Corrections

page 119, legend to figure 4.4, last line read: " whereas the overexpression of of the *tsl1*$^+$ cDNA severely inhibited growth in *cdc21* cells at 25°C and abolished it at 32 and 36°C".

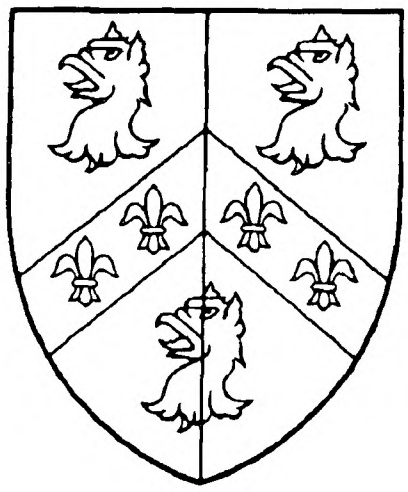
page 175, figure 7.2 (b); change scale bar from 1kb to 1.3 kb

page 176, scale bar= 14μm;

Role of *cdc21+* and related genes in eukaryotic chromosome replication

A thesis submitted for the degree of
Doctor of Philosophy
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Trinity College



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"Considerate la vostra semenza: Fatti non fuste a vivere come bruti ma per seguire virtute e conoscenza"

Abstract

The *Schizosaccharomyces pombe cdc21*$^+$ gene product is related to the Mcm2-3-5 family of replication proteins.

By phylogeny analysis of their protein sequences and screening for *cdc21*$^+$-related sequences using molecular probes I have suggested that at least six types of *cdc21*$^+$-related genes may be present in the yeast genome.

The isolation of interaction suppressors of the *cdc21*^{ts} mutant was attempted by overexpression of an *S. pombe* cDNA library. Two cDNAs were isolated, *tsl1*$^+$ and *dom1*$^+$, whose overexpression specifically affected the viability of *cdc21*^{ts} cells under certain conditions. The predicted dom1 protein is 60% identical to the budding yeast HMG-like Nhp2 protein.

I have studied the phenotype of *S. pombe* cells overexpressing the *cdc21*$^+$ gene and amino-terminal truncations of it. Overexpression of the *cdc21*$^+$ gene caused cell elongation but cells were not significantly affected in growth rate. Cells overexpressing the carboxyl-terminal part of *cdc21*$^+$ arrested in S phase and also entered mitosis in the absence of nuclear division.

The possibility that chromosomes in *cdc21*^{ts} arrested cells may be damaged was investigated by pulsed field gel electrophoresis. No differences could be found compared to wild-type chromosomes. I have also studied the arrest phenotype of *cdc21 rad1* and *cdc21 cdc2.3w* double mutants. Both strains entered mitosis at the restrictive temperature indicating that *cdc21*^{ts} cells arrest in S phase and may contain DNA damage.

I have generated two new mutant alleles of *cdc21*$^+$. The first allele was made by deleting most of the *cdc21*$^+$ open reading frame (ORF). The second allele was constructed by placing the *cdc21* $^+$ ORF under control a regulatable promoter. The resulting construct was used to complement the *cdc21* deletion. Both mutants were inviable under appropriate conditions arresting in S phase as elongated cells, although a proportion of them (15-20%) entered mitosis in the absence of nuclear division.

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Strangely enough, I do not find it difficult to look back over my years in Oxford, meaning that indeed I had a very pleasant time over here. Here is a *resumé* of this adventure, unfortunately limited for obvious reasons. Hence I do apologise in advance to all those people (far too many!) that I had the pleasure to meet and have not cited in this page.

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Abbreviations

ATP	adenosine-5'-triphosphate,
APS	ammonium persulphate
B. subtilis	*Bacillus subtilis*
BSA	bovine serum albumin
bp	base pair(s)
Ci	curie
cps	counts per second
dH_2O	distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	dATP, dCTP, dGTP, dTTP
D. melanogaster	*Drosophila melanogaster*
DAPI	4,6-Diamidino-2-phenylindole
DNase	deoxyribonuclease
DMSO	dimethyl sulfoxide
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
H. sapiens	*Homo sapiens*
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2ethanesulfonic acid])
kb	kilobase
kDa	kilodalton
krpm	thousand revolutions per minute
LB	Luria broth
M	molarity
MeOH	methanol
MOPS	(3-[N-morpholino]-2-hydroxypropanesulfonic acid)
mA	milli Ampére
mRNA	messenger RNA
MW	molecular weight

nm	nanometers
OD	optical density
PEG	polyethyleneglycol
PIPES	(piperazine-N,N'-bis[2-ethanesulfonic acid]; 1,4-piperazinediethanesulfonic acid)
PFGE	pulsed field gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate buffer saline
RNA	ribonucleic acid
rNTPs	ribonucleotides tri-phosphate
rpm	revolutions per minute
S. pombe	*Schizosaccharomyces pombe*
S. cerevisiae	*Saccharomyces cerevisiae*
ssDNA	single stranded DNA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethylaminomethane)
X. laevis	*Xenopus laevis*
w/v	weight by volume

Chapter One

Introduction

Preface

DNA replication is an essential and irreversible cellular event which ensures the faithful perpetuation of genetic information. An elegant and simple molecular model of how a DNA molecule may replicate was suggested immediately after the elucidation of its three-dimensional structure (1953) and was experimentally proved 5 years later. Ironically, things started to become more complicated when people began to ask what makes a DNA molecule replicate and how cells regulate the onset of this process. To date, more than 40 years later, we have a detailed knowledge of how DNA replicates in the bacterium *Escherichia coli*, but the regulation of this process is still poorly understood. Significantly less is known about the DNA replication and its regulation in eukaryotic systems. The latter field lacks an *in vitro* system able to support a round of semi-conservative replication at a chromosomal origin of DNA replication using highly purified proteins, as has been developed for *E. coli*. The development of an eukaryotic *in vitro* system based on the Simian Virus 40 (SV40) origin of replication and initiator protein (large T antigen) has represented an outstanding contribution to this field of research. This system has allowed the assay of factors with a suspected function in DNA replication and the elucidation of the biochemical mechanisms of the initiation of viral DNA replication.

In the following introduction I shall attempt to review our current knowledge of DNA replication in eukaryotic systems, and how DNA replication is co-ordinated with the cell cycle.

Initiation of DNA replication *in vitro*

DNA replication can be basically divided into two consecutive and separate steps: initiation and elongation. The first is a rate-limiting step required to set up optimal conditions required for the subsequent step which ensures bulk DNA synthesis.

The early events of DNA replication in bacteria and eukaryotic cells, or "pre-initiation mechanisms" I will discuss, have been elucidated studying the process of amplification of the DNA of prokaryotic and eukaryotic viruses. This is mainly because the replication of some viral genomes has proven amenable to analysis in cell-free systems, which makes feasible to study replication mechanisms at the molecular level. Basic early events in the initiation step of DNA replication seem to have been conserved from prokaryotic to eukaryotic cells (Stillman, 1994a, provides a complete review). Therefore, I will summarise the early events of DNA replication in a generalised model mentioning distinctions and differences between prokaryotes and eukaryotes when necessary.

The initiation events at the origin of replication of the *E. coli* chromosome (*oriC*) have been elucidated by reconstitution of an *in vitro* replication system with highly purified proteins (Kaguni and Kornberg, 1984; Bramhill and Kornberg, 1988a). It was predicted that the basic mechanism of initiation of DNA replication in all *Enterobacteriacae* is comparable to that of *E. coli* (Bramhill and Kornberg, 1988b). Recently also a *B. subtilis in vitro* system has been set up (Moryia *et al.*, 1994).

The eukaryotic counterpart of the *E. coli in vitro* replication system is based on animal virus replication systems (see Challberg and Kelly, 1989 for review) the more valuable perhaps being the Simian Virus 40 (SV40) cell-free system (Li and Kelly, 1984) which, except for one viral protein (large T antigen), this system entirely relies on the replication machinery of the host cell. Moreover, the DNA of SV40 is assembled in a chromatin structure indistinguishable from that of the host cell and bi-directional semi-conservative replication of the viral genome takes place in the nucleus.

Common early events

Normally, the initiation of DNA replication is not a random process but it depends on chromosomal sites which have been termed 'replicators' (Stillman, 1994b). This would be distinct from the site where DNA synthesis begins, which should be called the *ori* (origin). This distinction seems to be rather important as there are many examples in prokaryotes and eukaryotes, where regions required for initiation (as genetically mapped) do not overlap with the initiation site (for review see Stillman, 1994b). For instance, DNA replication in early embryonic cell cycles does not apparently need a replicator (Harland and Laskey, 1980). However, I will not adopt

this terminology (in general) and I will refer to the 'origin' in its broad sense to indicate regions of DNA involved in initiation events.

In both *E. coli* and SV40 the origin is a short and very well defined genetic element which binds a *trans*-acting factor (the initiator protein) which is absolutely required for the initiation event (Stillman, 1993). Initiator proteins include the DnaA protein of *E. coli* (Fuller *et al.*, 1984) and the large T antigen of SV40 (DeLucia *et al.*, 1983; Deb *et al.*, 1986). The binding of the initiator protein to the origin causes some structural changes in the adjacent DNA so that it locally unwinds (Bramhill and Kornberg, 1988a; Borowiec *et al.*, 1990). This conformational change mediated by the binding of initiator proteins at the origin is a key event in the activation of initiation of DNA replication and therefore this process is likely to be highly regulated. At *oriC* , the local unwinding occurs in an ATP-dependent fashion; after binding of DnaA, local unwinding is coupled to a slow hydrolysis of ATP (Bramhill and Kornberg, 1988a). In the SV40 system ATP is required for the binding of T antigen to the origin (Mastrangelo *et al.* 1989), whereas an optimum phosphorylation of T antigen seems to be important in the unwinding reaction. Hyperphosphorylated T antigen is inactive in this process (see Fanning, 1994, for review). Thus the unwinding activity of DnaA at *oriC* is ATP-dependent and that of large T antigen most probably dependent on the activity of a protein kinase, suggesting that the initiator protein *per se* is not sufficient to drive the initiation reaction.

Extensive unwinding of the DNA duplex is provided by the DnaB helicase at *oriC* (Baker *et al.*, 1987) and T antigen itself at the SV40 origin (Stahl *et al.*, 1986). Physically separated DNA strands are kept apart by the subsequent binding of proteins with high specificity for single stranded DNA (ssDNA); these are the Single Strand Binding proteins (SSB) in prokaryotes and Replication Factor (or Replication Protein) A in eukaryotes (RF-A or RP-A or HSSB). Although RPA is absolutely required for replication, prokaryotic SSB can substitute for RPA in the unwinding reaction *in vitro* (Wold and Kelly, 1988). Unlike prokaryotic SSB, RP-A is made of three distinct subunits of 70, 34 and 11 (14) kDa. The ssDNA binding activity of RP-A is confined to the larger subunit whereas the role of the two other subunits is still unknown. This suggests that RP-A may have some other role in DNA replication.

SSB or RP-A co-operate in conjunction with the initiator protein to direct the loading of other replication proteins required for the pre-priming step at the origin. A network of protein-protein interactions ensures that the replicative DNA polymerase(s) are correctly loaded on the DNA at the origin.

Unlike eukaryotes, in *E. coli* DNA primase is not physically associated with DNA polymerase and it has to be assembled in a subsequent step through interaction with another multiprotein complex, including the polymerase accessory proteins (e.g., β, γ, δ, δ' and τ) (Hurwitz *et al.*, 1990). In eukaryotes, DNA polymerase α-primase

interacts with large T antigen and RP-A (Dornreiter *et al.*, 1992). Loading of the polymerase complex is necessary in order to initiate the synthesis of short RNA primers, catalysed by the activity of the DNA primase in the presence of rNTPs. This is because the DNA polymerase α enzyme on its own cannot synthesise DNA chains *de novo*, but it needs a 3'-OH end to start synthesis moving in a 5' to 3' direction in the presence of dNTPs.

This step in *E. coli* is fulfilled by the loading of the processive DNA polymerase III holoenzyme (Baker and Kornberg, 1992). In the SV40 system DNA polymerase α is not very processive as it is only able to synthesize short fragments of DNA 100-200 bp in length (Okazaki fragments). The loading of another DNA polymerase, (δ), on the newly synthesised DNA duplex ensures processivity. This step is catalysed by the presence of DNA polymerase δ accessory proteins, such as RF-C and PCNA (Tsurimoto, *et al.*, 1990; Waga and Stillman, 1994).

After these early events, replication proceeds continuously on one DNA strand (leading strand) and discontinuously on the complementary (lagging) strand. DNA polymerase δ seems to play an important role in extending chains on both strands; thus it appears that the only role of the DNA polymerase α-primase complex is in starting short chains, or in the multi pre-priming step on the lagging strand (Waga and Stillman, 1994). In prokaryotes all the synthesis reactions are performed by the DNA polymerase III holoenzyme (Baker and Kornberg, 1992). RNA primers are then removed by the action of RNaseH and perhaps by the exonuclease activity of PolI (RNase H and MF1 5'-3' exonuclease in the SV40 system; Waga and Stillman, 1994) the gaps between elongated chains filled-in by the processive polymerases (pol III holoenzyme or DNA polymerase δ) and Okazaki fragments are joined together by the action of a DNA ligase (DNA ligase I in the SV40 system). Duplicated DNA molecules are decatenated by the action of the DNA gyrase in *E. coli* or by a type I or II topoisomerase in the SV40 system.

Initiation of DNA replication *in vivo*

The replication of DNA *in vivo* is co-ordinated with the cell cycle. Usually only one round of DNA synthesis is allowed in each cell cycle, whereas viruses (with one exception; see the licensing factor hypothesis) have evolved to escape this control. Accordingly, failure to complete S phase caused by mutations in genes required for DNA synthesis causes cell cycle arrest (Hartwell and Weinert, 1989). As pointed out in the previous paragraph, the presence of the initiator protein is necessary but not sufficient to trigger the initiation event at the origin. Both DnaA and large T antigen can be in excess in the cell and even bound to the origin without promoting extra rounds of

DNA replication within the same cell cycle (Sekimizu *et al.*, 1988; Roberts and Weintraub, 1986). Therefore, the initiation activity of the initiator protein may be regulated. Evidence for such a mechanism has been recently provided. A cold sensitive mutant of the *dnaA* protein has been recently isolated and shown to promote hyperactive initiation events at *oriC in vivo* (Katayama and Kornberg, 1994). This may represent a constitutive active form of DnaA. Thus it is likely that the regulation of the activity of the initiator protein may be under cell cycle control. There is no evidence, however that modification of the DNA at the origin contributes to this regulation mechanism in higher eukaryotes.

In order to study DNA replication in co-ordination with the cell cycle, an homologue eukaryotic cell-free system able to support a single round of bidirectional DNA replication in a semi-conservative fashion is needed.

The Xenopus cell-free system

The *E. coli* and SV40 *in vitro* systems represent very valuable tools to gather important information about the detailed molecular mechanisms of virus and chromosomal DNA replication. To bridge the gap between the *in vitro* and *in vivo* observations a system designed to study DNA replication within the cell cycle has been developed. A cell-free extract made from fertilised *Xenopus* eggs able to replicate DNA *in vitro* was described (Lohka and Masui, 1983). Such a system could support a single round of semiconservative DNA replication within each cell cycle using interphase blocked extracts (Blow and Laskey, 1986; Blow and Watson, 1987) and not mitotic blocked extract (Blow and Sleeman, 1990). DNA from any source used in this assay was assembled in a nuclear structure and replicated just like in the intact egg (Harland and Laskey, 1980; Forbes *et al.*, 1983). Assembled nuclei replicated independently from each other even if incubated in the same extract. Using this system, it was for the first time possible to study DNA replication within its functional unit, the nucleus. A similar system has been recently developed in *Drosophila*, (Crevel and Cotterill, 1991), although this has not been very well characterised yet. This system would be potentially interesting given the enormous genetic knowledge accumulated in *Drosophila*.

Experiments carried out by Newport *et al.*, (1990) have shown that in the *Xenopus* system lamin III is not necessary for the formation of a nuclear envelope, whilst it is necessary to maintain and allow growth of the nuclear envelope. Nonetheless, the nuclear lamin III was absolutely required for DNA replication, suggesting that it may have a direct role in DNA replication. Extracts depleted of nuclear lamin III (LIII) could support chromatin decondensation, a pre-requisite for initiation of DNA replication, but they did not support initiation of DNA synthesis, even though enzymes required for DNA replication where still detectable inside the nucleus (Meyer *et al.*, 1991). These authors proposed that LIII may have a direct role in organising the replication clusters. Given that assembly of a nuclear structure is important to promote DNA replication in the *Xenopus* system (Blow and Watson, 1987; Blow and Sleeman, 1990), the next step is to identify components, within the extract, able to promote DNA synthesis. Cox, (1992) has exploited the importance of nuclear transport in DNA synthesis. She has found that blocking nuclear import with wheat germ agglutinin (WGA) inhibits replication of sperm and somatic nuclei, suggesting that active nuclear import of replication components is a requirement for DNA replication. In an attempt to identify putative initiator proteins in these extracts, Cox and Leno studied the ability of oocyte and activated egg extracts to promote DNA replication (Cox and Leno, 1990). They found that oocyte extracts did not inhibit replication when mixed with egg extracts and could decondense sperm chromatin but could not form an intact nuclear envelope. This suggested that oocyte extracts contain important components for DNA replication but they do not contain the activity required to initiate such a process.

DNA replication in a simple eukaryote: yeast

Yeast has proved to be an amenable system for the study of the molecular genetics of DNA replication and its regulation within the cell cycle. The biochemical activity of some purified yeast replication proteins has also been tested in the SV40 system. DNA replication in the budding yeast *Saccharomyces cerevisiae*, shows similarities with prokaryotic and animal virus replication, in that specific origins of DNA replication have been identified and strong candidates for initiator proteins have been put forward.

Specific origins of replication (*ARS*; Autonomously Replication Sequences; Struhl *et al.*, 1979; Hsiao and Carbon, 1979) have been isolated in budding yeast as genomic sequences able to confer high frequencies of DNA transformation of yeast cells. *ARSs* have been shown to be origins in both plasmids and chromosomal locations (Brewer and Fangman, 1987; Huberman *et al.*, 1987), although it is now clear that not all *ARSs* which are functional on a plasmid are also functional in the chromosome (Reynolds *et al.*, 1989). This suggests that a) they may function as origins in some cell cycles and not in others; b) they may be redundant.

Genetic dissection of *ARSs* has revealed the presence of three elements, A, B and C. Extensive genetic analysis has demonstrated that within the A element of all *ARSs* there is a minimal 11 base pair consensus sequence which is essential for *ARS* function called the ACS (Ars Consensus Sequence; Celinker *et al.*, 1984; Kearsey, 1983, 1984; Broach *et al.*, 1983). Mutations within this consensus sequence abolish *ARS* function (Kearsey, 1984; vanHouten and Newlon, 1990). The B domain has been functionally divided in three sub-domains, B1, B2 and B3. These sub-domains are individually not essential for *ARS* function and any two are sufficient to co-operate with the essential element A to support *ori* activity (Marahrens and Stillman, 1992).

B3 contains the binding site for the multifunctional protein ABF1 (Ars Binding Factor1), whose binding is required for function of certain *ARSs in vivo* (Rhode *et al.*, 1992). Interestingly, ABF1 has been shown to influence positively the functioning of an *ARS* also if located within 1.2 kb from the ACS (Walker *et al.*, 1990) and can be exchanged with a binding site for other transcription factors without affecting *ARS* activity (Marahrens and Stillman, 1992). This suggests that the role of this element may be either in interacting with other elements of the *ARS* or in regulating the chromatin structure of the *ori*. As with the SV40 origin, the ACS and flanking sequences are nucleosome-free *in vivo*. Alteration of the nucleosome positioning around *ARS1* results in *ARS* inactivation (Simpson, 1990), suggesting that this region has to be maintained in a nucleosome-free status in order to be functional.

The roles of elements B1, B2 and C are not yet clear. The influence of 3'-flanking sequences on the functionality of *ARSs* has also been shown to be important. Usually, an AT-rich element is found at the 3' end of every *ARS*. This is similar to the AT-rich element of *oriC* in *E. coli* (Bramhill and Kornberg, 1988b). Based on studies of thermal stability of these sequences, Umek and Kowalski, (1988) have suggested that this region may be important in the unwinding reaction of the *ARS*. The low helical stability element has been proposed to be called DUE (Distal Unwinding Element). Studies of reversion of mutated *ARS* on a plasmid revealed that the reversion events all mapped to sequences within a vector DNA sequence derived form the bacteriophage

M13, suggesting that new *ARSs* can be generated by creating a close match to the ACS (Kipling and Kearsey, 1990).

S. cerevisiae initiator protein(s)

Genetic and a biochemical approaches have been undertaken in order to identify putative initiator proteins in this system.

Two genetic screens aimed to isolate *ARS* binding factors as suppressors of *cis*-acting *ars* mutations were devised. A similar screening had previously been used to isolate suppressors of point mutations in the 65-bp minimal replication origin region of SV40. These second-site revertants all mapped in the large T antigen gene (Shortle *et al.*, 1979).

Kearsey and Edwards, (1987) isolated extragenic suppressors able to increase the mitotic stability of a plasmid containing a mutated *ars*. This allowed the isolation of the *RAR1-5* complementation groups. Characterisation of the *rar3-5* mutants has shown that they may affect general transcription (*rar3* ; Kipling *et al.*, 1991) and RNA processing (*rar5*; Kearsey and Kipling, 1992). Interestingly, *RAR1* encodes for an essential function (Kearsey and Edwards, 1987).

Thrash-Bingham and Fangman (1989) have used a similar screen using an *ARS1* mutated in the C domain. This led to the identification of four complementation groups, one of which, *amm1* (altered minichromosomes maintenance) was found to encode a transcription factor allelic to *TUP1* (Schultz and Carlson, 1987) which may be involved in the organisation of repressive regions of chromatin (Cooper *et al.*, 1994). The role of these proteins in DNA replication is to date unclear.

Strong candidates for initiator proteins have been recently proposed. These are the multiprotein complexes ORC (Origin Recognition Complex) and CBF (Core Binding Factor), both biochemically identified.

ORC was isolated as a complex of 6 polypeptides (120, 72, 62, 56, 53 and 50 kDa respectively) specifically binding to an *ARS* sequence *in vitro* (Bell and Stillman, 1992); such an activity was shown to be present also *in vivo* (Diffley and Cocker, 1992). A number of strong correlations support a role for the ORC as the initiator protein at *ARSs*. Biochemical data show that the binding of ORC at the ACS is highly specific and ATP-dependent for all the *ARSs* tested, although ATPase activity cannot be detected in purified preparations of ORC (Bell and Stillman, 1992). From data gathered following DNAseI and chemical cleavages experiments it has been proposed that the ACS and B1 element may be wrapped around the multiprotein complex, just like the interaction between the *E. coli* initiator protein DnaA and *oriC* (Diffley and Cocker, 1992).

Mutations which inactivate *ARS* function abolish ORC binding *in vitro* (Bell and Stillman, 1992). A temperature-sensitive mutant in the 72 kDa subunit of the ORC complex (*orc2-1*) shows phenotypes typical of mutants in DNA replication (Bell *et al.*, 1993). Intriguingly the *orc2-1* mutant has been shown to be allelic to *rrr1*, a mutant defective in the transcriptional silencing of mating-type genes (Micklem *et al.*, 1993; Foss *et al.*, 1993). This observation is difficult to correlate with DNA replication but it may help to uncover mechanisms that couple transcription and DNA replication in eukaryotes. The gene encoding the 50 kDa subunit of ORC (*ORC6*) has been genetically isolated in a screen aimed at identifying proteins binding the ACS *in vivo* . The overexpression of *ORC6* exacerbated the lethal phenotype of yeast strains harbouring mutations in the *CDC6* or *CDC46* genes, both functions required for the initiation of DNA replication (Hogan and Koshland, 1992; Bueno and Russel, 1992; Hennessy *et al.*, 1991), suggesting a genetic interaction with the *ORC6* gene (Li and Herskowitz, 1993).

There are some basic questions which need to be answered. What is the specific function of ORC at the *ARS*? Does ORC work like *E. coli* DnaA? Are all the subunits of ORC required to bind the *ARS*? What is the relationship between ORC and transcriptional silencing?

ORC seems to be bound to the *ARS* throughout the cell cycle (Diffley and Cocker, 1992). It has been suggested that this multiprotein complex may have a role in marking the origin of replication, acting as a landing pad for other replication proteins, perhaps present in their active form at a specific stage of the cell cycle (Stillman, 1994b). The presence of cell cycle-regulated components interacting with the ORC complex has been predicted from analysis of the chromatin structure of *ARSs in vivo* (Diffley *et al.*, 1994). These studies have demonstrated that such components may transiently interact with the ORC complex at the initiation of DNA replication and are not bound at other stages of the cell cycle. In this view the assembly of a pre-replication complex at an *ARS* may be carried out in two steps. First modification of the ORC complex during G1 followed by activation of DNA replication at the G1/S transition. Circumstantial evidence indicate that these components may include Cdc6 and members of the Mcm2-3-5 protein family (Hennessy *et al.*, 1990; see the Mcm protein family section) as recently suggested (Newlon, 1993; Huberman, 1994). The identification of a kinase activity interacting with the ORC complex at an origin (Dowell *et al.*, 1994) may indicate that phosphorylation of these components may be crucial in the activation of DNA replication.

CBF is as a protein complex able to bind ACS *in vitro* (Estes *et al.*, 1992). An ABF1-specific affinity resin was used to purify this complex. The binding obtained by incubation of *ARS121* probes with two fractions eluted from the affinity column (0.4 and 0.8M KCl fractions) was greatly reduced by addition of the flow-through fraction

in an ATP-dependent fashion, suggesting that specific binding of the complex is ATP-dependent. The relationships between CBF and ORC are not clear, as CBF has not been purified yet and *ARS121* is slightly different from *ARS1*.

Characterised S. cerevisiae replication proteins

Several *S. cerevisiae* replication proteins with well characterised functions have been biochemically and genetically identified.

Three essential DNA-dependent DNA polymerases (α, ε, δ) have been identified in this yeast (see Campbell and Newlon, 1991, for review). The presence of an additional DNA polymerase, predicted to be non-essential (*REV3*), has been inferred from genetic and DNA sequence data (Morrison *et al.*, 1989). The product of the non-essential *MIP1* gene encodes the mitochondrial DNA polymerase (Foury, 1990). The four-subunit DNA polymerase α holoenzyme (Badaracco *et al.*, 1983; Plevani *et al.*, 1984, 1985) also contains DNA primase activity (Plevani *et al.*, 1985; Brooks and Dumas, 1989; Lucchini *et al.*, 1987) which has been shown to be confined to the 48 kDa subunit (Santocanale *et al.*, 1993). There seems to be species-specific requirements for DNA polymerase α *in vivo*, as the human homologue cannot complement *S. cerevisiae* and *S. pombe* strains mutated for DNA polymerase α (Francesconi *et al.*, 1993).

The B-subunit of the DNA polymerase α (70 kDa subunit; Foiani *et al.*, 1994) is of particular interest as the human counterpart has been proposed to mediate the interaction between large T antigen and the DNA polymerase α-primase complex (Collins *et al.*,1993). The role of polymerase ε in DNA replication has not yet been clarified.

Also, putative RP-A and RF-C homologues have been purified from whole-cell yeast extracts. The yeast RP-A has been shown to have the same structure and activity of the human RP-A *in vitro* (Brill and Stillman, 1989). Interestingly, an essential role for this protein in DNA repair and recombination in budding yeast has also been proposed (Longhese *et al.*, 1994). RF-C was similarly shown to be chromatographically and enzymatically equivalent to the human counterpart (Fien and Stillman, 1992). Topoisomerase II has also been identified (Goto and Wang, 1982) together with three additional DNA topoisomerase genes (see Newlon and Campbell, 1991, for review). An important component of the replication fork yet to be found is the DNA helicase. Only one DNA-dependent helicase, ATPaseIII, has been described and shown to stimulate activity of DNA polymerase α (Sugino *et al.*, 1983).

DNA replication in another yeast: *Schizosaccharomyces pombe*

The yeast *S. pombe* is becoming an increasingly popular model system as it shares the genetic amenability of budding yeast but it has some features of its cell biology that are more common to higher eukaryotes (see Forsburg and Nurse, 1991, for review). The study of DNA replication in this system has more limitations than budding yeast for two major reasons: 1) an origin of DNA replication as a defined genetic element has not been found- fission yeast origins being functionally and structurally different from those described in budding yeast; 2) relatively few fission yeast replication proteins have been isolated so far.

S. pombe ARSs

Fission yeast *ARSs* were isolated using the same assays employed for budding yeast. However, following deletion analysis a well defined *cis*-acting element in the origin has not been found (Johnston and Baker, 1987; Maundrell *et al.*, 1988). Intriguingly, the efficiency of yeast transformation correlates with the length of the *ARS* insert. A consensus of 11 bp could be identified in a set of fission yeast *ARSs*, however its deletion did not affect *ARS* function (Maundrell *et al.*, 1988). Thus the minimal *S. pombe ARS* element seems to be much larger than that of *S. cerevisiae*. Mapping techniques have suggested that initiation occurs in a broad zone of the origin rather than at specific sites (Zhu *et al.*, 1992). Recent studies on the *ARS* associated with the *ura4*$^+$ locus have suggested that within the broad zone of initiation discrete sites of initiation can be identified (Dubey *et al.*, 1994). Interestingly such discrete origins seem to interfere with each other's function. These features, show some similarities to metazoan replication origins, and perhaps this yeast will be an important and amenable model system to investigate the properties of higher eukaryotic origins.

S. pombe characterised replication proteins

Four fission yeast replication proteins have been genetically identified and characterised so far. These are the catalytic subunit of the DNA polymerase α (Damagnez *et al.*, 1991; Park *et al.*, 1993), DNA polymerase δ (Francesconi *et al.*, 1993; Park *et al.*, 1993), PCNA (*pcn1*$^+$; Waseem *et al.*, 1992) and DNA ligase (*cdc17*$^+$; Nasmyth, 1977). Mutations in these proteins display phenotypes associated with DNA replication defects. The gene encoding topoisomerase II has also been isolated (Uemura *et al.*, 1986). The biochemical data on these proteins are limited. The

human PCNA gene was able to complement a null mutant of *pcn1*, whereas the budding yeast structural homologue (*POL30*) failed to do so (Waseem *et al.*, 1992).

DNA replication in higher eukaryotes

Studies of DNA replication in higher eukaryotes has been limited to biochemical approaches such as the biochemical purification of proteins displaying DNA replication activity *in vitro*. The SV40 system has represented an invaluable tool in the characterisation of such mammalian replication proteins (as earlier discussed). A great gap in the field is represented by the difficulty in identifying specific origins of DNA replication.

Mammalian origins of DNA replication

After the pioneering studies of Huberman and Riggs (1968) who showed that DNA replication in mammals proceeds bidirectionally from initiation sites spaced about 100kb apart, a great deal of effort has been expended in trying to map these sites within the chromosomes. However, the size of the region which was supposed to contain an origin was dependent on the mapping technique used (see Linskens and Huberman, 1990). In parallel studies other groups have tried to identify mammalian origins using an *ARS*-type assay, as used in budding yeast. Mammalian sequences able to confer autonomous replication could be isolated if a viral nuclear retention signal was also present (Krysan and Calos, 1991). It was later shown that human sequences smaller than 12kb or bacterial DNA replicated poorly (Heinzel *et al.*, 1991) and that, as for *S. pombe*, the efficiency of transformation was increased by increasing the size of the insert. These data together with the observation that assembly of the DNA in a nuclear structure is the major pre-requisite for efficient DNA replication in *Xenopus* cell-free extract (Newport, 1987) suggested that chromatin structure and chromosomal context are important in this process.

To date three metazoan origins of DNA replication have been identified and one of them, the origin associated with the DHFR gene, has been extensively characterised (for review see Hamlin, 1992; DePamphilis, 1993). The second origin is the one found associated with the chorion genes in *Drosophila* (Heck and Spradling, 1990). The last one is represented by the origin associated with the upstream sequences of the human β-globin gene region. A deletion of the 5' upstream region of this locus abolished bidirectional DNA replication at this site as seen in the wild-type configuration and leads to replication coming from a unidentified origin outside the β-globin locus

(Kitsberg *et al.*, 1993a). Interestingly, the temporal pattern of transcription and replication of this locus overlap. This may represent a good example of presence of specific sites of initiation in metazoan chromosomes. Due to the limits of resolution of the available mapping techniques and to the large size of the initiation zone, it is difficult to identify origin-specific elements. Nevertheless, within an initiation zone ranging from 0.5 to 12 kb, elements such as transcription factor binding sites, major DUEs, pyrimidine tracts and Scaffold Associated Regions (SARs) seem to be the major common elements of mammalian origins. If compared to bacteria and budding yeast origins it may be said that perhaps the modular structure of the origin has been conserved in evolution, although on a different scale (for review see Benbow *et al.*, 1992). Studies in *Xenopus* embryos have shown that any DNA can be replicated (Blow and Laskey, 1986; Newport, 1987; Méchali and Kearsey, 1984), although the embryo may be an exception as early embryos cleavages are very rapid and there may be a need to relax the initiation requirements for DNA replication. Nevertheless, DNA still replicates in co-ordination with the cell cycle.

Cell cycle regulation of DNA replication *in vivo*

Integration of DNA replication within the cell cycle

Within the eukaryotic cell cycle, DNA replication takes place during a specific period of interphase, the synthetic period or S phase. In order to allow co-ordination with other cellular processes, a fine regulation of ordered events is needed. For instance, cells prepare themselves for entry of S phase by synthesis (or activation) of S phase-specific proteins during the G1 phase (see Johnston, 1990, for review). Also duplication of the DNA has to be completed before chromosome segregation and subsequent mitosis (M phase) take place. Failure to co-ordinate distinct phases of the cell cycle results in catastrophic events, such as premature mitosis (see Hartwell and Weinert, 1989, for review).

The licensing factor hypothesis

One important application of the *Xenopus in vitro* system has been the study of the regulation of the once-per-cell cycle replication of the DNA. As previously discussed (see the *Xenopus* cell-free system paragraph) interphase blocked egg extracts support a single round of DNA replication in this system. It was found that for the DNA to re-replicate in this *in vitro* system, a passage through mitosis was necessary, as if the DNA had to acquire an important factor required to reset the programme of DNA

replication, in agreement with the results of Rao and Johnson (1970), who have shown that G1 and not G2 nuclei are competent to replicate. A set of experiments aimed in identifying a modification of the nuclei which would allow re-replication in a single S phase was performed. Protein synthesis was shown to be important to allow re-replication. The inability of cellular extracts treated with cycloheximide to re-replicate was however reversed by addition of MPF (see page 17), an activity which causes nuclear envelope breakdown and mitotic changes. Therefore, it was tested if the integrity of the nuclear membrane was important for allowing re-replication. Nuclei were permeabilized with agents such as lysolecithin and it was found that the DNA was able to re-replicate in these extracts (Blow and Laskey, 1988). As the re-replication was independent on the DNA used (Blow and Laskey, 1986) it does not appear to be dependent on a specific DNA sequence element. A model put forward to explain these results postulates the existence of a positive factor, Licensing Factor (LF), which is required to 'license' chromatin for the initiation of DNA replication. LF can only gain access to the chromatin at mitosis when the nuclear envelope breaks down. LF must support a single round of DNA replication and has to be destroyed or modified after the initiation event.

If nuclear permeabilization allows re-replication within a single S phase, the entry of a positive factor or the escape of a negative one can be postulated. The presence of a positive factor is much simpler than to imagine the escape of a negative one which *a priori* cannot be excluded. Interestingly the licensing activity has been shown to be inhibited by treatment of metaphase-arrested egg extracts with 6-DMAP, an inhibitor of cdc2-like protein kinases (Blow, 1993), consistent with previous findings that a such a kinase may be involved in the initiation of DNA replication in *Xenopus* (Blow and Nurse, 1990).

This model fits very well the observations made in the *Xenopus* system but has some limitations when applied to other systems. There are certain organisms, such as yeast, where the nuclear envelope does not break down at mitosis. In yeast the putative LF might gain access to the nucleus during the M phase as a result of regulated nuclear import.

Re-replication with no intervening mitoses occurs in certain tissues, such as salivary glands in *Drosophila* and in processes of gene amplification (Spradling and Rubin, 1981). Moreover, cells treated with some chemical agents such as staurosporine analogues (Usui *et al.*, 1991) and the mammalian *ts41* mutant (Handeli and Weintraub, 1992) also re-replicate with no intervening mitoses. Mutations in the *cdc2* protein kinase or in its regulative subunit have been shown to cause over-replication. Broek *et al.*, (1991) have shown that temperature-sensitive mutants of *cdc2*, under certain conditions, can induce diploidisation in fission yeast. They proposed that the destruction of the cdc2-cyclin B complex was responsible for such a phenotype and

proposed that indeed the cdc2 protein kinase, in combination with cyclins, may be the indicator of specific stages of the cell cycle. Consistent with this model is the observation that mutant forms of cyclin B (*cdc13*) induce diploidisation in fission yeast (Hayles *et al.*, 1994). Another point to be considered is the requirement for a *cis*-acting element. It has never been shown that such a requirement does not exist in systems other than *Xenopus*, where it may be very relaxed. On the contrary there is the example of the Bovine Papilloma Virus in which the replication of the viral genome is under the once-per-cell cycle regulation and is dependent on a *cis*- acting viral sequence binding site for viral encoded proteins (Roberts and Weintraub, 1986).

These observations have two important consequences;

1) they suggest the presence of a negative component more than a positive one which normally suppresses the repeated rounds of DNA replication within the same S phase; this model would also explain phenomena as gene amplification;

2) the protein kinase cdc2 or cdc2-like kinases seem(s) to have an important role in regulating this process.

In order to explain these observations, the licensing factor model has been very recently modified in that LF is assumed to be the natural modifier of a pre-existing repressor of replication on the DNA (hit and run model; Coverley and Laskey, 1994). Following this model, DNA replication is allowed to initiate by transient relief of such a repression that occurs only once-per-cell cycle at the G1/S boundary. Cyclin-dependent kinases might be candidates for a catalytic licensing role.

Based on their similarity with LF, the budding yeast Cdc46/Mcm5 and related-proteins (see the Mcm protein family) were put forward as best candidates for molecular licensing factors (Leno *et al.*, 1992). The recent findings of Kimura *et al.*, (1994) and Todorov *et al.*, (1995) have shed light on the mechanism by which the Mcm proteins may play a role on this process (see the Mcm protein family section, page 28).

Cell division cycle (cdc) mutants

Study of a number of yeast mutants blocked at specific stages of the cell division cycle (*cdc* mutants) has proven to be an invaluable tool in the identification of molecular components involved in this process. Usually inactivation of functions required for transition from one phase to another by mutation, results in the arrest of the cell in that stage of the cell cycle where the specific function is required. The arrest is dependent on a surveillance mechanism which ensures the ordered dependency of cell cycle events (Hartwell and Weinert, 1989; Murray, 1992). Analysis of the terminal phenotype of arrested cells has helped in screening of mutants affecting the transition of specific stages in the cell cycle. Among a number of lethal-conditional *cdc* mutants

isolated (Hartwell *et al.*, 1974; Hartwell, 1978) few were originally indicated as required for the G1 to S transition or for S phase execution in budding yeast. These include the products of the *CDC4, 7, 8, 21* and *28* genes. The *cdc4, 7, and 28* mutants arrest before DNA synthesis, whereas the *cdc8* and *21* mutants arrest in S phase (Hereford and Hartwell, 1974; Hartwell, 1976). Genetic analysis of the *cdc4, 7 and 28* mutants has provided the basis for the concept of START in yeast, also known as restriction point in higher eukaryotes (Hartwell, 1974; Pringle and Hartwell, 1981) and by analogy this concept has been adopted for fission yeast (Bartlett and Nurse, 1990; Nasmyth, 1990). Once cells have passed through START they become irreversibly committed to the mitotic cell cycle. This implies that at a point before START, that is in the G1 phase, a choice between meiosis or mitosis in yeast (and remaining quiescent or undertaking the mitotic cell cycle in higher eukaryotes) has to be taken. Moreover, before going through START cells must reach a critical size, making the G1 phase expandable to adjust this. Therefore, START is a major rate-limiting step in the eukaryotic cell cycle. A direct link between growth and commitment in the mitotic cell cycle has been recently proposed (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994). These authors have shown that high levels of cAMP are able to repress the expression of rate-limiting factors (G1 cyclins, see below) required to trigger START in budding yeast.

In addition to the above mentioned genes, other *CDC* genes required at the G1 to S transition have been identified in budding and fission yeast and some of them have been characterised at the molecular level. Because budding and fission yeast regulate their cell cycle in different ways they will be discussed separately. The different regulation of the cell cycle in the two yeast has been reviewed by Forsburg and Nurse (1991).

G1/S phases in budding yeast

The major regulation point of the budding yeast cell cycle is at the G1 to S transition. The product of the *CDC28* gene is required for this transition (Hartwell *et al.*, 1973; Reed, 1980) as well as for the G2 to M transition (Piggott *et al.*, 1982; Reed and Wittenberg, 1990). *CDC28* encodes a 34 kDa serine/threonine protein kinase ($p34^{CDC28}$) which associates *in vitro* and *in vivo* with proteins known as cyclins. These are organised in a conserved super family of structurally related proteins and represent the regulative subunits of the prototype *CDC28* kinase of yeast and related kinases in higher eukaryotes (Cdk_s) (reviewed by Hunt, 1989). Cyclins are periodically expressed and destroyed at specific stages of the cell cycle. It is thought that their stability may allow co-ordination of cell cycle events (Nasmyth, 1993).

Budding yeast cyclins expressed in G1 (G1 cyclins) are the Cln_S 1-3, Hcs26 and Orfd gene products. Up to two *CLN1-3* genes can be deleted without affecting viability, although the length of G1 is extended, whereas the triple deletion is lethal and leads to G1 arrest (Surana *et al.*, 1991; Richardson *et al.*, 1992). This demonstrates that the Clns 1-3 are redundant. In addition to the above mentioned G1 cyclins, six B-type cyclins (Clb1-6) associate with Cdc28 and may regulate progression through S and M phase (Nasmyth, 1993).

The Cdc28/cdc2 protein kinase, in combination with a M-phase specific cyclin (maturation promoting factor, MPF) has been shown to regulate the G2 to M transition in yeast and higher eukaryotes, by activation of a wide range of substrates (reviewed by Coleman and Dunphy, 1994). Thus, by analogy, it is thought that Cdc28/cdc2 in combination with G1 cyclins may have a similar activity on substrates required for the initiation of DNA replication. Recent work has suggested that the B-type cyclin kinase inhibitor $p40^{SIC1}$ could control the G1 to S transition in *S. cerevisiae* (Schwob, *et al.*, 1994). The p40 protein would inhibit the activity of the Clb-Cdc28 kinase by direct binding. Its proteolysis, mediated by the ubiquitin-conjugating enzyme Cdc34 (Goebl *et al.*, 1988), would promote entry into S phase.

The product of the *CDC7* gene is a protein kinase which is required for the initiation of DNA replication (Hollinsworth and Sclafani, 1990). The activity of this protein was shown to be required to replicate DNA *in vitro* (Jazwinski, 1988). Very recently this protein has indeed been shown to interact physically with ORC *in vivo* in combination with its regulatory subunit, Dbf4 (Dowell *et al.*, 1994).

Other gene products required for G1 to S transition are *CDC6, 34, 36, 37, 39, 45, 46, 54, 68*. The roles of some of them will be discussed in the following sections.

In budding yeast there are a number of S phase-specific genes that are periodically transcribed at the G1/S boundary. This control is given by the presence of a short hexamer element called the *Mlu*I box (it contains the consensus sequence for recognition by the *Mlu*I restriction enzyme). An activity, called MBF (*Mlu*I Binding Factor) or DSC1 (DNA Synthesis Control), specifically recognises and binds this sequence (see Merrill *et al.*, 1992, for review). This contains the Swi4 and Swi6 proteins, previously shown to be required for mating-type switching in yeast (Andrews and Herskowitz, 1989). The significance of this control element is unclear as its deletion from the promoter of the DNA polymerase α (*POL1*) gene does not affect ongoing DNA synthesis (Muzi-Falconi *et al.*, 1993). *CDC6* is one of the genes periodically transcribed at the G1/S boundary. The Cdc6 protein may be involved at an early step in the initiation of DNA replication and may perhaps have a role as mitotic inhibitor (Hogan and Koshland, 1992; Bueno and Russell, 1992).

The *CDC14* gene, despite its late execution point (late mitosis) may also be required for the initiation of DNA replication (Hogan and Koshland, 1992) and encodes a putative protein phosphatase (Wan *et al.*, 1992).

Other genetically identified functions required for S phase execution and thought to be required at an early step of chromosome replication are represented by the *MCM* complementation groups which will be discussed in a specialised section (see page 28).

G1/S phases in fission yeast

Unlike budding yeast, the major cell cycle control of fission yeast acts at the G2/M boundary. Thus the G1/S transition in this system is poorly understood. G1 in exponentially growing fission yeast cells is normally very short, similar to the cell cycle of the early embryos or to that of few cultured cells (Prescott, 1987). Also in this yeast the product of the *cdc2*$^+$ gene, homologous of the *CDC28* gene of budding yeast (Beach *et al.*, 1982), is required for the G1/S and G2/M transition (Nurse and Bissett, 1981). There is no evidence so far for G1 cyclins in fission yeast. Hence, if fission yeast regulates the G1/S transition as in budding yeast, then the partner of cdc2 is still unknown.

A number of genetically identified functions required for the G1/S phase transition have been described. One is the product of the *cdc10*$^+$ gene (Aves *et al.*, 1985), a transcription factor (Lowndes *et al.*, 1991) which activates transcription of S phase specific genes such as *cdc18*$^+$ (Kelly *et al.*, 1993), *cdc22*$^+$ (Gordon and Fantes, 1986; Lowndes *et al.*, 1992) and *cdt1*$^+$ (Hofmann and Beach, 1994). cdc10 shares structural homologies with the Swi4 and Swi6 proteins of budding yeast, and binds the *Mlu*I box of the *cdc18*$^+$ and *cdc22*$^+$ genes suggesting that the DSC1 activity has been conserved between the two yeasts (see previous paragraph). The sct1/res1 gene product has been shown to be physically associated with cdc10 in the binding to the *Mlu*I box (Tanaka, *et al.*, 1992; Caliguri and Beach, 1993). An additional *res*$^+$ function, *res2*$^+$/*pct1*$^+$, has been identified as partner of the cdc10 protein (Miyamoto, *et al.*, 1994; Zhu *et al.*, 1994). *res2*$^+$ is not an essential gene. It may partially overlap in function with *res1*$^+$ and probably has a role in pre-meiotic and meiotic DNA synthesis. Thus the *cdc10*$^+$/*res1*$^+$ are thought to be components of START in fission yeast.

The functions of some genes under control of the cdc10 protein has been reported. The *cdc22*$^+$ gene, and its suppressor *suc22*$^+$, encode respectively the large and small subunits of ribonucleotide reductase, enzyme involved in the synthesis of nucleotides (Fernandez-Sarabia *et al.*, 1993). The *cdc18*$^+$ gene has been shown to be required for S phase onset and restraint of mitosis during S phase (Kelly *et al.*, 1993).

The cdc18 protein shares homology with the Cdc6 protein of budding yeast and contains an NTP binding motif.

Another function required for the onset of S phase is represented by the product of the *cut5*$^{+}$/*rad4*$^{+}$ gene (Saka and Yanagida, 1993). The role of this protein seems to be similar to that of the cdc18 protein in that both functions are required for S phase onset and restraint of mitosis until completion of DNA replication. Interestingly, the cut5/rad4 protein is not under control of the cdc10 protein (Saka *et al.*, 1994). Very recently an additional target of the cdc10/sct1 transcription factor activity has been identified (Hofmann and Beach, 1994). This is the *cdt1*$^{+}$ gene required for DNA synthesis and presumably in the restraint of mitosis until completion of DNA replication.

Thus, in *S. pombe*, it is not clear yet how the cdc10/sct1 activity is regulated and therefore how this activity is relevant to the initiation of DNA synthesis.

Other functions required for S phase execution in fission yeast are the *cdc19*$^{+}$, *20*$^{+}$, *21*$^{+}$, *23*$^{+}$ and *24*$^{+}$ gene products (Nasmyth and Nurse, 1981). The role of these proteins is still unclear. Two of them, *cdc19*$^{+}$ and *cdc21*$^{+}$ have been shown to be structurally related to the budding yeast Mcm protein family (Coxon *et al.*, 1992; Forsburg and Nurse, 1994; see specific section , page 28).

There is no evidence to date for a factor similar to MPF, which regulates the G1/S transition in yeast or higher eukaryotes (S phase promoting factor). Although the p34$^{cdc2/CDC28}$ protein and related Cdks in higher eukaryotes are required for such a transition, it has still to be determined how this kinase triggers DNA replication.

Higher eukaryotes

Isolation of cell cycle mutants in mammalian cell lines has been attempted with not much success. Several problems arise in this system due to the difficulty in doing genetics (see Simchen, 1978, for review). Thus cell cycle studies in mammalian cells have been largely limited to biochemical studies.

The finding that a mammalian homologue of the yeast p34^{cdc2} protein kinase can substitute for the yeast kinase *in vivo* (Lee and Nurse, 1987) has strongly suggested that cell cycle mechanisms have been conserved in evolution (Nurse, 1990). Strong evidence for a requirement of p34^{cdc2} in initiation of DNA replication has been provided. An unwinding activity from mammalian cells which appeared at a discrete point in the cell cycle, just before cells entered S phase, was identified. This activity was required, in conjunction with SV40 T antigen, for specific initiation at the SV40 origin *in vitro* (Roberts and D'Urso, 1988). The purified activity (S phase replication factor; RF-S) was shown to contain a human homologue of the yeast p34^{cdc2} protein

kinase as well as cyclins A and B (D'Urso *et al.*, 1990). Interestingly, p34^{cdc2} was shown to be monomeric in G1 and also present in a large complex (ca. 250 kDa) in S phase extracts, suggesting that the activation of DNA replication by p34^{cdc2} required its association with other components. It is not clear why a mitotic cyclin (B) would stimulate the initiation of DNA replication in this *in vitro* system.

Nevertheless, a growing number of cdc2-like protein kinases and cyclins have been identified (see Heichman and Roberts, 1994; Sherr, 1994, for review). There are six distinct Cdks known so far and eight classes of cyclins, designated A-E, G-H, with multiple members, in animal cells. Two of these cyclins, D and E are considered G1 cyclins and indeed they rescue mutations in budding yeast G1 cyclins. Human cyclin F can partially complement the defect of a *cdc4* mutant in budding yeast (Bai *et al.*, 1994).

Regulation of cell proliferation in higher eukaryotes

Regulation of cell cycle progression in higher eukaryotes is certainly far more complex than in yeast. Mammalian cells have to respond to a variety of external signals which may stimulate or repress the mitotic cell cycle (e.g., growth factors; see Pardee, 1989, for review). This usually takes place before passage through the G1 restriction point. Mammalian cells can also enter a quiescent state called G_0 soon after mitosis, whereas fission yeast cells have been shown to enter quiescence either before G1 or before mitosis (Costello *et al.*, 1986). The passage through the restriction point requires the participation of a large number of genes, some of them similar to the yeast ones (see Pardee, 1989 for review). Not surprisingly, some molecules, not previously described in lower eukaryotes, have been identified and shown to have key roles in this process. Two of these are the retinoblastoma and the p53 proteins. Both proteins were originally identified as tumour suppressor genes, in that they negatively regulate cell proliferation. Mutations in these genes have been associated with occurrence of a carcinogenic process (Levine, 1990). Although an enormous effort has been directed towards understanding the precise molecular mechanism by which these molecules prevent cell proliferation, their specific roles remain unclear. Moreover two new molecules related to Rb and p53 have been identified. These are the Rb-related protein p107 and the p53-dependent protein p21.

The retinoblastoma gene

The retinoblastoma gene product (Rb protein) is a nuclear phosphoprotein of 110 kDa (Xu *et al.*, 1989; Horowitz *et al.*, 1990) which was originally shown to act as a negative regulator of cellular proliferation (Marshall, 1991). It has been proposed that Rb may act as a tumour suppressor by inhibiting cell cycle progression, arresting certain tumour cells in G1 while having no effect on other cell types (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Fung *et al.*, 1993). Extensive biochemical studies have shown that the Rb protein associates with different partners in the G1 phase of the cell cycle (for review see Wang *et al.*, 1994). One set of these partners comprises the E2F family of transcription factors, activities which have been shown to activate a variety of G1/S-specific genes (for review see Nevins, 1992). E2F-related proteins are thought to be mammalian counterparts of the yeast DSC1 protein complex (Merrill *et al.*, 1992). Rb-bounded E2F would be inactive, thus unable to activate transcription of G1/S phase genes. Another partner is the SV40 large T antigen (DeCaprio *et al.*, 1988; Ewen, *et al.*, 1989). In normal cells, cell cycle progression is thought to require the cyclical phosphorylation of Rb by the cyclin-dependent kinases (see previous paragraphs). The Rb protein is poorly phosphorylated in G1 and hyperphosphorylated in the remaining phases of the cell cycle. E2F, as well as T antigen, binds the hypophosphorylated form of Rb. Thus it seems that the Rb protein has a role in regulating cell cycle progression at a restriction point in G1 phase by regulating the availability of factors important for this transition.

A protein which shares about 30% similarity with Rb, the p107 protein, has been identified and also shown to bind SV40 large T antigen (Ewen *et al.*, 1989; Dyson *et al.*, 1989) and to inhibit its ability to initiate DNA replication in a similar way to p53 (Amin *et al.*, 1994). It is not known whether both Rb and p107 bind similar proteins or perhaps different ones, but it is a possibility that there may be a redundant control in G1 in higher eukaryotes.

The p53 protein

There is evidence that the p53 protein may play an important role in cell cycle regulation. Wild-type p53 can suppress the transformation of cells in culture by viral or cellular oncogenes (Finlay *et al.*, 1989). Although the p53 protein seems to have properties similar to the Rb protein, as a negative regulator of the cell cycle, some mutants of p53 act to stimulate cell division (Levine, 1990). Interestingly, and unlike the Rb protein, the levels of the p53 protein are variable during the cell cycle, accumulating in the G1 to S phase of proliferating cells. (Bischoof *et al.*, 1990). These

authors have also reported a cell-cycle dependent change in the phosphorylation of the protein. The protein was found highly phosphorylated in S phase, later than the peak of its accumulation. p53 has been shown to bind SV40 large T antigen (Friedman, 1990; Wang *et al.*, 1989) and to specific sequences adjacent to the origin thus inhibiting the initiation of DNA replication (Bargonetti *et al.*, 1991). The p53 protein is also a transcription factor which regulates the synthesis of a set of S phase genes. DNA damage increases the levels of p53 protein, resulting in elevated synthesis of the cyclin-dependent kinase inhibitor p21 (el-Deiry, *et al.*, 1993). The p21 protein can also inhibit the replication of SV40 DNA *in vitro* by directly binding to PCNA (Waga *et al.*, 1994). Interestingly, this association seems to do not cause inhibition of the PCNA-dependent nucleotide-excision repair (Li *et al.*, 1994).

Thus the emerging picture is that the p53 protein is not required for normal cell division, but it may be required to arrest the cell cycle in response to DNA damage or to other abnormal signals which may lead to uncontrolled cell proliferation (Lane, 1992). However, attempts to identify p53-like genes in invertebrates and lower eukaryotes such as yeast has been unsuccessful (Soussi *et al.*, 1987)

Dependency of M phase upon S phase: checkpoint controls

That the cell cycle may be organised in a temporally ordered series of events each one dependent upon the execution of previous ones was suggested by the elegant experiments involving cell fusions of Rao and Johnson, (1970). G2 cells fused to S phase cells caused a mitotic delay in the G2 nucleus until completion of DNA replication. Also these experiments demonstrated that replicated chromosomes (G2) may be different from non-replicated chromosomes (G1) in that the first could not re-replicate on fusion with S phase cells.

The dependency between independent events appears to be mediated by a checkpoint control (Hartwell and Weinert, 1989) able to maintain the dependency of late events upon the correct execution of early ones. Accordingly yeast mutants in functions required for DNA replication normally arrest in S phase and do not enter mitosis (Hartwell and Weinert, 1989).

The presence of a checkpoint control able to monitor the progression of S phase was suggested following the characterisation of the *rad9* mutant of budding yeast. The *RAD9* gene is not essential for growth but *rad9* mutant cells are UV sensitive and also unable to arrest in the presence of DNA damage resulting in rapid loss of viability, whereas they arrested normally in the presence of the DNA replication inhibitor hydroxyurea (Weinert and Hartwell, 1988).

Similar mutants have been isolated in fission yeast, namely *rad* (*rad*iation sensitive) and *hus* (*h*ydroxy*u*rea *s*ensitive) mutants.

rad and *hus* mutants have similar phenotypes, both are UV sensitive and unable to arrest following DNA damage or a DNA replication block. However, of the original *rad* mutants isolated, only the *rad1*, *3*, *9*, and *17* mutants show checkpoint deficiencies. The hus5 gene product is a novel member of the E2 family of ubiquitin-conjugating enzymes (UBCs), therefore it is not directly involved in checkpoint control (Al-Khodairy *et al.*, 1995). Some of the *rad*$^+$ genes have already been cloned but none of the sequences is informative except for the rad17 protein which has limited homology with the *S.cerevisiae* RF-C (Hoekstra and Carr, 1995). A new screen for such a mutants has produced one new gene, *rad26* and new *hus* alleles (Al-Khodairy *et al.*, 1994).

Another function involved in the checkpoint pathway is the *chk1*$^+$/*rad27*$^+$ gene of fission yeast which is predicted to be a protein kinase (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994). Isolated as a high-copy suppressor of a cold sensitive mutant of *cdc2*, the *chk1::ura4*$^+$ null mutant was showed to be incapable of arresting in S phase in the presence of unligated DNA or following UV irradiation. However, the UV sensitivity of the mutant could be reversed by imposing a G2 delay, suggesting that the protein may have no role in DNA repair. Also a double mutant of *chk1::ura4*$^+$ and *cdc10*^{ts} could not execute S phase but entered a premature mitosis, suggesting a disturbance of the surveillance mechanism which prevents mitosis in the absence of DNA synthesis (checkpoint control; Hartwell and Weinert, 1989). Because the *cdc10* mutant arrests pre-START, *chk1* has been proposed to be a component of the pre-START checkpoint. Evidence for the presence of a pre-START checkpoint have also been provided by Moreno and Nurse, (1994). Mutant cells lacking the *rum1*$^+$ gene cannot arrest in the G1 phase. The *rum1*$^+$ gene seems to be required to define the length of the G1 phase in fission yeast. A double mutant of *rum1::ura4*$^+$ and *cdc10*^{ts}arrested at the G1/S boundary entered mitosis, while the *rum1* null mutant arrested at a stage after START with the DNA replication inhibitor hydroxyurea did not (Moreno and Nurse, 1994).

Additional functions involved in the checkpoint control have been identified in *S. cerevisiae* through a screen for S phase arrest-defective mutants (*sad*; Zhou and Elledge, 1992). One of these, the *SAD1/RAD53/SPK1/MEC2* gene, encodes a protein kinase which may control multiple checkpoints in *S. cerevisiae* (Allen, *et al.*, 1994). The Sad1 gene product is epistatic to the Dun1 protein kinase, which is involved in the transcriptional response to DNA damage. A recent screen for checkpoint mutants in budding yeast has produced new mutants and novel complementation groups (*mec*, *mi*tosis *e*ntry *c*heckpoint; Weinert *et al.*, 1994). Of these, the Mec1 function was shown

to have a role in both DNA replication and DNA damage checkpoints, while the Rad9, 17, 24 and Mec3 proteins were proposed to have a role in the DNA damage checkpoint only.

Disturbance of the checkpoint control has been also reported in cells mutated in the RCC1 function, required for chromosome decondensation in mammalian cells (Nishitani, *et al.*, 1991). Rcc1 is a 45 kDa chromatin-associated protein which is found in a complex with the small GTP exchanging protein Ran1. It has been proposed that the Rcc1-Ran1 complex may act as a sensor of DNA replication (Roberge, 1992). However the effect on the checkpoint caused by the mutations in the *RCC1* gene may be due to pleiotropic effects. The Rcc1 structurally-related protein of fission yeast, dcd1/pim1 has been proposed to be required for the mitosis-interphase transition (Sazer and Nurse, 1994; Matsumoto and Beach, 1991). This protein, contrary to a previous report (Matsumoto and Beach, 1991) may have no role in the mitotic checkpoint in this yeast (Sazer and Nurse, 1994).

Mutations in the cdc2 protein kinase have also been shown to cause checkpoint deficiencies. This was of great interest because of the central role of this protein kinase in regulating cell cycle progression in fission yeast and higher eukaryotes (Nurse, 1990). Biochemical studies have established that entry into mitosis in fission yeast and most probably in higher eukaryotes is coupled with dephosphorylation of cdc2 at tyrosine-15 residue, achieved by the activity of the cdc25 protein phosphatase. The *cdc2.3w* mutant is a semi-dominant gain of function mutant which undergoes mitosis at a reduced size (Fantes, 1981) and it is insensitive to the presence of a DNA replication defect (Enoch and Nurse, 1990). A similar phenotype was obtained by overexpression of the mitotic inducer $cdc25^{+}$ (Enoch and Nurse, 1990). These observations have suggested a simple model by which cells can monitor progression of S phase. The checkpoint system would be made up of a sensor, a receiver and a transducer. Signals produced by the presence of unreplicated or damaged DNA (detected by the sensor) would be sent to the cdc2 protein kinase through a biochemical pathway which includes the rad^{+} and hus^{+} functions (signals transducers). These signals would inhibit the activity of the cdc2 protein kinase (receiver) by down-regulation of a tyrosine phosphatase or up-regulation of a tyrosine kinase. A similar regulatory mechanism appears not to be present in *S. cerevisiae*, where the phosphorylation of tyrosine-19 of the Cdc28 protein kinase (the equivalent of cdc2 tyrosine-15) is not limiting for entry into mitosis (Amon *et al.*, 1992). Likewise, mutations in the cdc28 protein kinase or in the mitotic cyclins do not abolish mitotic restraint. Thus, the two yeasts may regulate the mitotic checkpoint using different mechanisms.

Evidence for controls similar to *S. pombe* in higher eukaryotes has been provided by Smythe and Newport, (1992). Checkpoint deficiencies were observed by treatment of *Xenopus* cell-free extracts with the protein serine/threonine phosphatase

inhibitors caffeine and okadaic acid. These authors have shown that these compounds were able to inactivate a tyrosine kinase acting on p34^{cdc2} thus demonstrating that p34^{cdc2} is one component of the S phase feed-back control.

It has been proposed that in fission yeast there may exist two distinct pathways through which signals produced by DNA damage or a DNA replication block may inhibit the activation of the cdc2 protein kinase (Sheldrick and Carr, 1993). Also in *S. cerevisiae* there is evidence for two distinct checkpoints (Weinert *et al.*, 1994).

It has been suggested that the presence of replication complexes on the DNA may be a way to monitor progression of S phase (Kelly *et al.*, 1993; Li and Deshaies, 1993). Evidence for this have been very recently provided. Certain temperature-sensitive mutants in the DNA polymerase ε of budding yeast are checkpoint deficient (Navas *et al.*, 1995). Interestingly, these mutant alleles of DNA polymerase ε were identified as *dun* mutants (*dun2*), previously isolated as *d*amage *uni*nducible mutants (Zhou and Elledge, 1993). Similar phenotypes have been observed in mutants in the DNA polymerase α of fission yeast (G. D'Urso, pers. comm.) and in other mutants thought to be required for the initiation of DNA replication (Kelly *et al.*, 1993; Saka and Yanagida, 1993; Hofmann and Beach, 1994).

Although this field is in rapid expansion and more checkpoint functions are being identified it is becoming clear that the dependency of M phase upon S phase may be controlled by different pathways, some of them overlapping in function.

Temporal and spatial organisation of DNA replication in the nucleus

If the basic molecular mechanisms of the initiation of DNA replication have been strikingly conserved from bacteria to higher eukaryotes *in vitro*, important and fundamental differences arise *in vivo*. First of all, both prokaryotes and eukaryotic viruses rely mainly on a single origin for the duplication of their small genome. In mammals the presence of some 50,000 origins of replication has been estimated (Huberman and Riggs, 1968) as a natural answer to the increased size of the genome. Secondly, the synthesis of chromosomal DNA occurs at a specific stage of the cell cycle, that is the synthetic or S phase period. This means that all the DNA has to be replicated within the length of S phase through co-ordinated firing of a large number of origins within the chromosome. A third point to be considered is that the DNA is packaged into chromatin in higher eukaryotes. Experiments of reconstitution of DNA replication systems *in vitro* have been done using naked DNA (see Stillman, 1989, for review). Furthermore, a cellular process such as DNA replication *in vivo* has also to take account of higher order organisation of chromatin.

It has been known for many years that in mammalian nuclei chromosome replication is temporally regulated. This was observed in regard to the replication of the highly condensed portion of the chromosomes, that is the heterochromatin. Heterochromatic masses replicate always in late S phase if compared to the euchromatic regions. Another observation came from studies of transcriptionally active and inactive alleles of a specific locus in the chromosomes. It was found that most of the time the active allele of a certain locus replicated earlier than the inactive one and this was later extrapolated to all those regions of the genome which are transcriptionally active (Goldman *et al.*, 1984; Hatton *et al.*, 1988). Therefore, a correlation was found between active transcription and early DNA replication. This explained the late replication timing of heterochromatin. More recently, sophisticated analysis of S phase synchronised nuclei has shown that indeed DNA replication of Chinese Hamster ovary cells (CHO) and HeLa cells is subjected to a temporal order in that following BrdU incorporation, five distinct patterns of DNA replication could be identified (O'Keffe *et al.*, 1992).

Use of specific probes has also shown a correlation between imprinting and DNA replication. The paternal allele of four imprinted genes was found always early replicating compared to the maternal one, although both parental alleles were equally transcribed (Kitsberg *et al.*, 1993b). One interpretation is that the structure of the chromatin may be important for the temporal regulation of DNA replication. This possibility has been in part answered by studying the temporal order of activation of *ARSs* in the yeast chromosome. *ARSs* which replicated early and late were identified. By moving an early replicating *ARS* to a late replicating region it was elegantly shown that this temporal regulation was dependent on the position of the *ARS* in the chromosome (Ferguson *et al.*, 1991). This effect required proximity of a yeast telomere (Ferguson and Fangman, 1992). It would be of great interest to identify *ARSs* which replicate late independently of the position in a chromosome.

Detection of the actual sites of DNA replication within the nucleus was first showed by Nakamura *et al.*, (1986). He observed that incorporation of the nucleosides analogue 5-bromodeoxyuridine (BrdU) into nascent DNA chains, visualised by staining nuclei with an antibody against BrdU, was not dispersed all over the nucleus but localised in specific foci. Interestingly the intensity and size of the foci increased for about one hour after which more foci became visible. Similar results were obtained using the *in vitro Xenopus* system (Mills *et al.*, 1989). Because this pattern of replication occurred also if bacteriophage lambda was used as a DNA source, this experiment demonstrated that assembly of pre-initiation clusters and initiation of DNA replication in *Xenopus* is sequence independent (Cox and Laskey, 1991). This observation inspired the idea that initiation of DNA replication might take place in specialised nuclear sub-compartments where DNA replication proteins assemble. This

idea was also supported by earlier observations indicating that a proteinaceous nuclear scaffold structure would give a physical support to the chromosomes (Paulson and Laemmli, 1977) and the suggestion that processes such as DNA replication and transcription might be physically more efficient if the DNA swivels through the enzymes, following the fixed polymerase model of Cook (1991). The presence of a scaffold and other histone-like protein complexes acting as chromosome skeleton has been severely criticised during the past years due to the different results obtained using different extraction procedures; in addition, components of this complex seem to have the tendency to spontaneously aggregate if removed from their natural loci (Cook, 1988; Roberge *et al.*, 1988). A more physiological method to show the presence of such a structure has been developed by Jackson and Cook (1986), who encapsulated living cells within small agarose beads making possible the preparation of nuclei in more physiological salt conditions. Isolated nuclei were able to replicate and transcribe in a way similar to living cells. Additionally, it was predicted that if the replication foci were associated with a nuclear structure, after digesting the chromatin with a restriction enzyme and eluting it off the nucleus, nuclei would be still able to replicate DNA *in situ*. This prediction turned out to be correct as incorporation of BrdU was detected in these digested nuclei (Hozak and Cook, 1994). Moreover, this activity was also found aphidicolin-sensitive, demonstrating that it was DNA polymerase α-dependent. Despite the evidence that sites of DNA replication may be localise to specific sub-nuclear compartments, where some replication proteins are accumulated, a DNA replication system can be reproduced *in vitro* without any added skeleton of matrix proteins (Li and Kelly, 1984; Waga and Stillman, 1994). Hence, it may be argued that the nucleoskeleton is not necessary for DNA replication *per se* although it may important *in vivo* for co-ordination with other cellular processes, such as transcription and recombination. Very recently it has been shown that sites of DNA replication and transcription co-localise (Hassan *et al.*, 1994) suggesting that the two processes may be coupled and that perhaps transcription foci may be centre of nucleation for DNA replication foci. As conflicting results have been reported (Wansink *et al.*, 1994), more work is needed to elucidate the relationship between these two important cellular processes.

The Mcm protein family

These proteins were identified in a screen for minichromosomes maintenance mutants (Maine *et al.*, 1984; Gibson *et al.*, 1990). A summary of the properties and genetic interactions of these mutants is provided in Table 1.1. The *mcm*$^-$ mutants were divided into 18 complementation groups and two phenotypic classes. The first class affected the stability of all circular minichromosomes employed, independently of the *ARS* present, whereas the second class of mutants showed an *ARS* specific *mcm*$^-$ phenotype. Four mutants belonging to the second phenotypic class (*mcm1-2-3* and *5*) have been extensively characterised.

The *MCM1* gene has been shown to encode a multifunctional transcription factor mediating the transcriptional activation of many genes in combination with different co-factors (Passmore *et al.*, 1988, 1989). The Mcm1 protein does not have any sequence homology with the Mcm2-3-5 proteins but is instead a member of a very well conserved class of DNA binding proteins related to serum response factors (SRF; Sprague, 1990). Genetic evidence supports a role for Mcm1 in DNA replication. The lethal-conditional mutant *mcm1-110L* arrests at the restrictive temperature as large budded cells generally with a single nucleus and partially unreplicated DNA (Elble, and Tye, 1992). The *mcm1-110L* mutant also shows high rates of plasmid loss and hyper-recombination, phenotypes associated with a defect in DNA replication. Interestingly, Mcm1 binding sites have been identified in a set of DNA replication genes, such as *MCM2*, *RPA1*, and *CDC28*, although it is not known whether these genes depend upon Mcm1 for their expression (Elble and Tye, 1992).

The *mcm2-3-5* mutants all display similar phenotypes. The *mcm2-1* allele is not lethal but has slow growth at 35°C and a temperature-dependent *mcm*$^-$ phenotype. Only minichromosomes bearing specific *ARSs* were unstable at 25°C whereas all *ARSs* were affected at 35°C. The defect caused by the *mcm2-1* mutation was shown to disturb replication rather than the segregation of minichromosomes (Maiti and Sinha, 1992). A null mutation of *mcm2* is lethal. Cells arrest at the large bud stage with a single nucleus not generally localised at the bud neck. Cells, however, arrested at the end of S phase with almost completely replicated DNA (Yan *et al.*, 1991). It has been very recently shown that the *mcm2-1* mutant arrests with damaged DNA in a *RAD9*-dependent manner (Ray and Shina, 1995). The *mcm3-1* mutant is conditional-lethal and showed phenotypes similar to the *mcm2* null mutant at 37°C (Gibson *et al.*, 1990).

The *MCM2* and *MCM3* genes were found to encode structurally related proteins of a predicted size of 101 and 107 kDa respectively, although they show an electrophoretic mobility consistent with a size of ca. 125 kDa (Gibson *et al.*, 1990; Yan

et al., 1991). There is evidence that these proteins genetically interact. Double mutants of *mcm2* and *mcm3* are inviable at their permissive temperature; moreover overexpression of the Mcm2 protein partially complements the *mcm3-1* mutation, whereas the overexpression of the Mcm3 protein was lethal in *mcm2-1* cells (Yan *et al.*, 1991). The Mcm2 and Mcm3 proteins have been shown to be required for the initiation of DNA replication at chromosomal origins *in vivo* and to be chromatin associated (Yan *et al.*, 1993).

The *MCM5* gene (Chen *et al.*, 1992) was found to be identical to the *CDC46* gene which is required at an early stage of DNA replication in budding yeast (Hennessy *et al.*, 1991). The *CDC46* gene encodes a predicted protein of 95 kDa also structurally related to the Mcm2-3 proteins. The *CDC46* mRNA is periodically transcribed in S phase, whereas the Cdc46 protein is stable throughout the cell cycle. Remarkably the Cdc46 protein was shown to be localised largely in the nuclear compartment at late mitosis, persisting there until a stage in the cell cycle overlapping with the initiation of DNA replication, after which was excluded from the nucleus (Hennessy *et al.*, 1990). The observation that the Cdc46 protein is required for the initiation of DNA replication and that its exit from the nucleus is coincident with the initiation of DNA replication has suggested that it may be the regulator, or a component of the mechanism which limits S phase to only one round of DNA synthesis per cell cycle (Leno *et al.*, 1992; see also the licensing factor hypothesis in this Chapter). A similar regulation has been reported for the Cdc46-related proteins Mcm2-3 (Yan *et al.*, 1993) and Cdc47 (Dalton and Whitbread, 1995), suggesting that they are all under the similar regulatory control.

Interestingly, the *cdc46-1* mutant, as well as the *cdc47-1* mutant, was isolated as an extragenic suppressor of the *cdc54* and *cdc45* cold-sensitive mutants whose wild-type gene products are required for DNA replication (Moir *et al.*, 1982). Double mutants of *cdc46* and *cdc47*; *cdc46* and *cdc54*; *cdc47* and *cdc54* are inviable at the permissive temperature (synthetic lethals). This network of suppression and synthetic lethality has suggested that these proteins may all interact (Hennessy *et al.*, 1991). The Cdc54 protein may be a *S. cerevisiae* homologue of the fission yeast cdc21 protein (Whitbread and Dalton, 1995; see also Chapter Three).

Proteins related to Mcm2-3-5 have been genetically identified in fission yeast. These are the cdc19, cdc21, nda1, nda4 and mis5 proteins.

The *cdc21*^{ts} and *cdc19*^{ts} mutants were isolated during a screen for mutants affecting the cell division cycle and DNA replication. The *cdc21-M68* mutant, which is the main subject of this dissertation, was shown to have an altered round of DNA replication at the restrictive temperature and was proposed to affect the initiation step of DNA replication (Nasmyth and Nurse, 1981). Cdc21 is a nuclear protein which, unlike the Mcm2-3-5 proteins of budding yeast, is localised into the nucleus throughout the cell cycle (Maiorano *et al.*, in preparation).

Unlike *cdc21*, the *cdc19*^{ts} mutant does not affect the kinetics of DNA replication after shift to the restrictive temperature (Nasmyth and Nurse, 1981). However, a null allele of *cdc19* manages to replicate the DNA, although at a very slow rate, suggesting that the cdc19 protein may not be absolutely required for DNA replication (Forsburg and Nurse, 1994). The cdc19 protein may be a fission yeast homologue of the budding yeast Mcm2 (Forsburg and Nurse, 1994). The *cdc19*$^{+}$ gene was found to be identical to the previously identified *nda1*$^{+}$ gene (Miyake *et al.*, 1993), previously isolated in a screen for mutants showing an early nuclear division arrest phenotype at the restrictive temperature (Toda *et al.*, 1983). The *nda1-108* allele was shown to arrest in the G1/S phase (Miyake *et al.*, 1993), although in another study the same allele was shown to arrest in late S phase (Forsburg and Nurse, 1994)

The *nda4* cold sensitive mutant has been shown to have altered kinetics of DNA replication after shift to the restrictive temperature. Moreover, the *nda4-108* mutation was shown to be partially suppressed by Ca^{2+} (Miyake *et al.*, 1993). The nda4 protein may be a fission yeast homologue of the budding yeast Cdc46.

The *mis5-268* mutant was isolated in a screening for mutants causing minichromosomes instability (Takahashi *et al.*, 1994). The *mis5-268* mutant affects the maintenance of minichromosomes, is hypersensitive to hydroxyurea and sensitive to UV irradiation at the permissive temperature. Mutant cells shifted at 36°C show cell cycle arrest and an aberrant round of DNA replication. Chromosomes of the arrested cells were found to be damaged indicating that DNA synthesis was defective. The effect on DNA replication was similar if cells were released at the restrictive temperature after nitrogen starvation (Takahashi *et al.*, 1994). These data are consistent with a role for the mis5 protein in DNA replication and/or repair.

Double mutants of *cdc21* and *cdc19* or *cdc21* and *mis5* are inviable at the permissive temperature (Forsburg and Nurse, 1994; Takahashi *et al.*, 1994). Thus it is likely that, as for budding yeast, fission yeast *MCM2-3-5* related-genes may interact.

Mammalian structural homologues of the Mcm proteins have also been described.

The first mammalian Mcm protein to be characterised was the P1 protein, closely related to the budding yeast Mcm3 protein (Thömmes *et al.*, 1992). Interestingly, P1 was identified during a purification step of a DNA polymerase α-primase holoenzyme complex as a co-purifying protein. However it is not clear whether the association between the P1 protein and the DNA polymerase complex is specific and functionally significant. It has been very recently pointed out that the P1 protein may have been a contaminant of the DNA polymerase α-primase complex preparation due to some similar chromatographic properties (Burkhart *et al.*, 1995). Polyclonal antibodies raised against the P1 protein localise the antigen into the nucleus of mouse

cells. No cell cycle dependent subcellular localisation has been reported for this protein so far. A cDNA encoding a *Xenopus* P1-related protein was identified, as inducible by thyroid hormone in limb (Buckbinder and Brown, 1992). Further investigation of the properties of this protein have not been reported to date.

Another mammalian Mcm protein has been characterised, namely the BM28 protein which has a very close similarity with the budding yeast Mcm2 protein (Todorov *et al.*, 1994). Its cDNA was isolated by screening of a colon carcinoma cDNA expression library using an antibody raised against proliferating nuclear antigens (Todorov *et al.*, 1991). Microinjection of BM28 affinity-purified antibodies resulted in inhibition of DNA synthesis and cell division (Todorov *et al.*, 1994). Based on these results these authors have suggested that the protein may have two execution points, one at a very early stage of S phase, and the second at mitosis. This might be different from yeast where the Mcm2-3 proteins have been shown not to be required for mitosis (Yan *et al.*, 1993; Ray and Shina, 1995).

A similar approach has been adopted to identify Mcm-related proteins in human cells (Hu *et al.*, 1993). Eight cDNA clones were found to potentially encode structural homologues of the budding yeast Mcm3 and Cdc46 proteins and fission yeast Cdc21 protein. Interestingly, the translation of 3 partially overlapping cDNA sequences of a total length of 1.8kb, produced a predicted protein with 46% similarity to the human and mouse P1 protein and designated P1.1^{Mcm3}. Very recently the human P1 and Cdc46 proteins have been shown to interact physically *in vitro* and *in vivo* (Burkhart *et al.*, 1995). These authors have reported that P1/Cdc46 complexes occurs as dimers and in high-molecular-mass complexes (ca. 500 kDa); the high molecular mass complexes dissociate at 0.5M NaCl releasing P1/Cdc46 complexes. Thus it may be likely that the Mcm proteins physically interact and that this interaction may be important to stimulate DNA replication.

A P1/Mcm3-like protein, the B_{24} protein, has been identified in newts as associated with cell proliferation (Bucci *et al.*, 1993). A cDNA encoding for the B_{24} protein was isolated by screening of an ovary cDNA expression library with a monoclonal antibody raised against proteins of the frogs oocytes. Curiously, the protein is apparently not expressed in somatic tissues. A role for this protein in the assembly of snRPs of the oocyte has also been proposed (Bucci *et al.*, 1993).

Despite the extensive genetic characterisation of the Mcm proteins in yeast, to date, it is not clear what the specific role of the Mcm proteins in DNA replication may be.

New insights have been provided by the recent work of Kimura *et al.*, (1994) and Todorov *et al.*, (1995).

Kimura and co-workers raised a polyclonal antibody against the mouse P1 protein and showed that this was located in the nucleus throughout the cell cycle. However, the intra-nuclear distribution of this protein was shown to change during S phase. Its pattern of distribution did not overlap with that of the DNA polymerase δ-associated protein PCNA, neither with that of cdk2 nor cyclin A. On the contrary, the P1 staining was brighter in the heterochromatic regions during late S phase. These observations have been reproduced in an independent study (Starborg *et al.*, 1995). From these observations it was concluded that the P1 protein may be associated with unreplicated DNA. This in agreement with earlier observations made by Todorov *et al.*, (1994) showing that the BM28 protein did not colocalise with sites of active DNA replication. In addition, the P1 protein has been shown to be phosphorylated at a level which increased during S phase. Thus, there may be two populations of P1 in the cell; the first form, hypophosphorylated and bound to nuclear non-chromatin structures; the second is hyperphosphorylated form, is free in the nucleoplasm. Kimura *et al.*, (1994) propose that the hyperphosphorylation of P1 may be coincident with its release from the nuclear structure after DNA replication. Finally, microinjection of affinity purified antibodies in 3T3 cells inhibited DNA synthesis. A similar behaviour has been recently reported for the human P1 protein (Schulte *et al.*, 1995).

Similar results, although with some differences, have been independently obtained by studying the human BM28 protein. The BM28 protein, like the budding yeast Mcm2 protein (Yan *et al.*, 1993), is chromatin associated. The BM28 protein is a phosphoprotein present in the nucleus of human cells in two different isoforms, differing in their phosphorylation state. Interestingly, the relative abundance of the two isoforms changes during the cell cycle, the mobility of the protein moving from a slow migrating form to a fast migrating form as cells proceed from the G1 to the G2 phases. The extractability of the BM28 protein from nuclei is also cell cycle-dependent; the protein is tightly bound to chromatin during the G1 phase and becomes more soluble as DNA replication proceeds. Todorov *et al.*, (1995) have proposed a model in which the BM28 protein, alone or in combination with other proteins, is chromatin bound in the G1 phase and it is displaced from chromatin as DNA replication proceeds, perhaps by the movement of the replication fork. Post-translational modifications of the BM28 protein may have a key role in regulating its re-association with chromatin (Todorov *et al.*, 1995).

In summary the information available so far on the Mcm proteins indicate that:

a) Members of the Mcm protein family are essential for DNA replication. This may not be true for all of them;

b) Mcm proteins may physically interact to stimulate DNA replication;

c) Some members of the Mcm protein family (e.g., yeast Mcm2 and Mcm3; BM28) are chromatin associated, other members (e.g., P1 and Cdc46) may instead be associated with nuclear non-chromatin structures;
d) members of the Mcm protein family are usually not associated with replication foci; they may be associated instead with unreplicated chromatin;

e) The cell cycle-dependent nuclear localisation of members of the Mcm protein family seen in budding yeast has not been conserved in higher eukaryotes, or in fission yeast.

Koonin, (1993) has recently pointed out that the Mcm proteins share a conserved NTPase motif which is conserved in a class of prokaryotic transcription regulators related to NtrC protein which is a DNA-stimulated ATPase (Weiss *et al.*, 1991; Austin and Dixon, 1992), making attractive the idea that these proteins may have a role in the unwinding step of DNA replication acting as helicases. However, the shared epitope is not perfectly conserved. Furthermore, there is one case where a prokaryotic protein containing a perfect match to a helicase motif apparently lacks helicase activity (Selby and Sancar, 1993). It remains to be shown whether the structural homology between members of the cdc21 protein family and these prokaryotic helicases has also a functional significance.

Allele	Arrest phenotype	Cell cycle arrest	Genetic interactions	References
S. cerevisiae				
cdc45-1	cs	2C (15°C) 1C (12°C)	suppressed by *cdc47-1* and *cdc46-1*	Hennessy *et al.*, 1991
*cdc46-1/ mcm5**	ts	1C (37°C)	suppresses *cdc45-1*; lethal to *cdc 47-1* and *cdc54-1*; synthetically lethal with *orc6-1*	Hennessy *et al.*, 1990 and 1991; Chen *et al.*, 1992 Li and Herskowitz 1993
*cdc46-5/ mcm5**	ts	2C (37°C)	suppresses *cdc54-1*	Hennessy *et al.*, 1991
*cdc47-1**	ts	2C (37°C)	suppresses *cdc45-1*; lethal to *cdc54-1*	Hennessy *et al.*, 1991
*cdc54-1**	cs		suppressed by *cdc46-5*; lethal to *cdc46-1* and *cdc47-1*	Hennessy *et al.*, 1991
cdc45-1 cdc47-1	ts/cs	intermediate beteewn 1C and 2C (37°C)		Hennessy *et al.*, 1991
mcm1-110-L	*mcm*⁻;ts	almost 2C (36°C)	transcription factor, interacts with a and α proteins;	Passmore *et al.*, 1989; Elble and Tye, 1992
*mcm2-1**	*mcm*⁻; slow growth at 37°C	S/G2 (null)	synthetically lethal with *mcm3-1*; lethality by Mcm3 OP	Yan *et al.*, 1991
*mcm3-1**	ts; *mcm*⁻	2C (37°C)	synthetically lethal with *mcm2-1*; suppressed by Mcm2 OP	Yan *et al.*, 1991
S. pombe				
*cdc21-M68**	ts	2C (37°C)	synthetically lethal with *cdc19-P1*	Nasmyth and Nurse, 1981; Coxon *et al.*, 1992; Forsburg and Nurse, 1994;
*nda4-108**	cs	intermediate between 1C and 2C (20°C)	suppressed by Ca^{2+}	Miyake *et al.*, 1993;
*nda1-376**	cs	almost 2C (20°C)		Miyake *et al.*, 1993;
*cdc19-P1**	ts	2C (36°C)	synthetically lethal with *cdc21-M68*	Nasmyth and Nurse, 1981; Forsburg and Nurse, 1994
*mis5-268**	ts; HU and UV hyper-sensitive	intermediate between 1C and 2C (36°C)	synthetically lethal with *cdc21-M68*	Takahashi *et al.*, 1994

Table 1.1

Phenotypes and genetic interactions of *S. cerevisiae* and *S. pombe* *mcm*⁻ and related mutants.

Structurally related genes are indicated by a star (*). The cell cycle arrest phenotype refers to the DNA content of the mutant cells measured by flow cytometry. 1C implies a cell cycle arrest at the G1/S boundary; 2C implies a cell cycle arrest at the end of S phase or in G2; intermediate values imply an arrest in S phase. OP= overexpression; HU= hydroxyurea. cs=cold sensitive; ts= temperature-sensitive.

Aim of this work

The aims of this thesis were to clarify the role of the fission yeast *cdc21*$^+$ and related genes in chromosome replication. Previous work had established that a number of proteins structurally related to the fission yeast cdc21 protein were present in various organisms (Coxon *et al.*, 1992). More cdc21-related proteins have been identified in a wide range of eukaryotes during the past three years (see Chapter One). Nevertheless, the question of how many *cdc21*$^+$-related genes can be found in each organism had not yet been addressed. A small sized organism, such as yeast, represents a suitable model system that can be used to approach this problem. I have investigated this issue by performing phylogeny analysis using the sequences of cdc21-related proteins and by searching for *cdc21*$^+$-related sequences in the yeast genomes. The specific biochemical role of these proteins in DNA replication is to date obscure. Genetic data had shown that each distinct protein is essential and that these proteins may genetically interact, consistent with their involvement in the same genetic pathway or in the same protein complex. I have shown that although related in structure, members of this protein family fail to complement each other, consistent with previous studies.

Genetic characterisation of the *cdc21* temperature-sensitive mutant indicated that the *cdc21*$^+$ gene may be required at an early step of chromosome replication (Nasmyth and Nurse, 1981). However, determination of the DNA content of this mutant strain showed that DNA replication was not blocked at the restrictive temperature (Coxon *et al.*, 1992), implying the either the *cdc21*$^+$ gene may be not absolutely required for DNA replication or that the *cdc21-M68* allele is not a severe an allele of *cdc21*. Hence I have constructed new mutant alleles of *cdc21* in order to determine if they display phenotypes similar to the original temperature-sensitive allele.

The identification of proteins interacting with cdc21 was also attempted. This was in order to gain insight into the biochemical function by which cdc21 may act. Two cDNAs encoding two functions which could potentially interact with the *cdc21*$^+$ gene product were also identified. Characterisation of these functions was attempted.

Materials and Methods

Reagents

Reagents were generally obtained from Sigma or BDH and were of AnalaR or equivalent grade. DNA modifying enzymes were obtained from New England Biolabs or Pharmacia. The remaining reagents were obtained from Fluka or Bethesda Research Limited.

40% Acrylamide

190g acrylamide
10g N,N'-methylene-bis-acrylamide
Made up to 500ml, stirred with 10g Amberlite MB-1 resin, then filtered through sintered glass. Stored at 4°C in the dark.

Acrylamide Top Mix for sequencing gels

urea	153.6g
40% acrylamide	48ml
10x TBE	16ml
dH_2O to 320ml	

Components were mixed with moderate heating to help the urea to dissolve. The solution was filter sterilised, degassed and then stored at 4°C

Acrylamide bottom mix for sequencing gels

urea	57.6g
sucrose	12g
10x TBE	15ml
40% acrylamide	18ml
bromophenol blue	5mg

Made up to 120ml with dH_2O as above and stored at room temperature in the dark.

Agarose gel loading dye

0.25% bromophenol blue
40% w/v sucrose
6x TBE
Stored at room temperature.

Alkaline lysis solution 1

50mM glucose
25mM Tris pH 8.0
10mM EDTA
Made up to 100ml in dH_2O, autoclaved and stored at 4°C.

Alkaline lysis solution 2

0.2M NaOH
1% SDS
Made up fresh to 1-10ml in dH_2O.

APS (25%)

dissolved in dH_20 and filter sterilised. Stored at 4°C and used within one week.

Breaking buffer

1% Triton X-100
1mM PMSF
20µg/ml leupeptin
40µg/ml aprotinin
Made up fresh in dH_2O.

Calcofluor

1mg/ml stock made up in dH_2O and stored at 4°C in a dark glass bottle.

Citrate/Phosphate

(50mM solution)

Na_2HPO_4	7.1g/l
citric acid	11.5g/l

pH 5.6 with NaOH

Autoclaved and stored at room temperature.

CSE

20mM Citrate/Phosphate

40mM EDTA

1.2M sorbitol

pH 5.6 with NaOH

Autoclaved and stored at room temperature.

Denhardt's solution

1% Bovine serum albumin (Sigma, fraction V)

1% Ficoll 400

1% polyvinylpyrrolidone 40

Made up in dH_2O and stored at -20°C.

50mM dNTPs

dTTP 312mg/10ml in TE (10mM Tris pH 8.5, 0.1mM EDTA)

dCTP 296mg/10ml in TE

dGTP 316mg/10ml in TE

dATP 295mg/10ml in TE

dNTP chase

this was made up using 0.25mM of each dNTP using 50mM stock and TE.

ETS

0.25M EDTA
5mM TrisHCl
1% SDS

10X Hin buffer

100mM Tris pH 7.5
500mM NaCl
100mM $MgCl_2$
prepared fresh.

HYB

30ml 20X SCP
4ml 25% sodium lauryl sarcosine
62ml dH_2O
Stored at room temperature.

NDS

0.5M EDTA pH 9.5
1% sodium lauroyl sarcosine
0.5mg/ml Proteinase K (Boehringer Mannheim)
Made up fresh.

Oligolabelling solution O

1.25M Tris-Cl pH 8.0
0.125M $MgCl_2$
Made up in dH_2O and stored at -20°C.

Oligolabelling solution A

1ml oligolabelling solution O
18µl β-mercaptoethanol
10µl 50mM dTTP
10µl 50mM dGTP
10µl 50mM dATP
Stored at -20°C.

Oligolabelling solution B

2M HEPES pH 6.6 (pH with 4M NaOH). Autoclaved and stored at 4°C.

Oligolabelling solution C

Random hexanucleotides (Pharmacia) were dissolved at 90 OD_{260} units/ml in TE and stored at -20°C.

Oligolabelling Buffer (OLB)

Oligolabelling solutions A:B:C were mixed in the ratio 100:250:150 and stored at -20°C.

RNase A

Ribonuclease A stocks (Sigma) were made up at 10mg/ml and boiled for 10 minutes to remove any residual DNase activity. Stored at -20°C in aliquots.

Salmon sperm DNA

100mg of salmon sperm DNA was dissolved in 15ml of dH_20. When dissolved (after 2-3 days) the DNA was sonicated and then heated to 100°C for 20 minutes. EDTA was added to 0.1mM, Tris pH 8.0 to 10mM, and stored at -20°C.

20X SCP

2M NaCl
0.6M Na_2HPO_4
0.02M Na_2EDTA
The reagents were dissolved in 700ml of dH_2O and the pH adjusted to 6.2 with HCl before making up to 1 litre. The solution was autoclaved and stored at room temperature.

Sodium lauryl sarcosin

Made up as a 25% solution in dH_2O by heating at 65°C.
Stored at room temperature.

20X SSC

3M NaCl
0.3M *tri*-sodium citrate
Made up to 1 litre with water, autoclaved and stored at room temperature.

20X TAE

97g Tris Base
6.75g Na_2EDTA
dissolve in 800ml of dH_2O, adjust to pH 8.0 with glacial acetic acid and make up to 1 litre with dH_2O. Autoclave and store at 4°C.

10X TBE

0.9M Tris base
0.9M boric acid
25mM Na_2EDTA
Made up to 1 litre with water, autoclaved and stored at 4°C in the dark.

TE

10mM Tris-HCl
1mM EDTA (pH7.5-8.0 or 8.5)
stored at room temperature

TFB I

10mM $CaCl_2$
100mM $RbCl_2$
50mM $MnCl_2.4H_2O$
30mM potasssium acetate
15% (v/v) glycerol
pH was adjusted to 5.8 with 0.2M acetic acid and filter sterilised. Stored at 4°C.

TFBII

10mM MOPS or PIPES
10mM $RbCl_2$
75mM $CaCl_2.2H_2O$
15% (w/v) glycerol
pH was adjusted to 7.0 with KOH and filter sterilised.
Stored at 4°C.

TSE

10mM Tris·HCl
0.125 M EDTA
0.9M Sorbitol
pH 7.6 with HCl
Autoclaved and stored at 4°C.

Cell culture

Escherichia coli

Media

LB or antibiotic plates

tryptone	10g
yeast extract	5g
NaCl	10g
agar	15g

dH_2O was added to 1 litre, the media autoclaved and when cool (below 60°C) 1ml of 50mg/ml stock of ampicillin was added.

2X TY

tryptone	16g
yeast extract	10g
NaCl	5g

Made up to 1 litre with dH_2O and autoclaved.

Schizosaccharomyces pombe

The fission yeast, *Schizosaccharomyces pombe* , is considered as the best defined yeast after the related *Ascomycete S. cerevisiae*. Most of the molecular techniques employed for *S. cerevisiae* have also been adapted for *S. pombe*, with appropriate modifications. General methods of maintenance and growth of *S. pombe* cells as well as general molecular genetics techniques described in this Chapter have been adapted from those recommended by Moreno *et al.*, (1991).

Media

1) Edinburgh minimal medium (EMM)

	g/l
KH phthallate	3.0
Na_2HPO_4	2.2
NH_4Cl	5.0
1M NaOH	1ml
(agar	20g)

dH_2O to 850ml

Medium was autoclaved and then the following components were added:

Stock	ml
glucose (20% solution)	100
salts	20
vitamins	1
minerals	0.1

Supplements (as required) were added from a 7.5g/l stock solution:

adenine	10ml
histidine	10ml
leucine	10ml
uracil (3.75g/l)	20ml

1:1:2 chlorobenzene: dichloroethane: chlorobutane was added after autoclaving as a preservative. 4ml of phloxin B stock solution (10mg/ml) was added to one litre of agar if required.

2) EMMG

As EMM using 5g/l or 1g/l sodium glutamate instead of NH_4Cl as a nitrogen source.

3) Yeast Extract Supplemented (YES)

	g/l
Difco Yeast Extract	5.0
Glucose	30.0
agar	20.0

All supplements (also lysine for YES solid media) were added at the stated concentration after autoclaving. 4ml of Phloxin B stock solution was added to one litre of agar if required.

4) Malt Extract medium (ME)

	g/l
Difco Bacto-malt extract	30
agar	20

All supplements (as for YES except lysine); adjust to pH 5.5 with NaOH and autoclave.

5) Nitrogen Starvation Media

	g/l
Yeast nitrogen base without amino acids or $(NH_4)SO_4$	6.8
Glucose	20.0

6) Salts Stock

$MgCl_2.6H_2O$	53.5
$CaCl_2.2H_20$	0.74
KCl	50.0
Na_2SO_4	2.0

Made up to 1 litre with dH_2O and autoclaved.

7) Minerals x 10,000

	g/l
H_3BO_4	0.5
$CuSO_4.5H_2O$	0.04
KI	0.1
$FeCl_3.6H_2O$	0.2
$MnSO_4.H_2O$	0.4
$Na_2MoO_4.2H_2O$	0.2
$ZnSO_4.7H_2O$	0.4

Made up to 100ml with dH_2O and filter sterilised.

8) Vitamins x 1000

	g/l
Calcium pentothenate	0.1
Nicotinic acid	1.0
Mersoinositol	1.0
Biotin	0.001

Made up to 100ml with dH_2O and filter sterilised.

9) Amino Acids

	g/l
Adenine	7.5
Histidine	7.5
Leucine	7.5
Uracil	3.75

The stocks were made up with dH_2O and sterilised by autoclaving. This stock was used in all media at a concentration of 0.075g/l except where stated otherwise.

S. pombe life cycle

S. pombe is a rod-like shaped haploid organism which grows around 3-4 μm in diameter and 7-15 μm in length by tip extension and reproduces asexually by medial fission producing two equally sized daughter cells (see Figure 2.1). Haploid cells can be of two mating types, called h^- and h^+. A mixed population of h^- and h^+ cells can only mate if arrested in the G1 phase of the cell cycle (e.g., by nitrogen starvation). Nuclear fusion takes place thus producing diploid zygotes. These zygotes are usually very unstable and immediately enter the meiotic cell cycle followed by sporulation, unless transferred to rich medium. The product of meiosis is a four-spored ascus (zygotic

ascus) which when put in favourable growth conditions releases its content and the four liberated spores (ascospores) can now germinate and re-enter the mitotic cell cycle. In certain conditions of growth diploid cells can be also propagated. These cells, if induced to sporulate by nitrogen starvation, will produce a four-spored ascus (azygotic ascus) morphologically distinct from the zygotic ascus (see Figure 2.1). Haploid ascospores released form the azygotic ascus can re-enter the mitotic cell cycle under favourable conditions of growth.

S. pombe cell cycle

In standard conditions of growth (32°C in minimal medium, EMM) the *S. pombe* cell cycle is about 2 hours and 30 minutes long. S phase and mitosis are separated by two gaps, G1 and G2 (Figure 2.2). In G1 cells have to grow in order to reach a critical size required to enter S phase. M, G1 and S phase are short, taking each one-tenth of the cell cycle. After mitosis cells go straight into S phase which is almost completed by the time septation and cytokinesis has occurred, so 1C cells are not seen if the DNA content / cell is measured for a rapidly growing population.

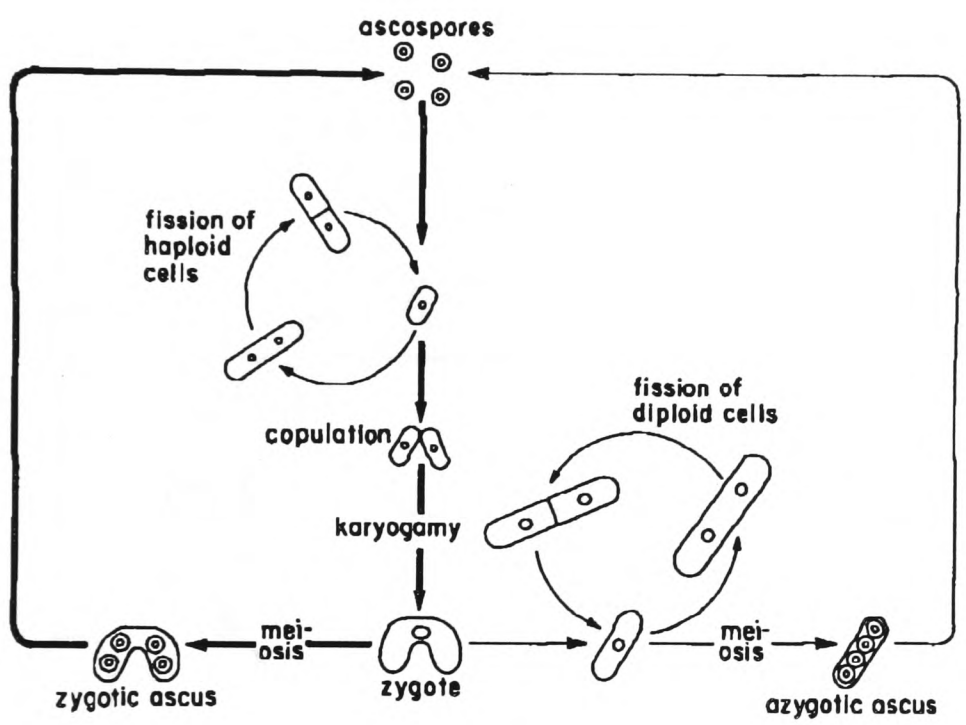


Figure 2.1

Life cycle of fission yeast, *Schizosaccharomyces pombe*.

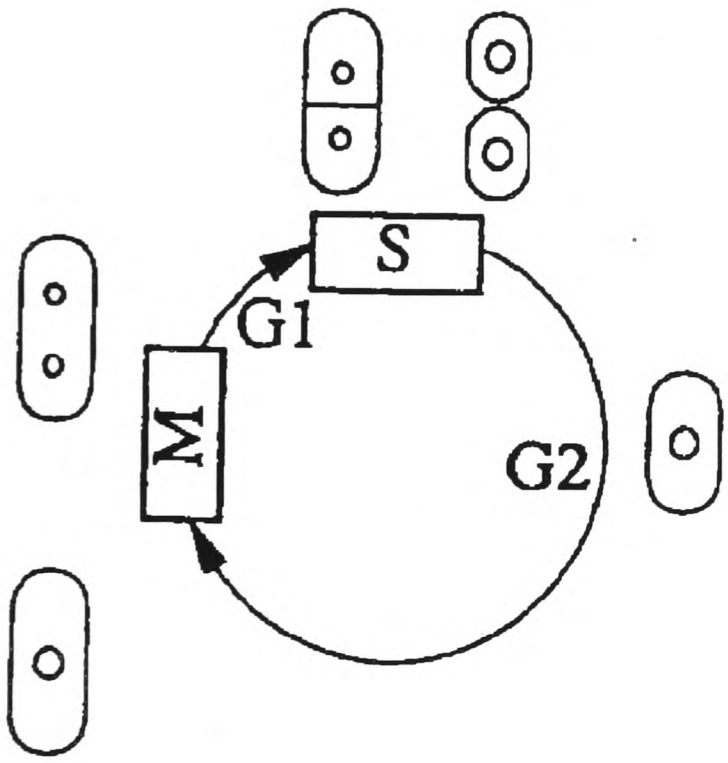


Figure 2.2

The cell cycle of S*chizosaccharomyces pombe*

General methods

Purification of genomic DNA

Yeast

A sample of *S. cerevisiae* genomic DNA (extracted from strain P700: a *trp1 ura3-52 his3 leu2 ade2*) was kindly provided by S. Kearsey.

Large scale preparations of fission yeast genomic DNA were made as described by Moreno *et al.*, (1991).

Small scale preparations of *S. pombe* genomic DNA were made using a modified protocol obtained from Dr. K.Labib (Zoology Department, Oxford University) as follows.

10 ml culture of stationary *S. pombe* cells were collected by centrifugation at 3 krpm for 5 minutes at room temperature in a Jouan bench top centrifuge and resuspended in 1ml of 50mM Citrate/Phosphate pH 5.6; 40mM EDTA pH 8.0; 1.2M sorbitol containing 2.5 mg of Zymolyase-20T.

Cell walls were digested by incubating cells at 37°C for 30-60 minutes. Once digestion was completed (as judged by monitoring cell lysis under light microscopy in the presence of 1% SDS), cells were pelleted for 10 seconds in a microfuge and resuspended in 0.55ml of 1X TE, 1% SDS solution. 175µl of 5M KAc solution were added and the sample was incubated on ice for 15 minutes.

After incubation cells were spun down at 14 krpm for 15 minutes at 4°C and the supernatant was collected in a fresh tube avoiding cell debris. At the supernatant were added 0.5ml of ice-cold isopropanol, mixed gently and incubated at -20°C for 10 minutes.

The sample was then spun at 14krpm for 15 minutes at 4°C and briefly washed with 1ml of cold 70% EtOH. The pellet was briefly air-dried and resuspended in 0.35ml of 1X TE containing 50µg/ml of RNaseA and incubated at 65°C for 10 minutes.

Nucleic acids were purified from proteins by extracting the sample twice with an equal volume of phenol/chloroform 1:1, adding 1/10 volume of 3M NaAc and 2.5 volumes of absolute EtOH, by incubating on a dry-ice EtOH bath for 10 minutes.

The DNA was finally collected by centrifugation at 10 krpm at 4°C for 10 minutes in a microfuge and the pellet washed with 1ml of ice-cold 70% EtOH solution. The pellet was vacuum dried and resuspended 1n 20-50µl of 1X TE pH 8.0.

X. laevis

A DNA sample was kindly provided by Dr. J. Slack, ICRF Developmental Biology Unit, Department of Zoology, Oxford University.

H. sapiens

A DNA sample from Hela cells was kindly donated by Dr. Martin Pera, CRC Growth Factors Group, Department of Zoology, Oxford University.

D. melanogaster

DNA was purified from wild-type flies (kindly provided by Anita Taylor), ICRF, Developmental Biology Unit, Department of Zoology, Oxford University), using the quick fly DNA procedure described below.

About 100 frozen flies were homogenised in a Dounce in 2 ml of "quick fly" buffer (80mM EDTA; 100mM Tris; 160mM sucrose; 0.5% SDS). The dounce was rinsed with 1ml of the same buffer and the homogenised flies were incubated at 65°C for 30 minutes. After incubation the homogenate was left to cool at room temperature, 0.45ml of a 8M potassium acetate solution was added and then incubated on ice for 30 minutes.

The sample was therefore spun at 10 krpm for 10 minutes at 4°C in a microfuge. The supernatant was clarified by centrifugation as described above, collected in a fresh tube and left to equilibrate at room temperature. An equal volume of absolute EtOH was added, mixed gently by inversion and spun at 10 krpm for 5 minutes at 20°C. Pellet was resuspended in 1ml of TE and 0.1ml of a 4M NaCl solution was added. After careful mixing the sample was spun at 10 krpm for 10 minutes at 4°C. An equal volume of EtOH was added to the supernatant and incubated at room temperature for 5 minutes. Sample was spun at 10 krpm for 5 minutes at 20°C and the pellet washed twice with a 70% EtOH solution. The vacuum dried pellet was finally resuspended in 0.2ml of TE. 15µl of DNA were used for restriction enzyme digestion and appropriate dilutions were used for PCR analysis.

Isolation and purification of restriction fragments

Isolation

This method has been adapted from that of Heery *et al.*, (1990). DNA digested with restriction enzymes was fractionated on an agarose gel containing 1µg/ml of EtBr. In the mean time the bottom of a 0.7ml eppendorf tube was pierced with a needle and a very small amount of glass wool placed in it. This tube was then placed inside a 1.5ml lidless eppendorf tube. After electrophoresis the products of the digestion were visualised using a long wave UV lamp and the desired band was cut out the gel in small volume of agarose using a scalpel blade.

Purification

The excised band was placed into the 0.7ml eppendorf tube and incubated at -70°C for 15 minutes. Sephadex G-50 column was set up in a 1ml plastic syringe as follows: a syringe was plugged with glass wool and Sephadex G-50 resin was poured inside until it had reached the top. The syringe was then spun down in a 15ml Falcon tube at 3 krpm for 5 minutes at room temperature in a Jouan benchtop centrifuge and then equilibrated with 100µl of sterile water. After incubation at -70°C, the 0.7ml tube containing the DNA was spun down in a microfuge inside the 1.5ml tube at 12 krpm for 5 minutes at room temperature and the eluate directly loaded onto the G-50 column. The DNA was spun down for 5 minutes at 3 krpm at room temperature and the eluate EtOH precipitated at -70°C for 30 minutes and resuspended in a suitable volume of TE (pH 7.5).

Southern blot analysis of DNA

Genomic DNA digested was digested with restriction enzymes and subjected to electrophoresis on agarose gel as described (Sambrook *et al.*, 1991). After electrophoresis, the gel was blotted to a nylon membrane as follows.

The DNA was first depurinated by soaking the agarose gel in a 0.25M HCl solution for 12 minutes. The gel was briefly rinsed in water and then the DNA denatured for 30 minutes at room temperature by addition of a 0.5M NaOH, 0.5 NaCl solution.

After rinsing in water, the denaturation solution was neutralised by addition of a 0.5M TrisHCl, (pH 7.5), 10X SSC solution and incubated at room temperature for 40 minutes. The gel was then blotted for 16-24 hours in 10X SSC as described (Sambrook *et al.*, 1991). After blotting the membrane was rinsed in 6X SSC for 5 minutes at room temperature and then air dried. DNA was cross-linked to the membrane by incubation at 80°C under vacuum for 2 hours. The efficiency of blotting was determined by staining the agarose gel with 1μg/ml of EtBr in 2X SSC. Nylon membranes were usually stored at room temperature until ready to be hybridised.

Hybridisation of genomic DNA to radio-labelled probes

Labelling DNA fragments

a) dsDNA probes

Radio-labelled probes, unless otherwise indicated, were prepared using the multiprime DNA labelling method of Feinberg and Vogelstein (1984) with some modifications. Reactions were set up as follows: about 50ng of DNA was denatured at 100°C for 3 minutes and then left to cool down by incubation at 37°C for at least 10 minutes. The labelling reaction was set up in the following order: water to 50μl; 10μl of OLB solution; 20μg of BSA (New England BioLabs); 50μCi of [α-^{32}P]dCTP (5 μl) (NEN); 2 Units of large *E. coli* DNA polymerase fragment (Klenow enzyme; Pharmacia). Incorporation of the label was performed at 37° for 20 minutes (instead that over-night at room temperature) and then stopped by addition of 2μl of 0.5M EDTA pH 8.0. Probes were purified from the unincorporated label by chromatography on a Sephadex G-50 column.

b) ssDNA probes

Probes described in the legend of Figure 3.5 were synthesised as follows.

Annealing reaction

About 200ng of ssM13mp9 A50 and A136 DNAs (given by A. Coxon; see table 3.4) were mixed with 1pg of M13 ssDNA primer (17-mer, forward; Pharmacia) in 1X Hin buffer (final volume of 10μl) by heating the mixture at 75°C for 1 minute and then leaving to cool down to 38°C. The mixture was kept on ice until used.

Extension reaction

At the annealing reaction was added

1µl of 1.2mM dGTP, dATP and dTTP nucleotides mix;
1µl of 0.12mM dCTP;
30µCi of [α-^{32}P]dCTP (3µl);
1 unit of Klenow fragment

The mixture was incubated at room temperature for 7 minutes, after which the incorporation of the label was chased by addition of 1µl of a 1.2mM dCTP solution. Reaction was further incubated at room temperature for 15 minutes and the Klenow enzyme was heat inactivated at 70°C for 5 minutes.

Digestion

The newly synthesised and labelled dsDNA was digested with *Hin*dIII (24 units) in a final volume of 20µl by incubation at 37°C for 20 minutes. Reaction was stopped by addition of an equal volume of formamide dyes (sequencing kit STOP solution; USB) and kept on ice before loading onto a denaturing polyacrylamide preparative gel on a 3cm wide slot.

Electrophoresis

Samples were denatured at 100°C for 2 minutes and chilled on ice. Samples were then loaded onto a 4% polyacrylamide gel containing 7M Urea in 1X TBE buffer. Electrophoresis was carried out at 30mA, 1200V for 2 hours at room temperature.

Probes isolation

After electrophoresis the top plate was removed and the gel covered with cling film. Bits of opaque tape were added round the edge of the gel to facilitate the orientation and the gel was laid down on a piece of pre-flashed X-ray film (Fuji). Labelled DNA fragments were visualised by autoradiography for 2 minutes. Gel and film were aligned on a light box and the bands cut out with a number 11 scalpel blade. The cling film was removed before bands were isolated and put in a weighing boat containing a drop of water to avoid drying out.

Probes purification

Isolated bands were purified from the polyacrylamide gel by electroelution. Bands were transferred to a dialysis bag containing water. The excess water was poured off leaving 1.5 ml, the bag sealed with a plastic clamp and the sample electroeluted in a minigel apparatus, containing 90ml of 0.5X TBE buffer, at 40mA for 90 minutes. Probes were recovered by removing the eluate from the bag into 15ml Corex tubes and the bag was rinsed with eluate. Probes were placed on ice until used. About $5x10^5$ cpm per each probe were used in hybridisation.

Hybridisation

Nylon membranes (Hybond™-N, Amersham) were hybridised either in plastic hybridisation bags or in rollers units of a hybridiser oven (Techne). All the hybridisation experiments described in Chapter Three have been performed in hybridisation bags.

Hybridisation bags

Nylon membranes containing immobilised DNA were pre-hybridised in HYB solution (1ml per cm^2 of membrane) in the presence of 1mg of denatured salmon sperm DNA for at least 6 hours at 52°C. The probe (denatured for 5 minutes at 100°C prior hybridisation if double stranded) was added directly to a pre-hybridisation mix. Hybridisation was carried out at 52°C in a shaking bath for 12-16 hours. After hybridisation filters were washed in:

30mM NaCl
60mM TrisHCl (pH 8.0)
2mM EDTA (pH 8.0)
twice at room temperature for 5 minutes;

300mM NaCl
60mM TrisHCl (pH 8.0);
2mM EDTA (pH 8.0)
1% SDS
twice at 60°C for 30 minutes;

30mM NaCl;
6mM TrisHCl (pH 8.0);
0.2mM EDTA (pH 8.0);
twice at room temperature for 30 minutes.

Hybridiser oven

The hybridisation experiments described in Chapter Four and Seven were performed in hybridiser oven.

Nylon membranes containing immobilised DNA were pre-hybridised in 15ml of pre-hybridisation mix (6X SSC, 5X Denhardt's, 0.5% SDS) in the presence of 0.1mg/ml of denatured salmon sperm DNA for at least 6 hours at 65°C. Hybridisation was carried out at 65°C for 12-16 hours. Hybridised nylon membranes were rinsed briefly in 2X SCP, 1% SDS at 50°C twice and then left to wash for more than 10 minutes. Membranes were rinsed briefly in 3mM Tris HCl before drying.

Removing probes from hybridised nylon membranes

Probes were removed using the SDS method as described by Sambrook *et al.*, (1991). Briefly, hybridised membranes were immersed in about 800ml of a solution containing 10mM Tris HCl pH 7.5 at 100°C. The solution was then removed from the heat source and SDS was added at a final concentration of 0.1% -1%. This procedure was repeated 2-3 times until more than 90% of the counts had been removed, as judged by monitoring the membrane with a Geiger counter. Filters were then rinsed briefly at room temperature in 5mM Tris HCl pH 7.5, briefly wiped between two layers of adsorbent tissues and exposed at -70°C for at least 24 hours.
The predicted size of the hybridisation bands were calculated using the StatView SE+Graphics™ programme.

Polymerase Chain Reaction

Purified lyophilised primers were resuspended in 1 ml of sterile deionized water and stored as 100 ml aliquots at -20°C.
PCR reactions were set up in a 10µl final volume containing 200mM of each dNTPs (Pharmacia); 1µg of each primer; 0.35 units of Taq DNA polymearse (Promega) and 1-10ng of genomic DNA. The mix was overlaid with 15µl of mineral oil and the PCR carried out in a Grant PCR machine. The conditions of amplification were as follows:

melting 95°C/1 minute ('); annealing 55°C/1'; extension 72°C/1'; melting 95°C/1.4' for 35-40 cycles, with a final step of annealing at 50°C 5' and extension at 72°C/5'. The products of the PCR reaction were visualised by EtBr staining (1μg/ml) after electrophoresis on a 1.5% agarose gel (w/v) in 1X TBE buffer.

Purification, isolation and cloning of PCR products

PCR products were gel purified using the glass wool method. Phosphorylation was carried out at 37°C for 30 minutes using 5 units of bacteriophage T4 polynucleotide kinase (Promega) in the presence of 0.2mM ATP in a final volume of 25μl. Phosphorylated DNA was treated with the large fragment of the *E. coli* DNA polymerase (Klenow enzyme) to remove 5' protruding ends as described (Sambrook *et al.*, 1991). Klenow treated PCR products were phenol/chloroform (1:1) treated, EtOH precipitated and resuspended in a suitable volume of TE (pH 7.5). Purified PCR products were ligated into the *Sma*I site of bovine alkaline phosphatase-treated (BAP) pUC18 vector (Pharmacia) in the presence of PEG as described (Sambrook *et al.*, 1991). Recombinant plasmids were recovered in the *E. coli* DH5α strain. The sequence of the PCR products was determined with the dideoxy termination chain method (Sanger *et al.*, 1977).

Preparation of E. coli competent cells

A pre-culture (5ml) of *E. coli* cells were grown over night at 37°C in LB medium. 0.1ml of the over-night culture was grown in 6.5ml of LB with good aeration until cloudy. 5ml of this culture was inoculated in 100ml of LB in 1 litre flask until OD_{550}=0.2-0.3. Cells were then chilled on ice for 20 minutes and spun down at 2 krpm for 20 minutes at 4°C in a bench top centrifuge. Pellet was resuspended in 2/5 of the original volume of TFBI and incubated on ice for 30 minutes. Cells were spun down as above and resuspended in 1/25 of the original volume of TFBII using cold pipettes. Cells were dispensed in 200μl aliquots in 1.5ml eppendorf tubes in a tray containing dry ice and absolute EtOH and stored at -70°C.

Transformation of E. coli competent cells

Competent *E. coli* cells were transformed as described (Hanahan, 1985). Frozen (-70°C) stored *E. coli* cells were thawed on ice and mixed with the DNA. Cells were then incubated on ice for 30 minutes, and heat-shocked at 42°C fro 2 minutes.

Cells were placed back on ice for at least 2 minutes and then 1ml of LB medium was added. Cells were transferred to 37°C with shaking for 30-45 minutes and appropriate dilutions plated on ampicillin selective plates. Colonies were scored after 16-20 hours of incubation at 37°C.

Isolation and purification of plasmid DNA

Plasmid DNA was recovered from *E. coli* bacterial strains using either the small scale preparation (mini prep) or a mid-scale preparation (midi prep) essentially as described by Sambrook *et al.*, (1991).

Mini preps (alkaline lysis method)

This method gives clean DNA for plasmids amplified in the *E. coli* DH5α strain. For the *E. coli* JA226 strain phenol/chloroform purification is needed.

1.5-5ml of bacteria grown over night in 2X TY medium at 37°C were transferred to an eppendorf tube and harvested by centrifugation in a microfuge (by a quick spin) or directly spun down in 15ml Falcon tubes in a bench top Jouan centrifuge at 3krpm for 15 minutes at 4°C. Pelleted bacteria were resuspended in 100µl of alkaline lysis solution 1, in a 1.5ml eppendorf tube and incubated at room temperature for 5 minutes. In the mean time 1-10ml of alkaline lysis solution 2 was prepared and 200µl of this was added. After a very gentle mix the tube was incubated on ice for 5 minutes after which 150µl of alkaline solution 3 was added and left on ice for 5 more minutes. The lysate was clarified by centrifugation in a microfuge at 14 krpm for 5 minutes. At this stage, if necessary (e.g., for JA226 strain), nucleic acids were purified by addition of and equal volume of a 1:1 solution of phenol/chloroform and vigorous vortexing. The upper phase was precipitated with 2 volumes of absolute EtOH. After vigorous vortexing and incubation at room temperature for 2 minutes, nucleic acids were recovered by centrifugation at 14 krpm for 5 minutes and the pellet washed with 1ml of ice-cold 70% EtOH solution. The pellet was then vacuum dried and resuspended in 25-50µl of TE (pH 8.0) containing 20µg/ml of DNase-free RNase A. This mini preparation of DNA is immediately suitable for restriction enzyme digestion analysis and does not need further purification. The yield is around 10-30µg of plasmid DNA per preparation.

Midi preparation (Qiagen method)

About 25-50ml of bacteria were grown as described above with good aeration. Bacterial cells were lysed following the alkaline lysis procedure and the supernatant applied to a Qiagen column (Qiagen). Eluted plasmid DNA was precipitated with an equal volume of isopropanol and immediately spun down at 12 krpm for 30 minutes at room temperature in a Corex tube. Pellet was washed with 70% EtOH, briefly air dried and resuspended in 100-300µl of TE (pH 8.0).

Setting up sequencing reactions

Sequencing reactions were performed with the dideoxy termination chain method of Sanger *et al.*, (1977) using the Sequenase® kit (United States Biochemical).

Denaturation step

About 2-5 µg of purified supercoiled plasmid DNA was resuspended in 18µl of water and denatured for 10 minutes at room temperature by addition of 2µl of a 2M NaOH, 2mM EDTA solution. The reaction was then neutralised by addition of 2µl of a 3M NaAc solution (pH 5.3) and precipitated by addition of 75µl of absolute EtOH. Denatured DNA was recovered by centrifugation at 14 krpm in a microfuge after incubation at -70°C for 15 minutes. The pellet was washed twice with 70% EtOH, vacuum dried and finally resuspended in 7µl of sterile distilled water.

Annealing step

2 µl of Rx reaction buffer and 1ng of M13 ssDNA primer (1µl) was added to the denatured DNA. The mixture was incubated at 37°C for 15 minutes and then left to cool at room temperature.

Labelling step

1µl of 0.1M DTT, 2µl of 1:5 diluted dGTP labelling mix, 0.5µl of [α-^{35}S] dATP (NEN biochemical) and 2µl of 1:8 diluted Sequenase enzyme was added at the annealing reaction. The mix was spun down very briefly in a microfuge and incubated at 19°C for 5 minutes.

Termination step

In the mean time 2.5µl of each dideoxynucleotide was aliquoted in a 96 well V-bottom microtiter plate and the plate pre-warmed at 37°C. 3.5µl of the labelling mixture was then added to the dideoxynucleotide containing well, spun down briefly and incubated at 37°C for 5 minutes. Termination reaction was stopped by addition of 4µl of formamide dye STOP solution and stored at 4°C. Reactions were denatured at 80°C for 3 minutes before loading onto a gradient 4% polyacrylamide denaturing gel.

Preparing a gradient acrylamide sequencing gel

9ml of acrylamide "Bottom mix", containing 18µl of APS and 18µl of TEMED and an equal volume of acrylamide "Top mix", containing 60µl of APS and 60µl of TEMED, were taken up with the same pipette and the two mixed by brief air bubbling. This mix was then poured into the gel plates and the remaining of the "Top mix" was taken up and poured with the same pipette, a comb put in place and a weight placed on top to ensure that the gel was even of thickness. Electrophoresis was carried out at 30mA for 1.5 hours in 0.5X TBE buffer. After electrophoresis the gel was fixed by soaking it in 10% Acetic Acid, 10% MetOH solution (about 1 litre), rinsed in water and propped on one side to allow water to drain. After transfer to a 3MM paper a layer of cling film was placed on top of the gel and then allowed to dry on a gel drier at 80°C for 60 minutes. The gel was exposed to an X-ray film (Fuji) at room temperature for 16-24 hours.

ExoIII progressive deletions

For this purpose the Erase-A-Base® system (Promega) was used. Progressive Exonuclease III (*Exo*III) deletions (Henikoff, 1984) of the pC_{25} plasmid were generated as follows. About 1µg of purified pC_{25} plasmid was digested with *Sal*I and *Kpn*I in a final volume of 20 µl. At the end of the digestion the reaction was diluted to 100µl with TE (pH 8.0) and extracted twice with phenol/chloroform. The extracted sample was EtOH precipitated and resuspended in 27 µl of sterile water. In the mean time, 7.5µl of S1 nuclease mix was dispensed in 11 test tubes on ice. 3µl of a 10X *Exo*III buffer was added. About 500 Units of *Exo*III were added and mixed thoroughly. The sample was then incubated at room temperature (25°C) for 11 minutes. 2.5 µl aliquots were removed every minute and mixed with 7.5µl of S1 nuclease mix on ice. At the end of the time course, the tubes kept on ice were moved to room temperature for 30 minutes to allow digestion of single stranded DNA by the S1 nuclease. Digestion was stopped by addition of 1µl of S1 stop buffer and heat inactivated at 70°C for 10 minutes. The extent of digestion was determined by loading 1.5µl of the mix on a 1.2% agarose gel, 1X TBE. S1 digested ends were filled-in with the large fragment of *E. coli* DNA polymerase (Klenow fragment). 1µl of Klenow mix was added to each sample at 37°C and incubated for 3 minutes after which 1µl of dNTP mix was added followed by an additional 5 minutes of incubation. 40µl of ligase mix were added to each sample and linearised plasmids were ligated at room temperature for 1 hour. 10µl of ligation mix per each time point were transformed into 100µl of competent *E. coli* DH5α cells. 11 plasmids, containing progressive deletions of ca. 100bp, were recovered.

S. pombe transformation procedures

Transformation using electroporation

The procedure used is very similar to that described by Prentice (1991). This method of transformation gives about $4\text{-}5x10^5$ transformants/µg of supercoiled DNA used with the P1 and P2 strains of *S. pombe*. It seems that this method may be strongly strain dependent (G. D'Urso, pers. comm.). Miniprep DNA works less efficiently. Sometimes sorbitol containing plates may increase the efficiency of transformation, perhaps because cells recover better from the electroshock in this medium.

Preparation of electrocompetent cells

Cells were usually grown in 100ml of YES medium (EMM can also be used) until late log phase [Optical Density (OD) of 1.8-2.0 at a wavelength of 595_{nm}]. This may be important if a high efficiency of transformation is needed.

Cells were washed 3 times in 50 ml of ice-cold 1.2M sorbitol. Finally cells were resuspended in 1 ml of ice-cold 1.2M sorbitol and kept on ice until ready to be electroporated.

Electroporation

200µl of the cell suspension was used in each electroporation. Cells were mixed with the DNA in an eppendorf tube immediately before electroporation. This mixture was transferred to an ice-cold electroporation cuvette (Bio-Rad), briefly tapped on the bench and electroporated as follows: 2.0kV (Voltage), 25µF (capacitance), 200Ω (resistance). The time constant usually varies between 4.0 and 4.5 ms. Immediately after pulse, cells were diluted with 0.5ml of ice-cold 1.2M sorbitol in the electroporation chamber and left on ice. Appropriate dilutions (recommended not more than x5) were made in ice-cold 1.2M sorbitol and cells spread on very dry selective plates. Colonies were usually scored after 5 days at 25°C or 3 days at 30°C.

Transformation of S. pombe using the Lithium Chloride procedure

This method was used to transform the P180 diploid strain as described by Moreno *et al.*, (1991).

Plasmid stability test

Typically, isolated transformants stored at 4°C as small patches under selective conditions, were streaked out as small patches on YES plates divided in sectors and left to grow for 3-5 days at the appropriate temperature. After growth cells were streaked out at single colonies on the same YES plate and left to grow as above. After this second round of growth on YES, cells were finally replica-plated on selective EMM plates and YES plates (at the permissive and restrictive temperature respectively if needed to determine co-segregation of Ts^+ and auxotrophy markers).

Recovering plasmids from Schizosaccharomyces pombe

This method has been adapted from that by Moreno *et al.*, (1991) and was used in the recovery of plasmids from the putative suppressors isolated in the screenings described in Chapter Four.

Cells were grown up under selective conditions to an OD_{595} of approximately 1 (1-2 x10^7 cells/ml). Cells were then spun down at 3 krpm for 5 minutes at room temperature and resuspended in 1.5ml of 50mM citrate/phosphate (pH 5.6), 1.2M sorbitol, containing 2 mg/ml of Zymolyase-20T. Cells were then transferred to an 1.5ml eppendorf tube and incubated at 37°C for 1 hour to allow digestion of the cell wall. This was periodically monitored by light microscopy of a cell sample in the presence of 1% SDS. When digestion was completed cells were pelleted in a micro centrifuge for 30 seconds and resuspended in 300µl of TE (pH 8.0). 35µl of 10% SDS was added and the mix incubated at 65°C for 5 minutes. 100µl of 5M potassium acetate was added and then incubated on ice for 30 minutes.

The mix was spun down at 4°C for 10 minutes and the supernatant transferred to a fresh test tube. The supernatant was purified using the GeneClean procedure (GeneClean Kit, Stratatech Scientific Ltd.).

100µl of NaI solution was added to 50µl of the supernatant together with 5µl of glassmilk. After mixing thoroughly the sample was left to incubate 5 minutes at room temperature and then spun down for 5 second in a microfuge. The pellet was washed three times with 400µl of ice-cold NewWash solution by carefully resuspension and finally resuspended in 10µl of TE (pH8.0). The DNA was then eluted at 55°C for 3 minutes. Glass milk was spun down and the supernatant was recovered. Glassmilk was eluted again with 10µl of TE (pH 8.0) in order to improve the efficiency of recovery.

5µl of the eluate was used to transform 100µl of competent *E. coli* JA226 cells.

Staining S. pombe nuclei

Nuclei were visualised using the fluorescent dye DAPI (Sigma, D-1388) after cells fixation using a) paraformaldehyde or b) EtOH fixation (Moreno *et al.*, 1991). Cells can also be fixed following c) the rapid heat fixation procedure;

a) paraformaldehyde. This procedure may be useful to prevent alteration of nuclear morphology due to the fixation manipulations. 10ml of exponentially growing cells were fixed by mixing with an equal volume of 8% paraformaldheyde followed by incubation at room temperature for 90 minutes. After incubation cells were spun down

5 minutes at 2 krpm on a bench top centrifuge (Jouan) and resuspended in 1ml of PBS containing 1% Triton X-100. Cells were incubated at room temperature 1-10 minutes and then spun down as above. Pellet was washed 3 times in PBS and finally resuspended in 50-200 µl of PBS. Cells can be stored at 4°C for one week.

b) EtOH fixation. Cells were scraped from a vegetative plate and resuspended directly in 100µl of 95% EtOH. Cells were fixed for 5 minutes at room temperature and then washed 3 times in PBS. Cells were then resuspended in 10-50 µl of PBS and stored at 4 °C.

c) the rapid heat fixation procedure. 5-7µl of cells were spotted on a microscopic slide and put on a hot plate at 70°C until the sample had dried out. 3µl of a 0.3 µg/ml DAPI solution was spotted on a coverslip and mounted on the dried cells. The mounted coverslip was then sealed with polish nail in order to prevent drying out of the sample. Stained cells were viewed under incident-light fluorescence using a Zeiss Axioskop (Zeiss, West Germany) and photographed using a Kodak T-MAX 400 film.

Visualising S. pombe nuclei and septa by DAPI and Calcofluor double staining

This staining procedure works very well in combination with the quick heat fixation method described above.

A mounting solution containing 25% of 0.3 µg/ml DAPI and 50% 50 µg/ml Calcofluor solutions was prepared. Round coverslip (9m/m) were mounted with 3µl of the mounting solution. Residual mounting solution was drained using the corner of a tissue before mounting.

Physiological methods for S. pombe

S. pombe cells were routinely grown in YES or EMM medium as described (Moreno *et al.*, 1991). In experiments involving temperature shift, cells were grown to mid-log phase, spun down at 2 krpm for 5 minutes at room temperature, and resuspended in pre-warmed medium. In experiments involving hydroxyurea, cells were processed as above and then resuspended in pre-warmed medium in the presence or absence of 10mM of hydroxyurea (from 100mM stock solution).

Viability experiments

Viability of *S. pombe* cells was performed as described (Enoch *et al.*, 1991) with some modifications.

Cells were grown in YES liquid medium (instead of EMM) at the appropriate temperature until they had reached mid-log phase (1-2 x 10^6 cells/ml). Appropriate dilutions were made in sterile distilled water in order to seed about 500 cells per plate. Diluted cells were sonicated briefly to eliminate clumping at setting 5 for 15 seconds using a tip sonicator (MSE ultrasomicator).

In temperature shift experiments 100µl of the diluted cells were plated out on pre-warmed YESP plates and incubated at 36°C. Plates were thus moved back to 25°C after 0-9 hours of incubation at the restrictive temperature. Colonies were usually scored after 3-5 days of incubation at 30 or 25°C respectively.

In experiments involving incubation in hydroxyurea cells growing in liquid culture were collected periodically, diluted and sonicated as described above before plating on YESP plates. Colonies were scored after 3 days of incubation at 30°C.

Viability of the cells from each time point was determined as percentage of cells surviving the treatment. Data were plotted using the Cricket Graph™ programme.

Monitoring septation index

Septation index was monitored by haemocytometer analysis. The septation index was calculated as the percentage of cells displaying a septum versus the total number of cells in the field. At least 500 cells were counted for each strain.

Monitoring aberrant mitoses

This was determined by double staining of nuclei and septa with DAPI and Calcofluor. Cells displaying aberrant mitoses (termed "cut") were identified as single septated cells in which one daughter cell contained one nucleus and the other was anucleate, or as cells containing a septum bisecting the nucleus. Physically separated cells but connected by a string of DNA bridging two visible and asymmetric nuclei were also considered as "cut". The number of cells displaying aberrant mitoses was expressed as percentage of aberrant mitoses versus the total of cells counted (500).

Random spore analysis

This was performed as described by Moreno *et al.*, (1991) with no modifications.

Spore germination experiment

A large scale preparation of spores was prepared as described by Moreno *et al.*, (1989) with some modifications.

Sporulation and Helicase digestion of unsporulated cells

Diploid *Schizosaccharomyces pombe* cells were grown in 100-200 ml of EMMG medium supplemented with leucine and glutamate (5g/l instead) for 7 days at 29°C with gentle shaking. Cells reached saturation after 2-3 days of incubation and began to sporulate. When more than 90% of the diploid cells had sporulated, cells were collected by centrifugation at 3 krpm for 5 minutes at room temperature and washed once in sterile distilled water (SDW). Cells were resuspended at a density of $1x10^7$ cells/ml in SDW in the presence of a 1/500 dilution of Helicase enzyme juice (IBF Biotechnics, Paris, France) in SDW and incubated at 29°C over-night. Complete digestion of vegetative cells was monitored by light microscopy; digested cells are not usually visible, whereas spores look like bright spherical shaped cells. When hardly any vegetative cells were left, spores were purified as described in the next paragraph.

Spores purification

Flasks containing spores were left undisturbed at room temperature on the bench until most of the cell debris produced by the digestion of the ascus wall and vegetative cells had sedimented to the bottom of the flask (about 10-15 minutes). Spores were then transferred to 50 ml Falcon tubes by carefully pipeting them from the surface of the culture, taking care to avoid cell debris. Spores were then pelleted at 1 krpm for 10 minutes at room temperature and the pellet washed with an equal volume of SDW by vigorous vortexing. Spores were then spun down at 1 krpm for 15 minutes at room temperature and washed as above and finally spun down at 2 krpm for 10 minutes. Spores were resuspended in 10-15 ml of SDW and generally speaking to an OD_{595}=1.0 (of a 1:10 dilution) so that 1/100 of this spore inoculum was used in the following experiments. Spores can be stored in these conditions at 4°C for many months.

Spore germination

Spores were inoculated in EMM lacking uracil at an OD_{595}= 0.1 and germinated at 29°C with gentle shaking. At this temperature spores begin to germinate after 7-9 hours and enter the first mitosis after 13-14 hours of incubation.

Determination of the DNA content of S. pombe cells by flow cytometry.

The DNA content was determined by following the method of Sazer and Sherwood (1990). Cells were grown to mid-log phase (OD_{595}= 0.2-0.5) in EMM. Haploid G1 arrested *cdc10-129* cells or nitrogen starved wild-type cells were used as 1C DNA content standard. Exponentially growing haploid cells were used as 2C DNA content standard.

Fixation

About 10^7 cells were spun down at 2 krpm for 5 minutes at 4°C, washed once in 1 ml of sterile distilled water and finally resuspended in 0.3 ml of sterile distilled water. 0.7 ml of ice-cold absolute EtOH was slowly added at the sample while vortexing. Fixed cells are suitable to be frozen down at -70°C or stored indefinitely at 4°C with no harm.

Staining

0.3 ml of cells were washed once with 3-5 ml of 50mM sodium citrate buffer and then resuspended in 0.5 ml of the same buffer containing 0.1 mg/ml of RNase A (DNase free). Cells were then incubated at 37°C for 2-3 hours to allow complete RNA digestion and directly stained by addition of 0.5ml of a 4µg/ml propidium iodide solution in sodium citrate buffer. These cells can be stored at 4°C in the dark. Cells were sonicated for 45 seconds at setting 5 using a tip sonicator (MSE ultrasonicator) to reduce clumping. The DNA content of these cells was finally determined by running the samples in a Beckton-Dickinson FACS-SCAN cell sorter and data were analysed using the Lysis II acquisition software. 1C and 2C standards were prepared, using nitrogen starved haploid and exponentially growing cells, respectively. These were used singularly (1C or 2C) or as a mixed population (1C+2C) for reference in every experiment involving flow cytometry of fission yeast cells. When cells are arrested in the cell cycle, flow cytometry measurements show a slight drift in the position of the peak of cells with a

division, or a non-specific effect of increased cell size (Sazer and Sherwood, 1990). The effect is however rather small, in comparison with the larger increases produced by re-replication and an increase in ploidy.

Pulsed field gel electrophoresis (PFGE) of S. pombe chromosomes

Purification of intact chromosomes

Intact *S. pombe* chromosomes included in agarose plugs ready to be analysed for PFGE were prepared as described (Waseem *et al.*, 1992) with some modifications.

Typically, 100-500 ml of cells were grown in EMM medium and collected during early log phase by centrifugation at 2 krpm for 10 minutes at room temperature. Cells were washed twice in 25-50 ml of CSE solution containing 150 mM β-mercaptoethanol and then resuspended in 10 ml of CSE containing 30 mM β-mercaptoethanol in the presence of 2 mg/ml of zymolyase 20-T. Cell wall was digested at 37°C for 1 hour. Digestion of the cell wall was monitored under light microscopy in the presence of 1% SDS. When all the cells were spheroplasted, they were spun down at 186 rpm for 10 minutes at room temperature and resuspended at a density of $6x10^8$ cells/ml in TSE. Cells were equilibrated at 37°C, mixed with an equal volume of 1% Low Melting Point Agarose in TSE and dispensed in plug moulds. Plugs were left to set on ice and lysed in ETS at 55°C for 90 minutes. Lysis was continued at the same temperature for 48 hours in NDS in the presence of 0.5 mg/ml of Proteinase K. Plugs were stored at 4°C in NDS indefinitely. Plug moulds were cleaned by soaking over-night in 0.25M HCl, rinsed several times in dH_2O and dried at 65°C for 10-15 minutes.

Pulsed field gel electrophoresis

After removal of EDTA and Proteinase K from the plugs by washing them three times in TE buffer (pH 8.0) at room temperature, for 15 minutes during each wash, plugs were loaded on a 0.8% agarose gel in 0.5X TAE buffer. To avoid plugs coming out of the wells during electrophoresis, they were sealed with a solution of 0.8% LMP agarose gel in 0.5x TAE. Electrophoresis was carried out at 3V/cm, 14°C using a switching time of 4500 seconds for 72 hours with recirculation of the electrophoresis buffer in a LKB 2015 Pulsaphor™ electrophoresis unit (Pharmacia LKB Biotechnology). After electrophoresis chromosomes were visualised by staining with 1µg/ml of EtBr solution in deionized water for 15 minutes and 30 minutes destaining at room temperature in 0.5x TAE buffer with several changes.

Screening of libraries

Genomic library.

About 1µg of fission yeast genomic library constructed into the vector pUR18 (Barbet *et al.*, 1992) was transformed into the P28 strain by electroporation. About $1x10^4$ transformants were selected on ura$^-$ EMM plates at 25°C and replica-plated at 36°C under the same conditions of selection. Putative suppressors were scored and analysed as described in Chapter Four.

cDNA expression library

A cDNA expression library made into the pREP3X vector under control of the fission yeast inducible promoter *nmt1* (Maundrell, 1990) was kindly donated by C. Norbury (Cell Cycle Group; ICRF, London; Kelly *et al.*, 1993). The *S. pombe* P1 strain was transformed with about 1µg of the library by electroporation and cells were plated out on leu$^-$ EMM plates in the presence of 5µg/ml of thiamine (promoter off). About $3x10^5$ transformants were scored at 25°C after 5 days of incubation and then replica-plated at 25°C in the same conditions of selection in the absence of thiamine for 16-18 hours to allow derepression of the *nmt1* promoter (Maundrell, 1990). Cells were then subjected to another round of replica-plating to eliminate residual growth and then replicated at 36°C on pre-warmed plates for 3-5 days in the absence of thiamine. Putative suppressors were identified as colonies able to grow in the absence of thiamine (promoter on).

Tetrads analysis

Diploid *Schizosaccharomyces pombe* cells were streaked out as small patches on a YES plate and incubated at 30°C for 2-3 days. A loopful of cells was spread on a ME plate and cells were incubated at 25°C in order to allow sporulation. This was monitored after 2-3 days of incubation by re suspending a small loopful of cells in sterile water and viewing under a normal light microscope to determine the presence of

monitored after 2-3 days of incubation by re suspending a small loopful of cells in sterile water and viewing under a normal light microscope to determine the presence of asci containing four distinct spores. Cells were left to incubate until sporulation was greater than 90%.

Dissection of asci and spores was performed using a Zeiss micromanipulator on a inverted very thin YES plate. The dissection plate was marked with two parallel lines about 5mm apart, and offset from the centre. A loopful of sporulated diploid cells was mixed well in a drop of sterile distilled water to the end of the outer line and a single streak was made along the line. This to ensure that single asci can be observed and therefore easily isolated. Asci were visible as irregular shaped cells containing within them 4 bright spores. Only mature asci containing 4 well distinct spores were isolated.

Individual asci were isolated from associated cells by positioning them on the second line and the position of the asci was recorded using the microscope vernier, so that they could be easily found the day after.

Asci walls were allowed to break down usually by incubation of the dissection plate at 18-20°C overnight. An alternative method used was to store the dissection plate overnight at 4°C and then incubating at 35°C for few hours the day after. Asci that had released their content were then dissected by separating the spores and moving them at a distance of 5mm from each other. The position of each individual spore was recorded using the microscope vernier. Dissected spores were then allowed to germinate at 30°C for 4 days.

Making S. pombe protein extracts

The protocol used has been adapted from that suggested by Moreno *et al.*, (1991).

10 ml of cells were grown to mid-log phase and spun down at 2 krpm for 5 minutes at 4°C in a 15 ml Falcon tube. Cells were resuspended in 100 µl of Breaking Buffer and an equal volume of acid-washed glass beads (0.5 mm diameter, Sigma, G-9268) was added. Cell walls were broken by constant vortexing for 3 minutes and the lysate was transferred to an 1.5 ml eppendorf tube. An equal volume of Laemmli buffer (Laemmli, 1970) was added and the lysate was then clarified by spinning down for 5 seconds in a bench top microfuge. Protein extracts were stored indefinitely at -20°C.

Fission and budding yeast nomenclature

S. pombe gene names are referred to using italics and lower case e.g. *cdc21*$^{+}$. An allele name, or mutation, may be added to designate a specific mutant e.g., *cdc2.3w*, whilst a particular class of mutants is indicated with superscript e.g. *cdc21*^{ts}. A gene deletion is indicated as follows: *cdc21::ura4*$^{+}$. When the gene product is being referred to, italics are not used e.g., cdc21. If the molecular weight of the protein is known, this may be shown and the gene name is superscript in italics e.g. p34^{cdc2}.

For *S. cerevisiae* , gene names are referred to using capital letters e.g. *CDC46*. Mutant names are still indicated using lower case if recessive, as for fission yeast mutants e.g., *cdc46-1*. Gene products are referred to putting the first letter of the name in capital e.g. Mcm2.

Table 2.1

***Schizosaccharomyces pombe* strains used in this thesis**

Strain	Genotype	Reference/Source
P1	*h⁻cdc21-M68 leu 1-32*	K. Maundrell, Glaxo, Switzerland
P2	*h⁻leu 1-32*	Cell Cycle Group, ICRF, London
P28	*h⁻cdc21-M68 leu1-32 ura4-294 ade6 -704 ars1⁺*	S. Kearsey
P72	*h⁻cdc2.3w leu1-32 ade*	Tamar Enoch, Harvard Medical School
P73	*h⁺rad1-1 leu1-32*	T. Carr, Sussex
P86/87	*h⁻cdc21-M68 cdc2.3w*	S. Kearsey, unpublished
P119	*h⁻cdc21-M68 rad1-1*	S. Kearsey, unpublished
P135	*h⁺/h⁻leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216*	Cell Cycle Group, ICRF, London
P136	*h⁺/h⁻leu1⁺/leu1-32 ura4⁺/ura4-D18 de6⁺/ade6-704*	Cell Cycle Group, ICRF, London
P139	*h⁻nda1 leu1-32*	M. Yanagida
P155	*cdc23-M36 leu1-32*	P. Nurse, Cell Cycle Group, ICRF, London
P156	*cdc24-M38 leu1-32*	P. Nurse, Cell Cycle Group, ICRF, London

P176	*h*$^{+N}$*pol*δ^{ts}3 *leu1-32 ura4-D18 ade6-M216*	Francesconi *et al.*, 1992
P177	*h*$^{-}$*nda4 leu1-32*	M. Yanagida
P178	*h*$^{-}$*cdc19-P1 leu1-32 ura4-D18 ade6-M210*	S. Forsburg, Salk Institute, San Diego, CA (USA)
P183	P1[pREP1; *LEU2 ars1*$^{+}$]	this work
P184	P2[pREP3X;*LEU2 ars1*$^{+}$]	this work
P185	P2[pREP*3X::21*$^{+}$; *LEU2 ars1*$^{+}$]	G. Blom
P180	*cdc21*$^{+}$*/cdc21::ura4*$^{+}$*ura4-D18* (other markers as in P135)	this work
P181	*cdc21::ura4*$^{+}$[*81Xnmt1:cdc21*$^{+}$ *LEU2 ars1*$^{+}$]; other markers as P180	this work
P182	*h*$^{+}$ or *h*$^{-}$*cdc21::ura4*$^{+}$[pAC1*LEU2 ars1*$^{+}$] *ade6 leu1-32 ura4-D18*	this work

Table 2.2

***E. coli* strains used in this thesis**

Strain	Genotype	Reference/source
DH5α	[*supE*44D*lac*U169 (ϕ80*lacZ*DM15) *hsdR*17 *recA*1 *gyrA*96*thi*-1*relA*1 Ap^s]	(Hanhan, 1983; Bethesda Research Laboratories 1986)
JA226	*reBC* Ap^s	F. Verde, ICRF, Cell Cycle Group, London

Table 2.3

Yeast shuttle plasmids used in this thesis

Plasmid	Construction	Reference/ Source
S. cerevisiae		
YCP101.*MCM2*	*MCM2 LEU2 ARS1*	B. Tye, Cornell University, USA
YCP50.*MCM3*	*MCM3 URA3 ARS1 CEN4/ARS4*	B. Tye, Cornell University, USA
S. pombe		
pIRT2	*LEU2 ars1*$^+$	Hindley *et al.*, 1987
pIRTU	*ura4*$^+$ *ars1*$^+$	S. Kearsey
pD*MCM2*	*S. cerevisiae MCM2* in pIRT2	this work
pD*MCM3*	*S. cerevisiae MCM3* in pIRT2	this work

pAC1	*cdc21*$^+$ in pIRT2, *LEU2 ars1*$^+$	A. Coxon, D. Phil. thesis
pREP1	*nmt1 LEU2 ars1*$^+$	Maundrell, 1990
pREP3X	3X*nmt1 LEU2 ars1*$^+$	Susan Forsburg
pREP*81X::21*$^+$	81X*nmt1* promoter replaces 3X*nmt1* promoter of pREP*3X::21*$^+$ as the *Pst*I-*Xho*I fragment; other markers as in pREP*3X::21*$^+$	this work
pREP3X library	*S. pombe nmt1::cDNA* library in pREP3X	C. Norbury, Cell Cycle Group, ICRF, London
p*dom1*$^+$	pREP3X*nmt1::dom1*$^+$ (*LEU2 ars1*$^+$)	this work
pC_{25}	pREP3X*nmt1::tsl1*$^+$	this work
pC_{27}	pREP3X*nmt1tsl2*$^+$	this work
pOE1	*cdc21*$^+$ *Bam*HI fragment in pREP1	this work
pOE2	*cdc21*$^+$ *Nsi*I fragment in pREP1	this work
pOE3	*cdc21*$^+$ *Hae*II-*Pst*I fragment in pREP1	this work
pUR18 library	*S. pombe* genomic library in pUR18 (*ura4*$^+$ *ars1*$^+$)	Barbet *et al.*, 1992
pUR1a	*cdc21*$^+$ 1a genomic clone in pUR18	this work
pUR6a	*cdc21*$^+$ 6a genomic clone in pUR18	this work
pREP*3X::21*$^+$	*cdc21*$^+$ full length in pREP3x	G. Blom

pKOXX	pAC1 in which the *Nsi*I restriction fragment of *cdc21*$^+$ is replaced by *ura4*$^+$	this work

Table 2.4

Other plasmids used in this Thesis

Plasmid	Construction	Reference/Source
pUC18	Amp^r *ori laci lacz*	Pharmacia
pM13A$_{50}$	Amp^r fl*ori laci lacz*; A$_{50}$ insert from *cdc21*$^+$ shotgun bank	A. Coxon, D.Phil. thesis
pM13A$_{136}$	Amp^r fl*ori laci lacz*; A$_{136}$ insert from *cdc21*$^+$shotgun bank	A. Coxon, D.Phil. thesis
pBlueScript	Amp^r fl*ori laci lacz*	Stratagene
pUC*cdc21*$^+$	*cdc21*$^+$ PCR fragment iserted into the *Sma*I site of pUC18	this work
pUC*nda1*$^+$	*nda1*$^+$ PCR fragment iserted into the *Sma*I site of pUC18	this work
pUC*nda4*$^+$	*nda4*$^+$ PCR fragment iserted in the *Sma*I site of pUC18	this work
pUC*MCM2*	*MCM2* PCR fragment iserted in the *Sma*I site of pUC18	this work
pUC*MCM3*	*MCM3* PCR fragment iserted in the *Sma*I site of pUC18	this work

pUCES	370bp *Esp*I-*Sma*I fragment of *dom1*$^+$ cDNA in the *Sma*I site of pUC18	this work
pUCD*Esp*	400bp *Esp*I fragment of *dom1*$^+$ cDNA in the *Sma*I site of pUC18	this work
pUC*CDC46/MCM5*	*CDC46/MCM5* PCR fragment inserted into the *Sma*I site of pUC18	this work

Chapter Three

Sizing the *cdc21+* gene family

Introduction

As extensively discussed in Chapter One (page 28), the fission yeast cdc21 protein shares similarities with the yeast and metazoan Mcm2-3-5 and related replication proteins (Coxon *et al.*, 1992). The overall level of identity between cdc21 and the three budding yeast proteins is about 25% with a degree of similarity up to 60% in a central 200 amino-acid region of the aligned proteins (see Figure 3.1). An up-to date summary of the Mcm proteins identified so far in a variety of eukaryotes is provided in Table 3.1.

In constructing models to explain the function of cdc21-related proteins, it would be helpful to know whether the number of family members has been conserved in evolution (as for the histones) or whether there has been diversification in higher eukaryotes.

Based on phylogeny trees drawn for cytochrome c, it has been estimated that *S. cerevisiae* and *S. pombe* diverged 880 million years ago, whereas these two species diverged from mammals 1200 million years ago (Wu *et al.*, 1986). Given the evolutionary distance, one may ask whether diversification of these proteins has occurred between fission yeast and budding yeast. Using the sequences of cdc21-related proteins available in the database I have constructed phylogeny trees to determine how many distinct members can be identified in this conserved protein family.

In this Chapter I have also attempted to identify *cdc21+*-related sequences in the yeast genome using *cdc21+* gene family-conserved and non-conserved DNA sequences as molecular probes on Southern blots, as well as to amplify *cdc21+*-related sequences by PCR.

Results

Phylogeny analysis of cdc21-related protein sequences suggests the presence of six distinct members

To determine how many distinct members of the cdc21-related protein family have been identified so far, the cdc21 predicted protein sequence was used as a probe to search various protein databases for related sequences. This was done by using the BLASTP programme of the Genetics Computer Group (GCG, version 8). The highest score was found with the Cdc54 protein from the yeast *S. cerevisiae* (Whitbread and Dalton, 1995). The second high score was with the *S. pombe* nda4 protein (Miyake *et al.*, 1993). Among the already known yeast Mcm proteins a close match was also found with the *YBR1441* open reading frame of the chromosome II of *S. cerevisiae* (Bussereau *et al.*, 1992) which has now been shown to be identical to the *CDC47* gene (Dalton and Whitbread, 1995).

Phylogeny analysis was performed using the CLUSTALV programme (Higgins *et al.*, 1991) which uses the neighbour-joining method (Saitou and Nei, 1987) for calculating relationships (see Table 3.2). A tree was derived from this analysis and is shown in Figure 3.2. This tree is shown unrooted and branch lengths are proportional to estimated phylogenic distance. There are six distinct branches which appear to be equally distant from the centre of the tree. Thus this analysis suggests that the proteins in the family fall into six distinct groups, implying that six distinct types of Mcm proteins have been identified so far.

Determination of the minimum size of the *cdc21*$^+$ gene family in *S. cerevisiae* by Southern blotting

This approach may be valuable as pseudogenes are very rare in yeast (Olson, 1991), although there are certain limitations. Restriction fragments belonging to different genes might co-migrate and a single gene may give more than one restriction fragment that can be recognised by the probe. Moreover, results from hybridisation analysis need to be confirmed by isolation and DNA sequencing of such putative related sequences from the yeast genome. It is thus clearly difficult to infer the gene number accounted from the number of hybridisation bands. Nevertheless, the approach may be

useful in order to gather a very approximate estimation of the smallest number of *cdc21*$^+$-related genes in the yeast genome.

As a first approach to this study, a budding yeast *cdc21*$^+$-related gene, the *S. cerevisiae MCM3* gene (Gibson *et al.*, 1990) was used as a molecular probe to detect *MCM3*-related sequences in yeast as well as in a variety of eukaryotes. This was done by low-stringency hybridisation of the *MCM3* gene to chromosomal DNA immobilised on a nylon membrane (Southern blotting). Genomic DNA extracted from different organisms was digested with the restriction enzyme *Hin*dIII, subjected to electrophoresis and blotted to a nylon membrane (see Chapter Two). This was then probed with the full *MCM3* gene (see legend to Figure 3.3).

Although the hybridisation pattern forms a continuum (Figure 3.3, panel a, lane 4), twelve discrete hybridisation bands can be distinguished in the lane loaded with *S. cerevisiae* genomic DNA. These are more evident in a low exposure of the filter (panel b). The hybridisation bands can be classified as strong and weak, probably due to the different degree of homology and overlap of the probe to *MCM3*-related sequences, as well as to co-migration of more than one restriction fragment . The size of each band was estimated (Table 3.3) and assigned to each five known *MCM* gene by comparison with its restriction map (Figure 3.4). From this analysis, described in Table 3.3, the number of unaccounted bands (U_B) is four. These bands may correspond to at least one new *MCM3*-related sequence, suggesting that there may be at least six *MCM3*-related genes in budding yeast.

In the lane loaded with genomic DNA from fission yeast (Figure 3.3, panel a, lane 5), the *MCM3* probe recognises five distinct hybridisation bands (although four more very faint bands, three above 6.5 kb and one below 2.7 kb, may be also present). The estimated size of these bands is shown in Table 3.3 and the restriction map of fission yeast *cdc21*$^+$-related genes is shown in Figure 3.4. The number of restriction fragments detected in this experiment is identical to the number of restriction fragments expected for the known genes (U_B=0, Table 3.3), suggesting the presence of at least 5 *MCM3*-related genes in *S. pombe*.

Faint hybridisation bands are also detectable in lanes loaded with *X. laevis*, *D. melanogaster* and *H. sapiens* DNA (Figure 3.3, lanes 1, 2 and 3 respectively) which may represent related genes. No attempt was made to count genes in these large genomes owing to the weakness of the signals and the inherent limitations of this approach.

Because the recognition site for *Hin*dIII occurs frequently in the *MCM* genes of budding yeast, I have repeated the experiment described above choosing a combination of two restriction enzymes with a less frequent site usage, that is, *Mlu*I and *Sal*I.

The result of the hybridisation is shown in Figure 3.5. Five hybridisation bands are observed. This number is lower than the number of restriction fragments expected

(U_B= -1, see Table 3.4) suggesting that some of the hybridising restriction fragments may be co-migrating or contain more than one *MCM3*-related sequence and therefore at least 5 *MCM3*-related sequences may be present in this organism.

To summarise the data of this section, there may be a minimum of six *MCM3*-related sequences in budding yeast and a minimum of five in fission yeast.

Determination of the minimum size of the *cdc21*$^+$ gene family in *S. pombe* by Southern blotting

A *cdc21*$^+$-specific probe and a *cdc21*$^+$-gene family-specific probe were used for this study. Ideally the *cdc21*$^+$-specific probe is expected to recognise sequences very close to the *cdc21*$^+$ gene, whereas the *cdc21*$^+$-gene family specific probe is expected to hybridise only to related genes in the family (see Figure 3.4). The size of the probes was chosen as short as possible in order to increase specificity and to simplify the interpretation of the results (see legend to Figure 3.6). Initially the probes were made by *in vitro* synthesis, as for making single strand (ss) DNA probes suitable for S1 mapping experiments (see Chapter Two). The ssDNA probes synthesised *in vitro* were purified from a polyacrylamide gel (Figure 3.6, panels a and b) and used to probe *S. pombe* genomic DNA digested with the restriction enzymes *Mlu*I and *Sal*I immobilised to a nylon membrane (panels c and d; see also Chapter Two for experimental details).

As can be seen in Figure 3.6 the *cdc21*$^+$ gene family-specific probe (panel b) recognises 3 distinct bands on *S. pombe* DNA (panel d), whereas the *cdc21*$^+$-specific probe (panel a) recognises only one band (panel c). The three bands of panel c have different intensities probably due to either the extent by which the probe cross-reacts with *cdc21*$^+$-related sequences or to the presence of more than one related sequence in each restriction fragment. The more intense band is the 12 kb band (top band, panel d) and may correspond to the *cdc21*$^+$ gene. For this experiment the hybridisation bands are less than the number of expected restriction fragments (U_B= -2). There are no recognition sites for *Mlu*I or *Sal*I in the probed regions suggesting that each band may correspond to a single gene.

Thus, this experiment suggests that the *cdc21*$^+$ gene does not have a very close relative (i.e., conserved in the N-terminal region in the fission yeast genome) and that the number of *cdc21*$^+$-related genes is small.

Although this approach uses highly specific probes the efficiency of recovery of the probe after the purification steps is low and this may not allow the detection of all the *cdc21*$^+$-related DNA sequences even after long exposures. I therefore decided to increase the specific activity of the *cdc21*$^+$ gene family-specific probe by using the

random priming labelling method (see Chapter Two). A *cdc21*$^+$ gene family-conserved probe was isolated from the pAC1 plasmid (which contains a genomic clone of the *cdc21*$^+$ gene, A. Coxon, D.Phil. thesis; see legend to Figure 3.7). This labelled DNA fragment was used to hybridise *S. pombe* genomic DNA digested with *Hin*dIII, *Eco*RI or *Bam*HI. As can be seen in Figure 3.7 the maximum number of hybridisation bands that can be counted is 6 (*Eco*RI lane). Intriguingly, the band expected for the *mis5*$^+$ gene digested with *Eco*RI (0.6 kb) is not detectable. The calculation of the U_B for these digests is described in the legend of Table 3.6. The highest U_B obtained is with the *Eco*RI or *Bam*HI digests (=2). Thus the minimum number of fission yeast *cdc21*$^+$-related sequences that can be predicted by this study is six.

Amplification of *cdc21*$^+$- related sequences by PCR

Another approach I have used to investigate the number of *cdc21*$^+$-related genes in yeast has been by amplification of *cdc21*$^+$-related sequences using the Polymerase Chain Reaction technology (PCR; Saiki *et al.*, 1988). Degenerate primers (Figure 3.8) specific for the 200 amino-acids highly conserved region in the protein family were synthesised and used in a PCR reaction with DNA purified from different organisms as substrate (see Chapter Two). These primers were able to amplify a band of the expected size of 280 bp from *S. pombe* (Figure 3.9, panel a) and *S.cerevisiae* genomes (panel b, lane 2). The bands amplified from the yeast genomes were isolated and their DNA sequence determined. The results of such a screening are shown in Table 3.7.

A DNA sequence amplified from the *S. cerevisiae* genome, that could potentially encode for the Mcm2 protein, was found to contain some discrepancies from the one published by Yan *et al.*, (1991). The triplet GCA at position 1846 was instead CGA and that the triplet CAT at position 1861 was instead GAT. These changes turn the R^{578} to an A^{578} and the D^{583} to a H^{583}; this was true for three independent PCR products sequenced. Interestingly, the new amino-acids introduced are conserved between members of the yeast protein family (compare proteins in the alignment of Figure 3.10), whereas the previous were not. The differences found in the nucleotide sequence of *MCM2* also alter the restriction map of the gene (see Table 3.8). Since these are the only changes scored in the *MCM2* sequence amplified by PCR, it is unlikely that the this *MCM2* sequence is a new distinct member of the gene family. Thus this leaves the possibility that the original *MCM2* sequence published by Yan *et al.*, (1991) either represents a polymorphism or may contain a reading mistake.

While this work was in progress the cloning and characterisation of putative *S. pombe* homologues of the budding yeast *MCM2* and *CDC46/MCM5* genes were published. They are the *nda1*$^+$/*cdc19*$^+$ (Forsburg and Nurse, 1994) and *nda4*$^+$ genes

(Miyake *et al.*, 1993) which encode structural homologues of the Mcm2 and Cdc46 proteins of *S. cerevisiae* (Miyake et al. 1993; Forsburg and Nurse, 1994). In both cases the sequences I have amplified by PCR perfectly matched those which have been published.

Discussion

Whatever the involvement of cdc21 and related proteins in S phase, they are presumably very important as they have been very well conserved in evolution (see Chapter One). The number of cdc21-related proteins identified in yeast and higher eukaryotes so far is small. Phylogeny analysis performed on the protein sequences available in various databases to date suggests the presence of six distinct members in yeast. The P1.1^{Mcm3} protein, not included in the phylogeny analysis because it is not a complete sequence, may represent a sub-class of the P1/Mcm3 proteins, suggesting the presence of more than one type of Mcm in higher eukaryotic cells. The P1.1^{Mcm3} protein (Hu *et al.*, 1993) has not been identified in any other organisms as yet.

I have attempted to determine the minimum number of *cdc21*$^+$ family-related genes in yeast by cross-hybridisation on Southern blots using as a probe either the *S. cerevisiae MCM3* or the *S. pombe cdc21*$^+$ gene. The data suggest that a minimum number of six genes can be predicted for the budding yeast genome. The suggested number of *cdc21*$^+$-related genes in fission yeast using both budding yeast *MCM3* or *cdc21*$^+$ itself as probes is also six. The results also suggest that there may be only one type of *cdc21*$^+$ in fission yeast.

PCR analysis failed to identify new homologues of *cdc21*$^+$, but this could be merely due to a non-suitable degeneracy of the primers used. I was able to identify DNA sequences which could potentially encode for fission yeast structural homologues of the Mcm2 and Mcm5/Cdc46 proteins of budding yeast. While this work was in progress, these have been published, as the nda1/cdc19 and nda4 proteins respectively (Miyake *et al.*, 1993; Forsburg and Nurse, 1994).

These data are consistent with the presence of a small number of Mcm proteins in yeast which may be similar in both *S. cerevisiae* and *S. pombe*. One may ask if there may be a difference in number and quality of Mcm proteins between yeast and higher eukaryotes. The use of completely degenerate primers and low stringency of annealing and ultimately the screening of cDNA libraries will be required to saturate the screening. Also the completion of the sequence of the yeast genome would give the final answer to the number of *cdc21*$^+$-related genes. Certainly the finding that two different versions of the Mcm3 protein is present in the same organism opens new and interesting possibilities and inevitably raises more questions: Do these proteins have

similar functions? Are these proteins ubiquitous or perhaps they are differentially expressed in certain cell types?

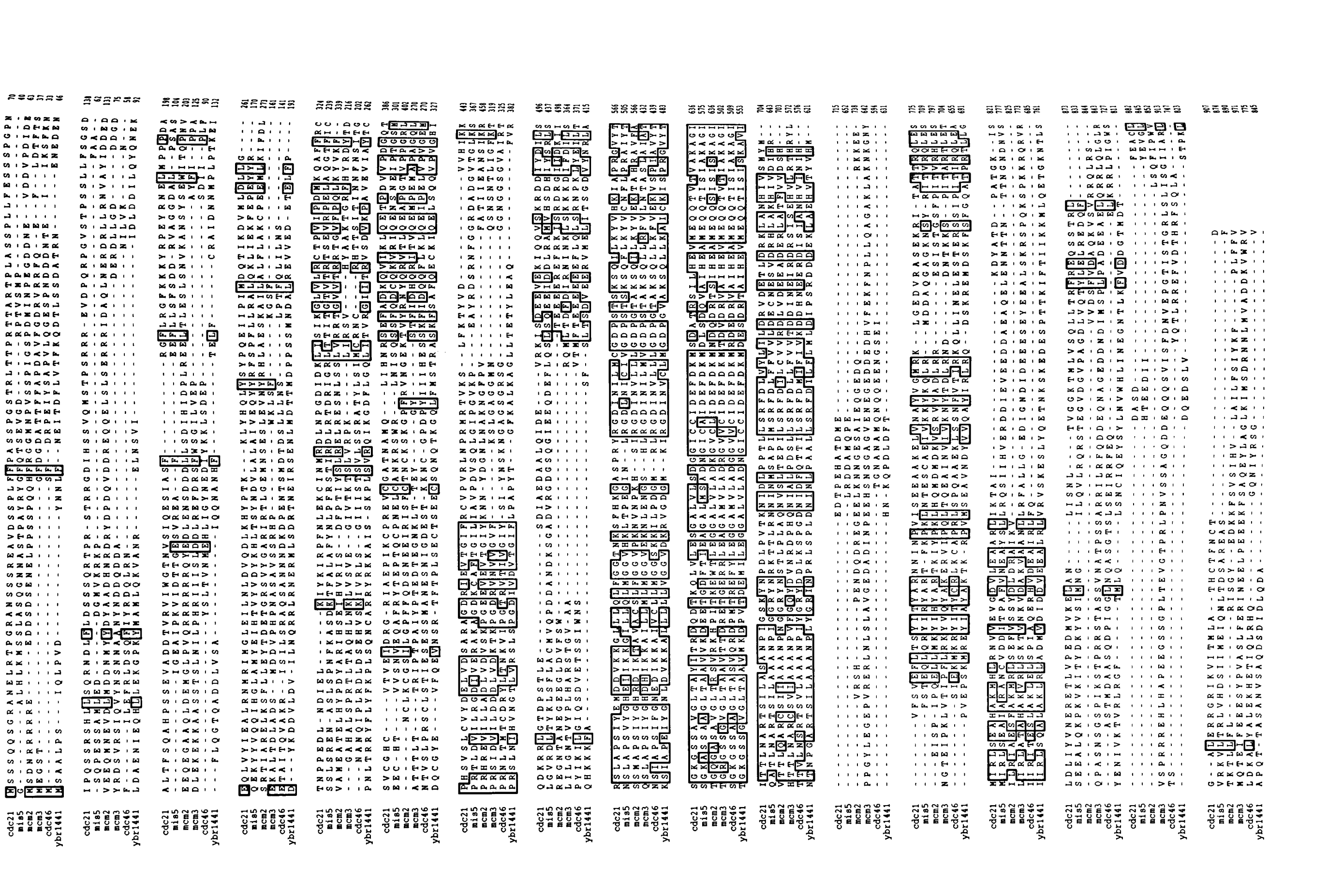


Figure 3.1

The cdc21 protein is structurally related to the Mcm protein family

Alignment of budding and fission yeast cdc21-related proteins. Sequences were retrieved from various databases using the accession numbers indicated in Figure 3.4. Alignment was created using the programme CLUSTALV (fixed gap penalty = 10; floating gap penalty = 10; protein weight matrix = pam 250). Identical/similar amino-acids were displayed boxed using the prettyplot programme of the Genetics Computer Group (GCG, version 8). Pad characters ('-') were inserted to optimise the alignment.

	Organism				
	S. cerevisiae	*S. pombe*	*X. laevis*	*M. musculus*	*H. sapiens*
Protein					
Mcm2	+(1)	nda1(8) cdc19(9)	?	?	(Bm28) (17)
Mcm3	+(2)	+*(10)	P1(12,13)	P1 (14,15,16)	(P1Mcm3) (14) (P1.1Mcm3) (18)
Mcm5/Cdc46	+(3,4)	nda4 (8)	?	+(16)	+(18)
Cdc21	Cdc54(5)	+(10)	+*(10)	+(16)	+(18)
Ybr1441/Cdc47	+(6,7)	?	?	?	?
Mis5	?	+(11)	?	?	?

Table 3.1

Summary of cdc21-related proteins identified in a variety of organisms

Individual members of the Cdc21 protein family identified so far in a variety of organisms (shaded regions). Superscript asterisks indicate putative Mcm homologues identified as PCR fragments. Unidentified members are indicated by a question mark. Proteins with the same name in different organisms are indicated by (+). References: (1) Yan *et al.*, 1991; (2) Gibson *et al.*, 1990; (3) Hennessy *et al.*, 1991; (4) Chen *et al.*, 1992; (5) Whitbread and Dalton, 1995; (6) Bussereau *et al.*, 1993; (7) Dalton and Whitbread, 1995; (8) Miyake *et al.*, 1993; (9) Forsburg and Nurse, 1994; (10) Coxon *et al.*, 1992; (11) Takahashi *et al.*, 1994; (12) Laskey, R., pers. comm.; (13) Buckbinder and Brown, 1992; (14) Thömmes *et al.*, 1992; (15) Starborg *et al.*, 1995; (16) Kimura *et al.*, 1994; (17) Todorov *et al.*, 1994; (18) Hu *et al.*, 1993.

	cdc21	cdc54	mis5	Mcm2	BM28	nda1	Mcm3	mP1	nda4	Cdc46	Cdc47
cdc21	100	*52.48*	25.74	20.22	21.39	25.82	18.85	21.84	27.08	23.48	24.73
Cdc54	100		25.17	23.48	22.17	26.75	20.48	22.09	27.36	25.9	25.32
mis5	100			20.13	21.62	26.35	23.57	19.31	24.4	25.1	24.8
Mcm2	100				*41.34*	*61.72*	22.69	23.1	26.25	26.45	22.6
BM28	100					*48.3*	18.95	22.85	24.3	23.35	22.36
nda1	100						26.2	22.78	24.72	26.49	24.76
Mcm3	100							*39.26*	28.33	26.83	23.20
mP1	100								25.70	23.87	22.85
nda4	100									*54.58*	27.36
Cdc46	100										25.16
Cdc47	100										

Table 3.2

Percentage of similarity between cdc21-related proteins

The numbers are expressed as a percentage of the similarity and were calculated using the CLUSTALV programme. Italic underlined numbers indicate cdc21-related proteins with a degree of similarity significantly higher than the average (25%) and may represent homologous relationships.

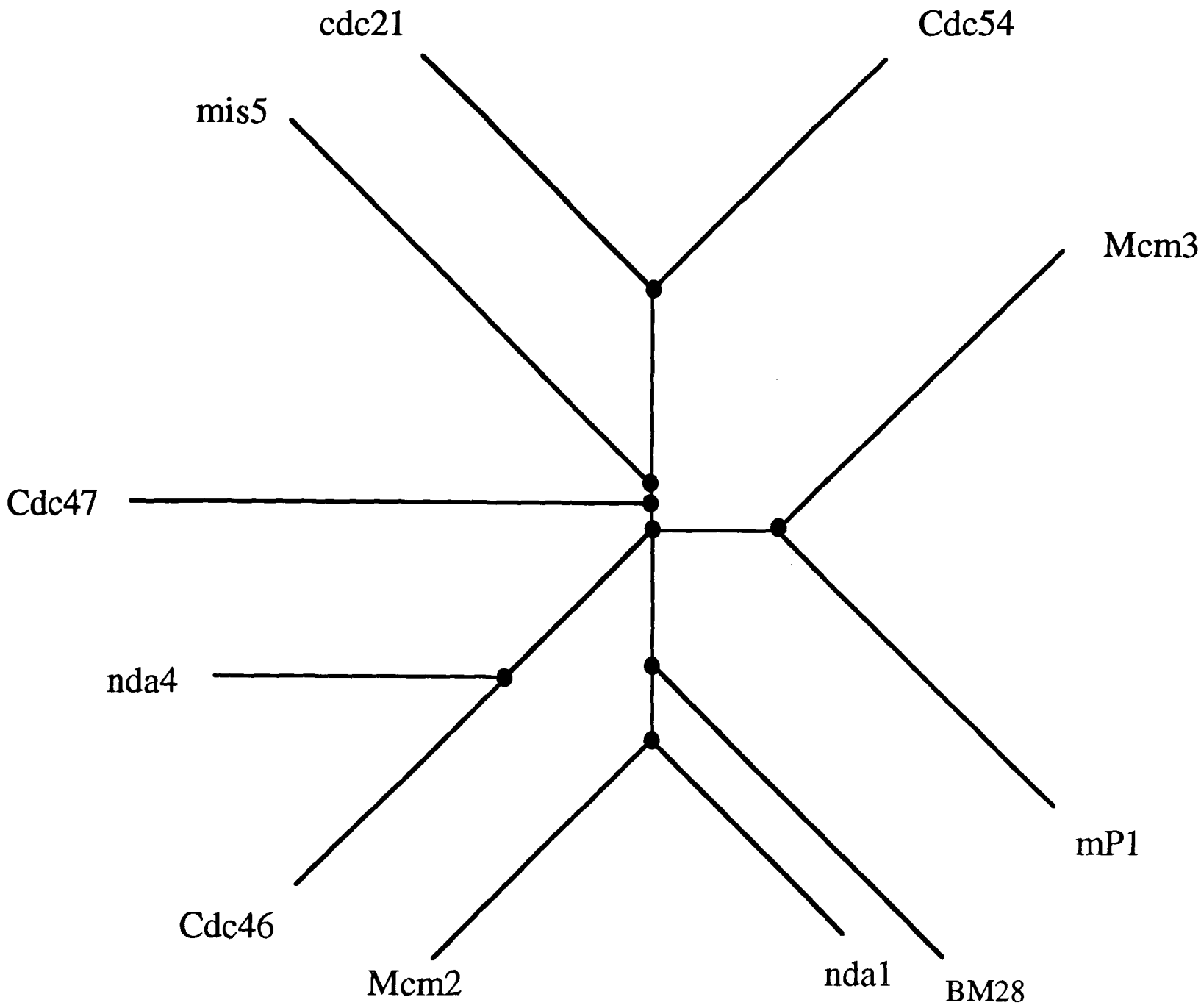


Figure 3.2

Phylogeny analysis of cdc21-related proteins

This unrooted tree was derived using the neighbour-joining method (in CLUSTALV) to calculate branch lengths and estimated phylogenic distances.

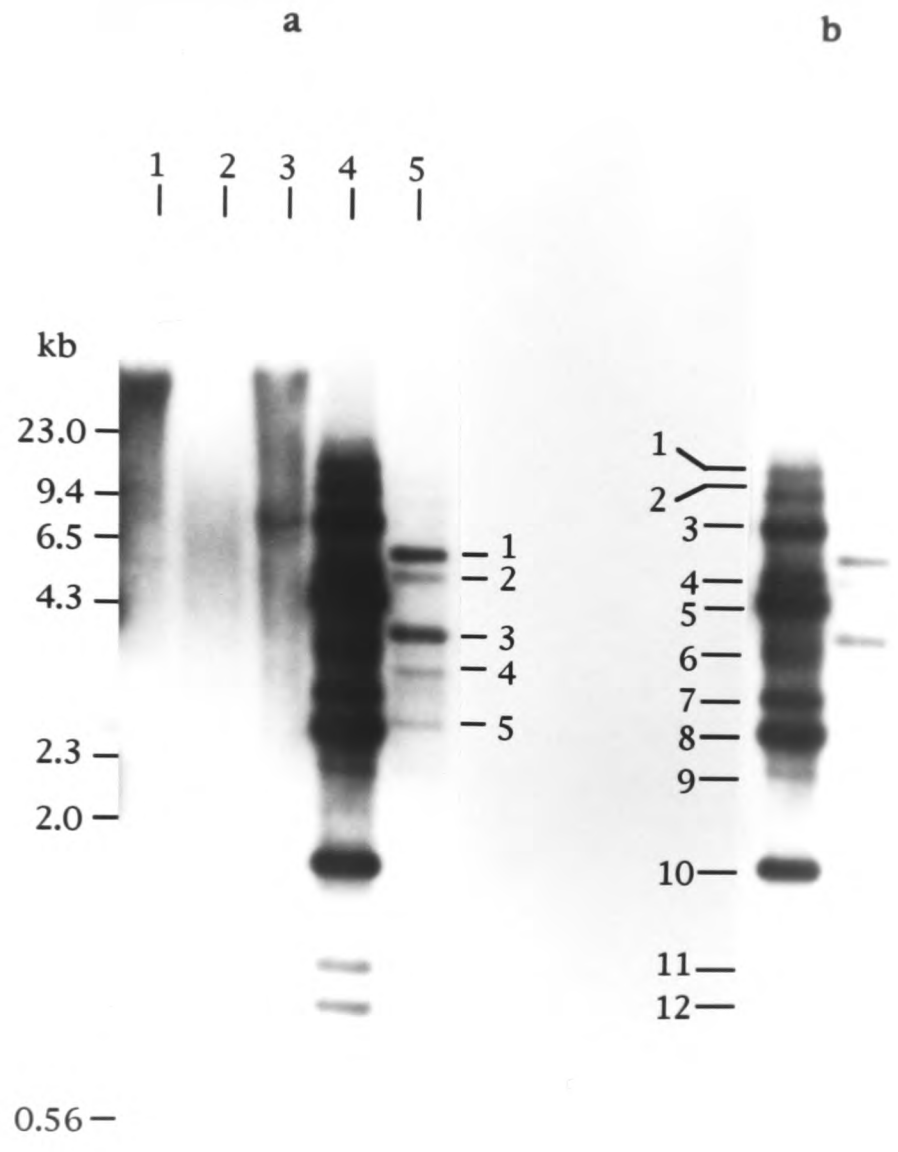
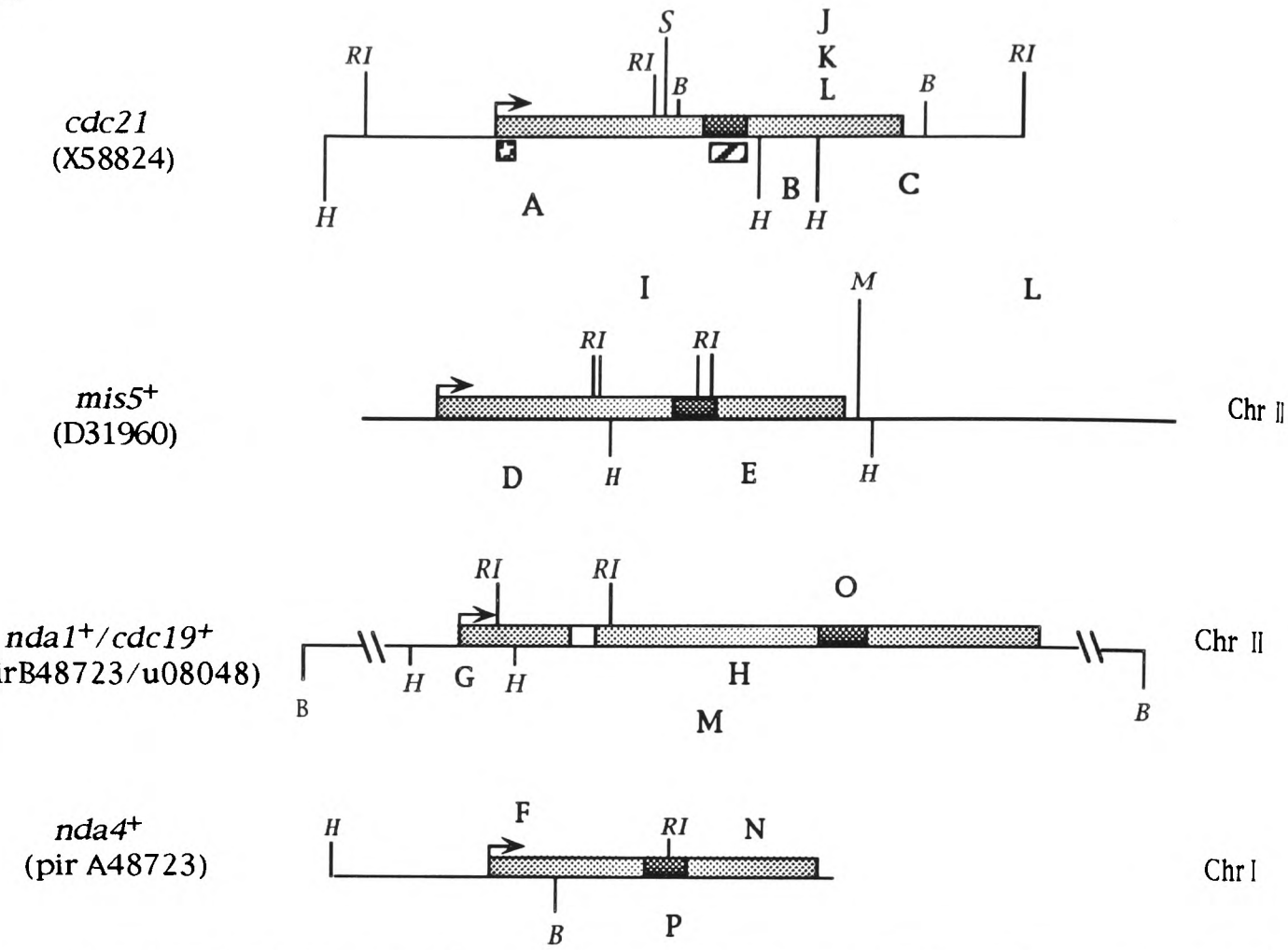


Figure 3.3

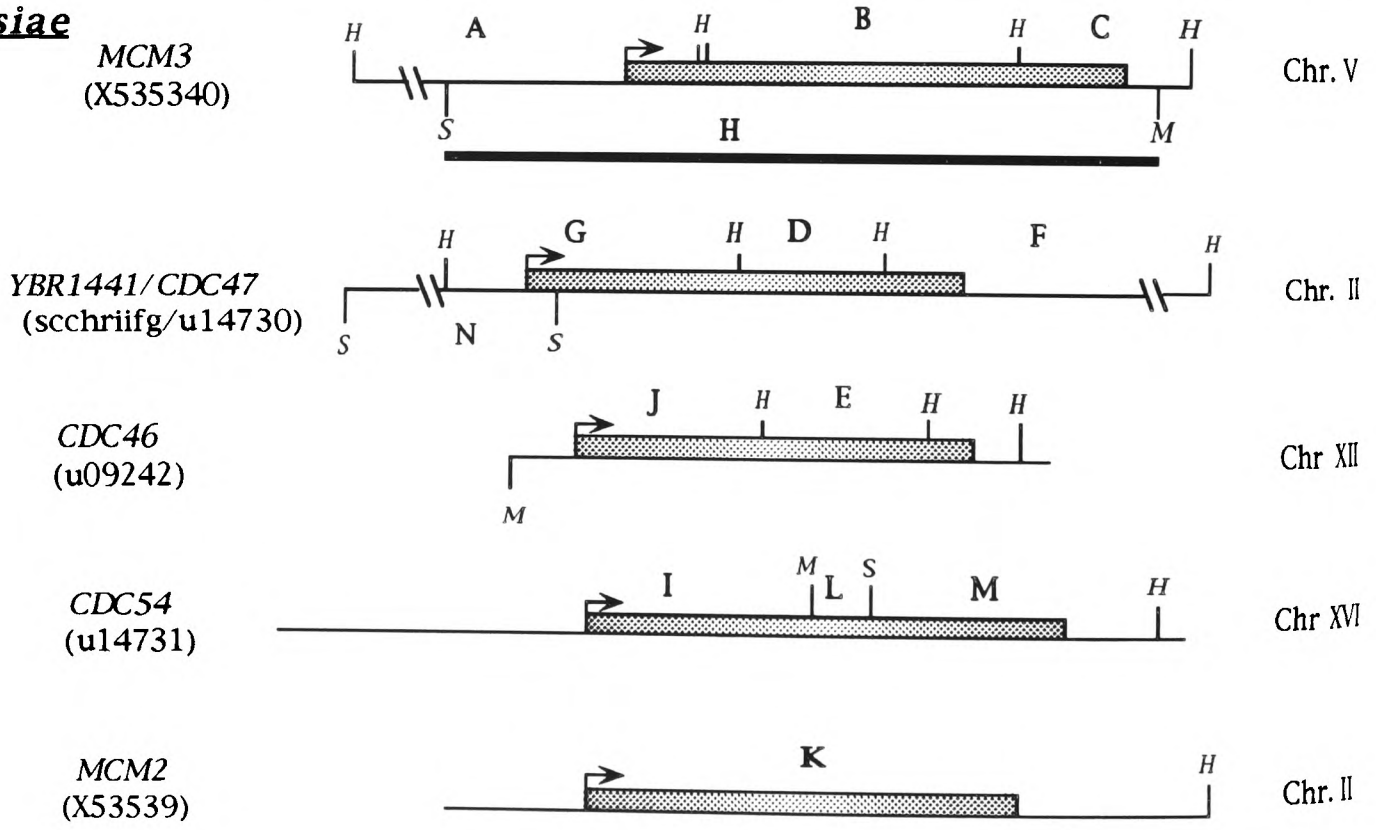
The budding yeast *MCM3* gene cross-reacts with putative related sequences in yeast and higher eukaryotes genomes

Genomic DNA was digested with the restriction enzyme *Hin*dIII, subjected to electrophoresis and transferred to a nylon membrane as described in Chapter Two. After transfer, the filter was probed with [α-^{32}P]dCTP random primed labelled *S. cerevisiae MCM3* gene isolated as the *Mlu*I-*Sal*I fragment from the YCP50.*MCM3* plasmid (Gibson *et al.*, 1990). Hybridisation was carried out as described in Chapter Two. **Panel a**; lane **1**: *X. laevis* DNA; lane **2**: *D. melanogaster* DNA; lane **3**: *H. sapiens* DNA; lane **4**: *S. cerevisiae* DNA; lane **5**: *S. pombe* DNA. **Panel b**; low exposure of lanes **4** and **5** of panel **a**. Numbers on the right and left hand side of panel **a** and **b** respectively, indicate the bands whose size is reported in Table 3.3.

S.pombe



S.cerevisiae



Key:

=*cdc21*$^+$-specific probe;

= *cdc21*$^+$ family-specific probe;

= *MCM3* probe

= intron; = Mcm_s highly conserved region = Open Reading Frame

RI= *Eco*RI; H= *Hin*dIII; B= *Bam*HI; S= *Sal*I; M= *Mlu*I = direction of transcription

1kb

Figure 3.4

Restriction maps of *cdc21*$^+$-related genes in *S. cerevisiae* and *S. pombe*

This was constructed by retrieving the correspondent sequences from various databases (database accession numbers are indicated underneath the name of each gene) and searching for restriction recognition sites using the Nucleotide Interpretation Programme (NIP) of Staden (1986). Letters in bold in between two restriction enzymes recognition sites (plain letters) indicate the restriction fragment generated by digestion with those restriction enzymes. The chromosomal location of the genes (Chr.), when known, is also indicated. A large genomic clone of the budding yeast *MCM3* gene was also found in the database as the open reading frame 23 on chromosome V (accession number L10830).

S. cerevisiae *Hin*dIII			*S. pombe* *Hin*dIII		
Band no. on gel	calculated size	similar to restriction fragment	Band no. on gel	calculated size	similar to restriction fragment (of expected size)
1	13170		1	5906	
2	10279		2	4913	
3	7414		3	3792	**A**
4	5159		4	3299	
5	4692	**A**	5	2766	
6	3610				
7	3119				
8	2840	**F**	U_B= [5 - (1 + 4)]= 0		
9	2288				
10	1674	**B** **G**			
11	1106	**E** **C**			
12	907	**D**			
U_B = [12 - (5 + 3)] = 4					

Table 3.3.

Predicted size of the hybridisation bands of lane 4 (a or b) and 5 of Figure 3.3

Letters in bold correspond to restriction fragments of the size predicted for the known *MCM* genes digested with the restriction enzyme *Hin*dIII (see Figure 3.4).

S. cerevisiae. Five distinct genes encoding cdc21-related proteins have been described in this yeast (see Figure 3.4). Five out of the twelve observed bands (5, 8, 10, 11 and 12) can be assigned to restriction fragments of three genes in the family (*CDC46*, *MCM3* and *YBR1441/CDC47*). Note that some of them (B+G and E+C) may co-migrate in the same band. Three more restriction fragments (I + L + M, J and K) are expected for the *CDC54*, *CDC46* and *MCM2* genes and are of unknown molecular weight (see restriction map of Figure 3.4). Fragments smaller than 400 bp are not detectable in this experiment as they have most probably run off the gel.

S. pombe. Four distinct genes encoding cdc21-related proteins have been described in this yeast. Only one band (3) can be assigned to the *cdc21*$^+$ gene digested with *Hin*dIII (restriction fragment A in Figure 3.4). The sizes of the four remaining restriction fragments (C, D, F and H) are unknown. The U_B is calculated as:
Total observed bands - [assigned bands + expected fragments of unknown size].

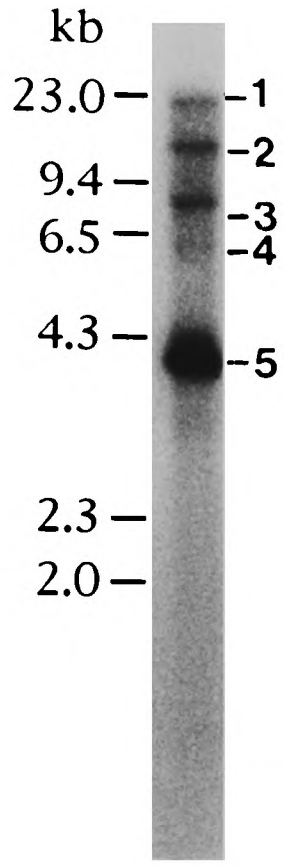


Figure 3.5

The number of *MCM3*-related genes in budding yeast is small

Genomic *S. cerevisiae* DNA was digested with the restriction enzymes *Mlu*I and *Sal*I, blotted and probed as described in Chapter Two. Hybridisation signals were detected after 7 days of exposure to an X-ray film at -70°C. Only one band of unknown size per gene is expected for this digest except for the *MCM3* (restriction fragment H) and the *CDC54* genes (restriction fragment L). Two restriction fragments (L and N of 0.47 and 4.4 kb respectively) are not detectable in this blot, the second probably due to the short extent of overlap with the probe used.

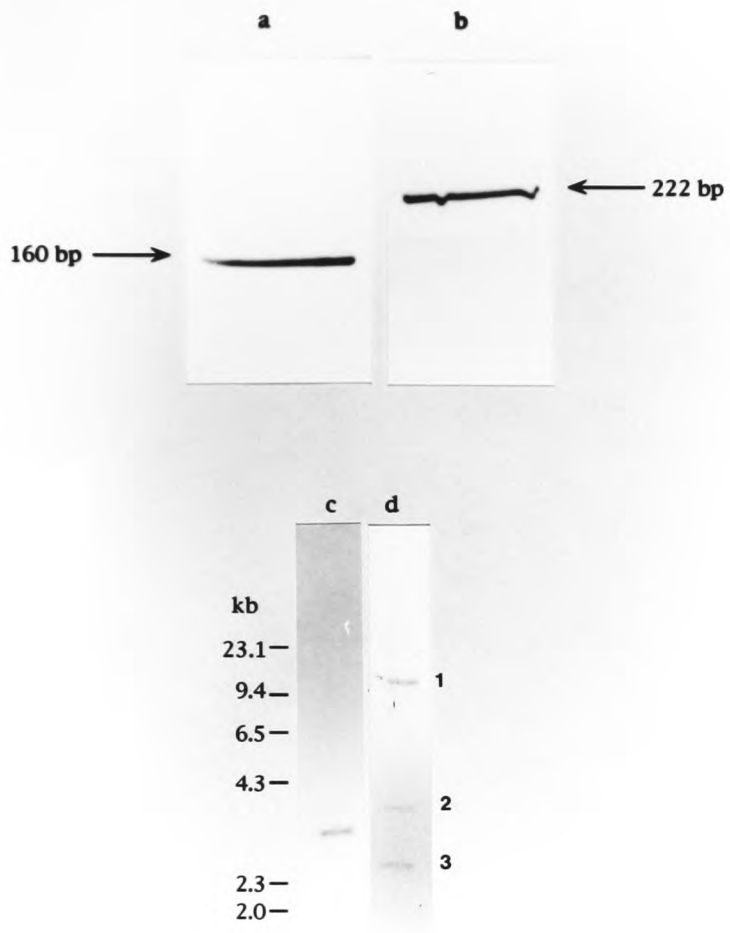


Figure 3.6

Synthesis of *S. pombe cdc21*$^+$-specific and *cdc21*$^+$ gene family-specific ssDNA probes and hybridisation to fission yeast DNA.

cdc21$^+$-specific (panel **a**) and *cdc21*$^+$ gene family-specific (panel **b**) ssDNA probes were synthesised *in vitro* in the presence of [α-^{32}P]dCTP as described in Chapter Two. The DNA templates used to prepare these probes were selected from a *cdc21*$^+$-shotgun bank made in the M13mp9 vector (A.Coxon, D.Phil thesis) as the A136 clone (160 bp, *cdc21*$^+$-specific) and the A50 clone (222 bp , *cdc21*$^+$ gene family-specific) (see Figure 3.4). These probes were purified and used to hybridise *S.pombe* genomic DNA (see Chapter Two) digested with the restriction enzymes *Mlu*I and *Sal*I (panel **c** and **d**). Hybridised nylon membranes were exposed to an X-ray film with double screen intensifier for 1 month at -70°C.

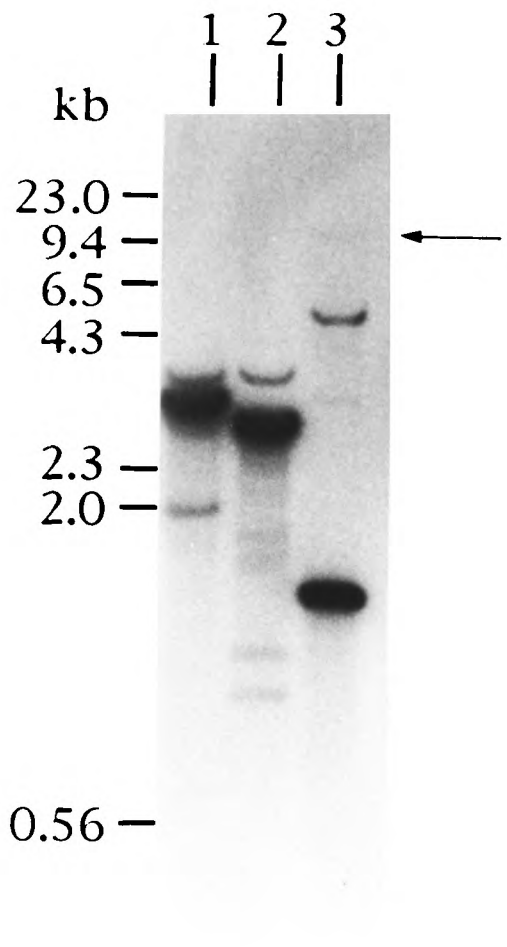


Figure 3.7

The number of *cdc21*$^+$-related sequences in fission yeast is small

S. pombe genomic DNA was digested with the restriction enzymes *Hin*dIII, *Eco*RI or *Bam*HI, and processed as described in the legend of Figure 3.3. A 500 bp *cdc21*$^+$ gene family-specific DNA fragment (see Figure 3.4) was purified from the pAC1 plasmid (A. Coxon, D.Phil. thesis) by digestion with the restriction enzymes *Nde*I and *Eco*RV, labelled and used to hybridise the nylon membrane as described in Chapter Two. The nylon membrane was exposed for 7 days at -70°C with double intensifier screen. Lane **1**: *Hin*dIII; lane **2**: *Eco*RI; lane **3**: *Bam*HI.

S. cerevisiae *MluI-SalI*	
Band	calculated size
1	16593
2	11177
3	7437
4	5931
5	**3969H**
U_B=[5 - (1 + 5)]= -1	

Table 3.4

Predicted size of the hybridisation bands of the Southern blot experiment of Figure 3.5

The letter and number in bold correspond to the size of the restriction fragment expected for the *MCM3* gene (Gibson *et al* ., 1990).

S. pombe *MluI-SalI*		
Band	calculated size	
	panel c	**panel d**
1		12611
2	3424	3741
3		2701
U_B= [3 - (0 + 5)] = -2		

Table 3.5.

Predicted size of the hybridisation bands of the Southern blot experiment of Figure 3.6

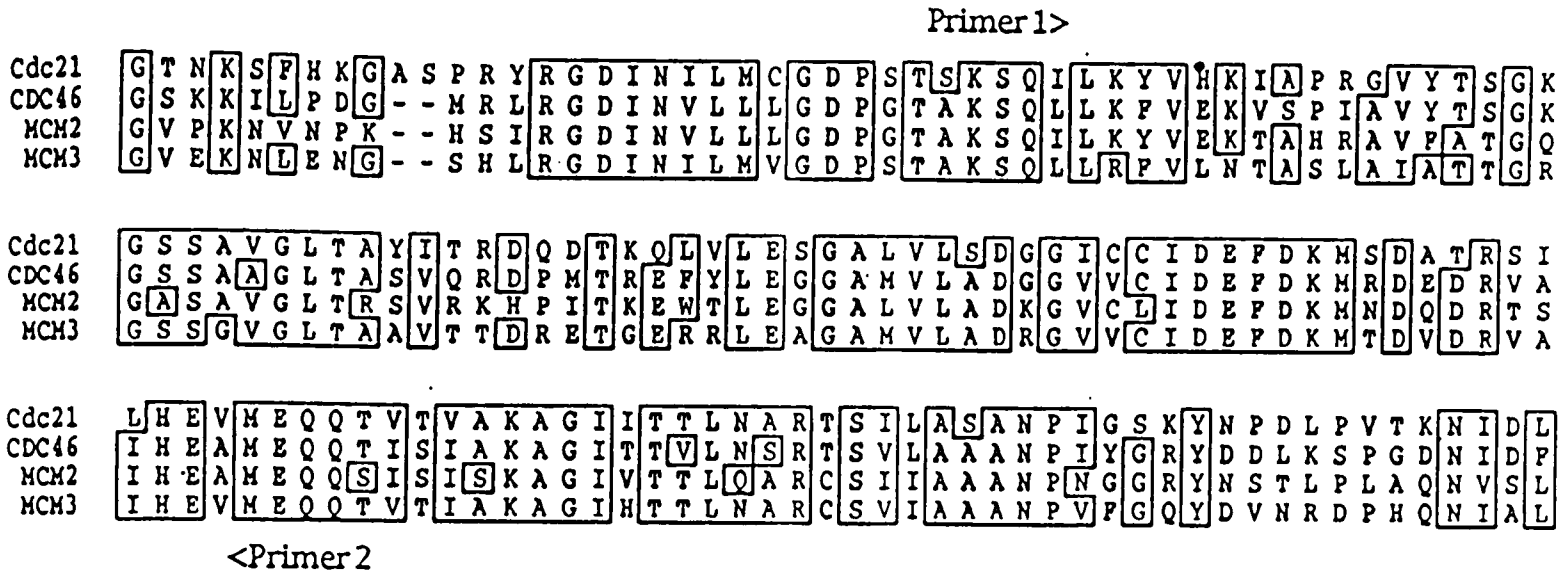
The ordering of the bands was similar to that adopted in Table 3.3. There are no bands of known molecular weight expected for *cdc21*$^+$ and related genes in fission yeast digested with the indicated enzymes (see restriction map in Figure 3.4).

	S. pombe		
Band	*Hin*dIII	*Eco*RI	*Bam*HI
	calculated size		
	1	2	3
1	3987	3795	14984
2	3457**A**	3170**K**	5686**M**
3	3050	1815	3457
4	2050	1635	1467**L**
5		1020	
6		816	
	$U_B = 1$ [4- (1+2)]	$U_B = 2$ [6- (1+3)]	$U_B = 2$ [6- (2+2)]

Table 3.6

Predicted size of the hybridisation bands of the Southern blot experiment of Figure 3.7

Letters (superscript and in bold) correspond to the restriction fragments of the restriction map in Figure 3.4.



G D P G T A K W/S Q

Primer1 5'- GGG GAT CCA GGT ACT GCT AAR TXN CAA - 3'

(forward) *Bam*HI

T Q P D M V/A E

Primer2 5' - TC CGT TTG TGG ATC CAT NRC YTC RT - 3'

(reverse) *Bam*HI

Figure 3.8

Synthesis of degenerate primers used to amplify *cdc21*$^+$-related sequences from yeast genome

The sequences of cdc21-related proteins were aligned in the highest region of homology as described in Figure 3.1. Sequences were retrieved using the accession numbers indicated in Figure 3.4. Degenerate primers specific for this region were designed. Codons were biased to the *S. pombe* codon usage. R= G or A; X= G or C; N= any nucleotide; Y= C or T. Amino-acids are indicated on top of each primer (one letter one amino-acid). The Oligo programme (Rychlik and Rhoads, 1989) was employed to determine the melting temperature (T_m) and the probability of self-annealing of each primer. Primers were designed to contain a *Bam*HI restriction site to facilitate the cloning and screening procedure.

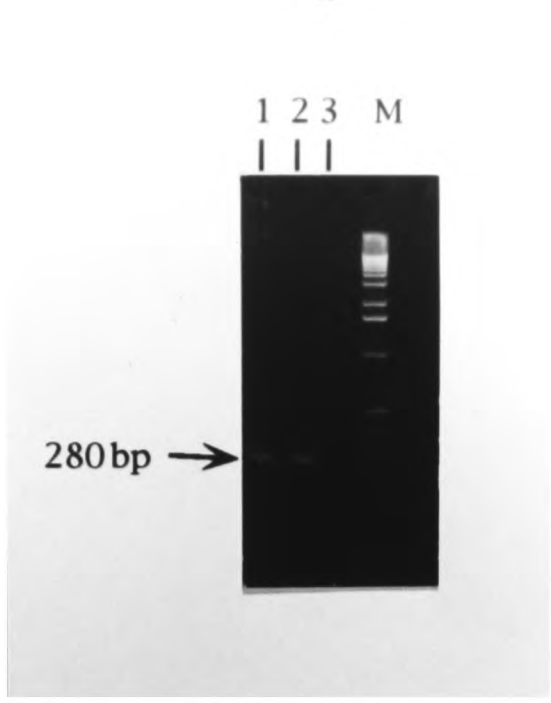


Figure 3.9

Amplification of *cdc21*$^+$-related sequences in yeast and higher eukaryotes by PCR

Degenerate primers (Figure 3.8) were used in a PCR reaction with genomic DNA purified from different organisms (see Chapter Two). Conditions of amplification are described in Chapter Two. The annealing temperature was 55°C for PCR on *S. pombe* DNA (a) and 58°C on *S. cerevisiae* DNA (b). The expected size of the DNA fragment to be amplified is of 280 bp (arrow). **Panel a**. Amplification of *cdc21*$^+$-related sequences from fission yeast genome. Lane **1** and **2**: fission yeast DNA; lane **3**: no DNA; **M**: 1kb DNA ladder molecular marker. **Panel b**. Amplification of *cdc21*$^+$-related sequences from budding yeast (lane **2**), *E. coli* (lane **3**), *D. melanogaster* (lanes **4** and **5**) and *H. sapiens* genomes (lanes **6** and **7**) DNA. Lane **1**: no DNA control. **M**: 1kb DNA ladder.

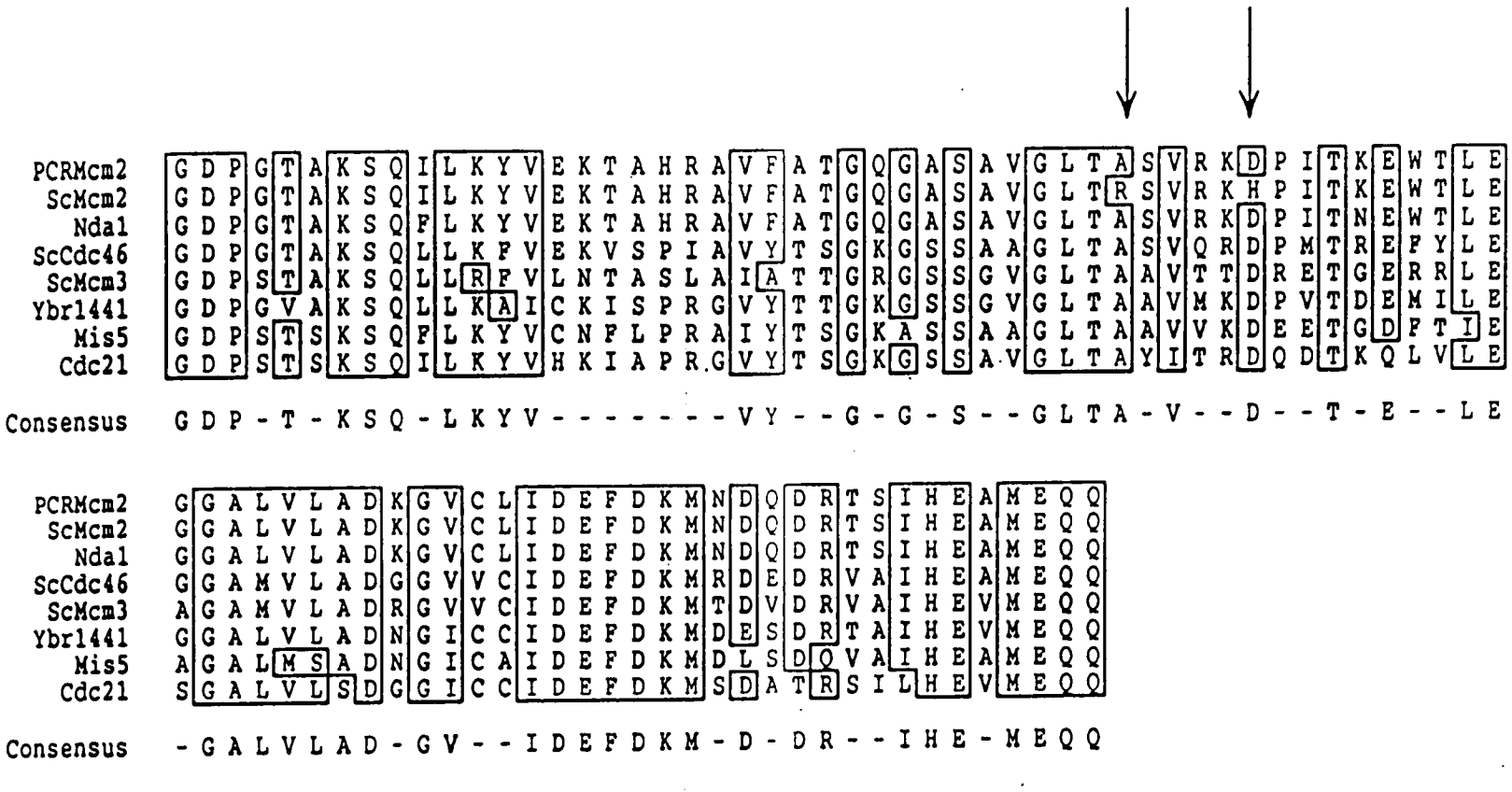


Figure 3.10

Comparison of the *S. cerevisiae MCM2* gene product identified by PCR with members of the cdc21 protein family

Sequences were aligned as described in Figure 3.1. Arrows indicate the amino-acids changes introduced by the DNA sequence of the *MCM*2 sequence amplified by PCR from *S. cerevisiae* genomic DNA (PCRMcm2).

PCR	clones sequenced	sequences amplified			
		*MCM2** */nda1*$^+$	*MCM3* *Spmcm3*$^+$	*CDC46* */nda4*$^+$	*cdc21*$^+$/ *CDC54*
S. cerevisiae	20	3	5	12	0
S. pombe	39	10	0	26	3

Table 3.7

Summary of the PCR screening aimed to amplify *cdc21*$^+$-related sequences in yeast

Amplified DNA fragments were isolated and ligated into the polylinker of the pUC18 plasmid as described in Chapter Two. Recombinant plasmids were recovered in *E. coli* and sequenced (see Chapter Two for experimental details). The sequence of *S. cerevisiae MCM2* (*) amplified was found different from that one published by Yan *et al.*, (1991).

RE	*MCM2* (Yan *et al.*, 1991)	*MCM2* (this work)
*Alw*I	102	116
*Bsty*I/*Xho*II	none	1861
*Fok*I	108	94
*Mbo*I	107	121
*Sfa*nI	none	97

Table 3.8

Restriction map of the DNA sequence of the highly conserved region of budding yeast *MCM2* (Yan *et al.*, 1991) and of budding yeast *MCM2* amplified by PCR. RE: restriction enzyme. Map positions are indicated in bp.

Chapter Four

Genetic suppression of the *cdc21*^{ts} mutation.

Introduction

The fission yeast *cdc21-M68* strain was isolated as a recessive temperature-sensitive (ts) mutant causing *c*ell *d*ivision *c*ycle arrest and altered kinetics of DNA replication after shift to the restrictive temperature (Nasmyth and Nurse, 1981). *cdc21* mutant cells arrest in interphase with an undivided nucleus and almost completely replicated DNA (Coxon *et al.*, 1992). The *cdc21* mRNA produced by the mutant cells is indistinguishable from that of wild-type cells at both permissive and restrictive temperature on Northern blots (A.Coxon, D. Phil Thesis). The *cdc21*$^{+}$ gene product is a predicted 101 kDa protein whose function is unknown. Its early transition point in the cell cycle and its similarity to the Mcm2-3-5 proteins (see Chapter One) are consistent with a role in the initiation step of DNA replication (Nasmyth and Nurse, 1981; Coxon *et al.*, 1992). The specific role of cdc21 and related proteins in DNA replication has still to be determined.

One way to gain more insight on the function of cdc21 is to identify interacting proteins. This can be done genetically by isolation of interaction suppressors, like compensatory mutations in an interacting function (induced by mutagenesis or by alteration of the copy number of the interacting function) which may restore the defect of the *cdc21*^{ts} mutant. This is a widely used an approach to identify interaction suppressors (for review see Maurer and Botstein, 1982).

In addition by-pass suppressors may be identified, which cause the activation of a new pathway that substitutes for the pathway blocked. This may be achieved by overproduction of a function which now activates a secondary pathway in the mutant strain. The wild-type version of the mutated allele may also suppress the conditional allele when overexpressed.

Interacting functions can also be identified through the isolation of unlinked non-complementing mutants which are non-viable under conditions that are permissive for either of the single mutants alone (synthetic lethality). This comes from the observation that two recessive mutations in unlinked genes that encode interacting proteins may fail to complement (Stearns and Botstein, 1988; Guarente, 1993). In some cases this has been shown to be true at a biochemical level (Ludeña, *et al.*, 1977).

In this Chapter I have tried to determine if proteins structurally related to cdc21 can cross complement yeast lethal-conditional mutants in members of the *cdc21*$^{+}$ gene family.

I have also attempted to identify functions genetically interacting with *cdc21*$^{+}$ in the hope of isolating a gene with a known function, perhaps in DNA replication. This was done either by screening for multi-copy suppression of the *cdc21*^{ts} mutation or for mutants that exacerbate the phenotype of the *cdc21-M68* allele in permissive conditions (synthetic lethality).

Results and Discussion

Suppression of the *cdc21*^{ts} mutation by budding yeast and fission yeast *cdc21*$^{+}$-related genes

Two *Saccharomyces cerevisiae* genes related to fission yeast *cdc21*$^{+}$, namely the *MCM2* and *MCM3* genes (Yan *et al.*, 1991; Gibson *et al.*, 1990), obtained as the budding yeast multi-copy plasmids YCp101.*MCM2* and YCP50.*MCM3*, were introduced into wild-type and *cdc21-M68* strains of fission yeast as described in Chapter Two. The fission yeast replicative vector pIRT2 (Hindley *et al.*, 1987) was included as a control of efficiency of transformation and transformants were selected at the *cdc21*^{ts} permissive temperature (25°C). It was found the budding yeast plasmids were not able to transform the wild-type or the *cdc21-M68* strain, while the pIRT2 plasmid was able to transform fission yeast at high frequency (see Table 4.1), indicating that the budding yeast vectors could not propagate in fission yeast. This result is not surprising since *ars* function is only partly conserved between *S. pombe* and *S. cerevisiae* (see Chapter One).

Therefore, the full *MCM2* and *MCM3* genes were sub-cloned into the multi-copy *S. pombe* pIRT2 vector (see Figure 4.1). The recombinant plasmids obtained, pD*MCM2* and pD*MCM3*, and the pAC1 plasmid (pIRT2, bearing a wild-type copy of the *cdc21*$^{+}$ gene; A. Coxon, D.Phil. thesis) were transformed into the *cdc21*^{ts} strain. Transformants were recovered at 25°C and replica-plated at the restrictive temperature (36°C). Unlike pAC1, the pD*MCM2* and pD*MCM3* plasmids were not able to suppress the *cdc21*^{ts} mutation (see Table 4.1). This failure may be due to non-expression of the budding yeast genes themselves as a consequence of differences in transcription initiation mechanisms between the two yeasts (Russell, 1983). However this seems unlikely since the *MCM2* and *MCM3* genes cannot suppress the *cdc21*^{ts} mutation even if under control of a fission yeast promoter (I.Todorov, pers. comm., see also Table 4.1). One interpretation of these results is that individual members of the Mcm protein family, although structurally related, have unique functions and may need species-specific requirements in order to function.

Moreover the ability of the *cdc21*$^{+}$ gene to suppress two mutations in two fission yeast genes related to *cdc21*$^{+}$, namely *nda1* and *nda4* (Miyake *et al.*, 1993) was also tested. For this purpose the pREP*81X::21*$^{+}$ construct (in which *cdc21*$^{+}$ is under control of the fission yeast 81X*nmt1* thiamine-repressible promoter; Basi *et al.*, 1993; see Chapter Seven) was transformed into the above strains and transformants were selected at the permissive temperature for these strains (33°C). No colonies

could be scored at their restrictive temperature (20°C) indicating that the pREP*81X::cdc21*$^{+}$ construct cannot suppress these mutants.

These results show that, although related in structure, individual cdc21-related proteins are not functionally interchangeable.

Suppression of the *cdc21*^{ts} mutation by an *S. pombe* genomic library

I tried to suppress the *cdc21*^{ts} mutation by altering the dosage of fission yeast DNA sequences in the *cdc21*^{ts} mutant strain. A fission yeast genomic bank ligated into the pUR18 vector (Barbet *et al.*, 1992) was transformed into the *cdc21-M68* strain, transformants were recovered at the permissive temperature and suppressors were selected at 36°C. The results of such a screen are reported in Table 4.3. Eight putative transformants able to form colonies at 36°C were isolated from a total of 10,000 colonies (Table 4.2). Genetic analysis on these transformants indicated that the Ts^{+} phenotype of six out of the eight transformants was plasmid-dependent. The remaining two Ts^{+} transformants could have then been intra or extra-genic suppressors of the *cdc21*^{ts} mutation. It has been previously shown that such revertants may acquire new phenotypes (e.g., cold sensitivity; Jarvick and Botstein, 1975; Moir *et al.*, 1982). This was also tested for the *cdc21*$^{ts+}$ strains by incubation at 18°C for 7 days. None of these strains was found to be cold sensitive, making the genetic analysis of these mutants difficult. Plasmids recovered from the six transformants which showed instability of the Ts^{+} phenotype were analysed by restriction digestion and found to contain inserts. The restriction pattern of these plasmids fell into two categories (Figure 4.2A). The two distinctive classes of plasmids were called respectively pUR1a (groups 1, 3, 4, 5 and 6) and pUR6a (group 2). Each digest pattern was compared with the one of a plasmid containing the *cdc21*$^{+}$ gene (pAC1, A.Coxon, D.Phil. thesis). The restriction digest pattern of the recovered plasmids matched that of *cdc21*$^{+}$ (Figure 4.2B), suggesting that the two plasmids may contain two *cdc21*$^{+}$ genomic inserts . The restriction pattern of these plasmids, after further analysis (Figure 4.3), was consistent with that of plasmids containing two overlapping genomic DNA fragments of the *cdc21*$^{+}$ gene. That the inserts contained the *cdc21*$^{+}$ gene was confirmed by sequencing using a *cdc21*$^{+}$-specific primer (see legend to Figure 4.3).

This screen thus failed to produce non-*cdc21*$^{+}$ genomic sequences able to suppress the *cdc21*^{ts} mutant. As suppression might be allele specific (Stearn and Botstein, 1982) one limitation of this genetic screening has been the availability of just one allele of *cdc21* (Nasmyth and Nurse, 1981). Genetic evidence indicates that

the *cdc21* protein produced by the *cdc21-M68* mutant may be partially defective (see Chapter Seven) which may make the isolation of extragenic suppressors more difficult. Another possible explanation may be that high copy suppression of mutations in genes of the *MCM* family may not be feasible. No such suppressors have been reported so far.

Isolation of genes interacting with *cdc21*$^+$ by screening for 'synthetic lethality'

I have attempted to isolate functions that showed a synthetic lethal phenotype with the *cdc21-M68* allele by overexpression of a library of fission yeast cDNAs. This complementary screen may lead to the identification of interacting genes (Botstein and Maurer, 1982; Li and Herskowitz, 1993). The rationale behind this approach is that abnormal levels of interacting functions, produced by overexpression of their correspondent cDNAs, could in principle give rise to lethality only in the *cdc21*^{ts} strain. This is because the *cdc21*^{ts} mutant in some way may not be completely functional even at the permissive temperature, thus an additional defect in the same pathway (overexpression of upstream interacting genes; increased levels of cdc21 substrate; overexpression of a physically interacting protein) may exacerbate the temperature-sensitive phenotype of the *cdc21*^{ts} strain. An *S. pombe* cDNA library (kindly donated by C. Norbury, ICRF, London, UK) under control of the *nmt1* inducible promoter (*n*o *m*essage in *t*hiamine; Maundrell, 1990) was employed. The *nmt1* promoter is a widely used inducible expression system in fission yeast and it is particularly suitable since thiamine is not essential for growth. The *nmt1* promoter is fully activated within 16 hours of thiamine starvation, whereas repression takes place within 3 hours upon addition of thiamine to the growth medium (Maundrell, 1990). As the 'synthetic lethal mutant' phenotype can be modulated by the induction/repression of the cDNA library from the *nmt1* promoter, this constitutes a valuable tool for such a screening.

cdc21^{ts} cells were transformed with the *S. pombe nmt1::cDNA* expression library and transformants obtained at 25°C were replica plated at the same temperature in the absence of thiamine, to select transformants that become lethal in the absence of thiamine.

The results of such a screening are reported in Table 4.4. Two strains, namely C_{25} and C_{27}, were isolated as putative '*cdc21* synthetic lethals'. Lethality was seen only in the absence of thiamine, suggesting that the effect was presumably dependent on the overexpression of cDNAs.

A truncated *cdc21*$^+$ cDNA (as a *Bam*HI fragment) was expected to be isolated in this screening as it gives rise to lethality when overexpressed in the *cdc21* mutant strain at the permissive temperature (see Chapter Five), suggesting that the screening was not saturated.

Characterisation of the putative cdc21 synthetic lethals

A stability test was performed to determine if the synthetic lethality phenotype was plasmid-dependent and this was shown to be the case. Plasmids from the two putative *cdc21* synthetic lethals were recovered in *E. coli* (see Chapter Two). These plasmid were named pC_{25} and pC_{27}. If the overproduced functions are interacting with *cdc21*$^+$ then the effect must be seen only in the *cdc21*^{ts} strain and not in a wild-type strain. In order to confirm that the synthetic lethal phenotype was indeed conferred by the presence a plasmid overexpressing a cDNA, the presence of inserts was determined by restriction digestion. Both plasmids had inserts, of 0.9 and ca. 4.3 kb for pC_{25} and pC_{27} respectively (see Table 4.4). These plasmids were back-transformed into *S. pombe* wild-type and *cdc21*^{ts} strains (see Chapter Two). Transformants obtained in the presence of thiamine (*nmt1* promoter off) were replica-plated at 25, 32 and 36°C in the absence and in the presence of thiamine. As can be seen in Figure 4.4, the pC_{27} plasmid produced lethality in both wild-type and *cdc21*^{ts} strains in a thiamine-dependent fashion. The pC_{25} plasmid instead caused a severe inhibition of growth only when overexpressed in the *cdc21*^{ts} strain grown at 25°C, while cells could normally grow if thiamine was present in the medium. Complete absence of growth at 32 and 36°C was seen in the absence of thiamine.

This result suggests that the overexpression of the pC_{25} cDNA lowers the restrictive temperature of *cdc21* mutant cells, thus making it an interesting candidate as interacting gene. The cDNA of this plasmid was named *tsl1*$^+$ (for *t*wenty-one *s*ynthetic *l*ethal).

In order to get more insight on the function of the *tsl1*$^+$ cDNA, its nucleotide sequence was determined.

The *tsl1*$^+$ cDNA was sub-cloned (Figure 4.5a) and sequenced by progressive 3' end deletions generated by a time-course digestion with exonuclease III (*Exo*III, Henikoff 1984; Figure 4.5b) as described in Chapter Two. A translation of this cDNA is shown in Figure 4.6. The sequence was analysed for the presence of open reading frames (ORF) and an ORF of 624 bp, the largest that could be found in this cDNA (Figure 4.7), was identified. This ORF could encode for a predicted protein of 208 amino-acids and a predicted molecular weight of 23.9 kDa. There are multiple

stop codons upstream of the first methionine, suggesting that this may be the 'bona fide' first methionine of the tsl1 protein. The large region of untranslated DNA upstream of the start codon (293 bp) may be due to multiple transcription start sites. A long poly $(A)^+$ tail was also found at the 3' end of the cDNA sequence. The protein is acidic (pI= 4.66), and has no obvious similarities with any other proteins available in the database. No obvious revealing motifs were identified. It is difficult at this stage to suggest a possible role for the tsl1 protein which may in turn help to explain the synthetic lethal phenotype with the *cdc21-M68* mutant.

Suppression of the *cdc21*^{ts} mutation by an *S. pombe* cDNA expression library

Mutant alleles of the *mcm* genes show a network of allele specific suppression and synthetic lethality which is consistent with these genes being involved in the same genetic pathway (Moir and Botstein 1982; Hennessy *et al.*, 1991). Very recently a physical interaction between mammalian P1 (an Mcm3-like protein) and Cdc46 proteins has been reported (Burkhart *et al.*, 1995). Interestingly a genetic interaction has also been shown for the *cdc46-1* mutant and the *orc6-1* mutant (although this was restricted to a single allele of *cdc46* ; Li and Herskowitz, 1993), suggesting that members of the Mcm protein family may have a direct role at origins of DNA replication. If the Mcm proteins are organised in a consecutive genetic pathway, constitutive expression of downstream target(s) dependent upon the cdc21 protein would become now independent of cdc21 so that they may rescue the *cdc21-M68* mutant. In another possible situation (branched pathway), constitutive expression of a factor might activate a genetic pathway parallel to that one of cdc21 but leading to the same final product (by-pass). Total or partial complementation of the *cdc21*^{ts} mutation may also occur if the proteins physically interact.

The *cdc21*^{ts} strain was transformed with the *nmt1::cDNA* library (see previous section). Transformants were recovered at 25°C in the presence of thiamine (*nmt1* promoter off) and were replica-plated at 36°C in the absence of thiamine to select for suppressors as detailed in Chapter Two.

Eighteen independent *cdc21*$^{ts+}$ transformants were isolated from three independent screenings (see Table 4.6). None of these transformants was found to be thiamine-dependent for growth at the restrictive temperature.

One Ts^- transformant (E3) which showed residual growth at 36°C arrested with a phenotype which was different from the one expected for *cdc21*^{ts} cells (Figure 4.8). Small cells accumulated in the absence of thiamine (panel B) whereas elongated *cdc*-like cells were more abundant in the presence of thiamine (panel A). This

phenotype was initially interpreted as an ability to overcome the arrest in interphase of *cdc21*^{ts} cells, presumably due to overexpression of a cDNA. This strain was provisionally called *dom1* (for *d*ominant *o*nset of *m*itosis).

Although the *cdc21*$^{ts+}$ transformants isolated are all thiamine-independent for growth at 36°C, it is possible that the *cdc21*$^{+}$ cDNA itself or putative interacting functions may be able to suppress the *cdc21*^{ts} mutation also in the presence of thiamine, given that the *nmt1* promoter has a basal level of transcription when shut off (Forsburg, 1993). A plasmid stability test (see Chapter Two) indicated that Ts^{+} phenotype of the putative suppressors of *cdc21*^{ts} was not plasmid-dependent. The phenotype of the E3 strain was however unstable and co-segregated with the Leu^{+} phenotype (see Table 4.5), suggesting that it was plasmid-dependent. The Ts^{+} phenotype of the putative *cdc21*^{ts} suppressors was instead stable and never linked to the Leu^{+} phenotype indicating that these were likely to be intra- or extra-genic suppressors of the *cdc21*^{ts} mutation.

Only one type of plasmid, containing an insert of ca. 0.9 kb (see Table 4.6), was recovered from the E3 strain (p*dom1*$^{+}$). The p*dom1*$^{+}$ plasmid was back-transformed into *S. pombe* wild-type and *cdc21-M68* cells and transformants showed the same phenotype of the original strain, thus confirming that it was p*dom1*$^{+}$ plasmid-dependent. This strain (PDOM1) was retained for further analysis and its preliminary characterisation will be described in the following section.

One limitation of the cDNA library employed in this screening is that it is not fully representative; synthesised cDNAs were digested with *Bam*HI and size selected (C. Norbury, pers. comm.), thus making the isolation of the *cdc21*$^{+}$ cDNA unlikely. However, the smallest *Bam*HI fragment of the *cdc21*$^{+}$ gene (1.5 kb) cannot complement the *cdc21*^{ts} mutation (see Chapter Five) implying that this could not be isolated in this screen.

Phenotypic characterisation of the PDOM1 strain.

The arrest phenotype of PDOM1 cells incubated at 36°C in the absence of thiamine is consistent with a) by-pass of the interphase block imposed by the *cdc21*^{ts} mutation; this would cause low cell viability as a result of the onset of mitosis in cells which are not prepared to do so (aberrant mitosis); b) the overexpression of the *dom1*$^{+}$ cDNA may be toxic, and therefore interfere with growth and cell elongation.

Because no aberrant mitotic events could be detected in the absence or in the presence of thiamine in this strain, as shown in Figure 4.8, by-pass of the interphase block of *cdc21* mutant cells may be not likely. Therefore the viability of this strain in

both conditions of repression or induction of the *dom1*+ cDNA is expected to be the same.

In order to test the first possibility the viability of PDOM1 cells grown at 36°C in the absence (*dom1*+ on) and in the presence (*dom1*+ off) of thiamine was determined and compared to the viability of a set of control strains (see legend to Figure 4.9). Intriguingly the E3 strain loses rapidly viability at 36°C only in the absence of thiamine (Figure 4.9). This was comparable to the viability of the *cdc21 rad1* double mutant strain cultured in the same conditions (see Chapter Four). This experiment suggests that the overexpression of the *dom1*+ cDNA lowers the viability of the *cdc21-M68* strain incubated at the restrictive temperature.

The effect of the overexpression of the *dom1*+ cDNA in wild-type and *cdc21*ts strains at 25 °C was also determined. As can be seen in Figure 4.10 the growth of both *cdc21-M68* and wild-type strains was not affected by the overexpression of the *dom1*+ cDNA, arguing that the effect seen in the *cdc21-M68* strain at the restrictive temperature is likely to be *cdc21* specific.

Taken together these results indicate that the two functions, *cdc21* and *dom1*, in certain conditions, interfere with each other, suggesting that they may interact. It is possible that the overexpression of the *dom1*+ cDNA may interfere with cell elongation when the *cdc21*+ function is inactivated.

Analysis of the *dom1*+ cDNA.

As for the *tsl1*+ cDNA, the *dom1*+ cDNA was sub-cloned (Figure 4.11) and sequenced (see legend of Figure 4.12a) to see if it may have similarities with previously identified gene products. Translation in all possible frames showed the presence of an open reading frame (ORF) of 462 bp. This ORF (+1, forward orientation) was the largest found (Figure 4.13) and could potentially encode for a protein of 154 amino-acids with a predicted molecular weight of 17.2 kDa.

Search through various protein databases using the programme BLASTP of the Genetic Computer Group (GCG, version 8) revealed that the dom1 predicted protein was 60% similar to the budding yeast protein Nhp2, or high mobility group-like (HMG) protein (Kolodrubetz and Burgum, 1991).

Nhp2 is an essential protein originally identified in budding yeast as similar to vertebrate HMG proteins based upon the size, the high content of basic/highly acidic amino-acids, and the methods used to extract it from nuclei (Kolodrubetz *et al.*, 1988). The HMG are ubiquitous proteins which are chromatin associated and play important roles in DNA transcription (see Grosschedl, *et al.*, 1994, for review). However, Nhp2 was found not to share any significant similarity with the sequenced

vertebrate HMG proteins, suggesting that it may be a novel member of a subclass of HMG proteins (Kolodrubetz and Burgum, 1991).

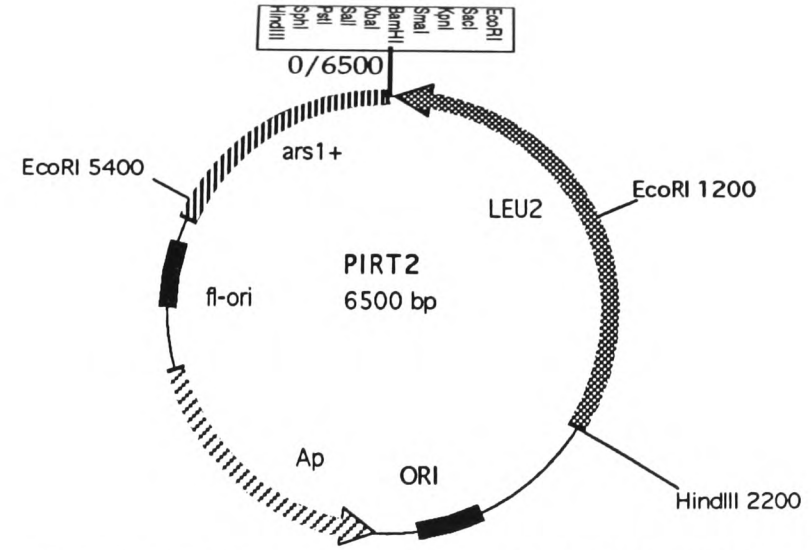
The second highest score (27%) was found with a predicted yeast protein (Yelo26p) derived from the translation of a random sequence of the budding yeast chromosome. The next highest scores were with the ribosomal protein S6 from *Halobacterium marismorium* (17%, Kimura *et al.*, 1987), and the ribosomal protein L4 from budding yeast (20%, Yon *et al.*, 1991).

Diagonal plots, graphically displaying the similarity of these proteins, are shown in Figure 4.14. Sequence comparison showed similarity between dom1 and Nhp2 proteins from the amino-terminal to the carboxyl-terminal of the aligned proteins, suggesting that they may be homologous (Figure 4.15a). This will be tested by the ability of the *dom1*$^+$ cDNA to complement a null mutant of the *nhp2* gene.

The similarity of dom1 with Yelo26 and the two ribosomal proteins from bacteria and yeast is instead limited to a central region of the protein sequences (Figure 4.15b). There are two very well conserved motifs between Nhp2, dom1 and Yelo26. These are the PLA (amino-terminus) and LCED--VPY (central portion) motifs, which do not correspond to previously identified functional domains of the ribosomal proteins (e.g., RNA binding domains).

Given the high similarity with the Nhp2 protein, it is very likely that the *dom1*$^+$ cDNA isolated in this screening may be full length. There is however a large untranslated region at the 3' end of this cDNA. To exclude possible rearrangements, the *dom1*$^+$ cDNA was labelled with [α-^{32}P]dCTP and used to probe fission yeast genomic DNA by Southern blotting. As shown in Figure 4.16 the *dom1*$^+$ cDNA recognises a single band in each digest, thus indicating that the 3' untranslated region is co-linear with the *dom1*$^+$ open reading frame. The predicted size of the hybridisation bands is shown in Table 4.7.

The similarity of the Nhp2 and dom1 proteins with some bacterial and eukaryotic ribosomal proteins is intriguing. Nhp2 (pI=10.0) and dom1 (pI=9.8), are highly basic proteins, whereas the ribosomal protein S6a is acidic (pI=3.88; Kimura *et al.*, 1987). Nhp2 does not share significant similarity with previously identified proteins loosely associated with ribosomes in yeast (Kolodrubetz and Burgum, 1991), nor can it be detected in the ribosomal cellular fraction, instead is enriched in the nuclear fraction (Kolodrubetz *et al.*, 1988), suggesting that it may be a nuclear protein. And indeed, the dom1 protein contains a bipartite nuclear localisation signal (NLS) which is also present in the Nhp2 protein (depicted in Figure 4.12b). Sub-cellular localisation of the dom1 protein may be important to shed light on its cellular function.



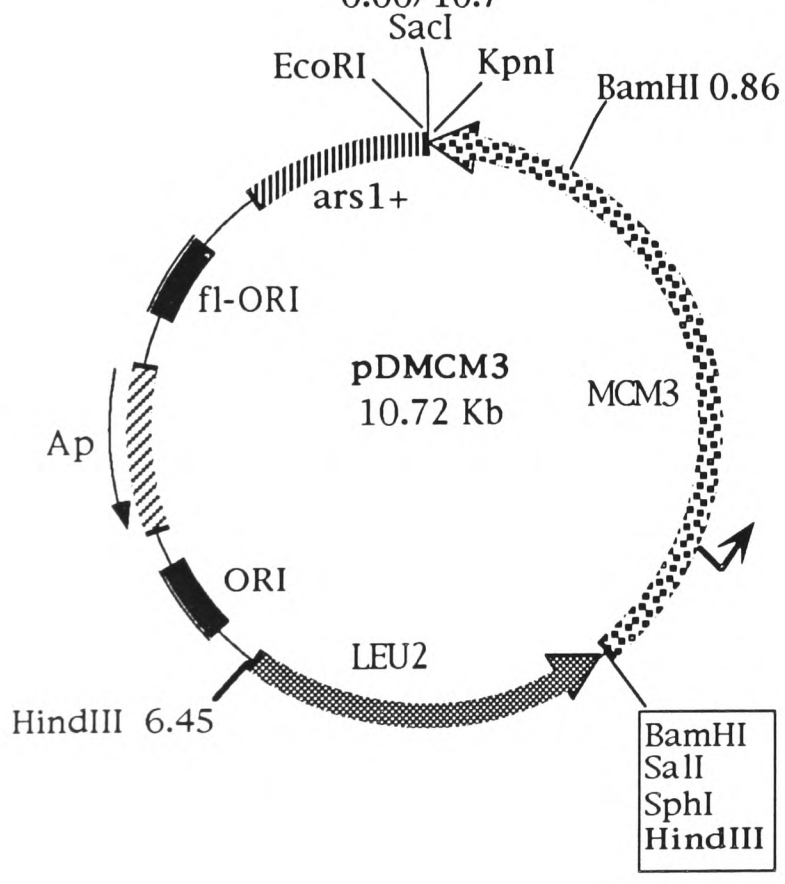
YCP50.*MCM3* x MluI-SalI

Klenow treatment

ligatein
pIRT2 x SmaI

YCP101.*MCM2* x SacI-HindIII

Klenow treatment



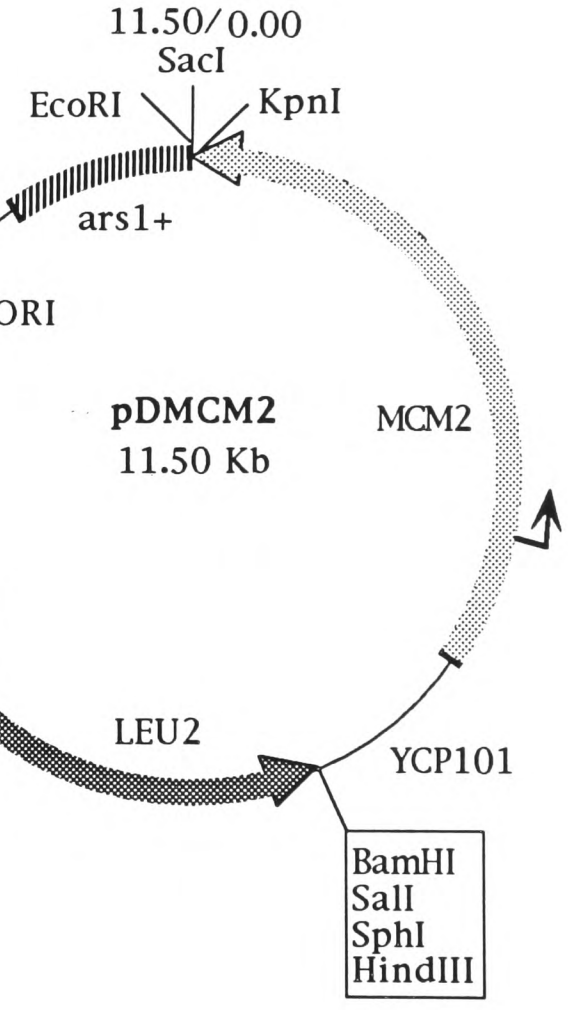


Figure 4.1

Sub-cloning of the MCM2 and MCM3 budding yeast genes in the S. pombe replicative vector pIRT2

The *MCM2* and *MCM3* genes were obtained in the vectors YCP101.*MCM2* (*LEU2*) and YCp50.*MCM3* (*URA3*) respectively. The *S. cerevisiae* selectable markers of these vectors can complement the *leu1-32* and *ura4-D28* mutants of fission yeast respectively.

The complete *MCM2* gene was isolated from the YCp101.*MCM2* plasmid (Yan *et al.*, 1991; B.Tye; unpublished) as the 5 kb *Sac* I-*Hin*d III restriction fragment, after the 3' protruding ends had been removed with the bacteriophage T4 DNA polymerase and the 5' protruding ends filled-in with the large subunit of the *E. coli* DNA polymerase (Klenow fragment) as described (Sambrook *et al.*, 1991). This fragment was ligated into the *Sma*I site of the pIRT2 vector (Hindley *et al.*, 1987) and recombinant plasmids were screened by restriction digestion.

The full *MCM3* gene was isolated from the YCp50.*MCM3* plasmid (Gibson *et al.*, 1990) as the 4.3 kb *Mlu*I/*Sal*I restriction fragment after the 5' protruding ends were filled-in with the *E. coli* Klenow fragment (Sambrook *et al.*, 1991). This DNA fragment was then ligated into the *Sma*I site of the pIRT2 vector. Recombinant plasmids screened for the presence of the expected insert as described above.
The major restriction sites are displayed in the figure. Arrows on top of the open reading frames indicate the approximate position of transcription start sites.

Plasmid	Suppression of *cdc21* (36°C)	*nda1* (20°C)	*nda4* (20°C)	Reference/Source
YCP101.*MCM2*	does not transform fission yeast			B.Tye, Cornell Univ., Ithaca. USA
YCP50.*MCM3*	does not transform fission yeast			B.Tye, Cornell Univ., Ithaca. USA
pIRT2	-	n.d.	n.d.	Hindley *et al.*, 1987
pD*MCM2*	-	n.d.	n.d.	This work
pD*MCM3*	-	n.d.	n.d.	This work
pAC1	+	-	-	A.Coxon D.Phil. thesis, 1992
pREP*81X::21*$^+$	+*	-	-	This work, Chapter 5
pREP*MCM2*	-*	-	-	I.Todorov, unpublished
pREP*MCM3*	-*	-	-	I.Todorov, unpublished
pREP*BM28*	-*	-	-	I.Todorov, unpublished

Table 4.1

Suppression of fission yeast *mcm*$^-$ mutants by structurally related genes

The indicated plasmids were tested for their ability to suppress the lethality of the listed fission yeast mutants at their restrictive temperature (indicated in brackets). The star (*) where present, indicates that the same result was obtained in conditions of both repression and expression of the inducible promoter *nmt1*. n.d.= not determined.

Strain	Growth at 36°C	Stability Leu$^+$	Stability ts$^+$	Plasmid isolation	Assigned ts$^+$ phenotype
pUR1a	+++	-	-	+	Plasmid dep.
pUR6a	+++	-	-	+	Plasmid dep.
pUR7d	+++	-	-	+	Plasmid dep.
pUR10b	+++	-	-	+	Plasmid dep.
pUR13c	+++	-	-	+	Plasmid dep.
pUR16f	+++	-	-	+	Plasmid dep.
pUR20.1	+++++	-	+	-	Revertant
pUR22.1	+++++	-	+	-	Revertant

Table 4.2

List of the cdc21^{ts+} strains isolated from a *S. pombe* genomic library introduced in the *cdc21-M68* strain

About 1µg of *S. pombe* genomic library made in the pUR18 vector (Barbet *et al.*, 1992) was introduced in the *cdc21-M68* strain by electroporation and transformants recovered on minimal selective plates (ura$^-$). Putative *cdc21*^{ts} suppressors were selected at 36°C and tested for instability of the Ura$^+$ and Ts$^+$ phenotypes as described in Chapter Two. The Ts$^+$ phenotype of putative suppressors is listed as plasmid dependent (plasmid dep.) or due to chromosomal reversion (revertant). Plasmids were recovered in *E. coli*.

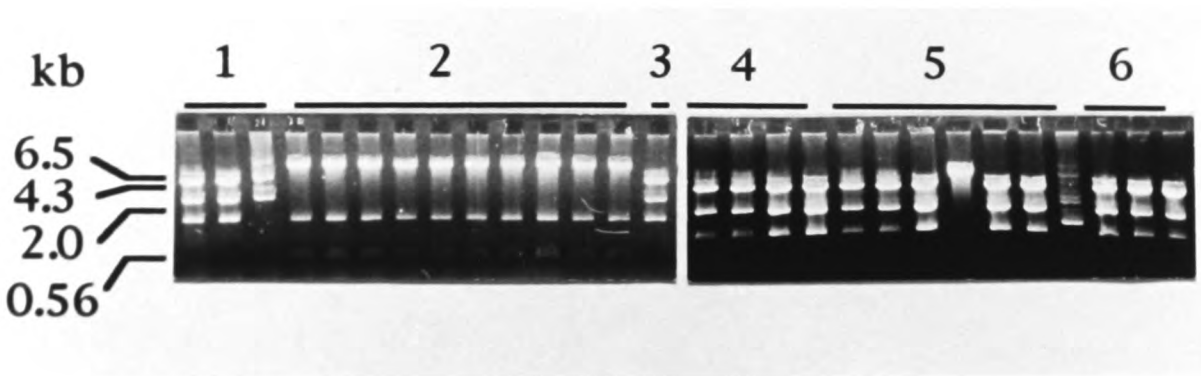
Tot. transformants	Tot Ts$^+$ strains	Unstable Ts$^+$	Plasmids recovered	Size of the insert (kb) 1a	Size of the insert (kb) 6a	Revertants
1x10^4	8	6	6	4.3	11.3	2

Table 4.3

Summary of the screening for suppressors of *cdc21* by high copy number of an *S. pombe* genomic library

Eight independent Ura$^+$ *cdc21*$^{ts+}$ strains were isolated. Ts$^+$ clones which did not show co-segregation with the Ura$^+$ phenotype were considered as revertants. Plasmids were recovered from the unstable Ts$^+$ clones. Revertants were never cold sensitive.

A


kb
1
2
3
4
5
6
6.5
4.3
2.0
0.56

B

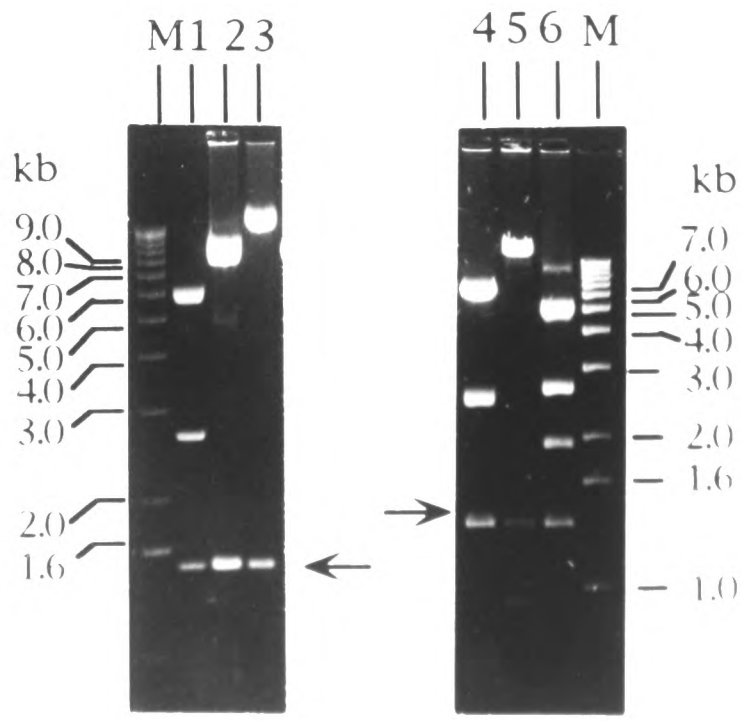

M 1 2 3
4 5 6 M
kb
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.6
kb
7.0
6.0
5.0
4.0
3.0
2.0
1.6
1.0

Figure 4.2

Restriction digest of the plasmids recovered from the putative suppressors of *cdc21*

(A)

Plasmids recovered in *E. coli* from the putative suppressors of *cdc21* indicated in Table 4.5 were digested with *Bam*HI and the products of the reaction fractionated by electrophoresis on a 0.8% (w/v) agarose gel in 1X TBE buffer containing 10µg/ml of EtBr. Plasmids recovered from each putative suppressor are grouped by a line bar on top of the wells of the gel. **1**= pUR1a; **2**= pUR6a; **3**= pUR7d; **4**= puR10b; **5**= pUR13c; **6**= pUR16f. Note that within the groups of plasmids 1 and 5 there are some plasmids whose restriction pattern is different from the rest in the group, and may be due to rearrangement. These rearranged plasmids could not back-transform *cdc21-M68* cells at both 25 or 36°C.

(B)

The pUR1a (lane 1,4), pUR6a (lane 2,5) and pAC1 (lane 3,6)[which contains the *cdc21*$^+$ gene; A.Coxon, D.Phil. Thesis] plasmids were digested with *Bam*HI (lanes 1 -3) or *Kpn*I (lanes 4-6). In both digestions the three plasmids produce a co-migrating fragment of 1.5 kb and of 1.4 kb for the *Bam*HI and *Kpn*I digest respectively (arrows). The 0.6 kb *Bam*HI fragment of the pUR6a plasmid has run off the gel.

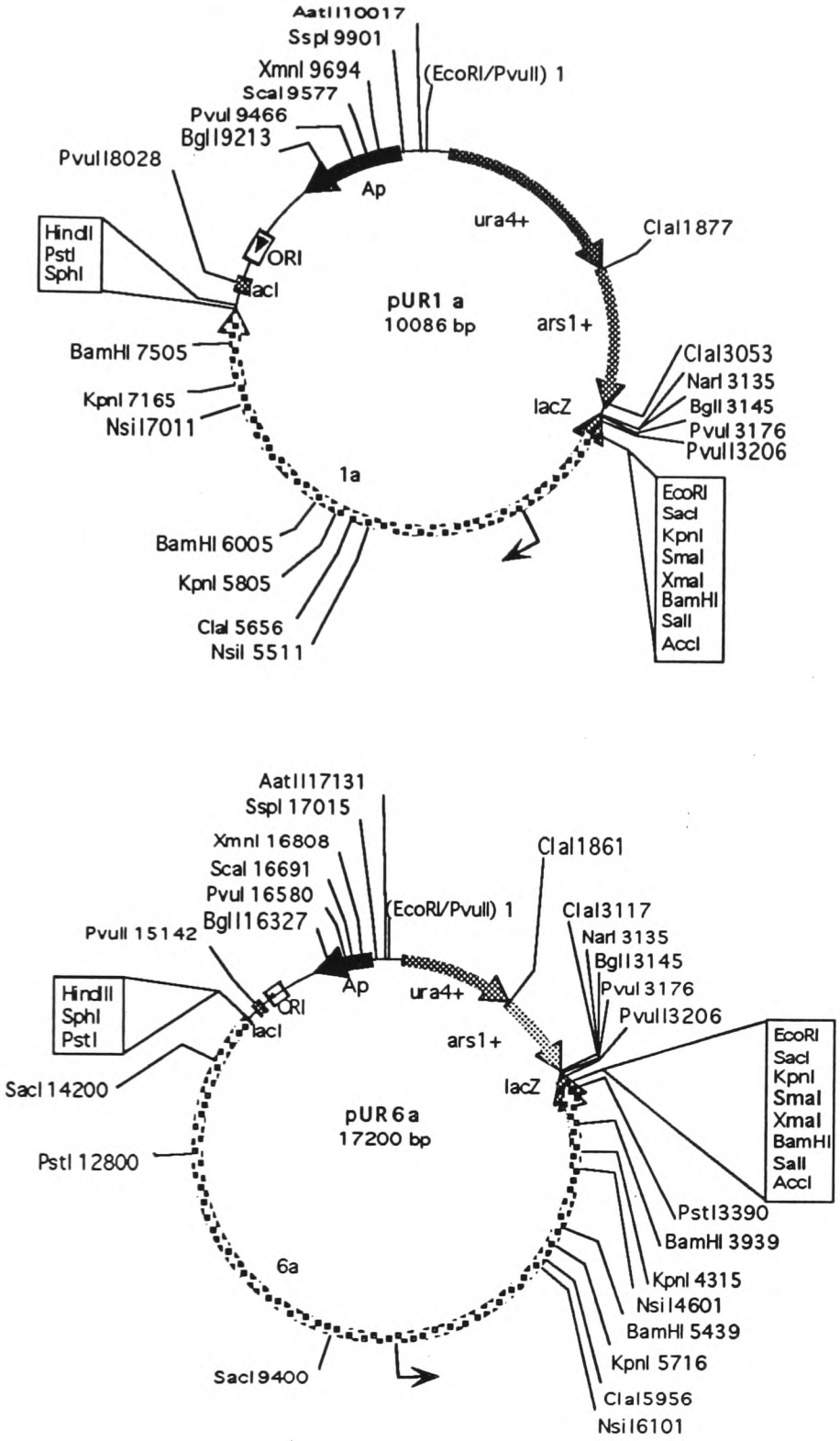


Figure 4.3

Restriction map of two overlapping *cdc21*$^{+}$ genomic clones

The restriction map of the two plasmids (pUR1a and pUR6a) isolated from the putative *cdc21*$^{ts+}$ unstable strains is shown. The pUR1a plasmid contains an insert of about 4.3 kb (1a) whereas the pUR6a plasmid contains an insert of about 11.3 kb (6a). The arrow on top of the inserts indicates the cdc21+ start codon. The occurrence of the indicated restriction sites was determined by digestion of the DNA with the corresponding restriction enzymes. The partial sequences of the 1a and 6a inserts were determined using a 25 bp *cdc21*$^{+}$ single stranded DNA primer specifically designed to anneal the *cdc21*$^{+}$ DNA sequence at position 1753 on the reverse strand (5'-GGAGTTTAATAACTTATCGGC-3') (given by Dr. Blom). Within 200bp sequenced the sequence of both inserts was found identical to that of the *cdc21*$^{+}$ gene (Coxon et al., 1992).

Total transformants	Putative 'synthetic lethals'	Unstable 'synthetic lethals'	Plasmids rescued	Size of the insert (kb)		Confirmed 'synthetic lethals'
				pC25	pC27	
$1x10^4$	2	2	2	0.9	4.3	1

Table 4.4

Summary of the screening for *cdc21* synthetic lethals isolated by overexpression of an *S. pombe nmt1::cDNA* expression library

The data reported refer to a single screening. Putative *cdc21* synthetic lethals were isolated as colonies unable to grow at 25°C in the absence of thiamine. Plasmids recovered from the 2 putative '*cdc21* synthetic lethals' were back-transformed into both wild-type (P2) and *cdc21-M68* (P1) strains by electroporation (see Chapter Two) and strains were grown in the presence or absence of thiamine on solid media. Only those cDNAs that gave rise to lethality in the *cdc21-m68* strain and not in the wild-type strain when overexpressed were considered as true '*cdc21* synthetic lethals'.

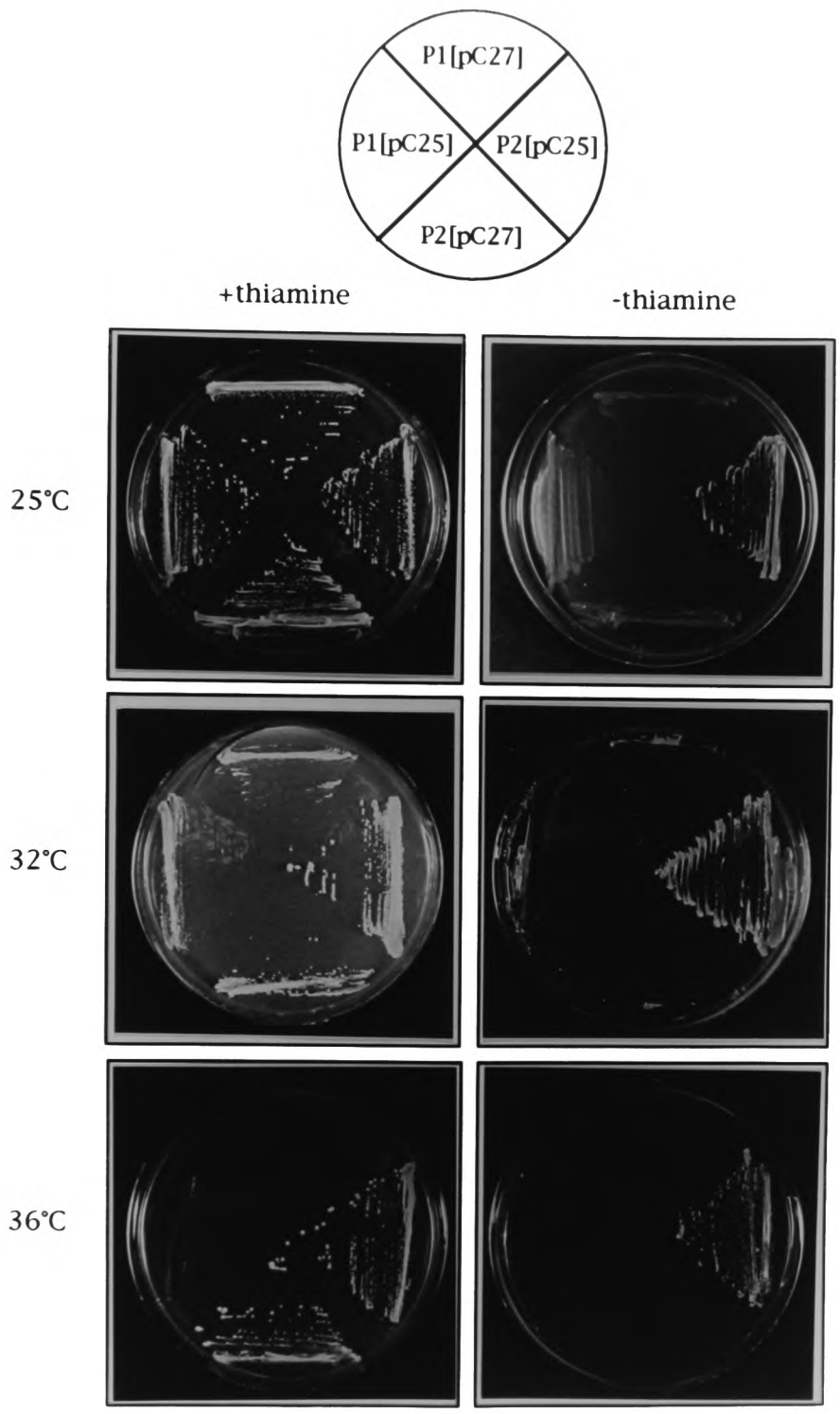
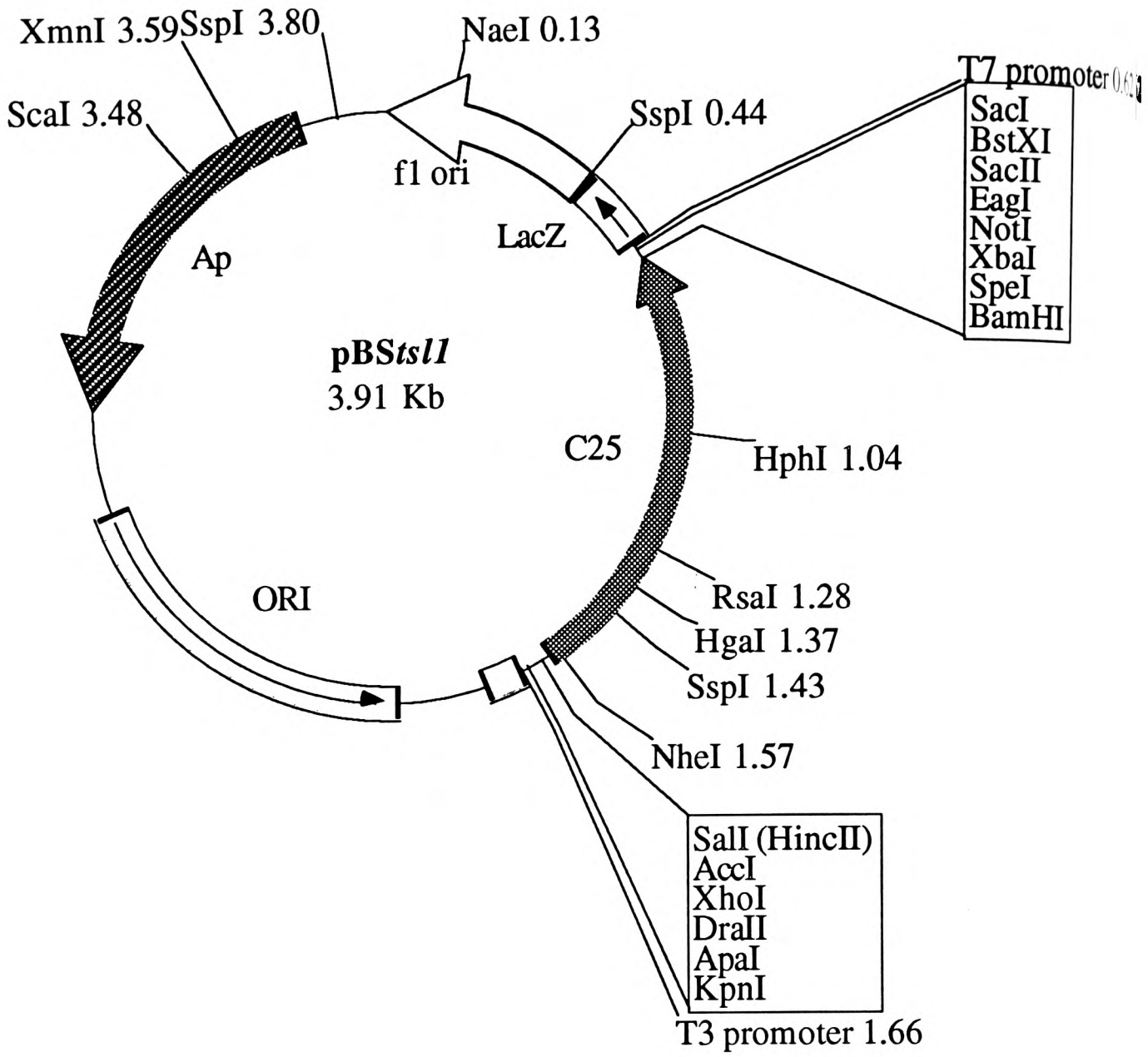


Figure 4.4

The overexpression of the *tsl1*$^+$ cDNA causes lethality in a *cdc21-M68* strain

S. pombe wild-type (P2) and *cdc21-M68* cells (P1) were back-transformed with the pC_{25} and pC_{27} plasmids recovered from the C_{25} and C_{27} strains respectively (see Chapter Two). Transformants were recovered and streaked out on minimal selective plates (leu^-) in the absence of thiamine at 25°C for 18-24 hours to allow full induction of the *nmt1* promoter and then re-streaked in the presence or in the absence of 5µg/ml of thiamine at 25, 32 and 36°C. Growth was scored after 5 days at the indicated temperatures. Overexpression of the C_{27} cDNA gave rise to lethality in both wild type and mutant cells at all the temperatures tested, whereas the overexpression

(a)


XmnI 3.59
SspI 3.80
NaeI 0.13
ScaI 3.48
SspI 0.44
T7 promoter 0.63
SacI
BstXI
SacII
EagI
NotI
XbaI
SpeI
BamHI
f1 ori
LacZ
Ap
pBStsl1
3.91 Kb
C25
HphI 1.04
ORI
RsaI 1.28
HgaI 1.37
SspI 1.43
NheI 1.57
SalI (HincII)
AccI
XhoI
DraII
ApaI
KpnI
T3 promoter 1.66

(b)

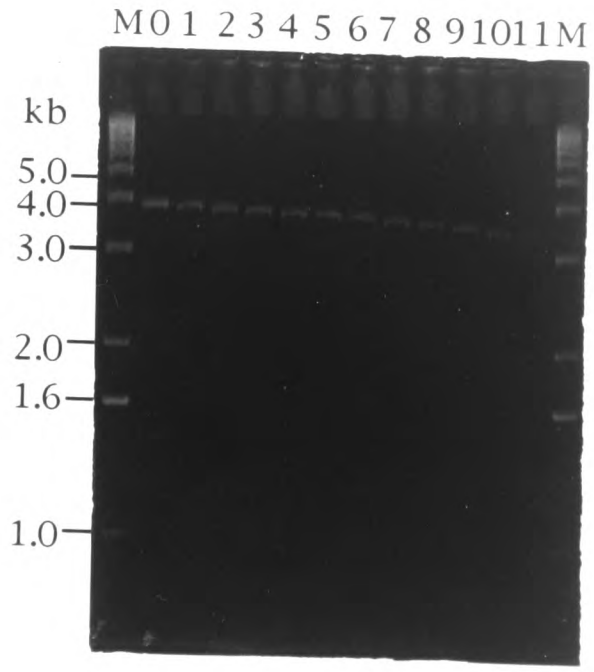

M 0 1 2 3 4 5 6 7 8 9 10 11 M
kb
5.0
4.0
3.0
2.0
1.6
1.0

Figure 4.5

(a)

Restriction map of the pBS*tsl1* plasmid

The *tsl1*$^+$ cDNA was sub-cloned as the ca. 0.9 kb *Sal*I-*Bam*HI DNA fragment in the Bluescript plasmid (KS+; Stratagene). The recombinant plasmid, pBS*tsl1* was used to generate progressive deletions of the *tsl1*$^+$ cDNA. The occurrence of the major restriction recognition sites is reported.

(b)

Generations of progressive deletions of the *tsl1*$^+$ cDNA using *Exo*III

Progressive deletions of the *tsl1*$^+$ cDNA were generated in a time course digestion with the *Exo*III exonuclease (Henikoff, 1984) in the pBS*tsl1* plasmid as described in Chapter Two. An aliquot of each time point was loaded on a 1.2% agarose gel in 1X TBE buffer containing 10µg/ml of EtBr and subjected to electrophoresis. **M**= 1kb DNA ladder molecular size marker; **0-11**= time points (minutes) of *Exo*III digestion at room temperature. The rate of *Exo*III digestion at this temperature is of ca. 80-90bp per minute.

```
TCGAGGTCGACGTTAATTAAGCCTATTGTTTACATGTTGTGTTTCAACTGCTAAATTAAC      60

AGGAGAAGGAGAATAATTGTAAGCTAGCAGATACTGTGTGGAGCGATCTTTGTCGAACAG     120

GCAGTATTCGTTTTGTCTGGAGACCTTACCTGAGATTCTGATACTAAACGTTGTAATCAT     180

CTTAGAGTAACTGGATCACTCGTTTAATTCTTTAAAAGTGAACTGAAAAATATTTTTACC     240

                                                  T   T   M   R
ATTTAACTCATTGCACGTTTGAGGATGCAAGATTCTTCCAAAAATAAACAACCATGCGTC     300

L   M   E   S   F   S   F   K   S   E   S   M   S   P   D   D   V   S   R   L
TCATGGAAAGTTTCTCCTTTAAAAGCGAAAGCATGTCTCCTGATGATGTCTCAAGGCTTT     360

F   C   K   S   S   T   S   S   A   T   R   K   H   D   P   F   H   K   L   W
TTTGTAAATCCAGTACTAGTAGCGCTACTCGGAAGCATGATCCTTTTCATAAGCTATGGG     420

D   R   L   Q   P   K   A   Q   S   T   I   Q   R   T   S   S   L   P   V   P
ATCGCTTACAACCAAAAGCACAATCCACTATTCAACGGACATCTTCATTGCCTGTTCCTT     480

S   S   S   N   F   K   E   R   L   N   N   I   G   G   L   K   R   S   R   T
CGTCAAGTAATTTTAAAGAGAGATTAAATAATATAGGAGGACTTAAGAGGTCAAGGACAT     540

L   E   S   S   Y   E   D   E   T   E   T   A   N   R   L   S   R   V   S   S
TGGAAAGCTCCTATGAAGATGAAACAGAGACTGCAAATAGACTGTCACGGGTTTCAAGTT     600

L   V   S   V   I   R   Q   T   I   D   R   K   K   S   L   E   R   R   V   R
TGGTGAGCGTGATACGACAAACTATAGATCGCAAAAAATCACTAGAAAGAAGGGTAAGGG     660

E   E   Q   E   E   K   T   D   N   E   D   D   N   D   V   E   I   S   T   Q
AAGAACAAGAAGAGAAGACAGATAATGAGGATGATAATGATGTCGAAATATCTACTCAGG     720

E   S   L   E   N   N   G   L   A   E   K   K   D   D   T   S   S   L   A   T
AATCCCTGGAAAACAATGGTTTAGCGGAAAAGAAGGATGATACTTCATCACTTGCTACGT     780

L   E   D   D   I   E   G   Q   E   F   S   F   D   D   Q   D   L   Q   M   L
TGGAAGATGACATTGAGGGCCAAGAATTTTCGTTTGATGACCAAGATTTACAAATGCTTC     840

Q   D   I   E   D   Q   W   L   S   S   Q   K   Q   Q   G   S   P   L   T   S
AGGACATAGAAGACCAATGGCTCAGTTCTCAAAAACAGCAAGGTTCTCCTCTAACATCAG     900

D   H   I   S   K   *
ATCATATAAGCAAATAGAAAACGCTATTCGT      931
```

Figure 4.6

DNA sequence of the *tsl1*$^+$ cDNA from *Schizosaccharomyces pombe*

The complete sequence of the *tsl1*$^+$ cDNA is shown. The translation of the longest open reading frame is shown on the top of the sequence. The complete sequence determined by sequencing of *Exo*III deletions of the *tsl1*$^+$ cDNA was reconstructed using the Staden sequence assembly programme (SAP). The original sequence data of the deletions contig are shown in the Appendix I.

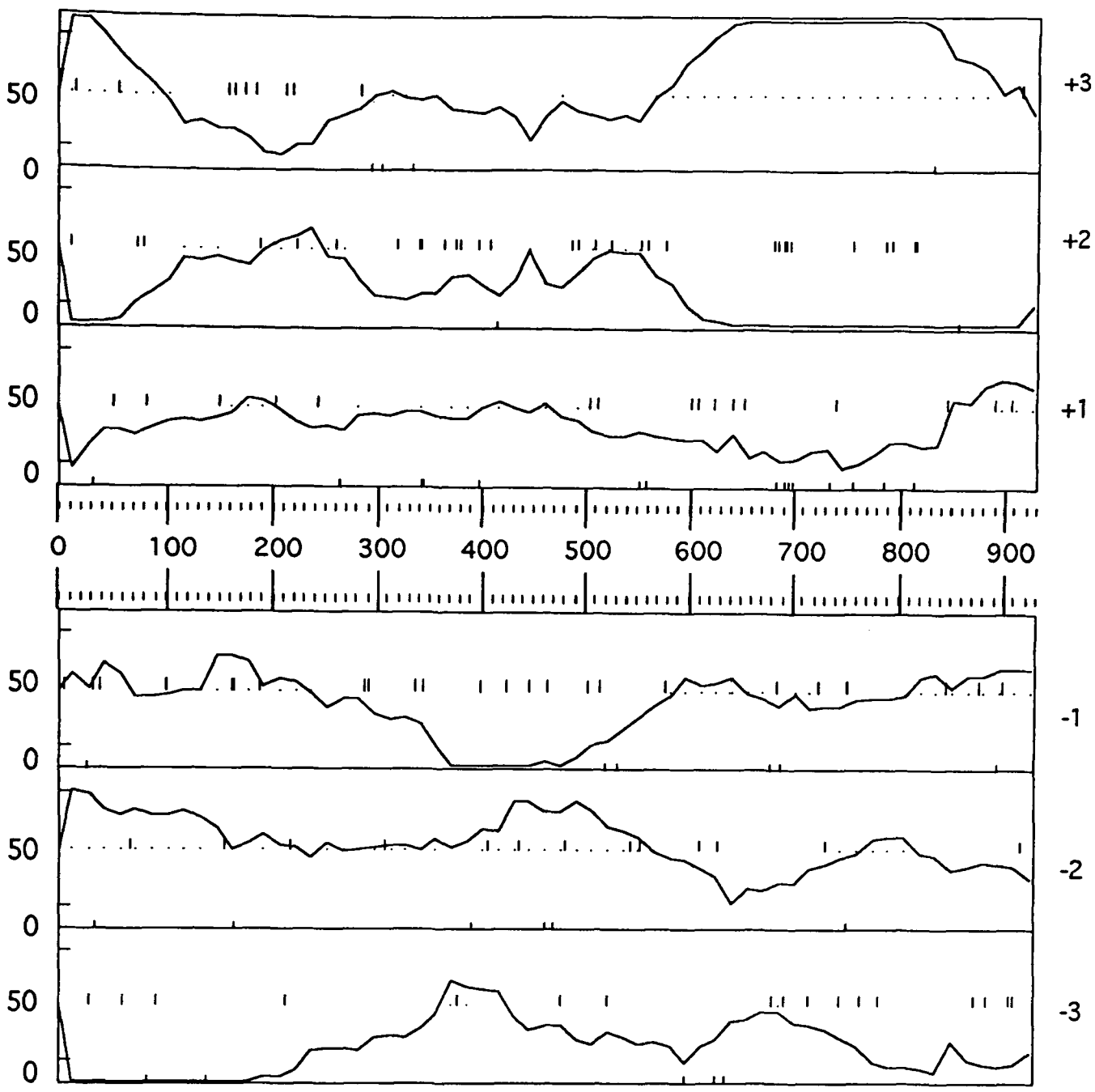


Figure 4.7

Predicted open reading frame of the *tsl1*$^+$ cDNA

This analysis was performed using the positional base preference method of Staden, (1986) on both strands (Forward and Reverse) of the *tsl1*$^+$ cDNA using the Staden Nucleotide Interpretation Programme (NIP). The number of base pairs are shown on the horizontal axis (spacing 10 bp) and the level of probability (0-100) of that sequence coding is indicated on the vertical axis. Start codons are represented by small vertical lines that bisect the 0% level of probability. Stop codons are depicted by small vertical lines that bisect the 50% level of probability. The cDNA relies on the presence of an 'in frame' endogenous methionine for expression as the nmt1 promoter of the vector used to make the cDNA library lacks such a codon. The *tsl11*$^+$ open reading frame was found on frame +3.

Strain	Growth at 36°C		Stability		Plasmid rescue	Ts$^+$ phenotype
	+thi	-thi	Leu$^+$	Ts$^+$		
C1	+++	++	-	+	-	plasmid indep.
C2	++	++++	-	+	-	plasmid indep.
E2	++	+++	-	+	-	plasmid indep.
35.1	++	++	-	+	-	plasmid indep.
35.2	+/-	+/-	-	+	-	plasmid indep.
35.3	++	++	-	+	-	plasmid indep.
E3*	-	-	-	+/-	+	Ts$^-$ (*dom*)
29	++	++	-	+	-	plasmid indep.
on.2	+++	+++	-	+	-	plasmid indep.
E6	+	+	-	+	-	plasmid indep.

Table 4.5

List of the *cdc21*$^{ts+}$ strains isolated by overexpression of an *S. pombe* *nmt1:: cDNA* expression library in the *cdc21-M68* strain

The *cdc21-M68* strain was transformed with about 1µg of the *nmt1::cDNA* library by electroporation and transformants were selected at 25°C on minimal selective plates (leu$^-$) containing phloxin B, in the presence of 5mg/ml of thiamine (see Chapter Two). Suppressors were selected at 36°C by replica-plating in the absence of thiamine. The strains listed in the table were isolated from three independent screenings. The instability and co-segregation of the Leu$^+$ and Ts$^+$ phenotypes was determined following a stability test (see Chapter Two). Recovery of plasmids from both stable and unstable strains was attempted. The Ts$^+$ phenotype of putative suppressors is listed as plasmid independent (plasmid indep.). *= PDOM1 strain was able of residual growth at 36°C but could not be sub-cultured at 36°C (see text and Table 4.6). ++++= growth comparable to wild-type cells; - = absence of growth; +/- very slow growth.

Tot transformants	Tot ts+ strains	Non sub-culturable at 36°C	Plasmids recovered	Size of the insert (kb)	Rev.
2-3 x10^5	18	10	1*	0.9	8

Table 4.6

Summary of the screening for *cdc21*$^{ts+}$ by overexpression of an *S. pombe* *nmt1::cDNA* expression library

A summary of the data of Table 4.2 is reported. Ts + strains in which the leu+ and Ts+ phenotype did not co-segregate were considered as revertants (Rev.). Revertants were tested for cold-sensitivity and found negative. *One unstable strain (E3), was found to alter the arrest phenotype of *cdc21* cells at 36°C in a thiamine dependent fashion, yet incapable to rescue the *cdc21*^{ts} mutant. Revertants were never cold sensitive.

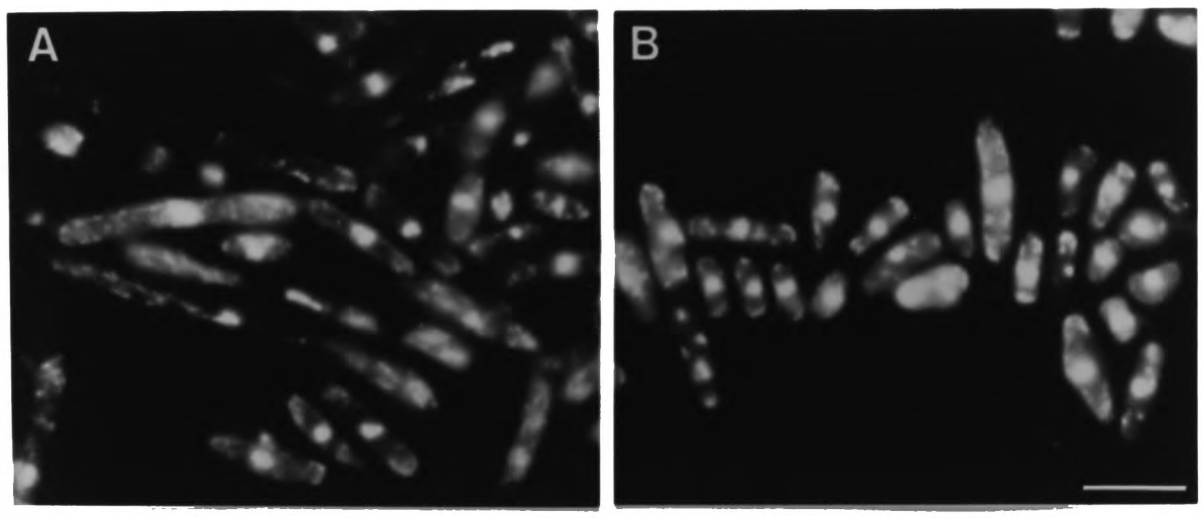


Figure 4.8

Overexpression of the *dom1*$^{+}$ cDNA alters the arrest phenotype of *cdc21*^{ts} cells

The E3 strain was grown to mid-log phase, cells were washed three times in sterile water, resuspended in fresh medium without (A) or with 5µg/ml of thiamine (B) and incubated at 25°C for 20 hours in order to allow full expression from the *nmt1* promoter. After this period of time cells were shifted at 36°C for 24 hours and nuclei were stained with DAPI (see Chapter Two). Bar= 10µm.

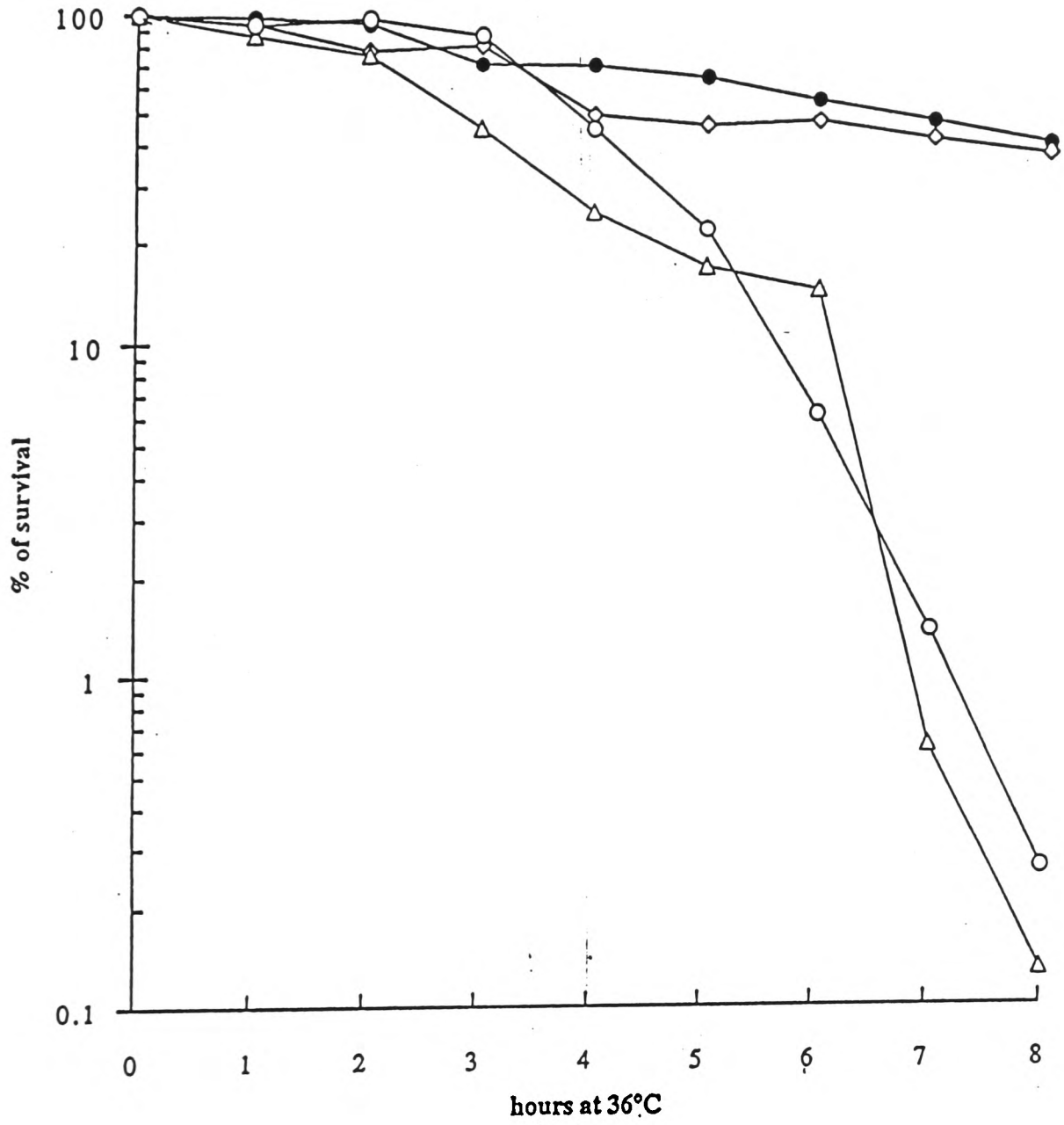


Figure 4.9

Overexpression of the *dom1*$^{+}$ cDNA lowers the viability of *cdc21*^{ts} cells at the restrictive temperature

Cells were shifted to the 36°C for the period of time shown and tested for their viability as described in Chapter Two. The E3 strain, grown in the absence of thiamine (Δ), showed loss in viability after 3 hours at 36°C, whereas no such an effect was seen if the same cells were shifted to 36°C in the presence of 5mg/ml of thiamine (◊) or in the *cdc21*^{ts} strain (P1, •). The loss in viability was similar to that of the *cdc21 rad1* double mutant (P119, o; see Chapter Six). Data points were average of three sets of data.

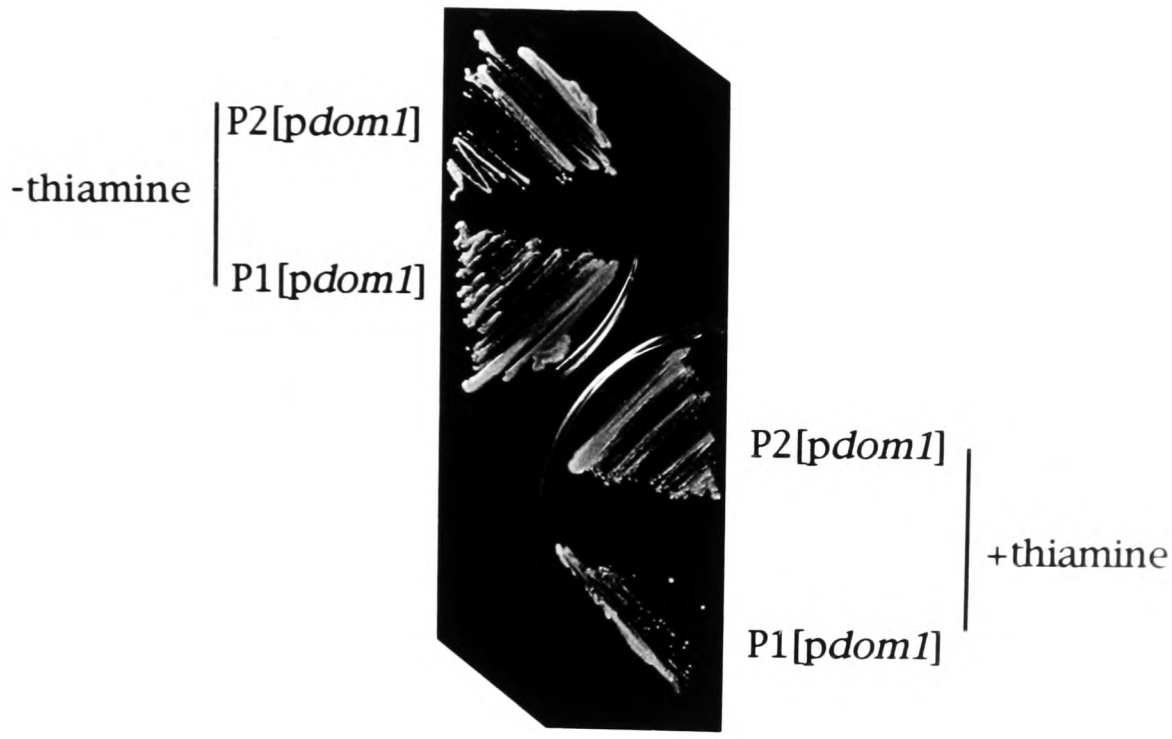
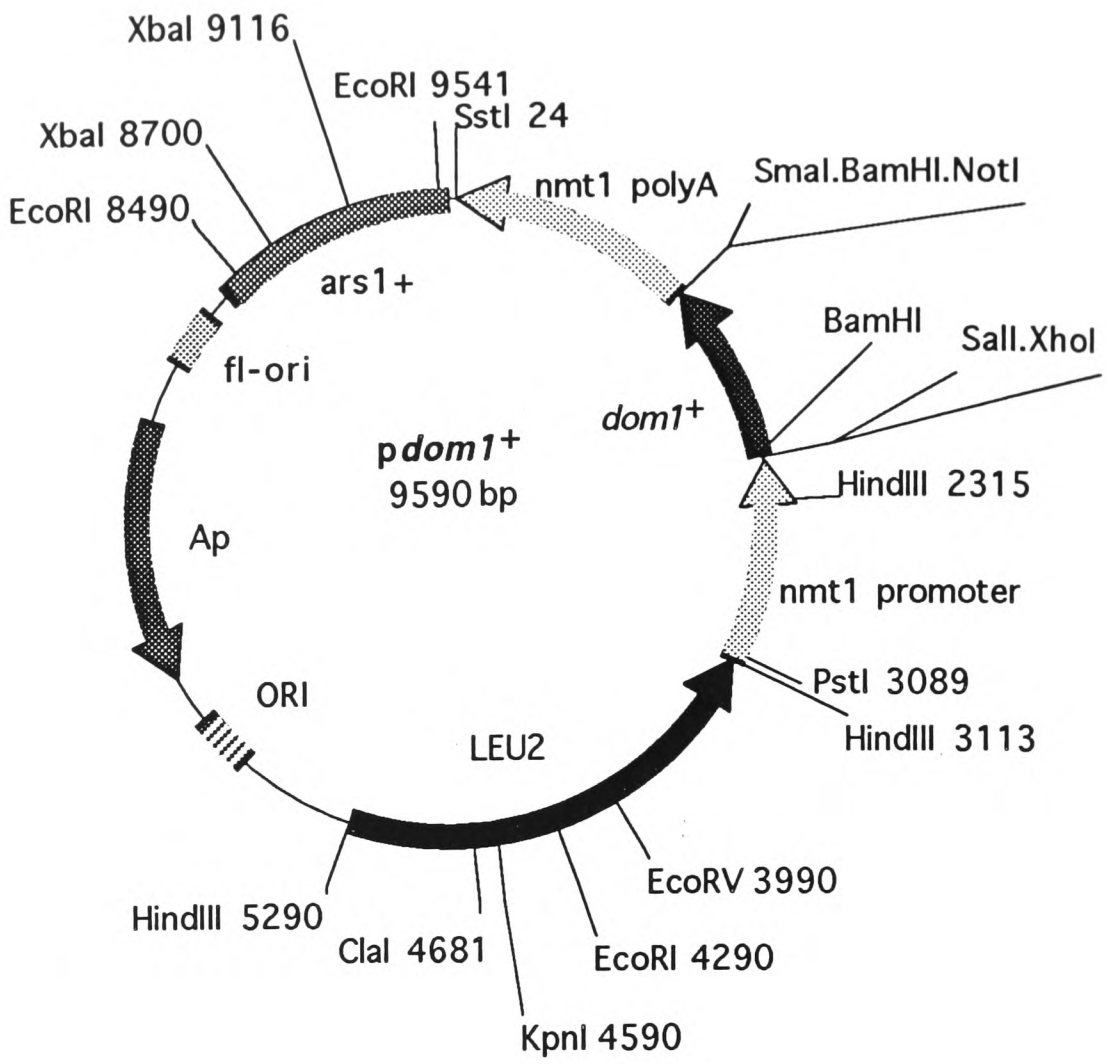


Figure 4.10

Overexpression of the *dom1*$^+$ cDNA does not interfere with the growth of both wild-type and *cdc21-M68* strains

Wild-type (P2) and *cdc21-M68* (P1) strains transformed with the plasmid containing the *dom1*$^+$ cDNA (p*dom1*$^+$) were streaked out on minimal selective plates with or without thiamine and incubated at 25°C for 5 days. Both strains were found viable in both conditions of growth.

(a)


XbaI 9116
EcoRI 9541
SstI 24
XbaI 8700
EcoRI 8490
nmt1 polyA
SmaI.BamHI.NotI
ars1+
BamHI
SalI.XhoI
f1-ori
dom1+
p dom1+
9590 bp
HindIII 2315
Ap
nmt1 promoter
PstI 3089
ORI
HindIII 3113
LEU2
EcoRV 3990
HindIII 5290
ClaI 4681
EcoRI 4290
KpnI 4590

(b)

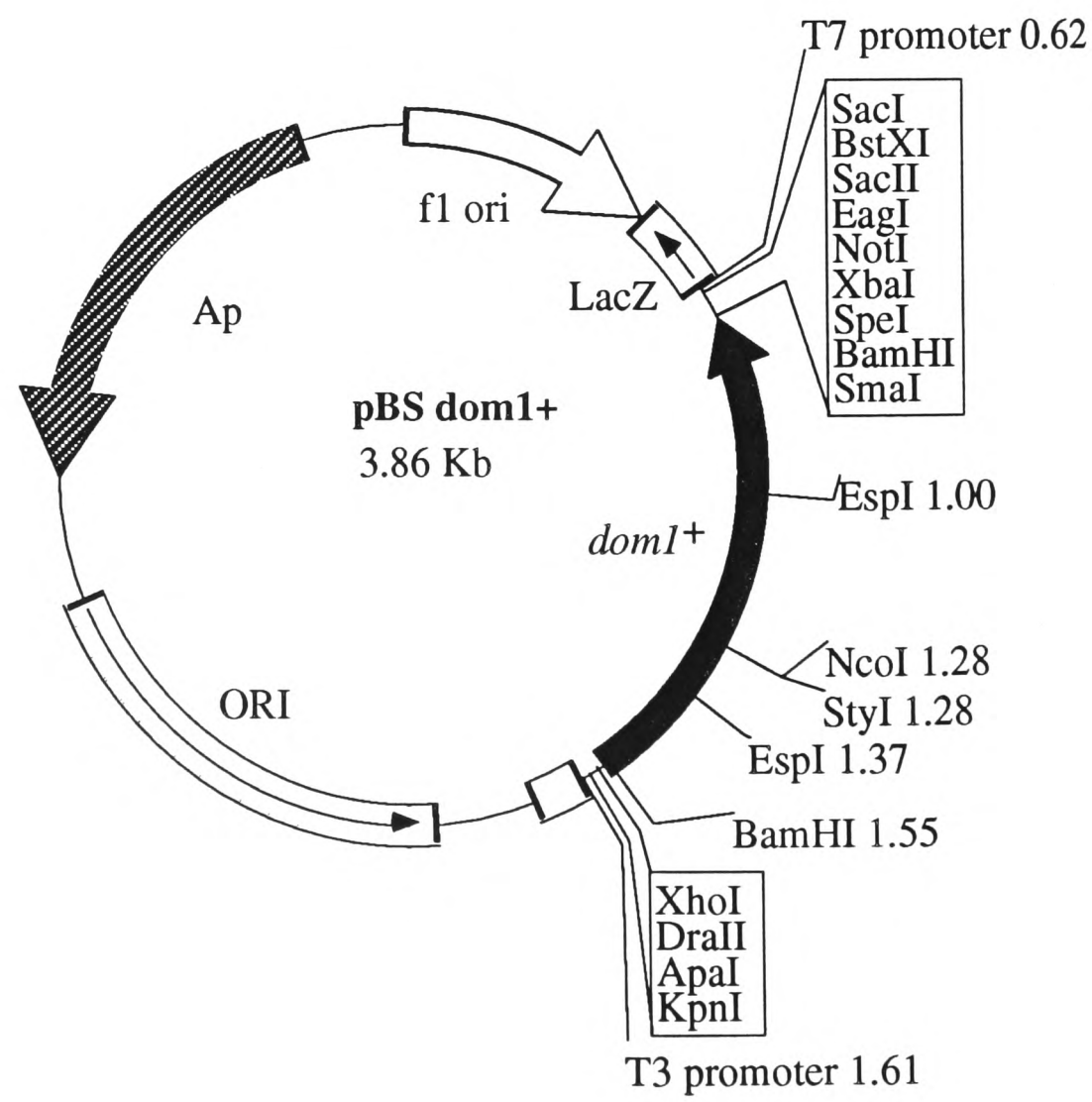

T7 promoter 0.62
SacI
BstXI
SacII
EagI
NotI
XbaI
SpeI
BamHI
SmaI
f1 ori
LacZ
Ap
pBS dom1+
3.86 Kb
dom1+
EspI 1.00
NcoI 1.28
StyI 1.28
ORI
EspI 1.37
BamHI 1.55
XhoI
DraII
ApaI
KpnI
T3 promoter 1.61

Figure 4.11

(a)

Restriction map of the p*dom1*$^+$ plasmid

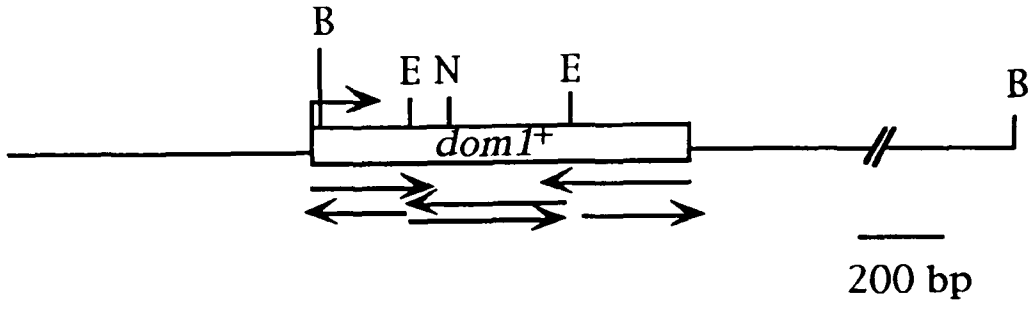
The cDNA library was constructed so that the cDNAs were inserted in the *Sal*I and *Bam*HI restriction sites of the pREP3X vector, a modified version of the REP3 vector (Maundrell, 1990) in which the ATG of the *nmt1* gene has been destroyed (Kelly *et al.*, 1993). The restriction sites of the vector, *Xho*I and *Sma*I are indicated outside the cDNA.

(b)

Construction of the pBS*dom1*$^+$ plasmid

The plasmid p*dom1*$^+$ was digested with *Bam*HI. This digest produced a fragment of ca. 0.9 kb. Thus the cDNA was isolated as the ca. 0.9 kb *Sma*I-*Xho*I DNA fragment and sub-cloned into the polylinker of the Bluescript KS (+) vector digested with *Sma*I and *Xho*I. The occurrence of the major restriction sites on the *dom1*$^+$ cDNA are reported

(a)



(b)

```
                                                        M  A  K  D  K  K  D
GTTAATTAAGCTGAGAGGATCCTTCTCCATTTCTTTATCATGGCAAAAGATAAAAAAGAC    60
                  BamHI
 H  K  H  S  G  S  T  E  D  E  Y  D  S  Y  L  P  A  L  M  P
CATAAGCATAGTGGAAGCACCGAAGATGAGTATGATTCATATCTTCCTGCTTTAATGCCA   120

 I  A  K  P  L  A  P  K  K  L  N  K  K  M  M  K  T  V  K  K
ATTGCTAAGCCTTTAGCTCCTAAAAAATTGAACAAAAAAATGATGAAGACTGTCAAGAAG   180
   EspI
 A  S  K  Q  K  H  I  L  R  G  V  K  E  V  V  K  A  V  R  K
GCTTCGAAGCAGAAACACATCCTTCGTGGGGTCAAAGAGGTTGTAAAGGCTGTTAGAAAA   240

 G  E  K  G  L  V  I  L  P  G  D  I  S  P  M  D  V  I  S  H
GGAGAGAAGGGTCTTGTAATTCTTCCTGGTGATATTTCTCCCATGGATGTTATATCTCAT   300
                                                  NcoI
 I  P  V  L  C  E  D  N  N  V  P  Y  L  Y  T  V  S  K  E  L
ATTCCAGTATTATGTGAAGACAATAATGTCCCTTACTTATATACTGTTTCCAAAGAGTTG   360

 L  G  E  A  S  N  T  K  R  P  T  S  C  V  M  I  V  P  G  G
CTTGGTGAAGCATCAAACACCAAGAGACCTACTAGCTGTGTTATGATTGTTCCCGGTGGA   420

 K  K  K  D  M  S  K  V  E  E  Y  K  E  S  Y  E  E  I  I  K
AAAAAGAAGGACATGTCTAAAGTTGAGGAGTACAAGGAAAGCTACGAGGAAATTATAAAA   480

 E  V  P  A  L  E  V  *
GAAGTACCTGCTCTTGAAGTTTAATTGTTCAATATCTAAAAGATGCTTAGCCATTTAATG   540
                                                 EspI
TAGATTATTATATACTATTAAAATTTGGTATTTATCCTGGGATTTAACTTTTGGTATATC   600

ATGTCTGTTTGCTTGATAGTAAGCGAAAAGAAGTTTTGCTTAATGGTCGGTTTTAAGGTT   660

TCAAAAAATTTCCTTTTTAATCTAGTGTTTTATCATTCTTTCTTATGCCCTAGTATTTTA   720

TGGGTTTAGACTGGTTTGCGAGCAAACTAATCACTATACCTCCAAAACTTTGTTAGCAAT   780

AATATCGTATCATTTACCAACTTATATGAGAATGGTATATCGCTGTAACAGCGATGAAAT   840

ATCAACTGATTTTTCCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA              890
```

dom1 **KR**PTSCVMIVPGG-**KKK**
Nhp2 **KR**PTSVVFIVPGSN**KKK**

Figure 4.12

(a)

Strategy used to sequence the *dom1*$^+$ cDNA

A partial sequence of the *dom1*$^+$ cDNA was obtained by sequencing the insert of the p*dom1*$^+$ plasmid in both forward and reverse directions using the M13 universal primers (see Chapter Two). A restriction map of this sequence was then constructed by searching for restriction enzymes recognition sites using the Nucleotide Interpretation Programme (NIP) of Staden. Two *Esp*I sites were mapped in the insert; one at position 124 and a second *Esp*I site at position 525. There are no *Esp*I sites in the Bluescript plasmid. Digestion of the p*dom1*$^+$ plasmid with the restriction enzyme *Esp*I produced a fragment of about 400bp in length. The ca. 0.4kb *Esp*I fragment and the p*dom1*$^+$ plasmid deleted of the *Esp*I fragment were then gel purified and treated with the large fragment of the *E. coli* DNA polymerase enzyme (Klenow fragment). The deleted plasmid was re-ligated whereas the *Esp*I fragment was ligated into the *Sma*I site of the pUC18 plasmid. Two recombinant plasmids pUCΔ*Esp*F and pUCΔ*Esp*R were recovered in *E. coli* (a restriction map of these constructs is available in Appendix II). These two plasmid differ only for the orientation for the *Esp*I insert (Forward or Reverse). The DNA sequence of the insert of both plasmids was determined. The p*dom1*$^+$ plasmid deleted of the *Esp*I fragment was also recovered. The DNA sequence of the insert was determined on both strands. One *Nco*I restriction site was mapped at position 281 in the p*dom1*$^+$ cDNA. The pBS*dom1*$^+$ plasmid was then digested with *Esp*I, *Nco*I and *Sma*I and the ca. 300bp *Sma*I-*Esp*I restriction fragment was gel purified. After treatment with Klenow enzyme, this DNA fragment was ligated into the *Sma*I site of the pUC18 plasmid. The DNA sequence of the insert was then determined. The orientation of this insert was worked out by comparison with the partial sequence obtained from the 3' end of the cDNA in the p*dom1*$^+$ plasmid. Arrows underneath the *dom1*$^+$ open reading frame (boxed region) show the strategy used to sequence the cDNA. B: *Bam*HI; E: *Esp*I; N: *Nco*I.

(b)

DNA sequence of the *dom1*$^+$ cDNA from *Schizosaccharomyces pombe*

The complete sequence of the *dom1*$^+$ cDNA is shown. The translation of the longest open reading frame found is shown on the top of the sequence. Underlined amino acids indicate the putative bipartite nuclear localisation signal (NLS; Dingwall and Laskey, 1991) conserved in the Nhp2 protein The long stretch of adenines at the 3' end of the cDNA represents the polyadenylation signal of the correspondent mRNA. The sequence of each position was determined at least once on each strand. The original sequence data, reconstructed using the Staden sequence assembly programme (SAP), is presented in Appendix II.

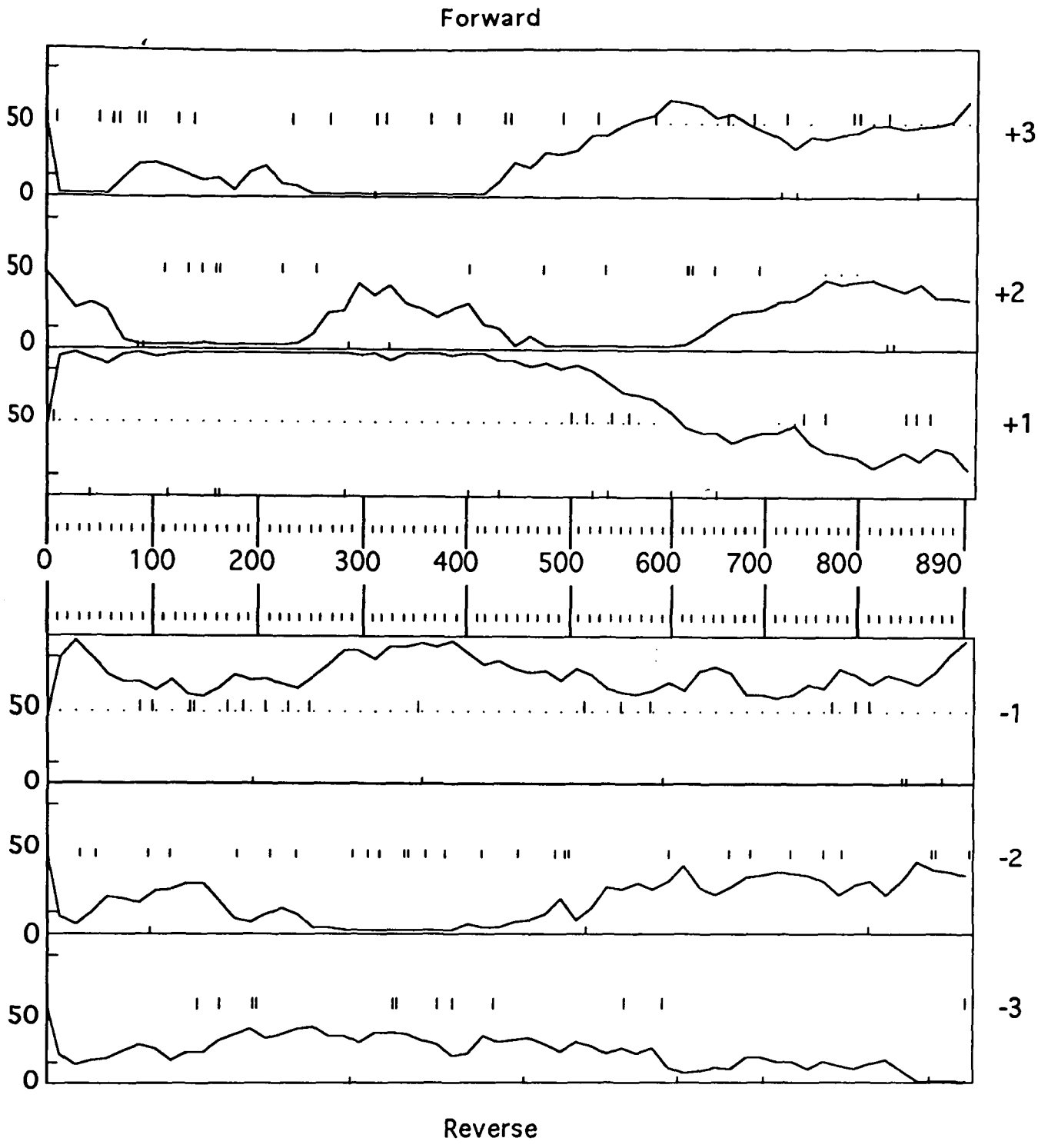


Figure 4.13

Predicted open reading frame of the *dom1*$^+$ cDNA

This analysis was performed as described in Figure 4.7. The *dom1*$^+$ open reading frame was found on frame +1.

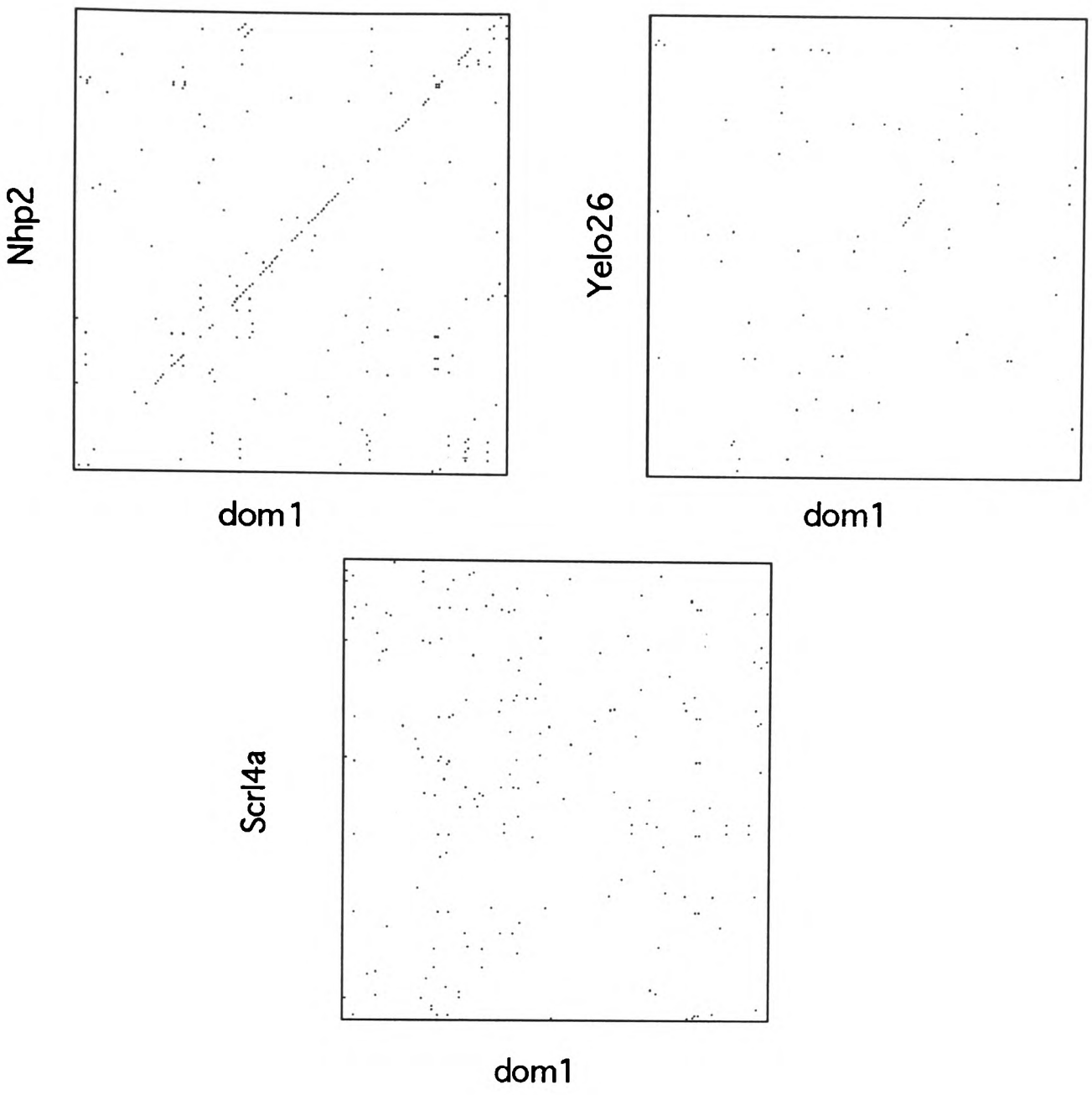


Figure 4.14

The dom1 protein may be the fission yeast homologue of the *S. cerevisiae* Nhp2

These diagonal plots were constructed using the Staden Similarity Investigation Programme (SIP). The method employed to derive these plots is based on a proportional algorithm whereby similar amino acids are compared. A single dot is plotted at each identical residue within a matching span. The odd span length used was 11 and the proportional score 132. Note the similarity of the dom1 protein with the Nhp2 protein which is extended all over the protein sequence, although with little conservation in the far N-terminal region; whereas only a short region of similarity is found with the predicted Yelo26 protein and a very weak similarity with the *S. cerevisiae* L4a protein.

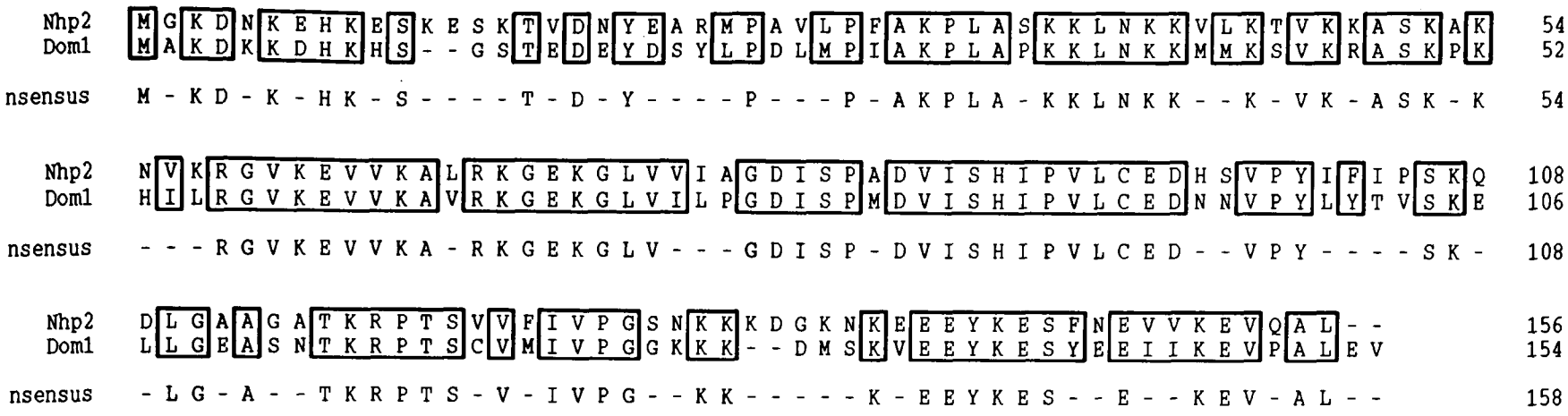


Figure 4.15 (a)

Alignment of the Nhp2 and dom1 proteins

The alignment of the two sequences was created using CLUSTALV. Identical/similar residues are shown boxed; this was generated using the Prettyplot programme of Genetics Computer Group (GCG). Padding characters ('-') have been inserted to optimise the alignment.

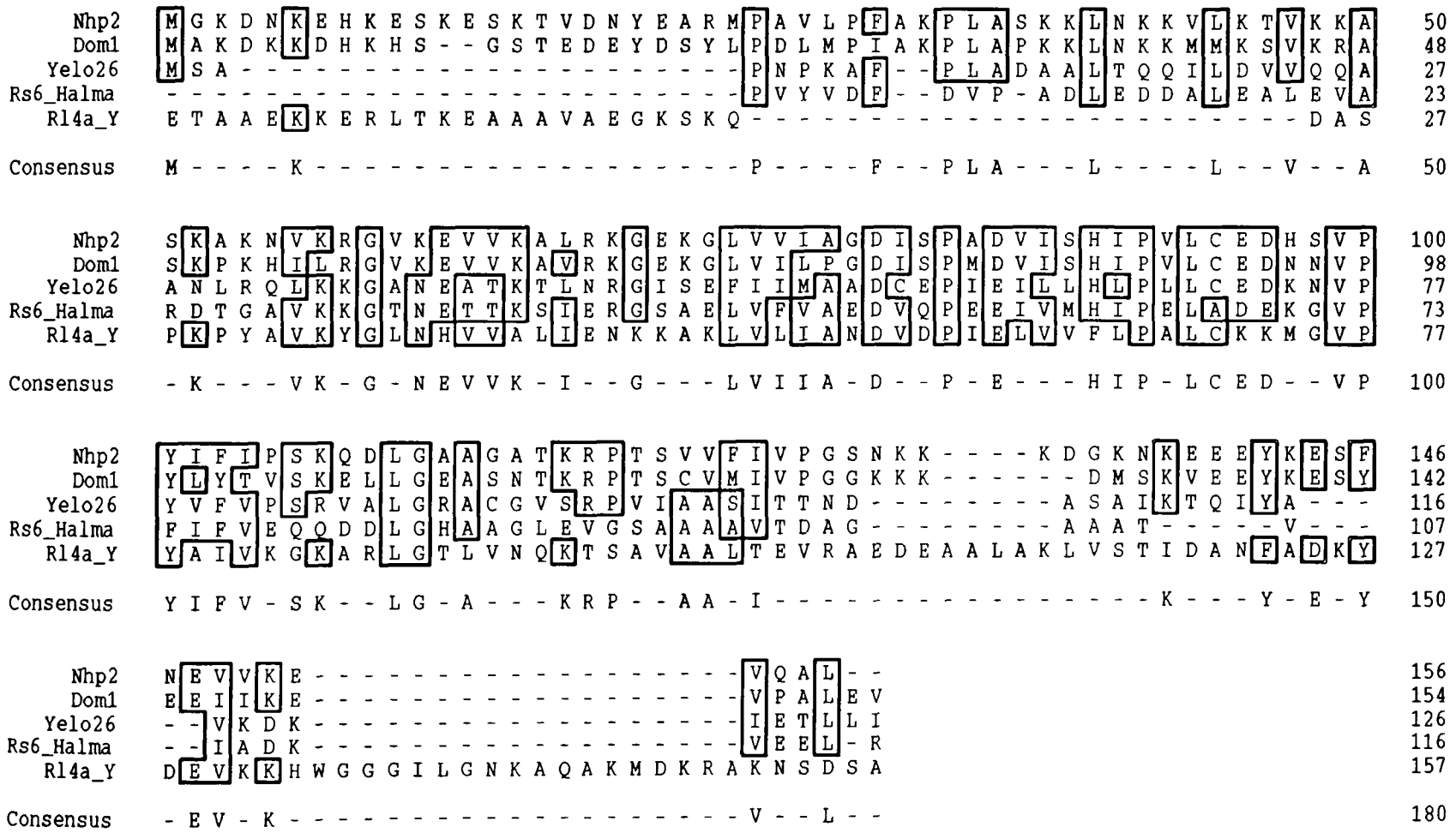


Figure 4.15 (b)

Alignment of the proteins showing similarities with dom1

The alignment was generated as described in (a).

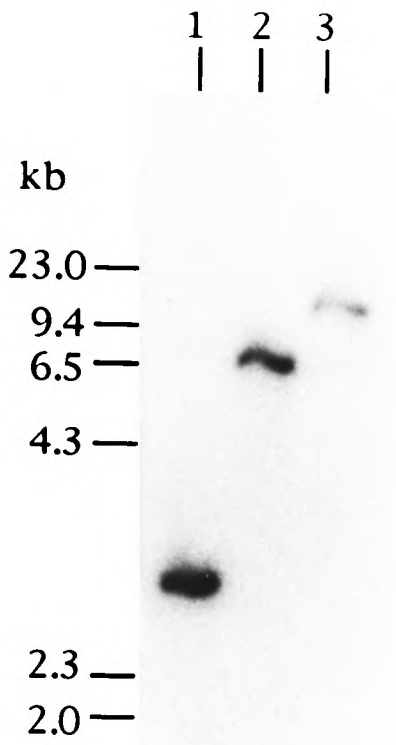


Figure 4.16

Hybridisation of the *dom1*$^+$ cDNA to *S. pombe* DNA

Total DNA was extracted from wild-type haploid *S. pombe* cells (P2 strain), digested with the indicated restriction enzymes, loaded on a 0.8% (w/v) agarose gel and subjected to electrophoresis in a 1X TBE buffer containing 10µg/ml of EtBr as described in Chapter Two. After electrophoresis the gel was blotted to a nylon membrane (see Chapter Two) and hybridised as described in Chapter Two. **1**= *Bam*HI; **2**= *Hin*dIII; **3**= *Eco*RI.

Digest	Predic. MW (bp)
*Bam*HI	2761
*Eco*RI	6384
*Hin*dIII	18897

Table 4.7

Predicted molecular weight of the hybridisation signals produced by hybridisation of the *dom1*$^+$ cDNA to *S. pombe* DNA

The predicted size of the hybridisation signals of the Southern blot experiment of Figure 4.16 was calculated by extrapolation of their relative mobility compared to that of a molecular size standard.

Chapter Five

Studies on the overexpression of *cdc21+*

Introduction

If *cdc21*$^+$ has a positive role in DNA replication, as suggested by earlier phenotypic analysis of the *cdc21*^{ts} mutant (Nasmyth and Nurse, 1991; Coxon *et al.*, 1992), it would be of interest to determine if its overexpression may cause perturbation of S phase. The overexpression of *cdc21*$^+$-related genes in budding yeast has been reported. Overexpression of *MCM3* causes an *mcm*$^-$ phenotype (Gibson *et al.*, 1990), whereas the overexpression of another related gene, *MCM2*, has no phenotype (Yan *et al.*, 1991). Therefore I have studied the effect of the overexpression of *cdc21*$^+$ on the progression of S phase in fission yeast.

Inspection of the primary sequence of the cdc21 protein suggests the presence of three distinct regions (see Figure 5.1).

The first region (I), confined to the amino terminus of cdc21, is hydrophilic and contains many serine, arginine and proline residues which resemble PEST sequences (PEST region; Rogers *et al.*, 1986). These motifs have been associated with short protein half lived *in vivo* involved in proteins turnover *in vivo* (Rechneister, 1988).

The second region (II) comprises a short stretch of amino-acids which contains a motif reminiscent of a zinc finger DNA binding motif, although the sequence does not strictly match a classical zinc finger (see Coleman, 1992 for review). This motif ($CX_2CX_{18}CX_4C$) is absolutely conserved in the yeast Cdc54, Cdc47 and mis5 proteins and is also present in the yeast Mcm2/nda1 and mammalian BM28 proteins as the $CX_2CX_{19}CX_2C$ variant. This motif may serve to co-ordinate a metal ion, perhaps zinc (Berg, 1990).

The third region (III), limited to the central part and carboxy-terminus of the cdc21 protein, contains three areas of homology (A, B and C) with the Mcm proteins (Coxon *et al.*, 1992). The region IIIB has been recently shown to contain a) a putative degenerate ATP/GTP binding site and b) a weak homology to a family of prokaryotic transcription factors in a region spanning the ATP/GTP binding site (Koonin, 1993; Todorov *et al.*, 1994; see also Chapter One).

Regions IIIB and C have been shown to be essential as the *cdc21*$^+$ gene lacking these regions (*Bam*HI deletion) cannot complement the *cdc21-M68* mutation (A. Coxon, D.Phil. thesis). Because these parts of the proteins have been very well conserved in evolution it is a possibility that the functional domain of cdc21 may lie within the regions conserved in the Mcm proteins. If this is the case, the carboxyl-terminal of cdc21 may be able to complement the *cdc21*^{ts} mutation.

I have generated and expressed amino-terminal truncations of the *cdc21*$^+$ gene. These were tested for their ability to rescue the *cdc21-M68* mutant at the restrictive temperature.

Moreover these truncated versions of *cdc21*$^+$ were introduced into fission yeast wild-type cells to see if they could behave as lethal-dominant mutants. As these mutants may display new phenotypes this approach represents an additional way to gain insight in the function of a protein *in vivo* (Herskowitz, 1987).

Results

Overexpression of *cdc21*$^+$

The effect on DNA replication and cell cycle progression of overexpressing *cdc21*$^+$ was investigated. A fission yeast wild-type strain bearing the pREP*3X::21*$^+$ plasmid (which contains the *cdc21*$^+$ gene under control of the thiamine repressible 3X*nmt1* promoter, given by Dr.Blom; Basi *et al.*, 1992) was used. The *nmt1* promoter was induced by thiamine deprivation as described in the legend of Figure 5.2. Cells were examined for DNA content, morphology and number in a time course experiment following induction.

As shown in Figure 5.2 after 16 hours of thiamine deprivation the growth rate of this strain (*nmt1::cdc21*$^+$ on) is similar to that of the control strains (see legend to Figure 5.2). However, two hours later, when full derepression of the *nmt1* promoter has taken place (Maundrell, 1990) the growth rate of cells overexpressing the *cdc21*$^+$ gene appears to show a temporary lag after which the rate recovers to a level similar to that of the control strains. The growth rate of the control strain (*nmt1* on) was not affected by thiamine starvation.

Microscopic observation of cells revealed that after about 24-28 hours of *nmt1* induction cells bearing the pREP*3X::21*$^+$ construct (Figure 5.3, panel C) were elongated dividing at about twice the size of cells bearing the vector alone (about 28μm versus 14μm). Most of the elongated cells contained one nucleus. Altered nuclear morphology (arrow) and faint DAPI staining, similar to the phenotype of *cdc21-M68* arrested cells (see Chapter Six; Coxon *et al.*, 1992) were also seen in these cells. No such alterations were seen in the control cultures (panels A and B). These result suggest that overexpression of the *cdc21*$^+$ gene may delay entry into mitosis.

To determine if the cell cycle delay was due to lengthening of the S or G1 phases, the DNA content of cells overexpressing *cdc21*$^+$ was determined by flow cytometry (Sazer and Sherwood, 1990). Exponentially growing *S. pombe* cells are mostly in the G2/M phases and contain fully replicated DNA (2C DNA content per haploid cell); G1 and S phases are usually short (Fantes and Nurse, 1977; see also Chapter One). Cells delayed in the G1 phase are expected to accumulate unreplicated DNA (1C) while cells delayed in S phase should have a content of DNA in between 1C and 2C.

As shown in Figure 5.4 before induction of the *nmt1* promoter most cells are in the G2 phase of the cell cycle, indicated by the presence of a 2C peak of DNA content

(*nmt1::cdc21*$^+$ off). After 24 hours of *nmt1* induction (24h) the proportion of cells with a 2C DNA content does not change implying that the length of the G1 or S phases is not affected in these cells. Control cells (*nmt1* on and off) behave similarly.

These results taken together suggest that cells overexpressing the *cdc21*$^+$ gene may be delayed in progression through the G2/M phases of the cell cycle.

Overexpression of amino-terminal truncations of the *cdc21*$^+$ gene

In order to identify possible functional domains of the cdc21 protein and to determine if truncated forms of *cdc21*$^+$ may confer a lethal dominant phenotype, three different regions of the *cdc21*$^+$ gene were fused to the inducible promoter of the *nmt1*$^+$ gene (Maundrell, 1990). The criteria chosen to make the constructs were essentially dependent on the availability of suitable restriction endonuclease cleavage sites which could restore the frame of the cdc21 protein when placed under control of the fission yeast *nmt1* gene.

The construction of the vectors (pOE1-3) is described in the legend of Figure 5.5.

These constructs were transformed into the *S. pombe* wild-type and *cdc21-M68* strains (see Chapter Two). Transformants were recovered on selective plates in the presence of thiamine (*nmt1* off). The expressed proteins were detected in protein extracts by using a polyclonal antibody raised against the carboxyl-terminal of the cdc21 protein (G.Blom, unpublished) in collaboration with Dr. G.Blom (Zoology Department, Oxford University). The antibody (Figure 5.6) recognised an endogenous band of about 100 kDa in all the lanes (1-6). This band was particularly enhanced in the lane loaded with extracts made from cells overexpressing the full *cdc21*$^+$ gene (lane 2). Other bands at lower molecular weights, not present in the other lanes, are also recognised by the antibody and they are likely to be degradation products of the overproduced cdc21 protein. This antibody did not recognise an amino-terminal part of the *cdc21*$^+$ gene as expected (lane 3; G.Blom, unpublished). The antibody instead recognised bands of a molecular weight very similar to that predicted for the recombinant proteins oe1-3 (lanes 4-6). These bands were not detected in conditions of *nmt1* repression (G.Blom, pers. comm.).

Therefore, these results demonstrate that the pOE1-3 vectors were able to express the expected truncated forms of the cdc21 protein.

The pOE1-3 constructs were introduced in *cdc21-M68* and wild type *S. pombe* cells and tested for both complementation and overexpression-induced lethality. The

results of this experiment are shown in Figure 5.7. None of the constructs gave rise to lethality when overproduced in a wild-type strain, nor did they complement the *cdc21*^{ts} mutant when grown at 36°C. Surprisingly, overexpression of the oe1 truncation of cdc21 produced lethality in the *cdc21-M68* strain at the permissive temperature in a thiamine-dependent manner.

To determine if the lethality was specific to the *cdc21-M68* strain the pOE1 construct was transformed in a set of *cdc* strains (Table 5.1). The pREP*81X*::*21*$^{+}$ vector (see Chapter Four) containing the full *cdc21*$^{+}$ open reading frame under control of the 81X*nmt1* inducible promoter (Basi *et al.*, 1993) was included as a control. Transformants were recovered at the permissive temperature in the presence of thiamine and tested for their ability to grow on plates lacking thiamine (*nmt1*::*cdc21*$^{+}$ on). Both the full length *cdc21*$^{+}$ and its truncated form o*e1*$^{+}$ were found not to cause lethality if overproduced in all the strains tested at their permissive temperature, strongly arguing against any possible non-specific effect caused by overexpression in a lethal-conditional genetic background. This strain was therefore retained for further characterisation.

Phenotype of *cdc21-M68* cells overexpressing the *cdc21*$^{+}$ carboxyl-terminal truncation *oe1*$^{+}$

The arrest phenotype of *cdc21-M68* cells overexpressing the *oe1*$^{+}$ truncation of *cdc21*$^{+}$ was studied.

cdc21-M68 cells bearing the pOE1 construct were grown in selective liquid medium in the absence and in the presence of thiamine at the permissive temperature; cell number, nuclear morphology and DNA contents were monitored prior and after thiamine starvation. Monitoring cell number showed that *cdc21*^{ts} cells carrying the pOE1 construct were still dividing (doubling time of 4 hours) after 24 hours of thiamine deprivation. Cell division started to slow down after about 28 hours of thiamine starvation and ceased after 38 hours (Figure 5.8a).

Staining of nuclei with DAPI after 24 hours of *oe1*$^{+}$ overexpression revealed the presence of elongated and small cells with one nucleus (Figure 5.8b, 24h). After 30-34 hours of induction about 25% of the cell population entered an abnormal mitosis. Septa were formed across an undivided nucleus or completely missed the nucleus, thus producing a cell with one nucleus and an anucleate one. Also, one or more than one septum was found in elongated and small cells. (aberrant mitoses; Figure 5.8b, 34h). The phenotypic description and the frequencies of the aberrant mitoses are listed in Table 5.2. These phenotypes were never seen when cells were grown in the presence

of thiamine (0h) or in the control strain grown in the absence of thiamine (C). These phenotypes are similar to those seen in cells lacking the *cdc21*$^+$ gene (see Chapter Seven).

The DNA content of these cells after 34 and 36 hours of thiamine deprivation was determined by flow cytometry. As shown in Figure 5.9, after 34-36 hours of *oe1*$^+$ overexpression cells accumulated in the G1/S phases, whereas they were in the G2 or M phases before thiamine starvation suggesting that they could not complete S phase.

These results taken together indicate that the lethality caused by the overexpression of the carboxyl-terminal of the *cdc21*$^+$ gene in the *cdc21-M68* strain at the permissive temperature is likely to be specific and due to cell cycle arrest in S phase. Surprisingly, a significant proportion of these cells entered mitosis, suggesting they may have lost the correct dependency of mitosis upon completion of S phase.

Discussion

I have further characterised the fission yeast wild-type strain overexpressing the *cdc21*$^+$ gene originally made by Dr. G.Blom (unpublished). As I have shown, the growth rate of *S. pombe* wild-type cells was affected by the overexpression of *cdc21*$^+$ and cells also became elongated suggesting a cell cycle delay. Most of the elongated cells were found to be mononucleate indicating that cells were not delayed after nuclear division. Flow cytometry analysis on these cells showed that the G1 or S phases were not affected, consistent with a delay occurring at the G2/M phase transition. The significance of this delay is not clear. It is possible that it may be due to a non-specific effect caused by the overexpression of the *cdc21*$^+$ gene. More experiments are necessary to show the specificity of such an effect. A similar phenotype has been reported for the overexpression of genes involved in DNA replication and the restraint of mitosis in fission and budding yeast. Overexpression of the *S. cerevisiae CDC6* gene causes a cell cycle delay in the G2 phase (Bueno and Russell, 1992). The overexpression of the fission yeast *cdc18*$^+$ gene, which has structural similarity with the *CDC6* gene, shows similar phenotypes (Kelly *et al.*, 1993). In contrast, overexpression of two budding yeast *cdc21*$^+$-related genes, *MCM2* and *MCM3* has been reported not to cause a cell cycle delay (Yan *et al.*, 1991; Gibson *et al.*, 1990)

I have also studied the effect of overexpressing truncated versions of the *cdc21*$^+$ gene. None of the three truncated forms of cdc21 could rescue the lethality of the *cdc21-M68* strain at the restrictive temperature. These results, taken together with the finding that the overexpression of the amino terminus of cdc21 cannot complement the lethality of the *cdc21-M68* strain (G.Blom, pers. comm.), suggest that perhaps the

entire cdc21 protein is needed to carry out its function. This would be further confirmed by showing that these truncated forms cannot complement a null mutant of *cdc21*.

The overproduction of the carboxyl-terminal of the cdc21 protein oe1, gave rise to lethality if overproduced in the *cdc21*^{ts} strain at the permissive temperature. The lethality of *cdc21-M68* cells overexpressing *oe1*$^{+}$ took place slowly (about 38 hours after thiamine deprivation) and can be split into two steps; initially (20-24 hours after thiamine deprivation) about 40% of the cell population become elongated presumably as a result of cell cycle delay in S phase. This delay was evident at a later stage (34-36 hours) when also about 25% of the cell population enter an abnormal mitosis.

The lethality is likely to be specific for the following reasons. i) overexpression of *oe1*$^{+}$ did not cause lethality when overexpressed in a set of *S. pombe cdc* mutant strains whose wild-type function is required in S phase ii) The arrest phenotype of this strain is similar although not exactly the same, to that of *cdc21-M68* incubated at the restrictive temperature.

One interpretation is that overexpression of the *oe1*$^{+}$ truncation of the *cdc21*$^{+}$ gene may interfere with DNA replication, thus exacerbating the defect of the *cdc21-M68* mutant. Failure to activate the system which monitors the progression of S phase (checkpoint; Hartwell and Weinert, 1989; see also Chapter One) may be thus explained as interference with the initiation of DNA replication, as suggested for a set of mutant in fission yeast showing similar abnormalities (see Nurse, 1994, for review).

Another interpretation is that the oe1 protein may also titrate out a cellular factor which is normally required to restrain mitosis during S phase and with which the wild-type cdc21 protein may normally interact. This factor may be more available or abundant in the *cdc21-M68* strain compared to a wild-type strain. Immunoprecipitation experiments will be necessary to give a biochemical support to this hypothesis.

The oe3 form of cdc21 cannot produce lethality in the *cdc21*^{ts} strain. This may be simply due to lower levels of expression or to higher turnover of this truncated version of cdc21. This may be a possible explanation of why the oe1 protein does not give rise to lethality in wild-type cells.

The truncated form oe2 cannot be compared with the other two peptides as it lacks a small part of the carboxyl-terminal of cdc21. It might only suggests that the entire carboxyl-terminal of cdc21 is needed in order to give rise to lethality in the *cdc21*^{ts} strain at the permissive temperature.

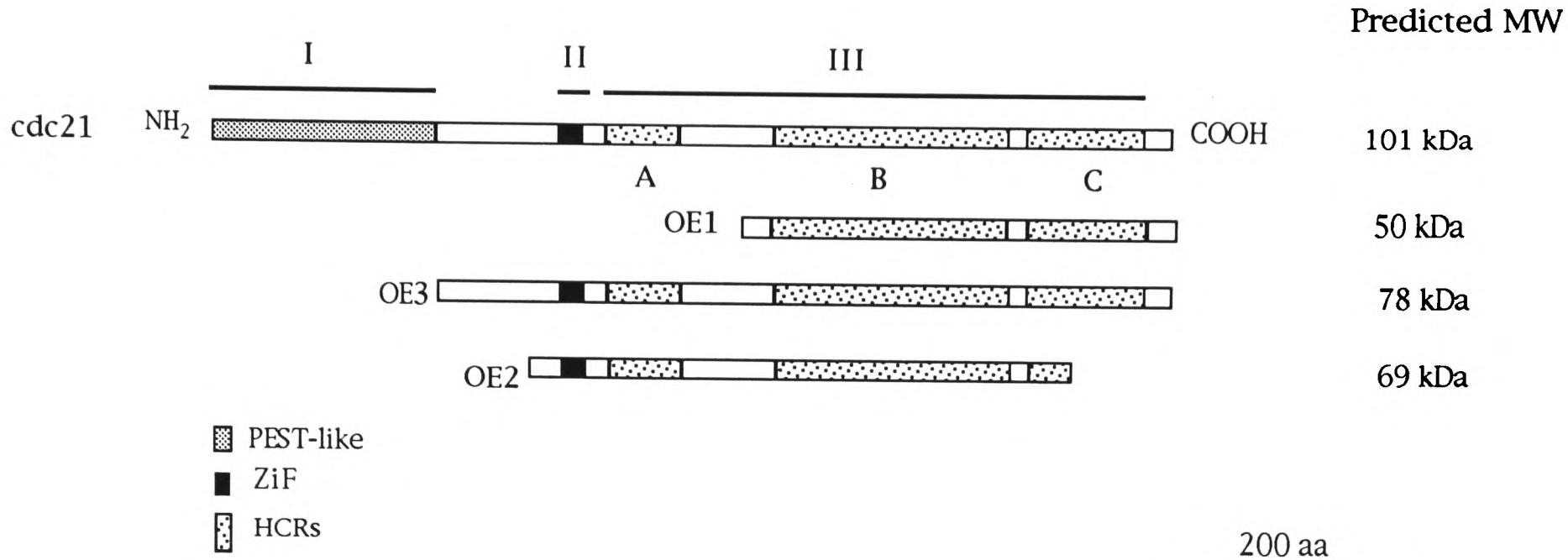


Figure 5.1

Schematic representation of the cdc21 protein and its amino-terminal deletion mutants oe1-3

I, II and III indicate the three regions containing-motifs of the cdc21 protein. IIIA, B and C indicate the three areas of similarity with the Mcm protein family. PEST-like= PEST-like region (see text); ZiF= putative zinc finger motif; HCRs= highly conserved regions among the Mcm proteins. The calculated molecular weight of cdc21 and deletion mutants oe1-3 is indicated.

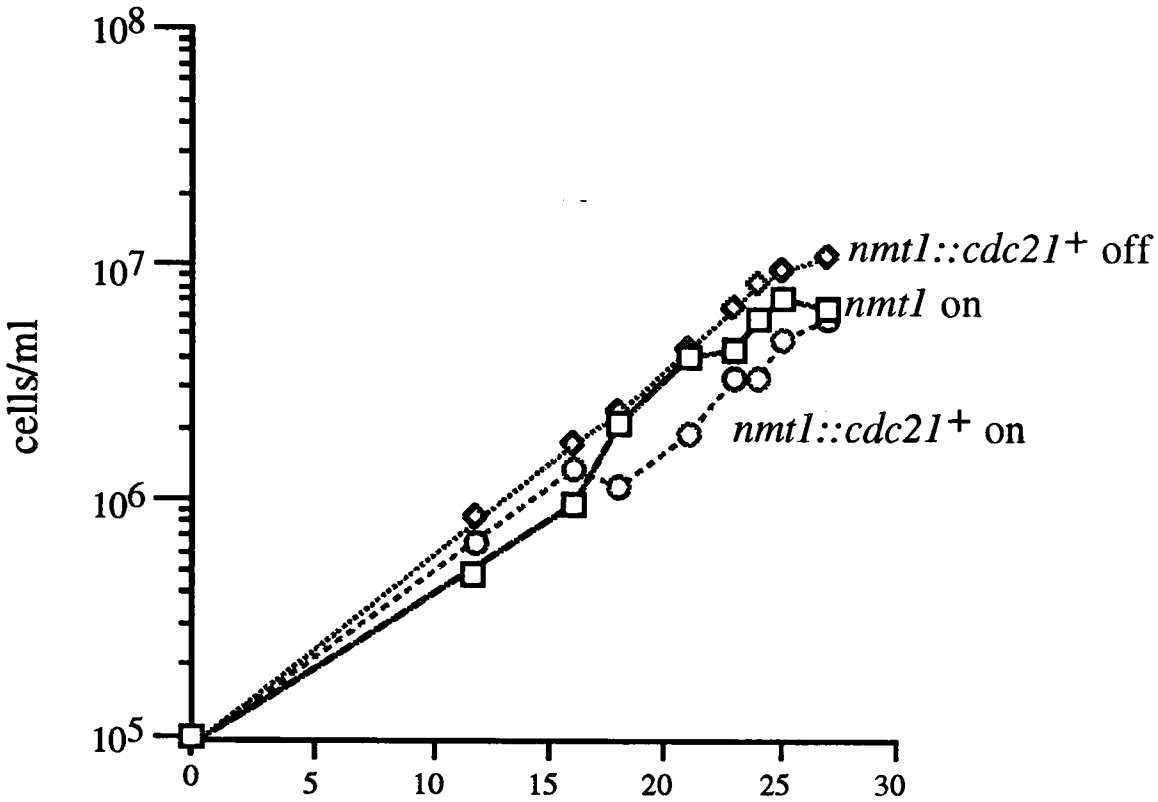


Figure 5.2

Growth rate of *S. pombe* cells overexpressing the *cdc21*$^+$ gene

S. pombe wild-type cells (P2 strain; see Chapter Two) bearing the pREP*3x::21*$^+$ or the pREP3X plasmids were grown in minimal medium lacking leucine in the presence of 5µg/ml of thiamine (*nmt1* off). Cells were washed in sterile water and resuspended in fresh medium in the absence and in the presence of thiamine. Cell number was monitored as described in Chapter Two 12-24 hours after induction of the *nmt1* promoter. *nmt1 on*: P2[pREP3X] -thiamine; *nmt1::cdc21*$^+$ off: P2[pREP*3x::21*$^+$] + thiamine; *nmt1::cdc21*$^+$ on: P2[pREP*3x::21*$^+$] -thiamine.

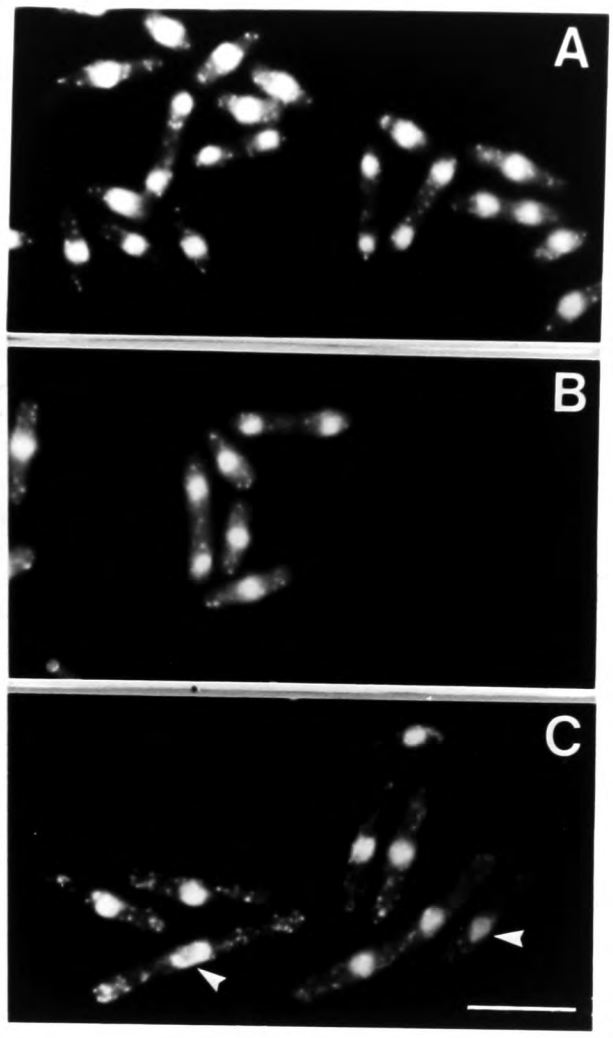


Figure 5.3

Overexpression of *cdc21*$^+$ causes cell elongation

Cell and nuclear morphology of *S. pombe* cells overexpressing *cdc21*$^+$. *S. pombe* cells bearing the pREP*3X::21*$^+$ vector (3X*nmt1::cdc21*$^+$, B and C) and the pREP3X*nmt1* vector (A) were stained with DAPI (see Chapter one). Cells were sampled after 28 hours of growth in the absence (A and C) or in the presence (B) of thiamine and heat fixed as described in Chapter One. Arrowheads indicate cells with altered nuclear morphology. Bar indicates 10μm.

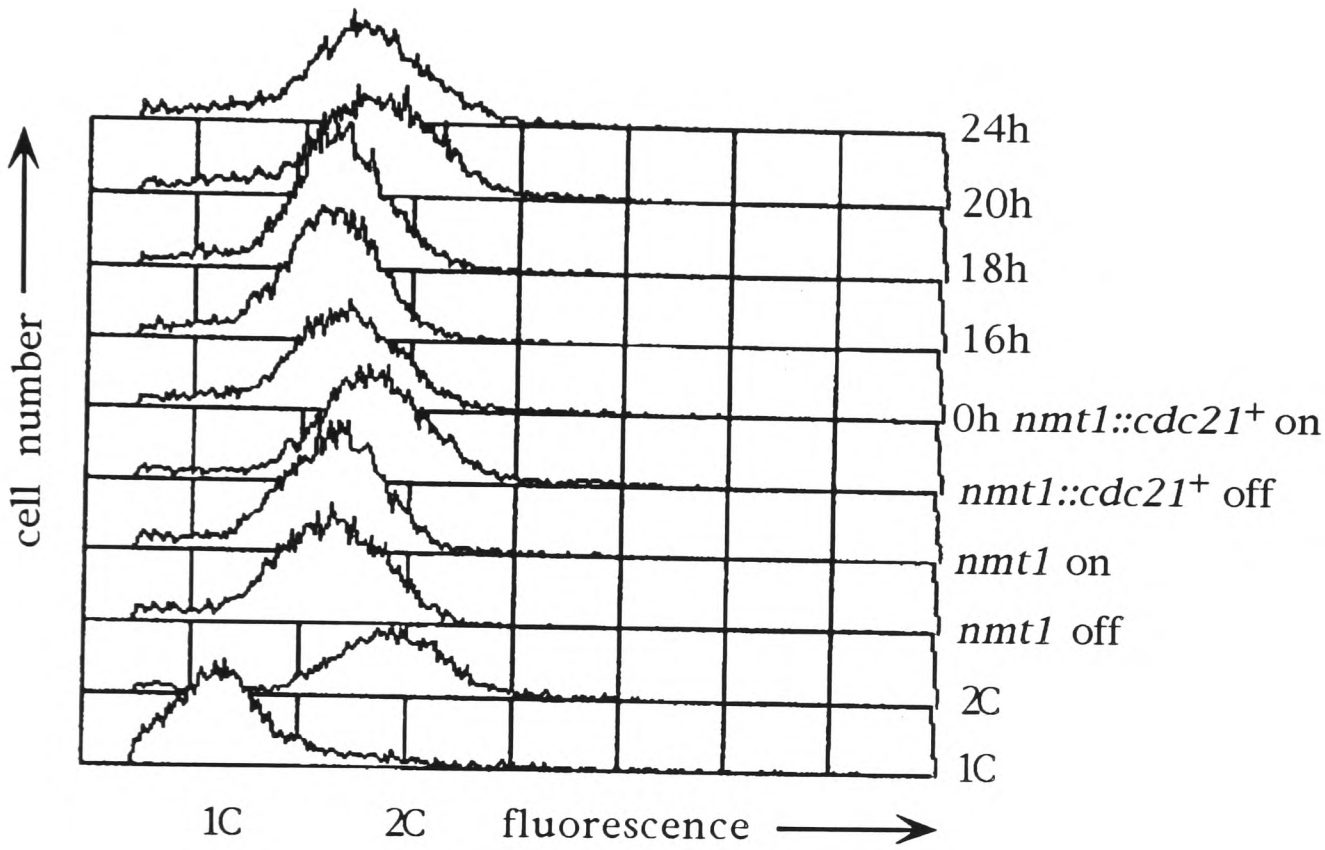


Figure 5.4

DNA content of cells overexpressing the *cdc21*$^+$ gene

The DNA content of wild-type haploid *S. pombe* cells overexpressing (*nmt1*::*cdc21*$^+$ on) or not overexpressing (*nmt1*::*cdc21*$^+$ off) the *cdc21*$^+$ gene was determined by flow cytometry (see Chapter Two). Cells overexpressing *cdc21*$^+$ were sampled after 0, 16, 18 and 24 hours of thiamine deprivation. Wild-type haploid fission yeast cells bearing the *nmt1* promoter alone (pREP3X vector) non induced (*nmt1* off) and after 24 hours of induction (*nmt1* on) were included as a control. 1C and 2C DNA content standards were included (see Chapter Two for details). The data were plotted in linear scale.

Figure 5.5

Construction of the pOE1, pOE2 and pOE3 recombinant plasmids

Amino-terminal truncations of the *cdc21*$^+$ gene were ligated 'in frame' to the promoter of the fission yeast *nmt1*$^+$ gene (Maundrell *et al.*, 1990). The recombinant plasmids pOE1-3 can potentially express truncated versions of the cdc21 protein upon thiamine deprivation. The sequence of the pREP1 polylinker is shown in an inverted direction in the figure (arrow).

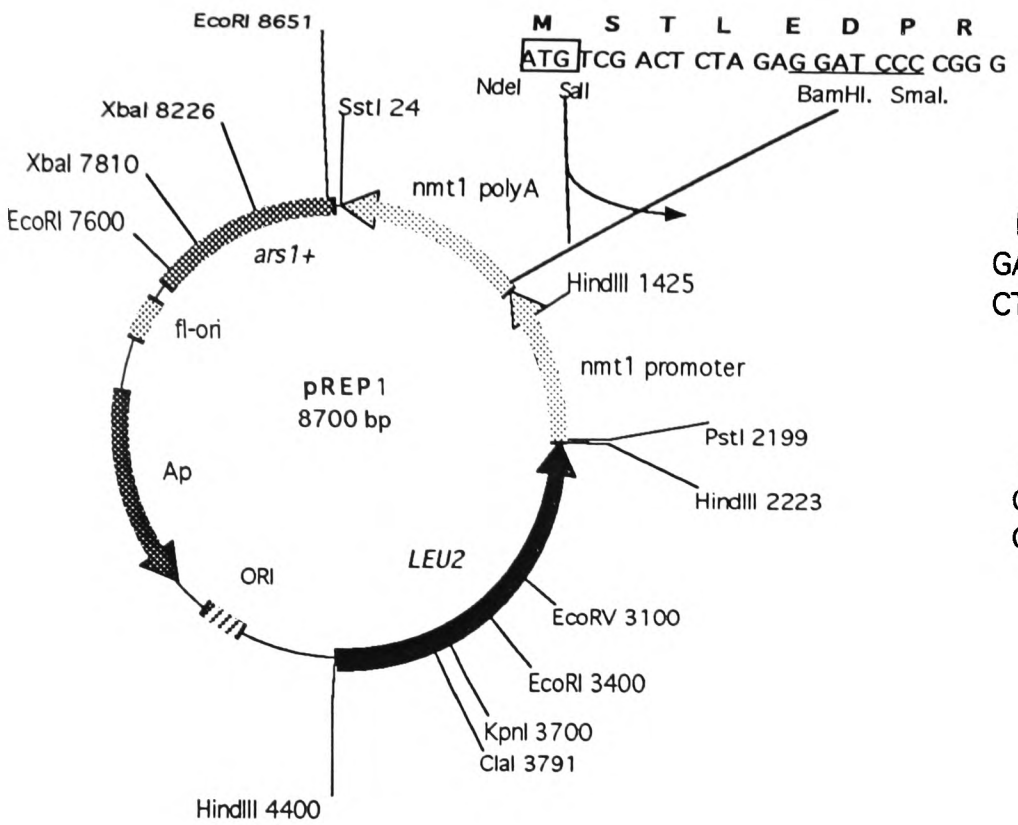
pOE1 was obtained by ligating the *cdc21*$^+$ 1.5 kb *Bam*HI restriction fragment isolated from the pAC1 plasmid (A. Coxon, D.Phil. thesis) into the unique *Bam*HI restriction site of the pREP1 expression vector containing the *nmt1* promoter.

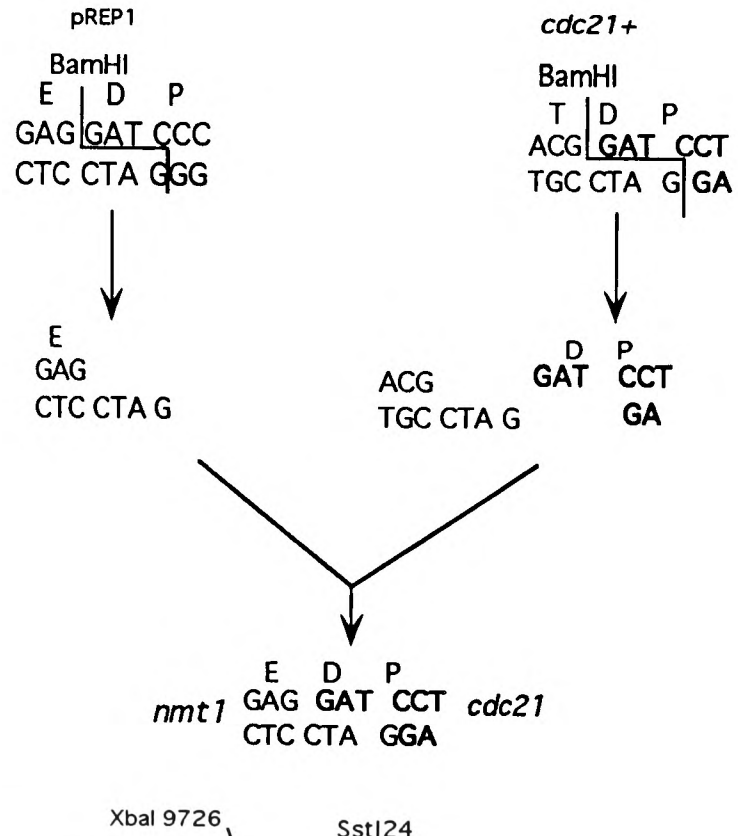
pOE2 was created by ligating the *cdc21*$^+$ 1.5 kb *Nsi*I restriction fragment isolated from the pAC1 plasmid into the unique *Sma*I restriction site of the pREP1 vector, after the 3' protruding ends produced by the *Nsi*I digestion had been removed with the bacteriophage T4 DNA polymerase enzyme as described (Sambrook *et al.*, 1991).

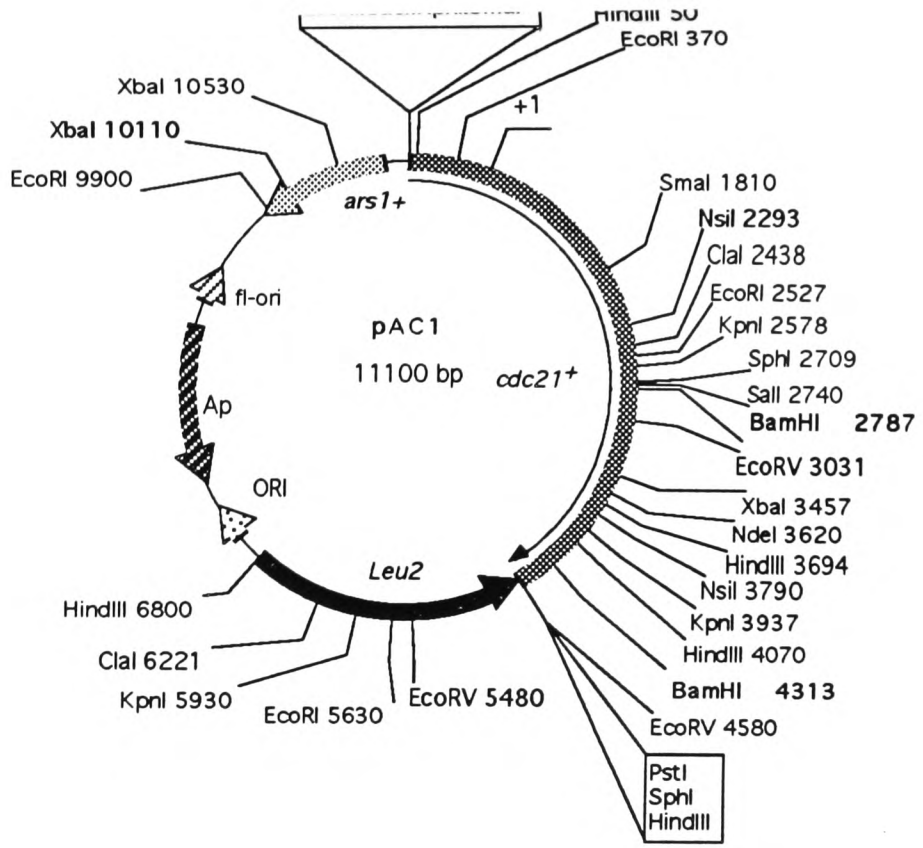
pOE3 was generated by ligating the *cdc21*$^+$ 2.8 kb *Hae*II-*Pst*I restriction fragment, isolated from the pAC1 plasmid, into the unique *Sma*I restriction site of the pREP1 vector, after the 3' protruding ends produced by *Hae*II and *Pst*I had been removed with the bacteriophage T4 DNA polymerase enzyme.

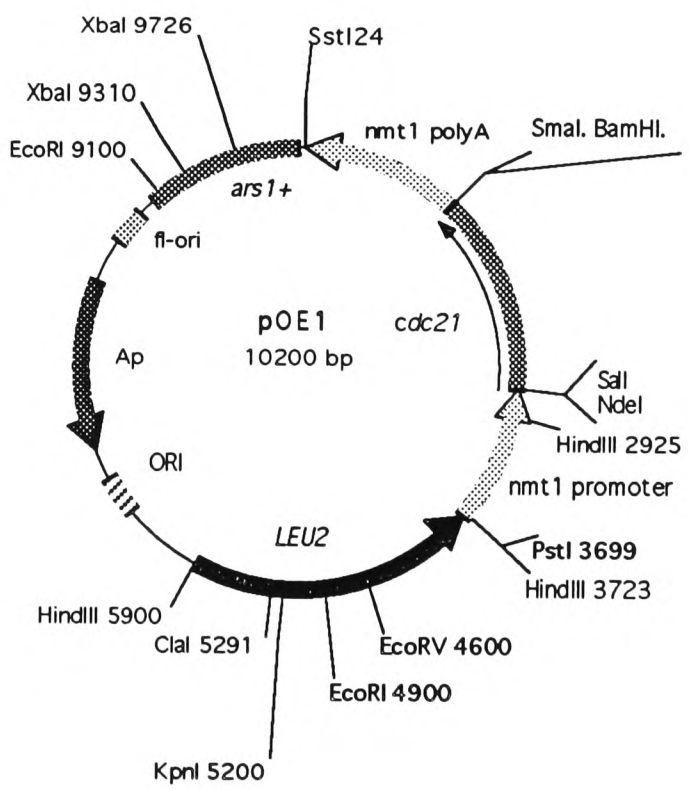
The first 5 amino acids (MSTLE) of the oe1, the first 8 (MSTLEDPL) of the oe2 and the first 7 (MSTLEDP) of the oe3 proteins have been introduced by the gene fusion procedure.

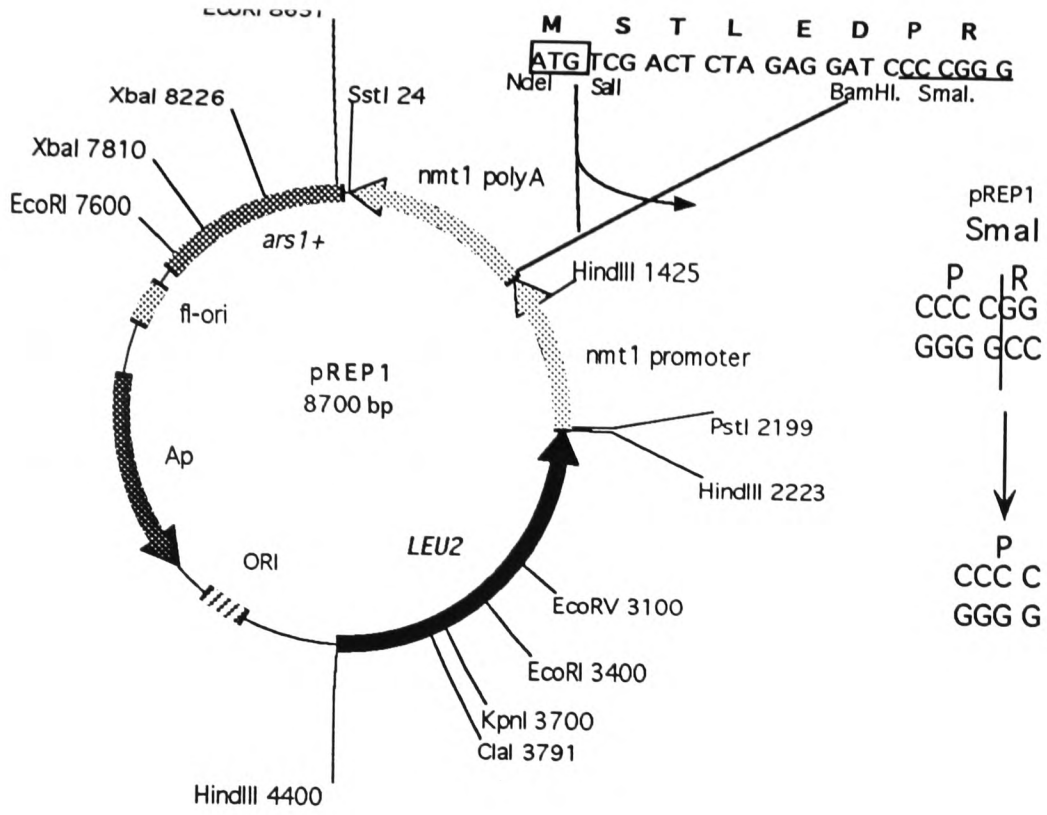
These recombinant plasmids were screened by restriction digest to confirm the presence of the expected insert in the correct (sense) orientation. The *cdc21*$^+$ frame of the pOE2 and pOE3 plasmids was verified by DNA sequencing using a 25 bp *cdc21*$^+$ single stranded DNA primer specifically designed to anneal the *cdc21*$^+$ DNA sequence at position 1753 on the reverse strand (5'-GGAGTTTAATAACTTATCGGC-3') (given by Dr. Blom).

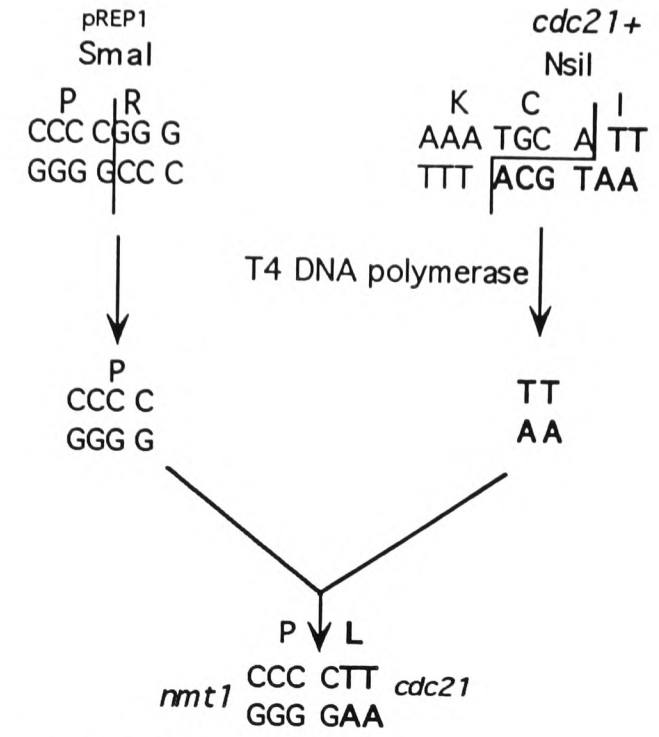

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NdeI SalI BamHI. SmaI.
XbaI 8226
SstI 24
XbaI 7810
nmt1 polyA
EcoRI 7600
ars1+
HindIII 1425
fl-ori
nmt1 promoter
pREP1
8700 bp
PstI 2199
Ap
HindIII 2223
ORI
LEU2
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ClaI 3791
HindIII 4400

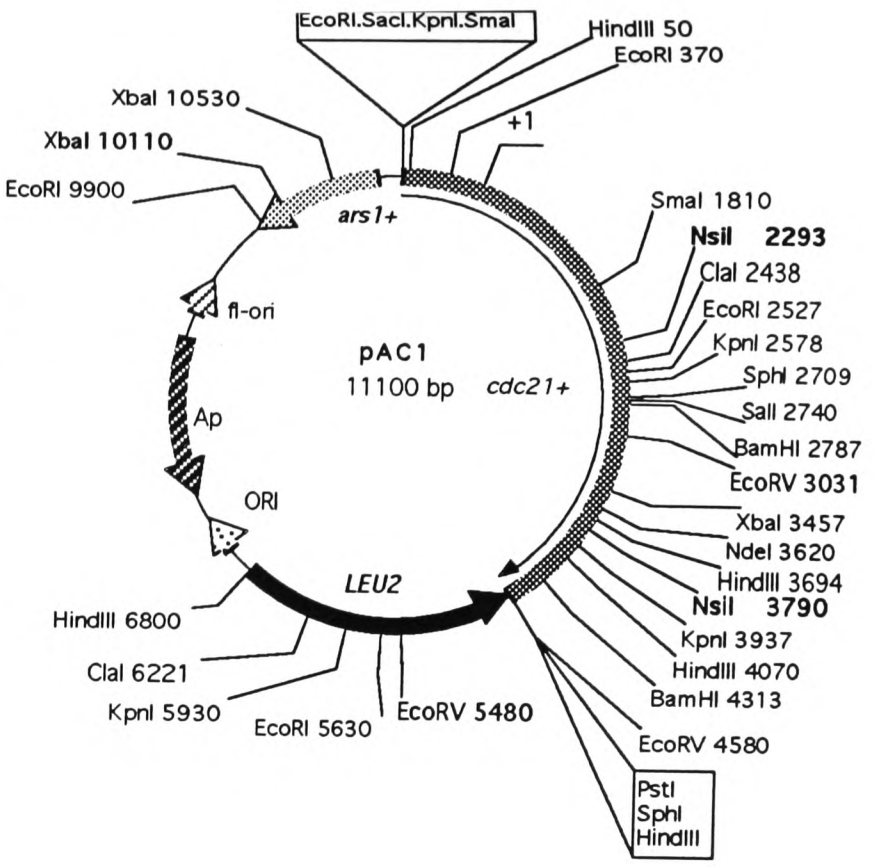

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CTC CTA GGG
E
GAG
CTC CTA G
cdc21+
BamHI
T D P
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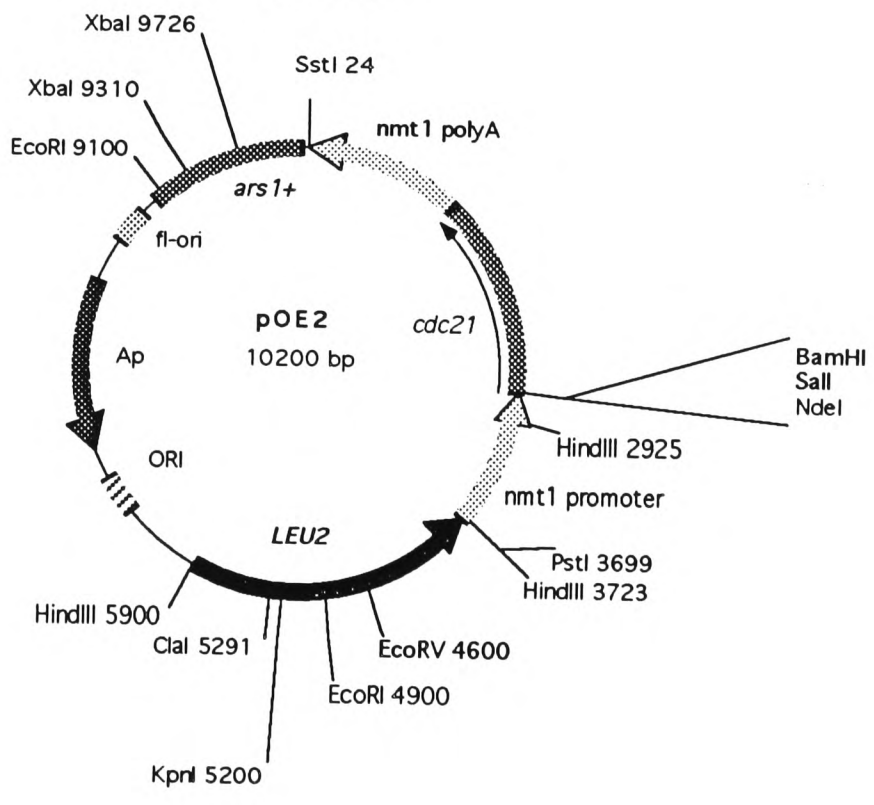

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XbaI 10530
+1
XbaI 10110
EcoRI 9900
SmaI 1810
ars1+
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EcoRI 2527
pAC1
KpnI 2578
11100 bp
cdc21+
SphI 2709
SalI 2740
Ap
BamHI 2787
EcoRV 3031
ORI
XbaI 3457
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HindIII 3694
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KpnI 3937
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SphI
HindIII

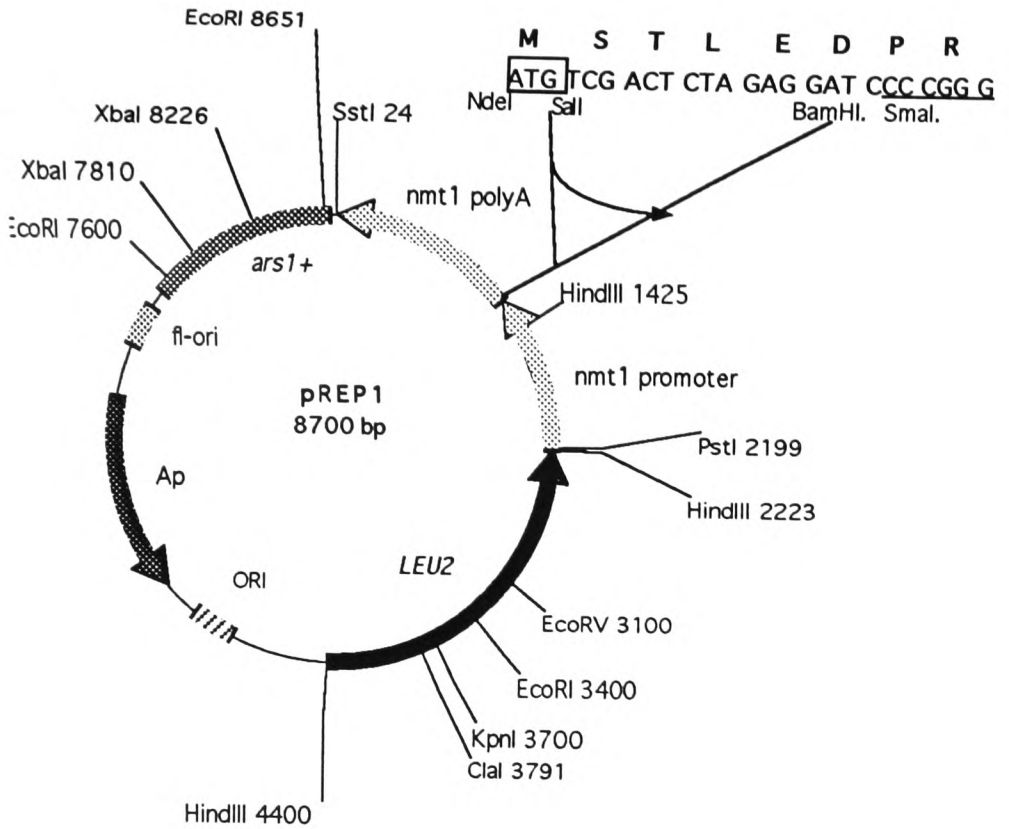

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SstI24
XbaI 9310
nmt1 polyA
SmaI. BamHI.
EcoRI 9100
ars1+
fl-ori
pOE1
cdc21
Ap
10200 bp
SalI
NdeI
HindIII 2925
ORI
nmt1 promoter
LEU2
PstI 3699
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EcoRI 4900
KpnI 5200

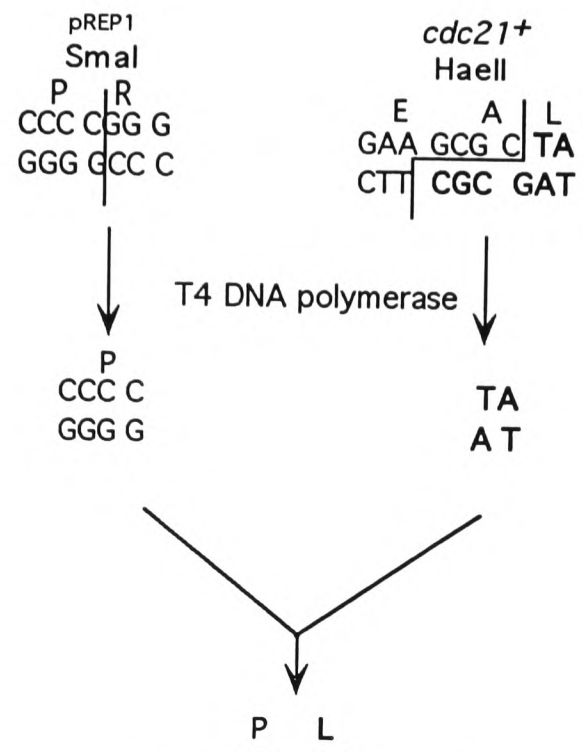

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NdeI SalI BamHI. SmaI.
XbaI 8226
SstI 24
XbaI 7810
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EcoRI 7600
ars1+
HindIII 1425
fl-ori
nmt1 promoter
pREP1
8700 bp
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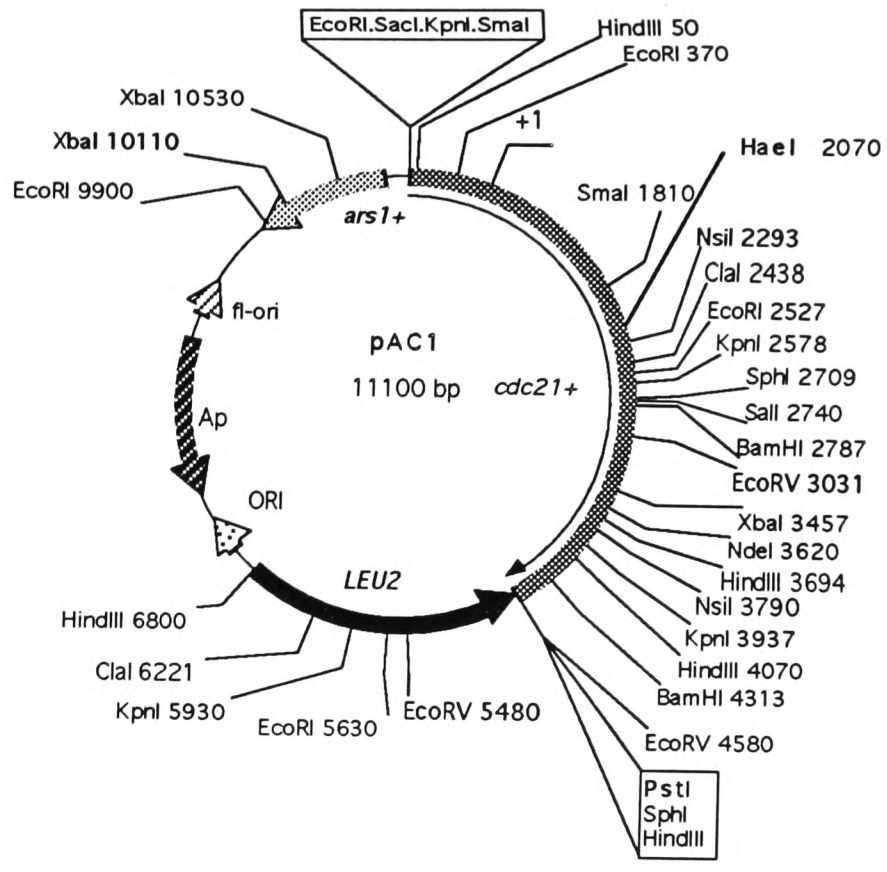

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GGG GCC C
P
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GGG G
cdc21+
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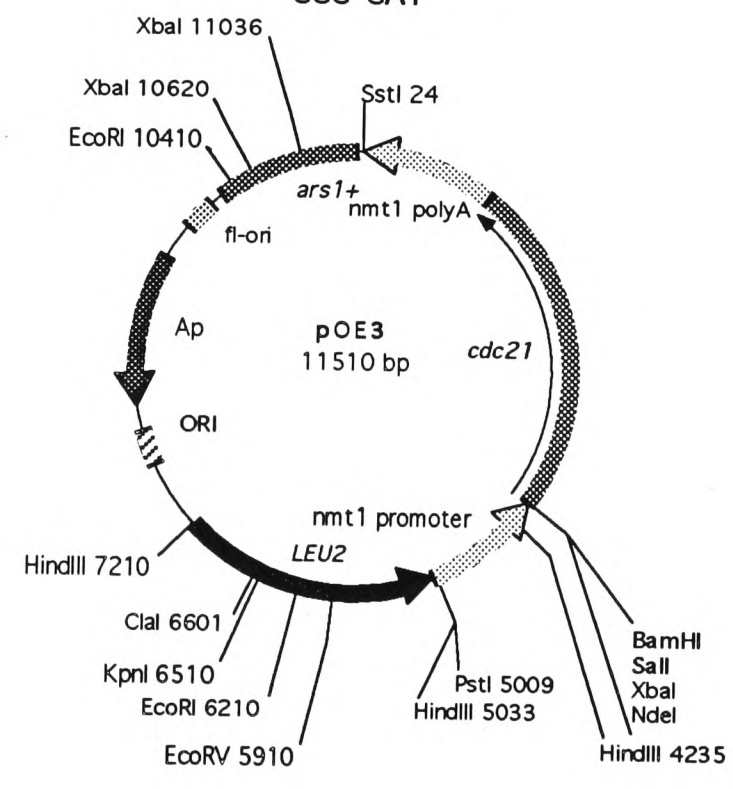

EcoRI.SacI.KpnI.SmaI
HindIII 50
EcoRI 370
XbaI 10530
XbaI 10110
+1
EcoRI 9900
ars1+
SmaI 1810
NsiI 2293
ClaI 2438
fl-ori
EcoRI 2527
KpnI 2578
pAC1
11100 bp
cdc21+
SphI 2709
SalI 2740
Ap
BamHI 2787
EcoRV 3031
XbaI 3457
ORI
NdeI 3620
HindIII 3694
LEU2
NsiI 3790
KpnI 3937
HindIII 6800
HindIII 4070
ClaI 6221
BamHI 4313
KpnI 5930
EcoRI 5630
EcoRV 5480
EcoRV 4580
PstI
SphI
HindIII


XbaI 9726
SstI 24
XbaI 9310
nmt1 polyA
EcoRI 9100
ars1+
fl-ori
pOE2
cdc21
Ap
10200 bp
BamHI
SalI
NdeI
HindIII 2925
ORI
nmt1 promoter
LEU2
PstI 3699
HindIII 3723
HindIII 5900
ClaI 5291
EcoRV 4600
EcoRI 4900
KpnI 5200


EcoRI 8651
M S T L E D P R
ATG TCG ACT CTA GAG GAT CCC CGG G
NdeI SalI BamHI. SmaI.
XbaI 8226
SstI 24
XbaI 7810
EcoRI 7600
nmt1 polyA
ars1+
HindIII 1425
fl-ori
nmt1 promoter
pREP1
8700 bp
PstI 2199
Ap
HindIII 2223
ORI
LEU2
EcoRV 3100
EcoRI 3400
KpnI 3700
ClaI 3791
HindIII 4400


pREP1
SmaI
P R
CCC CGG G
GGG GCC C
cdc21+
HaeII
E A L
GAA GCG C TA
CTT CGC GAT
T4 DNA polymerase
P
CCC C
GGG G
TA
AT
P L
nmt1 CCC CTA cdc21
GGG GAT


EcoRI.SacI.KpnI.SmaI
HindIII 50
EcoRI 370
XbaI 10530
XbaI 10110
+1
HaeI 2070
EcoRI 9900
SmaI 1810
ars1+
NsiI 2293
ClaI 2438
fl-ori
EcoRI 2527
pAC1
KpnI 2578
11100 bp
cdc21+
SphI 2709
SalI 2740
Ap
BamHI 2787
EcoRV 3031
ORI
XbaI 3457
NdeI 3620
LEU2
HindIII 3694
NsiI 3790
HindIII 6800
KpnI 3937
ClaI 6221
HindIII 4070
KpnI 5930
BamHI 4313
EcoRI 5630
EcoRV 5480
EcoRV 4580
PstI
SphI
HindIII


XbaI 11036
XbaI 10620
SstI 24
EcoRI 10410
ars1+
nmt1 polyA
fl-ori
Ap
pOE3
11510 bp
cdc21
ORI
nmt1 promoter
LEU2
HindIII 7210
ClaI 6601
KpnI 6510
PstI 5009
BamHI
SalI
XbaI
NdeI
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HindIII 4235

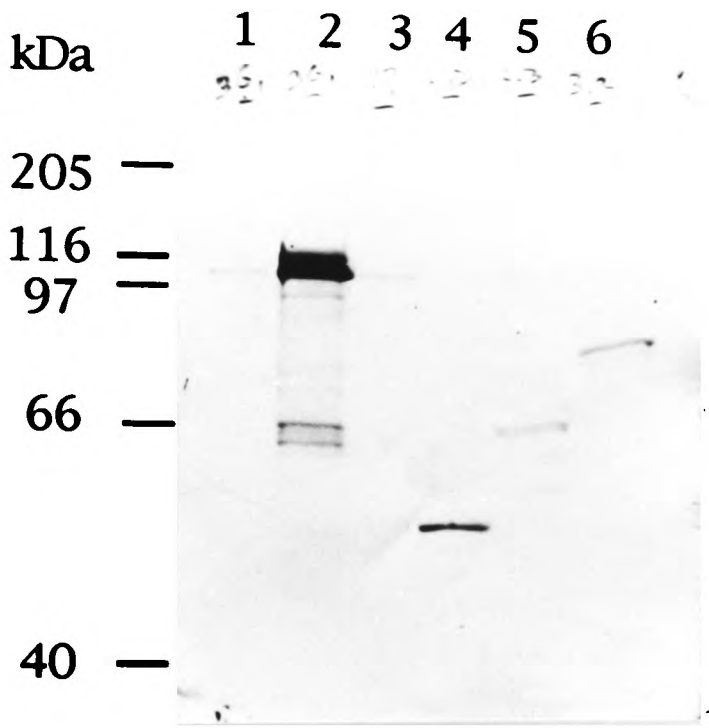
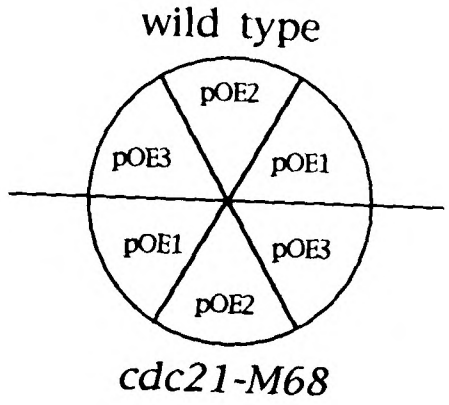


Figure 5.6

Detection of the expressed oe1-3 proteins by immunoblot.

S. pombe cells were grown to $1x10^7$ cells/ml in selective (leu^-) minimal medium without thiamine at 32°C. Protein extracts were made from cells carrying the pOE1-3 constructs (lanes 4-6), the pREP*3X::21*$^+$ expression vector (3X*nmt1::cdc21*$^+$; lane 2), the pREP3X vector (lane 1) or from cells overexpressing a carboxyl-terminal truncation of *cdc21*$^+$ (lane 3). Total proteins were fractionated by SDS-PAGE, blotted to a nitro-cellulose filter and the filter stained with a cdc21 polyclonal antibody raised against the carboxyl-terminal of the cdc21 protein.



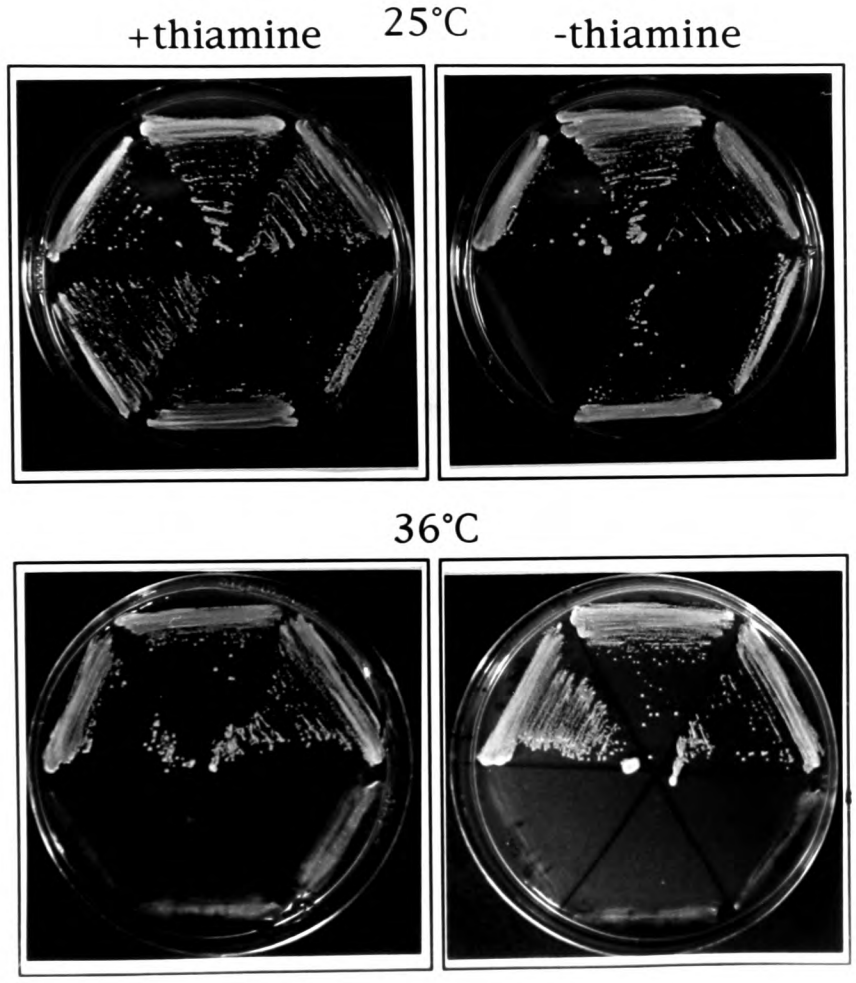
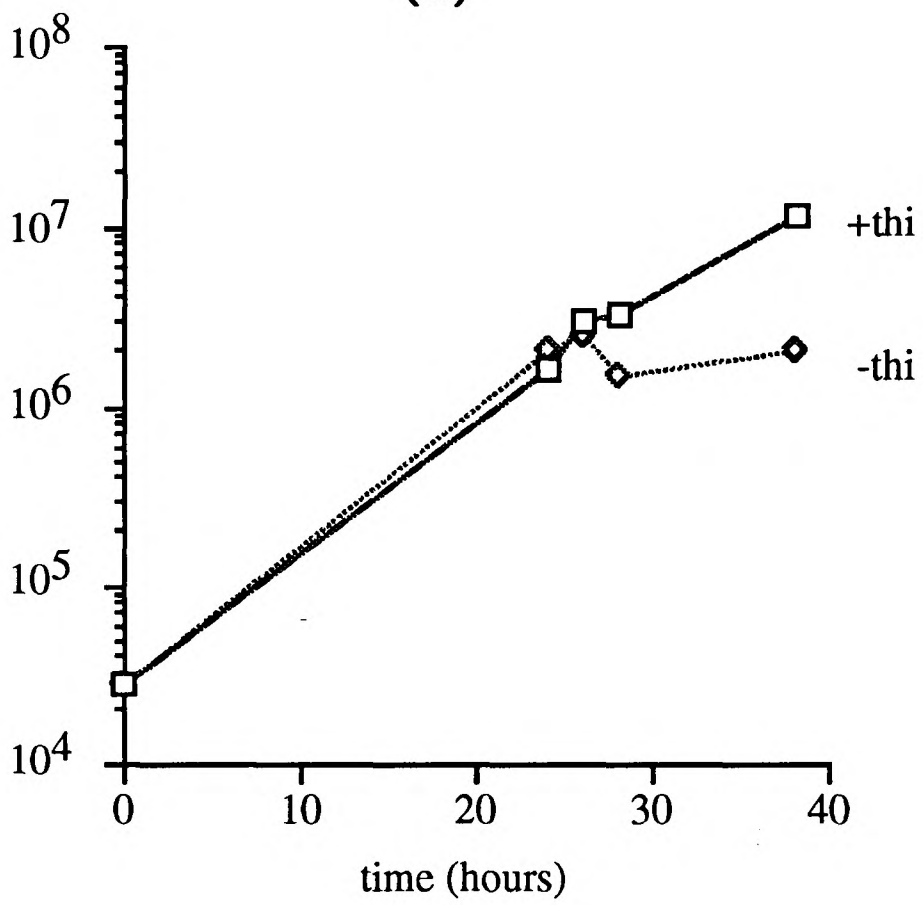


Figure 5.7

Overexpression of the *cdc21*$^{+}$ carboxyl-terminal causes lethality in *cdc21-M68* cells

pOE1-3 constructs were introduced in both wild-type and *cdc21-M68 S. pombe* cells and induced to overexpress the *cdc21* truncations by streaking them out on minimal selective (leu $^{-}$) plates in the absence of thiamine at 25 and 36°C. Cells were also streaked out on thiamine-containing plates as a control. Growth was scored after 5 days of incubation at the indicated temperatures.

(a)


10^8
10^7
10^6
10^5
10^4
+thi
-thi
0
10
20
30
40
time (hours)

(b)

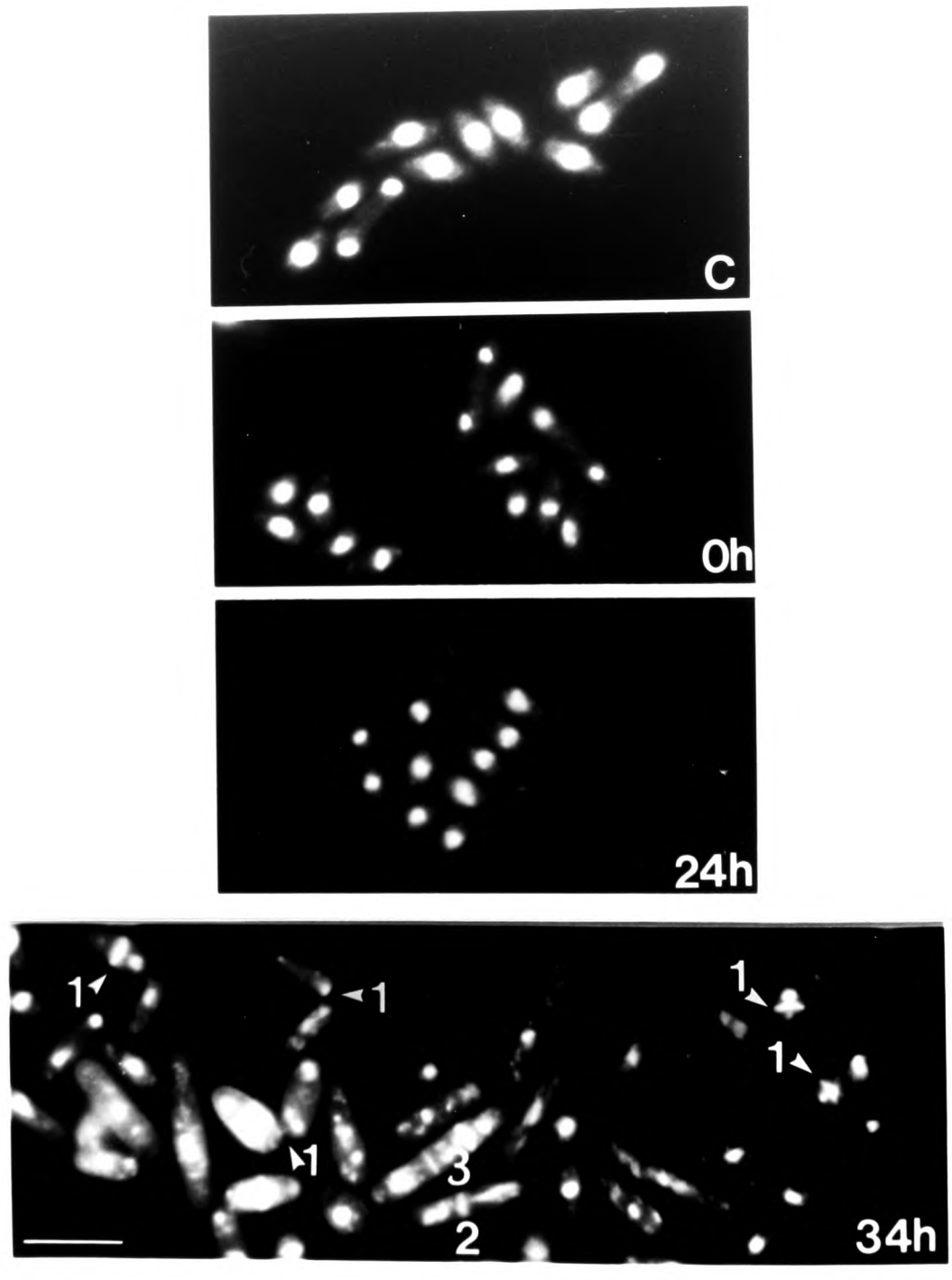

C
0h
24h
1
1
1
1
1
3
2
34h

Figure 5.8

Arrest phenotype of *cdc21-M68* cells overexpressing the *cdc21*$^+$ carboxyl-terminal.

(a)

Growth rate of *cdc21-M68* cells overexpressing the *cdc21*$^+$ carboxyl-terminal. Cells were grown in minimal medium lacking leucine to mid-log phase in the presence of 5μg/ml of thiamine, washed with thiamine free medium and resuspended in fresh medium with (oe1 +thi) or without thiamine (oe1 -thi). Cell number was determined for both cultures after 0-40 hours upon dilution as described in Chapter Two.

(b)

Nuclear morphology of *cdc21-M68* cells bearing the induced pREP vector (panel C) or the pOE1 vector before (panel 0h) and after 24-34 hours of thiamine deprivation (panel 24h and 34h) was monitored by DAPI staining as described in Chapter Two. Calcofluor was included to show septa (panel 34h). Arrowheads indicate aberrant mitoses. Numbers indicate the types of aberrant mitoses classified in Table 5.2. Bar indicates 10μm.

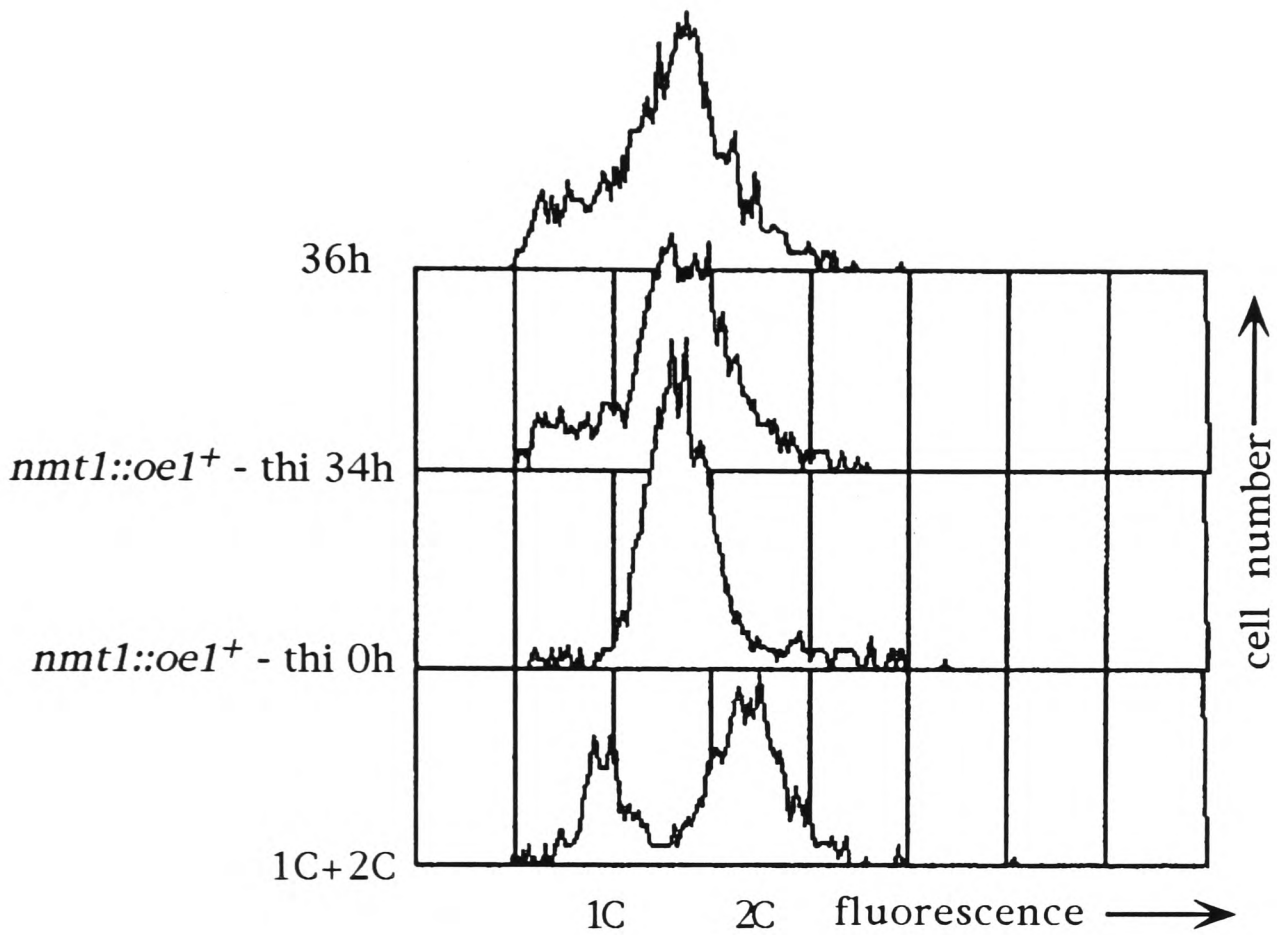


Figure 5.9

DNA content of *cdc21-M68* cells overexpressing the carboxy -terminus of *cdc21*+

The flow cytometry profiles of *cdc21-M68* cells bearing the pOE1 construct before (*nmt1::oe1*+ 0h) and after 34 and 36 hours of thiamine (thi) starvation (*nmt1::oe1* 34h and 36h) were fixed and processed as described in Chapter Two. *cdc21-M68* cells bearing the pREP1vector induced (*nmt1* on) and standards of DNA content (1C + 2C) were included as controls. The data were plotted in linear scale.

	Strain	Growth + thiamine	Growth - thiamine
P2	wild-type (30°C)	+	+
P1	*cdc21*^{ts} (25°C)	+	-
P155	*cdc23*^{ts} (25°C)	+	+
P156	*cdc24*^{ts} (25°C)	+	+
P139	*nda1*^{cs} (33°C)	+	+
P177	*nda4*^{cs} (33°C)	+	+
P176	*pol*δ^{ts} (25°C)	+	+

Table 5.1

Overexpression of the *cdc21*$^{+}$ carboxyl-terminal is not lethal in S phase *cdc* mutant cells

The pOE1 and the pREP*81X::cdc21*$^{+}$ constructs were introduced in a set of fission yeast *cdc* strains required for S phase execution. Growth was scored on selective (leu^{-}) plates after 5 days of incubation at the indicated temperatures, in the absence or in the presence of 5µg/ml of thiamine. cs= cold sensitive strains. ts= temperature-sensitive strains.

Phenotypes	Frequency (%) 24 hours	34 hours
normal cells	40	6
small cells	20	37
elongated, 1nucleus	40	32
elongated 2 nuclei	0	0
aberrant mitoses	0	25
Types of aberrant mitosis		
cut (1)		16
2 nuclei + septum (2)		7.7
2 nuclei + 2 septa (3)		1.3

Table 5.2

Summary of phenotypes of *cdc21*^{ts} cells after 24 and 34 hours of *oe1*$^{+}$ overexpression.

Phenotypes associated with the overexpression of the *oe1*$^{+}$ truncation in *cdc21-M68* cells at 25°C were scored by DAPI staining (see Chapter Two) after 24 and 34 hours of thiamine starvation in minimal medium lacking leucine. Aberrant mitoses were scored as presence of one or more septa in a single cell containing an undivided nucleus or of a septum bisecting an undivided nucleus ('cut' phenotype; Hirano *et al.*, 1986). Numbers in brackets refer to the numbers of Figure 5.8, panel 34h, illustrating the aberrant mitoses.

Chapter Six

Analysis of the cell cycle arrest of the *cdc21-M68* strain

Introduction

The *cdc21*$^+$ gene has been shown to be required for cell cycle progression and most probably for chromosomal DNA replication. The *cdc21-M68* mutant arrest as elongated cells at the restrictive temperature (36°C) indicating a defect in the progression of the cell cycle. Cells also have slow kinetics of DNA replication but they eventually replicate most of their DNA (Nasmyth and Nurse, 1981). Further analyses have shown that arrested cells have an interphase array of microtubules and do not show signs of chromatin condensation, suggestive of a block in late S or G2 phases (Coxon *et al.*, 1992). Determination of the DNA content of arrested cells by flow cytometry has confirmed that the DNA may be completely replicated (Coxon *et al.*, 1992). In order to explain the arrest phenotype of *cdc21* mutant cells, it has been proposed that the *cdc21* mutant may be defective in the replication of a small portion of the genome (Nasmyth and Nurse, 1981). It is then possible that chromosomes of *cdc21*^{ts}-arrested cells may be partially replicated or damaged in a way which is undetectable by flow cytometry. Thus I have used a more sensitive technique, namely pulsed field gel electrophoresis (PFGE), to determine possible abnormalities of chromosomes of *cdc21* arrested cells.

Moreover, if the arrest in interphase of *cdc21* cells is due to DNA damage or unreplicated DNA this presumably is mediated by the surveillance mechanism which prevents the onset of mitosis before completion of S phase (mitotic checkpoint; Hartwell and Weinert, 1989; see also Chapter One).

In fission yeast the *rad1*$^+$, *3*$^+$, *9*$^+$, *17*$^+$ and the *cdc2*$^+$ genes have been shown to be components of such a mechanism (see Chapter One). Mutants in these loci are deficient in the arrest caused by DNA damage (e.g.: UV irradiation) or by a DNA replication defect (such as a hydroxyurea block or mutations in enzymes required for DNA replication; Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992a; Enoch and Nurse, 1990). Removal of such a mechanism in *cdc21*^{ts} cells arrested at the restrictive temperature should allow mitotic events to occur in cells which are not prepared to do so, resulting in a aberrant mitosis and low cell viability.

It has been proposed that in fission yeast, the S phase arrest produced by a defect in DNA replication may be detected by a pathway distinct from that required for arrest caused by DNA damage. This possibility comes from the experimental observation that a semi-dominant mutant of the protein kinase *cdc2* , the *cdc2.3w* mutant, has an apparent normal arrest after UV irradiation whereas mutations in the *wee1* gene increase sensitivity to radiation (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992b; Sheldrick and Carr, 1993; see also Chapter One).

Thus in order to attempt to identify the nature of the possible DNA damage of *cdc21* arrested cells I have studied the arrest phenotype of double mutants of *cdc21 rad1* and *cdc21 cdc2.3w*.

Results

Analysis of chromosomes of *cdc21*^{ts}-arrested cells

To determine if chromosomes of *cdc21*^{ts}-arrested cells are damaged despite the apparent complete replication (Coxon *et al.*, 1992), these were analysed by PFGE (see Chapter Two). Damaged or partially unreplicated chromosomes are not resolved by PFGE (Hennessy *et al.*, 1991; Waseem *et al.*, 1992; Saka *et al.*, 1994).

As can be seen (Figure 6.1) chromosomes prepared from the *cdc21*^{ts} strain grown at the restrictive temperature (lane 4) are still able to enter the gel, indicating the absence of extensive DNA damage. In contrast, chromosomes prepared from a wild-type strain grown in the presence of the specific inhibitor of DNA replication hydroxyurea (HU; lane 2) are not resolved by PFGE.

Chromosomes prepared from a mutant in a gene related to *cdc21*$^{+}$, *nda1-108* (Toda *et al.*, 1983) were also analysed by PFGE. This mutant was first reported to arrest with a 1C content of DNA at the restrictive temperature, indicating a role in the onset of S phase (Miyake *et al.*, 1993). However conflictual results have been reported by Forsburg and Nurse, (1994). They have shown that the same mutant was transiently delayed in the G1 phase and finally arrested with a content of DNA very close to 2C.

As can be seen in Figure 6.1 (lane 11) chromosomes of the *nda1*^{cs} strain shifted to its restrictive temperature (20°C) could be resolved by PFGE, even though they were fainter if compared to the same strain grown at the permissive temperature. This observation indicates that chromosomes of *nda1*^{cs} arrested cells may not be extensively damaged or unreplicated.

These results thus suggest that chromosomes of *cdc21* arrested cells (or of the related *nda1-108* mutant cells) have no major damage comparable for instance to that seen for the budding yeast *cdc46-1* mutant (Hennessy *et al.*, 1991), consistent with previous studies (Coxon *et al.*, 1992; Forsburg and Nurse, 1994). However it is still possible that chromosomes are damaged in a way which in undetectable by PFGE (e.g. by nicks). One interpretation of this experiment is that *cdc21*$^{+}$ may not be absolutely required for DNA replication and it may have some role in the onset of mitosis. However this is not consistent with earlier genetic studies and the phenotype of *cdc21 rad1* double mutants (see next section).

Characterisation of the *cdc21 rad1* double mutant

Double mutants having both the *cdc21*^{ts} allele and *rad1, 3, 7, 9* or *17* mutations attempt mitosis, arresting as small cells, some of them with damaged DNA (S.Kearsey, pers. comm.). I have studied in detail the phenotype of the *cdc21 rad1* double mutant. The *rad1* strain cultured in the presence of hydroxyurea and the *cdc21*^{ts} mutant alone were included for comparison.

The kinetics of viability of single and double mutant cells incubated at the restrictive temperature were studied (see Chapter Two and legend to Figure 6.2 for experimental details). The *cdc21 rad1* double mutant lost rapidly viability at 36°C (Figure 6.2) in agreement with the findings of Ohi *et al.*, (1994). The viability of the *cdc21 rad1* double mutant was higher than that of the *rad1* strain incubated in the presence of hydroxyurea and correlates with the higher frequency of aberrant mitoses or 'cut' phenotypes (Hirano *et al.*, 1986) of the latter (Table 6.1).

Staining of nuclei of arrested double mutant cells with DAPI (see Chapter Two) revealed the presence of a high proportion of cells entering mitosis (Figure 6.4). These events led to the production of small cells some of them with a 'cut' phenotype. Intriguingly about 20% of the cells did not display a 'cut' phenotype but had a very disorganised chromatin not seen in the control cultures. *rad1* cells cultured in HU (panel E), displayed about 55% of aberrant mitoses under these experimental conditions- the frequency of 'cut' phenotypes being higher than that of *cdc21 rad1* double mutants (see Table 6.1). On the other hand, chromosomes of the double mutant *rad1 cdc21*^{ts} arrested at the restrictive temperature (Figure 6.1, lane 5) fail to enter the gel, in agreement with the lower viability and the arrest phenotype of the *cdc21*^{ts} *rad1* double mutant compared to that of *cdc21*^{ts} mutant itself.

These observations indicate that: a) *cdc21*^{ts} cells shifted to the restrictive temperature arrest in S phase; b) *cdc21*$^{+}$ is not required for mitosis. Cell cycle arrest of *cdc21*^{ts} cells mitotic arrest is mediated by the *rad* checkpoint, consistent with the presence of DNA damage or incomplete DNA synthesis.

Unlike the *cdc21 rad1* double mutant a double mutant of *cdc21* and *cdc2.3w* has the same viability as the *cdc21* mutant

In fission yeast some dominant mutants of *cdc2*, such as *cdc2.3w*, show premature mitosis (Fantes, 1981) and are insensitive to DNA damage caused by a DNA replication defect (Enoch and Nurse, 1990). Thus, if arrested *cdc21*^{ts} cells have a DNA replication defect a double mutant of *cdc21* and *cdc2.3w* is expected to behave as the *cdc21 rad* double mutants, that is, it should have low viability and arrest as small cells at the restrictive temperature.

I have performed a viability experiment, similar to that described in the previous paragraph, for the *cdc21 cdc2.3w* double mutant. The *cdc2.3w* and the *cdc21* single mutants were included as controls.

The viability of the *cdc21 cdc2.3w* double mutant was found to be similar to that of the *cdc21* mutant when incubated at 36°C (Figure 6.2). *cdc2.3w* cells were not affected by the temperature shift. The septation index was also monitored during the course of the experiment as indication of cell proliferation. In *cdc21 cdc2.3w* cells the septation index dropped after 4 hours but increased significantly after 5 and 6 hours, whereas that of *cdc21* dropped dramatically after 3 hours and remained low after 6 hours of incubation (Figure 6.3) indicating cell cycle arrest. As indicated by the viability data of Figure 6.3, the increase of the septation index seen in *cdc21 cdc2.3w* cells after 4 hours at 36°C apparently did not lower the viability of this strain.

The arrest phenotype of *cdc21 cdc2.3w* cells, examined by staining of nuclei with DAPI (Figure 6.4), was different from that of *cdc21 rad1*. Cells showed a very low frequency of 'cut' phenotypes although they were still arresting at a small size, but bigger than *cdc2.3w* alone. Moreover the nuclear morphology of these cells was very similar to that of wild-type cells.

These results then show that in *cdc21 cdc2.3w* arrested cells the mitotic restraint is lost but the phenotype is different to that obtained with *cdc2.3w* cells incubated in the presence of hydroxyurea.

Discussion

Earlier analyses of the arrest phenotype of the *cdc21*^{ts} mutant have suggested that cells may manage to replicate DNA, although at a slow rate (Nasmyth and Nurse, 1981; Coxon *et al.*, 1992). Nevertheless, cells do not enter mitosis nor attempt nuclear division and arrest as elongated cells in interphase (Coxon *et al.*, 1992). These phenotypes are consistent with a partial defect in DNA replication.

By PFGE fractionation of chromosomes of *cdc21*^{ts} arrested cells I have shown that there are no detectable defects in replication of specific chromosomes, even though the possibility cannot be ruled out that the DNA may still be damaged in a way which is undetectable by PFGE (e.g. nicks). Since the relative intensity of the bands corresponding to distinct chromosomes is similar to that of cells grown in permissive conditions and to that of wild type cells, this experiment also excludes the possibility that *cdc21*^{ts} cells may fail to replicate one chromosome, as previously suggested (Nasmyth and Nurse, 1981). Chromosomes of the *nda1-108* fission yeast mutant, related to *cdc21*$^{+}$ (Miyake *et al.*, 1993) arrested at the restrictive temperature, were also able to enter a pulsed field gel, suggesting that this mutant may cause a transient delay in the S phase in agreement with the recent results of Forsburg and Nurse (1994). On the other hand chromosomes of the *cdc18-K46* mutant, whose arrest phenotype is similar to that of *cdc21-M68* , have been shown not to be able to enter a pulsed field gel. A null mutant of *cdc18* fails to replicate its DNA (Kelly *et al.*, 1993).

If no DNA damage can be detected in *cdc21*^{ts} arrested cells, it is a possibility that the *cdc21*$^{+}$ gene may not be absolutely required for DNA replication and it may also perform some other role in exit from S phase. However, unlike a double mutant of *cdc25-22* and *rad1*, a double mutant of *cdc21* and *rad1* enters mitosis and rapidly loses viability at the restrictive temperature (Ohi *et al.*, 1994). Because *cdc25-22* blocks after DNA replication, these results demonstrate that *cdc21* cells arrest in S phase and that the *cdc21*$^{+}$ gene is not required for mitosis, consistent with earlier ·etic studies (Nasmyth and Nurse, 1981). Intriguingly the viability of the *cdc21* ·nt was higher than that of *rad1* and resembled that of *cdc2.3w* when cultured in hydroxyurea. The nuclear morphology of *cdc21 rad1* c peculiar, showing together with classical 'cut' phenotypes, disorgani about 20% of the cell population. This was seen for all the combination *rad* mutants tested (S.Kearsey, pers. comm.). This may suggest that the r exacerbates the defect caused by the *cdc21* mutation. It has been proposed t functions may have an additional role in S phase which is independent of the

checkpoint (Enoch *et al.*, 1991). If so, then the presence of a *rad* mutation in a *cdc21* background may compromise recovery from S phase. Consistent with these observations, and despite the modest frequency of 'cut' phenotypes scored in this strain, chromosomes of *cdc21 rad1* double mutant cells cultured in non-permissive conditions are severely damaged as assayed by PFGE. Another possibility is that this extensive DNA damage may be due to re-initiation of replication in the next S phase following the aberrant mitosis.

Surprisingly *cdc21* and *cdc2.3w* double mutant cells showed the same viability of *cdc21* mutant cells incubated in non-permissive conditions, in contrast to the combinations of *cdc2.3w* and *cdc* functions defective in DNA replication (e.g. DNA ligase) which show a fast drop in viability at the restrictive temperature (Enoch and Nurse, 1990). A small fraction of cells, significantly less than that of a *cdc21 rad1* double mutant, showed aberrant mitoses which did not affect cell viability. One explanation is that most of the septa seen in the *cdc21 cdc2.3w* double mutant miss the nucleus entirely, thus not causing apparent DNA damage. This has been proposed to explain why *cdc2.3w* mutant cells are more viable than *rad1* cells if cultured in hydroxyurea (see Table 6.1; Enoch *et al.*, 1992) and may also help to explain why the increase in septation index of *cdc21 cdc2.3w* cells at the restrictive temperature does not correlate with inviability. The nuclear morphology of this double mutant was comparable to that of wild-type cells, suggesting that the peculiar nuclear morphology of the *cdc21 rad1* double mutant is likely to be relevant to an additional defect imposed by the *rad* mutation.

Another phenotype of *cdc21 cdc2.3w* arrested cells is a delay of entry into mitosis if compared to *cdc2.3w* cells, where mitosis is premature (Fantes, 1981). This could be interpreted as mitotic delay caused by the *cdc21* mutation. A similar phenotype has been reported for the *hus3-12*, *hus3-26* and *hus4-16* mutants which are thought to be also component of the mitotic checkpoint in fission yeast (Enoch *et al.*, 1992). The above mentioned mutants were classified as 'late-cut' mutants in that they entered mitosis with delayed kinetics if compared to that of *cdc2.3w* cells in the presence of hydroxyurea. The transient delay in the onset of mitosis in *cdc21 cdc2.3w* cells at the restrictive temperature is difficult to interpret as the molecular mechanism by which the *cdc2.3w* mutation alters the timing of mitosis remains to be determined.

The results present a paradox, in that *cdc21*^{ts} cells incubated at the restrictive temperature have no detectable DNA damage but appear to arrest at the end of S phase. These data suggest three possible explanations. First, in *cdc21* arrested cells there may be DNA damage which is detected by the *rad* checkpoint and signalled to the mitotic apparatus via a pathway that is altered in the *cdc2.3w* mutant. It is not clear if this defect is the result of the lack of cdc21 protein or if it is the product of the leakiness of

the *cdc21*^{ts} allele. The second possibility is that there is no DNA damage and that the activation of the *rad* checkpoint is triggered by the slow kinetics of DNA replication. A third possibility is that *cdc21*$^{+}$ may have an additional function in S phase distinct from DNA replication, that is required to exit S phase. The *cdc21-M68* mutant may be defective in one of these functions or in both.

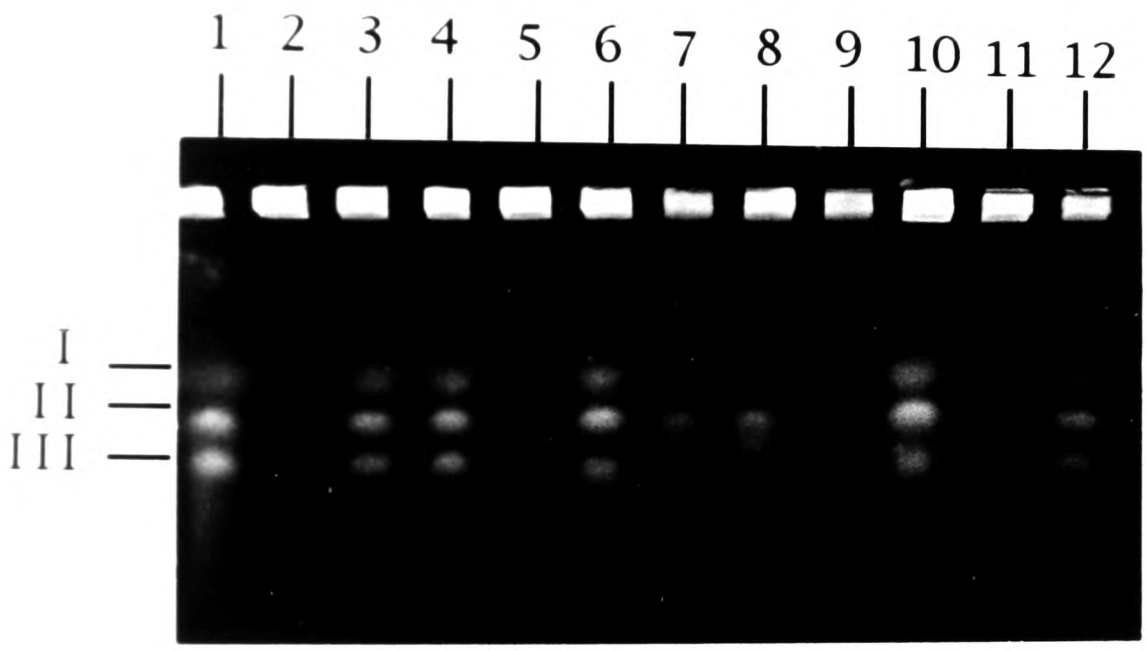


Figure 6.1

Pulsed Field Gel Electrophoresis of *S. pombe* chromosomes

Chromosomes of *S. pombe* wild-type and mutant cells included in agarose plugs were prepared and fractionated by PFGE as described in Chapter Two. Lane **1**: wild-type (P2); lane **2**: wild type cultured 3 hours in the presence of 10mM hydroxyurea; lane **3**: *cdc21*^{ts} (P1) at 25 °C; lane **4**: *cdc21*^{ts} 4 hours at 37°C; lane **5**: *cdc21 rad1* (P119) 4 hours at 37°C; lane **6**: *rad1* at 25°C; lane **7**: *rad1* (P73) 4 hours at 37°C; lane **8**: *cdc21 rad1* at 25°C; lane **9**: *rad1* cultured 3 hours in the presence of 10mM of hydroxyurea; lane **10**: *nda1*^{cs} (P139) at 33°C; lane **11**: *nda1*^{cs} shifted 4 hours at 20°C; lane **12**: wild type. **I, II,** and **III** indicate *S. pombe* chromosomes I, II and III respectively.

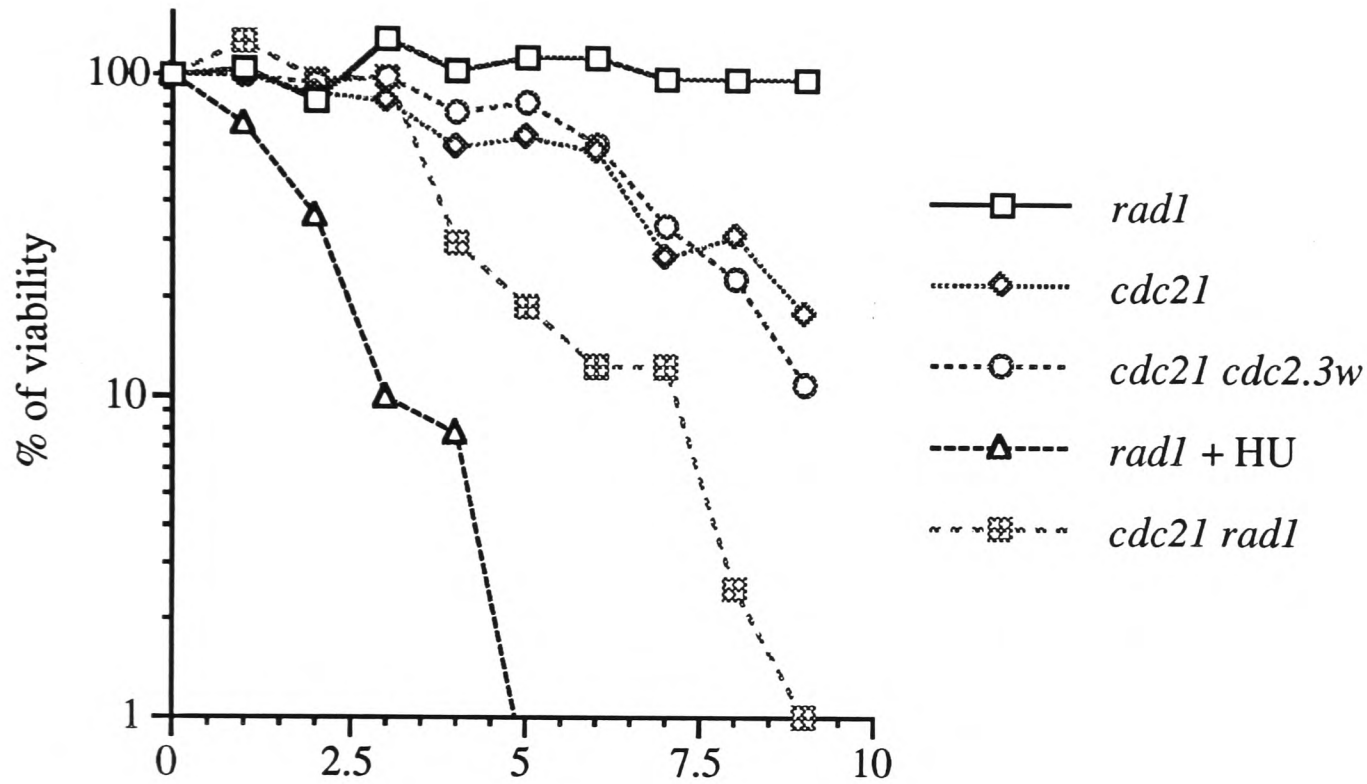


Figure 6.2

Viability of *cdc21-M68*, *cdc21-M68 rad1* and *cdc21-M68 cdc2.3w* mutant strains

The viability of *S. pombe rad1* (P73), *cdc21-M68* (P1), *cdc21-M68 cdc23.w* (P86) and *cdc21-M68 rad1* strains after shift at 36°C was determined as detailed in Chapter Two. Cells were grown to mid-log phase in rich liquid medium and appropriate dilutions were plated on rich plates containing the red dye phloxin B (YESP). For the hydroxyurea (HU) treatment, cells were spun down and resuspended in fresh medium in the presence or in the absence of 10mM HU. Samples were removed at one hour intervals, diluted, briefly sonicated and plated on YESP plates. Plates were incubated at 25/30°C and growth scored after 3-5 days of incubation. Each time point is the average of three independent counts.

Strain	Viability	Septation Index (as % after 4 hours at 36°C)	Cuts
cdc21-M68	59.7	0	0
cdc21-M68 cdc2.3w	77.1	9	1.3
cdc2.3w + HU*	38.0	15.6	29.3
cdc21-M68 rad1	30	31.4	26.8
rad1 +HU*	<1	55.2	58.4
rad1	103	0	0
cdc2.3w	100	0	0

Table 6.1

Correlation between viability, septation index and aberrant mitoses in *S. pombe* mutant cells

Summary of frequency of aberrant mitoses (cuts), septation index and the cell viability of the experiment of Figure 6.2 after 4 hours of incubation at 36°C (for the strains *cdc21-M68*, P1; *cdc2.3w*, P71; *cdc21 cdc2.3w*, *rad1*, P73; *cdc21 rad1*, P119) or 3 hours of incubation in 10mM* hydroxyurea (HU) (for the strains P71 and P73).

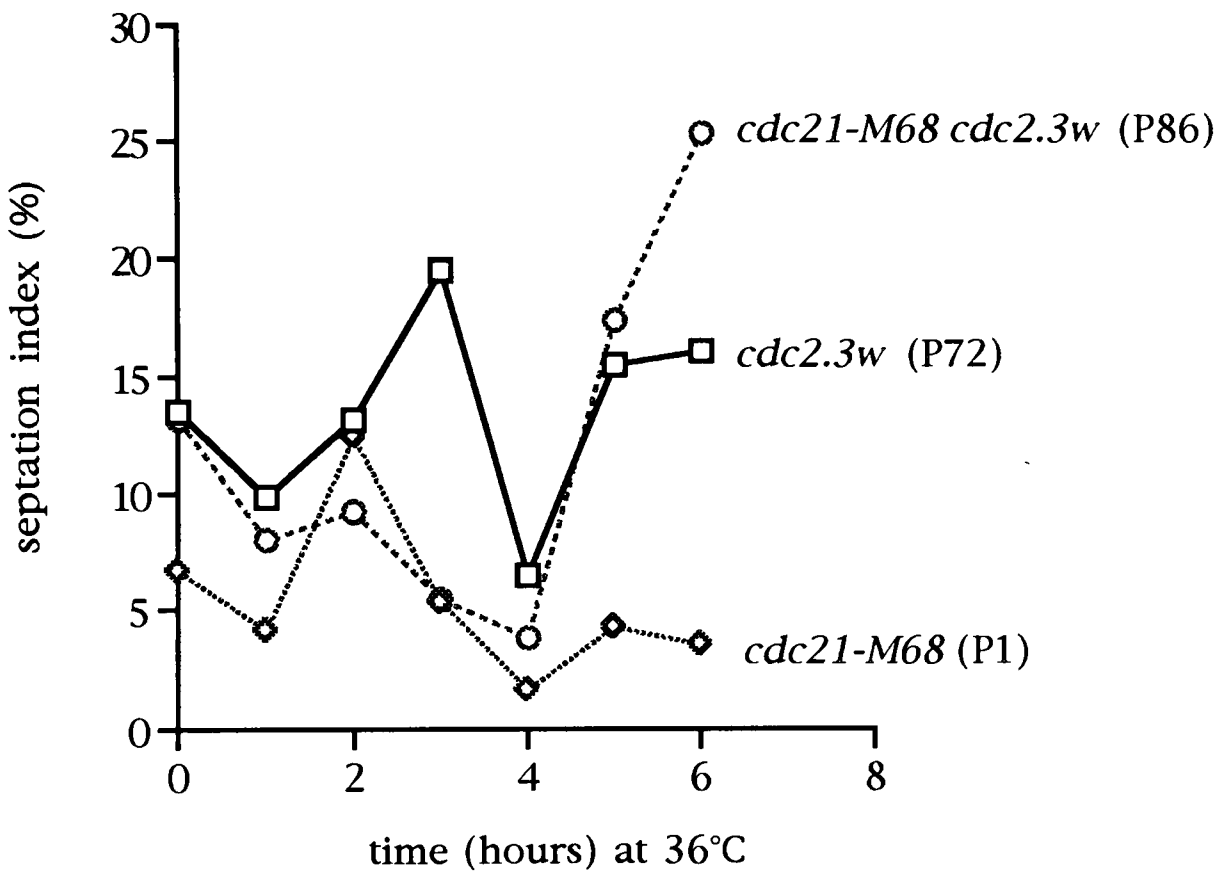


Figure 6.3

***cdc21-M68 cdc2.3w* double mutant cells have a delayed entry into mitosis**

The septation index of the *cdc21-M68 cdc2.3w* (P86), *cdc2.3w* (P72) and *cdc21* (P1) strains after shift at 36°C was determined as detailed in Chapter Two. About 500 cells per each time point were counted and the fraction of septated cells was calculated as percentage of the whole population. Septated cells in the process of cytokinesis were counted as two.

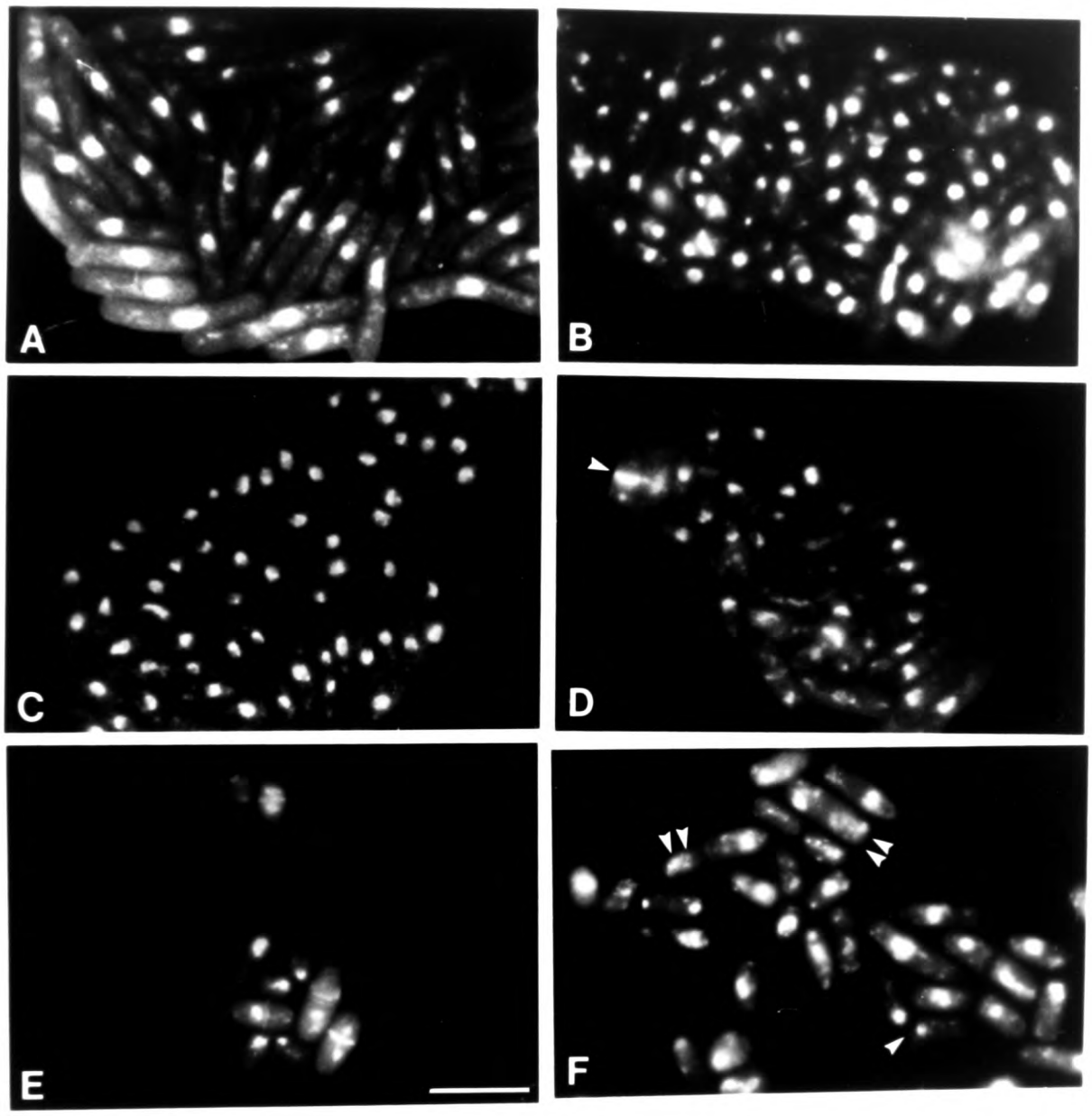


Figure 6.4

Nuclear morphology of *S. pombe* mutant cells

Cells of the experiment of Figure 6.2 were stained with DAPI and Calcofluor to show nuclei and septa respectively (see Chapter Two). Panel **A**: *cdc21*^{ts} (P1) 4 hours at 36°C; panel **B**: *cdc2.3w* (P72) cultured 3 hours in 10mM hydroxyurea; panel **C**: *cdc21 cdc2.3w* (P86) after 4 hours at 36°C; panel **D**: *cdc21 cdc2.3w* after 6 hours at 36°C; panel **E**: *rad1* (P73) cultured 4 hours in 10mM hydroxyurea; panel **F**: *cdc21 rad1* after 4 hours at 36°C. Single arrowheads show aberrant mitoses. Double arrowheads show cells with disorganised chromatin. Scale bar, 10μm.

Chapter Seven

Effects of a *cdc21* null allele on DNA replication and cell cycle arrest

Introduction

As previously shown *cdc21-M68* mutant cells synchronised in the G2 phase of the cell cycle and released in non-permissive conditions (36°C) enter S phase and replicate most of their DNA, but with a reduction in the rate of DNA synthesis in the last third of S phase (Nasmyth and Nurse, 1981).

Moreover, *cdc21-M68* cells recover from a hydroxyurea block when released at the restrictive temperature, although at a slower rate compared to wild-type cells, and arrest in the next cell cycle. These experiments have suggested that the *cdc21*$^{+}$ gene is required before (or independent of) the hydroxyurea sensitive step, i.e. at the initiation of DNA replication.

The DNA replication defect of the *cdc21*^{ts} mutant is compatible with failure to initiate DNA synthesis at a subset of replication origins (Nasmyth and Nurse, 1981). This hypothesis is consistent with the apparent ARS-specific defect in DNA replication of *cdc21* and mutants in members of the *cdc21*$^{+}$ gene family (Coxon *et al.* 1992; Maine *et al.*, 1984).

Mutant alleles of *cdc21*$^{+}$-related genes have similar phenotypes (see Chapter One, Table 1.1). Conditional-lethal/null mutants in the budding yeast *mcm2-3-5* and fission yeast related genes arrest at the end of S phase with almost completely replicated DNA (Gibson *et al.*, 1990; Yan *et al.*, 1991; Miyake *et al.*, 1993; Forsburg and Nurse, 1994). The *cdc19/nda1* null mutant completes most of the S phase but with a reduced rate compared to wild-type cells (Forsburg and Nurse, 1994). The fission yeast *mis5-268* mutant executes an abnormal S phase at the restrictive temperature (36°C; Takahashi *et al.*, 1994). Nonetheless, the Mcm2 and Mcm3 proteins have been recently shown to be required for the initiation of DNA replication at replication origins *in vivo* (Yan *et al.*, 1993).

The budding yeast *cdc46-1* mutant is the only allele in the *S. cerevisiae MCM2-3-5* gene family that arrests with unreplicated DNA. However a less severe mutant of *cdc46*, *cdc46-5*, is not completely blocked in DNA replication (Hennessy *et al.*, 1991), suggesting that the study of a single mutant allele may be misleading. A *cdc47*^{ts} allele alone has almost no inhibition on DNA synthesis (Moir *et al.*, 1982), however its arrest

in S phase is improved in a *cdc45*^{cs} mutant background. The *cdc45* mutant arrests in the G1 phase and genetically interacts with *cdc46-1* and *cdc47-1* mutants (Hennessy *et al.*, 1991). It is not known whether the *CDC45* gene product is related to the Mcm protein family.

Thus if the *cdc21*$^{+}$ gene is required for chromosomal DNA replication, the *cdc21*^{ts} mutant might be partially defective in this process at the restrictive temperature, i.e. it may be a leaky allele. The other possible conclusion is that the *cdc21*$^{+}$ gene, as with the related *cdc19*$^{+}$/*nda1*$^{+}$ gene (Miyake *et al.*, 1993; Forsburg and Nurse, 1994) may not be absolutely required for DNA replication, suggesting a partially redundant role of *cdc21*$^{+}$-related genes in this cellular process.

In order to shed light on the role of the *cdc21*$^{+}$ gene in DNA replication I have generated new alleles of *cdc21*.

Results

Cdc21 is essential for cell viability.

A null mutant of *cdc21* was constructed by disrupting one copy of the *cdc21*$^+$ gene in a diploid strain of *S. pombe* . For this purpose the *Nsi*I 1.5 kb internal fragment of the *cdc21*$^+$ gene was replaced *in vitro* with the *S. pombe ura4*$^+$ gene resulting in the plasmid pKOXX (see legend to Figure 7.1). The *Sac*I-*Pst*I linear DNA fragment containing the *cdc21*$^+$ gene interrupted by the *ura4*$^+$ gene was used to transform an *S. pombe* diploid strain to uracil prototrophy (see legend to Figure 7.2). Stable Ura$^+$ transformants were isolated and the integration of the *cdc21::ura4*$^+$ construct at one *cdc21*$^+$ locus was verified by Southern blotting. The total DNA, extracted from wild-type and from four *cdc21::ura4*$^+$ putative disruptants strains, was probed with the *cdc21*$^+$ gene labelled with [α-^{32}P]dCTP (Figure 7.2, *cdc21*$^+$ panel). Three out of four integrants (Δ1, Δ3 and Δ4) showed a hybridisation pattern consistent with a site-specific integration event of the targeting construct at the *cdc21*$^+$ locus (see legend to Figure 7.2). Another integrant (Δ2) seemed to have integrated randomly in the genome and was not further investigated. To confirm further that the *cdc21*$^+$ locus was disrupted by *ura4*$^+$, the same filter was probed with the *ura4*$^+$ gene, labelled as described above, after the *cdc21*$^+$ probe had been removed. As can be seen (Figure 7.2, *ura4*$^+$ panel) the *ura4*$^+$ probe hybridised only to the uppermost band in all the integrants, whereas it did not hybridise to the DNA of the wild-type strain completely delete for *ura4*$^+$, as predicted.

In order to show that the disruption of the *cdc21*$^+$ gene is essential for cell growth and to analyse the terminal phenotype of the arrested cells, two independent *cdc21* disruptant diploid strains were induced to sporulate and the products of meiosis were dissected (see Chapter Two). Thirty-one tetrads were analysed (the statistics of such an experiment are shown in Table 7.1). Most of the tetrads gave 2 viable spores and they were always ura$^-$ as expected, indicating that the *cdc21::ura4*$^+$ disruption was lethal. An example of dissected spores is shown in Figure 7.3, panel A. The phenotype of the arrested cells (shown in Figure 7.3, panels B and C) is one of elongated cells, some of them (about 10%) going through mitosis and cytokinesis and then arresting as elongated or small cells.

This experiment demonstrates that the *cdc21*$^+$ gene is essential for cell viability and that spores disrupted for *cdc21*$^+$ produce elongated cells, similar to the phenotype of the original temperature-sensitive strain, indicating a cell cycle arrest.

Spores of the *cdc21* disrupted strain show aberrant onset of mitosis and are deficient in the onset of DNA replication

Spores from one *cdc21* disruptant strain were prepared and germinated on a large scale as described in Chapter Two. Spores prepared from a wild-type diploid strain heterozygous for the *ura4-D18* mutation (P136) were included as a control for germination. In fact only 50% of the spores of both strains can germinate in minimal medium lacking uracil (Figure 7.4). These spores were inoculated in minimal medium lacking uracil and enriched with leucine, and germinated at 29°C. Samples were taken in a time course experiment to be analysed for DNA content, cell number and nuclear morphology.

Spores of the strain heterozygous for the *ura4-D18* mutation (wild-type spores) started to germinate after 9-11 hours as judged by the appearance of a germination tube (Figure 7.5). Cells entered mitosis after 13-14 hours producing two identical daughter cells containing a single nucleus (panel A); cell number increased accordingly (Figure 7.6). Spores disrupted for the *cdc21*$^+$ gene (panels B-E) also germinated but they produced elongated cells with a single nucleus. Additionally about 15-20% of the germinated spores underwent mitosis in the absence of nuclear division (aberrant mitosis) at the same time as wild-type spores (Figure 7.5, arrowheads). Septa were observed in both elongated and non-elongated cells. Septa were either bisecting (panel D) or entirely missing the nucleus (panel C). The frequency of aberrant mitoses is reported in Table 7.2. Germinating spores which did elongate showed an abnormal nuclear morphology and entered mitosis after 16-18 hours of germination.

These results show that *cdc21*$^+$ is required for cell cycle progression although a small proportion of spores disrupted for the *cdc21*$^+$ gene enter mitosis in the absence of nuclear division.

During the same experiment the DNA content of germinating spores was analysed by flow cytometry (Shazer and Sherwood, 1990). The results are shown in Figure 7.7. Four hours after germination, both wild-type and *cdc21*-disrupted spores had a 1C DNA content as judged by comparison with 1C+2C fluorescence standards. Wild-type spores began to replicate their DNA after 10 hours of incubation as judged by the appearance of a clear G2+M (2C) peak of fluorescence. In contrast *cdc21*-disrupted spores did not show any signs of DNA replication even after 16 hours of germination. No 1C peak could be detected 2 hours before germination, thus demonstrating that ungerminated spores do not contribute to the fluorescence of the 1C peak.

These results taken together strongly indicate that germinating spores lacking the *cdc21*$^+$ gene fail to replicate the DNA and also can show aberrant onset of mitosis.

Construction of the *cdc21* "shut off" mutant.

To determine if the phenotypes of the *cdc21::ura4*$^+$ disruption seen in germinating spores were also present in mitotically cycling cells, I have constructed a new conditional allele of the *cdc21* gene (*cdc21* "shut off" strain). In this strain, the *cdc21*$^+$ gene, placed under control of the *nmt1* thiamine regulatable promoter on a plasmid (Maundrell, 1990), complements the chromosomal *cdc21::ura4*$^+$ disruption (see Figure 7.8b).

The pREP*3X::21*$^+$ vector, which contains the *cdc21*$^+$ open reading frame under control of a strong version of the *nmt1* promoter (3X; G.Blom, unpublished) was found able to rescue the *cdc21* null mutant also in conditions of repression (G.Blom, S.Kearsey and D.Maiorano, unpublished obs.), indicating that this promoter cannot shut off completely. For this reason the weakest version of the *nmt1* promoters series, the 81X*nmt1* promoter was used (Basi *et al.*, 1993).

The 81X*nmt1*::*cdc21*$^+$ gene fusion was generated by replacing the 3X*nmt1* promoter of the pREP*3X::21*$^+$ plasmid with the 81X*nmt1* promoter of the pREP81X vector as described in the legend of Figure 7.8a. The recombinant plasmid *81X::21*$^+$ was used in all the experiments described below.

The 81X::21$^+$ *gene fusion complements the lethality of the cdc21-M68 mutant allele both in the presence or in the absence of thiamine*

The *81X::21*$^+$ plasmid was transformed into the *cdc21-M68* strain (see Chapter Two) and tested for its ability to complement the *cdc21*^{ts} mutation both in the presence and in the absence of thiamine under non-permissive conditions. The pREP81X vector alone was included as a control. Transformants, isolated in the presence of thiamine (promoter off) were streaked out at the restrictive temperature (36°C) in the presence and in the absence of thiamine. As can be seen in Figure 7.9 cells containing the *81X::21*$^+$ construct formed colonies both in the presence and in the absence of thiamine at non-permissive conditions while no colonies were scored if cells contained the vector alone. This result indicates that the 81X*nmt1* promoter also has a basal level of transcription in conditions of repression. This experiment demonstrates that the *81X::21*$^+$ vector indeed expresses the correct *cdc21*$^+$ message.

Isolation of the cdc21 "shut off" mutant strain

Although basal transcription of the *81X::21*$^+$ gene fusion can still complement the lethality of the *cdc21*^{ts} strain (see previous paragraph), this might not be sufficient to rescue a null mutant of *cdc21*, particularly as there are indications that the *cdc21-M68* allele may be a leaky allele (see previous section). To test this possibility, the *81X::21*$^+$ construct was transformed into the *cdc21*$^+$/*cdc21::ura4*$^+$ diploid strain using the LiCl procedure (see Chapter Two). The pAC1 plasmid, which contains *cdc21*$^+$ under control of its own promoter, was transformed into the same strain as a control. Two independent diploid transformants were sporulated in the absence of thiamine and a spore suspension was prepared (see Chapter Two). Spores were plated out on selective plates and the *cdc21* "shut off" mutant isolated (Figure 7.8b).

This strain was challenged to grow when the expression of the *cdc21*$^+$ gene was shut off by the addition of thiamine. Haploid *cdc21* disrupted cells kept viable by the presence of the pAC1 plasmid were included as a control. The *cdc21* "shut off" strain was able to form colonies on thiamine-free plates whereas no growth was scored on plates containing thiamine (Figure 7.9). Cells disrupted of the *cdc21*$^+$ gene containing the pAC1 plasmid were able to grow in both conditions. Thus, shut off of *cdc21*$^+$ gene expression from the 81X*nmt1* promoter was sufficient to arrest the growth of cells in a *cdc21*-disruption background.

Effects of *cdc21*$^+$ "shut off" on DNA replication

Cell number and kinetics of DNA replication of the *cdc21* "shut off" strain grown in the presence and in the absence of thiamine was monitored in liquid culture. Cells were grown to mid-log phase in complete minimal medium lacking thiamine (*cdc21*$^+$ on) and then divided into two separate cultures. In the first one thiamine was added (+thi) whereas no thiamine was added in the other (-thi). The two cultures were incubated at 32°C and cell number was monitored. The cell number in the culture (+thi) increased at the same rate as for the (-thi) culture (1 duplication every 4 hours) during the first nine hours after addition of thiamine. However, after 9 hours the generation time of cells in the (+thi) culture increased 2-fold and then ceased (Figure 7.10). Cells in the control culture (-thi) continued to grow normally. Thus it took about three generations for the *cdc21* "shut off" mutant to arrest.

The DNA content of these cells was analysed by flow cytometry. The result of this experiment is shown in Figure 7.11a. At the time of addition of thiamine exponentially growing cells showed a sharp 2C peak of fluorescence indicating that they were in the G2/M phases (time point 0). Nine hours after the addition of thiamine, the 2C peak

widened towards the 1C position, indicating a cell cycle delay. Later in the time course the widening increased and cells finally accumulated with a DNA content between 1C and 2C.

The nuclear morphology of cells sampled from the (-thi) and (+thi) cultures was analysed by DAPI staining, also including Calcofluor to show septa. At the time of thiamine addition, most of the cells were normal, although modestly elongated cells could also be seen (Figure 7.11b). After 9-15 hours of addition of thiamine, elongated cells containing an undivided nucleus and cells containing septa were seen. These and other phenotypes described below were prominent after 17-21 hours.

Three distinct phenotypes could be observed (Figure 7.11b), namely: 1) elongated cells with one nucleus, similar to *cdc21-M68* arrested cells (Coxon *et al.*, 1992); 2) elongated and small cells with a classical 'cut' phenotype (Hirano *et al.*, 1986) and 3) elongated cells with two nuclei and more than one septum, similar to those of *cdt1* "shut off" mutant arrested cells (Hofmann and Beach, 1994). Small cells were also observed, most probably products of the aberrant mitoses (see Chapter 4). The frequency of these phenotypes is reported in Table 7.3. It was often difficult to assign a specific phenotype to the arrested cells due to the presence of extensive cytoplasmic staining and to the absence of well defined nuclei. Over-replication of mitochondrial DNA, often seen in arrested *cdc* mutants, may have contributed to the cytoplasmic staining (Sazer and Sherwood, 1990).

Hence, mitotically cycling cells, as germinating spores, lacking the *cdc21*$^+$ gene are deficient in DNA replication but do not always arrest in interphase.

Discussion

To obtain evidence that the *cdc21*$^+$ gene is required for DNA replication I have generated new alleles of *cdc21*.

A null allele was constructed by disrupting one copy of the *cdc21*$^+$ gene in an *S. pombe* diploid strain. The products of meiosis of such a strain indicated that *cdc21*$^+$ is dispensable for viability, as expected. By making a null allele of *cdc21* I have shown that the *cdc21*$^+$ gene is indeed required for chromosome replication. Cells with no *cdc21*$^+$ function failed to replicate their DNA and they did elongate indicating cell cycle arrest. Surprisingly, a small proportion of cells were able to escape such an arrest as they went through mitosis in the absence of DNA replication and nuclear division. This may indicate that the absence of the *cdc21*$^+$ gene may also interfere with the control mechanism which mediates inhibition of mitosis in cells which have not executed S phase (mitotic checkpoint; Hartwell and Weinert, 1989; see also Chapter One). These phenotypes are similar to those of three fission yeast mutants, *cdc18* (Kelly *et al.*, 1993); *cut5/rad4* (Saka and Yanagida, 1993) and *cdt1* (Hofmann and Beach, 1994)

required for DNA replication and restraint of mitosis until completion of S phase (see Nurse, 1994, for review). Perhaps the *cdc21*$^+$ gene may play similar roles in S phase.

Similar phenotypes were also seen in the *cdc21* 'shut off' mutant strain, in which a chromosomal disruption of the *cdc21*$^+$ gene is complemented by a wild-type copy of *cdc21*$^+$ under control of the *nmt1* regulatable promoter on a plasmid. Such a strain arrested three generations after repression of the *cdc21*$^+$ expression from the *nmt1* promoter, with a marked increase in the generation time during last division (10 hour versus 4). Thus, this represents a new conditional-lethal allele of *cdc21*. Given that the *nmt1* mRNA is undetectable within three hours after addition of thiamine (Maundrell, 1990) this suggests that three generations are necessary to dilute out the cdc21 protein, which may reflect its cellular abundance and/or turnover. The possibility cannot be ruled out that the mRNA produced by the *nmt1*::*cdc21*$^+$ gene fusion may be more stable than that of the *nmt1*$^+$ gene alone. Similar kinetics of arrest have been reported for the budding yeast *mcm2* and *mcm3* "shut off" mutants (Gibson *et al.*, 1990; Yan *et al.*, 1991).

These observations, taken together demonstrate that the phenotype of a null allele of *cdc21* is not the same as that of the *cdc21-M68* allele.

The apparent failure of cells lacking the *cdc21*$^+$ gene to activate the mitotic checkpoint may be explained as i) failure to assemble replication complexes, as proposed for *cdc18*, *cut5/rad4* and *cdt1* mutants of fission yeast (Li and Deshaies, 1993) ii) a second role of the *cdc21*$^+$ gene in the restraint of mitosis until completion of S phase. However, the frequency of aberrant mitoses in *cdc21* mutant cells is about 3-fold lower than that seen in *cdc18*, *cut5/rad4* or *cdt1* mutants, suggesting that perhaps a role of the *cdc21*$^+$ gene in the mitotic checkpoint is unlikely. Such a role has never been reported for members of the *cdc21*$^+$ gene family.

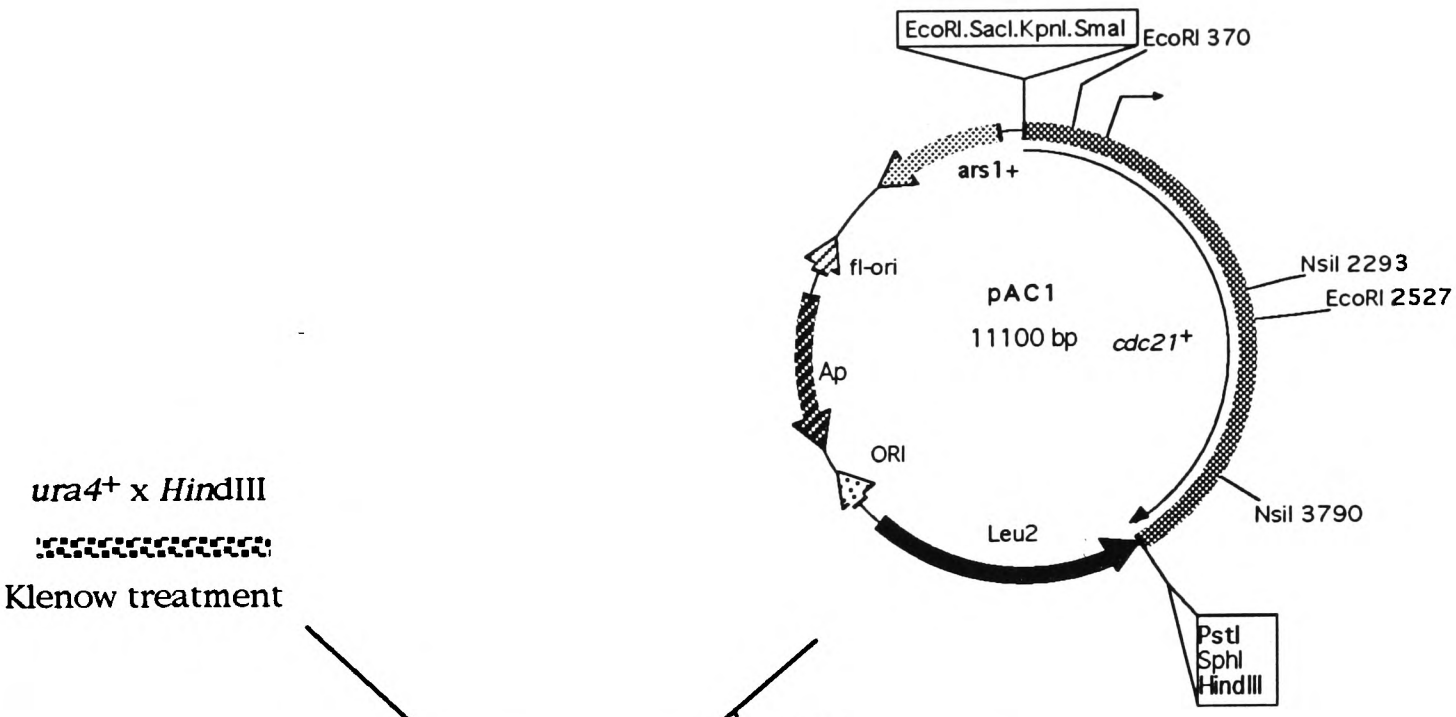
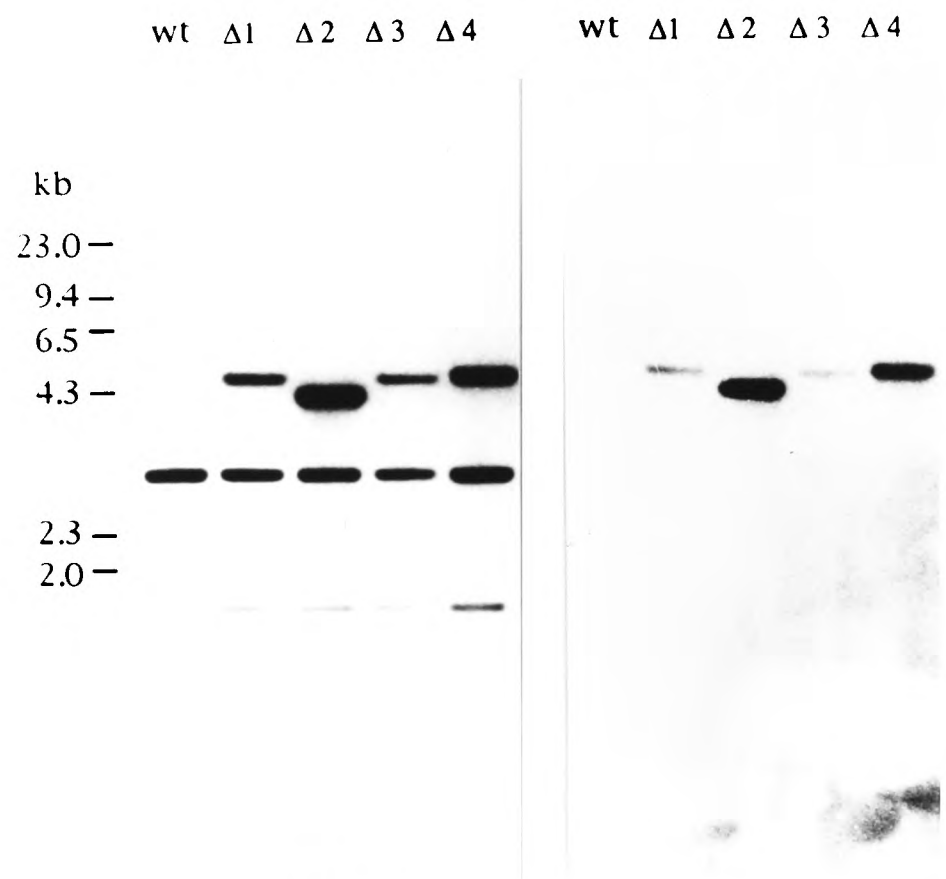

EcoRI.SacI.KpnI.SmaI
EcoRI 370
ars1+
fl-ori
pAC1
11100 bp
cdc21+
Ap
ORI
Leu2
NsiI 2293
EcoRI 2527
NsiI 3790
PstI
SphI
HindIII
ura4+ x HindIII
Klenow treatment
NsiI
T4 DNA polymerase
Ligate to
pAC1ΔNsiI
NsiI
NsiI
EcoRI.SacI.KpnI.SmaI
EcoRI 370
ars1+
cdc21
NsiI 2330
pKOXX
11400 bp
Ap
ura4+
NsiI 3130
ORI
Leu2
HindIII.SphI.PstI
digest SacI-PstI
EcoRI 370
SacI
PstI
ura4+
M1
K287
Transform S.pombe ura-/ ura- diploid

Figure 7.1

Strategy used to generate a *cdc21* gene disruption *in vitro*.

A 1.5 kb internal deletion of the *cdc21*$^+$ gene was generated by digestion of the pAC1 plasmid, which contains the *Bgl*II-*Pst*I genomic clone of *cdc21*$^+$ (A. Coxon, D.Phil. thesis) with *Nsi*I. The 3' protruding ends generated by the *Nsi*I digestion were recessed with the bacteriophage T4 DNA polymerase enzyme as described (Sambrook *et al.*, 1991). The full-length *ura4*$^+$ gene of fission yeast, isolated from the pIRT2U plasmid (see Chapter Two) as the 1.8 kb *Hin*dIII DNA fragment, was ligated into the *Nsi*I deletion of pAC1 after the *Hin*dIII 5' protruding ends were filled-in with the large subunit of the *E. coli* DNA polymerase enzyme (Klenow fragment). The resulting plasmid pKOXX was then digested with *Sac*I and *Pst*I and the 5.1 kb DNA fragment, containing the *cdc21* gene interrupted by the *ura4*$^+$ gene, was isolated. This DNA fragment was used to disrupt one copy of the *cdc21*$^+$ gene in an *S. pombe* wild-type diploid strain (see Chapter Two for details). The boxed recognition sites for restriction enzymes represent sequences of the polylinker of the backbone plasmid (pIRT2; Hindley *et al.*, 1987). The start of the *cdc21*$^+$ open reading frame is indicate by a small arrow on the *cdc21*$^+$ open reading frame.

(a)


wt Δ1 Δ2 Δ3 Δ4
wt Δ1 Δ2 Δ3 Δ4
kb
23.0
9.4
6.5
4.3
2.3
2.0
cdc21+
ura4+

(b)

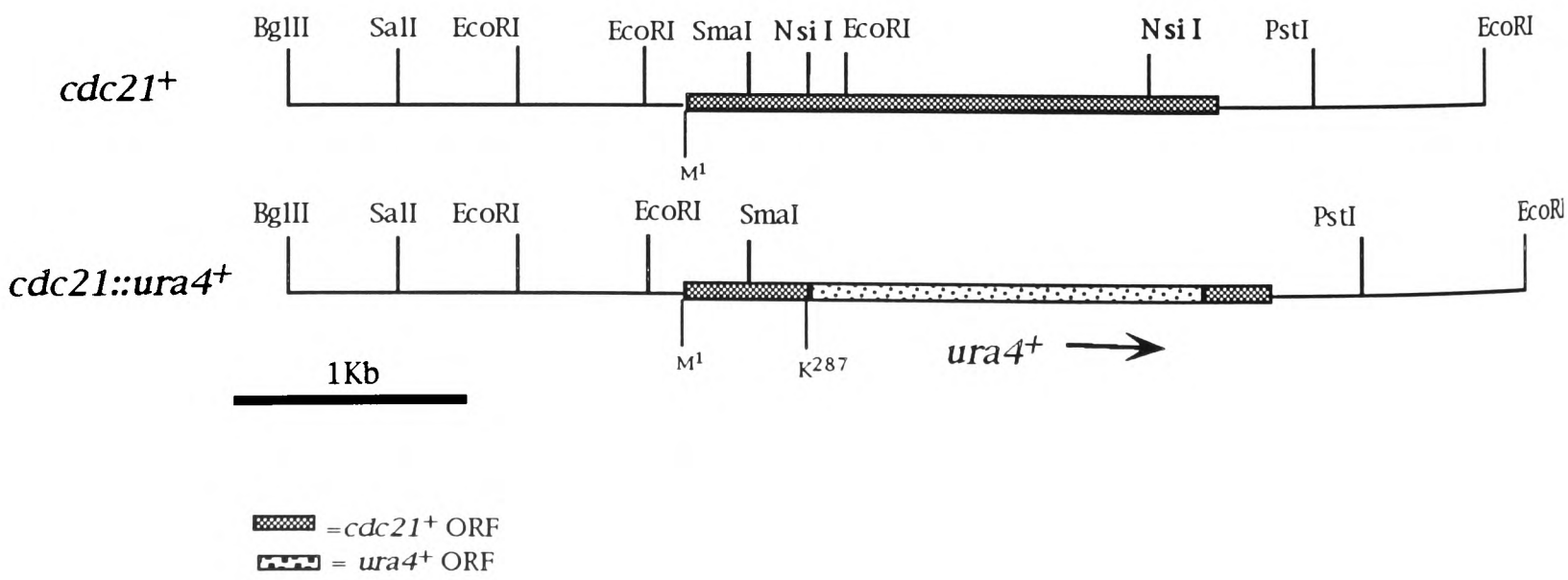

BglII SalI EcoRI EcoRI SmaI NsiI EcoRI NsiI PstI EcoRI
cdc21+
M1
BglII SalI EcoRI EcoRI SmaI PstI EcoRI
cdc21::ura4+
M1
K287
ura4+
1Kb
=cdc21+ ORF
= ura4+ ORF

Figure 7.2
Southern blot analysis of the *cdc21::ura4*$^+$ disruptants

(a)

About 1µg of genomic DNA extracted from diploid wild-type (P135; wt) and 4 independent transformants stable for the Ura$^+$ phenotype (Δ1-4) was digested with *Eco*RI and loaded on a 0.8% agarose gel in 1X TBE buffer. After electrophoresis the gel was blotted and probed with [α-^{32}P] dCTP random primed labelled *S. pombe cdc21*$^+$ (*Sac*I-*Pst*I fragment from pAC1). The probe hybridised to two *Eco*RI bands of 3.1 and 1.7 kb in the lane loaded with wild type and *cdc21*-disruptants strains DNA, whereas one additional band of 5 kb, which is of the same size expected for the disruption of the *cdc21*$^+$ gene by the targeting construct (5.1 kb), appears only in the lanes loaded with DNA prepared from three of the disruptants strains (Δ1, Δ3 and Δ4). The size of the additional band seen in the disruptant Δ2 is not of the size expected for the disruption of *cdc21*$^+$ and may be the result of a random event of integration. The same filter was probed with the *S. pombe ura4*$^+$ gene labelled as above (*Hin*dIII fragment) after the *cdc21*$^+$ probe had been removed as described in Chapter Two.

Hybridisation was carried out as described in Chapter Two. Nylon membranes were exposed to an X-ray film for 3 days at -70°C.

(b)

Physical map of the *cdc21*$^+$ genomic loci in the *S. pombe cdc21*$^+$*/cdc21::ura4*$^+$ diploid strain. The 1.5kb *Nsi*I internal fragment of the *cdc21*$^+$ gene was replaced with the *ura4*$^+$ gene as detailed in Chapter Two and above. ATG= first codon of the *cdc21*$^+$ open reading frame. The arrow in the figure indicates the direction of transcription of the *ura4*$^+$ gene.

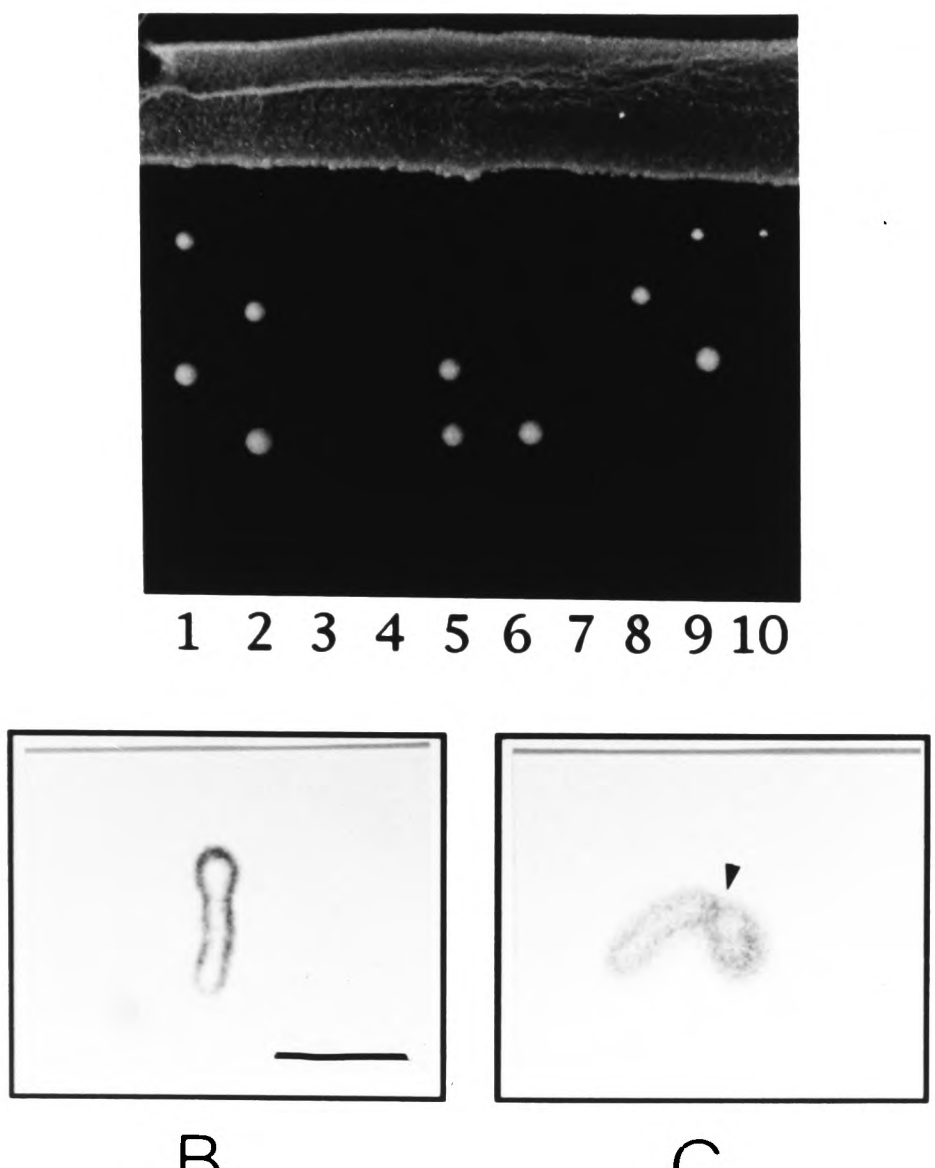


Figure. 7.3

The *cdc21*$^+$ gene is required for cell viability

Ten asci produced by sporulation of the *cdc21*$^+$/*cdc21::ura4*$^+$ diploid strain (1-10; see Chapter Two) were dissected on a vegetative plate using a micromanipulator (panel A). After dissection spores were allowed to germinate at 30°C for 5 days. The phenotype of non-germinated spores was observed by light microscopy and photographed (panels B and C). The cell wall of asci 3, 4 and 7 never broke down; in some cases spores had slow growth (spores of asci 9 and 10). The streak on the top are undissected germinated spores.

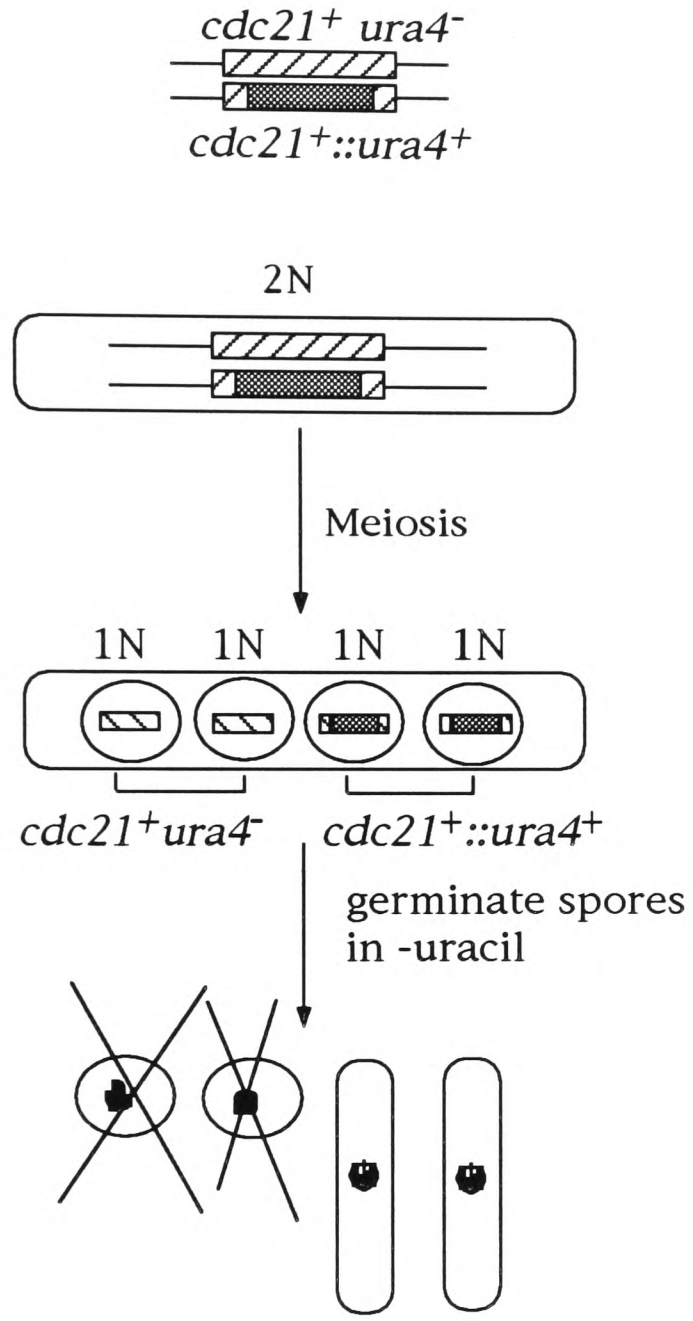


Figure 7.4

Strategy used to obtain and germinate spores deleted for the *cdc21*$^+$ gene.

Spores of the *cdc21* disruption diploid strain (P180; *h*$^+$/*h*$^-$ *cdc21+/cdc21::ura4*$^+$*ura4-D18 leu1-32 /leu1-32 ade6-M210/ade6-704*) were prepared as detailed in Chapter Two. Diploid cells were sporulated in minimal medium supplemented with leucine (75 mg/l) containing glutamate (5g/l) as a carbon source. Non-sporulated vegetative cells were killed by over-night incubation with helicase and spores were purified as described in Chapter Two. Spores were inoculated in minimal medium lacking uracil so that just *ura4*$^+$ spores could germinate. Spores prepared from the *S. pombe* diploid strain (P136, wild-type; *h*$^+$/*h*$^-$ *leu1*$^+$/*leu1-32 ura4*$^+$/*ura4-D18 ade6*$^+$/*ade6-704*) were prepared using the same procedure described above.

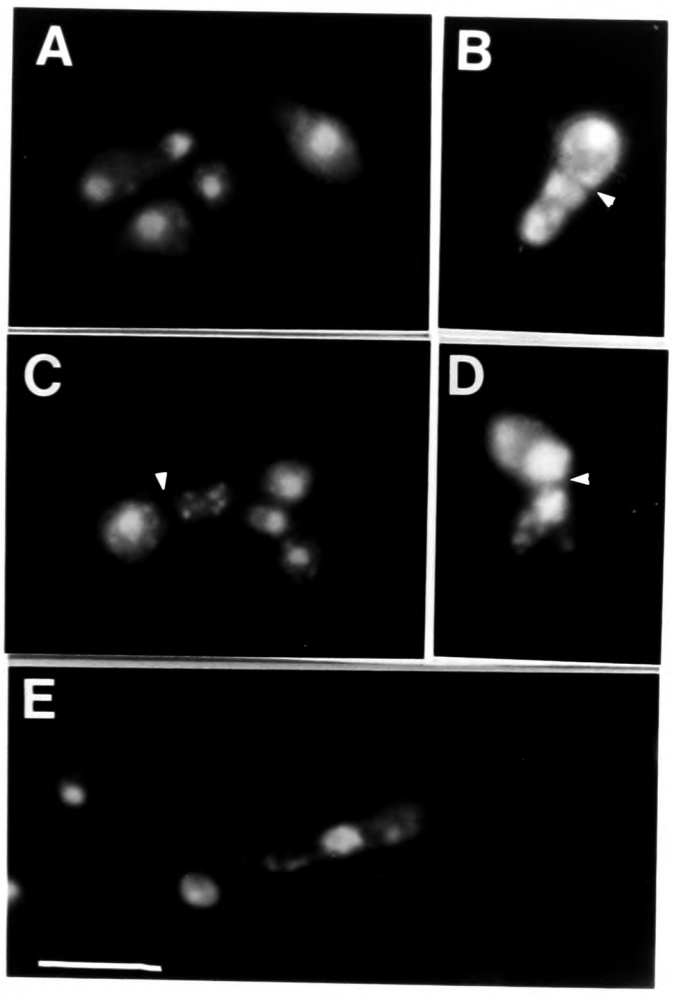


Figure 7.5

Arrest phenotypes of germinating spores derived from the *cdc21* disruption strain

The nuclear morphology of wild-type (*cdc21*$^{+}$ *ura4*$^{+}$) and *cdc21::ura4*$^{+}$ heat fixed germinating spores was observed by DAPI staining (see Chapter Two). Spores were germinated in minimal medium lacking uracil supplemented with 300mg/l of leucine at 29°C. Calcofluor was omitted to allow a better observation of the nuclear morphology. The phenotypes shown are representative samples of each time point (11-16 hours). Arrowheads indicate aberrant mitoses. Bar= 5μm.

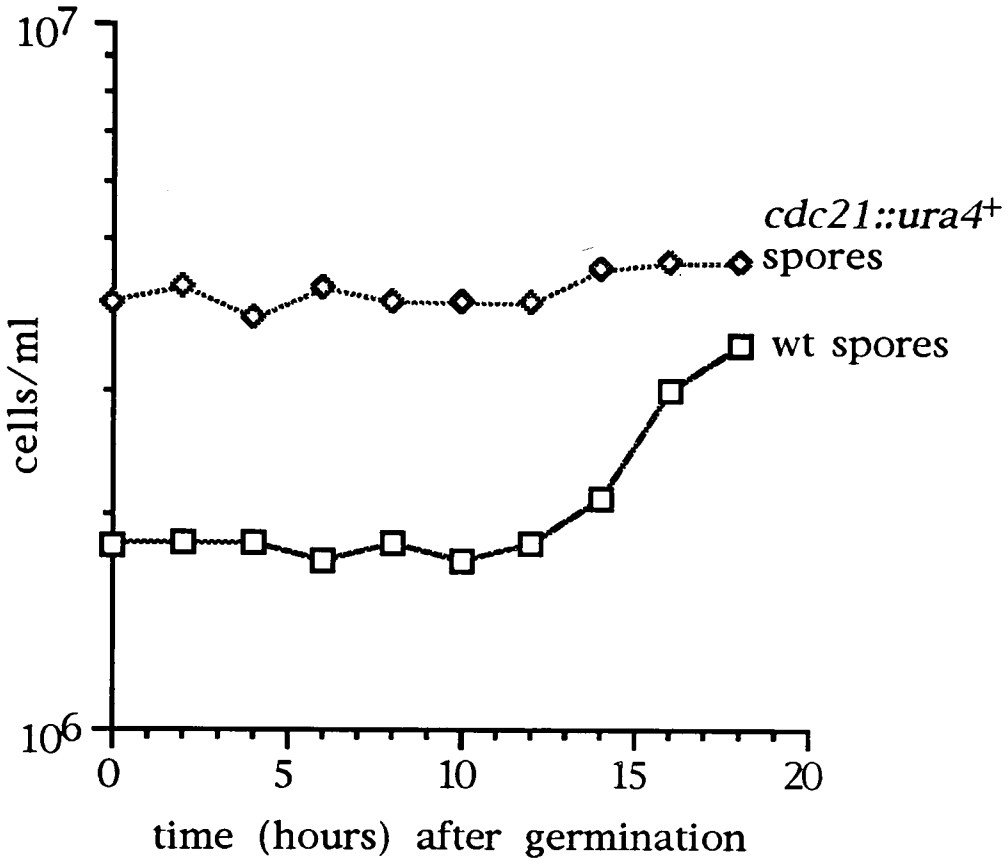


Figure 7.6

Growth rate of *S. pombe* wild-type and *cdc21::ura4*+ spores

Cell number of germinating spores prepared from wild-type (P136) and *cdc21* disruption (*cdc21::ura4*+) diploid strains was determined, by sampling an aliquot of germinating spores every 2 hours as described in Chapter Two. The cell number of the P136 strain increased after the first mitosis, whereas that of the *cdc21::ura4*+ spores did not and remained stable over time.

cdc21::ura4^{+}

wild-type

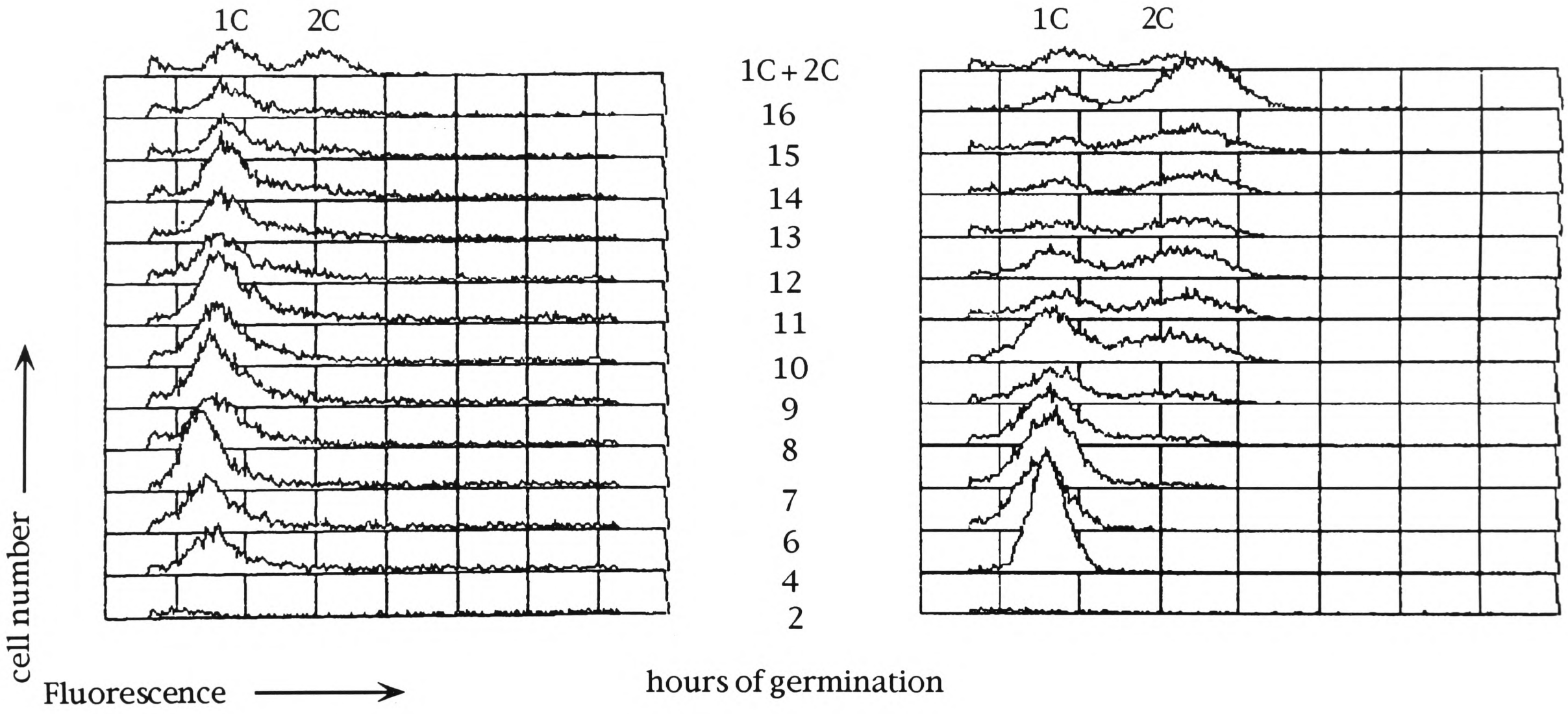


Figure 7.7

Germinating spores lacking the *cdc21*$^+$ gene are deficient in DNA replication

The DNA content of wild-type and *cdc21::ura4*$^+$ germinating spores was determined by flow cytometry. Samples of germinating spores were collected at different time intervals and processed as described in Chapter Two. 1C and 2C indicate the fluorescence standards of cells containing 1C and 2C DNA contents. Data were plotted on a linear scale on both axes.

Figure 7.8

Strategy used to generate the *cdc21*$^+$ "shut off" construct and the *cdc21* "shut off" mutant

(a)

Construction of the 81X::21$^+$ *gene fusion*

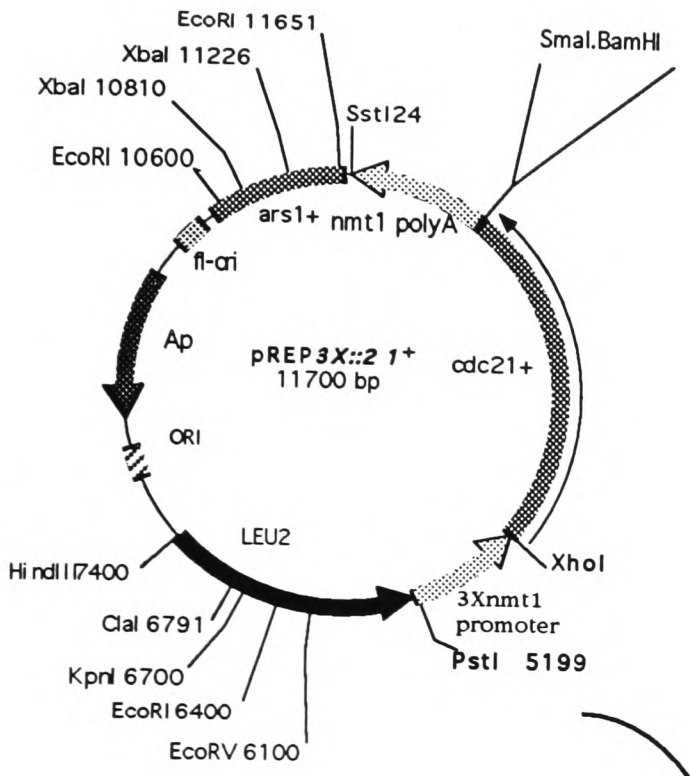
The *cdc21*$^+$ gene was placed under control of the 81X*nmt1* regulatable promoter of fission yeast (Basi *et al.*, 1993). This was done by promoter replacement. The 3X*nmt1* promoter of the pREP*3X::21*$^+$ plasmid (3X*nmt1::cdc21*$^+$) was replaced by the 81X*nmt1* promoter as follows. The pREP*3X::21*$^+$ plasmid was digested with *Pst*I and *Xho*I and the vector deleted of the 1.2kb *Pst*I-*Xho*I DNA fragment (that is the 3X*nmt1* promoter) was gel purified. The *Pst*I-*Xho*I 81X*nmt1* promoter was then ligated to the vector thus generating the pREP*81X::cdc21*$^+$ (*81X::21*$^+$) recombinant plasmid.

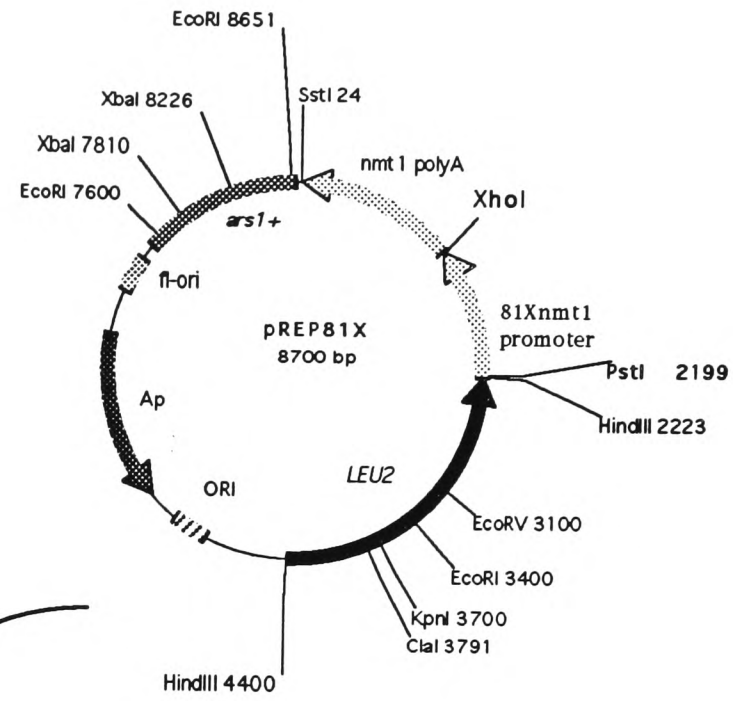
(b)

Isolation of the cdc21::ura4$^+$*[81X::21*$^+$*] shut off strain*

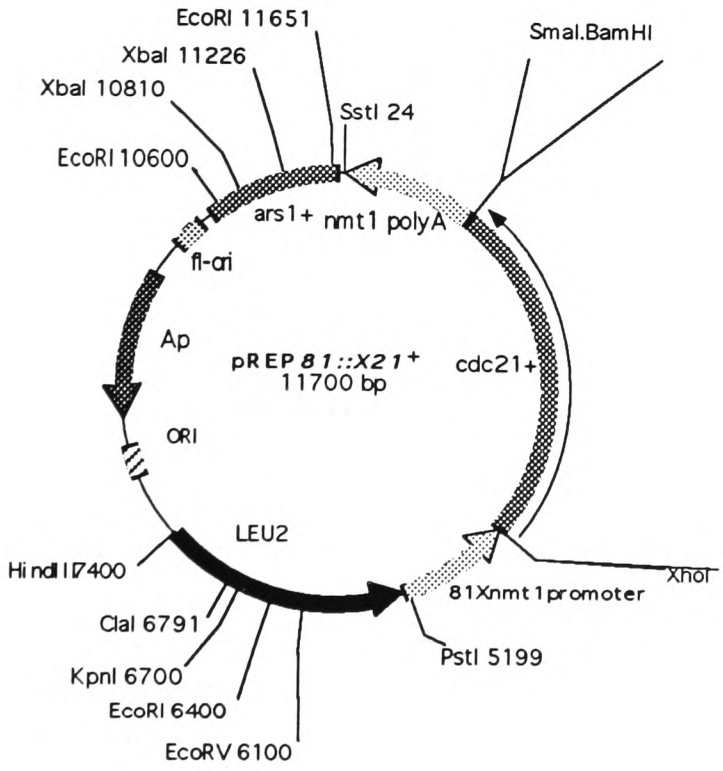
The *81X::21*$^+$ (*LEU2*) construct was transformed in the *cdc21* disrupted diploid strain (*cdc21*$^+$*/cdc21::ura4*$^+$ *ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216 h*$^+$*/h*$^-$) using the LiCl procedure (see Chapter Two) and Ura$^+$ Leu$^+$ Ade$^+$ transformants were selected on minimal selective plates. These diploids were sporulated by growing them until saturation on leu$^-$ ade$^-$ minimal plates in the absence of thiamine (*nmt1* on). Cells were scraped off the plate and resuspended in 1 ml of sterile water. Spores were prepared and plated out as described for the random spore analysis procedure (Moreno *et al.*, 1991; see also Chapter Two). Spores were plated out on selective leu$^-$ ura$^-$ plates in the absence of thiamine and a haploid strain harbouring the *cdc21::ura4*$^+$ deletion and kept viable by the expression of *cdc21*$^+$ due to the presence of the pREP*81X::21*$^+$ plasmid was then selected. Colonies were scored after 3 days of incubation at 30°C. Transformants were tested for their ability to grow on plates lacking of adenine and found to be all ade$^-$, thus demonstrating they were genetically haploid. Measure of the cell length by light microscopy confirmed the genetic prediction.

(a)

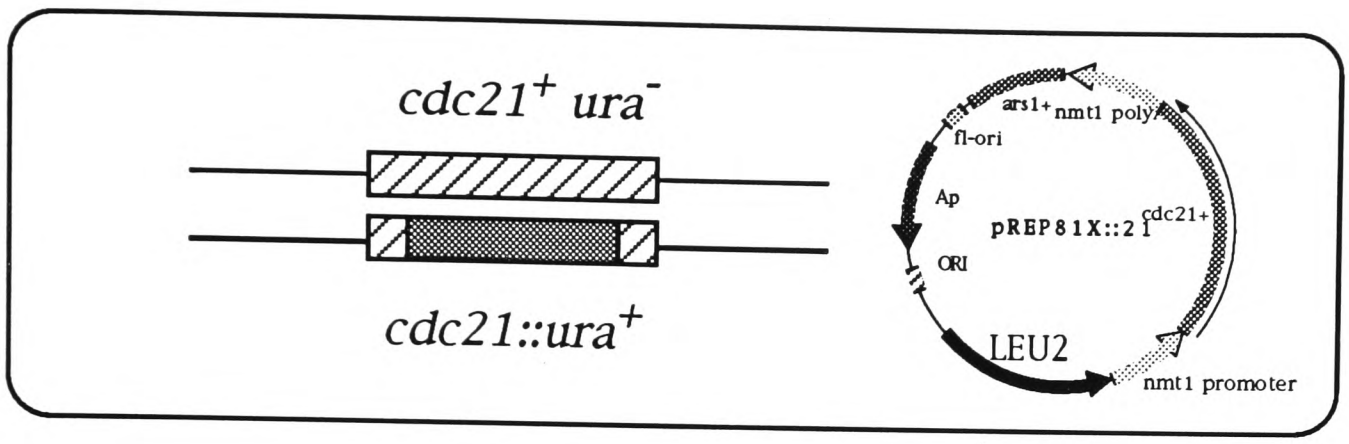




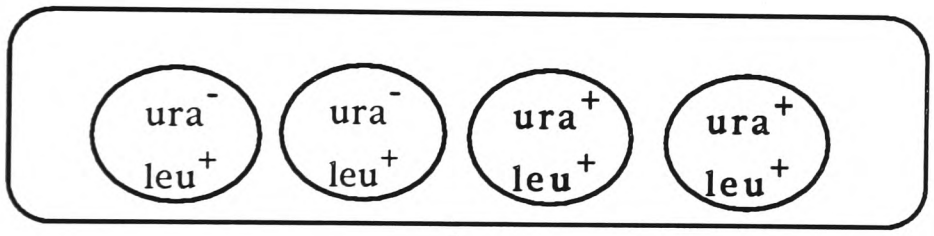
digest *Pst*I-*Xho*I
ligate 81Xnmt1 promoter
to pREP*3X::21*$^{+}$ deleted of the
3Xnmt1 promoter



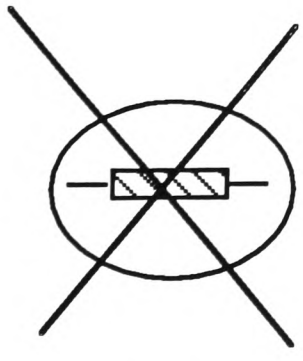
(b)

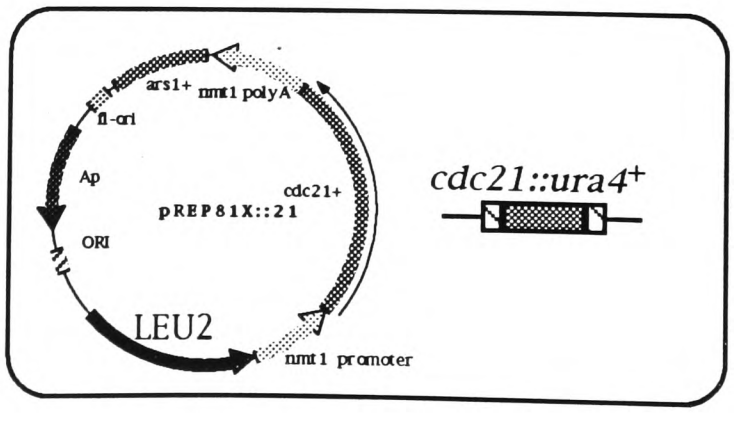


Meiosis



select on
-ura -leu





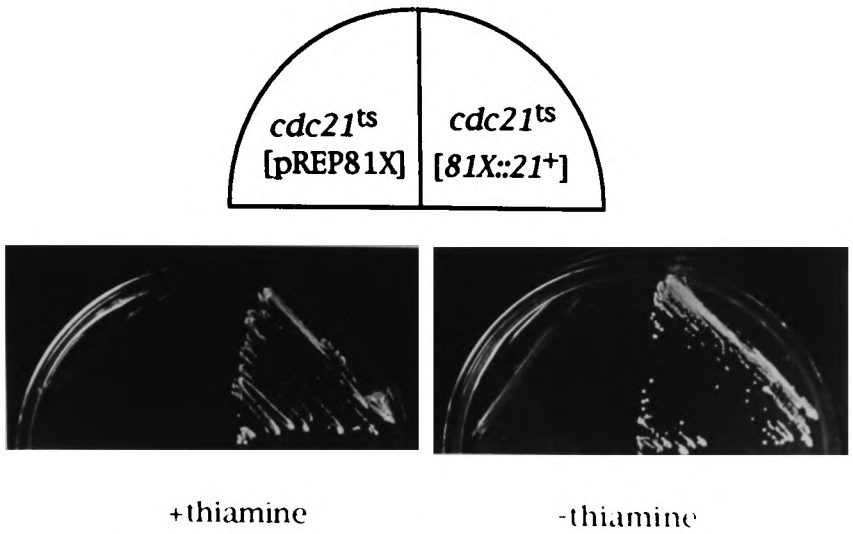


Figure 7.9

Shut off of the expression of the *cdc21*$^{+}$ gene from the *81X::21*$^{+}$ construct complements the *cdc21*^{ts} but not the *cdc21::ura4*$^{+}$ deletion mutant

The pREP*81X::cdc21*$^{+}$ construct was transformed into the *cdc21-M68* strain (P1; *cdc21*^{ts} *leu1-32*) by electroporation. The *cdc21*^{ts} [pREP81X] strain was included as control. *cdc21*^{ts} [*81X::21*$^{+}$ *LEU2*] transformants were selected on minimal leu^{-} plates and challenged to grow at 36°C in the absence or in the presence of 5µg/ml of thiamine. *cdc21::ura4*$^{+}$[*81x::21*$^{+}$] transformants were streaked out on minimal complete plates in the absence or in the presence of thiamine and tested for the ability to grow at 32°C. The *cdc21::ura4*$^{+}$ [pAC1] strain was included as control. Growth was scored after 4-5 days at the indicated temperature.

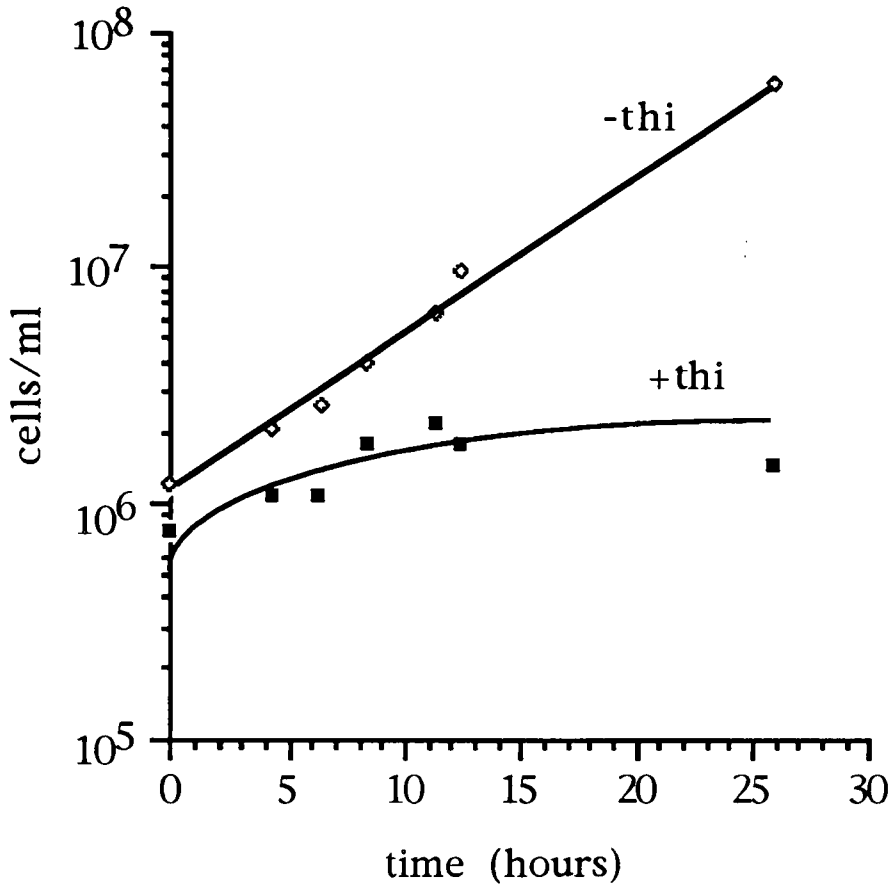
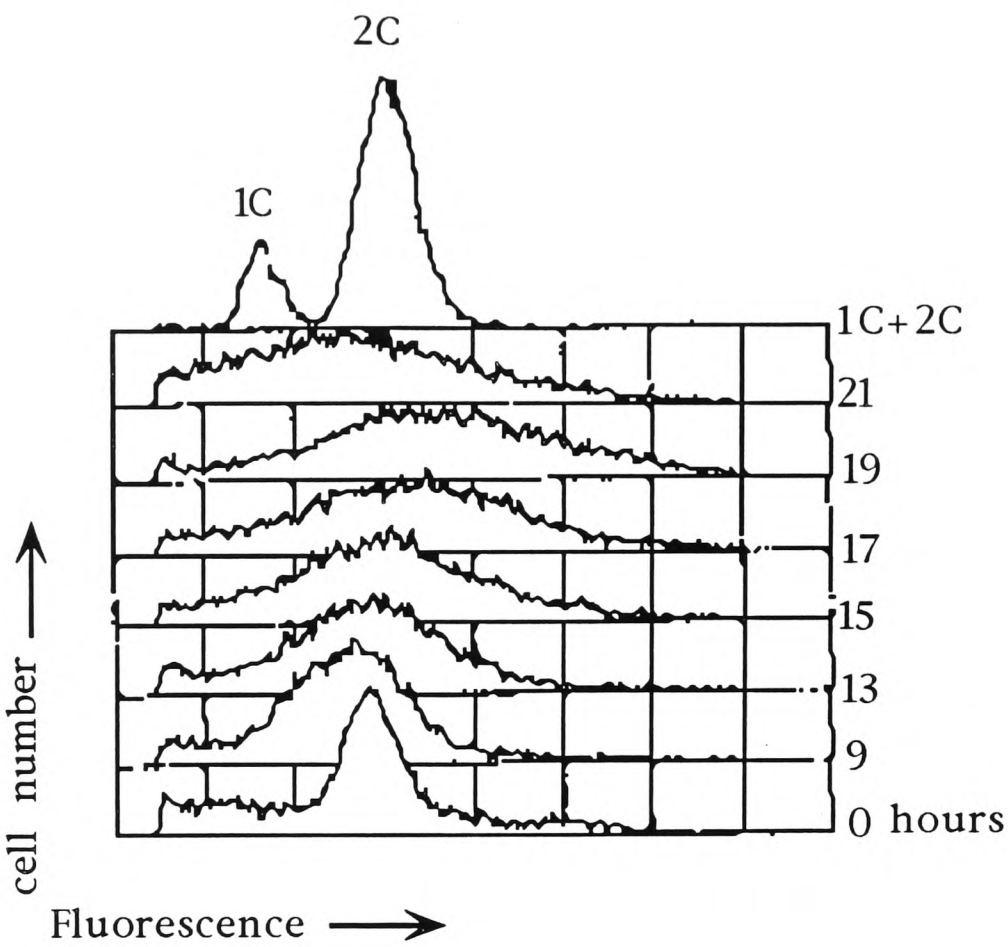


Figure 7.10

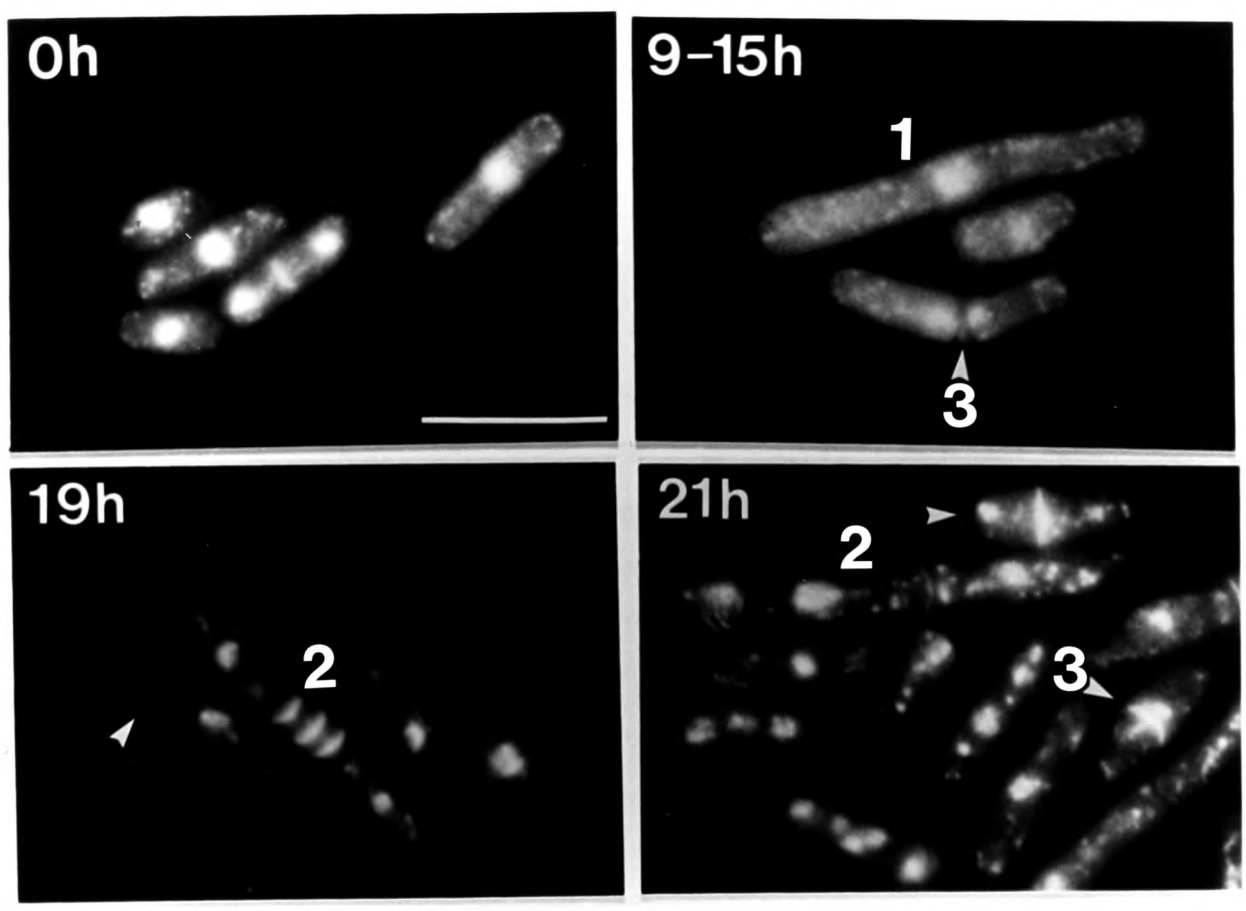
The *cdc21* "shut off" mutant strain arrests 3 generations after thiamine addition

Growth curve of *cdc21* "shut off" mutant cells grown in the presence and in the absence of thiamine. Cells were grown in minimal complete liquid medium in the absence of thiamine to $1x10^6$ cells/ml. The culture was split in half and thiamine was added in one culture (+thi; 5µg/ml) while no thiamine was addede in the other one (-thi). Growth was allowed to continue at 32°C for about 2 generations (9 hours) after that cells were diluted back in fresh medium (time 0 in the plot). Cells in the (+thi) culture continued to divide for 1 generation after dilution then arrested.

a


2C
1C
1C+2C
21
19
17
15
13
9
0 hours
cell number
Fluorescence

b


0h
9–15h
1
3
19h
2
21h
2
3

(-thi). Growth was allowed to continue at 32°C for about 2 generations (9 hours) after that cells were diluted back in fresh medium (time 0 in the plot). Cells in the (+thi) culture continued to divide for 1 generation after dilution then arrested.

Figure 7.11
The *cdc21* "shut off" mutant arrests with incomplete replicated DNA and shows aberrant mitoses

(a)

The DNA content of exponentially growing *cdc21* "shut off" mutant cells in the absence (*nmt1::cdc21*$^+$ on) and in the presence of 5μg/ml of thiamine (*nmt1::cdc21*$^+$ off, 0-21 hours) was determined by flow cytometry. Samples were collected periodically and fixed as described in Chapter Two. 1C and 2C fluorescence DNA contents were also included as standards.

(b)

The arrest phenotype of *cdc21* "shut off" mutant cells was monitored by DAPI and Calcofluor staining (see Chapter Two). Exponentially growing cells were sampled from the (+thi) culture at 0, 9, 15, 19 and 21 hours after addition of 5μg/ml of thiamine. Arrowheads indicate the occurrence and the products of aberrant mitotic events.

Total tetrads	'nr'	2:2	1:3	genotype
31	10	12	9	ura$^-$

Table 7.1

Results of the dissection of the asci obtained by sporulation of three *cdc21*$^+$/*cdc21::ura4*$^+$ diploid strains

Asci were dissected on rich plates as described in Chapter Two and growth was scored after 5 days at 30°C. The ratio of viable:dead spores is also reported in the table. Colonies were replica-plated on minimal selective (ura$^-$) plates and growth scored after 5 days at 30°C. ' nr' = asci which never released spores.

Phenotype	Frequency (%)
elongated, 1 nucleus	84.5
elongated 2 nuclei	0
aberrant mitoses	15.5

Table 7.2

Phenotype of *cdc21::ura4*$^+$ germinating spores

Cells (about 500) were observed under fluorescence microscopy and the frequencies of the different phenotypes scored after 13 hours of germination at 29 °C. The frequencies are expressed as the percentage (%) of the germinated spores. Germinating spores displaying aberrant mitoses were scored as spores containing one septum bisecting an undivided nucleus or missing completely the nucleus, similar to the 'cut' phenotype described by Hirano *et al.*, (1986).

Phenotype	Frequency (%) after	
	9-15h	19-21h
normal shaped cells	48	30
elongated 1 nucleus (1)	40	30
elongated 2 nuclei	10	0
1 or more septa (2)	1	30
'cut' (3)	1	10

Table 7.3

Phenotypes associated with the *cdc21*$^+$ "shut off"

Frequencies of phenotypes associated with the shut off of *cdc21*$^+$ as determined by DAPI and Calcofluor staining 9-15 and 19-21 hours after addition of thiamine. Very close but physically divided cells with asymmetric positioned nuclei were considered product of a 'cut' event and counted as one. Numbers in brackets indicate phenotypes shown in Figure 7.10b and also described in the text.

Chapter Eight

Summary and general discussion

The cdc21+ gene is required for chromosomal DNA replication

Although the *cdc21+* gene was isolated in a screen aimed at identifying new mutants in DNA replication, no conclusive evidence had so far been available demonstrating that it is essential for DNA replication. Previous genetic characterisation of the *cdc21-M68* temperature-sensitive mutant has suggested that the *cdc21+* gene may be involved in the replication of a portion of the genome (Nasmyth and Nurse, 1981; Coxon *et al.*, 1992). Evidence presented in this thesis indicates that the *S. pombe* *cdc21+* gene is essential for all chromosome replication. This was demonstrated by generating new alleles of *cdc21.*

Haploid spores containing a null allele of *cdc21* germinated when put in conditions favourable for growth; however they failed to replicate their DNA.

Likewise, a new conditional allele of *cdc21*, bearing a chromosomal disruption of *cdc21* and the pREP*81X::21+* construct, arrested in S phase upon repression of the *cdc21+* gene, although with a DNA content apparently different from that of germinating spores lacking the *cdc21+* gene. A similar discrepancy has been reported for a null mutant of the fission yeast *cdt1+* gene which shows a phenotype similar to that of the *cdc21* null allele (Hofmann and Beach, 1994). This apparent difference may be due to residual transcription from the repressed 81X*nmt1* promoter and perhaps to different cdc21 protein levels in spores and mitotically growing cells. Lowering the *cdc21+* gene dosage by integration of the pREP*81X::21+* construct in the chromosome may give a tighter arrest phenotype for this mutant allele. The *cdc21* disruption mutant and the *81X::cdc21+*construct will be useful for the isolation of new recessive and perhaps dominant alleles of *cdc21.*

DNA synthesis was also inhibited in the *cdc21-M68* strain at the permissive temperature by overexpression of the carboxyl terminal region of the *cdc21+* gene.

Phenotypes not seen in *cdc21-M68* cells arrested at the restrictive temperature, were seen in cells lacking the *cdc21+* gene. A small proportion of cells (15-20%) entered mitosis in the absence or before completion of DNA replication, suggesting that the system which normally prevents the onset of mitosis during S phase (checkpoint

controls; Hartwell and Weinert, 1989; see Nurse, 1994, for review) may be disturbed in these cells. *cdc21-M68* cells overexpressing the *cdc21*$^+$ carboxyl-terminal region arrested with a similar phenotype.

These results taken together clearly demonstrate that the phenotype of a *cdc21* null mutant is not completely similar to that of the *cdc21-M68* mutant, arguing that the latter is not a particular severe allele of *cdc21*. Paradoxically, despite its early transition point in the cell cycle, the *cdc21-M68* strain arrested at the end of S phase with apparently duplicated DNA (Coxon *et al.*, 1992). I have indicated that this arrest may be due to a defect in replication, neither detectable by flow cytometry (Coxon *et al.*, 1992), nor, as I have shown, by pulsed field gel electrophoresis. Double mutants of *cdc21* and *rad1* or *cdc21* and *cdc2.3w*, which cannot arrest following DNA damage or in the presence of unreplicated DNA, entered mitosis. It is a possibility that the arrest in S phase of the *cdc21-M68* mutant is caused by the slow kinetics of DNA replication. Consistent with this, *cdc21 cdc2.3w* arrested cells are much more elongated than *cdc2.3w* cells, indicating that a delay, independent of the pathway which is altered in the *cdc2.3w* mutant, is imposed in these cells. Experiments using a synchronised population of cells will be necessary to finally confirm the presence of such a delay.

dom1$^+$ *and tsl1*$^+$ *may genetically interact with cdc21*

My search for functions which may genetically interact with *cdc21*$^+$ has so far not been helpful in clarifying the role of the *cdc21*$^+$ gene. In one approach I have isolated a cDNA, *tsl1*$^+$, whose overexpression specifically lowered the restrictive temperature of the *cdc21-M68* strain, suggesting a sort of genetic interaction between the two functions. Unfortunately the nucleotide sequence of this cDNA was uninformative. Further experiments are necessary to determine how the overexpression of the *tsl1*$^+$ cDNA may interfere with the *cdc21* mutation. It needs to be shown that the overexpression of the *tsl1*$^+$ cDNA does not interfere with growth when overexpressed in other *cdc* mutants of fission yeast. Determination of the DNA content of *cdc21*^{ts} cells overexpressing the *tsl1*$^+$ cDNA and ultimately the generation of a null allele is needed to establish whether this function may interfere with DNA replication. In another approach a cDNA, *dom1*$^+$, was isolated and shown to cause *cdc21* mutant cells to arrest as small cells in a thiamine-dependent manner. The *dom1*$^+$ cDNA encodes a protein which is probably the homologue of the budding yeast HMG-like protein Nhp2 (Kolodrubetz and Burgum, 1991) whose cellular role is unknown. This will be proved by complementation of a null mutant of the *nhp2* gene by the *dom1*$^+$ cDNA. This finding is potentially very interesting as Nhp2 may be a chromatin-associated protein

(Kolodrubetz and Burgum, 1991) and cdc21-related proteins have been shown to be also chromatin associated (Yan *et al.*, 1993; Todorov *et al.*, 1995).

Does cdc21 play a role in the replication checkpoint?

One unexpected result was the ability of a small proportion of cells lacking the *cdc21*$^+$ gene to enter mitosis in the absence of DNA synthesis. A number of yeast DNA replication mutants showing similar phenotypes have been described. These include the *cdc18* (Kelly *et al.*, 1993), *cut5/rad4* (Saka and Yanagida, 1993) and *cdt1* (Hofmann and Beach, 1994) mutants of fission yeast. It has been suggested that they may play a dual role, one in the initiation of DNA synthesis and another one in the restraint of mitosis until completion of S phase.

The rad4/cut5 protein may have a direct role in the restraint of mitosis as *cut5* mutant cells incubated at the restrictive temperature enter mitosis even when DNA replication is inhibited by hydroxyurea. Because UV irradiation did inhibit cell division in this mutant at the restrictive temperature the cut 5 protein may have an important role in the feedback mechanism which links DNA replication to mitosis (Saka *et al.*, 1994).

The *cdc18*$^+$ is a target of the cdc10 transcription factor and it is therefore periodically transcribed at the G1/S boundary (see Chapter One; Kelly *et al.*, 1993). cdc18 is required for DNA replication and most probably for mitotic restraint until completion of DNA replication. It is not known whether the mitotic restraint deficiency is caused by the failure to replicate DNA or by an additional function in the replication/damage checkpoint (Kelly *et al.*, 1993). The *cdt1*$^+$ gene, also a target of the cdc10 transcription factor, may have a similar role (Hofmann and Beach, 1994). The budding yeast Cdc6 protein, which shows close similarity to the fission yeast cdc18 protein, may also play similar roles, although a direct evidence for an involvement in the mitotic checkpoint has not been provided yet (Bueno and Russel, 1992).

Thus, as recently pointed out (Li and Deshaies, 1993; Nurse, 1994), one way to explain the arrest phenotype of these mutants is to speculate that cells may monitor the progression of S phase by monitoring the presence of replication complexes. Evidence for this has been provided (see Chapter One). Thus the arrest phenotype of cells lacking the *cdc21*$^+$ gene can be explained as failure to assemble (pre)replication complexes and consequently failure to activate DNA synthesis. It is possible that the cdc21 protein may interact with proteins required to assemble a pre-replication complex, such as the cdc18, cdt1 and/or cut5/rad4 proteins.

The possibility cannot be ruled out that cdc21 may also have a separate role in the restraint of mitosis until completion of S phase. The low frequency of aberrant mitoses seen in cells lacking the *cdc21*$^+$ gene compared to that of the *cdc18* or *cut5/rad4* mutants may be not completely consistent with this hypothesis. However, if the cdc21 protein is required for this control, the presence of damaged DNA should not suppress the aberrant onset of mitosis of cells lacking the *cdc21*$^+$. Thus if spores deleted for the *cdc21*$^+$ gene are irradiated with UV light prior germination they should be still able to enter mitosis in the absence of DNA replication.

Role of cdc21 and related proteins in DNA replication

What is the function of *cdc21*$^+$ in DNA replication?

The data presented in this thesis are consistent with a role in the initiation step of DNA replication, although a role also in elongation cannot be excluded. In support of this, *cdc21-M68* cells released at 36°C from a DNA replication block imposed by the presence of hydroxyurea, go on to divide but are delayed if compared to wild-type cells (Nasmyth and Nurse, 1981). Also, the non-uniform arrest phenotype of the *cdc21* disruption and 'shut off' mutants, as well as that of the *cdc21-M68* mutant overexpressing the *cdc21*$^+$ carboxyl terminal are consistent with such a role.

The cdc21 protein shares structural similarity with a set of essential replication proteins, the Mcm protein family (Coxon *et al.*, 1992). By constructing a phylogeny analysis using the protein sequences of members of the Mcm protein family I have shown that there are 6 distinct members identified in yeast so far. From the data obtained by Southern blot and PCR it is likely that at least six distinct members of this protein family may be present in yeast. The *MCM* genes have been shown to interact genetically (Hennessy *et al.*, 1991; Yan *et al.*, 1991; Takahashi *et al.*, 1994; Forsburg and Nurse, 1994). Very recently, a physical interaction between the mammalian P1 and Cdc46 proteins has been reported. These proteins were found in the nucleus as heterodimers and as part of a large multi-protein complex which may include other Mcm proteins (Burkhart *et al.*, 1995). Thus it is tempting to speculate that only a species-specific combination of at least two Mcm proteins may be functional. This would be one way to explain why conserved homologues do not cross-complement (Forsburg and Nurse, 1994; I.Todorov, pers. comm.). It would be then of interest to determine the effect on DNA replication of combining two *mcm2-3-5* mutants. Double mutants of *mcm2* and *mcm3* (Yan *et al.*, 1991), or *cdc46-1* and *cdc54-1* are inviable (Hennessy *et al.*, 1991). In contrast, a double mutant of *cdc21* and *nda4* is viable (Blom and Kearsey, pers. comm.). It has been reported that a cross between *cdc21* and

cdc19 (Forsburg and Nurse, 1994) or *cdc21* and *mis5* (Takahashi *et al.*, 1994) produces inviable progeny and therefore it cannot be studied. The viability of the spores is recovered by introducing a wild-type copy of one of the genes on a plasmid (Forsburg and Nurse, 1994). The availability of the pREP*81X::21*$^+$ construct now makes the study of these double mutants of fission yeast feasible. The lethality and most importantly the kinetics of DNA replication of these double mutants can be studied by performing the cross with the *cdc21-M68* mutant or with the *cdc21* deletion strain containing the pREP*81X::21*$^+$ construct as the expression of *cdc21*$^+$ can be tightly regulated. The biochemical role of the Mcm proteins is still unclear. It has been suggested that these proteins contain a helicase motif, implying that they may have a role in DNA replication as helicases (Koonin, 1993), although there is no biochemical evidence.

The Mcm proteins are considered good candidates for factors involved in the control that limits DNA replication to one round per cell cycle (licensing factors, see Chapter One; Blow and Laskey, 1988; Leno *et al.*, 1990; Tye, 1994). This is essentially based on the observation that in *S. cerevisiae* these proteins appear to gain access to the nucleus only at late mitosis, in agreement with the licensing factor hypothesis. Evidence for such a role has been recently provided. Some mammalian members of the Mcm protein are associated with unreplicated chromatin and are displaced from the DNA by the ongoing DNA replication. Post-translational modification of the displaced proteins (in part due to phosphorylation) perhaps would prevent their binding to replicated chromatin, thus also preventing re-replication within the same S phase (Kimura *et al.*, 1994; Todorov *et al.*, 1995). However, unlike the budding yeast homologues, the Mcm proteins in higher eukaryotes have been found in the nucleus throughout the cell cycle, and a similar situation applies in fission yeast (Maiorano *et al.*, in preparation). This observation can be accommodated within the licensing factor theory assuming that another cell-cycle regulated component is required for the licensing reaction or that cell-cycle regulated post-translational modifications of the Mcm proteins may be required for this control. If members of the Mcm protein family play a similar role in fission yeast, the cdc21 protein may be an absolutely necessary component of this control mechanism.

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Appendix I

tsl1+ DNA sequence contig

The DNA sequence contig shown below is that of the fission yeast *tsl1*+ cDNA, the open reading frame is described in Chapter Four. The contig was generated using the Staden sequence assembly programmes (SAP).

```
                        10        20        30        40        50
1   C25.GEL1;3   TCGAGGTCGACGTTAATTAAGCCTATTGTTTACATGTTGTGTTTCAACTG
2   C25A2.GEL2                                          TGTTTCAACTG
    CONSENSUS    TCGAGGTCGACGTTAATTAAGCCTATTGTTTACATGTTGTGTTTCAACTG

                       60        70        80        90        100
1   C25.GEL1;3   CTAAATTAACAGGAGAAGGAGAATAATTGTAAGCTAGCAGATACTGTGTG
2   C25A2.GEL2   CTAAATTAACAGGAGAAGGAGAATAATTGTAAGCTAGCAGATACTGTGTG
3   C25B2.GEL2                   GGAGAATAATTGTAAGCTAGCAGATACTGTGTG
    CONSENSUS    CTAAATTAACAGGAGAAGGAGAATAATTGTAAGCTAGCAGATACTGTGTG

                      110       120       130       140       150
1   C25.GEL1;3   GAGCGATCTTTGT
2   C25A2.GEL2   GAGCGATCTTTGTCGAACAGGCAGTATTCGTTTTGTCTGGAGACCTTACC
3   C25B2.GEL2   GAGCGATCTTTGTCGAACAGGCAGTATTCGTTTTGTCTGGAGACCTTACC
    CONSENSUS    GAGCGATCTTTGTCGAACAGGCAGTATTCGTTTTGTCTGGAGACCTTACC

                      160       170       180       190       200
2   C25A2.GEL2   TGAGATTCTGATACTAAAC
3   C25B2.GEL2   TGAGATTCTGATACTAAACGTTGTAATCATCTTAGAGTAACTGGATCACT
6   C25C4.GEL4                                  TTAGAGTAACTGGATCACT
    CONSENSUS    TGAGATTCTGATACTAAACGTTGTAATCATCTTAGAGTAACTGGATCACT

                      210       220       230       240       250
3   C25B2.GEL2   CGTTTAATTCTTTAAAAGTGAACTGAAAAATATTTT
6   C25C4.GEL4   CGTTTAATTCTTTAAAAGTGAACTGAAAAATATTTTTACCATTTAACTCA
5   C25D6.GEL2                        CTGAAAAATATTTTTACCATTTAACTCA
    CONSENSUS    CGTTTAATTCTTTAAAAGTGAACTGAAAAATATTTTTACCATTTAACTCA

                      260       270       280       290       300
6   C25C4.GEL4   TTGCACGTTTGAGGATGCAAGATTCTTCCAAAAATAAACAACCATGCGTC
5   C25D6.GEL2   TTGCACGTTTGAGGATGCAAGATTCTTCCAAAAATAAACAACCATGCGTC
10  C25E8.GEL6                                                  TC
    CONSENSUS    TTGCACGTTTGAGGATGCAAGATTCTTCCAAAAATAAACAACCATGCGTC

                      310       320       330       340       350
6   C25C4.GEL4   TCATGGAAAGTTTCTCCTTT
5   C25D6.GEL2   TCATGGAAAGTTTCTCCTTTAAAAGCGAAAGCATGTCTCCTGATGATGTC
10  C25E8.GEL6   TCATGGAAAGTTTCTCCTTTAAAAGCGAAAGCATGTCTCCTGATGATGTC
    CONSENSUS    TCATGGAAAGTTTCTCCTTTAAAAGCGAAAGCATGTCTCCTGATGATGTC

                      360       370       380       390       400
5   C25D6.GEL2   TCAAGGCTTTTTTGTAAATCCAGTACTAGTAGCGCTACTCGGAAGCATGA
10  C25E8.GEL6   TCAAGGCTTTTTTGTAAATCCAGTACTAGTAGCGCTACTCGGAAGCATGA
    CONSENSUS    TCAAGGCTTTTTTGTAAATCCAGTACTAGTAGCGCTACTCGGAAGCATGA
```

```
                        410        420        430        440        450
 5  C25D6.GEL2   TCCTTTTCATAAGCTATGGG
10  C25E8.GEL6   TCCTTTTCATAAGCTATGGGATCGCTTACAACCAAAAGCACAATCCACTA
    CONSENSUS    TCCTTTTCATAAGCTATGGGATCGCTTACAACCAAAAGCACAATCCACTA

                        460        470        480        490        500
10  C25E8.GEL6   TTCAACGGACATCTTCATTGCCTGTTCCTTCGTCAAGTAATTTTAAAGAG
 9  C25G5.GEL5                                                  AAAGAG
    CONSENSUS    TTCAACGGACATCTTCATTGCCTGTTCCTTCGTCAAGTAATTTTAAAGAG

                        510        520        530        540        550
10  C25E8.GEL6   AGATTAAATAATA
 9  C25G5.GEL5   AGATTAAATAATATAGGAGGACTTAAGAGGTCAAGGACATTGGAAAGCTC
    CONSENSUS    AGATTAAATAATATAGGAGGACTTAAGAGGTCAAGGACATTGGAAAGCTC

                        560        570        580        590        600
 9  C25G5.GEL5   CTATGAAGATGAAACAGAGACTGCAAATAGACTGTCACGGGTTTCAAGTT
    CONSENSUS    CTATGAAGATGAAACAGAGACTGCAAATAGACTGTCACGGGTTTCAAGTT

                        610        620        630        640        650
 9  C25G5.GEL5   TGGTGAGCGTGATACGACAAACTATAGATCGCAAAAAATCACTAGAAAGA
11  C25F4.GEL7                                              TCACTAGAAAGA
    CONSENSUS    TGGTGAGCGTGATACGACAAACTATAGATCGCAAAAAATCACTAGAAAGA

                        660        670        680        690        700
 9  C25G5.GEL5   AGGGT
11  C25F4.GEL7   AGGGTAAGGGAAGAACAAGAAGAGAAGACAGATAATGAGGATGATAATGA
    CONSENSUS    AGGGTAAGGGAAGAACAAGAAGAGAAGACAGATAATGAGGATGATAATGA

                        710        720        730        740        750
11  C25F4.GEL7   TGTCGAAATATCTACTCAGGAATCCCTGGAAAACAATGG
 4  C25B9.GEL3             TCTACTCAGGAATCCCTGGAAAACAATGGTTTAGCGGAAA
 8  C25E4.GEL5                                                 GCGGAAA
12  C25B7.GEL3                                                     AAA
    CONSENSUS    TGTCGAAATATCTACTCAGGAATCCCTGGAAAACAATGGTTTAGCGGAAA

                        760        770        780        790        800
 4  C25B9.GEL3   AGAAGGATGATACTTCATCACTTGCTACGTTGGAAGATGACATTGAGGGC
 8  C25E4.GEL5   AGAAGGATGATACTTCATCACTTGCTACGTTGGAAGATGACATTGAGGGC
12  C25B7.GEL3   AGAAGGATGATACTTCATCACTTGCTACGTTGGAAGATGACATTGAGGGC
    CONSENSUS    AGAAGGATGATACTTCATCACTTGCTACGTTGGAAGATGACATTGAGGGC

                        810        820        830        840        850
 4  C25B9.GEL3   C
 8  C25E4.GEL5   CAAGAATTTTCGTTTGATGACCAAGATTTACAAATGCTTCAGGACATAGA
12  C25B7.GEL3   CAAGAATTTTCGTTTGATGACCAAGATTTACAAATGCTTCAGGACATAGA
    CONSENSUS    CAAGAATTTTCGTTTGATGACCAAGATTTACAAATGCTTCAGGACATAGA

                        860        870        880        890        900
 8  C25E4.GEL5   AGACCAATGGCTCAGTTCTCAAAAA
12  C25B7.GEL3   AGACCAATGGCTCAGTTCTCAAAAACAGCAAGGTTCTCCTCTAACATCAG
 7  C25C2.GEL4                                                 TCTAACATCAG
    CONSENSUS    AGACCAATGGCTCAGTTCTCAAAAACAGCAAGGTTCTCCTCTAACATCAG

                        910        920        930
12  C25B7.GEL3   ATCATATAAGCAAATAG
 7  C25C2.GEL4   ATCATATAAGCAAATAGAAAACGCTATTCGTAAAAAAAAAAAAAAAAAA
    CONSENSUS    ATCATATAAGCAAATAGAAAACGCTATTCGT
```

Appendix II

dom1+ DNA sequence contig

The DNA sequence contig shown below is that of the fission yeast *dom1*+ cDNA, the open reading frame is described in Chapter Four. The contig was generated using the Staden sequence assembly programmes (SAP).

```
                        10        20        30        40        50
 3  on1r.gel3;  GTTAATTAAGCTGAGAGGATCCTTCTCCATTTCTTTATCATGGCAAAAGA
 1  on1r.gel1;   TTAATTAAGCTGAGAGGATCCTTCTCCATTTCTTTATCATGGCAAAAGA
 2  on1r.gel2;   TTAATTAAGC*GAGAGGATCCTTCTCCATTTCTTTATCATGGCAAAAGA
  CONSENSUS     GTTAATTAAGCTGAGAGGATCCTTCTCCATTTCTTTATCATGGCAAAAGA

                       60        70        80        90       100
 3  on1r.gel3;  TAAAAAAGACCATAAGCATAGTGGAAGCACCGAAGATGAGTATGATTCAT
 1  on1r.gel1;  TAAAAAAGACCATAAGCATAGTGGAAGCACCGAAGATGAGTATGATTCAT
 2  on1r.gel2;  TAAAAAAGACCATAAGCATAGTGGAAGCACCGAAGATGAGTATGATTCAT
  CONSENSUS     TAAAAAAGACCATAAGCATAGTGGAAGCACCGAAGATGAGTATGATTCAT

                      110       120       130       140       150
 3  on1r.gel3;  ATCTTCCTGATTTAATGCCAATTGCT
 1  on1r.gel1;  ATCTTCCTGATTTAATGCCAATTGCTAAGCCTTTAGCTCCTAAAAAATTG
 2  on1r.gel2;  ATCTTCCTGATTTAATGCCAATTGCTAAGCCTTTAGCT
10  on1r.gel5;                                              TG
11  on1r.gel8;                                              TG
  CONSENSUS     ATCTTCCTGATTTAATGCCAATTGCTAAGCCTTTAGCTCCTAAAAAAATG

                      160       170       180       190       200
10  on1r.gel5;  AACAAAAAAATGATGAAGACTGTCAAGAAGGCTTCGAAGCAGAAACACAT
11  on1r.gel8;  AACAAAAAAATGATGAAGACTGTCAAGAAGGCTTCGAAGCAGAAACACAT
  CONSENSUS     AACAAAAAAATGATGAAGACTGTCAAGAAGGCTTCGAAGCAGAAACACAT

                      210       220       230       240       250
10  on1r.gel5;  CCTTCGTGGGGTCAAAGAGGTTGTAAAGGCTGTTAGAAAAGGAGAGAAGG
11  on1r.gel8;  CCTTCGTGGGGTCAAAGAGGTTGTAAAGGCTGTTAGAAAAGGAGAGAAGG
  CONSENSUS     CCTTCGTGGGGTCAAAGAGGTTGTAAAGGCTGTTAGAAAAGGAGAGAAGG

                      260       270       280       290       300
10  on1r.gel5;  GTCTTGTAATTCTTCCTGGTGATATTTCTCCCATGGATGTTATATCTCAT
11  on1r.gel8;
 5  on1r.gel9;    CTTGTAATTCTTCCTGGTGATATTTCTCCCATGGATGTTATATCTCAT
 4  on1r.gel7;                          atattctcccatggatgttatatctcata
  CONSENSUS     GTCTTGTAATTCTTCCTGGTGATATTTCTCCCATGGATGTTATATCTCAT

                      310       320       330       340       350
10  on1r.gel5;  ATTCCAGTATTATGTGAAGACAATAATGTCCCTTACTTATATACTGTTTC
 5  on1r.gel9;  ATTCCAGTATTATGTGAAGACAATAATGTCCCTTACTTATATACTGTTTC
 4  on1r.gel7;  attccagtattatgtgaagacaataatgtcccttacttatatactgtttc
  CONSENSUS     ATTCCAGTATTATGTGAAGACAATAATGTCCCTTACTTATATACTGTTTC

                     360       370       380       390       400
 4  on1r.gel7;  caaagagttgcttggtgaagcatcaaacaccaagagacctactagctgtg
 6  on1r.gel10  caaagagttgcttggtgaagcatcaaacaccaagagacctactagctgtg
 9  on1r.gel13  caaagagttgcttggtgaagcatcaaacaccaagagacctactagctgtg
  CONSENSUS     CAAAGAGTTGCTTGGTGAAGCATCAAACACCAAGAGACCTACTAGCTGTG
```

```
                          410        420        430        440        450
  4  on1r.gel7;   ttatgattgttcccggtggaaaaaagaaggacatgtctaaagttgaggag
  6  on1r.gel10   ttatgattgttcccggtggaaaaaagaaggacatgtctaaagttgaggag
  9  on1r.gel13   ttatgattgttcccggtggaaaaaagaaggacatgtctaaagttgaggag
   CONSENSUS      TTATGATTGTTCCCGGTGGAAAAAAGAAGGACATGTCTAAAGTTGAGGAG

                          460        470        480        490        500
  4  on1r.gel7;   tacaaggaaagctacgaggaaattataaaagaagtacctgctcttgaagt
  6  on1r.gel10   tacaaggaaagctacgaggaaattataaaagaagtacctgctcttgaagt
  9  on1r.gel13   tacaaggaaagctacgaggaaattataaaagaagtacctgctcttgaagt
 18  on1r.gel20                                              CCTGCTCTTGAAGT
   CONSENSUS      TACAAGGAAAGCTACGAGGAAATTATAAAAGAAGTACCTGCTCTTGAAGT

                          510        520        530        540        550
  4  on1r.gel7;   ttaattgttcaatatctaaaagatgctta
  6  on1r.gel10   ttaattgttcaatatctaaaagatgctta
  9  on1r.gel13   ttaattgttcaatatctaaaagatgctta
-16  on1r.gel18                                  gccatttaatgtagattatta
 18  on1r.gel20   TTAATTGTTCAATATCTAAAAGATGCTTAGCCATTTAATGTAGATTATTA
  8  on1r.gel12                                     ATTTAATGTAGATTATTA
  7  on1r.gel11                                     ATTTAATGTAGATT
   CONSENSUS      TTAATTGTTCAATATCTAAAAGATGCTTAGCCATTTAATGTAGATTATTA

                          560        570        580        590        600
 18  on1r.gel20   TATACTATTAAAATTTGGTATTTATCCTGGGATTTAACTTTTGGTATATC
-16  on1r.gel18   tatactatt
  8  on1r.gel12   TATACTATTAAAATTTGGTATTTATCCTGGGATTTAACTTTTGG*ATATC
 15  on1r.gel17                                  GGGATTTAACTTTTGGTATATC
   CONSENSUS      TATACTATTAAAATTTGGTATTTATCCTGGGATTTAACTTTTGGTATATC

                          610        620        630        640        650
  8  on1r.gel12   ATGTCTG
 15  on1r.gel17   ATGTCTGTTTGCTTGATAGTAAGCGAAAAGAAGTTTTGCTTAATGGTCGG
   CONSENSUS      ATGTCTGTTTGCTTGATAGTAAGCGAAAAGAAGTTTTGCTTAATGGTCGG

                          660        670        680        690        700
 15  on1r.gel17   TTTTAAGGTTTCAAAAAATTTCCTTTT
 13  on1r.gel15                aaatttccttttaatctagtgttttatcattctt
 12  on1r.gel14
   CONSENSUS      TTTTAAGGTTTCAAAAAATTTCCTTTTTAATCTAGTGTTTTATCATTCTT

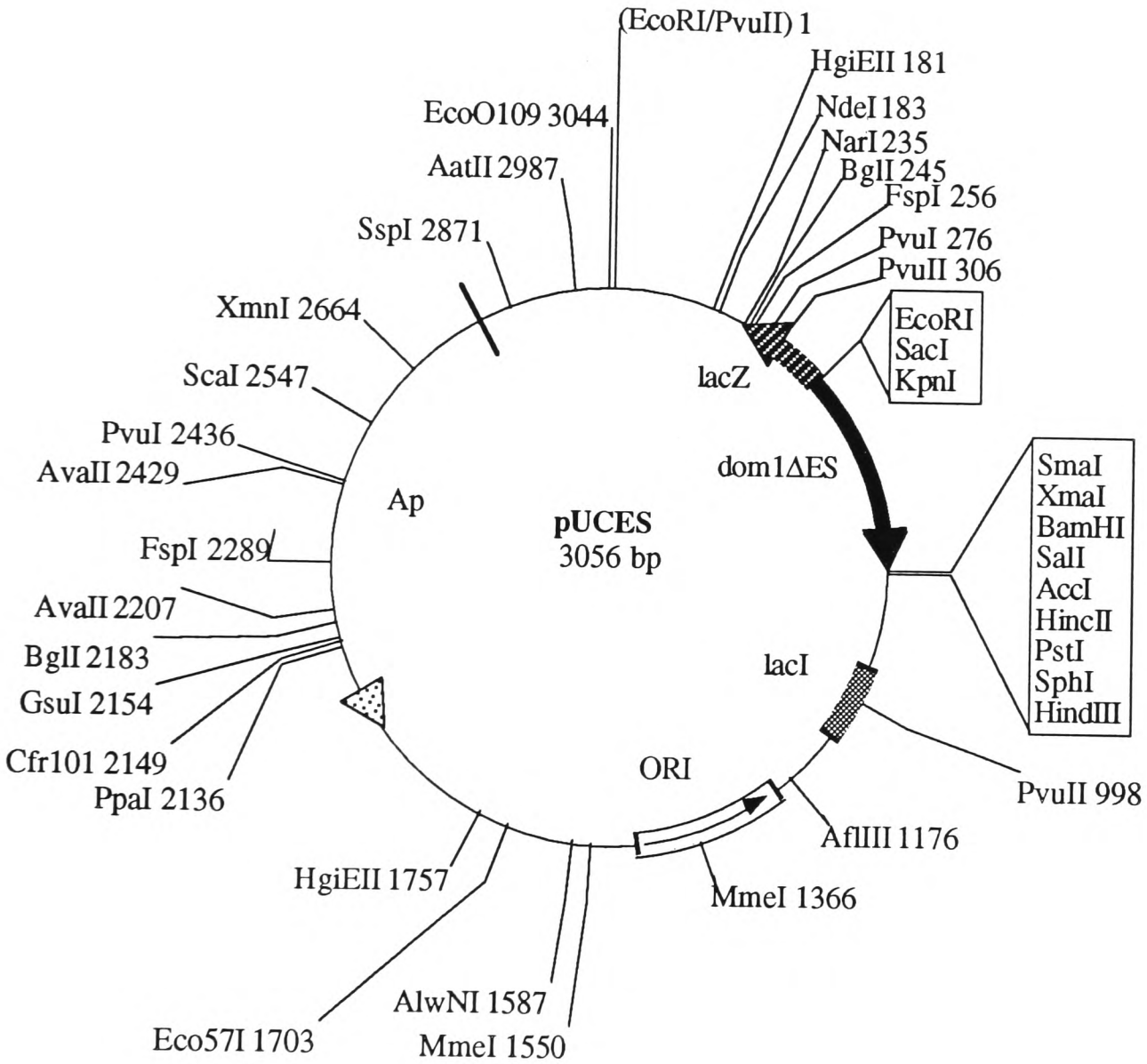
                          710        720        730        740        750
 13  on1r.gel15   tcttatgccctagtattttatgggtttagactggtttgcgagcaaactaa
 12  on1r.gel14   tcttatgccctagtattttatgggtttagactggtttgcgagcaaactaa
 14  on1r.gel16         GCCCTAGTATTTTATGGGTTTAGACTGGTTTGCGAGCAAACTAA
   CONSENSUS      TCTTATGCCCTAGTATTTTATGGGTTTAGACTGGTTTGCGAGCAAACTAA

                          760        770        780        790        800
 13  on1r.gel15   tcactatacctccaaaactttgttagcaataatatcgtatcatttaccaa
 12  on1r.gel14   tcactatacctccaaaactttgttagcaataatatcgtatcatttaccaa
 14  on1r.gel16   TCACTATACCTCCAAAACTTTGTTAGCAATAATATCGTATCATTTACCAA
   CONSENSUS      TCACTATACCTCCAAAACTTTGTTAGCAATAATATCGTATCATTTACCAA

                          810        820        830        840        850
 13  on1r.gel15   cttatatgagaatggtatatcgctgtaacagcgatgaaatatcaactgat
 12  on1r.gel14   cttatatgagaatggtatatcgctgtaacagcgatgaaatatcaactgat
 14  on1r.gel16   CTTATATGAGAATGGTATATCGCTGTAACAGCGATGAAATATCAACTGAT
   CONSENSUS      CTTATATGAGAATGGTATATCGCTGTAACAGCGATGAAATATCAACTGAT

                          860        870        880        890        900
 13  on1r.gel15   ttttcctcaaaaaaaaa
 12  on1r.gel14   ttttcctcaaaaaaa
 14  on1r.gel16   TTTTCCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
   CONSENSUS      TTTTCCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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The restriction map of the pUCES plasmid is shown below. The construction of this plasmid is described in Chapter Four (Figure 2.6). The direction of the arrow indicates the orientation of the insert relative to the the *dom1*$^{+}$ cDNA transcritpion. Boxed restriction sites indicate the pUC18 multi-cloning sites.



The restriction map of the pUCΔ*Esp* (F or R) plasmid is shown below. Its construction is described in Chapter Four (Figure 2.6). Boxed restriction sites indicate the pUC18 multi-cloning sites.

