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Cell cycle control of DNA synthesis in budding yeast

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The primary cell cycle control over DNA synthesis lies in an event called START. At this point in late G1 the cell assesses the environment and if conditions are appropriate START is traversed and cells become committed to the initiation of DNA replication and completion of the cell cycle. Genetic analysis in the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*, has shown that passing START requires the products of the *CDC28* and *cdc2*$^+$ genes, respectively (1–3). These genes encode a protein of some 34 kilodaltons (kd) with serine/threonine protein kinase activity (p34^{cdc2}) that is very highly conserved in evolutionary terms. It occurs in all organisms and it is also the active component in M-phase promoting factor (MPF), that controls the G2/M transition (for a review see ref. 4). Activation of this kinase is probably governed by its association with unstable proteins known as cyclins that accumulate at particular points in the cell cycle. It is the so-called 'G1-cyclins', encoded by the *CLN1, 2* and *3* genes in budding yeast, that are responsible for the activation of p34^{cdc2} at START.

p34^{cdc2} is controlled by a positive feed-back loop at START

The control of p34^{cdc2} at START has been well reviewed recently (5, 6) and will only be briefly summarised and updated here. The G1 cyclins are rate limiting for traverse of START and are believed to accumulate in late G1 leading to activation of p34^{cdc2} at the appropriate time. The accumulation of the Cln1 and Cln2 proteins can be accounted for by the cell cycle regulated transcription in late G1 of the *CLN1* and *CLN2* genes, together with the known instability of the proteins (reviewed in ref. 6). *CLN3*, however, is expressed constitutively and control of its activity is not understood.

Expression of *CLN1, CLN2* and a fourth G1 cyclin, *HCS26*, is under control of two transcription factors encoded by the *SWI4* and *SWI6* genes (7, 8). These genes were first characterised as being essential for the periodic expression in late G1 of the *HO* gene (9) encoding an endonuclease required for mating type switching. *HO* is not an essential gene yet deletion of both *SWI4* and *SWI6* is lethal (9). Moreover, *SWI4* is essential for viability of haploids at high temperature (at least in some strains) and of a/α diploids at all temperatures (8). The essential role of these genes has turned out to be the control of *CLN1* and *CLN2* (7,8). Surprisingly, it seems that *SWI4* and *SWI6* are also necessary for function of *CLN3* although its transcript is constant throughout the cell cycle (7).

Expression of the *SWI4* and *SWI6* genes is dependent (indirectly) on the *CDC28* gene (9), encoding the budding yeast p34^{cdc2} kinase. In turn, the p34^{cdc2} enzyme depends on the *CLNs* for activity, as outlined above, and these depend on *SWI4* and *SWI6* for their expression (7, 8). These dependencies can be resolved by invoking a positive feed-back loop (Fig.1) whereby low levels of active p34^{cdc2} would allow some *SWI4* and *SWI6* expression, leading to increased *CLN* synthesis, more active p34^{cdc2} and so on (7, 8, 10). The idea of a positive feed-back loop had been previously suggested by Reed (2) and Cross and Tinkelenberg (11).

A positive feed-back circuit of this sort could lead to a very rapid activation of p34^{cdc2} in response to environmental conditions, giving a virtual binary system which may be a paradigm for other developmental switches. A number of questions still remain unanswered, for instance what specifically triggers the initial activation of the loop and how it finally switches to the 'off' position.

p34^{cdc2} and initiation of S phase

Traversing START is of course essential for initiation of DNA synthesis and the completion of S phase. Activation of p34^{cdc2} and execution of START is not, however, a decision specifically to enter S phase. Rather, it is a commitment to enter and complete all the events of the cell cycle including M phase. In budding yeast other START-dependent events occur in late G1 (Fig.1). For instance, spindle pole body duplication occurs (analogous to centriole duplication in vertebrate cells which is also initiated early in the cell cycle); there is cell wall growth leading to bud emergence at about the beginning of S phase (for a review see ref. 12) and expression of the *HO* gene also takes place (13). Moreover, a number of genes are known to act in late G1 between START and S phase (see below), so that these processes are also genetically distinct. Theoretically, therefore, DNA synthesis could occur after these events, as part of a dependent sequence, without any direct involvement of p34^{cdc2}. However, experiments done in other systems do suggest that p34^{cdc2}, or a related enzyme, may have a direct role in controlling S phase.

Extracts of *Xenopus* eggs depleted for p34^{cdc2} are incapable of initiating DNA replication although they efficiently elongate replication forks that have already been initiated (14). Readdition of an eluate enriched for p34^{cdc2} allows initiation to proceed, arguing that p34^{cdc2}, or a homologue, is directly involved. In other experiments human extracts were used to study the cell free replication of SV40 DNA (15). These extracts were only active in supporting DNA synthesis when prepared from cells as they left G1 and entered S phase. Purification of the factor necessary for this *in vitro* replication activity showed it to be p34^{cdc2}, or a homologue. This again points to a direct role for this kinase in initiation, with the caveat that SV40 may not be

a perfect model for initiation studies, due to its unique use of T-antigen.

The precise form of the $p34^{cdc2}$ complex required for initiation of DNA synthesis in yeast is not yet clear. It may be the form activated at START which simply phosphorylates the S phase specific substrates as they are generated. Alternatively, it may be a different form of the kinase, perhaps associated with a different S phase-specific cyclin, to generate a S phase promoting factor, sometimes called SPF by analogy with MPF (see ref. 6). SPF would presumably have a different substrate specificity from the form active at START and would now recognise specific initiation proteins.

Cell cycle control of S phase also involves regulation of DNA synthesis genes

In *S.cerevisiae*, and perhaps other organisms as well, there is at least one other level of control to consider, namely regulation of genes encoding proteins required for DNA synthesis. In budding yeast, all of the genes required for DNA synthesis so far examined are expressed under cell cycle control at, or near to, the G1/S phase boundary, as are some of the genes required for initiation of replication (for reviews see refs. 16–18). All of these genes are coordinately regulated through a common cis-acting sequence, ACGCGT, an *Mlu*I restriction site that has become known as the '*Mlu*I cell cycle box' or MCB (19–21). This sequence is recognised by a transcription factor, DSC1 (for DNA Synthesis Control) (20), and it is this *Mlu*I-activation system (MAS) that controls the S phase specific genes (see below).

This system has in some way to be integrated with the controls exerted by $p34^{cdc2}$. Before this can be considered it is necessary to review in depth this system of gene expression and also to consider in detail the genes acting in late G1, some of which may be targets for $p34^{cdc2}$.

Genes acting in late G1

Genes required for initiation of DNA replication would be expected to act in late G1/early S phase and in budding yeast many such genes have been identified (Fig.2). Whether any of their encoded proteins are substrates for $p34^{cdc2}$ is not known, however some of them do contain the target sequence for this kinase, S/TPXZ, where X is a polar amino acid and Z is usually a basic amino acid (22).

CDC4 and CDC34. Temperature-sensitive *cdc4* and *cdc34* mutant cells have a similar phenotype and block in the cell cycle slightly later than *cdc28* at the restrictive temperature (Fig.2). The *CDC4* protein has homology with the *CDC36* gene product, required for START, and part of the *V-ets* oncogene protein, whilst *CDC34* encodes a ubiquitin conjugating enzyme that can use histones H2A and H2B as *in vivo* substrates (reviewed in refs. 5, 23). Since the *cdc4* mutant prevents expression of the histones H2A and H2B (24), conceivably the two genes could be associated with histone metabolism and exert an indirect effect on DNA synthesis. However, there is no firm evidence for any direct involvement with histones (the effect of *cdc4* on histone expression could be indirect and due to a cell cycle dependency), and both genes could function in an event other than DNA synthesis.

CDC7 and DBF4. The *CDC7* gene functions very close to the initiation of DNA synthesis and there has been much speculation, but little evidence, that it is involved in initiation (reviewed in refs. 5, 23, 25). The gene encodes a protein having homology to serine/threonine protein kinases (26) that has been shown *in vitro* to have kinase activity (27). Moreover, the protein contains the sequence SPQR, a potential target for $p34^{cdc2}$ (see above), suggesting it could be controlled directly by *CDC28*. The S phase function of *CDC7* can also be completed in the absence of protein synthesis (reviewed in 23), consistent with some form of post-translational control.

The *DBF4* gene functions immediately before *CDC7* but, unlike *CDC7*, following *DBF4* function subsequent protein synthesis is required before S phase (28). The Dbf4 and Cdc7 proteins appear to interact with one another (29). Each gene when present in high copy number is able to suppress defects in the other gene and whilst cells mutant for either gene alone grow normally at a permissive temperature, cells containing both mutants are inviable at any temperature. The *DBF4* gene is expressed transiently under cell cycle control at the G1/S phase boundary (30) and the upstream sequence contains the MCB elements, so that it is likely to be controlled as part of the MAS.

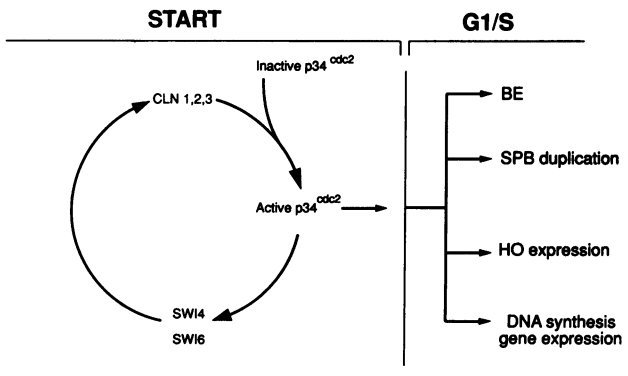

Figure 1. $p34^{cdc2}$ is controlled by a positive feed-back loop. Active $p34^{cdc2}$ (encoded by *CDC28* in budding yeast) is required for expression of the *SWI4* and *SWI6* genes encoding transcription activators which regulate periodic expression of *CLN1* and *CLN2* in late G1. *SWI4* and *SWI6* also regulate *CLN3* by some as yet unknown mechanism. The *CLN* gene products ('G1' cyclins) in turn associate with inactive $p34^{cdc2}$ to give the active protein kinase. Once activated, $p34^{cdc2}$ controls, either directly or indirectly, the events of late G1 which include bud emergence (BE), spindle pole body (SPB) duplication, expression of the *HO* gene and initiation of DNA synthesis.

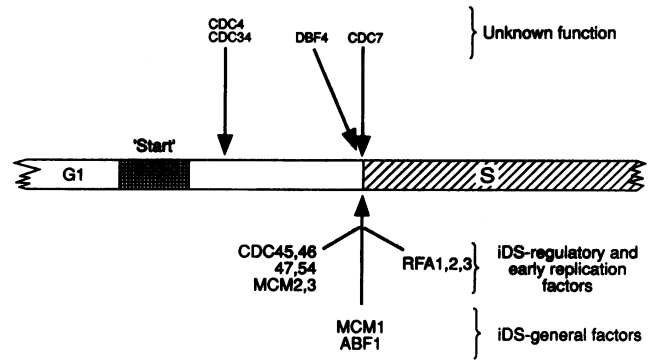

Figure 2. Budding yeast genes acting in late G1 and their approximate order of function. iDS = initiation of DNA synthesis.

MCM1, 2 and 3. These three genes were first identified as mutants defective in the maintenance of minichromosomes, their phenotypes suggesting that the gene products are involved in the initiation of replication at autonomously replicating sequences (ARSs, yeast chromosomal origins of replication, see refs. 23, 31) (32–34). Mcm1 appears to be a general transcription factor that in addition functions at ARSs (35, 36), it therefore seems unlikely to be involved in primary control of initiation.

The *Mcm2* mutation causes the loss of minichromosomes rather than their mis-segregation, consistent with a defect in replication (33). In addition, *mcm2* and *mcm3* mutants have pleiotropic phenotypes characteristic of other mutants defective in DNA synthesis, including increased levels of mitotic recombination and chromosome loss (33, 37). The Mcm2 and Mcm3 proteins are strikingly homologous over their entire lengths and Mcm2 might interact directly with DNA since it contains a zinc-finger domain that is essential for function (38). Cells mutant in *MCM2* or *MCM3* do synthesise substantial amounts of DNA and they have a premitotic arrest point (37, 38). Mcm2 and Mcm3 therefore might not affect initiation of all replicons but each may be specific for particular sets of ARS elements. They also have homology with the *nda4*$^+$ and *cdc21*$^+$ gene products in *S.pombe* and, significantly, with the protein encoded by *CDC46* (*S. cerevisiae*) (38), another gene with a role in late G1 (see below). We note that the Mcm3 protein has no fewer than four potential p34^{cdc2} target sites in the C-terminal part of the protein; SPQK, SPKK, TPRR and SPRR.

CDC45, 46, 47 and 54. Cdc45 and *54* were isolated as cold-sensitive cell cycle mutants and *cdc46* and *47* are temperature-sensitive suppressors of *cdc45* and *54* (39). Double mutant and suppression analysis indicates that this group of genes interacts with one another and that they are therefore acting in the same pathway (40). *Cdc45, 46* and *47* are defective in initiation of DNA synthesis and at the restrictive temperature cells accumulate with a DNA content equivalent to a single genome (40, 41). Like *mcm2* and *mcm3* and consistent with a defect in DNA replication, *cdc45, 46* and *54* show increased levels of recombination and chromosome loss (40). Cdc46-specific antibodies recognise proteins in both fission yeast and in mouse. The equivalent *S.pombe* protein may be encoded by the cell cycle gene *nda4*$^+$ since its gene product has extensive homology to Cdc46 protein (40). As mentioned above Cdc46 also has substantial homology with Mcm2 and Mcm3 and their similar phenotypes provide general support for the notion that they are all involved in initiation of DNA replication.

One other property of the Cdc46 protein is of particular interest, its nuclear localisation is cell cycle regulated (41). Interestingly, it is not present in the nucleus during S phase. Cdc46 is predominantly nuclear in G1 prior to bud formation, which is roughly coincident with onset of S phase, but then it migrates to the cytoplasm. The protein reappears in the nucleus in late mitotic cells. It has been suggested that this entry into the nucleus triggers initiation of DNA replication in the next cell cycle, in effect coupling two cell cycles (41). Presumably the nuclear migration is simply a prerequisite for DNA replication since additional controls at START are obviously necessary to maintain the option of leaving the cell cycle. The behaviour of the *CDC46* gene product is consistent with the model of Blow and Laskey (42) for restricting the initiation of DNA replication to only one event per cell cycle (for additional discussion of this see ref. 43).

The *CDC46* gene is also expressed under cell cycle control and the authors conclude that the transcript peaks in late mitosis (41). *CDC46* has a MCB in its upstream and all of the other genes that have this sequence, at least eighteen in number, are expressed at the G1/S phase boundary (see below). No comparison was made with any of these other genes but since all of them are involved in S phase it would seem likely that *CDC46* is expressed as part of the MAS.

RFA1, 2 and 3. Human RF-A is a protein complex with single-stranded DNA binding activity that cooperates with SV40 large T antigen and DNA topoisomerase I to unwind the SV40 origin of replication *in vitro* (for reviews see 43–45). It is composed of 70-, 34- and 11-kd subunits and a similar yeast complex with 69-, 36- and 13-kd subunits has been identified (46). These yeast subunits are encoded by the *RFA1, 2* and *3* genes, respectively,

Table 1. Budding yeast genes expressed in late G1 under control of the *Mlu1*-activation system.

Gene	Gene Product	Reference
POL1	DNA polymerase I	59
POL2	DNA polymerase II	60
POL3	DNA polymerase III	61
POL30	PCNA	61
DPB2	DNA polymerase II subunit B	62
DPB3	DNA polymerase II subunit C	63
PRI1	DNA primase I	64
PRI2	DNA primase II	65
CDC6	?	66
CDC8	Thymidylate kinase	24
CDC9	DNA ligase	67
CDC21	Thymidylate synthase	68
RNR1	Large subunit of ribonucleotide reductase	69
RFA1		
RFA2	Replication factor A	47
RFA3		
DBF4	Interacts with CDC7	30
RAD51	?	a.

a. G.Basile, pers. comm.

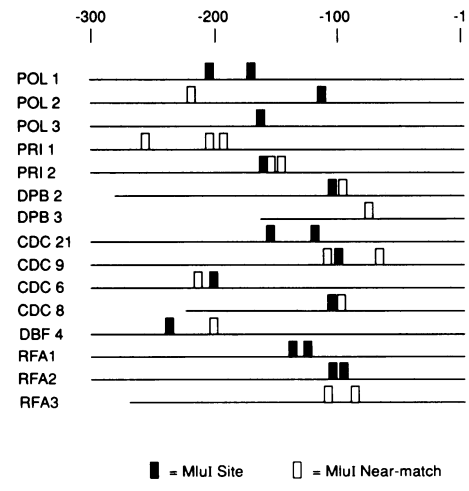

Figure 3. The distribution of MCBs and near-matches in the upstream of various DNA synthesis genes. The continuous lines represent upstream sequence of the genes and the numbers given are with respect to the ATG codon.

and each gene is essential for viability (47). The 36/34 kd subunit from both human and yeast is phosphorylated in a cell cycle dependent manner suggesting that RF-A could have a regulatory role, perhaps linking initiation of replication to cell cycle control (48). Reinforcing the idea that RF-A could have a regulatory role, each of the genes for the three subunits in yeast have MCB elements in their promoter regions and hence are expressed under cell cycle control at the G1/S phase boundary (47).

ABF1. The Abf1 protein, first identified by binding to ARS1 (49), is now known to act at a large number of ARS elements and to be identical to OBF1 (50). Its binding sites are not confined to ARS elements (51) and, like Mcm1, it seems to be a general transcription factor that also functions at origins of replication.

SWI4 and SWI6. These genes were originally identified as mutants defective in mating type switching and they are required for transcription of the *HO* gene (9). Neither is an essential gene but deletion of both is lethal (9). The nuclear localisation of Swi6 is cell cycle regulated (52) and both Swi4 and Swi6 are components of a complex that binds to CACGAAAA sequences found upstream of the *HO* and the *CLN* genes (7, 8, 52–54). Through their regulation of the *CLN* genes, *SWI4* and *SWI6* are involved in the positive feed back loop that controls the $p34^{cdc2}$ protein kinase at START (7, 8, 10) (Fig.1). Both *SWI4* and *SWI6* are also involved in control of *CTS1* (55), encoding chitinase, and, in addition, Swi6 is a component of the DSC1 transcription factor that regulates DNA synthesis genes (see below).

Swi4 and Swi6 have homology to one another and also to the fission yeast Cdc10 protein (56, 54). The homology is most significant within two 33 amino acid repeats (56) that also occur in a number of metazoan proteins including those encoded by the *Drosophila* Notch gene, several *Caenorhabditis* genes and the erythrocyte ankyrin gene (for a review see ref. 17). These repeats have become known as the 'cdc10-SWI6 motif' or, alternatively, as the 'ankyrin motif'. The Swi6 protein contains two sequences that could be $p34^{cdc2}$ targets.

DBF2 and DBF3. These genes were originally identified as temperature-sensitive mutations affecting DNA synthesis under restrictive conditions(57, 28). More detailed analysis has shown that each mutation causes no more than a forty minute delay in DNA synthesis and, at least in a *dbf2* mutant, the cell cycle block occurs after S phase (58). Therefore they probably do not have a primary defect in DNA synthesis. They may have some role in events preceding DNA synthesis which, when blocked, lead to a delay in DNA synthesis as a secondary consequence. Conceivably, this delay may be due to a pre-S phase cell cycle check point.

	1.	2.	3.	4.	5.	6.	7.	8.
	A_{30}	A_0	A_0	A_0	A_0	A_5	A_8	A_{20}
	C_0	C_{31}	C_0	C_{31}	C_0	C_3	C_8	C_2
	G_0	G_0	G_{32}	G_0	G_{32}	G_1	G_6	G_7
	T_2	T_1	T_0	T_1	T_0	T_{23}	T_{10}	T_3
Summary:	$A_{30/32}$	$C_{31/32}$	$G_{32/32}$	$C_{31/32}$	$G_{32/32}$	$T_{23/32}$	N	$A_{20/32}$ $G_{7/32}$

Figure 4. Homology between *MluI* hexamers, near-matches and their surrounding sequence. The sequence from a total of 32 MCB elements together with some 40 bp of surrounding sequence was compared.

The cell cycle regulation in late G1 of genes required for DNA synthesis

All of the genes required for DNA synthesis in budding yeast examined so far are expressed under cell cycle control at G1/S phase (Table 1). Since a total of 18 genes involved in all aspects of replication are controlled in this way, it seems very likely that all DNA synthesis genes will be regulated in the same way. We have shown that *CDC8, CDC9, CDC21, PRI1, POL1, POL2, DPB2, DPB3* and *RNR1* are expressed at the identical point in the cell cycle in whatever manner the cells were synchronised (refs. in Table 1). Moreover, when *cdc28, cdc4, dbf4* or *cdc7* mutants were used to block cells at various points in G1 (Fig.2), those DNA synthesis genes examined were either all expressed together or were all switched off (24, unpubl. obs.). Thus the genes appear to be coordinately regulated.

Of the genes listed in Table1, *DBF4, CDC46* and *RAD51* are not required directly in DNA replication. *DBF4* and *CDC46* may be involved in controlling S phase (see above) whilst *RAD51* participates in recombinational repair of DNA (70). Its expression together with the DNA synthesis genes under control of the MAS suggests it may also have a role in S phase, possibly in repair of post-replication lesions. Significantly, the *rad51* mutation causes an increase in spontaneous rates of both mutation frequency and chromosome loss (71, 72). If *RAD51* does have a role in repair of replication errors it seems very likely that other post-replication repair genes may also be expressed under cell

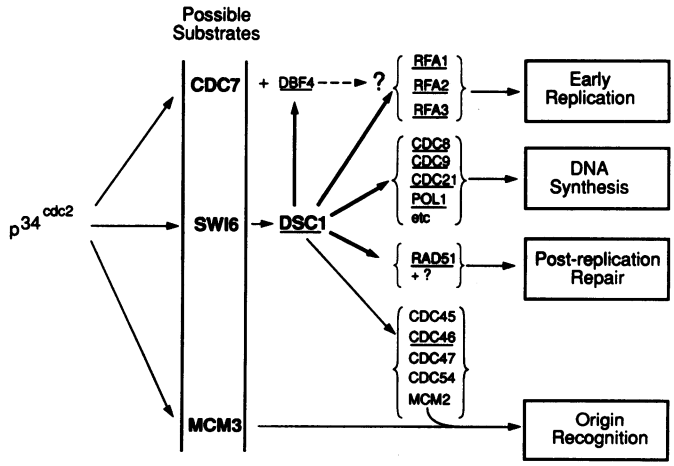

Figure 5. A hypothetical scheme showing the possible coordination of events in late G1. The *CDC7, SWI6* and *MCM3* genes encode proteins that contain target sequences for $p34^{cdc2}$ (see text). The Cdc7 protein is a serine-threonine protein kinase and the *DBF4* gene product interacts with it. No substrate for Cdc7 is known. The Swi6 protein is a component of the DSC1 transcription complex which regulates a large number of DNA synthesis genes (underlined). These genes are involved in all aspects of DNA replication so that DSC1 must play a central role in controlling or coordinating the events associated with S phase. The *MCM3* gene product is involved in initiation of replication and probably interacts with the Mcm2 protein and also the proteins encoded by the genes grouped with *MCM2*. Thus if $p34^{cdc2}$ did regulate the *CDC7, SWI6* and *MCM3* gene products, it could exert direct control over the whole of S phase. Note that the form of $p34^{cdc2}$ active at this stage of the cell cycle may not be the form active at START.

cycle control at G1/S phase. Hence, the genes of the MAS can be regarded as being specific for S phase rather than for DNA replication alone.

A hexamer, ACGCGT, controls the periodic expression of the genes

In the three genes examined in detail *CDC21*, *CDC9* and *POL1* the regulation is primarily transcriptional (19, 73, 74). As mentioned above, these three genes, and most of those in Table1, have in their upstreams a 6 bp sequence in common, ACGCGT, the MCB element. These elements lie between −90 and −250 with respect to the ATG in most of the genes (Fig.3). *PRI1*, *DPB3* and *RFA3* lack canonical ACGCGTs but do have a degenerate form of the sequence (Figs. 3, 4). Deletion of sequences containing the MCBs from *CDC21*, *CDC9* or *POL1* abolishes periodic expression of the genes (19, 20, 74). Synthetic oligonucleotides containing ACGCGT sequences inserted into appropriate test plasmids act as strong enhancers and, more important, these sequences are sufficient for correctly timed periodic expression of a reporter gene (19, 20). MCB elements are therefore responsible for both the periodic expression and the coordinate regulation of the DNA synthesis genes.

A survey of all the MCBs from the genes listed in Table1 emphasises the importance of the central CGCG (Fig.4). This survey and the analysis of McIntosh *et. al.* (19), indicates the essential core to be ACGCGNNPu, with a preference for T and A residues at positions 6 and 8, respectively.

The minimal sequence necessary for correctly timed expression appears to be tandem repeats of the *Mlu*I core itself. Three synthetic copies of the ACGCGT sequence were sufficient for cell cycle expression of a *lacZ* reporter gene at G1/S phase (20). Expression was, however, early in the first cycle of each of the two synchronous cultures examined, including an elutriated culture. Possibly transcription of the reporter gene from the high copy number vector used is affected by the perturbations which are present in the first cycle of all synchronised cultures. Alternatively, sequences in addition to the minimal ACGCGT could be required for correctly timed expression in the first cycle, for instance, the additional purine at position 8 (Fig.4). McIntosh *et. al.* (19) found that a single MCB with this additional purine was an effective enhancer, whereas in our experiments a single minimal MCB was not, at least two such sequences being required (20). We have recently inserted MCB elements with an additional A residue at position 8 into a test plasmid (20) but again, single elements were not active (our unpubl. obs.). Possibly somewhere on the vector used by McIntosh *et. al.* (19) there might be a degenerate form of an *Mlu*I sequence (or even an unrelated sequence) which can act cooperatively with the single inserted synthetic copy to stimulate expression.

Consistent with the need for mutiple MCBs the great majority of the genes have at least two of the elements (Fig.3). Only *POL3*, *POL30* and *DPB3* have single MCBs but in each case only limited upstream sequence is available. Apart from the purine at position 8 there is no significant conservation of sequence surrounding the MCBs. A high proportion do have tracts of As or Ts associated with them but their significance, if any, is not clear.

Proteins binding to the MCBs

The key to the periodic expression of the DNA synthesis genes is of course the transcription factor that recognises the MCBs. Using a synthetic substrate in gel retardation assays a protein complex, DSC1, that specifically binds to ACGCGT sequences was detected (20). Consistent with the sequence analysis of the MCBs described above, this complex did not bind to the sequence AC*ta*GT in which the central nucleotides of the MCB were changed. Significantly, the binding of DSC1 is cell cycle regulated, with the peaks of binding activity coinciding roughly with expression of the DNA synthesis genes (20). Binding was, however, early in the first cycle of both types of synchronous culture examined and in fact followed the precise pattern of expression of the MCB-driven *lacZ* reporter gene (see above). So *in vivo* there might be other regulatory elements that modulate the control exerted by the MCBs and/or DSC1.

Partial purification of yeast extracts has also led to the identification of a protein that binds specifically to *Mlu*I sequences (MCBF = MCB binding factor) (75). In contrast to DSC1, MCBF is able to bind to a single MCB element. MCBF is a 17kd protein that DNaseI footprinting showed to specifically recognise *Mlu*I sequences. The relationship between DSC1 and MCBF is not at all clear. DSC1 causes a very much larger retardation of its substrate than does MCBF suggesting it contains larger proteins or more species. DSC1 is also of physiological significance. Its binding is regulated in the cell cycle and the *SWI6* transcription factor is a component of DSC1 (see below). Moreover, a similar complex has been detected in fission yeast containing the *cdc10*$^+$ protein which has homology with Swi6 (see below). Conceivably, MCBF may correspond to a proteolytic fragment of a DSC1 component that has retained some aspects of DNA binding.

Swi6 is a component of DSC1

The Swi4 and Swi6 transcription factors control the expression of the *HO* and *CLN* genes which are expressed in late G1 at about the same time as the DNA synthesis genes. *swi4* and *swi6* mutants were therefore screened for any effect on DSC1 and whilst *swi4* had no detectable effect, in *swi6* mutants DSC1 was undetectable (N.F. Lowndes, L. Breeden, L.H. Johnston, unpubl. obs.). Moreover, the use of antibodies against Swi6 showed that rather than controlling DSC1, Swi6 is a component of the complex.

The sequences recognised by the Swi4/Swi6 complex in the upstreams of the *HO* and *CLN* genes is $CACGA_4$ (7−9), compared to the MCB sequence ACGCGT. Presumably, therefore, another component of DSC1, perhaps analogous to Swi4, must be largely responsible for DNA sequence recognition and Swi6 may interact with this by means of the *SWI6*−*cdc10*$^+$ motifs. A structure for such a heteromeric transcription factor has been proposed (76).

The *SWI6* gene appears to be the common feature in expression of the *HO*, *CLN*, *CTS*1 and *DNA* synthesis genes (77). Swi6 might therefore be the molecule that senses cell cycle position and it might be responsible for the timing of the expression in late G1 of all these genes. Since Swi6 has target sequences for the $p34^{cdc2}$ kinase and is part of the positive feedback control of *CDC28* (Fig.1), it may be a substrate for this kinase and the phosphorylation event may be the means by which cell cycle position is assessed.

The *Mlu*I activation system is conserved in fission yeast

In *S.pombe* the *cdc22*$^+$ gene, encoding a subunit of ribonucleotide reductase (P.Fantes, pers. comm.), is the only gene so far known to be expressed at the G1/S phase boundary (78). Sequencing of *cdc22*$^+$ revealed no fewer than seven *Mlu*I elements in its promoter region (79). Moreover, as in budding yeast, synthetic ACGCGT sequences confer correctly timed cell

cycle expression on a reporter gene (79). Crude extracts from *S.pombe* also contain an activity that binds to ACGCGT sequences with a specificity indistinguishable from that of DSC1, indicating that the MAS is conserved in fission yeast(79).

As DSC1 controls cell cycle genes essential for DNA synthesis, certain mutants defective in DSC1 might themselves be cell cycle mutants with execution points in late G1. In *S.pombe* only *cdc2*$^+$ and *cdc10*$^+$ are known to act in late G1 and, significantly, the *cdc10*$^+$ protein has homology with both the *SWI4* and *SWI6* gene products (54, 56) and, in addition, the *cdc10*$^-$ mutation affects expression of *cdc22*$^+$ (quoted in ref. 79). When assayed by gel retardation the DSC1-like activity was undetectable in *cdc10*$^-$ mutants. As in the case of *SWI6*, the use of antibodies to the *cdc10*$^+$ protein showed that it was a component of the *S.pombe* DSC1-like complex (79).

The *cdc10*$^-$ mutation leads to a block at START (80) suggesting that it may control other genes in addition to *cdc22*$^+$. *SWI4* and *SWI6* have an essential role in the control of budding yeast G1 cyclins so that *cdc10*$^+$ may control the *S.pombe* homologues of these genes. Indeed by analogy with *SWI4* and *SWI6*, *cdc10*$^+$ might have a wide spectrum of targets and by analogy with *SWI6*, specifically, these genes may not necessarily have *Mlu*I sequences in their upstream. Like Swi6, the *cdc10*$^+$ protein has recognition sequences for phosphorylation by p34^{cdc2} (81), indeed it is known to be a phosphoprotein (82). Again, like Swi6, Cdc10 may be the connection between START and the expression of genes in late G1.

Fission yeast has diverged very widely in evolutionary terms from budding yeast (83) so that cell cycle controls conserved in both organisms may well be widespread in nature. Thus the conservation of the MAS in this organism argues that it may be present in other eukaryotes.

Physiological significance of the *Mlu*I activation system

Although the MAS is conserved in fission yeast it is far more limited in extent. For instance, *cdc22*$^+$ is the only DNA synthesis gene known to be expressed at G1/S phase and the fission yeast DNA ligase gene, *cdc17*$^+$, is not cell cycle regulated unlike *CDC9* its budding yeast counterpart (67). Moreover, many of the DNA synthesis genes that are expressed under cell cycle control in budding yeast are not periodic in metazoan cells although some are, including thymidylate synthase and ribonucleotide reductase (for a more detailed discussion and references see 16, 84). Whether the periodic metazoan genes are regulated by sites related to ACGCGT, together with a DSC1-like activity is not yet clear. The nearest analogous metazoan transcription factor may be E2F/DRTF (for a review and references see 85), which has the target sequence TTTCGCGC (86). E2F is active in late G1 and has binding sites in the DHFR gene (86) which is expressed under cell cycle control (87). At most, therefore, it seems that the MAS may only be partly conserved in other organisms.

This limited conservation of the MAS, even in fission yeast, raises the question of why it should be so extensive in budding yeast. It is certainly hard to see why a gene like *CDC9* should be periodically expressed as it encodes an extremely stable protein (88). Indeed of all the genes in the MAS in *S.cerevisiae*, only thymidylate synthase and ribonucleotide reductase are so far known to encode unstable products that seem to be synthesized *de novo* each cycle in an exponential culture (for a more detailed discussion and references see 16). One possible explanation is that the MAS plays a coordinating role in budding yeast allowing a more rapid completion of late G1 events and a quicker onset of S phase. In short, it may be an adaptation to provide a faster growth rate. Being a single-celled saprophyte that is dispersed passively, there certainly would be selective pressure for rapid growth in budding yeast to take full advantage of any nutrients encountered. Alternatively, the MAS may be explained by the fact that yeast cells in the wild must spend a great deal of time in a non-growing state, awaiting a supply of nutrients. In effect they are in very late stationary phase for much of their life cycle. Many of the proteins necessary for S phase may decay during this time and resynthesis would then be essential prior to S phase in the first cell cycle.

A third consideration is that many of these DNA synthesis genes are also induced by damage to DNA (for example see 56, 69, 88), presumably to carry out the necessary repair synthesis. As we have suggested before (88), this inducibility might be an adaptation to spending long periods in stationary phase and although the relationship between the MCBs and DNA damage inducibility is not yet clear, the two systems may have evolved in an inter-related manner.

Coordination of events controlling S-phase

The initiation of DNA replication can be considered as three separate processes; production of the necessary proteins, the sequence specific recognition of origins and the establishment of replication forks by the 'early replication proteins' (probably including RF-A, a helicase and topoisomerase I, refs. 43–45). In none of these three areas has an *in vivo* substrate for the p34^{cdc2} protein kinase been demonstrated. However, in yeast potential targets for the kinase do exist, namely Swi6, Mcm3 and Cdc7 and the link between START and S phase could occur by means of these proteins (Fig.5). So, for instance, phosphorylation of Mcm3 could control the recognition of at lease a subset of replication origins.

In the case of Cdc7, also a serine/threonine kinase, phosphorylation by p34^{cdc2} would presumably activate the kinase activity. The *DBF4* protein, which interacts with Cdc7, could be a subunit of the Cdc7 activity and might, for instance, influence its specificity. Note that *DBF4* is itself regulated since the gene is expressed as part of the MAS, so that *CDC7* could be subject to dual regulation. No likely substrate for Cdc7 is known. Incidentally, if Cdc7 had a role in controlling *DSC1*, and since *DBF4* in turn is controlled by DSC1, there is the possibility of a positive feed-back loop regulating DSC1.

Control of DSC1 almost certainly involves Swi6. It is a vital component of the complex, it has phosphorylation sites for p34^{cdc2} and it is an *in vitro* substrate for p34^{cdc2} (unpubl. obs). The related protein in fission yeast encoded by *cdc10*$^+$ also contains p34^{cdc2} sites and is known to be a phosphoprotein. Moreover, *SWI6* is expressed at START as part of the positive feedback loop controlling p34^{cdc2}. *SWI6* also regulates the *HO*, *CTS1* and *CLN* genes and phosphorylation of Swi6 might be the means by which cell cycle position is assessed in order to control expression of these genes in late G1. As argued above, DSC1 and the whole MAS may play a coordinating role to facilitate late G1 events. It controls expression of genes participating in all aspects of replication (Fig.5) but how many of these also involve the *de novo* synthesis of the protein concerned is not known. Whether expression of any genes required for bud emergence or spindle pole body metabolism are integrated into late G1 events through control by the MAS, or by some other aspect of *SWI6* regulation, is also not clear.

p34^{cdc2} might directly control all the events associated with initiation if it regulates DSC1 via Swi6 as well as regulating Cdc7 and Mcm3. Preparation for S phase could perhaps occur largely in G1 before START and then once p34^{cdc2} was activated there could be immediate initiation of S phase. This view would be consistent with the experiments of d'Urso *et al.* (15) on the *in vitro* replication of SV40. However, at least in yeast there are other G1 events to be considered, that is, bud emergence and spindle pole body metabolism together with expression of the DNA synthesis genes. Expression of these genes in even the best synchronous cultures lasts at least 20 minutes. However, allowing for the degree of synchrony in the culture (for example, by the time taken for complete bud emergence), expression can be seen to occupy well under one minute in individual cells. Translation then has to occur, at least for those enzymes such as thymidylate synthase and ribonucleotide reductase that are synthesised *de novo* in each cell cycle, which would overlap transcription and occupy a few minutes at most. Thus, in principle, and assuming that bud emergence and spindle pole body metabolism are not limiting, DNA synthesis could occur shortly after START in yeast, perhaps under direct control of the p34^{cdc2} activated at START. Alternatively, other events in late G1 may be limiting, indeed there may be as yet uncharacterised regulatory events, when there may be no direct link between START and initiation of DNA synthesis. In this case, the putative S phase-specific form of the kinase would be more likely to take over from the START-specific form.

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