Genome wide comparative comprehensive analysis of *Plasmodium falciparum* MCM family with human host

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**Introduction**

The minichromosome maintenance (MCM) genes were first identified in the yeast *Saccharomyces cerevisiae* by a genetic screen for minichromosome maintenance-defective mutants. The mutants defective in each of these genes exhibit high rates of mitotic chromosome loss and recombination. MCM4 (CDC54) and MCM7 (CDC47) were isolated as cell cycle division mutants and MCM6, as a chromosome segregation mutant (mis5) was originally isolated in *Schizosaccharomyces pombe*. The MCM proteins are essential replication initiation factors consisting of six sequence-related subunits MCM2 to 7, which are evolutionally conserved in all eukaryotes and archaea. Because of their involvement in a fundamental cellular process, all six of these genes are essential for viability and renamed MCM2 through MCM7, the name MCM1 had already been categorized as a transcription factor and it does not belong to the MCM2-9 family. It has been suggested that MCM family in human is constituted of eight members, falling into two different groups, one constituted by the MCM2-7 complex and the other by MCM8 and MCM9, which are present only in higher eukaryotes. Each MCM protein is essential for DNA replication because deletion of any one of the six subunits results in cell death in yeast. The six eukaryotic MCM proteins share significant sequence similarity with one another, concentrating on a nearly 250- amino-acid region that encodes the ATPase active site (AAA domain). MCMs drive the formation of prereplicative complexes (PRCs) during the G1 phase of the cell cycle. It has been well established that the conversion from the cell cycle to G0 phase (quiescence) is due to the downregulation of the MCM2-7 protein complexes.

AAA or AAA+ is an abbreviation for ATPases associated with diverse cellular activities. They have a common conserved module of approximately 230–250 amino acid residues. They contain ring-shaped P-loop NTPases, which exert their activity through the energy-dependent remodeling or translocation of macromolecules. MCM family is a large and functionally diverse protein family belonging to the AAA+ superfamily. AAA proteins form ATPase active sites at clefts between two subdomains: one containing a series of parallel β-strands (P loop) and a second called the lid. Both subdomains contain conserved active-site motifs. The P loop contains motifs involved in binding ATP (Walker A box) and positioning the nucleophilic water molecule (Walker B box and sensor 1), while the lid domain contains motifs that contact the γ-phosphate of ATP (arginine finger and sensor 2). Three indispensable motifs known as Walker A or motif I, Walker B or motif II and arginine finger are characteristic of all of the 8 MCM proteins.

During the G1 phase of the cell cycle, MCM2-7 is carried to origins of replication in an inactive state by Cdt1 to form the prereplication complex (pre-RC), a well ordered assembly that contains the AAA+ proteins Cdc6 and the hetero-hexameric origin recognition complex (Orc1-6). All the six MCM subunits 2 to 7 co-localize to origins of replication during pre-RC formation. The inactivation or loss of any of the six MCM subunits during G1 phase inhibits pre-RC formation in vivo in yeast. The loading of MCM2-7 onto DNA requires ATP hydrolysis by both Orc1-6 and Cdc6. Once the loading has been achieved, Orc1-6 and Cdc6 are no more required for MCM2–7 retention at the origin and they are unnecessary for subsequent DNA replication.
Table 1. MCM family of Plasmodium falciparum

| S.No | Protein name | PlasmoDB No. | Location (chrom.No/Position) | Size in Kda (Pf/Hs) | Exon/intron | Identity with HsMCM (%) |
|------|--------------|--------------|------------------------------|---------------------|-------------|------------------------|
| 1    | MCM2         | PF14_0177    | 14/ 753,388 to 756,439       | 111/91              | 2/1         | 42                     |
| 2    | MCM3         | PE1345c      | 5/1,125,094 to 1,128,141     | 110/91              | 2/1         | 41                     |
| 3.   | MCM4         | PF13_0095    | 13/712,146 to 715,163        | 115/80              | 1/0         | 43                     |
| 4    | MCM5         | PF0580w      | 12/521,681 to 524,464        | 86/82               | 4/3         | 41                     |
| 5    | MCM6         | PF13_0291    | 13/2,183,768 to 2,186,557    | 106/92              | 1/0         | 39                     |
| 6    | MCM7         | PF07_0023    | 7/320,354 to 322,819         | 94/81               | 1/0         | 43                     |
| 7    | MCM8         | PF0560c      | 12/498,518 to 502,223        | 133/81              | 3/2         | 36                     |
| 8    | MCM9         | PF0790c      | 4/718,075 to 722,472         | 171/127             | 1/0         | 49                     |

MCM proteins are related to cell proliferation therefore they serve as useful biomarkers for cancer screening, surveillance and prognosis. The deregulation of MCM expression disrupts genetic stability and the overexpression of MCM subunits has been reported in various premalignant dysplastic lesions and cancers. It has been reported that MCM2, MCM3 and MCM7 expression are frequently deregulated in medulloblastoma (MB). MCM family of proteins was reported to be upregulated in meningiomas and it was suggested that these can be used as diagnostic markers. Significant increase in expression in meningiomas was reported for MCM2 (8-fold), MCM3 (4-fold), MCM5 (4-fold), MCM6 (3-fold), and MCM7 (5-fold). Although the roles of many proteins involved in the initiation of replication are understood, the role of MCM10 remains controversial. MCM family of proteins was reported to be expressed only in gametocytes. However presence of all the six subunits of the MCM complex in the P. falciparum genome has been reported. In the present manuscript we present a comprehensive silico analysis of MCM family of proteins from P. falciparum and their comparison with human host.

Results and Discussion

The genome of P. falciparum, available at www.plasmodb.org was investigated using ‘MCM’ as query. The results of this search presented in Table 1 showed a total of 8 hits. The PlasmoDB number for MCM29 and other details are listed in Table 1. All the 8 MCM members from this list were used to BLAST with H. sapiens and the comparative analysis are presented under different categories in the following sections. MCM proteins show considerable sequence conservation particularly in a 200 amino acid residue domain, which is located almost in the center of these large proteins. Walker A motif contains the consensus sequence [(G)xxxxGK[S/T]], where x is any residue and lysine residue is present which is characteristic of all the ATP-binding proteins. It has been observed that the Walker A motif sequence in all the MCMs is slightly deviated from the consensus and the glycines in the motif GKS/T are substituted by serine or alanine. The Walker B motif [hnhh(D/E)] has conserved nucleotide phosphate-binding motif (where h is a hydrophobic residue). These are the characteristic feature of members of the P-loop NTPase domain superfamily. The Walker A motif binds the β-γ phosphate moiety of the bound nucleotide (typically ATP or GTP) and Walker B motif binds with the Mg²⁺ cation. All the MCMs contain a Zn finger and another short motif SRFD, which is present approximately 70 residues after the Walker B motif and defines an arginine finger motif.

MCM2. It has been well established that HsMCM2 contributes to a variety of nuclear functions in addition to DNA replication. The detailed biochemical characterization of HsMCM2 showed that the C-terminal region of HsMCM2 contains ssDNA- binding activity that inhibits the DNA helicase activity. On the other hand using pulldown analysis it was reported that two fragments from the central region were mainly responsible for the interaction between HsMCM2 and HsMCM4. The gene with PlasmoDB number PF14_0177 is a homolog of human MCM2 and is located on chromosome 14. PfMCM2 protein is slightly larger in size as compared with its human
contribute to complex assembly. The arginine finger motif of MCM2 of *P. falciparum* and human are highly conserved (Fig. 1A and B). The expression of PfMCM2 predominantly in late trophozoites and during schizont maturation, concur with DNA replication in *Plasmodium*. MCM3. The phosphorylation of HsMCM3 has been well studied. It was reported that cyclin B–CDK1 catalyzes phosphorylation of HsMCM3 at Ser-112, thereby regulating HsMCM3 association with other HsMCM2–7 subunits and loading of HsMCM3 onto chromatin. In a recent study it has been reported that MCM3 is a substrate of cyclin E/Cdk2 and can be phosphorylated by cyclin E/Cdk2 at Thr-722. The HsMCM3 T722A mutant binds chromatin much less efficiently as compared with wild type HsMCM3 suggesting that homolog (Table 1). It contains additional 163 amino acids in comparison to the human counterpart. The difference in size mainly lies in the N-terminal extension (Fig. 1A and B). The sequence of all the domains and the comparison with the human counterpart shows that Walker A motif of PfMCM2 follows the consensus sequence but in human counterpart A is present instead of G (Fig. 1A and B). In motif II i.e., Walker B motif PfMCM2 and HsMCM2 obey the consensus sequence, although there is little sequence dissimilarity (Fig. 1A and B). Similar to other eukaryotic MCMs, PfMCM2 also contains a putative C4-type zinc finger domain at its N-terminal region. The zinc finger domain may have a role in the binding of PfMCMs to chromatin because these domains are known to be responsible for protein-DNA and protein-protein interactions and therefore contribute to complex assembly. The arginine finger motif of MCM2 of *P. falciparum* and human are highly conserved (Fig. 1A and B). The expression of PfMCM2 predominantly in late trophozoites and during schizont maturation, concur with DNA replication in *Plasmodium*. MCM3. The phosphorylation of HsMCM3 has been well studied. It was reported that cyclin B–CDK1 catalyzes phosphorylation of HsMCM3 at Ser-112, thereby regulating HsMCM3 association with other HsMCM2–7 subunits and loading of HsMCM3 onto chromatin. In a recent study it has been reported that MCM3 is a substrate of cyclin E/Cdk2 and can be phosphorylated by cyclin E/Cdk2 at Thr-722. The HsMCM3 T722A mutant binds chromatin much less efficiently as compared with wild type HsMCM3 suggesting that
this phosphorylation site is involved in HsMCM3 loading onto chromatin. The knockdown of HsMCM3 does not affect the S phase entry and progression, indicating that a small fraction of HsMCM3 is sufficient for normal S phase completion. These studies strongly suggest that HsMCM3 phosphorylation is a critical posttranslational modification that regulates assembly and activity of the HsMCM2-7 complex.

The gene with PlasmoDB number PFE1345c is a homolog of human MCM3 and expressed specifically in the sexual erythrocytic stages indicating that PfMCM4 may be involved in gametogenesis where DNA is replicated. It was also reported that PfMCM4 contains some unique protein-DNA interactions. In the case of Walker A motif, the consensus sequence i.e., GXXXXGK(S/T) is not followed in the protein-DNA interactions. In the case of Walker A motif, only Walker A motifs (motif I) all the other domains are identical (Fig. 1C and D).

MCM4. The importance of phosphorylation for the activity of HsMCM4 has been well studied. The phosphorylation at sites 3 and 32 of HsMCM4 required CDK2 in HeLa cells and this phosphorylated HsMCM4 had several distinct and site-specific roles in MCM function. It was reported that the central region of HsMCM2, which contains zinc finger and ATPase motif interacts with HsMCM4. In a recent study the first human mutation in MCM4 gene has been reported and it has been shown to be associated with adrenal insufficiency, short stature, and natural killer (NK) cell deficiency. In a related study it was reported that the partial HsMCM4 deficiency results in a genetic syndrome of growth retardation with adrenal insufficiency and selective NK deficiency.

The gene with PlasmoDB number PF13_0095 is a homolog of human MCM4 and is located on chromosome 13 and is larger in size as compared with its human homolog (Table 1). It contains 197 additional amino acids in comparison to human homolog. The N-terminal and C-terminal of PfMCM4 has 56 and 38 amino acid more than the human counterpart (Fig. 1E and F). The conserved motif of PfMCM4 showed differences in sequences in Walker A motif (motif I) while Walker B (motif II) and arginine finger motifs (motif III) are identical to HsMCM4 (Fig. 1E and F). PfMCM4 contains the zinc finger domain at the N-terminal region. This domain may have a role in the protein-DNA interactions. In the case of Walker A motif, the consensus sequence i.e., GXXXXGK(S/T) is not followed by both PfMCM4 and HsMCM4 (Fig. 1E and F). In a previous study it has been reported that PfMCM4 contains some unique features such as it is the largest of all the MCM4 and contains insertions at few places within its entire sequence including the zinc finger domain. It was also reported that PfMCM4 is expressed specifically in the sexual erythrocytic stages indicating that PfMCM4 may be involved in gametogenesis where DNA is replicated.

MCM5. Cyclin E has been shown to directly interact with and colocalize on centrosomes with HsMCM5 in a centrosomal localization sequence (CLS)-dependent but Cdk2-independent manner. The HsMCM5 domain responsible for interaction with cyclin E is reported to be highly conserved in MCM5 proteins from yeast to mammals. The expression of HsMCM5 or its cyclin E-interacting domain significantly inhibits over-duplication of centrosomes indicating that proteins involved in DNA replication might also regulate centrosome duplication. In a related study it was reported that cyclins E and A sequentially prevent centrosome reduplication throughout interphase by recruitment of HsMCM5 and Orc1. HsMCM-5 expression has been reported to be associated with clinicopathological parameters in gastric adenocarcinoma.

The gene with PlasmoDB number PFL0580w is a homolog of HsMCM5. PfMCM5 is almost similar in size and is located on chromosome number 12 (Table 1). The comparative analysis of their motifs showed that only Walker A motifs (motif I) are identical and other two motifs have little dissimilarity in their sequences (Fig. 1G and H).

MCM6. MCM proteins are essential for DNA replication and MCM6 is one subunit of HsMCM2-7 complex that serves as the replicative helicase in DNA replication. It is well established that Cdt1 physically interacts with the MCM complex and this interaction mainly occurs between Cdt1 and MCM6 in human cells. The detailed analysis indicated that the C-terminal 79 residues of hCdt1 interact with the C-terminal 113 residues of HsMCM6 while the large N-terminal Orc6-binding domain recruits Cdt1/MCM2-7 to ORC complex. HsMCM6 has been suggested to be a suitable marker for evaluation of the proliferation potential of craniopharyngioma. The study of expression of HsMCM6 and its correlation with recurrence of the tumor in craniopharyngiomas suggested no difference in HsMCM6 expression between the primary and recurring tumors.

The gene with PlasmoDB number PF13_0291 is a homolog of HsMCM6. PfMCM6 is larger by 108 amino acids (Table 1). The difference in size lies in the N-terminal region of PfMCM6 (Fig. 1I and J). The comparative analysis of the conserved motifs showed slight differences in their amino acid sequences in all three motifs (Fig. 1I and J). The zinc finger domain in PfMCM6 is also present at its N-terminal region and may play a role in the binding of PfMCM6 to chromatin. Walker A motifs of both MCM6 do not obey the consensus sequences (Fig. 1I and J). In a previous study the expression of PfMCM6 was observed predominantly in late trophozoites and during schizont maturation. During the trophozoite stage, the antibody against PfMCM6 pulls down PfMCM2 very inefficiently. It was also reported that only PfMCM6 is associated with the chromatin fraction at all stages of growth and PfMCM6 is also present in both the nucleosolic and cytoplasmic fractions. It might be possible that the PfMCM6 subunit makes direct contact with chromatin while other subunits are associated through protein–protein interactions.

MCM7. Recent studies suggest that in a variety of human malignancies MCM7 is amplified and overexpressed. It has been reported that dysregulation of MCM7 expression is observed in various types of cancer and it correlates with a negative outcome in patients with non-small cell lung cancer after surgical resection. Therefore MCM7 can be explored as a potential therapeutic and prognostic target in lung and other cancers.

The gene with PlasmoDB number PF07_0023 is a homolog of HsMCM7 and it is located on chromosome 7 (Table 1). PfMCM7 contains additional 102 amino acids as compared...
putative C4-type zinc finger domain at its N-terminal region. This domain probably has a role in the binding of PfMCM7 to DNA. It has been shown that during the trophozoite stage, the antibody against PfMCM6 does not pull-down PfMCM7. The expression of PfMCM7 polypeptides was predominantly observed in late trophozoites and during schizont maturation and decreased in the ring stages, which is in agreement with DNA replication in Plasmodium. It was also reported that its human counterpart. These amino acids are distributed on both the N-terminal and C-terminal sides of the protein (Fig. 2A and B). Walker A motifs (motif I) in both Pf and HsMCM7 do not agree with the consensus sequences (Fig. 2A and B). The comparison of HsMCM7 with PfMCM7 indicates that Walker B motifs (motif II) are identical but the arginine finger motif (motif III) displays variation in one amino acid (Fig. 2A and B). Similar to other MCMs, PfMCM7 contains
PfMCM7 is found in the nucleosolic and cytoplasmic fractions throughout the intraerythrocytic life cycle and increased in both fractions from ring to schizont stages.33

MCM8. MCM8 is a new evolutionarily conserved family member but its homolog is not present in yeast.48 It may function alone or with other family members in DNA metabolism. HsMCM8 mRNA accumulates during the G1/S to G2/M phase, while its protein product is detectable throughout the normal cell cycle.50 MCM8 is a crucial component of the pre-RC and the interaction between HsMCM8 and hCDC6 is required for pre-RC assembly.49 In this study MCM8 appears to participate in pre-RC formation in human cells, although this is not the case in other systems. It has been reported that MCM8, like MCM7, colocalizes on a specific DNA segment of the c-Myc replication initiation zone (c-Myc replicator) with Cdc6.50 The association of both MCM proteins with Cdc6 continues even after DNA replication is complete. It was reported that MCM8 may play a role in elongation and was strongly suggested that MCM8 functions with other known replication proteins in processes which accompany initiation of DNA replication.50

The gene with PlasmoDB number PFL0560c is a homolog of HsMCM8 and PfMCM8 is located on chromosome 12 (Table 1). There is a large difference in their size; PfMCM8 is larger by 400 amino acids in comparison to HsMCM8. The difference in size is mainly attributable to a longer N-terminal region in PfMCM8 (Fig. 2C and D). The comparative analysis of the conserved motifs showed that only arginine finger motif (motif III) is highly conserved and identical in HsMCM8 and PfMCM8 but there are some differences in Walker A (motif I) and Walker B motifs (motif II) of HsMCM8 and PfMCM8 (Fig. 2C and D).

MCM9. HsMCM9 is a novel member of MCM family.51,52 Similar to HsMCM8, HsMCM9 is only present in the genome of higher eukaryotes. It showed 24–31% total amino acid identity with HsMCM2–MCM8 proteins and contains a unique C-terminal domain which has only weak homology to MCM2-7 and MCM8 but is conserved within MCM9 homologs.53 The phylogenetic analysis on MCM family members indicated that MCM9 was most closely related to MCM8.53 Further studies reported that HsMCM9 is expressed and required for loading the HsMCM2-7 helicase onto chromatin during pre-RC formation.53

PFD0790c is the PlasmoDB number of the homolog of HsMCM9 (Table 1). PfMCM9 has additional 322 amino acids as compared with HsMCM9. PfMCM9 has extra 685 amino acid at its N-terminal while its C-terminal is shorter by 321 amino acid as compared with HsMCM9 (Fig. 2E and F). The motif comparison showed that only the arginine finger motif (motif III) is identical in HsMCM9 and PfMCM9 but there are some variations in Walker A (motif I) and Walker B (motif II) motifs of HsMCM9 and PfMCM9 (Fig. 2E and F).

To further analyze the conserved motifs in P. falciparum protein family, MEME Suite-GLAM2 version 4.8.0 was used. This analysis indicated that in P. falciparum MCM family in Walker A motif (motif I), 5 out of 11 amino acids are highly conserved and others are variable to some extent (Fig. 2G). On the other hand in Walker B motif (motif II), two out of six amino acids are highly conserved and others are slightly variable (Fig. 2G).

For structural modeling the sequence of PfMCM9 was submitted to the Swissmodel homology-modeling server (swissmodel.expasy.org/). PfMCM9 primary sequence residues 405 to 650 showed only:16.4% sequence identity to a MCM protein homolog from archael Methanobacterium thermoautotrophicum.54 The structural modeling of the PfMCM9 was therefore done using the known crystal structure of this homolog as the template (PDB number 1LTL at www.rcsb.org). The ribbon diagram of the template is shown in Figure 2I and the predicted structure of PfMCM9 is shown in Figure 2J respectively. When the modeled structure of PfMCM9 was superimposed, it is clear that these structures superimpose partially (Fig. 2K). Molecular graphic images were produced using the UCSF Chimera package (www.cgl.ucsf.edu/chimera) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).55

It is well established that post translational modification especially phosphorylation plays a very important role in regulating the function of various MCMs. The phosphorylation of MCM2, MCM3, MCM4, MCM6 and MCM7 has been observed in vivo and in vitro in different eukaryotic cells56,57 however the same is not reported in case of PfMCMs.58 Therefore the phosphorylation potential of all the HsMCMs and PfMCMs was analyzed using NetPhosK at www.cbs.dtu.dk/services/NetPhosK.58 The results of this analysis suggested that all of these proteins are prone to phosphorylation and contain the recognition sites mostly for PKC, followed by PKA, CKII and only few contain the sites for phosphorylation by PKB or cdk5 (Fig. 3). This analysis suggested that although there is a large variation in the size of PfMCMs and HsMCMs, the phosphorylation sites are almost equal in Hs and PfMCM3, MCM5, MCM6, MCM7 and MCM9 (Fig. 3b and f, d and h, i and m, j and n, l and p respectively). Furthermore it is interesting to note that PfMCM2 contains only 9 sites as opposed to HsMCM2, which contains 12 sites (Fig. 3a and e) and PfMCM4 and PfMCM8 each contain 20 phosphorylation sites as compared with HsMCM4 and HsMCM8, which contain only 14 sites each (Fig. 3c and g, k and o respectively). Overall in this manuscript, we have presented a comparative systematic analysis of MCM family from P. falciparum and human. These studies pave the way for further studies on this important family of proteins.

Materials and Methods

All the sequence data used in the analysis were downloaded from Plasmodium genome database available at www.plasmodb.org. The downloaded sequences were used as query and then matched with the human homolog using BLAST search (www.ncbi.nlm.nih.gov). The corresponding human sequences were retrieved and then their conserved domains were searched by using online CD search tool available on www.ncbi.nlm.nih.gov/Structure/
Figure 3. Phosphorylation sites in Hs and PfMCMs. The sequence of each MCM was submitted to www.cbs.dtu.dk/services/NetphosK for predictions of kinase specific eukaryotic protein phosphorylation sites. A threshold of 0.7 was used and the sites predicted for each MCM are presented in the figure. CKII: casein kinase II, PKA: protein kinase A, PKB: protein kinase B, PKC: protein kinase C.
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