Identification of EhTIF-IA: The putative *E. histolytica* orthologue of the human ribosomal RNA transcription initiation factor-IA

Ankita Srivastava[1], Alok Bhattacharya[2], Sudha Bhattacharya[1] and Gagan Deep Jhingan[3,*]

[1]*School of Environmental Sciences and* [2]*School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110 067, India*

[3]*National Institute of Immunology, Aruna Asaf Ali marg, New Delhi 110 067, India*

**Corresponding author (Fax, +91-11-26742125; Email, gaganjhingan@hotmail.com)*

Initiation of rDNA transcription requires the assembly of a specific multi-protein complex at the rDNA promoter containing the RNA Pol I with auxiliary factors. One of these factors is known as Rrn3P in yeast and Transcription Initiation Factor IA (TIF-IA) in mammals. Rrn3p/TIF-IA serves as a bridge between RNA Pol I and the pre-initiation complex at the promoter. It is phosphorylated at multiple sites and is involved in regulation of rDNA transcription in a growth-dependent manner. In the early branching parasitic protist *Entamoeba histolytica,* the rRNA genes are present exclusively on circular extra chromosomal plasmids. The protein factors involved in regulation of rDNA transcription in *E. histolytica* are not known. We have identified the *E. histolytica* equivalent of TIF-1A (EhTIF-IA) by homology search within the database and was further cloned and expressed. Immuno-localization studies showed that EhTIF-IA co-localized partially with fibrillarin in the peripherally localized nucleolus. EhTIF-IA was shown to interact with the RNA Pol I-specific subunit RPA12 both *in vivo* and *in vitro*. Mass spectroscopy data identified RNA Pol I-specific subunits and other nucleolar proteins to be the interacting partners of EhTIF-IA. Our study demonstrates for the first time a conserved putative RNA Pol I transcription factor TIF-IA in *E. histolytica*.

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1. Introduction

Ribosomes are the primary site for protein synthesis in all living organisms and consist of highly conserved ribosomal proteins and ribosomal RNAs (Hernandez-Verdun *et al.* 2010). In eukaryotes, rDNA transcription in the nucleolus is performed by RNA polymerase I (Pol I) transcriptional machinery, which regulates rRNA production in response to growth stress (Lempiainen and Shore 2009; Drygin *et al.* 2010; Grummt and Voit 2010). The basal transcription machinery of RNA Pol I differs significantly between mammals and yeast; in mammals it consists of promoter selectivity factor, e.g. mouse TIFIB or human SL1, along with upstream binding factor (UBF)(Gorski *et al.* 2007; Grummt 2003), while in yeast it consists of core factor (CF), Upstream activating factor (UAF) and TBP (TATA-binding protein) (Steffan *et al.* 1998).

One interesting similarity is the evolutionary conservation of mammalian transcription initiation factor (TIF-IA) and its yeast counterpart Rrn3p (RNA polymerase I-specific transcription initiation factor)(Bodem *et al.* 2000). TIF-IA mediates the interaction of general transcription factors with RNA Pol I to initiate transcription from rRNA promoters (Yuan *et al.* 2002). The mammalian

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and yeast TIF-IA homologs range in size between 68 and 74 kDa and contain a conserved LARAK motif which is responsible for interaction with TIFIB/SL1.The C-terminus of TIF-IA has been shown to interact with RNA Pol I subunits (Yuan *et al.* 2002). In mammals and yeast, growth-dependent rDNA transcription is shown to be regulated by the phosphorylation status of TIF-IA/Rrn3p and its association with a transcriptionally active subpopulation of RNA Pol I molecules in the cell (Szymanski *et al.* 2009; Yuan *et al.* 2002). In yeast, translational inactivation results in reduced levels of Rrn3p via ubiquitin mediated proteasomal degradation pathway, resulting in reduction of Pol I–Rrn3p complexes (Philippi *et al.* 2010). In mammals, under growth-regulated conditions, various kinases like ERK, RSK, JNK and TOR have been shown to participate in regulation of rRNA synthesis via phosphorylation of TIF-IA (Zhao *et al.* 2003; Mayer *et al.* 2004, 2005; Bierhoff *et al.* 2008; Hoppe *et al.* 2009). Rrn3p has been shown to be an essential gene in yeast. Genetic inactivation of TIF-IA has been shown to result in the early death of mouse embryos (Yamamoto *et al.* 1996; Yuan *et al.* 2005).

Although some detailed work on RNA Pol I transcription machinery has been done in *Acanthamoe bacastellanni* and *Trypanosoma brucei* (Radebaugh *et al.* 1994; Al-Khouri and Paule 2002; Bric *et al.* 2004; Gogain and Paule 2005; Brandenburg *et al.* 2007; Park *et al.* 2011; Nguyen *et al.* 2012), TIF-IA homologs have not yet been identified in protozoan parasites. The *A. castellanni* TIF-IA has been biochemically purified from the cell extracts and shown to be essential for RNA Pol I transcription but its complete protein sequence has not been reported till date. On the other hand, *T. brucei* RNA Pol I transcription machinery contains novel CITFA (class I transcription factor A) complex but lacks any TIF-IA homolog (Nguyen *et al.* 2012).

Unlike most model systems where rRNA genes are arranged as chromosomal head-to-tail repeats, the rRNA genes in the early branching parasitic protest *Entamoeba histolytica* are located on extra chromosomal circles, in about 200–400 copies (Bhattacharya *et al.* 1989, 1992; Mittal *et al.* 1991, 1992; Ramachandran *et al.* 1993; Sehgal *et al.* 1994). *E. histolytica* also demonstrates a unique nucleolar organization at the nuclear periphery, as well as recently reported circular etsRNA (external transcribed spacer RNA) from the 5′-external transcribed spacer, which respond to growth stress (Jhingan *et al.* 2009; Gupta *et al.* 2012).

Here we have identified the *E. histolytica* RNA Pol I transcription factor EhTIF-IA based on its sequence similarities with mammalian TIF-IA and yeast RRN3. We demonstrate EhTIF-IA interaction with RNA Pol I under *in vitro* and *in vivo* conditions and its co-localization with RNA Pol I subunit and fibrillarin protein in the peripheral nucleolus of *E. histolytica*.

2. Materials and methods

2.1 *Strains and cell culture*

All experiments were carried out with *E. histolytica* strain HM-1:IMSS clone 6. The cells were maintained and grown in TYI-33 medium supplemented with 15% adult bovine serum, 1X Diamond's vitamin mix and antibiotics (0.3 units/mL penicillin and 0.25 mg/mL streptomycin) at 35.5°C (Diamond *et al.* 1978). G418 (Sigma) was added at 10 mg/mL for maintaining the transfected cell line.

2.2 *Bioinformatics analysis*

Functionally validated *S. cerevisiae* [CAA81966], *S. pombe* [CAA92389], and *H. sapiens* [NP_060897] protein sequences were used as a query to identify putative EhTIF-IA containing similar sequence and protein domains in *E. histolytica* database. We performed initial searches using Blastp algorithm with default search parameters against all predicted ORFs of *E. histolytica* available in AmoebaDB (*http://amoebadb.org*) database. ORF's identified with the highest percent coverage and identity was used for further analysis and validation. PSI Blast was performed against *S. cerevisiae*, *S. pombe*, and *H. sapiens* TIF-IA to identify distant conservation. Conserved domains and motifs were identified by CD-search (*http://www.ncbi.nlm.nih.gov/BLAST*), Pfam (*http://pfam.sanger.ac.uk/search*) and Interpro (*https://www.ebi.ac.uk/interpro*). The results were compared with yeast as well as that of human. The determination of amino acid composition and multiple alignments (ClustalW) were performed using the BioEdit sequence alignment editor (version 7.0; Tom Hall).

2.3 *Cloning, expression, antibody preparation and Western blot analysis*

The genomic sequence of putative EhTIF-IA (GeneDB, EHI_035130; uniprot, C4M4Z3_ENTHI) was identified by homology search with other eukaryotic homologues. Genomic PCR was performed to amplify EhTIF-IA full length sequence and further cloned in pET30b vector. The recombinant protein was induced with 0.2 mM IPTG at 18°C for 16 h and purified using Ni^{2+}-affinity resin as recommended by the supplier (Qiagen). To raise antibodies, purified recombinant protein was separated on SDS-PAGE and the protein band was cut out and emulsified with adjuvant. Immunizations were done in rabbit and mice to produce antigen-specific antibodies. Pre-bleed were taken before any immunization schedule. To analyse the specificity of these antibodies, recombinant protein from *E. coli* or cellular

extracts from *E. histolytica* were analysed on immunoblots using ECL detection system (Amersham).

2.4 *Immunocytochemical analysis*

*E. histolytica*trophozoites were grown to mid-log phase and were fixed with chilled methanol (−20°C) for 10 min and permeabilized with chilled acetone for 5 min. Cells were then washed with PBSand further blocked with 1% BSA/PBS for 30 min, followed by primary antibody incubation at 37°C for 1 h and subsequently incubated with secondary antibody (Alexa Flour 488 or 555) for 45 min at 37°C. The preparations were further washed with PBS and mounted on a glass-slide using prolong gold anti-fade reagent (Invitrogen, USA). Fluorescence images were monitored using an Axioscope microscope (Carl Zeiss, Germany) equipped with epifluorescence and Axiocam camera system coupled with Axio Vision software (Carl Zeiss, Germany) and images were processed using Axiovision Rel4.6 (Carl Zeiss).

Confocal images were visualized using an Olympus Fluoview FV1000 laser scanning microscope.

2.5 *Immunoprecipitation*

Anti-EhTIF-IA antibody was conjugated to CNBr-activated Sepharose for immunoprecipitation. 1 gm of CNBr-activated Sepharose-4B (Pharmacia) was activated and processed as per supplier's protocol. The immunoglobulin conjugated CNBr-Sepharose beads were incubated with *E. histolytica* nuclear extracts (200 μg) for 4 h at 4°C. The beads were washed three times with wash buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM Imidazole, 1 mM Magnesium acetate, 2 mM β-ME and protease inhibitor cocktail). Beads were further washed with 60 mMTris-Cl (pH 6.8) and 100 mM NaCl and finally with 60 mM Tris-Cl (pH 6.8). The bead pellet was resuspended in 2X SDS-PAGE buffer, boiled for 5 min and after brief spin the proteins in the supernatant were analysed by western blotting.

Anti-HA pull-down assays were performed with commercially available anti-HA beads (sigma). Nuclear extracts (200 μg) were incubated with HA beads for 4 h at 4°C. The beads were washed as described earlier and elution of bound protein complex was performed with 0.2 M glycine (pH 2.5) followed with immediate neutralization with 1 M Tris (pH 8.0).

2.6 *Protein digestion*

The pull-down elutes were dried and re-dissolved in freshly prepared digestion buffer (8 M urea in 25 mM NH_4HCO_3). Samples were reduced with 5 mM TCEP (45 min, 37°C) and alkylated with 50 mM iodoacetamide (60 min, 37°C) in the dark. Samples were diluted with 25 mM NH_4HCO_3 to a final concentration of 1M urea and digested overnight at 37°C with sequencing grade trypsin gold (1 μg, Promega USA). After digestion, peptide mixtures were acidified to pH 2.8 with Formic acid and desalted using minispin C18 columns (Nestgrp, USA). Samples were dried under vacuum and re-solubilized in 0.1% formic acid and 2% acetonitrile before mass spectrometric analysis.

2.7 *LC-MS/MS Analysis*

The tryptic peptide samples were separated by reverse-phase chromatography for each experiment via Thermo Scientific Proxeonnano LC using a self-packed C18 picofrit analytical column (360 μm OD, 75 μm ID, 10 μm tip, Magic C18 resin, 5 μm size, Newobjective, USA. The HPLC was coupled to an LTQ-OrbitrapVelos mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto the column with Buffer A (2% acetonitrile, 0.1% Formic acid) and eluted with 120 min linear gradient from 2% to 40% buffer B (80% acetonitrile, 0.1% Formic acid). After the gradient the column was washed with 90% buffer B and finally equilibrated with buffer A for next run. The mass spectra were acquired in the LTQ Orbitrapvelos with full MS scan (RP 60,000) followed by 20 data-dependent MS/MS scans with detection of the fragment ions in the ITMS mode.

2.8 *Data analysis*

Data analysis was performed using Proteome Discoverer software suite (version 1.3). For the search engine SEQUEST, the peptide precursor mass tolerance was set to 10 ppm, and fragment ion mass tolerance was set to 0.8 Da. Carbamidomethylation on cysteine residues was used as fixed modification, and oxidation of methionine along with N-terminal acetylation was used as variable modifications. Spectra were queried against the *E. histolytica* uniprot database. In order to improve the rate of peptide identifications percolator node in proteome discoverer was utilized with the false discovery rate (FDR) set to 0.01 for peptide and protein identifications.

The identified peptide and protein tables are provided as supplementary table 2.

3. Results

3.1 *Identification of putative EhTIF-IA*

The sequence of TIF-IA/RRN3 gene is not highly conserved phylogenetically (Yamamoto *et al.* 1996; Bodem *et al.* 2000; Moorefield *et al.* 2000) which makes it difficult to search for

corresponding homologs in other organisms. We have utilized the well characterized yeast RRN3, and human TIF-IA gene sequences to identify a putative EhTIF-IA using BLAST search of the *E. histolytica* genome database. In this approach, we have performed PSI-BLAST using human TIF-IA, *S. cerevisiae* and *S. pombe* RRN3 as query sequences to derive position-specific scoring matrix from multiple distant homologs to improve the scoring of weak homologs. The position-specific approach was used to search the database for matches by further iterations (figure 1A). This iterative profile generation is capable of detecting distant sequence similarity (Altschul *et al.* 1997). The PSI-BLAST using *S. pombe* RRN3 sequence identified a putative EhTIF-IA with e-value ($3e^{-25}$). Comparison of EhTIF-IA with other divergent species was also performed and results are provided in supplementary table 1. The alignment of EhTIF-IA with *S. pombe* Rrn3 protein was the best match and showed relatively longer stretches of sequence matches (figure 1B).

We further verified the identified protein via Interpro domain analysis which proved that the RRN3 domain architecture (Pfam ID: PF05327, Panther ID: PTHR12790 and interpro ID: IPR00791) (supplementary figure 1) was present in putative EhTIF-IA (figure 1C).

3.2 *EhTIF-IA colocalizes with fibrillarin in the nucleolus*

EhTIF-IA gene was cloned and expressed in *E. coli* as a 6XHIS-tagged protein, which was purified to raise polyclonal antibodies in rabbit. The antibody was used in western blots of *E. histolytica* total cell lysates (TCL) and nuclear extracts (NE) and detected the expected band size of predicted MW (molecular weight) of EhTIF-IA (53.2 kDa in TCL, NE) in Western blot (figure 2A). As a control the blot was also probed with preimmune sera in order to check antibody specificity (data not shown). Nucleolus is primarily dedicated to the transcription of pre-rRNA, its processing into mature rRNAs and assembly into ribosomal particles (Venema and Tollervey 1999; Cheutin *et al.* 2002; Huang 2002). The *E. histolytica* nucleolus has been shown to be located at the nuclear periphery using RNA Pol I antibodies and nucleolar marker Fibrillarin (Jhingan *et al.* 2009). Since mammalian and yeast TIF-IA is known to be involved in regulation of Pol I transcription we expect the EhTIF-IA to be nucleolar-localized. We utilized the specific antibodies raised against EhTIF-IA to study its subcellular localization. Double immunostaining of trophozoites was performed with anti-EhTIF-IA and anti-EhFibrillarin (EhFIB) antibodies which showed that EhTIF-IA colocalized at the nuclear periphery along with EhFIB. Significant staining of the cytoplasm was also observed with anti-EhTIF-IA antibodies (figure 2B).

3.3 *EhTIF-IA interacts with the RNA Pol I subunit EhRPA12*

To demonstrate EhTIF-IA interaction with RNA Pol I *in vitro*, recombinant EhTIF-IA protein was incubated with RNA Pol I-specific small subunit, i.e. recombinant EhRPA12 (Jhingan *et al.* 2009) in different combinations. Binding of the two proteins was confirmed by immunoprecipitation using CNBr-conjugated anti-EhTIF-IA beads followed by detection of bound proteins on immunoblots using antibodies raised against the two proteins. EhRPA12 was found to interact with EhTIF-IA bound to the beads as well as in the solution (figure 3A; lane 1). The controls used in the assay included EhRPA12 alone, BSA with EhRPA12 and CNBr-beads alone, and none of these showed any non-specific signal in our binding assay (figure 3A; lane 2–4).

Pull-down experiments with nuclear extracts of *E. histolytica* using CNBr-conjugated anti-EhTIF-IA antibody were performed and bead-bound EhTIF-IA complex was further probed with antibodies against EhTIF-IA, EhRPA12 and EhFIB. Preimmune sera did not enrich EhTIF-IA from the nuclear extracts but immune sera detected EhTIF-IA protein at expected size in the western blot (figure 3B; left panel). Further, the complex showed EhTIF-IA interaction with RNA Pol I subunit RPA12 and nucleolar protein fibrillarin (figure 3B; right panel). As a negative control, we also probed the blots with anti-EhCaBP6 (calcium binding protein 6) which is nuclear localized (Grewal *et al.* 2013), but has no obvious relationship with rRNA metabolism and it showed no interaction with EhTIF-IA. We conclude that the putative EhTIF-IA is a bonafide interactor of the RNA Pol I subunit EhRPA12 and the nucleolar protein fibrillarin.

3.4 *Identification of EhTIFIA pull-down complex using mass spectroscopy*

Affinity purification and mass spectroscopy (AP-MS) proteomics is very efficient methodology to study protein complexes and discovering new protein-protein interactions (Blagoev *et al.* 2003; Eberl *et al.* 2013). In order to study the interacting partners of EhTIF-IA we utilized a tag-based approach where we generated a constitutively expressing stable cell line of EhTIF-IA containing a C-terminal HA tag (figure 4A). Pull-down experiments were performed with nuclear extract of *E. histolytica* (untransfected) and EhTIFIA cell line. The HA-bead-bound protein complex was analysed by western blotting as well as mass spectroscopy. Western blot analysis demonstrated specific interaction of EhRPA12 with ectopically expressed EhTIFIA-HA protein (figure 4B). As a negative control, nuclear EhCaBP6 was not present in bead-bound protein complex. Both EhRPA12 and EhCaBP6 were undetectable in 50 μg of total cell extracts due to low

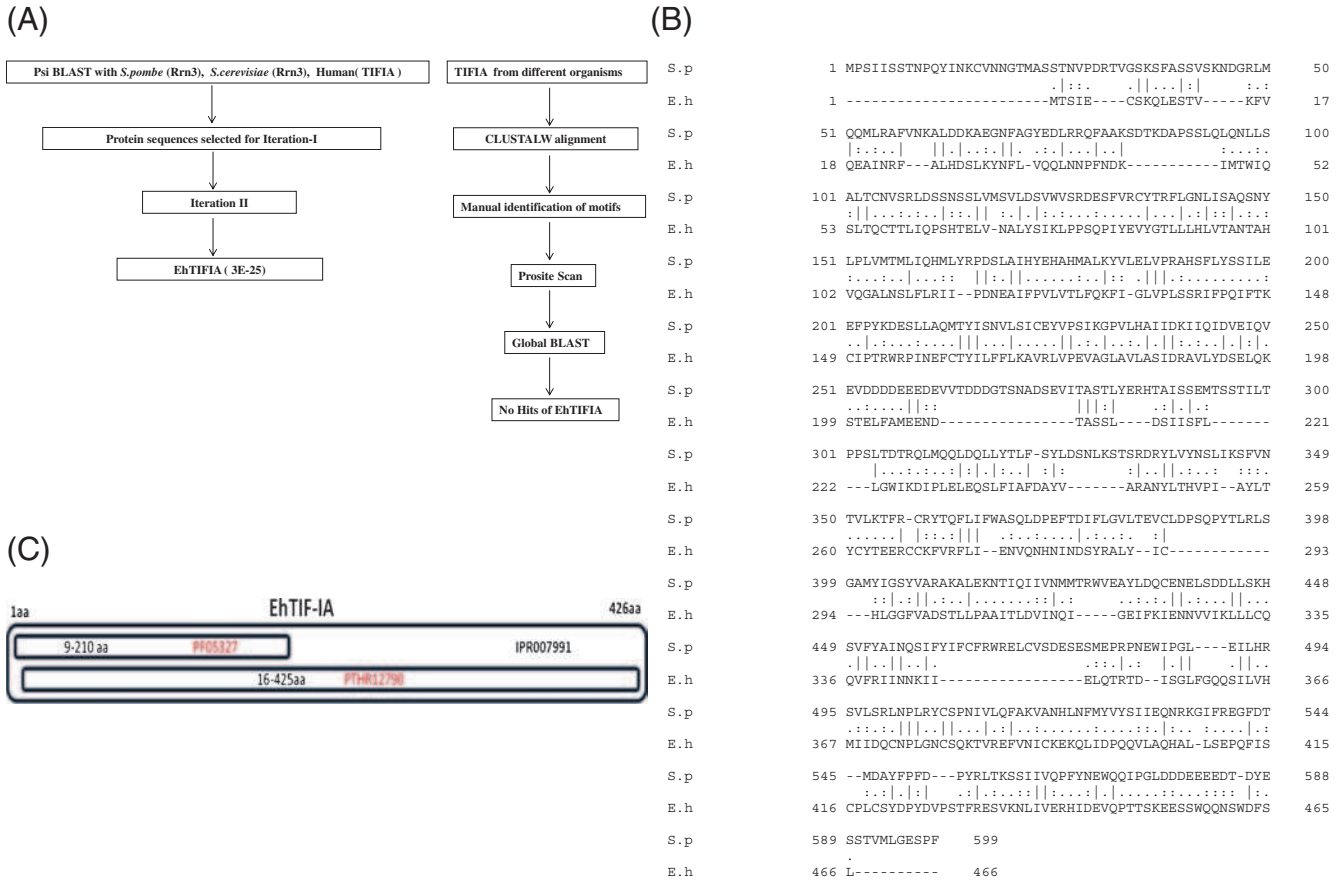

Figure 1. Multiple iteration-based identification of TIFIA/RRN3 counterpart in *E. histolytica*: (**A**) Flow chart. Steps followed in bioinformatic analysis of putative EhTIFIA. (**B**) Pairwise alignment of EhTIFIA with *S.pombe* Rrn3 protein. (**C**) EhTIFIA protein demonstrating RNN3 domain architecture using Interpro analysis (Pfam ID: PF05327, Panther ID: PTHR12790 and interpro ID: IPR00791) *S.p-S.pombe* and *E.h-E.histolytica*. Line (|) and dots (., :) represent identity (18.2%) and similarity (36.0%) respectively.

levels of these proteins in the cell. As a control, Anti-HA antibody did not recognized any non-specific protein in total cell lysates, nuclear extracts and immunoprecipitates from *E. histolytica*untransfected extracts (data not shown). In order to identify detailed constituents ofEhTIF-IA protein complex the pull-down material was digested with trypsin and further analysed by LC-MS/MS mass spectroscopy. Many nucleolar proteins were identified and among them RNA Pol I-specific subunits were present with ~50% or more sequence coverage (supplementary table 2). No RNA Pol II- and III-specific subunits were detected in the identified proteins. Other nucleolar proteins, i.e. fibrillarin, Nop56, Nop10, RPB10, RPB5 and RPB8 were also identified, as listed in figure 5A. The CID (collision induced dissociation) MS/MS fragmentation mass spectra of EhTIF-IA, and RNA Pol I subunits, i.e. RPA1, 2 and 12, are shown in figure 5B. From this analysis we can conclude that the putative EhTIF-IA interacts preferentially with RNA Pol I subunits and nucleolar architectural proteins.

4. Discussion

Ribosome biogenesis in any type of cell is highly energy consuming process which responds to general metabolism and specific environmental challenges in a tightly regulated manner (Grummt and Voit 2010). RNA Pol I transcribes rRNA genes as pre-rRNA followed by processing to remove (5′ and 3′) ETS (external transcribed spacer)and ITS (internal transcribed spacer) (1 and 2) leading to mature rRNA (18S, 5.8S, 28S) (Grummt 2003; Henras *et al.* 2008). Experimental evidence suggests that ribosome biogenesis is correlated with altered rates of rDNA transcription rather than rRNA processing and stability (Hannan *et al.* 1995).

Initiation of rDNA transcription is mediated by specific multi-protein complex at rDNA promoter, along with a number of auxiliary proteins. The role of TIF-IA/Rrn3 is to serve as a bridge between Pol I and TIF-IB/SL1 or CF (core factor in yeast). It interacts with RPA43 (a unique

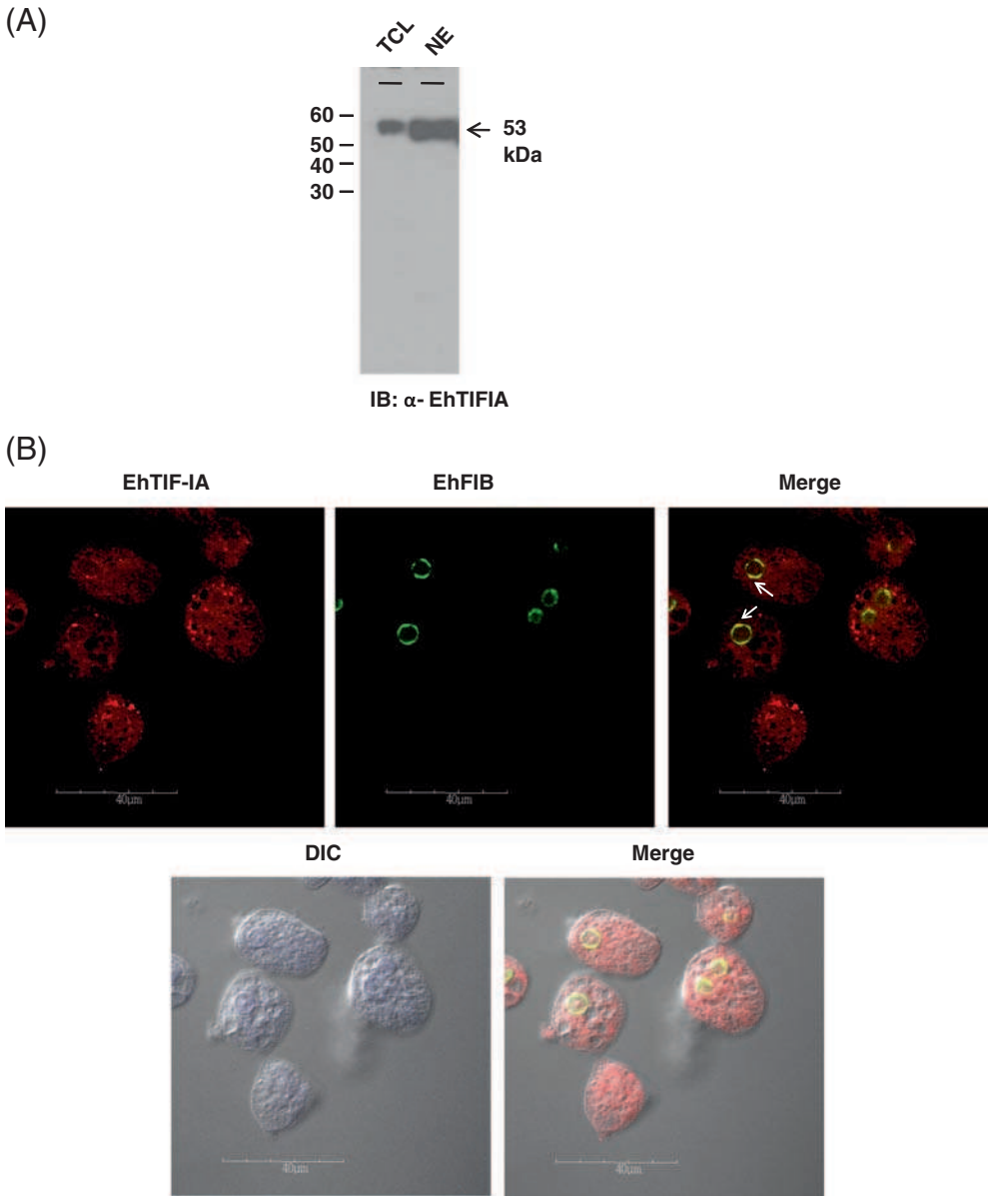

Figure 2. Expression and immunolocalization of EhTIFIA in *E. histolytica* cells: (**A**) Total cell lysates (100 μg), nuclear extracts (20 μg) of *E. histolytica*and recombinant TIFIA (100 ng) were separated on 10% SDS-PAGE and probed after electrotransfer (IB) with preimmune and immune sera at 1:2000 dilution. The expected size of recombinant protein (53.7 kDa) and cellular EhTIFIA (53.2 kDa) is indicated along with arrow. (**B**) For colocalization, trophozoites were double-labelled for EhTIF-IA and EhFIB proteins using antibodies raised in rabbit and mice. Confocal laser scanning microscopy was carried out with Olympus FluoViewTM FV1000 microscope. Colocalization is apparent as yellow signal in the merged panel. Arrows indicate strong colocalization position.

subunit of RNA Pol I) and with PAF67 (polymerase-associated factor), and with two TAF_I (TATA-binding protein-associated factor) subunits (Peyroche *et al.* 2000; Seither *et al.* 2001; Cavanaugh *et al.* 2002; Yuan *et al.* 2002; Claypool *et al.* 2004). Thus, by associating with both RNA Pol I and the promoter selectivity factor, TIF-IA may link both protein complexes.

Initiation of pre-rRNA synthesis is best characterized in human and yeast. Corresponding information in protozoan parasites is rudimentary, and detailed pathways have not yet been worked out (Polakowski and Paule 2002; Bric *et al.* 2004; Gogain and Paule 2005; Nguyen *et al.* 2007, 2012; Park *et al.* 2011). A previous study in our lab had focused on identification of RNA Pol I

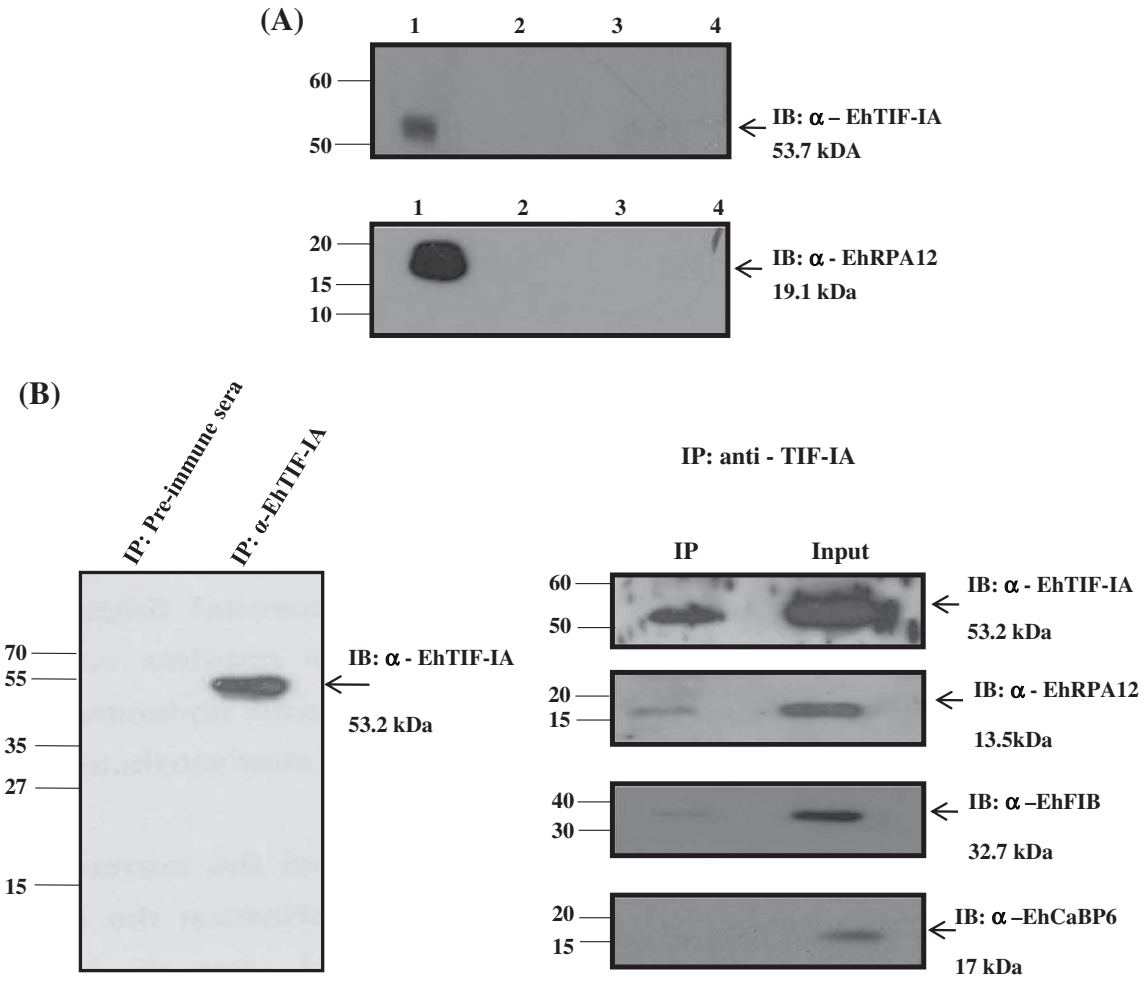

Figure 3. Interaction studies of EhTIF-IA with RNA Pol I subunit EhRPA12: (**A**) *In vitro* interaction of EhTIF-IA and EhRPA12. Recombinant EhTIF-IA protein was incubated with CNBr-conjugated anti-EhTIF-IA antibody beads and interaction was demonstrated by Western blot analysis using α-EhTIF-IA and α-EhRPA12 antibodies. Lane 1, Recombinant EhTIF-IA (1 μg) incubated along with EhRPA12 (1 μg) for 2 h followed by immunoprecipitation. Lane 2, Recombinant EhRPA12 alone passed through beads. Lane 3, BSA incubated with recombinant EhRPA12 for 2 h followed by immunoprecipitation. Lane 4, Only CNBr Beads. (**B**) Immunoprecipitation of EhTIF-IA complex from nuclear extract of *E. histolytica* using CNBr-conjugated anti-EhTIF-IA antibody. Left panel: Immunopreciptation and detection of EhTIF-IA from 200 μg nuclear extract of *E. histolytica* using CNBr-conjugated anti-EhTIF-IA (rabbit) antibody and CNBr-conjugated pre-immune. Right panel: The nuclear extract Input (10%) and pull-down complex (IP) were probed for the presence of EhTIF-IA (53.2 kDa), EhRPA12 (19.1 kDa), EhFIB (32.7 kDa) and EhCaBP6 (17 kDa) by respective antibodies. Molecular weights of protein of interest are indicated by arrows.

subunits and understanding of the nucleolar organization in *E. histolytica* (Jhingan *et al.* 2009). In the present study we have identified a putative EhTIF-IA and attempted to identify its cellular localization and interaction partners in the cell. By using well-characterized sequences of TIF-IA homologs from yeast and human, EhTIF-IA was identified using homology search. EhTIF-IA sequence was further confirmed using Psi BLAST with *S. pombe* Rrn3, which detects distant sequence similarity (Altschul *et al.* 1997).

The transcription, processing, and assembly of pre-ribosomal particles takes place in the nucleolus (Venema and Tollervey 1999). Ultrastructural studies on *E. histolytica* nucleus by electron microscopy showed that chromatin, apart from being diffusely distributed in the nucleoplasm, is concentrated in two zones, namely the peripheral chromatin occupying almost the entire nuclear periphery and a centrally located spherical karyosome (el-Hashimi and Pittman 1970; Miller *et al.* 1961). In *E. histolytica* the rDNA circles are located at the nuclear

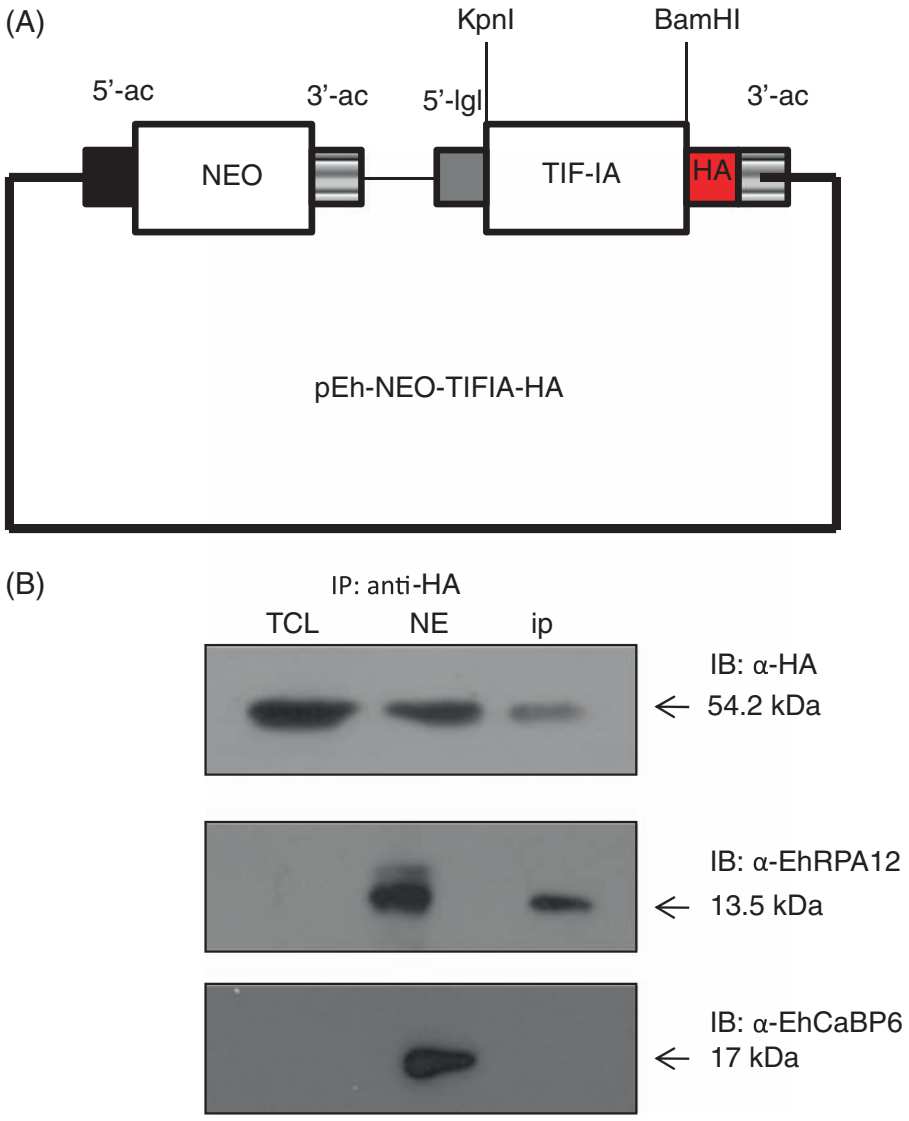

Figure 4. Ectopic expression and immunoprecipitation of EhTIF-IA-HA complex: (**A**) Schematic representation of EhTIF-IA-HA construct. EhTIF-IA-HA tagged gene (1428 bp) was cloned in the KpnI-BamHI site of pEh-NEO-GFP vector after removal of the GFP gene. (**B**) Immunoprecipitation of ectopically expressed EhTIFIA-HA from nuclear extract of *E. histolytica*. EhTIFIA-HA was immunoprecipitated from the nuclear extract (200 µg) using anti-HA beads. The total cell lysate (TCL), nuclear extract (NE) and Immuno-pull-down (ip) of EhTIFIA-HA tagged cell line was probed for the presence of EhTIFIA-HA (54.2 kDa), EhRPA12 (19.1 kDa) and EhCaBP6 (17 kDa) by their respective antibodies. Molecular weights of proteins of interest are indicated by arrows.

periphery (Michel *et al.* 1995; Willhoeft and Tannich 1999). Previous study from our lab has shown that nucleolus in *E. histolytica* is located at the nuclear periphery (Jhingan *et al.* 2009). Nucleolus was identified by using antibody against fibrillarin, a highly conserved protein known to be a marker of nucleolus where EhFIB completely co-localized with RNA Pol I-specific subunits in *E. histolytica*. Immunoflorescence studies presented here showed that putative EhTIF-IA was also localized to the nucleolus. In addition, pull-down experiments using EhTIF-IA antibodies showed that EhTIFIA could associate with Pol I subunit EhRPA12 as well as nucleolar architectural protein EhFIB. This is expected from its putative role as a regulatory molecule of RNA Pol I transcription. Like its corresponding yeast and mammalian counterparts, localization studies showed that EhTIF-IA is present in both cytoplasm as well as nuclear periphery (Mayer *et al.* 2004; Philippi *et al.* 2010). Mammalian TIF-IA is known to translocate to cytoplasm during

(A)

Description protein	Score	Coverage (%)
EHI_035130, EhTIFIA	2653.72	86.91
EHI_095890, EhRPA1	873.63	55.54
EHI_186020, EhRPA2	460.06	41.23
EHI_044620, RPA12	102.7	75.41
EHI_142090, EhRPB5	48.95	25.98
EHI_122780, EhRPB10	43.68	49.32
EHI_122740, Nucleolar protein Nop56	27.60	18.32
EHI_118840 , Fibrillarin	38.71	29.84
EHI_038570, EhRPB8	28.41	22.38
EHI_131470 , Ribosome biogenesis protein Nop10	2.13	16.95

(B)

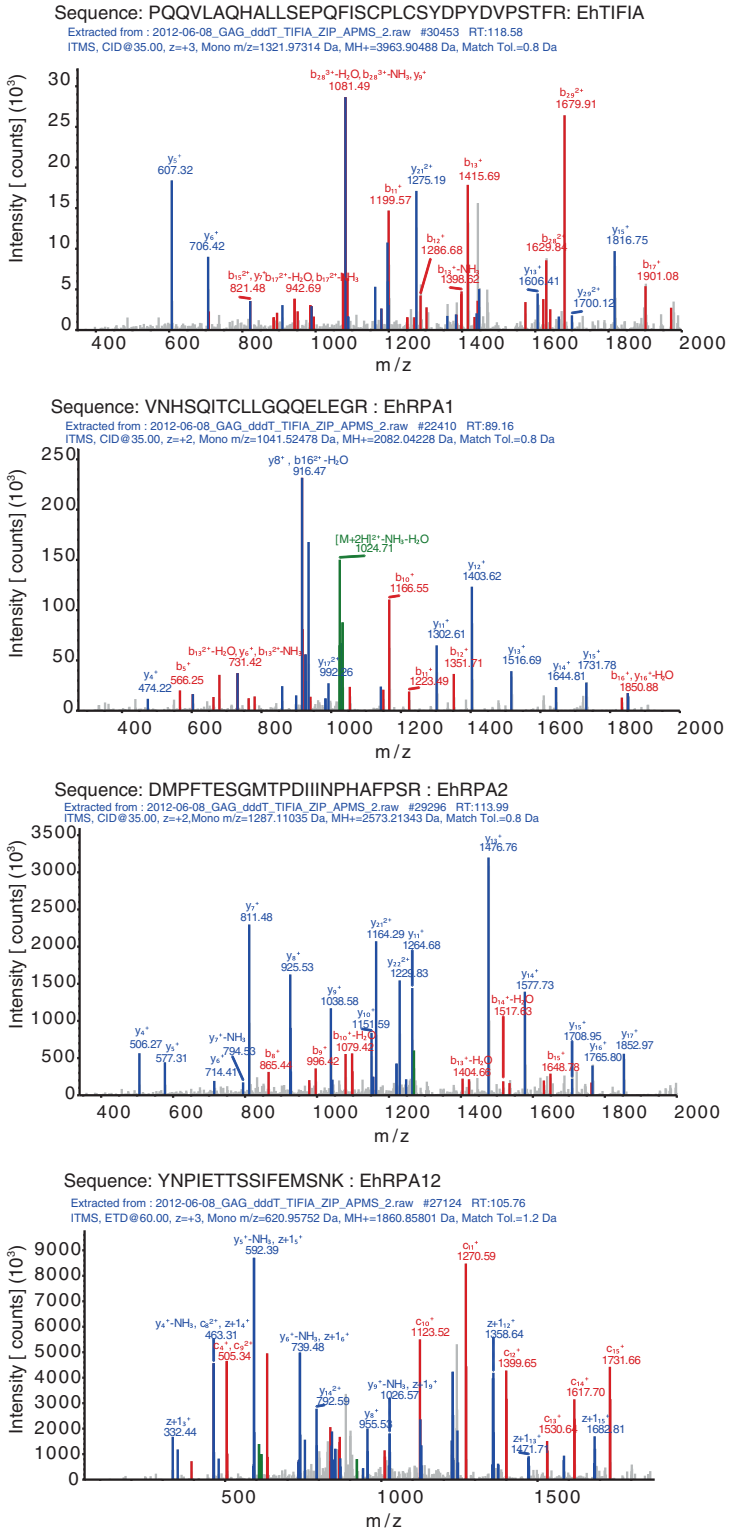

Figure 5. Mass spectrometric identification of interaction partners of EhTIFIA-HA: (**A**) Table showing major interaction partners of TIFIA-HA. (**B**) LC-MS/MS data showing collision-induced fragmentation mass spectra identifying EhTIF-IA and three RNA polymerase subunits, i.e. EhRPA1, EhRPA2 and EhRPA12. The MS/MS spectrum of precursor peptides (sequence shown above each panel) from all four proteins is evident by the observation of the 'b' and 'y' ion series.

rapamycin-mediated stress conditions. Our data show that EhTIF-IA localizes to the cytoplasm even in normal growth conditions and the physiological relevance of this observation remains to be understood.

RNA Pol-I is 14 subunit enzyme in yeast whose activity is the major cause of cell growth. The core of Pol-I includes the two largest subunits, i.e. A190 and A135, that forms DNA binding cleft together with five subunits present in all nuclear RNA polymerases (Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12) along with AC40–AC19 heterodimer which is shared with Pol III and the homologous Rpb3–Rpb11 in Pol II. A subunit formed by two zinc ribbons A12.2, completes the core which participates in RNA cleavage (Kuhn *et al.* 2007; Fernández-Tornero *et al.* 2013).

Our group has already identified homologue of RNA Pol I, II, III subunits in *Entamoeba histolytica* (Jhingan *et al.* 2009), these homologues may have similar role as in yeast which further needs to be studied. Detailed proteomics based pull-down complex analysis have identified several RNA Pol I subunits and nucleolar proteins as part of the EhTIF-IA complex inside the cell. EhTIF-IA seems to be part of a big protein complex which not only contained RNA Pol I subunits but also many other proteins of different functions. Earlier studies performed with rat RNA Pol I holoenzyme have shown the presence of many kinases and DNA modification enzymes as part of RNA Pol I transcriptional complex (Hannan *et al.* 1998, 1999). We have identified EhTIF-IA among the top hit during our mass spectrometry analysis followed by RNA Pol I subunits, e.g. RPA12, which was also used in this work for Pol I interaction studies. The other constituent's contained nucleolar proteins, actin/myosin and several kinases. Actin and myosin are known to promote RNA pol I transcription (Ye *et al.* 2008). Nop56 is the component of the C/D Box SnoRNP complex that have role in pre-rRNA maturation (Watkins *et al.* 2004) in yeast. Nop10 is also a part of the H/ACA small nucleolar ribonucleoprotein (H/ACA snoRNP) complex, which catalyses pseudouridylation of rRNA (Wang and Meier 2004). In *Entamoeba histolytica* Nop56 have already identified which have conserved C/D Box (Srivastava *et al.* 2014). Further analysis of the pre-rRNA processing machinery in *E. histolytica*, may help in understanding the evolution of this conserved function.

In summary, this study was aimed to understand a specific component of the rRNA transcription machinery in *E. histolytica* which may be important for regulation of RNA Pol I transcriptional initiation. We have been able to identify putative EhTIF-IA and have also generated its specific antibodies. These antibodies showed EhTIF-IA colocalization and interaction with EhRPA12 and other RNA Pol I subunits along with nucleolar proteins. To our knowledge this is the first report of putative EhTIF-IA in *E. histolytica*. Future efforts will be aimed to understand the role of putative EhTIF-IA in rRNA transcription and regulation.

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