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Fission yeast *cdc21*$^+$ belongs to a family of proteins involved in an early step of chromosome replication

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ABSTRACT

The *cdc21*$^+$ gene of *Schizosaccharomyces pombe* was originally identified in a screen for *cdc* mutants affecting S phase and nuclear division. Here we show that the *cdc21*$^+$ gene product belongs to a family of proteins implicated in DNA replication. These include the *Saccharomyces cerevisiae* MCM2 and MCM3 proteins, which are needed for the efficient function of certain replication origins, and *S.cerevisiae* CDC46, which is required for the initiation of chromosome replication. The cdc21 mutant is defective in the mitotic maintenance of some plasmids, like *mcm2* and *mcm3*. The mutant arrests with a single nucleus containing two genome equivalents of DNA, and maintains a cytoplasmic microtubular configuration. Activation of most, but not all, replication origins in the mutant may result in failure to replicate a small proportion of the genome, and this could explain the arrest phenotypes. Using the polymerase chain reaction technique, we have identified new *cdc21*$^+$-related genes in *S.cerevisiae*, *S.pombe* and *Xenopus laevis*. Our results suggest that individual members of the *cdc21*$^+$-related family are highly conserved in evolution.

INTRODUCTION

Relatively little is known about proteins that are required for the initiation of eukaryotic DNA replication. From analyses of prokaryotic and viral replication systems, it seems likely that this initiation event will involve proteins that associate with replication origins to prepare the DNA template for initiation. This function can involve the melting of the origin region and binding to other replication proteins, so that a replisome capable of elongation is built up at the initiation site. In the replication of the *E.coli* chromosome, a key initiator protein is dnaA (reviewed in ref. 1). This protein recognizes the origin region, helps to denature the DNA, and may assist the binding of the dnaB-dnaC protein complex which can unwind the DNA helix further via a helicase function. In the replication of the SV40 chromosome, T-antigen not only recognizes the origin, but uses a helicase activity to denature the DNA, and a subsequent interaction with DNA polymerase α-primase may then allow replication to start (2). It would clearly be interesting to identify host proteins with analogous roles and to determine their involvement in the regulation of chromosome replication.

One genetic method for identifying replication proteins involves the analysis of yeast *cdc* mutants that are conditionally affected in their ability to execute S phase normally (reviewed in refs. 3, 4). Analysis of such mutants, mainly in the budding yeast, has shown that some *CDC* genes encode enzymes directly involved in DNA replication, such as DNA polymerases (*S.cerevisiae* CDC2 (5) and *CDC17* (6)). Other *cdc* gene products are implicated in early stages of chromosome replication, but their functions are less clear. These include fission yeast cdc10, which appears to function as a transcription factor (7), and budding yeast CDC4(8); both these proteins contain repetitive motifs that may be important for interacting with other proteins. Potential regulatory factors include the serine/threonine protein kinases encoded by *cdc2/CDC28* (for review, see ref. 9) and *CDC7* (10).

A second genetic approach involves the analysis of mutations that affect replication origin function, detected by monitoring the mitotic stability of minichromosomes. Thus mutations that decrease the mitotic stability of yeast minichromosomes (such as Mcm$^-$ mutants, (11)) may identify factors that are needed for origin activation, and mutations that suppress replication origin defects (such as Rar$^-$ mutants, (12)) are also implicated in some aspect of replication initiation. Some of the budding yeast genes identified in this kind of analysis encode transcription factors such as MCM1 (13), GAL11/RAR3 (14), and TUP1 (15), suggesting that in common with eukaryotic viral replication, transcription factors may play a role in replication origin activation. Curiously, both *cdc6* and *cdc14* mutants of *S.cerevisiae* show a minichromosome loss phenotype which is suppressed when extra replication origins are inserted (16); this may reflect an involvement of these proteins in initiation.

In addition to using genetics, the detailed characterization of replication origins in budding yeast allows direct biochemical methods to be used to identify proteins that interact with important initiator sequences. Sequences that can function as replication

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origins in *S.cerevisiae* contain an essential core element, related to the consensus sequence $^{T}_{A}TTTA^{TA}_{CG}TTT^{T}_{A}$, and other closely linked sequences which contribute to the efficiency of replication origin function (for review, see ref. (17). Proteins binding to sequences that are important for the efficient functioning of some replication origins include ABF-1, which appears to function in both transcription and replication (18,19, 20), providing further evidence for a role of transcription factors in initiation. Proteins have also been described which interact with the essential ARS consensus region, although the function of these proteins in replication has yet to be established (21, 22, 23).

In this paper we describe the molecular genetic characterization of the *cdc21*$^+$ gene of fission yeast, which we selected for further analysis since earlier studies suggested a role in the initiation of DNA replication. The *cdc21*$^+$ gene was originally identified in a screen for *cdc* mutants that arrest as elongated cells with undivided nuclei (24). The mutant has an early transition point in the cell cycle, and shows an altered round of DNA replication at 36°C, in that the first two-thirds of S phase occurs with a similar timing to wild type, but the last one-third takes much longer to complete. Releasing *cdc21*^{ts} cells from a hydroxyurea block allows them to go on to divide at the restrictive temperature, consistent with the notion that the cdc21 protein functions before the elongation step of DNA replication. Here we find that cdc21 belongs to a family of yeast proteins which have been implicated in an early stage of chromosome replication by both Cdc phenotypes and minichromosome stability assays. This gene family has been highly conserved in evolution, and we have identified new related genes in yeast and vertebrate genomes using the polymerase chain reaction technique.

MATERIALS AND METHODS

Yeast molecular genetics

Haploid strains of *Schizosaccharomyces pombe* used were: RP1: *h*$^-$, *leu1*, *cdc21*; RP2: *h*$^-$ *leu1*. The diploid strain of *S.pombe* used was 972/972: *h*$^-$/*h*$^-$. Media and general *S.pombe* techniques were as described in (25). General nucleic acid techniques were as described in (26).

DNA sequencing

The *cdc21*$^+$ gene was sequenced using the M13/dideoxy-nucleotide chain termination method following the cloning of random fragments generated by sonication (27). Analysis of DNA sequences and protein sequences, and database searches were performed using the STADEN (28) and University of Wisconsin GCG (29) packages. Multiple sequence alignments were carried out using CLUSTALV (30).

Immunofluorescence microscopy

S.pombe microtubular structure was examined by indirect immunofluorescence using the anti-tubulin antibody TAT1 (31), which was a gift of R.Bartlett. Cells were fixed, permeabilized and incubated with primary (TAT1) and secondary antibody (antimouse IgG coupled to fluorescein, Sigma), essentially as described in (32), and mounted in 0.15 μg/ml Hoecsht 33258 (bisbenzimide) to stain DNA.

Determination of DNA content by flow cytometry

The cells were fixed in ethanol by the method described by (33). An RNase A digest was performed, followed by staining with propidium iodide (10mg/ml) as described in (34). Flow cytometric analysis was performed using a Beckton Dickinson FACScan.

Minichromosome stability assays

S.pombe strains containing *S.pombe* ARS plasmids (35) were grown under selection to late log phase and then 10^6 cells were added to 5 mls of rich non-selective medium and grown to saturation at 25°C. Cells were plated out on non-selective plates and replica plated to selective plates to determine the proportion of plasmid-containing cells. Plasmid stabilities shown are means of three assays on independent transformants.

Polymerase chain reaction and phylogeny analysis

Two 26 nucleotide PCR primers were designed to amplify a conserved region the gene family. The sequences of the primers were: CCCAATATTTTGATGTGTGGTGA(T/C)CC; and GCTCGAGTTTGTTGTTCCATNAC(T/C)TC. The predicted size of the PCR product from the *cdc21*$^+$ gene is 279 bp. PCR reactions were carried out using Promega Taq DNA polymerase (36) and a Grant PCR machine with a thermal cycle of: 95°C, 1.2′; 52°C, 0.8′; 72°C, 1′; for 30 cycles; then final annealing and extension steps of 52°C, 5′; 72°C, 5′. DNA products were purified from gels, and cloned into the SmaI site of pUC18 for subsequent DNA sequencing. For phylogeny analysis the neighbour-joining method of Saitou and Nei was used (37), as implemented in CLUSTALV.

RESULTS

Molecular cloning of the *cdc21*$^+$ gene

A plasmid containing a 9 kb fragment capable of complementing the temperature-sensitive *cdc21* mutation was derived from a gene library cloned in the vector pDB248X. Further dissection localized the gene to a 4.4 kb region (Fig. 1). A *LEU2* plasmid containing the 9 kb Pst I fragment was integrated into homologous sequences in the *leu1 cdc21* strain RP1, which was subsequently crossed to a *leu1 cdc21*$^+$ strain. Random spore analysis from this cross showed close linkage between the *cdc21* locus and the site of integration (Cdc$^+$, Leu$^+$: 269; Cdc$^+$ Leu$^-$: 162; Cdc$^-$ Leu$^+$: 1; Cdc$^-$ Leu$^-$: 8), implying that the cloned fragment contains the *cdc21*$^+$ gene.

The DNA sequence of the BglII-PstI *cdc21*$^+$-containing fragment revealed a single large reading frame of 909 codons, capable of encoding a 101 kDa protein. Disruption of this reading frame abolished the ability of the DNA fragment to complement the *cdc21* mutation (Fig. 1). Northern analysis using a fragment

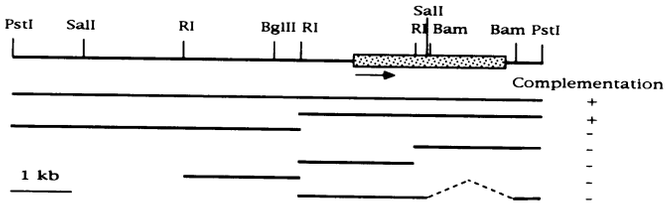

Figure 1. Restriction map and location of the *cdc21* gene. The ability of DNA fragments to complement the *cdc21* mutation is shown. '▨' indicates the extent of an open reading frame in the BglII- Pst I fragment; the direction of transcription is indicated. The *cdc21*^{ts} mutation is probably 5′ to the EcoRI site in the reading frame, since fragments containing this region can give a delayed rescue of the mutant phenotype, presumably by gene conversion.

from the coding region as a probe showed a single transcript of 3.1 kb, which is large enough to encompass the predicted *cdc21*$^+$ reading frame (data not shown). Incidently, monitoring the level of this transcript through the cell cycle showed no evidence for periodic changes in the mRNA level (data not shown).

Simliarity of cdc21 to MCM2, MCM3 and CDC46

Through database comparisons we established that the *cdc21*$^+$ gene product is related in sequence to three *S.cerevisiae* proteins, MCM2, MCM3 and CDC46, that are implicated in DNA replication (Fig. 2). *MCM2* and *MCM3* are essential genes that were identified by mutations that lower the mitotic stability of minichromosomes (11, 38, 39). *Mcm2* and *mcm3* mutant strains appear to affect only the stability of plasmids containing certain ARS elements, and for *mcm2* it has been shown that the cause of instability is failure to replicate rather than nondisjunction (40, 41) This implicates the gene products in the function of some but perhaps not all replication origins. The *CDC46* gene was identified as a temperature-sensitive suppressor of two cold-sensitive *cdc* mutations, *cdc45* and *cdc54* (42). *Cdc46* mutant cells arrest with unreplicated DNA, and the gene product executes

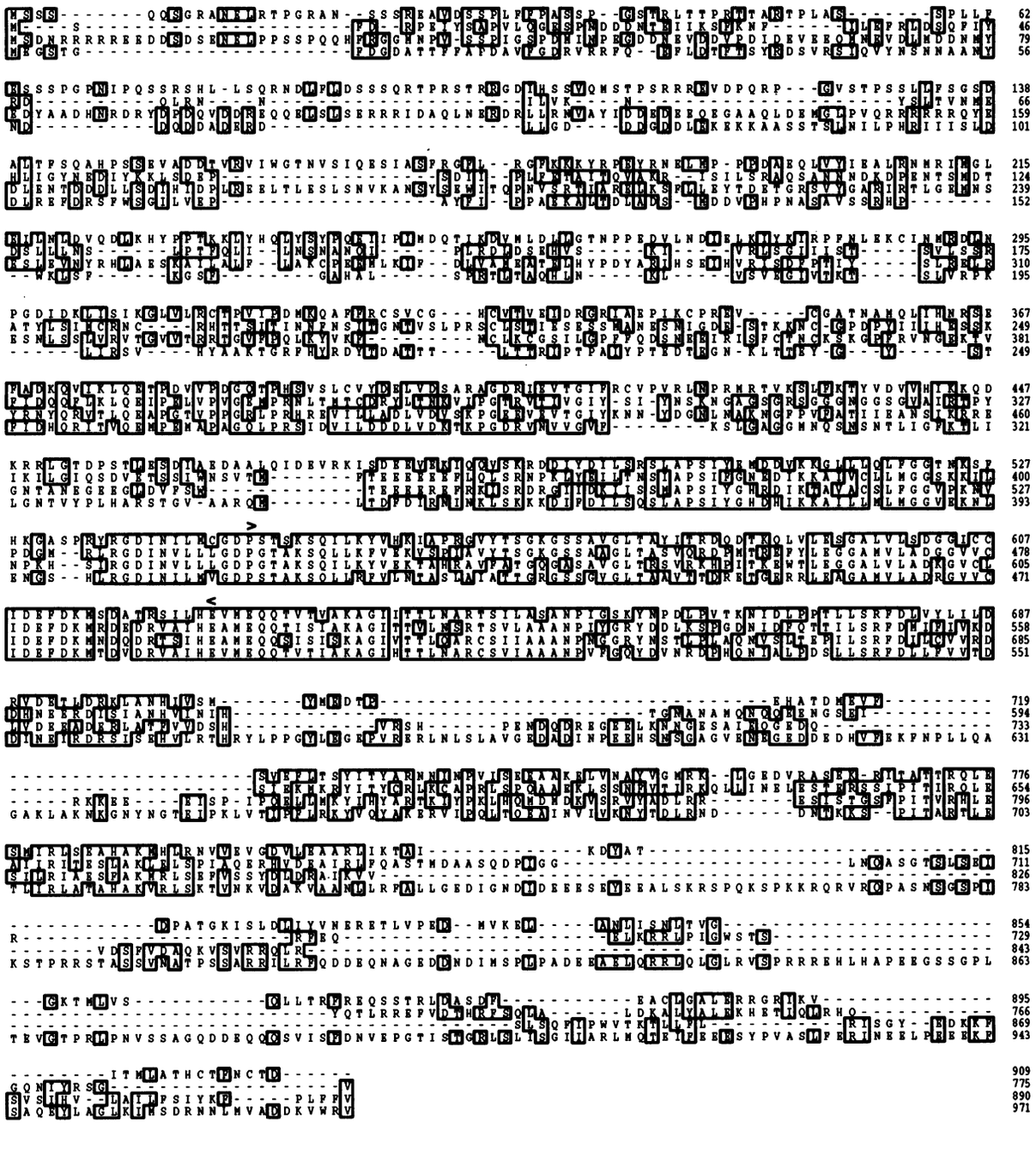

Figure 2. Comparison of cdc21 sequence with related proteins. The cdc21 protein sequence (top) is shown, and compared to (order top to bottom) CDC46, MCM2 and MCM3. Residues are boxed to show identities. The alignment of the four sequences was generated using CLUSTALV (fixed gap penalty = 10; floating gap penalty = 10; protein weight matrix = pam 250). '−' indicates padding characters inserted to optimize the alignment. The arrows indicate the region amplified by PCR analysis in Fig. 8. The DNA sequence of the *cdc21*$^+$ gene is available from the EMBL database, accession number X58824.

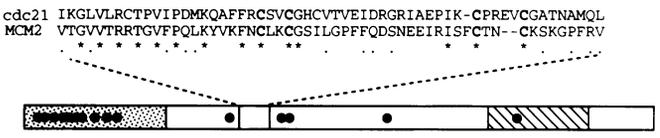

Figure 3. Protein sequences features of the *cdc21*$^+$ gene product. The sequence shows an N-terminal hydrophilic region (indicated by '▨') that is particularly rich in serine, arginine and proline. This region contains elements resembling PEST sequences (51), and it is of interest that the CDC46 protein also has features that may indicate relative instability in vivo (43). Cdc21 contains many(S/T)PXX motifs (indicated by '●'), concentrated in the N-terminus, which have been implicated in nucleic acid binding (52). Two of these motifs have the sequence TPXR, and are thus potential phosphorylation sites for the cdc2 protein kinase (reviewed in (53)). '▧' indicates the region of a possible four-α-helix bundle, which is conserved between cdc21, MCM2, MCM3 and CDC46. Also indicated is a region of the MCM2 sequence that contains a possible zinc finger motif, which is partially conserved in cdc21 (shown), but not in MCM3 and CDC46. In MCM2 this motif is implicated in its function by mutational studies (39). The cdc21 sequence does not correspond to known zinc-finger structures, but it is possible that this region of the protein has a role in coordinating a metal ion.

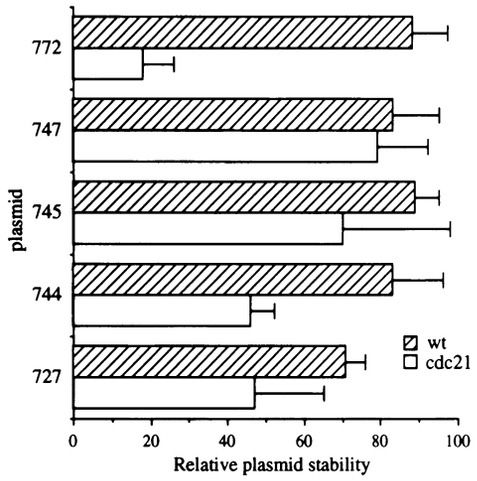

Figure 4. Mitotic stability of ARS plasmids in *cdc21* and wild type strains. The graph shows the percentage of cells retaining the *S.pombe* ARS-containing plasmids after approximately 9 generations in non-selective medium. Values shown are averages and standard deviations of three determinations on independent transformants. Presence of monomeric automously replicating plasmid in the strains used was confirmed by Southern blotting (data not shown).

its function before the hydroxyurea-sensitive elongation step (43). Intriguingly, the *CDC45* and *CDC54* genes are also required for an early step in chromosome replication (43).

Fission yeast cdc21 is approximately equally related to the three budding yeast proteins, the overall level of identity being about 25%. The degree of similarity is most impressive in a central 200 amino-acid region of the aligned proteins, where approximately 55% of amino-acids are identical in pairwise alignments, and 37% of amino-acids are identical between all four proteins. These conserved regions do not appear to correspond to previously recognized domains involved in, for instance, DNA binding or protein-protein interactions. However, from a consideration of the predicted secondary structure of the conserved regions in the alignment, it is possible that the C-terminal regions of the proteins contain a four-α-helix bundle

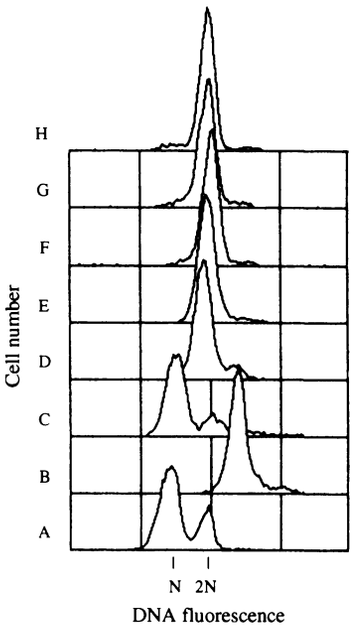

Figure 5. Flow cytometric analysis of the *cdc21* mutant. A: Nitrogen-starved haploid wild-type showing fluorescence peaks corresponding to one-genome (N) and two-genome equivalents (2N) of DNA. B: Wild-type (strain 972/972) diploid in log phase; C. Nitrogen-starved strain P1 (*cdc21*) at 25°C; D: *cdc21* cells in log phase at 25°C; E, F, G H: as D, but shifted to 36°C for 1, 2, 3 and 4 hours respectively. A *cdc21*$^+$ strain (RP2) showed a similar profile to the *cdc21* strain at 36°C (data not shown).

(Fig.3, (44)). The cdc21 protein has a neutral pI (pI=7.0), and is thus less acidic than the related proteins. Sequence motifs of possible significance are described in Fig. 3.

Phenotypic analysis of the *cdc21* mutant

Given the similarity of cdc21 to the MCM2 and MCM3 proteins, we analyzed the *cdc21* mutant to see if it exhibits a defect in its ability to maintain autonomously replicating plasmids. Plasmids containing different *S.pombe* ARS elements (35) were transformed into wild-type and *cdc21* strains, and the mitotic stabilities of the plasmids under nonselective conditions were compared. Out of five plasmids tested, two are clearly less stable in the *cdc21* strain compared the the wild-type (Fig. 4). In addition, hybridization analysis shows that the pARS772 plasmid has a lower copy number in the *cdc21* strain compared to the wild type, when grown under selective conditions (data not shown). This mitotic instability, which is particularly marked with the pARS772 plasmid, may reflect a defect in replication origin activity in the *cdc21* mutant, although we are unable to distinguish between a defect in segregation or replication from this data.

Since the *cdc46* and *mcm2* mutants also show DNA replication defects that are detectable by flow cytometry (43, 39), we used this technique to analyze *cdc21* cells at the nonpermissive temperature to see if a change in cell DNA contents could be detected (Fig.5). In log phase at the permissive temperature, haploid *cdc21* cells show a predominant '2N' peak of DNA fluorescence as expected, reflecting the brevity of the G1 phase in *S. pombe*. When shifted to 36°C, cells can still enter S phase, since an 'N' peak of fluorescence does not appear (Fig. 5). We cannot detect a significant change in the profile of cell DNA

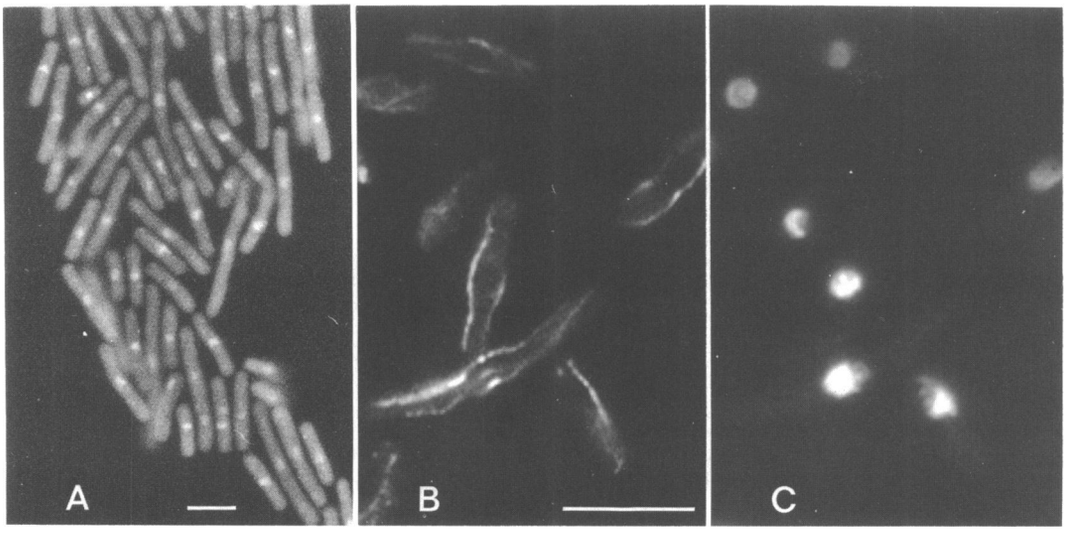

Figure 6. Terminal phenotype of the *cdc21* mutant. A: *cdc21* cells were grown to log phase at 25°C, and shifted to 36°C for 6 hours before fixation and stained to show DNA. Bar = 10 μm. B & C: As in (A) except cells were arrested for 5 hours, and then permeabilized for indirect immunofluorescence detection of microtubules using the TAT1 antibody, (31) shown in B; C shows DNA staining. Bar = 10 μm. At the permissive temperature, microtubule staining of the *cdc21* strain is similar to wild type (not shown).

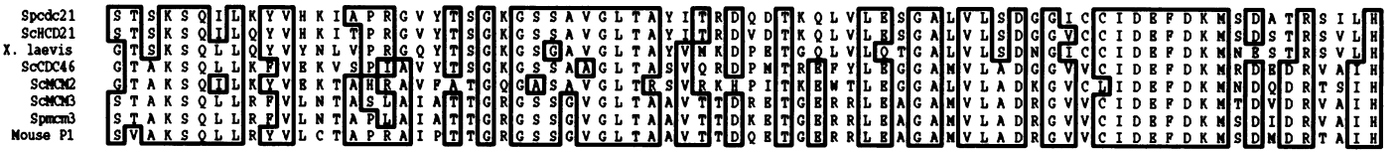

Figure 7. Comparison of cdc21-related protein sequences derived by PCR. The translation of DNA sequences, obtained from PCR of *S. pombe* (Sp *mcm3*$^+$), *S.cerevisiae* (Sc *HCD21*) and *X. laevis* DNA, is compared to sequences of the cdc21, CDC46(43), MCM2(39), MCM3 (38), and mouse P1 (45) proteins. The DNA sequences derived by PCR have the following EMBL accession numbers: Z15032 (Sc HCD21); Z15033 (X. laevis sequence); Z15034 (*S.pombe mcm3*+).

contents compared to a wild-type strain, implying that the mutant cells are able to replicate most of their DNA at the restrictive temperature, and do not arrest in G1. A defect in replicating a small fraction (10−20%) of the genome would probably be difficult to detect in this analysis.

We also examined microtubules in *cdc21* cells by immunofluorescence microscopy (Fig. 6). Arrested cells show a microtubular array typical of interphase cells (32), together with an undivided nucleus, implying that the cells are arrested in interphase rather than an early mitotic phase.

Identification of *cdc21*$^+$-related genes in yeast and vertebrates

The identification of *cdc21*$^+$-related genes in two distantly related yeasts suggests that similiar genes may be found in a wide range of eukaryotic genomes. Indeed, a partial cDNA encoding a mouse protein related to CDC46 has been reported (43), and very recently Thömmes et al. have identified mammalian proteins which appear to be homologues of MCM3(45). To gain insight into the size of the family of *cdc21*$^+$-related genes we used the polymerase chain reaction (PCR) to identify similar genes in yeast and other eukaryotic genomes. Protein sequences that are highly conserved in the *cdc21*$^+$-related family were chosen for the design of the primers (see Fig. 2). The primers were degenerate at their 3′ ends to increase the number of genes that might be identified in PCR reactions. A PCR product of the predicted size was generated from *S.pombe* DNA (data not shown). Cloning and sequencing of this product revealed the *cdc21*$^+$ sequence as expected, and a related sequence which encodes a product more closely related to the *S.cerevisiae* MCM3 sequence than any other protein in the family (Figs. 7 and 8). Using *S.cerevisiae* DNA, the PCR reaction generated a fragment identical in size to that obtained with *S.pombe* DNA (data not shown). Clones of this product gave the *S.cerevisiae MCM3* sequence and a novel sequence which on translation is more closely related to cdc21 than the other gene products (Figs. 7 and 8). The new sequences may correspond to an *S.pombe* homologue of the *MCM3* gene (provisionally designated *mcm3*$^+$), and an *S.cerevisiae* homologue of the *cdc21*$^+$ gene (provisionally designated *HCD21*), but clearly the isolation and sequencing of the entire genes would be necessary to substantiate this.

PCR reactions using *Xenopus laevis* also generated a fragment identical in size to the yeast products (data not shown). Cloning and sequencing showed that the encoded protein is more closely related to cdc21 than other proteins in the family. The degree of similarity between this sequence and *S.pombe* cdc21 is somewhat less than that between the mouse P1 and MCM3 proteins in this region (Fig. 8), so it is not clear whether this

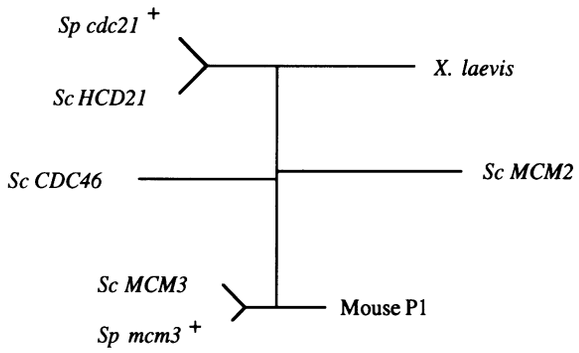

Figure 8. Phylogeny analysis of the alignment of cdc21-related sequences shown in Fig. 8. Branch lengths are proportional to estimated phylogenic distance. The unrooted tree was derived using the neighbour-joining method (37).

Table 1.

	cdc21$^+$-related genes			
	cdc21	*CDC46*	*MCM2*	*MCM3*
S.pombe	+ *cdc21*$^{+a}$	+ *nda4*$^{+b}$		+? *mcm3*$^{+a}$
S.cerevisiae	+? *hcd21* [a]	+ *CDC46*[c]	+ *MCM2*[d]	+ *MCM3*[e]
X.laevis	+?[a]			
Mouse		+? [f]		+ P1[g]
Human				+ P1[g]

+ = gene or probable homologue found (name given).
+? = possible homologue identified by limited sequencing of gene.
References: [a]This work; [b]M.Yanagida. personal communication; [c](43); [d](39); [e](38); [f](54), (43), (55); [g](45).

vertebrate gene represents a homologue of fission yeast *cdc21*$^+$. A phylogenic comparison of all the sequences shows that they fall into four discrete subgroups (Fig. 8), implying that there are at least four distinct types of protein in this family.

DISCUSSION

Cdc21 function

The *S.cerevisiae* proteins MCM2, MCM3 and CDC46, that are related in sequence to cdc21, are implicated in an early phase of S phase execution from the effect of mutations on the overall extent of DNA replication, and on minichromosome replication. A *cdc46* mutant arrests with one genome equivalent of DNA (43). *Mcm2* and *mcm3* mutants are unable to maintain plasmids containing certain ARS elements as if some replication origins work poorly in the mutants (40, 38). Although the *cdc21* mutant replicates most of its DNA at the restrictive temperature this is not inconsistent with a role for the protein in initiation. If a mutation caused a failure to initiate at only a subset of replication origins, the replication origins that did function might be sufficient to allow replication of most of the genome. This situation may occur with certain, perhaps less tight, alleles of *cdc46*, when cells arrest having replicated some of their DNA(43). Also, depletion of the *MCM2* and *MCM3* gene products does not prevent chromosome synthesis (38, 39). A partial defect in initiation in *cdc21* would thus be consistent with the transition point of the mutant, the apparent effect on plasmid replication, and the terminal phenotype.

If replication origin activation is only partially defective in the *cdc21* and related mutants, why does this lead to an arrest of the cell cycle? One possibility is that the gene products are additionally required for mitosis but this is not supported by the hydroxyurea sequencing data for *cdc21* (24) and *cdc46* (46) mutants. An alternative explanation is that complete DNA replication cannot be achieved from the activation of a subset of origins, and this defect is detected by a 'checkpoint' system which prevents mitosis and nuclear division in the absence of complete DNA replication (47). Certain large regions of the genome, such as that containing the rRNA gene repeats, may be dependent for replication on an identical type of replication origin. A defect in replicating this region from the internal origins would not be efficiently suppressed by invading replication forks from flanking DNA, owing to the relatively slow rate of fork movement. Alternatively, certain regions of the genome may be dependent for replication on specific origins owing to the presence of elements which impede the progress of replication forks. Such replication fork barriers have been identified in *S.cerevisiae* in the rRNA gene repeat (48), and may also exist near the telomere in chromosome V (49). The residual unreplicated DNA might be difficult to detect using the flow cytometric analysis we have used here.

If the cdc21 protein is required for replication origin activation, it will be interesting to determine whether it is needed for all initiation events, or just those at a subset of origins. In *S.cerevisiae*, certain replication origins appear to be activated late in the S phase (49), and this late activation might reflect the participation of specific initiator proteins that are not involved at earlier stages. Although the plasmid stability assay hints at a *cdc21* phenotype that is origin-specific, an overall reduction in replication origin efficiency might reduce the stability of plasmids containing weak origins more so than ones containing efficient replication origins. If cdc21 is universally required for replication, it should be possible to get tighter alleles that cause an arrest with unreplicated DNA.

The cdc21-related protein family

The conservation of protein sequence between members of the family is impressive but has given no clues as to biochemical functions. In *S.cerevisiae*, there are at least four genes related to *cdc21*$^+$, one of which may be a *cdc21*$^+$ homologue (Table 1). In *S.pombe*, apart from *cdc21*$^+$, we have identified a possible *MCM3* homologue, and another gene has been identified which appears to be a homologue of *CDC46* (*nda4*$^+$, M.Yanagida, personal communication). Related genes are also found in vertebrates; perhaps there are eukaryotic homologues for each member of the gene family found in yeast?

The similarity of mutant phenotypes and protein sequences relating to the *cdc21, MCM2, MCM3* and *CDC46* genes may reflect the fact that these proteins function independently to carry out the same biochemical function, but for some reason are unable to substitute for each other's activity. An alternative explanation is that they may interact, perhaps physically, to allow a specific function, such as initiation of replication, to be carried out. In either situation, the degree of protein sequence conservation may reflect the existence of protein domains involved in protein-protein, or protein-DNA interaction. By suppressor analysis, three *cdc* genes have been identified that interact with *CDC46*, and which appear to function at the same stage of the cell cycle (42, 43). In addition, a mammalian homologue of the MCM3 protein co-purifies with DNA polymerase α (45). Thus there is

preliminary evidence that *cdc21*$^+$-related gene products function in complexes with a number of other proteins.

One interesting feature of the CDC46 protein is the change in its nuclear localization during the cell cycle. Cells are born with CDC46 in the nucleus, but the protein disappears from this compartment at a point close to the initiation of DNA replication, and the protein continues to be excluded until mitosis (46). This behaviour mirrors that of the hypothetical 'licensing factor', in a model which accounts for the once-per-cell-cycle replication of DNA (50). It would clearly be of interest to determine whether fission yeast relatives of CDC46 show a similar behaviour, and we are currently investigating this possibility.

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