The Regulation of Pre-Replicative Complex Formation in the Budding Yeast Cell Cycle

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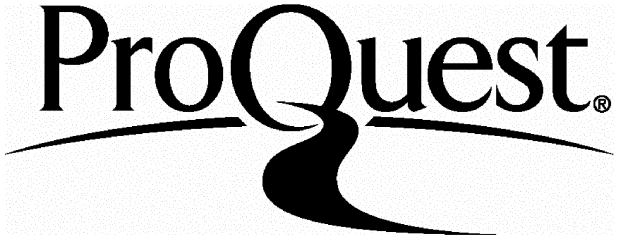

Abstract

DNA replication must occur once and only once in every cell cycle to ensure that mitosis produces two daughter cells with the same complement of genomic DNA. Initiation of DNA replication depends upon pre-replicative complex (pre-RC) assembly at origins during G1. The pre-RC includes a six-subunit origin recognition complex (ORC) and Cdc6p which together load a complex of the Mcm2-7p family of putative helicases onto chromatin. Only one ORC subunit, Orc6p, is not required for binding of the others to origins *in vitro*. Using a temperature sensitive *orc6* mutant we have shown *in vivo* that although Orc6p is dispensable for binding of other ORC subunits to chromatin, it is essential for pre-RC formation and may function as an "adapter" between ORC and other pre-RC components.

The activity of cyclin dependent kinases (CDKs) regulates pre-RC formation in the cell cycle. CDKs inhibit the formation of pre-RCs during S phase, G2 and M phase, suggesting that inactivation of CDKs at the end of mitosis is essential for pre-RC formation in G1. Other mitotic events may also be important for pre-RC formation. In particular, the anaphase promoting complex (APC/C), which targets mitotic proteins for proteolysis and the mitotic exit network which activates a mitotic phosphatase, Cdc14p, have been implicated in pre-RC formation. We have found that inactivation of CDKs during mitosis bypasses the requirement for the APC/C and the mitotic exit network in DNA replication. This suggests that the only essential role for these proteins for pre-RC formation is to bring about CDK inactivation at the end of mitosis.

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In memory of Annie Noton (née Peaker)
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Abbreviations

ACS	ARS consensus sequence
APC/C	anaphase promoting complex or cyclosome
ARS	autonomously replicating sequence
ATP	adenosine tri-phosphate
bp	base pair
CDC	cell division cycle
CDK	cyclin dependent kinase
C-terminus	carboxy-terminus
CHX	cycloheximide
6-DMAP	6-dimethylaminopurine
DNA	de-oxy ribonucleic acid
dNTP	di-deoxy nucleotide triphosphate
FACS	fluorescent activated cell sorter
Gal	galactose
Glu	glucose
HU	hydroxyurea
kbp	kilo base pair
MBF	MluI element binding factor
MCM	minichromosome Maintenance
Noc	nocodazole
N-terminus	amino-terminus
OBD	origin of bi-directional replication
ORC	origin recognition complex
PCR	polymerase chain reaction
Post-RC	post-replicative complex
Pre-RC	pre-replicative complex
Raf	raffinose
Rel	release
RNA	ribonucleic acid
SBF	SCB element binding factor
SCF	Skp1-Cullin-F-box protein

SPF	S phase promoting factor

D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
X. laevis	*Xenopus laevis*

Nomenclature

scGenep	*S. cerevisiae* protein
spGene	*S. pombe* protein
xlGene	*X. laevis* protein
hsGene	Human protein
cgGene	Hamster protein
dmGene	*D. melanogaster* protein

S. cerevisiae:

GENE	wild type gene
gene	mutant gene
Genep	protein product

S. pombe:

gene+	wild type gene
gene	mutant gene
Gene	protein product

Other eukaryotes:

Gene	wild type gene
gene	mutant gene
Gene	protein product

E. coli:

gene	gene
gene	protein product

Chapter One: Introduction to Control of DNA Replication in Mitotic Cell Cycles

The growth of an organism depends on production of new cells by mitotic cell division. A mitotic cell division involves growth of the cell, duplication of the organelles and division to produce two genetically identical daughter cells. In particular, a single accurate copy of the genome must be produced before cell division occurs. In all organisms new copies of the genome are generated directly from existing copies by semi-conservative DNA replication. The two new copies of the genome are segregated equally during cell division so that each daughter receives an identical copy of the genome. It is essential that the genome remains unchanged through cycles of DNA replication and segregation during cell division as even tiny alterations in the content of the genome can be catastrophic for the organism. The mitotic cell cycle of eukaryotic cells generally consists of 4 phases including two gap phases (called G1 and G2), S phase (when DNA replication occurs) and M phase (where the 2 copies of the genome are equally segregated and cell division takes place). Checkpoint controls ensure that inappropriate progression through the cell cycle does not occur. The rate of entry of cells into the cell cycle is controlled by extra-cellular signals from other cells and by the availability of nutrients necessary for cell growth.

This thesis will deal with some of the regulatory mechanisms which exist in eukaryotic cells to ensure that the genome is replicated only once in each mitotic cell cycle, using the yeast *Saccharomyces cerevisiae* as a model organism. The initiation of DNA replication in eukaryotes occurs from multiple origins and initiation from each origin is restricted to once per cell cycle. Our understanding to date of the initiation reaction at a molecular level is detailed below.

Initiation of DNA replication in Escherichia coli, bacteriophage and SV40

Before initiation of DNA replication was understood at a molecular level, the replicon model predicted that it involves recognition of DNA

sequences near the start site of DNA replication (known as the replicator), by a factor (called the initiator), which then induces assembly of the replication machinery (Jacob et al., 1963). The initiation reaction in *E. coli* is relatively well understood (Kornberg and Baker, 1992). The *E coli* genome is a single circular chromosome and DNA replication initiates at a single origin, *oriC*, and proceeds until specific termination sequences are reached. The functional elements of *oriC* are contained within a 245bp sequence which includes four 9bp sequences which the initiator protein, dnaA, binds in a sequence specific manner (Fuller et al., 1984). Around 20-40 dnaA subunits bind to the origin in a co-operative manner, and footprinting studies have demonstrated that it protects 200bp of the origin sequence from DNase I (Fuller et al., 1984). After binding to the origins, dnaA then induces melting in three 13mers in the origin sequence (Bramhill and Kornberg, 1988) and this allows 2 molecules of the DNA helicase, dnaB to bind as a complex with another protein, dnaC (Wahle et al., 1989). The activity of the helicase induces origin unwinding and assembly of a replication fork (Baker et al., 1987). dnaA function in initiation of replication is dependent on it being bound to ATP (Sekimizu et al., 1987). A polymerase III processivity factor, the sliding β clamp induces conversion of active ATP-bound dnaA to replicatively inactive ADP-bound dnaA (Kurokawa et al., 1999). In this way assembly of an active replication fork is linked to inactivation of the initiator protein, thus preventing immediate re-initiation.

The initiation reaction in the bacteriophage λ and the mammalian specific SV40 virus have also been intensively studied (Kornberg and Baker, 1992). Four 19bp elements in the bacteriophage λ origin is recognised by the phage protein O, which melts a flanking A/T rich sequence (Schnos et al., 1988). O then interacts with the λP protein which acts in a similar way to *E. coli* dnaC to recruit dnaB helicase to the origins (Dodson et al., 1986; Dodson et al., 1985; Zylicz et al., 1984). In SV40 replication, a single viral protein, the T-antigen, is sufficient to recognise four repeats of a 5bp sequence in the viral origin and induce origin melting in a 15bp palindrome, (reviewed Bullock, 1997; Kornberg and Baker, 1992). T-antigen helicase activity is sufficient for viral replication. Other replication proteins are provided by the host. Initiation of DNA replication in *E. coli*, bacteriophage and SV40 all involve sequence specific

recognition of an origin by an initiator protein which then assembles a replication fork and therefore conforms to the replicon model described above. Of eukaryotic cells, the initiation reaction in the budding yeast *Saccharomyces cerevisiae*, also conforms to the replicon model and is described below.

Origins in S. cerevisiae

In *S. cerevisiae*, origins of replication have been identified as sequences which were required for extra-chromosomal maintenance of plasmids (Stinchcomb et al., 1979). Such sequences are known as autonomously replicating sequences (ARS) and are approximately 100-150bp long. ARS sequences function as origins in the plasmids which they are required to replicate (Brewer and Fangman, 1987; Huberman et al., 1987). ARS have an A/T rich 11 base pair consensus sequence called the ARS Consensus Sequence (ACS) (Broach et al., 1983) which is essential for origin function (Deshpande and Newlon, 1992; Rivier and Rine, 1992). In addition to the ACS, surrounding DNA sequences are also important for origin function of ARS sequences. Analysis of the usage of ARS sequences using 2-D gel analysis has revealed that some but not all ARS sequences act as origins during a normal S phase (Dubey et al., 1991; Newlon et al., 1993). Origin firing occurs in a temporal program throughout S phase and there is now evidence that chromosomal ARS sequences which do not fire during a normal S phase are simply very late firing origins which are normally replicated by replication forks initiating from other origins before they fire themselves (Santocanale et al., 1999).

The best characterised origin in yeast is *ARS1* which has an ACS (or A element), essential for its function in vivo plus three additional functional elements known as B elements, B1, B2 and B3. No single B element is essential for origin function, however deletion of any 2 B elements severely disrupts origin use (Marahrens and Stillman, 1992). In addition a C element has been described which only affects ARS function in the absence of a B element (Celniker et al., 1984). Other *ARS* elements have a similar structure. Replication initiation point mapping has now identified the origin of bi-directional DNA replication (OBD) in *ARS1* to be a single site located between the B1 and B2 elements (Bielinsky and Gerbi, 1999). Identification and characterisation of

origin sequences in *S. cerevisiae* was crucial for the identification of origin binding proteins which initiate the assembly of a replication fork.

Identification of origin binding proteins in S. cerevisiae

The first ARS binding protein identified was a transcription factor called Abf1p which binds to the B3 element of *ARS1 in vivo* and *in vitro* (Diffley and Stillman, 1988; Diffley and Cocker, 1992; Diffley and Stillman, 1989). *abf1* mutants have a plasmid loss defect (Rhode et al., 1992) and *ARS1* sequences with mutated B3 element function less efficiently as origins compared to wild type *ARS1* (Marahrens and Stillman, 1992). The B3 element in *ARS1* can be substituted with binding sites for other transcriptional activators (Marahrens and Stillman, 1992) although the role of transcription factors in origin firing is still not well understood.

Biochemical purification of ARS binding proteins also resulted in the identification of another protein complex known as the origin recognition complex or ORC which binds all ARS sequences examined (Bell and Stillman, 1992). ORC binding to *ARS1* requires the A and B1 elements, furthermore DNase I footprinting has shown that ORC protects the A and B1 elements of *ARS1* and induces a hypersensitive site in the B1 region (Bell and Stillman, 1992). ORC subunits can be chemically cross-linked to A and B1 regions on both strands of the DNA (Bell and Stillman, 1992; Lee and Bell, 1997) and it has been suggested that origin DNA wraps around the ORC complex, perhaps facilitating ORC function. By analogy with dnaA of *E. coli* and T-antigen of SV40, it might be expected that ORC has some DNA melting properties, although no evidence for this has been described.

ORC consists of six polypeptides, Orc1-6p, all of which are essential *in vivo* (Bell et al., 1995; Hardy, 1996; Li and Herskowitz, 1993; Loo et al., 1995; Micklem et al., 1993). DNase I footprinting and chromatin immuno-preciptitation techniques have demonstrated that ORC binds ARS sequences *in vivo* as well as *in vitro* (see below for details) (Aparicio et al., 1997; Diffley and Cocker, 1992; Rowley et al., 1995; Tanaka et al., 1997). Analysis of ORC mutants demonstrated that it has roles in both initiation of DNA replication and silencing at yeast mating type loci, although the latter will not be discussed further here

(Loo et al., 1995; Bell et al., 1993; Micklem et al., 1993; Foss et al., 1993; Fox et al., 1995). *orc2* and *orc5* mutants have a reduced efficiency of origin firing and do not complete DNA replication at the non-permissive temperature (Bell et al., 1993; Fox et al., 1995; Liang et al., 1995; Loo et al., 1995; Micklem et al., 1993). Furthermore *orc2*, *orc5* and *orc3* mutants have a plasmid loss defect, which can be rescued by addition of multiple origins to the plasmid(Hardy, 1996; Loo et al., 1995) and such a phenotype suggests a defect in initiation of DNA replication. In conclusion ORC clearly has an important role in initiation of DNA replication in *S. cerevisiae.*

Origins of replication and ORC in other eukaryotes

Unlike *S. cerevisiae*, the origins of replication in other eukaryotes are much less well defined. In *Schizosaccharomyces pombe* ARS sequences about 500-1000bp long have been identified. No consensus sequences essential for ARS function have been identified, although A/T rich regions about 20-50bp long are important for the function of some ARS sequences (Clyne and Kelly, 1995). Purification of proteins associating with tandem repeats of a conserved element in *S. pombe* ARS has identified two proteins, Abp1 and Abp2 neither of which has been shown to have a role in DNA replication (Murakami et al., 1996; Sanchez et al., 1998). A six-subunit complex containing homologous polypeptides to the ORC subunits of *S. cerevisiae* has now been identified in *S. pombe* (Moon et al., 1999). Although no sequence specificity for spORC binding to origins has been demonstrated, it binds A/T rich sequences using an 'AT hook' in Orc4 (Chuang and Kelly, 1999). This does not appear to be a common mechanism for ORC binding to DNA as no other Orc4p homologues have more than one copy of this motif. spORC appears to bind origins of DNA replication *in vivo* as Orp1 can be cross-linked to functionally important elements in *ars2004* and to *ars3002* (Ogawa et al., 1999). Analysis of mutants of *orp1* and *orp2* (*ORC1* and *ORC2* homologues respectively) in *S. pombe* has demonstrated that these genes have an important role in DNA replication(Grallert and Nurse, 1996) (Leatherwood et al., 1996; Muzi-Falconi and Kelly, 1995). Therefore spORC apparently functions in a similar way to ORC in *S. cerevisiae.*

The nature of metazoan replication origins is controversial (DePamphilis, 1999). No ARS sequences have been identified and origin mapping techniques give conflicting data about the location of origins. Over a dozen replication origins have now been identified in metazoans however very few have been demonstrated to direct initiation DNA replication in alternative chromosomal locations. In *Drosophila melanogaster*, 430bp of the *ACE3* region which is responsible for amplification of the chorion gene in follicle cells can direct initiation within a 7.7kb P element (Orr-Weaver et al., 1989). In addition an 8kb region of the human β globin locus functions as an origin when integrated at two different sites in the simian genome. In the latter case a minimum of 2kb of the human β globin locus can act as an origin, dependent on proximal 5′ or 3′ auxiliary sequences (Aladjem et al., 1998). These experiments suggest that, as in yeast, specific sequences direct initiation of DNA replication in metazoans, however this may not always be the case. At one extreme *Xenopus laevis* oocytes will replicate random sequences of DNA injected into them with apparently no sequence specificity (Harland and Laskey, 1980). The existence of specific initiation sites in metazoan genomes rather than broad 'initiation zones' is controversial (for example compare Dijkwel and Hamlin, 1995 ;Kobayashi et al., 1998), as is the notion that DNA sequence rather than chromatin structure directs initiation. In addition it is possible that sequences distant from origins may be important for replication, for example, deletion of sequences 50kb away from the human β globin locus disrupts origin function, (Aladjem et al., 1995) although these sequences were not required for initiation from the 8kb replicator described above. In *D. melanogaster* chorion gene amplification, the *ACE3* element acts 1.5kb away from an origin in or near the *AER-d* amplification control element (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). Origins of replication are also developmentally regulated. For example in *X. laevis* the pattern of origin firing in rDNA genes alters with the activation of transcription at the mid-blastula transition (Hyrien et al., 1995).

Despite the confusion surrounding the nature of metazoan origins of replication, complexes which contain several homologues of ORC subunits have now been identified in *D. melanogaster* (Chesnokov et al., 1999) and *X. laevis* (Romanowski et al., 1996b; Rowles et al., 1996; Tugal et al., 1998). In mammalian

cells interactions have been described between Orc2p, Orc4p and Orc5p homologues (Quintana et al., 1997; Quintana et al., 1998). In *D. melanogaster* ORC specifically binds to *ACE3* and *AER-d* DNA elements (Austin et al., 1999), suggesting that there is at least some specificity for ORC binding to origins of replication in metazoans. In addition ORC subunits localise with elements undergoing gene amplification *in vivo* (Austin et al., 1999; Royzman et al., 1997). No association of ORC with origins of replication in other metazoans has been described to date.

ORC appears to be functionally conserved throughout eukaryotes, for example, immuno-depletion of ORC from *X. laevis* egg extracts results in a defect in initiation of DNA replication (Carpenter et al., 1996; Romanowski et al., 1996b; Rowles et al., 1996). *In vitro* experiments using *D. melanogaster* have demonstrated that extracts immuno-depleted for ORC have a defect in initiation of replication (Chesnokov et al., 1999). Furthermore a *D. melanogaster* mutant in the gene encoding an Orc2p homologue has a defect in chorion gene amplification (Landis et al., 1997). It has not been directly demonstrated whether ORC is required for DNA replication in mammalian cells.

Other factors important for initiation of DNA replication

Genetic screens in fission and budding yeasts have been invaluable for identification of other genes which have a role in initiation of DNA replication. Two types of screens have been especially useful; those for genes which are important for cell cycle progression (the CDC screen) and those for genes which have a role in minichromosome maintenance (the MCM screen). The *S. cerevisiae* gene, *CDC6* and in the *S. pombe* homologue *cdc18+*, were identified in screens for genes which have a role in cell cycle progression (Hartwell, 1976; Nasmyth and Nurse, 1981). Mutants in both genes, have a defect in initiation of DNA replication as measured by FACS analysis, pulsed field gel electrophoresis and 2-D gel analysis (Bueno and Russell, 1992; Kelly et al., 1993; Liang et al., 1995; Piatti et al., 1995). Furthermore *cdc6-1* mutants have a plasmid loss defect which can be rescued by addition of multiple origins to the plasmid (Hogan and Koshland, 1992). In *S. pombe*, over-expression of *cdc18+* induces multiple rounds of DNA replication in the absence of mitosis (Muzi-Falconi et al., 1996; Nishitani and

Nurse, 1995), suggesting that *cdc18+* has a key role in controlling initiation of DNA replication. Cdc6p and Cdc18 homologues have been identified in human cells (Williams et al., 1997)and in *X. laevis* (Coleman et al., 1996). *In vitro* studies using *X. laevis* egg extracts confirm that xlCdc6 is required for DNA replication (Coleman et al., 1996)and over-expression of a dominant negative *cdc6* mutant, or micro-injection of anti-Cdc6 antibodies in cultured mammalian cells also induces a replication defect (Hateboer et al., 1998; Herbig et al., 1999; Yan et al., 1998). In conclusion Cdc6p homologues are important for initiation of DNA replication throughout eukaryotes.

Another group of proteins which are important for DNA replication are the Mcm2-7p family, which form a multimeric complex (see below). In *S. cerevisiae MCM2, MCM3* and *MCM5* were identified in a screen for mutants which have an ARS specific defect in the maintenance of minichromosomes (Maine et al., 1984). A screen for cell cycle genes identified *CDC54* which was subsequently designated *MCM4* (Moir et al., 1982). Mutants in *cdc46* and *cdc47* were identified as suppressers of *cdc54* mutants and *CDC46* was found to be identical to *MCM5* (Chen et al., 1992; Moir et al., 1982), while *CDC47* was designated *MCM7*. Homologues of these genes were identified in *S. pombe* in similar genetic screens. For example, a screen for cell cycle genes identified *nda1+* and *nda4+* (Miyake et al., 1993) which are homologues of *MCM2* and *MCM5* respectively, and *nda1+* was also identified independently as *cdc19+* (Forsburg and Nurse, 1994). Another *S. pombe* cell cycle gene, *cdc21+* is a homologue of *MCM4* in *S. cerevisiae* (Coxon et al., 1992). Finally, a screen for mutants with a minichromosome loss defect in *S. pombe* identified *mis5+* (Takahashi et al., 1994) which was subsequently designated *MCM6*. FACS analysis of *mcm2-7* mutants shown that they typically arrest with incompletely replicated DNA (Hennessy et al., 1990; Hennessy et al., 1991; Labib et al., 2000). Furthermore 2-D gel analysis reveals that *mcm2* and *mcm3* mutants in *S. cerevisiae* have a reduced efficiency of origin firing, consistent with the observed plasmid loss defect (Gibson et al., 1990; Yan et al., 1991; Yan et al., 1993). These observations suggest that Mcm2-7p have an important role in initiation of DNA replication.

In mammalian cells the first homologue of Mcm2-7p (named P1) was found to physically interact with the replication machinery (polymerase α) (Thommes et al., 1992). In *X. laevis*, Mcm2-7 are essential for initiation of replication(Kubota et al., 1997; Madine et al., 1995a; Thommes et al., 1997). Injection of antibodies raised against a mammalian homologue of Mcm2-7 into cultured cells resulted in a defect in DNA replication (Kimura et al., 1994; Todorov et al., 1994). In *D. melanogaster*, mutants in *mcm2* and *mcm4* homologues also have defects in DNA replication (Feger et al., 1995; Treisman et al., 1995), and injection of antibodies against dmMcm4 and dmMcm5 into *D. melanogaster* embryos induces a phenotype consistent with partial inhibition of DNA replication (Su et al., 1997). These experiments suggest that the function of Mcm2-7p is conserved between eukaryotes.

cdt+ was originally identified in a screen in *S. pombe* for genes regulated by a cell cycle transcription factor (Cdc10) (Hofmann and Beach, 1994). spCdt1 and the *X. laevis* homologue were both found to be essential for initiation of DNA replication, (Maiorano et al., 2000; Nishitani et al., 2000). No *S. cerevisiae* homologue of Cdt1 has been characterised to date.

Genetic interactions between the genes encoding ORC, Cdc6, Mcm2-7 and Cdt1 have been described. For example, in *S. cerevisiae*, over-expression of *CDC6* can rescue the origin firing defect of an *orc5-1* mutant (Liang et al., 1995) and synthetic lethality has been observed between *cdc6-1*, *cdc54-1* or *cdc47-1* and either *orc2-1* or *orc5-1* (Liang et al., 1995; Loo et al., 1995). In *S. pombe* over-expression of *cdt1+* promotes *cdc18+* induced re-replication (Nishitani et al., 2000). Over-expression of *ORC6* in *S. cerevisiae* is synthetically lethal with *cdc6*, and *mcm5* mutants (Kroll et al., 1996). Taken together these results suggest that ORC, Cdc6, Cdt1 and Mcm2-7 act at a common step in initiation of DNA replication.

Formation of the pre-replicative complex

In budding yeast for much of the cell cycle (after origins fire in S phase, G2 and M phases) the DNase I footprinting pattern at origins of replication is identical to that produced when purified ORC binds origins of replication *in vitro*, suggesting that ORC alone is bound to origins as these times (Diffley and

Cocker, 1992; Rowley et al., 1995). Consistent with this, Orc1-6p are associated with chromatin throughout the cell cycle (Donovan et al., 1997; Liang and Stillman, 1997)and chromatin immuno-precipitation has demonstrated that Orc1p, Orc2p and Orc3p are associated with origins throughout the cell cycle (Aparicio et al., 1997; Tanaka et al., 1997). Interaction of ORC with origins in *S. cerevisiae* requires an intact *ARS* element (Aparicio et al., 1997; Rowley et al., 1995; Tanaka et al., 1997). Similar results have been obtained in *S. pombe;* chromatin binding analysis has demonstrated that Orp1, Orp2 and Orc5 are associated with chromatin throughout the cell cycle (Lygerou and Nurse, 1999) and that Orp1 specifically immuno-precipitates with origins throughout the cell cycle (Ogawa et al., 1999). In metazoans the pattern of ORC chromatin binding is less clear. In human cell culture hsOrc2 is associated with chromatin throughout the cell cycle (Ritzi et al., 1998) as is cgOrc2 in hamster cells, however cgOrc1 becomes removed from origins during mitosis (Natale et al., 2000). Some studies have also suggested that the nature of *X. laevis* ORC association with origins changes during that cell cycle (Romanowski et al., 1996a; Rowles et al., 1996; Rowles et al., 1999). In conclusion, although in yeast ORC clearly associates with origins throughout the cell cycle, this may not be the case in higher eukaryotes.

In vivo DNase I footprinting studies of origins of DNA replication in both *S. cerevisiae* and human cells have shown that the chromatin structure at origins changes throughout the cell cycle (Abdurashidova et al., 1998; Diffley et al., 1994). In *S. cerevisiae* during G1, the DNase I footprinting pattern at origins changes and an extended region of protection appears at both active and inactive origins of replication (in *ARS1* this extends over the A, B1 and B2 elements), and the hypersensitive sites induced by ORC binding disappears (Diffley et al., 1994; Santocanale and Diffley, 1996). This alteration in the DNase I footprint is due to binding of a protein complex, called the pre-replicative complex (pre-RC) at origins. Mutants in *orc2, cdc6, mcm4* and *mcm7* have a defect in pre-RC formation (Cocker et al., 1996; Detweiler and Li, 1997; Santocanale and Diffley, 1996) (Karim Labib and John F. X. Diffley, unpublished data). Chromatin immuno-precipitation has demonstrated that ORC, Cdc6p and Mcm2-7p are all present at

origins during G1 in *S. cerevisiae* (Aparicio et al., 1997; Ogawa et al., 1999; Tanaka et al., 1997), consistent with the idea that all these proteins are part of the pre-RC.

Chromatin binding studies and chromatin immuno-precipitation studies in yeast and *X. laevis* have provided a model for pre-RC assembly (Aparicio et al., 1997; Coleman et al., 1996; Donovan et al., 1997; Romanowski et al., 1996b; Rowles et al., 1996; Tanaka et al., 1997). These studies have found that ORC binding to chromatin is required for binding of Cdc6p to chromatin, while both ORC and Cdc6p are required for loading of Mcm2-7p onto origins. In budding yeast around half of the Cdc46p, Cdc47p and Mcm2p proteins present in a cell become loaded onto chromatin during G1 (Donovan et al., 1997). In *X. laevis* and *S. pombe* Cdt1 is essential for Mcm2-7 chromatin binding but not for ORC and Cdc18 loading onto chromatin (Maiorano et al., 2000; Nishitani et al., 2000). In *S. cerevisiae* an unrelated protein, Mcm10p has some role in Mcm2-7p binding to chromatin, but it is unclear if this represents a step in pre-RC formation since Mcm10p is present on chromatin throughout the cell cycle and binding of Mcm10p to chromatin does not require ORC (Homesley et al., 2000). Estimations suggest that a single ORC complex and 10-20 Mcm2-7p complexes are loaded onto each origin of replication prior to initiation, suggesting that ORC and Cdc6p (and possibly Cdt1p) actively load multiple Mcm2-7p complexes onto origins of replication.

It is not understood how ORC and Cdc6p load Mcm2-7p onto origins although since Orc1p and Cdc6p have homology to each other (Bell et al., 1995), it has been suggested that they form a complex which loads Mcm2-7p. Consistent with this Cdc18 associates with one of the ORC subunits, Orp2, in fission yeast (Leatherwood et al., 1996) and purified, recombinant ORC and Cdc6p from *S. cerevisiae* form a complex dependent on the presence of origin DNA (Mizushima et al., 2000). In human cells hsOrc1 and hsCdc6 co-purify away from the hsOrc2 subunit (Saha et al., 1998), suggesting that ORC may not necessarily function as a six subunit complex in mammals. Cdc6p, Orc1p, Orc4p and Orc5p contain Walker A and B motifs which are thought to be important for ATP binding and hydrolysis respectively (Neuwald et al., 1999). The ATP binding and hydrolysis motifs in Cdc6p and ORC appear to be important for the function of ORC and Cdc6p in DNA replication. In *S. cerevisiae*, ORC binding to

origins requires the presence of ATP, but not ATP hydrolysis (Bell and Stillman, 1992) and yeast which contain mutations in Orc1p predicted to inhibit ATP binding are inviable and these mutations abolish sequence specific ORC binding to ARS sequences *in vitro* (Klemm et al., 1997). Cdc6p mutants in residues predicted to be important for ATP binding will not support DNA replication or load Mcm2-7p onto chromatin (Wang et al., 1999; Weinreich et al., 1999). Expression of an allele of *cdc6* which encodes a protein predicted to have non-functional ATP hydrolysis motifs has a dominant negative phenotype in *S. cerevisiae* and human cells, suggesting that this protein can interact with other pre-RC components (Herbig et al., 1999; Perkins and Diffley, 1998). Mutation of conserved ATP hydrolysis motifs in Cdc6p allows pre-RCs to form, but bulk Mcm2-7p loading onto chromatin is disrupted (Perkins and Diffley, 1998). This may be explained if the mutant is able to load a single Mcm2-7p complex onto chromatin but no more. This interpretation is controversial, as one group has reported that mutation of the ATP hydrolysis motif of Cdc6p from *S. cerevisiae* does not affect the role of Cdc6p in DNA replication (Weinreich et al., 1999). Despite this, recent studies have demonstrated that association of Cdc6p with ORC *in vitro* induces an increased specificity of ORC for ARS DNA dependent on ATP binding and hydrolysis motifs in Cdc6p (Mizushima et al., 2000). In addition biochemical remodelling of Orc2p and Orc6p, occurs upon association of ORC and Cdc6p. This is dependent on Cdc6p ATP binding motifs and is inhibited in the presence of non-hydrolysable ATP analogues (Mizushima et al., 2000), however the functional significance of these *in vitro* observations has not been addressed. Taken together, these data suggest that the ATPase activity of ORC and Cdc6p may be important for Mcm2-7p loading onto chromatin.

Loading of Mcm2-7p onto origins appears to complete the role of Cdc6p and ORC in initiation of DNA replication. After pre-RC formation, in both *X. laevis* and *S. cerevisiae*, ORC may be removed from chromatin with high salt washes (Donovan et al., 1997; Rowles et al., 1999), while Mcm2-7p chromatin binding is maintained. Furthermore, in *X. laevis* extracts depletion of both ORC and Cdc6p after Mcm2-7p loading but before activation of S phase does not affect DNA replication (Rowles et al., 1999). In budding yeast most Cdc6p is rapidly degraded prior to entry into S phase but it is not known whether the

Cdc6p present in pre-RCs is removed by this degradation (Drury et al., 1997). Contrary to these observations, inactivation of a temperature sensitive allele of *cdc6* in *S. cerevisiae* cells arrested in G1 disrupts the pre-replicative footprint (Cocker et al., 1996; Detweiler and Li, 1997), suggesting that some Cdc6p and ORC may be required to maintain pre-RCs in *S. cerevisiae*.

Loading of Mcm2-7p onto chromatin is essential for initiation of DNA replication, although chromatin binding analysis in *X. laevis* has demonstrated that Mcm2-7 are gradually removed as replication proceeds. In fission yeast and *X. laevis* Mcm2-7 are predominantly found in hetero-hexameric complexes (Adachi et al., 1997; Chong et al., 1996; Kubota et al., 1997) although sub-complexes of Mcm4, 6, 7 and Mcm3, 5 have also been identified. The role of these sub-complexes are unclear since all Mcm2-7 subunits are required for the function of Mcm2-7 in DNA replication (Prokhorova and Blow, 2000). Mcm2-7 have a region of homology known as an 'Mcm2-7 box' which includes the Walker A and B motifs characteristic of ATPases (Koonin, 1993). A single homologue of the eukaryotic Mcm2-7 proteins has been identified in the archaebacterium *Methanococcus thermoautotrophicum*. This archaebacterial Mcm2-7 forms a double-hexameric complex *in vitro* and has helicase activity sufficient to displace 500bp of DNA (Chong et al., 2000; Kelman et al., 1999; Shechter et al., 2000). These observations suggest that Mcm2-7 proteins in eukaryotes may function as a helicase in DNA replication. Consistent with this idea, sub-complexes of hsMcm4,6,7 purified from human cells have been shown to have limited helicase activity (Ishimi, 1997; You et al., 1999), although hexameric complexes of Mcm2-7 purified from *X. laevis* and *S. pombe* have never been found to have any helicase activity (Adachi et al., 1997). In *E. coli* and SV40 systems, helicases (dnaB and T-antigen respectively) open the DNA around an origin during initiation and this stimulates the assembly of an active replication fork and it is possible that Mcm2-7 function in a similar way. In *S. cerevisiae* Mcm2-7p are not only involved in initiation of DNA replication, they are required for elongation of the replication fork (Labib et al., 2000) and chromatin immuno-precipitation analysis has shown that Mcm2-7p move away from origins of replication after initiation (Aparicio et al., 1997). This suggests that Mcm2-7p are

the first known components of the replication fork to be loaded onto origin sequences and this step links initiation and elongation of DNA replication.

S phase promoting factor

Loading of Mcm2-7p is the last known event at origins before S phase. Cell fusion studies predicted that a factor in S phase cells can promotes initiation of DNA replication in G1 phase nuclei (Rao and Johnson, 1970). This factor was called S phase promoting factor or SPF. We now know that at the end of G1 two kinases essential for origin firing, cyclin dependent kinases (CDKs), and Cdc7/Dbf4 become active and it is likely that these proteins are SPF. In *S. cerevisiae* mutants which arrest prior to activation of CDKs and Cdc7/Dbf4 arrest with pre-RCs at origins (Diffley et al., 1994). The two kinases are similar; both have a catalytic subunit which is constitutively expressed and regulatory subunits with cell cycle regulated expression patterns.

CDKs

In all eukaryotes CDKs control progression through the cell cycle. CDK activation requires binding of a regulatory cyclin subunit and phosphorylation of the CDK catalytic subunit by CDK activating kinase stabilises cyclin binding. Successive activation of different CDK/cyclin complexes controls cell cycle progression. This is achieved through regulated transcription of cyclin genes and regulated degradation of cyclin proteins. In addition, CDK/cyclin activity can be controlled through dephosphorylation of threonine and tyrosine residues in the ATP binding domain of the CDK partner. Proteins which specifically bind and inhibit CDKs also control cell cycle progression. For a review of the control of CDKs see (Morgan, 1997). CDK/cyclin complexes bind their substrates through contacts between the cyclin and cy (also called RXL) motifs in the substrate (Adams et al., 1999; Adams et al., 1996; Schulman et al., 1998). CDK/cyclin activity is necessary to enter both S phase and mitosis and at the end of mitosis CDKs are inactivated and remain low until the end of the following G1.

The major CDK in *S. cerevisiae*, Cdc28p, associates with at least nine cyclin subunits to control S phase and mitosis (Nasmyth, 1993; Nasmyth, 1996).

The first cyclins to become activated in the cell cycle are the G1 cyclins, Cln1-3p which control progression through G1 (Cross, 1988; Hadwiger et al., 1989; Nash et al., 1988; Richardson et al., 1989). Subsequently six B-type cyclins, Clb1-6p become expressed. The first B type cyclins to become activated at the G1/S transition are Clb5p and Clb6p and these trigger initiation of DNA replication (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). Later during S phase and mitosis *CLB3* and *CLB4* and then *CLB1* and *CLB2* become expressed (Fitch et al., 1992; Richardson et al., 1992; Surana et al., 1991). Clb3p and Clb4p probably promote spindle formation, while Clb1p and Clb2p are important for entry into mitosis. Deletion of *CLB5* and *CLB6* results in a delayed entry into S phase, presumably until Cdc28/Clb3 and Cdc28/Clb4 become activated (Schwob and Nasmyth, 1993). Cdc28/Clb kinase activity is required throughout S phase for activation of both early and late origins. In particular Clb5p is required for late origin firing (Donaldson et al., 1998b). Therefore sequential activation of different CDK/cyclin complexes control progression through the cell cycle.

In *S. pombe* a single CDK, Cdc2 controls progression through S phase and mitosis (Nurse and Bissett, 1981). The three major cyclins in *S. pombe* are Cig1, Cig2 and Cdc13. Cdc13 is essential for completion of mitosis and Cig1 also appears to have some role in mitosis. Initiation of DNA replication is probably triggered by Cig2, the first cyclin to become activated after mitosis (Martin-Castellanos et al., 1996). In strains where the *cig2+* gene is deleted, the mitotic cyclin Cdc13 is able to trigger initiation of DNA replication (Fisher and Nurse, 1996; Mondesert et al., 1996).

In vertebrates, the control of cell cycle progression is much more complex with several CDKs associating with multiple cyclin subunits (for review see Pines, 1993). Progression through G1 requires CDK4/ cyclin D, followed by Cdk2/cyclin E in late G1. Cdk2/cyclin A is important for progression through S phase while CDK1/cyclin A and CDK1/cyclin B trigger mitosis. In *X. laevis* egg extracts, immuno-depletion of Cdk2 or cyclin E inhibits DNA replication (Fang and Newport, 1991; Jackson et al., 1996). In mammalian cells micro-injection of anti-cyclin A, anti-cyclin E or anti-Cdk2 antibodies inhibits DNA replication (Girard et al., 1991; Tsai et al., 1993) (Ohtsubo et al., 1995; Pagano et al., 1992) and addition of cyclin A is sufficient to activate SV40 replication in a G1 extract

(D'Urso G et al., 1990). Over-expression of cyclin E is cultured cells decreases the length of G1 in serum depleted conditions (Ohtsubo and Roberts, 1993), while expression of dominant negative alleles of Cdk2 arrests cells in G1 (Vandenheuvel and Harlow, 1993). Therefore both Cdk2/cyclin A and Cdk2/cyclin E have important roles in S although the precise role of these two kinases in S phase is not understood.

Cdc7/Dbf4

CDC7 and *DBF4* were originally identified in *S. cerevisiae* in screens to identify cell cycle genes. Cdc7p is a kinase which requires Dbf4p for activity (Hollingsworth and Sclafani, 1990; Jackson et al., 1993; Yoon and Campbell, 1991). Although Cdc7p levels are constant during the cell cycle, Dbf4p levels oscillate, being low during G1, but becoming induced as cells enter S phase, coincident with an increase in Cdc7/Dbf4 kinase activity (Cheng et al., 1999; Godinho Ferreira et al., 2000; Oshiro et al., 1999). Cdc7 and Dbf4 are required throughout S phase for both early and late origin firing but do not play a role in progression through mitosis (Bousset and Diffley, 1998; Donaldson et al., 1998a). Homologues of Cdc7pwhich bind Dbf4p-like regulatory subunits have now been identified in mammals, fission yeast, *X. laevis* (Cdc7p but not Dbf4p) and *D. melanogaster* (a Dbf4p homologue) and they are all important for initiation of DNA replication (Brown and Kelly, 1998; Jiang and Hunter, 1997; Landis and Tower, 1999; Sato et al., 1997; Takeda et al., 1999). Furthermore in mammals and fission yeast, Dbf4 levels are regulated with a similar periodicity to *S. cerevisiae*(Kumagai et al., 1999) (Takeda et al., 1999).

Progression through G1 in S. cerevisiae

In *S. cerevisiae* S phase promoting CDKs and Cdc7/Dbf4 are maintained in an inactive state by three mechanisms during early G1. Firstly, Clbs and Dbf4p are targeted for ubiquitin mediated proteolysis by the anaphase promoting complex (APC/C) (see below) (Amon et al., 1994; Cheng et al., 1999; Godinho Ferreira et al., 2000; Irniger et al., 1995; Oshiro et al., 1999). In addition a specific inhibitor of Cdc28/Clb kinase, the Sic1 protein, is expressed in early G1 (Donovan et al., 1994; Mendenhall, 1993; Nugroho and Mendenhall, 1994;

Schwob et al., 1994). Lastly, *CLB1-6* and *DBF4* are not transcribed during early G1 (Chapman and Johnston, 1989; Fitch et al., 1992; Richardson et al., 1992; Schwob and Nasmyth, 1993).

When cells become committed to the cell cycle at start in late G1 Cdc28/Cln kinases become activated. This activation depends upon transcription of *CLN1* and *CLN2* by the cell cycle regulated transcription factor SBF, which comprises Swi4p and Swi6p (for review of G1 specific transcription see Lee, 1999). For much of the cell cycle Cdc28/Clb kinase activity inhibits SBF (Amon et al., 1993), but in late G1 SBF mediated transcription of *CLN2* and *CLN2* is activated by Cdc28/Cln3 (Dirick et al., 1995; Tyers et al., 1993). Cdc28/Cln3 also activates the transcription factor, MBF (Tyers et al., 1993), which activates transcription of *CLB5*, *CLB6* and *DBF4* (Chapman and Johnston, 1989; Epstein and Cross, 1992; Schwob and Nasmyth, 1993). Cdc28/Cln1 and Cdc28/Cln2 bring about activation of Cdc28/Clb kinase in two ways; Cdc28/Cln1 and Cdc28/Cln2 inhibit the APC/C, and thereby inactivate Clb proteolysis (Amon, 1997). In addition Cdc28/Cln targets the Cdc28/Clb inhibitor, Sic1p, for ubiquitin mediated proteolysis by the SCF.

Ubiquitin mediated proteolysis plays a crucial role in regulating entry into S phase. It involves covalent attachment of ubiquitin residues to lysine residues in the target proteins followed by proteolysis of ubiquitinated proteins by the proteosome (reviewed Hershko and Ciechanover, 1992; Hershko and Ciechanover, 1998). Attachment of ubiquitin to target proteins requires an E1 enzyme which activates ubiquitin, an E2 enzyme which is a ubiquitin conjugating enzyme and an E3 protein which covalently attaches ubiquitin specifically to the target protein. The E3 enzymes include the SCF (for Skp1-cullin-F-box protein) and the anaphase promoting complex or cyclosome (APC/C) and these provide specificity in the ubiquitination reaction. SCF comprises Cdc34p, an E2 enzyme, Cdc53p, Skp1p, Rbx1p and an F-box protein (Feldman et al., 1997; Kamura et al., 1999; Patton et al., 1998a; Seol et al., 1999; Skowyra et al., 1997). The F-box proteins, (which share a region of homology known as the F-box), provide SCF with specificity and recognition of substrates by F-box proteins generally requires the substrate proteins to be phosphorylated (Bai et al., 1996). Examples of F-box proteins important in cell cycle control

include Cdc4p (which targets Cdc6p, Far1p (an inhibitor of Cdc28/Cln) and Sic1p for ubiquitination) and Grr1p (which targets Cln1p and Cln2p for ubiquitination) although SCF substrates are not only involved in cell cycle control (reviewed Patton et al., 1998b). SCF dependent degradation of Sic1p at the end of G1 requires phosphorylation of Sic1p by Cdc28/Cln kinases (Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997a; Verma et al., 1997b). SCF dependent proteolysis of Sic1p is essential for entry into S phase (Schneider et al., 1996; Schwob et al., 1994; Tyers, 1996).

Progression through G1 in other eukaryotes

In *S. pombe*, CDK activity is low during G1 due to APC/C mediated proteolysis of Cdc13, Cig1 and Cig2 (Blanco et al., 2000; Kominami et al., 1998b; Stern and Nurse, 1998; Yamaguchi et al., 2000). In addition Cdc2/Cig2 and Cdc2/Cdc13 kinase activity is inhibited by Rum1 during G1 (Correa Bordes and Nurse, 1995; Martin-Castellanos et al., 1996). A homologue of the SCF containing a Cdc53p homologue, Pcu1 and two F-box proteins Pop1 and Pop2/Sud1 may be important for degradation of phosphorylated Rum1 at the G1/S transition (Benito et al., 1998; Kominami and Toda, 1997; Kominami et al., 1998a; Wolf et al., 1999). In addition Cdc2 inhibits APC/C dependent proteolysis of Cig1 and Cdc13 (Blanco et al., 2000; Yamaguchi et al., 2000). It not understood how the balance between inhibition of Cdc2 kinase activity and activation of Cdc2 becomes tipped in favour of Cdc2/Cig2 activation of the G1/S transition.

In metazoans, entry into S phase also involves activation of a transcriptional program and degradation of CDK inhibitors. In quiescent cells mitogenic signals induce Cdk4/cyclin D which phosphorylates and inhibits the Rb protein which is a negative regulator of the E2f transcription factors (reviewed Dyson, 1998; Sherr, 1996; Sherr and Roberts, 1999). Targets of E2f transcription factors include cyclin E, and cyclin A (Botz et al., 1996; Geng et al., 1996; Ohtani et al., 1995; Schulze et al., 1995). At the G1/S transition an inhibitor of Cdk2/cyclin E, p27 (also known as Kip1), is phosphorylated by Cdk2/cyclin E and targeted for ubiquitin mediated proteolysis in a Cul1 (a Cdc53p homologue), Skp1 and Skp2 (an F-box protein) dependent manner (Carrano et al., 1999;

Montagnoli et al., 1999; Tsvetkov et al., 1999). A homologue of Cdc34p, Ubc3 may also be involved (Pagano et al., 1995). In *X. laevis* extracts, inhibition of DNA replication by a dominant negative Ubc3 protein can be overcome by Cdk2/cyclin E, suggesting that degradation of Cdk2/cyclin E inhibitors is essential for entry into S phase (Yew and Kirschner, 1997). In conclusion, although the proteins involved in triggering activation of S phase in metazoans are different from those in yeast the, ways they are activated are similar.

Origin Firing in SV40

The viral SV40 system has been used as a model system for studying origin firing *in vitro*, and all the components necessary for DNA replication in this system have been purified (Tsurimoto et al., 1989; Waga et al., 1994a). After origin recognition and unwinding by the T-antigen, a single stranded DNA binding protein (RPA) stabilises the unwound DNA (reviewed Borowiec et al., 1990), (Wold and Kelly, 1988). DNA primase and polymerase α are then recruited by T-antigen and synthesise RNA and DNA primers (Gannon and Lane, 1987; Murakami et al., 1986). Polymerase δ together with a processivity factor, PCNA, is the only polymerase required for elongation of the primed DNA (Prelich et al., 1987). In addition a PCNA loading factor, RFC is required for replication (Tsurimoto and Stillman, 1989). Resolution of okazaki fragments requires ligase I, a DNA ligase and FEN1 an exonuclease (Waga et al., 1994a; Waga and Stillman, 1994). Topoisomerases I and II accommodate unwinding of the DNA at the replication fork and Topo II allows the two daughter molecules of DNA to be resolved (Yang et al., 1987). The proteins involved in the recognition of chromosomal origins of DNA replication and origin unwinding are clearly different from those of SV40 and in addition replication of DNA involves additional proteins which are not required for replication of SV40 *in vitro*. Nevertheless origin firing in SV40 provides a useful model for understanding origin firing in eukaryotes.

Origin firing in eukaryotes

Cdc7/Dbf4 probably acts directly at origins to trigger initiation of DNA replication since in *S. cerevisiae* Dbf4p binds to chromatin in an ORC dependent

manner and interacts with origins in a 1-hybrid analysis using ARS sequences as bait (Dowell et al., 1994; Pasero et al., 1999). Reciprocal shift experiments have suggested that activation of Cdc7/Dbf4 requires prior activation of Cdc28/Clb in *S. cerevisiae* (Nougarede et al., 2000), however observations in *X. laevis* suggest that Cdc7p activation does not require prior activation of CDKs and that Cdc7p binds to chromatin through the Mcm2-7 proteins (Jares and Blow, 2000). Although the target of CDKs in activation of origin firing is not well understood, there is now convincing evidence that the essential target of Cdc7/Dbf4 in initiation of DNA replication is the Mcm2-7p complex. For example in *S. cerevisiae*, the function of Cdc7/Dbf4 in origin firing can be bypassed by an *mcm5* mutant (*bob1*) (Hardy et al., 1997) and allele specific suppression has been described between *dbf4* and *mcm2* mutants (Lei et al., 1997). In addition, Mcm2p, Mcm3p, Mcm4p and Mcm6p from *S. cerevisiae* can all be phosphorylated *in vitro* by Cdc7/Dbf4 (Lei et al., 1997; Oshiro et al., 1999). Mcm2p has also been found to be phosphorylated *in vitro* in a Cdc7p dependent manner in *S. pombe*, *X. laevis* and mammalian cells (Jares and Blow, 2000; Roberts et al., 1999; Sato et al., 1997; Takeda et al., 1999). Taken together these results suggest that Mcm2-7p are a target for Cdc7/Dbf4p throughout eukaryotes.

The next known step in origin firing requires binding of Cdc45p (which has *S. cerevisiae* and *X. laevis* homologues) to origins of replication. Activation of both Cdc7/Dbf4 and Cdc28/Clb is appear to be important for the interaction of Cdc45p with origins (Owens et al., 1997; Zou and Stillman, 1998; Zou and Stillman, 2000), although this is controversial as some studies have reported that Cdc45p is bound to origins during G1 (Aparicio et al., 1997). Both results may be explained by a change in the nature of Cdc45p's association with origins upon activation of Cdc7/Dbf4 and Cdc28/Clb. Cdc45p can be phosphorylated *in vitro* by a kinase which associated with Cdc7p, although it is unclear whether Cdc45p is an *in vivo* target of Cdc7/Dbf4 (Nougarede et al., 2000). Cdc45p has close biochemical and genetic associations with Mcm2-7p proteins, (Dalton and Hopwood, 1997; Hopwood and Dalton, 1996; Zou et al., 1997), suggesting that Cdc7/Dbf4 dependent phosphorylation of Mcm2p is a key step in allowing Cdc45p to bind to origins. The function of Cdc45p in replication forks in unknown but, like Mcm2-7p proteins, Cdc45p moves away from origins as

replication initiates and is essential for the elongation phase of DNA replication (Aparicio et al., 1997; Tercero et al., 2000), suggesting that Cdc45p is part of the replication fork.

The following steps in establishment of a replication fork have been elucidated by chromatin immuno-precipitation analysis in *S. cerevisiae* (Aparicio et al., 1997; Tanaka and Nasmyth, 1998) and chromatin binding and recent studies of origin unwinding in *X. laevis* (Jares and Blow, 2000; Walter and Newport, 2000). Cdc45 loading is essential for binding of RPA to chromatin and consequently for origin unwinding (Walter and Newport, 2000). Origin unwinding is essential for association of the polymerase α-primase complex with DNA and consistent with this, Cdc45 is essential for polymerase α-primase loading (Mimura and Takisawa, 1998; Walter and Newport, 2000). In *S. cerevisiae* Cdc45p has genetic and biochemical interactions with Dbp11p and polymerase ε. Dbp11p is required for association of polymerase ε and polymerase α with ARS sequences (Masumoto et al., 2000). The role of Dbp11p and polymerase ε in replication is not well understood and neither protein is required for replication in SV40. Analogy with DNA replication in *E. coli* and SV40 would suggest that after primer synthesis by polymerase α, polymerase δ loads via interactions with RFC and PCNA using a polymerase switching mechanism (Tsurimoto and Stillman, 1991; Yuzhakov et al., 1999). The composition of a replication fork and the function of the proteins within in it is a long way from being understood in eukaryotic cells.

Association of Cdc45p, polymerase ε, and polymerase α with origins of DNA replication is temporally controlled during S phase, with binding occurring first at early origins and later at late origins (Aparicio et al., 1999). The chromosomal location of an origin has some influence over whether it will fire early or late within S phase (for example origins close to telomeric sequences fire late (Ferguson and Fangman, 1992)), but the importance and regulation of the timing of origin firing remain obscure. If replication is disturbed during S phase, for example if an inhibitor of ribonucleotide reductase, hydroxyurea, or a DNA damaging agent is present, a Rad53p and Mec1p dependent checkpoint ensures that replication ceases (Paulovich and Hartwell, 1995). This intra-S phase checkpoint halts S phase, in part by inhibiting late origin firing (Santocanale and

Diffley, 1998; Shirahige et al., 1998; Tanaka and Nasmyth, 1998). It is still not understood how late origin firing is prevented in this checkpoint or whether replication elongation is also inhibited.

Replication foci

Many observations of the incorporation of nucleotide analogues during S phase suggest that replication occurs at discrete foci within nuclei which may represent the association of many replication forks (Cox and Laskey, 1991; Leno and Laskey, 1991; Mills et al., 1989; Nakamura et al., 1986; Nakayasu and Berezney, 1989; Newport and Yan, 1996; Pasero et al., 1997). Several replication proteins including RPA, PCNA and polymerase α, cyclin A and Cdk2 co-localise with sites of DNA replication in the nucleus (Bravo and Macdonald-Bravo, 1987) (Adachi and Laemmli, 1992; Cardoso et al., 1993; Hozak et al., 1993). In *X. laevis* RPA foci can be observed before activation of Cdk2 kinase (Yan and Newport, 1995). The pattern of replication foci characteristically alters during S phase (Bravo and Macdonald-Bravo, 1987; Fox et al., 1991; Hozak et al., 1994; Nakayasu and Berezney, 1989; Okeefe et al., 1992), although the significance of this is not well understood. Regulation of replication foci assembly may represent another pathway through which initiation of replication can be controlled, although little is understood about this at present.

Cell cycle control of pre-RC formation: cell fusion studies

Some of the controls over DNA replication during the cell cycle were first demonstrated by cell fusion studies. One of the most elegant of these studies involved fusion of HeLa cells arrested at different cell cycle stages (Rao and Johnson, 1970). Fusion of a G1 phase cell to an S phase cell induced the G1 nucleus to initiate DNA replication prematurely, while fusion of a G2 phase cell to an S phase cell did not induce DNA replication in the G2 nucleus. Therefore a diffusible factor exists in S phase cells which can induce G1 but not G2 nuclei to undergo DNA replication. This implies that conversion of a G2 nucleus into a G1 nucleus during mitosis "re-sets" the nucleus so that it is competent to initiate DNA replication in the following cell cycle.

Licensing factor

The nature of the dependency of S phase upon mitosis was partly identified through *in vitro* studies using *X. laevis* oocytes. Extracts released from meiotic metaphase form a nucleus around added sperm chromatin, undergo a round of DNA replication and can be arrested in the following mitosis by addition of inhibitor of protein synthesis. When a metaphase arrested nucleus was exposed to fresh interphase extract another round of DNA replication could only occur upon permeabilisation of the nuclear envelope. This observation lead to the licensing factor model for control of DNA replication (Blow and Laskey, 1988). In this model a 'licensing factor' essential for DNA replication can only enter the nucleus during nuclear envelope breakdown during mitosis. This licensing factor then remains nuclear until replication occurs, during which it is excluded from the nucleus. The licensing factor would only then be able to re-enter the nucleus during the following mitosis.

The licensing factor model has lead to biochemical identification of some factors which are inactive in an extract prepared in the presence of an inhibitor of licensing factor. 6-DMAP, a kinase inhibitor, inhibits both replication licensing and release from metaphase arrest in *X. laevis* egg extracts (Blow, 1993). The ability of 6-DMAP treated extract to replicate DNA can be restored upon simultaneous addition of DNA and untreated interphase extract (Blow, 1993). Two biochemical fractions of *X. laevis* interphase extract, RLF-M and RLF-B have been found to be necessary for licensing in the presence of 6-DMAP (Chong et al., 1995). The RLF-M fraction contains xlMcm2-7 (Chong et al., 1995; Thommes et al., 1997). xlORC, xlCdc6 or xlCdt1 present in 6-DMAP treated nuclei are competent to initiate DNA replication, suggesting that they do not form part of licensing factor (Carpenter et al., 1996; Maiorano et al., 2000; Rowles et al., 1996; Tada et al., 1999). RLF-B is required for Mcm2-7 loading, and may be a novel component of pre-RCs, or a factor which overcomes inhibition of pre-RC formation (perhaps by geminin or CDKs, see below) in 6-DMAP treated extracts.

Consistent with the above results, studies using HeLa cell nuclei incubated in *X. laevis* egg extracts and *X. laevis* sperm chromatin incubated in embryonic *D. melanogaster* extracts demonstrated that perforation of the nuclear

membrane was necessary to re-set G2 nuclei with respect to initiation of DNA replication (Crevel and Cotterill, 1991; Leno et al., 1992). Mcm2-7 present in the *X. laevis* extract was found to be necessary for the re-replication observed in HeLa nuclei (Madine et al., 1995a). The intact HeLa nuclear membrane was permeable to xlMcm2-7, although nuclear membrane permeabilisation was required for xlMcm2-7 loading onto chromatin, suggesting that the nuclear localisation of some other factor is important for pre-RC formation (Madine et al., 1995b). Permeabilisation of the G2 nuclear membrane followed by repair of the membrane is not sufficient to allow another round of DNA replication upon exposure to a fresh interphase extract, suggesting that entry of a positive factor is required during nuclear envelope breakdown, rather than dilution of a negatively acting one (Coverley et al., 1993). These observations suggest that the licensing factor model may be generally applicable to eukaryotic cells.

The licensing factor model in the form described above does not include all the different ways in which DNA replication is controlled with respect to mitosis. This was always likely to be the case since certain organisms, such as yeast, do not undergo nuclear envelope breakdown during mitosis, yet DNA replication is still tightly regulated with respect to mitosis in these organisms. Furthermore, regulated DNA replication has been observed in the absence of a nuclear membrane in *X. laevis* extracts (Walter et al., 1998). Nevertheless the concept of a positively acting 'licensing factor' has been useful for understanding how DNA replication might be co-ordinated with mitosis.

DNA replication can initiate only once from each replication origin during a mitotic cell cycle and consistent with this Mcm2-7p are not present at origins after origin firing. This fits well with the observation that the Mcm2-7p proteins form part of licensing factor. Upon initiation Mcm2-7p proteins travel with the replication fork and are gradually displaced from the nucleus (in *S. cerevisiae)* and chromatin as replication progresses (Aparicio et al., 1997; Krude et al., 1996; Kubota et al., 1997; Labib et al., 1999; Labib et al., 2000; Madine et al., 1995a; Madine et al., 1995b; Nguyen et al., 2000; Thommes et al., 1997). Furthermore DNase I footprinting studies in *S. cerevisiae* demonstrated that concomitant with origin firing, the pre-replicative footprint is replaced by the post-replicative footprint which represents binding of ORC alone to the origin (Diffley et al.,

1994; Santocanale and Diffley, 1998). Thus after initiation of DNA replication, Mcm2-7p move away from origins and the pre-RC is then prevented from forming until completion of the following mitosis. The inability to form pre-RCs during S phase and G2 could partly explain why fusion of a G2 cell to an S phase cell does not induce DNA synthesis in the G2 nucleus. Control of pre-RC formation during the cell cycle is a key step to ensure that DNA replication occurs once and only once per cell cycle.

Control of pre-RC formation by CDKs

We now know that the cyclin dependent kinases have a key role in regulating pre-RC formation during the cell cycle. The first indication that CDK activity may have a negative as well as a positive role in control of DNA replication came from observations in *S. pombe* which demonstrated that *cdc2* mutants can be induced to undergo re-replication under certain conditions (Broek et al., 1991) and that yeast deleted in the mitotic cyclin *cdc13* underwent over-replication of the genome (Hayles et al., 1994). Furthermore over-expression of the mitotic CDK inhibitor Rum1 also resulted in over-replication (Moreno and Nurse, 1994). Similar experiments in *S. cerevisiae* demonstrated that inhibition of Cdc28/Clb kinase activity in G2/M arrested cells by over-expression of the Cdc28/Clb kinase inhibitor Sic1p induces pre-RC formation. Subsequent re-activation of Cdc28/Clb kinase then induces an additional round of DNA replication (Dahmann et al., 1995). Premature induction of Cdc28/Clb kinase activity in G1 arrested cells by over-expression of Clb2p was found to inhibit pre-RC formation (Detweiler and Li, 1998) and in addition Cdc6p can only function to induce pre-RC formation if it is expressed prior to activation of Cdc28/Clb kinase activation at G1/S (Piatti et al., 1996). Taken together these experiments suggest that Cdc28/Clb activity inhibits pre-RC formation and that pre-RC formation is restricted to G1, when Cdc28/Clb kinase is inactive. Recent data also suggests that Cdc28/Cln kinase activity as well as Cdc28/Clb kinase activity inhibits pre-RC formation.

There is now considerable evidence that CDKs negatively regulate pre-RC formation in metazoans as well as yeast. In *D. melanogaster* constitutive expression of cyclin E in endo-reduplicating tissues (which undergo multiple

rounds of DNA replication in the absence of mitosis), inhibits DNA replication (Follette et al., 1998; Weiss et al., 1998). Furthermore, G2/M arrested HeLa nuclei treated with the CDK inhibitor 6-DMAP can be induced to load Mcm2-7p onto chromatin and undergo an additional round of DNA replication (Coverley et al., 1996; Coverley et al., 1998). Interestingly, this is not dependent upon nuclear permeabilisation. Conditional alleles of *CDC2* have been used to generate rounds of re-replication in cultured human cells (Itzhaki et al., 1997). Thus oscillating levels of CDK activity are important for re-setting origins of replication.

CDKs may regulate the activity of pre-RC components, their stability, their sub-cellular localisation and their transcription. The many redundant mechanisms by which pre-RC formation is inhibited illustrates the importance of preventing re-replication during the cell cycle.

Regulated transcription of pre-RC components

CDK activity regulates the transcription of genes which have a role in pre-RC formation. For example, in *S. cerevisiae CDC6* transcription is at least partly under the control of the transcription factor Swi5p (Piatti et al., 1995). The sub-cellular localisation of Swi5p is regulated by Cdc28/Clb kinase such that phosphorylation of the N-terminus of Swi5p by Cdc28/Clb inactivates its nuclear localisation signal (Jans et al., 1995), and inhibits its ability to regulate *CDC6* transcription.

Regulated stability of pre-RC components

Ubiquitin mediated proteolysis controls the levels of proteins important in cell cycle progression. The stability of the pre-RC component Cdc6p in *S. cerevisiae* is the best understood example of how CDK dependent ubiquitin mediated proteolysis can control pre-RC formation. A 2-hybrid screen first demonstrated an interaction between Cdc6p and Cdc4p, an F box protein of the SCF (Drury et al., 1997). Substrates of the SCF are often targeted for destruction by phosphorylation and this is also the case for Cdc6p. Cdc6p interacts physically with Cdc28/Clb5 and a Cdc6p in which consensus phosphorylation sites for Cdc28p kinases have been mutated to non-phosphorylatable residues is

considerably more stable than the wild-type protein (Drury et al., 2000; Elsasser et al., 1999; Elsasser et al., 1996). Surprisingly, both Cdc28/Clb kinase activity and Cdc28/Cln kinase activity are able to target Cdc6p for proteolysis. Over-expression of *CDC6* in *S cerevisiae* does not induce re-replication, suggesting that other mechanisms negatively regulate DNA replication in the cell cycle (Drury et al., 1997).

In *S. pombe* Cdc18 is stability is regulated by Cdc2 kinase activity (Baum et al., 1998; Jallepalli et al., 1997; Jallepalli and Kelly, 1996; Lopez-Girona et al., 1998). Cdc18 proteolysis requires Pop1 and Sud1 (also called Pop2), which are homologues of Cdc4p (Jallepalli et al., 1998; Kominami et al., 1998a; Kominami and Toda, 1997). Mutation of CDK sites in Cdc18 stabilise the protein *in vivo* and abolish the interaction between Sud1 and Cdc18 (Jallepalli et al., 1998). Over-expression of *cdc18+* in *S. pombe* induces re-replication (Muzi-Falconi et al., 1996; Nishitani and Nurse, 1995), suggesting that control of Cdc18 levels is the major mechanism by which pre-RC formation is regulated. However this may be an over-simplification as expression of Cdc18 with mutated CDK phosphorylation sites enhances the over-replication phenotype, associated with over-expression of wild-type Cdc18, even when the proteins are present at comparable levels, suggesting that Cdc2 also inhibits Cdc18 function (Jallepalli et al., 1997; Lopez-Girona et al., 1998).

Regulation of protein localisation

The licensing factor model for control of DNA replication in the cell cycle suggests that at least one of the factors required for initiation of DNA replication becomes excluded from the nucleus as replication proceeds, until nuclear envelope breakdown at the end of mitosis. As Mcm2-7 proteins were found to be part of replication licensing factor in *X. laevis* it was possible that they have a regulated nuclear localisation. However in both *X. laevis*, and human cells Mcm2-7 proteins are constitutively nuclear (Thommes et al., 1992; Todorov et al., 1994) demonstrating that nuclear envelope breakdown at mitosis is not required for entry of Mcm2-7 into the nucleus.

In *S. cerevisiae*, which does not undergo nuclear envelope breakdown during mitosis, the sub-cellular distribution of Mcm2-7p proteins is cell-cycle

regulated. All six Mcm2-7 proteins have the same pattern of nuclear localisation; during G2/ M phase Mcm2-7p are excluded from the nucleus. However as cells exit M phase, Mcm2-7p re-localise to nuclei where they remain throughout G1 and the beginning of S, phase, becoming re-localised to the cytoplasm upon completion of S phase (Hennessy et al., 1990; Yan et al., 1993; Labib et al., 1999; Nguyen et al., 2000). Cdc28/Clb has been conclusively shown to inhibit accumulation of Mcm2-7p in the nucleus (Labib et al., 1999; Nguyen et al., 2000) and there is some evidence to suggest that Cdc28/Cln also does (Labib et al., 1999). Its is not understood how CDKs regulate the nuclear localisation of Mcm2-7p, for example whether CDKs inhibit entry of the Mcm2-7p to the nucleus or promote their nuclear export.

In mammalian cells the sub-cellular localisation of Cdc6 is regulated so that it is excluded from the nucleus during S phase. Cdk2/cyclin A appears to regulate this subcellular localisation and specifically phosphorylates Cdc6 *in vivo* and *in vitro* (Fujita et al., 1999; Jiang et al., 1999; Peterson et al., 1999; Saha et al., 1998). Mutation of consensus phosphorylation sites for CDKs in Cdc6 to non-phosphorylatable residues (alanines) results in accumulation of nuclear Cdc6p, while mutation of these residues to those which mimic phosphorylation results in constitutively cytoplasmic Cdc6. Therefore regulation of the subcellular distribution of proteins is one way in which pre-RC formation is controlled by CDKs.

Regulation of protein function by CDKs

In *S. cerevisiae* Cdc6p dependent loading of Mcm2-7p onto *ARS1* sequences *in vitro* has been observed in extracts prepared from G1 arrested cells. However, extracts prepared from G2/M arrest cells will not load Mcm2-7p onto *ARS1* sequences, even in the presence high levels of Cdc6p (T. Seki and J. F. X. Diffley, unpublished data). This suggests that in *S. cerevisiae*, regulation of Mcm2-7p localisation and regulation of Cdc6p levels are redundant with other controls of pre-RC formation. It is possible that direct modification of pre-RC components by Cdc28/Clb contributes to this control. For example two subunits of ORC, Orc2p and Orc6p have multiple consensus CDK phosphorylation sites and Orc6p is phosphorylated in a Cdc28/Clb dependent manner (Liang and

Stillman, 1997). Since ORC is constitutively bound to origins, any modification by CDKs may regulate ORC function in Mcm2-7p loading or binding to Cdc6p

In *S. pombe* Orp2 is associated with Cdc2 (Leatherwood et al., 1996) and in *X. laevis* Orc1 and Orc2 specifically associate with Cdk2/cyclin A (Romanowski et al., 2000). Studies using hamster cells have found that cgOrc1 dissociates from chromatin during mitosis (Natale et al., 2000) and consistent with this in *X. laevis* egg extracts high levels of Cdk2/cyclin A can induce dissociation of ORC from chromatin (Hua and Newport, 1998) and exposure of licensed interphase chromatin to metaphase extracts induces dissociation of xlOrc1p, xlOrc2p and xlCdc6p, from chromatin (Rowles et al., 1999). In conclusion CDK activity may modify the interactions of ORC with chromatin in metazoans.

Mcm2-7p may also be a target for negative regulation by CDKs despite the fact that levels of Mcm2-7p are constant throughout the cell cycle and the proteins are constitutively nuclear. Phosphorylation of hsMcm4 inhibits the helicase activity associated with hsMcm4,6,7 complexes purified from HeLa cells (Ishimi et al., 2000). In *X. laevis* and *S. pombe* mitotic extracts various Mcm2-7 have been found to be phosphorylated, but the functional significance of these observations is not understood (eg (Nishitani et al., 2000; Pereverzeva et al., 2000).

Other pathways important for initiation of DNA replication

The loading of polymerase α–primase onto chromatin is regulated during the cell cycle. CDK phosphorylation of the p86 subunit may regulate the association of polymerase α-primase with chromatin during G1, although the functional significance of this interaction is entirely unclear(Desdouets et al., 1998). It is possible that this represents a pre-RC independent pathway through which CDKs can negatively regulate DNA replication.

CDKs control pre-RC formation and DNA replication in multiple ways. Although these mechanisms are not precisely conserved across species, the same types of control are used, for example. transcription, and proteolysis are used to control different pre-RC components in different species. The multiple

redundant mechanisms by which CDKs control pre-RC formation reflects the importance of inhibiting re-replication in the cell cycle.

CDK control of DNA replication: a model

The results described above suggest the following model for control of pre-RC formation within the cell cycle. During G1 Cdc28p kinase activity is low and this allows formation of pre-RCs at origins. When CDK and Cdc7p kinase activity rises at the end of G1, assembly of the replication fork is completed and initiation of DNA replication occurs. Upon initiation of DNA replication the Mcm2-7 proteins move with the replication fork away from origins leaving ORC alone bound at origins. CDK activity remains high throughout S, G2 and M phase and inhibits further pre-RC formation until CDKs are inactivated at the end of mitosis. This model not only explains why completion of mitosis is important for pre-RC formation in the following cell cycle, but also provides a mechanism whereby DNA replication is restricted to a single round in each mitotic cell cycle.

Cell cycles where S Phase is not coupled to mitosis

In most cell cycles DNA replication is tightly coupled to nuclear division, however this is not always the case. For example, when haploid gametes are produced during meiosis a single S phase is followed by two cycles of nuclear division in the absence of an intervening round of replication. In some cell types, the majority of the genome is replicated multiple times in the absence of nuclear division. In other cells a particular region of the genome, usually encoding a highly transcribed genes is replicated multiple times while the rest of the genome is replicatively inactive. What is known about the control of DNA replication during these cell cycles is summarised below.

Meiosis

S phase is inhibited between the two meiotic divisions, meiosis I and meiosis II. Progression through meiosis I and II has been most intensively studied in *X. laevis* and requires Cdc2/cyclin B kinase activity. Activation of Cdc2/cyclin B between meiosis I and II requires the Mos MAP kinase and

depletion of Mos during meiosis results in DNA replication (Furuno et al., 1994). Furthermore, in starfish oocytes, micro-injection of dominant negative Cdc2 between meiosis I and II induces DNA replication (Picard et al., 1996). Therefore CDK activity appears to be important for prevention of DNA replication between meiosis I and meiosis II. This may well be due to inhibition of pre-RC formation by the mechanisms discussed above.

Endoreduplication

Endoreduplication cycles generally occur in specific cell types. One of the best understood is the endoreduplication observed in the larval tissues of *D. melanogaster.* These endocycles appear to require down regulation of Cdc2/cyclin A and Cdc2/cyclin B (Hayashi, 1996; Sigrist and Lehner, 1997). cyclin E is necessary to trigger S phase during endocycles (Knoblich et al., 1994), but constitutive expression of *cyclin E* inhibits endoreduplication (Follette et al., 1998; Weiss et al., 1998), suggesting that Cdk2/cyclin E inhibits origin re-setting. Endoreduplicative cycles also occur in the endosperm of maize embryos and in the mammalian megakaryocyte. In both maize endosperm and megakaryocytes, endoreduplication is accompanied by a drop in the activity of mitotic CDKs (Datta et al., 1996; Grafi and Larkins, 1995). These observations are consistent with a model where oscillating levels of CDK activity are required for origin re-setting and firing as described above.

Gene amplification

There are several examples of multiple rounds of specific gene amplification. In *D. melanogaster* follicle cells (which are responsible for synthesis of the egg), chorion genes are amplified. Chorion gene amplification requires dmOrc2, dmDbf4 (Chiffon), dmcyclin E and dmE2f1. dmOrc2 localises specifically at amplifying sequences (Landis et al., 1997; Landis and Tower, 1999; Royzman et al., 1997), suggesting that the normal DNA replication machinery is used to amplify the chorion genes. However chorion gene amplification can occur in cells where cyclin E is constitutively expressed, suggesting that chorion gene amplification over-rides negative controls on DNA replication imposed be CDKs in other cell cycles (Calvi et al., 1998). The mechanism for this is as present unknown.

Mitosis and pre-RC formation

One of the attractive features of a model where CDKs have a dual control over DNA replication is that it links mitosis with control of DNA replication. High CDK activity is essential for completion of S phase and entry into mitosis, whereas exit from mitosis requires inactivation of CDKs which allows pre-RC formation and DNA replication in the following cell cycle. However it is also possible that other aspects of mitosis are important for pre-RC formation. In *S. cerevisiae* entry into mitosis requires Cdc28/Clb2 or Cdc28/Clb1 activity, and progression through mitosis requires that activity of the anaphase promoting complex or cyclosome (APC/C) and a pathway of proteins called the mitotic exit network.

The APC/C and mitotic exit network in S. cerevisiae

The APC/C is essential for sister chromatid separation but also has an important role to bring about inactivation of CDKs at the end of mitosis which allows cytokinesis to proceed (reviewed Page and Hieter, 1999; Zachariae and Nasmyth, 1999). The APC/C consists of at least 11 core subunits which were identified in yeast as genes encoded by cell cycle mutants and by biochemical purification of known APC/C components (Irniger et al., 1995; Zachariae et al., 1998b; Zachariae et al., 1996). Two APC/C activating proteins have been identified; Cdc20p in budding yeast, which has been identified in *D. melanogaster* and in other metazoans as fizzy and Cdh1p (also called Hct1p) which has been identified as fizzy-related in other organisms. APC/C-Cdc20p is activated at the metaphase to anaphase transition, while APC/C-Cdh1p is activated during anaphase.

Substrates of the APC/C-Cdc20p include Pds1p which is an inhibitor of sister chromatid separation, (also known as a securin) (Cohen-Fix et al., 1996; Lim et al., 1998; Visintin et al., 1997). During metaphase sister chromatids become attached to the mitotic spindle but sister chromatid separation cannot occur until cohesion between them in destroyed. In *S. cerevisiae* degradation of Pds1p, activates a separin, Esp1p (Ciosk et al., 1998). Esp1p cleaves a cohesin Scc1p which is responsible for maintaining sister chromatids cohesion, from the time that chromosomes are replicated during S phase until the APC/C is

inactivated in mitosis (Guacci et al., 1997; Michaelis et al., 1997; Uhlmann et al., 1999; Uhlmann and Nasmyth, 1998). Inappropriate separation of sister chromatids is prevented by a checkpoint which prevents activation of the APC/C until bipolar attachments have been formed between spindle microtubules and kinetochores.

Another essential target of the APC/C-Cdc20p in *S. cerevisiae* is Clb5p. Clb5p proteolysis is important for inactivating CDKs at the end of mitosis (Shirayama et al., 1999). APC/C-Cdc20p also induces degradation of Clb3p and some degradation of Clb2p (Baumer et al., 2000; Yeong et al., 2000). APC/C-Cdc20p activity is regulated in the cell cycle by Cdc20p expression. Cdc20p is low during G1 but increases as cells enter mitosis. Premature expression of *CDC20* during the cell cycle induces premature degradation of Pds1p and expression of *CDC20* in nocodazole arrested cells (which have an active spindle checkpoint) induces premature sister chromatid separation (Shirayama et al., 1998). Cdc20p is itself an APC/C substrate and is unstable during G1 and early S phase (Prinz et al., 1998; Shirayama et al., 1998).

The APC/C-Cdh1p promotes exit from mitosis. Targets of APC/C-Cdh1p include Clb2p, Cdc5p, a member of the mitotic exit network and Ase1p, a spindle protein (Charles et al., 1998; Schwab et al., 1997; Shirayama et al., 1999; Shirayama et al., 1998; Visintin et al., 1997). The activity of APC/C-Cdh1p is controlled within the cell cycle by the phosphorylation status of Cdh1p (Zachariae et al., 1998a). Cdh1p is phosphorylated and inhibited by Cdc28/Cln and Cdc28/Clb kinases (Zachariae et al., 1998a). The mitotic exit network is important in bringing about dephosphorylation and therefore activation of Cdh1p.

In *S .cerevisiae* completion of mitosis requires activation of the 'mitotic exit network'. This includes Tem1p, a GTP binding protein, Lte1p, a putative guanine nucleotide exchange factor, the protein kinases Dbf2p, Dbf20p, Cdc5p, Cdc15p, a novel protein which associates with Dbf2p called Mob1p, and a dual specificity phosphatase, Cdc14p. The mitotic exit network is required after sister chromatid separation for exit from mitosis as mutants in mitotic exit network genes such as *cdc14, cdc5, cdc15* and *tem1* mutants arrest at the end of mitosis with separated nuclei (Johnston et al., 1990; Kitada et al., 1993; Shirayama et al.,

1994; Shirayama et al., 1996). Many genetic interactions have been described between the components of the mitotic exit network, suggesting that they act in a common signalling pathway (Jaspersen et al., 1998). Recent studies have suggested that Cdc14p is the downstream target of the mitotic exit network. For much of the cell cycle, Cdc14p is sequestered in the nucleolus by the 'RENT' complex which includes an inhibitor of Cdc14p activity, Net1p (also called Ctf1p), Sir2p (a regulator of trancriptional silencing) and Ypl126wp (Shou et al., 1999; Visintin et al., 1999). Release of Cdc14p from the nucleolus requires both the mitotic exit network, (Cdc5p, Tem1p, Dbf2p, Dbf20p and Cdc15p) and proteolysis of Pds1p (Shirayama et al., 1999; Shou et al., 1999; Visintin et al., 1999). Activation of the Cdc14p phosphatase is important for dephosphorylation of Cdc28/Clb substrates during exit from mitosis. Net1p itself may be a target of Cdc14p (Shou et al., 1999). In addition Cdc14p dephosphorylates and activates Cdh1p inducing a second wave of Clb2p proteolysis (Jaspersen et al., 1999). Consistent with this, Cdc5p is required for Clb2p proteolysis (Charles et al., 1998; Shirayama et al., 1998). Other Cdc14p targets are the Swi5p transcription factor which activates *CDC6* and *SIC1* transcription (Knapp et al., 1996; Piatti et al., 1995) and Sic1p, an inhibitor of Cdc28/Clb (Visintin et al., 1998). Sic1p is stabilised by Cdc14p dependent dephosphorylation since phosphorylation of Sic1p by Cdc28p targets it for SCF dependent ubiquitin mediated proteolysis (see above) (Visintin et al., 1998). Therefore the mitotic exit network brings about inactivation of Cdc28/Clb at the end of mitosis through the activation of a phosphatase, Cdc14p which has multiple substrates.

How the activity of components of the mitotic exit network is regulated during the cell cycle is not well understood. Cdc5p is known to be a target of the APC/C and Cdc5p kinase activity and protein levels peak in mitosis after Clb2p appears(Cheng et al., 1998; Hardy and Pautz, 1996). Dbf2p protein levels are constant during the cell cycle (Johnston et al., 1990) (and may be regulated by Dbf20p) but Dbf2p kinase activity peaks in very late mitosis after Cdc5p activity, but (like Cdc5p kinase) decreases sharply at the time Clb2p is degraded at the end of mitosis(Fesquet et al., 1999).

The APC/C and mitotic exit network in other eukaryotes

Progression through mitosis is not so well understood in other organisms. In all eukaryotes the Cdc25 phosphatase plays a crucial role in regulating entry into mitosis(reviewed Ohi and Gould, 1999). Cdc25 homologues are essential for activation of mitotic CDK activity by dephosphorylation of Cdc2 in *S. pombe*. The APC/C was first identified in metazoans through fractionation of the factors required for ubiquitination of cyclin B in *X. laevis* extracts, and subsequently human homologues have been identified (King et al., 1995; Peters et al., 1996; Tugendreich et al., 1995; Yu et al., 1998). The APC/C plays a similar role in other eukaryotes to that in *S. cerevisiae*. The APC/C is required for degradation of B-type cyclins at the end of mitosis. In *S. pombe*, sister chromatid separation requires APC/C mediated degradation of the Cut2 securin (Funabiki et al., 1996) which binds a homologue of Esp1p (Cut1) *in vivo* (Kumada et al., 1998). Degradation of *X. laevis* securin by the APC/C is also essential for separation of sister chromatids (Zou et al., 1999). Therefore the role of the APC/C is similar throughout eukaryotic organisms.

The role of homologues of the mitotic exit network proteins is much less well understood in other eukaryotes. Homologues of Cdc5p exist in fission yeast and metazoans and are known as Polo kinases. Polo kinases are not known to activate a Cdc14p homologue in these organisms although the homologues in *X. laevis* (Plx) and mammalian cells (Plk) have a role in targeting B-type cyclin s for ubiquitin mediated proteolysis. (Descombes and Nigg, 1998; Kotani et al., 1998). Polo kinases have other roles which are not shared by Cdc5p in *S. cerevisiae*. For example, Plx has been found to activate XCdc25C *in vitro*. In *S. pombe*, vertebrates and *D. melanogaster*, Polo-kinases are important in formation of a spindle, a role which is not shared by Cdc5p in *S. cerevisiae* (reviewed Nigg, 1998). Therefore Polo-like kinases have multiple roles in regulating progression through mitosis.

Two homologues of Cdc14p have been cloned from human cells and these can activate APC/C dependent proteolysis when ectopically expressed in mammalian cell culture (Listovsky et al., 2000). hsCdc14 is constitutively nuclear, and does not appear to be localised to the nucleolus (Li et al., 1997)

suggesting that Cdc14p activation in human cells is not controlled in the same way as *S. cerevisiae*. hsCdc14 is also capable of dephosphorylating the human checkpoint protein p53, although the functional significance of this has not been addressed (Li et al., 2000).

Several homologues of the mitotic exit network in *S. cerevisiae*, are found in *S .pombe* as part of the 'Sid' pathway. These include Spg1, Cdc7 and Sid2 which are homologues of *S. cerevisiae* Tem1p, Cdc15p and Dbf2p/Dbf20p respectively (Balasubramanian et al., 1998; Fankhauser and Simanis, 1994; Marks et al., 1992; Schmidt et al., 1997). However the phenotype of mutant alleles of the genes encoding in these proteins, suggest that the two pathways have different roles in cell cycle progression. Mutants in the *S. cerevisiae* mitotic exit network genes cannot exit from mitosis and arrest with high Cdc28/Clb kinase activity. However Sid pathway mutants from *S. pombe* are unable to undergo septation but are able to exit mitosis and undergo multiple rounds of DNA replication and mitosis in the absence of cytokinesis. Therefore the Sid pathway in *S. pombe* is thought to regulate the physical process of septation itself, rather than CDK activity, which is a target of the mitotic exit network.

The role of the APC/C and mitotic exit network in DNA replication

There is considerable evidence that the mitotic exit network and APC/C are important for DNA replication. Temperature sensitive mutants of *cdc14* and *cdc5* have a plasmid loss defect which is suppressed by addition of multiple origins to the plasmid (Hardy and Pautz, 1996; Hogan and Koshland, 1992). This mutant phenotype is characteristic of genes such as *CDC6* which have a role in pre-RC formation (Hogan and Koshland, 1992). Both *cdc14-1* and *cdc5* mutants are synthetically lethal with *orc2-1* (Hardy, 1996; Hardy and Pautz, 1996; Loo et al., 1995) and *cdc14-1* is synthetically lethal with *orc5-1* and *ORC6* over-expression (Kroll et al., 1996; Loo et al., 1995). Over-expression of *CDC5* suppresses *dbf4* mutants (Kitada et al., 1993), and Cdc5p and Dbf4p interact in two hybrid analysis and co-immuno-precipitate (Hardy and Pautz, 1996). Temperature sensitive mutants in the mitotic exit network arrest with post-RCs at origins (Diffley et al., 1994). These experiments suggest that the mitotic exit network proteins have an important role in controlling DNA replication.

The APC/C may also have a role in control of DNA replication. Recent studies in *X. laevis* oocytes and HeLa cells have shown that an inhibitor of Mcm2-7p loading, geminin, is degraded in an APC/C dependent manner (McGarry and Kirschner, 1998). In budding yeast, mutants in two APC/C subunits, *cdc16* and *cdc27*, have been shown to re-replicate at the restrictive temperature (Heichman and Roberts, 1996; Heichman and Roberts, 1998), although this observation is controversial (Pichler et al., 1997). The S phase promoting factor, Dbf4p, is also targeted for degradation by the APC/C (Cheng et al., 1999; Godinho Ferreira et al., 2000). Furthermore, a mutant in the *CDC16* gene, encoding an APC subunit, exhibits synthetic lethality with over-expression of *ORC6* (Kroll et al., 1996). *cdc16* and *cdc23* (encoding another APC/C subunit) mutants, like the mitotic exit network mutants, arrest without pre-RCs at origins (Diffley et al., 1994). These results suggest that the APC/C may play a role in origin re-setting in mitosis.

Conclusion

In eukaryotic cells DNA replication is precisely controlled in the cell cycle by multiple redundant mechanisms. In particular origin firing and pre-RC formation are respectively activated and inhibited by CDK activity. In this thesis I will address whether inactivation of CDK activity at the end of mitosis is the only mechanism by which pre-RC formation and DNA replication are coupled to completion of the previous mitosis. In addition this thesis will address the role of one of the ORC subunits, Orc6p in pre-RC formation.

Chapter Two: Experimental Procedures

Yeast strains and media

The yeast strains used are listed in Table 1. Strains were grown in YP (1% yeast extract, (Difco); 2% Bacto-peptone, (Difco)) containing either glucose, galactose or raffinose (Sigma) at 2% w/v. Wild type strains were grown at 30°C and temperature sensitive mutants were grown at 24°C. Exponentially growing cultures were arrested in G2/M using 5µg/ml nocodazole (Sigma) dissolved in DMSO, or in G1 using α factor (ICRF Peptide Service) at 5µg/ml. Cycloheximide (Sigma) was used at 100µg/ml (in ddH_2O), while hydroxyurea (Sigma) was used at 0.2M in the appropriate growth medium. Geneticin sulphate (G418) was used to select for the kanamycin marker at 200mg/l in YP medium. Minimal medium was used to select for auxotrophic markers (2% agar; 100ml yeast nitrogen base w/o amino acids (67mg/ml in ddH_2O); 20% (w/v) glucose or galactose; plus appropriate supplements: adenine 5mg/ml; uracil 2mg/ml; leucine 10mg/ml; tryptophan 2mg/ml; histidine 10mg/ml). Yeast strains were stored at -70°C in YP-glucose containing 15% glycerol.

Degron strains were grown at the permissive temperature in YP medium containing 0.1mM $CuSO_4$ to induce expression of the degron from the *CUP1* promoter. At the non-permissive temperature $CuSO_4$ was not added to the medium.

Yeast transformation

Cells were grown to logarithmic phase (approximately 10^7cell/ml) in YP-glucose. 10^8 cells were collected for each transformation by centrifugation in a bench-top centrifuge at 3000rpm for 3 min. The cells were then washed in 10ml ddH_2O, resuspended in 1ml ddH_2O, centrifuged at 12,000g in a microfuge, washed once in 1 ml sterile Li/TE (0.1M lithium acetate pH7.5; TE pH7.5 (1X TE: 10mM tris-HCl pH7.5; 1mM EDTA pH8), prepared fresh from 10X stocks of lithium acetate and TE) and resuspended at $2X10^9$ in Li/TE. 5µl herring sperm DNA (10mg/ml in ddH_2O) (phenol chloroform extracted and sonicated to

approximately 2kb fragments) and 1-2µg plasmid DNA were added to the cells, before vigorous mixing. 300µl Li/PEG (0.1M lithium acetate pH7.5; TE pH7.5, in 40% (w/v) polyethylene glycol 3550) prepared fresh from 10X stocks of lithium acetate and TE and 50% (w/v) polyethylene glycol 3550 was then added to the cells followed by vortexing to mix. Cells were then incubated at 30°C for 30 minutes, cooled on ice, and DMSO added to a final concentration of 10%. The mixture was briefly vortexed and heat shock was carried out at 42°C for 15 minutes. Cells were then cooled on ice, briefly spun in a microfuge, resuspended in ddH_2O and plated onto agar plates of the appropriate selective medium.

Growth of E. coli

E. coli strain DH5α was used for amplification of plasmids containing an ampicillin marker. Cells were grown in LB (1% (w/v) bacto-tryptone (Difco), 1% (w/v) bacto yeast extract (Difco), 0.17M NaCl, 2% (w/v) agar for solid medium), pH adjusted to 7 with NaOH. Ampicillin was used at 50µg/ml for selective growth. Plasmids pDK368-1 and pDK368-7 were amplified in *E. coli* strain MC1066 using the yeast *LEU2* marker o to complement the bacterial *leuB* mutation. The *E. coli* were grown in M9 minimal medium supplemented with uracil and tryptophan (for 500 ml: 400ml H_2O; 50ml 10x M9 salts (Sambrook et al., 1989); 10ml 20% glucose; 1ml 1M $MgSO_4$; 50µl 1M $CaCl_2$; 2ml 1% thiamine; 20ml 2%glucose; 4ml 10mg/ml tryptophan). Competent cells for transformation were prepared as described in Sambrook et al., 1989, using protocol I or II. Transformations were also performed as described in Sambrook et al., 1989.

Preparation of plasmid DNA

Plasmid DNA was prepared on a small scale either as described in Sambrook et al., 1989, using alkali lysis, or using a modified method, described below: A 2ml overnight culture of cells was grown, and the cells collected in 50µl of culture medium. 300 µl of TENS (10mM tris-HCl pH8; 1mM EDTA; 0.5% (w/v) SDS; 0.1M NaOH) was then added followed by vigorous vortexing for 3-4 seconds. 150 µl 3M NaOAc pH5.4 was then added followed by vortexing for 3-4 seconds. The mixture was then centrifuged briefly at 12,000g for 2min in a

microfuge, 500µl of the supernatant was removed and transferred to a fresh tube containing 900µl 100% ethanol. The mixture was then centrifuged as above, the supernatant discarded and the pellet washed with 1ml 70% ethanol. The ethanol was then removed and the pellet left to dry before being resuspended in 20 µl TE pH8 plus 50µg/ml RNaseA. 5µl of this was used for digestion with restriction enzymes.

Plasmid was prepared from *E. coli* on a larger scale using QIAGEN Maxi Plasmid Purification kit according to the manufacturers instructions.

Agarose gels

DNA was analysed on 1% (w/v) agarose gels made up with TAE (40mM tris-acetate, 1 mM EDTA pH8) and run in the same at 90V. The gels were stained using ethidium bromide (1µg/ml) in H_2O and DNA viewed on a UV transilluminator. DNA samples were loaded onto agarose gels in loading buffer (6X loading buffer was 0.1% bromophenol blue, 40% (w/v) ficoll). DNA was purified from gels prepared with low-melt agarose, using a Gene Clean II Kit (BIO 101 USA).

Restriction digests

Restriction enzymes were purchased from New England Biolabs and digested using buffers supplied by Boehringer Mannheim for use with enzymes supplied by the latter company according to their instructions.

Ligation

Digested fragments were ligated using bacteriophage T4 DNA ligase (New England Biolabs) using supplied buffer for 2 hours at room temperature.

Sequencing

Sequencing reactions were carried out using a BIG DYE ABI PRISM Terminator Cycle Sequencing Ready Reaction kit according to manufacturers recommendations using appropriate oligos of 20bp length. Products were precipitated with 100% ethanol and washed with 70% ethanol and analysed

PCR

PCR reactions were carried out using a Perkin Elmer Thermocycler

PCR Reaction Mix:

•1µl Vent Polymerase (New England Biolabs)

•10 µl 10X Vent Buffer (supplied by New England Biolabs)

•8µl 2.5mM dNTP mix

•10ng *S. cerevisiae* genomic DNA (prepared as footprinting DNA, without the DNase I treatment).

•0.2-1µM of each primer oligonucleotide

•H_2O to 100µl

Reaction mixes were overlaid with mineral oil and PCR reaction carried out for 30 cycles as below:

1 min 95°C, 1 min 50°C, 72°C 1min/Kb DNA amplified.

PCR reactions were purified by phenol:chloroform extraction and the nucleic acids recovered by ethanol precipitation and resuspended in ddH_2O.

Plasmids

pLD1 was derived from pMHTgal by subcloning *SIC1ΔNT* into BamHI restriction sites (Desdouets et al., 1998).

pLD2 was constructed by subcloning the full length *SIC1* gene into pMHTgal into using BamHI restriction sites.

pLIL3 was constructed by subcloning a fragment containing *SIC1ΔNT* and the *GAL1-10* promoter from pLD1 into pFA6 Kan MX4 (Wach et al., 1994) using PvuII and BglII restriction sites. This plasmid was integrated at the *SIC1* locus after digestion with BstIII.

pLIL10 The primers:

5'TACTCAAAGCTTCCGGAGGCATGTCCATGCAACAAGTCCAAC3'

5'AGACATCTCGAGGTAGTTCTGGTATTACGAACGAATC3'

were used to amplify the first 500bp of *ORC6*. The product was then digested with HindIII and XhoI and cloned into pPW66R (Dohmen et al., 1994) after, to replace the fragment of *CDC28* in this plasmid. This plasmid was named pLIL8. pLIL8 was then digested with NotI and XhoI to remove a

fragment encoding the *CUP* promoter, degron tag *(UBI1, DHFR* tag and *HA* tag) and the N-terminus of Orc6p. The fragment was then cloned into pRS304 (Sikorski and Hieter, 1989), the resulting plasmid being named pLIL10. pLIL10 was integrated into the *ORC6* locus after digestion with StyI.

Western Blotting

Samples of 10^8 cells were harvested and stored for up to 1 month at -70°C before extract preparation. Protein extracts were made by TCA precipitation (Foiani et al., 1994). Breifly, cell pellets were thawed quickly at 37°C and 0.3ml 20% tri-chloroacetic acid (TCA) and 0.3ml acid washed glass beads were added immediately to each sample. The cells were then vortexed vigorously for 1 minute at room temperature and the supernatant removed to a clean eppendorf. The beads were then washed with 0.3ml 5% TCA and the supernatant collected. The supernatant was then spun in a microfuge at 10,000 rpm for 10 minutes. The supernatant was discarded and the insoluble pellet was resuspended in Laemmli buffer (20% (w/v) glycerol; 15% β-mercaptoethanol; 9% SDS, 0.125% bromophenol blue; 187.5mM Tris base), then boiled for 3 minutes and stored at -20°C. The protein extracts were boiled again and spun briefly in a microfuge before being run on 10% polyacrylamide gels as described in (Sambrook et al., 1989). The gels were then equilibrated in Transfer Buffer (48mM tris base; 39mM Glycine; 0.0375% SDS (w/v); 20% methanol) for 15 min. Proteins were subsequently transferred to Hybond ECL nitrocellulose membrane (Amersham) (previously soaked in Transfer Buffer) using a semi-dry blotter at 15 Volts for 20 minutes. Total protein was visualised on the membrane with Ponceau S (2% (w/v) Ponceau S, 3% (w/v) trichloroacetic acid) for 1 min, followed by washing in H_2O.

In general, nitrocellulose membranes were washed in H_2O after Ponceau S staining, incubated in TBST (140mMNaCl; 2.5mMKCl; 25mM tris-HCl pH8; 0.1% tween 20 (Sigma)) with appropriate blocking conditions (see below) and then transferred to a solution of the primary antibody (see below). The membrane was then washed in TBST at least 3 times over at least 30 minutes, incubated in the secondary antibody (see below) and washed as above.

Immuno-reactive bands were detected with Enhanced Chemiluminesence (ECL) (Amersham) according to manufacturer's instructions.

Blocking conditions: Membranes were blocked for either1-2 hours at room temperature or for 14 hours at 4°C. Blocking conditions were as follows:

Sic1p, Mcm2p, Orc6p 5% dry milk (Marvel) in TBST

Clb3p 5% dry milk and 1% dried yeast extract (Difco) in TBST

Orc1p, 5% dry milk and 2.5% dried yeast extract in TBST

Orc2p 5% dry milk plus 5% dried yeast extract in TBST

Membranes were blocked for 2 hours room temperature or overnight at 4°C.

Primary antibodies: Membranes were incubated in primary antibody solutions with appropriate blocking conditions (see above) for either 1-2 hours at room temperature or for 14hours at 4°C. Sic1p was detected with polyclonal Sic1p antibody (a gift from Lee Johnston). Tagged Sic1ΔNTp was detected using mAb 9E10 (ICRF Cell and Media production). Clb3p was detected using a polyclonal Clb3p antibody (YC20) from Santa Cruz Biotechnology at 0.8µg/ml. Mcm2p was also detected with a polyclonal antibody from Santa Cruz Biotechnology used at 1:2,000. Other polyclonal antibodies used were JAB51 to detect Orc1p (1:250), JAB12 to detect Orc2p (1:500). The monoclonal antibody SB49 was used at 12.5µg/ml to detect Orc6p.

Secondary antibodies: Membranes were incubated in secondary antibody solutions (made up in 5% dry milk, TBST) for either 1-2 hours at room temperature or for 14hours at 4°C. Signals from polyclonal antibodies produced by Santa Cruz Biotechnology were detected was detected using anti-goat antibody conjugated to HRP, also from Santa Cruz Biotechnology, at 1/5000. All other signals from polyclonal antibodies were detected with protein A coupled to HRP (Amersham) at 1:8,000. All signals from monoclonal antibodies were detected using anti mouse IgG coupled to HRP at 1:10,000 (Amersham).

Footprinting

Sample preparation for genomic footprinting was performed as previously described (Santocanale and Diffley, 1997) with the following modifications: $5X10^8$ cells were collected for each sample. Extract preparation

and DNase I reactions were carried out as previously described (Santocanale and Diffley, 1997), followed by a single phenol chloroform purification step. In particular the DNA samples were mixed gently with phenol chloroform by repeated inversion for at least 6 minutes before separation of the two layers in a microfuge. After isopropanyl precipitation and washing in 70% ethanol, as previously described (Santocanale and Diffley, 1997), DNA samples were resuspended in 50µl Hepes/Na+ pH7.5; 0.1mM EDTA containing 1mg/ml RNaseA. Samples were then incubated at 37°C for 4 hours before DNA was carefully quantified on a 0.8% agarose gel (as(Santocanale and Diffley, 1997)). Primer CS305 was used to detect ARS305 (Perkins and Diffley, 1998). Primer labelling reactions were as follows 5µl 32P γ-ATP (5000Ci/millimole, 10mCi/ml), 1µl 23.3pmol/µl ARS305 oligo/H_20, 1µl 10X polynucleotide kinase buffer (supplied by New England Biolabs), 0.66µl polynucleotide kinase (New England Biolabs), 2.34µl H_20. The reactions were carried out and the labelled oligonucleotide was isolated as (Santocanale and Diffley, 1997). Extension reactions were carried out with Vent exo$^-$ DNA polymerase (New England Biolabs).

Flow cytometric DNA analysis (FACS)

10^7 cells were collected for each FACS sample, and fixed in 70% ethanol for at least 15 minutes at room temperature. Fixed cells were stored at 4°C for up to 1 month before processing. Fixed cells were washed with 1ml 50mM Tris-HCl pH7.8, resuspended in 0.5ml 50mM Tris-HCl pH7.8 with 0.4mg/ml RNaseA and incubated at 37°C for 4 hours. Cells were then pelleted, resuspended in 0.5ml 5mg/ml Pepsin (Sigma) dissolved in 55mM HCl and incubated at 37°C for 30 minutes. Cells were then washed once with FACS buffer (180mM Tris-HCl pH7.5, 190mM NaCl, 70mM $MgCl_2$) and resuspended in 0.5ml FACS buffer containing 50µg/ml Propidium Iodide (Sigma). Cells were then briefly sonicated and stored for up to 1 month at -20°C. 50µl of each sample was added to 1ml 50mM Tris-HCl pH7.8 before analysis on a Becton Dickenson FACS scan.

Chromatin binding analysis

Chromatin binding analysis was carried out as previously described (Donovan et al., 1997) with the following modifications: Extract preparation was carried out as previously described (Donovan et al., 1997) except that magnesium acetate was not included in the lysis buffer. 100µl of the whole cell extracts was then centrifuged in a microfuge at 14,000 rpm for 10 minutes. The supernatant was removed and the insoluble pellet was resuspended in 100µl lysis buffer. Protein was then precipitated from the pellet, supernatant and whole cell extract samples by addition of 100µl 10%Trichloroacetic acid solution. The samples were then centrifuged for 10 minutes at 10,0000 rpm at room temperature. The supernatant was then removed from each sample and the insoluble protein pellet was resuspended in 100µl Laemmli buffer (x1) and boiled for 3 minutes before loading onto a 10% polyacrylamide gel.

Plasmid loss assay

Cells were grown to log phase in minimal medium containing 2% galactose, yeast nitrogen base (6.7mg/ml); adenine (40µg/ml); tryptophan (40µg/ml) and histidine (40µg/ml) at 24°C and then diluted into YP galactose (2%) and grown for multiple generations at 30°C. Cells were plated onto minimal medium agar plates (2% galactose; yeast nitrogen base (6.7mg/ml); adenine (40µg/ml); tryptophan (40µg/ml) and histidine (40µg/ml) with or without leucine (80µg/ml)). The rate of plasmid loss was calculated as previously described (Dani and Zakian, 1983). Standard deviation values were calculated from three parallel cultures of each strain used.

Crude H1 kinase assay

$5X10^8$ cells were collected, washed once in ice cold HB (15mM $MgCl_2$, 25mM MOPS pH 7.2 1% triton X, 1mM AEBSF (Pefabloc SC); 10µg/ml leupeptin; 10µg/ml peptstatin; 10mM benzamidine HCl; 5mM EGTA, 0.1mM Na Vanadate; 60mM β-glycerophosphate; 1mMDTT), lysed by bead beating (3X30 seconds vortexing with 30 second rests on ice), the extract was then spun at 4°C 14,000rpm for 5 minutes at 4°C. 10µl of the supernatant was pre-incubated at 30°C for 10 minutes before 10µl reaction buffer (as HB but containing 1mg/ml

histone H1 plus 200mM γ-32P ATP (5000Ci/millimole, 10mCi/ml), (Amersham)) was added. The reaction was stopped after 15 minutes by addition of 1/3 volume 3X Laemmli buffer and boiling for 3 minutes. The samples were then loaded onto a 10% polyacrylamide gel, stained with Coomassie brilliant blue, and the phosphorylated proteins analysed using a Phosphoimager (Molecular Dynamics).

Table 1: Yeast strains

Strains	Genotype	Background	Source
K5014	***Mat a*** *ade2-1 ura3-1 his3-11,15, leu2-3,112, trp1-1, URA3::GAL-SIC1 (5X) can1-100.*	W3031a	(Dahmann et al., 1995)
YLD12	***Mat a,*** *ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1::pLD1 (URA3 pMHT GAL-SIC1DNT myc his tag), can1-100.*	W3031a	This study
YLIL4	***Mat a,*** *ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1::pMHTgal (URA3), can1-100.*	W3031a	This study
YLIL29	***Mat a,*** *cdc16-123, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1::pLD1 (URA3, GAL-SIC1ΔNT myc his tag), can1-100.*	W3031a	This study
YLIL18	***Mat a,*** *cdc16-123, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1::pMHTgal (URA3), can1-100.*	W3031a	This study
YKB7	***Mat a,*** *CDC28 ::pPW66R (CUP1-cdc28-td, URA3), ade2-1, his3-11,1leu2-3,112, trp1-1, ura3-1, can1-100, UBR1::pKL54 (HIS3, GAL-UBR1).*	W3031a	This study

YKL83	***Mat a**, ade2-1, his3-11,1, leu2-3,112, trp1-1, ura3-1, can1-100 UBR1::pKL54 (HIS3, GAL-UBR1).*	W3031a	(Labib et al., 1999)
YLIL7	***Mat a**, cdc15-2, ura3-1::pLD1 (URA3, GAL-SIC1ΔNT myc his tag).*	W3031a	This study
1993	***Mat a**, cdc15-2 ura3-1, leu2.*	W3031a	Kim Nasmyth
YLIL35	***Mat a**, cdc14-1, leu2, ura3, trp1, ura3-1::pLD1 (URA3, GAL-SIC1ΔNT myc his tag).*	15Dau (Bueno and Russell, 1992)	This study
YLIL36	***Mat a**, cdc14-1, leu2, trp1, ura3-1:pMHTgal (URA3).*	15Dau	This study
YLIL9	***Mat a**, cdc6-1, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1::pLD1 (URA3 pMHT GAL-SIC1ΔNT myc his tag), can1-100.*	W3031a	This study
YKB2	***Mat a**, cdc7-4, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100.*	W3031a	(Bousset and Diffley, 1998)
YLIL15	***Mat a**, cdc7-4, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1::pLD1 (URA3 pMHT GAL-SIC1ΔNT myc his tag), can1-100.*	W3031a	This study
KKY012	***Mat a**, msd2-1::URA3, leu2-3,112, trp1-289, ura3-52.*		(Kitada et al., 1993)
K4050	***Mat a** cdc6-1, ade2-1, his3-11,15 trp1-1, leu2-3,112, , can1-100.*		(Piatti et al., 1995)
YLIL39	***Mat a**, msd2-1::URA3, leu2-3,112, trp1-289, ura3-52, SIC1::pLIL3 (KAN MX4, GAL-SIC1ΔNT).*	KKYO21	This study

YLIL37	***Mat a**, leu2, trp1, ura3-1::pMHTgal (URA3), bar1Δ.*	15Dau	This study
YLIL53	***Mat a**, cdc14-1, leu2, trp1, ura3-1:pLD2 (URA3, pMHT, GAL-SIC1).*	15Dau	This study
8030	***Mat a**, cdc20-3*	W3031a	M. Shirayama
YLIL65	***Mat a**, cdc20-3, ura3-1:pLD1 (URA3, pMHT, GAL-SIC1ΔNT).*	W3031a	This study
YMIG03	***Mat a**, cdc23-1, ura3, trp1, leu2*	W3031a	
YLIL8	***Mat a**, cdc23-1, trp1, leu2, ura3-1:pLD1 (URA3, pMHT, GAL-SIC1ΔNT).*	W3031a	This study
YLIL51	***Mat a**, ade2-1, his3-11,1, leu2-3,112, trp1-1, ura3-1, can1-100 UBR1::pKL54 (HIS3, GAL-UBR1). ORC6:pLIL10 (TRP1, CUP1-orc6-td).*	W3031a	This study
YLIL5	***Mat a**,cdc4-1, trp1, leu2, ura3-1:pLD1 (URA3, GAL-SIC1ΔNT).*	W3031a	This study
W3031a	***Mat a**, ade2-1, his3-11,1, leu2-3,112, trp1-1, ura3-1, can1-100*		
W3031b	***Mat** α, ade2-1, his3-11,1, leu2-3,112, trp1-1, ura3-1, can1-100*		

Chapter Three: Orc6p is Essential for Pre-RC Formation but Not for Binding of Other ORC Subunits to Chromatin

Introduction

In *S cerevisiae* Orc6p, the smallest subunit of ORC was also identified as an origin interacting protein by one-hybrid analysis (Li and Herskowitz, 1993). Detailed biochemical analysis of recombinant *S. cerevisiae* ORC has shown that Orc6p is the only ORC subunit which is not essential for binding of the others to origins of DNA replication *in vitro* (Lee and Bell, 1997). This suggests that Orc6p may have unique role in ORC. To investigate the role of Orc6p in DNA replication, a temperature sensitive mutant of *ORC6* was constructed.

Construction of a temperature sensitive orc6 mutant

The 'degron' method was used to construct a temperature sensitive *orc6* mutant. In this method, a tag which confers temperature sensitive instability is attached to the protein of interest (Dohmen et al., 1994; Labib et al., 2000). This temperature sensitive instability is dependent upon the 'N-end rule' in which the half-life of a protein is determined by the identity of its N terminal amino acid (reviewed Varshavsky, 1992). An N-terminal arginine residue will target a protein for ubiquitin mediated proteolysis dependent on the presence of proximal lysine residues which allow covalent attachment of ubiquitin to the protein. N-end rule ubiquitin mediated proteolysis requires the E3 enzyme Ubr1p. The degron tag introduces a ubiquityl moiety, an arginine residue and a variant of DHFR in frame with the N-terminus of the gene of interest. After translation of the degron, the ubiquitin is rapidly cleaved to expose an arginine residue. The DHFR variant has an aspartate to proline mutation at amino acid 66 which is thought to induce unfolding of the DHFR module, increasing the accessibility of lysine residues for interaction with ubiquitin at the non permissive temperature. Over-expression of *UBR1* from the inducible *GAL1-10* promoter considerably increases the temperature sensitive instability of degrons at the non-permissive temperature (Labib et al., 2000). The *GAL1-10* promoter is

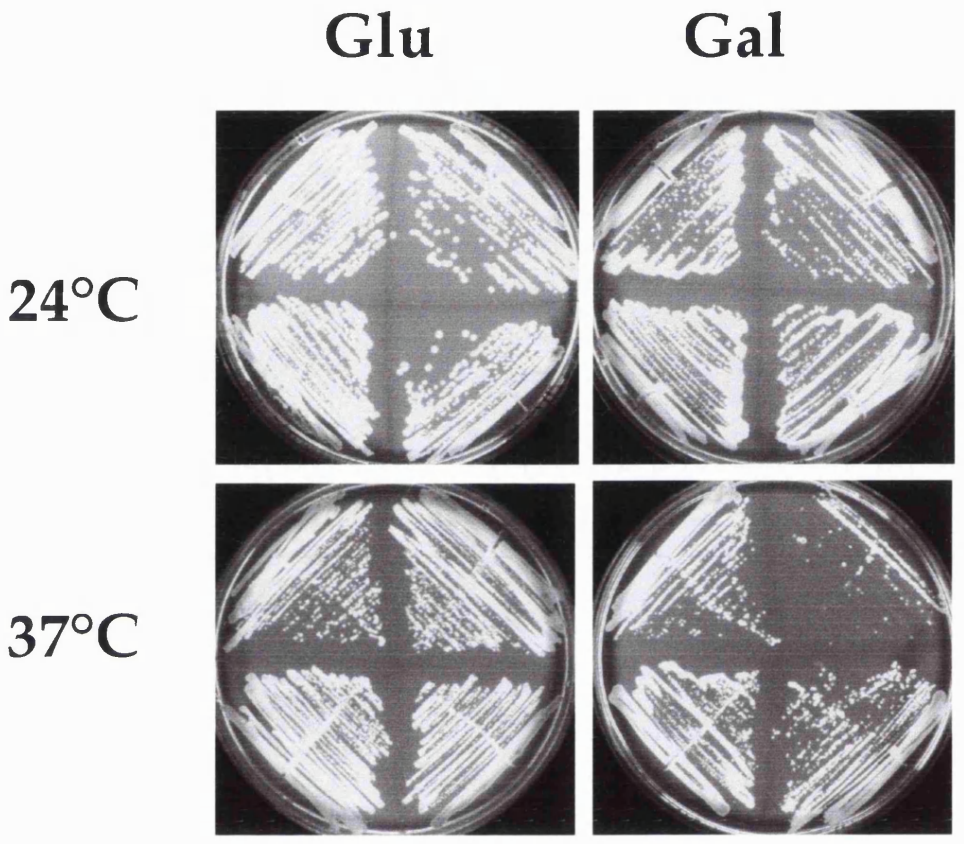
especially useful, as it is highly induced in the presence of galactose medium, repressed in the presence of glucose medium but neither induced not repressed in the presence of raffinose medium. A strain was constructed which contained both *UBR1* under control of the *GAL1-10* promoter (*GAL-UBR1*) and a single copy of *ORC6* with a degron tag. This strain was found to be viable at both 24°C and 37°C on glucose medium where expression of *UBR1* was repressed. When grown on galactose medium where expression of *UBR1* is induced, both the *orc6* degron strain and a wild type strain containing *GAL-UBR1* were viable at 24°C. However, when grown at 37°C on galactose medium, although the wild type strain grew well, growth of the *orc6* degron strain was inhibited. Therefore the *orc6* mutant strain was temperature sensitive dependent on over-expression of *UBR1* (figure 1). Diploid strains constructed by mating a wild type strain to either the *orc6* degron strain or the wild type strain with *GAL-UBR1*, were viable at 24°C and 37°C when grown on either glucose or galactose medium, suggesting that the *orc6-td* mutation is recessive (figure 1). The *orc6* degron allele was named *orc6-td*.

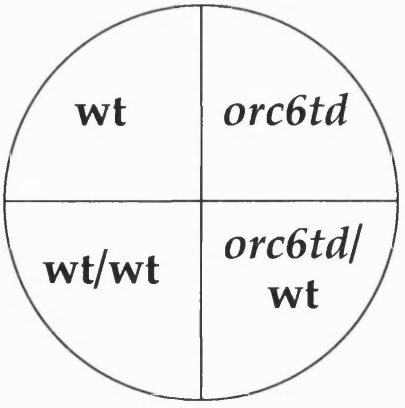
To determine whether the degron tag confers temperature sensitive instability on the Orc6 protein, a wild type strain with *GAL-UBR1* and an *orc6-td* strain with *GAL-UBR1* were grown to log phase in raffinose medium at 24°C. The cultures were then split and resuspended in either galactose medium to induce *UBR1* expression or in glucose medium to repress *UBR1* expression. After 2 hours the cultures were shifted to 37°C. Protein extracts were made from samples collected throughout the experiment and polyacrylamide gel electrophoresis followed by western blotting of Orc6p was carried out. This demonstrated that that after 90 minutes at 37°C in glucose medium there was no change in the expression of Orc6p in either the wild type or the *orc6-td* strain. In galactose medium both wild type and *orc6-td* strains had equivalent levels of Orc6p at 24°C. After 90 minutes at 37°C, there was no change in the level of Orc6p in the wild type strain however the majority of Orc6-tdp in the galactose culture had disappeared. This suggests that Orc6-tdp is degraded at 37°C in a Ubr1p dependent manner (figure 2A). It is possible that degradation of Orc6-tdp induces non-specific degradation of other ORC subunits, which may contribute to the phenotype of *orc6-td* at 37°C. Western blotting for Orc2p and Orc1p in this

Figure 1. Construction of a temperature sensitive orc6 mutant

A strain containing *GAL-UBR1* and *orc6-td* (YLIL51) and a wild type strain containing *GAL-UBR1* alone (YKL83) were mated to a wild type strain (W3031b). The four strains YLIL51 (*orc6-td*), YKL83 (wt), YLIL51/W3031b (*orc6-td*/wt) and YKL83/W3031b (wt/wt) were plated onto glucose medium (Glu) (*GAL* promoter repressed) or onto galactose medium (Gal) (*GAL* promoter active) and grown either at 24°C or at 37°C for several days until colonies were established. Although growth of the majority of cells in the *orc6-td* strain was inhibited at 37°C on galactose medium, some colonies grew well. These are likely to represent cells which have lost *GAL-UBR1*, or *orc6-td* as the medium on which the cells are grown is not selective.

Figure 1





experiment demonstrated that the expression of these proteins did not change in conditions where Orc6-tdp is degraded (figure 2B). Therefore in the *orc6-td* strain Orc6-tdp is specifically degraded at the non-permissive temperature in a *UBR1* dependent manner.

To address whether degradation of Orc6-tdp results in cell cycle arrest, FACS analysis was carried out on the above experiment and the cell density of the cultures was monitored to determine whether cell division took place (figure 2B). This analysis indicated that both the wild type strain and the *orc6-td* strain continued to divide after incubation in glucose medium at 37°C. In addition the wild type strain continued to divide when grown at 37°C in galactose medium. However when the *orc6-td* strain was grown at 37°C in galactose medium, cells accumulated in S phase with large buds (data not shown) and the cell density of the culture did not increase. This suggests that degradation of Orc6-tdp results in a defect in cell cycle progression.

Orc6p is essential for S Phase but not mitosis

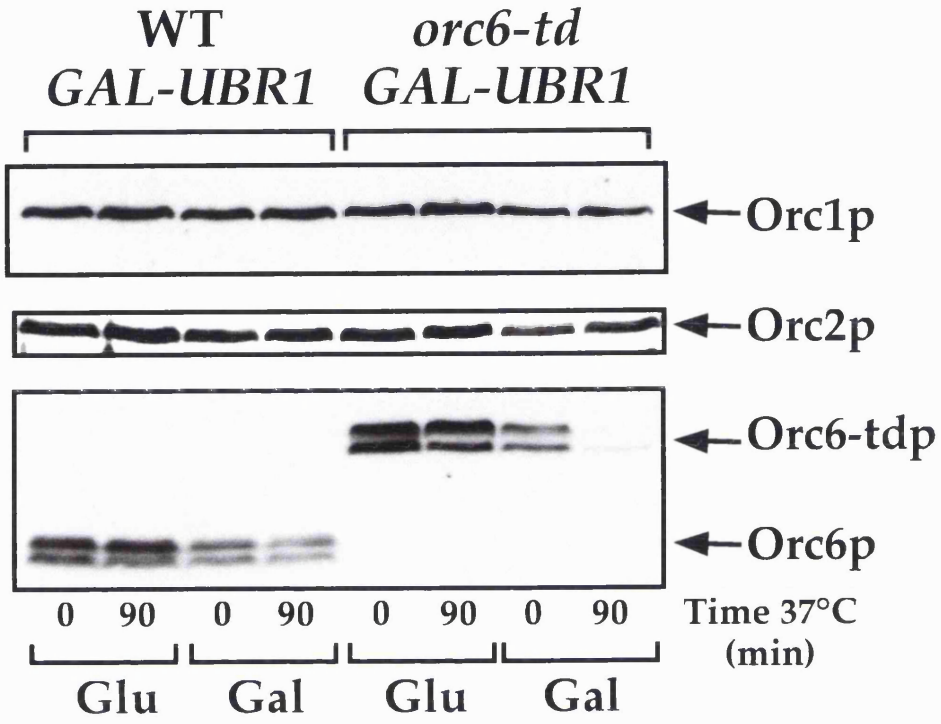
The above results suggest that Orc6p has a role in DNA replication. To characterise this further, we arrested cells at various points in the cell cycle, induced degradation of Orc6-tdp and then released cells from the arrest while following cell cycle progression using FACS analysis. To address whether Orc6p was required in G1, a wild type strain and an *orc6-td* strain both containing *GAL-UBR1* were grown to log phase in galactose medium and were arrested in G2/M using nocodazole (an inhibitor of microtubules). Degradation of Orc6-tdp was induced by raising the temperature of the culture to 37°C for 90 minutes. The cells were then washed to remove the nocodazole and resuspended in fresh galactose medium at 37°C. FACS analysis showed that both strains completed mitosis one hour after release from the G2/M arrest (figure 3A). The wild type strain had completed S phase two hours after release from the arrest, however three hours after release the *orc6-td* strain had completed very little DNA replication. This is consistent with an essential role for Orc6p for entry into S phase.

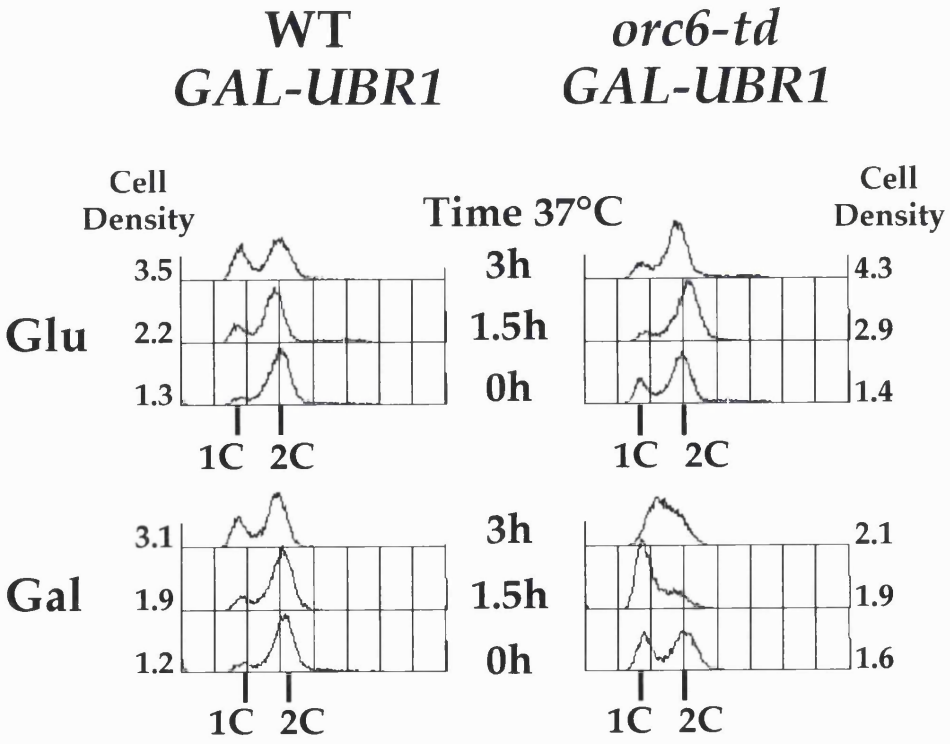
To address whether Orc6p function is important throughout G1, an *orc6-td* strain was arrested in G1 using alpha factor and the temperature raised to

Figure 2. Orc6-tdp is degraded at 37°C and induces an S phase arrest

A strain containing *GAL-UBR1* and *orc6-td* (YLIL51) and a wild type strain containing *GAL-UBR1* alone (YKL83) were grown to log phase in raffinose medium at 24°C. The cultures were then split and grown for 2 hours at 24°C in either glucose (Glu) or galactose medium (Gal). The medium was the exchanged for pre-warmed glucose or galactose medium at 37°C and the cells were incubated at 37°C for a further 90-180 minutes and samples for protein extract preparation were collected. Polyacrylamide gel electrophoresis followed by western blotting of the protein extracts was carried out. Orc1p and Orc2p were identified using polyclonal antibodies, while Orc6p was recognised using a monoclonal antibody (A). FACS samples were also taken and the cell density of the cultures was monitored throughout the experiment (B).

Figure 2


WT
GAL-UBR1
orc6-td
GAL-UBR1
Orc1p
Orc2p
Orc6-tdp
Orc6p
0 90 0 90 0 90 0 90
Time 37°C
(min)
Glu Gal Glu Gal


WT
GAL-UBR1
orc6-td
GAL-UBR1
Cell
Density
Time 37°C
Cell
Density
Glu
3.5
2.2
1.3
3h
1.5h
0h
4.3
2.9
1.4
1C 2C
1C 2C
Gal
3.1
1.9
1.2
3h
1.5h
0h
2.1
1.9
1.6
1C 2C
1C 2C

37°C for 90 minutes to induce degradation of Orc6-tdp. Cells were then washed to remove the alpha factor and resuspended in fresh medium at 37°C. Although a wild-type strain had completed S phase by one hour after release, an *orc6-td* strain had not progressed significantly through S phase by 3 hours after release from alpha factor (figure 3B). These results suggest that Orc6p has an essential function during G1 and is important for the initiation of DNA replication.

Recent reports have suggested that ORC has an essential function during mitosis (Dillin and Rine, 1998). However, the above results suggest that Orc6p is not required after a nocodazole (G2/M) arrest. To investigate whether Orc6p is required earlier in mitosis, cells were grown to log phase in galactose medium as above and then arrested in S phase using hydroxyurea. Degradation of Orc6-tdp was induced by increasing the temperature to 37°C for 90 minutes. Cells were then washed to remove the hydroxyurea and released into fresh medium at 37°C. Both wild type and *orc6-td* mutant cells had completed S phase by one hour after release from the hydroxyurea arrest and entered the next G1 two hours after release, although the *orc6-td* mutant was then unable to complete a second S phase (figure 3C). This result strongly suggests that Orc6p has no essential role in the later stages of S phase or mitosis, but is consistent with an essential role for Orc6p in initiation of DNA replication.

The viability of wild type and *orc6-td* strains was measured during the above arrest and release experiments and the results were similar in all three cases. The *orc6-td* strain and the wild type strain had similar viability in G2/M, G1 and S phase arrests at 24°C and 37°C. However, after release from the arrests, the *orc6-td* strain dramatically lost viability compared to the wild type strain. All three experiments induced an S phase arrest in the *orc6-td* strain and this suggests that entry into S phase in the absence of Orc6p is lethal (figure 3A-C, ii).

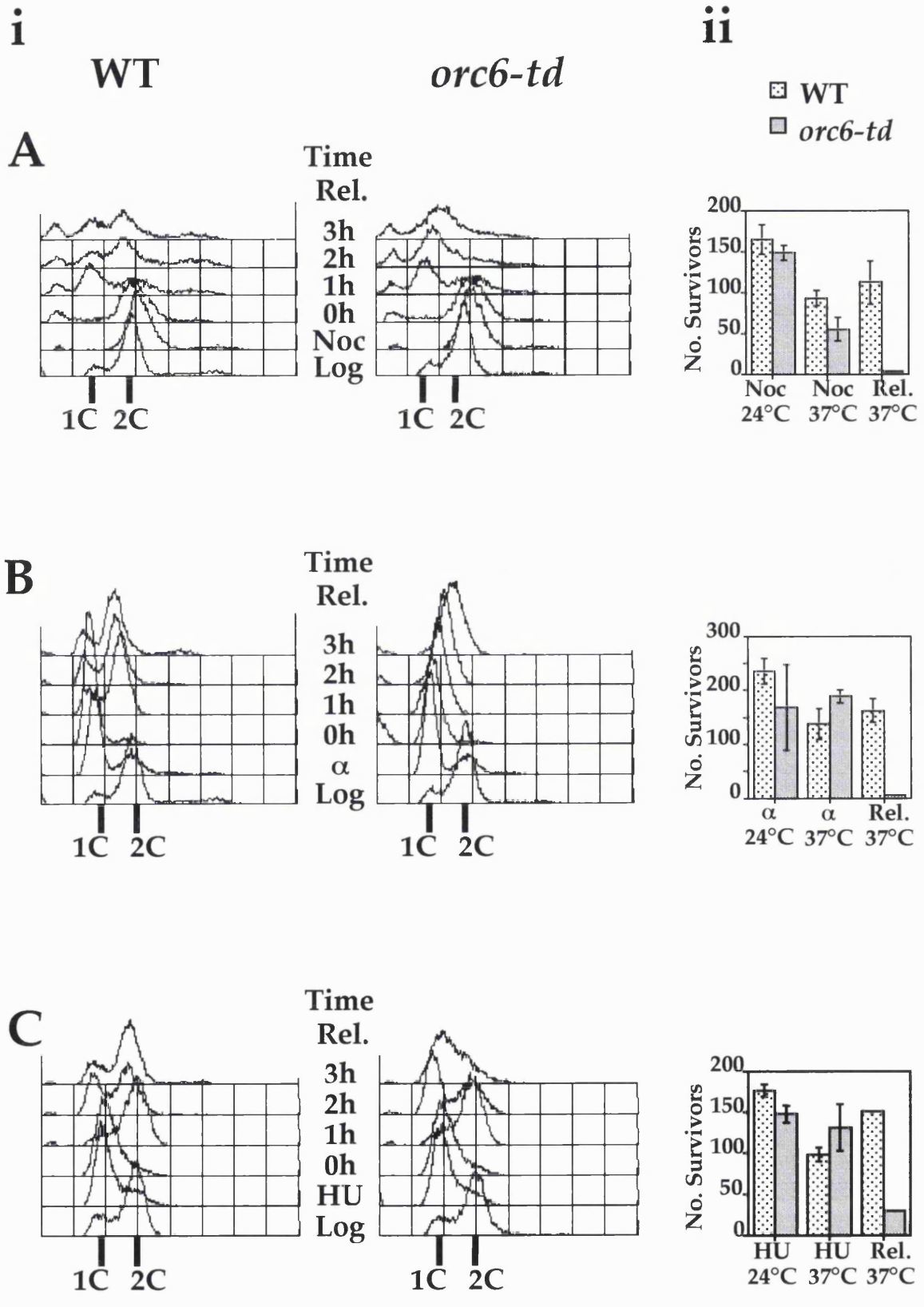
Orc6p is essential for Pre-RC formation

The above results strongly suggest that Orc6p has an essential role in initiation of DNA replication and since ORC is essential for formation of the pre-RC, we investigated whether Orc6p has an essential role in pre-RC formation. Single copy DNase I genomic footprinting at the chromosomal origin *ARS305*

Figure 3. Orc6p is required for S phase but not mitosis

A strain containing *GAL-UBR1* and *orc6-td* (YLIL51) and a wild type strain containing *GAL-UBR1* alone (YKL83) were grown to log phase in galactose medium at 24°C and cells were arrested either in G2/M using nocodazole (Noc) (A), in G1 using alpha factor (α) (B) or in S phase using hydroxyurea (HU) (C). The temperature was then raised to 37°C for 90 minutes to induce degradation of Orc6-tdp. Cells were then washed to remove the nocodazole/alpha factor/hydroxyurea and resuspended in fresh galactose medium at 37°C (Rel. 37°C). FACS analysis was carried out (i) and aliquots of equal numbers of cells were plated onto glucose medium in triplicate throughout the experiment. The plates were incubated at 24°C for 3 days, after which the number of colonies was counted (ii). The error bars indicate the standard deviation of the number of colonies on three plates.

Figure 3


i
WT
orc6-td
ii
WT
orc6-td
A
Time Rel.
3h
2h
1h
0h
Noc
Log
1C 2C
1C 2C
No. Survivors
200
150
100
50
0
Noc 24°C
Noc 37°C
Rel. 37°C
B
Time Rel.
3h
2h
1h
0h
α
Log
1C 2C
1C 2C
No. Survivors
300
200
100
0
α 24°C
α 37°C
Rel. 37°C
C
Time Rel.
3h
2h
1h
0h
HU
Log
1C 2C
1C 2C
No. Survivors
200
150
100
50
0
HU 24°C
HU 37°C
Rel. 37°C

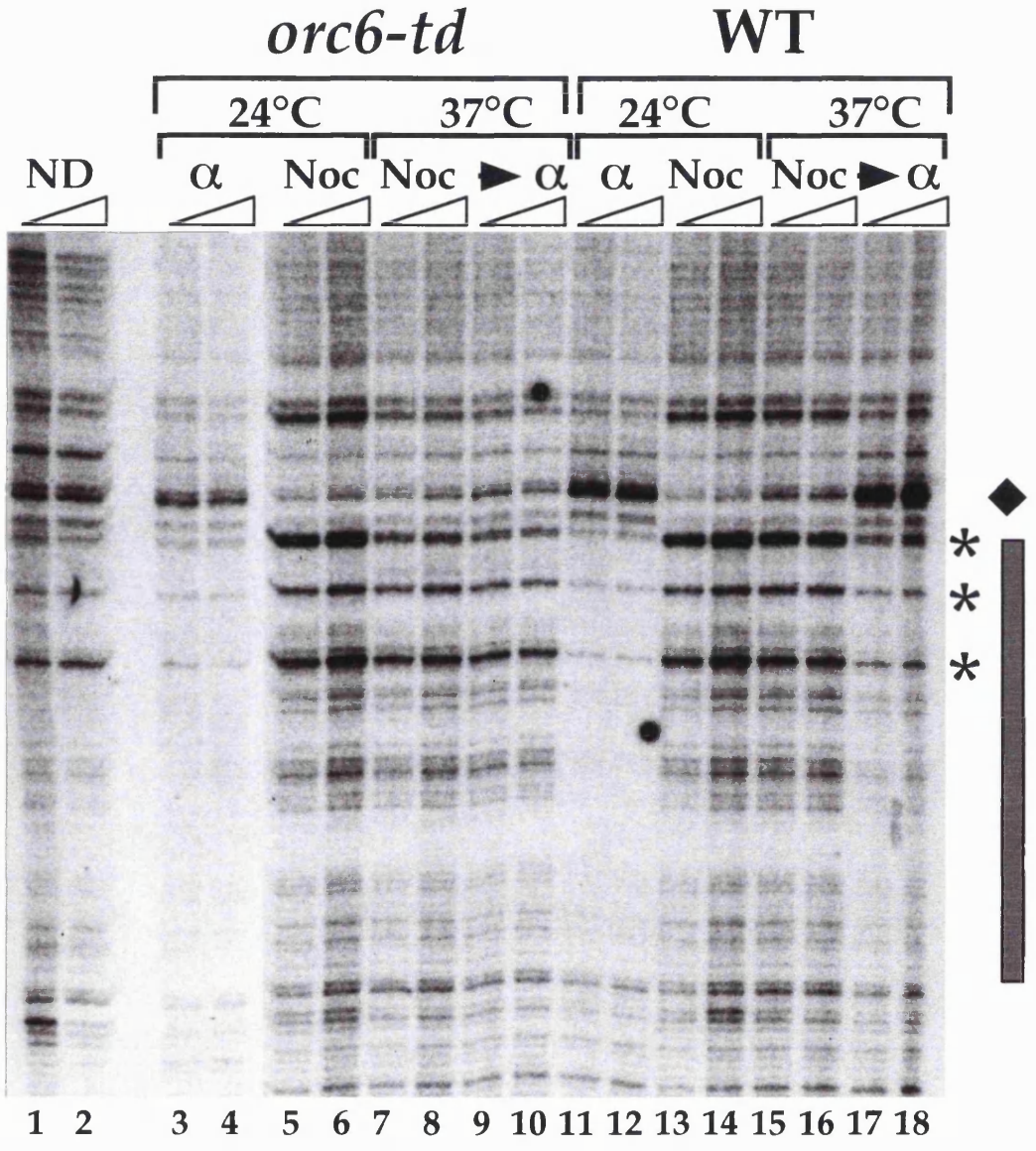
was used to establish whether this is the case. The post-replicative footprint of *ARS305* is characterised by three hypersensitive sites (*) (figure 4, lanes 5 and 6). The pre-replicative footprint is characterised by a region of protection (indicated by a grey box) and an additional hypersensitive site (◆) (figure 7A, lanes 3 and 4). The pattern of DNase I digestions on naked DNA is also shown in lanes 1 and 2). A wild type strain and an *orc6-td* strain were arrested in G2/M using nocodazole and the temperature was raised to 37°C for two hours to induce degradation of Orc6-tdp before cells were released into a G1 arrest at 37°C. In a wild type strain, the post-RC found at *ARS305* when cells are arrested in G2/M was clearly converted to a pre-RC when cells were released into the G1 arrest (figure 4, lanes 15-18). The *orc6-td* strain was clearly able to maintain a post-replicative footprint at origins in a G2/M arrest at the non-permissive temperature (lanes 7 and 8). However upon release into the G1 arrest, the characteristic pattern of protection and hypersensitive sites produced by the pre-replicative footprint at *ARS305* did not form (compare lanes 9 and 10 to lanes 3, 4, 11, 12, 17 and 18). The DNase I digestion pattern is clearly distinct from that produced by naked DNA (from which all proteins have been stripped prior to DNase I treatment) (lanes 1 and 2) and is most similar to a post-replicative footprint. Therefore although Orc6p seems to be essential for pre-RC formation *in vivo*, it does not appear to induce dissociation of other ORC subunits from chromatin since the post-replicative footprint is maintained.

To address whether this was the case, we took advantage of chromatin binding analysis to address which pre-RC components were able to bind to chromatin in an *orc6-td* mutant. In this technique, extracts made by gentle lysis of yeast cells are centrifuged to separate an insoluble pellet from a soluble supernatant. The insoluble pellet contains most of the DNA in the extract but approximately 5% of the protein and is believed to contain the chromatin present in the extract (Donovan et al., 1997). An *orc6-td* strain was synchronised in G1 at the permissive temperature and then released into S phase. When 90% of the cells had budded, degradation of Orc6-tdp was induced by increasing the temperature to 37°C and cells were synchronised in the following G1 using alpha factor. Extracts from a wild type strain treated in this way show that in cells arrested in G1 at 37°C, nearly all the Orc2p and Orc6p from in an extract

Figure 4. Orc6p is essential for pre-RC formation

A strain containing *GAL-UBR1* and *orc6-td* (YLIL51) and a wild type strain containing *GAL-UBR1* alone (YKL83) were grown to log phase in galactose medium at 24°C and then arrested either in G1 phase using alpha factor (α) or in G2/M using nocodazole (Noc). The temperature of the G2/M arrested cells was then raised to 37°C for 2 hours to degrade Orc6-tdp. Cells were then washed to remove the nocodazole and resuspended in galactose medium at 37°C containing alpha factor to synchronise the cells in the following G1 (α). Single copy DNase I footprinting was carried out at the chromosomal origin *ARS305*. Triangles indicate increasing amounts of DNase I. ND indicates naked DNA where proteins are removed from DNA before DNase I digestion. The post-replicative footprint is characterised by 3 hypersensitive sites (*) and the pre-replicative footprint is characterised by a single hypersensitive site (◆) and a region of protection form DNase I (indicated by a grey box.

Figure 4


orc6-td
WT
24°C
37°C
24°C
37°C
ND
α
Noc
Noc ► α
α
Noc
Noc ► α
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

precipitates with the chromatin, as does about half of the Mcm2p (figure 5, lanes 4-6). This is also the case in an *orc6-td* strain arrested in G1 at 24°C (lanes 7-9). In an *orc6-td* strain arrested in G1 at 37°C, nearly all the Orc6p is degraded, but despite this, nearly all of the Orc2p in the extract is found in the chromatin pellet and none of the Mcm2p is (lanes 10-12). This result strongly suggests that *in vivo* Orc6p is not required for the binding of Orc2p to chromatin, but that it is required for Mcm2p binding to chromatin.

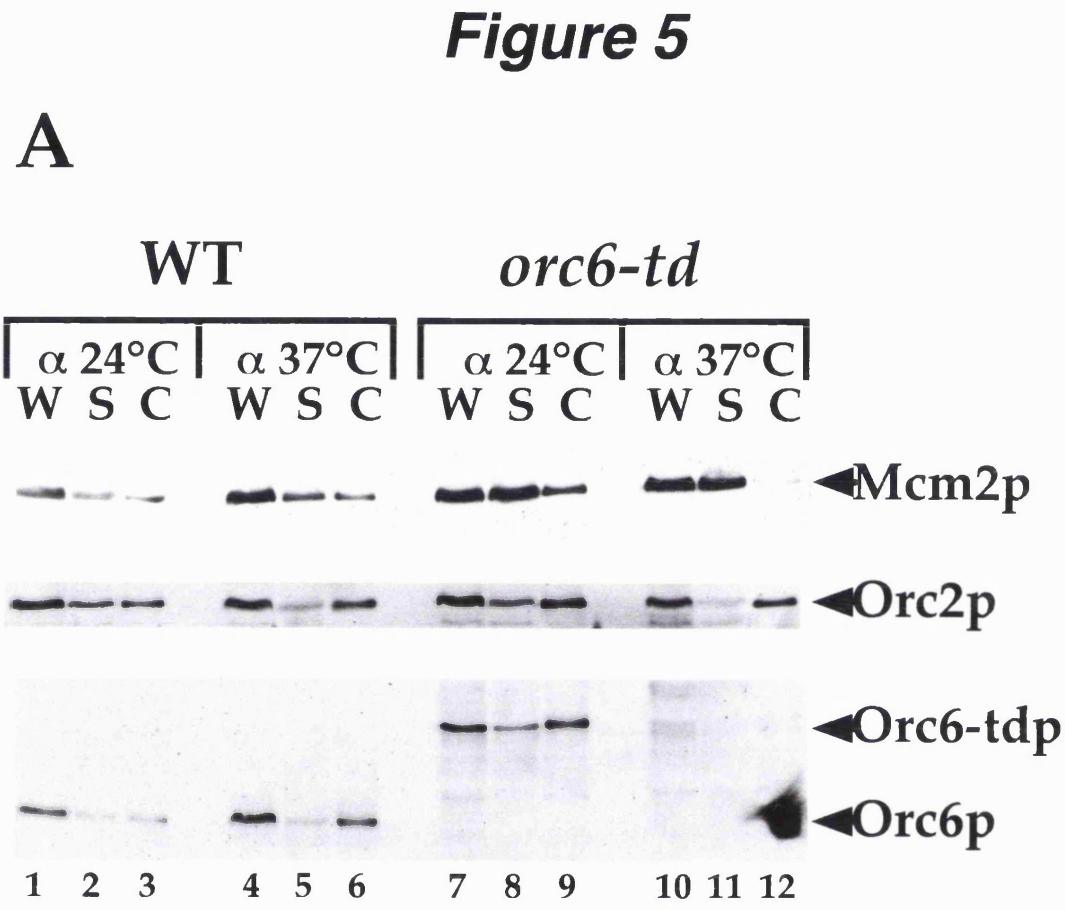
Conclusions

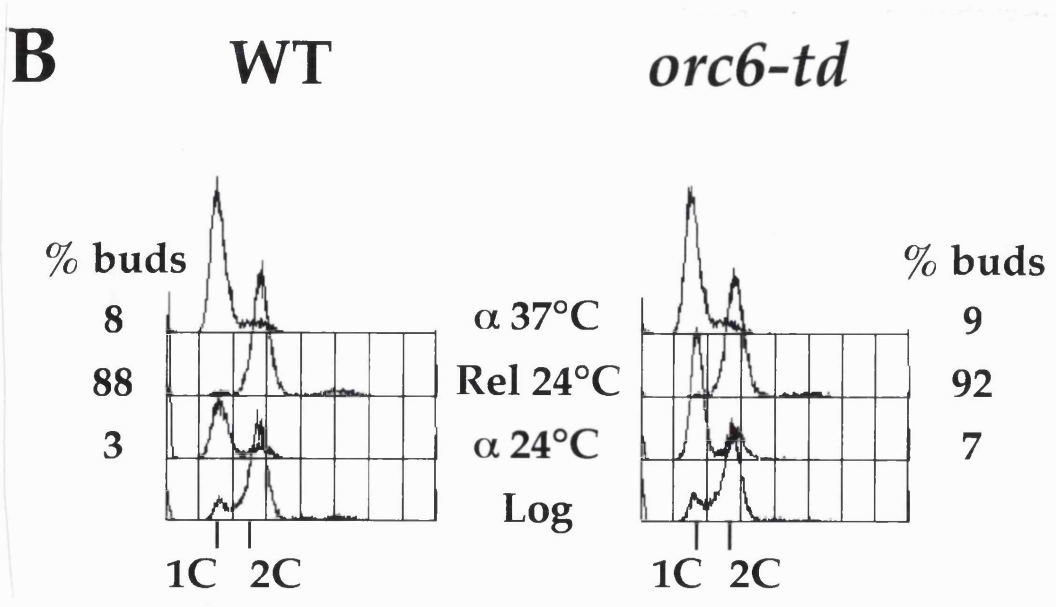
In this chapter a temperature sensitive degron mutant of *orc6* has been constructed and used to investigate the role of Orc6p in DNA replication. FACS data has indicated that Orc6p is important for progression through S phase but not for completion of mitosis. A small amount of DNA replication appears to occur in these experiments, and this may be due to incomplete inactivation of *orc6-td.* DNase I footprinting of *ARS305* and chromatin binding analysis demonstrate that Orc6p is essential for pre-RC formation but, consistent with *in vitro* data, demonstrate that Orc6p is not required for Orc2p chromatin binding for maintenance of the post -RC. In conclusion these investigations have established a role for Orc6p function in pre-RC formation after origin recognition by ORC.

Figure 5. Orc6p is essential for Mcm2-7p loading but not for ORC loading onto chromatin

A strain containing *GAL-UBR1* and *orc6-td* (YLIL51) and a wild type strain containing *GAL-UBR1* alone (YKL83) were grown to log phase in galactose medium at 24°C and then synchronised in G1 phase using alpha factor (α 24°C). Cells were then washed to remove the alpha factor and resuspended in galactose medium at 24°C. When approximately 90% of cells had buds, P(Rel. 24°C), the medium was then exchanged for galactose medium at 37°C which contained alpha factor to arrest cells in the following G1 (α 37°C). During the experiment samples were taken for FACS analysis and the budding index of the culture was monitored. Chromatin binding analysis followed by polyacrylamide gel electrophoresis and western blotting were carried out. Orc6p was identified using a monoclonal antibody. Orc2p and Mcm2p were identified with polyclonal antibodies. Equivalent amounts of whole cell extract (W), soluble supernatant (S) and insoluble chromatin pellet (C) were loaded onto the gel for each sample.

Figure 5


A
WT
orc6-td
α 24°C
α 37°C
α 24°C
α 37°C
W S C W S C W S C W S C
Mcm2p
Orc2p
Orc6-tdp
Orc6p
1 2 3 4 5 6 7 8 9 10 11 12


B
WT
orc6-td
% buds
8
88
3
α 37°C
Rel 24°C
α 24°C
Log
1C 2C
% buds
9
92
7
1C 2C

Chapter Four: Inactivation of Cdc28/Clb Kinase in S and G2/M Arrested Cells Drives Pre-RC Formation and Re-replication Dependent on Protein Synthesis.

Introduction

One model for control of DNA replication is that high Cdc28p kinase activity inhibits pre-RC formation from the point that origins fire during S phase until Cdc28/Clb is inactivated at the end of mitosis. In this way pre-RC formation is limited to G1 when Cdc28p kinase activity is low. This model makes several predictions about control of DNA replication in the cell cycle. For example, the model assumes that Cdc28p kinase activity inhibits pre-RC formation during S phase as well as during mitosis and suggests that the only mitotic event which is important for pre-RC formation is inactivation of Cdc28/Clb kinase activity is at the end of mitosis. These assumptions are tested here and in the following chapter using, a system where an additional round of DNA replication is induced in the absence of mitosis.

Sic1ΔNTp is a stable inhibitor of Cdc28/Clb kinase.

To investigate how mitotic events affect the control of DNA replication, we describe here a system where re-replication occurs in the absence of nuclear division. Previous studies have shown that inhibition of Cdc28/Clb kinase by over-expression of its inhibitor, *SIC1*, drives pre-RC formation and re-replication (Dahmann et al., 1995). In G2/M arrested cells phosphorylation of the amino terminus of Sic1p targets it for ubiquitination by the E3 enzyme SCF, and this leads to degradation by the 26S proteasome in a Cdc4p dependent manner (Verma et al., 1997a). Consequently it is difficult to express the gene to high levels after G1. To improve the efficiency of Cdc28/Clb kinase inhibition by *SIC1* in our experiments, we constructed *SIC1ΔNT*, in which 50 amino acids have been removed from the amino terminus of wild type Sic1p. Truncated Sic1ΔNTp lacks some of the phosphorylation sites which target wild type Sic1p for degradation and is therefore considerably more stable than the wild type protein

product. Sic1ΔNTp should inhibit of Cdc28/Clb kinase efficiently, as wild type Sic1p binds and inhibits Cdc28/Clb through its C-terminus (Hodge and Mendenhall, 1999; Verma et al., 1997b). *SIC1ΔNT* was placed under the control of the *GAL1-10* promoter (*GAL-SIC1ΔNT*). In previous experiments, five integrated copies of wild type *SIC1* under the control of the *GAL1-10* promoter (*GAL-SIC1*) were required to induce pre-RC formation in G2/M arrested cells (Dahmann et al., 1995). We compared the expression of Sic1ΔNTp in a strain containing a single copy of *GAL-SIC1ΔNT*, to expression of Sic1p in a strain containing five copies of *GAL-SIC1* (full length) in G2/M. Figure 6A shows that Sic1ΔNTp expressed from a single integrated copy accumulated to a considerably higher level than full length Sic1p expressed from five integrated copies in G2/M arrested cells.

We also investigated the stability of Sic1ΔNTp in G2/M and found that after one hour of expression from the *GAL* promoter, the levels of Sic1ΔNTp are high. However, if transcription from the *GAL1-10* promoter is repressed by exchanging the medium for glucose medium, the protein is gradually degraded (figure 6B). In a *cdc4-1* mutant at the non-permissive temperature, Sic1ΔNTp accumulates to much higher levels than in a wild type strain and persists for a greater period of time after addition of glucose (figure 6B). This suggests that although some of the residues which target Sic1p for Cdc4-dependent degradation are missing from Sic1ΔNTp, the protein is still degraded and Cdc4p plays a major role in this degradation.

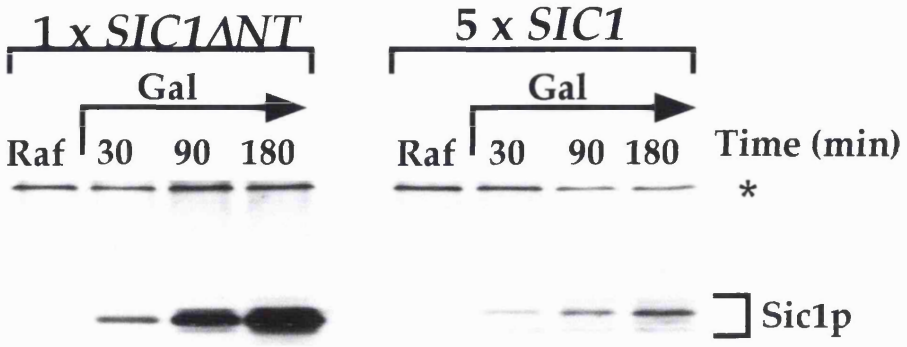
Constitutive over-production of Sic1ΔNTp is lethal (figure 6C) and arrests cell growth with a terminal phenotype similar to that seen in *cdc4, 34* and *53* mutants and similar to that induced by overproduction of 5X *GAL-SIC1* (Dahmann et al., 1995). In particular, the cells re-bud and buds elongate dramatically, which suggests that Cdc28/Clb kinase is inhibited while Cdc28/Cln kinase is activated (Lew et al., 1992; Lew and Reed, 1993). *SIC1ΔNT* expression in G2/M arrested cells also drives dephosphorylation of the p86 subunit of DNA polymerase α-primase, a Cdc28p substrate (Desdouets et al., 1998), suggesting that Sic1ΔNTp is an inhibitor of Cdc28/Clb kinase. Furthermore, extracts prepared from G2/M arrested cells over-expressing

Figure 6. SIC1ΔNT encodes a stable inhibitor of Cdc28/Clb

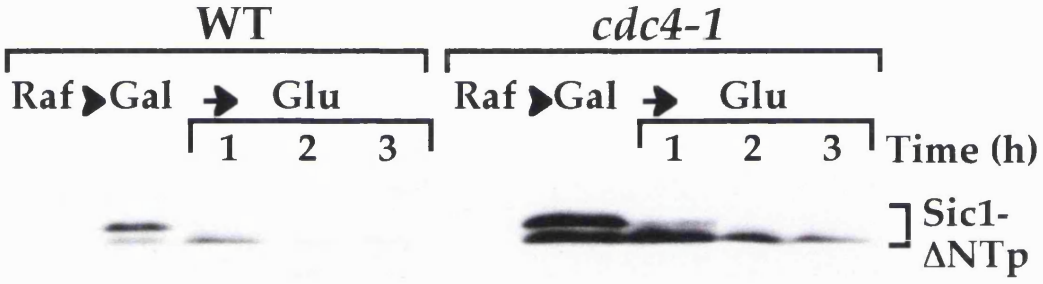
A. Log phase cultures of a wild type yeast strain with one copy of *GAL-SIC1ΔNT* (YLD12) and another wild type strain with five copies of *GAL-SIC1* (full length) (K5014) were arrested in nocodazole in raffinose medium (Raf). The raffinose medium was then exchanged for galactose medium containing nocodazole (Gal) to induce expression from the *GAL* promoter. Samples for protein extracts were taken throughout the experiment. Polyacrylamide gel electrophoresis and western blotting were carried out. Sic1p was identified using a polyclonal antibody. The asterisk indicates a non-specific polypeptide which cross-reacts with the Sic1p antibody and serves as a loading control.
B. YLD12 and a *cdc4-1* strain with *GAL-SIC1ΔNT*, (YLIL5), were arrested in nocodazole in raffinose medium (Raf) at 24°C. The raffinose medium was exchanged for pre-warmed galactose medium containing nocodazole (Gal) at 37°C to induce expression of *SIC1ΔNT* and inactivate *cdc4-1*. *SIC1ΔNT* expression was then repressed by replacing the medium with pre-warmed glucose medium containing nocodazole (Glu). Samples for protein extracts were taken throughout the experiment. Polyacrylamide gel electrophoresis and western blotting were carried out and Sic1ΔNTp was identified using the 9E10 antibody which recognises a myc epitope at the N-terminus of Sic1ΔNTp.
C. YLD12 and a wild type yeast strain (W303-1a) (Control) were grown on galactose medium at 30°C for 3 days.
D. YLD12 and a strain with *GAL* promoter alone (YLIL4) (Control) were arrested in nocodazole in raffinose medium (Raf) and medium was then exchanged for galactose medium containing nocodazole (Gal). After 2.5 hours in galactose medium samples for protein extracts were taken. The extracts were incubated with radiolabelled γ-ATP and histone H1 at 30°C. Polyacrylamide gel electrophoresis was carried out and labelled proteins were identified using a phosphorPP-imager, the most intensely labelled protein ran at the same position as histone H1.

Figure 6

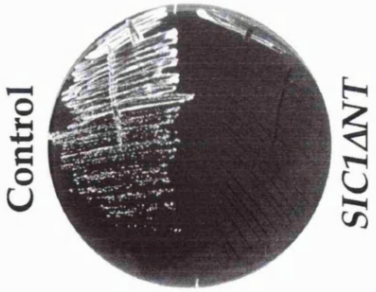
A



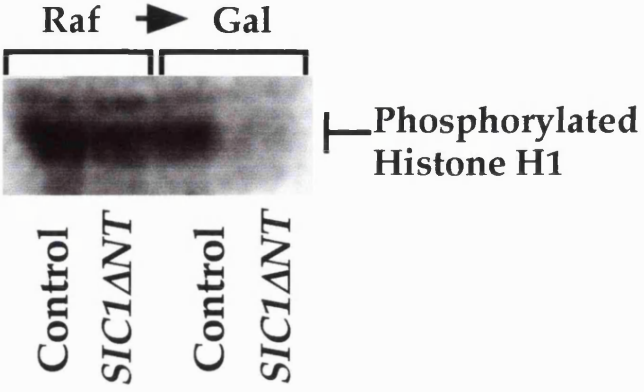
B



C



D



SIC1ΔNT have a much lower H1 kinase activity than control extracts prepared from cells which do not express *SIC1ΔNT* (figure 6D). Thus, Sic1ΔNTp is a stable inhibitor of Cdc28/Clb kinase activity in G2/M arrested cells.

Over-expression of SIC1ΔNT in G2/M arrested cells re-sets origins of replication

In order to test whether inhibition of Cdc28/Clb kinase by over-expression of *SIC1ΔNT* is sufficient to induce pre-RC formation in G2/M, a yeast strain containing *GAL-SIC1ΔNT* was arrested in G2/M and expression of *SIC1ΔNT* was induced from the *GAL* promoter. The formation of pre-RCs in this experiment was measured by DNase I footprinting of the genomic origin *ARS305*. This experiment shows that over-expression of *SIC1ΔNT* induced pre-RC formation in G2/M arrested cells (figure 7A, lanes 5 and 6). A control strain, which did not contain *GAL-SIC1ΔNT*, did not form pre-RCs when treated in the same way (figure 7A, lanes 7 and 8). This confirms previous data which suggested that inhibition of Cdc28/Clb kinase in G2/M arrested cells by over-expression of *SIC1* induces pre-RC formation at genomic origins of replication.

If inhibition of Cdc28/Clb kinase by *SIC1ΔNT* over-expression in G2/M induces pre-RC formation at the majority of origins, then these should fire when Cdc28/Clb kinase is re-activated, causing re-replication. We investigated whether transient inhibition of Cdc28/Clb kinase by *SIC1ΔNT* was sufficient to induce a further round of replication in G2/M arrested cells. A wild type yeast strain containing *GAL-SIC1ΔNT* was arrested in G2/M, *SIC1ΔNT* expression induced for one hour and then repressed. FACS analysis showed that when *SIC1ΔNT* was over-expressed, the cells maintained a 2C content of DNA (figure 7B). Subsequent repression of *SIC1* transcription induced re-replication so that cells acquired a 4C content of DNA. A control strain which did not contain *GAL-SIC1ΔNT*, did not re-replicate when treated in the same way.

The transient over-expression of *SIC1ΔNT* does not significantly reduce the viability of G2/M arrested cells compared to control cells which do not over-express *SIC1ΔNT* (figure 7C). Individual colonies of survivors from this experiment were grown to logarithmic phase and their DNA content was

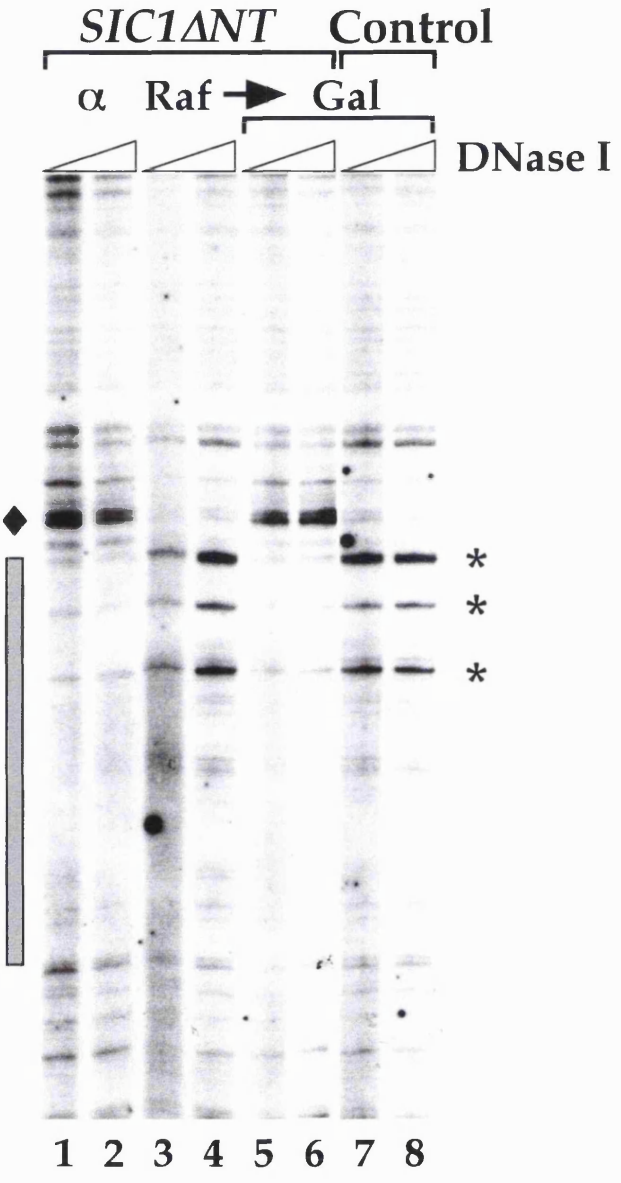
Figure 7A and 7B. SIC1ΔNT drives pre-RC formation and re-replication in G2/M arrested cells

A. A wild type yeast strain with *GAL-SIC1ΔNT* (YLD12) and a strain with the *GAL* promoter plasmid (pMHT-Gal) alone (YLIL4) (Control), were grown to log phase in raffinose medium and arrested in G2/M using nocodazole (Raf), or in G1 using alpha factor (α). G2/M arrested cells were transferred to galactose medium containing nocodazole (Gal) for 2.5 hours to induce *SIC1ΔNT* expression and DNase I footprinting analysis was carried out *in vivo* at the chromosomal origin *ARS305*. Triangles indicate increasing concentrations of DNase I.

B. (YLD12) and (YLIL4), (Control) were grown to log phase (Log) and arrested in G2/M with nocodazole (Noc) as above. Cells were transferred to galactose medium (Gal) containing nocodazole for one hour to induce *SIC1ΔNT* transcription and then to glucose medium containing nocodazole for 3 hours to repress *SIC1ΔNT* transcription. FACS analysis was carried out. Microscopy of propidium iodide stained cells from this experiment confirmed that nuclear division had not occurred (data not shown).

Figure 7A and 7B

A



B

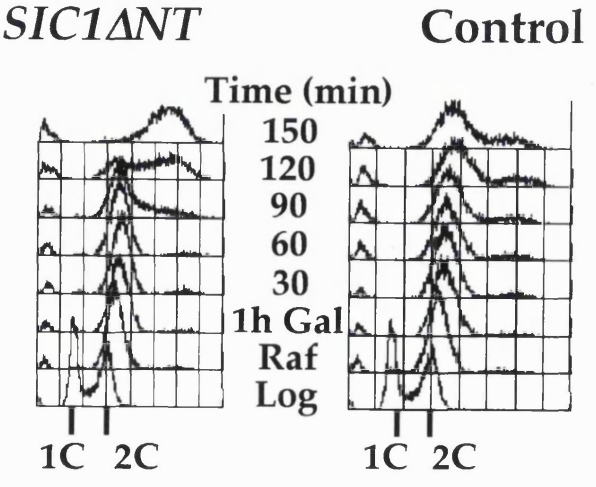


Figure 7C, 7D and 7E. SIC1ΔNT drives complete re-replication in G2/M arrested cells

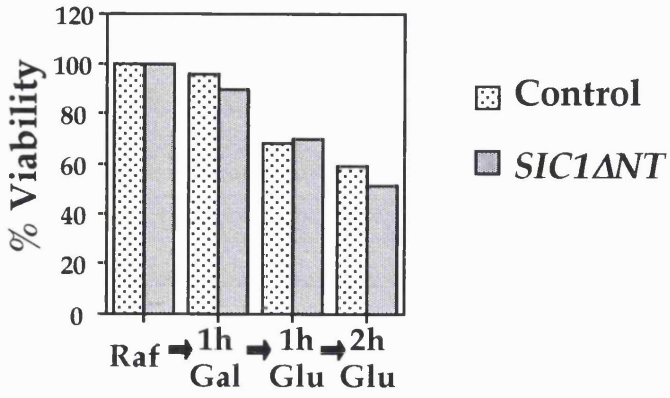
C. A wild type yeast strain with *GAL-SIC1ΔNT* (YLD12) and a strain with the *GAL* promoter plasmid (pMHT-Gal) alone (YLIL4), (Control) were grown to log phase (Log) and arrested in G2/M with nocodazole (Noc) as above. Cells were transferred to galactose medium (Gal) containing nocodazole for one hour to induce *SIC1ΔNT* transcription and then to glucose medium containing nocodazole (Glu) for 3 hours to repress *SIC1ΔNT* transcription. To calculate the viability of cells throughout the experiment, aliquots of cells were plated at equal density onto solid glucose medium at various times during the experiment and the numbers of colonies which grew were counted. The relative viabilities of cells at different stages in the experiment were calculated as a percentage of the number of colonies which grew from cells arrested in nocodazole at the start of the experiment compared to the number of colonies which grew from cells taken later in the experiment.

D Colonies of survivors plated onto YP-glucose after arrest in nocodazole (Raf), or after incubation galactose and glucose medium (1h Gal + 3h Glu) were grown to log phase and FACS analysis was carried out to determine their ploidy.

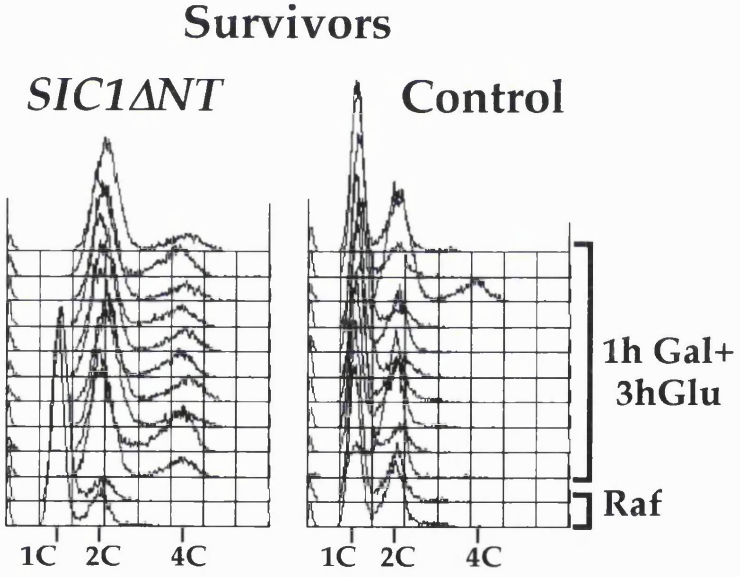
E Diploid survivors were grown to log phase in raffinose medium and arrested in G2/M using nocodazole (Raf). The medium was then replaced with galactose medium containing nocodazole to induce expression of *SIC1ΔNT* for 1 hour. *SIC1ΔNT* expression was then repressed by exchanging the medium for glucose medium containing nocodazole for 2 hours (1h Gal +2h Glu). Aliquots of cells were removed throughout the experiment and plated on glucose medium. When colonies grew, cells were removed and grown to log phase in liquid culture and FACS analysis was carried out.

Figure 7C, 7D and 7E

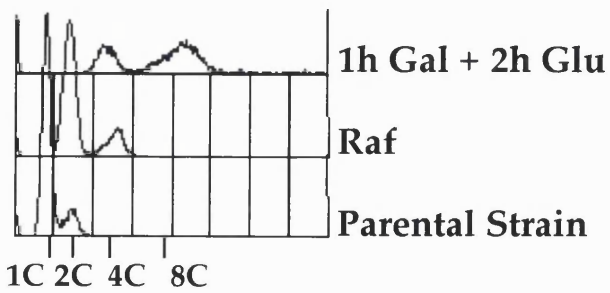
C



D



E



determined by flow cytometry. Although the parental strain was haploid, 90% of the survivors of transient Sic1ΔNTp over-production had a DNA content indistinguishable from that of a diploid strain (figure 7D). This diploidisation required Sic1ΔNTp over-production since the strain containing the vector alone produced only haploid survivors. The diploid strain could also be induced to undergo an additional round of DNA replication in the absence of mitosis by arresting cells in G2/M and transiently inducing *SIC1ΔNT* expression. Viable tetraploid colonies (figure 7E) grew when cells treated in this way were plated onto glucose medium. Therefore, transient inhibition of Cdc28/Clb kinase by over-expression of *SIC1ΔNT* induces origin resetting and apparently complete re-replication of the genome.

Transient over-expression of SIC1ΔNT is necessary to induce re-replication

The re-replication observed above predicts that repression of *SIC1ΔNT* transcription and re-activation of Cdc28/Clb kinase is required for re-replication. To investigate whether this is the case, a wild type strain was arrested in G2/M and expression of *SIC1ΔNT* was induced from the *GAL* promoter. After 1 hour the culture was split and in one half *SIC1ΔNT* expression was repressed by addition of glucose to the culture, in the other half *SIC1ΔNT* expression was maintained by incubation of the culture in galactose. In this experiment re-replication was only observed upon repression of *SIC1ΔNT* transcription (figure 8A). In a similar experiment the ability of cells to re-replicate in the absence of Cdc4p function was examined since degradation of *SIC1ΔNT* is, in part, dependent upon Cdc4p (figure 6B). Wild type and *cdc4-1* strains were arrested in G2/M and expression of *SIC1ΔNT* was induced. At the same time the temperature was raised to 37°C to induce inactivation of *cdc4*-1. After 1 hour, transcription of *SIC1ΔNT* was repressed by addition of glucose to the culture, while the temperature was maintained at 37°C. FACS analysis demonstrated that although re-replication occurred in the wild type strain, no re-replication occurred I the *cdc4-1* strain (figure 8B). These experiments suggest that after *SIC1ΔNT* induced pre-RC formation, re-replication requires a drop in the levels of Sic1ΔNTp and re-activation of Cdc28/Clb kinase.

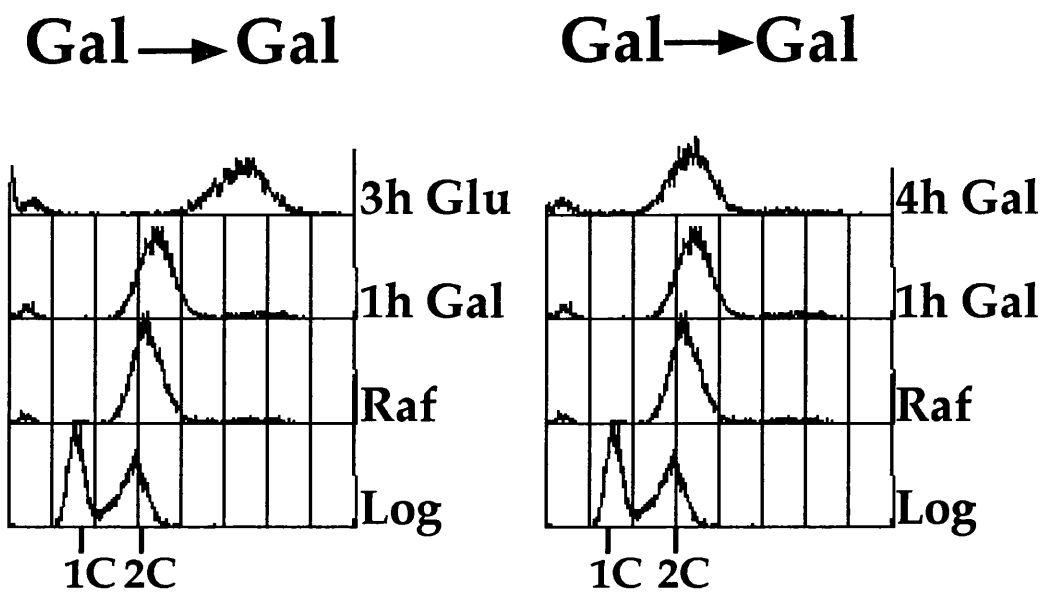
Figure 8. Transient expression of SIC1ΔNT induces re-replication

A. A wild type yeast strain with *GAL-SIC1ΔNT* (YLD12) was grown to log phase in raffinose medium and arrested in G2/M using nocodazole (Raf). Cells were transferred to galactose medium containing nocodazole (Gal) for 1 hour to induce *SIC1ΔNT* expression and the culture was then split. In one half *SIC1ΔNT* expression was repressed by transferring the cells to glucose medium containing nocodazole (Glu). In the other half, *SIC1ΔNT* expression was maintained by incubating the cells in galactose medium containing nocodazole (Gal) for a further 3 hours. FACS analysis was carried out.

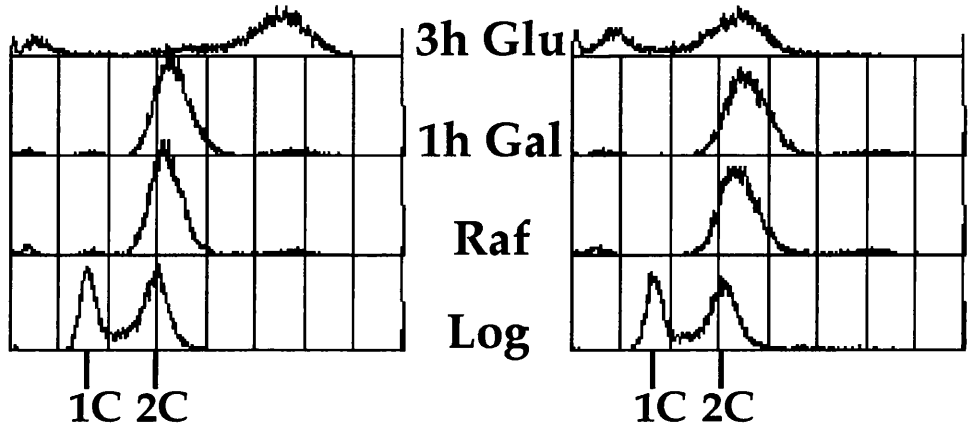
B. Log phase cultures of YLD12 and a *cdc4-1* strain, each with one copy of *GAL-SIC1ΔNT* (YLIL5) were arrested in nocodazole in raffinose medium (Raf) at 24°C. The raffinose medium was then exchanged for pre-warmed galactose medium containing nocodazole (Gal) at 37°C to induce expression from the *GAL* promoter and inactivate *cdc4-1*. *SIC1ΔNT* expression was then repressed by exchanging the medium for pre-warmed glucose medium containing nocodazole (Glu). Samples for FACS analysis were taken throughout the experiment.

Figure 8

A


Gal→Gal
Gal→Gal
3h Glu
1h Gal
Raf
Log
4h Gal
1h Gal
Raf
Log
1C 2C
1C 2C

B


WT
cdc4-1
3h Glu
1h Gal
Raf
Log
1C 2C
1C 2C

SIC1ΔNT induced re-replication is dependent upon CDC6 and CDC7

It is possible that *SIC1ΔNT* induced re-replication in G2/M arrested cells occurs by a different mechanism to replication in a normal S phase. In a normal cell cycle *CDC6* and *CDC7* are essential for DNA replication and therefore we investigated whether these two genes are required for *SIC1ΔNT* induced re-replication. A wild type strain and strains containing temperature sensitive mutations in either *CDC6, (cdc6-1)* or *CDC7 (cdc7-4)*, were arrested in G2/M and expression of *SIC1ΔNT* was transiently induced at the permissive temperature (24°C). All three strains underwent re-replication in this experiment (figure 9A). However, if cells were arrested in G2/M at the permissive temperature and then incubated at the non-permissive temperature (37°C) to inactivate *cdc6-1* and *cdc7-4* before expression of *SIC1ΔNT* was transiently induced, only the wild type strain underwent re-replication; neither *cdc6-1* nor *cdc7-4* mutants re-replicated (figure 9B). Therefore *SIC1ΔNT* induced re-replication is dependent on Cdc6p and Cdc7p.

In a normal cell cycle Cdc6p is essential for pre-RC formation, while Cdc7p is essential for origin firing after pre-RC formation. To investigate whether this is the case in *SIC1ΔNT* induced re-replication, *cdc6-1* and *cdc7-4* mutants were arrested in G2/M and incubated at 37°C to inactivate *cdc6-1* or *cdc7-4* before expression of *GAL-SIC1ΔNT* was induced. DNase I footprinting of the genomic origin *ARS305* showed that *SIC1ΔNT* over-expression in *cdc6-1* cells did not induce pre-RC formation (figure 9C, lanes 3 and 4), however, in *cdc7-4* cells, over-expression of *SIC1ΔNT* did induce pre-RC formation (figure 9C, lanes 7 and 8). A control *cdc7-4* strain which did not contain *GAL-SIC1ΔNT* did not induce pre-RC formation when treated in the same way (figure 9C, lanes 9 and 10). These results show that *CDC6* is essential for *SIC1ΔNT* induced pre-RC formation and *CDC7* is essential for re-replication. These results also demonstrate that *SIC1ΔNT* over-expression in G2/M can be used to distinguish between a defect in pre-RC formation and a defect in initiation of DNA replication.

Figure 9A and 9B. CDC6 and CDC7 are required for SIC1ΔNT induced re-replication

B. A wild type yeast strain with *GAL-SIC1ΔNT* (YLD12), a *cdc6-1* mutant with *GAL-SIC1ΔNT*, (YLIL9), and a *cdc7-4* mutant with *GAL-SIC1ΔNT* (YLIL15) were grown to log phase (Log) in raffinose medium and then arrested in nocodazole (Raf). *SIC1ΔNT* expression was then induced by addition of galactose medium containing nocodazole (Gal) for one hour and then repressed by addition of glucose medium containing nocodazole (Glu) for 2 hours and FACS analysis was carried out. Cells were maintained at 24°C throughout this experiment.

B. A wild type yeast strain with *GAL-SIC1ΔNT* (YLD12), a *cdc6-1* mutant with *GAL-SIC1ΔNT*, (YLIL9), and a *cdc7-4* mutant with *GAL-SIC1ΔNT* (YLIL15) were grown to log phase (Log) in raffinose medium and then arrested in nocodazole (Raf). Cells were then incubated at 37°C for one hour in pre-warmed raffinose medium containing nocodazole, to inactivate the temperature sensitive alleles (37°C). *SIC1ΔNT* expression was then induced by addition of pre-warmed galactose medium containing nocodazole (Gal) for one hour and then repressed by addition of pre-warmed glucose medium containing nocodazole (Glu) for 2 hours and FACS analysis was carried out.

Figure 9A and 9B

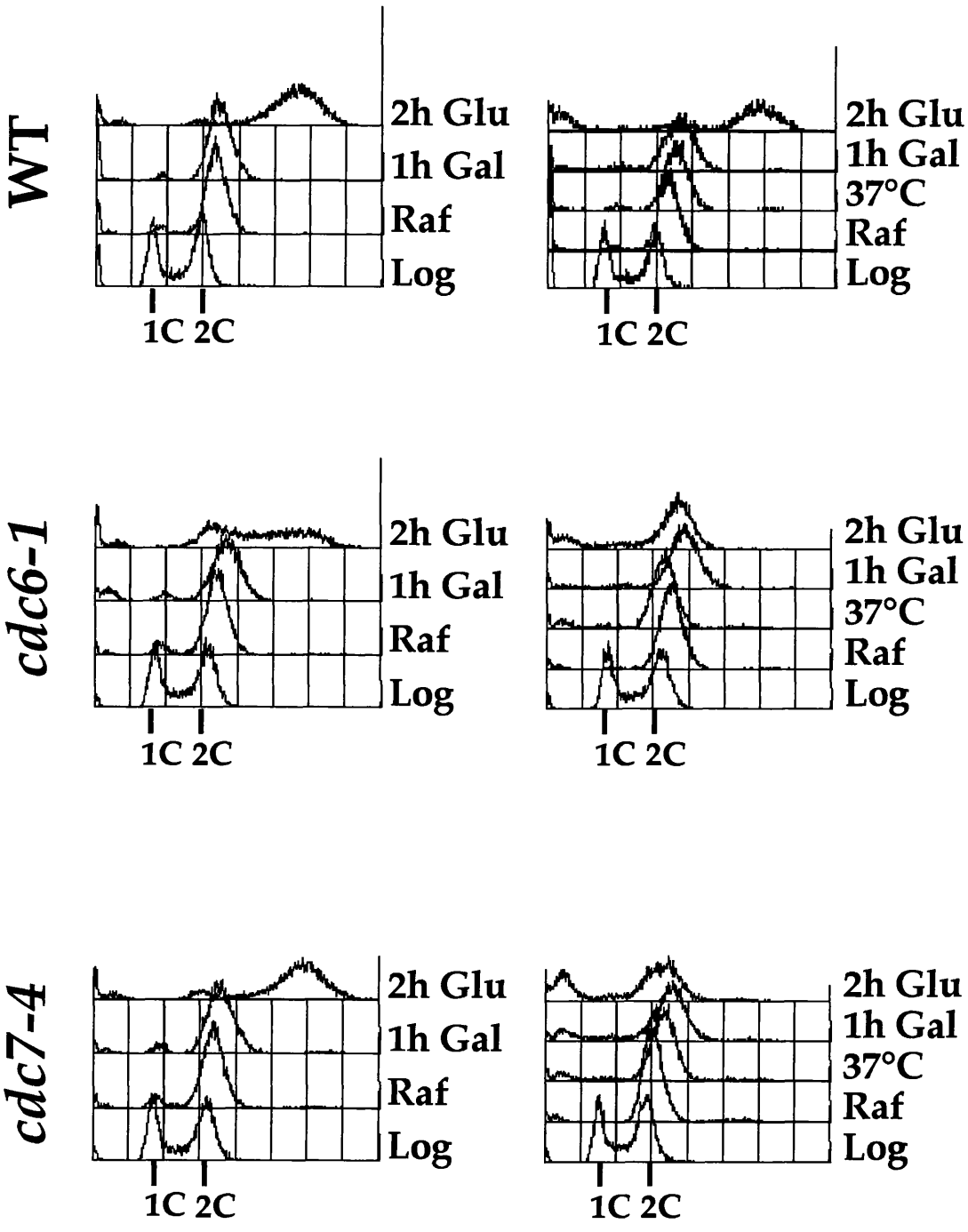
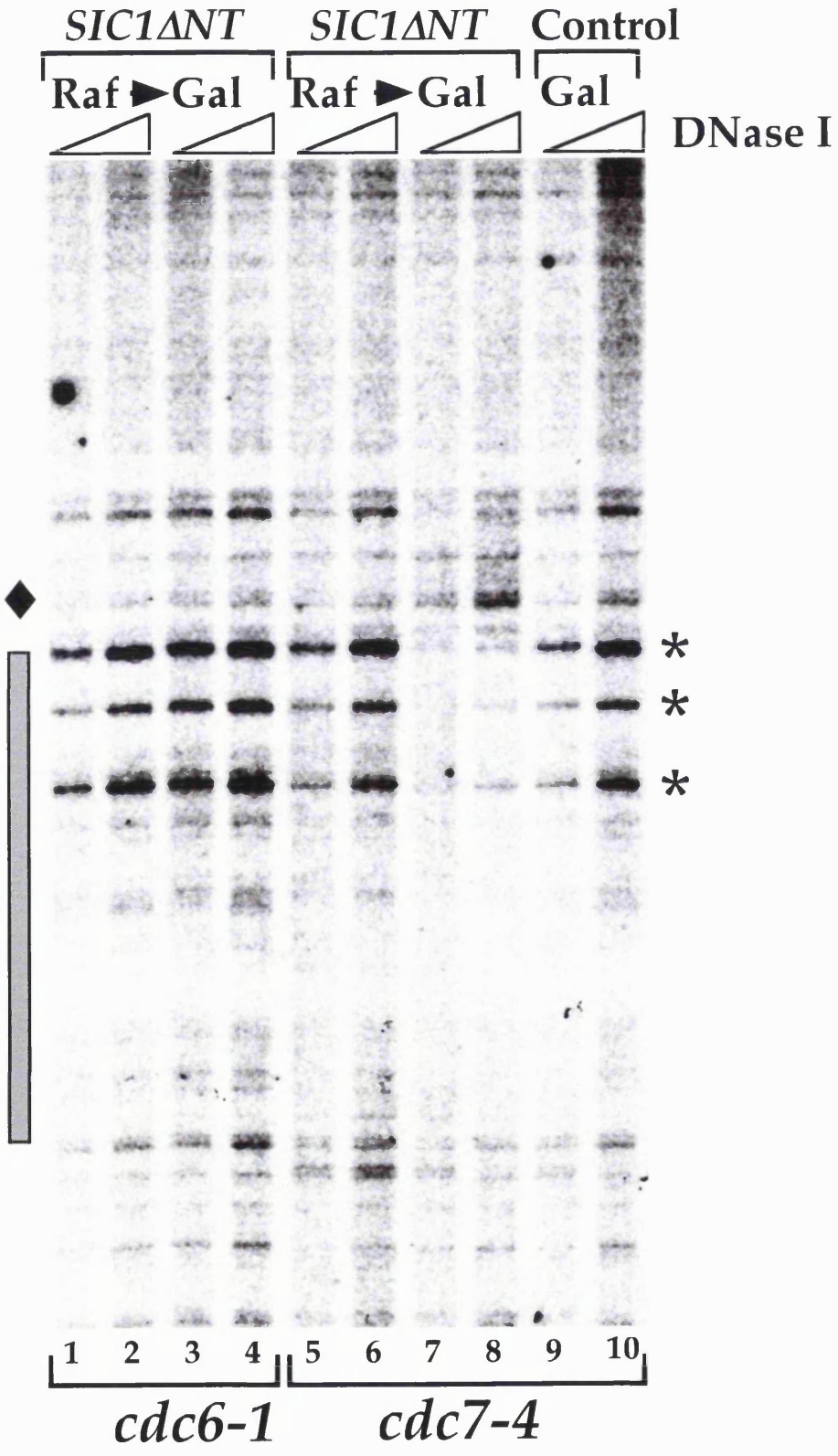


Figure 9C. CDC6 and CDC7 are required for SIC1ΔNT induced re-replication

C. A *cdc6-1* mutant with *GAL-SIC1ΔNT*, (YLIL9), and a *cdc7-4* mutant with *GAL-SIC1ΔNT* (YLIL15) plus a *cdc7-4* mutant without *GAL-SIC1ΔNT*, (Control), (YKB2), were arrested in nocodazole at 24°C. The cells were then incubated in pre-warmed raffinose medium at 37°C for 1 hour to inactivate the temperature sensitive alleles (Raf). *SIC1ΔNT* expression was then induced for 2.5 hours using pre-warmed galactose medium (Gal) at 37°C, containing nocodazole. Genomic footprinting of *ARS305* was carried out.

Figure 9C


SIC1ΔNT
SIC1ΔNT
Control
Raf ► Gal
Raf ► Gal
Gal
DNase I
*
*
*
1 2 3 4 5 6 7 8 9 10
cdc6-1
cdc7-4

Start is essential for SIC1ΔNT induced re-replication

Inhibition of Cdc28/Clb kinase by over-expression of *SIC1ΔNT* in G2/M arrested cells induces re-budding which suggests that Cdc28/Cln kinase becomes activated in these experiments and the cells have bypassed mitosis and entered a G1 like state. It is possible that inhibition of Cdc28/Clb kinase in G2/M accelerates cells to a point equivalent to either pre-start G1 (figure 10A, i), or to a point after start but prior to DNA replication (figure 10A, ii). If the former is true, then the mating pheromone alpha factor, which inhibits start, should inhibit *SIC1ΔNT* induced re-replication. To investigate whether this is the case, cells were arrested in G2/M, and *SIC1ΔNT* expression was induced for one hour in the presence of alpha factor, and then repressed, also in the presence of alpha factor. FACS analysis showed alpha factor inhibited *SIC1ΔNT* induced re-replication (figure 10B), suggesting that over-expression of *SIC1ΔNT* induces a pre-start G1 like state, and that some aspect of start is essential for re-replication in G2/M.

Direct inactivation of a temperature sensitive allele of cdc28 induces pre-RC formation and re-replication in G2/M

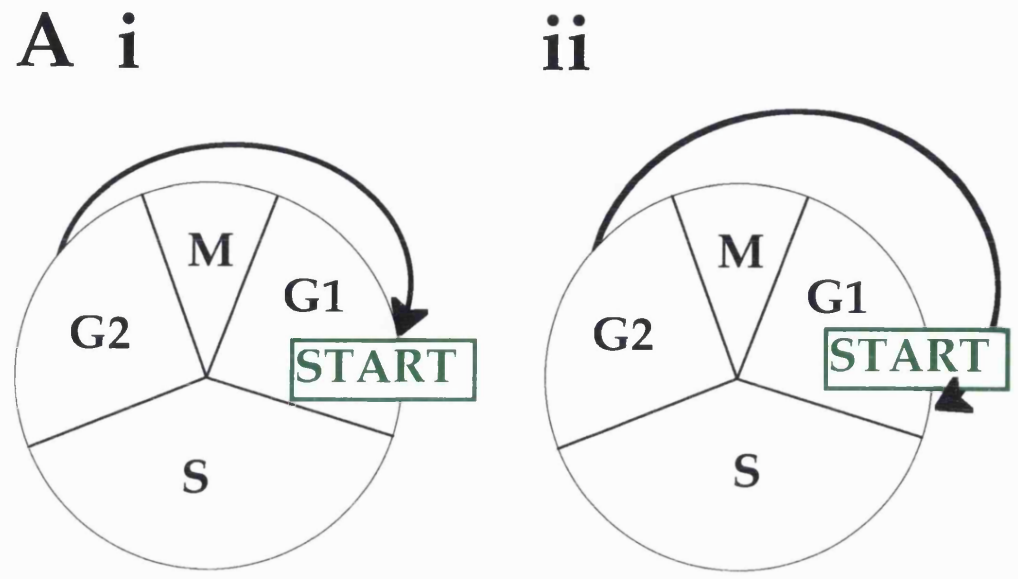
In human cells, an inhibitor of cyclin dependent kinases, Cip1, also inhibits PCNA, a component of the replication machinery (Harper et al., 1993; Waga et al., 1994b; Xiong et al., 1993). Although there is presently no evidence that Sic1p interacts with the replication machinery of *S. cerevisiae*, it is possible that massive over-expression of *SIC1ΔNT* re-set origins by a mechanism independent of its ability to inhibit Cdc28/Clb kinase. Therefore we investigated whether direct inactivation of Cdc28p induced pre-RC formation.

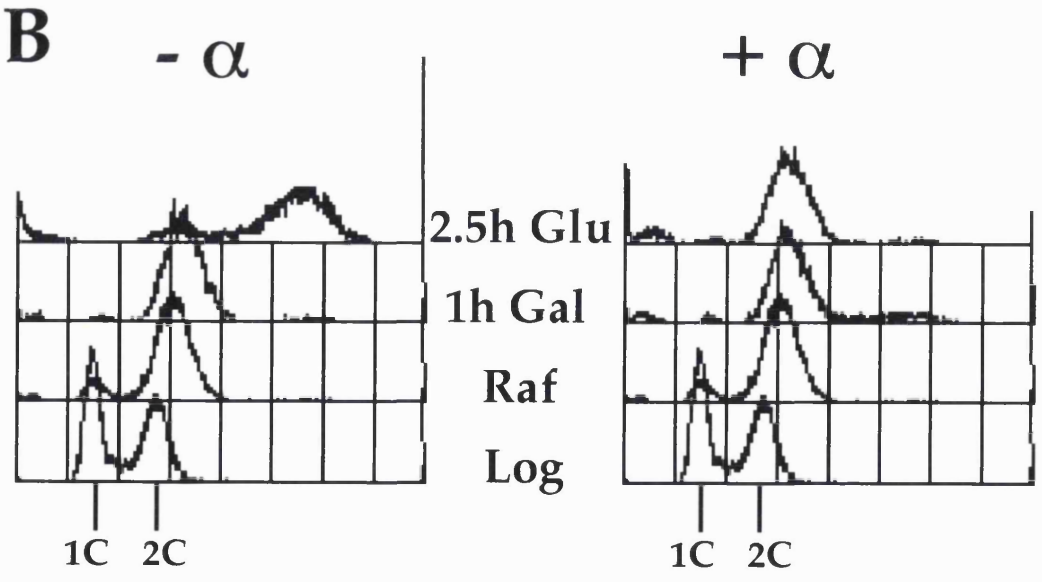
Previous attempts to induce pre-RC formation in G2/M arrested cells by inactivation of temperature sensitive mutants of *CDC28* have been unsuccessful (John F. X. Diffley unpublished results). This is likely to be due to incomplete inactivation of Cdc28p at the non-permissive temperature. To overcome this problem we used a "degron" allele of *CDC28* (*cdc28-td*) (Dohmen et al., 1994), which is selectively targeted for proteolytic degradation at 37°C (see

Figure 10. Start is essential for SIC1ΔNT induced re-replication

B. A wild type strain with *GAL-SIC1*, (YLD12), was grown to log phase (Log) and arrested in raffinose medium containing nocodazole (Raf). Galactose medium (Gal) containing nocodazole was then added to induce *SIC1ΔNT* expression and at the same time the culture was split and alpha factor (α) was also added to half of the cells. After one hour the medium was replaced with glucose medium containing nocodazole or with glucose medium containing nocodazole and alpha factor (Glu). FACS analysis was carried out.

Figure 10


A i
ii
M
G1
G2
START
S
M
G1
G2
START
S


B
- α
+ α
2.5h Glu
1h Gal
Raf
Log
1C
2C
1C
2C

chapter 3 for an explanation of construction of temperature sensitive mutants using the degron). The degron allele of *CDC28* (*cdc28-td*) is selectively unstable at 37°C, and this effect is enhanced if the ubiquitin protein ligase, *UBR1*, is over-expressed concomitantly (Kristine Bousset, Karim Labib and John F. X. Diffley, unpublished results). A strain which has *cdc28-td* as its only copy of *CDC28*, and *UBR1* under control of the *GAL1-10* promoter, was arrested in G2/M at the permissive temperature. Expression of *UBR1* was then induced for 30 minutes before degradation of Cdc28p was initiated by increasing the temperature to 37°C. DNase I footprinting of *ARS305* showed that degradation of Cdc28p induced pre-RC formation (figure 11A, lanes 7 and 8), however, a wild type strain treated in the same way did not (figure 11A, lanes 5 and 6). This experiment shows that inactivation of *CDC28* induces pre-RC formation and it confirms our results observed with *SIC1ΔNT* over-expression. In addition, it suggests that Cdc28/Cln kinase is not required for the formation of pre-RCs. FACS analysis showed that if *cdc28-td* was inactivated for one hour in G2/M arrested cells and then the temperature lowered to allow Cdc28tdp to re-accumulate, re-replication occurred (figure 11B). These experiments confirm that when Cdc28/Clb kinase is inactivated in G2/M arrested cells, either by *SIC1ΔNT* or *cdc28-td*, origins of DNA replication are re-set so that re-replication occurs when Cdc28/Clb kinase is reactivated.

Protein synthesis is essential for pre-RC formation in G2/M

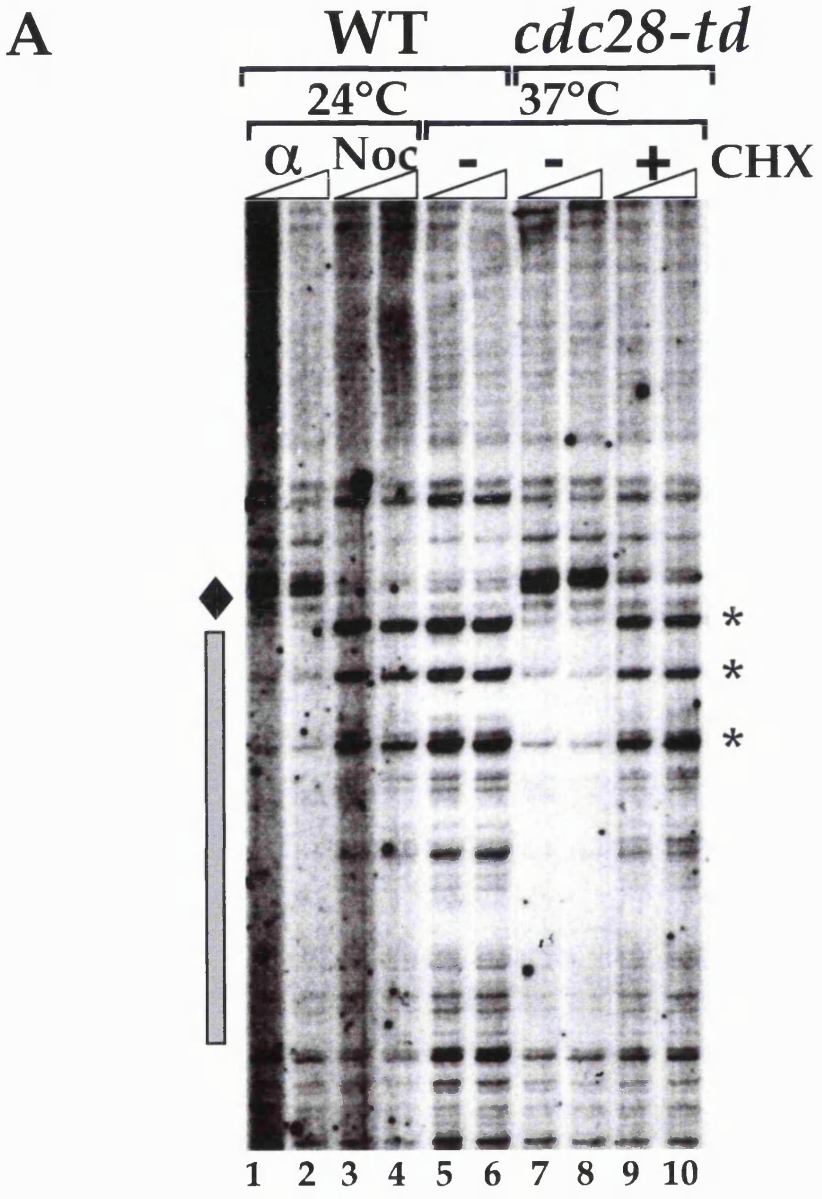
In *S. cerevisiae* at least one component of pre-RCs, *CDC6*, is synthesised *de novo* in each cell cycle (Bueno and Russell, 1992; Piatti et al., 1995; Zhou and Jong, 1990; Zwerschke et al., 1994). To investigate whether *cdc28-td* induced pre-RC formation is dependent on protein synthesis, a *cdc28-td* strain was arrested at G2/M and expression of *GAL-UBR1* was induced. The temperature was then raised to cause degradation of Cdc28p, and at the same time an inhibitor of protein synthesis, cycloheximide, was added to the culture. DNase I footprinting of *ARS305* showed that pre-RC formation did not occur when *cdc28-td* was inactivated in the presence of cycloheximide (figure 11A, lanes 9 and 10). Therefore synthesis of Cdc6p or some other component of pre-RCs is required for *cdc28-td* induced pre-RC formation to occur.

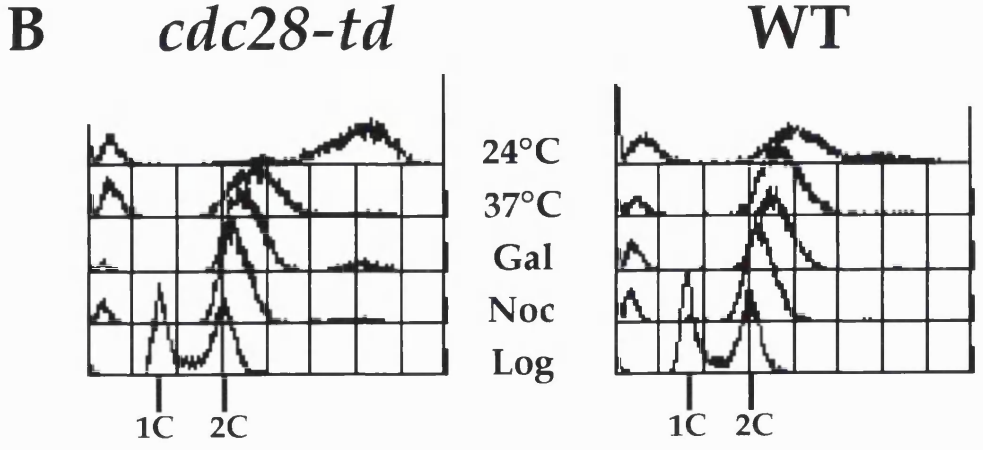
Figure 11. Inactivation of cdc28-td induces re-replication

A A strain containing *cdc28-td* and *GAL-UBR1* (YKB7), together with a wild type strain containing *GAL-UBR1*, (YKL83) were arrested either in G2/M using nocodazole or in G1 using alpha factor (α), at 24°C. Expression of *UBR1* was then induced in the G2/M arrested cells by transferring the cells to galactose medium containing nocodazole for 30 minutes at 24°C (Noc). Pre-warmed glucose medium at 37°C (containing nocodazole) was then added to induce Cdc28p degradation and repress *UBR1* expression. At the same time the *cdc28-td* culture was split and cycloheximide (CHX) was added to one of the cultures. After 1.5 hours, footprinting at *ARS305* was carried out (A).

B. YKB7 and YKL83 were arrested in nocodazole (Noc) as above and *UBR1* expression was induced for 30 minutes at 24°C (Gal). Pre-warmed glucose medium at 37°C was then added to inactivate *cdc28-td*, and after one hour (37°C) this was replaced with glucose medium containing nocodazole at 24°C, for 2.5 hours to induce re-synthesis of Cdc28p (24°C). FACS analysis was carried out to assess the DNA content of the cells.

Figure 11


A
WT
cdc28-td
24°C
37°C
α Noc
-
-
+
CHX
*
*
*
1 2 3 4 5 6 7 8 9 10


B
cdc28-td
WT
24°C
37°C
Gal
Noc
Log
1C 2C
1C 2C

Protein synthesis is essential for re-replication in G2/M

In addition to proteins involved in pre-RC formation, two proteins required for origin firing, Clbs and Dbf4p, are regulated in the cell cycle, in part by their periodic degradation in an APC/C dependent manner (Amon et al., 1994; Cheng et al., 1999; Godinho Ferreira et al., 2000; Irniger et al., 1995; Oshiro et al., 1999). It is likely that transcription of these genes is required in every cell cycle and we investigated whether protein synthesis is required for re-replication in G2/M. A wild type strain containing *GAL-SIC1ΔNT* was arrested at G2/M and *SIC1ΔNT* expression was induced for 2.5 hours. *SIC1ΔNT* expression was then repressed, and at the same time the culture was split and cycloheximide was added to one half to inhibit protein synthesis. FACS analysis of this experiment indicated that *SIC1ΔNT* induced re-replication did not occur in the presence of cycloheximide (figure 12A). To confirm that origin firing was inhibited by the addition of cycloheximide, the experiment was repeated and genomic footprinting of *ARS305* was carried out. As above, over-expression of *SIC1ΔNT* induced pre-RC formation (figure 12B, lanes 5 and 6). When *SIC1ΔNT* expression was then repressed in the absence of cycloheximide, the pre-RC was replaced by a post RC (figure 12B, lanes 7 and 8), consistent with origin firing. However, the pre-RC remained after *SIC1ΔNT* transcription was repressed in presence of cycloheximide (figure 12B, lanes 5, 6, 9 and 10), suggesting that cycloheximide inhibits origin firing. This result also demonstrates that pre-RCs are stable over a reasonably long period of time. It is possible that protein synthesis is required for Sic1p degradation and that stable Sic1p inhibits replication. However, when the levels of Sic1p were examined in the above experiment, Sic1p was degraded with normal kinetics (figure 12C). Furthermore, treatment of the cells with cycloheximide did not results in any loss of viability compared to the control experiment where no cycloheximide was used (figure 12D).

The requirement for protein synthesis in origin firing may be due to APC/C mediated degradation of Dbf4 and Clbs. To address this a strain containing a temperature sensitive mutation an APC/C subunit (*cdc16-123*), together with *GAL-SIC1ΔNT* was arrested in G2/M, *cdc16-123* function was

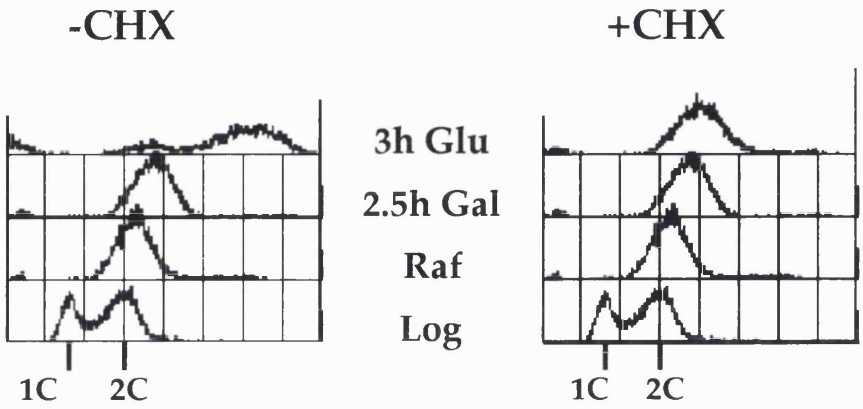
Figure 12A and 12B. Protein synthesis is required for SIC1ΔNT induced re-replication

A, A wild type strain with *GAL-SIC1ΔNT*, (YLD12) was grown to log phase (Log), in raffinose medium and arrested in G2/M using nocodazole (Raf). *SIC1 ΔNT* expression was then induced by addition of galactose medium containing nocodazole (Gal). After one hour the culture was split and glucose medium containing nocodazole (Glu) was added to repress *SIC1ΔNT* expression. Cycloheximide (CHX) was added to one half of the culture. FACS analysis was carried out.

B YLD12 was grown to log phase in raffinose medium and arrested in at G2/M in nocodazole (Raf) or in G1 using alpha factor (α). *SIC1ΔNT* expression was then induced for 2.5 hours by addition of galactose medium containing nocodazole (Gal). The culture was then split and *SIC1 ΔNT* expression was repressed by addition of glucose medium containing nocodazole (Glu), either with or without cycloheximide (CHX) for 2.5 hours. Genomic footprinting at *ARS305* was then carried out.

Figure 12A and 12B

A



B

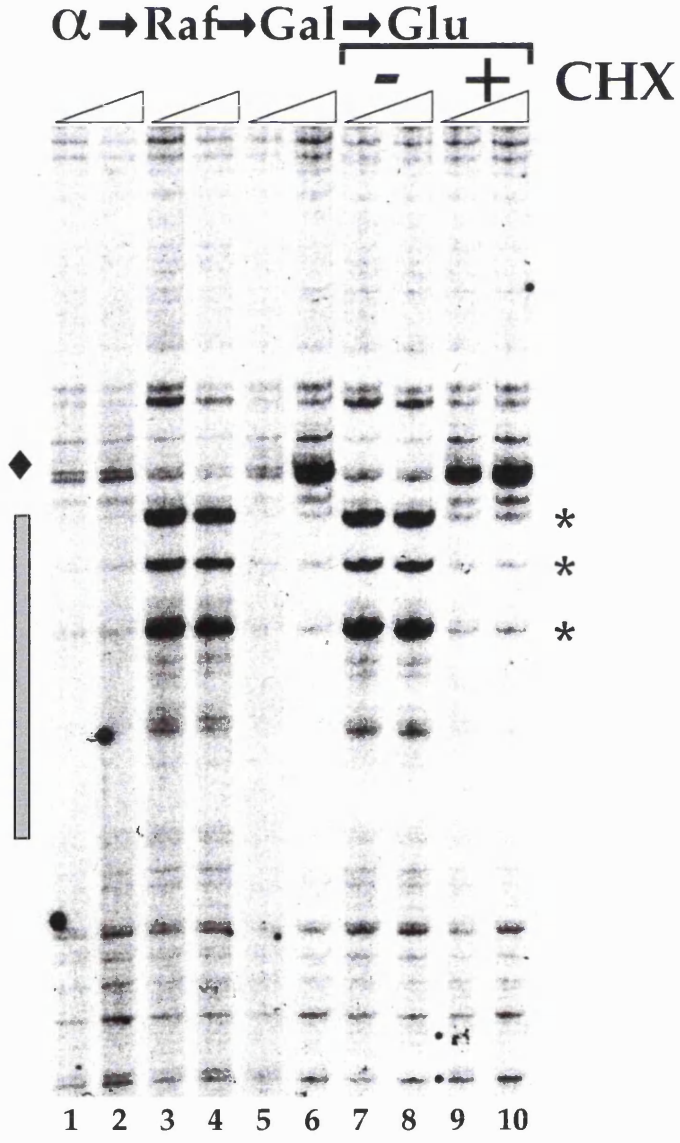
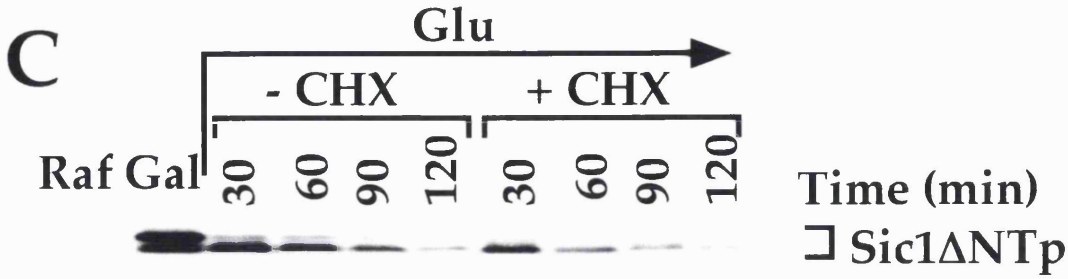


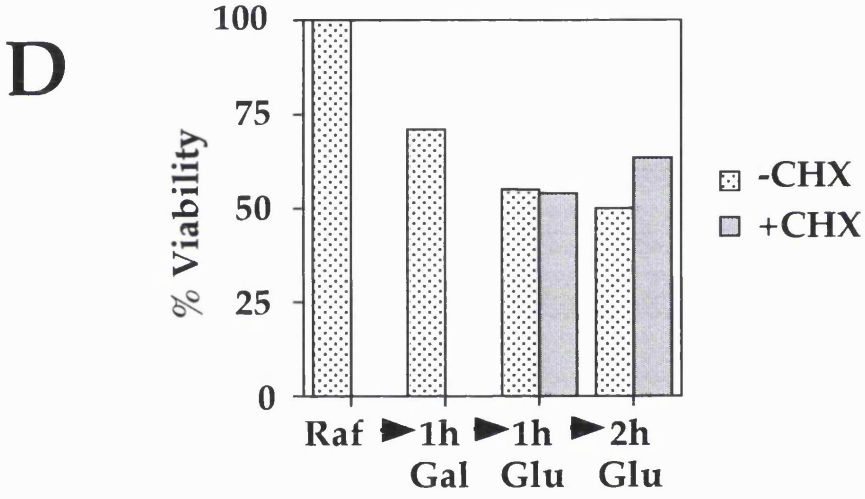
Figure 12C, 12D and 12E. Protein synthesis is required for SIC1ΔNT induced re-replication

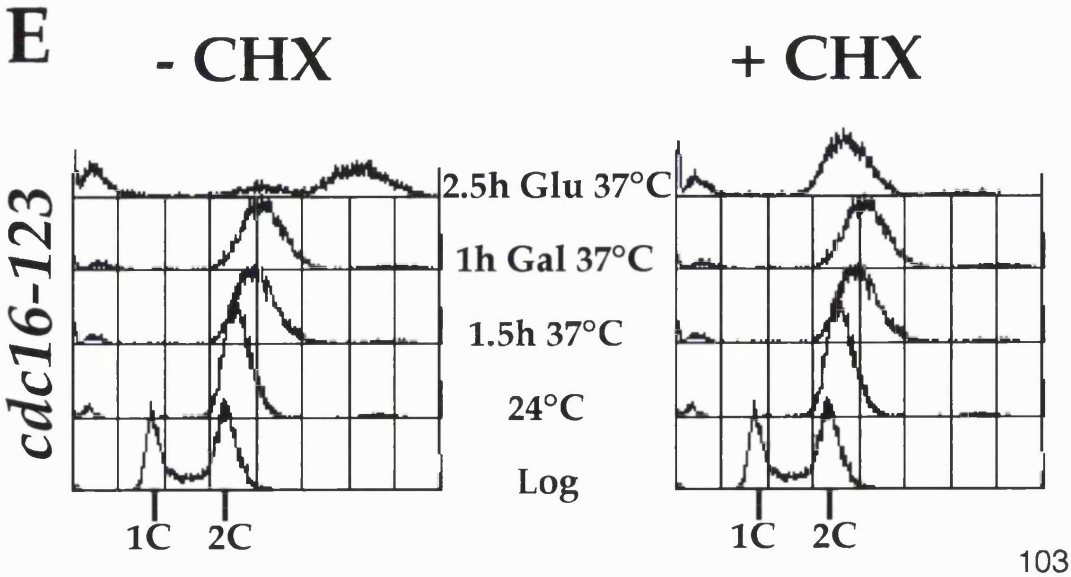
C and D YLD12 was grown to log phase (Log), in raffinose medium and arrested in G2/M using nocodazole (Raf). *SIC1 ΔNT* expression was then induced by addition of galactose medium containing nocodazole (Gal). After one hour the culture was split and glucose medium containing nocodazole (Glu) was added to repress *SIC1ΔNT* expression. Cycloheximide (CHX) was added to one half of the culture. Aliquots of cells were removed and plated onto glucose medium at equal density. Samples were taken throughout the experiment for protein extracts. Polyacrylamide gel electrophoresis and western blotting were carried out and Sic1ΔNTp was identified with the 9E10 antibody which recognises a myc epitope at the N terminus of Sic1ΔNTp (C). The viability of cells throughout the experiment was calculated as in figure 7C (D).

E YLD12 and a *cdc16-123* strain containing *GAL-SIC1ΔNT* (YLIL29) were grown to log phase (Log) in raffinose medium and then arrested in nocodazole (Noc). Cells were then incubated at 37°C for 1.5 hours in pre-warmed raffinose medium containing nocodazole, to inactivate *cdc16-123*. *SIC1ΔNT* expression was then induced by addition of pre-warmed galactose medium containing nocodazole (Gal) for one hour and then repressed by addition of pre-warmed glucose medium containing nocodazole (Glu). The cultures were split at this time and cycloheximide was added to one half to inhibit further protein synthesis. FACS analysis was carried out.

Figure 12C, 12D and 12E







inactivated by incubating the cells at 37°C for 1.5 hours (see results chapter 3), before expression of *SIC1ΔNT* was transiently induced. Re-replication occurred efficiently in the *cdc16-123* mutant (see results chapter 3), but was still inhibited by addition of cycloheximide to the culture while *SIC1ΔNT* expression was repressed (figure 12E).

Cdc28/Clb kinase inhibits pre-RC formation during S phase

The model proposed in the introduction to this chapter suggests that Cdc28/Clb kinase activity inhibits pre-RC formation during S phase as well as G2/M. Hydroxyurea (HU) arrests cells after the firing of early origins such as *ARS305*. Late firing origins are blocked from firing in this arrest by the checkpoint proteins Mec1p and Rad53p (Santocanale and Diffley, 1998). We therefore asked whether inactivation of Cdc28/Clb kinase could drive re-assembly of pre-RCs at *ARS305* in HU arrested cells. Figure 13A (lanes 2, 3, 8 and 9 show that *ARS305* is in the post-replicative state in HU arrested cells consistent with the fact that it has already fired. Inactivation of Cdc28/Clb kinase in these cells by over-expression of Sic1ΔNTp clearly induces the re-assembly of pre-RCs at *ARS305* (figure 13A lanes 4 and 5). If Sic1ΔNTp expression is then repressed and cells are released from HU into nocodazole, cells arrest with a DNA content which is greater than 2C but less than 4C (figure 13 B) and the pre-RC is lost from *ARS305* (figure 13A, lanes 6 and 7), consistent with the re-firing of a subset of origins (the early origins) during a single S phase. Inactivation of Cdc28/Clb kinase in HU arrested cells is accompanied by a loss of viability (figure 13C), suggesting that initiation from early origins of replication twice during S phase is lethal. However if cells are arrested in HU and a temperature sensitive allele of *cdc6* is inactivated, subsequent *SIC1ΔNT* over-expression still induces a significant loss of viability. This suggests that the effects of Cdc28/Clb inactivation during S phase is not necessarily confined to origins.

Conclusions

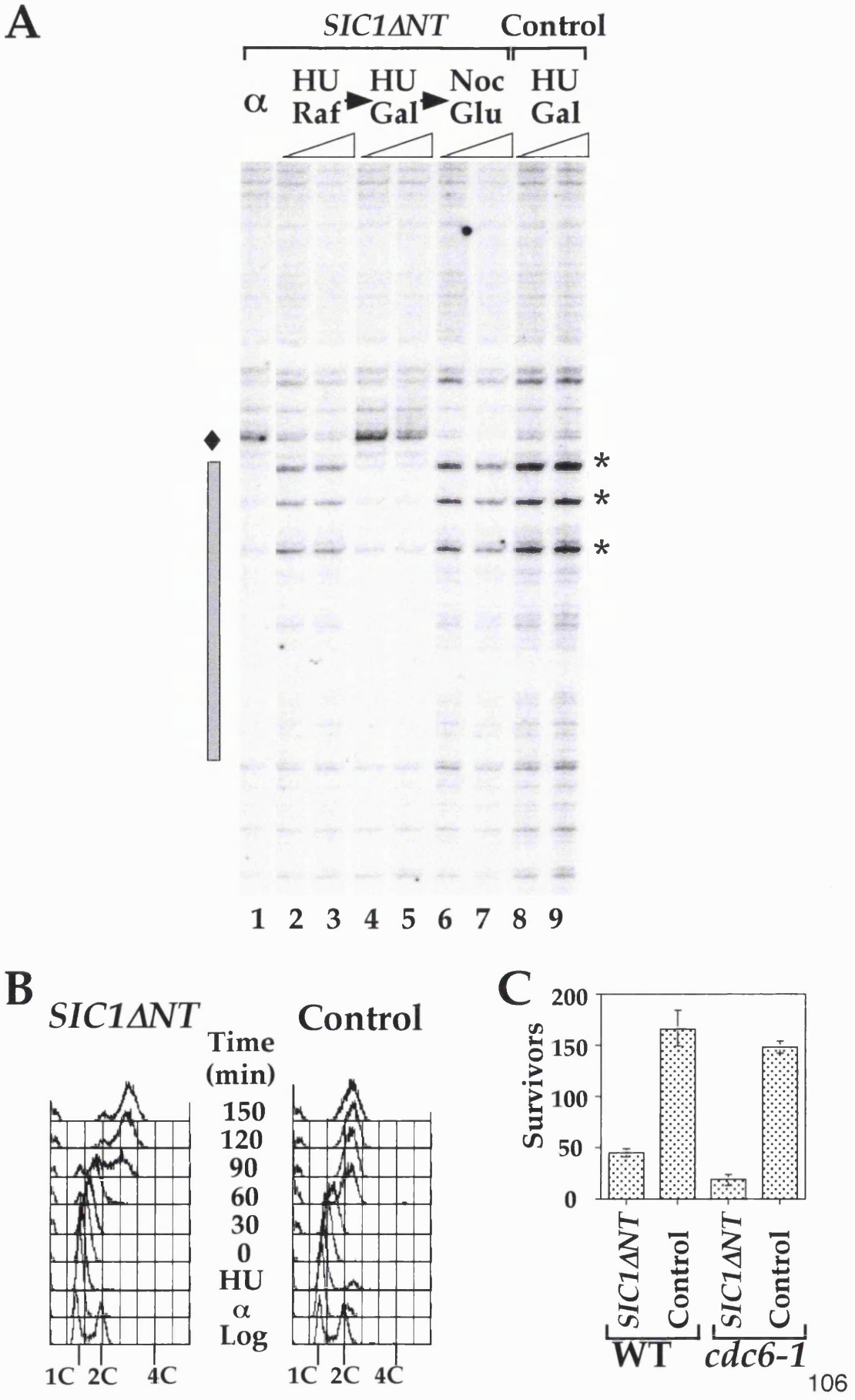
The results described here demonstrate that pre-RC formation and re-replication of DNA can be induced in G2/M phase using two independent

Figure 13. Over-expression of SIC1ΔNT drives pre-RC formation in S phase

A and B. A wild type yeast strain with *GAL-SIC1ΔNT* (YLD12) and a control strain with the *GAL* promoter alone, (YLIL4), (Control) were grown to log phase in raffinose medium and arrested in G1 using alpha factor (α). The alpha factor was removed by washing and the cells were arrested in early S phase using hydroxyurea (HU Raf). The medium was then exchanged for galactose medium (HU Gal) containing hydroxyurea for 2.5 hours. After this time the cells were washed to remove the hydroxyurea, and *SIC1ΔNT* expression was repressed by addition of glucose medium (Glu). At this time nocodazole (Noc) was also added to the culture to arrest the cells in G2/M. DNase I footprinting at the chromosomal origin *ARS305* and FACS were carried out.

C. YLIL4, YLD12 a *cdc6-1* mutant (K4050) and a *cdc6-1* mutant with *GAL-SIC1ΔNT*, (YLIL9) were grown to log phase (Log) in raffinose medium and arrested in α factor at 24°C. The alpha factor was removed by washing and the cells were arrested in early S phase using hydroxyurea (HU Raf). The cells were then incubated in pre-warmed raffinose medium containing nocodazole at 37°C for one hour to inactivate *cdc6-1*. Expression of *SIC1ΔNT* was then induced by exchanging the medium for pre-warmed galactose medium containing hydroxyurea. After 2 hours, aliquots of cells were taken, equal numbers of cells plated onto glucose medium and then incubated at 24°C until colonies were established. The number of colonies established was counted and a mean was calculated from 3 parallel plates. The error bars represent the standard deviation between the number of colonies on each plate.

Figure 13


A
SIC1ΔNT
Control
α
HU Raf
HU Gal
Noc Glu
HU Gal
◆
*
*
*
1 2 3 4 5 6 7 8 9
B
SIC1ΔNT
Control
Time (min)
150
120
90
60
30
0
HU
α
Log
1C 2C 4C
1C 2C 4C
C
Survivors
200
150
100
50
0
SIC1ΔNT
Control
SIC1ΔNT
Control
WT
cdc6-1

methods to inhibit Cdc28/Clb kinase activity; over-expression of *SIC1ΔNT* or direct inactivation of a temperature sensitive allele of *cdc28*. *CDC6* and *CDC7* are required for *SIC1ΔNT* induced re-replication suggesting that *SIC1ΔNT* induced re-replication is similar to S phase. Both pre-RC formation and origin firing in G2/M arrested cells require protein synthesis, demonstrating the importance of cell cycle regulated protein synthesis for control of DNA replication. Lastly, my results demonstrate that inactivation of Cdc28/Clb kinase in S phase arrested cells can drive pre-RC formation (Noton and Diffley, 2000).

Chapter Five: The Role of the Mitotic Exit Network and Anaphase Promoting Complex in DNA Replication

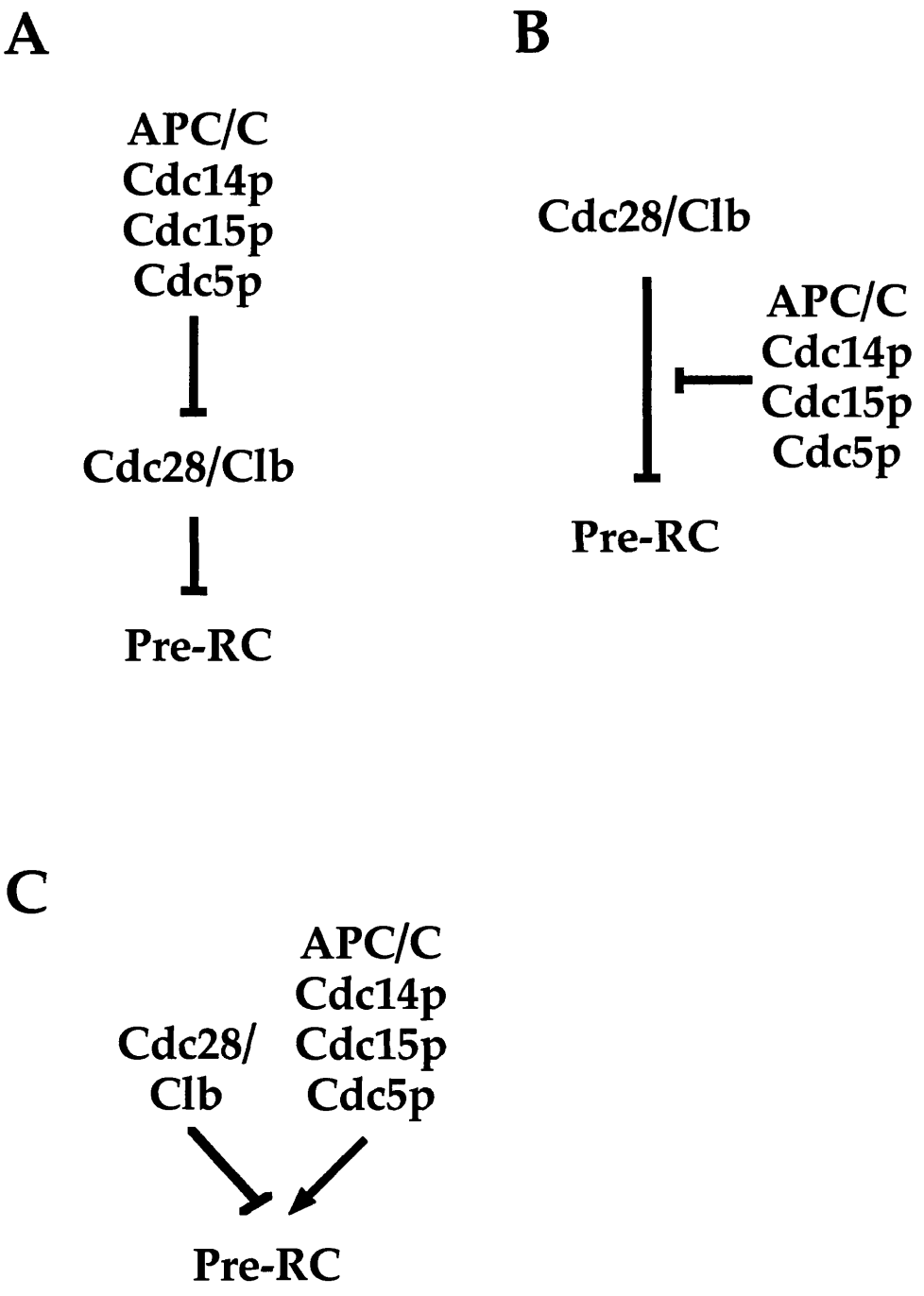
Introduction

There is considerable evidence that the APC/C and mitotic exit network are required for DNA replication. However the role of the APC/C and mitotic exit network in DNA replication has always been difficult to assess as it is impossible to separate the mitotic function of these proteins from their potential role in DNA replication. Inhibition of Cdc28/Clb kinase in G2/M arrested cells induces a round of pre-RC formation and DNA replication in the absence of mitosis. By combining this system with temperature sensitive mutants in the APC/C and mitotic exit network, it is possible to determine whether these genes have an essential role in DNA replication.

Possible roles of the APC/C and mitotic exit network in DNA replication

There are three ways that the APC/C and mitotic exit network might affect DNA replication and these are illustrated in figure 14. The mitotic exit network and APC/C both have important roles in Cdc28/Clb kinase inactivation at the end of mitosis. One of the important consequences of Cdc28/Clb inactivation in mitosis is pre-RC formation in the following G1. Therefore it is possible that this alone explains the role of the mitotic exit network and APC/C in DNA replication (figure 14A). Another likely possibility for the role of the mitotic exit network and APC/C in DNA replication is to reverse inhibition of pre-RC formation by Cdc28/Clb kinase by acting directly on pre-RC components (figure 14B). For example, Cdc14p is a phosphatase and may reverse inhibitory phosphorylation of pre-RC components by Cdc28/Clb kinase. Indeed, Cdc14p can reverse Cdc28/Clb dependent phosphorylation of the Swi5p transcription factor which drives *CDC6* transcription in late mitosis. A third possibility is that the mitotic exit network and APC/C act independently of Cdc28/Clb kinase to promote pre-RC formation (figure 14C). For example, the APC/C may degrade a geminin-like inhibitor of pre-RC formation in budding yeast. To address which of the above models for the role the APC/C and mitotic

Figure 14


A
APC/C
Cdc14p
Cdc15p
Cdc5p
Cdc28/Clb
Pre-RC
B
Cdc28/Clb
APC/C
Cdc14p
Cdc15p
Cdc5p
Pre-RC
C
Cdc28/
Clb
APC/C
Cdc14p
Cdc15p
Cdc5p
Pre-RC

exit network in DNA replication is correct, we asked whether inactivation of Cdc28/Clb kinase in G2/M arrested cells could bypass the function of the APC/C and mitotic exit network in DNA replication.

Inactivation of temperature sensitive alleles of the APC/C and mitotic exit network genes

In the following experiments we determine whether strains with an inactivated APC/C or mitotic exit network are able to undergo *SIC1ΔNT* induced re-replication in G2/M arrested cells. Before beginning these experiments it was important to determine conditions in G2/M arrested cells where temperature sensitive alleles of the APC/C or mitotic exit network could be reliably inactivated. To address this strains containing a temperature sensitive mutation in one of the APC/C genes or mitotic exit network genes, were arrested in G2/M using nocodazole at the permissive temperature of 24°C. When the cells arrested, the temperature of the culture was raised to 37°C. At various times after the shift to 37°C, the cells were washed to remove nocodazole and resuspended in fresh medium at 37°C. The cell density of these cultures was then monitored to determine whether cell division took place. Figure 15A shows that when a wild type strain was released from a nocodazole arrest after incubation at 37°C for 90 minutes it underwent cell division. A strain carrying a mutation in the APC/C gene, *cdc16*, treated in the same way did not undergo either cell division (figure 15A) or nuclear division (data not shown), suggesting that the APC/C had been inactivated. Similar experiments with strains containing temperature sensitive mutations in other components of the APC/C, (*cdc20-3* and *cdc23-1*) and components of the mitotic exit network (*cdc14-1*, *cdc5ts* (*msd2-1)* and *cdc15-1*) demonstrated that incubation at 37°C for 60 minutes in a G2/M arrest was sufficient to inactivate these mutant alleles (data not shown).

As an additional control to demonstrate that the APC/C is inactive in the experiments described below, we compared the levels of Clb3p, an APC/C substrate, in wild type and strains with the mutations *cdc16-123* or *cdc20-3*. Cells were arrested in G2/M using nocodazole at the permissive temperature and then incubated at 37°C (in nocodazole) for 90 minutes or 60 minutes respectively to inactivate the temperature sensitive mutations. The cultures were

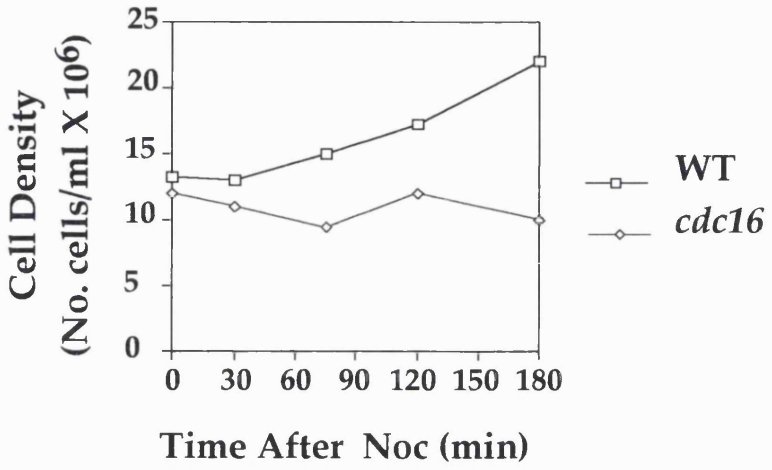
Figure 15. Inactivation of cdc16-123 and cdc20-3

A A wild type strain containing *GAL-SIC1ΔNT* (YLD12) and a *cdc16-123* strain containing *GAL-SIC1ΔNT* (YLIL29) were arrested in nocodazole at 24°C. Cells were then incubated in pre-warmed raffinose medium containing nocodazole at 37°C for 90 minutes. The cells were then washed to remove the nocodazole and transferred to pre-warmed glucose medium at 37°C and the cell density of the cultures was recorded.

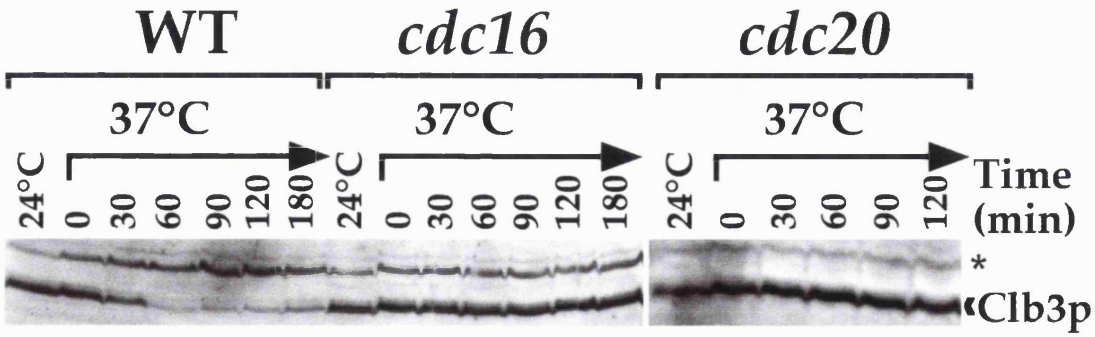
B YLD12, YLIL29 and a *cdc20*-3 strain containing *GAL-SIC1ΔNT* (YLIL65) were arrested in nocodazole at 24°C (24°C). Cells were then incubated in pre-warmed raffinose medium containing nocodazole at 37°C for 90 minutes (WT and YLIL29), or for 60 minutes (YLIL65), to inactivate *cdc16-123* and *cdc20-3* respectively (37°C). The cells were then washed to remove the nocodazole and transferred to pre-warmed glucose medium containing alpha factor at 37°C for 2 or 3 hours. Wild type cells were arrested in G1, however the *cdc16* and *cdc20* mutants did not complete mitosis (data not shown). Samples were taken for protein extracts and polyacrylamide gel electrophoresis and western blotting were then carried out. Clb3p was identified using a polyclonal antibody. The asterisk indicates a non-specific background band which serves as a loading control.

Figure 15

A


Cell Density
(No. cells/ml X 10^6)
25
20
15
10
5
0
0 30 60 90 120 150 180
Time After Noc (min)
WT
cdc16

B


WT
cdc16
cdc20
37°C
37°C
37°C
24°C
0 30 60 90 120 180
24°C
0 30 60 90 120 180
24°C
0 30 60 90 120
Time
(min)
*
Clb3p

then washed to remove nocodazole and resuspended in fresh medium at 37°C containing alpha factor to arrest cells in the following G1. As expected the wild type cells arrested as unbudded cells (data not shown) with relatively low levels of Clb3p, whereas the *cdc16-123* and *cdc20-3* mutants did not complete mitosis and retained high levels of Clb3p. (figure 15B). These experiments suggest that these conditions are sufficient to inactivate the temperature sensitive mutants used in the experiments below.

The APC/C is not required for SIC1ΔNT induced pre-RC formation and re-replication

Studies in *X. laevis* and HeLa cells have shown that an inhibitor of MCM loading, geminin, is degraded in an APC/C dependent manner during mitosis (McGarry and Kirschner, 1998). In *S. cerevisiae* the APC/C is essential for degradation of Clbs and Pds1p, an inhibitor of sister chromatid separation. In order to determine whether APC/C mediated degradation is an essential part of the origin re-setting event in mitosis, we investigated whether the activity of the APC/C is necessary for *SIC1ΔNT* induced pre-RC formation and re-replication.

A yeast strain containing a temperature sensitive allele of the APC/C gene *CDC16 (cdc16-123)*, was arrested in G2/M phase and *cdc16-123* was then inactivated by incubating the cells at the non-permissive temperature. Inhibition of Cdc28/Clb kinase was then induced by expression of *GAL-SIC1ΔNT* and DNase I footprinting of the genomic origin *ARS305* was carried out. This demonstrated that *SIC1ΔNT* over-expression in wild type and *cdc16-123* strains, induced pre-RC formation (figure 16A, lanes 5, 6, 9 and 10). Control strains which did not have *GAL-SIC1ΔNT* did not form pre-RCs (figure 16A, lanes 7, 8, 11 and 12). This experiment suggests that APC/C dependent proteolysis of mitotic proteins is not required for *SIC1ΔNT* induced pre-RC formation.

It is possible that a substrate of the APC/C could inhibit firing of origins of replication. We investigated whether degradation of an APC/C substrate is essential for *SIC1ΔNT* induced re-replication. Strains with temperature sensitive mutations in the APC/C genes *cdc16-123, cdc20-3* or *cdc23-1* were arrested in G2/M and the temperature sensitive mutations inactivated by incubation of the cells at the non-permissive temperature, as described above. Expression of

Figure 16A. CDC16 is not required for SIC1ΔNT induced pre-RC formation

Wild type strains containing either *GAL-SIC1ΔNT* (YLD12), or the *GAL* promoter alone (YLIL4) (Control), and *cdc16-123* strains containing either *GAL-SIC1ΔNT* (YLIL29) or the *GAL* promoter alone (YLIL18) (Control), were grown to log phase in raffinose medium at 24°C and arrested in G2/M using nocodazole (Raf), or in G1 using alpha factor (α). G2/M arrested cells were then incubated in raffinose medium at 37°C for 90 minutes to inactive *cdc16-123*. The medium was then replaced with pre-warmed galactose medium (Gal) containing nocodazole at 37°C for 2.5 hours to induce *SIC1ΔNT* expression. DNase I genomic footprinting at *ARS305* was then carried out.

Figure 16A

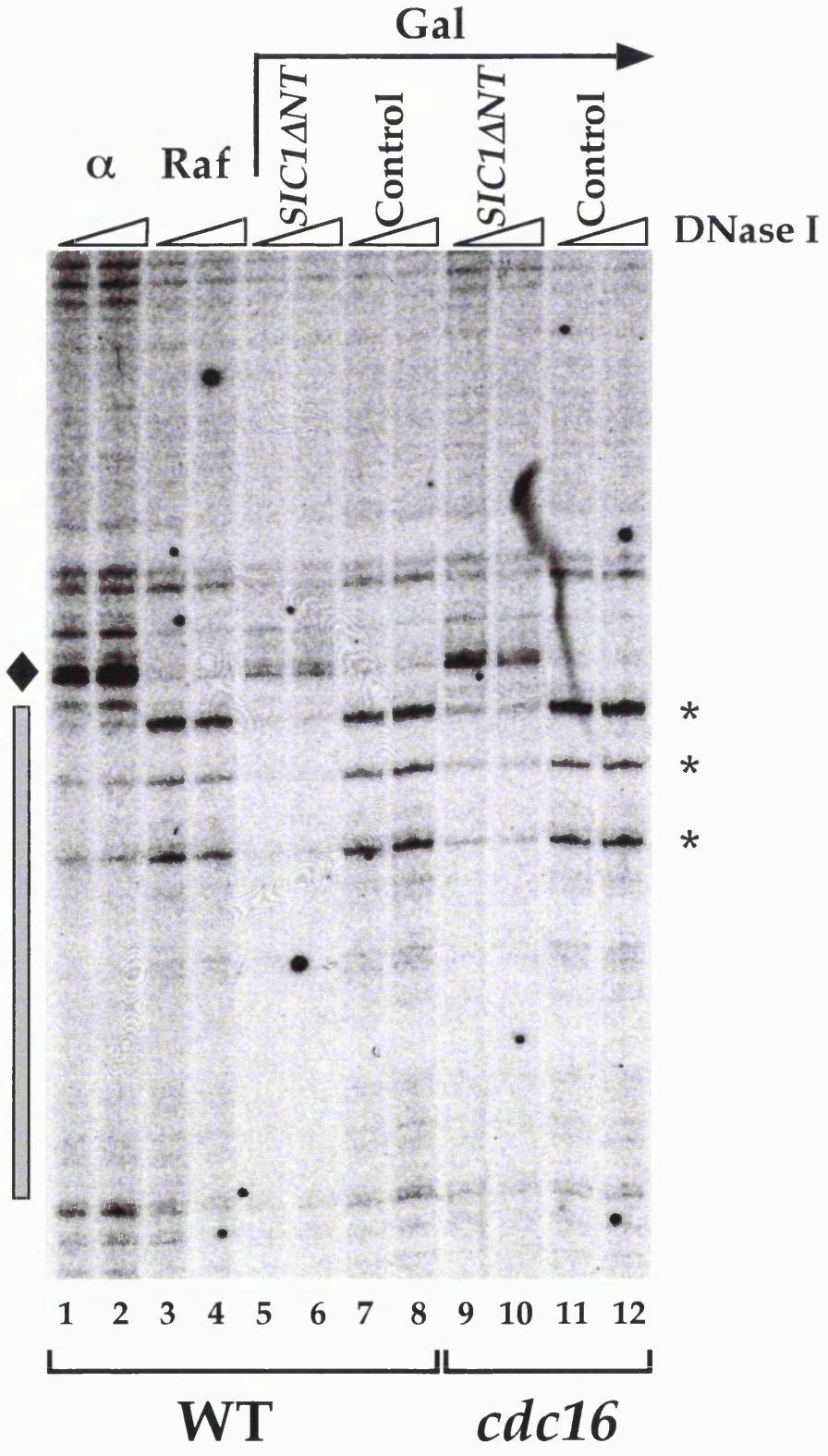
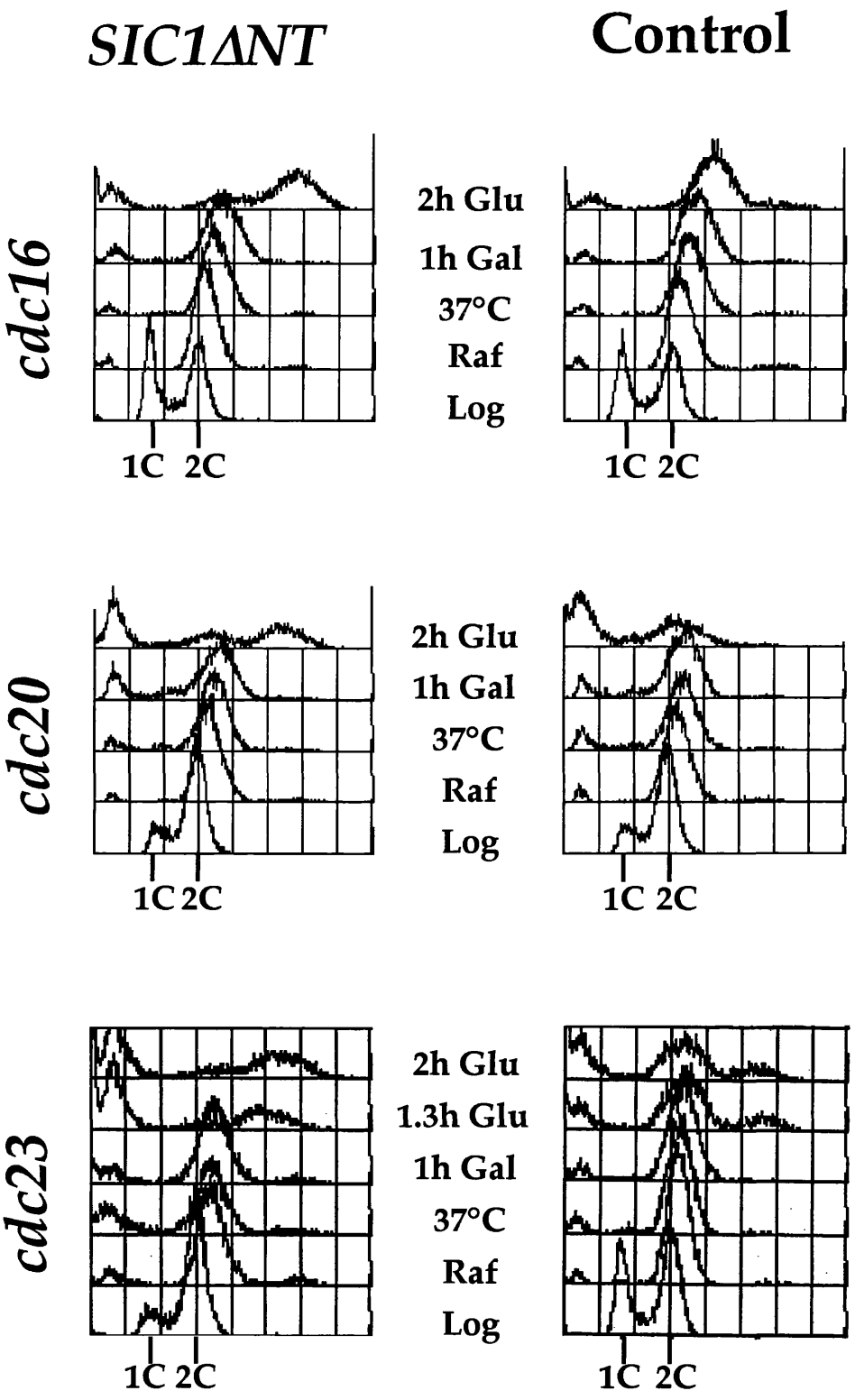

Gal
α
Raf
SIC1ΔNT
Control
SIC1ΔNT
Control
DNase I
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12
WT
cdc16

Figure 16B. The APC/C is not required for SIC1ΔNT induced re-replication

cdc16-123, *cdc20-3* and *cdc23-1* strains containing *GAL-SIC1ΔNT* (YLIL29, YLIL65 and YLIL8 respectively), a *cdc16-123* strain containing the *GAL* promoter alone (YLIL29) (Control), *cdc20-3* and *cdc23-1* strains without *GAL-SIC1ΔNT* (8030 and YMIG03 respectively) (Control) were grown to log phase (Log) in raffinose medium at 24°C and arrested in G2/M phase with nocodazole (Raf). As above the medium was replaced with raffinose medium at 37°C for 90 minutes to inactivate *cdc16-123*, or for 60 minutes to inactivate *cdc20-3* and *cdc23-1* (37°C). This was then replaced with pre-warmed galactose medium (Gal) for one hour to induce *SIC1ΔNT* expression. *SIC1ΔNT* transcription was subsequently repressed by transferring the cells to pre-warmed glucose medium (Glu). Nocodazole was present in the medium throughout this experiment. FACS analysis was carried out to monitor the DNA content of the cells.

Figure 16B


SIC1ΔNT
Control
cdc16
cdc20
cdc23
2h Glu
1.3h Glu
1h Gal
37°C
Raf
Log
1C
2C

SIC1ΔNT was then induced for one hour before being repressed and cells were maintained at the non-permissive temperature throughout the experiment. FACS analysis showed that control strains which did not contain *GAL-SIC1ΔNT* did not undergo any additional DNA replication. However transient over-expression of *SIC1ΔNT* induced re-replication in all three temperature sensitive mutants (figure 16B). This data suggests that APC/C dependent degradation is not required for *SIC1ΔNT* induced pre-RC formation, or re-replication in G2/M arrested cells.

CDC14, CDC5 and CDC15 are not required for SIC1ΔNT Induced pre-RC formation and DNA replication

To address the role of the mitotic exit network in *SIC1ΔNT* induced pre-RC formation, strains with the temperature sensitive mutations *cdc14-1*, *cdc5ts* (*msd2-1*) or *cdc15-2* were arrested in G2/M and the temperature sensitive mutations were inactivated by incubation at 37°C for one hour. Expression of *SIC1ΔNT* was then induced for 2.5 hours and DNase I footprinting at the chromosomal origin *ARS305* was carried out. Pre-RC formation clearly occurred in all three mutants, however control strains which did not express *SIC1ΔNT* did not form pre-RCs (figure 17A, lanes 7, 8, 11, 12, 13, and 14). This suggests that the mitotic exit network is not essential for pre-RC formation.

The role of the mitotic exit network in other aspects of DNA replication was also investigated. The strains used above were arrested in G2/M, and the temperature sensitive mutations inactivated as previously described. Expression of *SIC1ΔNT* was then transiently induced for one hour. Cells were maintained at the non-permissive temperature throughout. FACS analysis demonstrated that re-replication occurred in *cdc14-1*, *cdc5ts* and *cdc15-2* mutants containing *GAL-SIC1ΔNT*, however control strains without *GAL-SIC1ΔNT* did not re-replicate when treated in the same way (figure 17B). This data suggests that *CDC5*, *CDC14* and *CDC15* have no direct role in DNA replication and models B and C of figure 14 are incorrect. It is therefore likely that the role of *CDC5*, *CDC14* and *CDC15* in DNA replication is to inhibit Cdc28/Clb kinase at the end of mitosis (figure 14A).

Figure 17A. The mitotic exit network is not required for SIC1ΔNT induced pre-RC formation

cdc14-1, *cdc5*ts (*msd2-1)* and *cdc15-2* strains with *GAL-SIC1ΔNT* (YLIL35, YLIL39 and YLIL7 respectively), and isogenic control strains without *GAL-SIC1ΔNT*, (YLIL36, KKY021 and K1993) (Control) were grown to log phase in raffinose and then arrested in G2/M using nocodazole, or in G1 using alpha factor (α). The G2/M arrested cells were transferred to pre-warmed raffinose medium for one hour (Raf) to inactivate the temperature sensitive mutations. (If cells are washed at this point to remove nocodazole and then resuspended in glucose medium, they do not complete mitosis, indicating that the mutations have been inactivated (data not shown)). The medium was then replaced with pre-warmed galactose medium containing nocodazole (Gal) to induce *SIC1ΔNT* expression for 2.5 hours. DNase I genomic footprinting at *ARS305* was carried out.

The post-replicative footprint at *ARS305* in *cdc14* and *cdc5* strains has only two hypersensitive (*) sites compared to the three hypersensitive sites displayed by other strains used in our experiments, (which are all based on W303-1a). This is due to a polymorphism in *ARS305* rather than a replication defect since the wild type strain isogenic to the *cdc14-1* mutant strain also lacks the third hypersensitive site. Sequencing of *ARS305* in W303-1a, *cdc14-1* and *cdc5* strains revealed a single base pair difference at the position of the third hypersensitive site (data not shown).

Figure 17A

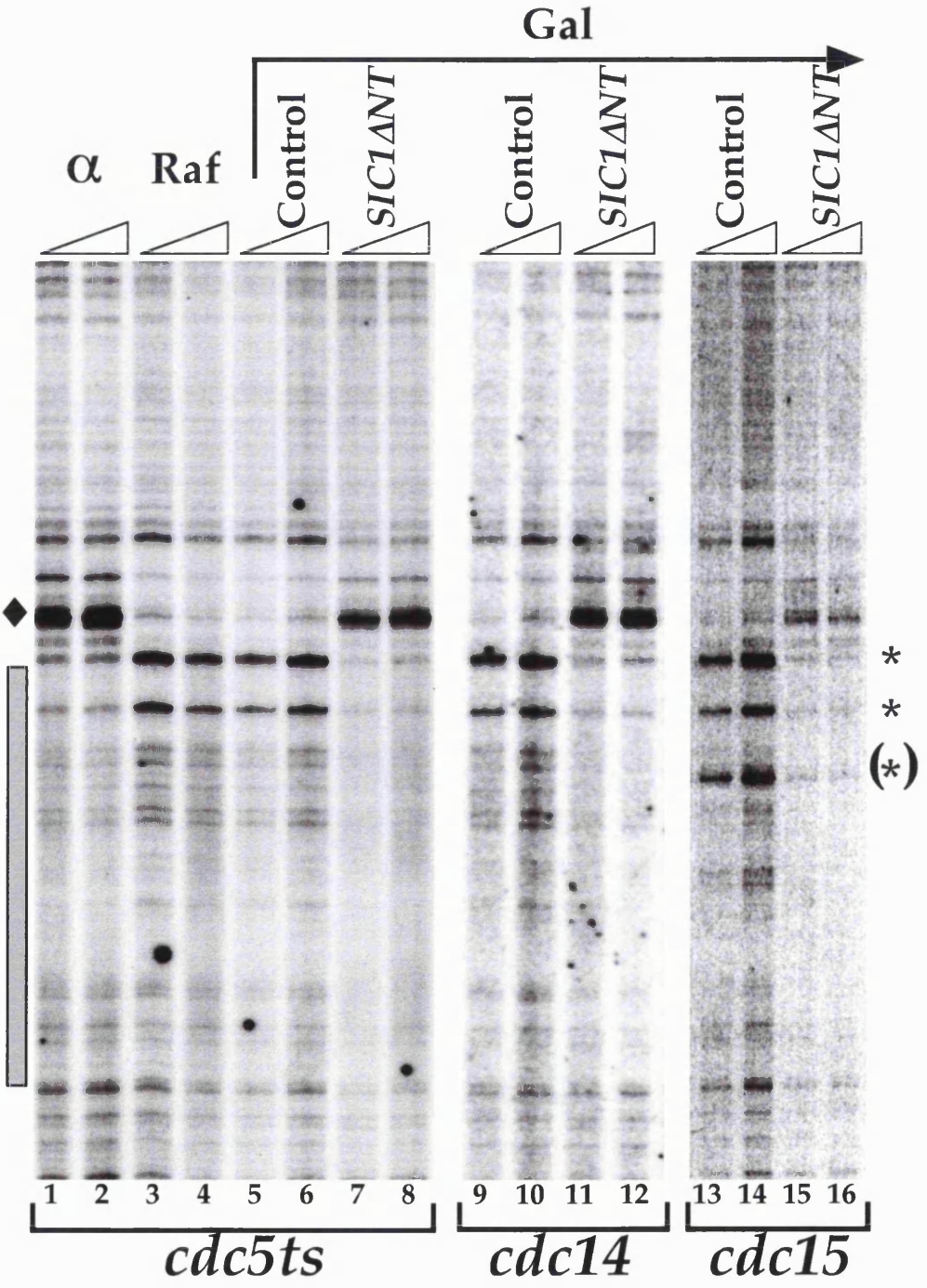
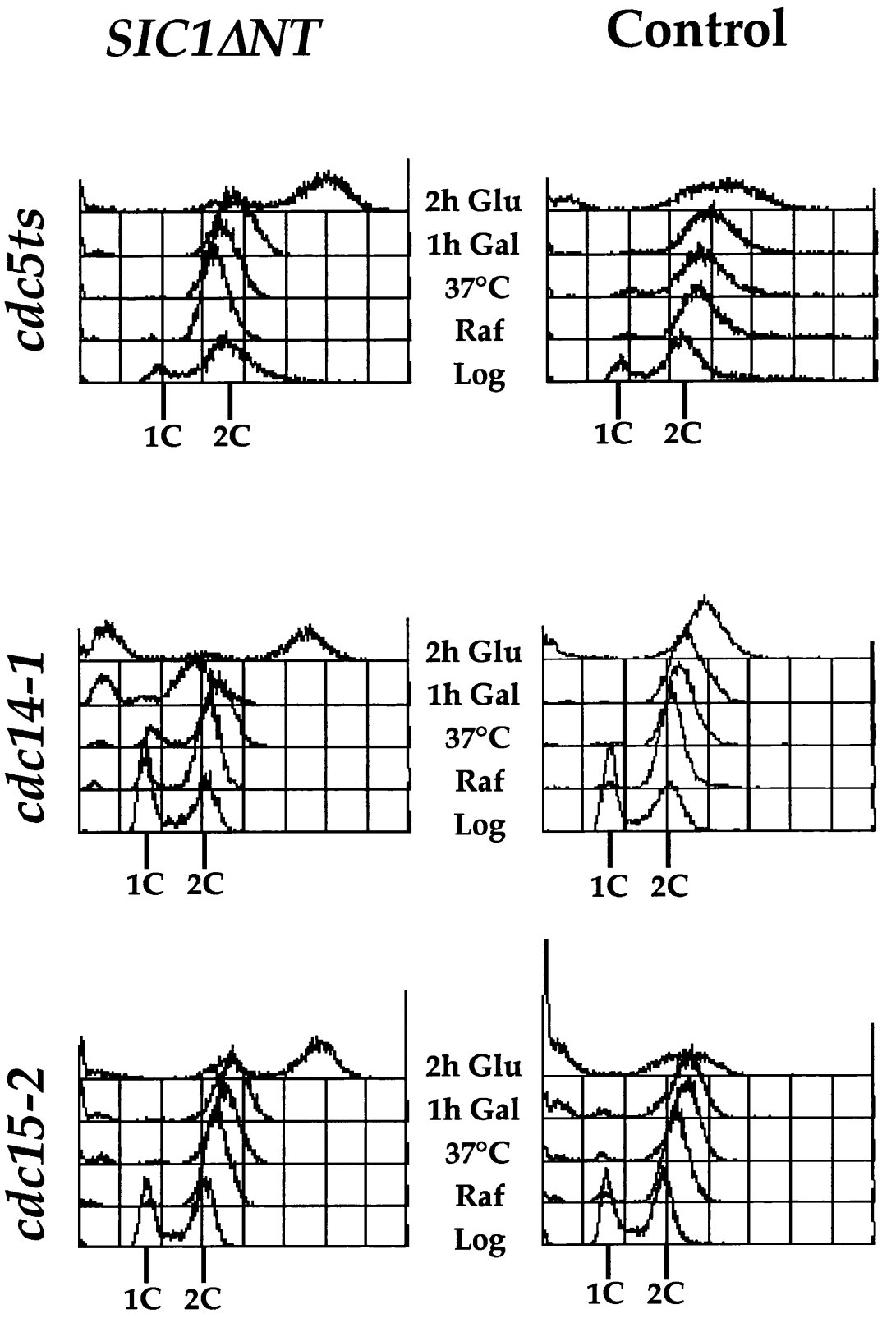

Gal
α
Raf
Control
SIC1ΔNT
Control
SIC1ΔNT
Control
SIC1ΔNT
◆
*
*
(*)
1 2 3 4 5 6 7 8
9 10 11 12
13 14 15 16
cdc5ts
cdc14
cdc15

Figure 17B. The mitotic exit network is not required for SIC1ΔNT induced re-replication

cdc14-1, *cdc5*ts (*msd2-1)* and *cdc15-2* strains with *GAL-SIC1ΔNT* (YLIL35, YLIL39 and YLIL7 respectively), and isogenic control strains without *GAL-SIC1ΔNT*, (YLIL36, KKY021 and K1993) (Control) were grown to log phase (Log) and arrested in nocodazole (Raf). The temperature sensitive mutations were then inactivated by incubation of the cells in pre-warmed raffinose medium for one hour (37°C). This medium was then replaced with pre-warmed galactose medium (Gal) containing nocodazole to induce *SIC1ΔNT* expression for one hour, before the medium was replaced again with pre-warmed glucose medium (Glu) at 37°C for 2 hours. Nocodazole was present in the medium throughout the experiment and the DNA content of the cells was analysed using FACS analysis.

Figure 17B


SIC1ΔNT
Control
cdc5ts
cdc14-1
cdc15-2
2h Glu
1h Gal
37°C
Raf
Log
1C
2C

The plasmid loss defect of cdc14-1 is rescued by over-expression of SIC1

The results presented above suggest that the major role of *CDC14*, *CDC5* and *CDC15* in DNA replication is to inhibit Cdc28/Clb kinase at the end of mitosis. However, it is possible that although Cdc14p might not be essential for replication, it may improve the efficiency of pre-RC formation. A sensitive method for identifying mutants which have a reduced efficiency of DNA replication is by measuring the rate of loss of a plasmid which contains a selectable marker over multiple generations. Characteristically, a yeast strain which has a defect in initiation of DNA replication, will have a plasmid loss defect which can be rescued by addition of multiple origins to the plasmid. (Indeed both *cdc14* and *cdc5* mutants have such a phenotype (Hardy and Pautz, 1996; Hogan and Koshland, 1992)). The data presented in figure 17 predict that the plasmid loss defect of *cdc14* and *cdc5* mutants is due to inefficient pre-RC formation caused by incomplete inactivation of Cdc28/Clb kinase activity at the end of mitosis. Is this is the case, constitutive expression of the Cdc28/Clb kinase inhibitor Sic1p should rescue the plasmid loss defect of *cdc14-1* mutants. Therefore we examined the rate of loss of a centromeric plasmid containing two ARS elements and a selectable marker (pDK368-1), (Hogan and Koshland, 1992) from both wild type and *cdc14-1* strains over five to ten generations in the absence of selection. A *cdc14-1* strain with the *GAL* promoter alone was found to have a plasmid loss rate of around 20% per generation at the semi- permissive temperature of 30°C, compared to a wild type strain which had a plasmid loss rate of around 0.5% per generation. The plasmid loss rate of *cdc14-1* mutants was suppressed to around 7% per generation in a strain containing a plasmid with 8 ARS elements (pDK368-7) and in a strain which *SIC1* is constitutively expressed from the *GAL* promoter (figure 18). This result confirms that the origin firing defect of *cdc14ts* strains is due to incomplete inactivation of Cdc28/Clb kinase at the end of mitosis.

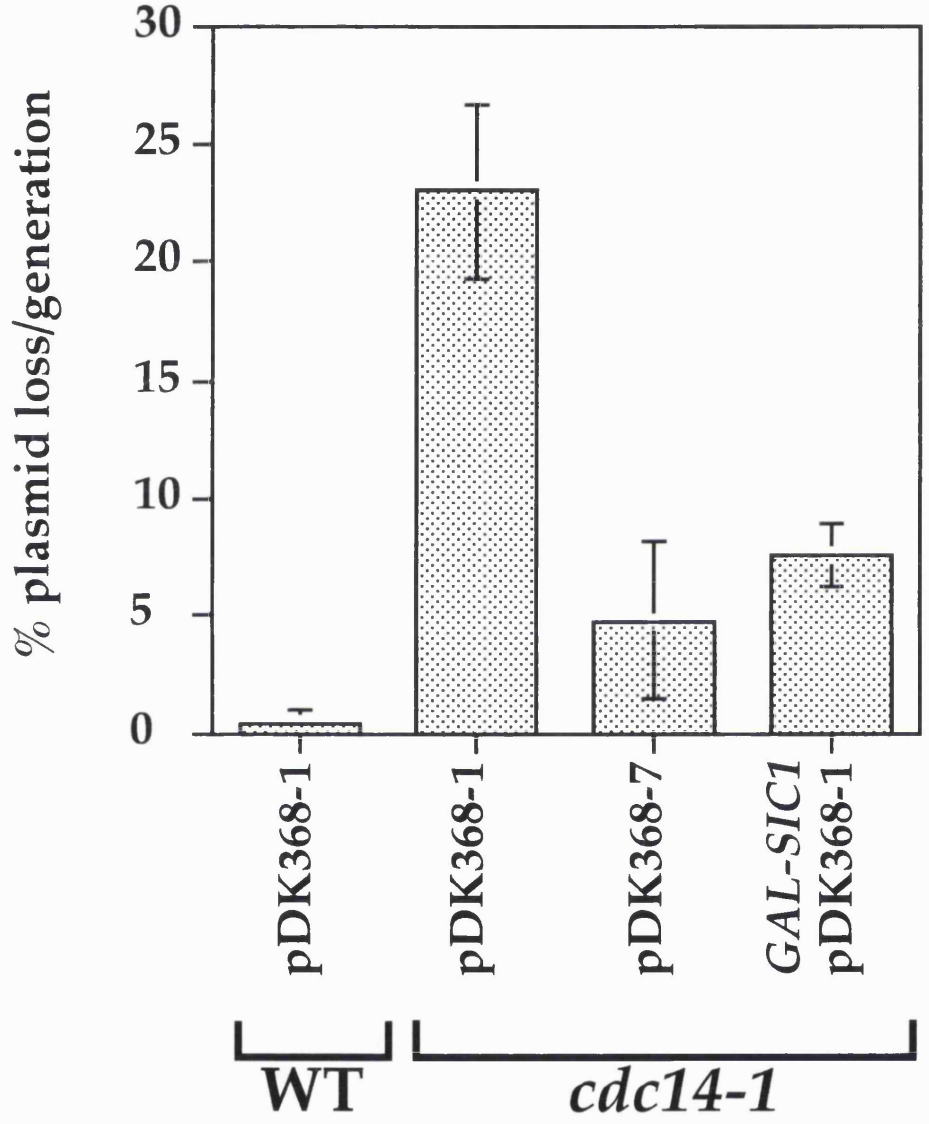
Conclusion

We have examined the mitotic requirements for re-setting origins of replication using *SIC1ΔNT* to induce re-replication in G2/M arrested cells. Our results demonstrate that inactivation of Cdc28/Clb kinase bypasses the

Figure 18. Over-expression of SIC1 rescues the plasmid loss defect of cdc14-1

A wild type strain containing the *GAL* promoter (YLIL37) plus pDK368-1 (*LEU2*, 2 ARS elements), a *cdc14-1* strain containing the *GAL* promoter (YLIL36) plus either pDK368-1 or pDK368-7 (*LEU2*, 8 ARS elements), and a *cdc14-1* strain with *GAL-SIC1* (YLIL53) plus pDK368-1 were grown to mid-log in selective medium containing galactose at 24°C. The cells were then diluted into non-selective medium containing galactose and grown at 30°C for between five and ten generations. To calculate the percentage of cells containing the plasmid, cells were plated onto selective medium with or without leucine before and after the experiment. The graph shows the means percentage plasmid loss per generation calculated from three parallel cultures. The percentage plasmid loss per generation (X) was calculated using the formula $X=1-e^r$, where $r=\ln(A/B)/N$. N is the number of generations, A is the % of cells containing the plasmid before the experiment and B is the % of cells containing the plasmid after the experiment [Dani, 1983 #5864]. % plasmid loss per generation is plotted in 6C. The Y error bars indicate standard deviation calculated from three parallel cultures.

Figure 18


% plasmid loss/generation
30
25
20
15
10
5
0
pDK368-1
pDK368-1
pDK368-7
GAL-SIC1
pDK368-1
WT
cdc14-1

requirement for the APC/C and the late mitotic genes, *CDC14*, *CDC5* and *CDC15* in DNA replication (Noton and Diffley, 2000). This strongly implies that the only role for the these genes in origin re-setting is to inactivate Cdc28/Clb kinase in mitosis, and that inhibition of Cdc28/Clb kinase at the end of mitosis is the major event required to re-set origins of DNA replication from one cell cycle to the next.

Chapter Six: Discussion

Several conclusions can be drawn from the work presented here. The first is that Cdc28/Clb kinase activity inhibits pre-RC formation during G2/M phase and during S phase. Secondly, the major role of the mitotic exit network and APC/C in DNA replication is to inactivate Cdc28/Clb kinase at the end of mitosis thereby driving pre-RC formation in the subsequent G1. Thirdly, cell cycle regulated protein synthesis is important both for pre-RC formation and origin firing. Lastly these results demonstrate that *in vivo* Orc6p is not required for ORC binding to chromatin, but is essential for pre-RC formation.

CDK activity inhibits pre-RC formation

The results presented here support a model where pre-RC formation is restricted to G1 when CDK activity is low and is inhibited during S and G2/M when CDK activity is high. Inactivation of Cdc28/Clb kinase activity in G2/M either by over-expression of a stable inhibitor, Sic1p, or by direct inactivation of a temperature sensitive allele of *cdc28* is sufficient to drive pre-RC formation. This result is consistent with previous studies and suggests that CDK activity the major factor preventing pre-RC formation in G2/M.

Regulation of DNA replication by protein synthesis

Cdc28/Clb kinase activity inhibits pre-RC formation in multiple different ways (see introduction). Reversal of CDK mediated phosphorylation of pre-RC components is not sufficient to induce pre-RC formation in G2/M arrested cells as protein synthesis is essential for both pre-RC formation and origin firing in these experiments. This suggests that cell cycle regulated gene expression is an important control over DNA replication. Although in *S. cerevisiae* many replication genes are transcribed in a cell cycle dependent manner, Cdc6p is the only known pre-RC component that is periodically expressed in the cell cycle. Cdc6p is present during G1, but disappears as cells enter S phase, only to re-appear in the next G1. *CDC6* is transcribed at the end of mitosis in a Swi5p dependent manner (Piatti et al., 1995). As discussed in the

introduction, Swi5p is activated in late mitosis when Cdc28/Clb is inactivated. Therefore it is possible that this transcription is activated when Cdc28/Clb activity is inhibited in G2/M. An additional wave of *CDC6* transcription is observed in cells released from a G1 arrest (McInerny et al., 1997; Piatti et al., 1995). Cdc6p is a highly unstable protein and Cdc6p stability is regulated by Cdc28p kinase activity. (Drury et al., 1997; Drury et al., 2000; Elsasser et al., 1999; Elsasser et al., 1996). Our finding that protein synthesis is required for pre-RC formation in G2/M arrested cells is consistent with the notion that Cdc6p must be newly synthesised in every cell cycle. However, over-expression of stable Cdc6p is not sufficient to rescue the requirement for protein synthesis for pre-RC formation in G2/M (data not shown). There could be many reasons for this, for example the Cdc6p may not be expressed to a high enough level, or there may be an unknown requirement for protein synthesis in pre-RC formation. Consistent with this over-expression of Cdc6p in G2/M arrested cells does not induce pre-RC formation (Drury et al., 1997), although a *cdc6* allele which induces limited re-replication has been isolated (Liang and Stillman, 1997).

Protein synthesis is essential for origin firing in G2/M arrested cells after origins have been re-set by over-expression of *SIC1ΔNT*. Many replication genes such as *CLB5*, *CLB6*, *DBF4*, genes encoding enzymes involved in nucleotide biosynthesis such as *RNR1* and *TMP1* and DNA polymerase subunits such as *POL1* and *DPB2* are transcribed in a cell cycle dependent manner reviewed (Johnston and Lowndes, 1992). In particular, Clb and Dbf4p levels are known to vary during the cell cycle and regulate the activity of Cdc28/Clb and Cdc7/Dbf4 (see introduction). *CLB5* and *CLB6* are transcribed in late G1 in a *CLN* dependent manner (Epstein and Cross, 1992; Schwob and Nasmyth, 1993) and it is possible that this mode of transcription is activated when Cdc28/Clb kinase is inhibited by *SIC1ΔNT* in G2/M arrested cells. Consistent with this, inhibition of Cdc28/Cln kinase activity by addition of alpha factor to the culture, inhibits *SIC1ΔNT* induced re-replication. Both Clbs and Dbf4p are degraded in an APC/C dependent manner during late mitosis/early G1. However, in an APC/C mutant, where Clbs and Dbf4p should be stabilised, protein synthesis is still essential for *SIC1ΔNT* induced re-replication suggesting that synthesis of other replication proteins may be required for origin firing. Taken together these

results suggest that regulated protein synthesis has an important role in control of DNA replication during the cell cycle.

Control of protein expression is very important for control of DNA replication in other organisms, for example, in *E. coli*, levels of dnaA link DNA replication to growth rates. In *S. pombe*, re-replication induced by over-expression of Rum1 is dependent on the transcription factor Cdc10 (Moreno and Nurse, 1994). Cdc10 activated transcription occurs at the end of mitosis and regulated genes include *cdc18+* and *cdt1+* (Hofmann and Beach, 1994; Kelly et al., 1993) and *cdc18+* has been identified as a suppresser of *cdc10* mutants (Kelly et al., 1993). Control of *cdc18+* transcription is vitally important for control of DNA replication in the cell cycle, since over-expression of *cdc18+* (Muzi-Falconi et al., 1996; Nishitani and Nurse, 1995) is sufficient to drive multiple rounds of DNA replication and over-expression of *cdt1+* enhances this phenotype (Nishitani et al., 2000). This suggests that control of *cdt1+* and *cdc18+* transcription is a major point of replication control in *S. pombe*. Both Cdc18 and Cdt1 levels fluctuate during the cell cycle (Muzi-Falconi et al., 1996; Nishitani et al., 2000), Cdc18 being targeted for ubiquitin mediated proteolysis by two Cdc4p homologues, Pop1 and Sud1 in a Cdc2 dependent manner. Therefore in *S. pombe* and *S. cerevisiae* control of Cdc18 and Cdc6p levels by regulated transcription and proteolysis is very similar.

In *D. melanogaster ORC1* transcription is regulated by E2f. Constitutive expression of *ORC1* leads to ectopic DNA synthesis in imaginal discs (Asano and Wharton, 1999). In mammalian cells the transcription of *ORC1 CDC6* and *MCM2-7* can be induced in serum starved cells by expression of E2f1, E2f2 or E2f3, (Hateboer et al., 1998; Humbert et al., 2000; Leone et al., 1998; Ohtani et al., 1998; Yan et al., 1998) although only transcription of *CDC6* , *MCM2* and *MCM6* is actually cell cycle regulated in proliferating cells. Mcm2-7 and Cdc6 levels are constant during the cell cycle. Therefore the importance of regulated protein synthesis in control of DNA replication in higher eukaryotes remains to be demonstrated.

Phosphatases

Pre-RC formation is clearly inhibited by CDKs in many ways and in several cases this is directly caused by CDK dependent phosphorylation of pre-RC components. In principle this phosphorylation can be overcome in two ways, either by synthesis of new unphosphorylated protein, or by dephosphorylation of the protein by a phosphatase. Orc6p and the p86 subunit of polymerase α are phosphorylated in a Cdc28/Clb dependent manner during the cell cycle (Foiani et al., 1995). To date no functional interactions between pre-RC components and phosphatases have been described, although in mammalian cells a putative regulatory subunit of PP2A interacts with Cdc6p but the significance this has not been demonstrated (Yan et al., 2000). In *X. laevis* the phosphatase PP2A is essential for initiation of DNA replication downstream of Mcm2-7 loading (Lin et al., 1998). The activity of phosphatases may represent an important level of control over pre-RC formation.

The role of Orc6p in pre-RC formation

The work presented here has demonstrated that a potential target of Cdc2/Clb kinase, Orc6p, has an essential role in pre-RC formation. Consistent with observations made *in vitro* (Lee and Bell, 1997), we have found that Orc6p is not required for loading of other ORC subunits onto chromatin *in vivo*. Orc6p is essential for Mcm2-7p loading onto chromatin *in vivo* suggesting that Orc6p may mediate interactions between ORC and other components of the pre-RC.

It is not known how ORC and Cdc6p function in Mcm2-7p loading, although Orc1p, Orc4p, Orc5p and Cdc6p have homology with a family of 'clamp loaders' of the AAA+ family of ATPases (Neuwald et al., 1999; Perkins and Diffley, 1998; Tugal et al., 1998). The homology extends over 8 conserved boxes including ATP binding and hydrolysis motifs which are known to be important for ORC and Cdc6p function. The 'clamp loader' proteins include the 5 RFC subunits which load PCNA onto DNA at primed sites and the γ and δ' proteins of the *E. Coli* γ complex which loads the β clamp onto DNA. The biochemical activity of the *E. coli* clamp loader is well understood and may help to explain how ORC and Cdc6p function in *vivo*.

The minimal *E. coli* γ complex for loading of the β clamp are two to four γ subunits and one each of δ′ and δ. One model for γ complex activity is that binding of ATP by the γ subunits allows the δ subunit to interact with the β clamp causes the β clamp to 'open'. The δ′ subunit which has non-functional ATP binding and hydrolysis motifs may modulate this conformational change in the γ complex. The γ complex and β clamp then recognise primed DNA and ATP hydrolysis induces a conformational change in the complex, such that the β clamp is released from the δ subunit and closes around the primed DNA, leaving the γ complex free to load more β clamps at other primed sites (Turner et al., 1999).

This model for γ complex function may have relevance to ORC and Cdc6p function. It has been proposed that ORC and Cdc6p act together as an ATPase to load multiple Mcm2-7p complexes at origins (see introduction). One interpretation of this reaction is as follows; association of ATP-bound Cdc6p with ORC allows ORC/Cdc6p to bind a single Mcm2-7p complex. Hydrolysis of ATP by Orc1p/Cdc6p may then induce loading of the Mcm2-7p complex onto DNA around the origin. The Mcm2-7p complex may then dissociate from ORC/Cdc6p while remaining associated with the origin. The ORC/Cdc6p complex would then be free to load another Mcm2-7p complex. This model is consistent the requirements for ATP binding and hydrolysis motifs in ORC and Cdc6p described in the introduction.

How does this relate to the role of Orc6p in pre-RC formation? Orc6p does not have ATP binding or hydrolysis motifs and does not function in sequence specific recognition of origin DNA, however it is essential for loading of Mcm2-7p onto DNA. Orc6p may function in an analogous way to the δ subunit of the γ complex. For example, upon association of ATP-bound Cdc6p with ORC, a conformational change in Orc6p may enable it to interact Mcm2-7p. Hydrolysis of ATP by Cdc6p may then may then induce loading of Mcm2-7p onto chromatin. Alternatively, Orc6p may play no direct role in the loading of Mcm2-7p by ORC/Cdc6p, and may instead be required for formation of an ORC/Cdc6p complex by functioning as an adapter between other ORC subunits and Cdc6p. There is no direct evidence to support the models for pre-RC

formation discussed above, for example no biochemical interactions have been described between Orc6p and Cdc6p or Mcm2-7p, and they are based entirely upon analogy to the activity of clamp loaders. One way to address this question would be use the chromatin immuno-precipitation assay, or an *in vitro* assay for pre-RC formation to address whether Cdc6p binding is disrupted in an *orc6-td* mutant.

S. cerevisiae Orc6p shares very little homology with the smallest ORC subunit from either *S. pombe* or *D. melanogaster* and this may reflect a different function of Orc6p in these organisms (Chesnokov et al., 1999; Moon et al., 1999). Both *S. pombe and D. melanogaster* have homologues of Cdt1, which is required for Mcm2-7 loading but not ORC loading onto chromatin (Nishitani et al., 2000). Cdt1 does not have an *S. cerevisiae* homologue and it may be the case that *S. cerevisiae* Orc6p subserves the same function as Cdt1 in *S. pombe*. The expression of *S. pombe* Cdt1 is cell cycle regulated and this may be an important control over pre-RC formation. Orc6p is not known to be cell cycle regulated but it is apparently phosphorylated in a Cdc28/Clb dependent manner during the cell cycle and this may modify its function.

Pre-RC stability during the cell cycle

Several experiments have suggested that loading of Mcm2-7p complexes onto origins of DNA replication completes the function of ORC and Cdc6p in pre-RC formation (see introduction). However, inactivation of *cdc6-1* during a prolonged G1 arrest causes the pre-replicative footprint at *ARS1* to disappear and cells lose the ability to replicate their DNA (Cocker et al., 1996; Detweiler and Li, 1997). Like Cdc6p, Orc6p is required for pre-RC formation, but not binding of ORC to origins and inactivation of *orc6-td* after pre-RC formation in G1, results in a replication defect. One possibility is that Orc6p and Cdc6p are required to maintain Mcm2-7p loading during G1. Alternatively, Mcm2-7p loading may be sufficiently stable to be maintained independently of ORC and Cdc6p activity during a normal G1, but if G1 is artificially prolonged the pre-RC may be gradually lost and unable to reform in the absence of either Orc6p or Cdc6p, resulting in the S phase defect observed in *cdc6-1* and *orc6-td* mutants. Another interpretation of these results is that Orc6p is required for origin firing.

One piece of data to support this is that ORC is required for Dbf4p association with chromatin and in 1-hybrid analysis Dbf4p binding requires a consensus *ARS* sequence (Dowell et al., 1994; Pasero et al., 1999). This could be addressed by determining whether Orc6p has a role in Dbf4p binding to chromatin.

During an S phase arrest, pre-RCs are maintained at late origins despite inhibition of pre-RC formation by Cdc28/Clb (Santocanale and Diffley, 1998). Interestingly, degradation of Orc6-tdp in S phase arrested cells did not result in a replication defect, upon release from the arrest. This suggests that late origins fire and that pre-RCs are maintained at late origins in the absence of Orc6p activity during an S phase arrest (however late origin firing should be confirmed in a direct way, for example by 2-D gel analysis). This observation is consistent with the observation that pre-RCs are maintained at late origins until origin firing, while the majority of Cdc6p is rapidly degraded as cells enter S phase. Furthermore it provides a explanation why during an S phase arrest pre-RCs can be maintained at late origins while pre-RC formation is prevented at early origins.

Control of origin firing during S phase

Transient inactivation of Cdc28/Clb kinase by over-expression of *SIC1ΔNT* in S phase arrested cells induces pre-RC formation at early origins. However upon release from the S phase arrest and repression of *SIC1ΔNT* expression DNA is only partially re-replicated. This observation can be explained in two ways. Either only some of the 16 chromosomes of *S. cerevisiae* have early origins which have fired in an early S phase arrest and can form pre-RCs upon inactivation of Cdc28/Clb, or firing from the same origin twice during a single S phase disrupts progression of the replication forks. For example, two replication forks heading in the same direction may produce a complex termination structure, or the 2 forks may 'catch' each other at replication pause sites and produce a DNA structure which cannot be resolved. The drop in viability associated with Cdc28/Clb inactivation in S phase arrested cells may be explained by the inability of these cells to complete re-replication of the genome. However, inactivation of temperature sensitive alleles of either *cdc6*, of *cdc7* before Cdc28/Clb kinase inactivation in S phase arrested cells does not rescue

the drop in viability, (Kristine Bousset and John F. X. Diffley, unpublished data). This suggests that maintenance of Cdc23/Clb kinase activity in S phase arrested cells is not only important for inhibition of pre-RC formation, but has additional roles.

The role of the mitotic exit network and APC/C in control of DNA replication

Although inactivation of Cdc28/Clb kinase in S phase or G2/M arrested cells drives pre-RC formation, this does not necessarily mean that upon inactivation of Cdc28/Clb, all aspects of mitosis are bypassed in pre-RC formation. It is plausible that upon inactivation of Cdc28/Clb kinase the mitotic exit network and APC/C become activated. For example it is known that Cdc28p dependent phosphorylation of Cdh1p inactivates the APC/C, and inactivation of Cdc28/Clb kinase may well activate APC/C-Cdh1p in G2/M arrested cells. Accordingly the role of the mitotic exit network and APC/C in DNA replication was addressed using temperature sensitive mutants of *cdc15, cdc5, cdc14* and the APC/C . It was found that none of these alleles disrupted *SIC1ΔNT* induced re-replication at the non-permissive temperature. One of the concerns raised in this experiment was that the APC/C or mitotic exit network were not completely inactivated by the temperature sensitive mutations. However, appropriate controls were carried out to ensure that the temperature sensitive alleles used in these experiments were inactivated. In total three temperature sensitive alleles of mitotic exit network genes and three temperature sensitive alleles of APC/C genes were used and none of them were found to have a defect in *SIC1ΔNT* induced re-replication. The activities of the APC/C and mitotic exit network are interdependent, for example Pds1p degradation is essential for activation of the mitotic exit network, while Cdc14p dependent dephosphorylation of Cdh1p activates the APC/C-Cdh1p. Therefore the observations that none of the components of the APC/C or mitotic exit network examined here are required for *SIC1ΔNT* induced DNA replication are consistent.

As described in the introduction, Cdc5p has both genetic and physical interactions with Dbf4p. I have found that Cdc5p is not required for *SIC1ΔNT* induced re-replication, whereas Cdc7p, the catalytic partner of Dbf4p

responsible for S phase activation, is required for re-replication. This suggests that the putative interaction between Cdc5p and Dbf4p is independent of the role of Cdc7/Dbf4 in DNA replication and predicts that any functional interaction between Dbf4p and Cdc5p may important for Cdc5p function during mitosis. However, reciprocal shift experiments suggest that Dbf4p is not required either after an S phase arrest (HU) or after a G2/M arrest (nocodazole), (Miguel Godhino Ferreira unpublished data), although Dbf4p is bound to chromatin in a *cdc15-1* arrest (Pasero et al., 1999). Published data suggest that the expression of Dbf4p and Cdc5p overlaps only for a short period of time in late mitosis, if at all (compare Shirayama et al., 1998 with Cheng et al., 1999). In conclusion, this study cannot explain the interactions described between Cdc5p and Dbf4p.

The results presented here strongly suggest that degradation of an APC/C substrate is not essential for re-setting origins of DNA replication in *S. cerevisiae.* However this might not be the case in other eukaryotes as in *X. laevis* and HeLa cells an inhibitor of DNA replication, geminin, has been identified as a substrate of the APC/C (McGarry and Kirschner, 1998). Our model does not rule out the existence of a geminin-like factor in budding yeast, for example geminin may inhibit pre-RC formation by activating Cdc28/Clb kinase rather than by acting directly on pre-RC components. Alternatively, there may be no geminin-like inhibitor of pre-RC formation in budding yeast, consistent with the absence of a geminin homologue in the yeast genome.

APC/C dependent degradation of securins such as Pds1p from *S. cerevisiae* is essential for separation of sister chromatids in anaphase throughout eukaryotes (see introduction). The APC/C-Cdc20p does not appear to be required for *SIC1ΔNT* induced replication suggesting that sister chromatid separation is not essential for pre-RC formation and DNA replication. We have not examined whether sister chromatid cohesion is actually maintained throughout *SIC1ΔNT* induced pre-RC formation and re-replication and it is possible that inactivation of Cdc28/Clb or progression of the replication fork disrupts pre-existing cohesion between sister chromatids.

My results suggest a model where the APC/C, Cdc14p, Cdc5p and Cdc15p bring about origin re-setting by inhibition of Cdc28/Clb kinase activity.

This suggests that the replication defect of *cdc14* and *cdc5* mutants is due to incomplete inactivation of Cdc28/Clb activity during G1. Consistent with this conclusion, in *S. cerevisiae*, *cdc14*, *cdc15* and APC/C mutants arrest with high Cdc28/Clb kinase activity (Fitzpatrick et al., 1998; Surana et al., 1993). Yeast strains deleted for *SIC1* have a plasmid loss defect which can be suppressed by increasing the number of replication origins on the plasmid, similar to the phenotype of *cdc14* and *cdc5* mutants. *cdc5* and *cdc15* mutants are rescued by *SIC1* expression from a multicopy plasmid and in a similar way *SIC1* over-expression in *cdc14* mutants causes cells to bypass cytokinesis, and undergo re-replication in the following cell cycle (Charles et al., 1998; Donovan et al., 1994; Fitzpatrick et al., 1998). Our finding that over-expression of *SIC1* rescues the plasmid loss defect of *cdc14* mutants strongly supports the notion that incomplete inactivation of Cdc28/Clb kinase in G1 results in the replication defects of mitotic exit network mutants. Therefore the replication defect of *cdc5* and *cdc14* mutants can be explained as a defect in pre-RC formation caused by incomplete inactivation of Cdc28/Clb kinase during G1.

Recent data suggests that Cdc28/Cln kinase activity can inhibit pre-RC formation in late G1 through destabilisation of Cdc6p and inhibition of Mcm2-7p nuclear accumulation (Drury et al., 2000; Labib et al., 2000). In addition over-expression of *CLN2* in G2/M arrested cells inhibits *SIC1* induced pre-RC formation (Seiji Tanaka and John F. X. Diffley, unpublished data) (although it is not clear whether Cdc28/Cln3 as well as Cdc28/Cln1,2 inhibits pre-RC formation). This implies that there must be a time during early G1 when very little CDK activity if any is present. Down-regulation of pre-RC formation precedes origin firing in this model, and this may be important to ensure that pre-RC formation and origin firing do not overlap.

In a normal cell cycle several apparently redundant mechanisms ensure that Cdc28/Clb activity is low in late mitosis/early G1. In particular, Clbs are degraded by the APC/C, and Sic1p is stable. The results presented here suggest that APC/C dependent Clb degradation is not required for pre-RC formation in the presence of Sic1p. Consistent with this, yeast strains deleted for *SIC1* are viable and strains deleted for *CDH1*, can exit mitosis and initiate DNA replication, dependent on *SIC1* (Nugroho and Mendenhall, 1994; Schwab et al.,

1997). The existence of several redundant mechanisms for inactivation of Cdc28/Clb kinase can explain results which have suggested that mutants of APC/C subunits including *cdc16* mutants undergo re-replication in the presence of apparently high Cdc28/Clb kinase activity (Heichman and Roberts, 1996; Heichman and Roberts, 1998). Periodic inhibition of Cdc28/Clb kinase activity by Sic1p or another inhibitor of Cdc28/Clb kinase in *cdc16* mutant cells could induce origin re-setting and subsequent initiation of DNA replication over a substantial period of time.

The redundancy of Cdc28/Clb inhibition during G1 may act as a safeguard to ensure that cells are able to form pre-RC before Cdc28p becomes activated at the end of G1. Yeast with mutations in *cdc6* or *sic1* exhibit increased rates of genomic instability, (Bruschi et al., 1995; Nugroho and Mendenhall, 1994), suggesting that pre-RC formation is very important for maintaining genomic integrity from one cell cycle to the next.

Conclusion

In conclusion my results have demonstrated the essential role of Orc6p in pre-RC formation and have provided data to support a model for control of DNA replication in the cell cycle, where during early G1, low Cdc28/Clb and low Cdc28/Cln activity drives pre-RC formation. Activation of Cdc28/Cln in late G1 then discourages further pre-RC formation, and activation of Cdc28/Clb and Cdc7/Dbf4 induce origin firing. Pre-RCs are stable during S phase until they fire, upon which they are disassembled and prevented from re-forming by Cdc28/Clb. Cdc28/Clb kinase activity then remains high through G2 and M phase until the activity of the APC/C and mitotic exit network bring about Cdc28/Clb inactivation, allowing exit from mitosis and formation of pre-RCs in the following G1.

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