Chapter 5

Dynamics of nucleolus organization in

\textit{E. histolytica} and \textit{E. invadens}
5.1. RESULTS

Dynamics of nucleolus organization in *E. histolytica* and *E. invadens*

The nucleolus is the site of rDNA transcription, pre-rRNA processing and modification and various steps of pre-ribosome assembly (Venema and Tollervey, 1999). In higher eukaryotes, nucleoli undergo a cycle of disassembly–reassembly during mitosis (Leung et al., 2004; Olson et al., 2000). Nucleoli disappear during prophase and reform at the end of telophase around specific chromosomal regions termed nucleolar organizer regions (NORs), which contain tandem repeats of ribosomal RNA (rRNA) genes. Since RNA and proteins are densely concentrated in the nucleolus, it is readily distinguished from the surrounding nucleoplasm as the most conspicuous nuclear structure observed by light and electron microscopy (Lam et al., 2005). In actively growing *Xenopus laevis* cells, RNA Pol I drives pre-rRNA synthesis at the border between the fibrillar centres and the dense fibrillar compartment, which accounts for about 50% of the total cellular RNA production (Reeder and Roeder, 1972). RNA Pol III and RNA Pol II in the nucleoplasm (extranucleolus) couple with the Pol I activity to synthesize 5S rRNA and mRNA for more than 70 ribosomal proteins, respectively (Gorlich and Mattaj, 1996).

Previous studies on nuclear ultrastructure of *E. histolytica* showed two distinct regions – a peripheral chromatin consisting of beaded structures lining the inner nuclear membrane, and a centrally located, small spherical karyosome (or endosome). The rDNA genes in this organism are located almost exclusively on extrachromosomal circular DNA molecules (Sehgal et al., 1994). These circular episomes were shown to be preferentially localized all along the inner nuclear membrane in the trophozoite stage, appearing to be part of the peripheral chromatin (el-Hashimi and Pittman, 1970; Miller et al., 1961; Zurita et al., 1991). Previous work based on in *vivo* labeling of nucleic acids showed that DNA was distributed and synthesized primarily throughout the nucleus, while RNA was synthesized (or accumulated) primarily in the peripheral chromatin; lesser amounts were evenly distributed (Albach, 1989; Albach et al., 1977; Albach et al., 1980). In another report, utilizing FISH technology it was shown that ribosomal episomes are located in the vicinity of the nuclear membrane and their segregation appears to precede the separation of chromosomes during nuclear division (Willhoeft and Tannich, 2000).
From these studies it was proposed that ribosomal RNA synthesis may occur in the peripheral chromatin and that the episome could be considered as a nucleolar organizer, and the peripheral chromatin may be the functional equivalent of a nucleolus in *E. histolytica*.

In this present work, we have tried to understand nucleolar organization in *E. histolytica* in much more detail. For the first time, we have utilized antibodies against proteins known to be localized in the nucleolus as specific markers to distinguish nucleolus from the rest of the nucleus.

5.1.1. **EhFibrillarin is localized at the nuclear periphery of *E. histolytica* cells.**

Fibrillarin is one of major components present in the fibrillar compartments of the nucleolus (Ochs et al., 1985b; Parker and Steitz, 1987) and is involved in pre-rRNA processing and ribosome assembly. *E. histolytica* fibrillarin (EhFIB) gene was identified by sequence homology, cloned and expressed in *E. coli*. Polyclonal antibodies were raised against EhFIB in both mice and rabbit (details in chapter 4) and used to localize EhFIB by immunofluorescence. Trophozoites were fixed in methanol, immunostained with anti-FIB antibody and analyzed by fluorescence microscopy. Control bleed (preimmune sera) did not give rise to any nuclear or cytoplasmic staining, while anti-FIB antibody prominently stained the nuclear periphery (Fig. 5.1A). No significant staining of nucleoplasm and cytoplasm was observed (Fig. 5.1B). In the picture shown, the trophozoite is bi-nucleate and in each nucleus fibrillarin is localized to an arc of the nuclear periphery. However in many nuclei (shown in subsequent pictures) fibrillarin occupies almost the entire nuclear periphery.

5.1.2. **RNA polymerase I subunits colocalizes with EhFIB at nuclear periphery.**

Fibrillarin, being a major protein in the nucleolus, colocalizes with other proteins that are known to be components of the nucleolus (Scheer and Hock, 1999; Scheer et al., 1993; Shaw and Jordan, 1995). One of the major components of nucleolus is RNA polymerase I itself. *E. histolytica* RNA Pol I is composed of 12 subunits (details in chapter 3). We
Fig. 5.1. Immunolocalization of Ehfibrillarin (EhFIB) in *E. histolytica*.
Trophozoites grown for 60 hours were transferred to pre-warmed, acetone-washed cover-slips for 10 minutes at 37°C. The attached cells were then fixed with chilled methanol and acetone for 10 min each, followed by immunostaining with antibodies. Amoebae were treated with (A) mouse preimmune serum (B) mouse anti-EhFIB antiserum, and anti-mice Alexa-green secondary antibody. DNA was stained with Hoechst. Scale bar: 5 μm. EhFIB localization shown by white Arrows at nuclear periphery. 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)
generated polyclonal antibodies against three subunits of Pol I: RPA1, RPA2 and RPA12 (details chapter 4). Double staining of trophozoites was done with antibody against EhFIB raised in rabbit and the Pol I subunits in mice. The results showed that EhFIB and all three RNA pol I subunits colocalized at the nuclear periphery and no significant staining of nucleoplasm was observed (Fig. 5.2). Colocalization of the nucleolar architectural protein fibrillarin along with RNA Pol I subunit on nuclear periphery allows us to conclude that: Nuclear periphery (peripheral chromatin) is indeed the nucleolar equivalent in *E. histolytica*.

5.1.3. Nucleolus is located in the form of complete ring along the nuclear periphery

Studies with EhFIB immunofluorescence in trophozoite showed various patterns of peripheral EhFIB on nuclear membrane. EhFIB was present in shapes ranging from incomplete to complete ring in various cells of the same culture population. Even in the same cell sometimes two different nuclei showed totally different patterns (Fig. 5.3). To understand how EhFIB is distributed along the nuclear periphery, we utilized confocal microscopy to take various sections of a single cell containing three nuclei. It was noticed that for this cell two nuclei were present in one plane while the third was in a different plane (Fig. 5.4). For each nucleus the staining pattern of EhFIB and EhRPA1 changed from complete ring to incomplete arcs as the plane in view moved away from the center of nucleus in different sections. Each nucleus at one particular plane showed a complete ring of nucleolus at nuclear periphery. In normal fluorescence microscopy also it was possible to view a complete nucleolar ring in each nucleus by manually changing the plane in focus. In many cases the thickness of ring was not uniform along the nuclear membrane. Sometimes extra thick bulges were noticed, although they were found to be extensions of the complete nucleolar ring in a different section.
Fig. 5.2. Co-immunolocalization of RNA polymerase I and EhFIB in *E. histolytica*.
Amoebae were double labeled for EhFIB and (A) EhRPAl (B) EhRPAl2 and (C) EhRPAl2. RPA subunits were labeled with Alexa-red conjugated secondary antibody (mouse) and EhFIB with Alexa-green secondary antibody (rabbit). Colocalization is apparent as yellow signal in the merged panel. Confocal laser-scanning microscopy was carried out with a Zeiss LSM510 META confocal microscope (Oberkochen, Germany).
Fig. 5.3 Detection of various patterns of EhFIB on nuclear periphery of healthy growing trophozoites (60 day culture). Microscopy pictures were taken in three different fields. EhFIB localization (red) is shown by white arrows at nuclear periphery.
Fig. 5.4. Confocal optical section stacks showing colocalization (yellow) of EhFIB (green) and EhRPA1 (red) of a single trophozoite. (A) Colocalization images of twenty five sections of trophozoite taken at 0.37 μm interval. (B) Enlarged and split image of 4.08 μm section demonstrating ring shape pattern of EhFIB and EhRPA1.
5.1.4. RNA Pol II is present exclusively in nucleoplasm while Pol III is present predominantly in nucleoplasm with a minor component at the nuclear periphery.

If the nuclear periphery of *E. histolytica* is indeed equivalent to the nucleolus, then RNA Pol II and III, which are not normally involved in rRNA gene transcription, are not expected to be localized at the nuclear periphery. In mammals, RNA Pol II has been shown to localize throughout the nucleoplasm, excluding the nucleolus (Dirks and Snaar, 1999; Mortillaro et al., 1996; Neugebauer and Roth, 1997). We analyzed the distribution of Pol II and III in *E. histolytica* using antibodies specific for the Pol II subunit EhRPB9 and the Pol III transcription factor EhBRF (details in chapter 4). In case of EhRPB9, trophozoites revealed a granular pattern throughout the nucleoplasm. Confocal imaging was also performed on the same slide. Double staining was done with anti-FIB antibodies and no colocalization was observed (Fig. 5.5. A, B). In yeast the 5S rRNA genes (which are Pol III-transcribed) are physically linked with rRNA genes and hence are located in the nucleolus. In higher the transcriptionally active 5S rRNA genes and tRNA genes are nucleolar localized while inactive copies are present in nucleoplasm (Highett et al., 1993; Kaplan et al., 1993; Mertens et al., 2001; Metzenberg et al., 1985; Montijn et al., 1999; Thompson et al., 2003). In *E. histolytica*, EhBRF which is a Pol III transcription factor revealed a punctate pattern throughout the nucleoplasm (Fig. 5.6. A, B). Unlike Pol II antibody which stained only the nucleoplasm, Pol III antibody showed a weak staining of the nuclear periphery also, although the predominant staining was in the nucleoplasm. Genome sequencing project of *E. histolytica* has revealed that almost all the tRNA genes are organized into tandem arrays that make up over 10% of the genome. The 25 distinct array units contain up to 5 tRNA genes each and some also encode the 5S rRNA (Clark et al., 2006; Tawari et al., 2008). Like yeast and higher eukaryotes, we also observed Pol III localization predominantly in nucleoplasm. It is tempting to propose that, the faint peripheral EhBRF staining may be due to transcriptionally active tRNA and 5S RNA gene arrays localized to the nucleolus.

These results indicate that in *E. histolytica* Pol I, II and III are located in different nuclear domains, and further confirm that nucleolus is at the nuclear periphery.
Fig. 5.5. Immunolocalization of EhRPB9 in *E. histolytica*.

(A) Immunofluorescence microscopy of trophozoites double labeled for EhRPB9 (red) along with EhFIB (green).

(B) Confocal microscopy of the same slides (enlarged picture).
Fig. 5.6. Immunolocalization of EhBRF in *E. histolytica*.

(A) Immunofluorescence microscopy of trophozoites double labeled for EhBRF (red) along with EhFIB (green).

(B) Confocal microscopy of same slides (enlarged picture).
5.1.5. Transcription factor EhTBP is present in both nucleolus and nucleoplasm

TBP (TATA binding protein) is a constituent of SL1, TFIID and TFIIIB- the basal factors required for transcriptional assembly in Pol I, II and III respectively (Hahn et al., 1989; Paule and White, 2000; Radebaugh et al., 1994). Previous work in *E. histolytica* has reported the cloning and expression of EhTBP gene (Luna-Arias et al., 1999). Using immunofluorescence microscopy it was further shown that EhTBP is located throughout the nucleus. Recombinant EhTBP was shown to form specific complexes with TATA variants found in *E. histolytica* gene promoters (de Dios-Bravo et al., 2005). In order to see if EhTBP is also localized to the nucleolus, we have generated EhTBP-specific antibodies in both mice and rabbit. EhTBP was found to localize throughout the nucleoplasm. Most interestingly, we also observed a strong colocalization signal of EhTBP and EhFIB at nuclear periphery (Fig. 5.7A). Confocal imaging of same slides showed much higher resolution of TBP localization within the nucleus (Fig. 5.7B). EhTBP was localized both to the nucleoplasm and to the nuclear periphery. At the periphery the staining of anti-FIB and anti-TBP antibodies was found to co-localize. The result is expected of a transcription factor which is a conserved component of all three Pols.

5.1.6. Effect of stress on Nucleolar organization in *E. histolytica*

5.1.6.1. Cycloheximide treatment

Cells treated with cycloheximide (CHX), a protein synthesis inhibitor have reduced need for ribosomes. Therefore CHX- treated cells generally show inhibition of rRNA synthesis and processing (Ghosh and Paweletz, 1994; Kay and Korner, 1966; Taber and Vincent, 1969). Nucleolar organisation was studied in CHX-treated rat kangaroo cells in which after 1 h of treatment most nucleoli became loose and revealed a nucleolonemal network (Ghosh and Paweletz, 1994). In another study no significant differences were registered in the mean number of nucleoli per cell after CHX treatment, but occurrence of large fibrillar centres, along with formation of microspherules and small intranucleolar
Fig. 5.7. Immunolocalization of EhTBP in *E. histolytica*.

(A) Immunofluorescence microscopy of trophozoites double labeled for EhTBP (red) along with EhFIB (green).

(B) Confocal microscopy of same slides (enlarged picture).
vacuoles were reported (Lafarga et al., 1994). Previous work in our lab showed that in *E. histolytica* 100µg/ml of CHX treatment inhibits protein synthesis to 85% by one hour. It was also observed that prolonged incubation with CHX (>4hr) leads to rounding of cells and finally cell death.

To study the effect of this inhibitor on nucleolar architecture, 100µg/ml of CHX was added to two and a half days (mid logarithmic phase) grown trophozoites for different time intervals i.e. 30min, 1hr, 2hr, 4hr, 6hr and 8hr. Trophozoites were taken after different time intervals and double stained for EhFIB and EhRPA1. As compared to normal cells, CHX- treated cells showed a rapid cytoplasmic appearance of EhRPA1, while EhFIB was also observed in the cytoplasm at later time points (Fig. 5.8). The nucleolar structure was maintained till 6 hrs of treatment as judged by anti-EhFIB staining, and RPA1 continued to colocalize with EhFIB, but was found in the cytoplasm in large amounts. After 6-8 hrs of treatment, drastic changes in cell morphology in terms of nuclear shape and cytoplasmic appearance were observed. Breakage of peripheral nucleolus, distortion in nucleus shape, uneven DNA staining as well as cell rounding was seen at 8 hrs when cells began to die (Fig. 5.8).

The main conclusion that can be drawn from this experiment is that *E. histolytica* nucleolus is maintained, even after translational inhibition. However, RNA Pol I is affected by loss of translation activity and high levels of it accumulate in the cytoplasm.

### 5.1.6.2. Heat and oxygen stress

In mammals, striking nucleolar changes occur in cultured cells after exposure to supranormal temperatures (Simard and Bernhard, 1967). These alterations are reversible when the cells are returned to 37°C and are associated with the reappearance of an exaggerated amount of intranucleolar chromatin and granular RNP (Simard and Bernhard, 1967). Ultrastructural studies of the heated cells indicated that the fibrillar component of the nucleolus became diffusely distributed throughout the nucleolus and that the granular component of the nucleolus almost completely disappeared. Assembly and export of ribosomes was found to be blocked (Love et al., 1970; Pelham, 1984).
Fig. 5.8. Effects of translational inhibition on nucleolar architecture in *E. histolytica*. Immunofluorescence microscopy of trophozoites treated with CHX (100 µg/ml) for different time periods as indicated in top panel. Trophozoites were double labeled for EhRPA1 (red) along with EhFIB (green).
E. histolytica cells growing at 35.5°C were subjected to heat shock by transferring to 42°C. In a time-course of temperature shift a gradual cytoplasmic appearance of both EhRPA1 and EhFIB was observed, although the nucleolar structure was maintained at 1 hr of treatment with diffuse cytoplasmic staining. When cells were restored to normal growth conditions (35.5°C), it took about one hour for the cytoplasmic signal to go down (Fig. 5.9). From this experiment we conclude that E. histolytica nucleolus is maintained under heat stress, but as in yeast, substantial relocation of EhFIB and EhRPA1 from nucleolus to cytoplasm took place under stress conditions.

E. histolytica is amitochondriate, microaerophilic organism which uses fermentation enzymes like those of bacteria to survive anaerobic conditions within the intestinal lumen (Fahey et al., 1984). However, it encounters a high-oxygen environment, during invasive amoebiasis (Variyam, 2007). Nucleolar changes were monitored in E. histolytica cells subjected to high oxygen stress (Fig. 5.10).

The results obtained were quite similar to heat stress and recovery. The only difference was that even after 1 hr of recovery, there was residual presence of EhFIB and EhRPA1 inside the cytoplasm.

5.1.6.3. Serum starvation

It has been shown that perturbation of growth by serum starvation causes changes in the ribosome content, along with total RNA and protein levels (Sienna et al., 1996). During serum starvation alterations in the localization of some nucleolar components has also been observed (Chan et al., 1985; Chatterjee et al., 1987; Matsumura et al., 1990; Seither et al., 1997b).

The results obtained with serum starvation (0.5% serum) and replenishment (15% serum) on trophozoites were quite similar to heat and oxygen stress and recovery. Cytoplasmic localization of RPA1 was especially acute in 24 hr serum starved cells. The pattern was reversible, as seen in cells grown in normal serum for 1 hr (Fig. 5.11).
Fig. 5.9. Effects of heat stress on nucleolar architecture in *E. histolytica*.

Immunofluorescence microscopy of trophozoites subjected to heat (42°C) for different time periods as indicated in top panel. Normal growth conditions were restored after 1 hr heat stress. Trophozoites were double labeled for EhRPA1 (red) along with EhFIB (green). Same abbreviations employed as mentioned in previous figures.
Fig. 5.10. Effect of oxygen stress on nucleolar architecture in *E. histolytica*. Immunofluorescence microscopy of trophozoites exposed to oxygen for different time periods as indicated in top panel. Normal growth conditions were restored after 2 hours oxygen stress. EhRPA1 (red) along with EhFIB (green).
Fig. 5.11. Effect of serum starvation on nucleolar architecture in *E. histolytica*.
Immunofluorescence microscopy of trophozoites subjected to serum starvation for different time periods as indicated in top panel. Normal growth conditions were replenished after 24 hr of treatment. Trophozoites were double labeled for EhRPA1(red) along with EhFIB (green).
5.1.7. Effects of kinase inhibitor rapamycin and staurosporine on nucleolar organization

**Rapamycin** is a highly specific inhibitor of the protein TOR, the Target Of Rapamycin. Inhibition of TOR by rapamycin or nutrient starvation causes rapid dephosphorylation of TOR (Beck and Hall, 1999; Bertram et al., 2000), leading to expression of many starvation-responsive genes. Rapamycin treatment inhibits rDNA transcription in both mammalian and yeast cells and leads to significant nucleolar size reduction (Leicht et al., 1996; Mahajan, 1994; Powers and Walter, 1999; Tsang et al., 2003; Zaragoza et al., 1998).

Presence of TOR kinase homologs in *E. histolytica* genome database indicates that this kinase might be involved in regulation of nutrient sensing in this organism also. Treatment of trophozoites with rapamycin leads to changes in nucleolar organization (Fig. 5.12, 5.13). In most of the cells the peripheral nucleolar ring was diminished or absent (Fig. 5.13). There was diffuse staining of FIB and RPA1 antibodies in the nucleoplasm. In some cells the nucleolar ring was fragmented into discrete spots. There was also some staining of the cytoplasm. It remains to be seen whether TOR kinase signaling is functional in *E. histolytica*, and whether it mediates the rapamycin effect on *E. histolytica* nucleoli.

**Staurosporine** is a potent inhibitor of many kinases including protein kinase A, C and G (Matsumoto and Sasaki, 1989; Tamaoki et al., 1986). Previous studies have shown the effects of staurosporine on the cell cycle progression, nuclear elongation, nuclear condensation and fragmentation (Bruno et al., 1992). It is potent antiproliferative agent and is commonly used as apoptotic inducer. In NIH/3T3 cells, incubation with staurosporine resulted in nuclear condensation and fragmentation (Liu et al., 2001). Staurosporine also impairs TOR signalling by dephosphorylation of p70 S6 kinase, which lies downstream of the TOR kinase (Tee and Proud, 2001).

In *E. invadens*, staurosporine treatment leads to inhibition of growth and cyst formation. (Makioka et al., 2003; Makioka et al., 2000a; Makioka et al., 2000b; Meza, 2000). Another study in *E. histolytica* showed inhibition of erythrophagocytis upon
Fig. 5.12. Effects of rapamycin and staurosporine on nucleolar architecture in *E. histolytica*. Trophozoites were treated with 1 μM of rapamycin and staurosporine for 8 hr and 2 hr respectively. Trophozoites were double labeled for EhRPA1 (red) along with EhFIB (green). Same abbreviations employed as mentioned in previous figures.
Fig. 5.13. Rapamycin treatment leads to nucleolar changes, including foci formation in some cells. Top panel is low magnification image (40X) showing EhFIB (green) and EhRPA1 (red). Bottom panel shows multiple images taken from treated cells where foci formation has been observed.
staurosporine treatment, probably due to its effect on protein kinase C (Batista Ede and de Souza, 2004). In order to demonstrate the effect of a broad range kinase inhibitor like staurosporine on nucleolar architecture, we treated trophozoites with 1μM inhibitor for 2 hrs and double labeled the cells as previously described. We observed major changes in nuclear morphology after treatment, where the nuclei became elongated in shape when compared to untreated cells (Fig. 5.12, 5.14). In some cells there was prominent nuclear shrinkage. In many cells the nucleolus was at one end of the nucleus. Staurosporine also leads to major changes in cell morphology with vacuole formation inside the cytoplasm. Our results show major changes in nucleolus repositioning as well as nuclear morphology within 2 hrs of staurosporine treatment.

5.1.8. Effect of trophozoite to cyst transition on nucleolar organization in *E. invadens*

Understanding biology and pathogenesis of the human parasite *E. histolytica* has been limited by the lack of efficient procedures to induce axenic encystation in the laboratory. However, such methods have been developed for *E. invadens*, a reptilian parasite, for which encystation can be induced by resuspension of trophozoites from growth medium into a diluted glucose-free medium (47% LG) containing 5% adult bovine serum (ABS). Changes in trophozoite morphology appear after 12 hrs of shift to LG medium, and complete cyst formation takes place after 72 hrs (Garcia-Zapien et al., 1995; Lopez-Romero and Villagomez-Castro, 1993; Turner and Eichinger, 2007; Vazquezdelaracasneros and Arroyo-Begovich, 1984).

Recently, ultrastructural analysis of the encystation and excystation processes in *E. invadens* was performed using electron microscopy, but the main focus of authors was cyst wall formation and dissolution (Chavez-Munguia et al., 2003; Chavez-Munguia et al., 2007).

In the present study, we have looked at nucleolar changes associated with trophozoite to cyst transition (Fig. 5.15). EhRPA1 antibodies failed to crossreact with *E. invadens*. This may be due to sequence divergence between both species. Fibrillarin is a very well conserved protein across various species, and as shown in chapter 4 anti- EhFib
Fig. 5.14. Staurosporine treatment leads to elongated nuclei.
Left panel shows EhRPA1(red) colocalization with DNA staining (blue)
Right panel shows magnified multiple images taken from treated cells labeled for EhRPA1 (red) and EhFIB (green)
Fig. 5.15. Effect of trophozoite to cyst transition on nucleolar architecture in *E. invadens*
Numbers on top of each column indicate the time after shift to LG medium.
First row, staining with EhFIB antibody; second row, merge with FIB and Hoechst staining; third row, Hoechst; fourth row, DIC.
antibodies crossreact very well with *E. invadens*. Using these antibodies, we were able to observe major changes within 12 hrs of LG medium induction, after 12 hrs the ring shape of nucleolus become non uniform (Fig. 5.16). From 36 hours till 48 hours many small and big foci starts accumulated inside the nucleoplasm along with much diminished peripheral nucleolar staining. Till 48 hours, there was a mixture of population of cells which had different pattern with respect to nucleoplasmic and non uniform ring forms (Fig. 5.17-5.19).

But at 72 hours, majority (90%) of the cells contained multiple foci in the nucleoplasm while no peripheral localization of nucleolus was observed (Fig. 5.20). Technically, it becomes difficult to permeabilize the cells after 72 hours, but we were able to stain some mature cyst (96hours) which contained discrete foci in all four nuclei along with some cytoplasmic staining (Fig. 5.21).

The main outcome of present study is that physiological changes which results in trophozoite to cyst transition are also accompanied with major changes in nucleolar architecture.
Fig. 5.16. Magnified images of cells stained with EhFIB (red) after 12 hrs in LG medium.
Fig. 5.17. Magnified images of cells stained with EhFIB (red) after 24 hrs in LG medium.
Fig. 5.18. Magnified multiple images showing EIFIB (red) localization in trophozoites after 36hrs LG medium induction. Same abbreviations employed as mentioned in previous figures.
Fig. 5.19. Magnified multiple images showing EIFIB (red) localization in trophozoites after 48hrs LG medium induction. Same abbreviations employed as mentioned in previous figures.
Fig. 5.20. Magnified multiple images showing EIFIB (red) localization in trophozoites after 72 hrs LG medium induction. Same abbreviations employed as mentioned in previous figures.
96 hrs (cyst) post induction LG medium

Fig. 5.21. Magnified image showing EIFIB (red) localization in cyst after 96 hrs LG medium induction. Same abbreviations employed as mentioned in previous figures.
5.2. DISCUSSION

The nucleolus has been viewed as a symbiotic mini life form with its own minigenome (predominantly rDNA), origin of replication and replication fork block (Higashinakagawa et al., 1992; Kobayashi et al., 1998; Mais et al., 2002; Muller et al., 2000; Walmsley et al., 1984; Wiesendanger et al., 1994). This mini life form is primarily dedicated to the transcription of pre rRNA, its processing into mature rRNAs and assembly into ribosomal particles (Cheutin et al., 2002; Huang, 2002; Venema and Tollervey, 1999). To perform these functions the nucleolus is organized in a highly structured fashion as revealed by ultrastructural studies (Colau et al., 2004; Haaf and Schmid, 1991; Sato, 1985; Scheer and Hock, 1999; Trumtel et al., 2000; Verheggen et al., 2001). In cells undergoing mitosis this structure has to disassemble at chromosomal sites (nucleolar organizer regions) which harbor the ribosomal gene repeats. Thus, the nucleolus is a dynamic structure. It is intriguing that the shape and structure which the nucleolus adopts is typical of every cell type. How is this governed? Does it depend on the distribution of rRNA genes (located on one or several chromosomes; located on small extrachromosomal circular or linear molecules; present in a few or large number of copies, etc.)? Is it influenced by structural features of the nucleolar architectural proteins like fibrillarin, nucleophosmin, nucleolin etc.? Is it influenced by the specific organization of the rDNA unit, e.g. relative length of rRNA gene versus the intergenic spacer? To answer these questions more information is required about nucleolar dynamics in a large variety of organisms.

In this thesis we report the identification of nucleolus in the unicellular protistan parasite *E. histolytica*. The rRNA genes in *E. histolytica* are located on extrachromosomal circles, in about 200-400 copies (Bhattacharya et al., 1992; Bhattacharya et al., 1989; Mittal et al., 1992; Mittal et al., 1991; Ramachandran et al., 1993; Sehgal et al., 1994). rRNA genes are an integral part of the nucleolus and it may be expected that the two would colocalize; although inactive gene copies may be excluded from the nucleolus. In an earlier study, the *E. histolytica* rDNA circles were localized to the nuclear periphery by in situ hybridization (Zurita et al., 1991). Very early ultrastructural analysis had shown a clearly defined peripheral chromatin in the *E.*
*Entamoeba histolytica* nucleus, in addition to a central karyosome (el-Hashimi and Pittman, 1970; Miller et al., 1961). Autoradiography of cells labeled with $^3$H-uridine showed maximum signal in the peripheral chromatin (Albach, 1989; Albach et al., 1977; Albach et al., 1980). From these studies, it was conjectured that the peripheral chromatin could be the nucleolus in *E. histolytica*. We have raised antibodies against the *E. histolytica* homologue of fibrillarin, a highly conserved protein known to be a marker for nucleolus in a wide variety of organisms. These antibodies cross-reacted strongly with the nuclear periphery forming a peripheral ring with very little labeling of nucleoplasm and cytoplasm. In addition there was complete colocalization of the fibrillarin pattern with the signal obtained by antibodies against *E. histolytica* RNA Pol I. This is strong evidence that the nucleolus in *E. histolytica* is indeed located at the nuclear periphery.

Extrachromosomal rRNA genes (either on circular or linear molecules) are found in a variety of unicellular eukaryotes, e.g. *Trypanosoma brucei* (Ogbadoyi et al., 2000), *Physarum polycephalum* (Guttes and Guttes, 1969; Tanaka, 1973), *Paramecium tetraurelia* (Tucker et al., 1980), *Naegleria gruberi* (Schuster, 1975), *Tetrahymena thermophyla* (Gorovsky, 1970) and *Euglena gracilis* (Leblond and El-Alfy, 1998). Of these a peripheral nucleolar ring was seen in *T. thermophyla* and *E. gracilis*. In *P. polycephalum, P. tetraurelia* the nucleolus was towards the centre of the nucleus. Thus the mere existence of rRNA genes on extrachromosomal molecules does not lead to the peripheral location of nucleolus. In *Saccharomyces cerevisiae* and *Plasmodium falciparum* where the rRNA genes are on chromosomes the nucleolus is located at one end of the nucleus, forming a crescent shaped cap-like structure, instead of a ring as seen in *E. histolytica* (Figueiredo et al., 2005; Senok et al., 2006; Tsang et al., 2003). In yeast during stress and ageing accumulation of extrachromosomal ribosomal DNA circles (ERC) have been reported in many studies (Defossez et al., 1998; Kaeberlein et al., 1999; Park et al., 1999). None of these studies have found peripheral nucleolar localization under these conditions. In a related study plasmids carrying single rRNA copy were introduced in *S. cerevisiae* cells and nucleolus was found to spread throughout the nucleus implying that each plasmid rDNA gene can serve as a nucleolar organizer (Nierras et al., 1997). Although extrachromosomal rDNA is not sufficient to result in a peripheral nucleolar ring, it is noteworthy that we have not encountered any report of
such a nucleolar arrangement in organisms with rRNA genes on linear chromosomes. In
the latter case the nucleolus is found either in the centre or at one side of the nucleus. It is
possible that the peripheral nucleolar ring forms only in those organisms where the
extrachromosomally rDNAs can diffuse and occupy the entire nuclear periphery.

We used antibodies against components of *E. histolytica* Pol II and III as
comparison with our results with Pol I. Both of them were located throughout the
nucleoplasm. However, a minor component of Pol III was observed at the nuclear
periphery also. This is not surprising since Pol III is involved in 5S rRNA gene
transcription inside the nucleolus, as described in other organisms (Haeusler and Engelke,
2006; Thompson et al., 2003; Wang et al., 2005).

One of the best-characterized subnuclear domains is the nuclear periphery.
Localization of parts of the genome to the nuclear periphery has important effects on
transcription. Constitutive heterochromatin localizes to the nuclear periphery in many cell
types (Comings, 1980; Haaf and Schmid, 1991), as do other silenced loci, such as the
immunoglobulin genes in hematopoietic progenitors of B lymphocytes (Kosak et al.,
2002). Localization of repressed parts of the genome to the nuclear periphery has been
thoroughly explored in *S. cerevisiae*, in which regions of transcriptionally silent
chromatin, such as telomeres and the mating type loci, associate with the nuclear
envelope (Cockell and Gasser, 1999). Recent work, however, indicates that
transcriptional activation also takes place at the nuclear periphery in yeast. Several
dynamically regulated genes are randomly distributed in the nucleoplasm when repressed
but are recruited to the nuclear periphery when activated. These genes include many
highly expressed genes such as INO1 (encoding an enzyme involved in phospholipid
biosynthesis), HSP104 (a molecular chaperone), HXK1 (hexokinase), SUC2 (invertase),
GAL1 (galactokinase), GAL2 (a hexose transporter), GAL10 (glucose epimerase) and
mating pheromone-induced genes (Abruzzi et al., 2006; Brickner and Walter, 2004;
Cabal et al., 2006; Casolari et al., 2005; Casolari et al., 2004; Sarma et al., 2007; Taddei
et al., 2006).

The mouse β-globin locus is transcriptionally active at the nuclear periphery before its
relocalization toward the nuclear interior during erythroid maturation (Ragoczy et al.,
2006). This indicates that localization at the nuclear periphery is not incompatible with
transcription in mammalian cells. Also, the transcriptional upregulation of the X chromosome in males in Drosophila requires nuclear pore components Mtor (also called TPR) and Nup153 and the X chromosome localizes at the nuclear periphery (Mendjan et al., 2006). Genome-wide chromatin Immunoprecipitation studies reveal that many transcriptionally active genes physically interact with components of the nuclear pore complex (NPC) and associated factors (Casolari et al., 2005; Casolari et al., 2004). Intriguingly, the nuclear pore component Nup2 is necessary and sufficient (when tethered to DNA directly) to create boundary elements that block the spread of heterochromatin (Burgess-Beusse et al., 2002; Dilworth et al., 2005; Ishii et al., 2002). Nup2 also interacts with transcriptionally active loci (Casolari et al., 2004), suggesting that the protein might help to shield these regions from neighboring silenced DNA. Therefore the nuclear periphery could represent a subnuclear domain that is itself composed of distinct microenvironments with roles in either gene silencing or activation.

Nucleolus has been proposed to be a stress sensor inside the cell. Various stress stimuli have been shown to induce changes of the cellular localization of nucleolar proteins. Tumor suppressor proteins like VHL and p53 are stabilized by sequestration in the nucleolus (Mekhail et al., 2004; Rubbi and Milner, 2003). Likewise, nucleolin and nucleophosmin are translocated from the nucleolus into the nucleoplasm, where they play a regulatory role in inducing cell cycle arrest by p53 mediated mechanism (Daniely et al., 2002; Kurki et al., 2004). We employed various stress conditions in *E. histolytica* to check their effect on peripheral nucleolar localization. Cycloheximide is well known as a translational inhibitor (Ghosh and Paweletz, 1994; Lafarga et al., 1994). Upon cycloheximide treatment, we noticed a gradual accumulation of RNA Pol I inside the cytoplasm, although nucleolus maintained its shape. The other conditions employed for the same study were stress due to heat, oxygen and serum starvation. A gradual accumulation of Pol I and Fibrillarin inside the cytoplasm was noticed with increasing time of treatment but the peripheral nucleolar ring was not lost. These changes were found to be completely reversible once cells were provided normal growth conditions. *E. histolytica* genome database contains homologs of TOR kinase. We treated cells with Rapamycin, which is a well known inhibitor of TOR kinase (Beck and Hall, 1999; Mahajan, 1994; Zaragoza et al., 1998). The results obtained after 8 hrs of rapamycin
treatment were very pronounced as compared to the previous stress conditions employed. Nucleolar ring fragmentation into discrete foci was observed for both Pol I and Fibrillarin. Some staining of cytoplasm was also observed under these conditions. It would be very interesting to check the mechanism behind this observation and any role TOR kinase plays in this process.

The second kinase inhibitor used in this series of experiments was staurosporine (Bruno et al., 1992; Matsumoto and Sasaki, 1989; Tamaoki et al., 1986). In mammalian cells, this inhibitor has been shown to interfere with cell cycle progression as well as nuclear size and shape (Baran et al., 2003; Bruno et al., 1992; Liu et al., 2001; Matsumoto and Sasaki, 1989; Tamaoki et al., 1986). The effects of staurosporine treatment again were quite dramatic. We found overall changes in nuclear shape. Normal nuclei were round in shape but after staurosporine treatment they got elongated along with some size reduction. In most of the cells nucleolus was located at one far end of the nucleus instead of the continuous ring. This result suggests that multiple signaling pathways are active in E. histolytica whose blockage leads to dramatic changes in overall nuclear and nucleolar shape. It will be interesting to see whether these inhibitors also alter the distribution of rDNA circles within the nucleus.

The next obvious question is which mechanism is responsible for these dramatic changes. One of the detailed mechanism was shown in yeast where rDNA chromatin undergoes dramatic remodeling and becomes condensed in the presence of rapamycin. In this model, TOR regulates accessibility of Rpd3–Sin3 HDAC (Histone deacetyl transferase) complex to the rDNA chromatin domain in response to nutrient availability. During nutrient starvation or rapamycin treatment, conditions that inhibit TOR, Rpd3–Sin3 HDAC becomes associated with rDNA chromatin, thereby causing histone H4 deacetylation, rDNA chromatin condensation and reduced nucleolar size. This chain of events further leads to RNA Pol I delocalization from the nucleolus and inhibition of rDNA transcription. Inhibition of rDNA transcription may be the result of both rDNA chromatin condensation and RNA Pol I delocalization. A smaller nucleolus could further provide spatial hindrance to rDNA transcription and ribosome biogenesis (Rohde and Cardenas, 2003; Tsang et al., 2003).
*E. histolytica* in its normal life cycle exists as trophozoite and cyst forms. However, this differentiation cannot be achieved in culture. In *E. invadens*, a reptilian parasite, encystation can be induced in culture by resuspension of trophozoites from growth medium into a diluted glucose-free medium (LG). These conditions mimic the natural life cycle of the organism. We investigated whether the nucleolus shows any changes during differentiation. After transfer to LG medium complete cyst formation takes around 96 hrs (4 days). Anti-fibrillarin antibodies generated by *E. histolytica* protein were shown to cross-react with *E. invadens* nuclear extracts (chapter 4). These antibodies were utilized in immunofluorescence analysis of *E. invadens* trophozoites and revealed a ring pattern for fibrillarin localization. This showed that nucleolar architecture is conserved between the two *Entamoeba* species. Trophozoites to cyst transition stages showed gradual breakage of peripheral nucleolar ring into small foci. Due to technical limitation we were not able to label the mature cyst stage. However, we could clearly observe nucleolar changes related to the conversion of trophozoite to cyst stage. What is the significance of this nucleolar breakdown just before cyst formation is an open question. What happens to rDNA circles and to rDNA transcription during this transition? Future studies utilizing rDNA specific FISH probes, and biochemical analysis of transcription are being planned to answer many of these interesting questions.