CHAPTER - 2

MATERIALS AND METHODS
SUBJECTS

All human subjects were from the Indian population and belonging to both sexes.

CHEMICALS

Highly polymerised calf thymus DNA, Bovine serum albumin, Adenosine 5'-Triphosphate (ATP), Histopaque, N-methyl-N'-nitro-N-nitroso guanidine (MNNG), Ethidium Bromide, Ethylene Diamine Tetraacetic Acid (EDTA), Sodium Lauryl sulfate (SDS), Phytohemeagglutinin (PHA), Hydroxyurea (HU), Ribonuclease A, Leupeptin, Pepstatin, Phenylmethylsulfonyl Fluoride (PMSF), Tizma base, Dithiothreitol (DTT), penicillin, streptomycin, and Agarose were purchased from Sigma Chemical Co., St. Louis, MO, USA. Unlabelled nucleotides, 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate (dCTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and thymidine 5'-triphosphate (TTP) were purchased from Pharmacia Fine chemicals, Uppsala, Sweden. Poly (dA). Oligo (dT)12-18 was supplied by Midland Certified Reagent Company, Midland, TX, USA. Tritiated TTP (specific activity 30 Ci/mmol) was purchased from (Radiochemicals) Life Science, Amersham, England and Tritiated Thymidine (specific activity 17 Ci/mmol) and radio-phosphorous y-labelled ATP (specific activity 3000 Ci/mmol) was purchased from BRIT, Bombay, India. Restriction enzyme Hinf I and T4 Polynucleotide Kinase were purchased from Bangalore Genei, Bangalore, India. RPMI-1640 was purchased from Hi-media, India. Proteinase K and nylon membrane was purchased from Bohringer Mannheim GmbH., Mannheim, Germany 2,5-Diphenyl-1,3-Oxazole (PPO) and 2,2'-p-Phenylen-bis[5-Phenylloxazole] (POPOP) were purchased from Beckman instruments inc., Fullerton, CA, USA. Foetal Calf serum was purchased from Biological industries, Kibbutz Beit Haemek, Israel. β-Mercaptoethanol was purchased from Biorad laboratories, Hercules, CA, USA. 1 Kb ladder was purchased from Gibco BRL Life Technologies Inc., Green Island, NY, USA. GF/C filters were purchased from Schleicher and Schüll, Dassel,
Germany. Probe (TTAGGG)$_4$ was obtained from Rama Biotechnologies India Pvt. Ltd., Hyderabad, India. All other chemicals used were of analytical grade.

**LYMPHOCYTE ISOLATION**

Lymphocytes from anticoagulated blood were isolated by Ficoll-paque density gradient centrifugation (Boyum, 1976 and Smith et al., 1987).

Anticoagulated peripheral blood collected aseptically is mixed 1:1 with sterile physiological saline and overlaid carefully onto 0.5 volume of Ficoll-paque. The gradient centrifugation was done at 400 xg for 30 minutes at room temperature. The lymphocytes appear a clear white ring above the ficoll-paque layer while the other leukocytes form the interface with the ficoll-paque and the erythrocytes sediment down. The lymphocytes were harvested into ice-cold RPMI-1640 medium with 2 mM L-Glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum and washed thrice with medium and each time harvested by centrifugation at 400 xg for 8 minutes. The viability of the cells was determined by trypan blue exclusion and found to be >95%.

**PHA-STIMULATION**

This was done at a concentration of 2 μg PHA (M-form)/$10^6$ cells and incubating for 72 hours at 37°C in a CO$_2$ incubator. The cells were harvested and washed as described above.

**ULTRAVIOLET (UV) IRRADIATION**

Aliquots of the cells at a concentration of $10^6$ cells/ml medium without FCS were taken in petriplates (3.4cm diameter), placed on ice and exposed, in the presence of 5mM HU, to UV light (254 nm) at a dose of 20 J/m$^2$ and 40 J/m$^2$ using Phillips TUV 8, 15W germicidal lamp adjusted to deliver at the rate of 1 J/m$^2$ per second. The cells were collected by centrifugation at 400 xg for 10 minutes. These cells were used for the assessment of various DNA-repair parameters alongwith unexposed controls processed identically.
N-METHYL-N'-NITRO-N-NITROSO GUANIDINE (MNNG) TREATMENT

Aliquots of the cells at a concentration of $10^6$ cells/ml medium without FCS were taken and treated with $50 \mu M$ MNNG in 1% DMSO, in the presence of 5mM HU, and incubated at 37°C for 30 minutes. The cells were collected by centrifugation at 400 xg for 10 minutes and washed once with fresh medium containing 5mM HU, to remove the MNNG. These cells were used for the assessment of various DNA-repair parameters along with unexposed controls.

UNSCHEDULED DNA SYNTHESIS

Unscheduled DNA synthesis [UDS] was assessed as described earlier (Rao et al., 1996).

The cells were incubated at a concentration of $10^6$ cells/ml in fresh RPMI-1640 medium containing 10% FCS and 5 mM hydroxyurea and 5$\mu Ci$ $^3$H-thymidine for 2 hours at 37°C and the reaction was stopped with 1 ml ice cold 10% TCA. The cells were washed thrice with cold 5% TCA containing 10 mM sodium pyrophosphate and twice with 95% Ethanol on GF/C filters and the dried filters processed for liquid scintillation counting using a toluene based fluid containing 5g PPO and 0.5g POPOP per litre.

The UDS was expressed as femtomoles of $^3$H-thymidine incorporated per $\mu g$ of DNA.

ASSAY PROCEDURE FOR DNases

UV and AP DNases were assayed essentially according to the procedure of Rao and Rao, 1984, with slight modification.

The cells were homogenised in ice-cold double distilled water at a concentration of $10^6$ cells/ml. The homogenate was used for the assay of both the DNases.
UV DNase ASSAY

The reaction mixture contained in a final volume of 0.6 ml, 200 μg of UV irradiated DNA, 0.1 M Sodium acetate buffer, pH 5.0 and the homogenate. At the end of 2 hr incubation at 37°C in a water bath shaker, the reaction was terminated by the addition of 0.4 ml of 1 4N perchloric acid (PCA). The tubes were kept in ice for 10 minutes, after which they were centrifuged at 4,000 rpm for 5 minutes. The absorbence of the supernatant was measured spectrophotometrically at 260 nm against an appropriate blank.

The enzyme activity was expressed as μg of acid soluble DNA-phosphorous (DNA-P) liberated per mg protein or DNA in 2 hours at 37°C.

AP DNase ASSAY

The reaction mixture volume, method of assay and the expression of activity and other details are the same as in the case of UV DNase, except that the reaction mixture consisted of depurinated DNA as the substrate while 0.05 M Tris-HCl, pH 8.25 was used as buffer.

PREPARATION OF SUBSTRATES

UV irradiated DNA was prepared by irradiation of highly polymerised calf thymus DNA (2mg/ml water). The DNA solution was taken into petriplates as a thin layer, kept on ice and UV (254 nm) irradiated at a dose of $2 \times 10^4$ J/m² using Phillips TUV 8, 15 W germicidal lamp.

Apurinic (AP) or Depurinated DNA was essentially prepared as described by Sharper and Grossman, 1980.

Native calf thymus DNA (2mg/ml water) was mixed with equal volume of depurination buffer containing 40 mM Sodium Citrate, 40 mM NaCl, 40 mM Potassium Phosphate, pH 5.0 and incubated at 70°C for 15 minutes.
PREPARATION OF DNA POLYMERASE ENZYME EXTRACT

The homogenates of the cells were prepared by homogenising them at 10^6 cells/ml homogenisation medium containing 20 mM Tris (pH 7.5), 0.1 mM DTT, 0.1 mM EDTA, 1.0 mM MgCl₂, 5% Glycerol, 1% triton X-100, 0.5 mM PMSF, 1μg/ml Leupeptin, 1μg/ml Pepstatin A and 0.5 M KCl. The homogenate was kept on ice for 1 hour, to aid the complete extraction of the DNA polymerase enzymes. Then it was centrifuged at 1,00,000 xg for 1 hour. The clear, particle-free supernatant thus obtained was used as the enzyme source of DNA polymerases.

DNA POLYMERASES ASSAY

The activity of total DNA polymerase was assayed according to the procedure of Prapurna and Rao, 1996 and the activity of DNA polymerase e was according to the procedure of Prapurna and Rao, 1997.

DNA Total POLYMERASES ASSAY

The reaction mixture contained in a final volume of 50μl, 40 mM Tris-HCl, pH 7.5, 8mM MgCl₂, 1mM β-mercaptoethanol, 4mM ATP, 100μM each of dATP, dCTP, dGTP, 25μM TTP, 1µCi of 3H-TTP, 5µg of ‘activated DNA’ and the enzyme. The incubation is carried out at 37°C for 20 minutes. At the end of incubation, 200μg each of DNA and BSA were added as carrier and the reaction was stopped by the addition of 1ml of ice-cold 10% TCA containing 10mM Sodium pyrophosphate. The precipitate was washed thrice with cold 5% TCA and twice with 95% Ethanol on GF/C filters and the dried filters processed for liquid scintillation counting using a toluene based fluid containing 5 g PPO and 0.5 g POPOP per litre.

The specific activity is expressed as picomoles of ^3H-TMP incorporated into the acid insoluble portion/mg protein or DNA/hour.
DNA POLYMERASE β ASSAY

The reaction mixture volume, method of assay and the expression of activity and other details are the same as in the case of Total DNA polymerases, except that the reaction was run with activated DNA as template primer at pH 8.25 (pH optimum for DNA polymerase activity).

DNA POLYMERASE ε ASSAY

The reaction mixture volume, method of assay and the expression of activity and other details are the same as in the case of DNA polymerase (total), except that the reaction mixture contained 40 mM Tris-HCl, pH 7.5, 0.8mM MgCl₂, 5μg bovine serum albumin, 2% Glycerol, 2mM DTT, 4μM TTP, 1μCi of ³H-TTP, 0.025 (A 260) units of poly (dA). oligo (dT)₁₂₋₁₈ as template primer and the enzyme.

DENATURED DNA

Calf thymus DNA dissolved in distilled water at a concentration of 2 mg/ml was kept in boiling water bath for 10 minutes and then rapidly cooled in ice.

PROTEIN ESTIMATION

Untreated cells were homogenised in ice-cold double distilled water at a concentration of 10⁶ cells/ml. The homogenate was used for protein estimation, according to the method of Lowry et al., 1951.

DNA ESTIMATION

Untreated cells were homogenised in ice-cold double distilled water at a concentration of 10⁶ cells/ml. The homogenate was incubated in buffer (pH 8.0) containing 10mM Tris.Cl (pH 8.0), 1 mM EDTA (pH 8.0), 20μg/ml RNase A at 37°C for 30 minutes and 100μg/ml Proteinase K at 50°C for 1 hour. The cell lysate was precipitated with ethanol and dissolved in TE buffer and the absorbence was read at 260 nm.
ISOLATION OF HIGH MOLECULAR MAMMALIAN DNA

DNA extraction was according to the method of Blinn and Stafford as given by Sambrook et al., 1989.

The cells were suspended at a concentration of $10^6$ cells/ml extraction buffer containing (10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% SDS, 20 μg/ml pancreatic RNase) and incubated at 37°C for 1 hour. Then Proteinase K was added to a final concentration of 100 μg/ml and incubated at 50°C for overnight (~16 hours). The solution was allowed to come to room temperature and equal volume of buffer saturated phenol was added and gently mixed. The phases were separated by centrifugation in a swing out rotor at 5000 x g for 15 minutes. The aqueous layer was separated and re-extracted once with phenol, twice each with Phenol:Chloroform:Isoamyl alcohol in ratio of 25:24:1 and with Chloroform:Isoamyl alcohol in the ratio of 24:1. After all the extractions, NaCl was added to the aqueous phase, to a final concentration of 0.2 M. Then two volumes of ice-cold isopropanol was added and left at -20°C overnight (~16 hours) for precipitation. The DNA was pelleted down at 5000 x g for 30 minutes. To the pellet was added 70% cold isopropanol and allowed to stand at room temperature for 30 minutes and the DNA was harvested by pelleting at 5000 x g for 30 minutes, air dried and then dissolved in Tris-EDTA (TE) buffer (pH 8.0). The concentration and purity of the DNA was estimated by measuring the absorbence at 260 nm and 280 nm against TE buffer. The quality of DNA was tested on 0.5% agarose gels.

RESTRICTION DIGESTION OF DNA

Restriction digestion of an aliquot of DNA was carried out at 37°C for 12-16 hours with 2 units Hinf I/μg DNA. To check the digestion, about 5 μl of sample was electrophoresed on a 0.5% agarose minigel (10 cms - 50 ml) at 30V using 1x TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) for a period of 3-5 hours, stained with ethidium bromide and visualised under UV (300 nm). If the digestion was complete, the reaction was stopped with 1μl 0.5M EDTA (pH 8.0) and the
restricted DNA was precipitated with 0.2M NaCl and two volumes of ice-cold isopropanol, left at -20°C overnight, and pelleted the DNA at 5000 xg for 20 minutes at 4°C and then washed with cold 70% isopropanol. The DNA pellet was dried briefly, and dissolved in TE buffer, pH 8.0. The DNA was quantified by absorbence of the samples at 260 nm and 280 nm against TE buffer.

**DETERMINATION OF AVERAGE TELOMERIC LENGTH**

**Electrophoresis**

On an 0.5% agarose gel (volume 200 ml, length 25 cm) in 1x TAE buffer (0.04 M Tris acetate, 0.001 M EDTA), equal amounts (3 to 5 µg) of restriction enzyme digested DNA in 6x Neutral loading dye (0.25% bromophenol blue, 40% (w/v) sucrose in water) was loaded with the standard 1 Kb DNA ladder on either sides; electrophoresed at 25 V at 4°C until the dye front travels to two-third of the gels length (usually this may take about 24 hours). The gel was stained with ethidium bromide solution 0.5µg/ml of water, observed under UV light (310 nm) and the standards were marked. After complete destaining of the gel with several changes of water, it was denatured with 0.5N NaOH, 0.15M NaCl for 15 minutes and then with a change allowed to denature for another 20 minutes. Then the gel was set up for Southern transfer.

**Southern Transfer**

Alkaline Transfer of DNA onto Nylon membrane using the VacuGene XL unit [Pharmacia Biotech] was done at 30 mbars for 1 hour. The nylon membrane was pretreated by floating the membrane on the surface of a dish of deionised water until it wets completely from beneath, and then immersing the membrane in transfer solution (1 M NaOH) for at least 5 minutes. After the transfer is over the nylon membrane was washed with 2x SSC for 10 minutes at room temperature to remove any adhering gel particles, air dried, UV cross linked for 2 minutes at 254 nm and then baked at 70°C for 2 hours. The membrane is then processed for hybridisation.
Hybridisation:

Prehybridisation of the nylon membrane was in Church buffer (1 % Bovine Serum Albumin, crystalline grade, 7 % SDS, 1 mM EDTA, 0.5 M Phosphate buffer, pH 7.2) at 42°C for 24 hours. After prehybridisation the labelled probe (16 picomoles end-labelled with 50 μCi γ-32P-ATP using 10 units of T4 Poly nucleotide kinase, incubated at 37°C for 1 hour and purified through Sephadex G-50 column), was added and the hybridisation carried out at 42°C for 24 hours. After hybridisation the membrane was washed with 0.5x SSC + 1% SDS four times of 10 minutes each at 42°C, air dried and subjected to autoradiography to visualise telomeric DNA. The autoradiograms were scanned with UVP gel documentation system (Mitsubishi) and the average length of the telomeric DNA (ATL) is calculated from the formula

$$\text{ATL} = \frac{\sum (MW_i \times O_{di})}{\sum (O_{di})}$$

where $O_{di}$ is the densitometer output and $MW_i$ is the length of the DNA at position $i$. 