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Constitutive association of Mcm2-3-5 proteins with chromatin in *Entamoeba histolytica*

Suchismita Das,† Chandrama Mukherjee, Pratima Sinha and Anuradha Lohia*
Department of Biochemistry, Bose Institute, P1/12 CIT Scheme VIIM, Kolkata 700054, India.

Summary

Eukaryotic cells duplicate their genome once and only once per cell cycle. Our earlier studies with the protozoan parasite, *Entamoeba histolytica*, have shown that genome reduplication may occur several times without nuclear or cellular division. The Mcm2–7 protein complex is required for licensing of DNA replication. In an effort to understand whether genome reduplication occurs due to absence or failure of the DNA replication licensing system, we analysed the function of Mcm2-3-5 proteins in *E. histolytica.* In this study, we have cloned *E. histolytica* (*Eh*) *MCM2* and *Eh MCM5* genes, while *Eh MCM3* was cloned earlier. The sequence of *Eh MCM2-3-5* genes is well conserved with other eukaryotic homologues. We have shown that *Eh* Mcm2,3 proteins are functional in *Saccharomyces cerevisiae*. Our studies in *E. histolytica* showed that *Eh* Mcm2-3-5 proteins are associated with chromatin constitutively in cycling cells and during arrest of DNA synthesis induced by serum starvation. Alternation of genome duplication with mitosis is regulated by association–dissociation of Mcm2–7 proteins with chromatin in other eukaryotes. Our results suggest that constitutive association of Mcm proteins with chromatin could be one of the reasons why genome reduplication occurs in *E. histolytica*.

Introduction

The precise timing and fidelity of S-phase are achieved through multiple regulatory mechanisms that involve positive, negative and feedback controls to co-ordinate DNA synthesis with different stages of the cell cycle. The DNA replication licensing system is one of the crucial mechanisms, which ensures the alternation of S-phase with mitosis in most cells (Tye, 1999). Licensing of replication origins for the initiation of DNA synthesis is achieved by the formation of the pre-replication complex (pre-RC) at the replication origin. Formation of pre-RC requires the ordered assembly of the origin recognition complex (ORC), Cdc6, Cdt1 and the Mcm2–7 proteins. Loading of Mcm2–7 to replication origins requires the binding of the ORC to the replication origin (Stillman, 1994) and recruitment of Cdc6 (Diffley, 1996) and Cdt1 (Nishitani *et al*., 2000). The pre-RC is activated by protein kinases Cdc7 and cyclin-dependant kinase (CDK) (Dahmann *et al*., 1995) for initiation to occur. Other factors that regulate the transition from pre-RC to replication initiation are Mcm10p and Cdc45p (Merchant *et al*., 1997; Wohlschlegel *et al*., 2002; Gregan *et al*., 2003). Both these proteins also have a role in elongation (Bell and Dutta, 2002). Replication origin licensing is inactivated during S-phase but Mcm2–7 may function as a helicase that unwinds DNA ahead of the replication fork during S-phase (Labib and Diffley, 2001). Once S-phase has begun, the formation of new pre-RC is kept in check by high CDK activity and by the activity of the protein geminin (Bell and Dutta, 2002).

In most organisms, Mcm proteins remain in the nucleus throughout the cell cycle, associating with chromatin during replication licensing and dissociating after replication termination. In special cases, where polyploidy is achieved through multiple rounds of DNA replication without alternating cell division, such as the polytene chromosomes of *Drosophila*, the same network of protein complexes regulates re-initiation of DNA synthesis by association–disassociation of the pre-RC (Su and Farrel, 1998).

Genes encoding the Mcm2–7 proteins are conserved over 50–70% of the total sequence in different organisms ranging from yeast to humans (Chong *et al*., 1996). The size of this six-member family ranges from 776 amino acids to 1016 amino acids in *Saccharomyces cerevisiae* (Tye, 1999). Interestingly, Mcm proteins have been found in Archaea (Smith *et al*., 1997), suggesting that the function of these genes may have evolved before the emergence of eukaryotes.

Received 23 June, 2004; revised 19 August, 2004; accepted 23 August, 2004. *For correspondence. E-mail amoeba@boseinst.ernet.in; Tel. (+91) 33 2334 7430; Fax (+91) 33 2334 3886. †Present address: Department of Molecular and Cell Biology, Goldman School of Dental Medicine, Boston University Medical Centre, Boston, MA, USA.

Entamoeba histolytica is a pathogenic protozoan parasite, which causes amoebiasis in humans. Earlier reports have shown that unlike most eukaryotes, *E. histolytica* cells reduplicate their genome several times before cell division occurs (Gangopadhyay *et al*., 1997a). DNA reduplication may also occur without nuclear division so that single nuclei may contain 1X–6X or more genome contents (Das and Lohia, 2002). Multinucleated cells may also accumulate more than 2X genome contents in each nucleus of one cell. Thus, DNA reduplication occurs several times before nuclear division and two to three nuclei may form before cell division, which suggests that eukaryotic cell cycle checkpoints or regulatory mechanisms, which ensure that reduplication of the genome is prevented, are either absent or turned off during the proliferation of this protozoan parasite. We were therefore interested to identify whether homologues of DNA replication licensing factors were functional in *E. histolytica*.

In this study, we have cloned *E. histolytica* (*Eh*) *MCM2* and *Eh MCM5* genes. *Eh MCM3* gene has been reported earlier (Gangopadhyay *et al*., 1997b). In an effort to understand the molecular basis of genome reduplication in *E. histolytica* cells, we have analysed the expression and localization of *Eh* Mcm2-3-5 proteins during DNA synthesis in *E. histolytica.*

Results

Molecular cloning, sequencing and characterization of Eh MCM2 *and* MCM5

While looking for *MCM* homologues in *E. histolytica*, we used oligonucleotide primers to the conserved amino acid region of *MCM2-3-5* from *S. cerevisiae* and *Schizosaccharomyces pombe* (Gangopadhyay *et al*., 1997b) to isolate a 220 bp product by polymerase chain reaction (PCR). We identified different products from a single PCR-amplified DNA fragment which were homologous to *MCM2*, *MCM3*, *MCM5*. We have earlier reported the cloning of the genomic and cDNA sequences of *Eh MCM3* (Gangopadhyay *et al*., 1997b). Analysis of the *Eh* Mcm3 open reading frame (ORF) showed that it was significantly shorter than other Mcm3 homologues (Gangopadhyay *et al*., 1997b). The 5′ and 3′ ends of *Eh MCM3* were confirmed by sequencing of cDNA and genomic clones of *Eh MCM3* and *Eh PAK* (immediately upstream of *Eh MCM3*) and by analysing transcriptional start sites (Gangopadhyay *et al*., 1997b).

In this study, we have isolated *Eh MCM2* and *Eh MCM5* from the *E. histolytica* genomic and cDNA libraries. The *Eh MCM2* gene was 2.65 kb long and the predicted ORF encoded a protein of 883 amino acids. By comparing the sequences with the help of BLAST search, it was found to have the conserved zinc finger motif of the type CX_2CXnCX_2C (Tye, 1999) at the N-terminal end from amino acid number 389–417. The Walker type ATPase motif is very well conserved and based on amino acid sequence homology, *Eh MCM2* has 40% identity and 60% similarity with other *MCM2* genes from *S. cerevisiae*, *Caenorhabditis elegans*, *S. pombe* and *Drosophila melanogaster*. [Sequence alignment of *Eh* Mcm2 and *S. cerevisiae* (*Sc*) Mcm2 is shown in Fig. S1].

The *Eh MCM5* gene contained an intron as deduced from analysing the nucleotide sequence of the genomic clone. *Eh MCM5* gene shares 41% identity and 60% similarity with other eukaryotic *MCM5* homologues from *Neurospora crassa*, *S. cerevisiae*, *S. pombe*, *Xenopus laevis*, etc. The Walker type ATPase motif is conserved at the C-terminal end of this gene also. The predicted ORF encodes a protein of 639 amino acids, unlike other Mcm5 homologues, which range from 724 to 776 amino acids. (Sequence alignment of *Eh* Mcm5 and *Sc* Mcm5 is shown in Fig. S2). Thus, *Eh* Mcm5 is shorter than its homologues and this is supported by Western analysis using an antibody to *Eh* Mcm5 (shown below).

We were unable to clone homologues of *MCM4-6-7* from the *E. histolytica* genomic or cDNA libraries but have recently identified putative homologues of these genes from the unannotated *E. histolytica* genome sequencing projects at the TIGR and Sanger Sequencing Centre. Presently available data suggest that the *Eh* Mcm4 (609 aa), *Eh* Mcm6 (675 aa) and *Eh* Mcm7 (691 aa) are also significantly smaller than their yeast homologues (data not shown) but show a high homology with the conserved residues. *Eh* Mcm2 was the only member of this family of six proteins, which was similar in size to the *Sc* Mcm2 and other Mcm2 homologues. Our analysis suggests that the *E. histolytica* genome encodes homologues of all six members of the Mcm2–7 family. We next investigated whether some of these *Eh* Mcm proteins could functionally complement *S. cerevisiae mcm* mutants.

Functional complementation of yeast mutants by Eh *Mcm proteins*

The *Sc mcm2* mutant M46-3C (Sinha *et al*., 1986) was transformed with *Eh MCM2* cloned in pACT2, while the *Sc mcm3* mutant R61-2B (Gibson *et al*., 1990) was transformed with *Eh MCM3*, also cloned in pACT2. Both the strains were also transformed with the vector alone as control. Both *Sc mcm2* and *Sc mcm3* mutants transformed with *Eh MCM2* and *Eh MCM3*, respectively, could grow at 36°C while mutants transformed with vector alone did not grow suggesting that the *Eh MCM2* and *Eh MCM3* genes could rescue the growth defect at the non-permissive temperature (Fig. 1).

In order to confirm whether the *Eh* Mcm2-3 proteins could stabilize minichromosomes, these transformants

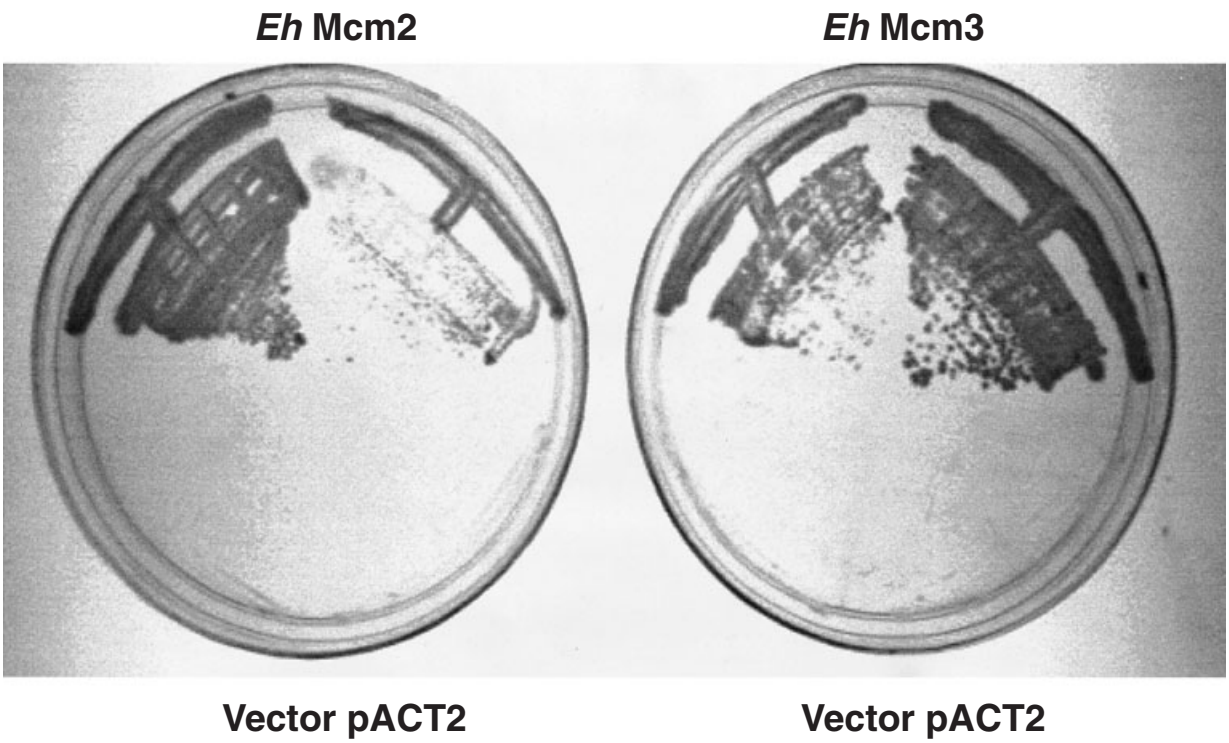

Fig. 1. Functional complementation of yeast mutants by *Eh* Mcm proteins. The *Sc mcm2* mutant M46-3C was transformed with *Eh MCM2* cloned in pACT2, while the *Sc mcm3* mutant R61-2B was transformed with *Eh MCM3*, also cloned in pACT2. Both the strains were also transformed with the vector alone as control. Streaks show two independent transformants for both *Eh MCM2* and *Eh MCM3* respectively. Growth was monitored at 36°C.

were further transformed with YCplac33 (Gietz and Sugino, 1988), which served as a minichromosome. The mitotic stability of YCplac33 was determined in the presence of *Eh MCM2* and *Eh MCM3* genes as described in *Experimental procedures*. Table 1 shows that the minichromosome had a high mitotic stability when maintained in the presence of *Eh MCM* genes but gave a low stability in the presence of the vector alone.

Our data clearly show that both *Eh* Mcm2 and *Eh* Mcm3 could complement yeast mutants and function to stabilize minichromosomes in budding yeast. The missing amino acids from the N- and C-terminal ends of *Eh* Mcm3 (Gangopadhyay *et al*., 1997b), compared with *Sc* Mcm3, did not compromise its ability to functionally complement the yeast mutant. We next investigated whether these proteins were expressed in *E. histolytica*. Antibodies to recombinant *Eh* Mcm2-3-5 were raised in rabbits, purified (data not shown) and used to study the expression and cellular localization of *Eh* Mcm2-3-5.

Cellular localization of Eh *MCM2-3-5 proteins*

To identify the cellular localization of *Eh* Mcm proteins, *E. histolytica* trophozoites were fractionated to separate the nucleus and the cytoplasm. The nucleus was further separated into Triton X-100-soluble and the detergent-insoluble fractions. The cytoplasmic fraction (CS), detergent-soluble nuclear fraction (NS) and the detergent-insoluble nuclear pellet (NP) were purified and analysed on an agarose gel. It may be seen that the detergent-insoluble nuclear fraction contained chromatin (Fig. 2A, NP), while the other two fractions contained RNA only (Fig. 2A, CS and NS). This was confirmed by treatment of each of the fractions with DNase I (lane 2) and RNase (lane 3) as described in the text.

Table 1. Functional complementation of *S. cerevisiae* mutants by *Eh MCM2* and *Eh MCM3*.

Yeast strains	Constructs	Mitotic stability of YCplac33
Sc mcm2 mutant (M46-3C)	Vector	0.05%
	Eh Mcm2	85%
Sc mcm3 mutant (R61-2B)	Vector	0.03%
	Eh Mcm3	90%

The *S. cerevisiae mcm2* mutant strain M46-3C was transformed with *Eh MCM2* cloned in pACT2 vector, with *LEU2* selection and then transformed with the minichromosome YCplac33 using *URA3* selection. Similarly *Sc mcm3* mutant strain R61-2B was transformed with *Eh MCM3* and YCplac33. As a control, the mutant strains were transformed with pACT2 vector and YCplac33. The mitotic stability of YCplac33 in these transformants was determined as described in the text.

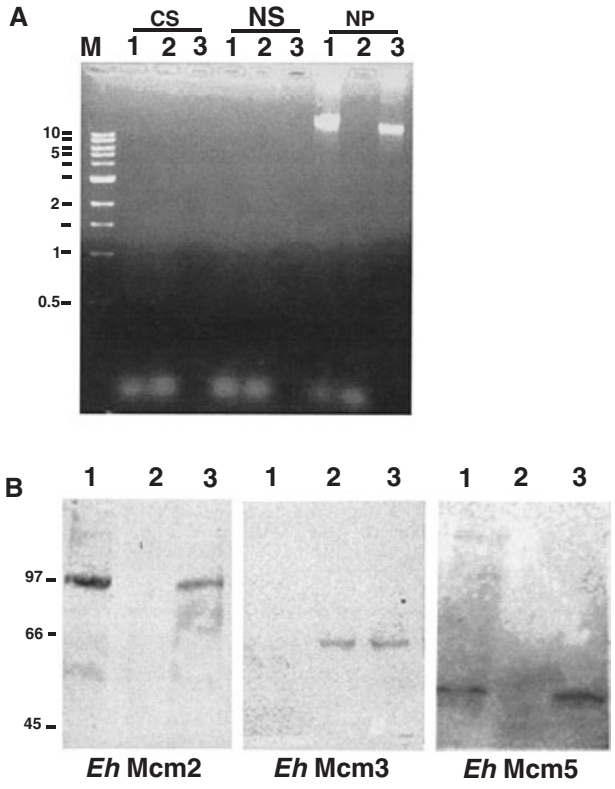

Fig. 2. Subcellular localization of *E. histolytica* Mcm2-3-5 proteins.
A. Ethidium bromide-stained agarose gel of cytoplasmic fraction (CS), detergent-soluble nuclear fraction (NS) and detergent-insoluble nuclear pellet (NP). Lane 1, nucleic acid profile of isolated fraction; lane 2, DNase I-treated fractions; lane 3, Rnase-treated fraction. Molecular sizes (M) are indicated on the left.
B. Western analysis of cytoplasmic fraction – lane 1, detergent-soluble nuclear fraction – lane 2, detergent-insoluble nuclear fraction – lane 3, hybridized with *Eh* Mcm2 antibody, Mcm3 antibody and Mcm5 antibody respectively. Molecular sizes are shown on the left.

Proteins from the three fractions were run on SDS-PAGE, Western transferred and hybridized with anti-*Eh* Mcm2-3-5 antibodies. *Eh* Mcm2 and *Eh* Mcm5 (Fig. 2B) were seen in the chromatin-bound detergent-insoluble nuclear pellet and in the cytoplasm whereas *Eh* Mcm3 was detected in the chromatin-bound detergent-insoluble nuclear pellet and in the detergent-soluble nucleoplasm (Fig. 2B). Cytoplasmic localization of *Eh* Mcm2 and *Eh* Mcm5 suggests that it may provide a pool for replenishing these proteins when necessary or these proteins may perform other functions apart from DNA replication licensing. Western analysis shows that the molecular size of the *Eh* Mcm2 protein was comparable with the size of the predicted ORFs from the DNA sequence. *Eh* Mcm5, however, showed a faster mobility and smaller molecular size (57 kDa) than its predicted size of 65 kDa. This difference may result from post-translational modifications.

Localization by confocal microscopy showed that *Eh* Mcm2 and *Eh* Mcm5 were both nuclear and cytoplasmic while *Eh* Mcm3 was predominantly nuclear (Fig. 3). This confirmed the results obtained from Western analysis in Fig. 2. It may be noted that the localization of *Eh* Mcm2-3-5 was evenly dispersed in the nucleus. To identify whether the localization of Mcm proteins was altered during DNA synthesis, we incubated cells with 5′-bromo-2′ deoxyuridine (BrdU) before fixing and staining them. Anti-BrdU antibody (FITC) showed colocalization of newly synthesized DNA with anti-Mcm2-3-5 antibodies (TRITC) as well as presence of *Eh* Mcm proteins in the rest of the nucleus. Colocalization of *Eh* Mcm proteins with DNA was also seen in nuclei not synthesizing DNA.

Localization of Eh *Mcm proteins in serum-synchronized cells*

We synchronized *E. histolytica* cells by serum starvation followed by serum addition and analysed DNA synthesis by BrdU incorporation and multiparameter flow cytometry. Figure 4A shows the DNA synthesis profiles of cells, which were serum starved for 12 h (panel 0) and after addition of serum (panels 2 and 4). BrdU incorporating cells were demarcated as R5. As described earlier (Gangopadhyay *et al*., 1997a; Das and Lohia, 2002), we demarcated G1 cells as those cells that were not synthesizing DNA (R1) and whose DNA content was less than those of BrdU incorporating cells (Fig. 4A). Our data shows (Fig. 4A) that the genome content of *E. histolytica* cells ranged from 1X (R1), 2X (R2), 4X (R3) and 8X (R4).

It may be seen that DNA synthesis is arrested in serum-starved cells as BrdU incorporation was seen in 0.65% of the cells only (Fig. 4A, panel 0; R5). Approximately 16% of the cells show induction of DNA synthesis 2 h after the addition of serum (Fig. 4A, panel 2; R5). Four hours after the addition of serum, 70% of the cells were estimated to synthesize DNA (Fig. 4A, panel 4; R5). It is interesting that the percentage of BrdU incorporating cells (R5) whose genome content is greater than 2X (R3 and R4) is significantly higher at 4 h after serum addition than at 2 h. The generation time of *E. histolytica* cells is ≈8 h in which S-phase lasts for 5–6 h (Gangopadhyay *et al*., 1997a). This study shows that a significant population of DNA-synthesizing cells accumulates greater than 2X genome contents within a single S-phase without cell division.

We examined the localization of *Eh* Mcm2-3-5 proteins when DNA synthesis was arrested (0 h) and after DNA synthesis was initiated (2 h and 4 h). Figure 4B shows that *Eh* Mcm2 and *Eh* Mcm5 were constitutively present in the chromatin-bound detergent-insoluble nuclear pellet and in the cytoplasm while *Eh* Mcm3 was present constitutively in the chromatin-bound nuclear pellet and the detergent-soluble nucleoplasm both during serum starvation and after serum addition (Fig. 4B). The expression and localization of *Eh* Mcm2-3-5 proteins did not show any cell

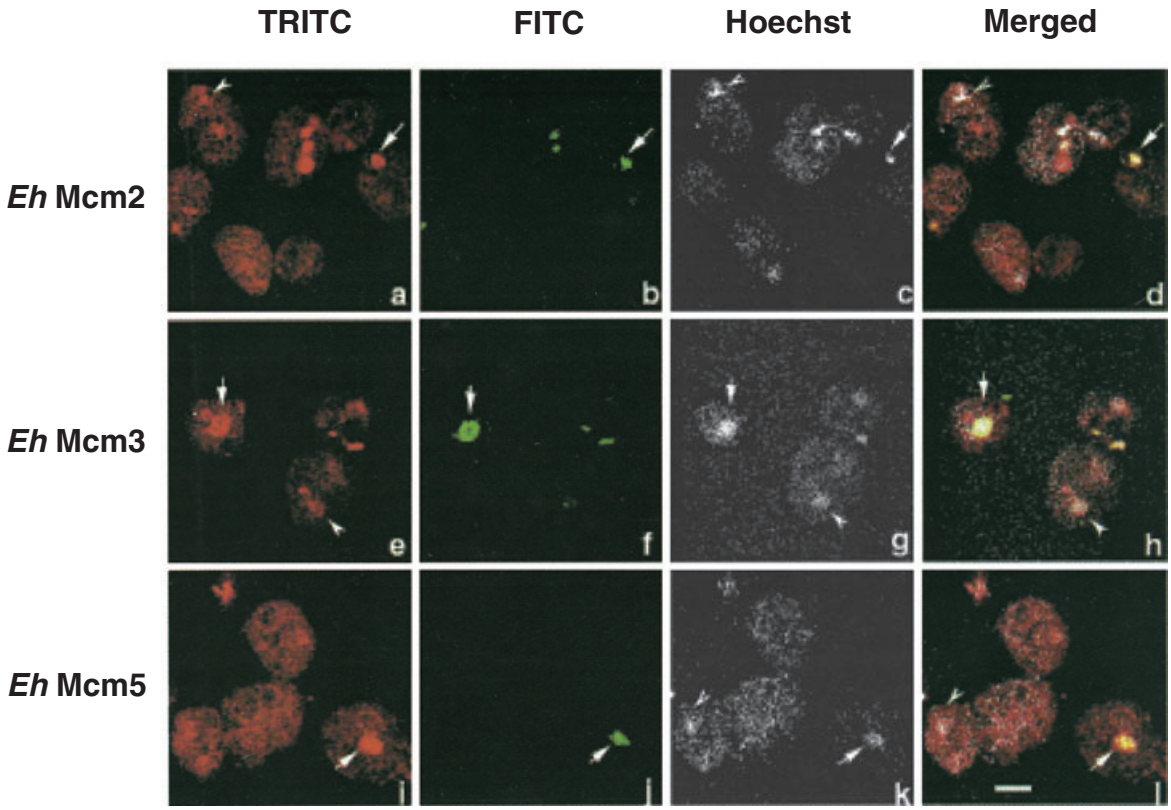

Fig. 3. Immunofluorescence localization of *E. histolytica* MCM2-3-5. *E. histolytica* cells (grown for 48 h) were incubated with 0.1 mM BrdU for 30 min and fixed. These cells were stained with FITC-conjugated anti-BrdU mouse monoclonal antibody, followed by rabbit polyclonal *Eh* Mcm2-3-5 antibodies and TRITC-labelled goat anti-rabbit antibody. DNA was stained with Hoechst 33258. (a–d) shows cells stained with *Eh* Mcm2 antibody (TRITC), anti-BrdU antibody (FITC), DNA (Hoechst) and merged images; (e–h) shows cells stained with *Eh* Mcm3 antibody (TRITC); (i–l) cells stained with *Eh* Mcm5 antibody (TRITC). Arrowheads show staining with Mcm antibodies in nuclei not synthesizing DNA while arrows show localization of Mcm proteins in nuclei which were synthesizing DNA. Bar, 10 μ.

cycle-dependant changes when analysed at 60 min intervals for 10 h after addition of serum (data not shown).

Discussion

In this study, we have cloned and analysed the *MCM2-3-5* genes from *E. histolytica*. Our data show that *Eh* Mcm2-3-5 proteins are associated with chromatin constitutively, in contrast to other organisms where an association–dissociation cycle of these proteins alternates DNA replication with mitosis. It was interesting that *Eh* Mcm2-3-5 proteins were chromatin associated during serum starvation. It is likely that inhibition of DNA synthesis after 12 h of serum starvation could result from depletion of precursors or cofactors. Association of *Eh* Mcm2-3-5 proteins possibly maintains the chromatin in a replication licensed state, so that DNA replication is initiated after addition of serum. It is possible that lack of dissociation from chromatin allows continued DNA synthesis without mitosis in *E. histolytica.* As discussed earlier, licensing of DNA replication is regulated by the ordered assembly of many proteins which form the pre-RC. At this time, it is not clear whether a pre-RC similar to that observed in yeast and higher eukaryotes is formed in *E. histolytica*. Recent data available from the genome sequencing projects of *E. histolytica* indicate that sequence homologues of *CDC6*, *CDT1*, *ORC2–7* are missing from the amoeba genome. Other genes, which have been shown to prevent over-replication of the genome in yeast, are *CDK* (Nguyen *et al.*, 2001), *CDC16*, *CDC27* (Heichman and Roberts, 1996) and *rum1* (Moreno and Nurse, 1994). We could not identify sequence homologues of *rum1* or *CDC27* in the amoeba genome but an ORF with low sequence homology to *S. cerevisiae CDC16* was present (C. Mukherjee and A. Lohia, unpubl. obs.). However, it is possible that functional orthologues of the missing genes may be present in *E. histolytica*, even though they lack sequence similarity. Indeed, homologues of Cdt1 and Rum1 show limited sequence similarity in budding yeast. Oscillation in CDK activity is another control mechanism, which regulates licensing of DNA replication (Nguyen *et al.*, 2001; Blow and Hodgson, 2002). We have earlier cloned a

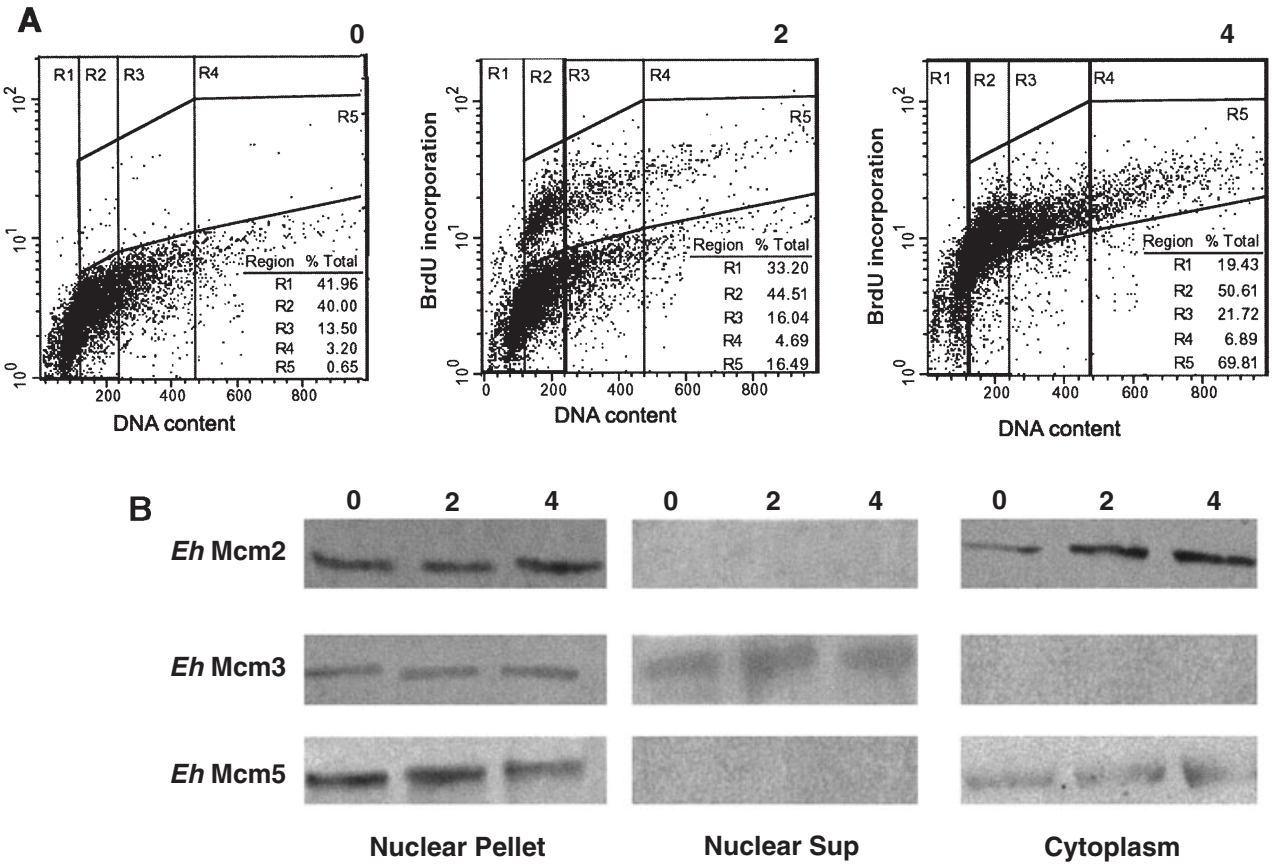

Fig. 4. Localization of *Eh* Mcm proteins in serum-synchronized cells. *E. histolytica* HM1:IMSS cells were serum starved for 12 h (0 h) after which serum was added and cells were analysed after 2 h and 4 h.
A. At 0 h, 2 h and 4 h, 0.1 mM BrdU was added for 15 min at 37°C before harvesting. Cells were then fixed and stained with anti-BrdU antibody (mouse monoclonal, at 1:40 dilution) and FITC-conjugated goat anti-mouse secondary antibody (1:100). DNA content and synthesis was estimated by multiparameter flow cytometry. Electronic gates were set for demarcating cells with 1X genome content (R1), 2X genome content (R2), 4X genome content (R3) and 8X genome content (R4). Multiple genome contents were estimated by the doubling of fluorescence values on the *x*-axis. R5 shows cells, which had incorporated BrdU as these were detected by an increase in FITC fluorescence. The percentage of cells in each of the gates was estimated and the values are shown in the inset tables of each dotplot. The added values for R1–R4 are equal to 100%, while R5 overlaps with R2–R4 and represents a subfraction of cells in gates R2–R4. Dotplots 0, 2, 4 show the DNA content and BrdU incorporation in serum-starved cells (0); 2 h after addition of serum (2); and 4 h after addition of serum (4). The *x*-axis represents the total DNA content (PI fluorescence) and the *y*-axis represents the BrdU-incorporated DNA-synthesizing cells (FITC fluorescence).
B. At the above time points, cells were harvested and fractionated to separate the cytoplasm, the detergent-soluble nuclear supernatant (Sup) and the detergent-insoluble nuclear pellets. They were run on SDS-PAGE, Western blotted and hybridized with *Eh* Mcm2, Mcm3 and Mcm5 antibodies respectively.

CDC2 homologue from *E. histolytica* (Lohia and Samuelson, 1993). Recently we have identified several other CDK homologues from the genome sequence database of *E. histolytica* and cloned a second CDK gene (C. Mukherjee and A. Lohia, unpubl. obs.). Kinase activity of the two cloned genes remained constitutively high during the cell cycle (C. Mukherjee and A. Lohia, unpubl. obs.). All these observations suggest that the controls, which ensure alternation between DNA synthesis and mitosis in other eukaryotes, are either absent or different in *E. histolytica.*

Although the function of individual replication proteins and the overall catalytic mechanism of DNA replication appear to be conserved, recent findings suggest considerable evolutionary differences in the regulation of these mechanisms across species (Kearsey and Cotterill, 2003). For example, it has been observed that mechanisms blocking chromatin association of Mcm2–7 proteins after replication initiation may vary widely in different eukaryotes (Kearsey and Cotterill, 2003). It has been observed that presence of Mcm2–7 proteins at a replication origin is sufficient to license it to fire, as origins depleted of ORC and Cdc6p are still capable of initiating DNA replication (reviewed in Bell and Dutta, 2002). If indeed ORC and Cdc6p homologues are missing from the amoeba genome, our present data show that chromatin-associated *Eh* Mcm proteins are sufficient for replication licensing.

While it is not possible yet to determine whether genome reduplication in *E. histolytica* occurs due to absence of some regulatory proteins or due to absence of mechanisms which regulate Mcm proteins in proliferating cells, *E. histolytica* presents a novel situation where the eukaryotic paradigm of cell cycle control is altered as S-phase regulation and its link to mitosis is absent. Obviously, this protozoan parasite must contain other regulatory mechanisms to ensure that its genome is maintained and transmitted with fidelity unlike organisms which exercise this control by ensuring that their genome is duplicated once and only once in each cell cycle.

Experimental procedures

Materials

BrdU, Propidium iodide (PI) and other reagents were purchased from Sigma. Anti-BrdU antibody was purchased from Becton and Dickinson. DNase I and RNase were purchased from Roche Applied Science. DNA molecular weight markers were purchased from New England Biolabs.

Cell culture and maintenance

Entamoeba histolytica HM1:IMSS trophozoites were maintained axenically in TYIS 33 (Diamond *et al*., 1978) medium at 37°C. Cells were routinely subcultured for maintenance every 72 h.

Cloning and sequencing of Eh MCM2 *and* Eh MCM5 *genes*

Segments of putative *Eh MCM2* and *Eh MCM5* genes had been identified earlier (Gangopadhyay *et al*., 1997b). These 220 bp fragments were radiolabelled and used to screen a λZAPII genomic and cDNA libraries. Positive genomic clones were sequenced by the cycle sequencing method. The 3′ end of *Eh MCM5* was confirmed by sequencing of the cDNA clone. The GenBank accession numbers are AF203971 for *Eh MCM2*, AF211951 (partial cDNA) and AY506845 (genomic clone) for *Eh MCM5*. *Eh MCM3 has* been reported earlier (Gangopadhyay *et al*., 1997b). Alignment of amino acid sequences were carried out using BLAST (Altschul *et al*., 1990).

Functional complementation of yeast mutants

The *Sc mcm2* mutant strain M46-3C (*MATα leu2-3*, *112 his3-11*, *15 ura3-52* μ*m2-1*) (Sinha *et al*., 1986) was transformed with *Eh MCM2* cloned in pACT2 vector, with *LEU2* selection and then transformed with YCplac33 (Gietz and Sugino, 1988) with *URA3* selection. Similarly *Sc mcm3* mutant strain R61-2B (*MATa leu2-3*, *112 ura3-52 his3-11*, *15* μ*m3-1*) (Gibson *et al*., 1990) was transformed with *Eh MCM3* and YCplac33. As a control, the mutant strains were transformed with pACT2 vector and YCplac33. All four transformants were streaked for single colonies on CM-leu-ura plates. Figure 1 shows two independent transformants from each strain. One colony from each transformant was inoculated in YPD broth and grown till saturation at room temperature. Single colonies from these cultures were isolated on YPD plates. Fifty colonies from each transformant were streaked as patches on CM-leu and CM-ura plates. Mitotic stability of YCplac33 at room temperature was defined as the percentage of Leu^+ cells which were also Ura^+.

Raising anti-sera to Eh *Mcm2p,* Eh *Mcm3p and* Eh *Mcm5p*

Partial coding sequences from *Eh MCM2*, *Eh MCM3* and *Eh MCM5* were subcloned in pET20b (+) and expressed in *Escherichia coli* by standard techniques. The recombinant proteins were purified and injected into rabbits. Polyclonal anti-serum was purified on a Protein A – Sepharose column. The three antibodies were then checked for cross-reactivity among the three Mcm proteins and further purified by adsorption on membranes containing the other two proteins. Non-cross-reacting anti-sera for each of the proteins was isolated and used in all experiments.

Entamoeba histolytica *cell fractionation*

Entamoeba histolytica trophozoites were lysed with 0.03% NP-40 to separate the nuclei and cytoplasm as described earlier (Banerjee and Lohia, 2003). Nuclei were washed with extraction buffer and lysed with 1% Triton X-100 at room temperature. The nuclear membrane was isolated by centrifugation at 10 000 r.p.m. for 10 min in an Eppendorf microfuge and separated from the Triton X-100-soluble nucleoplasm. All procedures were carried out in the presence of the protease inhibitor E-64 (1 mM) and PMSF (1 mM) at 4°C unless specified. The cytoplasmic extract, Triton X-100-soluble nuclear extract and Triton X-100-insoluble nuclear membrane pellet were divided into three aliquots each. One aliquot of each was treated with RNase (0.1 μg μl^{-1}) at 37°C for 30 min, while another aliquot was treated with DNase I (0.2 unit μl^{-1}) at 37°C for 30 min.

Indirect immunofluorescence by confocal microscopy

BrdU was added at a final concentration of 0.1 mM to *E. histolytica* trophozoites for 15 min at 37°C (Das and Lohia, 2002). Cells were then fixed and stained with anti-BrdU antibody (mouse monoclonal, at 1:40 dilution) and FITC-conjugated goat anti-mouse secondary antibody (1:100). These cells were subsequently hybridized with rabbit polyclonal antibodies to either *Eh MCM2*, *MCM3* or *MCM5* and TRITC-conjugated goat anti-rabbit antibodies. DNA was stained with Hoechst 33258. Cross-over correction for each of the fluorochromes was performed separately and confocal sections were collected in an Ultima Meridian microscope at CCMB, Hyderabad.

Analysis of DNA synthesis by multiparameter flow cytometry

To study DNA synthesis, BrdU (0.1 mM) was added to the cultures 15 min before harvesting at every time point (Gangopadhyay *et al*., 1997a) and fixed in 70% ethanol on ice for 15 min. For the measurement of BrdU incorporation, fixed cells were washed with phosphate-buffered saline (PBS) and treated with 1.6N HCl and pepsin (0.2 mg ml^{-1}) at 37°C for 30 min for simul-

taneous hydrolysis and denaturation of DNA. The cell suspension was then neutralized with 1M Tris-HCl, pH 8.0, to stop hydrolysis and washed again with PBS. Next, the cells were resuspended in PBS containing 0.5% Tween 20 and stained with FITC-conjugated anti-BrdU antibody (1:100 dilution) for 30 min at room temperature in the dark. Cells were subsequently washed in PBS containing 0.5% Tween 20 and treated with RNase (0.3 mg ml^{-1} for 16 h) and PI (0.5 mg ml^{-1} for 30 min) in the dark. Cells were analysed for DNA synthesis and DNA content by flow cytometry. Flow cytometric analysis was carried out using a FACSCalibur flow cytometer (Becton and Dickinson) equipped with a single laser system (6W Innova 90-6 argon ion laser). For simultaneous measurement of DNA synthesis with DNA content, cells were excited with 488 nm light and emission was measured through 525 DF20 (FITC-conjugated anti-BrdU antibody – FITC fluorescence; FL1) and 575 DF20 (for PI fluorescence; FL2). Data from 10 000 cells were recorded for each experiment and all these sets were analysed using CellQuest software. Electronic gates (R1–R4) were set to demarcate cells with 1X (R1), 2X (R2), 4X (R3) and 8X (R4) genome contents based on the PI fluorescence. Gate R5 showed cells with FITC fluorescence or BrdU incorporation. Percentage of cells in each region is given in the table inset in each dotplot (Fig. 4). It may be noted that the total value of cells add up to more than 100% as cells in R5 are overlapping with cells in R2–R4.

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Supplementary material

The following material is available from
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Fig. S1. Alignment of *Eh* Mcm2 and *Sc* Mcm2.

Fig. S2. Alignment of *Eh* Mcm5 and *Sc* Mcm5.

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