Material and Methods
3. MATERIALS AND METHODS

3.A MATERIALS

Chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise mentioned. Molecular biology reagents were purchased from New England Biolabs (NEB, Beverly, MA, USA). Plasmid DNA has sometimes been purified with the help of Wizard Mini-Prep Kit from Promega Corporation.

Chemicals to prepare medium for S. pombe cells have been purchased from Invitrogen, Sigma, Difco and Merck. PVDF and nitrocellulose membranes were procured from Amersham (UK). Goat anti-rabbit and anti-mouse alkaline phosphatase conjugated antibodies were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GFP antibody, Anti-myc antibody, Alexa flour red and Alexa flour green conjugated anti-rabbit and anti-mouse antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Anti-HA antibody (Roche) was a kind gift from Prof. Asis Datta, Director, NCPGR, New Delhi Monoclonal antibodies against p32 were a kind gift from Dr. A. R. Krainer, Cold Spring Harbour Laboratory, Cold Spring Harbour, USA. EZ-Link™ Sulfo-NHS-LC-Biotin used for biotinylating HABP1 was purchased from Pierce (Rockford, IL, USA).

Water used for preparing media and reagents was either autoclaved triple distilled (distilled in our laboratory) or autoclaved Milli Q (obtained from water purification system, Millipore, MA, USA).

The Schizosaccharomyces pombe strains used in this study, BJ 7468 was a kind gift from Prof Asis Datta, Director, NCPGR, New Delhi; YSAB 199 was a kind gift from Carlos R Vázquez de Aldana, Instituto de Microbiología Bioquímica. Departamento de Microbiología y Genética.
CSIC/Universidad de Salamanca. 37007, Salamanca, Spain. Strain JM 2645 was kind gift from Jonathan B. A. Millar, Division of Yeast Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK. and Strain KGY 2432 and KGY 2422 was the kind gift from Kathleen L. Gould, Howard Hughes Medical Institute, and Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA.

3.A GROWTH MEDIUMS USED IN S. pombe CULTURE

a) Edinburgh Minimal Medium (EMM) leu:

Potassium hydrogen phthalate (3 g/litre), 2% (w/v) glucose, ammonium chloride (5 g/litre), disodium hydrogen phosphate (2.2 g/litre), 20 ml/litre salts (50X stock), 1 ml/litre vitamins (1000X stock), 0.1 ml/litre minerals (10000X stock) and supplements (50–250 mg/litre adenine and uracil). Solution were autoclaved before use.

Salts stock (50X):

52.5 g/litre MgCl2.6H2O, 0.735 mg/litre CaCl2.2H2O, 50 g/litre KCl and 2 g/litre Na2SO4. Solution was to be filter sterilized before use.

Vitamins stock (1000X):

1 g/litre pantothenic acid, 10 g/litre nicotinic acid, 10 g/litre myo-inositol and 10mg/litre biotin. Solution was to be filter sterilized before use.

Minerals stock (10000X):

5 g/litre boric acid, 4 g/litre MnSO4, 4 g/litre ZnSO4.7H2O, 2 g/litre FeCl2.6H2O, 0.4 g/litre molybdic acid, 1 g/litre KI, 0.4 g/litre
CuSO₄·5H₂O and 10 g/litre citric acid. Solution was to be filter sterilized before use.

b) **Yeast Extract + Supplements (YES):**

0.5% yeast extract, 3% (w/v) glucose, 50–250 mg/ml leucine, lysine, histidine, adenine and uracil. Solution was to be autoclaved before use.

c) **Stop Buffer:**

150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1mM NaN₃.

The solution to be used ice-cold.

d) **Breaking Buffer:**

150 mM NaCl, 50 mM PBS, 50 mM NaF, 1 mM sodium orthovanadate (Na₃VO₄), 1 mM PMSF (Phenyl Methyl Sulphonyl Fluoride), 10% glycerol, 5 μg/ml each of pepstatin, aprotinin and leupeptin.
3.B METHODS

3.B.i PROCESSING OF DIALYSIS TUBING

Convenient length of dialysis tubing were boiled in 1 mM EDTA, pH 8.0 for 10 min and thoroughly washed in distilled water and stored at 4°C for subsequent use.

3.B.ii PREPARATION OF HA-SEPHAROSE-4B AFFINITY MATRIX

Hyaluronic acid (HA) from human umbilical cord (grade 1) was coupled to EAH-Sepharose-4B beads by acid catalysed condensation reaction using N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide (EDC) as the coupling reagent. Here, EDC acts as a homo-bifunctional reactant, which conjugates the carboxy group of the ligand (HA), to amino group of the gel beads. Reaction was performed in distilled water adjusted to pH 4.5-6.0 for 12 h. For 20 ml of EAH-Sepharose-4B (7-10 μmole-NH₂/ml), 310 mg of EDC and 50 mg of HA were added and the pH was checked intermittently and kept between 4.5-6.0. The slurry was washed thoroughly with water and then alternatively with 0.2 M Glycine-HCl, pH 2.2 and 0.1 N NaHCO₃ containing 0.5 M NaCl. The activated Sepharose was blocked by 1 M acetic acid, pH 4.0 for 4 h at 4°C under constant stirring condition and again washed as before. The amount of HA bound per ml of gel was estimated by carbazole test (Bitter and Muir, 1962) using glucuronic acid as standard. Column was packed with HA-Sepharose-4B matrix and equilibrated with 0.01 M phosphate buffered saline (PBS), pH 7.2.
3.B.iii PURIFICATION OF HYALURONAN-BINDING PROTEIN (HABP1)

3.B.iii.a INDUCTION AND EXPRESSION OF HYALURONAN-BINDING PROTEIN 1 (HABP1) IN E. coli

Sequence analysis of hyaluronan-binding protein (HABP1) was reported earlier and found to be identical to that of p32, a protein co-purified with the splicing factor SF2. p32 cDNA clone (in the bacterial expression vector pT7.A.A32) was a kind gift from Dr. Adrian Krainer, CSHL, USA. pT7A. A32 plasmid construct harbouring the 209 amino acids long mature p32 protein (Krainer et al., 1991; Honore et al., 1993) under the control of T7 promoter was expressed in BL21 (DE3) cells according to Krainer et al (1991). Pre-innoculum of the BL21 (DE3) cells transformed with pT7A.A32 was grown overnight at 37°C in LB medium containing 50 µg/ml ampicillin, 0.2% glucose. One percent of the pre-innoculum was transferred to fresh LB medium containing 50 µg/ml ampicillin, 0.2% glucose and grown until OD_{600} reached 1.0. Then, 0.4 mM IPTG was added to the culture and it was grown at 37°C for the next 3 h. The cells were pelleted by centrifugation at 5000 x g for 30 min at 4°C and either stored at -70°C or immediately processed for protein purification.

3.B.iiib PURIFICATION OF RECOMBINANT HABP1 USING HA SEPHAROSE AFFINITY

Purification of HABP1 was carried out according to the procedure described by Deb and Datta, 1996. Bacterial pellet obtained from 50 ml culture was washed with PBS and suspended in 15 ml PBS. Triton X-100 (0.01%, v/v) was added and left at room temperature for 45 mins. Complete lysis was achieved by sonication (10-15 bursts each of 30 sec) on ice. Cellular debris were pelleted down and the supernatant was dialysed against PBS and the volume made upto 50 ml with PBS and loaded onto the HA-Sepharose-4B column. After washing with 20 bed-volumes of PBS (pH 7.2) containing 0.15 M NaCl and then, with 20 bed-
volumes of 0.01 M phosphate buffer (pH 7.2) containing 0.5 M NaCl; the column was eluted with 0.2 M Glycine-HCl (pH 2.2) at a flow rate of 20 ml/h and the eluent was collected as 2 ml fractions. The protein-containing fractions were identified by measuring their absorbance at 280 nm. The peak fractions were pooled and concentrated. Whole purification process was carried out essentially at 4°C. Functional activity of purified rHABP1/p32 was confirmed by its binding to biotinylated HA in vitro.

3.B.iv BIOTINYLIATION OF HABP1

For biotinylation of rHABP1, it was incubated with N-hydroxy succinimide biotin (Pierce) in dimethyl sulfoxide followed by incubation with ammonium chloride and then dialyzed against PBS (pH 7.2) and stored for further analysis with 20% glycerol at -20°C (Yang et al., 1994).

3.B.v GROWTH, MAINTAINENCE AND MANIPULATION OF S. pombe CELLS

Wherever the reference has not been mentioned in this section, the protocol has been adapted from Moreno et al. (1991).

3.B.v.a BASIC PROCEDURE OF S. pombe CELL CULTURE

Transformed S. pombe cells were maintained on EMM leu plates and grown when needed in EMM leu Liquid Medium (composition given earlier) at 29°C with constant shaking at 200 rpm in shake flasks. From the plates, a 5 ml EMM leu primary culture was grown for 72 hours and then a suitable volume of secondary culture was inoculated at a starting OD$_{595nm}$ of 0.1. The OD$_{595nm}$ of the secondary culture was measured at given time intervals and the cell number was calculated from the OD$_{595nm}$ for experiments where cells had to be taken out from the culture and processed further. Wherever mentioned, thiamine was added to the medium while growing the cells.
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3.B.v.b CELL GROWTH ASSAY FOR S. pombe CELLS

Cells were grown as given above and their OD_{595nm} checked and plotted against time in hours.

3.B.v.c TRANSFORMATION OF S. pombe CELLS WITH PLASMID DNA

i. Preparation of competent S. pombe cells:

This protocol is an adaptation from Sherman et al (1986) and Ito et al (1983). S. pombe cells are streaked on a YES (0.5% yeast extract; 3% glucose; 50 mg per litre of leucine, adenine and uracil) agar (2%) plate and grown at 29°C for 2-3 days. When individual colonies appeared, a single colony was picked up and was used to inoculate 10 ml YES medium and was grown again at 29°C overnight with proper shaking. Five ml of this primary culture was used to inoculate 50 ml of YES medium which was grown at 30°C until the optical density of the culture reaches 0.55 to 0.65. The cells were then pelleted at 7000-8000 rpm for five minutes and washed carefully with sterile MQ water, repelleted and finally dissolved in 1/100^{th} original culture volume with lithium acetate buffer (pH 4.9) (lithium acetate 11.2 gm/litre and pH adjusted with glacial acetic acid). The cells were once again washed again with lithium acetate buffer and finally resuspended in 0.5 ml lithium acetate buffer and stored in the refrigerator, until further use.

ii. Transformation of competent S. pombe cells with Plasmid DNA:

Transformation of S. pombe cells were carried out according to the protocol of Sherman et al (1986). 100 μl of the S. pombe cell suspension in the lithium acetate buffer was taken and 4 μl of Salmon Sperm DNA (10 μg/μl) and 1-2 μg of the recombinant pRep1 plasmid DNA was added to it. This mixture was incubated at room temperature for ten minutes. Following the incubation, 0.5 ml of PEG 3350 in lithium acetate buffer
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(pH 4.9) was added to the mixture. The contents were incubated at 29°C for 45 minutes with tapping from time to time. The tube was then kept at 46°C for 25 minutes. After that the tube was allowed to cool down to room temperature. The tube was centrifuged at 4500 rpm for 5 minutes to pellet down the cells. The supernatant was discarded and the pellet was dissolved in 300 μl of MQ water. This cell solution was then plated on EMM-Agarose (Leu –) plate for selection along with EMM-Agarose (Ade –) and EMM-Agarose (Ura –) plates (as negative controls) to test for transformed colonies. Here it must be pointed out that the strains of S. pombe used in this study are cells that do not grow in the absence of adenine or uracil or leucine. But when they are transformed with pRep1 or any of the other clones, they can grow in leucine free medium as the transforming plasmid has the gene Leu2 which enables the cells to grow in leucine free medium and this is used for selection.

3.B.v.d FIXING S. pombe CELLS WITH ETHANOL

From an exponentially growing culture about 10⁷ are pelleted down at 2000 rpm for 5 minutes. The supernatant was discarded. The cells were then resuspended in 1 ml distilled water, vortexed well and then centrifuged for 15 sec to spin them down and resuspended in 1-ml cold 70% ethanol. The microcentrifuge tube was vortexed briefly and the cells were stored at 4°C till further use.

3.B.v.e FIXING S. pombe CELLS WITH PARAFORMALDEHYDE

First, 17.5% of paraformaldehyde is prepared as follows: 8.75 grams of paraformaldehyde is weighed out and added to 50 ml PBS, 1 ml of 1M NaOH is added to it and the mixture is incubated at 65°C for 20 minutes. The solution is shaken for solubilization and centrifuged for 5 minutes at room temperature at 3000 rpm to remove the polymers. The clear supernatant is taken and used to fix cells.
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The OD of the growing cells is checked at 595 nm and approximately $10^8$ cells are taken and paraformaldehyde is added to it such that its volume is one fourth the volume of the cell culture taken. The mixture is then rotated slowly on a wheel for 30 minutes for thorough mixing. Following this, the cells are centrifuged at 3000 rpm for 5 minutes at room temperature and washed three times with PBS. These fixed cells are stored in PBS having 1mM sodium azide at 4° C until further use.

3.B.v.f MOUNTING OF S. pombe CELLS

To avoid rolling of the cells between glass slide and cover slip and to attain better stability of cells, the S.pombe cells were mounted on to the glass slides precoated with poly-l-lysine (1mg/ml) and dried.

3.B.v.g PROPIDIUM IODIDE/ CALCOFLUOR STAINING OF S. pombe CELLS

Three hundred microlitres of ethanol fixed cells are taken (containing around $2 - 3 \times 10^6$ cells, after washing), centrifuged to remove the ethanol and the cells are rehydrated by resuspending in 1 ml of 50 mM sodium citrate. The cells are centrifuged briefly at 14000 rpm for one minute at room temperature) and the supernatant was discarded. The pellet was then resuspended in 0.5 ml 50 mM sodium-citrate containing 0.1 mg / ml RNase A incubated at 37°C for at least 2 hr. To this suspension of cells, 0.5 ml 50 mM sodium citrate containing 4 µg / ml propidium iodide (PI), was added bringing the final concentration of PI to 2 µg / ml (PI can be added together with the RNase if desired). Next the suspension was centrifuged at full speed for 4 minutes and most of the supernatant is discarded leaving around 50 µl of the liquid. From this suspension about 4 µl of was put on the slide and the slide was heat fixed at 70° for two minutes. Then the slide was cooled to room temperature and 1 µl of Calcofluor working stock (Calcofluor 50 µgm/µl in
1mg/ml PNPP and 50% glycerol) was added to the slide and then the cover slip was sealed with nail varnish and observed under suitable excitation in a Zeiss Axipscope II Fluorescence Microscope.

3.B.v.h HOECHST STAINING OF LIVE S. pombe CELLS

Transformed S. pombe cells belonging to the strain MBY 624 cells were grown in EMM leu- medium and their cell growth rates were measured spectrophotometrically at 595 nm. Accordingly approximately $10^7$ cells were taken out and washed in sterile distilled water and resuspended in 50 mM sodium citrate Buffer. Four microlitres of this suspension was heat fixed at 70° C on a glass slide and cooled to room temperature and 1 µl of DAPI (4',6-diamidino-2-phenylindole. 2HCl) working solution (DAPI 50 µlg/ml, PNPP 1mg/ml, 50% glycerol) was added onto it. Then a cover slip was put on it, sealed with nail varnish and observed under a fluorescent microscope (Zeiss Axioscope II).

3.B.v.i STAINING OF ACTIN POLYMERS IN S. pombe BY RHODAMINE CONJUGATED TO PHALLOIDIN

The protocol for staining actin polymers was done according to that given by Balasubramanian et al., 1997. Twenty milliliters of growing cells were taken at various time points and 5 ml of 17.5% formaldehyde solution (prepared as mentioned earlier) was added to it and incubated at the same temperature at which the cells were growing for 30 minutes. The cells were then centrifuged at 2000 rpm for five minutes and resuspended in 1 ml PBS in a microcentrifuge tube and centrifuged at 2000 rpm. This step was repeated three more times. The cells were then suspended in PBS containing 1% NP-40 and mixed gently for 1 minute. The cells were again centrifuged for five minutes at 2000 rpm at room temperature and washed with PBS as done earlier and resuspended in 1 ml of PBS. From this suspension, 270 µl were taken and 30 µl of Rhodamine-conjugated phalloidin stock (0.1 mg/ml in PBS) was added.
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Then the microcentrifuge tube was wrapped in aluminium foil and incubated at room temperature on a rotator for 30 minutes. Then the cells are centrifuged at 2000 rpm at room temperature and washed three times with 1 ml PBS as done earlier and finally resuspended in 50 μl of PBS. Four microlitres of this suspension was put on the slide and heat fixed at 70 ° C for five minutes. After the slide cooled to room temperature 1 μl of DAPI and 1 μl of Calcofluor are put on the cells and then the cover slip was sealed with nail varnish and observed under suitable excitation in a Zeiss Axioscope II Fluorescence Microscope.

3. B.v.j INDIRECT IMMUNO-FLUORESCENCE OF S. pombe CELLS

The protocol followed here was developed by Moreno et al 1991. Cells are taken at various time points and fixed with formaldehyde as mentioned earlier. Following fixation, the cells they were suspended in PBS containing 1mM sodium azide. From this suspension the cells were centrifuged at 2000 rpm for 5 mins at room temperature and resuspended in PBS containing 1.2 M Sorbitol. To this Novozym and Zymolase 20T were added to a final concentration of 0.5mg/ml each. This suspension was then incubated at room temperature for 20 minutes, until most of the cell walls of the S. pombe cells have been digested. This was checked by mixing 10 μl of 10% SDS and checking under a phase contrast microscope. Cells, whose cell wall has been completely digested, lose their refringence. After this the tube was filled immediately with PBS containing 1% Triton X-100 and the cells were again centrifuged at 2000 rpm at room temperature. The supernatant was then discarded and the cells resuspended in PBS and washed thrice. After this the cells were resuspended in PBS containing 100mM lysine-HCl and 1% fatty acid free BSA (PBAL) and incubated on a rotator for 30 minutes at room temperature. Then the cells were pelleted at 2000 rpm at room temperature and resuspended in 100 μl of primary anti-body solution (dilution 1:100) and incubated on a rotator overnight at room
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temperature. The cells were again pelleted down at 2000 rpm at room temperature and washed thrice with PBAL. These washed cells were left on the rotator for 20 minutes. Then the cells were resuspended in 100 μl of secondary antibody (dilution 1:100) and kept on the rotator for 2 hrs at room temperature. Following this the cells were pelleted down at 2000 rpm at room temperature and washed thrice with PBAL as above. The cells were then pelleted and resuspended in 50 μl of PBAL. 4 μl of this suspension was taken on the slide and heat fixed at 70 °C for five minutes and 1 μl of anti-fade (1 mg/ml p-phenylenediamine in 50% glycerol) was put on the dried cells after cooling the slide to room temperature and then the cover slip was sealed with nail varnish and observed under suitable excitation in a Zeiss Fluorescence Microscope Axioscope II.

3.B.vi ANALYSIS OF PROTEINS

3.B.vi.a ESTIMATION OF PROTEINS

Estimation of proteins was done with the help of Bradford’s Reagent from Biorad following the manufacturer’s instructions (Bradford 1976).

3.B.vi.b EXTRACTION OF PROTEINS FROM S. pombe CELLS

Cells were centrifuged in SM 24 tubes and washed once in stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA and 1mM NaN₃) and stored in −80 °C till further use. When needed, the pellets were thawed on ice and a suitable amount of breaking buffer [150 mM NaCl, 50 mM Phosphate, 50 mM NaF, 1 mM sodium orthovanadate (Na₃VO₄), 1 mM PMSF (Phenyl Methyl Sulphonyl Fluoride), 10% glycerol, 5 μg/ml each of pepstatin, aprotinin and leupeptin] was added (100 μl of breaking buffer for every 10⁸ cells) and 1.5 ml of 50 micron glass beads were added and
vigorously vortexed 10 times for one minute each. Between two bursts, a gap of one minute was given to avoid over heating.

3.B.vi.c SDS-PAGE OF PROTEINS EXTRACTED FROM S. pombe CELLS

Polyacrylamide gel electrophoresis under denaturing condition (in the presence of 0.1% SDS) was performed according to the method of Laemmli (1970). The proteins were stacked at pH 6.8 in a stacking gel containing 3.5% acrylamide, 0.106% N, N'-methylene bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.01% TEMED and 0.1% ammonium persulfate. The running (separating) gel was made of 12.5% acrylamide, 0.33% N, N'-methylene bisacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.01% TEMED and 0.1% ammonium persulfate. The protein samples were electrophoresed in running buffer consisting of 0.025 M Tris-base, 0.192 M glycine, pH 8.3 and 0.1% SDS. The protein samples were prepared in sample buffer containing 0.0625 M Tris-HCl, pH 6.8; 2% SDS, 10% glycerol, and with or without 5% β-mercaptoethanol (Laemmli, 1970) and immersed in a boiling water bath for 5 min. For lysates prepared by the direct boiling method (in 0.5% SDS-PBS or 1X Laemmli buffer), the samples were loaded as such onto the gel. Standard molecular weight marker (Pharmacia Biotech Inc., Uppsala, Sweden) was also electrophoresed alongside to calculate the subunit molecular size of the proteins.

3.B.vi.d VISUALISATION OF PROTEINS ON SDS-PAGE:

a) Coomassie Brilliant Blue staining:

SDS-Polyacrylamide gels containing more than 2 µg protein concentration were visualized by standard Coomassie Brilliant Blue (CBB) staining (0.1% (w/v) CBB dissolved in 25% (v/v) methanol and 10% (v/v) acetic acid in water), followed by de-staining in 25% (v/v) methanol and 10% (v/v) acetic acid in water.
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b) Silver staining of proteins on SDS- Polyacrylamide gels:

SDS-Polyacrylamide gels containing very little amount of protein samples were detected by silver stain using the protocol described by Merril et al., 1984. Briefly, following electrophoresis, the gel was fixed for 2 x 30 min or overnight in ethanol (40%) and acetic acid (10%). After fixation, the gel was washed 2 x 10 min in Milli-Q water and then sensitised in sodium thiosulphate pentahydrate solution (0.3 g/l) for 1 min. A brief wash (2 x 1 min) in Milli Q water was given after sensitisation, followed by a 30 min incubation in silver nitrate solution (2 g/l silver nitrate and 250 µl/l 37% formaldehyde). After staining, the gel was rinsed for about 10-20 sec in Milli-Q water and then the bands were developed with 30 g/l sodium carbonate, 250 µl/l 37% formaldehyde and 10 mg/l sodium thiosulphate pentahydrate till the desired intensity was reached. The gel was then rinsed several times with Milli Q water and then photographed. Caution was taken not to touch the gel with bare hand while processing.

3.B.vi.e IMMUNO-BLOT ANALYSIS

Proteins were separated on 12.5% SDS-PAGE under reducing conditions and electroblotted onto a Hybond-C™ nitrocellulose membrane or PVDF (Amersham, UK) in a buffer containing 0.025 M Tris, 0.192 M glycine, 0.037% SDS and 20% methanol, pH 8.3 following the procedure described by Towbin et al (1979). When PVDF membrane was used then the membrane was equilibrated once with methanol before equilibrating it in the transfer buffer. After blocking in 3% (w/v) BSA in phosphate buffered saline with detergent (PBST – 10mM phosphate, pH 7.2; 0.15 M NaCl; 0.05 % Tween-20) at room temperature, the blots were probed with primary antibody for 1 h at room temperature. All dilutions were made in 1.5 % (w/v) BSA-PBST. Membranes were then washed 5 x 5 min in PBST and further incubated with secondary antibody (goat-anti-
rabbit IgG conjugated to alkaline phosphatase (AP) or goat-anti-mouse IgG conjugated to AP; 1:10,000 in 1.5% BSA-PBST) for 1 h at room temperature. The bound antibody was detected with the NitroBlue Tetrazolium (NBT) / 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP) colour system. Ten microliters of NBT (30 mg/ml) and 10 μl of BCIP (15 mg/ml) in 10 ml of AP buffer was normally used for the same. The reaction was continued until the bands developed to desired intensity.

3.B.vi.f FAR-WESTERN BLOTTING

The process for Far-Western was exactly the same as in immuno-blotting as given above but instead of the primary anti-body, biotinylated HABP1 was used as the primary probe with was detected by the secondary anti-body extravidin labeled AP from Santa Cruz (Macgregor et al., 1990).

3.B.vi.g CO-IMMUNO-PRECIPITATION

First S. pombe cells were lysed as given above the cell debris removed by centrifugation and the total protein content estimated. Samples containing 1mg/ml was prepared from it and to this suitable dilution of the first antibody was added and incubated with gentle rocking on ice for 2 h, following which 100 μl protein A sepharose beads (Pharmacia) from a stock of 40 mg/ml was added. This was also incubated with gentle rocking on ice for 2 h. The beads were then pelleted and washed thoroughly by centrifugation at 3000 rpm for 10 min at 4°C. The beads were then washed for at least six times with PBS. The washed beads were boiled in a suitable volume of Laemmli’s buffer for 15 min. Finally the boiled samples were centrifuged at 12000 rpm for 10 min and resolved on 12.5% SDS-PAGE. Then the proteins were electro blotted onto a PVDF membrane and probed with the primary antibody of the other protein as processed as in immuno-blotting.
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3.B.vii DNA PREPARATION AND ANALYSIS

3.B.vii.a MINISCALE DNA PREPARATION BY MODIFIED ALKALINE LYSIS METHOD

Rapid mini scale DNA purification from *E. coli* was done by the method described by Ahn et al (2000). *E. coli* cells were grown in 2 ml of Luria Broth (LB) with appropriate antibiotic and the cells were harvested by centrifugation at 11000 X g for 1 min. Cells were then resuspended in 100 µl resuspension buffer containing 50 mM Tris, pH 8.0; 10 mM EDTA and 20 µg RNase A and then 100 µl lysis buffer (200 mM NaOH; 1% SDS) was added and mixed thoroughly. The lysed suspension was then neutralised with 120 µl neutralising buffer (3 M Potassium acetate, pH 5.5). It was mixed properly and incubated at room temperature for 3 min. The bacterial debris were removed by centrifugation at 11000 X g for 1 min. The supernatant was then added to 200 µl of isopropanol and incubated for 1 min at room temperature to precipitate DNA. The DNA pellet was collected by centrifugation at 11000 X g for 30 sec and washed with 500 µl of 70% (v/v) ethanol. Finally, the pellet was air dried and resuspended in 100 µl of sterile water or TE (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0).

3.B.vii.b MIDI SCALE DNA PREPARATION BY ALKALINE LYSIS METHOD

Medium scale DNA purification from *E. coli* was done by the method described by Birnboim and Doly (1979). *E. coli* cells were grown overnight in 50 ml LB medium containing ampicillin (50 µg/ml) or kanamycin (30 µg/ml) and collected by centrifugation (5000 X g at 4°C for 15 min). The cell pellet was resuspended in 1.5 ml of TES Buffer (50 mM Tris, pH 8.0; 10 mM EDTA) containing 2 mg/ml lysozyme, and kept on ice for 10 min. The lysed cells were subjected to 3 ml of denaturing solution (1% SDS and 0.2 N NaOH) on ice for 10 min. The chromosomal DNA-Protein complex was selectively removed by incubating with 1.6 ml
of 3 M sodium acetate (pH 4.6) for 20 min on ice followed by centrifugation at 12000 X g for 20 min. The RNA was digested by treating with 5 µl of RNase A for 45 min at 37°C. The crude preparation was further purified by extracting twice with an equal volume of phenol/chloroform/ isoamyl alcohol [25:24:1 (v/v/v)] and once with equal volume of chloroform/ isoamyl alcohol [24:1 (v/v)]. The crude plasmid DNA was precipitated by the addition of three volumes of pre-chilled ethanol, and the precipitate was collected by centrifugation at 12000 X g for 30 min at 4°C. The pellet was resuspended in 0.4 ml of nuclease-free water and 0.12 ml of 4 M NaCl. DNA was precipitated with 0.5 ml of 13% polyethylene glycol (average Mol. Wt. 8000), and collected by centrifugation at 12000 X g for 10 min at room temperature. The pellet was washed with 70% (v/v) ethanol, and dissolved in 50 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA).

3.B.vii.c AGAROSE GEL ELECTROPHORESIS FOR DNA

Agarose gel electrophoresis was carried out as described by Sambrook et al. (1989). For DNA samples, the required amount (0.8%) of agarose was melted by heating in 1 X TAE buffer, cooled to 55°C and ethidium bromide (0.5 µg/ml) was added prior to casting of the gel on the gel tray. One-sixth volume of DNA gel loading buffer was mixed with samples and loaded in the wells. The electrophoresis was performed at 5 V/cm in 1 X TAE buffer and the DNA fragments were visualised on an UV transilluminator at 302 nm.

3.B.viii GLUCANASE ASSAY

β-1,3-glucanase activity was assayed in cell extracts as described (Baladrón et al., 2002). Determination of the reducing sugars released in the reactions was performed by the methods of Somogyi (Somogyi, 1952) and Nelson (Nelson, 1957). One unit of activity was defined as the amount of enzyme that catalysed the release of reducing sugar groups.
equivalent to 1 mmol of glucose per hour, and specific activity was expressed as units per milligram of protein.

3.B.ix ASSAY FOR ACID POSPHATASES

Acid phosphatase secretion was assayed as follows (Craighead et al., 1993; Tanaka and Okayama, 2000). Cells were grown 48h to log phase in EMM leu- medium at 29°C, pelleted, washed twice with fresh medium, and again resuspended in fresh medium at 29°C. Samples were taken at 0 h (time of resuspension) and at hourly intervals thereafter. For each sample, 1 ml of culture was centrifuged, and 500 μl of the supernatant was added to 500 μl of substrate solution (2 mM p-nitrophenyl phosphate, 0.1 M sodium acetate, pH 4.0; prewarmed to 30°C) and incubated at 30°C for 5 min. Reactions were stopped by the addition of 500 μl of 1 M sodium hydroxide. The absorbance at 405 nm was measured, using the 0-h sample as a blank control.

3.B.x ROS ESTIMATION

Cellular ROS production was examined by a method dependent on intracellular deacylation and oxidation of 29,79-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 29,79- dichlorofluorescein (DCF). This probe was highly reactive with hydrogen peroxide and has been used in evaluating ROS generation in mammalian (Hagar et al., 1996., Rosenkranz et al., 1992) and yeast (Brennan, R. J., et al. 1993 ) cells. After preincubation of the yeast cells (10^7 cells/ml) in EMM Leu- medium with 40 mM DCFH-DA at 30°C for 60 min, the cell suspensions (1.0 ml) were then washed and resuspended in 100 ml of phosphate-buffered saline. Fluorescence intensity of the cell suspension (100 ml) containing 10^7 cells was read with a LS 55 Luminescence spectrometer (Perkin Elmer) with excitation at 480 nm and emission at 530 nm. The arbitrary units were based directly on fluorescence intensity.