MATERIALS AND METHODS

MATERIALS

Chemicals

Agarose, ampicillin, ammonium acetate, ammonium persulfate, 1-acetyl 2-phenyl hydrazine, β-mercaptoethanol, boric acid, calcium chloride, chloramphenicol, citric acid, coomassie blue G250, creatine phosphate, creatine phosphokinase, DEPC, dialysis tubing, disodium hydrogen phosphate, dithioerythritol, dithiothreitol, DMPG, DOPG, DMPA, EDTA, ethidium bromide, glucose, glycerol, GSSG, guanidine hydrochloride, heparin, haemin., HEPES, IPTG, kanamycin, L-glycine, L-arginine, lithium chloride, magnesium acetate, magnesium sulfate, MES, PEG 8000, potassium acetate, potassium chloride, RNase free BSA, SDS, sucrose, sodium acetate, sodium dihydrogen phosphate, spermidine, sodium bicarbonate, sigmacote, Tris base, Triton X-100, urea and uridine were obtained from Sigma chemical Co. (St. Louis, USA). Trizol reagent, PCR buffer, magnesium chloride solution for PCR, RPMI-1640, leucine free RPMI, DMEM, trypsin, Fetal calf serum, antibiotic-antimycotic solution were purchased from Life Technologies (Maryland, USA). NTPs, dNTPs, cation exchange resins: S-sepharose and SP-sepharose were obtained from Pharmacia Biotech (Uppsala, Sweden). Bromophenol blue, xylene cyanol, acrylamide, bis-

acrylamide, TEMED were obtained from Bio-Rad laboratories (California, USA). Coomassie Plus protein assay reagent was purchased from Pierce (Illinois, USA). All other chemicals were at least of analytical grade and were from Qualigens laboratories (Bombay, India). HSA was from alpha therapeutic corporation (California, USA). Bacto-tryptone, yeast extract, and bacto-agar were obtained from Difco laboratories (Detroit, USA).

Cell Lines and Bacterial Strains

Cancer cell lines of human origin, HUT102, T-cell leukemia; K562, erythroleukemia; COLO205; colon adenocarcinoma; MCF7, breast adenocarcinoma; A431, epidermoid carcinoma; A549, lung carcinoma and HeLa, cervical carcinoma and J774A.1, mouse monocyte-macrophage; and L929, mouse fibroblast were obtained from ATCC. All the cell lines were maintained in RPMI 1640 supplemented with antibiotic antimycotic solution, 2 mM glutamine and 10% heat inactivated foetal calf serum (Life Technologies, Maryland, USA). *E. coli* strain DH5α was used for DNA manipulation, cloning and mutagenesis. Strains CJ236 and DH5αF' were used for oligonucleotide mediated site directed mutagenesis. BL21 (λDE3) strain containing T7 RNA polymerase gene under the control of lac promoter, was used for expression of the recombinant proteins.

Radioisotopes

L-[3,4,5-^{3}H (N)]-leucine (143Ci/mmol), [35S]-dATPαS, ^{125}I-Na (350mCi/ml) were obtained from Amersham (England, UK).

Enzymes and Molecular Weight Markers

DNA restriction enzymes were purchased from New England Biolabs (Massachusetts, USA) and Life Technologies (Maryland, USA). Lysozyme and RNase A were obtained from Sigma. RNase T1, DNA ligase, RNA polymerase, Taq DNA polymerase, 1Kb DNA ladder and prestained molecular weight markers for proteins were obtained from Life Technologies (Maryland, USA). Other protein molecular weight markers were from Sigma chemical co. T4 polynucleotide kinase were purchased from Promega. T7 DNA polymerase was obtained from USB.

Oligonucleotides

Oligonucleotides used in this study were synthesized by Rama Biotechnologies (Hyderabad, India).

METHODS

Compositions of the different solutions used in this study are described in appendix.

Preparation of Competent Bacterial Cells

Competent cells of the different strains of *E. coli* were prepared as described by Cohen *et al.* (1972). An LB-agar plate was streaked with the desired strain, and a single colony was inoculated into 5 ml of LB medium. The culture was grown at 37 ^{0}C with continuous shaking at 200 rpm for 6 hours. A small inoculum from this culture was used to start a 100 ml culture in the same medium. At an OD_{600} of 0.3-0.4, when the culture reached early log phase, it was chilled on ice for 30 min., and centrifuged at 2000 g for 15 min. at 4 ^{0}C. The pellet was gently resuspended in 50 ml of chilled 50 mM calcium chloride and incubated on ice for 60 min. The cell suspension was centrifuged at 2000 g for 15 min. at 4 ^{0}C, and the pellet was gently resuspended in 5 ml of chilled 50 mM calcium chloride containing 20% glycerol. The competent cell suspension was immediately aliquoted in prechilled vials and stored at –70 ^{0}C.

Polymerase Chain Reaction

A standard PCR was set up as described below.

10X PCR buffer	10.0 µl
1.25 µM dNTP mix	16.0 µl
Template DNA (10 µg/ml)	10.0 µl
Primer #1 (25 nmoles/ml)	4.0 µl
Primer #2 (25 nmoles/ml)	4.0 µl
Taq DNA polymerase (5 U/µl)	0.5 µl
Sterile water	55.5 µl
Total volume	100 µl

The reaction was started by an initial hot start at 94 ^{0}C for 5 min., followed by a three-step amplification cycle. The amplification cycle consisted of a 1 min. denaturation at 94 ^{0}C, followed by a 2 min. annealing at 48 ^{0}C and an extension at 72 ^{0}C for 2 min. The cycle was repeated 30 times and the reaction mixture was incubated at 72 ^{0}C for additional 7 min. to allow for primer extension. The PCR amplified product was separated from the primers on a 1% agarose gel. A well was carved ahead of the required fragment and filled with 15% PEG-TAE solution (Zhen and Swank, 1993). The DNA was electro-eluted in 15% PEG-TAE solution, and purified further by phenol/chloroform extraction and ethanol precipitation.

DNA Digestion and Ligation

The pure PCR amplified product, and the vector were digested with required restriction enzymes in the reaction buffer as per supplier's recommendation. Ten units of enzyme were used to digest 1 µg of DNA and the samples were incubated for three hours at appropriate temperature. The vector was dephosphorylated with calf intestinal phosphatase (0.2 units/µg of DNA) for 30 min. at 37 ^{0}C. After digestion, relevant fragments were gel purified in 15% PEG-8000-TAE solution as described by Zhen and Swank (1993). Ligation of the vector and insert DNA was performed in a reaction volume of 20 µl using 400 units of T4 DNA ligase in the recommended ligation buffer at 16 ^{0}C for 12 h. A control ligation reaction without the insert was also done keeping the other components same. The concentration of insert was eight to ten times more than the vector. The ligated sample and control mix was later used for transformation.

Transformation of Bacterial Host

Transformation of the bacterial host with an appropriate plasmid was performed using the method of Mandel and Higa (1970). A vial of competent bacterial cells was thawed on ice. The plasmid DNA was added at a concentration 1ng/25 µl of competent cells and the mixture was allowed to stand on ice for 30 min. The cells were given a heat shock by incubating the mixture at 42 ^{0}C for 90 sec, followed by a 2 min. incubation on ice. The mixture was diluted 10-fold with LB and incubated at 37 ^{0}C for 1 h. Afterwards the cells were plated on the LB-agar containing the antibiotic whose resistance marker was present in the plasmid.

Site-directed Mutagenesis

Site-directed mutagenesis was done according to the method employed by Kunkel *et al.*, 1987.

Preparation of Uracil containing phagemid: E. coli strain CJ236 was transformed with the required template DNA and grown on LB plates containing the antibiotics, ampicillin 100 µg/ml and chloramphenicol 30 µg/ml (stock solution made in alcohol). Further, the plates were incubated at 37 ^{0}C for 12 h and a single colony was picked from the center of the plate, inoculated in 5 ml LB containing ampicillin and chloramphenicol. The liquid culture was grown at 37 ^{0}C overnight with vigorous shaking. About 500 µl of the culture was diluted 40-times with LB containing ampicillin and chloramphenicol and grown at 37 ^{0}C with vigorous shaking (200 rpm) upto an OD_{600} of 0.25-0.3. The speed of shaker was reduced to 100 rpm and the culture was left for 30 min. for the F pilus to grow. Afterwards, it was infected with VCS M13 helper phage at an MOI of 1:20. The cells were grown for 30 min. in a stationary culture to allow the phages to infect, followed by slow shaking (100 rpm) for one hour. Subsequently, the culture was diluted 10-times with 2X YT medium containing ampicillin and chloramphenicol and grown in the presence of 0.25 µg/ml uridine and 50 µg/ml kanamycin at 37 ^{0}C overnight with vigorous shaking. The following day, the culture was chilled on ice for 10 min. and centrifuged at 12,000 rpm for 10 min. at 4 ^{0}C in a Sorvall RC5C centrifuge using a GSA rotor. The pellet was discarded and the supernatant was centrifuged again in fresh GSA bottles. A small aliquot of about 1 ml from the supernatant was saved for titration and the precipitation of the single stranded phagemid was carried out using 0.15 volume of 16.67% PEG in 3.3 M NaCl followed by incubation on ice for 4 h. The mixture was centrifuged at 12,000 rpm at 4 ^{0}C for 30 min. using a GSA rotor and the pellet was resuspended in 3 ml TE buffer. The suspension was centrifuged at 15,000 rpm at 4 ^{0}C for 10 min. using a SS34 rotor. The supernatant was ultracentrifugated at 100,000 g at 4 ^{0}C for 2.5 h. The pellet was resuspended in 500 µl TE buffer followed by phenol-chloroform extraction and precipitation of the single stranded DNA with ethanol for 30 min. at –70 ^{0}C. The DNA pellet was washed with 70% ethanol, dried and dissolved in 200 µl TE buffer. The uracil containing template was quantitated by analysing on an agarose gel.

Titration of uracil containing template: The crude preparation of the phagemid DNA (1ml aliquot), was used to titrate the uridine incorporation in the template. The strains CJ236 (ung^- dut^-) and DH5αF'(ung^+ dut^+) of *E. coli* were transformed with the diluted template DNA. The CJ236 cells, plated in the presence of ampicillin and chloramphenicol while DH5αF' cells, plated in the presence of ampicillin alone, were grown overnight on LB agar plates. The good incorporation of uridine gave no colonies or very few colonies in DH5αF' cells whereas with CJ236 several colonies were obtained. A ratio of 10^3-10^4 between the number of colonies in CJ236 to that in DH5αF' cells was considered ideal for an efficient incorporation of uridine.

Phosphorylation of the mutagenic oligonucleotide: The components of a standard reaction to carry out the phosphorylation are described below

Oligonucleotide (180 nmol/ml)	1.0 µl
10X Kinase buffer	2.5 µl
10 mM ATP	1.0 µl
10 mM spermidine	0.25 µl
100 mM DTT	1.25 µl
T4 polynucleotide Kinase	0.5 µl
H_2O	18.5 µl
Total	25.0 µl

The constituents were mixed thoroughly, incubated at 37 ^{0}C for 30 min. and subsequently, the enzyme was denatured by heat inactivation at 70 ^{0}C for 10 min.

Annealing of the mutagenic oligonucleotide: 750 ng (approximately) of the uracil containing single stranded template and 1 µl of the phosphorylated oligonucleotide were taken in 1X annealing buffer making up the total reaction volume 20 µl. A control reaction, was also carried out simultaneously, lacking the oligonucleotide. The contents were mixed by vortexing and incubated at 95 ^{0}C for 10 min. in a water bath. The reaction mixtures were further incubated at 80 ^{0}C for 10 min. in a heat block and the heat block was transferred to ambient temperature, cooled slowly to about 30 ^{0}C over a period of 30-60 min.

Complementary DNA strand synthesis: The oligonucleotide annealed uracil containing template was used for complementary strand synthesis in the following reaction.

Annealed DNA	10.0 µl
10X synthesis buffer	2.5 µl
10X ligation buffer	1.0 µl
dNTP mix (100 mM)	1.0 µl
ATP (100 mM)	0.25 µl
T7 DNA polymerase (5 U/µl)	0.5 µl
T4 DNA ligase (400 U/µl)	0.5 µl
H_2O	9.25 µl
Total	25.0 µl

A control reaction was done with the control annealed DNA lacking the oligonucleotide. The constituents were mixed and incubated on ice for 2 min. afterwards, at room temperature, for 5 min. The reaction was further carried out at 37 ^{0}C for 2 h followed by heating at 70 ^{0}C for 10 min. 12.5 µl of the reaction products were analyzed on an agarose gel along with annealed samples to check the complementary strand synthesis. The samples were diluted 10-times with water and 5 µl of the diluted sample was used to transform 50 µl of *E. coli* host strain, DH5α cells. The suspension was plated on 2 LB agar plates containing ampicillin. Single colonies were picked, grown in liquid culture and miniprep screening of DNA was done to select the positive clones.

Mini Plasmid Preparation and Screening

5 ml LB containing 100 µg/ml of ampicillin was inoculated with single bacterial colony picked from the culture plates. The culture was grown for 12 h at 37 ^{0}C with vigorous shaking. Cells were harvested from 3 ml of culture by centrifugation at 3000 rpm in a microfuge (Plastocraft) at 4 ^{0}C for 15 min. Added 200 µl of TEG buffer was added to the cells, and they were gently resusupended to get a uniform suspension and kept on ice for 5 min. 400 µl of freshly prepared alkaline-SDS solution was added to the cell suspension and mixed well by inverting the tubes followed by an incubation on ice for 10 min. Subsequently, 300 µl of chilled potassium acetate solution was added and mixed thoroughly by vortexing. The mixture was centrifuged at 10,000 rpm at 4 ^{0}C for 15 min.. The supernatant was collected and phenol-chloroform extraction was performed followed by precipitation

of the plasmid DNA with 0.5 volume of cold isopropanol. The mixture was kept on ice for 10 min. and centrifuged at 12,000 rpm at 4^{0}C for 15 min. DNA pellet thus obtained was washed with 80% ethanol, dried and dissolved in 50 µl TE buffer (pH 8.0). Minipreps were screened by restriction digestion. 5 µl of plasmid DNA was incubated with 5 units of appropriate enzyme(s) and 150 units of RNaseT1 for 2 h and the products were analyzed on an agarose gel to identify the positive clones.

Large Scale Plasmid DNA Preparation

Plasmid DNA was prepared by alkaline lysis method of Ish-Horowicz (1981). 5 ml cultures were grown as described for small scale plasmid preparation. 0.5 ml from the growing culture was inoculated into 250 ml of LB containing ampicillin. The culture was grown for 12 h at 37 ^{0}C with vigorous shaking, centrifuged at 3000 g, at 4 ^{0}C, for 15 min. and the bacterial pellet was resuspended gently in 10 ml TEG buffer. The mixture was incubated at room temperature for 10 min., followed by addition of 20 ml of freshly prepared alkaline-SDS solution. The contents were mixed by inversion and the mixture was kept on ice for 10 min., followed by the addition of 15 ml of chilled potassium acetate solution. The contents were mixed by inverting the tube, and incubated on ice for 10 min. The lysed cell suspension was centrifuged at 5000 g, at 4 ^{0}C, for 20 min. The supernatant was taken, and nucleic acids were precipitated by adding 0.6 volume of chilled isopropanol. The mixture was incubated on ice for 10 min. followed by centrifugation at 5000 g at 4 ^{0}C, for 10 min.. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

The plasmid DNA was purified further to remove the contaminating proteins and RNA following the PEG purification protocol as described by Sambrook *et al* (1989). Equal volume of chilled 5 M lithium chloride solution was added to DNA suspension, mixed well and incubated on ice for 10 min. The precipitate was removed by centrifugation at 10,000 g at 4 ^{0}C, for 10 min. DNA was precipitated from the supernatant by adding equal volume of isopropanol. The mixture was centrifuged at 10,000 g for 10 min. at 4 ^{0}C and the pellet was washed with 70% ethanol. The DNA thus obtained was incubated in TE buffer containing 20 µg/ml of DNase free RNase A for 30 min. at room temperature. Afterwards, equal volume of 1.6 M NaCl containing 13% (w/v) PEG 8000 was added to DNA solution. The contents were thoroughly mixed and centrifuged at 10,000 g, at 4 ^{0}C, for 10 min.. The pellet was

resuspended in 0.2 ml TE, and extracted successively with phenol, phenol-chloroform, and chloroform. In the aqueous phase, 0.25 volume of 10 M ammonium acetate and two volumes of chilled ethanol were added and the mixture was incubated at room temperature for 5 min. to precipitate the plasmid DNA. The pure plasmid DNA was recovered by centrifugation at 12,000 g for 10 min. at 4 ^{0}C, washed with 70 % ethanol, dried and resuspended in TE buffer (pH 8.0). The amount and the purity of the DNA was done spectrophotometrically by recording the absorbance at 260 nm.

DNA Sequencing

Sequencing of cloned inserts was done by Sanger's dideoxy chain termination method (Sanger *et al.,* 1977) using Sequenase version 2.0 kit from USB. 5 µl of 1mg/ml suspension of plasmid DNA was incubated in denaturation buffer at 37 ^{0}C for 30 min. in a reaction volume of 50 µl, and then the precipitation was carried out in the presence of 5.5 µl of 3 M sodium acetate and 4 volumes of chilled ethanol at –70 ^{0}C for 30 min. The pellet, obtained by centrifugation at 10,000 g, for 20 min., at 4 ^{0}C, was washed with 70% ethanol and resuspended in 7 µl of sterile water. 1 pmole of sequencing primer in 1 µl water and 2µl of 5X sequenase reaction buffer were added to denatured DNA and the reaction mix was incubated at 65 ^{0}C for 5 min. for primer annealing. The reaction mixture was cooled slowly to about 35 ^{0}C, by putting the heat block at room temperature. For labeling, 1 µl of 0.1 M DTT, 1 µl radioactivity containinig 10 µCi of ^{35}S dATP, 2 µl labeling mix diluted 5-fold in sterile water and 2 µl sequenase enzyme diluted 8-fold in sequenase dilution buffer were added to the primer annealed DNA. Incubated the reaction mixture at room temperature for 2-5 min. and added 3.5 µl to each of the 4 different tubes containing 2.5 µl dideoxy nucleotides ddATP, ddTTP, ddCTP, and ddGTP separately. The mixture was incubated at 37 ^{0}C for 5 min. and finally, the reaction was stopped by adding 4 µl of stop solution to each tube. Reaction products were separated on a 6% polyacrylamide sequencing gel made in TBE buffer containing 7.5 M urea. The samples were heated at 75 ^{0}C for 2 min. and immediately loaded on the gel. The gel was run at a constant power of 60 watts maintaining the temperature of gel between 50-55 ^{0}C, dried and exposed to an X-ray film.

Isolation and Purification of Proteins from the Inclusion Bodies

Heterologus expression of various proteins was done in BL21 (λDE3) strain of *E. coli.* Bacterial cells were transformed with the desired construct and grown in Super broth (pH 7.2) containing 100 μg/ml ampicillin, at 37 ^{0}C with continous shaking in a gyratory shaker at 225 rpm. The cultures were induced, at A_{600} of 2.0, with 1mM IPTG, and harvested two hours later by centrifugation at 4000 g, at 4 ^{0}C, for 20 min.. The recombinant proteins were purified from the inclusion bodies using the procedure described by Buchner *et al* (1992). The total cell pellet from a liter of culture was homogenized in 180 ml of inclusion bodies washing buffer containing 8 ml of freshly prepared lysozyme solution (5 mg/ml). The suspension was incubated at room temperature for 1 hr with intermittent shaking. Added 20 ml each of 5M NaCl and 25% Triton X-100 were added to the suspension and incubated at room temperature for 30 min. with vigorous shaking. The suspension was centrifuged at 13,000 g at 4 ^{0}C, for 50 min. and the pellet was resuspended, in the washing buffer containing 1% Triton X-100, using a polytron homogenizer and centrifuged at 13,000 g for 50 min. The pellet was washed four times with washing buffer without Triton X-100. The pellet containing inclusion bodies was solubilized in 6 M guanidine hydrochloride by incubating for 2 hours at room temperature. The solubilized protein was centrifuged at 50,000 g, at 4 ^{0}C, for 30 min. and the protein concentration was adjusted to 10 mg/ml in the supernatant with 6 M guanidine hydrochloride. The denatured protein thus obtained was reduced by adding 65 mM dithioerythritol and incubated at room temperature for 2 h. To renature, the protein was diluted 100-fold in the refolding buffer and incubated at 10 ^{0}C for 48 h without stirring or shaking. Renatured material, after dialysis in 20 mM MES buffer, pH 5.0 containing 100 mM urea, was loaded on a S-Sepharose column, and the protein bound to the column was eluted with a 0-1 M NaCl gradient in 20 mM MES on an FPLC system (Pharmacia). The fractions containing the desired protein were pooled and concentrated, and the protein was further purified to homogeneity by gel filtration chromatography on a TSK 3000 column (LKB) in PBS, pH 7.4.

SDS-PAGE

The purified proteins were analysed by SDS-PAGE as described by Laemmli (1970). Restrictocin and its mutants were analysed on 12.5% resolving gels in 0.375

M Tris-HCl pH 8.8, containing 4% stacking gels in 0.125 M Tris-HCl pH 6.8. The gels were run in SDS-PAGE running buffer at a constant current of 40 mA. Proteins were visualized by staining the gels with Coomassie brilliant blue.

Western Blotting

The proteins were resolved on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane, in the transfer buffer, at a constant current of 300 mA for 2 h. The membrane was incubated in blocking buffer for 45 min. at room temperature with continous shaking. The membrane was further incubated in anti-restrictocin antibody diluted in PBS, pH 7.4, containing 0.1% Tween 20 (PBST), for 45 min.. The membrane was washed thrice with PBST, followed by incubation in anti-rabbit IgG-HRP conjugate diluted in PBST for 30 min. with shaking. After repeated washings with PBS, colour was developed by incubating the membrane with the chromogenic substrate 0.5 mg/ml of DAB.4HCl (diaminobenzidine tetrahydrochloride dihydrate) and 1 µl/ml of H_2O_2 in PBS.

Structural Characterization of Proteins by CD Spectroscopy

Circular Dichroic spectra of various proteins were recorded at room temperature, using a JASCO J710 spectropolarimeter fitted with a thermostated cell holder. 150 µg protein was dissolved in 3 ml of 10 mM sodium phosphate buffer (pH 7.0), and the samples were scanned in the far-UV range (200-250 nm). A cell with a 1 cm optical path was used to acquire the spectra at a scan speed of 50 nm/min. with a sensitivity of 50 mdeg and a response time of 1 sec. The sample compartment was purged with nitrogen, and spectra were averaged over 10 accumulations. The CD spectra were normalized to mean residue ellipticity curves using Jasco software. Yang's reference parameters were used to perform secondary structure analyses from CD measurements using Jasco Secondary Structure Estimation Programme (Yang *et al.*, 1986).

Preparation of rabbit reticulocyte lysate

The rabbit reticulocyte lysate was prepared as described by Sambrook *et al* (1989). A young male NZW rabbit weighing 2-2.5 Kg was subcutaneously injected for five consecutive days respectively with 2 ml, 1.6 ml, 1.2 ml, 1.6 ml and 2.0 ml of

1.2% acetylphenylhydrazine (APH) solution to render it anemic. The APH solution was prepared in sterile water and pH was neutralized to 7.0 with 1 M HEPES buffer (pH 7.5). The rabbit was allowed to recover for 5 days, after which it was bled. The blood was collected in a sterile tube, containing an equal volume of prechilled salt solution and the mixture was filtered through a cheese cloth. Filtrate was centrifuged at 2000 g for 10 min. at 4 ^{0}C. Supernatant was discarded, the pellet was washed twice with salt solution without heparin and finally, resuspended in equal volume of chilled sterile water. It was kept on ice for a minute and centrifuged at 20,000 g for 20 min. at 4 ^{0}C. The supernatant, containing the lysate, was immediately stored in liquid Nitrogen in 0.5 ml aliquots.

Specific Ribonucleolytic Activity Assay

The specific ribonucleolytic activity of restrictocin and its mutants was followed by detecting the release of the characteristic 400 nucleotide long α-fragment from 28S rRNA of eukaryotic ribosomes. All the reagents, water and glassware used during the experiment were treated with 0.1% DEPC to get rid of contaminating ribonucleases. Rabbit reticulocyte lysate (30 µl) was incubated with different concentrations of the toxin in 40 mM Tris-HCl (pH 7.5) containing 10 mM EDTA at 37 ^{0}C for 30 min. in a 50 µl reaction volume. The control reaction did not contain any toxin. The reaction was stopped by adding 2 µl of 10% SDS and incubated at ambient temperature for 5 min. Total RNA was extracted using Trizol reagent. 200 µl of the reagent was added to the reaction mixture, mixed well and incubated at room temperature for 5 min. Subsequently, 50 µl chloroform was added to each tube, mixed thoroughly and incubated at ambient temperature for 2 min. followed by centrifugation at 12,000 rpm, at 4 ^{0}C, for 15 min. in a microfuge (Plastocraft). The aqueous phase was mixed with 125 µl isopropanol to precipitate the RNA, allowed to stand at ambient temperature for 10 min. and centrifuged at 12,000 rpm at 4 ^{0}C for 15 min. The RNA pellet was washed with 75% ethanol, dried in air, dissolved in 10 µl of 0.5% SDS solution and electrophoresed on a 2% agarose gel after heating at 65^{0}C for 2 min. The RNA was visualized by ethidium bromide staining and photographed using Polaroid camera. The photographs were scanned, printed using a laser printer to present as figures in this thesis.

Assay for Inhibition of *in Vitro* Protein Synthesis

Cell-free protein synthesis inhibitory activity of restrictocin was assayed as described by Harlow and Lane (1988). The frozen rabbit reticulocyte lysate was thawed on ice in the presence of 30 µl of haemin solution per 0.5 ml vial. The toxin was diluted in 0.2% RNase free BSA and several concentrations were incubated with 10 µl lysate, 1 mM ATP, 0.2 mM GTP, 75 mM KCl, 2 mM magnesium acetate, 3 mM glucose, 10 mM Tris-HCl, pH 7.6, 4 µM amino acid mixture without leucine, 0.16 µCi [^{3}H] leucine, 1.33 mg/ml creatine phosphokinase, and 2.66 mg/ml creatine phosphate in a reaction volume of 30 µl. The reaction was carried out at 30 ^{0}C for one hour and terminated by adding 0.25 ml of 1 N NaOH containing 0.2% H_2O_2. The reaction mixture was further incubated for 10 min. at 37 ^{0}C, BSA added to a final concentration of 65 µg/ml, and the proteins were precipitated with 15% trichloroacetic acid. The mixture was left on ice for 30 min. for complete precipitation and harvested onto 26 mm glass fibre filters. The filter discs were placed in a manifold harvester (millipore) and rinsed with chilled 5% TCA, before the addition of reaction contents. The filters were thoroughly washed with chilled acetone and dried at 37 ^{0}C, for one hour. The dried filters were immersed in organic scintillation fluid, and counted using a liquid scintillation counter (Packard).

Assay of Cytotoxicity of Chimeric Toxins

Cytotoxic activity of restrictocin, its mutants and chimeric toxins was checked on a variety of cell lines. The cellular protein synthesis was assayed in the absence and presence of various concentrations of toxin by measuring [^{3}H] leucine incorporation in the newly synthesized proteins. Adherent cell lines namely A431, A549, HeLa, L929 and MCF7 were plated in RPMI 1640 containing 10% FCS at a density of $5x10^3$ cells per well in 96 well culture plates and allowed to adhere for 12 h at 37 ^{0}C in the presence of 5% CO_2. Next day, the medium was replaced with 200 µl of leucine free RPMI medium containing 10% serum. The leucine free RPMI containing 10% FCS was used for seeding partially adherent cell lines, COLO205 and J774A.1. Cells were allowed to adhere for 12 h and the toxin was added in the same medium. The suspension cell lines, HUT102 and K562 were plated at a density of $5X10^3$ cells/well in 80% leucine free RPMI containing 18% complete RPMI and 2% serum immediately before use. The medium used at various stages was supplemented

with appropriate antibiotics. Serial dilutions of the toxin, made in 0.2% HSA were added to the cells and incubated for indicated time periods. After incubation, the adherent cells were labeled with 0.25 μCi [^{3}H] leucine per well and the suspension cells with 0.75 μCi [^{3}H] leucine per well for 3 h at 37 ^{0}C. The cells were harvested on to filtermats using an automatic cell harvester, and the incorporation of the [^{3}H] leucine in the newly synthesized proteins was estimated using LKB β-plate counter. Activity was expressed as percentage of control where no toxin was added to the cells. To check the specificity of chimeric toxins for TFR, 10 μg of anti-TFR antibody (HB21) was added to each well prior to the addition of the fusion protein in the competition experiments.

Kinetics of Protein Synthesis Inhibition

The kinetics of intoxication by different restrictocin containing chimeric toxins was investigated by assaying the cytotoxic activity at various time points. HUT102, K562 and A431 cell lines were seeded at a density of 1X10^4 cells per well in 200 μl medium in a similar manner as described in cytotoxicity assay. The various concentrations of chimeric toxins were added and the cells were incubated at 37 ^{0}C in the presence of 5% CO_2 for different time points. At the end of each incubation period cells were pulsed with [^{3}H] leucine and the protein synthesis was measured as described above. The results were expressed as percentage of control where no toxin was added to the cells.

Binding Studies

Competition binding analysis was performed to compare the affinity of the chimeric toxins with native antibody. Anti-transferrin receptor antibody (HB21) was iodinated using iodogen method as described by Harlow and Lane, (1988). Adherent cell lines, namely, A431 and A549 were plated at 4X10^5 cells per well in a 24 well plate and used 16 h later for the assay. 500 μl medium, same as described in cytotoxicity assay, was used to dispense the cells. HUT102 cells were also plated at the same density in microfuge tubes and used immediately. After 2 washes with binding buffer (0.1% BSA in DMEM), various dilutions of toxin, along with 3 ng of labeled antibody in binding buffer, were added to the cells. The cells were incubated at 25 ^{0}C for 2 h with mild shaking, washed three times with binding buffer and lysed

in 10mM Tris/HCl (pH 7.4)/ 1mM EDTA/ 0.5% SDS. The radioactivity associated with the cells was counted in a γ-counter (LKB).

Trypsin Treatment

Tryptic hydrolysis of restrictocin and the chimeric toxins was carried out at neutral pH in 25 mM HEPES, pH 7.4 containing 1mM $CaCl_2$, 0.5 mM EDTA. For acidic pH, the buffer containing 200 mM sodium acetate, pH 5.2 and 1 mM $CaCl_2$ was used. Trypsin diluted in 50 mM PBS, pH 7.4 was added to the protein and the samples were incubated at 37 ^{0}C for specified time periods. The reaction was terminated by the addition of SDS-PAGE sample buffer. The digestion products were analyzed on a 12.5% SDS-PAGE using Tris-Glycine buffer system and restrictocin containing fragments were detected by western blotting with anti-restrictocin antibodies.

Preparation of Lipid Vesicles

The organic solution of phospholipid was made in a 1:1 mixture of chloroform and methanol at a concentration of 5 mg/ml. To get a thin and uniform lipid film, lipid was dried down onto the walls of a round bottom flask by rotating for 30 min. in a rotary evaporator fitted with a cooling coil and a thermostatically controlled water bath. The temperature of the water bath was maintained 10 ^{0}C above the phase transition temperature of the respective phospholipid. The film was stored under vacuum for 12 h to remove the traces of the solvent after flushing with nitrogen. Subsequently, lipid film was dispersed in 30 mM Tris-HCl buffer containing 0.1M NaCl, pH 7.0 at a concentration of 1 mg/ml of lipid using glass beads of 0.5 mm-3 mm in diameter. Multilamellar vesicles (MLVs) were obtained by manual swirling for 2 h at room temperature (Bangham *et al.*, 1965). Nitrogen was flushed into the flask and the vesicle suspension was left overnight at 4 ^{0}C. The following day, maintaining the temperature 10 ^{0}C above the phase transition temperature of the corresponding phospholipid, MLVs were sonicated in a water bath sonifier (Branson 3210) for 45 min. to get the small unilamellar vesicles (SUVs) (Huang C, 1969).

Aggregation of Lipid Vesicles

The aggregation of the phospholipid vesicles, induced by ribotoxins, was assayed by titrating 40 nmoles of freshly prepared lipid vesicles with various molar concentrations of restrictocin and its mutants. The samples of 1 ml volume were prepared in 30 mM Tris-HCl buffer, pH 7.0, containing 0.1M NaCl and incubated at 37 ^{0}C for 1h. The change in absorbance due to an increase in turbidity of the lipid suspension was measured at 400 nm in Lambda Bio 20 spectrophotometer (Perkin Elmer) in the cells of 1 cm optical path length. Appropriate control proteins, not interacting with the membranes and the vesicles without proteins were included in all the experiments. The change in absorbance of the lipid suspension was plotted against the toxin concentration.