CHAPTER II

General methods
A) **Plasmid pBR322 supercoiled DNA isolation**

**Transformation**

A single colony of *E. coli* strain DH5α was inoculated into a 100 ml liquid broth (LB) medium containing 1 g tryptone, 1 g NaCl and 0.5 g yeast extract. The culture was incubated overnight at 37 °C with vigorous shaking (200 revolutions/min). 0.5 ml of the overnight culture was inoculated into a fresh 100 ml LB medium and further incubated for 3-4 h at 200 revolutions/min.

The cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C. The cell pellet was resuspended in 20 ml of 10 mM CaCl₂ and kept on ice for 10 min. Again the cell pellet was collected by centrifugation and resuspended in 2 ml of 10 mM CaCl₂. These cells are referred to as competent cells.

200 µl of competent *E. coli* cells were transferred into a 1.5 ml micro-centrifuge tube containing 10-20 ng of plasmid (pBR322). The tube was kept on ice for 30 min and then transferred to a water bath set at 45 °C for 90 sec. Immediately after, the tube was kept on ice. To this 800 µL of SOC medium (100 ml of LB medium containing 10 mM Glucose and 10 mM MgSO₄) was added and incubated for 45 min at 37 °C. 200 µl of the transformed cells were poured onto a SOC agar (SOC medium contain 1.5% agar) plate containing appropriate antibiotic (40 µg/ml ampicillin or 10 µg/ml tetracyclin). The plate was kept in an incubator set to 37 °C for **17-20 h**.
Purification of supercoiled plasmid DNA

2-3 pBR322 transformed E. coli colonies were inoculated in to a 100 ml of LB medium containing 40 μg/ml ampicillin and incubated overnight at 37 °C. 1 ml of this overnight culture was inoculated into 1 L LB medium containing 40 μg/ml ampicillin and further incubation was continued further for 17-20 h.

The cells were harvested by centrifugation at 4000 rpm for 15 min at 4 °C. The cells were lysed in 40 ml lysis buffer (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 10 mg/ml Lysozyme) at room temperature for 10 min. To this, 80 ml of freshly prepared alkaline solution (0.2 N NaOH and 1% SDS) was added and mixed. The solution was kept on ice for 10 min. The proteins were precipitated by adding 50 ml of saturated ammonium acetate, mixed well and kept on ice for 10 min.

The precipitate was removed by centrifugation at 10 000 rpm for 15 min at 4 °C and (poured the supernatant through glass wool. The crude DNA was precipitated by adding 0.7 volume of ice cold isopropanol and the solution was kept on ice for 20 min. The crude DNA pellet was collected at 12 000 rpm for 15 min at 4 °C. The supernatant was discarded and pellet was dried. The DNA pellet was dissolved in 25 ml of acid extraction buffer (0.75 M NaCl, 0.3 M sodium acetate pH 4.2 and 10 mM EDTA). To this, equal volumes of water saturated phenol was added and mixed well to remove both RNA and DNA binding proteins. The aqueous and the phenol layers were separated by centrifugation at 10 000 rpm for 15 min at 4 °C. The aqueous layer was removed without disturbing the interphase. The phenol layer was extracted once again with acid extraction buffer.
To the phenol layer 7.5 ml of reverse extraction buffer (1.5 M Tris base and 5 mM EDTA pH 8.0) and 7.5 ml of chloroform were added. The aqueous layer was collected and once again extracted with equal volumes of chloroform. The DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate and 0.7 volumes of isopropanol and the DNA pellet was collected as before. The DNA pellet was washed with 70% ethanol and dissolved in 0.5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0)

**B) Purification and characterization of topoisomerase II**

*Pure nuclei isolation*

All procedures were carried out at 4 °C. 500 g of liver was collected from 60 day old rats (Wistar strain) and homogenized in 3 L of homogenization buffer (10 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 25 mM KCl, 0.34 M sucrose and 0.1 mM PMSF, Buffer A) by applying 10-15 strokes at 3000 rpm. The mixture was centrifuged at 5000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in 900 ml of Buffer A containing 2.2 M sucrose. Nuclei were collected by centrifugation at 26 000 rpm for 1 h in a Beckman SW 26 rotor and supernatant was discarded. The pellet was washed with 300 ml of Buffer A containing 1 M sucrose, followed by 250 ml of Buffer A containing 0.1% Triton X-100. The nuclei pellet was dissolved in TGM buffer (10 mM Tris-HCl pH 7.5, 8 mM MgCl$_2$, 0.1 mM EDTA, 0.2 mM DTT and 40% glycerol) and stored frozen at -20 °C.
**Topoisomerase II purification**

The nuclear pellet was centrifuged, the supernatant was discarded and the pellet was resuspended in lysis buffer (5 mM potassium phosphate pH 7.5, 100 mM NaCl, 10 mM 2-mercaptoethanol and 0.5 mM PMSF). The nuclei were lysed using a Branson sonicator with a macroprobe for 30 s, with 1 min intervals over a period of 5 min. The progress of lysis was monitored by phase contrast microscopy. After lysis was completed, freshly prepared 10% polymin P (pH 7.8) was slowly added to the mixture over a period of 15 min, while stirring to a final concentration of 0.35%. The resultant precipitate was collected by centrifugation at 6000 rpm for 10 min. The pellet was resuspended in 250 ml of PR buffer (20 mM potassium phosphate pH 7.5, 10 mM NaHSO₃, 10% glycerol, 10 mM 2-mercaptoethanol and 0.5 mM PMSF). Proteins were extracted from the chromatin-Polymin P pellet by 0.55 M NaCl, with continuous stirring over a period of 30 min. Following this, the nucleic acids were reprecipitated with the addition of Polymin P to a concentration of 0.7%. The suspension was stirred again for 15 min and the pellet was removed by centrifugation as described above. The supernatant was filtered through glass wool, to which solid ammonium sulfate was added to a final concentration of 60% with continuous stirring. After 1 h, the resultant precipitate was collected by centrifugation at 10 000 rpm for 20 min. The pellet was resuspended in 100 ml of PR buffer and dialysed against 5 x 1 L of PR buffer over a period of 15 h. The precipitate formed during dialysis was removed by centrifugation at 26 000 rpm for 20 min in Beckman SW 28 rotor.

**Chromatography on Hydroxyapatite**

The clarified dialysed supernatant was loaded onto a Biogel-hydroxyapatite (Biogel-HTP) column (2 x 10 cm) which had been equilibrated with a buffer containing 200 mM
potassium phosphate in PR buffer. The column was washed with the equilibration buffer until there was no A$_{280}$ absorbing material. The bound proteins were eluted with a linear gradient of potassium phosphate (200 mM-700 mM) in PR buffer. The fractions that contained topoisomerase II activity were pooled and dialysed against PR buffer. The dialysed fractions were re-chromatographed on a second Biogel-HTP column and the procedure was repeated as described above. The fractions containing topoisomerase II activity were combined and dialysed against PR buffer. The dialysed fractions were concentrated by using a Centricon-10 microconcentrator.

**Gel-filtration chromatography**

The protein from chromatography on Biogel-HTP was layered on sephadex G100 gel-filtration column (2 x 100 cm) which was previously equilibrated with PR buffer. Fractions of 0.5 ml were collected and analyzed for topoisomerase II activity. The fractions containing topoisomerase II activity were pooled, dialysed against PR buffer and concentrated using Centricon-10 microconcentrator. The final enzyme preparation was dialysed against storage buffer (30 mM potassium phosphate pH 7.5, 50% glycerol, 0.1 mM EDTA, 0.5 mM DTT) and stored at -20 °C.

**Characterization of topoisomerase II**

1. **SDS-PAGE analysis**

Protein concentration was measured by Bradford method. 100 ng of protein was electrophoresed on 12% SDS-PAGE gel and silver stained according to Laemmli (1970) method (Figure 6 lanes 2 & 3).
Figure 6. SDS-PAGE analysis of topoisomerase II fractions

Lane 1. Molecular weight markers

Lane 2. Bio-gel HTP fraction

Lane 3. Gel filtration (G-100) fraction
2. **Immunoblotting analysis (Western blotting)**

Immunoblotting was performed by the method of Towbin *et al.*, (1979). Protein from SDS-PAGE gel was transformed to 0.2 \( \mu \text{m} \) nitrocellulose membrane using trans blot apparatus (Biorad, USA). Protein transfer was performed at 45 mA for 3 h in 25 mM Tris-HCl (pH 8.8), 192 mM glycine and 20\% methanol buffer. After blotting, the membrane was washed with TBS (10 mM tris-HCl pH 7.5 and 150 mM NaCl) and non-specific binding sites were blocked with 3\% (w/v) BSA for 2 h.

Then the immunoblot was incubated overnight at 4 °C in a polyclonal antibody directed against topoisomerase II. Following three washes with TBS, the blot was incubated with secondary antibody (goat anti-rabbit conjugated with alkaline phosphatase) for 1 h. After washing three times with TBS, immunoreactive topoisomerase II was visualised using 5-bromo - 4 - chloro - 3 - indoylphosphate/nitrobluetetrazoliumchloride (BCIP/NBT) chromogen (Figure 7).

3. **Enzyme Catalyzed Relaxation Assay**

The reaction mixture of 20 \( \mu \text{l} \) contained 50 mM Tris-HCl pH 8.0, 120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM MgCl\(_2\), 30 \( \mu \text{g/ml} \) BSA, 1 mM ATP and 0.6 \( \mu \text{g} \) of negatively supercoiled pBR322 plasmid DNA and with increasing concentrations of drugs. The reaction was initiated by addition of topoisomerase II to the concentration of 8 nM and incubated at 30 °C for 20 min. The reaction was stopped by addition of SDS to a final concentration of 0.5\%. To this reaction mixture, 3 \( \mu \text{l} \) of loading dye (60\% sucrose ,0.5\% bromophenol blue, 0.5\% xylene cyanol and 10 mM tris-HCl pH 8.0) was added. Products were separated on 1\% agarose gel in 0.5x tris-acetate buffer (40 mM tris acetate pH 8.3
Figure 7

Figure 7. Western blot analysis of topoisomerase II

Lane 1. Molecular weight markers

Lane 2. 170 kDa band corresponds to Topoisomerase II.
and 2 mM EDTA) at 50 V for 16 h. The gel was stained in ethidium bromide (10 \( \mu g/ml \)) and visualised under uv and photographed (Figure 8 panel A).

4. Cleavage Assay

The formation of cleavage complex was assayed by the procedure of Brigette et al (1996). The reaction mixture contained essentially the same buffer used in the relaxation assay with increasing concentrations of drugs. The reaction was initiated by addition of 0.6 \( \mu g \) pBR322 DNA and 40 nM of topo II and incubated at 30 °C for 15 min. The reaction was stopped by adding SDS to a final concentration of 0.5% and 2 \( \mu l \) of 250 mM EDTA. The DNA bound protein was digested by incubating the reaction mixture with 2 \( \mu l \) of 1 mg/ml solution of Proteinase K at 45 °C for 1 h. 3 \( \mu l \) of loading dye was added and the products were separated on a 1% agarose gel, stained in ethidium bromide and photographed under uv. The linear DNA band was quantified as percentage of total DNA in a UVP gel documentation system (Figure 8 panel B).

C) ATPase assay

a) Spectrophotometric assay

In the spectrophotometric assay, rapid conversion of ADP to ATP by pyruvate kinase and phosphoenolpyruvate, a reaction coupled to NADH oxidation, was used to measure the rate of ATP hydrolysis following the procedure described by Morrical et al (1985). Under steady-state conditions, the rate of ATP hydrolysis is directly proportional to the rate of the absorbance decrease observed at 340 nm. The assay was performed in a Shimadzu UV-650A spectrophotometer. The reaction mixture of 1 ml containing reaction buffer (20
Figure 8. Characterization of Topoisomerase II. (A) Topoisomerase II catalysed relaxation activity. Supercoiled pBR322 DNA (lane 1) was incubated with topoisomerase II in the absence (lane 2) or presence of 50, $\mu$M m-AMSA (lane 3). (B) Cleavage reaction was conducted by incubating pBR322 DNA (lane 1) with topoisomerase II (lane 2) in presence of 50$\mu$M m-AMSA (lane 3)
mM Tris-HCl pH 7.5, 0.1 mg NADH, 100 μM DTT, 1 mM ATP, 2 mM phosphoenol pyruvate, 4 mM MgCl₂) 12.5 units pyruvate kinase and 12.5 units lactate dehydrogenase was incubated at 37 °C for 5 min. The incubation was continued with further addition of 0.3 μg of DNA with increasing concentrations of drugs and 8 nM topo II for 30 min and absorbance was recorded at 340 nm.

b) Thin layer chromatography (TLC) assay

In the TLC method, reaction mixture of 20 μl containing the enzyme catalysed relaxation assay buffer with 1 mM [γ³²P ATP] (0.025 Ci/mMol), 8 nM of topo II and 0.6 μg of pBR322 DNA with increasing concentrations of drugs was incubated at 30 °C for 15 min. The reaction was stopped by adding 2 μl of 250 mM EDTA. The reaction mixture was spotted on TLC plastic sheets coated with Polyethyleneimine (DC-PlastikfolienPEI-Cellulose F, Merck) and chromatographed in freshly made 1 M lithium chloride. The bands were monitored with reflecting uv at 366 nm in a Photodyne gel documentation system. In lithium chloride solution, $^{32}$P migrates first, followed by ADP and ATP. The areas corresponding to reaction products were cut out of the chromatogram and counted in a Wallac scintillation counter using a toluene based scintillation fluid (Osheroff et al., 1983).

D) Immunoprecipitation assay

The cleavage reaction was conducted with cobalt drugs with the concentrations at which they form maximum 'Cleavage complex'. After 15 min, topo II in the cleavage complex and in free form was immunoprecipitated with 20 μl of 1x relaxation buffer containing
0.04 units of anti-topo II antibody. The mixture was rocked for 1 h at 4 °C before addition of 50 μl of protein-A agarose. After 1 h incubation, samples were washed five times with 1x relaxation buffer and the immunocomplexes were eluted from the protein-A agarose by adding 50 μL of 4 N HNO3. The sample volumes were made up to 5 ml and analyzed for parts per million of cobalt metal by Atomic Absorption Spectroscopy by a Hitachi AAS650 F spectrometer.

E) DNA-Drug Binding Studies

(a) temperature melting studies

Calf thymus DNA (sodium salt) was dissolved in 1 mM sodium phosphate buffer containing 1 mM sodium chloride. The concentration of DNA was adjusted such that 1 ml of DNA gives an absorbance of ~1.0 (150 μM). This DNA was used in melting temperature studies in the presence of drugs. The concentration of metal complexes was adjusted such that drug to DNA nucleotide ratios of 1:20, 1:10, 1:5, 1:2 and 1:1 respectively were maintained in 1 ml of phosphate buffer. The samples were incubated in 1 ml quartz cuvettes for 2 min to allow drug-DNA binding. The cuvettes were placed in a Hitachi 150-20 spectrophotometer, and the instrument was set to give a 1 °C rise in temperature per min, and the increase in absorbance was recorded. The absorbance was recorded from 40 °C to 90 °C. Tm was determined from these absorbance values and the data was plotted.
(b) *Circular dichroic spectral studies*

Circular dichroic spectra of pBR322 DNA (20 μg) was monitored in presence of 20 mM of cobalt and copper drugs in a Jasco J-715 spectropolarimeter. The DNA and drug concentrations respectively corresponded to 0.6 μg of DNA and the concentration of drugs at which complete inhibition of enzyme activity was achieved as per the topo II relaxation assay. 2.5 mM of m-AMSA corresponding to 60 μM (as used in the relaxation assays) was included as a positive control. The spectra were measured in a quartz cuvette of 1 cm path length. The data was presented graphically as molar ellipticity ([θ] X 10^3 deg-cm^2/dmole) versus wavelength (nm).