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Research Article

Analysis of Mcm2–7 chromatin binding during anaphase and in the transition to quiescence in fission yeast

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ABSTRACT

Mcm2–7 proteins are generally considered to function as a heterohexameric complex, providing helicase activity for the elongation step of DNA replication. These proteins are loaded onto replication origins in M-G1 phase in a process termed licensing or pre-replicative complex formation. It is likely that Mcm2–7 proteins are loaded onto chromatin simultaneously as a pre-formed hexamer although some studies suggest that subcomplexes are recruited sequentially. To analyze this process in fission yeast, we have compared the levels and chromatin binding of Mcm2–7 proteins during the fission yeast cell cycle. Mcm subunits are present at approximately 1×10^4 molecules/cell and are bound with approximately equal stoichiometry on chromatin in G1/S phase cells. Using a single cell assay, we have correlated the timing of chromatin association of individual Mcm subunits with progression through mitosis. This showed that Mcm2, 4 and 7 associate with chromatin at about the same stage of anaphase, suggesting that licensing involves the simultaneous binding of these subunits. We also examined Mcm2–7 chromatin association when cells enter a G0-like quiescent state. Chromatin binding is lost in this transition in a process that does not require DNA replication or the selective degradation of specific subunits.

Introduction

Mcm2–7 proteins are likely to function as the replicative helicase in the elongation step of DNA replication (reviewed in [1,2]). These proteins are loaded onto chromatin at replication origins during late mitosis or G1 in a tightly regulated step termed licensing or pre-replicative complex (pre-RC) formation (reviewed in [3,4]). This involves the DNA-associated origin recognition complex (ORC) and two regulatory factors, Cdc18/Cdc6 and Cdt1. Entry into S phase is subsequently triggered by activation of CDK and Hsk1/Cdc7 kinases. During this transition, additional factors bind to origins, such as Cdc45 and GINS, and Mcm2–7 helicase is activated, allowing DNA synthesis by the replicative DNA polymerases. Pre-RCs are disassembled during S phase and Mcm2–7 proteins are displaced from chromatin, probably when converging replication forks meet. Following onset of S phase, a number of regulatory mechanisms prevent Mcm2–7 proteins from re-associating with origins, thus restricting DNA replication to a single round per cell cycle.

Mcm2–7 proteins themselves are generally thought to function as a complex, most likely as single or double heterohexameric complexes, with each heterohexamer containing one of each subunit (reviewed in [2]). In *S. cerevisiae*, analysis of degron mutants suggests that all Mcm2–7 subunits are required for the elongation step of DNA replication [5], and in *Xenopus* all the Mcm2–7 proteins are needed for licensing [6]. However, Mcm subcomplexes, such as Mcm4, 6 and 7 and

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Mcm3 and 5 can be isolated in fission yeast [7,8] and other organisms [9,10] (reviewed in [2,11]) and only the Mcm(4,6,7)$_2$ complex has been shown to have helicase activity in vitro [12,13]. In a genome wide study of Mcm3, 6 and 7 localization, all three Mcms were detected at only around 60% of binding sites [14]. It remains an open question whether Mcm2–7 subunits or subcomplexes have specific functions in vivo. Mcm7 may have specific regulatory roles as a recent study showed that this protein has a replication checkpoint signaling function that is not shared with other Mcm subunits [15]. Also, Rb specifically interacts with Mcm7 in mammalian cells, which may provide a mechanism for inhibition of DNA replication [16].

The licensing process, which loads Mcm2–7 proteins onto DNA, requires ATPase activity of ORC and Cdc6 [17,18]. ORC/Cdc6 contains six AAA+ proteins and may function in a way analogous to RF-C [19,20], which loads the ring-shaped PCNA trimer onto DNA. Thus, ORC/Cdc6 may effect topological loading of a pre-formed Mcm2–7 complex onto DNA, perhaps by transient opening of the hexameric ring, which would close around the DNA. A recent study suggests that Mcm2–7 may associate with ORC/Cdc6 via salt-sensitive interactions before being more stably loaded onto DNA by Cdc6-mediated ATP hydrolysis [17]. There is also evidence that Mcm2–7 proteins assemble onto chromatin in a stepwise fashion, which could be a manifestation of sequential association of Mcm subunits with an ORC landing pad (reviewed by [21]). In *Xenopus*, Mcm 2, 4 and 6 appear to bind before Mcm3, 5 and 7, and chromatin association of Mcm4 and 6 is not inhibited by 6-DMAP, unlike the other Mcms [22]. In human cells, Mcm2–7 proteins have been reported to bind to chromatin with different kinetics and deregulation of cyclin E inhibits chromatin association of Mcm subunits differentially, with a dramatic effect on Mcm4 [23,24]. This evidence is controversial, however, as biochemical studies in *Xenopus* indicate that Mcm subcomplexes cannot license DNA when added to chromatin sequentially, and only the complete Mcm2–7 heterohexamer can bind productively to chromatin [6].

In this paper, we have examined the timing of chromatin association of individual Mcm2–7 subunits in the fission yeast cell cycle. Since any attempt to compare the timing of chromatin binding could be affected by large differences in the relative abundance of Mcm2–7 subunits, we first compared total and chromatin bound levels of the proteins. A single cell analysis was used to correlate the timing of chromatin association with progress through mitosis and this showed that Mcm2, 4 and 7 associate with chromatin at a similar time in anaphase B, with no evidence for sequential binding. We also analyzed the chromatin binding of Mcm2–7 proteins during exit from the cell cycle to a quiescent G0-like state and showed that displacement from chromatin occurs in a process that does not require DNA replication.

Materials and methods

Yeast strains

Fission yeast strains used were constructed by standard genetic methods and are shown in Table 1. Strains were grown in rich medium (YE3S) or minimal medium (EMM) [25].

Gene tagging

Mcm3 was tagged with GFP by amplifying a genomic *mcm3* fragment using the oligos 10 (gtaccgggcccttatgcatggtctcgagggtcaaagatgcaaaggctgcgg) and 11(cattaaagcttcagcaccagcaccggctccggcaccagcaccaccccgggcaccagatctaccctcgagaatacgataaaccacattatctg), and this product was cloned into pSMUG [26] as an *Apa*I, *Hin*dIII fragment to generate pSMUG2+Mcm3. The Mcm3 insert in this fragment was subcloned into pSMRG2+ [27] as an *Apa*I, *Xho*I fragment and the resulting plasmid was integrated at the *mcm3*$^+$ locus after linearizing with *Nhe*I.

An Mcm4-CFP expressing strain was constructed by PCR amplification of an *mcm4*-encoding region with the oligos 213 (atagggcccatgctacagatatggaggtc) and 487 (tttctcgagatcagtctgtgcaattgaacgtaca). The product was cloned into pSMUC2+ [28] as an *Apa*I, *Xho*I fragment. The plasmid was integrated at the *mcm4*$^+$ locus after linearizing with *Eco*NI.

A strain expressing Mcm7-GFP was constructed by amplifying a genomic *mcm7* fragment with oligos 17 (caagtgggcccgccgctgcgaaccccttata) and 18 (cttaccccgggcattctccatatgtaaatccg), and this product was cloned into pSMUG2+ as an *Apa*I, *Sma*I fragment to generate pSMUG2+Mcm7. This was integrated into the *mcm7*$^+$ locus after integrating with *Mlu*I. Mcm7-YFP was constructed by subcloning the *Apa*I, *Sma*I Mcm7 fragment from pSMUC2+Mcm7 [28] into pSMRY2+ [29]. The plasmid was integrated into the *mcm7*$^+$ locus after

Table 1 – *S. pombe* strains used

P643	*mcm4*$^+$*-GFP: :ura4*$^+$*cdc25-22 ura4-D10*	This study
P682	*mcm4*$^+$*-GFP: :ura4*$^+$	[27]
P990	*mcm3*$^+$*-GFP: :kanMX6 leu1-32 ura4-D18*	This study
P992	*mcm2*$^+$*-GFP: :kanMX6 ade6-M210 leu1-32 ura4-D18*	[27]
P1001	*mcm6*$^+$*-GFP: :kanMX6 ade6-M210 leu1-32 ura4-D18*	[27]
P1051	*mcm2-CFP: :ura4*$^+$*ade6-M210 leu1-32 ura4-D18 h*$^-$	[28]
P1054	*mcm7-CFP: :ura4*$^+$*ade6-M210 leu1-32 ura4-D18 h*$^-$	[28]
P1057	*mcm7*$^+$*-GFP: :ura4*$^+$*ade6-M210 leu1-32 ura4-D18*	[28]
P1083	*sna41(cdc45)-YFP: :ura4*$^+$*ade6-M210 leu1-32 ura4-D18 h*$^-$	[28]
P1416	*cdc18: :TAP: :kanMX6 pat1-114 h*$^-$	D. Hermand
P1424	*cdt1-18myc pat1-114 h*$^-$	H. Nishitani
P1433	*mcm2*$^+$*-CFP: :ura4*$^+$*cdc45-YFP: :ura4*$^+$	This study
P1471	*mcm7*$^+$*-CFP: :ura4*$^+$*ars1(mlu1): : pREP3X-GFP-atb2: :LEU2 cdc25-22*	This study
P1472	*mcm2*$^+$*-CFP: :ura4 ars1(Mlu1): : pREP3X-GFP-atb2: :LEU2 cdc25-22*	This study
P1513	*mcm4*$^+$*-CFP: :ura4*$^+$*ars1(mlu1): : pREP3X-GFP-atb2: :LEU2 cdc25-22*	This study
P1525	*mcm3*$^+$*-GFP: :kanMX6*	This study
P1526	*mcm6*$^+$*-GFP: :kanMX6*	This study
P1527	*mcm7*$^+$*-GFP: :ura4*$^+$	This study
P1545	*mcm6*$^+$*-GFP: :kanMX6 cdc25-22*	This study
P1640	*mcm4*$^+$*-CFP: :ura4*$^+$*mcm7*$^+$*-YFP: :kan*$^+$*MX6 ars1 (mlu1): :pREP3X-GFP-atb2: :LEU2 cdc25-22*	This study