The yeast DNA ligase gene *CDC9* is controlled by six orientation specific upstream activating sequences that respond to cellular proliferation but which alone cannot mediate cell cycle regulation

Julia H.M.White+, Anthony L.Johnson, Noel F.Lowndes and Leland H.Johnston*
Laboratory of Cell Propagation, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

Received August 22, 1990; Revised and Accepted December 7, 1990

ABSTRACT

By fusing the *CDC9* structural gene to the PGK upstream sequences and the *CDC9* upstream to *lacZ*, we showed that the cell cycle expression of *CDC9* is largely due to transcriptional regulation. To investigate the role of six ATGATT upstream repeats in *CDC9* regulation, synthetic copies of the sequence were attached to a heterologous gene. The repeats stimulated transcription strongly and additively, but, unlike conventional yeast UAS elements, only when present in one orientation. Transcription driven by the repeats declines in cells held at START of the cell cycle or in stationary phase, as occurs with *CDC9*. However, the repeats by themselves cannot impart cell cycle regulation to a heterologous gene. *CDC9* may therefore be controlled by an activating system operating through the repeats that is sensitive to cellular proliferation and a separate mechanism that governs the periodic expression in the cell cycle.

INTRODUCTION

The level of the transcript from the *Saccharomyces cerevisiae* DNA ligase gene *CDC9* fluctuates sharply in the cell cycle (1,2), reaching a peak in late G1 and declining to background levels in early S phase (2). Other DNA synthesis genes in yeast are regulated in the same way, including *CDC8* (thymidylate kinase) (3), *CDC21* (thymidylate synthase) (4), *POL1* (DNA polymerase I) (5) and *PRI1* (DNA primase I) (6) and in each case the transcripts peak at the same point in the cell cycle. Indeed, we believe that these genes are coordinately regulated as it has not proved possible to separate the expression of the genes from one another by blocking cells at various points in G1 or S phase (by use of appropriate cell cycle mutants). They are either all expressed together or none are expressed (3,5, J.H.M. White, PhD thesis, C.N.A.A., London). In contrast, expression of the histone H2A, which is also cell cycle regulated (7), can be readily separated from the DNA synthesis genes by this means (3), suggesting that they may be controlled by separate mechanisms.

At present little is known about the mechanism controlling expression of the DNA synthesis genes and we are therefore examining the regulation of *CDC9*. It is not even clear whether this regulation occurs at a transcriptional level or whether changes in message stability are responsible. Preliminary experiments on *CDC21* suggest that it is regulated largely at a transcriptional level (8). However, with histone H2A message turnover also plays a part (9). In this paper we therefore initially investigated the molecular basis of *CDC9* control and showed it to be transcriptional.

The upstream sequence of the gene has revealed six repeats of the hexamer ATGATT which may be significant in this control. These are scattered over some 650 bp of DNA and are reminiscent of yeast upstream activating sequences (UASs). These usually contain short repeat sequences which are found 200–450 bp upstream from the start of transcription (for reviews see 10,11,12). Generally, yeast UASs resemble mammalian enhancer sequences in that deletion of these elements significantly reduces transcription from that gene and they also function in either orientation and at long and variable distances from the gene. Frequently these elements not only stimulate transcription but are also involved in the control of gene expression.

In the case of yeast genes which are periodically transcribed in the cell cycle, the histone H2A-H2B gene pair has three repeats of the sequence GAGCGAAA (13) whilst the HO gene encoding an endonuclease involved in mating type switching, has ten repeats of the sequence CACGAAAA (14). In both cases these sequences have been shown to stimulate transcription when present in either orientation and to mediate, by themselves, the periodic transcription of a heterologous *lacZ* gene (15,16,13). This highlights the possible significance of the ATGATT repeats in *CDC9* regulation and we have therefore inserted synthetic copies upstream of the *lacZ* gene to assess their possible role. They stimulate transcription strongly and in an additive fashion

* To whom correspondence should be addressed

+ Present address: Department of Genetics, Glaxo Group Research, Greenford Road, Greenford, Middlesex UB6 OHE, UK

but, surprisingly, only when present in one orientation. By themselves the ATGATT sequences are not able to impart cell cycle regulation to a heterologous gene but they do mediate a form of proliferation control. When cells are held at START at the beginning of the cell cycle or in stationary phase, transcription driven by the repeats declines.

MATERIALS AND METHODS

Strains and Media

S.cerevisiae CG378 has the genotype *MATa ade5 can1 leu2-3,112 trp1-289 ura 3-52* and *S.cerevisiae* L130-2A is *MATa trp1 tyr1 ura3 cdc28-4*. All cultures were grown in Wickerhams minimal salts medium (17) or in Difco Yeast Nitrogen Base (0.6%) with the appropriate nutritional supplements at 20μg/ml.

Bud counts were determined microscopically and cell numbers were monitored by means of a particle counter (Coulter Electronics, Dunstable, England).

Constructions

i) *CDC9'*. The *CDC9* gene, together with 650 bp of upstream sequence were removed from plasmid pR12Sclig2 (18) as a 4.2 kb *Hin*dIII-*Sst*I fragment and ligated into pEMBLYi32 (19) also known as YIpU5. A unique 910 bp *Alu*I fragment from pBR322 was then inserted into the *CDC9* structural gene at a *Pst*I site (20), which had been blunt-ended with T4 DNA polymerase, to give pEMBLYi32-*CDC9'*.

ii) *pPGK-CDC9'*. The upstream sequences of *CDC9'* were removed from pEMBLYi32-*CDC9'* by a *Hin*dIII−*Mlu*I digestion (Fig.1), which leaves the start of transcription intact (18) and preserves the entire untranslated leader, and the PGK upstream sequences were isolated on a 1.5 kb *Hin*dIII−*Bgl*III fragment from plasmid pMA91 (21). Following an initial ligation, the linear pEMBLYi32-PGK-*CDC9'* construct was purified from an agarose gel, the *Bgl*II and *Mlu*I ends filled in with T4 DNA polymerase I and the plasmid was self-ligated to produce plasmid pPGK-*CDC9'*.

iii) *CDC9-lacZ*. The construction of this fusion between the *CDC9* upstream sequences and the *lacZ* structural gene has been described previously (22). For the experiments described here, the fusion has been inserted into vector pEMBLYe23 (19).

iv) *Insertion of the ATGATT oligonucleotide into pLG178*. Two complementary single-stranded oligonucleotides, 5'-TCGAG-ATGATTTTC-3' and 3'-CTACTAAAAGAGCT-5' were synthesised on a Beckman 'System 1 Plus' DNA synthesizer, so that when annealed, an ATGATTTT duplex representing an extended consensus of all six 'ATGATT' repeats, would be formed with *Xho*1 cohesive ends. To anneal them, 5μg amounts of each oligonucleotide were mixed, denatured at 60°C for 1 hour and then cooled slowly to room temperature. Following phosphorylation of the 5'termini, the fragments were ligated into plasmid pLGΔ178 (23), linearised with *Xho*I, in the presence of 15% polyethylene glycol 6000 to promote the multiple insertion of small oligonucleotides (24). The resulting ligation was transformed into *E.coli* DH5, and the transformants were screened for insertions by restriction analysis. The number and orientation of the ATGATT inserts were accurately determined using an M13-sequencing protocol (25).

Synchronisation procedures and cell cycle arrest experiments

Transformed *S.cerevisiae* CG378 was grown to 5×10^6 cells/ml at 25°C or 30°C in minimal media, and α-factor was added to a final concentration of 2μg/ml. The cells were incubated in α-factor for approximately one generation, and the α-factor was removed by rapid filtration and washing of the cells. Finally the cells were resuspended in an equal volume of fresh medium. For protracted arrest experiments 3−5μg/ml of α-factor was used.

For the arrest at the *cdc28* block-point, cells of strain L130-2A were grown to 5×10^6 cells/ml in minimal medium at 25°C. The culture was then shifted to 37°C, the restrictive temperature, and was incubated for a further three to five hours.

Samples of 10^8 cells were harvested, washed in saline and frozen rapidly in dry ice.

Northern hybridization

This has been described in detail previously (2) and only a summary is presented here. 5μg of total RNA was denatured with glyoxal and after electrophoresis in 1.5% agarose gels transferred to 'Genescreen' membrane (New England Nuclear).

Internal DNA fragments from the genes concerned were used as probes: *CDC9/CDC36*, a 2.73 kb *Sst*1 fragment from plasmid pR12Sclig2 (18); histone H2A/protein 1, a 2.3 kb *Sst*1 fragment from plasmid TRT1 (26); phosphoglycerate kinase (PGK), a 2.95 *Hin*dIII fragment from pUN121/PGK; *URA3*, a 1.12 *Hin*dIII fragment; *LacZ*, a 3.0 kb *Eco*R1 fragment from pMC1871 (27). Probe DNA was labelled with [^{32}P] TTP (111TBq/mmol; New England Nuclear) to an approximate specific activity of 10^9 cpm/μg using an oligolabelling protocol (28).

Autoradiography was carried out at −70°C, using Fuji RX X-ray film, together with X-ograph, Hi-speed-X Intensifying screens. Several exposures were carried out for each experiment so that every RNA species of interest was within the exposure range of the film.

β-galactosidase assays

10^8 cells in 100μl 50mM Tris-HC1 pH7.0, 5mM EDTA, 10% glycerol, 2mM DTT and 0.2mM PMSF were disrupted by shaking with 0.5mm glass beads for 4×30 seconds on a Whirlimixer. Cell debris was then removed by centrifugation at 30,000g for 20 minutes at 4°C and the protein concentration of the supernatant was determined by the method of Lowry *et al.*, (29).

β-galactosidase levels were determined using a modification of the method of Guarente (30). 50μg of crude protein extract was added to 0.75ml Z-buffer and 0.2ml O-nitrophenyl-β-galactopyranoside (ONPG) (4mg/ml in water). Incubation was at 30°C for 1.5 hr and 0.5ml of 1M Na_2CO_3 was added before determining the A_{420}.

RESULTS

The cell cycle regulation of *CDC9* is transcriptional

Initial attempts to determine whether *CDC9* regulation is transcriptional or whether it is regulated partly (or entirely) by changes in message stability used pulse-labelling. However, this was unsuccessful due to the relatively low level of the *CDC9* message (3). We have therefore fused the upstream sequences from the constitutively expressed phospho-glycerate kinase (PGK) gene to the *CDC9* structural gene and then determined the cell cycle regulation of the message.

For the above experiment, some means of differentiating the

native *CDC9* transcript from that driven by the PGK upstream is necessary. A 910 bp fragment from pBR322 was therefore inserted into a plasmid-borne copy of *CDC9* (see Methods) to give *CDC9'* (Fig.1). This of course produces a larger transcript that is separable from the native message by electrophoresis and levels of the two transcripts can then be readily compared by Northern hybridization. The *CDC9'* gene, in the vector pEMBLYi32 (19), was integrated at the chromosomal *URA3* locus (31) and S1-nuclease mapping (32) showed that the gene was correctly transcribed at both the 5' and 3' ends and, more importantly, it was found to be correctly cell cycle regulated (J.H.M. White, PhD thesis, CNAA, London, 1988). Thus, insertion at the *URA3* locus does not appear to affect regulation of *CDC9*, although it was expressed at a somewhat lower level.

The *CDC9'* upstream sequences were next replaced with those from the PGK gene (see Methods and Fig.1) and the resultant plasmid, pPGK-*CDC9'*, was again integrated at *URA3*. The levels of the *CDC9* and the PGK-directed *CDC9'* transcripts were then examined over two cell cycles of an α-factor synchronised culture (Fig.2). The resulting Northern blot was initially probed with DNA from *CDC36*, the transcript from which is invariant in the cell cycle (1; our unpublished observations). The level of this transcript therefore shows that loading of the gel is approximately even and that there are no RNA transfer artifacts. The slight decline in levels on α-factor release is normally observed with some strains. The *CDC9* transcript showed the expected periodicity with one peak at 20−30 min and another at 1hr 45 min−2 hr. In contrast, the level of the *CDC9'* transcript showed little sign of periodicity. Upon release from the α-factor there was a decline in level, followed by a gradual increase back to mid-log levels. One sample, 20 min, showed a slight increase above background (densitometry showed it to be 1.25-and 1.6-fold higher than samples on either side) but this did not occur in another experiment. It is therefore unlikely to be significant but it may indicate slight control at the level of transcript turnover as occurs with histone H2A (9). Overall, however, the *CDC9'* pattern after release resembled that of the PGK transcript, showing that under control of PGK sequences the *CDC9'* message behaves as does PGK, rather than behaving like *CDC9*. Thus the periodic expression of *CDC9* must be governed largely by its upstream sequences rather than by gross changes in message stability throughout the cell cycle.

To confirm this, we determined whether the *CDC9* promoter could impart cell cycle regulation to a heterologous gene. The entire upstream sequence was therefore fused to the *E.coli lacZ* structural gene but when integrated into the genome the transcript was present at a very low level. However, when located on a multi-copy plasmid the *lacZ* mRNA was readily detectable and was sharply periodic (Fig.3). More important, expression occurred in the same interval of the cell cycle as that in which *CDC9* was expressed. Hence the upstream sequences are sufficient for cell cycle regulation and *CDC9* control must be transcriptional.

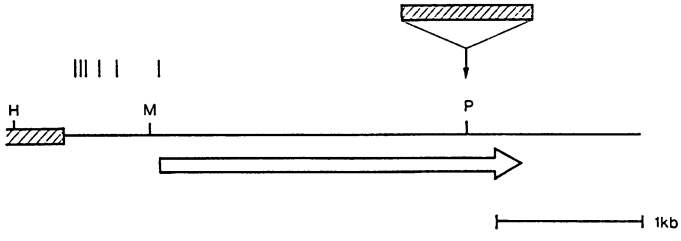

FIG.1 The *CDC9* gene showing relevant features. The thin line represents sequences from the *CDC9* locus while the hatched bars represent pBR322 DNA. The arrowed bar shows the *CDC9* transcript (20) and the vertical bars show the position of the ATGATT repeats. H=*Hin*dIII; M=*Mlu*I; P=*Pst*I.

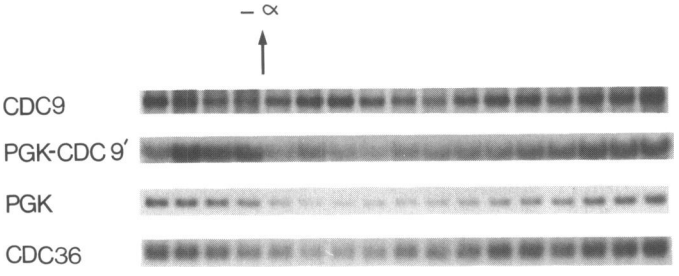

FIG.2 Regulation in the cell cycle of the *CDC9* structural gene fused to the PGK upstream sequences. A mid-log phase culture of CG378 at 30°C was sampled, α-factor was added and sampling continued at 40 min intervals to two hours. After α-factor removal, the culture was sampled at 10, 20, 30, 40, 55, 70, 90, 105, 120, 135, 150, 165 and 180 min. Total RNA was extracted from each sample, 5μg was denatured and following agarose gel electrophoresis, a RNA blot was prepared. This was probed with DNA from the genes shown and the resultant autoradiographs are shown above.

The ATGATT sequences act additively as orientation specific UAS elements

To study the effect of the ATGATT sequences on gene expression we used plasmid pLGΔ178 (23) which has the cytochrome C promoter fused to the *E.coli lacZ* gene but without any UAS elements. This plasmid has been used previously to characterise sequences that stimulate transcription since insertion of UAS elements upstream of the CYC promoter stimulates β-galactosidase synthesis. Indeed, pLGΔ178 was used in analysing the promoters of the cell cycle regulated HO and histone H2A/H2B genes (15,16,13). When synthetic copies of the ATGATT sequences were inserted into this plasmid a stimulation of β-galactosidase synthesis was observed. The number and orientation of the ATGATT sequences in the plasmid was determined by DNA sequencing (see Methods) and the presence of a single sequence inserted in the same orientation as in the *CDC9* upstream plasmid, pP1, leads to a dramatic nine-fold stimulation in β-galactosidase levels (Table 1). In contrast, when a single (pN1) or even two (pN2) ATGATT sequences are inserted in the opposite orientation, no such stimulation of β-galactosidase is seen. Instead, the enzyme activity remains low,

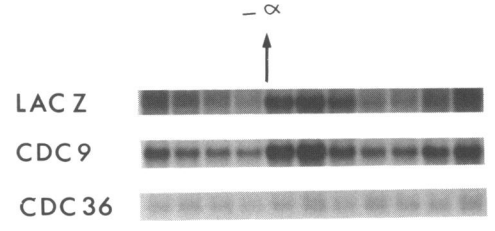

FIG.3 Regulation in the cell cycle of the *lacZ* structural gene fused to the *CDC9* upstream sequences. A mid-log phase culture of CG378 at 30°C was sampled, α-factor was added and sampling continued at 40 min intervals to two hours. After α-factor removal, the culture was sampled at 15, 30, 45, 60, 75, 90 and 105 min. For further details see legend to Fig.2.

at about the background level found in cells containing the parental pLGΔ178. This strongly suggests that an ATGATT sequence can act as a UAS element but, significantly, will do so only when present in the orientation found in the *CDC9* upstream.

When multiple insertions of ATGATT are considered (Table 1), there is a clear positive correlation between the number of correctly oriented repeats and β-galactosidase levels. For example, the single repeat in pPl results in 0.137 units of enzyme, while the three repeats in pP3Nl give 0.247 units and five repeats (pP5Nl) produce 0.321 units or some twenty times the level produced by pLGΔ178 alone. It is worth stressing that in no case of multiple insertions do the 'negatively' oriented repeats appear to have any consistent influence on the final level of the enzyme. They seem to be neutral, emphasising the orientation specific nature of the stimulation by the ATGATT sequences.

Stimulation by the ATGATT sequences is regulated at START

To determine whether the ATGATT sequences could have any role in regulating *CDC9* expression, we initially examined levels of transcription driven by the repeats in cells that were held at START, the beginning of the cell cycle. Under these conditions the *CDC9* transcript itself is known to decline in amount (1,2), presumably as cells cease dividing. A culture of cells containing plasmid pP5N1, with five 'positively' orientated repeats, was therefore treated in mid-log phase with α-factor, which blocks cells at START, and held for five hours. Levels of the *lacZ* and *CDC9* transcripts, together with various controls, was then examined by Northern hybridisation over this period (Fig.4A). The effect of the α-factor can be seen in the abrupt drop in level of histone H2A, together with the decline in level of *CDC9* (which is always slower than histone H2A, for example see references 2,3 and Fig.5). The level of the *lacZ* transcript also declined markedly and was comparable to that observed with the *CDC9* message. The relatively constant level of the 'Protein 1' transcript (2) shows that the loading of the gel is even. Moreover, the decrease in *lacZ* was not due to any substantial change in plasmid copy number since the level of the *URA3* transcript, the great bulk of which comes from the plasmid (strains carrying this plasmid contain 31× the level of *URA3* present in a strain without the plasmid), did not decline greatly in this experiment. Thus the transcriptional stimulation by the repeats is sensitive to α-pheromone.

To show that the decrease in the *lacZ* transcript is due to the block at START, rather than an α-factor specific effect, pP5N1 was transformed into the cell cycle mutant *cdc28-4*. At the restrictive temperature, cells of this mutant arrest at START and there is a rapid decline in *lacZ* transcript levels as division ceases

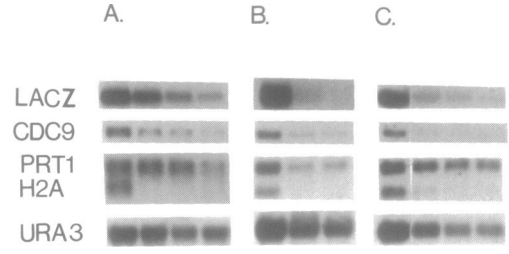

FIG.4 The transcriptional stimulation mediated by the ATGATT sequences is regulated at START. (A) Strain CG378 containing pP5N1 was treated in mid-log phase with 2.5μg/ml α-pheromone and sampled at 0, 1.5, 3 and 5 hours. (B) Strain L130-2A carrying pP5N1 was grown to mid-log at 25°C and transferred to 37°C and sampled at 0, 1.5 and 3 hours. (C) A mid-log phase culture of L130-2A with pP5N1 was sampled, grown to stationary phase (approximately 6×10^7 cells/ml in YNB) sampled once more and then again 8 hours and 24 hours later. RNA blots were prepared from these samples, as outlined in the legend to Fig.2, and the autoradiographs are shown above.

TABLE 1. The influence of the ATGATT sequences on expression of β-galactosidase in pLGΔ178

Construct	ATGATT repeats	β-galactosidase activity[a]
pLGΔ178	–	0.015±0.007[b]
pN1[c]	<[d]	0.031±0.018
pN2	<<	0.038±0.010
pP1	>	0.137±0.037
pP1N6	<<<<><<	0.164±0.045
pP2N6	>><<<<<<	0.191±0.037
pP2N3	><<><	0.167±0.025
pP3N1	>><>	0.242±0.079
pP5N1	>>>><>	0.321±0.124

a) Expressed as A_{420} units per 50μg of crude cellular protein after incubation with ONPG at 30°C for 90 minutes.
b) Approximately six independent transformants were assayed in duplicate for each construct. The average of these and the SD is given in each case.
c) Each construct is named according to the number and orientation of the ATGATT sequences. 'P' = positive repeats, ie. ATGATT with respect to a downstream promoter, while 'N' = negative repeats.
d) '>' represents positively oriented repeats and '<' negatively oriented repeats.

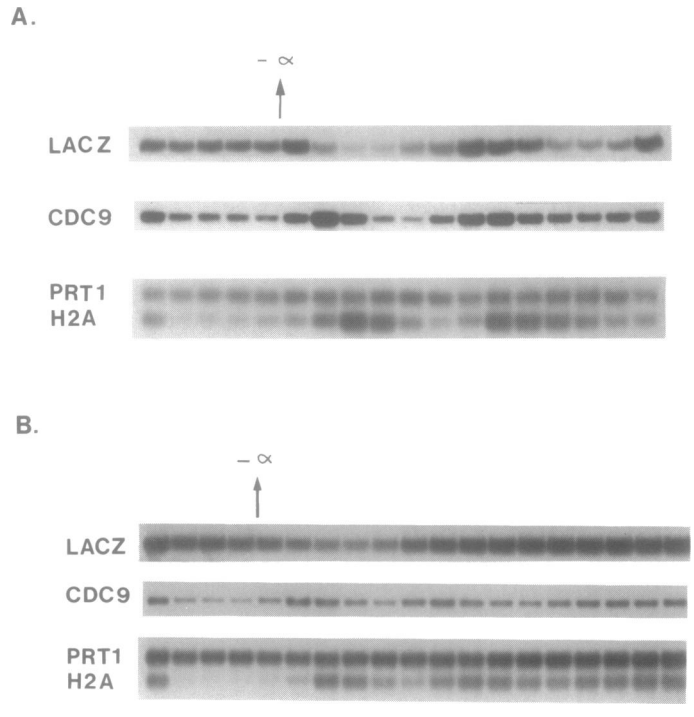

FIG.5 The ATGATT sequence does not mediate the cell cycle regulation of a heterologous gene. α-pheromone was added to a culture of CG378 carrying (A) pLGΔ178 containing a 477 bp fragment with most of the *CDC9* upstream (see text) or (B) plasmid pP1 (Table 1). Samples were taken (A) at 0, 45, 90, 180 and 240 minutes and (B) at 0, 45, 90 and 120 minutes. Following removal of the α-factor, further samples were taken (A) at 15 minute intervals to 180 minutes and (B) after 10, 25, 40, 55, 75, 95, 110, 125, 140, 155, 170, 185, 200, 220 and 240 minutes. RNA blots were prepared as outlined in the legend to Fig.2, and the resultant autoradiographs are shown above.

(Fig.4B). Again the *URA3* transcript serves as a control and although there is a slight decline in level during the experiment, it is certainly insufficient to account for the drop in level of *lacZ*.

Finally, a quite different growth condition was used to confirm that *lacZ* expression occurs only in cycling cells. As cells pass into stationary phase, cell cycle regulated transcripts rapidly decline in amount as cells stop dividing, as can be seen with *CDC9* and histone H2A (Fig.4C). The *lacZ* message driven by the ATGATT sequences behaved in the same way, the levels dropped sharply and were barely detectable in stationary phase cells (Fig.4C). This provides independent evidence that the transcriptional stimulation by the repeats requires cycling/dividing cells.

The ATGATT sequences do not mediate either the cell cycle regulation of *CDC9* or its induction by UV light

Before examining whether these repeats could impart cell cycle regulation to a heterologous gene, we showed that periodic expression of the *lacZ* gene located on pLGΔ178 could be detected. A 477 bp *CDC9* upstream fragment extending from an AluI site at −105, with respect to the ATG, to another AluI site at −582, was inserted upstream of the CYC promoter. This fragment imparted cell cycle regulation to the *lacZ* gene in pLGΔ178 (Fig.5A). However, the *lacZ* transcript did not decline during the α-factor holding and the peak in the first cycle after release was 15 minutes earlier than the peak of *CDC9* expression. The 477 bp fragment may therefore lack sequences important for the negative regulation of transcription during α-factor incubation. However, it is clear that in the second and even in the beginning of the third cell cycle, the *lacZ* transcript is indeed periodically expressed and, importantly, this is coincident with the *CDC9* transcript. Thus, periodic expression of the *lacZ* gene in pLGΔ178 coincident with the endogenous *CDC9* gene can be observed, confirming the validity of the assay.

To examine the cell cycle regulation of the *lacZ* message under the influence of the ATGATT repeats, an α-pheromone release experiment was carried out on a strain containing pPl (Fig.5). As judged by the budding profile and the periodic expression of histone H2A, two complete synchronous rounds of division were obtained with part of a third cycle in which the synchrony has decayed. The *CDC9* transcript shows two peaks, followed by loss of synchrony, and as expected, each peak precedes the corresponding histone peaks by some 15 minutes (2). However, the *lacZ* message showed no clear cyclic pattern. During the two hour incubation with α-factor the transcript level declined only slightly in this experiment (a longer incubation leads to loss of synchrony), and it only reached its lowest point some 40 minutes after the release from α-factor. This was, in fact, after the first peak in *CDC9* transcript levels and at about the time of the histone mRNA peak. Thereafter, a steady increase in *lacZ* message occurred until it reached a plateau in the second cycle. A second experiment, using pP5Nl instead of pPl, has given similar results, so it seems unlikely that the protein interacting with the ATGATT repeats can, by itself, mediate the cell cycle regulation of *CDC9*.

As well as being regulated in the cell cycle, *CDC9* is induced after irradiation with UV light (22) and neither the levels of *lacZ* message or β-galactosidase enzyme showed an increase after UV irradiation. Indeed, the *lacZ* mRNA initially declined in amount and only slowly re-established the original mid-log phase level, presumably as the cells recovered (result not shown). The ATGATT repeat can therefore play no specific role in mediating the UV-induction of *CDC9*.

DISCUSSION

The molecular basis for the regulation of *CDC9* could, in principle, be either transcriptional or some form of specific and regulated degradation of a constitutive message or to a combination of the two. Indeed, the cell cycle regulation of histone H2A-H2B relies on both mechanisms (9). However, fusion of the *CDC9* structural gene to the PGK promoter showed that the *CDC9* transcript is not subject to regulated turnover at a significant level. In turn, the *CDC9* upstream sequences can impart cell cycle regulation to a heterologous gene, so *CDC9* is therefore likely to be transcriptionally controlled. Thus regulation of the gene can be analyzed by fusion of *CDC9* upstream elements to a heterologous gene without complications arising from any disruption of regulated transcript turnover.

When inserted upstream of the *lacZ* gene in a test plasmid, a single synthetic ATGATT sequence acts as a UAS element and increases β-galactosidase levels by nine-fold. Presumably this occurs by means of a protein interacting with the sequence and stimulating transcription as has been proposed for other UAS elements (see for example, 11). Surprisingly, the ATGATT dependent stimulation occurs only when the repeats are in one orientation, namely ATGATT with respect to a downstream promoter. The alternative orientation, TTAGTA, has little or no effect on β-galactosidase production, even when it is repeated up to six times. To the best of our knowledge all other UAS elements that have been characterised are active in either orientation. Certainly, in the cell cycle regulated HO (15) and the histone H2A genes (13), the UAS elements are effective in either orientation. Presumably, the orientation specific properties of the *CDC9* UAS elements must reflect some particular aspect of the mechanism of its regulation.

The 'positively' oriented repeats appear to act in an additive manner, so that the level of β-galactosidase synthesised under their influence depends upon the number of the repeats. This additive effect might indicate the binding of protein molecules to each ATGATT repeat, however, the increase is not linear since the stimulation per repeat falls away with each additional ATGATT sequence. This non-linearity may be caused by steric hindrance to the binding of multiple proteins especially in a construct such as pP5Nl where four positively oriented repeats are juxtaposed. Alternatively, additional proteins may not be involved and the increased repeats may simply mean that more frequent, or productive, collisions take place between the protein and the sequences. In another case where the same vector was used in similar experiments to study the UAS activity of the upstream repeats from the HO gene (15,16) quite different kinetics were observed. One repeat had relatively little effect and the addition of a second and third repeat had far greater effects, with increases of much more than two- and three-fold, respectively. These different kinetics may merely indicate different binding affinities between the proteins concerned, or possibly, real differences in the regulatory mechanisms such as co-operative binding of the proteins interacting with the HO repeats, or a synergistic interaction between them.

There certainly are at least some mechanistic differences between the cell cycle regulation of HO and histones H2A-H2B, on the one hand, and of *CDC9* on the other. The ten repeats upstream of HO not only stimulate transcription but, in addition, when present in pLGΔ178 they mediate the precise cell cycle regulation of *lacZ* (16). Similarly, the three repeats associated with the histone H2A-H2B gene pair are able to mediate the cell cycle regulation of an attached heterologous gene (13). However,

the ATGATT repeats from *CDC9* are not, by themselves, able to direct the periodic expression of another gene. Since we have shown here that *CDC9* regulation is largely transcriptional there must be additional controlling sequences. Consistent with this, the pLGΔ178 construct containing the 477 bp AluI fragment produces four-times as much β-galactosidase as does pP5N1 (results not shown). Comparison with the upstream sequences of the other DNA synthesis genes co-regulated with *CDC9* has only identified either one or two ACGCGT sequences in common and preliminary data suggests that these are also UAS elements that may have a role in the cell cycle regulation of the gene (our unpublished observations).

The only physiological impact on the ATGATT stimulated expression of *lacZ* that we detected was the decline in transcript levels that occurred in non-cycling cells. This cannot have been due simply to a decline in RNA and protein synthesis, since these continue in cells blocked either by α-pheromone or by the *cdc28* mutation. Hence the protein interacting with the repeats to mediate the transcriptional stimulation of *CDC9* must be produced only in actively dividing cells or alternatively it is present constitutively but is only active in cycling cells. The *in vivo* role of this protein, and the repeats, may simply be to stimulate expression of *CDC9* when it is activated, either in the cell cycle or after DNA damage, or both. If it were absolutely required for the cell cycle expression it could have a role in exerting proliferation control on the gene i.e. the absence of active protein in non-cycling cells would ensure that the gene is not expressed inappropriately. The isolation of mutations in this protein should help clarify this and we are currently searching for such mutants.

REFERENCES

1. Peterson, T.A., Prakash, L., Prakash, S., Osley, M.A. and Reed, S.I. (1985) Mol. Cell. Biol. 5, 226–235.
2. White, J.H.M., Barker, D.G., Nurse, P. and Johnston, L.H. (1986) EMBO J. 5, 1705–1709.
3. White, J.H.M., Green, S.R., Barker, D.G., Dumas, L.B. and Johnston, L.H. (1987) Exptl. Cell Res. 171, 223–231.
4. Storms, R.K., Ord, R.W., Greenwood, M.T., Mirdamadi, B., Chu, F.K. and Belfort, M. (1984) Mol. Cell. Biol. 4, 2858–2864.
5. Johnston, L.H., White, J.H.M., Johnson, A.L., Lucchini, G. and Plevani, P. (1987) Nucleic Acids Res. 15, 5017–5029.
6. Johnston, L.H., White, J.H.M., Johnson, A.L., Lucchini, G. and Plevani, P. (1990) Molec. Gen. Genet. 221, 44–48.
7. Hereford, L., Osley, M.A., Ludwig, J.R. and McLaughlin, C.S. (1981) Cell 24, 367–375.
8. McIntosh, E.M., Ord, R.W. and Storms, R.K. (1988) Mol. Cell. Biol. 8, 4616–4624.
9. Lycan, D.E., Osley, M.A. and Hereford, L.M. (1987) Mol. Cell. Biol. 7, 614–621.
10. Guarente, L. (1984) Cell 36, 799–800.
11. Guarente, L. (1987) Ann. Rev. Genet. 21, 425–452.
12. Struhl, K. (1987) Cell 49, 295–297.
13. Osley, M.A., Gould, J., Sunyoung, K., Kane, M. and Herefore, l.M. (1986) Cell 45, 537–544.
14. Nasmyth, K. (1985) Cell 42, 225–235.
15. Breeden, L.G. and Nasymth, K.A. (1985) Cold Spring Harb. Symp. Quant. Biol. 50, 643–650.
16. Breeden, L.G. and Nasmyth, K.A. (1987) Cell 48, 389–397.
17. Wickerham, L.J. (1951) U.S. Dept. of Agriculture, Techn. Bulletin No.1029.
18. Barker, D.G. and Johnston, L.H. (1983) Eur. J. Biochem. 134, 315–319.
19. Baldari, C. and Cesareni, G. (1985) Gene 35, 27–32.
20. Barker, D.G., White, J.H.M. and Johnston, L.H. (1985) Nucleic Acids Res. 13, 8323–8337.
21. Mellor, J.M., Dobson, M.J. Roberts, N.A., Tuite, M.F., Emtage, J.S., White, S., Lowe, P.A., Patel, T., Kingsman, A.J. and Kingsman, S.M. (1983) Gene 24, 1–14.
22. Johnson, A.L., Barker, D.G. and Johnston, L.H. (1986) Curr. Genet. 11, 107–112.
23. Guarente, L. and Mason, T. (1983) Cell 32, 1279–1286.
24. Zimmerman, S.B. and Pheifer, B.H. (1983) Proc. Natl. Acad. Sci. USA. 80, 5852–5856.
25. Messing, J. (1983) Methods Enzymol. 10, 20–70.
26. Hereford, L., Fahrner, K., Woolford, J., Rosbash, M. and Kaback, D.B. (1979) Cell 18, 1261–1271.
27. Casadaban, M.J., Martinez-Arias, A., Shapira, S.K. and Chou, J. (1983) Methods Enzymol. 100, 293–308.
28. Feinberg, A.P. and Vogelstein, B. (1983) Analyt. Biochem. 132, 6–13.
29. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
30. Guarente, L. (1983) Methods Enzymol. 101, 181–191.
31. Orr-Weaver, T.L., Szostak, J.W. and Rothstein, R.J. (1981) Proc. Natl. Acad. Sci. USA 78, 6354–6358.
32. Berk, A.J. and Sharp, P.A. (1977) Cell 12, 721–732.