Cloning and characterization of the *Saccharomyces cerevisiae* CDC6 gene

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ABSTRACT

The yeast cell division cycle gene CDC6 was isolated by complementation of a temperature-sensitive cdc5 mutant with a genomic library. The amino acid sequence of the 48 kDalton CDC6 gene product, as deduced from DNA sequence data, includes the three consensus peptide motifs involved in guanine nucleotide binding and GTPase activity, a target site for cAMP-dependent protein kinase and a carboxy-terminal domain related to metallothionine sequences. A plasmid-encoded CDC6-β-galactosidase hybrid protein was located at the plasma membrane by indirect immunofluorescence. Disruption experiments indicate that the CDC6 gene product is essential for mitotic growth.

INTRODUCTION

DNA synthesis and nuclear division in *Saccharomyces cerevisiae* depends on the function of several cell division cycle genes, including *CDC4*, *CDC6* and *CDC7* (1-3). These genes execute their functions beyond "start", a G1 control point dividing the cell cycle into an uncommitted phase (decision between growth and sporulation in diploids, or growth and conjugation in haploids) and a committed phase, where DNA synthesis and mitosis is initiated (4). Temperature-sensitive cdc6 mutants arrest upon shift to restrictive temperature (36°C) as singly budded uninucleate G1 or G2 cells with complete short spindles in nuclei situated at the mother-bud neck (5), indicating a block of nuclear division. DNA synthesis continues up to a complete new round of replication (2) and remains sensitive to the DNA elongation inhibitor hydroxyl urea, but the newly made DNA appears to be faulty or incomplete, pointing to an incorrect initiation of replication at non-permissive temperature (3). Furthermore, cdc6 mutants tend to loose chromosomes under the same conditions. On the other hand, the size
distribution of single-stranded replication intermediates made at 36°C in cdc6 closely resembles that of wild type cells (6).

A more detailed knowledge of the CDC6 gene function requires the isolation and analysis of this gene. Here we report the molecular cloning, sequence analysis and transcription of the wild type CDC6 gene, as well as the intracellular localization of a CDC6-β-galactosidase fusion protein.

MATERIALS AND METHODS

Strains and Media

MATa. cdc6-1. ade1. ade2. ade6. uri1. his7. lys2. tyr1. gal1 (strain ts327) was from the Yeast Genetic Stock Center. The mutants MATa.ura3. MATa.ura3 and a yeast genomic library in the shuttle vector pFL1 (7) were kindly provided by F. Lacroute. cdc6-1.ura3 strains were constructed by crossing strain ts327 with MATa.ura3. Temperature sensitive, uracil requiring strains were complemented with four tester strains MATa.ura1. MATa.ura1. MATa.ura3. MATa.ura3 in order to identify cdc6-1.ura3 strains in both mating types. The procedure for molecular cloning has been described (8). The E. coli strain B15183 (F- recBC. end1. gal. met. str. thi. bio. hsd) was used for selection, propagation and isolation of plasmids, strain TG1 (K12 Δ(lac-pro). supE. thi. hsd. D5/F' traA36. proA+ B+. lacI Q. lacZAM15) for expression of β-galactosidase fusion protein. Yeast cells were grown in YPD medium, containing 1 % yeast extract, 2 % bactopeptone, 2 % glucose supplemented with 50 mg each of adenine and uracil per liter. Minimal medium (MM) containing 8 g yeast nitrogen base (with amino acid), 20 g glucose and 50 mg adenine per liter was used to select transformants. Sporulation was performed on 1.5 % agar containing 8 g potassium acetate per liter (SP2).

Mapping, Subcloning and Integration of Recombinant Plasmid

Restriction endonucleases were from Bethesda Research Laboratory. Klenow fragment and T4 DNA ligase was a gift of F. Eckstein; DNA polymerase I was provided by F. Grosse. Deletions were introduced either by single enzyme digestion and religation, or by double digestion, repair of single stranded ends and blunt ligation. By constructions of hybrid plasmids, restriction fragments were isolated from 1 % agarose gel by "geneclean-kit" (BIO 101) and ligated.
**DNA Sequencing**

A mixture of restriction fragments obtained by double or triple digestion was incubated at 25°C for 20 min with single kind of deoxynucleotide α-[32P] triphosphate (Amersham) and the Klenow fragment of DNA polymerase I in order to label only one 3' end of fragments by partial repair without adding unlabeled deoxynucleotide triphosphates. Labelled fragments were isolated from 5% polyacrylamide gel and cleaved according to the chemical method (9).

**Northern Analysis**

Total RNA was prepared according to Chomczynski at al. (10), incubated with formamide and formaldehyde (11), fractionated by 1% agarose gel containing 2.2 M formaldehyde, and transferred to HybondM filters (Amersham). The bands of the ribosomal RNAs were visualized by ethidium bromide staining and used as size markers. DNA probes were labelled with [32P]dCTP, using the Multiprime™ DNA labelling system (Amersham).

**Immunofluorescence of Yeast Cells**

Early log phase yeast cells were fixed with formaldehyde (12), treated with 0.2% Triton X-100 and digested with glusulase as described by Kilmartin and Adams (13). After washing, the cells were treated with 1:400 and 1:1200 diluted polyclonal rabbit β-galactosidase antibody overnight at 4°C. The next day the cells were washed 3 times with PBS-sorbitol and incubated 80 min long with fluorescein-isothiocyanate (FITC) conjugated sheep antibody to rabbit immunoglobulin G (SIGMA). After washing, the cells were resuspended in 50 ml glycerol and examined with a Zeiss photo microscope with standard FITC filter and photographed with KODAK PAN 400 film using a 100 x oil-immersion objective. The exposure times for FITC stained cells were between 20 and 50 sec.

**RESULTS**

**Molecular Cloning, Mapping and Integration of cdc6-Complementing Plasmids**

Test transformation of several MATa.cdc6-1.ura3 strains with the shuttle vector pFL1 revealed dramatic differences of transformability: four strains were not transformable, four other produced 200 - 1000 transformants/µg and two produced 5000 transformants/µg DNA. The two latter strains were complemented by a yeast wild-
A, Restriction map of the cdc6-complementing plasmid p68311, a pFL1 (7) derivative containing a 5.3 kb genomic insert. B, Localization of the complementing region by deletion mapping. C, Restriction map of the smallest complementing plasmid p6/l and sequencing strategy. Black box, genomic insert, open box, URA3 gene; waved line, 2 μD; ORF, open reading frame.

Figure 1. A, Restriction map of the cdc6-complementing plasmid p68311, a pFL1 (7) derivative containing a 5.3 kb genomic insert. B, Localization of the complementing region by deletion mapping. C, Restriction map of the smallest complementing plasmid p6/l and sequencing strategy. Black box, genomic insert, open box, URA3 gene; waved line, 2 μD; ORF, open reading frame.

type genomic library containing overlapping Sau3A fragments (up to 10 kb length) in the BamHI site of pFL1. Total cellular DNA was extracted from 12 individual transformants and introduced into E. coli, producing up to 400 ampicillin-resistant colonies. Plasmids were isolated and tested by back transformation into the strains cdc6, ura3 and ts327.

Figure 1A shows the restriction map of the recombinant plasmid p68311 containing a 5.3 kb genomic insert in pFL1. For locali-
zing the complementing region within the 5.3 kb insert, various parts of the plasmid were deleted by restriction and religation (Fig. 1B). Deletions of a 3 kb PvuII fragment and a 0.7 kb NHeI fragment did not alter the complementation activity, whereas the deletion of a 3 kb SphI fragment, a 4 kb XhoI fragment and a 3.7 kb PvuII/BclI fragment produced completely inactive plasmids.

Plasmid p6/1 (Figure 1C) was obtained by deleting the NheI and PvuII fragments from p68311 and used for sequencing. An integrative derivative (p6/int.) lacking the 2.2 kb EcoRI 2 μD fragment was linearized with BglIII and targeted into the insert-homologous chromosomal site. The map of the duplicated site was confirmed by genomic blotting (data not shown), and the site of integration relative to the cdc6 locus was determined by crossing CDC6::p6/int. (URA3), ura3 integrants with cdc6-1, ura3 strains of opposite mating types.

The phenotypical analysis of more than 100 spores resulted in a segregation pattern having a ratio of ts+,ura+ to ts-,ura- close to 1:1, which is expected from an integration of the URA3-containing plasmid into the CDC6 site. The same plasmid p6/int. linearized with BglII complements the cdc6-1 lesion as well, producing 50 ts+,ura+ integrants/μg DNA. We conclude from these observations that p6/int. carries the wild type allele of the cdc6 locus.

Nucleotide Sequence of the CDC6 Gene

Figure 1C shows the sequencing strategy of the complementing region, and the nucleotide sequence is presented in Figure 2. The sequence reveals an open reading frame of 1281 nucleotides (426 amino acid residues), coding for a protein of a calculated molecular mass of 48 kD. The 5' upstream region (nucleotides -402 to -255) contains several TATA boxes, the 3' downstream region (nucleotides 1507 - 1512) a polyadenylation signal AATAAA.

A search for similarities with protein sequences collected in the EMBL protein data base revealed sequence similarities with GTP-binding proteins and with metallothionine and high sulfur keratin (Figure 3). The CDC6 gene product contains the three peptide sequences GPPGTGK, DLNG and NSLD or NTGD (separated by 76, 68 and 132 residues, respectively) corresponding to the three consensus motifs found in all GTPase domains (14). A potential target site for cAMP-dependent protein kinase (KKTT, residues 126 - 129) is
Figure 2. Nucleotide sequence of the cdc6-complementing region and the deduced amino acid sequence of the CDC6 gene product.
Figure 3. A, Comparison of four CDC6 peptide regions with sequences conserved in human c-H-ras and in yeast GTP-binding protein families. Residues identical with the CDC6 sequences are boxed.

References: H-ras (30), RAS1,2 (31), RHO1,2 (32), IEH (33), SEC4 (34), GEA2 (37,36), YEFL (38,39).

B, Comparison of a carboxy-terminal CDC6 sequence with human metallothionine (MT1,2) and high sulfur keratin B2A (16). Most of the metallothionine residues are conserved in 10 other mammalian sequences (15).

Disruption of the CDC6 Gene

Cell cycle gene disruptions are not always lethal, as shown for CDC25 (17). The lethality of the CDC6 disruption was demonstrated by integrative transformation of an ura3/ura3 diploid strain with a DNA fragment, which contains the CDC6 gene interrupted by the URA3 gene. Plasmid p6/disr. shown in Figure 4 was constructed by first deleting from p6/1 two HindIII fragments containing 2 μD and URA3: a 310 bp XbaI-BstEII fragment within the coding region was then replaced by a pFL1 fragment containing the disrupted CDC6 gene. A 5.3 kb XhoI-PvuII fragment containing the disrupted CDC6 gene was introduced into the ura3/ura3 strain by single step replacement (genomic restriction maps of diploid transformants showing both the intact and the disrupted gene copy, data not shown), and six ura+ transformants were allowed to sporulate. The absence of viable haploid progeny indicates that the intact CDC6 gene is required for germination and viability.

Characterization of the CDC6 Transcript

Total cellular RNA was isolated from a cdc6,ura3 strain grown at permissive temperature or arrested at 37°C, as well as from
**cdc6.ura3** strains containing the *CDC6* gene on a multicopy plasmid (*cdc6.ura3.p68311*). Northern blots of these RNA preparations were hybridized with a labelled 0.2 kb BglII/XbaI *CDC6* gene fragment. Figure 5 shows that a labelled 1.2 kb RNA corresponding in size to the *CDC6* gene transcript is present in all RNA samples. The amount of *CDC6* mRNA in the *cdc6.ura3* strain does not significantly change after arrest at 37°C (lanes a,b), but is elevated in strains containing plasmid-amplified *CDC6* genes (lanes c,d). The latter two lanes also show a minor species of higher molecular weight which may originate by a read-through of termination signals on plasmid-amplified *CDC6* genes.

Control hybridizations with a labelled *HIS3* fragment as a single copy gene probe did not reveal significant differences of *HIS3* transcripts in the three RNA samples (data not shown).

**Subcellular Location of the CDC6 Protein**

The plasmids pMC6 and pMC6/2 shown in Figure 6 encode a fusion protein, where the carboxy-terminal 76 residues of the *CDC6* protein are replaced by ß-galactosidase. Plasmid pMC6 was con-
Figure 6. Restriction maps of CDC6-β-galactosidase hybrid plasmids used for immunochemistry. The construction is described in Results.

constructed by inserting the 3.8 kb EcoRI/BclI fragment of p6/1 between the EcoRI and BamHI site of the pMC1403 translation fusion vector (18). pCM6/2 was constructed by inserting a 8.2 kb XhoI/SalI fragment of pMC6 between the XhoI and SalI sites of p68311. The integrative plasmid pMC6 was linearized with BglII and introduced into cdc6,ura3 (5 ura+ colonies/µg DNA), whereas the autonomously replicating plasmid pMC6/2 was directly introduced into the same strain (500 ura+ colonies/µg DNA). Both types of transformants were temperature-sensitive, indicating that the CDC6 moiety of the fusion protein does not complement the cdc6 lesion.

The fusion protein expressed in both types of transformants was localized by indirect immunofluorescence. Figure 7 demonstrates that plasma membranes of cells expressing the fusion gene are stained by a fluorescing anti-β-galactosidase complex. No significant differences of the staining pattern was found between
Figure 7. Localization of CDC6-ß-galactosidase fusion protein by indirect immunofluorescence. A cdc6,ura3 strain transformed with plasmid pMC6 was grown at permissive temperature, and cells were stained with polyclonal antibody to ß-galactosidase and FITC-conjugated seep antibody to rabbit immunoglobulin G, as described in Materials and methods.

Transformants containing a single integrated or plasmid-amplified fusion gene, and between transformants incubated at permissive or restrictive temperature.

Control experiments were performed by staining fusion protein in transformants only with FITC, or by staining cells expressing only the intact CDC6 gene with anti-ß-gal and FITC. No significant staining was observed in these experiments (data not shown).

DISCUSSION

A comparison of the CDC6 protein sequence with that of nine functionally different GTP-binding protein families (Figure 3) reveals the presence of all three consensus elements conserved in these proteins, indicating that the CDC6 protein meets all structural requirements for GTP binding and hydrolysis (14). Based on X-ray data of human h-ras protein, the first two consensus elements are involved in the interaction with phosphate, whereas the third element is responsible for nucleoside specificity (19). Many ATP-binding proteins also contain a glycine-rich consensus element corresponding to the first GTPase element; however, the ATPase sites are less conserved and do not contain the other two GTPase elements (14).

The H-ras X-ray structure analysis has revealed a loop (10 GAGGVG) within the first element which appears to be responsible
for GTPase activity (20), and the substitution of the second glycine (residue 12) by other amino acids except proline endows the protein with transforming activity (21). The CDC6 protein contains two regions matching the third consensus element (175 NSLD and 239 NTGD). X-ray data indicate that only the asparagine and aspartic residues interact with the guanine ring, whereas the two other residues form a pocket (19,20), and there is no mutational evidence that the lysine residue is essential for forming the pocket. Therefore, both CDC6 sites are candidates for the third consensus element, although the surrounding sequences of the latter site are more similar to RAS-related proteins than those of the former site (Figure 3). Sequence comparison with all available GTPase proteins including the yeast families of RAS homologues, Ga subunits (SCG1 or GPA1) and peptide elongation factors (EF1 and a related protein GST1 required for the Gl-S transition, see legend to Figure 3) did not reveal further similarities except for the GTPase consensus elements.

Our immunohistochemical data suggest that the CDC6 protein is associated with the plasma membrane, like other GTP-binding protein families involved in signal transduction (e.g. Ga and RAS families). The RAS proteins and some members of the Ga family have conserved cysteine residues at their carboxy-terminal ends which are involved in membrane association (22). These cysteine residues are missing in the yeast Ga homologue SCG1 (GPA1) and in the CDC6 protein, suggesting a different mode of membrane association for these proteins. A hydropathy analysis (23) of the CDC6 protein has revealed a central hydrophobic domain (residues 157 to 177) which might be involved in membrane interaction (data not shown). Furthermore, the CDC6-lacZ fusion protein can only be attached to the membrane by an internal CDC6 region, because the carboxy terminus is replaced by a protein having no membrane affinity (24).

The CDC6 protein sequence exhibits a potential target site for cAMP-dependent protein kinase (25) which is also found in another yeast GTP-binding protein (GST1) required for the Gl/S transition of the cell cycle (26). The carboxy-terminal CDC6 region is related to metallothionines (15), suggesting a possible function in metal binding. It is interesting to note a similarity between metallothionine and high sulfur keratin (16). The functional implication of this observation is not clear.
A sequencial analysis of cell cycle functions relative to the DNA synthesis (S) phase has shown that the products of the three genes CDC4, CDC6 and CDC7 act at an early step beyond the G1 "start" phase, at or beyond the G1/S transition and near the time of bud initiation, and that the three gene functions are required for the initiation of replication (1-3). Interestingly all three gene products resemble components of GTP-dependent signal transduction: The CDC4 protein shares some similarity with the β subunit of transducin, a GTP-binding protein involved in visual signal transduction (27,28), the CDC6 protein shares a GTPase domain and membrane association with Gα and RAS proteins, and the CDC7 protein contains a protein kinase domain probably involved in the phosphorylation of replicative proteins (29). Whatever the inter-relation of these three CDC proteins might be, it remains to be shown whether initiation of replication is triggered by nutritional or other signals.

The early execution point of CDC6 at the initiation of replication is difficult to reconcile with the observation, that apparently correct DNA is continued to be synthesized in the absence of the CDC6 function, and that the cell cycle proceeds to a later stage, where chromosomes are to be distributed between mother and bud (3,5,6). The block of nuclear division as well as the frequent chromosome loss in arrested cdcc mutants might be the consequence of incorrectly initiated DNA, producing apparently normal replication intermediates (6). Alternatively, the CDC6 gene might execute its function(s) at two different stages of the cycle, like the S. cerevisiae CDC28 and the S. pombe CDC2 protein kinase (4).

The isolation and functional characterization of the CDC6 protein will be required to answer these questions.

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