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Modulation of Mcm2-7 activity by Cdt1 suggests novel roles for Cdt1 in the control of DNA replication

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By

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Graduate program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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Modulation of Mcm2-7 activity by Cdt1 suggests novel roles for Cdt1 in the control of DNA replication

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Abstract

Genome duplication occurs once and only once during each cell cycle. It is a highly ordered process and is separated into the formation of different multi-protein complexes. The pre-replicative complex (preRC) is formed during G1-phase and is composed of ORC, Cdc6, Cdt1 and Mcm2-7. Mcm2-7 is the replicative helicase in eukaryotic cells and is assembled on replication origins prior to S-phase. Cdt1 is an essential component of the preRC. Cdt1 has been shown to interact with Mcm2-7, however neither the requirements nor the effects of this interaction have explored. In this study, I show that Cdt1 forms a complex with Mcm2-7 without the need for other factors. Furthermore, Cdt1 modulates the helicase, ATPase and DNA binding activity of the Mcm2-7 complex. I propose a model where Cdt1 modulates Mcm2-7 helicase activity by inhibiting ATP hydrolysis by Mcm2-7, thus preventing premature DNA unwinding. Furthermore, the increase in dsDNA binding affinity for the Mcm2-7/Cdt1 complex (Mcm2-7•Cdt1) assists in its loading onto replication origins during preRC assembly. These results indicate a novel role for Cdt1 in Mcm2-7 modulation and DNA replication control.

Key words: DNA replication, Mcm2-7, helicase, Cdt1, ORC, Cdc6, preRC, Mcm2-7•Cdt1

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Abbreviations

ORC – Origin recognition complex

Mcm – Minichromosome maintenance

ARS – Autonomously replicating sequence

DNA – deoxyribonucleic acid

ssDNA – single stranded DNA

dsDNA – double stranded DNA

ACS – ARS consensus sequence

ATP – adenosine triphosphate

ADP – adenosine diphosphate

CDK – cyclin dependent kinase

preRC – pre-replicative complex

EDTA – Ethylenediaminetetraacetic acid

SDS – Sodium dodecyl sulfate

PAGE – polyacrylamide gel electrophoresis

SV-40 – Simian virus 40

preIC – pre-initiation complex

Chapter 1 – Introduction

1.1 DNA replication in context of the cell cycle

The cell cycle is a series of events that culminate in the production of two cells containing identical genomes (Figure 1.1; Howard and Pelc 1951). The cycle beings with Gap 1-phase (G1-phase) at which time the cell prepares for DNA synthesis (S-phase) followed by cell division (M-phase; Howard and Pelc 1951; Smith and Martin 1973). It is during G1-phase, and the preceding M-phase, where many of the earliest events required for DNA replication occur (Diffley et al. 1995).

During G1-phase, the pre-replicative complex (preRC) assembles on replication origins (Diffley et al. 1995). The preRC complex loads replicative helicases onto origins. Replicative helicases are the proteins responsible for the unwinding of double stranded DNA (dsDNA) at replication forks throughout S-phase (Oakley 1980). Once the assembly of the preRC is complete, the cell is ready to progress into S-phase (Howard and Pelc 1951; Smith and Martin 1973). The origins that were prepared for replication in G1 must now transition from inactive to active forms, a process termed origin firing (Lei and Tye 2001; Araki et al. 2010). It is the firing that defines the beginning of S-phase (Lei and Tye 2001). Firing of origins is a process that involves the recruitment of multiple proteins that assemble at replication origins to form the replicative complex (MacNeill 2010). Post-translational modifications of some of the components of the replicative complex are also required for the start of DNA synthesis (Araki 2010).

The assembly of the preRC can only occur during G1-phase when the levels of two regulatory kinases, cyclin dependent kinase (CDK) and DumbBell forming 4 (Dbf4) dependent kinase (DDK) are low (Piatti et al. 1996; Lei and Tye 2001). CDK and DDK phosphorylate replication proteins to promote the assembly of factors required for helicase activation and DNA synthesis (Piatti et al. 1996; Devault et al. 2008; Tanaka et al. 2007; Muramatsu et al. 2010; Sheu and Stillman 2010). CDK also prevents re-assembly of preRCs by signaling the degradation and/or nuclear export of preRC components (Nguyen et al. 2000). These mechanisms control the timing of DNA replication.

Once DNA synthesis has been completed, the cells enter G2 phase. During G2, the cell continues to grow and prepare for mitosis that will result in two daughter cells. In G2, the DNA content of the cell is double that of G1 phase cells (Reviewed in Norbury and Nurse 1990).

Upon completion of G2, the cell enters mitosis or M-phase (Reviewed in Bucher and Britten 2008) whereby the genome is separated into two complete sets at which point the cell divides, resulting in two genetically identical daughter cells. In many cases, the cell cycle begins again where the M-phase gives way to a new G1-phase.

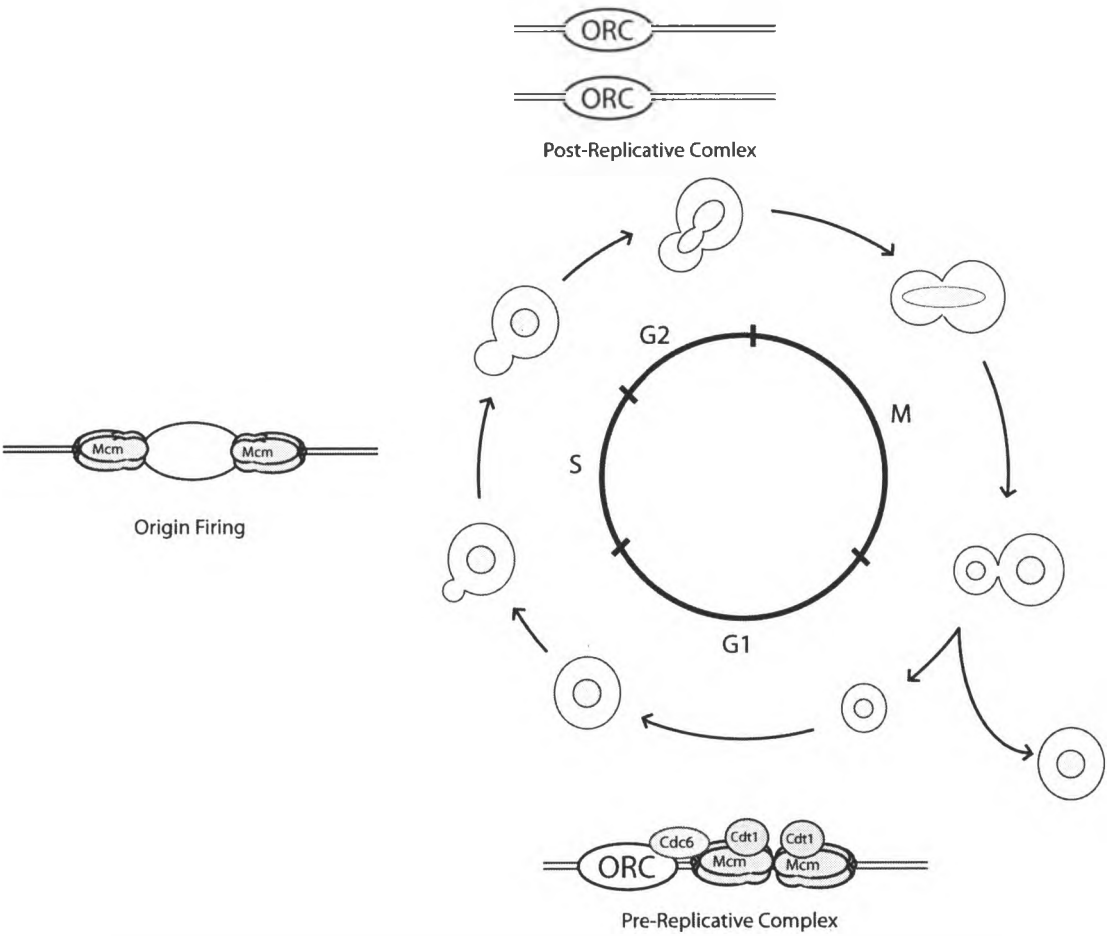

Figure 1.1: Representation of the *Saccharomyces cerevisiae* cell cycle. The cell cycle is divided into distinct phases, G1, S, G2 and M. DNA replication occurs during the S-phase. The yeast initiator protein, the origin recognition complex (ORC), binds origins throughout the cell cycle. The assembly of the preRC occurs primarily throughout G1. DNA unwinding and synthesis starts in S-phase and is completed before the start of G2. (Adapted from http://mpf.biol.vt.edu/research/budding_yeast_model/pp/index.php)

1.2 DNA replication

The mechanisms of DNA replication are highly conserved from eubacteria to eukaryotes (Sclafani and Holzen 2007). For example, an initiator protein binds replication origins throughout the genome nucleating the assembly of factors required for DNA replication (Reviewed in Mott and Berger 2007; Kawakami and Katayama 2010). The initiator along with a helicase loader recruits the replicative helicase to replication origins (Diffley and Cocker 1992; Romanowski et al. 1996). The eukaryotic initiator is termed the origin recognition complex (ORC) which binds origins and recruits the replicative helicases with the assistance of helicase loaders and accessory proteins (Seki and Diffley 2000; Lei and Tye 2001; Bell and Stillman 1992; Li and Herskowitz 1993; Aparicio et al. 1997; Tanaka et al. 1997). In all eukaryotes, the proteins required for unwinding and elongation must be found at the origins prior to initiation of DNA synthesis (Reviewed in Bell 2002).

1.3 Origins of Replication

Origins of replication are sites in the genome upon which replication machinery assembles prior to genome duplication and where DNA synthesis initiates (Huberman and Riggs 1966; Chan and Tye 1980). Not every origin of replication is used in each cell cycle, nor do they fire at the same time (Wyrick et al. 2001).

Replication origins in eubacteria are defined by a specific nucleotide sequence that appears only once in the genome (Marsh and Worcel 1977). This specific sequence is important for the function of the prokaryotic origin: providing the initiator proteins a site

to recognize and bind in the origin (Marsh and Worcel 1977; Meijer et al. 1979; Messer et al. 1979; Chakraborty et al. 1982).

Saccharomyces cerevisiae also utilizes a specific nucleotide sequence as a replication origin, however, a single origin is no longer sufficient, and the genome contains 429 predicted origins (Petes et al. 1974; Wyrick et al. 2001). Origins of replication in *S. cerevisiae* are referred to as autonomously replicating sequences (ARS). All ARS elements are defined by an 11-bp region termed the ARS consensus sequence (ACS or A; Cannon et al. 1990). The ACS, along with another nucleotide consensus sequence termed the B1 element, make up the core of the replication origin, marking the binding sites for ORC (Marahrens and Stillman 1992; Diffley and Cocker, 1992). Along with these two elements, other sequence elements are also present within 100 bp of some ARS elements, but are not essential for function (Marahrens and Stillman, 1992; Reviewed in Bell, 1995). Not all genomic regions containing the ACS sequence are used as origins of replication.

In the yeast *Schizosaccharomyces pombe,* there is limited sequence similarity from origin to origin throughout the genome, with a weak consensus sequence (Dubey et al. 1994; Clyne and Kelly 1995; Okuno et al. 1997; Kim and Huberman 1998). Regions composed of AT-rich stretches serve as ORC binding sites (Chuang and Kelly 1999; Moon et al. 1999; Kong and DePamphilis 2001).

In metazoans, the importance of nucleotide sequence specificity is reduced despite a greater number of origins to cope with the increased size of the genome

(Huberman and Riggs 1966). The search for discrete origins of replication in metazoan cells has yielded no defined conserved nucleotide sequence.

Metazoan replication origins that have been identified are zones of initiation, rather than discrete sequences, and are prevalently in close proximity to actively transcribed genes (Kitsberg et al. 1993; Dijkwel et al. 2002). DNA in cells exists as a protein-DNA structure termed chromatin which typically exists in two forms, namely protein accessible euchromatin and tightly packed heterochromatin (Reviewed in Khorasanizadeh 2004). This localization of origins is most likely due to the euchromatic status of these regions allowing replication machinery access to the DNA (MacAlpine et al 2004; Audit et al. 2009; Field et al. 2008; Zhou et al. 2005). Regions of the genome, termed heterochromatin, are thought to be inaccessible to replication machinery, however, replication in these regions must still occur for complete faithful transmission of genetic material (Cayrou et al. 2010). Evidence has demonstrated the need for chromatin remodeling in heterochromatin for the assembly of replication machinery in these regions (Collins et al. 2002; Cayrou et al. 2010).

Therefore, *S. cerevisiae* origins of replication are atypical in eukaryotes due to their sequence specificity.

1.4 The pre-replicative complex

The pre-replicative complex (preRC) is a multi-protein complex that assembles at replication origins during G1-phase (Diffley et al. 1994; Diffley et al. 1995; Cocker et al.

1996; Romanowski et al. 1996; Donovan et al. 1997). Assembly of the preRC culminates with the loading of the replicative helicase onto replication origins. The replicative helicase, termed the minichromosome maintenance 2-7 (Mcm2-7) complex, is responsible for the unwinding of duplex DNA during genome duplication (Reviewed in Bochman and Schwacha 2009). The loading of the replicative helicase is a process that must be completed prior to initiation of S-phase during the cell cycle. The first step of preRC assembly is the binding of the initiator, ORC (Bell and Stillman 1992; Diffley et al. 1994). ORC is bound to replication origins throughout the cell cycle and during G1 recruits other factors to origins to assemble the preRC (Diffley et al. 1994; Diffley et al. 1995; Cocker et al. 1996; Romanowski et al. 1996; Donovan et al. 1997). Mcm2-7 is loaded onto replication origins in an ATP-dependent manner and requires ORC, Cdc6 and Cdt1 (Figure 1.2; Cocker et al. 1996; Romanowski et al. 1996; Donovan et al. 1997; Bowers et al. 2004; Randell et al. 2006; Kawasaki et al. 2006; Remus et al. 2009; Evrin et al. 2009). Many Mcm2-7 complexes are loaded onto any single origin. In fact, there seems to be 10 to 40-fold excess Mcm2-7 complexes per origin (Lei et al. 1996; Edwards et al. 2002).

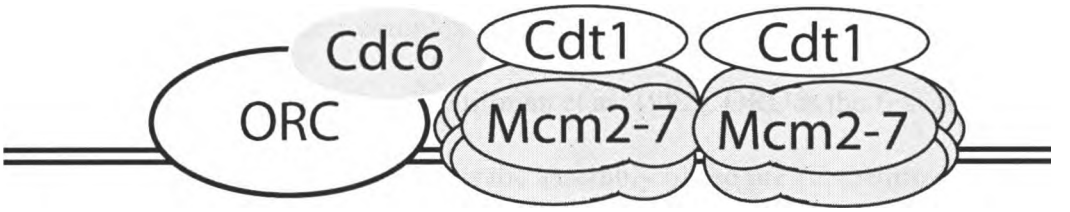

Figure 1.2: Schematic representation of the pre-replicative complex. The preRC is formed on double stranded DNA (Tanaka and Nasmyth 1998; Dimitrova et al. 1999; Costa and Onesti 2008). The minimal system requirements of ORC, Cdc6, Cdt1 and Mcm2-7 are shown. The Mcm2-7 complex, along with Cdt1, dimerizes upon helicase loading (Remus et al. 2009; Evrin et al. 2009).

1.5 Origin Recognition Complex

ORC is a multimeric complex that binds to replication origins scattered throughout an organism's genome (Stillman et al. 1992). ORC is the first factor present at the origins of replication and regulates the assembly of the preRC (Stillman et al. 1992; Diffley and Cocker 1992; Diffley et al. 1994).

ORC is composed of six subunits named sequentially Orc1-6 by their gel electrophoresis migration, from slowest to fastest. *S. cerevisiae* ORC (ScORC) recognizes the ACS and B1 elements of replication origins (Stillman et al. 1992; Diffley and Cocker 1992), while *S. pombe* ORC (SpORC) recognizes AT-rich regions of the genome, through an AT-hook motif on the Orc4 subunit (Chuang and Kelly 1999; Kong and DePamphilis 2001). The non-specific nature of SpORC binding likely explains why fission yeast do not have replication origins that are easily defined by sequence. ORC homologues are found in higher eukaryotes and all share the origin binding function of yeast ORC, but differ in the mechanisms by which the binding is achieved (Gossen et al. 1995; Carpenter et al. 1996; Quintana et al 1997). Unlike ScORC, ORC found in metazoans does not have a strong sequence requirement for DNA association (Muzi-Falconi and Kelly 1995). Other mechanisms may be responsible for ORC-origin interaction beyond nucleotide recognition. A conserved domain within Orc1, termed the bromo-adjacent homology domain, may be in part responsible for the interaction of ORC with chromatin, rather than a specific DNA sequence (Callebaut et al. 1999). In budding yeast, this domain has been implicated in ScORC-origin association independent of the ACS (Callebaut et al. 1999; Müller et al. 2010).

The concept of ORC's role in the assembly of the preRC has evolved from being a simple landing pad to an active participant in the loading reaction (Stillman et al. 1992; Bell and Stillman 1992; Reviewed in Diffley 1996; Bowers et al. 2004; Speck et al. 2005). ORC utilizes ATP to perform its function with the ATPase activity being essential for cell viability (Bowers et al. 2004).

ATP binding by ORC is essential for origin recognition (Klemm et al. 1997; Speck et al. 2005). Cdc6 associates with the ATP-bound form of ORC on origins of replication (Bowers et al. 2004). ORC-dependent ATP hydrolysis by Cdc6 drives Mcm2-7 origin loading in a Cdt1-dependent manner (Randell et al. 2006). Next, ATP hydrolysis by ORC is thought to release Cdc6 and Cdt1 from the origin (Randell et al. 2006; Kawasaki et al. 2006; Tsakraklides and Bell 2010). This hydrolysis event frees ORC to bind a new ATP molecule and drives another round of Mcm2-7 loading (Bowers et al. 2004; Randell et al. 2006). Recent observations suggest that Cdt1 may not be released from Mcm2-7 upon loading, but rather is no longer associated with ORC or Cdc6 (Remus et al. 2009). Interestingly, most evidence points to ScORC on origins throughout the cell cycle only briefly being displaced during synthesis in budding yeast (Diffley and Cocker 1992; Bell and Stillman 1992; Tsakraklides and Bell 2010). The temporary release of ORC from origins appears to be Cdc6-dependent (Bowers et al. 2004). Once the cell has entered S-phase the levels of Cdc6 are diminished (Drury et al. 2000; Kim et al. 2007). Therefore, ORC no longer needs to be released for reiterative Mcm2-7 loading once in S-phase and no longer displaces from the origin (Randell et al. 2006).

In mammalian cells, Orc1 binds chromatin in a cell cycle-dependent manner (McNairn et al. 2005). Orc1 dissociates from the origins once the cell has entered S-phase, at which point it is ubiquitinated, followed by its degradation (Natale et al. 2000; Fujita et al. 2002; Méndez et al. 2002; Sugimoto et al. 2009). The dissociation of Orc1 from replication origins is another form of replication control preventing the assembly of the preRC during the rest of the cell cycle.

ORC has functions in the cell other than those required for preRC assembly (Micklem et al. 1993; Foss et al. 1993; Bell et al. 1993; Fox et al. 1995; Loo et al. 1995; Bell et al. 1995; Triolo and Sternglanz 1996; MacAlpine et al. 2010). In yeast, ORC has been found to recruit factors responsible for gene silencing to sites containing the ACS (Micklem et al. 1993; Foss et al. 1993; Bell et al. 1993; Palacios DeBeer et al. 1999; Sutton et al. 2001; Rusché and Rine 2001; Sharp et al. 2003). The ORC subunits shown to be directly involved in silencing are Orc1, Orc2 and Orc5, with the whole ORC complex presumably taking part (Micklem et al. 1993; Foss et al. 1993; Bell et al. 1993; Fox et al. 1995; Loo et al. 1995; Bell et al. 1995; Triolo and Sternglanz 1996). Orc1 is also present at centrosomes and controls their copy number (Reviewed in Sasaki and Gilbert 2007; Hemerly et al. 2009).

1.6 Cell Division Cycle 6 (Cdc6)

Cdc6 is required for Mcm2-7 loading and was first identified in *S. cerevisiae* as essential for cell cycle progression (Lisziewicz et al. 1988). Cdc6 homologues exist from yeast to humans (Kawarabayasi et al. 2001; Coleman et al. 1996; Tugal et al. 1998; Saha

et al. 1998). Cdc6 is an ATPase, but requires interaction with ORC for hydrolysis activity (Speck et al. 2005). The ATP site of Cdc6 is essential for Mcm2-7 assembly at origins as described above and has been implicated in ORC stability and site selectivity on replication origins (Perkins and Diffley 1998; Mizushima et al. 2000; Randell et al. 2006).

1.7 Mcm2-7

DNA polymerases, enzymes responsible for DNA synthesis, use single stranded DNA as a template (Mitra and Kornberg 1966). However, DNA is typically double stranded and requires replicative helicases to melt the two strands (Reviewed in Coleman and Oakley 1980). Helicase function depends on its ability to utilize nucleotide hydrolysis as a source of energy to unwind the double helix (Hoffmann-Berling 1982). The replicative helicase in eukaryotic cells is Mcm2-7 (Labib et al. 2000; Labib and Diffley 2001, Takahashi et al. 2005; Forsburg 2008; Bochman and Schwacha 2009). Unlike other replicative helicases found in Archaea, eubacteria and viruses, which are homohexamers, the Mcm2-7 complex is a heterohexamer (Donmez and Patel 2006; Baily et al. 2007, Kelmen et al. 1999; Chong et al. 2000; Fletcher et al. 2003). The Mcm2-7 heterohexamer has the ability to bind and hydrolyze ATP and can be reconstituted from recombinant proteins (Lee and Hurwitz 2000; Schwacha and Bell 2001; Davey et al. 2003). The ATP hydrolysis activity is located in a 200-residue stretch that is conserved throughout the Mcms, termed the Mcm box (Reviewed in Koonin 1993). DNA binding

and unwinding are controlled through ATP binding and hydrolysis, respectively (Stead et al. 2009; Bochman et al. 2008; Bochman and Schwacha 2007, 2010).

ATP binding and hydrolysis is essential for Mcm2-7 helicase activity, however, the mechanism of DNA unwinding has not been determined. The six subunits of the complex form a ring that contains a central channel that is large enough to accommodate dsDNA (Brewster et al. 2008). Whether dsDNA or ssDNA travels through the channel is an area of study and there exist several models explaining how Mcm2-7 unwinds DNA. One such model has ssDNA passing through the central channel with the other strand passing on the outside of the hexameric ring (Figure 1.3B; Reviewed in Bochman and Schwacha 2009). Another related hypothesis has dsDNA entering the central channel with one strand exiting through a side channel while the first transits on through the centre (Figure 1.3C; Bochman et al. 2009). It is also possible that Mcm2-7 unwinds DNA not directly at the replication fork but at a distance, anchored to a structure, potentially the nuclear membrane. From there, Mcm2-7 proceeds to unwind DNA by changing the torsional strain on the duplex (Figure 1.3D). This model provides an explanation for observations in mammalian cells that localize Mcm2-7 away from the replication fork during DNA synthesis, termed the Mcm paradox (Reviewed in Laskey and Madine 2003). However, Mcm2-7 is at the replication fork in other organisms. In experiments in human cells it is possible that the signal from excess Mcm proteins found away from the fork drowns out the signal of those at the fork (Aparicio et al. 1997; Edwards et al. 2002, Takahashi et al. 2005). In another model, Mcm2-7 might be a drive motor for what is referred to as a ploughshare, which separates the two DNA strands (Figure 1.3E).

However, Mcm2-7 unwinds DNA without any other factors involved, making the ploughshare model less likely (Bochman and Schwacha 2008). Lastly, the Mcm2-7 complex may act in the same fashion as described for the SV 40 T-antigen helicase (Bochman and Schwacha 2008). In this model, Mcm2-7 dimerizes and threads dsDNA through its central core, with the ssDNA being released from the dimer interface or the side channels (Figure 1.3F, side channels depicted in A; Bochman and Schwacha 2008).

Cells have a pattern of regulated and coordinated events that load Mcm2-7 at the proper time and place, preventing inappropriate loading of Mcm2-7 onto replication origins (Blow and Dutta 2005). Mcm2-7 is held inactive at the origins once it has been loaded so as not to unwind DNA prematurely (Reviewed in Bochman and Schwacha 2009). Activation of Mcm2-7 is a multi-step process that includes post-translational modifications and the association of other factors such as Cdc45 and the GINS complex, (Japanese for 5 (Go), 1 (Ichi), 2 (Ni) and 3 (San) corresponding to Sld5, Psf1, Psf2 and Psf3; Takayama et al. 2003; Ilves et al. 2010).

Mcm2-7 complexes have also been implicated in other cellular functions due to their high copy number and observations that reduction of protein levels do not alter DNA replication (Todorov et al. 1998; Lei et al. 1996; Oehlmann et al 2004; Burkhart et al 2005; Crevel et al. 2007). The excess Mcm2-7 is required during times of replicative stress (Woodward et al. 2006; Ge et al. 2007; Ibarra et al. 2008). Mcm2-7 is also required for transcription regulation and chromatin remodeling (Zhang et al. 1998; Snyder et al. 2005; Holland et al. 2002; Snyder et al. 2009).

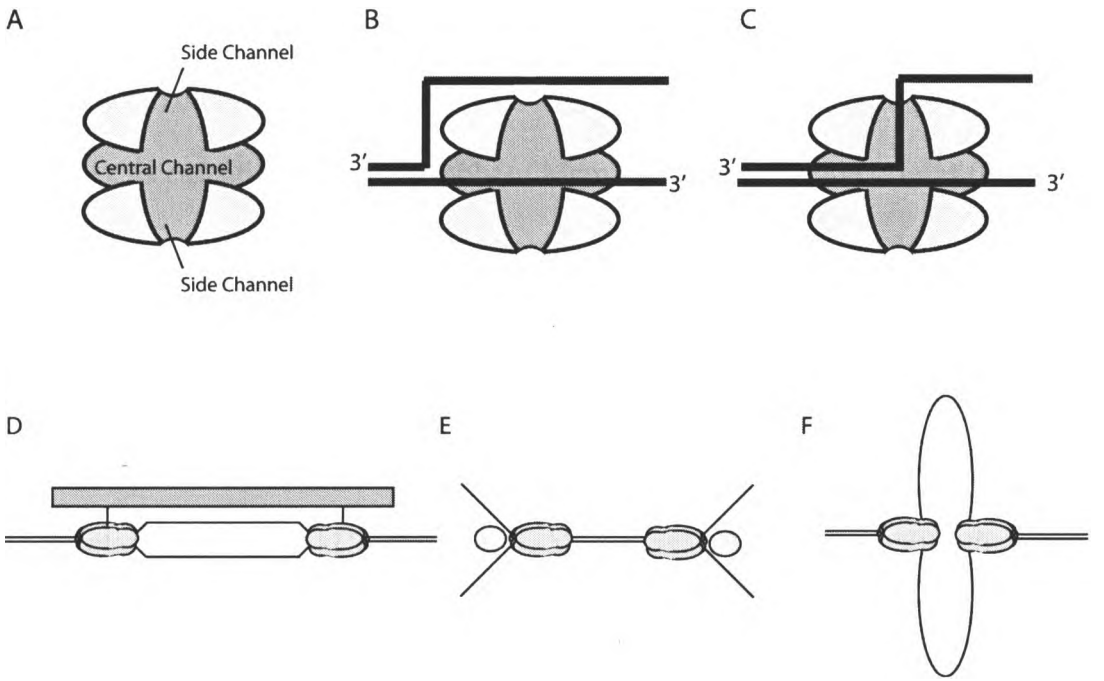

Figure 1.3: DNA unwinding models for Mcm2-7. (A-C; strand exclusion model) The Mcm2-7 complex is shown in cross-section from the side with only 3 subunits of the heterohexamer shown. (A) The Mcm2-7 complex is predicted to contain a central channel and side channels. (B) Only one strand enters the central channel with the other strand never entering the protein complex. The two DNA strands are separated by the entire protein complex. (C) Both DNA strands enter the central channel with one of the DNA strands exiting through one of the side channels. The separation of the two DNA strands occurs on the inside of the Mcm2-7 complex. (Adapted from Bochman et al. 2009). (D-F; the other Mcm2-7 models) (D) Mcm2-7 unwinds DNA at a distance from the origin of replication by being tethered to a scaffold, and changing the torsional strain on the DNA to create regions that melt. (E) Ploughshare model. Mcm2-7 acts as a drive motor that pulls along another component that separates the two strands of DNA. (F) T-antigen model. The Mcm2-7 complexes unwind DNA with dsDNA entering the central channel and exiting as a single stranded loop.

1.8 Cdt1

Cdc10-dependent transcript 1 (Cdt1), a protein component of the pre-replicative complex, is essential for the assembly of Mcm2-7 onto replication origins (Hofmann and Beach 1994; Nishitani et al. 2000; Maiorano et al. 2000). Cdt1 homologues have been found in all eukaryotes, from yeast to humans, and in all cases, Cdt1 is essential for Mcm2-7 loading (Hofmann and Beach 1994; Devault et al. 2002; Maiorano et al. 2000; Whittaker et al. 2000). Budding yeast Cdt1 (ScCdt1) is a 604 a.a. protein with a molecular weight of 68 kDa. Cdt1 has no known catalytic function unlike the other preRC components. Mammalian Cdt1 contains at least two domains, an N-terminal domain required for geminin interaction and DNA binding as well as a C-terminal domain required for interaction with Mcm2-7 (Yanagi et al. 2002). It is still unclear what mechanistic role Cdt1 plays within the context of the preRC (Hoffmann and Beach 1994; Maiorano et al. 2000; Nishitani et al. 2000, 2001; Yanow et al. 2001; Devault et al. 2002).

Cdt1 interacts directly Mcm2-7 in all organisms studied (Yanagi et al 2002; Cook et al. 2004; Teer and Dutta 2008; Wei et al. 2010; Jee et al. 2010). This interaction has been examined through co-purification, yeast two-hybrid assays, sucrose gradient sedimentation and x-ray crystallography (Kawasaki et al. 2004; Teer and Dutta 2008;You and Masai 2008; Remus et al. 2009; Wei et al. 2010; Jee at al. 2010). In mammals, the interaction is mediated through the C-terminal tail of Cdt1, at least in the context of Mcm6 (Teer and Dutta 2008; Jee et al. 2010; Wei et al. 2010). This region of Mcm6 contains a helix turn helix motif that binds to Cdt1 through residues Glu – 757, 763 and

766 (Wei et al. 2010). There is some evidence for Mcm2 to take part in this interaction (You and Masai 2008). The observation that Cdt1 co-purifies with Mcm2-7 from cell extracts in *Xenopus* and *S. cerevisiae* is indicative of complex formation *in vivo* (Kawasaki et al. 2006; Teer and Dutta 2008; Remus et al. 2009; Evrin et al. 2009). Further support for a Mcm2-7+Cdt1 complex (Mcm2-7•Cdt1) comes from co-dependent nuclear localization of Mcm2-7 and Cdt1 in budding yeast (Tanaka and Diffley 2002).

Interestingly, reconstitution of the pre-replicative complex using pure proteins required the Mcm2-7•Cdt1 complex (Remus et al. 2009; Evrin et al. 2009). When preRC assembly was attempted with recombinant Cdt1 and Mcm2-7 added separately, the preRC could not be reconstituted (Remus et al. 2009). This observation led Remus et al. (2009) to propose that an accessory factor could potentially be required for the assembly of the Mcm2-7•Cdt1 complex prior to loading. The activity of Mcm2-7 may be altered by the presence of Cdt1 upon complex formation that allows it to form the preRC.

Several functional sub-complexes of Mcm2-7 have been described *in vitro* including Mcm4/6/7, which gave insight into the activity of the Mcm2-7 helicase complex (Ishimi 1997; Lee and Hurwitz 2001; You and Masai 2005; Stead et al. 2009). Stimulation of helicase activity was observed when Cdt1 was added to the Mcm4/6/7 complex (You and Masai 2008). Mcm4/6/7 is not sufficient *in vivo*, Mcm2, Mcm3 and Mcm5 are also required (Pacek et al. 2004; Pacek et al. 2006). The effect of Cdt1 on Mcm2-7 may be different than that on Mcm4/6/7 (Reviewed in Bochman and Schwacha 2009). The ability to reconstitute helicase activity with Mcm2-7 *in vitro* provides a tool to study the effect of Cdt1 on Mcm2-7 activity directly, and answer many questions as to

the function of Cdt1 in the preRC (Bochman and Schwacha 2008). It is this aspect of Cdt1 function that this thesis addresses by ascertaining the modulatory effect of Cdt1 on Mcm2-7.

1.9 Preventing Re-replication

To prevent re-replication the preRC can only form during the G1-phase (Reviewed in Lei and Tye 2001; Labib 2010). One of the hallmarks of the transition between the G1-phase and S-phase is the accumulation of active B-type cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK; Lei and Tye 2001). The preRC transitions into the pre-initiation complex (preIC) which is composed of at least Sld2, Sld3, Dbp11, Cdc45, GINS, and Mcm2-7 and are all essential for the initiation of DNA synthesis (Reviewed in Teer and Dutta 2006). All but Sld2, Sld3 and Dbp11 are also required for elongation (Teer and Dutta 2006). CDK is responsible for phosphorylating Sld2 and Sld3, which are both required for preIC assembly (Masumoto et al. 2002; Tanaka et al. 2007; Zegerman and Diffley 2007). The phosphorylated forms of Sld2 and Sld3 interact with Dbp11 (Masumoto et al. 2002; Tanaka et al. 2007; Zegerman and Diffley 2007). This CDK-dependent interaction is what recruits Cdc45 to replication origins (Aparicio et al. 1999; Tercero et al. 2000; Zou and Stillman 2000; Tanaka et al. 2007; Zegerman and Diffley 2007). These events then lead to the interaction of GINS with Mcm2-7 and Cdc45 (Tanaka et al. 2007; Muramatsu et al. 2010).

Levels of active Dbf4-dependent kinase also rise at the G1/S-phase transition. DDK is responsible for the activation of Mcm2-7 by phosphorylating Mcm4, which

relieves helicase inhibition (Lei et al. 1997; Geraghty et al. 2000; Sheu and Stillman 2010). DDK is also required for the recruitment of Cdc45 to the replication origins (Yabuuchi et al. 2006). Some evidence suggests that Mcm2 is also an essential target of DDK (Bruck and Kaplan 2009). This conclusion is drawn from the observation that the expression of a phosphodefective Mcm2 causes lethality, however, overexpression of Mcm2 was required to observe this phenotype (Bruck and Kaplan 2009).

In addition to its role in initiating DNA replication, CDK also plays an integral role in preventing re-replication through the phosphorylation of the preRC components (Broek et al. 1991; Dahmann et al. 1995). The differing strategies that cells employ to prevent DNA re-replication are summarized in Figure 1.4. The signal to degrade is typically mediated through a poly-ubiquitination pathway through the action of E3 ubiquitin ligases (Kim and Kioreos 2007, 2008). These ligases are Cul4 in all metazoans, while the Skp2-mediated pathway is only observed in humans (Li et al. 2003; Nishitani et al. 2006; Kim and Kipreos 2007).

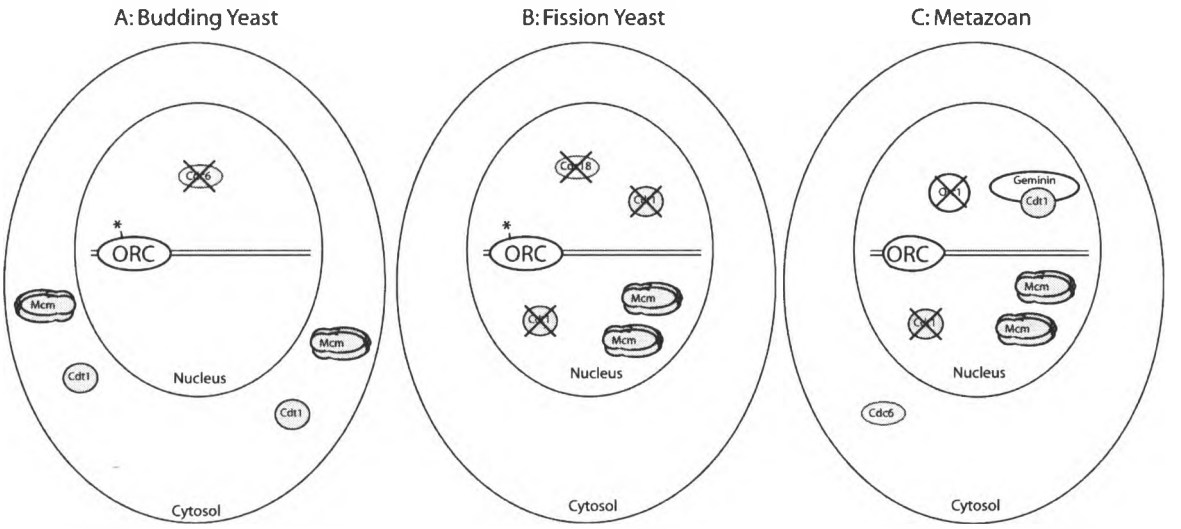

Figure 1.4: Differing strategies for preventing re-replication. A schematic illustrating the state of the preRC proteins post replication (A) *S. cerevisiae* targets Cdc6 for degradation and Cdt1 for export through CDK modifications. (B) *S. pombe* targets both Cdc18 (Cdc6) and Cdt1 for degradation in a CDK dependent manner. (C) Metazoan Cdc6 is exported from the nucleus in a CDK dependent manner. Cdt1 is both degraded through CDK phosphorylation and subsequent poly-ubiquitination. Cdt1 is also sequestered by geminin. Orc1 is phosphorylated by CDK, which dissociates it from chromatin. (Figure adapted from Feng and Kipreos 2003)

Cdc6

In budding and fission yeast, phosphorylation of Cdc6 by CDK targets Cdc6 for poly-ubiquitination and subsequent degradation, thereby preventing further Mcm2-7 loading (Kominami et al. 1997; Wolf et al. 1999; Drury et al. 2000; Kim et al. 2007). In *Caenorhabditis elegans,* Cdc6 is phosphorylated on multiple CDK sites at the N-terminus (Kim et al. 2007). This modification is essential for the nuclear export of Cdc6 at the onset of S-phase (Kim et al. 2007). Interestingly, this phosphorylation event is Cul4-dependent a reverse of the typical CDK modification followed by a Cul4 modification of a target protein (Kim et al. 2007). In vertebrates, phosphorylation of Cdc6 by CDK also causes its exclusion from the nucleus without directly leading to its degradation (Saha et al. 1998; Fujita 1999; Jiang et al. 1999; Petersen et al. 1999; Delmolino et al. 2001; Luo et al. 2003; Alexandrow and Hamlin 2004; Crevel et al. 2005). This phosphorylation of Cdc6 prevents its reassociation with preRC components (Mimura et al. 2004; Honey and Futcher 2007).

ORC

ORC subunits are also phosphorylated by CDK to prevent re-replication. In budding yeast, Orc2 and Orc6 are targets of CDK (Nguyen et al. 2001). This modification prevents further Mcm2-7 loading, yet, it does not alter the ability of ORC to bind DNA (Nguyen et al. 2001; Feng and Kipreos 2003). How phosphorylation of ORC is reversed so as to permit the next round of preRC formation has yet to be determined.

Mammalian Orc1 is modified by CDK which decreases the affinity of Orc1 for chromatin (Mendez et al. 2002). Upon phosphorylation, Orc1 dissociates from the rest of the ORC complex and from chromatin, in effect preventing further preRC formation (Mendez et al. 2002, Feng and Kipreos 2003). The modification by CDK is a prerequisite for Skp2-mediated poly-ubiquitination of Orc1 (Mendez et al. 2002, Feng and Kipreos 2003). When the cell transitions from M-phase to G1-phase, the preRC can be reformed on replication origins with newly-synthesized Orc1 (Mendez et al. 2002; Li et al. 2004; Reviewed in Blow and Dutta 2005).

Mcm2-7

Mcm2-7 is loaded onto DNA during G1-phase, however, the amount of Mcm2-7 is in great excess compared to the number of origins in all cells tested (Lei et al. 1996; Edwards et al. 2002; Fujita et al. 2002; Ghaemmaghami et al. 2003). In *S. cerevisiae,* Mcm2-7 complexes that are not involved in DNA unwinding are exported out of the nucleus after the transition from G1 to S-phase in a CDK-dependent manner (Labib et al. 1999). Export prevents promiscuous loading of Mcm2-7 onto origins that have already been activated (review Aparicio et al. 2006; Labib and Gambus 2007). Interestingly, *de novo* translated Mcm proteins are preferentially imported at the onset of G1 (Braun and Breeden 2007).

In mammalian cells, some data suggest that the phosphorylation of Mcm3 by CDK may decrease its ability to form a complex with the other Mcms (Lin et al. 2008).

The implications of this phosphorylation are still being explored, though it may be required for the cells to enter M-phase (Lin et al. 2008).

Cdt1

In budding yeast cells, Cdt1 is exported from the nucleus once it has been phosphorylated by CDK during the transition from G1 to S phase (Tanaka and Diffley 2002). This export prevents further Mcm2-7 loading until the next cell cycle whereby Cdt1 is either synthesized *de novo* or imported back into the nucleus (Kim and Kipreos, 2007). In all other systems examined, including fission yeast and mammals, Cdt1 is rapidly degraded during the onset of S-phase (Nishitani et al. 2000; Feng and Kipreos 2003).

Degradation of Cdt1 is a central theme that runs throughout metazoans. For instance, studies in human cancer cell lines have shown that overexpression of Cdt1 induces genome instability caused by re-replication (Nishitani et al. 2004; Tatsumi et al. 2006; Liu et al. 2007; Sugimoto et al. 2008). Re-replication can only be observed through overexpression, most likely due to the redundancy of the control mechanisms behind helicase loading, such as the Cdt1 inhibitor protein geminin (McGarry and Kirschner 1998; Wohlschlegel et al. 2000; Tatsumi et al. 2006).

Geminin binds tightly to Cdt1 and regulates its availability to promote preRC assembly (McGarry and Kirschner 1998; Wohlschlegel et al. 2000; Tada et al. 2001; Lee et al. 2004). As cells transition from G1-to S-phase, geminin accumulates in the nucleus and binds Cdt1. Once the cell begins to transition from mitosis, the levels of geminin

begin to diminish, thereby allowing preRC formation (Tada et al. 2001). A *S. cerevisiae* geminin homologue has not yet been identified.

In metazoans, phosphorylation of Cdt1 by CDK promotes poly-ubiquitination of Cdt1 by Cul4 E3 ligase targeting it to the lysosome (Zhong et al. 2003; Liu et al. 2004; Nishitani et al. 2006; Kim and Kipreos 2007). Therefore, once the cell transitions from the G1 phase, most of the Cdt1 is exported from the nucleus and degraded (Jin et al. 2006; Arias and Walter 2006). Human cells have another Cdt1 degradation pathway that appears to be unique compared to other metazoans (Li et al. 2003). This pathway, like the Cul4 pathway in yeast, is a poly-ubiquitination pathway that is mediated through Skp2, an E3 ligase, within the Skp2 Cullin F-box (SCF) complex (Liu et al. 2004; Sugimoto et al. 2004). It is still unclear why human cells contain two very similar pathways for Cdt1 degradation, possibly another form of redundant DNA replication control.

1.10 Thesis overview

Multiple proteins must assemble in the correct place at the right time for proper preRC assembly. Many redundant mechanisms prevent wanton preRC formation. Many of these mechanisms focus on the availability of Cdt1 at origins of replication.

Due to its central role in the assembly and regulation of the preRC, I set out to determine the role of Cdt1 within the pre-replicative complex. I hypothesized that Cdt1 modulates the activity of Mcm2-7 to create a functionally distinct complex from that of Mcm2-7. If this were the case, this would be a new role for Cdt1 during the assembly of the preRC.

To test my hypothesis, I reconstituted the Mcm2-7•Cdt1 complex and assessed the activities of this complex compared to Mcm2-7 alone. I discovered that indeed the Mcm2-7•Cdt1 complex is functionally distinct from Mcm2-7 alone, with Cdt1 decreasing the ATP hydrolysis and DNA unwinding activities of Mcm2-7. Furthermore, Mcm2-7•Cdt1 showed increased dsDNA binding affinity relative to Mcm2-7 alone. These results suggest that Cdt1 modifies the activity of the Mcm2-7 complex to allow Mcm2-7 to load onto dsDNA. It also reveals a novel helicase regulation mechanism by Cdt1 that prevents premature unwinding at replication origins prior to S-phase.

Chapter 2 - Materials and Methods

2.1 Purification of Mcms

Mcm2, Mcm4, Mcm5, Mcm6, and Mcm7 were purified as described before in Davey et al. (2003). $Mcm3^{PK}$ was purified as described in Stead et al. (2009).

2.2 Cdt1 purification

Cdt1 was purified from *E. coli* cells expressing a recombinant *S. cerevisiae* Cdt1 (12 L) grown to an OD_{600} 0.5-0.6, chilled to 15°C and then induced by addition of 1 mM IPTG. The cells were lysed in 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 2 mM DTT and 10% sucrose through a cell homogenenizer. Insoluble material was removed by centrifugation at 27,000 g for 30 min at 4 °C. Ammonium sulfate was added to the soluble fraction at a final concentration of 0.3 mg/ml. The precipitate was pelleted by centrifugation at 27,000g for 30 min at 4 °C then resuspended in 20 mM Tris-HCl pH 7.5, 10% glycerol, 0.1 mM EDTA, and 2 mM DTT (Buffer A). The solution was then dialyzed against Buffer A until the protein solution reached a conductivity equivalent to 100 mM NaCl. The solution (~120 mg of total protein) was applied to a 30 ml Fast Flow Q (FFQ) column equilibrated with Buffer A with 50 mM NaCl. Cdt1 was eluted with a linear gradient of 50 mM to 500 mM NaCl in Buffer A. Peak fractions were pooled (~60 mg of total protein) and dialyzed to a conductivity equivalent of 150-200 mM NaCl and applied to a 20 ml Heparin column equilibrated in Buffer A with 100 mM NaCl. Cdt1

was eluted with a linear gradient of 100 mM to 500 mM NaCl in Buffer A. Peak fractions were pooled and dialyzed against 20 mM HEPES pH 7.5, 10% glycerol, 0.1 mM EDTA and 2 mM DTT (Buffer H) with 100 mM NaCl to a conductivity equivalent to 100 mM NaCl. The protein solution (~29 mg of total protein) was then applied to a 1 ml MonoQ column equilibrated with Buffer H with 100 mM NaCl. Cdt1 was eluted with a linear gradient of 100 mM to 500 mM NaCl in Buffer H. Peak protein (~4 mg of total Cdt1) was determined by Bradford and SDS-PAGE.

2.3 Reconstitution of Mcm2-7 and Mcm2-7•Cdt1

Two nmol of each Mcm subunit with and without Cdt1 was mixed in 20 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, and 0.1 mM EDTA and concentrated in a 10,000 Da MW cutoff centrifugal ultrafiltration unit (Millipore, Billerica, Maryland) to a final volume of 200 µl and a conductivity equivalent to 300-400 mM NaCl. The mixture was then incubated at 16°C for 30 minutes followed by a 10-minute centrifugation at 16,000 x g. The protein mixture was then applied to a 26 ml Superose 6 10/300 GL size exclusion chromatography column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) equilibrated with 20 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, and 0.1 mM EDTA. Fractions of 250 µl were collected and analyzed by 8% SDS-PAGE stained with Coomassie Brilliant Blue.

2.4 DNA binding assays

DNA binding assays were performed in 100 µl with ^{32}P-labeled Mcm2-7^{3PK} (labeled as described in Stead et al 2009). Two hundred fmol protein sample was incubated with the amount of DNA substrate indicated (50-200 fmol; M13mp19 ssDNA and Y70-Cdt1 dsDNA (yeast LEU2 CEN/ARS), both plasmids approximately 8 kb in size) in the presence or absence of 5 mM ATP in Tris-HCl pH 7.5, 0.1 mM EDTA, and 10 mM magnesium acetate for 10 minutes at 37°C. The sample was then analyzed on a 5 ml, 4 % cross-linked agarose gel filtration column equilibrated with 20 mM Tris-HCl (pH7.5), 10 mM magnesium acetate, 0.1 mM EDTA, 100 mM NaCl, 10% glycerol, 50 µg/ml bovine serum albumin and 2 mM DTT. The amount of ^{32}P-Mcm2-7^{3PK} in each fraction was determined by scintillation counting. Analysis of the data (area under curve for recovery and ANOVA for comparison of mean DNA binding) were conducted in GraphPad Prism (La Jolla, CA)

2.5 Cdt1 polyclonal antibody

Polyclonal anti-Cdt1 antibody was raised and produced in New Zealand White rabbits by Pacific Immunology, Inc (Ramona, CA). The immune sera were tested against recombinant Cdt1 and in yeast protein extracts to verify that the antibodies generated were Cdt1-specific.

2.6 Co-immunoprecipitation

Protein G Dynabeads (Invitrogen, 100.03D; Carlsbad, California) were prepared following the instructions given by the supplier. Briefly, 50 µl of resin was combined with 10 µl of anti-Cdt1 polyclonal rabbit IgG sera in 200 µl phosphate buffered saline pH 7.4 (PBS) containing 0.02% Tween-20 (Poloxyethylene (20) Sorbitan Monolaurate; PBST). This mixture was then incubated at room temperature for at least 30 minutes with mixing. Following the incubation, the resin was washed once with 200 µl PBST and then resuspended in 200 µl PBST.

Purified proteins were used in the co-immunoprecipitation. Each protein was added to a final concentration of 5 pmol/µl, in a 50 µl volume, in 20 mM HEPES pH7.5, 100 mM NaCl, 2 mM DTT, 10 % glycerol, and 0.1 mM EDTA. The mixture was incubated at room temperature for 10 min and then 8 µl of anti-Cdt1 resin was added. The mixture was then incubated at room temperature for an additional 15 minutes and isolated via a magnet and washed 5 times with 200 µl 20 mM HEPES pH 7.5, 300 mM NaCl, 2 mM DTT, 10% glycerol, and 0.1 mM EDTA. Bound proteins were eluted by boiling for 10 min in 20 µl SDS sample buffer. Ten percent of the load and 50% of the elution was then analyzed by either immunoblotting or on an 8% SDS-PAGE gel stained with GelCode Blue (Thermo Scientific, 24590; Waltham, MA).

2.7 DNA unwinding assay

DNA unwinding was performed and quantified as described in Stead et al. 2009 with the following modifications. Assay buffer contained 5% polyethylene glycol 3350 (Mallinckrodt Baker; Phillipsburg NJ) and 100 mM sodium glutamate.

2.8 ATP Hydrolysis

ATP hydrolysis by the preRC components in isolation or in combination with DNA substrate and with each other was assayed through the use of thin-layer chromatography (TLC) as described in Stead et al. 2009. Each 12 µl reaction contained 20 mM Tris-HCl pH 7.5, 10mM magnesium acetate, 2mM DTT, 1mM ATP, with 5 µCi [γ-^{32}P]-ATP (Perkin Elmer Life Sciences, BLU002A250Ci; Waltham, MA). For the experiments where Cdt1 was added to Mcm2-7, the proteins were incubated on ice for 60 min before the addition of ATP. Samples were then incubated at 30ºC for the times indicated. Following this, 2 µl of the reaction was removed and quenched with 2 µl of stop buffer (40 mM EDTA, 1% SDS). A portion was spotted onto polyethyleneimine cellulose TLC sheets (EM Science, 5579/7; Damstadt, Germany) and the sheets were developed in 0.6 M potassium phosphate monobasic (pH 3.4) for 8 min. Once the sheets were dry, they were exposed to a PhosphorStorage screen and then scanned on a Storm 860 scanner (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Data analysis (linear regression for hydrolysis rates and ANOVA for mean rate comparisons) was conducted with GraphPad Prism (La Jolla, CA).

2.9 ORC purification

An *E. coli* strain that co-expresses all six of the ORC subunits each under the control of the T7 RNA polymerase promoter (M.J.Davey, J. Finkelstein and M. O'Donnell, unpublished) was grown to an OD_{600} of 0.5, chilled to 15°C and then induced by addition of 1 mM IPTG. After 18-20 hr shaking at 15°C, the cells (12 L) were harvested by centrifugation and resuspended (10 % w/v) in 20 mM HEPES pH 7.5, 150 mM KCl, 10% sucrose, 1 mM PMSF, 1 mM DTT, and 1.5 mM $MgCl_2$. The lysate was centrifuged at 27,000 xg for one hour and the supernatant was applied to an 40 ml SP Sepharose column equilibrated in 20 mM HEPES pH 7.5, 150 mM KCl and 10% glycerol and eluted with a 400 ml linear gradient of 150 mM KCl to 700 mM KCl in the above buffer. The peak fractions, as determined by SDS-PAGE, were pooled (approximately 40 mg in 110 ml) and loaded onto a 1 ml Hi-Trap (GE Healthcare, 17-0408-01; Little Chalfont, Buckinghamshire, UK) Ni-affinity column, equilibrated with 20 mM HEPES pH 7.5, 400 mM KCl, 5 mM imidazole and 10% glycerol. The column was then washed with 20 ml of 20 mM HEPES pH 7.5, 400 mM KCl, 60 mM imidazole, 10% glycerol, followed by elution with 20 ml of 20 mM HEPES pH 7.5, 400 mM KCl, 1 M imidazole, 10% glycerol. The peak fractions, as determined by SDS-PAGE, were pooled and loaded onto a P-100 (polyacrylamide gel filtration beads) column equilibrated in 25 mM Tris pH 8.0, 400 mM KCl, 5mM MgOAc, 10% glycerol, 0.02% NP-40, and 1 mM DTT. The resulting elution was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

2.10 Electrophoresis Gel Mobility Shift Assays

The oligonucleotide MD195 (5' – AAG CGC CAC GCT TCC CGA AG –3'; henceforth MD195*) was labeled at the 5' end using polynucleotide kinase (PNK; New England Biolabs, M0201L). MD195* along with MD196 (5' – Biotin – GTT GTA AAA CGA CGG CCA GTG –3') were used to amplify *ARS1*-containing fragments from pUC18 plasmids containing either *ARS1* or an *ARS1* fragment deleted between the *BamH*I-*Bgl*I restriction sites, removing the ACS (*ARS1ΔA)*. The amplified *ARS1* and *ARS1ΔA* DNA were then purified via ethanol precipitation. The DNA was then incubated with the indicated amounts of protein for 10 min at 37°C. The mixture was then analyzed by native PAGE, and the gel was dried and exposed to a PhosphorStorage screen before scanning on a Storm 860 scanner (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometry measurements were conducted using ImageQuant 5.2 (Molecular Dynamics, GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Chapter 3 – Results

3.1 Interaction of Cdt1 with Mcm2-7

I wished to test whether Cdt1 could interact and form a complex with Mcm2-7 *in vitro* using pure proteins. I combined each of the individual Mcm subunits and Cdt1 and then separated the complete complex from free proteins using a Superose 6 size exclusion chromatography column. The elution of the proteins from this column was analyzed by SDS-PAGE stained with Coomassie Brilliant Blue. Cdt1 co-eluted with Mcm2-7 proteins, with the peak of elution corresponding to a size of 670 kDa (Figure 3.1A). Notably, Cdt1 with Mcm2-7 eluted much earlier than Cdt1 alone, suggesting that a Mcm2-7•Cdt1 complex had formed. Cdt1 analyzed alone has an elution peak at fraction 35 with an apparent size of 131 kDa, which would be the predicted size of a dimer of Cdt1 (Figure 3.1B). Identification of the Cdt1 band was confirmed by western blotting using a polyclonal anti-Cdt1 antibody. Cdt1 is in a 1:1 ratio with each of the Mcm2-7 proteins as determined by densitometric measurement of the Coomassie-stained gels. Mcm2-7 without Cdt1 was also reconstituted (data not shown). The elution profiles of Mcm2-7 (Figure 3.1C) and Mcm2-7•Cdt1 (Figure 3.1A) are similar. The addition of 68 kDa in the form of Cdt1 does not change the elution profile of the 600 kDa Mcm2-7 complex, likely due to the resolution capabilities of the Superose 6 column.

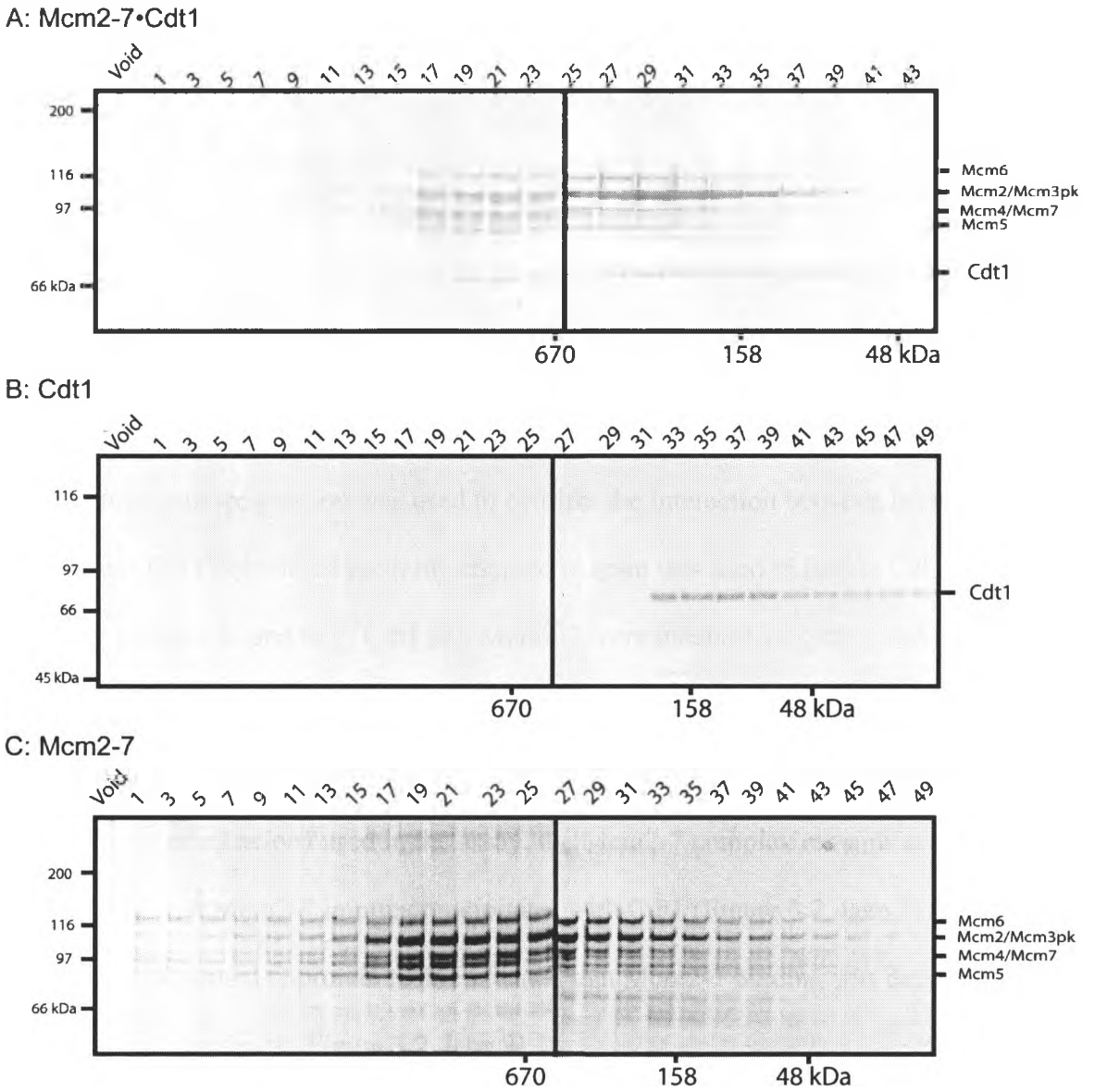

Figure 3.1: Mcm2-7, Cdt1 and a complex containing both were analyzed by gel filtration. Coomassie Brilliant Blue-stained SDS-PAGE (8%) of the indicated fraction shown for (A) Mcm2-7•Cdt1, (B) Cdt1 alone and (C) Mcm2-7. $Mcm3^{PK}$ is a engineered Mcm3 construct, containing a labelable N-terminal epitope tag. Peak elution of size-standards analyzed separately are shown below each panel.

This shift in elution of Cdt1 did not require any other protein components as all the components for complex reconstitution were purified separately from *E. coli*. It is therefore unlikely that any other factors are involved in the assembly of the Mcm2-7•Cdt1 complex.

3.2 Co-immunoprecipitation of Mcm2-7 and Cdt1

Co-immunoprecipitation was used to confirm the interaction between Mcm2-7 and Cdt1. Anti-Cdt1 polyclonal antibody coupled to resin was used to isolate Cdt1 along with any proteins bound to it. Cdt1 and Mcm2-7 were incubated together and subsequently added to the resin. After washing the anti-Cdt1 resin, proteins were eluted with SDS sample buffer and analyzed by western blotting for the presence of Mcm2. At the protein concentrations used in the assay, the Mcm2-7 complex remains as a hexamer. It was found that Mcm2-7 immunoprecipitates with Cdt1 (Figure 3.2, lane 3). A control assay with uncoupled G protein resin indicated that Mcm2-7 binding was dependent on Cdt1 and Cdt1 antibody (Figure 3.2, lane 2).

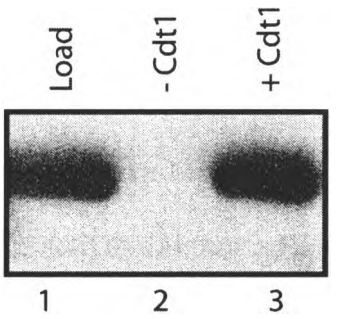

Figure 3.2: Co-immunoprecipitation of Mcm2-7 and Cdt1. Anti-Mcm2 immunoblot of co-immunoprecipitation of Mcm2-7 with Cdt1. (Lane 1) Analysis of the Mcm2-7 loaded onto the anti-Cdt1 resin. (Lane 2) Elution of Mcm2-7 incubated on resin not coupled to α-Cdt1 antibody. (Lane 3) Elution of Mcm2-7 and Cdt1 on αCdt1 antibody coupled resin.

3.3 Cdt1 inhibits ATP hydrolysis by Mcm2-7

To determine if Cdt1 modulates the ATP hydrolysis activity of the Mcm2-7 complex, I compared the ATPase activity of Mcm2-7•Cdt1 with Mcm2-7. Cdt1 itself does not have any ATP hydrolysis activity (Figure 3.3), but may modulate that of Mcm2-7. To test this idea, I incubated Mcm2-7•Cdt1 or Mcm2-7 in the presence of γ^{32}P-ATP and measured the amount of γ^{32}P (P_i) liberated. I first examined ATP hydrolysis of the individual gel filtration fractions of Mcm2-7 and Mcm2-7•Cdt1 (Figure 3.4). Two observations were made from this analysis. First, ATPase activity co-eluted with the fractions containing all six Mcm proteins, providing further evidence of complex formation. Second, despite equal amounts of protein in the peak fraction (fraction 21) less activity was observed with Mcm2-7•Cdt1 than with Mcm2-7 (Figure 3.4C).

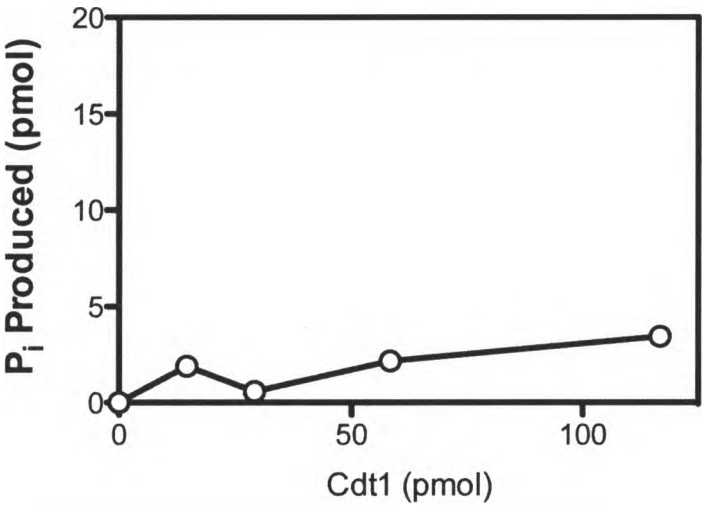

Figure 3.3: ATP hydrolysis activity of Cdt1. The amount of P_i liberated through the action of Cdt1 over an hour at increasing protein concentrations. The amount of ATP hydrolyzed is not significantly different from the no protein control ($P>0.05$). The highest protein level at 117 pmol of Cdt1 is representative of 50 fold higher protein level then used in subsequent ATP hydrolysis assays.

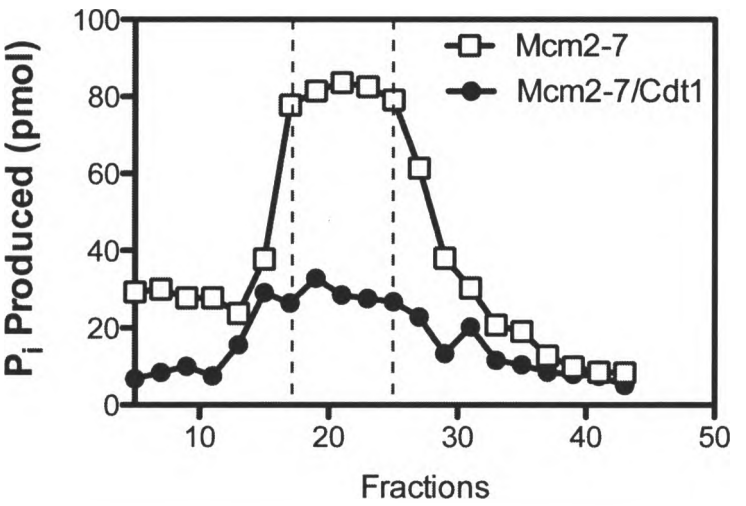

Figure 3.4: ATP hydrolysis analysis of the Mcm2-7 and Mcm2-7•Cdt1 complexes across all fractions from the Superose 6 column. The amount of ATP hydrolyzed over an hour was determined by counting the amount of liberated $\gamma^{32}P$ (P_i). The dashed line represents borders of the protein peak (fractions 17-25) eluted from the Superose 6 column as determined by SDS-PAGE.

To determine the rate of ATP hydrolysis for the two complexes, time courses for each complex were conducted (Figure 3.5A). The Mcm2-7 complex hydrolyses ATP at a rate of 1.51±.08 min^{-1} compared to the Mcm2-7•Cdt1 complex at 0.66 ±.03 min^{-1}. This decrease in ATP hydrolysis is substantial and represents half the activity of Mcm2-7 alone. The addition of Cdt1 to a pre-formed Mcm2-7 complex could not reconstitute helicase loading (Remus et al. 2009, Ervin et al. 2009), therefore I tested if Cdt1 could decrease the hydrolysis activity of a pre-formed Mcm2-7. The addition of Cdt1 to pre-formed Mcm2-7 did not have the inhibitory effect as was observed with the Mcm2-7•Cdt1 complex (Figure 3.5B). To ensure that this was not simply due to lower levels of Cdt1, I titrated increasing amounts of Cdt1 into a constant amount of Mcm2- 7. The Mcm2-7 ATP hydrolysis rate measured showed no difference even at the highest level of Cdt1 (Figure 3.5C).

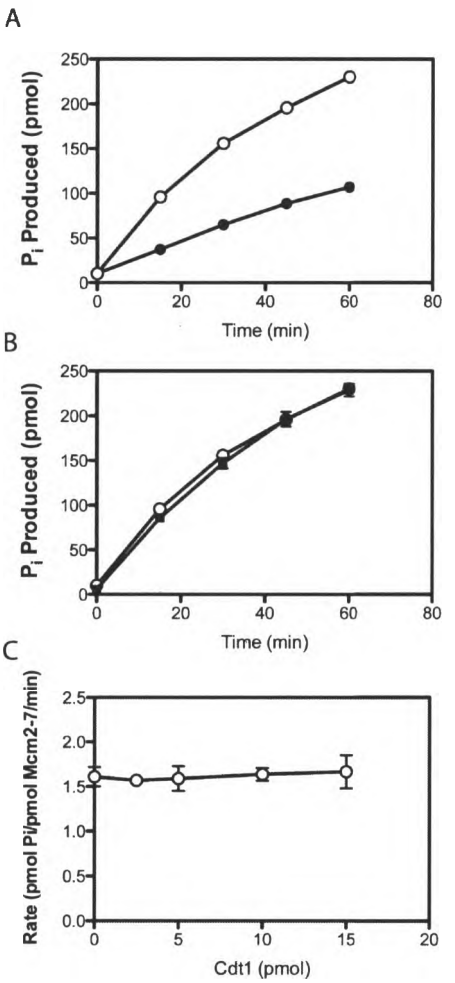

Figure 3.5: ATP hydrolysis of Mcm2-7 vs Mcm2-7•Cdt1. (A) ATP hydrolysis by Mcm2-7 (empty circle), and Mcm2-7•Cdt1 (filled circles) was measured. Reactions were quenched at 15 , 30, 45 and 60 min, and pmol of P_i produced at each time point was determined. (B) ATP hydrolysis of Mcm2-7 (closed squares) with and without Cdt1 (empty circles) added in solution. The reactions were performed in triplicate, and the mean and standard error of each time point were plotted. The error bars are hidden by the symbols. (C) Mcm2-7 ATP hydrolysis with the addition of increasing amounts of Cdt1. Increasing amounts of recombinant Cdt1 were added to a pre-formed Mcm2-7 complex and the rate of ATP hydrolysis was determined. The mean rate of ATP hydrolysis by Mcm2-7 is plotted against the amount of Cdt1 added.

3.4 Cdt1 inhibits DNA unwinding by Mcm2-7

To test whether Mcm2-7•Cdt1 unwinds DNA to the same extent as Mcm2-7, I performed helicase assays using an oligonucleotide substrate. The helicase assay consisted of a radiolabeled ssDNA oligonucleotide annealed to a complementary oligonucleotide creating a double stranded region. A single stranded poly-dT tail for initial helicase binding is also present (Figure 3.6). The radiolabeled substrate was incubated with either Mcm2-7 or Mcm2-7•Cdt1 and the samples were examined on a native polyacrylamide gel (Figure 3.6A). The amount of DNA unwinding at 100 nM of either complex appeared to be about the same (Figure 3.6B). As the amount of protein was increased from 100 nM to 400 nM, Mcm2-7 had higher DNA unwinding activity than Mcm2-7•Cdt1 (Figure 3.6B). The extent of DNA unwinding by 200 nM Mcm2-7•Cdt1 was approximately 3-fold lower than with Mcm2-7 alone (Figure 3.6C).

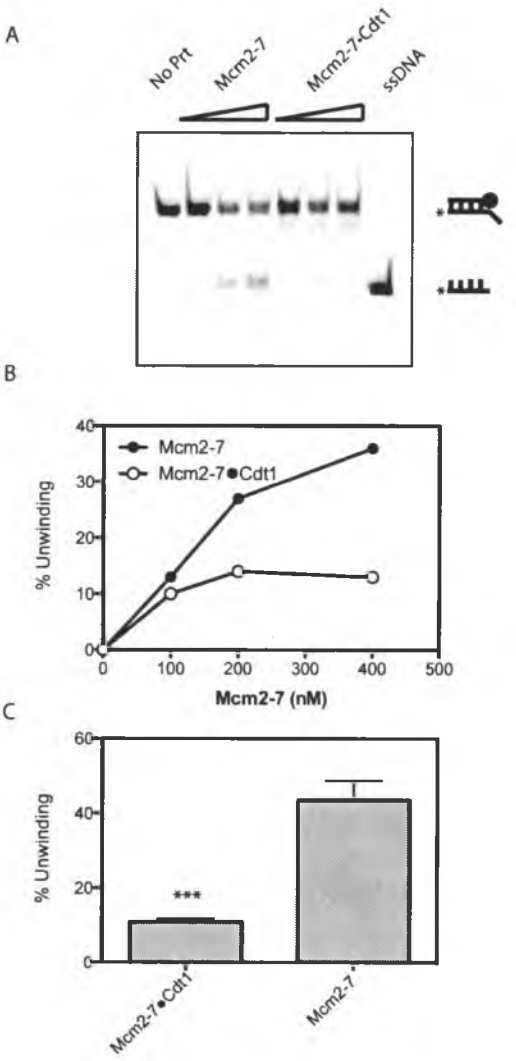

Figure 3.6: Effect of Cdt1 on Mcm2-7 DNA unwinding. (A) Representative gel of the DNA helicase assay. The upper band corresponds to the double stranded DNA substrate. The lower band is the single stranded DNA liberated after DNA unwinding. Increasing amounts of Mcm2-7 with and without Cdt1 were incubated with DNA substrate. (B) Quantification of panel (A). (C) Quantification of DNA unwinding activity of Mcm2-7•Cdt1 and Mcm2-7 at 200 nM protein. The reactions were performed in triplicate and the mean and standard error are shown. There is a significant difference between the means ($P<0.0001$).

3.5 DNA binding activity of Mcm2-7

I examined whether DNA binding by Mcm2-7 was altered in the presence of Cdt1. I utilized a gel filtration-based approach to measure DNA binding in which radiolabeled Mcm2-7 or Mcm2-7•Cdt1 was incubated with either circular single or double stranded DNA vector. Protein bound to DNA eluted early from the column (fractions 10-12) whereas free protein eluted later (fractions 15-31; Stead et al. 2009).

I first compared binding of Mcm2-7•Cdt1 and Mcm2-7 to ssDNA. Increasing amounts of ssDNA (50-200 fmol) were titrated into a constant amount of Mcm2-7•Cdt1 (Figure 3.7A) and Mcm2-7 (Figure 3.7B).The two complexes bound ssDNA to the same extent at all DNA concentrations tested (Figure 3.7A and B). At 200 fmol ssDNA for either Mcm2-7 or Mcm2-7•Cdt1, the extent of the binding was nearly identical with approximately 16 fmol of protein associating with ssDNA for both complexes (Figure 3.7C).

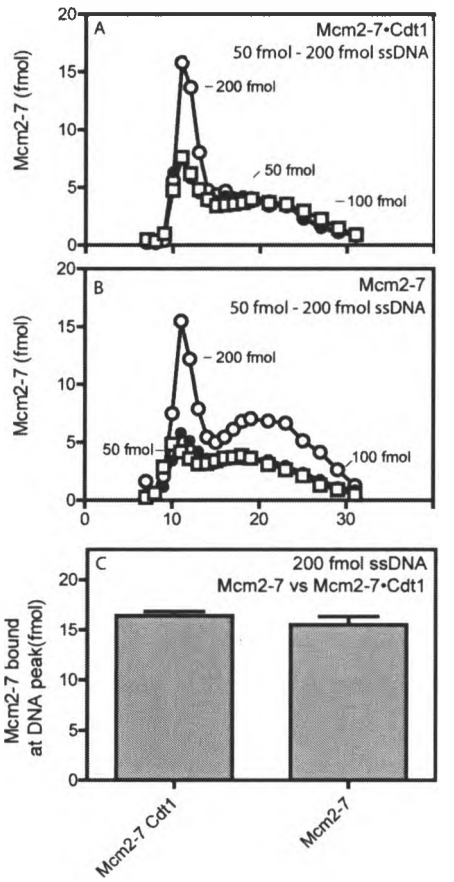

Figure 3.7: ssDNA binding activity of Mcm2-7 and Mcm2-7•Cdt1 complexes. (A) Mcm2-7•Cdt1 with 50 fmol (open squares), 100 fmol (filled circles) and 200 fmol (open circles) of single stranded DNA. (B) Mcm2-7 with 50 fmol (open squares), 100 fmol (filled circles) and 200 fmol (open circles) of single stranded DNA. (C) Comparison of the peak fraction (fr11) of Mcm2-7 and Mcm2-7•Cdt1 with 200 fmol ssDNA. Each DNA binding experiment was performed in triplicate, the mean has been plotted without error bars for clarity (A-B) standard error shown in C. Protein recovery ranged from 70% - 60% and the DNA-protein interaction is ATP dependent.

Double stranded DNA binding was consistent with other studies on Mcm2-7 (Bochman and Schwacha 2007; Stead et al. 2009). I observed little to no dsDNA binding by Mcm2-7 alone under conditions where there is robust ssDNA binding (Figure 3.8A). Only 1.7±1.1 fmol of Mcm2-7 eluted at the DNA elution peak at fraction 11 at 200 fmol DNA. The interaction was equally low for all dsDNA concentrations assayed with 1.9±1.1 fmol and 1.9±0.8 fmol at 100 fmol and 50 fmol of dsDNA, respectively, at the DNA elution peak (Figure 3.8A). In contrast, binding of Mcm2-7•Cdt1 was detected on dsDNA (Figure 3.8 B). Mcm2-7•Cdt1 binds dsDNA significantly better than Mcm2-7, with 6.0 ±1.1 fmol Mcm2-7•Cdt1 eluting at the DNA peak compared to 1.7±1.1 fmol Mcm2-7 (Figure 3.8E).

In the absence of DNA, both complexes migrate through the gel filtration column as is expected of unbound protein and elute in fraction 20 (Figure 3.8D). This profile suggests that the protein complexes are not aggregating, and thus not skewing the results by changing the elution profile of free protein. It is reasonable to conclude that the interaction of Mcm2-7•Cdt1 with dsDNA is significantly higher than that of Mcm2-7 alone (Figure 3.8E).

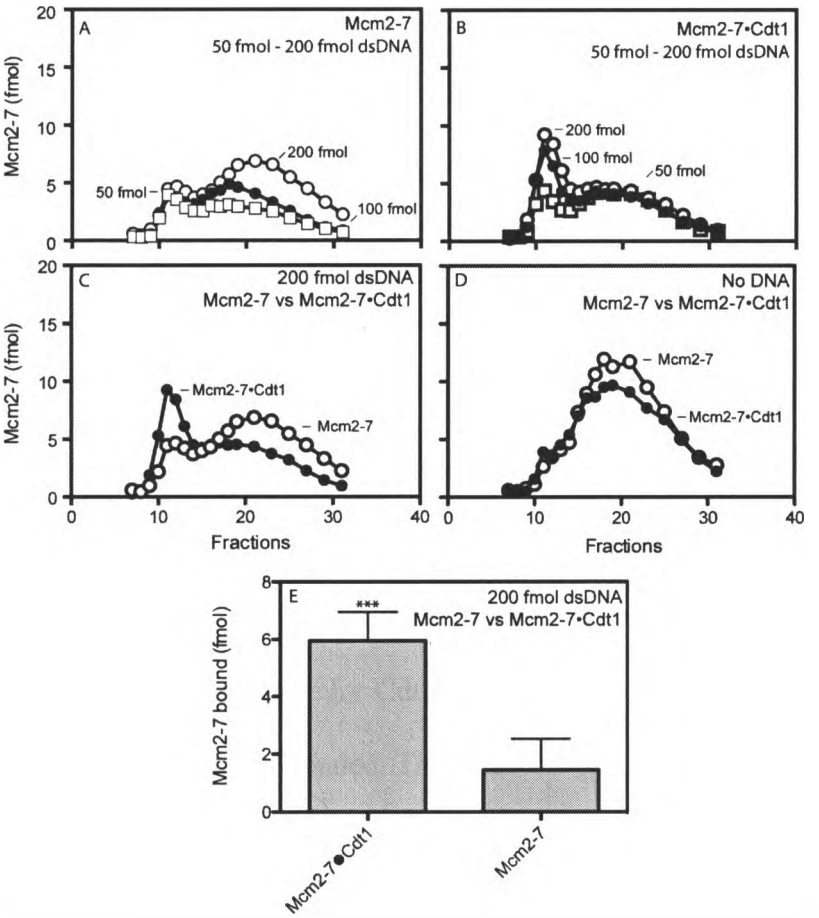

Figure 3.8: dsDNA binding activity of Mcm2-7 and Mcm2-7•Cdt1 complexes. Incubation of 50 fmol (open squares), 100 fmol (filled circles) and 200 fmol (open circles) double stranded DNA with (A) Mcm2-7 and (B) Mcm2-7•Cdt1. (C) Mcm2-7 (open circles) and Mcm2-7•Cdt1 (filled circles) binding curves for 200 fmol dsDNA. (D) The complexes were incubated in the absence of DNA to determine whether the proteins are stable and do not aggregate. (E) Comparison of the peak fraction (fr11) of Mcm2-7 and Mcm2-7•Cdt1 with 200 fmol of dsDNA with the mean difference in 200 fmol dsDNA binding being significantly different between Mcm2-7 and Mcm2-7•Cdt1 ($P<0.05$). Each DNA binding experiment was performed in triplicate, the mean has been plotted without error bars for clarity (A-D).

Chapter 4 – Discussion

Cdt1 is an essential component of the preRC and assists ORC and Cdc6 in the assembly of Mcm2-7 onto replication origins, thereby loading the helicase. This study shows that Cdt1 binds and forms a stable complex with Mcm2-7 in the absence of other proteins or post-translational modifications. When in complex with Mcm2-7, Cdt1 modifies the activity of Mcm2-7, making it distinct from Mcm2-7 alone. Mcm2-7•Cdt1 binds dsDNA with higher affinity than Mcm2-7 alone. In addition, Mcm2-7•Cdt1 hydrolyzes ATP and unwinds DNA at significantly lower levels compared to Mcm2-7. These observations reveal a novel role for Cdt1 in regulating the helicase activity of Mcm2-7 once the helicase has been loaded. They also unveil a mechanism that assists Mcm2-7 in dsDNA binding at replication origins.

4.1 Spontaneous assembly of the Mcm2-7•Cdt1 complex

In experiments used to reconstitute the pre-replicative complex *in vitro*, Mcm2-7 was purified from G1-arrested cells. In these preparations, Cdt1 was co-purified with Mcm2-7 in the form of Mcm2-7•Cdt1 complex (Kawasaki et al. 2006; Remus et al. 2009). This complex was able to support Mcm2-7 loading onto replication origins. When recombinant Cdt1 and Mcm2-7 were purified and added separately, no Mcm2-7 loading onto *ARS1* DNA was observed (Kawasaki et al. 2006; Remus et al. 2009).

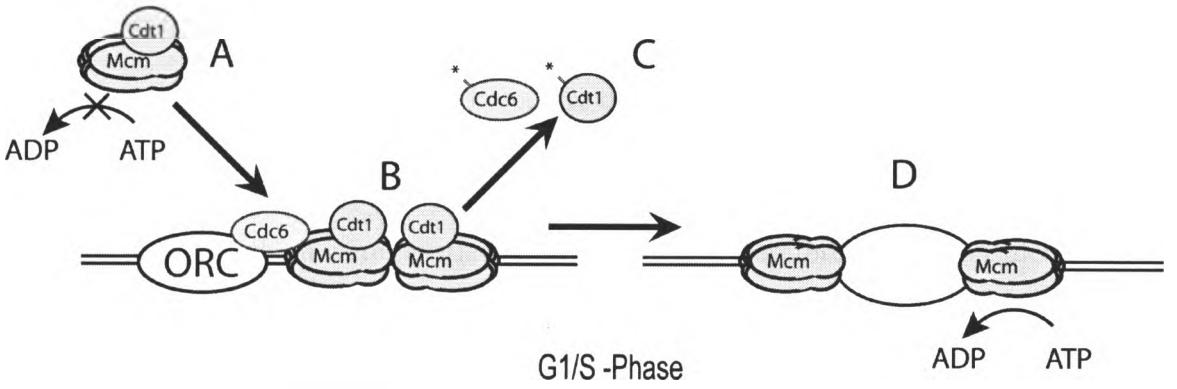

Figure 4.1: Proposed model for Mcm2-7•Cdt1. (A) Mcm2-7•Cdt1 is targeted onto origins of replication (B) ORC and Cdc6 along with Mcm2-7•Cdt1 complete the preRC by loading Mcm2-7•Cdt1 on dsDNA (Cdt1 is shown as stable but is most likely dynamically associating and dissociating with the complex). (C) Modified Cdt1 and Cdc6 are released from the preRC (D) The release of Cdt1 increases the ATP hydrolysis and DNA helicase activity of the Mcm2-7 complex.

This led to the proposal that Cdt1 and Mcm2-7 may require another factor for complex formation. I tested this hypothesis directly to determine if the Mcm2-7•Cdt1 could form without any other proteins or modifications. I reconstituted the Mcm2-7•Cdt1 from purified proteins which were purified from *E. coli*, and therefore can be considered to be unmodified, and are free of any other *S. cerevisiae* proteins. The reconstitution of the Mcm2-7•Cdt1 complex without the need for any other factors strongly suggests that the assembly of the Mcm2-7•Cdt1 can occur spontaneously *in vivo*. Whether every Mcm2-7 is in complex with Cdt1 is unknown. Protein measurements typically indicated more Mcm2-7 compared to Cdt1 molecules per cell (Lei et al. 1996; Edwards et al. 2002, Waga and Zembutsu 2006). However, those measurements were likely performed on asynchronous populations and the fluctuation of these proteins during the cell cycle can alter the results. Recent measurements when specifically looking at Mcm2-7 and Cdt1 have been more in the order of one Mcm2-7 to one Cdt1 (Kawasaki et al. 2006).

4.2 Cdt1 prevents premature DNA unwinding by Mcm2-7

Once loaded, Mcm2-7 remains at origins without unwinding DNA until S-phase commences (Geraghty et al. 2000; Sclafani and Holzen 2007; Evrin et al. 2009). Mcm2-7 however has been shown to have helicase activity *in vitro* without any other factors or modifications (Bochman and Schwacha 2008; Ilves et al. 2008). It would therefore follow that Mcm2-7's inherent helicase activity must be held in check prior to activation during S-phase or improper unwinding will result. Premature DNA unwinding would result in stretches of ssDNA that could potentially lead to dsDNA breaks and genome instability.

Genome instability is an indicator of potential tumorigenesis in mammalian cells, therefore preventing any form of genome instability is imperative for cell survival (Fujita 2006).

Studies examining the assembly of the preRC suggested that Cdt1 is released upon helicase loading along with Cdc6 (Kawasaki et al. 2006; Randell et al. 2006). However, in more recent studies on the assembly of the preRC using pure proteins, Cdt1 remains in the preRC (Remus et al. 2009). It has been suggested that Cdt1 is dynamically associated with origins of replication *in vivo* (Kawasaki et al. 2006; Randell et al. 2006; Xouri et al. 2007; Remus et al. 2009). This form of exchange could explain the two disparate observations for Cdt1 in preRC assembly.

Further evidence that Cdt1 remains associated with the preRC is the observation that Cdt1 is important for the recruitment of Cdc45 via an interaction between Cdt1 and Cdc7 (Ballabeni et al. 2009). The Cdt1-Cdc7 interaction not only results in the recruitment of Cdc45 to origins of replication, one of the steps involved in helicase activation, but also in the release of Cdt1 from Mcm2-7 (Ballabeni et al. 2009; Ilves et al. 2010). Cdt1 also appears to be required for DNA damage repair during S-phase (Lydeard et al. 2010).

You and Masai (2008) examined how Cdt1 interacted with Mcm proteins. They found that Cdt1 forms a complex with Mcm2-7 and Mcm4/6/7. The authors focused on the effects of Cdt1 on Mcm4/6/7. In their study, an increase in Mcm4/6/7 helicase activity was observed when in association with Cdt1 (You and Masai 2008). However,

they did not determine the effect of Cdt1 on Mcm2-7 due to the inability to form an active helicase with Mcm2-7 and did not pursue the ATP hydrolysis activity (You and Masai 2008). Mcm4/6/7 is not the Mcm complex found at the preRC and therefore activation of Mcm4/6/7 helicase activity by Cdt1 may not accurately recapitulate what occurs *in vivo* in the context of the preRC. These data presented in this thesis show that in the context of the complete Mcm2-7 complex, Cdt1 has an inhibitory role on the helicase activity of the Mcm2-7 complex.

4.3 Cdt1 inhibits ATP hydrolysis by Mcm2-7

Mcm2-7 has the ability to hydrolyze ATP without a DNA substrate (Matson 1986; Arai and Kornberg 1981; Davey et al. 2003). The rate of ATP hydrolysis is halved when Mcm2-7 is in complex with Cdt1. I propose two consequences for the decrease of ATP hydrolysis activity of Mcm2-7 by Cdt1. Firstly, the mechanism of helicase inhibition may be through the decrease in ATP hydrolysis activity by Mcm2-7•Cdt1. Alternatively, but not exclusively, non-functional hydrolysis activity is energetically unfavorable for a cell. There are approximately 30,000 Mcm2-7 complexes that at the catalytic rate determined would hydrolyze approximately 2.5% of total ATP pool per minute. Cdt1 in complex with Mcm2-7 would lower the energetic burden of the cell (Deeds et al. 2007; Lei et al. 1996).

Adding individually prepared Cdt1 to a preformed Mcm2-7 complex did not reconstitute the lower ATP hydrolysis activity seen with Mcm2-7•Cdt1. This could simply be a factor of not achieving high enough concentration of protein to form a

Mcm2-7•Cdt1 (Figure 3.5C). Alternatively, Mcm2-7•Cdt1 complex formation may require individual Mcm subunits to interact with Cdt1 to form the functionally distinct Mcm2-7•Cdt1 complex. The observation that Cdt1 co-immunoprecipitates with a pre-formed Mcm2-7 favors the concentration hypothesis due to the higher amount of protein used in immunoprecipitation assays compared to ATP hydrolysis assays (Figure 3.2).

4.4 DNA binding by Mcm2-7•Cdt1

This study demonstrates a higher affinity for dsDNA by Mcm2-7•Cdt1 than by Mcm2-7. In this thesis, the difference between ssDNA and dsDNA binding by Mcm2-7•Cdt1 is about half. Previously, dsDNA binding was shown to be 100-fold lower compared to ssDNA (Bochman and Schwacha 2007). This is in contrast to observations that indicate that Mcm2-7 loads onto dsDNA during preRC assembly (Tanaka and Nasmyth 1998; Dimitrova et al. 1999; Costa and Onesti 2008). The modulation of dsDNA binding activity of Mcm2-7 by Cdt1 may provide Mcm2-7 with the ability to associate with dsDNA, which is required for origin loading.

The increase in dsDNA binding affinity may be through Cdt1 binding to DNA. A study on human Cdt1 has demonstrated that Cdt1 associates with DNA on its own (Yanagi et al. 2002). It could be through Cdt1 that Mcm2-7•Cdt1 binds dsDNA. However, one cannot rule out the possibility that Cdt1 is altering Mcm2-7 DNA binding allosterically.

4.5 Model and Conclusions

This study demonstrates that the assembly of the Mcm2-7•Cdt1 complex occurs without other protein factors or post-translational modifications. The reconstituted Mcm2-7•Cdt1 is functionally distinct from Mcm2-7. Mcm2-7•Cdt1 complex has lower ATP hydrolysis activity, thereby diminishing energy loss (Figure 4.1A). The increased dsDNA binding activity helps target Mcm2-7•Cdt1 to origins to form the preRC with ORC and Cdc6 (Figure 4.1B). Mcm2-7 has lower helicase activity while in complex with Cdt1, which prevents unwanted and untimely DNA unwinding (Figure 4.1B). As the cell enters S-phase, CDK and DDK levels rise and modify preRC components causing the release of Cdt1 (Figure 4.1C). Upon dissociation of Cdt1, there is an increase in DNA helicase activity of the Mcm2-7 complex which also requires the association with other factors (Not shown; Figure 4.1D).

It would be interesting to determine how Cdt1 is modulating the dsDNA binding activity. Cdt1 may be directly binding DNA and recruiting Mcm2-7 to DNA or it may be modulating Mcm2-7 to bind DNA directly. One would first need to determine if *S. cerevisiae* Cdt1 binds DNA on its own through DNA binding assays such as EMSA. Secondly, if Cdt1 does bind DNA similarly to its mouse homologue, one could do mutational studies to determine the region required for this interaction and determine if mutations change the way that Mcm2-7•Cdt1 binds DNA (Yangani et al. 2002).

The difference between adding Cdt1 in solution to a preformed Mcm2-7 and the Mcm2-7•Cdt1 complex is most likely due to protein concentration. The assembly may

simply be a factor of protein affinity in assay conditions. Less likely, the differences observed may be due to some interacting surface changes during the assembly of the complex. Cdt1 may require the individual Mcm subunits for interaction.

Taken together, the results presented in this thesis indicate a novel role for Cdt1 in DNA replication control. They also begin to shed light on the mechanistic functions of Cdt1 with in the preRC.

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Appendix

A1 - Purification and assessment of ORC

A1.1 Purification of the origin recognition complex

My initial project goal was to reconstitute the pre-replicative complex *in vitro* to determine the minimal system requirements for preRC formation. To this end, I needed to purify the origin complex. The origin recognition complex is the core component of the preRC. It binds replication origins and nucleates the assembly of the preRC culminating in the loading of Mcm2-7 onto replication origins. Studies involving ORC have all been based on purifications from baculovirus or yeast cells (Bell and Stillman 1992; Lee and Bell 1997; Klemm and Bell 2001). Due to low yields, prohibitive costs and the potential for post transitional modifications, purification of ORC from *E. coli* would provide a foundation for studying the complex and its role in preRC assembly, where post-translational modifications are undesirable. I set out to purify a recombinant origin recognition complex.

To be able to purify the six ORC proteins, the ORC subunits were cloned into bacterial expression vectors and transformed into *E. coli.* (M.J.D, J. Finkelstein and M. O'Donnell, unpublished) Two of the six subunits, Orc5 and Orc6, contained a poly-histidine tag to facilitate purification of a complex.

The resulting purification was analyzed by gel electrophoresis (Figure 4.1) and the major bands corresponding to the correct sizes of Orc1-6 were further analyzed by

mass spectrometry. All six subunits of ORC were present and migrated as expected on SDS-PAGE. Orc4, Orc5 and Orc6 migrate together and due to the percentage of the gel, making the three proteins appear as a single band in certain lanes.

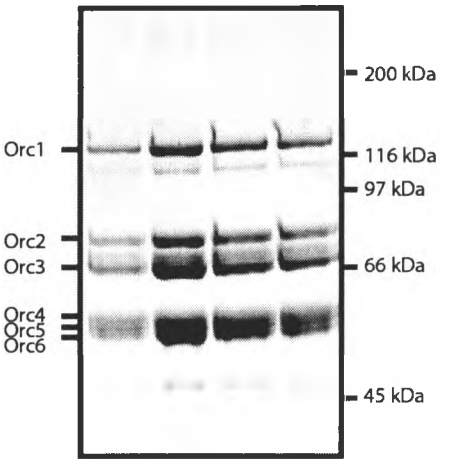

Figure A.1: Purification of the origin recognition complex. Coomassie Brilliant Blue stained SDS-PAGE (8%). Indicated on the left is the proteins as identified by mass spectrometry and on the right is the molecular weight marker.

A1.2 ORC DNA binding activity

The defining activity of ORC is to bind the origins of replication throughout the genome. ORC binds specifically the ACS with some limited affinity for the B2 region of *ARS1*. I wanted to test whether the DNA binding activity remained intact when the recombinant ORC is purified. To test this I used electrophoresis gel mobility shift assay (EMSA).

The EMSA relies on the protein complex interacting with DNA substrate. An interaction between the protein complex and the DNA substrate is visualized by the shift of the substrate into a slower migrating band. The slower band is indicative of an increased size of the DNA, which is due to the interaction with the protein in question.

The DNA substrate was amplified using a radiolabeled oligonucleotide containing the origin of replication, *ARS1*. A substrate with a deletion of part of the ARS sequence, which is thought to decrease affinity of ORC for DNA was also prepared to use as a control, *ARS1*-A.

Binding of ORC to both substrates was observed as seen by a change in mobility of the radiolabeled DNA fragments. Binding of ORC to wild type *ARS1* containing the complete ACS was about 3-fold better to *ARS1* than *ARS1*-A (Figure 4.2A and B).

Some binding was observed with the *ARS1*-ACS substrate, this however was consistent with published studies (Bell and Stillman 1992; Lee and Bell 1997; Remus et al. 2009).

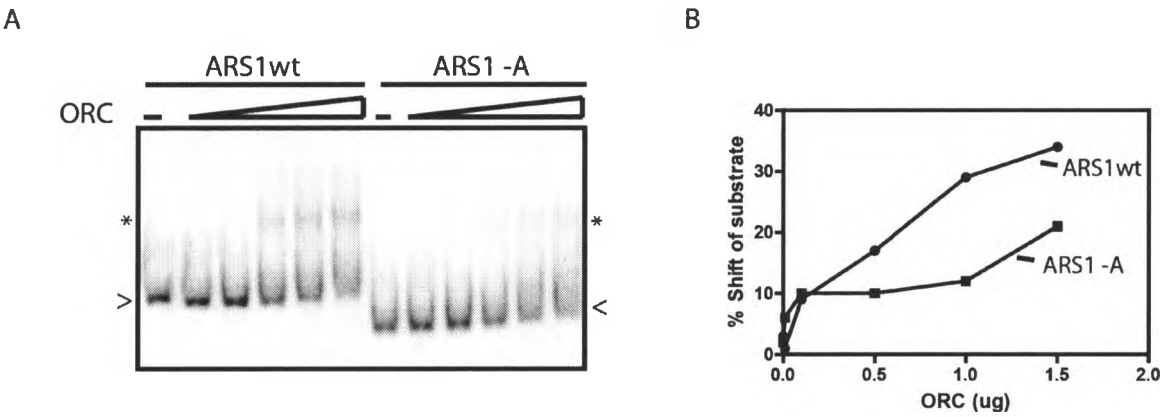

Figure A.2: Electrophoresis mobility shift assays with ORC and origin DNA substrate. (A) ORC was incubated with increasing amounts of *ARS1* and *ARS1*-A DNA substrate as indicated in the presence of ATP. The lower band for *ARS1* (>) and *ARS1*-A (<) is protein free DNA. The band indicated by (*) represents a shift to a slower migrating band indicating an interaction between ORC and the DNA substrate. (B) Quantification of the EMSA.

A1.4 References

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A2 - Cdt1 purification and antibody characterisation

A2.1 Cdt1 purification

The purification of Cdt1 was conducted according to the procedure described in Chapter 2. The sample of the load for each column represents the state of the protein following each step of the purification (Figure A.3).

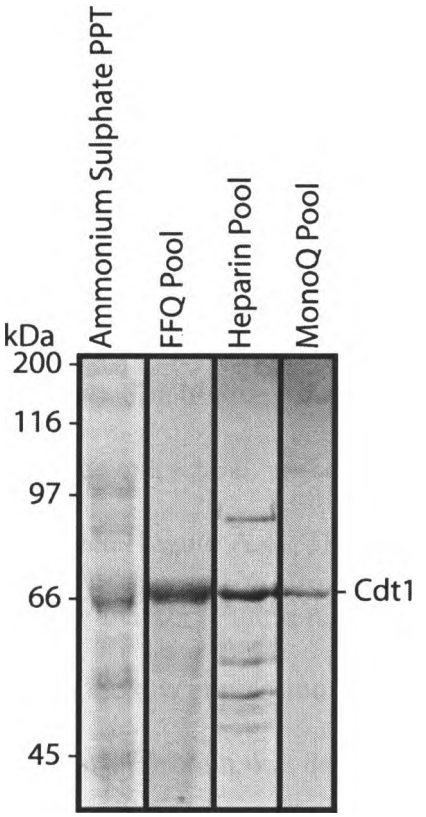

Figure A.3: Cdt1 purification. Each lane represents the protein pooled from the indicated purification step.

A2.2 Characterization of the Cdt1- antibody

The Cdt1 antibody was produced by Pacific Immunology Inc. from full length Cdt1 expressed and purified from *E. coli*. The identity of Cdt1 was confirmed by MALDI-MS. Antibodies were raised in New Zealand White rabbits. A pre-production bleed was included to determine whether there was any background Cdt1 binding antibodies in the rabbits (Figure A.4). The binding of sera from rabbits treated with Cdt1 to membranes containing yeast crude extracts, *E. coli* expressing Cdt1, *E. coli* without Cdt1 and purified Cdt1 was examined (Figure A.4). The purified Cdt1 band migrates as expected, directly below the 75 kDa marker (Figure A.4, Cdt1). No Cdt1 is detected in the yeast extract most likely due to the low expression levels of Cdt1 (Figure A.4, YE). In *E. coli* with Cdt1 expression a smear of protein was detected (Figure A.4, Induc.).

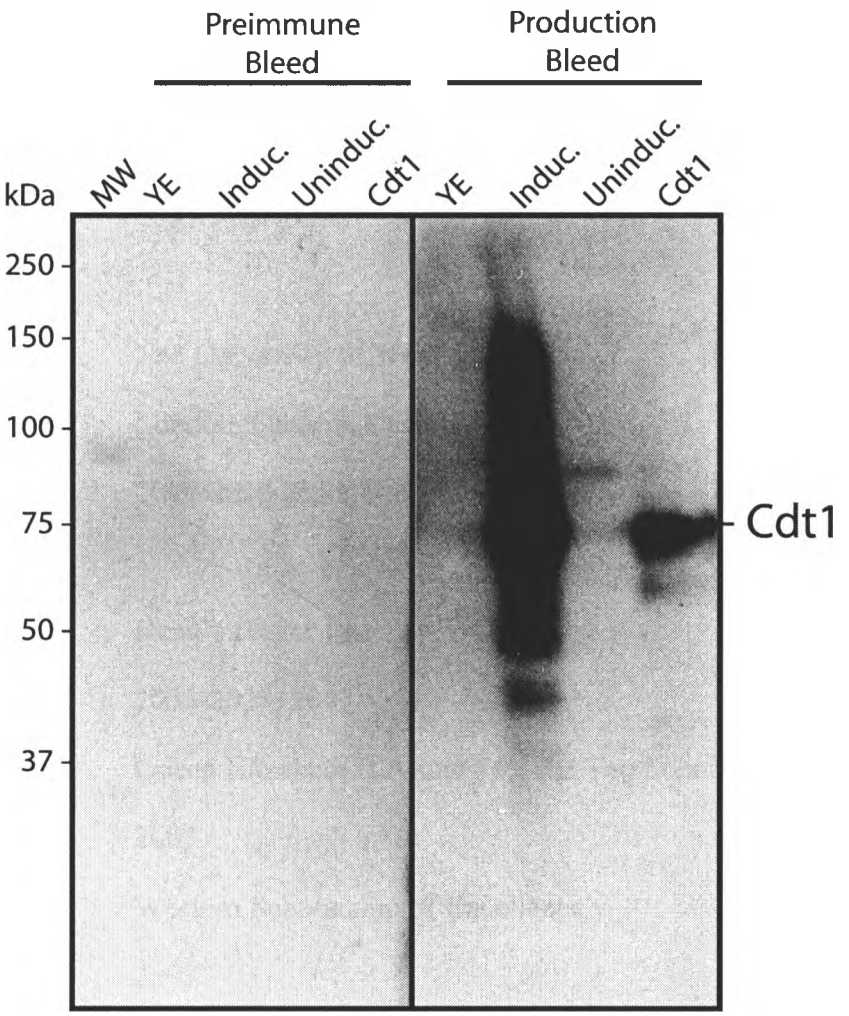

Figure A.4: Characterization of Cdt1 antibody. Both the preimmune bleed and production bleeds were tested on yeast extract (YE), *E. coli* containing a Cdt1 expression plasmid that was either induced (Induc.) or uninduced (Uniduc.) by the addition of IPTG and on a purified Cdt1.