labelled Oligonucleotides

Annealed double-stranded oligonucleotides for gelshifts were end-labelled with T4 polynucleotide kinase at 37°C for 10 min using γ-32P ATP. About 100 ng of the oligonucleotide was mixed with 1X polynucleotide kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl2 and 5 mM DTT), 10 units of T4 polynucleotide kinase and 40 μCi of γ-32P ATP in a 10 μl reaction volume. The labelling reaction was terminated with 1 μl of 0.5 M EDTA after 30 minutes. The volume was then made up to 100 μl with double-distilled water and the probe was purified using Sephadex G-25 or G50 spun column in order to remove unincorporated label. 2 μl of the sample was spotted on to Whatman No.3 paper extent of γ-32P incorporation was measured using scintillation counter.

2.2.16 Construction of pCAT-promoter Plasmids with Putative p53 Binding Sites Cloned as Enhancer Elements

Oligonucleotides corresponding to putative p53-binding sites were synthesized with ends corresponding to BamH I site. 2 μg each of the oligonucleotides were annealed by incubating at 65°C for 5 min in 10mM Tris (pH 8.0) and 50 mM NaCl solution, followed by cooling to 37°C in 40 μl volume. The annealed oligonucleotides

40
were diluted 1/10 in water. 1 μl of this diluted product was used for ligation with pCAT-promoter vector digested with BamHI.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3P1</td>
<td>5'-GATCTACAGACATGTGCCACCATGCCCTGGTT-3'</td>
<td>Site 1</td>
</tr>
<tr>
<td>C3P2</td>
<td>5'-GATCAACCAGGCATGGTCACATGTCTGTA-3'</td>
<td>Site 1</td>
</tr>
<tr>
<td>C3P3</td>
<td>5'-GATCAACCAGGCATGGTCACATGTCTGTA-3'</td>
<td>Site 2</td>
</tr>
<tr>
<td>C3P4</td>
<td>5'-GATCAACCAGGCATGGTCACATGTCTGTA-3'</td>
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</tbody>
</table>

The identity and integrity of the cloned oligonucleotides were confirmed by sequencing. The list of oligonucleotides cloned as putative p53-responsive enhancer elements is given above.

2.2.17 Mammalian Cell Culture

Cell lines were maintained in DMEM supplemented with 10% FBS with penicillin, streptomycin and kanamycin at 37°C in a humidified 5% CO₂ containing incubator. Sub-culturing was done by incubating the cells with trypsin-EDTA solution after rinsing with sterile 1X PBS.

2.2.18 Transfection of DNA in Mammalian Cells

Cell were transfected with required plasmids using Lipofectamine 2000 reagent according to the manufacturer's instructions. Cells were trypsinized and required number plated in antibiotic free medium the day before transfection. Plasmids (Total of 500 ng per cover slip and 2 μg for each 35 mm dish) and Lipofectamine 2000 (1 μl for coverslips and 4-5 μl for 35 mm dishes) were diluted in serum-free DMEM, mixed, incubated for 20 minutes and added on to the cells. Transfections were stopped after 5 hours by replacing transfection medium with complete DMEM.

2.2.19 Immunofluorescence

For immunofluorescence, the cells were grown on cover slips and processed as described by Radha et al., 1994. The cells, after required treatments or transfections, were washed with PBS and fixed with 3.7% formaldehyde in 1X PBS for 10 min at room temperature. They were then permeabilized using 0.5% Triton-X 100 and 0.05% Tween-20 in 1XPBS for 6 minutes at room temperature. The cells were then washed and incubated with PBS containing 2% BSA for 1 hour at room temperature for blocking. They were then incubated for required time with primary antibody that is diluted in PBS.
containing 2% BSA. After washing with PBS, cells were incubated with fluorophore-conjugated secondary antibody in blocking solution for 1 hour at room temperature. The cells were again washed with PBS and mounted on glass slides in mountant. In order to visualize mitochondria the cells were incubated with 500nM Mitotracker Red in serum free DMEM for 25 minutes. Subsequently the cells were washed with serum free medium to remove excess dye and stained using 3.7% formaldehyde in serum free medium. The cells were observed using an Olympus BX60 fluorescence microscope and images were captured with a CCD and analysed using the Image-Pro software.

2.2.20 Estimation of apoptosis

For estimation of apoptosis cells were plated on cover slips and required transfections or treatments were carried out. At appropriate time the cells were fixed and stained for required proteins by immunostaining. The cells were mounted in DAPI containing mountant to observe nuclear morphology. Apoptotic cells were detected by shrinkage, lower refractility and condensed chromatin. At least 200 expressing cells were counted from each cover slip. Mean and standard deviation of percentage of apoptotic cells from at least 3 independent experiments performed in duplicate were used in analysis. Untransfected apoptotic cells were also counted from each cover slip and this was used for calculating background apoptosis.

2.2.21 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE, as described by Laemmli, 1970, was carried out using a discontinuous buffer system. Stacking gel (0.125 M Tris-HCl pH 6.8, 5% acrylamide and 0.1% SDS) and the resolving gel (0.375 M Tris HCl pH 8.8, 10 or 12 % acrylamide and 0.1% SDS) were polymerised using TEMED and Ammonium per sulphate . The gels were run using buffer containing 0.025M Tris, 0.192M glycine and 0.1% SDS at 20 mA constant current. After stacking of proteins at the resolving front and subsequent entry into the resolving gel, current was increased to 30 mA till the end of the run.

2.2.22 Western Blotting

Proteins resolved using the SDS-PAGE gel were blotted onto nitrocellulose membranes (Hybond C from Amersham or Immobilon P from Millipore) using the semidry apparatus (Pharmacia). The semidry transfer buffer containing 39 mM glycine, 48 mM Tris-HCl, 0.0375% SDS and 20% methanol was used and transfer was carried out at constant current at 0.8 mA/cm² for 1-2 hours. After transfer, the proteins were stained using Ponceau S solution and the positions of molecular weight markers were
marked using pencil. Subsequently, blots were incubated in 5% Blotto (Santacruz) in TBST (10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 1 hour at room temperature. The blots were then incubated with the required dilution of primary antibody in 0.5% Blotto in TBST for 1-2 hours at room temperature or overnight at 4°C. Non-specifically bound primary antibody was removed by three 5-minute washes with TBST. Subsequently, blots were incubated with appropriate dilution of secondary antibody in 0.5% Blotto in TBST for 60 minutes. After three washes with TBST, blots were processed for alkaline phosphatase reaction or enhanced chemiluminescence (ECL) detection. For alkaline phosphatase colour reaction the blot was developed with 0.1M Tris HCl pH 9.0, containing 10 mM each MgCl₂ and NaCl, 0.005% BCIP and 0.01% NBT. ECL was done using the Western lighting chemiluminescence reagent from Perkin Elmer and different exposure times were given to get the right intensity of the signal.

2.2.23 Preparation of cytosolic extracts for western blot analysis of cytochrome c

Digitonin based permeabilization technique was used to release cytosol from cells. Transfected cells were trypsinised after 24 hours and suspended in permeabilization buffer (PBS with 250 mM sucrose, 40 mM KCl, 10 mM Hepes and 100 μg/ml digitonin (Sigma)). Under these conditions >95% cells were found permeabilized when stained with 0.2% trypan blue solution. After incubation for one minute on ice, the cells were centrifuged at 12000g for 5 minutes at 4°C. Supernatant was collected as cytosol, separated on 13% SDS-polyacrylamide gel and blotted on to Immobilon-P (Millipore Corp). Western blotting was carried out to detect relevant proteins.

2.2.24 β-Galactosidase Assay

The assay for β-galactosidase activity in cell extracts was done according to the method described in Promega protocols and applications guide. For each assay 20 μl of lysate was diluted to 100 μl with 1X RLB (Reporter Lysis Buffer). Equal volume of 2X β-Galactosidase assay buffer was added and the tube was incubated at 37°C until yellow colour developed (5-10 min). The reaction was stopped by the addition of 400 μl of 1M Na₂CO₃ and the absorbance was measured at 420 nm.

2.2.25 Chloramphenicol Acetyl Transferase (CAT) assay

Lysates from cells transfected in 35 mm dishes were made using 1X RLB (Promega). 200 μl of 1X RLB was added to each well after initially removing the medium and rinsing the cells with 1X PBS. The plate was kept in a shaker for 15 min and the cells were subsequently scraped using a policeman. Repeated pipetting was done to obtain a suspension and the debris was pelleted down by centrifuging at 10,000 rpm for
2 min at room temperature. The supernatant was used for β-galactosidase assays and CAT assays. Before setting up CAT assays, the lysates were heated at 60°C for 10 min to inactivate endogenous deacetylases. Heat-treated lysates were cleared of denatured proteins by centrifugation at 10,000 rpm for 1 min at room temperature and the supernatant was aspirated and used for CAT assays. Generally, a reaction containing 1 μl 14C-chloramphenicol (25 μCi/ml), 1 μl acetyl coenzyme A, 20 μl lysate and the volume was made up to 40 μl with 1X RLB. The reaction mix was incubated at 37°C for required time and the amount of acetylated chloramphenicol was assayed using scintillation counter. For this, the samples were vortexed with mixed xylenes (300 μl) for 30 sec followed by centrifugation at 12000 g for 2 min. The upper phase (mixed xylenes) was aspirated into fresh tube and subsequently extracted twice with 100 μl of Tris buffer (0.25M Tris-HCl pH 8.0). After extractions, a fixed volume (usually 200 μl) was added to 5ml of scintillation fluid followed by scintillation counting in the 14C channel.

2.2.26 Preparation of nuclear extracts

Nuclear extracts from MCF-7 cells treated with 500 ng/ml doxorubicin for 24 hours were prepared according to the procedure described in Hagenbuchle et al., 1992. Approximately 2-3 X 10^6 cells were harvested by scraping the culture in ice cold PBS with rubber policeman. After a brief spin the cells were suspended in 400 μl of hypotonic buffer A (10mM HEPES-KOH pH7.9, 1.5 mM MgCl₂ 10 mM KCl, 0.5mM DTT, 0.2 mM PMSF). The cells were allowed to swell on ice for 10 minutes and vortexed for 10 seconds. The nuclear pellet was collected by centrifugation at 12000 g for 1 minute at 4°C and resuspended in 100μl ice-cold high salt buffer C (20 mM HEPES-KOH pH7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂ 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). The nuclear pellet was incubated on ice for 20 minute in buffer C before centrifugation as above. The supernatants were stored at −70°C in aliquots of 20 μl each.

2.2.27 Electrophoretic Mobility Shift Assay (EMSA)

The PAGE purified oligonucleotides corresponding to the putative p53 binding sites were annealed, end-labelled and column purified. Binding reactions were performed in 10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 4% glycerol, 1 mM EDTA, 2 mM MgCl₂, 40 mM NaCl, 50 ng poly (dl-dC). Nuclear extract prepared from doxorubicin treated MCF-7 cells was then added followed by the addition of 6 ng of labelled probe
(10^5 cpm). The reaction mix was incubated at 30°C for 30 min. Samples were loaded on a 4% native acrylamide gel and subjected to electrophoresis in 0.5 X TBE buffer at 4°C.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Site</th>
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<tbody>
<tr>
<td>P1</td>
<td>5' -CTGGGAATTGTGGGGAAATTCCTGT-3'</td>
<td>Mutant</td>
</tr>
<tr>
<td>P2</td>
<td>5' -ACAGGAATTCCCACTTTCCACAG-3'</td>
<td>Mutant</td>
</tr>
<tr>
<td>P3</td>
<td>5' -CTGGGCATGGTGGGGCAGCCCTGT-3'</td>
<td>Wild type</td>
</tr>
<tr>
<td>P4</td>
<td>5' -ACAGGCATGCCCACCATGCCAG-3'</td>
<td>Wild type</td>
</tr>
<tr>
<td>Consensus 1</td>
<td>5' -CGAGAGACATGCCCAGGCCTGT-3'</td>
<td>Consensus</td>
</tr>
<tr>
<td>Consensus 2</td>
<td>5' -AGGCATGCCTGGGCATGTCTCTCG-3'</td>
<td>Consensus</td>
</tr>
</tbody>
</table>

The gel was subsequently dried and subjected to phosphor-imager analysis. Name and sequence of oligonucleotides that were used in EMSA are listed above.

2.2.28 Sequence analysis

NCBI programs BLASTn and BLASTp were used to analyze sequence similarity and database search. BL2seq was used to align two nucleotide sequences based on their similarity (http://www.ncbi.nlm.nih.gov/BLAST/). WebCutter 2.0 was used for analyzing restriction enzyme sites. MatInspector v2.2 (http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl) was used for identifying p53 binding sites in genomic sequences. Genomic sequence alignment with mRNA to identify intron-exon organization was carried out using Spidey at NCBI (http://www.ncbi.nlm.nih.gov/spidey/). Sequence Manipulation Suite was used to arrange and reverse complement sequences (http://www.ualberta.ca/~stothard/javascript/).