CHAPTER II

MATERIALS AND METHODS
2.1. MATERIALS

DNase I, protein A, molecular mass marker proteins for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric point (pI) calibration kit and ampholines were obtained from Pharmacia Fine Chemicals, Upsala, Sweden. RNase A, Hoescht 33258, sucrose, ethyleneglycol-bis-(β-aminoethylether)N, N, N', N'-tetraacetic acid (EGTA), β-mercaptoethanol, sodium dodecyl sulphate (SDS), Nonidet P-40, Triton X-100, Tween-20, bovine serum albumin (BSA), dithiothreitol (DTT), Coomassie Brilliant Blue R-250, adenosine 5-monophosphate, sodium pyrophosphate, horse radish peroxidase (HRP)-conjugated anti-rabbit IgG, HRP-conjugated antimouse IgG, 10X Ponceau Red concentrate and diaminobenzidine (DAB) were obtained from Sigma Chemical Company, St. Louis, U.S.A.

Acrylamide and bis-acrylamide were from Serva Fine Chemicals, Heidelberg, Germany. TEMED and Biogel-P2 were from Bio-Rad, Richmond, USA. Phenylmethanesulphonylfluoride (PMSF) was from Boehringer-Mannheim, Germany. Nitrocellulose membrane filters were from Schleicher & Schuell, Dassel, Germany. Biotinylated goat anti-mouse IgG, streptavidin-peroxidase conjugate and O-phenylenediamine were obtained from Bethesda Research Laboratories, Gaithersberg, USA, as part of a Streptavidin HyBRL screen TM Kit. Iodogen and N-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) were obtained from Pierce Chemicals, U.S.A. Na¹²⁵I (carrier-free) was obtained from Bhabha Atomic Research Centre, Bombay, India. ECL™ reagents 1 and 2 were obtained from Amersham International, UK.

All the chemicals required for cell culture and hybridoma work were of cell culture grade. ISCOVE's modified Dulbecco's medium (IMDM) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO Laboratories, New York, USA. L-glutamine, nystatin, polyethylene glycol (PEG 3500), hypoxanthine (6-
hydroxypurine), thymidine, aminopterin (4-aminofolic acid), penicillin, kanamycin, streptomycin and gentamycin were obtained from Sigma Chemical Company, USA. Fetal calf serum was from Flow Laboratories, England.

Chemicals required for peptide synthesis such as L-amino acids, nitroarginine, Merrifields's resin (polystyrene 1% divinyl benzene), trifluoroacetic acid (TFA), triethylamine (TEA), dicyclohexylcarbodiimide (DCC) and cesium carbonate were obtained from Sigma Chemical Company, USA. HPLC grade solvents were from Spectrochem, Bombay, India. Dichloromethane, methanol, 8-hydroxyquinoline and ethylacetate were obtained from Merck Chemicals, India.

All other chemicals were of analytical grade and were obtained from local suppliers.

2.2. METHODS

2.2.1. Preparation of nuclei

Nuclei were purified from the livers of mice or rats as described by Blobel and Potter (1966) with minor modifications. All the steps were carried out at 4°C unless mentioned specifically. Six mice or one rat was dissected to give ~8 g of liver. The tissue was washed with STM/PMSF buffer (0.25 M sucrose, 50 mM Tris. HCl,pH 7.4, 5 mM MgCl₂, 1 mM EGTA and 0.5 mM PMSF), minced and homogenized in eight volumes of homogenization buffer (250 mM sucrose, 50 mM Tris.HCl, pH 7.4, at 4°C, 5 mM MgCl₂, 1 mM EGTA, 0.5 mM PMSF, 25 mM KCl, and 2 mM DTT), by ten up and down strokes in a tight-fitting Dounce homogenizer. The homogenate was filtered through 6 layers of gauze cloth and centrifuged at 800 X g for 10 min at 4°C. The crude nuclear pellet was washed once with the same volume of STM/PMSF and then resuspended in ~ 20 ml of DS buffer (2.1 M sucrose, 50 mM Tris.HCl, pH 7.4, 5 mM MgCl₂, 1 mM EGTA, 0.5 mM PMSF). Six ml of resuspended pellet was loaded
over 6 ml of DS in 4 X 13 ml tubes of a Beckman SW41 rotor. Samples were centrifuged at 70,000 X g for 1 h in a Beckman 8M ultra-centrifuge. Nuclei formed a tight pellet at the bottom of the tube, whereas red blood cells and other contaminants remained suspended on top or at the interphase of the two layers. The supernatant was discarded carefully and sides of the tubes were wiped well with tissue paper. The nuclear pellet was resuspended in STM/PMSF (6 ml STM for each of the four pellets) and centrifuged at 800 X g for 10 min. Washed nuclei were resuspended in the required volume of desired buffer and counted by a hemocytometer. On an average, this protocol yielded ~ 5 X 10^8 nuclei per 8 g of liver.

### 2.2.2. Preparation of subcellular fractions

Liver tissue was homogenized as described in the previous section. The postnuclear supernatant obtained after sedimentation of nuclei at 800 X g for 10 min was processed as follows to isolate different fractions. To obtain partially purified mitochondria, the postnuclear supernatant was centrifuged at 10,000 X g for 10 min and mitochondrial pellet obtained was washed once with STM/PMSF. One aliquot of postmitochondrial supernatant was centrifuged at 100,000 X g for 30 min to pellet membranes. The supernatant after sedimenting membranes was taken as the cytosolic fraction.

Endoplasmic reticulum was prepared by the method described by Scheele et al. (1980). Postmitochondrial supernatant was obtained as described above and then layered over a discontinuous gradient of 1.3 M/2.25 M sucrose. Samples were centrifuged at 100,000 X g for 60 min. Rough endoplasmic reticulum banded at the 1.3 M/2.25 M sucrose interface.
2.2.3. Enzyme assays

2.2.3.1. 5'-Nucleotidase: 5'- Nucleotidase (EC 3.1.3.5) was assayed by the method of Aronson and Touster (1974). The reaction mixture consisted of 50 mM 5'-AMP, pH 7.0, 0.5 M glycine.NaOH, pH 9.1, 0.1 M MgCl₂ and H₂O mixed in the ratio of 1:2:1:5. 50 µl of sample (~30 µg protein) were added to 450 µl of the above reaction mixture and incubated at 37°C for 30 min. The reaction was terminated by addition of 2.5 ml of 8% TCA and inorganic phosphate was estimated by the method of Fiske and Row (1925) as follows. The above solution was chilled in ice for 10 min and centrifuged at 3000 X g for 5 min at 4°C, 2 ml of the supernatant was mixed with 2.3 ml H₂O, 0.5 ml of ammonium molybdate (2.5% in 5N H₂SO₄) and 0.2 ml of ANSA reagent (0.5 g α-naphthylsulphonic acid in 195 ml 15% NaHSO₃, 5.0 ml of Na₂SO₃·7H₂O). This was allowed to stand at room temperature for 10 min. Absorbance at 660 mM was measured in a Beckman spectrophotometer. Standard curves were obtained with sodium phosphate and used to calculate the phosphate released by the samples and hence the enzyme activity.

2.2.3.2 Succinate dehydrogenase: Succinate dehydrogenase (E.C. 1.3.99.1) was assayed by the procedure of Earl and Korner (1965). The reaction mixture consisted of 20 mM sodium succinate, 50 mM sodium phosphate buffer, pH 7.5, 1.0 mM KCN, 0.04 mM 2,4-dinitrophenol indophenol, and samples (~100 µg protein) in a total volume of 3.0 ml. The reaction mixture was incubated at room temperature for 20 min, and absorbance at 600 nm was measured after centrifuging the reaction mixture at 12,000 X g for 1 min in an Eppendorf microfuge. Quantitations were made on the basis of the relationship that the reduction of 0.0524 µmol of 2,4-dinitrophenol indophenol causes a change of absorbance of 0.1 unit.
2.2.3.3. **Pyrophosphatase**: Pyrophosphatase (EC 6.3.1.1.) was assayed by the procedure described by Shatton et al. (1981). The reaction mixture (0.5 ml) consisted of 40 mM histidine-HCl, pH 7.2, 4.0 mM MgCl$_2$, and 0.2 mM sodium pyrophosphate, to which 10 μg of sample protein was added. The mixture was incubated for 10 min at room temperature, and chilled in ice. To this reaction mixture, 50 μl of 0.6 M HClO$_4$ was added and contents were centrifuged at 3000 x g for 10 min. The supernatant was mixed with 0.45 ml of colour reagent, kept in ice for 10 min. Absorbance at 660 nm was measured. Standard curve was obtained with sodium phosphate. The colour reagent was prepared as follows: 30 ml of 0.1% malachite green and 10 ml of 4.2% ammonium molybdate were mixed by stirring at room temperature for 30 min and filtered through analytical grade filter paper prewashed with 1N HCl. Filtrate was chilled in ice and 1 ml of concentrated H$_2$SO$_4$ was added.

2.2.4. **Biochemical estimations**

2.2.4.1. **Protein**: Proteins were estimated by a modification of Lowry's method (Parnaik et al. 1983). One ml of alkaline tartrate solution (2% Na$_2$CO$_3$, 0.2% Na, K-tartarate and 0.002% CuSO$_4$ in 0.1 N NaOH), was added to 0.2 ml of protein solution. The mixture was allowed to stand at room temperature for 20 min. SDS (0.1 ml of 0.5%) and Folin-Ciocalteau reagent (0.1 ml of 2 N) were added in quick succession with rapid vortexing and the mixture was allowed to stand for 30 min at room temperature. Absorbance at 760 nm of the mixture was then measured in a spectrophotometer. A standard curve was obtained using BSA.

2.2.4.2. **Phospholipid**: Phospholipid content of different subcellular fractions was determined by the method of Stewart (Stewart 1980). Phospholipids were extracted from samples (100 μl) by adding chloroform and methanol in 2:1 ratio (100 μl), vortexing for 1 min, followed by microfuging the mixture for 1 min. The organic layer was
taken carefully and transferred to a clean, dry glass tube and evaporated in a vacuum desiccator. After drying, 2 ml of chloroform and 2 ml of dye (ammonium ferrothiocyanate) was added and mixed well for 1 min by vortexing. The mixture was allowed to stand for phase separation, after which the chloroform phase was taken and the optical density of the sample at 488 nm was determined. Standard curve was made using phosphotidylcholine.

2.2.4.3. RNA: The RNA content of nuclear envelopes and endoplasmic reticulum were determined by the procedure described by Schneider (1957). 0.5 ml of 10% TCA was added to sample suspensions (0.2 ml), and after mixing well, the mixture was microfuged for 2 min to remove acid soluble components. The pellet was washed again with 0.5 ml of 10% TCA and heated with 0.5 ml of 95% alcohol to remove lipoidal compounds. The sample was centrifuged and the pellet was again washed with 0.5 ml of 95% alcohol. The pellet obtained after alcohol extraction was suspended in 0.26 ml of water and 0.26 ml of 10% TCA, vortexed and heated at 90°C for 15 min with occasional stirring. The supernatant was collected after centrifugation and the pellet was washed once more with 0.5 ml of 5% TCA, and the supernatant combined with the previous one. This extract, which constitutes the nucleic acid fraction, was diluted to 1 ml and heated with 3 ml of orcinol reagent for 20 min in a boiling waterbath. Then the reaction mixture was cooled to room temperature, mixed with 3.0 ml of anhydrous acetone and absorbance at 660 nm measured. Standard curve was obtained using E.coli RNA. The orcinol reagent was prepared by dissolving 0.5 g of FeCl₃ and 1.0 g of orcinol in concentrated HCl.

2.2.4.4. DNA: DNA content was determined by the method of Labarca and Paigen (1980), which is based on the enhancement of fluorescence seen when bisbenzimidazole (Hoechst 33258) binds to DNA. Samples (0.02 ml) were diluted to 1.0 ml in 0.05 M Na₂PO₄ pH 7.4, 2M NaCl and mixed with 5 μl of the dye Hoechst 33258 (0.2 μg/ml in the same buffer). Change in fluorescence was monitored in a Hitachi
fluorimeter by keeping excitation at 356 nm and emission at 458 nm, with a 5 nm slit width. Standard curve was obtained by using known amounts of calf thymus DNA.

2.2.5. Microscopy

2.2.5.1. Phase contrast microscopy: Samples of nuclear envelopes for phase contrast microscopy were mounted directly in storage buffer (10 mM Tris. HCl, pH 7.4, 20% glycerol) and observed in a Zeiss Photomicroscope-3 microscope.

2.2.5.2. Electron microscopy: Samples for electron microscopy were processed as follows: Purified nuclei were sedimented at 800 X \( g \) for 10 min and nuclear envelopes obtained after second salt extraction were centrifuged at 1600 X \( g \) for 30 min. The pellets were fixed in 30% glutaraldehyde and post fixed in 1% osmium tetroxide. Samples were embedded and ultra thin sections were stained in 2% uranylacetate for 2 h, followed by 0.2% lead citrate for 5 min. Sections were examined in a JEOL 100 X electron microscope.

2.2.6. Gel electrophoresis

2.2.6.1 SDS-PAGE: SDS-PAGE was carried out by the method described by Laemmli (1970). Resolving gels of 10% or 8% acrylamide (10 ml or 8 ml acrylamide:bis-acrylamide at 30% : 0.8%, 0.15 ml of 20% SDS, 3.75 ml of 3 M Tris.HCl, pH 8.8, 15.8 or 17.8 ml of \( H_2O \) (depending on acrylamide percentage), 150 \( \mu l \) of freshly prepared 10% ammonium persulphate and 10 \( \mu l \) TEMED), were cast in a vertical gel apparatus (Bethesda Research Laboratories USA). A stacking gel of 4.5% acrylamide (2.25 ml of acrylamide:bis-acrylamide at 30%:0.8%, 1.9 ml of 1 M Tris.HCl, pH 6.7, 10.75 ml of \( H_2O \), 0.075 ml of 20% SDS, 90 \( \mu l \) of freshly prepared 10% ammonium persulphate and 10 \( \mu l \) of TEMED) was cast above the resolving gel. Protein samples were mixed with sample buffer (6.25 mM Tris. HCl, pH 6.8, 2%
SDS, 10% glycerol, 2% β-mercaptoethanol and 0.001% bromphenol blue), boiled for 3-5 min, and placed in the wells. Electrophoresis was carried out at constant current, 20 mA, for 3 h, and followed by 40 mA, for the next 2-3 h, in a buffer containing 25 mM Tris. HCl, pH 8.3, 192 mM glycine and 0.1% SDS. The gel was stained in 0.2% Coomassie Brilliant Blue R-250 dye in methanol:acetic acid:water (45:10:45) for 4-6 h. Subsequently the gels were destained in methanol:acetic acid:water (45:10:45). If autoradiography was required, gels were dried and exposed to X-ray film for 3-5 days at -70°C with intensifying screens.

2.2.6.2. Isoelectric focussing: Isoelectric focussing (IEF) of protein samples was done by the method of O' Farrell (1975). The gel mixture (10 ml) was prepared by mixing 5.5 g urea, 1.33 ml of acrylamide: bis-acrylamide at 30% : 1.6%, 2.0 ml of 10% NP-40, 1.97 ml of H₂O, 0.4 ml of ampholines (pH 5/8), 0.1 ml of ampholines (pH 3/10) and 10 µl of 10% ammonium persulphate in a side arm conical flask. This mixture was degassed under vacuum for 1 min; 7 µl TEMED was added, mixed, and the mixture was immediately poured into glass tubes (120 mm x 3 mm) blocked at the bottom with parafilm. Gels were overlayed with butanol. After the gels had polymerized, the butanol was replaced by 20 µl of lysis buffer (9.5 M urea, 2% w/v NP-40, 2% ampholines (16% pH 5/8, and 0.4% pH 3/10) and 5% β-mercaptoethanol). The parafilm was removed from the tubes and tubes were placed in a standard tube gel electrophoresis chamber. Lysis buffer and water were removed from the surface of the gels and 20 µl of fresh lysis buffer was loaded over each gel. The tubes were then filled with 0.05 M NaOH, which was also filled in the upper chamber. The anolyte lower chamber was filled with 0.025 M H₃PO₄ and the gels were pre-run at 200 volts for the first 15 min, then at 300 volts for 30 min and finally at 400 volts for 30 min. After the pre-run, the power was turned off, the upper reservoir was emptied and lysis buffer was removed from the gel surface. Samples prepared by suspending the sample pellet in 50 µl lysis buffer were loaded onto the tube gels, and over-
layed with 10 μl sample overlay solution (8 M urea, 1% ampholines (4:1 ratio of pH range 5/8: pH 3/10). The upper tank was then filled with NaOH, and electrofocussing was carried out at 400 volts for 12 h, followed by 800 volts for 1 h. The gels were then either stored frozen at -20°C or stained or subjected to second dimension analysis.

Staining of IEF tube gels was done as follows: tube gels were taken out from the glass tube and immersed in 5 ml of 10% TCA, 3% sulphosalicylic acid solution for 1 h with shaking. The gels were then transferred to isopropanol:acetic acid:water (50:5:45) and shaken till the white colour disappeared. Transparent gels were stained with Coomassie Blue and destained as described earlier.

For measuring the pH gradient, an electrofocussed tube gel was cut into 5 mm sections which were placed in individual glass vials with 2.0 ml of degassed H₂O. These vials were shaken for 5-10 min and the pH of each solution was measured with a pH meter. The pH was plotted against distance from one end.

2.2.6.3. Two-dimensional gel electrophoresis: Proteins separated on IEF gels were subjected to second dimensional separation by SDS-PAGE. Resolving and stacking gels were cast as described earlier. One well at one side of the stacking gel was made for the molecular mass markers. IEF tube gels were equilibrated in 5 ml of equilibration buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 62.5 mM Tris.HCl, pH 6.8) at room temperature for 30 min. The equilibrated IEF tube gel was carefully loaded over the stacking gel and sealed with 1% agarose gel (1% agarose in equilibration buffer). Protein molecular mass markers were loaded in the side well and the run was carried out as described for SDS-PAGE.
2.2.6.4. Electroelution of proteins from SDS-polyacrylamide gels:

Purification of the lamins was carried out by electroelution of bands from SDS-polyacrylamide gels as described by Hunkerpiller et al. (1983). Proteins were separated on 10% SDS-polyacrylamide gels, stained for 1 h in 0.2% Coomassie Blue, and destained as described before till the bands were seen clearly. Protein bands were cut out from the gel and minced, kept for 5 min in electrode buffer (0.005 M \( \text{NH}_4\text{HCO}_3 \), 0.1% SDS), and then transferred to the electroelution cell in soaking buffer (0.4 M \( \text{NH}_4\text{HCO}_3 \), 2% SDS, 0.1% DTT). Soaking buffer was filled till the top of the well, and then overlayed with electrode buffer. Electrotransfer was carried out in a Scientific Instrument Co. transfer apparatus for 24-48 h. Electroeluted samples which collected in the other well were taken out carefully, precipitated with two volumes of cold acetone and kept at -20°C. After 72 h at -20°C, the precipitate was recovered by spinning the tubes at 10,000 X g at 4°C. Precipitate was reconstituted in phosphate-buffered saline (PBS; 10 mM \( \text{Na}_2\text{PO}_4 \), pH 7.5, 150 mM NaCl) and quantitated by measuring its optical density at 280 nm.

2.2.7. Western blot analysis

Proteins were separated by SDS-PAGE as described previously, and then transferred to nitrocellulose membrane filters by electroblotting using an electrotransfer apparatus, Hoefer Scientific Instruments, in buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol, for 3-4 h at 300 mA. All steps were carried out at room temperature. Nitrocellulose filters were incubated in blocking buffer (0.3% BSA, 0.25% gelatin in PBS), for 2 h followed by incubation with first antibody in PBS/0.25% gelatin with shaking for 3-6 h, then washed with several changes of washing buffer (150 mM NaCl, 0.1% NP-40, 0.25% gelatin in PBS) over 2 h. After washing, blots were either incubated with the appropriate second antibody conjugated to HRP for 3 h and then washed for 2 h with washing buffer or treated with iodi-
nated protein A, as described later. When the HRP conjugate was used, blots were stained three different methods; (1) Blots were placed in 10 ml DAB (0.1 mg/ml in PBS) and H₂O₂ was added (100 μl). It took 5-10 min for color development. (2) Blots were placed in a solution containing 4-chloronaphthol; 100 μl of a 10 mg/ml solution in methanol was added to 4 ml of methanol and 16 ml of Tris-buffered saline (TBS) and then 100 μl H₂O₂ was added. Colour developed in 5 to 10 min. (3) For staining of blots with luminol, filters were incubated with ECL™ reagents 1 and 2 (1:1) for 1 min, followed by exposure to X-ray film for 10-40 sec at room temperature. X-ray films were then developed by standard procedures. When this staining procedure was used, blots were incubated with the primary and secondary antibody (1:5000) for only 1 h each.

For some experiments iodinated protein A (labelled as described in the next section) was used in place of second antibody-peroxidase conjugate. Hybridization and washing conditions were the same as described above. After washing, blots were exposed to X-ray film at -70°C using intensifying screens, and autoradiograms were developed.

To check the efficiency of transfer and for determination of molecular masses, nitrocellulose strips containing molecular mass markers or samples were stained in 0.1% Amido Black for 1-2 min and destained with methanol:acetic acid:water in the ratio of 45:10:45 or stained with 1 X Ponceau Red for 5 min and then destained with water.

2.2.8. Iodination of proteins

Radioiodination of proteins and peptides was carried out by the "iodogen" method (Fraker and Speck 1972, Markwell and Fox 1978). Iodogen in acetone (0.4 mg/ml) was added to dry, clean glass tubes (50 μl to each tube) and allowed to evaporate at room temperature. Iodogen-coated tubes were sealed with parafilm and
stored in a desiccator. Samples to be radioiodinated were suspended in PBS and added to iodogen-coated tubes, followed by addition of Na$^{125}$I. The reaction was carried out at 0°C for 10 min, with occasional shaking. After the reaction, labelled proteins or peptides were separated from unreacted Na$^{125}$I by gel filtration; and labelled nuclei were separated by centrifugation. For gel filtration of iodinated protein A, Sephadex G-50 was used, and for peptides, Biogel-P2 was used.

2.2.9. Immunization protocols

For raising polyclonal antibodies in rabbits, a Triton/highsalt extract of mouse nuclear envelopes or electroeluted proteins (~ 200 µg protein) were emulsified with complete Freund’s adjuvant and injected subcutaneously. Subsequent injections were done with the same amount of protein emulsified with incomplete Freund’s adjuvant, subcutaneously, at intervals of 4 weeks. Seven days after the third booster, animals were bled and serum was tested for antibody titre. The immunization protocol followed for monoclonal antibody production is described in Section 5.1.1.

2.2.10. Cell Culture Procedures

2.2.10.1. Preparation of stock solutions:

1) **Media**: ISCOVE’s modified Dulbecco’s Medium (IMDM) was used for most procedures. This medium is available as a dry powder, to be made up to one litre medium per packet. The contents of one packet was dissolved in 950 ml of H$_2$O (deionized) and 3.024 g of sodium bicarbonate was added to this medium. The other ingredients were added as indicated on the packet. For most batches of IMDM (Gibco), only sodium bicarbonate and β-mercaptoethanol had to be added. The medium was sterilized by filtering through a Millipore filter, using positive pressure, and stored in aliquots at -10°C.
To make complete IMDM, the medium was supplemented with additional glutamine, β-mercaptoethanol, antibiotics and fetal calf serum in the following manner.

(2) **Glutamine**: A stock solution of 100 X (200 mM) was prepared by dissolving 3.0 g of glutamine in 100 ml of PBS and sterilizing the solution by filtration. The solution was stored at -10°C in aliquots, and 1 ml of the stock was added to 100 ml of medium to give a final concentration of 2 mM.

(3) **β-Mercaptoethanol**: To prepare 100 X stock solution, 38 µl of β-mercaptoethanol was added to 100 ml of sterile PBS to make 5 X 10⁻⁵ M solution. Aliquots were stored at 8°C. 1 ml of this stock was added to 100 ml of medium to give a final concentration of 5 X 10⁻⁵ M.

(4) **Antibiotics:**

(a) **Penicillin G**: 100 X stock solution was prepared by dissolving 600 mg of penicillin G in 100 ml (10,000 unit/ml) of PBS. 1 ml of this stock was added to 100 ml of medium to give a final concentration of 100 unit/ml.

(b) **Streptomycin**: 100 X stock was made by dissolving 500 mg of streptomycin in 100 ml of PBS. 1 ml of this stock was added to 100 ml medium to give a final concentration of 50 µg/ml.

(c) **Kanamycin**: 100 X stock was made by dissolving 1 g of kanamycin in 100 ml of sterile PBS and 1 ml of this stock was added to 100 ml medium to give a final concentration of 100 µg/ml.

(d) **Nystatin**: A stock solution of 500 units/ml was prepared by suspending 1 mg of nystatin (5000 units/mg), in 10 ml of sterile PBS. 1 ml of this stock was added to 100 ml of medium to give a final concentration of 5 unit/ml.
2.2.10.2. Maintenance of cells: SP2/0 cells, hybridomas and other established cell lines were maintained in media containing 10% fetal calf serum at 37°C, in 5% CO₂ atmosphere in a CO₂ incubator.

2.2.10.3. Storage of cells: Cells were stored frozen in liquid nitrogen. Freezing was done by the following procedure. Cells grown to 70-80% confluency were harvested by flushing the medium with the help of a bent pipette, and centrifuged at 400 X g for 3-5 min. The cell pellet (~ 3 X 10⁶ cells) was first suspended in 0.2 ml medium, then in storage medium (2% DMSO, 50% fetal calf serum in IMDM), by adding the cell suspension dropwise with mixing. The suspension was transferred to storage vials. Vials were kept in ice initially and then in a thermacol box at -70°C. After 24 to 48 h, vials were transferred to a liquid nitrogen container.

To revive the cells, storage vials containing cells were transferred from liquid nitrogen to a waterbath at 40°C and were shaken till the contents thawed. The contents were quickly transferred to a sterile centrifuge tube containing 8 ml of medium (or PBS). Cells were recovered by centrifuging at 400 X g for 3 min. The cell pellet was resuspended in complete medium and plated in a flask for growth.

2.2.10.4. Growth on coverslips: The F-111 cell line (rat embryo fibroblasts) or the 215 cell line (mouse embryo fibroblasts) was grown on coverslips for immunofluorescence studies as follows. Cells were grown to 90% confluency in flasks, then medium was removed and cells were detached by treating with trypsin/EDTA (0.1% in PBS) for 2 min while tapping the flasks. Cells were collected in a sterile centrifuge tube, pelleted and resuspended in complete medium (10,000 to 30,000 cells/ml). 0.3 ml of this suspension was carefully poured over each glass coverslip kept in a sterile petri dish. Six hours later, cells were examined, and more medium was added to the petri dish to cover the coverslips. The cells were incubated at 37°C, in 5% CO₂, till the coverslips were 80% confluent.
2.2.10.5. Immunofluorescence assay: F-111 or 215 cells were grown on coverslips as described above, till 80% confluency. The cells were washed three times with PBS, and then fixed in acetone: methanol (2:1) at 0°C for 15 min. The cells were washed again 3 times with PBS. At this stage fixed cells could be kept at -20°C for further use, or processed for the assay immediately.

Fixed cells were incubated in blocking buffer (2% BSA in PBS) for 45 min at room temperature. After blocking, cells were washed three times with PBS, and then incubated with first antibody solution diluted in PBS (12 μl on each coverslip), for 1 h at room temperature. The samples were washed three times with PBS and incubated with second antibody (FITC-labelled anti-mouse or anti-rabbit IgG, depending on the source of the first antibody) at 1:100 dilution, for 1 h at room temperature, and washed again as described above. Incubation with second antibody and washings carried out in the dark. The coverslips were mounted in 10% glycerol and viewed under a Leitz Dialux 22 fluorescence microscope.

2.2.11. General methods used in peptide synthesis

2.2.11.1. Purification of solvents: The solvents and chemicals used during the course of peptide synthesis were purified as follows:

1. Dichloromethane (CH₂Cl₂) was distilled over P₂O₅.
2. Triethylamine (TEA) was refluxed over ninhydrin for 1 h and distilled.
3. Trifluoroacetic acid (TFA) was distilled before use.
4. Ethanol used for all synthetetic procedures was twice distilled commercial alcohol.

Petroleum ether used for precipitating peptides, dimethylformamide used for coupling reactions, ether, ethyl acetate, benzene, n-butanol, chloroform and methanol were used without any further purification.
2.2.11.2. Preparation of reagents and amino acid derivatives:

(1) **Boc azide (tert-butylcarbonyl chloride):** Boc azide was prepared by the procedure described by Carpino et al. (1959). t-Butyl carbazate (10 g) was dissolved in glacial acetic acid (8.8 ml) and water (12.5 ml). NaNO₂ (5.8 m) was added in small amounts over a period of 15 min. During the addition of NaNO₂, the solution was stirred vigorously and temperature was maintained at 0°C. After 90 min, the oily upper layer was separated from the aqueous layer. The aqueous layer was extracted with ether (3 X 100 ml). The ether extracts were mixed with the oily layer, washed with water and 1 M NaHCO₃ and dried over Na₂SO₄. On evaporating the ether extracts under reduced pressure, Boc azide was obtained as a golden yellow liquid. It was used directly without further purification.

(2) **Palladium black:** Palladium black was prepared by the method of Greenstein and Winitz (1961). Hydrochloric acid (6N) was boiled in a water bath, 0.1 g palladium chloride added and the mixture was heated in a boiling water bath. After a few minutes when a clear solution was obtained, formic acid (0.08 ml) was added, followed by 5N KOH till a pH of 10 was obtained. Formic acid was again added to bring the pH to 7.0. Palladium black was obtained as black granules. These granules were washed with distilled water and stored under water as these are pyrophoric.

(3) **Boc amino acids:** Boc amino acids were prepared by Schnable's procedure (Schnab e (1967). The amino acid (10 mmol) was suspended in 1:1 dioxane:water mixture (10 ml) and Boc azide (1.6 ml, 10 mmol) was added to it. The mixture was stirred at room temperature, maintaining the pH in the alkaline range with 4 N NaOH. After 24 h, 15 ml H₂O was added to the reaction mixture, and the solution was extracted with ether (3 X 10 ml). The aqueous layer was cooled in an ice bath, acidified with 2 N HCl, saturated with NaCl and extracted with ethyl acetate (3X20 ml). After drying over Na₂SO₄ and evaporating the
ethyl acetate under vacuum, the Boc amino acid was obtained. In case of 2-chloro-Z-lysine, the acidified aqueous layer was extracted with ether (3X20 ml). The yields of Boc amino acid preparations were approximately 60%.

(4) 2-Chloro-Z-lysine The ε-amino group of Lys was protected as described by Yajima and Fujii (1981). Lysine (3.6 gm, ~ 20 nmol) was dissolved in H₂O (50 ml), and to this was added CuCO₃ (3.6 g suspended in 120 ml H₂O). The mixture was boiled for 45 min and filtered. To the filtrate, NaHCO₃ (25 nmol) was added followed by slow addition (in 30 min) of 2-chlorobenzoxy-carbonyl succinamide (5.6 g, ~ 200 nmol) in 10 ml DMF, maintaining the pH between 8-9 with NaHCO₃. The reaction mixture was filtered through a sintered funnel and the light blue precipitate was washed with H₂O, methanol and ether (three times each). The precipitate was then dissolved in 2N HCl (20 ml), and to this solution EDTA (30 nmol) dissolved in water (50 ml) was added; followed by neutralization of the solution to pH 7.0 with 4N NaOH. After this the solution was kept at 4°C for 2 h for precipitation. The precipitate of pure 2-chloro-Z-lysine was recovered by filtration through a sintered funnel, washed with H₂O, methanol and ether (three times each).

The ε-amino group of 2-chloro-Z-lysine was blocked by Boc as described before.

2.2.1.3. Preparation of cesium salt of Boc-Gly: The cesium salt of Boc-Gly was prepared by the method described by Gisin (1973). Boc-Gly (0.2 g, ~ 1 nmol) was dissolved in 1 ml of H₂O: ethanol solution (1:9 ratio). To this was added a saturated solution of cesium carbonate (0.66 gm), dropwise, with continuous shaking and the pH was checked as the addition proceeded. The addition was stopped when a pH of 7 was achieved (stable for at least 1 h). Ethanol was evaporated under vacuum and the residue was dried by co-evaporation with benzene (3 X 10 ml) as an azeotrope.
under vacuum. A white powdery cesium salt was obtained, which was stored under vacuum till further use.

2.2.11.4. Picric acid test: The picric acid test was carried out as described (Gisin 1972) in order to monitor coupling efficiencies during peptide synthesis. Free aminoacyl or peptidyl (free base) resin (5 mg) was treated with 0.1 M picric acid in CH$_2$Cl$_2$ (3 x 2 ml, 2 min) on a 5 ml sintered funnel and washed with CH$_2$Cl$_2$ (3 x 2 ml, 2 min) to remove unbound picric acid. The picrate bound to the free amino group was eluted with 5% triethylamine (TEA) or diisopropylethylamine in CH$_2$Cl$_2$ (2 x 2 ml, 2 min) followed by CH$_2$Cl$_2$ (3 x 2 ml, 2 min). 0.2 ml of the picrate eluent was diluted to 2.0 ml with 25% ethanol and its absorbance was measured at 358 nm. From the extinction coefficient of picrate ($\epsilon$$_{358}$=14,500), the free amino groups were estimated and the substitution levels or coupling efficiencies was determined.

2.2.11.5. Preparation of dansyl chloride: Dansyl chloride was prepared by the method described by Mendel (1970). 2.5 g of 1 dimethylaminonapthalene-5-sulphonic acid was taken in a round bottom flask and to this 8 mol of POCl$_3$ was added in the hood. To this mixture was added 4 g of PCl$_5$, slowly with continuous stirring in the hood and the reaction was allowed to proceed for 16 h at room temperature. The reaction mixture was then poured over ice in a 500 ml beaker, with shaking. After the ice melted, the solution was extracted with benzene (three times). The benzene extract was washed twice with water, once with NaHCO$_3$ and dehydrated with CaCl$_2$. The dehydrated benzene extract was subjected to vacuum evaporation. To the orange-coloured semisolid material obtained; 10 ml petroleum ether was added, mixed and the mixture was kept at -10°C for 20 min. The solidified dansyl chloride was recovered by decanting the ether and drying the solid under mild vacuum.