The EMBO Journal vol.4 no.2 pp.457–463, 1985

Cloning, sequencing and transcriptional control of the *Schizosaccharomyces pombe cdc10* 'start' gene

Stephen J.Aves[1], Barbara W.Durkacz[2], Antony Carr[1] and Paul Nurse[1]

School of Biological Sciences, University of Sussex, Falmer, Brighton, East Sussex BN1 9QG, and Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

[1]Present address: ICRF, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK
[2]Present address: Cancer Research Unit, Royal Victoria Infirmary, Queen Victoria Road, Newcastle-upon-Tyne NE1 4LP, UK

Communicated by J.M.Mitchison

The *cdc10* **'start' gene from the fission yeast** *Schizosaccharomyces pombe* **has been cloned by rescue of mutant function. It is present as a single copy in the haploid genome. Hybridisation of the gene to Northern blots has identified a low abundance 2.7-kb polyadenylated RNA. Study of RNA extracted from cells both entering stationary phase and undergoing synchronous cell divisions suggests that commitment to the cell cycle is not controlled by regulation of** *cdc10* **transcript level. DNA sequence analysis of the gene has identified an open reading frame capable of encoding a protein of mol. wt. 85 400. The putative** *cdc10* **gene product shows no significant primary structure similarity with products of other fission and budding yeast cell cycle genes, or with other protein sequences in several databases.**

Key words: cell cycle/molecular cloning/*Schizosaccharomyces pombe*/sequence/start

Introduction

The major cell cycle control in both budding and fission yeasts is called start (Pringle and Hartwell, 1981; Reed, 1984; Fantes, 1984; Nurse, 1985). This is the point in G1 at which cells become committed to the mitotic cycle. Before this point cells can undergo alternative developmental pathways such as conjugation if challenged to do so. Once past this point cells are committed to the mitotic cycle in progress and are unable to initiate alternative developmental pathways until that cycle has been completed. In rapidly growing yeast cells, start occurs early in the cell cycle, soon after mitosis. In slower growing cells the G1 period becomes expanded and start occurs later in the cell cycle, just before S-phase but a long time after mitosis. As a consequence, in these poorer growth conditions cells spend a longer period of time before start in an uncommitted phase of the cell cycle. Therefore, start functionally divides the yeast cell cycle into two parts, an uncommitted phase during the first part of G1 and a committed phase including the last part of G1, S-phase, G2 and mitosis. Analogous controls have also been identified in mammalian cells (Pardee *et al.*, 1978). Nutrient deprivation blocks cells in G1 before the onset of DNA replication, and cells have also been found to proceed towards differentiation from certain blocks in G1 (Scott *et al.*, 1982).

In the fission yeast *Schizosaccharomyces pombe* two start genes, *cdc2* and *cdc10*, have been identified whose functions are required for the cell to become committed to the mitotic cell cycle (Nurse *et al.*, 1976; Nurse and Bissett, 1981). Temperature-sensitive mutants of these two genes become blocked in cell cycle progression but continue cellular growth when incubated at their restrictive temperature. Because of the block, cells stop dividing and become highly elongated. The *cdc2* gene function is complex, being required twice in the mitotic cell cycle – once at start and then again in G2 for the initiation of mitosis (Nurse and Bissett, 1981). Defects in the *cdc2* gene function can be rescued by the budding yeast start gene *cdc28* (Beach *et al.*, 1982a) indicating that the *cdc2* and *cdc28* genes perform equivalent functions in the two organisms. Both the *cdc28* and *cdc2* genes have been cloned and sequenced, and show a 62% identity in their predicted amino acid sequence (Lorincz and Reed, 1983; Hindley and Phear, 1984). The sequences also show ~20% identity to bovine cAMP-dependent protein kinase and the protein kinases of the *src* family. This homology is largely due to the presence of two consensus regions, one surrounding a putative ATP binding site, the other surrounding a putative phosphorylation site (Hindley and Phear, 1984; Nurse, 1985). These results suggest that the *cdc2* gene product may be a protein kinase, and that phosphorylation could play a role in its regulation.

Mutants in the *cdc10* gene have a more straightforward phenotype. Temperature-sensitive mutants in *cdc10* block only in the G1 phase of the cycle at start and, unlike *cdc2*, there is no second functon later in G2. The *cdc10* function is completed later in G1 during slow growth in chemostat cultures than at normal growth rates, consistent with the later occurrence of start in these conditions. Further analysis of these cultures shows that a critical cell size is required before the *cdc10* function can be completed and S-phase take place (Nasmyth, 1979). In poor growth conditions cell size at mitosis is reduced and cells have to grow for a longer period of time before attaining the size required at start for the *cdc10* gene function to take place. This accounts for the expansion of G1 phase before start observed in slow growing cultures of *S. pombe*.

Cloning a gene permits molecular characterisation of the gene and the products of its transcription. Such an analysis has proved very informative in the case of the *cdc2* and *cdc28* genes, giving clues as to the functions of the gene products. We decided to clone the *cdc10* start gene with a view to molecular analysis not only of the gene, which might elucidate the function of its product, but also of the transcript, to determine whether the temporal requirement for *cdc10* function is related to mRNA levels. Here we describe the isolation of the *cdc10* gene by its ability to rescue a defective *cdc10* function. The DNA region corresponding to the gene has been sequenced and found to contain a long open reading frame, and the *cdc10* gene transcript has been identified and its level monitored during the cell cycle and during a shift from exponential growth to stationary phase.

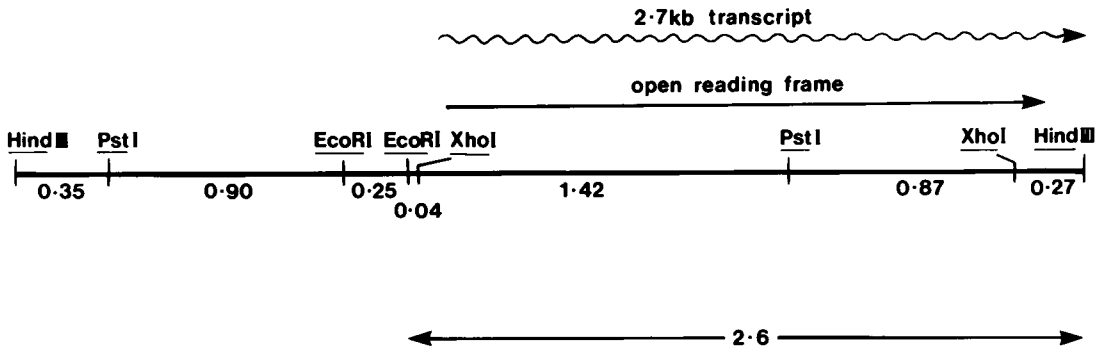

Fig. 1. Map of the 4.1-kb *Hind*III fragment containing the *cdc10* gene. The shortest sequence with full rescue activity is the 2.6-kb *Eco*RI-*Hind*III fragment indicated. Distances between restriction sites are given in kilobase pairs. The map position of the 5′ terminus of the 2.7-kb transcript has not been determined precisely with respect to the start of the open reading frame.

Results

Isolation of a plasmid containing the cdc10 gene

The *S. pombe* strain *cdc10-129 leu1-32 h*$^-$ is a leucine auxotroph temperature-sensitive *cdc10* mutant. It cannot form colonies at 36°C, but can at 29°C. This strain was transformed with two gene banks, a *Hind*III partial in pDB262 and a *Sau*3A partial in pDB248′ (Beach *et al.*, 1982b). Both these vectors contain the *S. cerevisiae leu2* gene which complements *S. pombe leu1-32* and part of the 2 μm circle which allows high frequency transformation of *S. pombe* (Beach and Nurse, 1981; Beach *et al.*, 1982a). Each gene bank produced ~25 000 transformants which could grow in the absence of leucine at 29°C. After replica plating, two yeast clones – one from each bank – were found to form colonies at the restrictive temperature of 36°C. The phenotypes were mitotically unstable and leucine prototrophy was found to cosegregate with the ability to grow at 36°C. DNA was prepared from both the yeast clones and used to transform the *recBC E. coli* strain JA226. Plasmids were isolated from both of the yeast clones and were shown to rescue *cdc10* mutant function after retransformation of the *cdc10-129 leu1-32 h*$^-$ strain.

The pDB262-derived plasmid was called pcdc10-2 and contained a 4.1-kb *Hind*III fragment. Its restriction map is shown in Figure 1. The insert contains no *Sal*I sites and two *Xho*I sites giving an *Xho*I fragment of 2.3 kb. The plasmid and *S. pombe* chromosomal DNA were cut with *Hind*III, *Xho*I and *Sal*I, and a Southern blot performed using nick-translated pcdc10-2 as probe (Figure 2). This showed that the chromosomal DNA contained unique *Sal*I and *Hind*III fragments of 6.2 and 4.1 kb, respectively, which hybridised to the probe. The *Xho*I digest contains a 2.3-kb fragment which hybridised. These data demonstrate that pcdc10-2 contains a unique sequence from the *S. pombe* genome which is not grossly rearranged in the isolated plasmid clones. The pDB248′ derived plasmid also contained the 2.3-kb *Xho*I fragment but as the plasmid had become rearranged during the cloning procedures it was not studied further.

To establish whether pcdc10-2 contained the *cdc10* gene it was integrated into the *S. pombe* chromosomes and mapped. The 4.1-kb *Hind*III insert was cloned into the plasmid pDAM6 which contains the *S. cerevisiae leu2* gene but no 2 μm circle sequences. This plasmid was transformed into the *cdc10-129 leu1-32 h*$^-$ strain, and the stabilities of the subsequent leucine prototroph transformants were tested. A stable one was selected as a putative integrant. This stable clone was crossed

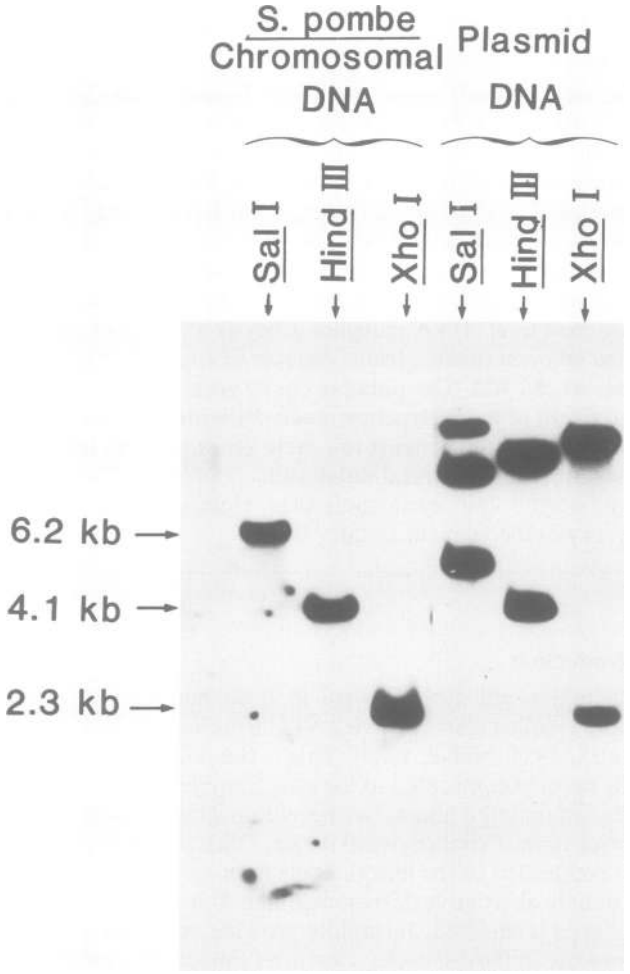

Fig. 2. Southern blots of chromosomal and plasmid DNAs probed with *cdc10*. *S. pombe* chromosomal DNA and pcdc10-2 DNA were cut with the restriction enzymes indicated (the *Sal*I digest of chromosomal DNA is partial), fractionated in agarose, blotted, and probed with nick-translated pcdc10-2. The vector contains two *Sal*I sites, one *Hind*III and no *Xho*I sites and therefore the plasmid blot shows that the 4.1-kb *Hind*III fragment contains no *Sal*I sites and two *Xho*I sites. The 4.1-kb *Hind*III fragment is also seen in the chromosomal DNA as is a 2.3-kb *Xho*I fragment (part of a doublet) corresponding in size to the band seen in plasmid DNA. A single *Sal*I fragment of 6.2 kb is also seen with chromosomal DNA.

to two strains: in cross A against *leu1-32 h*$^+$, and in cross B against *cdc10-129 leu1-32 h*$^+$. The first cross was to determine if the integrated plasmid containing the cdc$^+$ activity

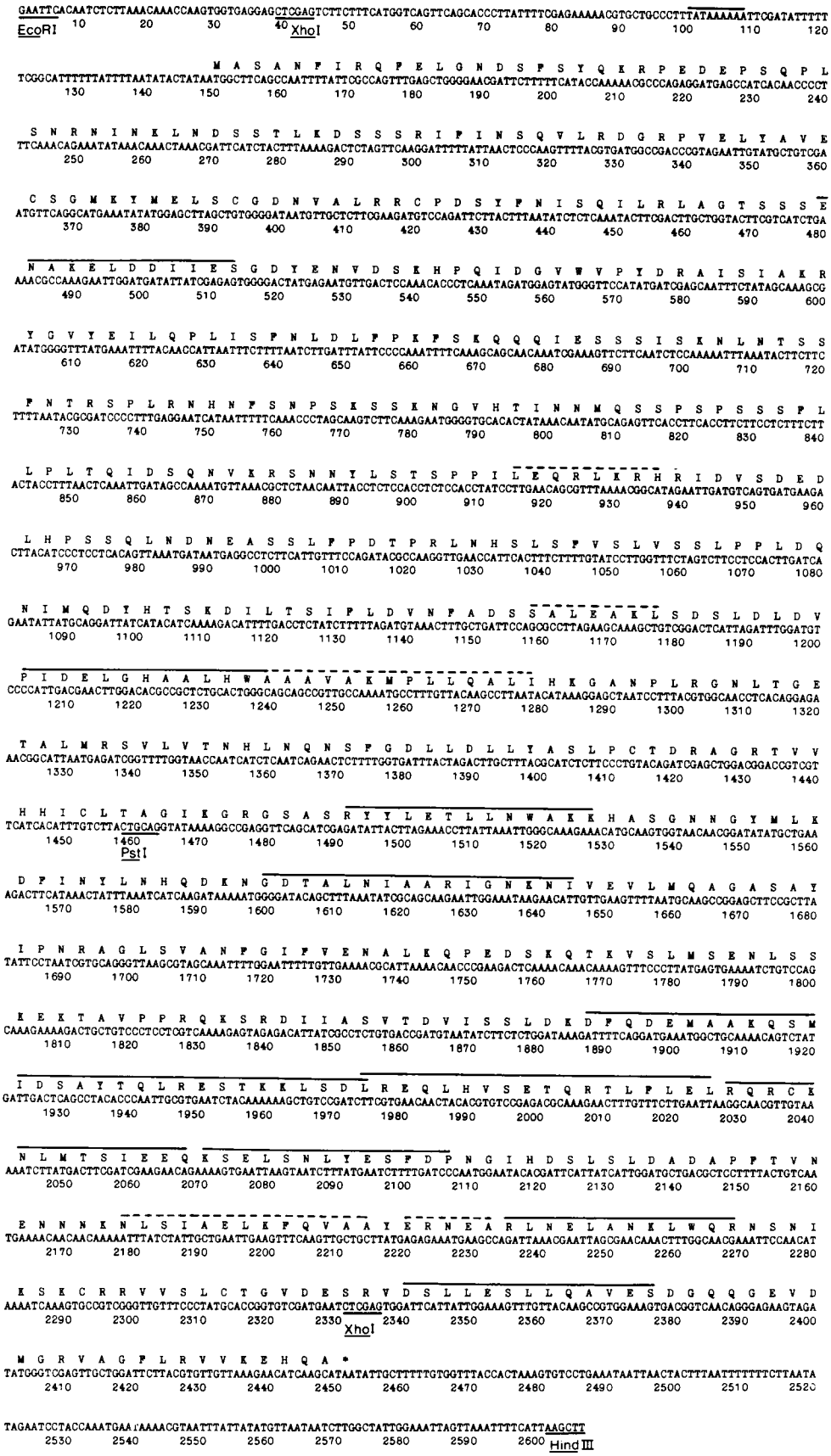

Fig. 3. DNA sequence of the 2.6-kb *Eco*RI-*Hind*III fragment. All sequence has been determined for both strands except for region 1307–1332, which has been cloned and sequenced three times. The translation of the long open reading frame is shown in one letter code; regions of predicted α-helix which could lie on the protein surface are shown overscored with solid lines. Other predicted regions of α-helix ($p > 0.6$) are overscored with broken lines. A TATA-like sequence upstream from the open reading frame is also overscored.

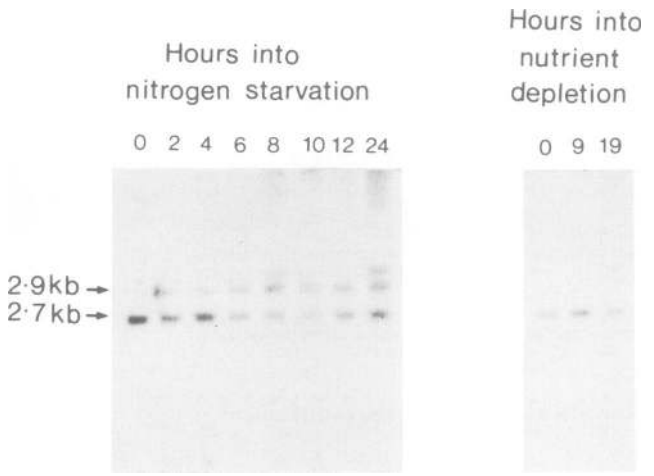

Fig. 4. *cdc10* transcript level on entry into stationary phase. 6 μg of total RNA extracted from asynchronous cells at various stages of entry into stationary phase was fractionated in agarose, blotted and probed with nick-translated 4.1-kb *Hind*III fragment. Stationary phase was induced either by nitrogen starvation at time = 0 or by general nutrient depletion. For nutrient depletion, cell densities were 0.6 x 10^7/ml at 0 h, 4.6 x 10^7/ml at 9 h and 12.9 x 10^7/ml at 19 h. For nitrogen starvation, cell density was 0.38 x 10^7/ml at 0 h, rising to 1.80 x 10^7/ml after 4 h and 2.20 x 10^7/ml after 6 h, but then remaining essentially unchanged (2.98 x 10^7/ml after 24 h).

was linked to the chromosomal map site of *cdc10*. Out of 630 spores analysed, all were cdc$^+$ indicating that the integrated plasmid mapped within 0.2 cM of the *cdc10* gene. The second cross was to establish if the *leu1*$^+$ gene on the plasmid was still linked to the cdc$^+$ activity. Out of 378 spores analysed, 212 were cdc$^+$ leu$^+$, 165 were cdc$^-$ leu$^-$, one was cdc$^+$ leu$^-$ and none were cdc$^-$ leu$^+$. This shows that the plasmid sequences conferring the leu$^+$ activity are tightly linked to the *cdc10*$^+$ gene. Both crosses together establish that the plasmid containing the 4.1-kb *Hind*III fragment had integrated at the chromosomal site of *cdc10* and therefore contains the *cdc10* gene.

Sub-cloning and sequencing of the cdc10 gene

The 4.1-kb *Hind*III fragment was subcloned into various vectors to determine the smallest clone which would rescue *cdc10* mutant activity. The smallest fragment with full activity was the 2.6-kb *Eco*RI-*Hind*III fragment (Figure 1). This was cloned into YIp5 which contains the *S. cerevisiae ura3* gene capable of complementing *S. pombe* mutant *ura4-294*. To increase transformation frequency this clone was co-transformed with pDB248′ into a *cdc10-129 ura4-294* strain (Sakai *et al.*, 1984). A smaller fragment with weaker rescue activity was also found. This was the 1.14-kb *Pst*I-*Hind*III fragment (Figure 1) which was subcloned into pDB262 and was tested by transformation into a *cdc10-129 leu1-32* strain. This clone required an extra day's incubation (2 days instead of 1 day) before growth was observed at the restrictive temperature. Because this smaller clone may have contained a truncated gene, the whole 2.6-kb *Eco*RI-*Hind*III fragment was sequenced to determine whether it contained an open reading frame.

Nested deletions of the 2.6-kb *Eco*RI-*Hind*III fragment were created with nuclease *Bal*31 and cloned directly into M13 vectors by an adaptation of the method of Poncz *et al.* (1982). Sequence was determined by the chain termination method (Sanger *et al.*, 1980). The complete sequence is shown in Figure 3 and reveals an open reading frame of 2301

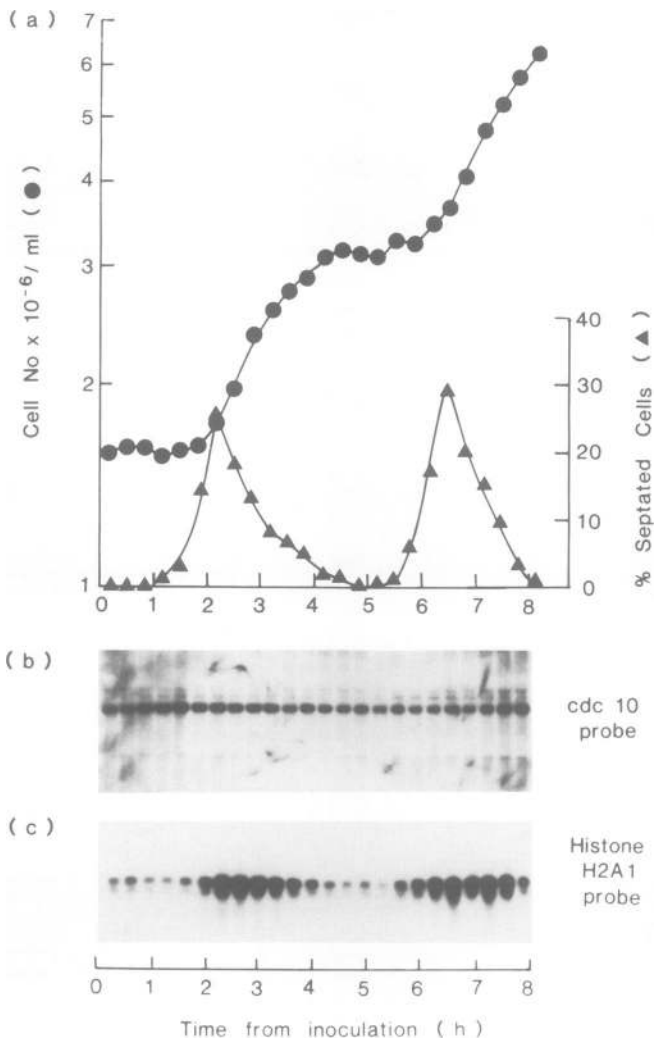

Fig. 5. *cdc10* transcript level during the cell cycle. Small cells were selected from an exponentially-growing culture after passage through an elutriator rotor as described in Materials and methods. (**a**) Cell density and percentage of septated cells after re-inoculation. (**b**) 6 μg RNA samples extracted from cells at the same times after inoculation, fractionated in agarose, blotted, and probed with nick-translated 4.1-kb *Hind*III fragment. Autoradiography was at −70°C with intensifying screen for 7 days. (**c**) Blot, as above, reprobed with histone H2A1 gene (obtained from M.Yanagida). Autoradiography was at −70°C with intensifying screen for 1 day.

nucleotides. This open reading frame exhibits features characteristic of other *S. pombe* genes which have been sequenced (see Discussion). A TATAAAAAA sequence is present 49 nucleotides upstream from the initiating ATG codon. The whole sequence was scanned for coding regions on the basis of codon usage by means of the computer program 'framescan' (Staden and McLachlan, 1982). With *cdc2* as the known gene, the whole open reading frame showed consistently high probability scores, whereas variable low scores were given for the flanking regions and other reading frames. This strongly suggests that the whole open reading frame codes for a protein. Computer analysis of the RNA sequence revealed that there are no regions of dyad symmetry within the open reading frame which could lead to the formation of stable secondary structure in an mRNA molecule, although a base-paired loop might be formed at the end of the coding region: nucleotides 2434−2449 share a 12/16 complementarity with nucleotides 2456−2471.

To confirm the presence of a transcript consistent with the

expression of this open reading frame, Northern blots of total RNA from exponentially growing cells were probed with nick-translated *cdc10* DNA. This hybridised principally to a 2.7 ± 0.15 kb RNA (Figures 4 and 5b). Fainter bands representing species of ~3.15 kb, 2.9 kb and 1.8 kb were also seen (these all hybridise to the 2.6-kb *Eco*RI-*Hind*III fragment, data not shown). At the present time we are unclear as to the relationship of these to the major transcript. They could represent precursor or breakdown products but it should be noted that two of them (3.15 kb and 1.8 kb) migrate at the same rate as rRNA. Further analysis showed that the major 2.7-kb RNA is polyadenylated, is formed by transcription in the same direction as the long open reading frame (left to right) and its 5′ end maps within the 2.6-kb *Eco*RI-*Hind*III fragment to the left of the *Pst*I site (data not shown).

The DNA sequence was scanned by computer in an attempt to detect introns. No sequences were found in the 2.6-kb fragment which are close to the consensus 5′ or 3′ splice sequences for *S. pombe* nuclear introns (Toda *et al.*, 1984; Hindley and Phear, 1984; Hiraok *et al.*, 1984; D.Barker and L.Johnston, personal communication). The open reading frame was therefore conceptually translated on the assumption that it contained no introns. This gave rise to a polypeptide with a mol. wt. of 85 400 comprising 767 amino acid residues (Figure 3). This putative product was found to contain no significant hydrophobic regions either internally or at the N terminus. Secondary structure prediction was performed by the method of McLachlan (1977). This indicated the presence of many regions of α-helix but little or no β sheet. More than half of the predicted α-helices would have opposite hydrophobic and hydrophilic sides characteristic of α-helices which lie on the surface of water-soluble proteins; these are shown in Figure 3. The whole polypeptide would have a small net negative charge at physiological pHs. The predicted polypeptide sequence was compared, in whole and in part, for similarity with other known protein sequences. Using the method of Wilbur and Lipman (1983) no marked global or local similarity was detected with sequences held in the Doolittle or National Biomedical Research Foundation databases or translated nucleic acid sequences in the EMBL database when these were probed with either the whole *cdc10* predicted product sequence, or with short regions of 100 amino acid residues. Comparison of the sequence was also made with that of the predicted products of the following cell division cycle genes: *cdc2* (start gene, Hindley and Phear, 1984); *cdc25* (involved in mitotic control, P.Russell, personal communication); *cdc28* and *cdc36* (budding yeast start genes, Lorincz and Reed, 1983; Peterson *et al.*, 1984) and part of the budding yeast gene *cdc4* which acts in G1 immediately after start (Peterson *et al.*, 1984). In no case was any significant similarity found.

Transcriptional control of cdc10

The *cdc10* gene function is required at start for commitment of the cell to the mitotic cycle, and commitment could be controlled by regulating the level of *cdc10* transcripts. To test this possibility *cdc10* transcript level has been monitored during a shift from exponential growth to stationary phase and during a synchronous culture. If changes occur in either of these situations then the level of *cdc10* transcript could act as an important commitment control.

RNA samples prepared from cells entering stationary phase induced either by nitrogen starvation or by nutrient depletion were blotted and probed with the 4.1-kb *Hind*III fragment. Figure 4 shows that in both cases there is little change in the level of the 2.7-kb transcript. Therefore exit from the cell cycle is not regulated by reducing the level of the major *cdc10* transcript. The 2.9-kb and 3.15-kb RNA transcript levels increase on nitrogen starvation but remain unaltered in nutrient-depleted cells. Because of this differing behaviour it is unlikely that changes in the levels of these transcripts are important for entry into stationary phase. If these species represent precursors of *cdc10* message then their increase could reflect a processing block during nitrogen starvation.

To investigate the level of transcripts through the cell cycle, RNA samples were prepared from cells synchronised by passage through an elutriator rotor. This technique separates cells on the basis of size. During the separation procedure the cells are continuously washed with fresh medium and so there is relatively little perturbation. Cells of small size (i.e., those near the beginning of the cell cycle) were selected and allowed to grow for two generations. During this period they retained synchrony as measured on the basis of increase in cell number and percentage of septated cells: two step-wise increases in cell number and two peaks of septated cells were observed (Figure 5a). RNA samples were blotted and probed as before. As controls, the transcripts of alcohol dehydrogenase and histone H2A1 were monitored. The former did not vary in level during the cell cycle (data not shown) whereas the latter was found to vary in a cell cycle-dependent manner with peaks at the expected timings of S-phase (Figure 5c). In contrast, no dramatic changes in the level of the *cdc10* 2.7-kb transcript were observed (Figure 5b). There was a small decrease in level between 4 and 5 h corresponding to the second half of G2 during the cell cycle. This period of decrease also correlated with the disappearance of the 2.9-kb transcript. The most likely explanation for these results is that there are some changes in the rate of transcription of the *cdc10* gene during the cell cycle but that these do not result in very significant changes in the level of the major *cdc10* transcript.

Figure 5 also demonstrates that the 2.7-kb transcript is not an abundant message. Comparing the specific activities of the probes and times of autoradiograph exposure indicate that the 2.7-kb transcript level is only a few per cent of the peak level of the histone H2A1 message.

Discussion

We have cloned the *cdc10* start gene from the fission yeast *S. pombe* by rescue of mutant function and shown it to be present as a single copy in the haploid gene. By subcloning we have shown that the gene is contained within a 2.6-kb DNA fragment. Sequencing of this has revealed the presence of an open reading frame of 2.3 kb and hybridisation of the fragment to cellular RNA has identified a low abundance 2.7-kb polyadenylated RNA species in the same sense as this open reading frame. Several lines of circumstantial evidence from the DNA sequence indicate that the whole open reading frame codes for a protein product: it terminates with a TAA triplet in common with all *S. pombe* genes investigated. The GC content of the open reading frame is 39% whereas that of the flanking regions is considerably lower (30% and 24%, respectively, for the 100 adjacent base pairs upstream and downstream): this pattern of nucleotide composition is typical for *S. pombe* genes. There is a TATAAAAAA sequence present 49 nucleotides upstream from the first ATG

codon which could function as a TATA box. The codon usage, while not the same as those for highly expressed genes such as alcohol dehydrogenase (*adh*) or cytochrome *c* (*cyc*) (Russell and Hall, 1982, 1983) is, however, very similar to the rather less biased usage exhibited by the *S. pombe* cell cycle genes *cdc2* and *cdc25* (Hindley and Phear, 1984; P.Russell, personal communication). In particular, there is a higher frequency of A than C at the third position in each triplet, in contrast to the highly expressed genes *adh* and *cyc* in which A occurs very seldom in this position. This difference in codon bias between genes which are expressed at different levels has also been found in the budding yeast, *S. cerevisiae* (Bennetzen and Hall, 1982). Finally, there are no regions of dyad symmetry within the open reading frame which could cause the formation of secondary structure in an mRNA, although such symmetries do exist in the flanking regions. Taking all this evidence together, it seems very likely that the *cdc10* protein product is coded for by the whole open reading frame.

The other start gene from *S. pombe, cdc2*, has been shown to contain four short (50 – 100 nucleotide) introns which are not found in the homologous *cdc28* gene from *S. cerevisiae* (Hindley and Phear, 1984 and unpublished results from this laboratory). Our data do not rule out the presence of introns within *cdc10*, but this seems unlikely since there are no sequences within the open reading frame which show close similarity with the consensus splice sequences derived from the *cdc2* introns and 11 other *S. pombe* introns. We are presently using S1 nuclease to test this prediction.

Some predictions about the nature and possible intracellular location of the putative *cdc10* product can be made from its primary structure. Since it does not contain any significant hydrophobic regions it is probably not membrane-bound or a secreted protein. The predicted presence of much surface α-helix, particularly in the C-terminal half of the molecule, suggests a globular, water-soluble protein. The observation that part of the *cdc10* gene on a plasmid can rescue (albeit poorly) the mutant *cdc10-129* gene may also throw some light on the product structure. This phenomenon could, in principle, be similar to intragenic complementation where two differently defective polypeptides associate as a multimeric protein and produce a functional product. If so, this implies that the *cdc10* product is a multimer. The *cdc10* product would have a mol. wt. of 85 400 and would be slightly acidic at physiological pHs.

In contrast to *cdc2*, which has homology with protein kinases, comparisons with other protein sequences have not revealed clues as to a possible function for the *cdc10* product since we have found no significant homology with other proteins. This is true even when small portions of the *cdc10* predicted product sequence are used for the comparisons in an effort to detect primary structure similarities reflecting functional regions, such as ATP binding sites. Some negative conclusions can be drawn from these comparisons, however: *cdc10* product is not homologous to other 'start' genes from either *S. pombe* or *S. cerevisiae*, or to the *S. pombe cdc25* or the *S. cerevisiae cdc4* genes. It does not contain the putative ATP binding site or the putative phosphorylation site found in *cdc2/cdc28*. The finding that *cdc10* predicted product shares no similarity with protein kinases suggests that it probably does not provide a function similar to *cdc2* at start.

Since the *cdc10* gene product is required for commitment of the cell to the mitotic cycle it is possible that the transition between cell cycle arrest during stationary phase and cell proliferation could be regulated at the level of *cdc10* gene transcription. However, there is a little change in the level of the major *cdc10* transcript on entry into stationary phase (Figure 4). This suggests that exit from the cell cycle is not regulated by reducing *cdc10* transcript level. There is a slight variation in the level of the 2.7-kb transcript through the cell cycle (Figure 5) but this is not likely to be significant for regulating cell cycle progress.

It is perhaps not surprising if the *cdc10* gene is not regulated at the level of transcription during the mitotic cycle. Most proteins and total polyadenylated RNA accumulate more or less continuously throughout the cell cycle (Fraser and Moreno, 1976). Probably only components required in relatively large amounts for completing specific stages of the cell cycle are likely to be regulated periodically at the transcriptional level. Two types of genes which fall in this category are the histones and tubulin. Histone gene transcripts peak in level during S-phase in vertebrate cells (Plumb *et al.*, 1983) in budding yeast (Hereford *et al.*, 1981) and in fission yeast (this study). Tubulin gene transcripts peak in level in late G2 during the *Physarum* mitotic cycle (Schedl *et al.*, 1984). Both histones and tubulin are structural components required for S-phase or mitosis, and so are required in large amounts at these two phases of the cell cycle. In the case of *cdc2* and the other start genes in fission and budding yeast, transcription appears to be at a low level suggesting that only low levels of the gene product are required. In these circumstances control at a post-transcriptional stage would be a more appropriate regulatory system. Therefore if *cdc10* does have a regulatory role in the cell cycle this is more likely to occur at the protein level. To test this possibility and also to probe the *cdc10* function further we are at present attempting to raise antibodies against the *cdc10* gene product.

Materials and methods

Strains

The gene banks were in *Escherichia coli* strain JA221, *recA1 leuB6 trpE5 HsdR⁻ HsdM⁺ lacY* C600 (obtained from J.Beggs). The strain JA226 (*recBC* instead of *recA*) was used for recovery of plasmids from yeast. The *S. pombe* strains used were 972 h^- wild-type, *cdc10-129 leu1-32* h^- or h^+ and *cdc10-129 ura4-292* h^-.

Cloning of cdc10

Procedures for gene bank construction, DNA isolation, transformation of *S. pombe* and recovery of plasmids in *E. coli* have all been described previously (Beach *et al.*, 1982b).

DNA sequencing

DNA to be sequenced was cloned into plasmid pUC12, a derivative of pUC8 (Vieira and Messing, 1982). The recombinant plasmid was cleaved adjacent to the insert with a restriction endonuclease, then treated with *Bal*31 nuclease (BRL) at 0.3 units/μg DNA for various lengths of time in a buffer containing 12 mM $CaCl_2$, 12 mM $MgCl_2$, 0.2 M NaCl, 20 mM Tris Cl pH 8.0, 1 mM EDTA, 250 μg/ml bovine serum albumin and 100 μg/ml DNA. The deleted inserts were excised by digestion with a second endonuclease and directly cloned into an appropriate bacteriophage M13 vector (Messing and Vieira, 1982) cleaved with the same restriction enzyme plus *Sma*I, which creates blunt ends. Alternatively, insert DNA was flush ended by treatment with DNA polymerase I large fragment (Klenow fragment) prior to excision and then ligated to *Bam*HI linkers (Amersham). After *Bam*HI digestion, unwanted linker fragments were removed by passage down a Sepharose CL-4B column (Pharmacia) and the remaining fragments cloned into the appropriate M13 vector cleaved with the same enzymes.

Chain termination sequencing was essentially as described by Sanger *et al.* (1980) using a 17 nucleotide synthetic primer (Amersham). Products of sequencing reactions were resolved on gradient polyacrylamide-urea gels (Biggin *et al.*, 1983).

Growth conditions

For all physiological experiments cells were grown in minimal medium (Nurse, 1975) with shaking at 25°C. For the nitrogen starvation experiment a mid-exponential culture was filtered on a membrane filter, washed, and resuspend-

ed in nitrogen-free minimal medium. For nutrient depletion an exponential culture was grown into stationary phase.

The synchronous culture was prepared using a prototype Beckman JE10X elutriator rotor. This rotor is similar in design to the JE6B elutriator rotor but is scaled up ten times. The basic procedure used followed that developed by Creanor and Mitchison (1979) with the smaller rotor. Four litres of the 972 wild-type strain were grown to 7 x 10^6 cells/ml and were pumped at 100–120 ml/min into the rotor which was spinning at 2100 r.p.m. in a J6B centrifuge. During the separation cells were kept at 25°C and flushed continuously with medium. After loading and separation, which took 40 min, the pump rate was increased by ~15% and a fraction of small cells at the beginning of the cell cycle were eluted off. A total of 1.5 litres of cells at 1.7 x 10^6/ml were collected representing a yield of ~8% of the total cells. These cells were in very early G2 as S-phase is completed during the process of cell separation.

RNA preparation

1–2 x 10^8 cells were harvested on Millipore 0.45 μm filters, washed with cold 150 mM NaCl and immediately frozen at −70°C for storage.

Cells were thawed and suspended in 0.5 ml of cold 0.32 M sucrose, 10 mM EDTA, 20 mM Tris-HCl pH 7.5, 0.5 mg/ml heparin, in a wide diameter glass tube. An equal volume of glass beads (0.5 mm diameter) was added and the cells disrupted by fast vortex mixing for 90 s followed immediately by addition of 5 ml of cold buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 mg/ml SDS, 0.5 mg/ml heparin, plus 5 ml of hot (65°C) phenol. Extraction was at 65°C for 5 min with vigorous agitation. After centrifugation, the aqueous phase plus interface were extracted twice more with hot phenol. A final extraction was performed with phenol-chloroform at room temperature. RNA was then precipitated with ethanol from the clear supernatant.

Poly(A)$^+$ RNA was prepared using oligo(dT)-cellulose as described by Aviv and Leder (1972) from total RNA obtained by a 5-fold scaling up of the above method.

Northern blots

RNA samples were denatured with glyoxal and fractionated according to size by electrophoresis in 1.2% agarose gels run in 10 mM sodium phosphate buffer pH 6.5 (McMaster and Carmichael, 1977). RNA was transferred to GeneScreen membrane (New England Nuclear) by blotting with 25 mM sodium phosphate buffer according to manufacturer's instructions. Subsequent treatment of the filter, hybridisation, washing and rehybridisation were according to manufacturer's instructions. Probe DNA was labelled either by nick translation (to 6 x 10^8 c.p.m./μg) or, for strand-specific probes, by the method of Hu and Messing (1982) to a specific activity of 4 x 10^8 c.p.m./μg.

Acknowledgements

We would like to thank Jim Creanor for assistance with setting up the elutriator rotor and Beckman Instruments Inc. for generously making a prototype model available. We also thank Mitsuhiro Yanagida and Seiji Matsumoto for providing the histone H2A1 probe prior to publication and Viesturs Simanis for helpful criticisms of the manuscript. Financial support was provided by MRC, SERC and ICRF.

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Received on 28 November 1984