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Cloning of *Drosophila* *MCM* homologs and analysis of their requirement during embryogenesis¹

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Abstract

MCM (minichromosome maintenance) gene family of *Saccharomyces cerevisiae* encodes essential DNA replication factors that participate in the initiation of DNA replication. In addition, their localization to the nucleus in a mitosis-dependent manner fueled the hypothesis that MCMs also act to couple DNA replication to mitosis. We report the identification of a *Drosophila* gene family with extensive sequence identity to the *MCM* genes. Results from antibody injection experiments suggest that MCMs play an essential role in DNA replication during embryogenesis. Evolutionary conservation of *MCM* sequences and function in *Drosophila* could potentially facilitate studies of how initiation of DNA replication is regulated and coupled to mitosis during metazoan development.

Keywords

Cell cycle; DNA replication; *MCM5*; *MCM4*

1. Introduction

Five *Saccharomyces cerevisiae* genes that were originally identified as cell division cycle (*CDC*) or minichromosome maintenance (*MCM*) mutations have been grouped into the *MCM* family on the basis of aa sequence similarity and their role in DNA synthesis (reviewed in Tye, 1994; Chong et al., 1996). Results from detailed analyses of *MCM* mutants suggest a role for the encoded proteins in the initiation of DNA replication. For example, *MCM5* (initially known as *CDC46*; we have followed the nomenclature of Chong et al. (1996) here) can complete its function prior to the elongation step of DNA synthesis, *MCM5* interacts genetically with a component of the origin recognition complex, *ORC6*, and mutations in *MCM2* and *MCM3* reduce origin firing in direct assays of chromosomal origin activity (Tye, 1994; Li and Herskowitz, 1993).

As might be expected for genes that participate in a fundamental process such as DNA replication, *MCMs* are conserved to other species. Multigene families with sequence similarity to *MCM* genes have been reported in *Schizosaccharomyces pombe*, *Xenopus laevis*, and mammals and these homologs appear to be involved in DNA replication. For example, mutants in *S. pombe* homologs have DNA replication defects, and a mammalian homolog of *MCM3* co-purified with DNA polymerase α (reviewed in Tye, 1994; Chong et al., 1996). Injection of antibodies against mammalian MCMs inhibited DNA synthesis, as did immunodepletion of MCMs from *Xenopus* extracts (Kimura et al., 1994; Todorov et al., 1994; Chong et al., 1995;

¹GenBank accession Nos.: *DmMCM5* cDNA, U83493; PCR1, U83491; PCR2, U83492.

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Madine et al., 1995; Kubota et al., 1995). Thus, MCMs are thought to play an important and conserved role in the initiation of DNA replication. Although origin sequences for DNA replication have been identified in *S. cerevisiae*, evidence for sequence-specific initiation of DNA replication is lacking in other organisms. Therefore, evolutionary conservation of essential proteins that function in initiation of DNA replications, such as MCMs, provides an access to mechanisms controlling initiation of DNA replication in multi-cellular eukaryotes.

We looked for MCM homologs in *Drosophila melanogaster* with four objectives: (1) to determine whether the individual members of the *MCM* family are independently conserved in *Drosophila*, (2) to test their involvement in DNA replication in another species, (3) to determine whether the pattern of nuclear localization is conserved as one might expect if these proteins have a conserved role in coupling DNA replication to mitosis, and (4) to investigate molecular events underlying developmental changes in regulation of DNA replication: these include the uncoupling of DNA synthesis from mitosis during endoreplication and chorion gene amplification as well as developmental changes in origin number and spacing (reviewed in Spradling and Orr-Weaver, 1987). We report here the identification of a *Drosophila* gene family with extensive sequence identity to the *MCM* genes. Injection of antisera against two MCM homologs into *Drosophila* embryos produced results that are consistent with a role of *Drosophila* MCM homologs in DNA replication.

2. Materials and methods

2.1. PCR

Two sets of PCR were performed using degenerate oligonucleotide primers. Sequences conserved in *S. cerevisiae* *MCM2*, *MCM3* and *MCM5* genes guided the preparation of degenerate oligonucleotide primers for the first set of PCR. The 5' primer was GGC GGA TCC GAT/C GAA/G TTT/C GAT/C AAA/G AUG. The 3' primer was GGC AGA TCT T/AGG A/GTT NGC NGC NGC (N=A, C, G or T). With the exception of restriction enzyme sites and 3 nt at the 5' end of each (underlined), 5' and 3' primers were based on the peptides DEFDKM and AAANP respectively (underlined in Fig. 1B). PCR with *Drosophila* genomic DNA as template gave a product of about 150 bp which was subcloned into pKS⁺ vector. Analysis of 97 clones showed four different sequences related to the *MCM* family (predicted aa sequences are shown in Fig. 1A).

Next, a second set of PCR was performed to obtain longer *MCM* sequences from a cDNA library (Brown and Kafatos, 1988). The 5' primer (GGC AGA TCT G/TCC AAA/G GCN GGT/C AT) was based on an internal region (AKAGI) common to all six sequences in Fig. 1A, while the 3' primer (GGC AGA TCT GGC CGC AGC ATT CGT TTT) was complementary to the junction between the poly(A) tail and the plasmid vector. The restriction enzyme sites and 3 nt at the 5' end of each primer are underlined. Details on PCR conditions are available upon request. Cloning and sequencing of a 1 kb PCR product identified an extension of 'PCR4' (Fig. 1A) towards the 3' end. The 1 kb PCR4 fragment was labeled with digoxigenin and used to screen a cDNA library from 0–4 h *Drosophila* embryos (Brown and Kafatos, 1988) using standard procedures. Four clones were obtained. Restriction analysis suggested that two of these are identical to each other and contained inserts of about 2.8 kb, while the others represent incomplete cDNAs. One of the longer inserts was subcloned into pSKII⁺ vector and sequenced on both strands. An extended open reading frame following the first AUG could encode a protein of 732 aa. The presence of a stop codon 66 bp before the first AUG suggests that this is the full reading frame.

2.2. Antibody injection

Rabbit polyclonal antibodies were raised against C-terminal portions of DmMCM5 (aa 466–732) and DmMCM4 (aa 132–866) produced in bacteria (Su et al., 1996; DmMCM5 and DmMCM4 were previously known as DmCDC46 and Dpa). Protein concentrations in injected sera were measured in a color assay using BSA as standard and were as follows, in mg/ml: DmMCM5 pre-immune, 426; DmMCM5 immune, 444; DmMCM4 pre-immune, 285; DmMCM4 immune, 311. Protein concentrations in purified antibodies (Su et al., 1996) were calculated from absorbance at 280 nm and were approx. 0.1 mg/ml. To neutralize antisera the bacterially produced DmMCM5 and DmMCM4 peptides were added to final concentrations of 0.025 and 0.020 mg/ml prior to injection. The volume of injected liquid was approx. 2% of the embryo. *Drosophila* embryos were collected for 20 min, aged for 20 min and injected according to standard procedures. Injected embryos were fixed in a two-phase mixture of heptane and 37% formaldehyde for 5 min. Fixed embryos were devitellinized manually, washed several times in PBT (PBS [Sambrook et al., 1989] with 0.2% Tween-20) and stained with 10 mg/ml bisbenzamid (Hoechst 33258) in PBT to visualize DNA. All embryos in each experiment were injected within about 5 min and approx. 3–4 min lapsed between the end of injection and delivery of embryos into fixative. Thus the time between injection and fixation ranged from about 4 to 9 min. Syncytial division cycles 3–10 average about 9 min, about 4 min of which is spent in S phase and the rest in mitosis (Foe et al., 1993). Thus defects in late anaphase to early interphase would have resulted from injection during the previous interphase (to produce anaphase bridges) or during previous interphase or mitosis (to produce interphase bridges). Embryos were assigned division cycles on the basis of nucleus number. Only those in cycle 11 (about 1560 nuclei) or earlier were scored, i.e., any chromosome bridges resulting from division 10 or earlier.

3. Results and discussion

3.1. PCR-mediated cloning of *Drosophila* MCMs

We used PCR to isolate DNA fragments with high sequence similarity to *S. cerevisiae* MCM genes (see Section 2.1 for PCR methods). Analysis of 97 PCR clones identified four different sequences, predicted aa sequences of which are shown in Fig. 1A. A cDNA clone for one of these was analyzed and found to have an open reading frame of 732 aa that has 46% identity with MCM5 (Fig. 1B) and much lower level of identity with the rest (for example, 25% identity with MCM2 and 21% with MCM3). In situ hybridization to polytene chromosomes revealed that the *Drosophila* gene, designated *DmMCM5*, is located at 86C (data not shown).

Identification of two *Drosophila* MCM homologs has been reported previously (Treisman et al., 1995; Feger et al., 1995). *DmMCM2* and *dpa/DmMCM4* show high sequence identity to MCM2 and CDC54/MCM4, respectively. While *DmMCM2* corresponds to ‘PCR3’ in Fig. 1A, *DmMCM4* does not correspond to any of the DNA fragments we isolated in our PCR screen. This result and the fact that our screen recovered some sequences only twice suggest that the *Drosophila* MCM family has more than five members. A sixth member of the *S. cerevisiae* MCM family has been recently identified (GenBank accession No. Z72723), as has the sixth member of the human MCM family (Holthoff et al., 1996), supporting this idea.

Note that individual *Drosophila* homologs have particularly high sequence similarity with individual members of the *S. cerevisiae* MCM family (e.g., *S. cerevisiae* MCM5 and *Drosophila* DmMCM5 have 46% aa identity). Similarly, MCM homologs from the fission yeast and vertebrates can be aligned with individual members of the budding yeast (reviewed in Chong et al., 1996). In contrast, the extent of aa identity among MCMs from each organism is about 20–30%. This is interesting from an evolutionary standpoint. Conservation of individual MCM family members among yeast, fly and mammals suggests, as proposed

previously, the existence of a similar gene family with each member in place prior to divergence of metazoa from single cellular eukaryotes about 500 million years ago (Chong et al., 1996; Kearsey et al., 1996). Recent identification of *MCM*-like sequences in archaeobacteria supports this idea (Bult et al., 1996). Independent conservation of individual family members suggests that each has a distinct function. Consistent with this idea, each *MCM* in fission and budding yeast, as well as DmMCM2 and DmMCM4 of *Drosophila* are essential for viability (Chong et al., 1996; Treisman et al., 1995; Feger et al., 1995).

3.2. Injection of anti-MCM antibodies disrupts early embryonic divisions

In *Drosophila* DmMCM4 and DmMCM2 mutants, defects in DNA replication were evident from mid-embryogenesis through larval development (Treisman et al., 1995; Feger et al., 1995). Early embryogenesis in these mutants proceeded normally. This could be either because *MCMs* are not required for early embryonic division cycles, or because a maternal supply of *MCM* function masks the zygotic defect until this deposit is depleted in mid-embryogenesis. We distinguished these possibilities by neutralizing maternally supplied *MCM* function and assaying for defects in embryonic divisions. We injected antisera against DmMCM5 and DmMCM4 (see Section 2.2 for injection methods) into syncytial embryos in nuclear cycles 3–10, before the onset of zygotic transcription (Edgar and Schubiger, 1986). At this stage in embryogenesis, cellularization has not taken place; nuclei share a common cytoplasm and undergo nuclear divisions synchronously. In embryos that have been injected with anti-*MCM* antisera, we observed pairs of nuclei that were connected by a ‘bridge’ of chromosomal material near the site of injection (shown for anti-DmMCM5 sera in Fig. 2A–D). The affected embryos were between late anaphase and early interphase (judging from nuclear morphology [Foe, 1989]), i.e., only nuclei that were progressing through division displayed the defect. Chromosome bridges have also been observed following injection of DNA polymerase inhibitor, aphidicolin, into pre-blastoderm *Drosophila* embryos (Raff and Glover, 1988). This is thought to arise from mitosis occurring prior to completion of DNA replication and a consequent failure to separate the incompletely replicated chromosomes. A DNA replication check-point is thought to be missing at this stage in development of *Drosophila* as in other species (Foe et al., 1993). Moreover, chromosome bridging is specific to aphidicolin and is not seen with inhibitors of transcription, protein synthesis and microtubule dynamics (Schubiger and Edgar, 1994). Observation of chromosome bridges after antiserum injection suggests that anti-MCM antibodies were naturalizing a process similar to the aphidicolin-sensitive event, namely, DNA synthesis.

3.3. The specificity of the effect of antibodies

Injection of buffer or pre-immune serum as controls resulted in a low background of bridges (< 1% of nuclei per embryo). In contrast, embryos injected with immune sera against DmMCM5 or DmMCM4 showed an average of about 20% of nuclei in bridges per embryo (for example, Fig. 2C), although this number reached as high as 50% in some embryos. Nearly all of the bridges occurred at or near the site of injection, suggesting that the effect of the antisera was limited to a fraction of the nuclei due to limitations on diffusion. For comparison among samples, the number of embryos showing $\geq 5\%$ of nuclei in bridges per embryo, i.e., five or more times the background, was expressed as a percentage of the total number of injected embryos for each sample (shown for DmMCM5 in Fig. 3). Note that the injection of immune serum increased the percentage of affected embryos by approximately 6-fold over the pre-immune control, to about 30% of total embryos. Because we detected chromosome bridges only in embryos in late anaphase to early interphase (see Section 3.2) and the average combined length of anaphase and telophase is about 15% of each division cycle (Foe et al., 1993), 30% may represent the maximum fraction of embryos (those in late anaphase, in telophase and in the early part of interphase) that could show the effect of antiserum injection. This effect was substantially reduced by co-injection of the DmMCM5 peptide (Fig. 3) but not the heterologous

peptide (DmMCM4; data not shown). Similar results were obtained with anti-DmMCM4 antisera as well as with affinity purified antibodies against both DmMCM5 and DmMCM4 (data not shown). Antiserum injections did not detectably reduce incorporation of a co-injected nt analog, BrdU (data not shown). However, a failure to replicate even a small percentage of the genome, while undetectable by BrdU incorporation, may prevent resolution of sister chromosomes and result in chromosome bridging. We conclude that antibody injection interferes with DmMCM5 and DmMCM4 functions and produces a phenotype consistent with a partial interference with DNA replication. Similarly, injection of antibodies against mammalian MCMs has been shown to impair DNA replication and cell division in cultured cells (Kimura et al., 1994; Todorov et al., 1994).

4. Conclusions

We report the existence of a *MCM* gene family in *Drosophila*. Sequence analysis of one *Drosophila MCM* indicates that evolutionary conservation of individual *MCM* genes extends to *Drosophila* as well. Previous analyses of *Drosophila MCM* mutants documented DNA replication defects in late embryogenesis (Treisman et al., 1995; Feger et al., 1995). *dpa/MCM4* mutants also showed severely reduced DNA synthesis in the CNS of the larvae (Feger et al., 1995). Antibody injection data presented here complement these analyses by uncovering a requirement for MCMs at an earlier time in development. On the basis of these results we suggest that MCMs function in DNA replication at all stages of *Drosophila* development.

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Abbreviations

aa	amino acid(s)
bp	base pair(s)
CDC	cell division cycle
CNS	central nervous system
<i>dpa</i>	<i>disc proliferation absent</i>
kb	kilobase(s) or 1000 bp
<i>MCM</i>	minichromosome maintenance
nt	nucleotide(s)
PCR	polymerase chain reaction

A

PCR1	ADQDRTAIHEVMEQQTISIAKAGIMTTLNARVSIL
PCR2	SDIDRTAIHEVMEQGRVTISKAGIHASLNARCSVL
PCR3	NDQDRTSIHEAMEQQSISISISKAGIVTSLQARCTVI
PCR4	REDDRVAIHEAMEQQTISIAKAGITTTLNSRCSVL
MCM2	NDQDRTSIHEAMEQQSISISISKAGIVTTLQARCSII
MCM3	TDVDRVAIHEVMEQQTVTIAKAGIHTTLNARCSVI
MCM5	RDEDRVAIHEAMEQQTISIAKAGITTVLNSRTSVL
consensus	-d-DRtaIHEaMEQQtisIa <u>KAGI</u> -ttLnaRcsvl

B

ScMCM5	M-SFDRPEIY SAPVLQESP NDDNT--EI IKSRKFILE FRLDSQFT--	45
DmMCM5	MEGFDDAGVF FSDNFGSDNQ QDAQINLQAV KKVKKEFIRT FN-EEMFVK	49
ScMCM5	YRDLLRNILL VKMYSLTVNM EALLGANEI YKKLSDEPSD IIPLFETAIT	95
DmMCM5	YRDLLKRNKL NGRYFLEIEM EDLVGFDETL -ADTDKQTE HLEIFEAAAR	98
ScMCM5	QVAKRTSILS RAQSANNNDK DPENTSMDDT SLLLSLPTF QLIINSNANQ	145
DmMCM5	EVADelta-P R-----PEHE-----EHMHDI QILSSNANP	128
ScMCM5	IPIRDLSEH VSKIVRLSGI IKTSVLSSR ATVLISICRN CRHTTSITIN	195
DmMCM5	TNIRDLSDC VSKLVKIAGI IVAASGISAK ATRMSIICLS C---SEVLP	174
ScMCM5	NFNSITG-NT VSLPRCLST IESESSMANE SNIGDESTKK NCBPPVITF	244
DmMCM5	NLKVNFLEG YALPRKC-NT -----EQAGRPF KCPLDPFFIM	209
ScMCM5	HESKFDLQ FLKLQELPEL VPGGEPRAL TMTCDRLTN KVLPGNIVIT	294
DmMCM5	PDKCKLVDFQ FLKLQELPDF VPGGEPRAL QLFCDRELCE RVMPGNIVIT	259
ScMCM5	VGIYSIYNSK NGAGSERGG GNGSGVAIR APYKILGID SDVETSSIWN	344
DmMCM5	QGIYSI--RK VCKFSRR--D GREKAVVGR APYMRVVGII -TVDSEGV-G	302
ScMCM5	SVTMF---TE EEEEFQLS RNPKLVELLT NSLAPSIFGN EDIKKAIIVCL	391
DmMCM5	AISRYSNITS DEEHLRMA ASGDIYERLS QSLAPSIFGS RDIKKAIICM	352
ScMCM5	LMGSSKKLP DGMRLRGDIN VLLLGDPGTA KSQLLKFVEK VPIAVYTSG	441
DmMCM5	LFGVSRKLP DGLCRRGDIN VLLLGDPGTA KSQLLKFVEK VPIAVYTSG	402
ScMCM5	KGSSAAGLTA SVDRPMTRE FVLEGGAMVL ADGGVVCIDE FDKMRDORV	491
DmMCM5	KGSSAAGLTA SVMKOPATRN FVVEGGAMVL ADGGVVCIDE FDKMRDORV	452
ScMCM5	AIHEAMEQQT ISIAKAGITT MLNSRISVLA AANHIGRVD DLKSPGNID	541
DmMCM5	AIHEAMEQQT ISIAKAGITT MLNSRISVLA AANHIGRVD DLKSPGNID	501
ScMCM5	FDITILSRFD MIFIVKDDIN EERDISIANI VINHTGNAN AMQNQQENG	591
DmMCM5	FMTILSRFD MIFIVKDDHD EERDITLAKH IINHLSSNK SAPSEPNE--	549
ScMCM5	SEISIEKMR VINYRLKCA FRLSPQAEK LSSNFVIRK QLLINELEST	641
DmMCM5	GEISLSTFKK VINYCRTHCG FRLSEAAEK LKRYVLMRE GAGQCEKASD	599
ScMCM5	ERESIPITR QLEAIRIRIE SLAKLELSPF ADEHVDEAL RLFQNSTDA	691
DmMCM5	KRLSIPITR QLEAVIRISE SLAKIRLDPF ADEHVDEAL RLFQNSTDA	649
ScMCM5	ABQDPICGLN Q-ASGTSLSE IRRFEELKR RLPFGWSTGY QTLRREFVDT	740
DmMCM5	AMTGLAGAE GFTTEEDQET LNRTEKQKR RFAIGSQVSE QNILQDEL-R	698
ScMCM5	HRFSQLALDK ALYALEKHET IQLRHSQNI YRSGV	745
DmMCM5	QKYEERTVMK VIHTMIRRG E LQRMORKML YRIC	732

Fig. 1.

Predicted aa sequences of *Drosophila* MCM homologs. (A) The aa encoded by four *Drosophila* genomic fragments (PCR1–4), along with the corresponding regions in the three budding yeast proteins, are shown. ‘PCR4’ corresponds to DmMCM5 and ‘PCR3’ to DmMCM2 (Treisman et al., 1995). The aa corresponding to primers for the second set of PCR (see Section 2.1) are underlined. A consensus for the region is shown; aa conserved in all seven sequences are capitalized while those occurring in four or more are shown in lower case. (B) Alignment of DmMCM5 and MCM5 using GeneWorks software. Identical residues are boxed. Regions corresponding to primers for the first set of PCR (see Section 2.1) are underlined. The region between the primers corresponds to that shown in A.

Fig. 2.

Injection of embryos with antisera against DmMCM5 and DmMCM4 prevents complete separation of chromosomes at mitosis. Embryos were injected either with pre-immune serum (**A** and **B**) or with immune serum (**C** and **D**) against DmMCM5. Injected embryos were fixed and stained for DNA with Hoechst 33258. The site of injection is indicated by an asterisk in **A** and **C**. Telophase nuclei in **A** showed no discernible defects following injection of pre-immune serum. In an embryo injected with anti-DmMCM5 antibody (**C**) nuclei, although as far apart as in **A**, were still connected by 'chromosome bridges' (brackets). Nuclei near the site of injection were more affected than those further away. **B** and **D** are higher magnifications of **A** and **C** respectively.

Fig. 3.

Quantitation of anti-DmMCM5 serum injection data. The number of embryos with $\geq 5\%$ of nuclei connected by chromosome bridges per embryo is expressed as a percentage of total injected embryos for each sample. *n* is the total number of embryos injected in two experiments.