Abstract

INTERACTION AND FUNCTIONAL ANALYSIS OF DROSOPHILA MCM10

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In eukaryotic cells, DNA replication and the subsequent packaging of DNA into specific chromatin states are essential processes for the transmission of genetic material and genomic stability which may be linked by interactions between DNA replication proteins and chromatin association proteins. The discovery of several proteins with roles in both processes is evidence for this. Mcm10 is one such protein that has shown evidence for multiple roles in DNA replication, heterochromatin formation, and chromosome condensation. The separation of the functions of Mcm10 are investigated in this study by the analysis of two mutant alleles. A hypomorphic allele of Mcm10 demonstrates that *Mcm10* has a role in heterochromatic silencing, chromosome condensation, and DNA replication. The analysis of a C terminal truncation allele indicates a role in endoreplication mediated through an interaction with Mcm2. The C terminal however, does not seem to be involved in heterochromatic silencing or chromosome condensation.

The second phase of this study involves the development of a novel yeast three-hybrid system. The yeast two-hybrid system is a useful tool for detecting interactions between two proteins and identifying novel protein interactions. However, one limitation to the system is that some two-protein interactions require a third protein to stabilize or facilitate the binding between the two. In this system, we present two novel vectors; pGBKTet and pHook (derived from

pDela) that are Gateway^{tm} compatible and allow screening for the third protein. Due to its numerous protein interactions and its exceptional abundance in the eukaryotic cell with approximately 40,000 molecules per haploid yeast cell, it has been proposed that Mcm10 not only has roles in DNA replication and heterochromatin formation, but also serves as a facilitator of other protein interactions. Using this system, it was shown that Mcm10 may participate in several ternary protein complexes.

INTERACTION AND FUNCTIONAL ANALYSIS OF DROSOPHILA MCM10

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-FOA	5-Fluoroorotic Acid
Aa	Amino acids
Amp	Ampicillin
DNA	Deoxyribonucleic Acid
CAF-1	Chromatin assembly factor 1 subunit
cDNA	complimentary deoxyribonucleic acid
Cdt1/Dup	Double parked
Cyo	Curly gene
DAPI	4',6-diamidino-2-phenylindole
Df(2L)	Deficiency on the long arm of the second chromosome
Dox	Doxycycline
Dp	Dumpy gene
EdU	5-ethynyl-2’-deoxyuridine
GFP	Green fluorescent protein
GINS	Go Ichi Nii San (5, 1, 2, 3)
GW	Gateway
HIS	Histidine
Hp1	Heterochromatin protein 1
Kan	Kanamycin
LacZ	Gene that codes for β-galactosidase
Leu	Leucine
MAT	Mating type

MCM	Minichromosomal maintenance
MCS	Multiple cloning site
Met	Methionine
Nm	Newton meter
ORC	Origin recognition complx
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEV	Position effect variegation
Pre-IC	Pre-initiation complex
Pre-RC	Pre-recognition
Pol α	Polymerase alpha
RPA	Replication protein A
Scim19	Sensitized chromosome inheritance modifier 19
Sir2/3	Silent mating-type Information Regulation-2
Tet	Tetracycline
Trp	Tryptophan
ul	microliter
Ura	Uracil
WT	Wild-type
Y3H	Yeast three-hybrid

CHAPTER 1: ANALYSIS OF TWO MUTANT ALLELES OF MCM10

INTRODUCTION

In eukaryotic cells, DNA replication and packaging of DNA into specific chromatin states are essential processes that are likely linked by interactions between DNA replication proteins and chromatin associated proteins. However, it is unclear how these processes are specifically linked. It is possible that DNA replication proteins interact with separate chromatin establishment factors. Another model suggests that DNA replication proteins themselves have roles in chromatin formation [1]. Both DNA replication and heterochromatin formation have long been known as important aspects in cancer biology. DNA replication is the central mechanism for the cell cycle and cell proliferation. Cancer is characterized by unregulated growth and loss of control of the cell cycle. The proper chromatin condensation of chromatin into chromosomes is critical for genome stability and any defects that are implicated in genome instability are associated with many cancers [2]. Also, the packaging of DNA into heterochromatin is known to be defective in tumor cells [3]. By understanding these two processes, it has allowed for a better understanding of cancer treatment and therapies. However, understanding how these two processes may be linked, can add new and useful information to ongoing cancer research.

Initiation of DNA synthesis occurs at replication origins scattered along the chromosome and are bound by the six-subunit origin recognition complex (ORC), which then directs the formation of protein complexes leading to the assembly of the bidirectional replication forks [4]. These events begin with the assembly of the pre-replicative complex (pre-RC) at the origins of replication. First, Cdc6 is recruited to the origin and is required for the heterohexamer complex Mcm2-7 to bind and act as a DNA helicase to melt the double helix. Another replication protein,

Cdt1, has been shown to associate with Cdc6 to cooperatively promote the association of Mcm2-7 to the chromatin. Together, these factors make up the pre-RC which is a key intermediate in replication initiation [4]. Before replication can be initiated, several other protein complexes must associate with the origin. Phosphorlyation of the Mcm2-7 complex by Dcd7-Dbf4 kinase allows the binding of Cdc45 which is necessary for the loading of DNA polymerase α on chromatin. Simultaneously the GINS complex is added and this leads to the formation of the pre-initiation complex (Pre-IC). Once DNA replication is initiated, Mcm2-7, Cdc45, and the GINS complex migrate with the replication fork [5]. A schematic of DNA synthesis from recruitment to activation of the MCM complex is shown in figure 1-1 [6]. How these proteins rearrange to become part of the replication fork is not fully understood, but likely involves an interaction with Mcm10 which may mediate the anchoring of the MCM complex [6] and help recruit DNA polymerase α to the chromatin for initiation of DNA replication [5].

The establishment of a chromatin state is dependent upon not only the replication of DNA but also the transmission of the epigenetic state to the newly formed chromatin. Epigenetic states are associated with modifications of the nucleotides and DNA associated proteins that result in an altered expression of genes. One example of a protein modification associated with specific epigenetic states is the modification of histones in the nucleosome [7]. Many DNA replication factors are necessary for the proper establishment of different states of chromatin, which supports the hypothesis that DNA replication factors themselves have roles in chromatin formation. For example, ORC, which is involved in the initiation of DNA replication, is also a structural component of heterochromatin in yeast and also interacts with heterochromatin protein 1 (Hp1) in *Drosophila* [1]. Support for the hypothesis that DNA replication factors interact with separate chromatin factors comes from CAF-1 (chromatin assembly factor 1) and its interaction

with PCNA to deposit H3 and H4 histones onto replicating DNA [8]. Identifying factors that have roles in both DNA replication and chromatin formation is crucial for understanding how the two processes are linked.

One such replication factor that is involved in establishing proper epigenetic states is Mcm10. It has been shown to interact with members of the Pre-RC, Pre-IC and Hp1 (Table 1-1) [1]. It was identified in a screen for strains with defects in plasmid stability which also isolated Mcm2, Mcm3 and Mcm5 [4] and was identified in budding yeast as a protein required for stable maintenance of minichromosomes [9]. In a study by Merchant et al [10], mutations in *Mcm10* of *Saccharomyces cerevisiae* showed a reduction in DNA replication initiation at origins and also pausing of replication forks during elongation, suggesting a role in DNA replication initiation and elongation. In a study by Liachko and Tye [9] using *S. cerevisiae*, *Mcm10* mutants displayed a defect in the maintenance of transcriptional silencing. Using a two-hybrid analysis, Mcm10 was found to interact with proteins Sir2 and Sir3 which are both known to maintain transcriptional silencing. These studies indicate that Mcm10 has more than one function in chromosome biology, and therefore would be an ideal candidate to study the links between DNA replication and chromatin dynamics. More evidence to support multiple roles for Mcm10 comes from an early observation that there are over 37,000 copies of this protein per cell in *S. cerevisiae* which indicates that Mcm10 is well in excess for having only a role in DNA replication [11][12].

Homologs of *Mcm10* have been identified in Drosophila, Xenopus, and human and are well conserved across different organisms [13] (Figure 1-2). The highly conserved core of Mcm10 is present in all eukaryotes and contains a zinc finger domain. In higher eukaryotes, Mcm10 is expanded and has a highly conserved C terminal domain with two zinc finger motifs

[1]. A structure of Mcm10 was determined by electron microscopy and single particle analysis [14] (Figure 1-3). The shape of the molecule is a double-layered ring with six-fold symmetry. The ring 'helicase-like' structure of human Mcm10 suggests a molecular model for its evolution in which Mcm10 served as an ancestral helicase that eventually lost its helicase activity to become a docking protein to facilitate interactions between other DNA replication proteins [14].

Overall, there is much evidence that Mcm10 has more than one function in chromosome biology. Previous studies indicate that Mcm10 would be a good target for study of the linkage between DNA replication and chromatin formation. However, what is still not understood is the link between DNA replication and chromosome biology. Do replication proteins such as Mcm10 participate in mechanisms outside their role in DNA replication? Or are the mechanisms intricately linked to one another? Due to its involvement in many stages of DNA replication, Mcm10 is an ideal target to study.

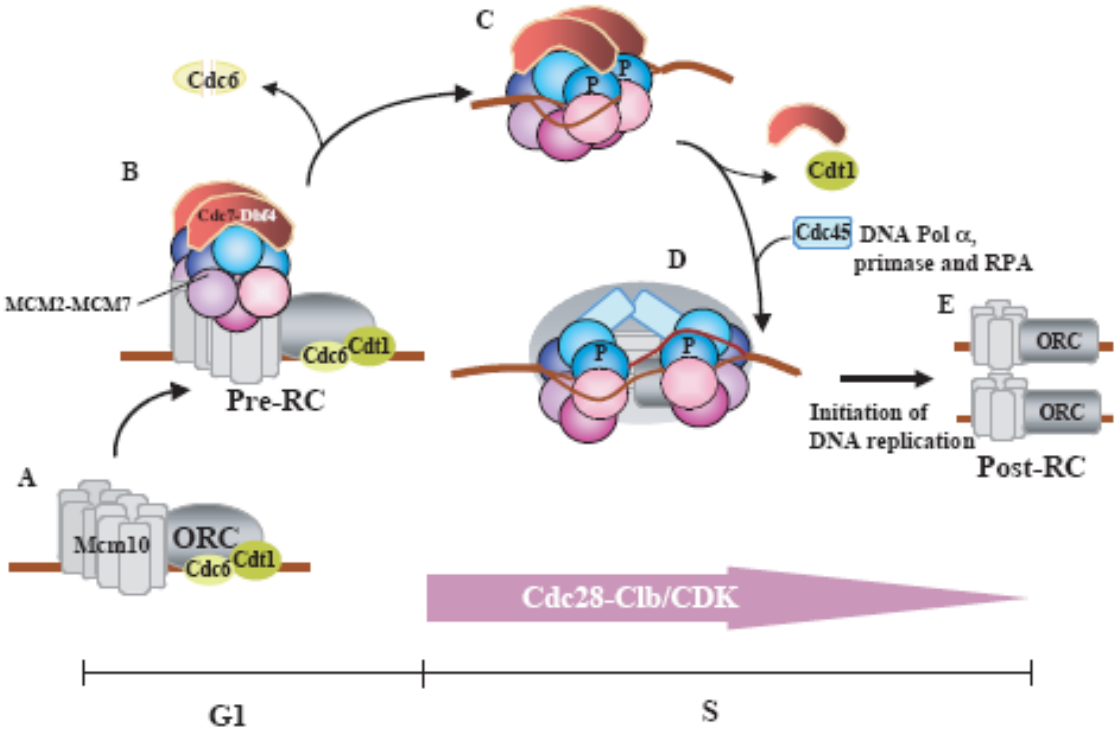


Figure 1-1. Initiation of DNA synthesis. (A) Cdc6 and Cdt1 are recruited to replication origins in G_1 phase where ORC and Mcm10 are bound. (B) Cdc6 and Cdt1 facilitate the loading of the MCM complex which is anchored through interaction with Mcm10. Cdc6 is removed once the MCM complex is recruited and the Cdc7-Dbf4 kinase is recruited to the origin. (C) Phosphorylation of the MCM complex by Cdc7-Dbf4 kinase during S phase is coupled to a conformational change which results in the melting of the DNA. (D) The MCM complex is converted to an active helicase due to the conformational change. Cdc45 is recruited and the disassociation of the MCM complex by Cdc45 from the Mcm10 anchor initiates DNA melting and recruits RPA, DNA polymerase α and primase to the origins. (E) Melting of the dsDNA induces a conformational change in ORC then the origins assume a post-replication chromatin state. Mcm10 is believed to stay bound to the origins throughout the cell cycle [6].

Protein	Function
HP1	Establishment of chromatin silencing
Orc2	DNA replication origin binding
Orc5	DNA replication origin binding
Cdc45	DNA replication
Mcm10	DNA replication and chromatin silencing
Mcm2	DNA helicase activity
Dup/Cdt1	DNA replication

Table 1-1. List of proteins that interact with Mcm10 and their function.

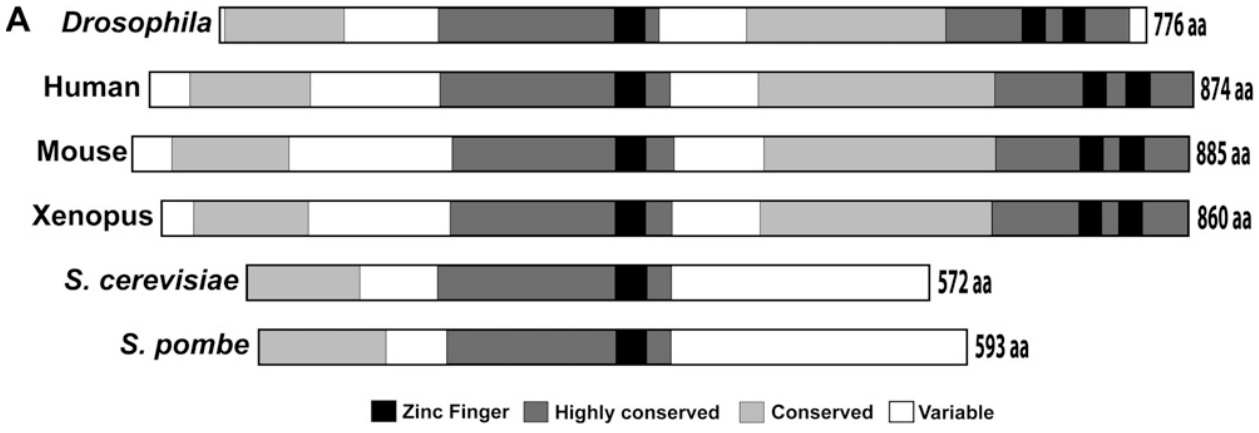


Figure 1-2. Alignment of Mcm10 from multiple species showing conserved zinc finger motifs (red), highly conserved regions (black) and moderately conserved regions (grey) [1].

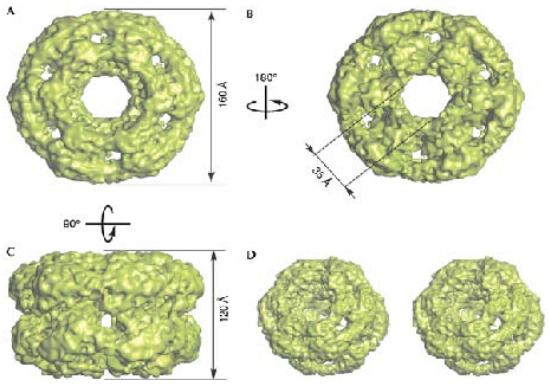


Figure 1-3. Structure of human Mcm10 by electron microscopy and single- particle analysis [14].

OBJECTIVES

The specific purpose of this research was to understand the multiple roles of Mcm10. Do DNA replication proteins have a role in chromatin dynamics and are these roles separable? There is a lot of research on the role for DNA in replication and chromatin formation but hardly any of the studies have attempted to separate these functions. Understanding Mcm10 is a good starting point for further studies on the link between these mechanisms. In order to begin to investigate the roles of Mcm10, two mutant alleles were used for this study.

The first mutant allele was identified in a literature search and is a P element insertion 76 base pairs upstream of the start codon of *Mcm10* [15] (Figure 1-4A). This allele exhibits a dominant minichromosome maintenance defect and is named Sensitized Chromosome Inheritance Modifier 19 ($Mcm10^{Scim19}$). Due to the close proximity of the P-element to the start codon of *Mcm10,* this allele is hypomorphic and reduces transcription by 74 % [1].

Another allele of *Mcm10* was identified in the Exelisis P element insertion which inserts a P element into the 3' region of the coding sequence of *Mcm10* and truncates the last 85 amino acids of the protein ($Mcm10^{d08029}$) (Figure 4A). Truncating the last 85aa causes the removal of one of the two conserved zinc finger motifs in the C terminal (Figure 1-4B). Both mutant alleles are homozygous viable [1].

Using these two mutants, there were several phases of the research to study the roles of Mcm10:

1. Assay the mutants for impacts on DNA replication.
2. Map the interaction domains of Mcm10 with known protein interactors.
3. Assay effects of a C-terminal truncation of Mcm10 with known protein interactors.

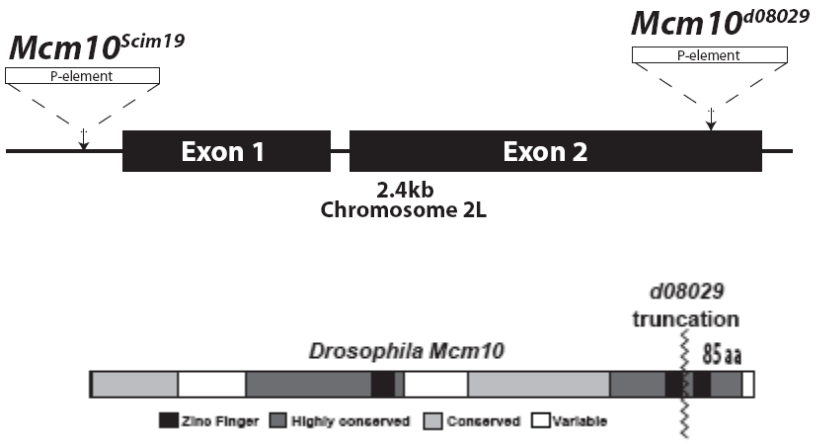


Figure 1-4. Two mutant alleles of Mcm10. A. Schematic of the two *Mcm10* mutant alleles on chromosome 2L with P element insertion sites indicated. B. The $Mcm10^{d08029}$ allele is predicted to cut off 85aa from the C terminal of the protein and remove a conserved zinc finger domain [1].

METHODS

Fly husbandry/Stocks: Fly stocks (*Mcm10*^{Scim19} Flybase ID: FBst0013070, y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Mcm10[KG00233]) and (*Mcm10*^{d08029} Flybase ID: FBst1011557, P{XP}Mcm10[d08029]) were obtained from the Bloomington Fly stock center and the Exelixis *Drosophila* Stock Collection at Harvard Medical School. Previously in the lab, the *Mcm10* P element insertions were confirmed by PCR (data not shown) and each line was backcrossed >7 times to w; Df(2L), b[82-2] / CyO to remove unwanted second site mutations. Fly stocks were maintained on Caltech media (US Biological # D9600-07) at room temperature [1].

Polytene Chromosomes: The size of the polytene chromosomes in the mutant flies were qualitatively compared to wild-type controls. This was done using the polytene chromosome protocol (Christensen 2008). With tweezers, the 3rd instar wandering larvae of the desired genotype were captured and placed in a well containing 100ul of 1X PBS pH 7.2 with 1% PEG 8000. The larvae were pulled apart by grasping the posterior end and the anterior mouth hooks with tweezers. The polytene glands were identified and placed in a fixative (50% acetic acid, 2% lactic acid, 3.7% formaldehyde) for two minutes. The polytene glands were then transferred to a microscope slide and a siliconized coverslip was pressed on top until the chromosomes were well spread. Using a digital torque wrench, 15 Nm of pressure was applied to the slide for two minutes. The slide was lowered into liquid nitrogen for one minute then placed in 100% ethanol for 10 seconds. Finally, Vectasheild with DAPI was added to the slide and a coverslip was placed on top. The polytene chromosomes were viewed under the confocal microscope in the Howell Science Complex.

Larval brain squashes/Mitotic index: The two mutant alleles were tested for competency in the more recognized cell cycle in the bran tissues of the wandering 3rd instar larvae. In order to measure chromosomes, brain squashes of third instar larvae of each mutant and wild-type were performed. This was done using a brain squash protocol (Christensen 2008). Third instar wandering larvae were placed in 1XPBS pH 7.2 with 1% PEG 8000 and pulled apart by grasping the posterior end and the mouth hooks using tweezers. The brain was identified and placed into a hypotonic solution (0.5% sodium citrate) for 10 minutes. It was then transferred to a fixative (acetic acid: methanol: water 11:11:2) for 20 seconds. The brain was transferred to a microscope slide and a siliconized cover slip was placed on top. Using the digital torque wrench, 15 Nm of pressure was applied then the slide was lowered in a container with liquid nitrogen then washed with ethanol. Vectashield with DAPI was placed on the slide then viewed using the confocal microscope. Mitotic indices were calculated by taking the number of cells in mitosis from 10 different random views at 60X divided by the total number of cells to determine if cells are delayed in S phase. *Mcm10*^{Scim19} and *Mcm10*^{d08029} were used to generate other mutants to analyze in order to ensure that the results from the mitotic indices were not due to other factors such as maternal loading. Mcm10Scim19 homozygous females were used to generate *Mcm10*^{Scim19}/+ and *Mcm10*^{Scim19}/Df(2L) by crossing to Df(2L)/CyO, GFP. *Mcm10*^{Scim19} homozygous females were also crossed to *Mcm10*^{d08029}/*Mcm10*^{d08029} to generate *Mcm10*^{Scim19}/*Mcm10*^{d08029}. *Mcm10*^{d08029}/+ and *Mcm10*^{d08029}/Df(2L) were generated as above except using *Mcm10d08029* homozygous females instead. Statistical analysis was performed using Minitab^{tm} [1].

Hatch rates: Recently eclosed Drosophila were allowed to deposit eggs on yeast dusted grape plates at 26°C for 2 hours. Eggs were counted then grape plates were incubated for 24 hours at

26°C. After the 24 hours, the unhatched eggs were counted. A minimum of 3 independent trials were conducted for each phenotype.

Yeast two-hybrid analysis: Yeast two-hybrid analysis was conducted according to standard protocols as in [13] and manufacture's protocols (Clonetech, Matchmaker^{tm} Yeast two-hybrid system). Yeast strain AH109 (Clonetech) containing the HIS3 reporter was used as the reporter strain. Plasmids pGBKT7 and pGADT7 (Clonetech) were converted to the Gateway^{tm} cloning system (Invitrogen) by insertion of a Gateway^{tm} cassette into the MCS were used as Gal binding domain and Gal activating domain, respectively. Also, the Kan^{R} gene of pGBKT7 was disrupted by Amp^{R} for use in the Gateway^{tm} system. All clones were sequence verified to ensure proper reading frame was maintained and there were no ectopic mutations.

RESULTS

Impact on DNA replication: Since it has been widely established that *mcm10* has a role in DNA replication [5][12][16][17][18][19], the impact of the two mutant alleles of *Mcm10* on DNA replication of polytene chromosomes and brain tissue were observed.

***Polytene Chromosomes*:** In *Drosophila*, there are different types of DNA replication that have different modes of regulation [20]. One common cell cycle variant is endoreplication, in which cells increase their genomic DNA content without dividing to generate polyploid tissues. Examples of this in *Drosophila* are the polytene chromosomes of the salivary gland tissues of wandering 3rd instar larvae. Some, but not all DNA replication proteins have a role in endoreplication [1]. In order to determine whether Mcm10 has a role in the process, polytene chromosomes from wild-tpe, $Mcm10^{d08029}$ and $Mcm10^{Scim19}$ were observed (Figure 1-5) [1]. The micrographs reveal that $Mcm10^{Scim19}$ polytene chromosomes appear normal compared to wild-type controls. This indicates that normal levels of Mcm10 are not required for endoreplication. However, polytene chromosomes of $Mcm10^{d08029}$ showed under-replication when compared to wild-type, suggesting that the last 85 amino acids of Mcm10 play a role in endoreplication.

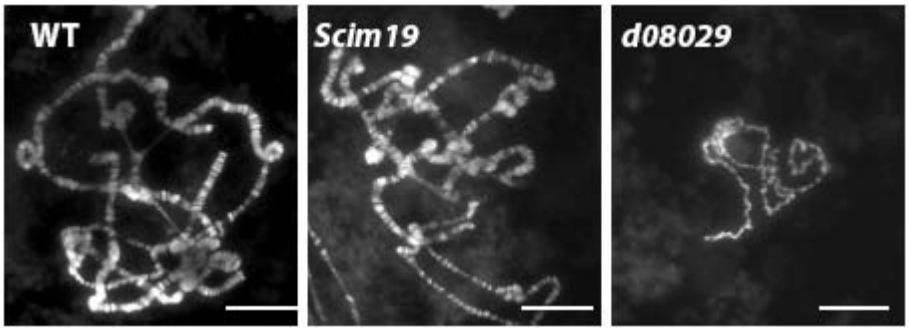


Figure 1-5. Confocal micrographs of polytene chromosomes from genotypes indicated. $Mcm10^{d08029}$ polytene chromosomes are under-replicated compared to $Mcm10^{Scim19}$ and wild-type [1]. (Note: micrographs were taken by other persons in the lab)

Brain squashes/Mitotic index: In addition to endoreplication, we sought to investigate the role of Mcm10 on the more traditional cell cycle of the brain. To test for a possible cell cycle delay, mitotic indices were done on genotypes indicated (Figure 1-6)[1]. *Mcm10*^{Scim19} showed significantly fewer cells in mitosis when compared to wild-type and the combinations of *Mcm10*^{Scim19} with *Mcm10*$^{+}$ and Df(2L) showed a dosage dependent trend with *Mcm10*^{Scim19}/Df(2L), *Mcm10*^{Scim19}/*Mcm10*^{Scim19}, *Mcm10*^{Scim19}/+, and *Mcm10*^{Scim19}/*Mcm10*^{Scim19}; p[*Mcm10*$^{+}$]/p[*Mcm10*$^{+}$] larvae having 6.0X, 3.6X, 2.6X and 1.1X respectively fewer nuclei in mitosis compared to wild-type. Since the trend is that the mutant over deficiency has the greatest defect, followed by the homozygote, then heterozygote, with the rescue mutant similar to wild-type, it can be concluded that *Mcm10*^{Scim19} represents a hypomorphic semi-dominant allele of *Mcm10*. Mitotic indices for combinations of *Mcm10*^{d08029} suggest that this allele is dominant for cell cycle delay as well as endoreplication as previously reported. The *Mcm10*^{d08029} allele differs from *Mcm10*^{Scim19} in that it required the addition of 3 wild-type copies of Mcm10 to rescue the mutant so that the number of nuclei in mitosis was similar to wild-type. Also, the mitotic indices for the *Mcm10*^{d08029} homozygote and *Mcm10*^{d08029}/Df(2L) were not significantly different from one another which suggests that the defects in this allele are not due to reductions in the level of protein, unlike the *Mcm10*^{Scim19} allele [1].

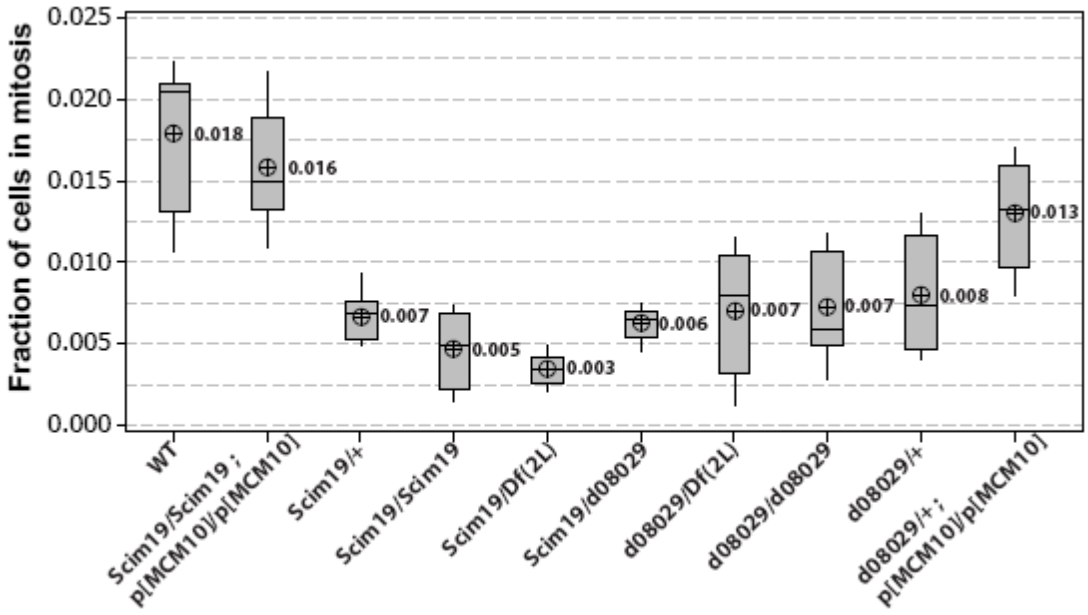


Figure 1-6. Graph of fraction of cells in mitosis from brain squashes of genotypes indicated [1].

Chromosome Condensation and Early Embryo: It had been previously reported in RNAi studies that depletion of *mcm10* in *Drosophila* KC cells results in under-condensed metaphase chromosomes [13]. However, in this study, metaphase chromosomes from 3rd instar larvae of $Mcm10^{d08029}$ and $Mcm10^{Scim19}$ did not show a defect in chromosome condensation (Figure 1-7A).

The effect of the mutant alleles on the early embryo cell cycle was investigated due to the fact that the early embryo cell cycles 10-12 differ from the larval brain which was already examined. The early embryo cell cycles occur in a syncytium, lack gap phases, are synchronous and occur rapidly over about 9 minutes [21]. Homozygous early embryos of both mutant alleles at cell cycles 10-12 were examined. $Mcm10^{d08029}$ embryos shoed normal synchrony and not a significant number of anaphase bridges while $Mcm10^{Scim19}$ embryos revealed asynchronous divisions and anaphase bridges in 29% of the embryos examined (Figure 1-7B). The asynchrony and bridging of the hypomorphic allele early embryos may have a negative effect on embryo

viability because the hatch rates for *Mcm10*^{Scim19} were only 46% compared to 68% for wild-type (Figure 1-7C). Hatch rates were also slightly lower for embryos with *Mcm10*^{d08029} background (Figure 1-7C). Since it was observed that *Mcm10*^{Scim19} shows an S phase delay (this study)[1], it is hypothesized that the asynchrony and anaphase bridges are a consequence of entry into mitosis before DNA replication is complete [1]. However, the S phase delay that has been observed with *Mcm10*^{d08029} does not come from asynchrony in the early embryo. It is possible that protein levels of Mcm10 are important in the early embryo for rapid DNA synthesis to occur and the last 85aa are dispensable for this process. (Note: chromosome condensation and hatching rates were performed by JA while early embryo experiments were done by other persons in the lab).

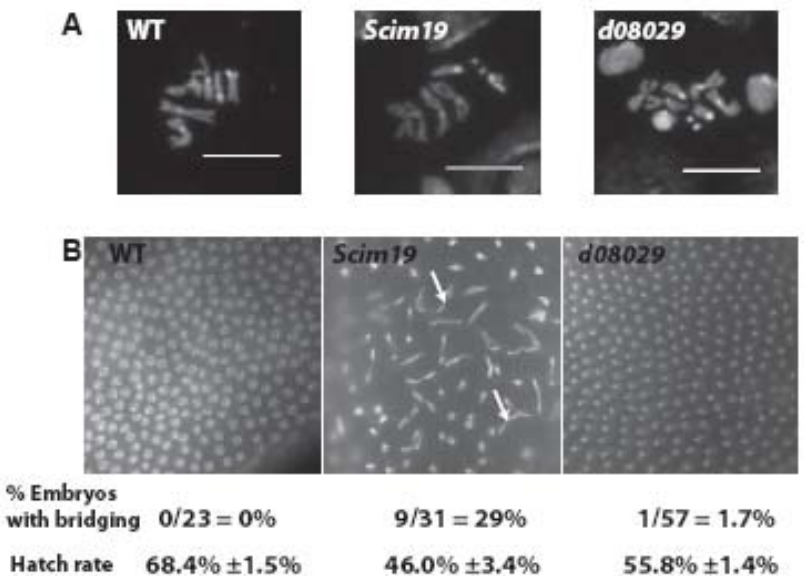


Figure 1-7. Chromosomal phenotypes in larval brains and early embryo. A. Fluorescent micrographs of mitotic figures from brain squashes of the indicated genotypes. B. Fluorescent micrographs o nuclei in early embryos and the percent of embryos showing 2 or more anaphase bridges and also hatching rates for genotypes indicated [1].

Dissection of interaction domains: As mentioned before, the central core of Mcm10 is conserved across different organisms while the C terminal is present in only higher eukaryotes. Determining which proteins interact with the different domains of Mcm10 could give insight into the multiple roles of Mcm10 that may have evolved over time. In order to determine the domains of Mcm10 responsible for protein interactions, a yeast two-hybrid analysis was performed using 200 amino acid fragments of Mcm10 overlapping by 100 amino acids against known protein interactors [13]. First, these known interactions were confirmed by yeast two-hybrid analysis (Figure 1-8A). Known protein interactors of Mcm10 (Mcm2, Orc2, Mcm10-100 aa, and Hp1) were fused to the Gal binding domain (pGBKT7 GW) and full length Mcm10 was fused to the Gal activation domain (pGADT7 GW). Mcm10 minus the first 100 amino acids was fused to the Gal binding domain because it has been previously shown that the first 100 amino acids have one-hybrid activity with the binding domain but not the activating domain [1]. Plasmids were then transformed into yeast strain AH109 and plated on complete media lacking histidine. Interactions were indicated by growth on the media as a result of the transcription of the HIS3 reporter construct. Growth was observed when Mcm10 was combined with Mcm2, Mcm10-100 aa, Orc 2 and Hp1 but not with the empty vector.

In order to determine the domains of Mcm10 responsible for protein interactions, a yeast two-hybrid analysis was performed using 200 amino acid fragments of Mcm10 overlapping by 100 amino acids against known protein interactors (Figure 1-8B). Fragments of Mcm10 were fused to the Gal activation domain (pGADT7 GW) and known protein interactors Mcm2, Mcm10, Orc2, and Hp1 were fused to the Gal binding domain (pGBKT7 GW). Plasmids were then be transformed into yeast strain AH109 then plated on complete media lacking histidine.

Interactions were indicated by growth on the media as a result of the transcription of the HIS3 reporter construct in the AH109 yeast strain.

Results show that Mcm10 interacts with Mcm2 via a region that contains the highly conserved core and central zinc finger as well as through the C-terminal of Mcm10 which includes one of the two conserved zinc finger domains (Figure 1-8C). Mcm10 self interaction occurs in the central region and C-terminal domain also. However, the Mcm10 interaction with Orc2 is mediated only through the C-terminal domain and the Hp1 interaction is mediated through an expanded portion of the C-terminal (Figure 1-8C.)

It should also be noted that one-hybrid activity was detected in the first 100 amino acids of Mcm10 (Figure 1-8B). In order to eliminate one-hybrid activity in further testing, clones were constructed without the first 100 amino acids.

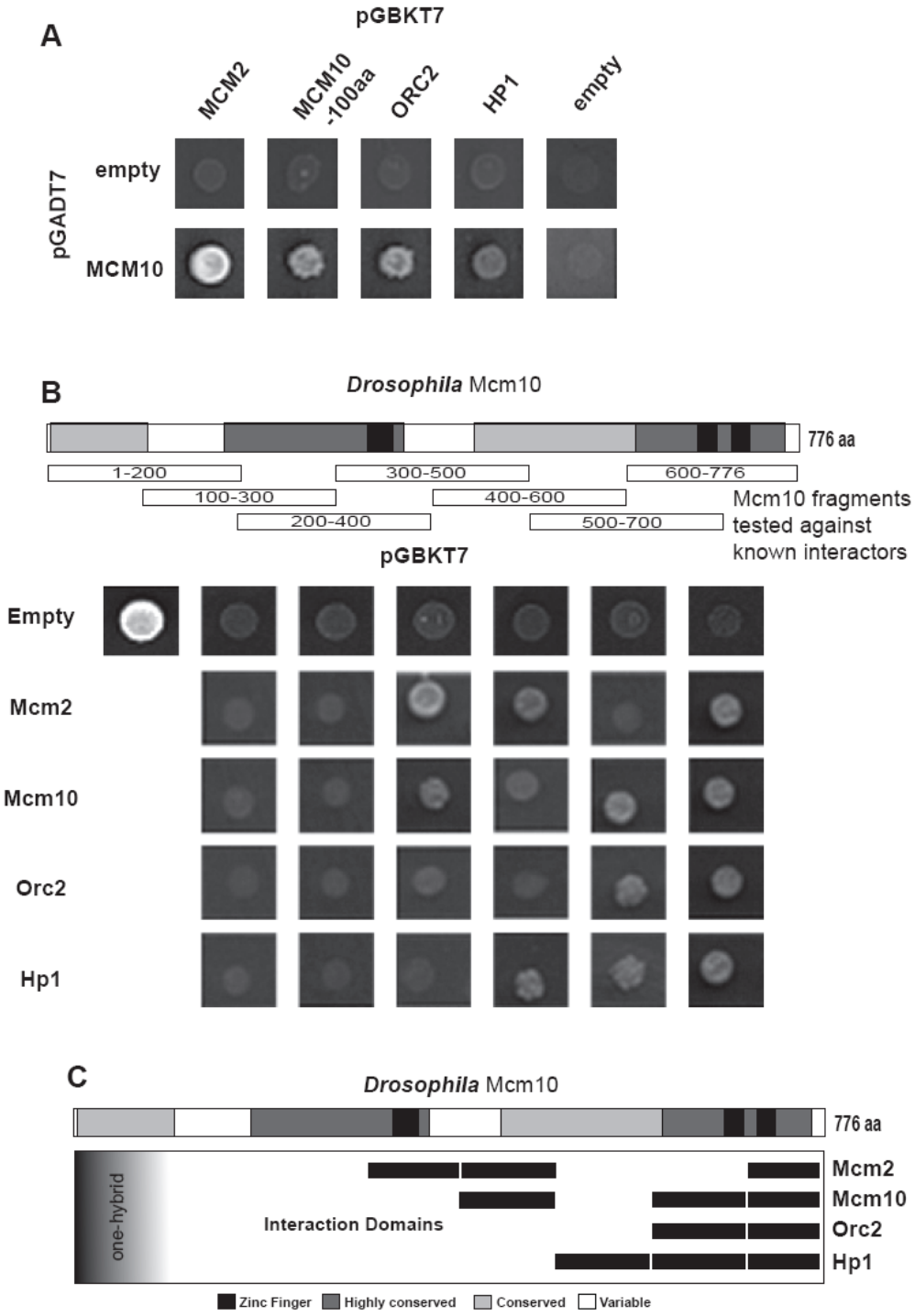


Figure 1-8. Dissection of interaction domains. A. Yeast two-hybrid analysis indicating interaction of Mcm10 with Mcm10, Mcm2, Orc2, and Hp1. Growth was not observed with one-hybrid controls. B. Schematic of the dissection of Mcm10 into 200 amino acid fragments,

overlapping by 100 amino acids and yeast two-hybrid analysis of each segment with known protein interactors from A directly below. The first 100 amino acids showed one-hybrid activity.

C. Schematic of the interaction domains of Mcm10 [1].

Impact of truncation on protein interactions: The dissection of the interaction domains of Mcm10 revealed that the C-terminal is involved in interactions with Mcm2, Mcm10, Orc2, and Hp1. Therefore, the next step was to determine the consequences of a C-terminal truncation of Mcm10 on its interactions with these proteins using the *Mcm10*^{d08029} allele which truncates the last 85 amino acids. This was done using a semi-quantitative yeast two-hybrid analysis using a clone of Mcm10 consisting of amino acids 101-691. The first 100 amino acids of the N-terminal were removed due to one-hybrid activity previously discovered and the last 85 amino acids correspond to the *Mcm10*^{d08029} allele. The truncated *Mcm10*^{d08029} and Mcm10 minus the first 100 amino acids were each fused to the Gal binding domain. Mcm2, Mcm10, Orc2, and Hp1 were each fused to the Gal activation domain and tested against the two Mcm10 clones (Figure 1-9). In order to semi-quantitatively measure the strength of the interaction, the yeast cultures were plated in 5 fold dilutions. The results indicate that a truncation of the last 85 amino acids of Mcm10 eliminates its interaction with Mcm2. It was also shown that the truncation weakened Mcm10 self interaction and interaction with Orc2. However, the interaction with Hp1 seems unaffected.

These results suggest that the last 85 amino acids are required for an interaction with Mcm2. In fact, within the C-terminal, Mcm2 only interacted with the last 76 amino acids of Mcm10 (Figure 1-8C) which was removed with the truncation. In contrast, Mcm10 self interaction and interaction with Orc2 occurred over a larger region in the C-terminal which may

explain a reduction, but not loss of interaction with Mcm10. Additionally, the observation that Hp1 remains unaffected by the truncation could be due to its interaction over a larger area of the C-terminal (276 amino acids). After the removal of the last 85 amino acids, the remaining 191 were sufficient for the interaction with Hp1. Lastly, the dissection of the interaction domains of Mcm10 revealed that Mcm2 and Mcm10 self interaction also occur at the core of Mcm10 (Figure 1-8C). It is possible that a false-positive occurred here since creating 200 amino acid fragments of Mcm10 could have exposed a binding domain that is normally folded within the protein.

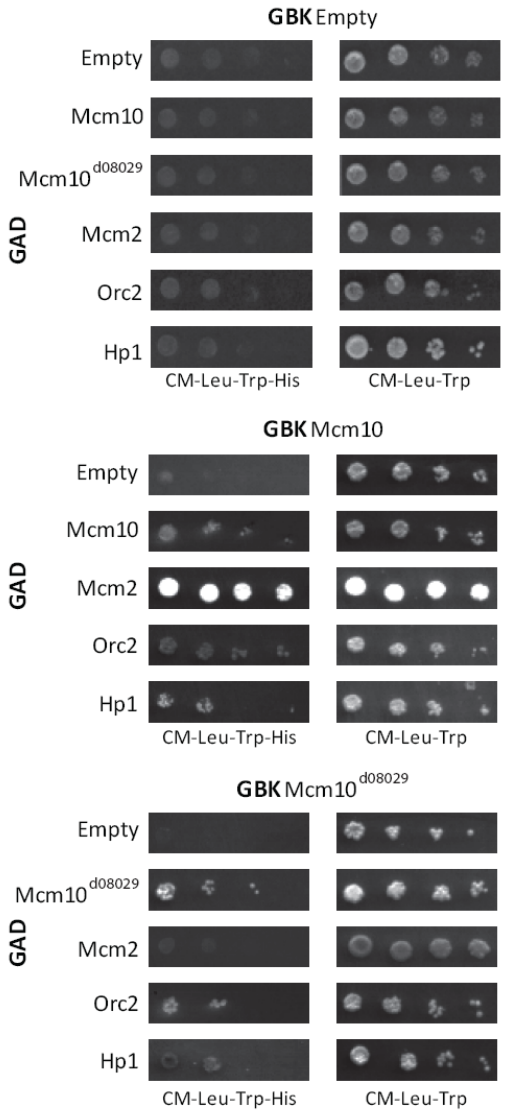


Figure 1-9. Serial dilution yeast two-hybrid showing the effect of a C-terminal truncation on Mcm10's interaction with other proteins. The first column is growth on media lacking histidine and the second column is a growth control. The top panel is a one-hybrid control showing that none of the clones interact with the empty vector. The second panel shows Mcm10 interacting with the proteins indicated and the third panel shows the effect of the truncation on these interactions [1].

Additional Results from Christensen Lab: Numerous experiments by others in the lab show support for multiple roles of Mcm10 in heterochromatin formation and DNA replication and add to the body of evidence shown above [1].

EdU Incorporation: Given that the mitotic indices of the two mutants showed a decrease in the number of cells in mitosis when compared to wild type, we sought to test for an S phase delay by utilizing EdU (5-ethynyl-2'-deoxyuridine) incorporation assays performed for 30 minutes on dissected 3rd instar brains. Two observations were made upon visualization of the brains. First, the brains of the wild-type larva were slightly larger than either *Mcm10*^{Scim19} or *Mcm10*^{d08029} which suggests that cell proliferation is slower in the mutants (Figure 1-10). Secondly, both mutants showed more EdU incorporation than the wild type (Figure 1-10) which is likely due to an S phase delay [1]. The decreased brain sizes, the increase in the number of cells in S phase and the reduced mitotic indices (Figure 1-6) are all consistent with a possible delay in S phase.

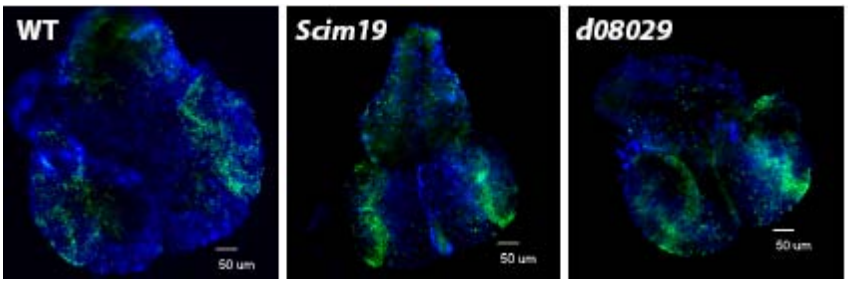


Figure 1-10. Fluorescent micrograph of wandering 3rd instar larval brain. DNA is shown in blue and EdU incorporation in green. WT brain is larger and shows less EdU incorporation than either mutant [1].

Position Effect Variegation (PEV) Analysis: Since Mcm10 has been shown to interact with Hp1 [2][1], a test was done to determine whether or not *Mcm10* has a role in heterochromatin formation. This was done by using variegating *dumpy (dp)* alleles that result in variable wing morphology due to the proximity of *dp* to centric heterochromatin [1]. Wing phenotypes were scored into categories of *dp* phenotypes by severity (Figure 1-11A). The impact of the two *Mcm10* mutant alleles on PEV were compared to wild type and an *Hp1* mutation (Figure 1-11A&B). The *Hp1* mutant served as a positive control and as expected, was able to suppress PEV of the *dp* wing phenotype when compared to wild-type (Figure 1-11A). Interestingly, when analyzing the two *Mcm10* mutant alleles, only the hypomorphic allele dominantly suppressed PEV and the truncated allele showed no suppression. Therefore, it is suggested that levels of Mcm10 are important for heterochromatin formation but the last 85aa are not involved [1].

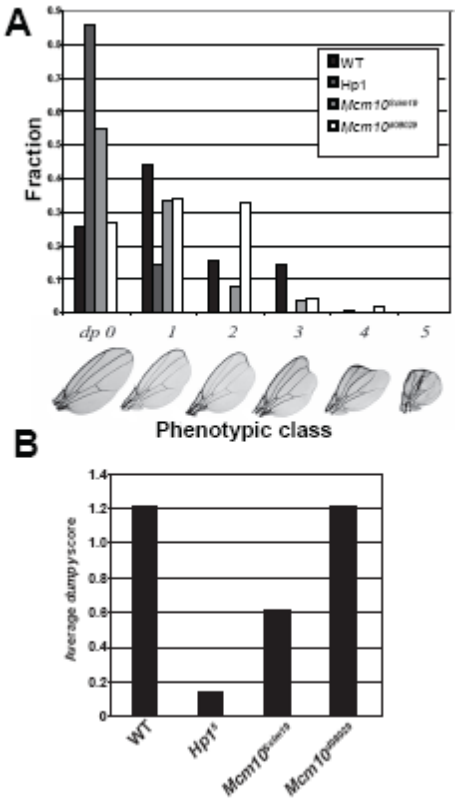


Figure 1-11. PEV analysis of *Mcm10* mutants and $Hp1^5$ mutant using a variegating *dumpy* reporter line. A. Fraction of flies scored in each phenotypic class (1-5) for genotypes indicated. B. Average "dumpy" score for each genotype. $Hp1^5$ and $Mcm10^{Scim19}$ show a significant suppression of dumpy PEV while $Mcm10^{d08029}$ shows no shift from wild-type [1].

DISCUSSION

Analysis of the two mutant alleles of *Mcm10* and its interaction analysis suggest multiple roles for Mcm10. It has long been established that Mcm10 has a role in DNA replication, but new information is revealing that Mcm10 is involved in many processes in the cell, including heterochromatic silencing [9], making Mcm10 an ideal candidate to study the link between DNA replication and heterochromatin formation. By utilizing a hypomorphic allele, $Mcm10^{Scim19}$, we were able to investigate the processes in which the majority of Mcm10 in the cell is involved. Since the C-terminal of Mcm10 was shown to be crucial for protein interactions, such as mediating the interaction between Sir2 and the Mcm2-7 complex [22], the truncated allele, $Mcm10^{d08029}$, provided insight into the significance of Mcm10's protein interactions.

Comparisons of the polytene chromosomes, mitotic indices, and early embryo synchrony of each reveal interesting results with regard to DNA replication. It appears that the last 85 amino acids of Mcm10 play a role in endoreplication since the truncation allele expressed under-replicated polytene chromosomes. However, only a small amount of Mcm10 is required for endoreplication since the hypomorph did not show any defect. The mitotic indices and the EdU incorporation assays suggest that both mutants are delayed in S phase, consistent with previous findings in human cell lines where depletion of Mcm10 resulted in S phase delay [16]. While both mutants showed a defect in DNA replication in the brain, the two mutants did not show the same trend when the alleles were combined with wild-type copies of Mcm10 and Df(2L). $Mcm10^{Scim19}$ revealed a dosage dependent trend with the fewest nuclei in mitosis when the dosage of Mcm10 was the lowest. The results of the mitotic indices for the combinations of $Mcm10^{d08029}$ infer that the defects observed were not due to reductions in levels of Mcm10. Unlike $Mcm10^{Scim19}$, the S phase delay in $Mcm10^{d08029}$ did not translate asynchrony in the early

embryo. Taken together, the results from the polytene chromosomes, mitotic indices and early embryos may indicate multiple roles for Mcm10 in replication. High protein levels of Mcm10, even if the C terminal is truncated, may be crucial for rapid DNA synthesis of the early embryo and S phase progression of the normal cell cycle while a small amount of Mcm10 is sufficient for endoreplication and relies on the C terminal. It is still unclear whether or not the S phase delays observed in the two mutants are due to the same defect, or reflect two separate roles for Mcm10 in S phase progression.

It is possible that Mcm10's highly conserved core which is present in all eukaryotes represents the ancient function of the protein and over time, Mcm10 acquired new roles with the conserved C-terminal domain in higher eukaryotes [1]. The observation that the last 85 amino acids appear to have a role in endoreplication may be evidence for this hypothesis. In addition to the under-replication of polytene chromosomes of the truncated allele, there is more evidence that the last 85 amino acids are involved in endoreplication. It was shown that fly larvae homozygous for the $Mcm10^{d08029}$ allele are, on average, about 16% smaller than wild type larvae and they have a lower hatching rate [1]. This is consistent with a defect in endoreplication since most of the larval growth occurs through this process in which polyploidy cells are enlarged through many rounds of DNA replication without mitosis.

Yeast two-hybrid analysis using the C terminal truncated allele, $Mcm10^{d08029}$, which removes one of the zinc-finger motifs, has been beneficial in beginning to understand these newer roles. The yeast two-hybrid results in this study revealed that the conserved C terminal is essential for Mcm10's interaction with Mcm2, since truncating the allele caused a complete loss of interaction with Mcm2. Taken with the results from the polytene chromosomes, it may be

possible that the last 85 amino acids are involved in endoreplication and that this function may be modulated through an interaction with Mcm2.

Studies have shown that the central core is responsible for essential functions of Mcm10 since mutations in this region affect cell viability and DNA replication [5][10][12]. There is also evidence that the central core is involved in heterochromatin silencing [9]. Since it was shown in this study that Mcm10 interacts with Hp1, the effect of $Mcm10^{Scim19}$ and $Mcm10^{d08029}$ on heterochromatin formation was investigated using position effect variegation (PEV) analysis. Results indicated that the last 85 amino acids were not involved in heterochromatin formation, but the hypomorph did show a defect in heterochromatin formation. If the role of Mcm10 in heterochromatin formation is mediated through its interaction with Hp1, it would make sense that the interaction with Hp1 would not be affected by the C terminal truncation. This is in fact consistent with what we observed in the yeast two-hybrid analysis.

Together with the DNA replication results above, a separation of function for Mcm10 is starting to become apparent. It is possible that the conserved core of the protein has a major role in DNA replication and heterochromatin formation and that the bulk of Mcm10 in the cell is used for this purpose. In higher eukaryotes, though, Mcm10 may have gained additional roles with the conserved C terminal in endoreplication and DNA replication. Understanding the multiple roles for Mcm10 may start to shed light onto the ways in which DNA replication and packaging of DNA into chromatin states are intertwined to maintain genome stability.

CHAPTER 2: DEVELOPMENT OF A NOVEL YEAST THREE-HYBRID (Y3H) SYSTEM

INTRODUCTION

The yeast two-hybrid system is a useful tool for detecting interactions between two proteins and identifying novel protein interactions. However, one limitation to the system is that some two-protein interactions require a third protein to stabilize or facilitate the binding between the two. In order to study these complexes, a number of yeast three-hybrid systems have been developed. Zhang and Lautar [23] constructed a plasmid, pDela, which is compatible with other yeast two-hybrid plasmids (pGBT9 and pGAD424) and contains a *URA3* gene for selection. Since the other two plasmids contain *TRP1* (pGBT9) and *LEU2* (pGAD424) genes for selection, triple transformants could be selected in a ura- trp- leu- yeast strain. The yeast strain used contained LacZ and His3+ reporter genes so interaction between the three proteins could be detected by β-galactosidase activity and histidine independent growth. This yeast three-hybrid system could be used to detect ternary complex formation or situations in which a third protein is required mediate an interaction between two proteins (Figure 2-1).

Another yeast three-hybrid system developed by Tirode et al [24] involved a third protein under control of a conditional promoter (Met25). This conditionally expressed third partner would allow or prevent the formation of the transcriptional activator. However, this system utilizes methionine depletion for the Met25 promoter which hampers basal growth of the yeast strain [25]. To circumvent this issue, Moriyoshi developed another yeast three-hybrid system in which expression was tetracycline (Tet)- regulated. Doxycycline (Dox), and inducing reagent for the Tet-regulated system, has no obvious effect on the phenotype. A novel vector, pBT, was constructed and contained all the Tet-OFF components in a single plasmid. One possible set-

back for this method is that having all the components in a single plasmid may be excessive. If one component of the system became compromised, it could easily result in a cascade effect causing the system to fail. Therefore, a novel yeast three-hybrid system has been developed that contains two new vectors: pGBKTet and pHook (derived from pDela) that are Gateway^{tm} compatible and allow for efficient screening for a third protein interactor.

Unlike the system developed by Tirode et al [24] which uses methionine depletion, this novel system does not use a method that affects the basal growth of the yeast cells. The system presented involves transformation of plasmids and growth on selective media to determine transcription of the *HIS3* reporter gene, neither of which has been shown to affect the basal growth of the yeast. The Tet-regulated system [25] may have resolved this issue, but in doing so may have created another. The novel yeast three-hybrid system is much less complex than the Tet-regulated system, allowing for fewer sources of error. However, there are set-backs to the novel system as well. Like any two-hybrid or three-hybrid system, it is still possible to get false positives when screening for proteins. The proteins detected from the screen may not actually interact *in vivo* because they don't come into contact in the cell or the interaction has no biological significance. Therefore, the system is most useful as an initial investigation but more follow-up procedures are required.

This system will be useful for detecting two-protein interactions that rely on a third protein. The protein in this research that will be investigated as a facilitator of interactions is *Drosophila Mcm10*. Due to its numerous protein interactions and its exceptional abundance in the eukaryotic cell with approximately 40,000 molecules per haploid yeast cell [11], it has been proposed that Mcm10 not only has roles in DNA replication and heterochromatin formation, but also serves as a facilitator of other protein interactions. In a previous study, it was shown that

Mcm10 mediates human RecQ4 association with Mcm2-7 helicase during DNA replication [26]. It was shown that Mcm10 is essential for the integrity of the RecQ4-MCM helicase/GINS complex and may regulate its DNA unwinding activity. It was suggested that RecQ4 activity requires tight regulation by Mcm10 to prevent unlicensed replication initiation [26]. It has also been reported that an interaction between Mcm10 and And-1 is required to load DNA polymerase α onto chromatin [27]. It was shown that human And-1 forms a complex with Mcm10 and pol α. However, what is not clear is whether or not they form a ternary complex. The model proposed suggests that the And-1-Mcm10 complex is part of a larger complex linking the Mcm2-7 helicase with the primase-containing pol α complex (Figure 2-2). The yeast three-hybrid system presented will be useful in screening for additional protein interactions in which Mcm10 is acting as a facilitator.

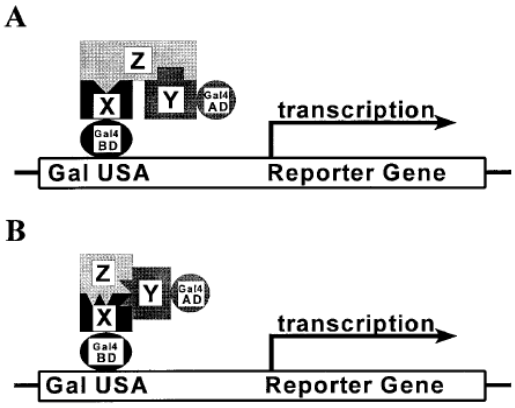


Figure 2-1. Schematic representation of three- protein complexes. A. Third protein (Z) is required for X and Y to interact. B. Y binds to a composite contour created by a combination of X and Z [23].

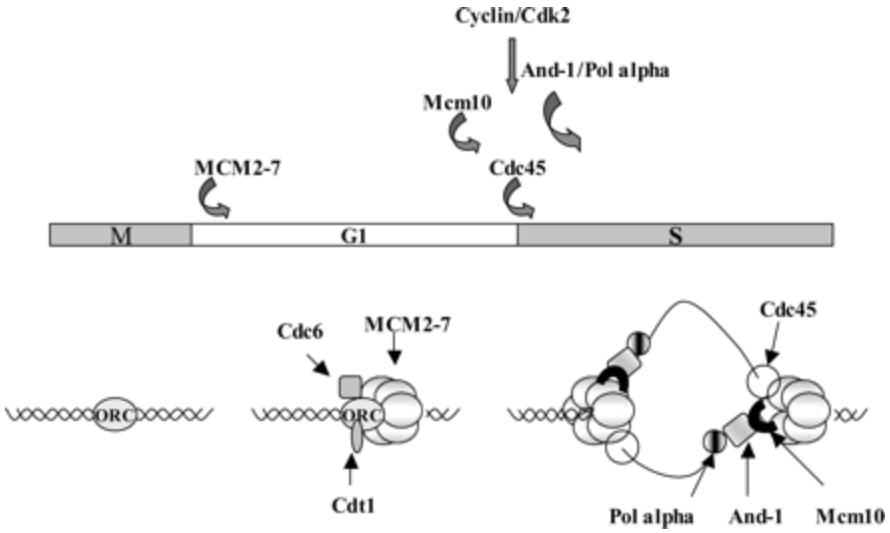


Figure 2-2. A model for the recruitment of pol α in which prior recruitment and interaction of Mcm10 and And-1 is required [27].

METHODS

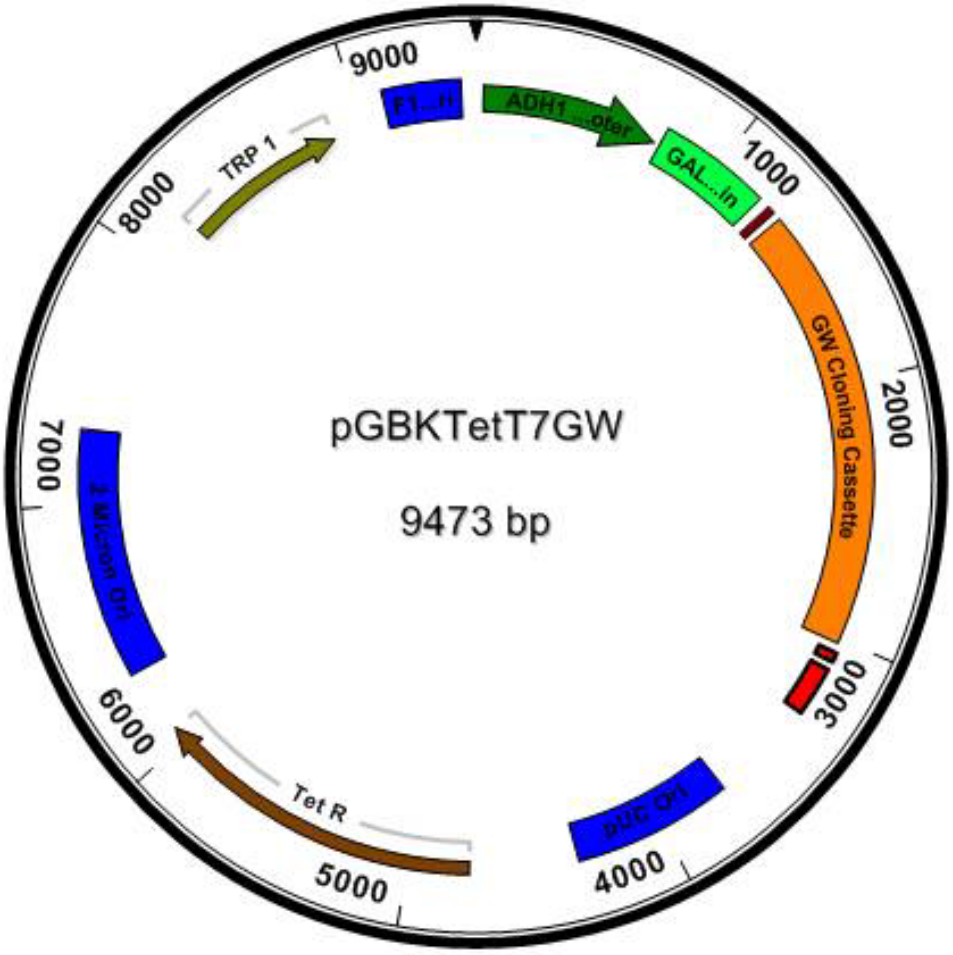
Yeast Strains

Yeast strains PJ694-a (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) and PJ694-α (Matα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (Clontech), which contain the HIS3 reporter, were used as the reporter strains. Transformation and selection were performed according to [13].

Construction of Novel Vectors

Two novel vectors were constructed for the system. The first vector, pGBKT7Tet, was constructed using the pGBKT7 vector (non-Gateway^{tm}) from Clonetech by replacing the kanamycin resistance with tetracycline resistance from the pBR322 plasmid. It was then converted to a destination vector by inserting a Gateway^{tm} cassette (Figure 2-3A). The second vector, pHook, was derived from pDela [23]. The Gateway^{tm} cassette and FLAG-tag from the pTFW plasmid were amplified and inserted into a multiple cloning site of pDela so that the FLAG-tag and Gateway^{tm} cassette were in fram to an existing nuclear localization signal (Figure 2-3B). Construction of these vectors was perfomed in the lab by Michael Reubens.

A.


pGBKTetT7GW
9473 bp
9000
1000
2000
3000
4000
5000
6000
7000
8000
TRP 1
ADH1 ...oter
GAL...in
GW Cloning Cassette
Tet R

B.

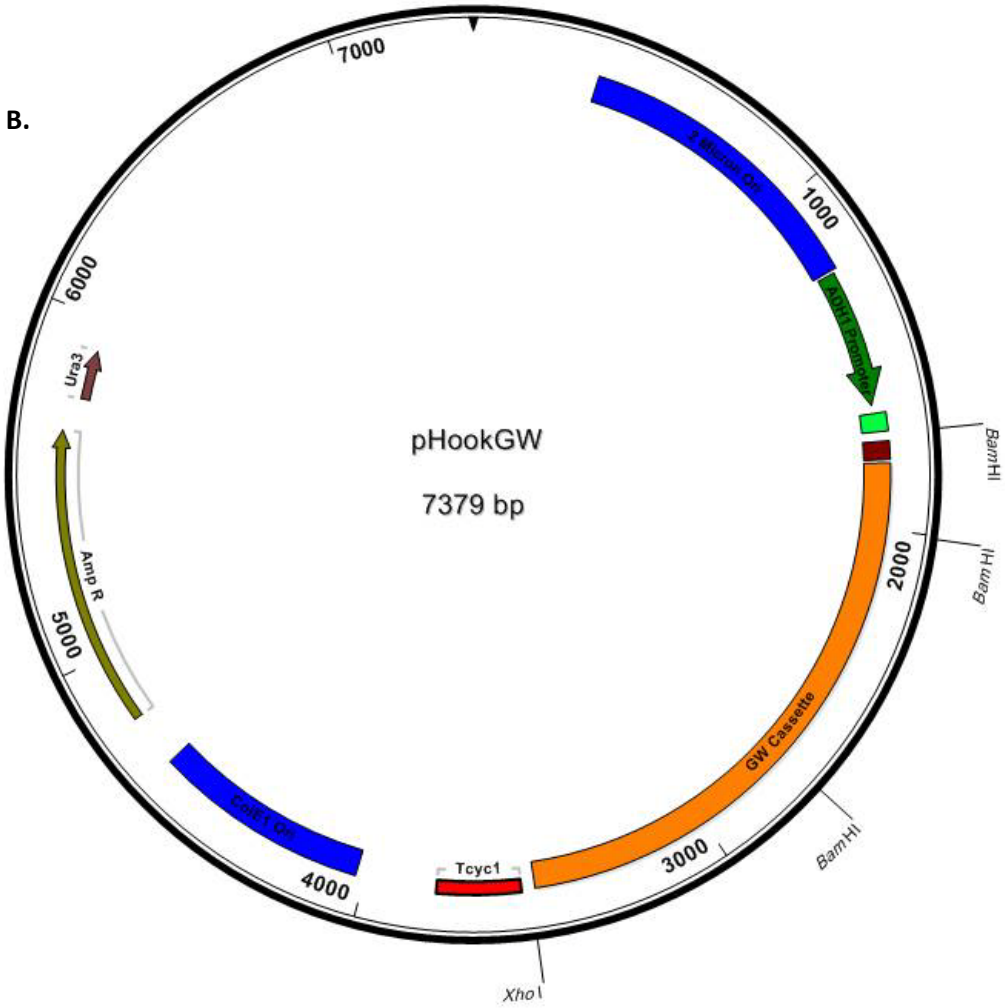


Figure 2-3. Novel three-hybrid system vectors. A. In addition to the TRP1 selection gene, pGBKTetT7GW contains a Gateway[tm] cassette and tetracycline resistance. pHookGW contains the URA selection gene in addition to a Gateway[tm] cassette and a FLAG-tag.

Yeast Three-hybrid Library

The library was created by using known interactors of Mcm10 that were identified in previous yeast two-hybrid screens in which Mcm10 was fused to pGBKT7 as a "bait" to screen an embryo derived cDNA library cloned into the pACT2 vector (Gal activation domain fusion) (Matchmaker^{tm} cDNA library Clonetech) (Table 2-1). By using a library of known interactors, it would narrow the yeast three-hybrid screen since Mcm10 has been known to interact with many proteins.

CG#	Name	Function
10221	Hrd3	Binding
10417		Serine/threonine phosphatase activity
7144	Lysine ketoglutarate reductase	Regulation of histone methylation
5486	Ubiquin-specific protease 64E	Proteolysis
32428		
3318	Dopamine N acetyltransferase (Dat)	Catecholamine metabolic processes
31708		
7023		Ubiquitin-dependent protein catabolic process
15023		
5468	TweedleM	
11378		
3446		Mitochondrial electron transport
6447	TweedleL	
10067	Actin 57B	Cytokinesis
16747	Ornithine decarboxylase antizyme (Oda)	Cell differentiation
1977	α Spectrin	Actin and microtubule binding
7178	Wings up A (wupA)	Muscle development
4609	Failed axon connections (fax)	Axonogenesis
6282		Lipid metabolic process
7611		
2238	Elongation factor 2b (Ef2b)	Mitotic spindle elongation/organization
3297	Minidiscs (mnd)	Leucine import
3209		Metabolic process
3680		Metabolic process
1803	Regulacin	
2614		Metabolic process
2493		Proteolysis
4236	Chromatin assembly factor 1 (Caf1)	Chromatin organization
4738	Nuclear pore protein 160 (Nup160)	
9261	Nervana 2 (nrv2)	Transmembrane transporter activity
4974	Division abnormally delayed (dally)	Organ development
4651	Ribosomal protein L13 (RpL13)	Mitotic spindle elongation/organization
1681		Glutathione transferase activity
1371		Carbohydrate binding
33214		
5476	TweedleN	
34003	Nimrod B3 (nimB3)	
6692	Cysteine proteinase-1 (Cp1)	Autophagic cell death
7490	Ribosomal protein LP0 (RpLP0)	Translation
7109	Microtubule star (mts)	Organelle organization, chromosome segregation
11180		Nucleic acid binding
17654	Enolase	Glycolysis
8938	Glutathione S transferase S1(GstS1)	Response to oxidative stress
8944		Zinc ion binding
14672	Spec2	
14057		tRNA processing, RNA metabolic process
7008	Tudor-SN	RNA interference
5642		

7111	Receptor of activated protein kinase C 1(Rack1)	Wing disc development, oogenesis
6480		
15118		
4625	Dihydroxyacetone phosphate acyltransferase (Dhap-at)	Metabolic process
1683	Adenine nucleotide translocase 2 (Ant2)	ATP/ADP transport
1651	Ankyrin	Cytoskeleton anchoring at plasma membrane
7538	Mcm2	DNA helicase activity
8409	Suppressor of Variegation 205	Chromatin silencing
18013	Psf2	DNA helicase activity
3658	Cdc45	DNA replication, chromosome condensation
3041	Orc2	DNA replication, chromosome condensation
7977	Ribosomal protein L23A (RpL23A)	Translation
	Pol α	DNA replication

Table 2-1. "Prey" library created for the three-hybrid screen. Each were identified previously in a two-hybrid screen using Mcm10 as "bait" and an embryo derived cDNA library cloned into the pACT2 vector.

RESULTS

The two main objectives for this research was to develop a yeast-three hybrid system that is Gateway^{tm} compatible and is capable of screening for third protein interactors using known protein and also to investigate Mcm10 as a facilitator of protein interactions. Mcm10 was cloned into the pHook vector to be used as the "hook" protein and a known protein interactor Psf2 was fused to the Gal binding domain (pGBKTet) to be used as "bait" for the screen. Both were transformed into PJ694-α yeast cells along with the library to screen for "prey" and plated on media lacking histidine. Clones were then streaked onto media containing 5-Fluoroorotic acid (5-FOA) to inhibit pHook which contains the URA gene for selection. In order to determine whether or not the "hook" (Mcm10) is required for the other two proteins to interact, a yeast mating assay was performed. An empty pHook and pHook Mcm10 were separately transformed into PJ694-a yeast cells and were grown in overnight liquid cultures. Overnight cultures of clones from the 5-FOA plate containing only the "bait" (Psf2) and the "prey" in PJ694-α were also grown. In a 96-well plate, 80 ul of the "bait" and "prey" culture and 80 ul of the pHook Mcm10 culture were mixed together and incubated at 30°C for 90 minutes to allow for the PJ694-a and PJ694-α to mate. This was also done with pHook empty and the "bait" and "prey". The mixtures were then plated on media lacking histidine (Figure 2-4 summarizes the protocol). Cells that grew in the presence of an empty pHook and in the presence of pHook Mcm10 indicated that Mcm10 was not required for the other two proteins to interact, but Mcm10 does participate in the interaction. If cells grew only in the presence of pHook Mcm10 and not with pHook empty, this indicated that Mcm10 might be required for the other two proteins to interact. All clones were sequenced to identify the "prey" from the screen (Table 2-2). This procedure was also performed using other "baits" for the screen. These included (in addition to Psf2) Sld5,

Cdc45, And-1, and Mcm2 which are all known interactors of Mcm10. Results from the screens suggest that Mcm10 is involved in numerous ternary protein complexes, but is not necessarily required for the interaction between the "bait" and "prey" (Figure 2-5).

Controls with empty vectors were also done to eliminate the possibility of one-hybrid activity (data not shown). To test one-hybrid activity, each individual pGADT7 plasmid was transformed into PJ694-α yeast. Also, pGBKTet empty was transformed into PJ694-a yeast. The two cultures were incubated to allow for mating similar to the above procedure and plated on media lacking histidine.

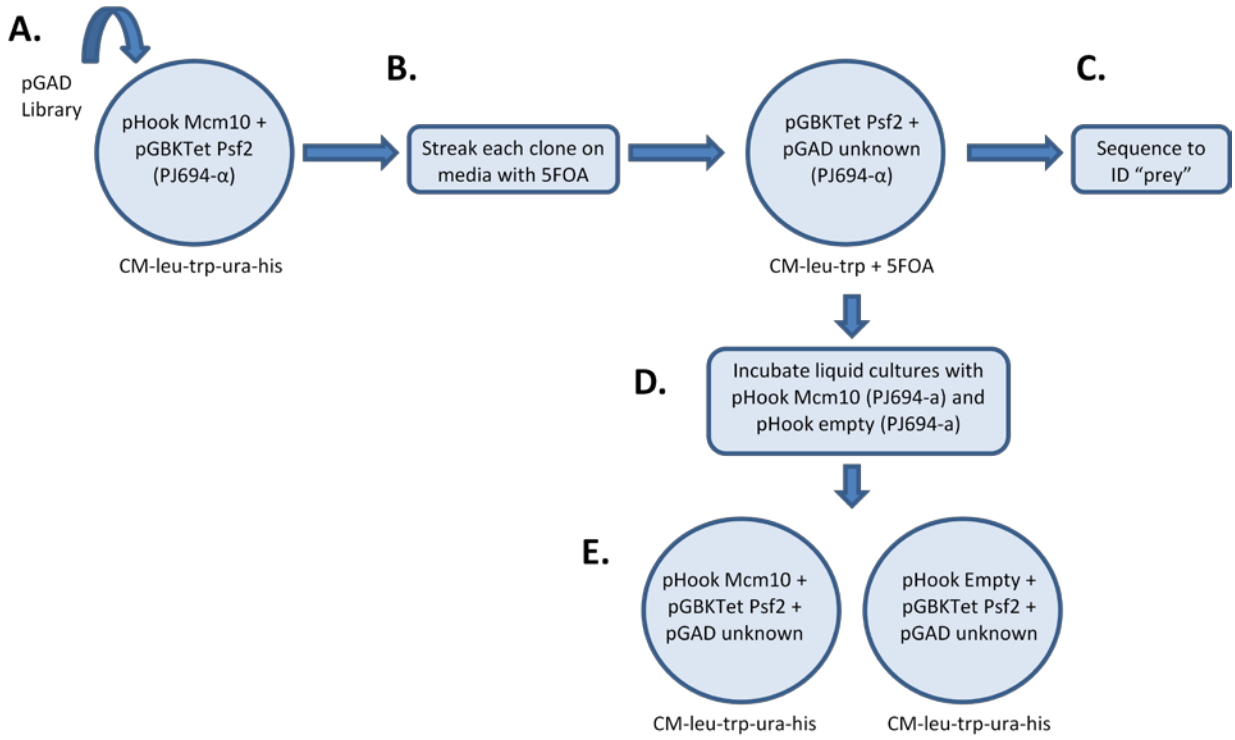


Figure 2-4. Summary of the yeast three-hybrid screen using Mcm10 as the "hook" and Psf2 as "bait" to screen a library of "prey". A. The pGADT7 library of known protein interactors is transformed into PJ694-α yeast cells containing pHook Mcm10 and pGBKTet Psf2. B. Each

clone is streaked onto media with 5FOA to kick out pHook Mcm10. C. Resulting clones are sequenced to identify the "prey". D. Resulting clones are also incubated in liquid with PJ694-a yeast cells containing pHook Mcm10 and also cells containing pHook empty to allow the yeast to mate. E. Cultures are then plated on media lacking histidine to determine whether Mcm10 is required for the other two proteins to interact.

"Bait" Used	"Prey" Identity	Name	Function	Is Mcm10 required?
Psf2	CG5486	Ubp64E	Proteolysis	No
Psf2	CG32428	-----	-----	No
Psf2	CG7111	Rack1	Oogenesis	No
Sld5	CG5642	-----	-----	No
Mcm2	CG4236	Caf-1	Chromatin assembly	No

Table 2-2. "Prey" proteins identified in the yeast three-hybrid screen using Mcm10 as the "hook" and indicated proteins as the "bait".

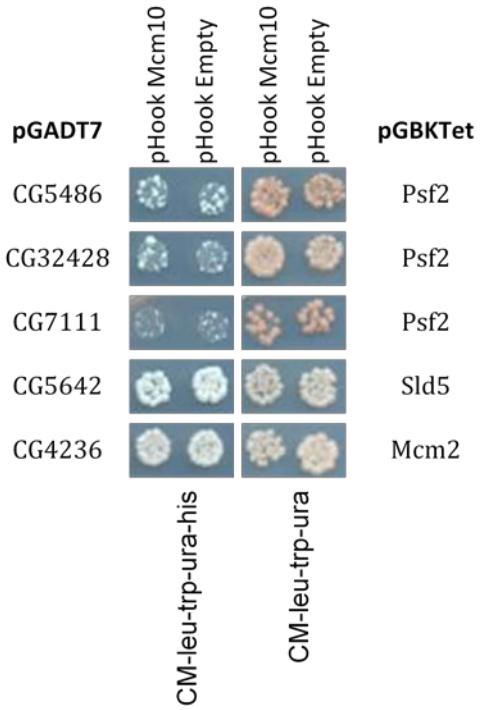


Figure 2-5. Assay to determine whether Mcm10 is required for the "bait" and "prey" interaction. "Bait" (pGBKTet) and "prey" (pGADT7) from the screen which were in PJ694-α yeast were mated with both pHook Mcm10 and pHook Empty which were in PJ694-a yeast. Mated yeast were then plated on media lacking histidine (left panel) and a growth control (right panel). All cultures grew in the absence of Mcm10, indicating Mcm10 is not required.

DISCUSSION

In this study, it is shown that this three-hybrid system can be useful in screening for ternary protein complexes in which the third protein participates but is not required for the other two to interact and may also be useful in identifying interactions in which a third protein is required to stabilize or facilitate an interaction between two others. In particular, we investigated the possibility that Mcm10 is one of these facilitators since it has been previously shown to interact with many proteins. However, the results indicate that Mcm10 may participate in several protein complexes, but is not necessarily required for the formation of these complexes. The question still remains as to whether or not Mcm10 has an essential role in facilitating interactions between other proteins. Although, there is still a large number of protein interactors of Mcm10 that should be used as "bait" in the screen which may yield the hypothesized result. Whether or not Mcm10 turns out to be a facilitator, the system presented could be a useful tool to investigate other proteins.

One of the major advantages of our system is that it is Gateway^{tm} compatible which makes it much more efficient to use in the lab. Investigators that have been using the yeast two-hybrid system can easily upgrade to the three-hybrid system by using our new vectors. The construction of the novel pGBKTet vector is beneficial when isolating the "prey" for identification (Figure 2-4). Originally, both pGBKT7 and pGADT7 contained ampicillin resistance so when isolating the vectors, both the "bait" and the "prey" were being selected. With the new pGBKTet vector containing tetracycline resistance instead of ampicillin, only the "prey" is being selected for. This means that fewer clones would need to be sequenced because the "bait" would be selected against.

Our system not only permits the screening of a cDNA library to identify third protein interactors, it also allows us to determine whether or not the "hook" is essential for the interaction by using a simple yeast mating assay. *S. cerevisiae* can stably exist as a haploid or diploid. Haploid cells can mate with other haploid cells of the opposite mating type (mating type *a* can mate with α and vice versa). Haploid cells respond to the pheromone only of the opposite mating type. This three-hybrid system takes advantage of this process by allowing PJ294-a and PJ694-α cells to mate then select for transcription of the HIS3 reporter gene by plating on media lacking histidine. The alternative would be to transform all three vectors into the yeast then select for transcription of the reporter gene which is not only more time consuming but also more difficult for the yeast to grow.

The three-hybrid system is useful when doing an initial screen for protein interactions that may be of interest in future studies. Therefore, the system is useful as an initial investigation into protein complexes but more follow-up research is required before making conclusions about their signficance.

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