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MCM proteins: evolution, properties, and role in DNA replication

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Abbreviations: ARS, autonomously replicating sequence; 6-DMAP, 6-dimethylaminopurine; cdc, cell division cycle; ECB, early cell cycle box; NLS, nuclear localization sequence; MCM, mini-chromosome maintenance; mis, mini-chromosome instability; nda, nuclear division arrest; ORC, origin recognition complex; PCNA, proliferating cell nuclear antigen; pre-RC, pre-replicative complex; RP-A, replication protein A

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1. Introduction

The replication of DNA is a fundamental step in the cell cycle, which must be coordinated with cell division to ensure that the daughter cells have the same ploidy as the parental cell. The control that commits a cell to a round of DNA replication is additionally responsive to a number of signals that reflect parameters such as cell size, nutritional status, cell–cell communication and DNA damage. In eukaryotic cells, DNA replication is initiated from a large number of replication origins, but initiation events must be restricted to once per cell cycle, to avoid overreplication of parts of the genome. This control demands a low error rate, since S phase in a higher eukaryotic cell may involve tens of thousands of initiation events which occur throughout S phase. Some eukaryotic organisms can vary the number of chromosomal replication origins that are active at different stages of the life cycle. For instance, cell proliferation is rapid and S phase is short in early embryos of creatures such as frogs and flies, to facilitate rapid development of the embryo. This is achieved by usage of many more origins of replication than are used in adult cells during S phase. The controls determining the order of S phase and nuclear division can also be disrupted in certain cell types. This occurs, for example, in meiosis where two nuclear divisions occur without an intervening S phase, or in the formation of polyploid cells during the development of some organisms, where multiple S phases occur in the absence of mitosis. Replication control can also be modified on a more local level, to allow replication origins in special parts of the genome to fire repeatedly, thus providing for selective gene amplification as occurs with the chorion genes of *Drosophila*.

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been useful for identifying proteins involved in the initiation of DNA replication, either via characterization of mutants affecting S phase (such as *cdc* or *mcm* mutants), or by isolating proteins that bind to origins of replication. Replication origins have been well characterized in budding yeast, and are marked throughout the cell cycle by the binding of a complex of six proteins, the origin recognition complex (or ORC), the function of which is essential for the initiation of DNA replication [1–5] (Section 5.2). It seems likely that ORC permits the loading of other replication factors onto origin DNA. One such protein is Cdc6 (and its fission yeast homologue cdc18), which has a key role in triggering initiation, and has been shown in *Xenopus laevis* egg extracts to associate with chromatin in an ORC-dependent fashion [6]. In budding yeast, Cdc6 has been shown to be specifically associated with origin DNA in G1 phase [7] and in fission yeast overexpression of cdc18 induces multiple rounds of S phase in the absence of mitosis, suggesting that Cdc6/cdc18 is central to the control limiting DNA replication to once per cell cycle [8,9]. Another essential group of replication proteins comprises the MCM family (MCM2–7). [1] Analysis of budding yeast *mcm* mutants has shown that these proteins function in the initiation step of DNA replication and, like Cdc6, are bound to chromatin around origins of replication during G1 phase, but are subsequently displaced during S phase and remain unbound until the end of mitosis (Section 3). This periodic association is thought to ensure that replication origins are only competent to fire at the end of G1 phase and can only fire once during S phase. Chromatin binding of MCM proteins requires other initiation proteins such as ORC and Cdc6/cdc18 (Section 5.2), and overall regulation of origin firing appears to be orchestrated by the protein kinases Cdk2/cdc2 and Cdc7–Dbf4 (Section 5.3). Elongation of replication forks away

[1] To avoid confusion, a simplified MCM nomenclature [10] will be used here; Table 1 shows the correspondence with original MCM protein or gene names. Note that *MCM1*, *MCM10* and *MCM17*, which were also identified in the original MCM genetic screen [11], share no sequence similarity to *MCM2–7*.

from individual replication origins is thought to disrupt the MCM-containing complex. Thus, reinitiation is prevented by a simple *cis*-regulatory mechanism which couples replication to the inactivation of a complex that is essential for initiation. Characterization of homologues of such proteins in higher eukaryotes has shown general conservation of the replication apparatus, and it seems likely that the basic mechanism of DNA replication evolved in a common ancestor of all eukaryotic cells.

The general area of DNA replication regulation and the more specific topic of the involvement of MCM proteins in this process has been extensively reviewed in recent years [10,12–23], but in the following discussion we shall try to emphasize primary experimental work.

2. The MCM protein family

2.1. Identification and phylogenetic analysis of MCM proteins

Genes encoding MCM proteins were originally identified in budding and fission yeast as mutants affected in the progression through the cell division cycle (*cdc* [24–26], *nda* [27]) or the replication of minichromosomes (*mcm* [11], *mis* [28]). Initial characterization of three *S. cerevisiae* genes (*MCM2* [29], *MCM3* [30] and *CDC46/MCM5* [24,31]) showed that they were all implicated in DNA replication and were related in sequence. This family rapidly grew to encompass the *S. pombe cdc21*$^+$(*mcm4*$^+$) [32] and *mis5*$^+$(*mcm6*$^+$) [28] genes, and the *S. cerevisiae CDC47*(*MCM7*) gene [33]. The complete genome sequence of *S. cerevisiae* indicates that there are six MCM-encoding genes, suggesting that there are six distinct classes of MCM protein in eukaryotes (Table 1).

Identification of MCM proteins in higher eukaryotes initially came from the detection of a murine protein related to *S. cerevisiae* Cdc46/Mcm5 [24] and isolation of the human P1 protein (homologous to Mcm3) that co-purified with DNA polymerase α [62], whilst the considerable sequence conservation of the family has made it easy to identify other higher eukaryotic homologues [32,56,65,74] (Table 1). The tendency of MCM proteins to interact with each other has made it possible in some cases to co-purify and characterize proteins in the family [46,55]. Higher eukaryotic MCMs have also emerged by characterizing mRNAs or antigens that are specifically associated with proliferating cells [43,60,71,75], or by screening for mutants affecting cell proliferation during development in *Drosophila* [42] or *Arabidopsis* [72]. All eukaryotic MCM sequences obtained thus far appear to be homologous to one or other of the six *S. cerevisiae* MCM proteins, suggesting that there were six distinct *MCM* genes in a primordial eukaryote and that the family has not diversified further.

MCMs were first implicated as possible regulators of DNA replication by the observation that the *S. cerevisiae* Cdc46(Mcm5) protein accumulates in the nucleus of G1 phase cells but rapidly disappears from the nucleus upon S phase onset [31]. Similar observations were also made for Mcm2, Mcm3 [34] and Cdc47(Mcm7) [33] leading to speculation about a relationship between 'licensing factor' and MCM proteins. The idea of a licensing factor first emerged from studies showing that permeabilization of the nuclear envelope is necessary for G2 phase chromatin to regain competence for another round of DNA replication in extracts of eggs from *Xenopus* [76]. It was suggested that a pre-replicative step (termed licensing) makes chromatin competent for initiation of DNA replication. This would involve the binding of licensing factor to chromatin at the end of mitosis, permitting a single initiation event at replication origins in the subsequent interphase, after which licensing factor is inactivated. Licensing factor was postulated to be unable to cross the nuclear membrane during interphase, so that licensing of DNA normally occurs only after nuclear membrane breakdown in mitosis and DNA replication is thus restricted to a single round per cell cycle. Subsequent analysis of MCM proteins in other eukaryotes has shown that these proteins remain nuclear throughout interphase, and probably can cross the nuclear membrane during interphase (Sections 3, 5.4). Although MCMs do not show the behaviour predicted for licensing factor, they do show a cell cycle change in chromatin binding which is likely to reflect their involvement in a licensing-type reaction. An alternative model to explain the rereplication results in *Xenopus* is that an inhibitor of the licensing reaction may be unable to

Table 1
The MCM protein family

MCM class	MCM2	MCM3	MCM4	MCM5	MCM6	MCM7
S. cerevisiae						
	MCM2	*MCM3*	*CDC54*	*CDC46 / MCM5*	*MCM6*	*CDC47*
	[29,34]	[29,30,34]	[35]	[24,31,36]		[33]
	P29469	P24279	P30665	P29496	P53091	P38132
S. pombe						
	nda1$^+$ */ cdc19*$^+$	*mcm3*$^+$	*cdc21*$^+$	*nda4*$^+$	*mis5*$^+$	*mcm7*$^+$
	[27,37–39]	[32]	[26,32,40]	[27,38]	[28]	[41]
	P40377	P30666 (partial)	P29458	P41389	D31960	AJ000065 (partial)
Drosophila						
	DmMCM2		*dpa*	*DmMCM5(DmCDC46)*		
	[42]		[43]	[44,45]		
	P49735		Q26454	U83493		
Xenopus						
	XMCM2	XMCM3	XCDC21	XCDC46/XMCM5	XMCM6	XMCM7
	[46,47]	[48–51]	[52,53]	[46,47]	[46]	[46,54]
	U44047	P49739	S64720	U44048	U44050	U44051
Mouse						
	mMCM2	mP1MCM3	mCdc21	mCDC46	mMis5	mCDC47
	[55]	[56,57]	[58]	[58]	[55]	[59]
	P97310	D26089	P49717	P26090	D86726	Q61881
Human						
	BM28/hMcm2	P1Mcm3	P1cdc21/hCdc21	P1Cdc46/hMcm5	p105Mcm/hMis5	p85Mcm/P1.1-MCM3/ hCdc47
	[60,61]	[62–64]	[65,66]	[65]	[67,68]	[69]
	P49736	P25205	P33991	P33992	U46838	D55716
Other						
	Arabidopsis	*Notophthalmus* B24		*Caenorhabditis*	*Rattus*	*Arabidopsis*
	Y08301	[70]		Q21902	[71]	PROLIFERA
		I51022			Q62724	[72]
	Aspergillus	*Triturus*			*Caenorhabditis*	P43299
	[73]	Y11554			P344647	
	AF014813	*Caenorhabditis*				
		Z81039				
		Zea ROA				
		[74]				
		Q43704				

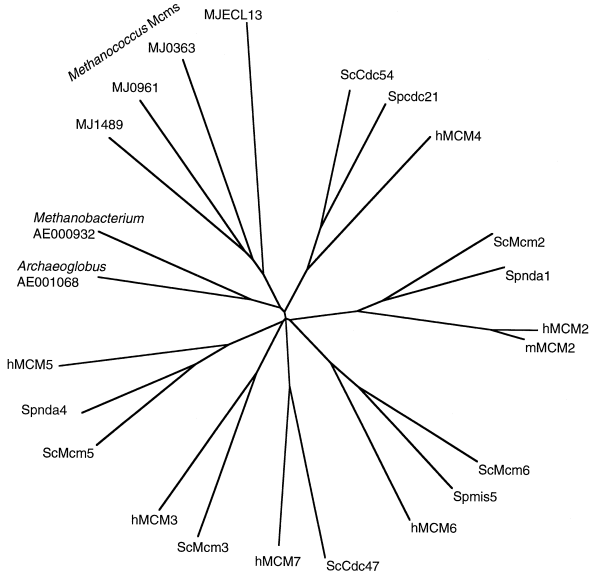


Fig. 1. Phylogenetic tree of MCM protein sequences. The tree was created from a CLUSTALW alignment, and the figure was generated using PHYLODENDRON.

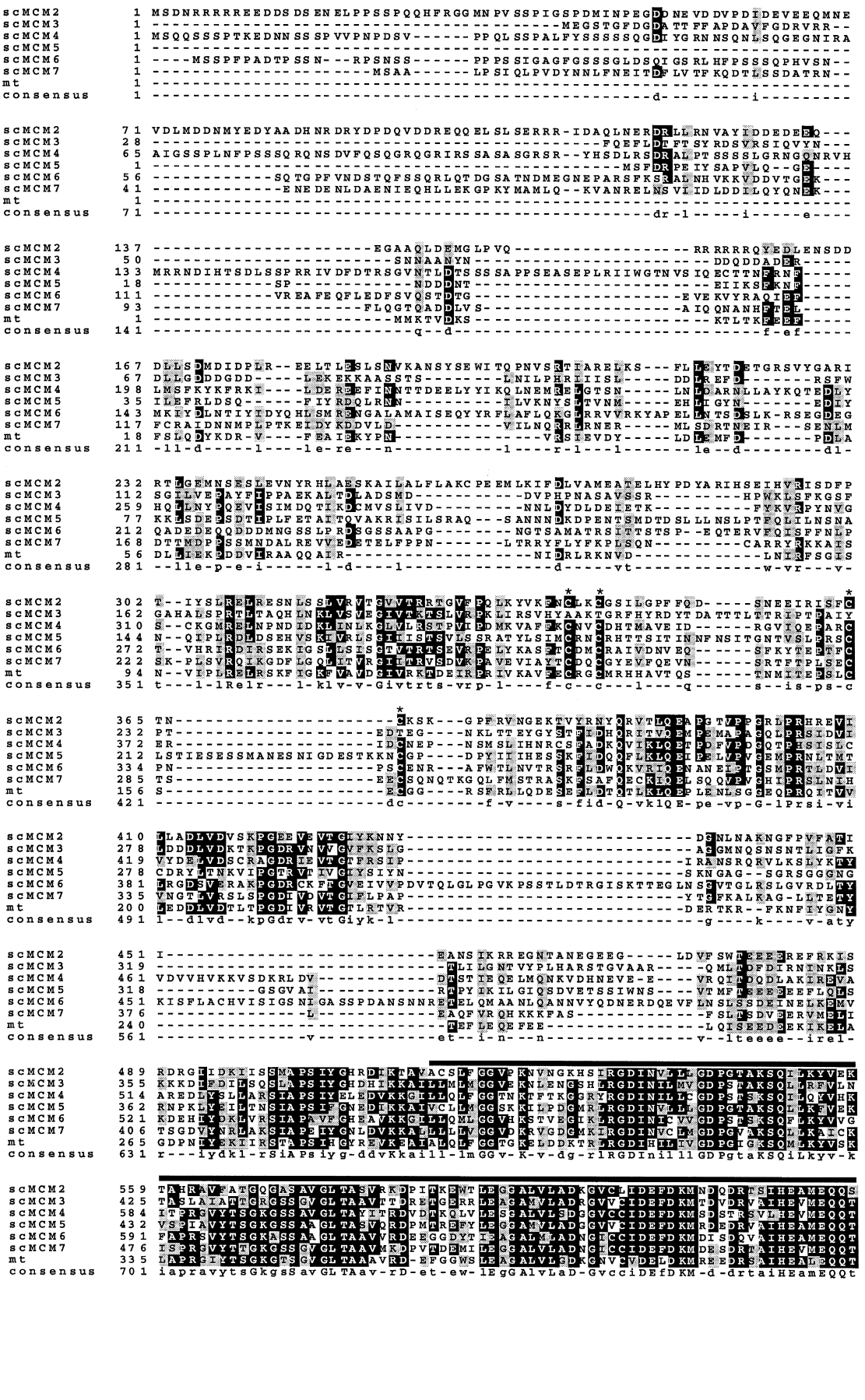
leave the nucleus before mitosis, thereby restricting DNA replication to one round per cell cycle. A good candidate for such an inhibitor would be some form of cyclin-dependent kinase activity, and there is some evidence to suggest that Cdk2/cyclin E may fulfil such a role in *Xenopus* egg extracts [77] (Section 5.3).

Phylogenetic comparison of eukaryotic MCM sequences shows that the six classes of MCM proteins are approximately equally related (Fig. 1). However, we now know that MCM proteins are not confined to eukaryotes. Although they are not found in eubacteria, sequencing of the complete genomes of a variety of Archaea has shown that MCM proteins are also found in this domain of living organisms. Archaea possess homologues of most of the key genes involved in eukaryotic DNA replication [78], implying that the eukaryotic mechanism of chromosome replication evolved in a common ancestor of eukaryotes and Archaea. Remarkably, whilst all eukaryotes appear to have six *MCM* genes, this is not true for Archaea. The *Methanococcus jannaschii* genome contains four genes that belong to the MCM family [79], all of which encode proteins that are more closely related to each other than to eukaryotic MCMs (Fig. 1). The *MCM* genes of *Methanobacterium thermoautotrophicum* [80] and *Archaeoglobus fulgidus* [81] are of exceptional interest, since both of these archaeons have just a single MCM. The archaeal MCMs form a sub-class that is marginally closer to MCM4 than the other eukaryotic MCM proteins (Fig. 1), and it is tempting to speculate that MCM4 may represent the most ancient of eukaryotic MCMs. Whilst it remains to be shown that the proteins encoded by these genes are indeed involved in DNA replication, it seems likely that these organisms may utilize a simplified version of the eukaryotic DNA replication apparatus. These organisms are thus potentially of great interest as model systems with which to study the central elements of initiation. A significant drawback is that they are obligate anaerobes requiring problematic growth conditions, but they certainly represent a challenge that may have much to contribute to our understanding of the mechanism of eukaryotic DNA replication.

2.2. Amino-acid sequence features of MCM proteins

The sequence conservation between MCM classes is mainly concentrated in a central ca. 200 amino acid

Fig. 2. Sequence conservation between *S. cerevisiae* MCM proteins. Black bars represent regions conserved between the *S. cerevisiae* MCMs and the single MCM protein of *M. thermoautotrophicum*, and grey bars represent regions conserved between yeast and mammalian MCMs of the same class. The figure was generated using sequence similarity presenter [82].



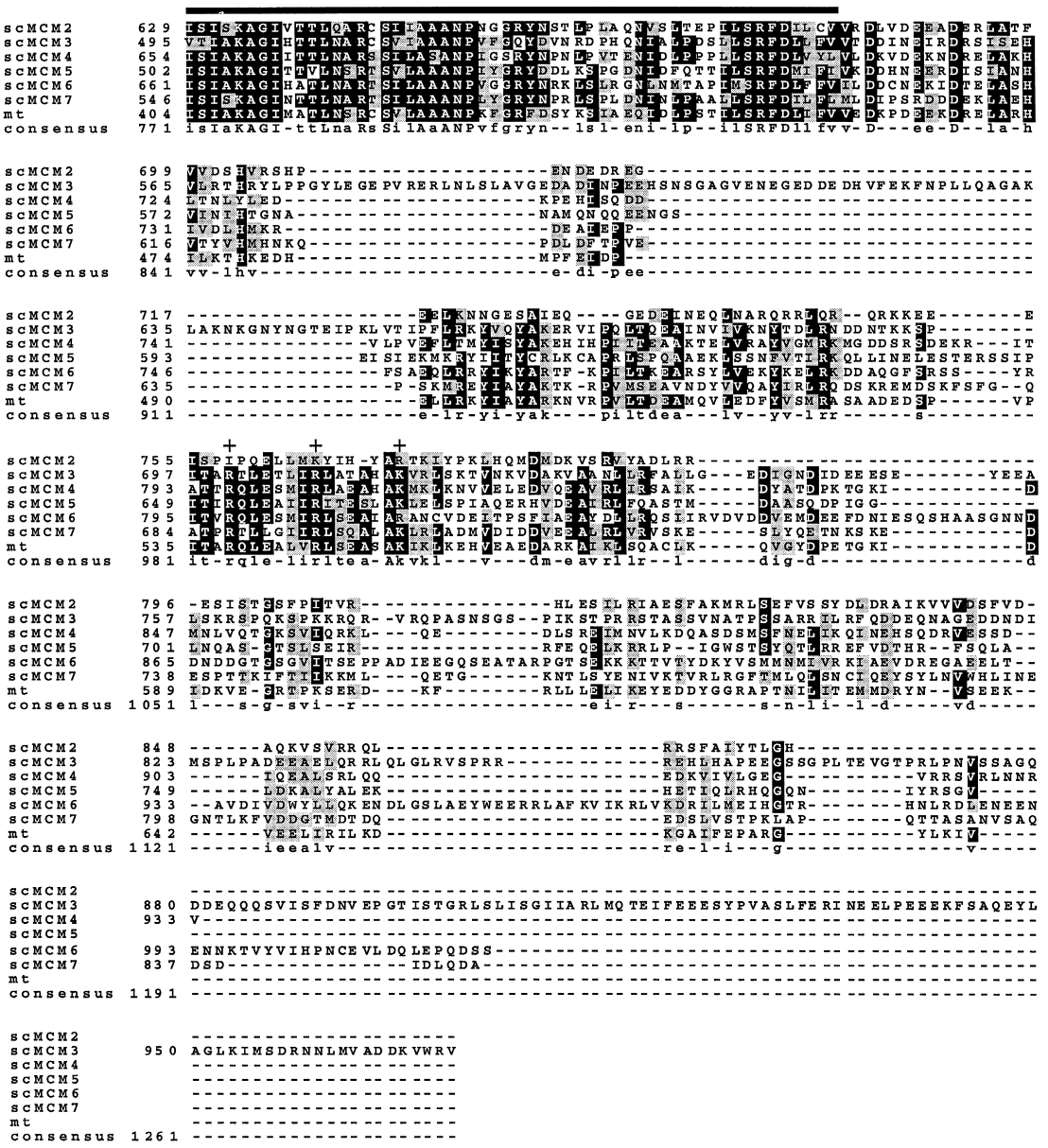


Fig. 3. Sequence conservation between *S. cerevisiae* and *M. thermoautotrophicum* MCM proteins. The figure was generated using CLUSTALW [83] and Boxshade 3.2. '*' indicates the cysteines conserved in the zinc-finger motif in MCM2, MCM4, MCM6 and MCM7 and '+' indicates conserved basic residues which occur as a heptad repeat. The thick line is above the region within the central conserved domain (or 'MCM box') that shows similarity to the NtrC family of transcription factors. In the consensus line, upper-case letters indicate residues identical in all seven proteins, while lower-case letters indicate residues conserved in a subset of the proteins.

region, while MCMs in the same class show more extensive similarity outside this region (Figs. 2 and 3). The highly conserved central region contains an element similar to the A motif of the Walker-type NTP-binding sequence, though depending on the MCM either alanine or serine replaces the second or third conserved glycine in the motif GXXGXGKS/T. This region shows moderate sequence similarity with two groups of (putative) prokaryotic ATPases [84]. These are the NtrC family of bacterial transcription factors and a set of proteins related to magnesium chelatases. The homology with NtrC transcription factors is particularly intriguing, since these proteins bind to specific promoter sites and stimulate the

formation of open complexes by the prebound σ^{54}-RNA polymerase complex (rather than stimulating polymerase binding in the conventional manner of transcription factors), and thereby facilitate the initiation of transcription [85–87]. It is tempting to imagine an analogous role for MCM proteins in the initiation step of DNA replication (Section 6).

In addition to the NTP-binding motif, four of the MCM proteins (MCM2, MCM4, MCM6, and MCM7) contain a zinc-finger-type motif of the form $CX_2CX_{18-19}CX_{2-4}C$. The functional importance of this motif has been suggested by mutagenesis studies [29] although it does not conform closely to standard DNA-binding zinc motifs. It is possible that it has a structural role or a role in mediating protein–protein interactions [88,89]. Other sequence motifs that have been noted suggest conservation of α-helical structure in the C-terminal region of the MCM proteins; these comprise a conserved heptad repeat [46] (Fig. 3) and a putative four-helix bundle [32]. Most of the MCMs show acidic regions, or alternately repeated clusters of acidic and basic amino acids, which may help to explain the anomalous migration of certain MCMs on SDS-PAGE, which is lower than would be expected from the predicted amino acid sequences [46] (Table 2).

3. Requirement for MCMs in chromosome replication

The essential requirement for MCM proteins in chromosome replication has been emphasized by a number of studies. Yeast *MCM* genes are essential and certain conditional alleles have been shown to undergo cell cycle arrest in non-permissive conditions with predominantly unreplicated DNA [24,40,73]; other *mcm* mutants show a less severe defect in S phase, but incomplete replication or a slowed rate of DNA synthesis can be demonstrated [24,26,28–30,37,40]. It is likely that MCM proteins are involved in the initiation step of DNA replication, since alleles of the budding yeast *MCM2* and *MCM3* genes show reduced efficiency in replication origin function in two dimensional gel electrophoresis [34]. Furthermore, the plasmid instability phenotype of *cdc46*(*mcm5*), *cdc47*(*mcm7*) and *cdc54*(*mcm4*) mutants can be suppressed by including multiple tandem copies of an ARS element in the plasmid, again indicating that the mutants are defective in the initiation of DNA replication [5]. The extensive DNA replication that occurs in many *mcm* mutants at the restrictive temperature may reflect the fact that Mcm proteins retain residual activity in these mutants, permitting some replication origins to function, but presumably an insufficient number for complete replication of the genome. Cell cycle arrest in *mcm* mutants depends on intact checkpoint control [29,37,40,97] suggesting that some signal is generated reflecting damaged DNA or incomplete replication. In fact, some *mcm* mutants show an incomplete block to mitotic entry [40,73] and there is evidence from *Aspergillus* that the *nimQ*(MCM2) function is required, probably via initiation of DNA replication, to trigger tyrosine phosphorylation of cdc2 and thus prevent premature entry into mitosis [73]. In mammalian cells, microinjection of anti-MCM2 antibodies [60], anti-MCM3 antibodies [56] or antisense oligomers against MCM7 mRNA [98] blocks DNA replication. In *Drosophila*, mutations in the *DmMCM2* [42] or *dpa*(*MCM4*) [43] genes inhibit cell proliferation and are lethal, with the mutants showing evidence of prolonged S phases.

In *Xenopus*, the availability of an in vitro system for DNA replication has facilitated a biochemical examination of the role of MCM proteins. When demembranated sperm nuclei are added to an interphase egg extract, MCM proteins are rapidly loaded onto chromatin, and this event precedes assembly of the nuclear envelope and the commencement of DNA replication [48–50]. Immunodepletion of MCM proteins from the extract before addition of sperm nuclei blocks DNA replication, showing that MCMs are essential for S phase in this system [49,50]. Immunodepletion using a single anti-MCM antibody can deplete all MCMs from *Xenopus* extracts, and restoration of DNA replication only occurs if all the MCMs are added back in approximately equal amounts [46,99]. Nuclei undergo a single round of replication in *Xenopus* extracts treated with cycloheximide to prevent the onset of mitosis, but can be induced to undergo a second complete round of replication by permeabilising the nuclear envelope. This allows MCM proteins to become reloaded onto chromatin [48], and permeabilized G2 phase nuclei do not replicate again when added to MCM-depleted

Table 2
Summary of MCM protein properties

	MCM2	MCM3	MCM4	MCM5	MCM6	MCM7
MW (kDa)						
S. cerevisiae calculated (observed)	99 (120)	107 (125)	105	86 (95)	113	94.8
S. pombe calculated (observed)	92.8 (115)		100 (110)	80 (80)	97	
Human calculated (observed) [68]	101 (125)	91 (105)	97 (97)	82 (90)	93 (105)	81 (85)
Human map location	3q21 [61]	6p12 [63]	8q12–q13 [68]	22q13.1–q13.2 [90]	2q14–q21 [68]	7q21.3–q22.1 [91]
pI (*S. cerevisiae)*	4.95	5.2	6.0	5.6	4.95	5.05
Abundance (molecules per cell)	*S. cerevisiae* 4×10^4 [92] *S. pombe* 3×10^3 (complexes also containing Mcm3-7) [41]	*S. cerevisiae* 2×10^5 [92]1.8×10^4 [93] Human 10^6 [64]		*S. cerevisiae* 1×10^4 [93] Human 10^5 [94]		*S. cerevisiae* 3.0×0^4 [93]
Substrate in vitro for protein kinase	Cdc7 [95,96]	Cdc7 [95,96]	cdc2 [53] Cdc7 [95]		Cdc7 [95]	

extracts [49]. MCMs are therefore required for the reestablishment of replication competence (or licensing) once S phase has been completed. It is not yet clear how many other replication proteins show similar properties, or to what extent MCM proteins have a unique role in determining replication competence of chromosomal DNA. We need to know whether other proteins such as DNA polymerases also show a periodic pattern of chromatin association similar to MCMs and, above all, need to establish the order of events in the initiation of chromosomal replication, and the dependency of the loading of each protein upon another.

Analysis of MCM protein localization during the cell cycle suggests that these proteins associate with chromatin before S phase itself and function in the initiation step of DNA replication, after which the proteins are displaced from chromatin. While initial observations in *S. cerevisiae* suggested that nuclear transport could be relevant to MCM function, subsequent analysis of MCM proteins in mammalian cells has shown that the proteins remain in the nucleus during interphase [54–56,60,63,67,100,101]. However, detergent extraction before fixation reveals two populations of MCM proteins, one which is freely extractable, the other which is bound to a nuclear structure. Since the bound population shows a similar nuclear distribution to that seen with DNA stains and can be released by DNase I digestion, the MCM proteins in this fraction are presumably bound to chromatin [55,94,101]. During the G1 phase, MCM proteins are predominantly, but not entirely, associated with chromatin since a small fraction is detergent extractable [54,94,100]. During S phase, MCM proteins become increasingly extractable and residual MCM proteins appear to be bound to regions of unreplicated DNA (such as late replicating heterochromatin) [55,56,101], suggesting that MCM proteins are displaced by ongoing DNA replication. During S phase, there is no observable colocalization of MCM proteins with replication foci or proteins involved in the DNA synthesis at the replication fork such as proliferating cell nuclear antigen (PCNA), which would have been expected if MCM proteins were involved in the elongation step of DNA replication [54,56,94,101]. From the end of S phase until mitosis, MCMs do not appear to be associated with chromatin since practically all the protein is detergent extractable. During mitosis, MCMs remain excluded from condensed chromatin and rebind in telophase, before reformation of the nuclear membrane [67,69]. This cell cycle variation in the nuclear binding of MCMs potentially provides an explanation for early cell fusion experiments, which demonstrated that G1 phase but not G2 phase nuclei could replicate in the environment of an S phase cell [102,103].

In *Xenopus*, similar results have been obtained regarding MCM-chromatin association. After mitosis, MCM4 (XCdc21) binds to chromatin before PCNA or replication protein A (RP-A), and before formation of the nuclear membrane [52]. When sites of RP-A staining appear later (termed pre-replication centres [104]) these do not colocalize with MCM4. As in mammalian cells, the chromatin displacement of MCMs occurs in S phase [46,48–52,54] and this can be prevented by aphidicolin [46,48,51,52], suggesting that the elongation step of DNA replication is required for displacement of the proteins. MCMs do not show S phase co-localization with sites of ongoing DNA replication and are rapidly displaced after the synchronous start of S phase [51,52,54], consistent with an exclusive role in initiation.

In both budding and fission yeasts, hydroxyurea can be used to block the elongation phase of DNA replication, arresting cells after early origins of replication have already fired. When temperature-sensitive *cdc46*(*mcm5*) and *cdc21*(*mcm4*) mutants are inhibited with hydroxyurea at the permissive temperature, and then released from the hydroxyurea block at the restrictive temperature for the mutant, S phase is completed and cells proceed with mitosis [26,31]. This would again suggest that MCMs function in the initiation of DNA replication, but are not required for later stages. However, the situation may not be quite so straightforward, as MCM2 function appears to be required after the hydroxyurea block in *Aspergillus* [73], and there is also some evidence that MCM proteins may in fact be associated with replication forks after initiation [105] (Section 5.2). It remains to be determined whether this does in fact reflect a role for MCM proteins after initiation.

The significance of the change in nuclear localization of budding yeast MCMs is also unclear at present. This does not seem to be related to the closed mitosis of fungi, since fission yeast MCMs show constitutive nuclear localization as in mammalian

cells [38,40]. One recent report, based on results from cellular fractionation, suggests that MCM proteins in *S. cerevisiae* may after all remain in the nucleus throughout the cell cycle as in other eukaryotes [106], though in either case the key point is that they show a change in chromatin binding through the cell cycle similar to that detected in mammalian cells [7,93,105–107].

4. Biochemical properties of MCM proteins

Insight into the function of MCM proteins has come from studies of how they interact with other replication and cell cycle control proteins. This section summarizes evidence that MCMs function together as a complex and describes biochemical or genetic data which suggest interactions with other proteins. Functional evidence for interactions between ORC, Cdc6/cdc18 and MCMs in effecting chromatin loading is summarized in Section 5.2, and Section 5.3 describes the evidence for regulation by protein kinases. A summary of all the proteins involved is given in Table 3.

4.1. Formation of complexes between different MCM proteins

It is clear from a variety of experiments in different systems that MCMs associate with one another, perhaps forming a variety of complexes with differing stoichiometries. A large MCM protein complex of around 450–600 kDa has been detected in extracts from budding yeast [92], fission yeast [41], *Drosophila* [44], *Xenopus* [46,53,99] and mammalian [55,116] cell extracts. Some studies support the simple notion that this complex represents a hexamer of MCM proteins, where each MCM type is present in equal stoichiometry. Immunoprecipitation with antibodies to a specific MCM protein in *Xenopus* extracts precipitates all six MCM proteins in approximately equal amounts [46], and similar results have been reported for HeLa cell extracts [116,117]. Glycerol gradient centrifugation of mammalian cell extracts also suggests that all six MCM proteins make up the protein complex [55,116]. In fission yeast, a 560 kDa MCM complex has been purified, containing approximately equal amounts of each of the six MCM protein types, again suggesting the proteins may form a heteromeric hexamer [41]. Ultrastructural studies of the purified complex indicate that it has a globular shape with a central cavity.

High molecular weight MCM complexes tend to be destabilized by high salt concentrations, often breaking down into a subcomplex composed of MCM3 and MCM5, and another consisting of three or four of the zinc-finger containing MCMs (MCM4, MCM6, MCM7 or MCM2, MCM4, MCM6, MCM7) [46,55,64,66,99,117]. MCM2 appears to be easily displaced in the MCM2, MCM4, MCM6, MCM7 complex [66], contributing to the variable composition of the larger complex.

Table 3
Proteins implicated in MCM function

S. cerevisiae	*S. pombe*	Vertebrate	Relationship				References
			Genetic interaction	Biochemical interaction	Kinase substrate in vitro	Required for chromatin binding	
Cdc28–Clb1–6	cdc2–cdc13, cig2, cig1	Cdc2–cyclin B			✓		[52,53]
Cdc45			✓	✓			[24,108–110]
Cdc7–Dbf4	hsk1	Cdc7	✓	✓	✓		[95,96,111]
Cdc6	cdc18	Cdc6/cdc18	✓			✓	[6,7,39,93,105]
		Histone H3		✓			[112]
Orc1	orp1	ORC1	✓	✓		✓	[16,105,113–115]
Cdc2 (Pol3)	pol3 (pol δ-ts1/ cdc6)	DNA polymerase δ large subunit	✓				[39]
Hys2	cdc1	DNA polymerase δ small subunit	✓				[39]

Other reports suggest that MCM proteins may interact to form more heterogeneous complexes, consisting of tetramers or hexamers with different composition [45,92,99]. Indeed, different MCM proteins are not equally abundant (Table 2), which is consistent with the possibility that MCMs may not be present in equal stoichiometries in complexes. Of particular interest is the observation by Ishimi [118] that a subcomplex of MCM proteins from human cells has an associated DNA helicase or strand displacement activity, a property which had already been suggested from sequence comparisons [84]. The purified MCM4, MCM6, MCM7 complex can displace an 18-mer oligonucleotide from ssDNA; on fractionation the activity is associated with fractions which have equal amounts of MCM4, MCM6 and MCM7, and no other co-purifying proteins are apparent. MCM2 protein weakly associated with this complex may possibly inhibit the activity of the helicase. Crosslinking studies suggest that a hexamer, consisting of two molecules each of MCM4, MCM6 and MCM7, may have helicase activity and this observation is intriguing given that other helicases, such as SV40 T antigen, function as hexamers. The complex requires hydrolysable ATP or dATP for activity, is stimulated by nucleic acids and either MCM4 or MCM6 can be affinity labelled with ATP. The relevance of this activity to replication remains unclear at present. The reported helicase activity is relatively weak and unprocessive, and cannot displace a 34-mer oligonucleotide. These are not the features predicted for a replicative helicase moving with replication forks. Other possible roles for such an activity exist, by analogy with current models for the initiation of transcription. For example, the DNA helicase activity associated with the transcription initiation factor TFIIH has been suggested to be required to allow the newly initiated RNA polymerase complex to 'escape' from the promoter region, without being required for subsequent elongation of transcripts. The helicase activity of TFIIH may help RNA polymerase to break away from the extensive protein–protein interactions established during initiation [119]. Perhaps a similar activity is required during the initiation of DNA replication, and MCMs could be involved in this process? Another caveat to the suggestion of MCM helicase activity is provided by the observation that the purified complex of MCMs from fission yeast, containing all six proteins in stoichiometrically equivalent amounts, has no detectable ATPase or helicase activity [41]. Whilst this may simply reflect conditions used during the purification, it is also possible that MCMs do not themselves have helicase activity, but are associated with a helicase that was present at very low amounts in the preparations isolated by Ishimi.

Another issue is whether the nature of MCM protein complexes changes during the cell cycle; for instance, do the same MCM complexes exist both on and off chromatin? In mammalian cells, soluble MCM proteins prepared either from interphase or mitotic cells occur in a ca. 600 kDa complex, perhaps consisting of one molecule of each of the six proteins [116]. In addition, the S phase fraction contains a smaller complex, probably organized as an MCM2, MCM4, MCM6, MCM7 tetramer. Solubilization of chromatin-bound MCMs using DNase I releases an MCM complex where all six MCMs can be precipitated by a single specific anti-MCM antibody, similar to results obtained with soluble (detergent-extractable) MCMs. No changes in the fission yeast 560 kDa complex were detected comparing G2 phase cells with cells arrested in G1 or S phase [41]. Similarly, in *Drosophila*, analysis of MCM complexes in single early embryos, where the cell cycles are synchronous, showed MCMs to be always present in 600 kDa complexes [45]. Thus the MCM complexes are presumably not sensitive to the cell cycle stage, since the embryos were randomly sampled and therefore in different stages of the cell cycle. Using *Xenopus* egg extracts, again no differences in multiprotein MCM complexes could be detected comparing samples taken at different stages during the cell cycle [53], and XMCM3 and XMCM7 show identical colocalization patterns on sperm chromatin [54]. Taken together, these observations suggest that putative hexameric MCM complexes may be the predominant form both on and off chromatin.

4.2. Interaction between MCMs and other proteins

Interactions have been described between MCMs and components of the origin recognition complex (ORC), which was identified by its ability to bind to the conserved ARS consensus sequence of replication origins in *S. cerevisiae* [1]. ORC binds replication

origins in vivo [120] and six components of the complex (encoded by the *ORC1–6* genes) have been identified [2–5,113,121]. Mutations in *ORC* genes cause defects in DNA replication, and reduce the efficiency of initiation at origins [2–5]. The *S. pombe* homologue of Orc1 has been shown to interact with cdc21 (MCM4) both genetically and biochemically [114], and genetic interactions have also been shown between the *CDC46*(*MCM5*) and *ORC6* genes [113]. In addition to this ORC interaction, both *CDC46*(*MCM5*) and *CDC47*(*MCM7*) were originally identified as extragenic suppressors of a mutation in the *CDC45* gene [25], which encodes another essential replication factor [24,108–110,122], and this interaction was both dominant and allele specific. This interaction is most easily explained if Cdc45 physically associates with MCM proteins and indeed the proteins have subsequently been shown to co-immunoprecipitate [108]. The role of Cdc45 in DNA replication is not known, but it also appears to interact at least genetically with ORC [109,123] (Section 5.3). MCM proteins are still loaded onto chromatin in a *cdc45* cold-sensitive mutant, suggesting that the link between Cdc45 and MCMs is not related to MCM chromatin binding, but may instead involve some other aspect of initiation or elongation [124].

In addition to interacting with ORC and other initiation factors, which would potentially allow origin-specific association with chromatin, MCMs may also associate less specifically with nucleosomes via an interaction with histones. The tetrameric MCM complex (MCM2, MCM4, MCM6, MCM7) interacts with histone H3, which could be important for stabilizing the interaction between MCM proteins and chromatin, or changing the stability of nucleosomes in the vicinity of replication origins [112]. The MCM2, MCM4, MCM6, MCM7 tetramer does not interact with histone H2A/H2B and the dimeric MCM subcomplex (MCM3, 5) appears to show no interaction with histones. The stability of the interaction between MCMs (MCM3 and MCM7) and chromatin in extracted cells has been shown to be increased in the presence of ATP or non-hydrolysable analogues of ATP [117] suggesting that ATP binding by MCMs or other chromatin proteins (e.g., components of ORC [125]) may in some way enhance the interaction with histones.

Human MCM3 was first described as a protein associated with DNA polymerase α-primase [62], although subsequent analysis failed to demonstrate a direct interaction between the two proteins [64]. However, genetic interactions have recently been documented between subunits of DNA polymerase δ and cdc19 (mcm2) in fission yeast [39].

5. Regulation of MCM proteins during the cell cycle

5.1. MCM protein levels in proliferating and non-proliferating cells

In *S. cerevisiae*, at least two of the *MCM* genes (*CDC46*(*MCM5*) [31], *CDC47*(*MCM7*) [33]) are periodically transcribed in the cell cycle, during mitosis and early G1 phase, and this control is dependent on an Mcm1-dependent promoter element (termed an early cell cycle box (ECB) element) [126]. In spite of this periodic transcription, levels of MCM proteins do not vary with the cell cycle (Mcm2 and Mcm3 [106], Cdc46(Mcm5) [31], Cdc47(Mcm7) [33]) which presumably reflects the stability of these proteins. Since transcription of the *MCM* genes is dependent on Mcm1, this potentially could explain the replication defect shown by *mcm1* mutants. However, periodic transcription of the *CDC6* gene also requires a ECB element, and since overexpression of *CDC6* can suppress the replication defect of an *mcm1* mutant it seems likely that the unstable Cdc6 protein links the replication defect to Mcm1 function [126].

Analysis of MCMs in mammalian cells presents a similar picture. mRNA levels are low in serum-starved cells and, on serum addition, peak at the late G1 phase [56,62,67,75,100]. A similar peak of late G1 phase transcription is seen in cells synchronized by mitotic or S-phase arrest suggesting that MCM transcription is periodic in cycling cells [58,63,67,69]. E2F binding sites are found in the promoter regions of a number of mammalian *MCM* genes, and a deletion analysis of the human *MCM6* gene suggests that E2F is partly responsible for promoter activity [67]. In spite of periodic transcription, levels of most MCM proteins also appear to remain constant during the mammalian cell cycle [63,66,69,75,117,127]. More direct analysis of synthesis rates and turnover of human MCM3 also indicates that this protein is relatively stable [63]. A comparison of human MCM6

protein levels with MCM2 and MCM3 levels in synchronized HeLa cells shows that, in contrast to most MCMs, MCM6 levels are periodic, peaking during the late G1 phase [67].

MCM expression has also been compared in quiescent and proliferating cells. In plants [72,74] and *Drosophila* [42], MCM mRNA levels go down dramatically in differentiated cells. MCM levels are present in high amounts during murine spermatogenesis when meiotic DNA replication is occurring, but are much reduced in pachytene spermatocytes [128]. Other studies on mammalian cells have shown that MCM mRNAs [69,71] and protein levels [69,127] are reduced in serum-starved G0 cells or in cells induced to differentiate. MCM3 and MCM5 appear to be even less abundant than other MCMs in the G0 state, although the proteins remain predominantly localized in the nucleus [127]. In this connection, antibodies against MCM proteins may be useful reagents for assessing the index of cell proliferation in tissue samples [129].

Thus de novo synthesis of MCM proteins may be important in the transition between the G0 to G1 phases, but in actively proliferating cells it seems likely that other mechanisms must be used for regulating MCM function. Consistent with this interpretation is the finding that while overexpression of individual MCM proteins in fission yeast may be deleterious, major disruption of replication control is not seen [38,40]. Both secondary modification by phosphorylation and interaction with unstable regulatory factors are probably relevant to understanding MCM function.

5.2. Involvement of ORC and Cdc6/cdc18 in binding of MCM proteins to chromatin

Purified MCM complexes do not appear to have DNA binding ability [41] and other proteins are needed to allow their interaction with chromatin. In *Xenopus*, immunodepletion of ORC blocks DNA replication [130], and prevents the association of MCM proteins with chromatin [115,131]. In contrast, depletion of MCM proteins has no effect on ORC binding [115], and treatments with protein kinase inhibitors (e.g., 6-DMAP) that prevent MCM association with chromatin have no effect on ORC binding [130,131].

Thus ORC is required for MCM binding, but since ORC remains bound to chromatin throughout interphase [107,115,130,131], this finding alone does not explain the periodic binding of MCMs. An additional factor, Cdc6/cdc18, may regulate this association. Cdc6/cdc18 is an unstable protein that is expressed in yeast during late mitosis/early G1 phase [126,132–134] and is essential for replication initiation [135–137]. Cdc6/cdc18 seems to associate physically with ORC [114,136,138], and shares sequence similarity with the largest subunit of ORC [121]. Analyzing the effects of Cdc6/cdc18 depletion in *Xenopus* extracts shows that, as with ORC, initiation of replication is inhibited, but Cdc6/cdc18 and ORC proteins show a different pattern of chromatin association [6]. Cdc6/cdc18 binds to chromatin in G1 phase but, unlike the situation with MCMs, this binding is not blocked by 6-dimethylaminopurine (6-DMAP). Additionally, Cdc6/cdc18 is displaced from chromatin during interphase in a reaction that is not prevented by aphidicolin, which does stop the displacement of most MCM proteins. Thus, Cdc6/cdc18 is apparently displaced either before replication, or during very early stages of replication which may not be inhibited by aphidicolin. Depletion of Cdc6/cdc18 does not affect ORC binding, but prevents MCM binding, suggesting that the order of chromatin association is first ORC, then Cdc6/cdc18 and finally MCMs [6].

Genomic footprinting in the vicinity of replication origins in *S. cerevisiae* demonstrates cell cycle changes which are likely to reflect the functional interactions detected in *Xenopus* extracts. During M-G1 phase, an extended region of nuclease protection is seen over replication origins [139]. This structure, termed the pre-replicative complex (pre-RC), may indicate that additional proteins have been recruited to ORC. During S phase, the pre-RC footprint changes to the standard post-replicative ORC-dependent footprint, in a reaction that requires initiation but not elongation, suggesting that pre-RCs are necessary for the initiation of DNA replication and may signal replication competence. Cdc6 is required for the formation of pre-RCs, and pre-RCs vanish in a conditional *cdc6* strain shifted to the restrictive temperature, suggesting that continued function of Cdc6 is required to maintain pre-RCs [140]. In a chromatin-binding assay for *S. cerevisiae* cells, Cdc6 has also

been shown to be necessary for binding of MCMs to chromatin, suggesting that pre-RCs may recruit MCMs [93]. Once MCMs have bound, however, continued function of Cdc6 is not required to maintain binding of MCMs to chromatin, and both ORC and Cdc6 can be extracted from chromatin in vitro by increased salt concentrations without removing MCM proteins [93].

Chromatin cross-linking studies have recently provided direct evidence that MCMs associate with replication origins in *S. cerevisiae*. Reversible chromatin crosslinking allows immunoprecipitation of DNA sequences associated with chromatin binding proteins, and the DNA enriched by this procedure can then be analyzed by PCR. In one study [7], Mcm7 was shown to be associated with chromatin in the vicinity of DNA sequences known to be functional origins, whereas no association was shown with DNA sequences only 2–4 kb away. The association was highest in G1 phase and is Cdc6 dependent, but MCMs were displaced from origin sequences in S phase and remained unassociated until telophase. At present it is not clear whether MCM binding contributes to the pre-replicative footprint observed at origins, or whether this merely reflects Cdc6 association. Overexpression of Cdc6 in cells arrested in G2/M phase by nocodozole does not lead to reformation of pre-RCs [141], though Cdc6 can be crosslinked to DNA in the vicinity of origins in these conditions [7].

A second crosslinking study of MCM chromatin association produced broadly similar results to those above. ORC and Cdc6 were shown to be important for the binding of Mcm4 and Mcm7 to origin DNA [105], consistent with the findings in *Xenopus* egg extracts. Intriguingly, it was observed that as S phase progresses, MCMs can be crosslinked to DNA further away from origins of replication, suggesting that they may associate with replication forks after initiation, and a similar conclusion was reached for Cdc45 [105]. This is difficult to reconcile with the results of MCM localization in vertebrate cells, which argues against a role after initiation, and also with the data discussed above (Section 3) showing that MCMs in at least some yeast mutants appear not to be required after a hydroxyurea arrest. Whether MCMs really do have a role after initiation remains to be clearly demonstrated, and is a key point for our understanding of the role of these proteins during chromosomal replication.

In both budding and fission yeast, MCM proteins are more abundant than ORC proteins [93] or estimates of the total number of replication origins [41,92] (Table 2). In *Xenopus*, MCMs do not appear to be limiting for replicon size in egg extracts, and each origin appears to recruit many MCM proteins [142,143]. Thus, there is the potential for large complexes of MCM proteins to form on chromatin at sites marked by ORC. Although this question has not been experimentally addressed, it is possible that MCMs may accumulate at individual origins to differing extents and this may affect the subsequent probability or timing of origin firing, and could even be relevant to the location and distribution of initiation sites in an origin region (see Section 6).

5.3. Role of phosphorylation in regulating MCM function

At least some MCM proteins are phosphorylated in a cell cycle specific manner. MCM2 [94,106], MCM3 [56,69,106], MCM4 [52,53,66] and MCM7 [127] have been shown to be phosphoproteins. MCM2, MCM3 and MCM4 proteins show a similar cell cycle pattern of phosphorylation, becoming dephosphorylated (or hypophosphorylated) on exit from mitosis, and being phosphorylated as cells enter S phase. MCM proteins bound to chromatin are hypophosphorylated compared to the displaced proteins suggesting that phosphorylation triggers or shortly precedes the displacement of MCMs, or alternatively that phosphorylation can only occur on displaced MCM proteins [53,56,66,94,116]. For at least two MCMs (MCM2 and MCM4), the proteins are phosphorylated first in S phase, but become hyperphosphorylated during mitosis [52,94] (see below).

In *Xenopus*, levels of Cdk2-cyclin E appear to be important for regulating MCM-chromatin binding [77]. Although overall levels of Cdk2-cyclin E levels are similar throughout the embryonic cell cycle, compartmentalization of the kinase in newly-formed nuclei gives rise to local concentrations around chromatin that are much higher than those in the cytosol. Low levels of Cdk2-cyclin E or cyclin A are essential to allow formation of replication-competent chromatin, apparently because MCM3 can only associate

with chromatin under these conditions, but no effect is seen on ORC binding. Once MCM3 is chromatin bound, increasing Cdk2-cyclin E (or cyclin A) does not displace the protein. Thus a window of low Cdk2 activity is essential, perhaps because Cdk2-mediated phosphorylation of MCM proteins themselves, or a necessary loading factor, such as Cdc6/cdc18, prevents MCMs from binding to chromatin (see Section 5.2). In a related study, Cdc2-cyclin B has also been shown to be an inhibitor of a component of replication licensing [143], and in *Drosophila*, mitotic degradation of cyclin A (but not cyclin B) is required for the rebinding of MCMs to chromatin [144].

In *S. cerevisiae* Cdks also seem to have a role in controlling the association of MCMs with chromatin. The ability of Mcm7 to associate with origins [7], or the ability of origins to form pre-RCs as defined by genomic footprinting [141] requires expression of Cdc6 during a period of low Cdk (Cdc28-Clb) activity. Cdk activity could in principle cause this effect by destabilising Cdc6 and thus preventing both its association with chromatin and the subsequent binding of MCMs. Overexpression of cdc18 in fission yeast perhaps overwhelms this regulatory step, accounting for the multiple rounds of replication of the genome without mitosis [8,9]. The situation in *S. cerevisiae* appears to be different, in that overexpression of Cdc6 does not cause rereplication [141]; under these conditions, Cdc6 can bind to origins during the G2 phase, i.e., after Cdk has been activated, and still pre-RCs do not form and MCMs are unable to bind [7]. Conceivably, there is some extra regulatory step in *S. cerevisiae* that prevents recruitment of MCMs to origins in the G2 phase and thus rereplication. Such a control may be abrogated by a recently reported dominant mutation of *CDC6*, which allows partial overreplication of the genome and promotes constant association of MCM proteins with chromatin through the cell cycle, despite high Cdk activity [107]. Although the details of replication control may differ in the two yeasts, it seems reasonable to infer that Cdk activity blocks the binding of MCMs to origins via an effect on Cdc6/cdc18.

The ability of Cdk activity to prevent the association of MCMs with chromatin is likely to provide an explanation for the observation that, during S and G2 phases, active kinase must be maintained to prevent additional rounds of DNA replication. Lowering cdc2 kinase activity in *S. pombe*, using mutations that affect the regulatory or catalytic subunits of the kinase [145–147], or by overexpression of the cdc2-inhibitor rum1 [148,149] can allow cells to undergo multiple S phases without going through mitosis, and related observations have been made in budding yeast [150], and *Drosophila* [151].

MCM4 may be phosphorylated by Cdk2/cdc2, since it contains consensus phosphorylation sites in the N-terminal region of the protein that are conserved amongst eukaryotes, and there is direct experimental evidence that it is a substrate in vitro. Examination of XMCM4 during the *Xenopus* cell cycle provides evidence for Cdk phosphorylation. XMCM4 is hypo- or unphosphorylated in G1 phase but chromatin-bound XMCM4 is phosphorylated in S phase before it is displaced by replication [52]. The identity of the kinase responsible for this step is uncertain, but candidates would be Cdk2-cyclin E and Cdc7–Dbf4 (see below). Later, hyperphosphorylation of the entire XMCM4 pool occurs in mitosis. This is probably brought about by the cdc2-cyclin B kinase and in vitro phosphorylation of chromatin-bound XMCM4 by cdc2-cyclin B kinase can displace this MCM (and probably the whole MCM complex) from nuclei isolated from cycling *Xenopus* extracts [53]. The significance of cdc2-mediated MCM displacement is not clear since replication alone can displace XMCM3 from chromatin, under conditions where cdc2-cyclin B activation is prevented [52]. Thus, replication would normally have displaced XMCM4 before the mitotic increase in cdc2-cyclin B activity. Perhaps this mitotic phosphorylation has a role in preventing the premature reassociation of MCM proteins with chromatin? Alternatively, this could reflect part of a programme of cdc2 phosphorylation of chromatin-bound proteins during mitosis, that causes their displacement and thus aids chromosome condensation [152].

While MCM4 is the only MCM which contains consensus cdc2 kinase sites that are conserved amongst eukaryotes, and for which in vitro phosphorylation has been shown, there is evidence that MCM2, MCM3, MCM4 and MCM6 may be phosphorylated by a distinct kinase, Cdc7. Cdc7 is required for initiation of replication, and functions in combination with a regulatory subunit, Dbf4, which may tether the kinase to ORC and stimulate the

activity of the catalytic subunit [153,154]. Cdc7 kinase is not required for MCM loading [93] and instead may trigger activation of prebound MCM complexes. Evidence for MCMs being the key targets of Cdc7 is provided by the fact that both the *CDC7* and *DBF4* genes can be deleted without causing lethality in cells carrying a particular *mcm5*(*cdc46*) mutation [111]. Although S phase may be slightly advanced in this suppressor strain, cells do not initiate S phase immediately after mitosis, showing that some other control must act somewhat redundantly with Cdc7, with regard to triggering the initiation of DNA replication. In addition, a *dbf4* allele can suppress an *mcm2-1* mutation and there is some evidence that Cdc7–Dbf4 physically interacts with Mcm2 [95]. The possibility that Cdc7 phosphorylates MCM is supported by the in vitro observations that yeast Cdc7–Dbf4 can phosphorylate Mcm2, Mcm3, Mcm4 and Mcm6 [95] and human Cdc7 expressed in COS7 cells (perhaps complexed to a homologue of Dbf4) can phosphorylate MCM2 and MCM3 [96].

There is considerable evidence that the cdc2 (in yeasts) or Cdk2 kinase is a major activator of DNA replication, which operates either independently or in the same pathway as the Cdc7–Dbf4 switch. In *S. cerevisiae*, inactivation of the Cdk (Cdc28–Clb) inhibitor Sic1 seems to be the last regulating step leading to S phase onset [155]. Similarly in fission yeast cdc2-cyclin B promotes entry into S phase [156–158]. In higher eukaryotes, rather than cdc2 fulfilling both S and M phase roles, a separate Cdk2 kinase is required for S phase entry. Immunodepletion of Cdk2 [159] or inhibition of its kinase activity by p21 inhibits DNA replication in *Xenopus* [160,161]. The key substrates of cdc2/Cdk2 for replication activation have yet to be identified, and this represents a central problem still to be addressed.

5.4. Relevance of the nuclear membrane to MCM function

The original candidature of MCMs as licensing factor has made it natural to consider the role of the nuclear membrane in their regulation. Given their constitutive nuclear localization in vertebrate and fission yeast cells, it seems unlikely that periodic access of MCMs to the nucleus is fundamentally important in all eukaryotes. However, it could still be important in embryonic situations, such as in *Xenopus* eggs, where large maternal stockpiles of replication components are present. Work in this area has produced conflicting reports. Kubota et al. [46] have reported that nuclei assembled in an XMCM3-depleted extract can only replicate when the XMCM protein complex is added back before nuclear membrane formation, since nuclear formation prevents the accumulation of XMCM3 in the nuclei. If this result reflects the failure of MCMs to bind chromatin, rather than a specific barrier to nuclear entry per se, it could be explained by accumulation of Cdk2-cyclin E in newly-forming nuclei (Section 5.3). Madine et al. [51] have shown that XMCM3 can cross the nuclear membrane but cannot bind to G2-phase chromatin unless the membrane is permeabilized. Rather than allowing access of a licensing factor, permeabilization of G2 phase-nuclei could allow release of Cdk2-cyclin E, and thus rebinding of MCMs. This explanation, though, fails to explain the report that XMCM3 can cross the nuclear membrane during interphase and still bind to G1-phase chromatin [49]; under these conditions intranuclear accumulation of Cdk2-cyclin E might be expected to prevent binding. Also, if G2 phase-nuclei are permeabilized and repaired before adding to egg extracts, they do not rereplicate, which would have been expected if replication were prevented by a diffusible inhibitor [162]. Although various possible explanations for these conflicting results can be envisaged, at present it is not possible to distinguish amongst them based on the available data.

Even if nuclear transport of MCMs is not generally relevant to their regulation, it is clear that transport of some MCM proteins into the nucleus requires formation of MCM complexes. When individually overexpressed, MCM4, MCM5, MCM6 and MCM7 are predominantly cytoplasmic in mammalian cells, in contrast to MCM2 and MCM3 which are nuclearly localized [55]. In mammalian cells, overexpression of both MCM2 and MCM6 results in nuclear localization of both proteins, and a similar result was found for the combination of MCM3 and MCM5. A nuclear localization sequence (NLS) motif is present in the MCM2 and MCM3 sequences so it seems possible that these proteins are able to transport other MCM proteins into the nucleus after formation of complexes (for instance MCM3 with MCM5, and MCM2 with MCM4,MCM6 and MCM7). In *S. cerevisiae*,

the MCM3 NLS has been identified by deletion analysis, and cells only expressing a version of MCM3 where the NLS is deleted are inviable [163].

6. Conclusions and speculations on MCM function

6.1. Model for MCM function in replication initiation

A cartoon summarizing a detailed model for the possible role of MCM proteins in the stages leading to formation of a replication fork is shown in Fig. 4. The model breaks down into two basic steps: the first involves the loading of MCM proteins onto chromatin at replication origins to make them competent for initiation; the second involves the firing of MCM-associated origins, which is brought about by the combined action of Cdc7 and Cdk kinases.

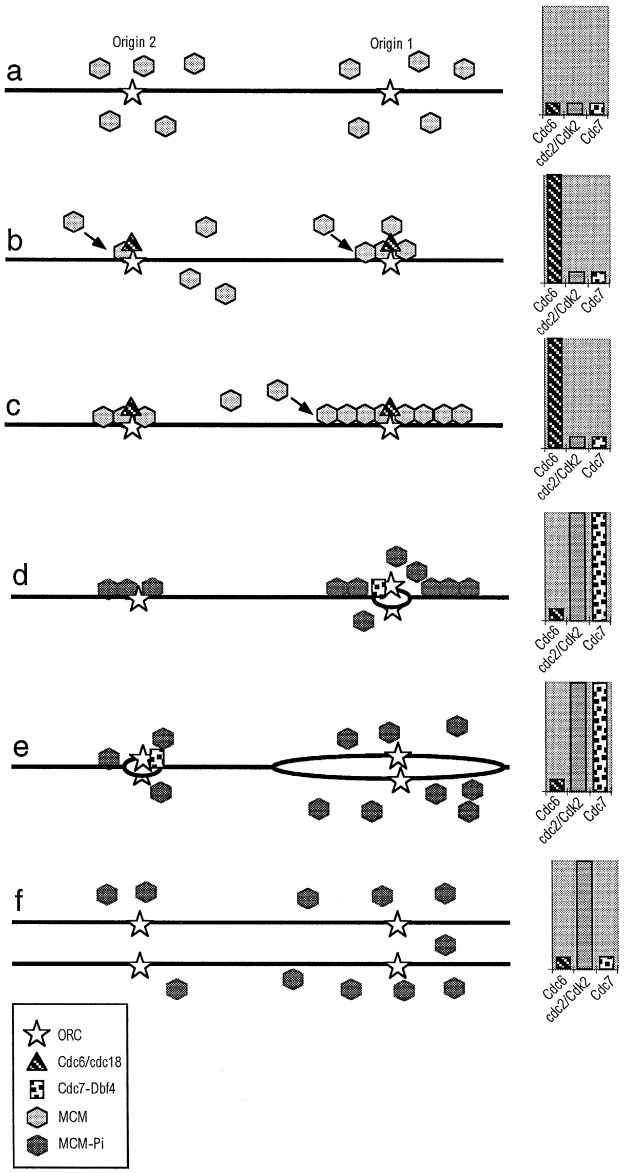
In the first step (Fig. 4b), ORC and Cdc6/cdc18 are needed to bring about the association of MCM proteins with chromatin. Cdc6/cdc18 can associate with origins in the absence of MCMs and may function to adapt origins so that MCMs can bind. In addition to the requirement for ORC and Cdc6/cdc18, Cdk2 or cdc2 (in yeasts) activity must be low. Since MCMs are considerably in excess over ORC in *S. cerevisiae*, each origin may attract a large complex of MCM proteins, which may form a larger target than the ORC alone for subsequent events leading to replication initiation. This first step corresponds to the licensing reaction in *Xenopus*, and is reflected by the formation of pre-RCs in *S. cerevisiae*.

In the second step (Fig. 4d), replication is initiated via activation of the Cdk2 (or cdc2 in yeasts) and Cdc7 protein kinases. The Cdc7 kinase associates with replication origins via its Dbf4 regulatory subunit, and its key target may be the MCM complex, given the genetic and biochemical evidence for their interaction [95,111]. In addition to Cdc7, Cdk2/cdc2 kinase activity is responsible for activating replication and also brings a halt to further binding of MCM proteins. The important substrate(s) of this kinase for replication activation are unknown, but could include ORC components [138,164], DNA polymerase α [165], Cdc6/cdc18 [166], as well as MCM proteins [52] while the negative role of Cdk2/cdc2 may be mediated through Cdc6/cdc18. In principle, phosphorylation of a key single factor could be responsible for both functions. Thus for instance, phosphorylation of Cdc6/cdc18 could activate the initiation of replication and subsequent local replication activity could displace MCMs from the origin. Phosphorylation of Cdc6/cdc18 may trigger its degradation, thus preventing rebinding of MCMs to origins and blocking reinitiation. In reality, the control could be far more involved than this basic mechanism, with the

Fig. 4. Model for MCM function in the initiation of eukaryotic DNA replication. The events shown at origin 1 present an orthodox view of the steps leading to initiation. We also speculate that origins may differ in their ability to recruit MCMs prior to replication, and this may affect for instance the timing of origin firing during S phase (compare events at origins 1 and 2), although there is no direct evidence for this aspect of the figure. (a) Chromatin with ORC bound, but otherwise in a 'ground' state, incompetent for replication. May correspond to chromatin in the G0 state in mammalian cells. (b) Increase in Cdc6/cdc18 levels permits MCM proteins (in hyposphosphorylated state) to bind to chromatin, at sites marked by ORC. (c) Later stage in G1 phase. Origin 1 is shown to be more efficient in recruiting MCM proteins than origin 2 (e.g., due to differences in local chromatin structure). (d) Start of S phase. Increase in the activity of Cdc7–Dbf4 kinase and the cdc2 (in yeasts) or Cdk2 kinase allows initiation of replication at sites on chromatin marked by MCMs. Initiation involves the association of the Cdc7–Dbf4 kinase at origins; it seems plausible that MCMs are the key substrate of Cdc7–Dbf4. The key substrate(s) of Cdc2/Cdk2 have not been identified but these could be Cdc6/cdc18, MCMs, other components of the pre-RC such as ORC, or factors involved in the elongation step of replication. As replication proceeds, MCM proteins disassociate from chromatin at origins probably as a result of elongation of replication forks, although there is one report that MCMs associate with replication forks [105]. Cdc2/Cdk2 phosphorylation also has a negative role in that it blocks reinitiation on already replicated DNA by preventing the rebinding of MCMs to chromatin. This may be via phosphorylation-induced inactivation of Cdc6/cdc18, but phosphorylation of other proteins, such as MCMs themselves, may also be relevant. Although the details of phosphorylation of individual MCMs probably differ, the MCMs are here shown to be phosphorylated shortly before displacement from chromatin. For simplicity, the activation of replication is shown to be triggered by a simultaneous rise in the activity of the two protein kinases, but in reality their activities may not be so coordinated. (e) Origin 2, marked with less MCM than origin 1, fires later in S phase. (f) G2 phase. MCM proteins have been displaced from chromatin by replication fork elongation and cannot rebind due to low Cdc6/cdc18 levels and perhaps also due to cdc2/Cdk2 phosphorylation.

phosphorylation of multiple substrates being relevant to replication activation and prevention of rereplication. Multiplicity of substrates for both activation of replication and blocking reinitiation could have the advantage of reducing the probability of inappropriate activation of replication origins, which would be expected to be very deleterious to the cell, even if occurring at a very low frequency.

Following this phosphorylation step, local unwinding of the DNA may occur in the vicinity of the origin, analogous to the formation of the 'open complex' during the initiation of transcription. This could be directly effected by MCM proteins themselves, or alternatively MCMs could use ATP hydrolysis to stimulate open complex formation by neighbouring proteins such as DNA polymerase α/primase, in a manner analogous to the action of NtrC-like transcription factors in prokaryotes (see Section 2.2). This initial unwinding would then allow the loading of other factors, and the formation of a replication fork. At present, this is a particularly obscure part of the initiation process. Origin-bound proteins may as-



sist this process by interacting with elongation factors and thus encouraging their binding, or by having a chaperonin-like function and aiding the disassembly of stable protein complexes to allow elongation components to move from the origin. During the elongation step of DNA replication MCM proteins are displaced from chromatin, and in the continuing presence of Cdk2 kinase activity, the MCMs are unable to rebind to chromatin, thus limiting DNA replication to a single round.

There are similarities between the role of MCM proteins in establishing domains of replication-competent chromatin and SIR proteins which establish a domain of chromatin that is transcriptionally repressed. Sir proteins (probably a polymer of a complex of Sir3 and Sir4) assemble onto chromatin from ORC via Sir1. The only function of ORC in this process appears to be as an assembly point for Sir1, since Sir1 that is tethered to DNA by fusing it to a DNA-binding domain can also establish a domain of silenced chromatin [167–169]; Thus the binding of Sir3 and Sir4 proteins to Sir1–ORC may be analogous to the binding of MCM proteins to Cdc6/cdc18–ORC. Perhaps the only purpose of ORC and Cdc6/cdc18 in DNA replication is to allow MCM proteins to associate with chromatin, with the key unwinding event in initiation being carried out by MCMs? In other words, ORC would be acting as a flag, marking out sites on the chromosomes at which MCMs can associate. If a large zone of bound MCMs were seeded by ORC, conceivably replication activation could occur anywhere in this zone. This interpretation might help to explain the pattern of initiation events seen in metazoan origins of DNA replication. Metazoan origins show a relatively discrete (0.5–2 kb) origin of bidirectional replication where most initiation events occur, surrounded by a larger region (6–55 kb) which in which some initiation events may occur (for review, see Ref. [170]). However, in budding yeast it has recently been shown that initiation at ARS1 occurs in a highly localized region coincident with DNase I hypersensitive sites induced by ORC, which is bound immediately adjacent, suggesting that perhaps ORC binding does indeed specify the site of initiation [171]. In higher eukaryotes we do not yet know the sequences that specify ORC binding, if such specific sequences do indeed exist, and the relatively broad zones of initiation observed at metazoan origins could reflect the fact that ORC binds at differing sites within such a zone, in different cells in a given population.

6.2. Determinants of replication origin function

In *S. cerevisiae* the sequence requirements for autonomous replication have been well characterized, and it is clear that origin function is not assured by the location in the chromosome of appropriate consensus sequences [172–174] or even by ORC binding or pre-RC formation [175]. Perhaps some sites in the genome assemble ORC, and even Cdc6/cdc18, but do not assemble MCM complexes and so do not become competent for initiation? In addition, origins are subject to other types of control. While some origins fire every S phase, others may have lower probabilities of functioning and initiation events do not occur synchronously at the start of S phase. In *S. cerevisiae*, origins seem to function early in S phase by default, and late replication seems to be a property imposed by *cis*-acting elements, such as telomeric sequences, which are distinct from genetic determinants required for origin function [176–178]. It is possible that the stepwise conversion of an ORC-associated origin to a replication-competent complex could be relevant to understanding some of these processes. Thus, for example one determinant of origin efficiency could be the ability of MCM proteins to associate with origins before S phase and a variety of factors, such as chromatin structure or the proximity of ancillary sequences which improve the efficiency of MCM binding to ORC, could affect this interaction. If the amount of MCM recruited by ORC varies at different origins, this might affect the efficiency or timing of replication activation during S phase (compare origins 1 and 2 in Fig. 4). There is no direct evidence for such a quantitative model of replication competence, but one recent observation suggests that ability of the telomere to effect late replication is imposed not during S phase but between mitosis and START, in other words when chromatin is converted into a replication-competent state [179]. Perhaps the efficiency of chromatin binding of MCM proteins at ORC-bound sites could be inhibited by the special chromatin structure propagated from the telomere, and MCM-poor origins

might be activated later in S phase than MCM-rich ones?

6.3. Future perspectives

There are clearly a large number of uncertainties connected with the involvement of MCM proteins in replication initiation and control. Details of the interactions between ORC, Cdc6/cdc18 and MCMs need to be worked out, as do the precise biochemical contributions of each of these components to initiation. Other genetically identified initiation factors, such as Cdc45 and Mcm10 [180], need to be fitted into the picture, and one very unclear area at the moment concerns how factors involved in the elongation step of DNA replication, such as DNA polymerases, associate with initiation sites. Concerning the overall regulation of replication, a fundamental uncertainty relates to the substrates of Cdc7 and Cdk2/cdc2 kinases, that must be phosphorylated for initiation to occur. In addition, the Cdk-inhibited substrates that are responsible for the block to reinitiation remain to be clearly determined. In fission yeast, perhaps cdc18 alone is inhibited by cdc2 to a significant level, since overexpression of cdc18 is sufficient to induce multiple rounds of S phase in the absence of mitosis [8,9]. Finally, as suggested in this summary, a better understanding of the biochemical events leading to initiation should help to shed light not just upon once per cell cycle replication, but also on more subtle processes that determine the timing of origin firing, and the distribution of initiation events in replication origins.

The recent discovery of archaeons with just a single *MCM* gene not only points to the antiquity of the initiation mechanism used in eukaryotic chromosomes, but also may provide a system for biochemical analysis of MCM function, stripped of some of the regulatory complexity found in eukaryotic cells. Comparing MCM function in Archaea and eukaryotes should give insight into the additional control mechanisms that eukaryotes have acquired, allowing the ordered replication of their large genomes.

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