2. Materials and Methods
2.1. Sources of materials

*E. coli* strain DH5α was obtained from Bethesda Research Labs (B.R.L., U. S. A.) and was used for all recombinant DNA work. Plasmid vectors pTZ18R and pBluescript II KS+ were obtained from United States Biochemicals (U. S. B., U. S. A.) and Stratagene (U. S. A.) respectively. Restriction enzymes and other molecular biological reagents were purchased from New England Biolabs (N. E. B., U. S. A.), Roche Biochemicals (Germany), Amersham Pharmacia (U. S. A.), Promega (U. S. A.) Sigma (U. S. A.) and Qualigens (India). Sequenase Version 2.0 DNA sequencing kit was obtained from Amersham; Random priming kit for labeling DNA was obtained from NEB; PCR kit was from Amersham; Oligonucleotide primers were synthesized by Microsynth (Switzerland); $^{32}$P-α dATP (specific activity ~3000 Ci/m mol) was obtained from Amersham, and $^{32}$P-γ ATP (specific activity ~5000 Ci/m mol) was obtained from Bhaba Atomic Research Centre (BARC, India). Adult Bovine Serum was purchased from Biological Industries (Israel) and *E. coli* media components were from DIFCO (USA). Diamonds Vitamin Mix for *Entamoeba* culture was purchased from Biosource International (U.S.A.). X-Ray films were from Konica and charged nylon membranes (GeneScreen plus) was obtained from New England Nuclear (N.E.N, USA) and nitrocellulose papers were obtained from Schleicher and Schuell (Germany).

(All concentrations indicated in percentage are in (w/v) basis unless stated otherwise. All solutions were prepared in double distilled water unless stated otherwise. Autoclaving was done at a pressure of 15lbs per square inch for 20 min.)

2.2. Organisms and growth conditions

*E. coli* DH5-α has the genotype: *SupE44 lacU169 (φ80 lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*. Cells from an agar stab or frozen glycerol stock were first streaked on an LB plate (containing the appropriate antibiotic wherever necessary) and allowed to grow overnight at 37°C. Liquid cultures in LB medium were initiated from a single colony and were grown with constant shaking at 225 rpm at 37°C. The cells were grown overnight, were used as inoculum for further growth by diluting 100 fold in fresh LB medium and grown with aeration at 37°C for 3-4 hr to obtain log phase cultures.

*E. histolytica* strain HM-1:IMSS clone 6 was obtained from Dr. William A Petri (University of Virginia, USA); all experiments were done with *E. histolytica* strain HM-1: IMSS clone 6. The
Materials and Methods

cells were maintained and grown in TYI-S-33 medium with 15% adult bovine serum, 2% Diamond's vitamin mix and antibiotic (125 μl of 250 U/ml Benzyl Penicillin and 0.25 mg/ml Streptomycin per 100 ml of medium). Hygromycin (Sigma) was added at 10 μg/ml for maintaining transgenic cell-lines pEhCaBP1-S and pEhCaBP1-AS. Cell viability was determined by microscopy in presence of trypan blue.

2.2.1. Measurement of cell number. Growth of trophozoites was measured by cell counting in triplicates using a hemocytometer (Neubauer, Marienfeld, Germany).

2.2.2. Luria Broth (LB) Composition per litre

- Tryptone: 10 g
- Bacto-Yeast extract: 5 g
- Sodium Chloride: 10 g

The components were dissolved in double distilled water and pH adjusted to 7.0 using 2N NaOH. The medium was sterilized by autoclaving.

2.2.3. LB Agar

LB agar was prepared by adding 1.5% (w/v) of Bacto-Agar to LB medium and sterilized by autoclaving. Ampicillin was added to a final concentration of 100 μg/ml (when required) after cooling the LB agar to around 55°C and plates poured.

2.2.4. TYI-S-33 medium Composition per 900 ml (10 units) (Diamond LS et al., 1978)

- Potassium phosphate, dibasic: 1.0 g
- Potassium phosphate, monobasic: 0.6 g
- Biosate peptone: 30.0 g
- Dextrose: 10.0 g
- Sodium chloride: 2.0 g
- L-Cysteine hydrochloride: 1.0 g
- Ascorbic acid: 1.0 g
- Ferric ammonium citrate: 22.8 mg
Materials and Methods

To these components 700ml of double distilled water was added and pH adjusted to 6.8 using 2 (N) NaOH. The volume was made up to 900 ml and filtered using Whatman #1 filter paper, aliquoted and autoclaved. Incomplete medium was stored at -20°C.

2.2.5. Heat Inactivation of Serum

Adult bovine serum was stored frozen at -20°C. Before heat inactivation the serum was thawed at room temperature and incubated in a water-bath at 37°C for 30 min with intermittent shaking. The serum was transferred to 55°C for 45 min with intermittent shaking for complement inactivation. The inactivated serum was stored at 4°C.

2.3. Plasmid DNA isolation

2.3.1. Boiling Method (mini-prep) (Holmes DS and Quigley M, 1981)

A single bacterial colony containing the plasmid was inoculated in 2 ml LB medium containing the appropriate antibiotic (50 µg/ml in case of Ampicillin) and grown overnight at 37°C with shaking at 225 rpm. The overnight culture was transferred to a 1.7 ml microfuge tubes and centrifuged at 6,000 rpm for 5 min. The medium was completely drained off and the pellet was resuspended in 0.3 ml of STET buffer (8% Sucrose, 0.5 % (w/v) Triton X-100, 50 mM EDTA, pH 8.0), containing 0.25 mg of lysozyme by vortexing. The tube was incubated on ice for 10 min and then kept in a boiling water bath for 3 min. To this 0.3 ml of ice-cold 5M LiCl was added and mixed by inverting and centrifuged at room temperature at 12,000 rpm for 10 min. The mucoid pellet was removed using a sterile toothpick. The DNA was precipitated by addition of 0.6 ml of isopropanol and mixed by inverting. The DNA was pelleted by centrifuging at 12,000 rpm for 10 min at room temperature. The pellet so obtained was washed with 200 µl of 70 % (v/v) ethanol, air dried and dissolved in 20 µl of T10E1 (10mM Tris, Cl, pH 8.0, 1mM EDTA, pH8.0) containing 0.1mg/ml RNAse A. The tube was incubated at 37°C for 45 min for RNAse digestion and 2 µl was loaded on a 0.8 % agarose gel to quantitate the DNA.

2.3.2. Alkaline lysis method (midi-prep) (Birnboim HC and Doly J, 1979)

Bacterial cells were grown overnight in 50 ml LB medium with the appropriate antibiotic and collected by centrifugation (6,000 rpm at 4°C for 8 min). The cell pellet was resuspended in lysis buffer (25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0, 15% Sucrose) containing 2 mg/ml lysozyme, and kept on ice for 10 min. To the lysed cells were added 3 ml of denaturing solution (freshly prepared solution containing 0.2 N NaOH and 1% SDS) and mixed gently by inverting.
To the tube was added 1.6 ml of 3 M sodium acetate (pH 4.6) and incubated on ice for 20 min followed by centrifugation at 12,000 rpm for 20 min at 4°C. RNA was removed by incubating the supernatant with 12-15 µl of RNAse A (10mg/ml) at 37°C for 45 min. The supernatant was extracted twice with phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)] and once with equal volume of chloroform:isoamyl alcohol [24:1 (v/v)]. The upper aqueous layer was transferred to an oakridge tube and the DNA precipitated by addition of 2.5 volumes of chilled ethanol and left for overnight incubation at -20°C or 45 min at -80°C. The DNA was precipitated by centrifugation at 12,000rpm for 20 min at 4°C. The pellet was resuspended in 0.4 ml of nuclease free water and to this was added 120 µl of 4M NaCl and 0.5ml 13% PEG 8000. The tube was incubated on ice for 1 h and centrifuged at RT at 12,000 rpm for 15 min. The pellet was washed with 200 µl of 70 % ethanol, dried in a 37°C incubator and resuspended in 50 µl of T10E1.

2.4. Preparation of competent cells and transformation (Hanahan D, 1983)

*E. coli* (strain DH5α) was grown overnight in 2ml LB medium and a 1% inoculum was added to 50 ml LB medium in 500 ml flask. The cells were grown to an OD600 of 0.38-0.42. The cells were vigorously shaken on ice water for 15 min and were thereafter collected by centrifugation at 6,000 rpm for 5 min at 4°C and resuspended in 25 ml ice-cold filter sterilized 0.1 M CaCl2. After 15 min incubation in ice with occasional shaking, the cells were collected again by centrifugation at 6,000 rpm, 5 min, 4°C. The halo shaped pellet was finally resuspended in 2 ml of ice-cold 0.1 M CaCl2. The competent cells were stored in 15% glycerol stocks in 100 µl aliquots at -70°C.

Competent cells were thawed on ice and to 100 µl cell suspension, 5-10 ng of plasmid DNA was added. The cells were incubated on ice for 45 min. Cells were then given a heat shock at 42°C for 90 seconds and incubated in ice for 2 min. 0.9 ml of LB was added to the cells and the cells were grown at 37°C for 1h at 225 rpm. Transformants were plated on LB agar plates with appropriate antibiotic and incubated at 37°C for 14-16 h.

2.5. Restriction enzyme digestion

Restriction enzyme digestions were carried out in small amounts, usually 20µl (50 µl in case of genomic DNA digests and insert isolations). Appropriate amount of DNA was digested in a reaction mixture containing enzyme buffer (as per manufacturer's instructions) and 5-10 units of
Materials and Methods

enzyme at the recommended temperature for 4-16 hours. After incubation the reaction mixtures were loaded with 1X gel loading buffer (GLB) (2.5 % Ficoll type-400, 0.04% bromophenol blue, 0.04% xylene cyanol FF) into agarose gel and run for appropriate times.

2.6. Insert isolation by n-Butanol method

The agarose slice containing insert was trimmed as much as possible and minced with a sterile blade. This was transferred to a microfuge tube to which was added 600-900 µl of T10E1. The gel mince was shaken at 225 rpm, 37°C for 45 min and then incubated at 4°C overnight. The contents were repeatedly frozen in 70% isopropanol slush and thawed at 37°C thrice. The gel was pelleted down by centrifugation at 15,000 rpm, 4°C for 20 min. The supernatant was carefully transferred to a fresh tube avoiding the gel pieces. The DNA was concentrated by repeated extractions with butanol till the volume of the lower DNA phase was reduced to about 250 µl. The DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v). The upper aqueous phase was transferred to a fresh microfuge tube and to this was added 1/10th volume 3M sodium acetate (pH 5.2) and 2.5 volumes ice cold ethanol. The DNA was precipitated and washed with 70% ethanol as mentioned earlier and the pellet dissolved in T10E1. Insert isolation and DNA purification kit from Amersham was also used.

2.7. PCR product purification.

The PCR reaction volume was increased to 200 µl and the PCR product was once extracted with equal volume of CHCl3:isoamyl alcohol (24:1). Precipitation of DNA from the aqueous phase was carried out with 1/20th volume of 10 M NH4OAc and equal volume of isopropanol. After incubation on ice for 30 min, the PCR product was precipitated by centrifugation and the pellet was washed with 70% ethanol. The pellet dissolved in nuclease free H2O.

2.8. Isolation of genomic DNA from Entamoeba trophozoites (Bhattacharya S et al., 1988)

Entamoeba cells (approximately 4 X 10⁷ cells) were harvested by chilling on ice for 10 min and centrifuged at 300 g at 4°C for 8 min. The cells were washed once with PBS # 8 [0.37% K2HPO4, 0.11% KH2PO4 and 0.95% NaCl, pH 7.2]. The cell pellet was resuspended in 4 ml of ice-cold NET 1 buffer [10 mM Tris-Cl pH 8.0, 100 mM NaCl, 10 mM EDTA, pH 8.0]. To this was added 10% SDS to a final concentration of 0.25%, gently mixed by inverting and transferred on to ice.
Materials and Methods

After 2-3 min when the cell suspension became viscous, equal volume of phenol was added and mixed thoroughly by gentle inversion. The phases were separated by centrifugation at 6,000 rpm, 4°C, and 20 min. The upper aqueous layer was transferred to a fresh tube and extracted once with phenol, once with phenol: chloroform: iso-amyl alcohol [25:24:1 (v/v/v)] and once with chloroform:iso-amyl alcohol [24:1 (v/v)]. All the centrifugation steps were done at 6,000 rpm, 4°C, 10 min. The DNA was precipitated with 1/10th volume of 4 M NaCl and 2.5 volumes of ethanol and incubated overnight at -20°C. The DNA was pelleted down by centrifugation at 12,000 rpm for 20 min at 4°C. The pellet was resuspended in 4 ml of NET 2 buffer [10 mM Tris-Cl pH 8.0, 10 mM NaCl, 10 mM EDTA, pH 8.0] and digested with RNase A (100 µg/ml) at 37°C for 30 min followed by Proteinase K (100 µg/ml) at 55°C for 45 min. The DNA was extracted with phenol:chloroform:isoamyl alcohol as described above but the temperature during centrifugation was 20°C. The DNA was precipitated with NaCl and ethanol as mentioned above and the pellet was washed with 70% ethanol. The pellet was dried and dissolved in 100 µl of T10E1.

2.9. Agarose gel electrophoresis

The agarose concentrations used in electrophoresis separation were chosen based on the size of the DNA to be resolved. Agarose was melted in 0.5X TBE [45 mM Tris-borate and 1mM EDTA, pH 8.0] by heating and was cooled to about 50°C before adding 0.5 µg/ml of ethidium bromide. The molten agarose was poured in a tray and allowed to gel. After the gel had set, DNA samples were loaded and electrophoresed in 0.5X to 1X TBE in appropriate electric field strength for optimum separation. The DNA was visualized at 302 nm using a UV trans-illuminator.

2.10. Southern Blotting (Southern EM, 1975)

DNA samples were digested with restriction enzymes and separated on agarose gel as described above. The fragments were transferred from agarose gels onto nylon membrane [GeneScreen Plus (GS+)] as described in product protocol (GS+). Briefly, the gel was removed from the tray and placed in a dish containing 0.25 N HCl for 15 min with intermittent shaking to depurinate the DNA. After depurinating, the DNA was denatured by incubation with 0.4 N NaOH containing 0.6 M NaCl for 30 min. The gel was then neutralized with 0.5 M Tris-Cl, pH 7.5,
containing 1.5 M NaCl for 30 min. The treated gel was placed on two sheets of Whatman 3 MM paper saturated with 10X SSC [1.5 M NaCl and 0.15 M Na-Citrate pH 7.0]. A sheet of GS+ membrane presoaked in 10X SSC was placed on the gel taking care that no air bubble(s) were trapped in between the membrane and the gel. On this was placed two sheets of Whatman 3 MM paper and a thick layer of dry absorbent paper (all cut to the size of the gel). Finally a weight was placed over the assembly to keep the papers firmly pressed on the gel. After 16-20 h the nylon membrane was carefully dislodged from the dry gel after marking the position of the wells and immersed in 0.4 N NaOH solution for 60 sec to ensure complete denaturation of immobilized DNA. The membrane was transferred to a neutralizing solution [2X SSC, 0.2 M Tris-Cl pH 7.5] for 60 sec and then air dried at room temperature. The membrane was then crosslinked in an UV Crosslinker (Gene Pulser, Bio-Rad) using the appropriate programme.

2.11. Hybridization of radiolabeled probes to immobilized nucleic acids
2.11.1. Preparation of radiolabeled DNA by (i) random priming method (Feinberg AP and Vogelstein B, 1983)

About 50-100 ng of linear DNA was denatured by heating in a boiling water-bath for 10 min and immediately chilled on ice. To the tube containing denatured DNA, 2 μl each of 0.5 mM dGTP, dCTP and dTTP, 5μl of hexanucleotide labeling mixture [containing random hexamers and reaction buffer at 10X concentration (2 M HEPES pH 6.6, 2 mM Tris-Cl pH 7.0, 0.1mM EDTA and 4 mg/ml BSA), 30-50 μCi (32P) α-dATP] and nuclease free water was added to make up the volume to 50 μl. The reaction was initiated by adding 5U of Klenow enzyme. All the components used were from Random priming kit, NEB, USA. Incubation was carried out for 2-3 hr at 37°C before stopping the reaction by addition of EDTA to a final concentration of 20 mM. Unincorporated dNTPs were removed by ethanol precipitation in the presence of 50 μg of carrier DNA (salmon sperm DNA) and 2.5 M ammonium acetate.

2.11.2. Strand-specific labeling using specific primers. For probing sense and antisense RNA, specific primers 5'-GGG GGA TCC CAT ATG GCT GAA GCA CT-3' and 5'-GGG GGT ACC AGT TTA GAG TGA AAA CTC A-3' were used to radiolabel the DNA strand and the mRNA strand, respectively. Radiolabeling was carried out in presence of the Klenow fragment of DNA Polymerase (minus 3'-5' exonuclease activity) and the corresponding Klenow buffer (NEB, USA).
2.11.3. Hybridization of radiolabeled probe to DNA immobilized on Gene Screen Plus nylon membrane

DNA blots were first incubated in prehybridization solution (1% SDS and 1 M NaCl, 0.3-0.4 ml per square cm of membrane) at 65°C in seal-a-meal pouches or hybridization bottles. After 3 h, heat-denatured radiolabeled probe (2 x 10^5 dpm/ml) and 100 μg/ml denatured salmon sperm DNA were added to the prehybridization mix. The hybridization was carried out at 65°C for 16-18 h. The membranes were washed sequentially to remove non-specifically bound probe using the following protocol: twice with 2X SSC at room temperature for 5 min each, twice with 2X SSC containing 1% SDS at 65°C for 20 min each and finally twice with 0.1X SSC at room temperature for 20 min each.

2.11.4. Removal of probe from nylon membrane for rehybridization

The probe was stripped off the membrane by incubating the membrane in a boiling solution of 0.1X SSC and 0.1% SDS for 20-30 min. Efficiency of the removal of probe was monitored by exposing the blot to an X-ray film before hybridization.

2.12. Autoradiography

After hybridization and washing, the blots were wrapped in saran wrap and mounted. Autoradiography was performed with an X-ray film and intensifying screens at -70°C for an appropriate amount of time.

2.13. Colony hybridization (Sambrook J et al., 1989)

2.13.1. Colony lifting and immobilization on nitrocellulose

The putative recombinants (white colonies) were consolidated by patching on 150 mm LB plates supplemented with 100 μg/ml of ampicillin and were transferred to nitrocellulose filters by placing a dry filter gently on the plate to avoid trapping air bubbles. The filter was removed from the agar surface and placed colony side up on a series of Whatman 3 mm sheets soaked in the following solutions:

(a) 10% SDS for 3 min.
(b) 0.5 M NaOH containing 1.5M NaCl for 5 min to lyse the cells and denature DNA.
(c) 0.5 M Tris-Cl (pH 7.5) containing 1.5 M NaCl for 5 min to neutralize.
(d) 2X SSC for 5 min.

After air drying, the DNA was fixed to the filter by baking for 2 h at 80°C in a vacuum oven.
2.13.2. Hybridization

The baked filters were soaked in 2X SSC for 5 min and pre-washed in 5X SSC containing 0.5% SDS and 1 mM EDTA (pH 8.0) for 30 min at 50°C. Pre-hybridization was performed in pre-hybridization mix [5X Denhardt's solution is 1% each of ficoll, polyvinylpyrrolidone and BSA (Pentax fraction V)]. After prehybridization for 3-4 h, 4 x 10^5 dpm/ml of heat denatured radiolabeled probe was added and incubation was continued at 68°C for 16 h. The blots were washed as described below:

(a) twice with 2X SSC, 0.1% SDS at room temperature for 5 min with one change
(b) twice with 0.2X SSC, 0.1% SDS at 68°C for 45 min with one change. Autoradiography was done as described earlier.

2.14. DNA dot blot

The DNA was denatured by addition of NaOH to a final concentration of 0.25 N, in a total volume of 200 µl. After keeping the DNA at room temperature for 30 min, it was transferred on to ice. The GS+ membrane cut to the required size was saturated in 0.4 M Tris.Cl pH 7.5 for 15 min and the DNA were spotted on to the membrane with the help of a mani-fold apparatus. The blots were air-dried and then used for hybridization after UV cross-linking.

2.15. DNA sequencing

2.15.1. Alkali denaturation of DNA template

1.5 pmoles of supercoiled plasmid DNA (approximately 10 µg) was denatured in 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37°C followed by neutralization with 0.1 volume of 3M sodium acetate pH 5.0. The denatured DNA was precipitated and collected as described before.

2.15.2. Sequencing reaction (Sanger F et al., 1977)

About 1.5 pmoles of primer was annealed to the denatured DNA in a 10 µl reaction volume containing 2 µl of 5X Sequenase buffer [40 mM Tris-Cl pH 7.5, 10 mM MgCl₂ and 50 mM NaCl] at 37°C for 30 min and mixed with 1µl of 0.1 mM DTT, 2 µl sequenase labeling mix diluted 1:5 (labeling mix contained 7.5 µM each of dGTP, dTTP and dCTP), 0.5 µl of ^35^S -α dATP. The labeling reaction was initiated by addition of 0.25 µl of sequenase followed by incubation at 25°C for 5 min. From this, 3.5 µl was transferred into four separate tubes containing 2.5 µl of dideoxy termination mixtures (G, A, T, C). These tubes were further
incubated at 37°C for 15 min. The reaction was stopped by addition of 4 µl of stop buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue).

2.15.3. Sequencing gel and electrophoresis conditions (Sambrook *et al* 1989):
The glass plates used for sequencing gels were thoroughly cleaned with detergent. The larger plate was siliconized with dimethyl-dichloro silane and the smaller plate was antisiliconized with α-methacryloxypropyl-trimethoxysilane. The sandwich was assembled as per manufacturer's instruction. 150 ml of the gel mix was prepared containing 6% acrylamide (acrylamide:bisacrylamide=19:1) and 7M urea in 1X TBE. The gel mix was filtered before adding 600 µl of freshly prepared 10% ammonium persulfate and 80 µl TEMED. The contents were mixed and poured immediately into the sandwich taking care that no air bubble was introduced. The gel was used 2-16 h after pouring. The temperature of the gel was brought upto 50° by performing a pre run at 60-80 W in 1X TBE for 30-45 min. Samples were denatured by incubating at 90°C for 2-3 min just before loading. The gel was run at 50°C at 60-80 W. Each sample was loaded twice with a second loading done when the xylene cyanol dye front of the first loading had migrated to the bottom of the gel. Electrophoresis was terminated when the bromophenol blue dye front of the second loading reached the bottom of the gel. After the gel run was over, the sandwich was dismounted and disassembled. The gel which was attached to the smaller plate, fixed in 10% (v/v) methanol and 10% (v/v) acetic acid for 30 min with intermittent shaking before drying at 55°C for 14-16 h.

2.16. Analysis of RNA
Glassware used for RNA isolation and analysis were treated with 0.1% (v/v) DEPC (Diethyl pyrocarbonate solution in water) for 10-16 hr at 37°C followed by baking at 180°C for 8 h as described by Sambrook *et al.* (1989). Electrophoresis chamber used for RNA samples was treated with 3% (w/v) hydrogen peroxide solution for 10 min in the dark and washed extensively with DEPC treated water.

2.16.1. Isolation of total RNA from *E. histolytica* (Chomczynski and Sacchi, 1987)
*E. histolytica* trophozoites were harvested and washed with PBS#8 as described for DNA isolation. Freshly washed cells (4 × 10^7) were resuspended in 4 ml of solution A [4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl] containing 72 µl of 2-mercaptoethanol and vortexed for 1 min at room temperature. The homogenate was mixed with
Materials and Methods

400 μl of 2 M sodium acetate (pH 4.0), 4 ml water saturated phenol and 2 ml of chloroform:isoamyl alcohol [49:1 (v/v)] followed by inverting the tube several times. The phases were separated by centrifugation at 10,000 x g for 20 min at 4°C. The crude RNA was recovered from the aqueous phase by precipitating with equal volume of isopropanol at -20°C for 1 h followed by centrifugation at 12,000 x g for 10 min at 4°C. The pellet was resuspended in 1.2 ml of solution A and the RNA re-precipitated with isopropanol, followed by washing with 75% (v/v) ethanol. The purified RNA preparation was vacuum dried and resuspended in DEPC treated water.

Tripure Isolation reagent (Roche Scientific, USA) was also used for some RNA preparations which essentially follow the procedure developed by Chomozynski and Sacchi.

2.16.2. Fractionation of RNA by agarose gel electrophoresis in Glyoxal / DMSO gel
The RNA (10 - 20 μg) was denatured by incubating with 6 M deionized glyoxal, 50% (v/v) DMSO and 1X gel running buffer [12 mM Tris-HCl, 6 mM sodium acetate and 0.3 M EDTA, pH 7.0] at 50°C for 15 min followed by chilling on ice. The denatured RNA sample was mixed with glyoxal/DMSO gel loading buffer [final concentration was 6% (w/v) glycerol, 1X gel running buffer, 0.03% bromophenol blue, and 0.03% xylene cyanol FF] and electrophoresed through a 1.0% agarose gel in 1X gel running buffer at 3 V/cm for 3 h. The tank buffer was changed every 30 min to prevent glyoxal from dissociating from RNA which occurs at pH > 8.0. λ - Hind III digest and pTZ18R - Hinf I digest, treated in the same way as the RNA sample, were used as molecular weight markers along with commercially available RNA markers. After electrophoresis, the portion of the gel containing the molecular weight markers and duplicate RNA samples was stained with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30 - 45 min. The excess stain was removed by incubating the gel in 0.1 M ammonium acetate for 15 min. The gels were visualized under UV illumination (302 nm).

2.16.3. Reverse Northern Analysis. DNA samples of genes to be analyzed were spotted onto a nylon membrane. In experiments involving comparisons of relative hybridization intensities, the same amount of DNA was spotted. Equal amounts of total RNA of different samples were labeled with [α³²P]dATP using reverse transcriptase (Promega, USA) and equal number of counts were used for each hybridization. After hybridization the filters were washed under stringent conditions. The amount of radioactivity hybridized was quantitated by a phosphoimager.
2.16.4. Northern blotting
The glyoxal / DMSO gel was used as such for northern transfer. RNA was transferred from the gel to nylon membrane essentially as described for Southern blotting. After the transfer, in the case of glyoxal / DMSO gel, the membrane was incubated in 50 mM NaOH for 15 sec for reversal of glyoxylation followed by neutralization in a solution containing 1X SSC and 0.2 M Tris-Cl (pH 7.5) for 30 sec. The membrane was then allowed to dry at room temperature and cross-linked in a UV cross-linker.

2.16.5. Hybridization and washing conditions for RNA blot
2.16.5.1. As per manufacturer's protocol
The blots were prehybridized in prehybridization solution (1 M NaCl and 1% SDS) at 65°C for at least 2 h. Denatured radiolabeled probe (3-4 x 10^5 dpm/ml) and salmon sperm DNA (100 µg/ml) were added and the incubation continued for 16 h. The blots were washed as described above for southern hybridization.

2.16.5.2. Formamide method
The blots were prehybridized in prehybridization solution (5X SSC, 50% formamide, 5X Denhardt’s reagent, 1% SDS, 10% Dextran sulfate) at 42°C for at least 2 h. Denatured radiolabeled probe (3-4 x 10^5 dpm/ml) was added and the incubation continued for 16 h. The blots were washed as described below:
(a) 2X SSC at room temperature for 15 min.
(b) 0.2X SSC at room temperature for 15 min.
(c) 0.1X SSC at room temperature for 15 min.

2.17. Protein Estimation:
(a) BCA method: The amount of protein in a sample was estimated by the bicinchoninic acid assay using BSA as the standard (Smith et al, 1985). The working solution was prepared by mixing bicinchoninic acid (Sigma) and 4% copper sulphate in a ratio of 50:1. To 10 µl of protein (appropriate dilutions) was added 200 µl of the working solution in a microtitre plate and incubated at 37°C for 30 min. The absorbance was taken at 560 nm using a microtiter plate reader (Bi0-Rad, USA).

(b) Lowry's method: Bio-Rad Protein Assay kit based on Lowry's method was used.
Materials and Methods

Sample preparation: 10 µl of sample + 990 µl H₂O + 250 µl Assay Reagent = OD₅₉₅ X 1000 X 20 (or 27 for crude extract) / x µl (sample volume) = µg/ml concentration.

2.18. SDS-Polyacrylamide gel electrophoresis (Laemmli UK, 1970)
SDS-PAGE was carried out under reducing conditions. The separating gels (10/14% acrylamide as per need) was prepared using acrylamide (acrylamide:bis-acrylamide=19:1) in 1.5% Tris-Cl pH 8.8, 0.1% (w/v) SDS, 0.04% (w/v) APS and TEMED. After polymerization of separating gel, stacking gel was poured. The stacking gel contained 4% acrylamide in 0.5% Tris-Cl pH 6.8, 0.1% (w/v) SDS, 0.04% (w/v) APS and TEMED. Prewarmed samples and 4X SDS-PAGE loading bye [ 125 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 10% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.2% (w/v) bromophenol blue] were mixed to 1X dye concentration and reboiled for 2 min. After electrophoresis, proteins were fixed in the gel by incubating in fixing solution (50% methanol, 7.5% acetic acid) and detected by Coomassie Brilliant Blue (0.25% CBB R-250 in fixing solution) staining for 1 hr. The gels were destained in the fixing solution and dried.

2.19. Transfer of proteins to nitrocellulose
Polyacrylamide gel to be transferred was incubated in transfer buffer for 15 min. The treated gel was placed on two sheets of Whatman 3 MM paper cut to the size of the gel, saturated with transfer buffer. A sheet of nitrocellulose presoaked in buffer was placed on the gel taking care that no air bubble(s) were trapped between the membrane and the gel. On this was placed two sheets of Whatman 3MM paper. For wet transfer (Hoefer Scientific Instruments, USA), the sandwich was placed between the grids before inserting into the tank containing buffer with gel side towards cathode (-ive). For semi-dry transfer (Owl Scientific, USA), the transfer was set on the bottom plate (-ive) with gel below the membrane, with wet Whatmann 3 MM sheets below and above them as described above.

Buffer for wet transfer (for 2 litre):
Tris-Cl- 4.84 g
Glycine- 28.8 g
Methanol- 400 ml
H₂O- 1.6 lit
pH 8.3
Materials and Methods

Buffer for semi-dry transfer (for 500 ml):
Tris-Cl 1.51 g
Glycine 7.2 g
Methanol 100 ml
pH 8.3

2.20. Preparation of amoebic extract

a. By cell-lysis: Crude cell extracts were prepared from amoeba in 1 mM Tris-Cl, pH 7.5, 1% SDS containing 2 mM PHMB, 1 mM PMSF, 6 mM Leupeptin, 1 mM N-ethyl-maleimide. The lysate was tapped well and then boiled for 3 min, followed by immediate chilling on ice. Quantitation was done before aliquoting to store at –20°C or analysis by SDS-PAGE. To separate membrane proteins from cytoplasmic fraction the cell extract was prepared by three cycles of freeze-thawing in 100 mM Na₂HPO₄ buffer containing the above mentioned protease inhibitors, ultracentrifuged at 100,000 X g, 30 min, resulting supernatant as cytoplasmic fraction and membrane fraction as pellet. Pellet was washed twice and resuspended in the same buffer containing 1% Triton X-100 and recentrifuged at 100,000 X g, 20 min, to separate the triton soluble fraction (cytoplasmic) from triton insoluble fraction.

b. By TCA precipitation: Cells washed with PBS was resuspended in 100 μl of PBS containing 0.1% BSA. To it was added equal volume of 15% TCA and incubated on ice for 10 min. The precipitated mass was collected on GFC (25 mm circles, Whatman) by filtration. The precipitate was washed well with 3% TCA followed by final rinse with 100% ethanol. The filter was dried in oven at 50°C, dried protein on membrane was solubilized in Cocktail ‘O’ and incorporated radioactivity was counted in a scintillation counter (Beckman, LS 1800).

2.21. Enzyme linked Immunoabsorbent Assay (ELISA)

2-5 μg of the Entamoeba total lysate in PBS was used to coat wells of a microtiter plate (Costar, USA) overnight at 4°C. Nonspecific sites were blocked with 3% (w/v) gelatin in PBS for 1 h at room temperature. Primary antibody incubation was carried out for 1 h at room temperature in 1% gelatin-PBS. Secondary antibody incubation was done for 30 min at room temperature with alkaline phosphatase linked IgG. The wells were washed thoroughly with PBS after every incubation. Bound antibodies were detected with using the colorimetric substrate p-nitro-
Materials and Methods

phenylphosphate (PNPP, Sigma) and monitoring the absorbance at 405 nm with a microtiter plate reader (Bio-Rad, USA).

2.22. Western analysis
For Immunodetection, SDS-PAGE separated proteins were transferred on to nitrocellulose membrane in 1X Towbin Buffer (0.025 M Tris, 0.192 M glycine, 20 % Methanol, pH 8.3) using Semi-dry Electroblotter, Owl Separation Systems. The membrane was blocked overnight at 4°C with 5% milk in PBS/ 0.1% Tween 20. Primary antibody followed by secondary antibody incubation was done in 1% milk in PBS/0.1% Tween20 with shaking at room temperature for 1 h and 30 min, respectively. Blots were washed thoroughly with PBS/0.1% Tween 20 after every incubation step with antibody. The second antibody used was horseradish peroxidase conjugated IgG. Band detection was done using ECL kit (Amersham). Antibody dilutions used: EhCaBP (polyclonal, rabbit); 1:5000, Actin (monoclonal, mouse, ICN); 1:500, Myosinlb (polyclonal, rabbit); 1:5000, PAK (polyclonal, rabbit); 1:5000, EhCaBPl; 1:1000, Anti-Rabbit Peroxidase (Nordic Immunology); 1:5000, Anti-Mouse Perodiase (Molecular Probes); 1:10,000.

2.23. Metabolic Labeling of Entamoeba histolytica:
E. histolytica trophozoites were labeled using [35S] methionine as described earlier (Bhattacharyya et al, 1990). Cells (2.5 X 10^6) were washed once with TYI-S-33 medium and then resuspended in the same medium at 5 X 10^5 cells/ml in 4ml screw-capped glass vials. The incubation was carried out for 1hr at 36°C followed by the addition of [35S]methionine (100-200μCi/ml). After 6hr of incubation, cells were harvested and washed twice with chilled medium and once with PBS#8. The radiolabeled cells were were resuspended in Buffer A (10 mM Tris.Cl, pH 7.5 and protease inhibitor cocktail) and lysed by freeze-thaw and sonication. All procedures were carried out at 4°C. The soluble cytoplasmic and particulate membrane fractions were prepared by ultracentrifugation of the cell lysate at 100,000 X g for 30 min at 4°C. The supernatant was used as the cytoplasmic fraction.

2.24. Immunoprecipitation.
The lysate (labeled or unlabeled) was spun to remove cellular debris. The lysate (1,00,000 cpn for labeled or 500 μg for unlabeled) preabsorbed on protein A-Sepharose beads, was incubated
with the antibody (at 1:20 dilution) for 2 h at 4°C in a reaction volume of 200 µl. Immune complexes were separated by using protein-A beads (50 µl suspension, Sigma, USA) followed by three washes with buffer 1: 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% ovalbumin (w/v), 0.1% Triton X-100 (w/v), 0.05% sodium azide (w/v) followed by buffer 2: 10 mM Tris-Cl, pH 7.5, 150 mM NaCl and buffer 3: 0.06 M Tris-Cl, pH 6.8. The pellet was resuspended in 50 µl of SDS-PAGE buffer (125 mM Tris-HCl 6.8, 2% SDS, 0.1 M DTT, 30% glycerol, 5% β-mercaptoethanol, and bromophenol blue), boiled for 5 min. The bound proteins were separated from beads by centrifugation for 5 min and the supernatant was analyzed by SDS-PAGE.

2.25. Fluorography and autoradiography:
For detection of [35S]-methionine labeled proteins, gels were subjected to fluorography as described by Laskey and Mills (1975). Briefly, proteins were fixed in gels and dehydration of gels carried out by incubating in DMSO with 2-3 changes of 45 min each. Finally, gels were impregnated with 20% PPO for 3 hr in the dark. The impregnated PPO in the gels was precipitated by water and the DMSO washed off by keeping the gel under running water. Subsequently, the gels were dried under vacuum and exposed on X-ray films.

2.26. EhCaBP1 protein purification
2.26.1. EhCaBP1 overexpression and purification:
A 5ml culture (LB + 100µg/ml ampicillin) of a single well-isolated p3N29 clone was given in a 50 ml glass tube (Borosil) and incubated overnight at 37°C at 225 rpm. A 50 ml culture (LB + 200µg/ml ampicillin) in 250 ml conical flask was inoculated with 1% of the above culture and grown at 37°C/225rpm till O.D.500nm =0.5. The bacterial culture was induced with 1mM IPTG and incubated for 3-4 hr at 37°C/225 rpm. The induced bacteria was collected at 5000g at 4°C for 5 min and washed once with Wash buffer (25 mM Tris.Cl, pH 7.5 and 100 mM NaCl). The cell pellet was suspended in 1/25th volume of the original culture in Buffer E (50 mM Tris.Cl pH 7.5 and 2 mM EGTA). The cells were lysed by freeze thawing thrice in liquid nitrogen followed by sonication (3 X 30sec, full burst, with 1min interval) on ice. The cell lysate was kept in a boiling water bath for 3min with constant shaking. This was done to coagulate and later precipitate the E. coli cellular proteins; EhCaBP1, being heat stable, will not get affected. The heat-treated lysate was chilled in an ice ethanol bath rapidly for 5min. Heat precipitated materials
Materials and Methods

in the lysate were spun down at 12,000 X g for 30 min at 4°C and the supernatant i.e. the heat stable fraction was loaded on to a packed DE 52 column.

2.26.2. Desalting and equilibration of protein samples:
Protein samples were desalted by dialysis against the appropriate buffer using a dialysis membrane of 3kDa molecular weight cutoff. Samples were dialyzed against 100 volumes of desired buffers at 4°C with minimum two changes.

2.27. Polymerase Chain Reaction (PCR)
Forward and reverse oligonucleotide primers flanking the desired region of interest were used for PCR. DNA template was in a total volume of 100 μl consisting of 50 mM KCl, 10 mM Tris.Cl pH 8.3, 2.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 μM each of each dNTPs, 20 pmole of each primer and 2.5 units of Taq DNA polymerase. The PCR cycle comprised of annealing at 5-10 degrees below the Tₘ of the primers used for 1 min, extension at 70°C for 1-2 min, and denaturation at 94°C for 1min. The last extension step at 70°C was done for an additional 10 min. The amplification reaction was carried out in a DNA thermal cycler (MJ Research Scientific Inc., USA) after overlaying the samples with 20 μl of mineral oil to prevent evaporation. The sample containing all reagents except the template DNA was treated as the negative control. For restriction site incorporation, first five cycles had annealing temperature 2°C below the Tₘ of the primer sequence without the restriction site. The size and integrity of the products were checked by electrophoresing 10 μl of the sample on a 0.8-1.2% agarose gel at V/cm for an appropriate time period.

2.28 Cloning of EhCaBP1 gene from E. histolytica in pEh-HYG-Tet-O-CAT
Plasmids containing EhCaBP1 gene in sense and antisense orientation were constructed and named pEhCaBP1-S and pEhCaBP1-AS respectively, using the shuttle vector pEhHYG-tetR-O-CAT (Hamman L et al., 1997. The CAT gene was excised using KpnI and BamHI and EhCaBP1 gene was inserted in its place in either the sense or the antisense orientation. Appropriate restriction enzyme sites were incorporated in the primers used for PCR amplification of the coding region of EhCaBP1 gene for this purpose. Antisense forward and reverse primers used were: 5'-GGG GGA TCC CAT ATG GCT GAA GCA CT-3' and 5'-GGG GGT ACC AGT TTA GAG TGA AAA CTG A-3'; sense forward and reverse primers were: 5'-GGG GGT ACC TTA GAG TGA AAA CTC A-3'.
Materials and Methods

CAT ATG GCT GAA GCA CTT-3' and 5'-GGG GGA TCC AGT TTA GAG TGA AAA CT-
3'.

Ligation of the two was carried out in the presence of 6 weiss units of T4 DNA ligase in ligase
buffer [50 mM Tris-Cl (pH 7.8), 10mM MgCl2, 10 mM dithiothreitol, 25 µg/ml bovine serum
albumin and 1mM rATP] at 16°C for 16 hours. 4-6 µl of the ligation reaction mixture was used
for transforming E. coli competent cells.

2.29. Transfection of E. histolytica trophozoites by electroporation

Tissue culture tubes containing trophozoites were harvested in log phase and detached by
chilling on ice for 10 min, mixed by inverting the tubes several times and then pelleted by
centrifugation at 0.275g for 3 min at 4°C. The medium was decanted and the cell pellet was
washed twice in 2 ml of PBS. The cell pellet was thereafter washed once in 2 ml of cytomix
buffer [10 mM K2HPO4/KH2PO4 (pH 7.6), 120 mM KCl, 0.15 mM CaCl2, 25 mM HEPES, 2
mM EGTA, 5 mM MgCl2. 12 ml glass tubes each containing 4x10⁶ trophozoites were pooled
together and resuspended in 4.8 ml of cytomix buffer. For electroporation, 0.8 ml was placed in a
cuvette (0.4 cm gap, BIO RAD) along with 4 mM Adenosine Triphosphate, 10 mM Glutathione
and 200 µg of plasmid DNA (for stable transfection). For “no DNA” control, 10 mM Tris buffer
was added. For stable transfection, two consecutive pulses were applied under conditions of
3000 V/cm (1.2 kV) and 25 µF, resulting in a time constant of 0.94 msec. Electroporated
trophozoites were transferred into tissue culture tubes containing 12 ml of TYI-S-33 (complete)
and were incubated in a slanted position at 36°C. Drug selection was started after 2 days of
transfection at a lower concentration of 5µg/ml. Transfected cells were monitored daily until the
cells looked healthy. Frequent change of medium was necessary along with appropriate
antibiotic selection to eliminate dead cells and cellular debris. The changes were determined by
examining the cultures under a microscope. After the cells were growing well, the drug
concentration was increased to 10 µg/ml. The transfected cell lines were stably maintained in the
presence of hygromycin.

2.30. mRNA Differential display.

mRNA differential display was carried out essentially as described by Liang P et al., 1993.
Briefly, total RNA was isolated from EhCaBP1-AS cells grown with or without tetracycline at 5
Materials and Methods

µg/ml and hygromycin at 10 µg/ml. Total RNA was reverse transcribed (RT) using the Superscript II reverse transcriptase (Life Technology, USA) and four different oligonucleotides [dT12MG/A/T/C (M represents either G, A or C)] as anchor primers. The products were then amplified by polymerase chain reaction using the corresponding oligo-dT and three different decamer oligonucleotides. They are H3 (AGACGTCCAC), P10 (TCCCGCCTAC) and Q7 (CAAACCGGATC). The amplified products were separated on a 6% denaturing polyacrylamide gel and specific bands were excised from the gel which were then reamplified with the same primer sets and reconfirmed for differential expression by reverse northern analysis before cloning in the pGEM Teasy vector (Promega, USA). Cloned DNA was purified and each putative gene was confirmed for differential expression by reverse northern hybridization. The nucleotide sequence was determined using Sanger’s di-deoxy chain termination method and the sequence was analysed by standard bioinformatics tools. Detailed reaction protocols are listed below:

1. RT reaction

   in µl

Buffer(5X)- 4
0.1 M DTT- 2
100 µM dNTP- 4
10 µM Primer- 2 (one out of the four dTMN, where M=A/C/G & N= A/T/G/C)
RNA- 2 (~200ng)
Superscript II- 1 (200U/µl)
H2O to 20µl.

Samples were heated without the enzyme for RT at 65°C for 10 minutes followed by immediately cooling to 37°C by incubating for 10 min at 37°C. RT enzyme was then added and incubated at 37°C for 1 h. Sample was denatured at the end by incubating at 95°C for 5 min (at this step sample can be stored at -20°C for a month).

2. PCR

   in µl

RT mix 2
Rt primer (10µM) 2 (as per previously used dTMN subtype)
10 mer(5µg/µl) 2.7
Taq Buffer 2  
DNTPs(20µM) 2  
S\textsuperscript{35} dATP 5 µCi per reaction  
Taq 1.5-1.6 U per reaction  
H\textsubscript{2}O to 20 µl.

In properly labeled tubes cocktail for the reactions were aliquoted and enzyme was added separately.

3. Gel
Gel mix:
Urea 50.4 g  
Acrylam:Bis (19:1) 18 ml  
10X TBE 12 ml  
H\textsubscript{2}O 48 ml  

Total volume to 120 ml.

To 100 ml gel mix add 220 µl freshly made APS and 50 µl TEMED.

4. Band excision and elution
The band was cut after aligning with the autorad. To be precise acrylamide piece containing the band was cut through slit in the autorad. The cut piece of gel was soaked in 100 µl sterile water for 10 minutes, followed by boiling for 15 minutes. Supernatant was collected in a fresh microfuge. To it was added 5 µl glycogen (10 mg/ml), 250 µl cold EtOH and kept overnight at –20°C. The sample was centrifuged at 12,000 rpm for 15 minutes, 4°C. The pellet was washed with 75% EtOH and dissolved in 10 µl H\textsubscript{2}O.

5. Band Reamplification

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Bfr</td>
<td>4 µl</td>
</tr>
<tr>
<td>DNTPs (100 µM)</td>
<td>8 µl (the specific one previously used)</td>
</tr>
<tr>
<td>RT primer</td>
<td>4 µl (&quot;&quot;)</td>
</tr>
<tr>
<td>10 mer (5 µg/ml)</td>
<td>5.4 µl</td>
</tr>
<tr>
<td>H\textsubscript{2}O</td>
<td>14.1 µl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.5 µl (10 U/µl)</td>
</tr>
<tr>
<td>Isolated DNA fragment</td>
<td>4 µl (of 10 µl)</td>
</tr>
<tr>
<td>Final volume</td>
<td>40 µl</td>
</tr>
</tbody>
</table>
Materials and Methods

PCR conditions: Denaturation - 94°C/ 30 sec, Annealing- 40°C/ 2 min, Elongation- 72°C/ 30 sec. 40 cycles followed by 5 min at 72°C.

6. Dot Blot
Dot blot of 5µl/ 10µl samples (gel purified/ PCR amplified) were loaded in duplicate on a dot-blot apparatus. The samples were denatured at 94°C for 10 min and cooled on ice for 10 min. The membrane was presoaked in 2X SSC for 10 min. before laying on the apparatus. Vacuum was applied after loading. The membrane was UV cross-linked before hybridization.

7. RT reaction for Reverse Northern

Content

<table>
<thead>
<tr>
<th>Item</th>
<th>in µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (5 µg) equal amount in both the reactions</td>
<td></td>
</tr>
<tr>
<td>5X RT Buffer</td>
<td>10</td>
</tr>
<tr>
<td>DTT</td>
<td>5</td>
</tr>
<tr>
<td>dNTP mix - dATP(20µM)</td>
<td>2</td>
</tr>
<tr>
<td>dATP (120µM)</td>
<td>2</td>
</tr>
<tr>
<td>oligo dT (18 mer,250 ng/ml)</td>
<td>2</td>
</tr>
<tr>
<td>depc d₂H₂O to Vf 50µl</td>
<td></td>
</tr>
<tr>
<td>RNAsin</td>
<td>1</td>
</tr>
<tr>
<td>Superscript II (200U/µl)</td>
<td>1</td>
</tr>
<tr>
<td>αP³² dATP (10µci/µl)</td>
<td>5</td>
</tr>
</tbody>
</table>

Sample was heated without the RT enzyme at 65°C for 10 minutes followed by immediate cooling to 37°C by incubating at 37°C for 10 min. RT enzyme was then added and incubated at 37°C for 1 h. Sample was denatured by incubating at 95°C for 5 min. 50 µl of 1X STE (0.1M NaCl, 20 mM Tris pH 7.5 and 10 mM EDTA) buffer was added to increase the reaction volume and passed through sephadex G-50 column (packed to 0.9 ml). The labeled cDNA was eluted by size exclusion by spinning at 3,500 rpm/ 4 min. 1 µl of eluate was taken to check incorporation. Equal amount of counts was taken for hybridization.

2.31. Co-sedimentation assay

Co-sedimentation assay was done following essentially the published conditions (Vargas M et al., 1997). Briefly, 5µM of rabbit muscle G-actin (Sigma) per reaction was polymerized for 60 min in polymerization buffer containing 100 mM KCl and 2 mM MgCl₂ at room temperature. After polymerization actin was mixed with 0.2 mM ATP and appropriate target protein (5 µM) in a total volume of 150 µl of buffer (10 mM Tris-Cl, pH 7.5, 1 mM CaCl₂, 2.5 mM β-Mercaptoethanol, 0.5 M KCl, 10 mM MgCl₂) and incubated for 2 h at room temperature. The
Materials and Methods

samples were centrifuged at 100,000 X g for 45 min at 4°C. The supernatant and the pellet were analyzed by 15% SDS-PAGE.

2.32. Solid Phase Assay
96-well plate was coated with 5 μM G-actin in PBS overnight at 4°C. Wells were blocked with 3% BSA in PBS for an additional 24 h. After washes with PBS-T (Tween-20, 0.05% w/v), EhCaBP1 was added in the wells in duplicates at concentrations ranging from 1.7 μM to 10 μM. Bound EhCaBP1 was detected with anti-EhCaBP antibody. Bound antibodies were detected with Alkaline phosphatase linked anti-rabbit IgG using the colorimetric substrate p-nitrophenylphosphate (PNPP, Sigma) and monitoring the absorbance at 405 nm with a microplate reader (Bio-Rad, USA). The concentrations of EhCaBP1 were calibrated to allow reading of the absorbance under a linear range of detection.

2.33. Immunostaining
E. histolytica resuspended in warm incomplete TYI-S-33 was transferred to prewarmed, acetone-cleaned coverslips placed in a petri-dish and allowed to adhere for 10 min at 37°C. The culture medium was removed and coverslips were fixed with 3.7% prewarmed paraformaldehyde (PFA) for 30 min. After fixation, the cells were permeabilized with 0.1% Triton PBS for 1 min, additional permeabilization for 3 min with -20°C cold ethanol was needed for myosin Ib staining. Cells were then washed with PBS and quenched for 30 min in PBS containing 50mM NH₄Cl. The cover-slips were blocked with 1% BSA/PBS for 30 min, followed by primary antibody incubation at 37°C for 1h. The coverslips were washed with PBS followed by 1% BSA/PBS before secondary antibody incubation of 30 min at 37°C. In cases of Phalloidin labeling for F-actin staining, methanol step is strictly omitted. Antibody dilutions used were: EhCaBP; 1:50, Phalloidin (Sigma, 1 mg/ml in methanol); 1:500, Myosin Ib; 1:30, Anti-Rabbit Alexa (Green); 1:200, Anti-Rabbit Alexa (Red); 1:300. The preparations were further washed with PBS and mounted on a glass-slide using DABCO (1,4-diazbicyclo(2,2,2)octane, SIGMA), 10 mg/ml in 80% glycerol. Sealing of the cover-slip edges was done with nail-paint to avoid drying.
Materials and Methods

2.34. Confocal Microscopy
Fluorescent samples were examined on an LSM 510 confocal laser scanning microscope (Zeiss, Germany) equipped with a 63X objective. Rhodamine-labeled samples were visualized after excitation at 543 nm using He/Ne Laser and Alexa-green labeled samples after excitation at 488 nm using Argon Laser. Focal planes of 0.8μ sections with a shift of objective by 1μm, captured for 20-30 planes from the bottom to the top of each cell. Pictures were processed using LSM 510 software, Zeiss.

2.35. Video-tracking of amoeba cells
2.35.1. Trophozoite preparation for video-tracking
pEhCaBP1-S and pEhCaBP1-AS transformant cells were grown for atleast 48 h in presence of hygromycin at 10 μg/ml and for the cells to be induced, with tetracycline at 5 μg/ml. Just before the recording 10^4 cells were transferred to a flat bottom 25 cm^2 flask containing fresh medium.

2.35.2. Computer-assisted analysis of cellular movement
pEhCaBP1-S and pEhCaBP1-AS transformant cells were prepared as described above and incubated at 36°C for 10 min before video-tracking. Fields containing over-crowded and close-contact cells hamper the computation by active-gradient contour method where a defined boundary around a moving cell tracks based on the segmentation of previous image. Amoebae were videotaped using a high-resolution Hammamatsu ORCA-ER video camera attached to an inverted microscope (Axiovert 200M, Zeiss, Germany). Analysis was done using previously published protocol of repulsive interactions between contours to handle cell contacts (Labruyere, E et al, 2003)

2.36. BCECF-labeling of Caco2 to assay for cytolytic activity of amoebae (Berringhauusen O and Leippe M, 1997).
For the cytolytic test, each cover-slip containing monolayer of differentiated human carcinoma Caco2 cells (1.3x10^6) in 35 mm tissue culture dish, was washed twice with sterile PBS, once with buffer A and incubated with 10 μM 2', 7'-bis(carboxyl)-5(6)-carboxyfluoresceinacetoxymethyl ester (BCECF-AM; Molecular probes) for 30 min in Labeling buffer-A (1 mM Na_2HPO_4, 1 mM sulfate, 150 mM NaCl, 2.5 mM KCl, 1 mM CaCl_2, 3.3 mM MOPS, 10 mM HEPES, pH 7.5). After the incubation period, the cells were washed twice with
buffer A, followed by incubation with $10^5$ amoebae for different time points to assay for label release. After the incubation period, supernatant was assayed for the fluorescent dye release in a fluorescence microtiter plate reader (Fluorotech, Merlin) using excitation and emission wavelengths of 485 and 538 nm, respectively.

2.37. Caco2 adhesion assay
For the adhesion test, each cover-slip containing monolayer of differentiated human carcinoma in 16 well (35 mm) tissue culture plate Caco2 cells ($1.3 \times 10^6$) was washed twice with sterile PBS and then covered with 1 ml of DMEM immediately before adding the suspension of amoebae ($3 \times 10^4$ trophozoites per well; one amoeba per four Caco2 cells). The Caco2 cell-amoeba were incubated at 37°C CO2 incubator for 15 min, followed by collecting the medium for estimation of amoebae left after adhesion to Caco2 cells. The cover-slips were washed twice by gentle swirling to remove non-adherent amoebae. The number of adherent amoebae, expressed as percent adhesion, was taken as the difference between the initial number of amoebae added and the total number of amoebae in the medium removed and in the wash liquid. Each experiment was carried out in duplicate on three different days.

2.38. Estimation of endocytosis using FITC-dextran.
The endocytosis of *E. histolytica* was studied by observing the uptake of FITC-dextran. The mid-log phase cells were harvested, washed and resuspended in fresh medium. The *Entamoeba* cells were incubated with FITC-dextran (2mg/ml) for 30 min at 36°C followed by harvesting and washing with PBS. The slides were prepared in presence of 70% glycerol in PBS containing 0.1% 2,5-diphenyl-1,3,4-oxadiazole (PPD). The uptake was observed in the presence or absence of tetracycline (5 μg/ml) for the EhCaBP1-S and EhCaBP1-AS cell lines under a Zeiss microscope with a fluorescence attachment. The total number of fluorescent vesicles engulfed in a cell was counted for 10 cells randomly from each slide at 100X magnifications and for each sample five such slides were counted. The amount of endocytosed material was also determined by measurement of total fluorescence in a cell using a fluorescent microscope (Varion, Cary).
2.39. Phagocytosis of red blood cells by trophozoites
To quantify the RBC ingested by amoebae, the colorimetric method of estimation with some modifications was followed (Rabinovitch M and Stefano MJd, 1971). Briefly, 1x10^8 RBC washed with PBS followed by TYI-S-33 were incubated with 1x10^6 amoebae for 10 min or as mentioned at 37°C in 0.2 ml culture medium. The amoebae and erythrocytes were pelleted down, non-engulfed RBC were bursted with cold distill water and recentrifuged at 1000 x g, 2 min. This step is repeated twice, followed by resuspension in 1ml formic acid to burst amoebae containing engulfed RBC. Samples were measured against a formic acid blank with a spectrophotometer at 400 nm.

2.40. Atomic force microscopy
In the contact mode AFM, also known as repulsive mode, the tip is mechanically contacted with the sample surface at an applied force. This applied force can be evaluated from the force-distance curve, measured when the tip is brought to and then retracted from the sample surface. A soft cantilever (Spring constant usually 0.01 ~ 1 N/m) is usually used in contact AFM. The cantilever is so soft that it can be pulled onto the surface due to high attractive force between them. After a mechanical contact between the tip and sample, there is repulsive force between them. This repulsive force is usually used as the feedback parameter (by maintaining a constant force through adjustment of the sample height while the tip is scanning the surface) to obtain AFM images. In the most common scheme, a laser beam bounces off the back of the cantilever, onto a position sensitive photodetector (PSPD). As the cantilever bends, the position of the laser beam on the detector shifts. The PSPD itself can measure displacements of light as small as 10Å^0. The ratio of the path length between cantilever and the detector to the length of the cantilever itself produces a mechanical amplification. AS a result, the system can detect sub-angstrom vertical movement of the cantilever tip.

The AFM data was collected employing contact mode AFM using the CP-Research model of Thermomicroscopes, USA. The cantilevers employed for this purpose had a force constant of 0.2 nN/m

2.40.1. Sample preparation
Before each experiment fresh F-actin were prepared by polymerization in presence or absence of EhCaBP1 as described above with some modifications. Briefly, 5 µM of rabbit muscle G-actin
(Sigma) per reaction was polymerized for 60 min in polymerization buffer containing 100 mM KCl and 2 mM MgCl₂ at room temperature. After polymerization actin was mixed with 0.2 mM ATP and appropriate target protein (5 µM) in a total volume of 150 µl of buffer (10 mM Tris-Cl, pH 7.5, 1 mM CaCl₂, 2.5 mM β-Mercaptoethanol, 0.5 M KCl, 10 mM MgCl₂) and incubated for 4 h at room temperature. The sample was diluted to 1:5000 in d₂H₂O before laying on freshly cleaved mica and air dried for 30 min at room temperature. The specimen was then mounted to a metal disc for imaging by AFM.

For immunogold AFM, the sample laid on freshly cleaved mica was air dried and fixed with 3.7% paraformaldehyde. The sample was then incubated with 1:50 dilution of anti-EhCaBP1 antibody (in PBS/2% BSA) for 1 h and 10 nm gold-conjugated anti-rabbit antibody for 30 minutes at dilution of 1:10,000 in PBS/2%BSA. Thorough washing was done after every incubation step with PBS/2% BSA. The processed mica was then mounted to a metal disc, air dried prior to AFM imaging.

2.40.2. AFM settings

Images were obtained at scan rate of 1.5 Hz. The set force in contact mode applied by the cantilever was kept below 5 nN. Topographical dimensions of actin strands were analyzed using the IP 2.1 software supplied Thermomicroscopes, USA.