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CDC33 Encodes mRNA Cap-Binding Protein eIF-4E of *Saccharomyces cerevisiae*

CHARLES BRENNER,[1] NAOKI NAKAYAMA,[1] MARK GOEBL,[2] KAZUMA TANAKA,[3] AKIO TOH-E,[3] AND KUNIHIRO MATSUMOTO[1]*

Department of Molecular Biology, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, California 94304[1]; Department of Genetics, University of Washington, Seattle, Washington 98195[2]; and Department of Fermentation Technology, Hiroshima University, Higashihiroshimashi 724, Japan[3]

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The *bcy1* mutation makes the *cdc33* start mutant arrest at random points in the cell cycle instead of only at G1. We cloned and sequenced *CDC33*. This coding sequence is identical to that of the gene encoding the *Saccharomyces cerevisiae* 24-kilodalton mRNA cap-binding protein, eIF-4E.

The mitotic cycle of the budding yeast *Saccharomyces cerevisiae* is initiated in the G1 phase of the cell cycle, at the stage called start. Start events are controlled by several cell division cycle (*CDC*) genes (12). According to the terminal phenotype shown by conditional *cdc* mutants, two classes of start mutants have been identified (13). Upon shifting to the nonpermissive temperature, class I mutants (*cdc28*, *cdc36*, *cdc37*, and *cdc39*) resemble mating-pheromone-arrested cells, while class II mutants (*cdc25*, *cdc33*, and *cdc35*) resemble nutritionally arrested cells. The *CDC35* gene is allelic with *CYR1* and codes for the catalytic subunit of adenylate cyclase (3, 8), whereas the *CDC25* gene product appears to be involved in positive control of adenylate cyclase activity (4). Cyclic AMP (cAMP) exerts its effect by binding regulatory subunits (*BCY1* gene product) of cAMP-dependent protein kinase, thereby freeing active catalytic subunits (9). *bcy1* mutations render cAMP-dependent protein kinases cAMP independent. Thus *bcy1* can suppress the growth defect of *cdc25* and *cdc35* (*cyr1*) (4, 9). In addition, *bcy1* cells fail to arrest in the G1 phase of the cell cycle upon nutrient starvation (7). These findings demonstrate that cAMP-dependent protein phosphorylation is required for cell cycle initiation. To investigate the role of other genes involved in the control of start, we have chosen to study *CDC33*.

The lengths of the G1 phase in *cdc33-1* and *CDC33*$^+$ strains were compared (Table 1). Since differences in the genetic backgrounds of strains have some effect on the kinetics of the cell cycle, such as the percentage of unbudded cells and doubling time, isogenic strains which differed only at the *CDC33* locus were constructed. From the proportions of unbudded (G1) and budded (S+G2+M) cells in exponentially growing cultures of *cdc33-1* and *CDC33*$^+$ cells, the length of the G1 phase of the total cell cycle time was calculated by the equation of Rivin and Fangman (14). The G1 period of exponentially growing *cdc33-1* cells was approximately twice as long as that of *CDC33*$^+$ cells. The duration of S+G2+M in *cdc33-1* cells was slightly longer than that in *CDC33*$^+$. These results indicate that even at a permissive temperature, the *cdc33-1* mutation affected the G1 phase. To test the effect of *bcy1* on *cdc33-1*, we constructed the isogenic strain CB103 (*bcy1::URA3 cdc33-1*). At the permissive temperature, the length of the G1 period in *bcy1::URA3 cdc33-1* cells was restored to that of *CDC33*$^+$. However, *bcy1::URA3 cdc33-1* cells had the same length of S+G2+M as *cdc33-1* cells. These results suggest that *bcy1* suppresses the defect of *cdc33-1* within the G1 phase. When exponentially growing cultures of *cdc33-1* and *bcy1::URA3 cdc33-1* cells were shifted from 25 to 35°C, the number of cells increased approximately twofold (data not shown). Upon cessation of growth, *cdc33-1* cells were arrested in the G1 phase of the cell cycle, whereas *bcy1::URA3 cdc33-1* cells stopped growth but the number of unbudded cells did not increase under the same conditions (Table 1). These results confirm that the *bcy1* mutation suppressed G1 arrest caused by *cdc33-1* but did not suppress the growth defect of *cdc33-1* at the restrictive temperature. These results suggest that *CDC33* plays an essential role in the G1 phase that can be overcome by constitutive activation of cAMP-dependent protein kinase but that it has another function essential for growth that is not specific for the G1 phase.

To address the function of the *CDC33* gene product, we cloned *CDC33*. Plasmids containing the *CDC33* gene were isolated by selection for complementation of a temperature-sensitive mutation. Strain CB101 (α *cdc33-1 leu1 ura3 trp1*) was transformed with a plasmid library carrying yeast genomic DNA averaging 8 kilobases (kb) inserted into the YCpN1 vector (10). Cells were plated at 37°C on medium selective for Trp$^+$. A total of 29 Trp$^+$ transformants were obtained at 37°C, two of which, when analyzed further, displayed coincident loss of the *TRP1* marker and the ability to grow at 37°C, thereby establishing that these markers were plasmid borne. Physical mapping of two clones with restriction enzymes revealed that they share a 3-kb *Cla*I restriction fragment. The 3-kb DNA fragment (Fig. 1), maintained in either YCpN1 or YEp24, complemented the growth defect of the *cdc33-1* mutation. Southern blot analysis of yeast genomic DNA cut with several restriction enzymes and probed with the 3-kb *Cla*I fragment showed that this fragment is unique in the genome (data not shown).

Proof that the cloned gene is actually *CDC33* depended on establishing that its genomic position coincides with that of *CDC33*. A 2-kb *Cla*I-*Hin*dIII fragment was cloned into YIp5 (Fig. 1). The resulting plasmid was linearized with *Hpa*I within the insert and targeted to its homologous genomic site by integrative transformation of CB101. A mitotically stable Ura$^+$ transformant was obtained at 37°C (CB102). To determine the site of integration relative to the *CDC33* gene, CB102 was crossed with KMY2-6B (**a** *CDC33*$^+$ *ura3 his3 leu2*). Fifteen tetrads of this diploid gave 4+:0− segregation

* Corresponding author.

TABLE 1. Effect of the *cdc33-1* mutation on the cell cycle

Strain	Genotype	Characteristics of exponential growth phase at 25°C[a]				% Unbudded cells when shifted to 35°C[b]
		% Unbudded cells	Doubling time (h)	Length (h) of phase(s): G1	Length (h) of phase(s): S+G2+M	
CB102	*CDC33*$^+$ *BCY1*$^+$	48	2.5	1.0	1.5	
CB101	*cdc33-1 BCY1*$^+$	61	3.8	2.0	1.8	93
CB103[c]	*cdc33-1 bcy1::URA3*	44	3.0	1.1	1.9	42

[a] Cells were grown in YPD (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories], 2% glucose) medium. The length of the G1 period was calculated from populations of unbudded cells in exponentially growing cultures at 25°C by the equation by Rivin and Fangman (14), $T[1 - \log(2 - F \cdot \text{unbud})/\log 2]$, in which T is the doubling time and F · unbud is the fraction of unbudded cells.

[b] Exponentially growing cultures in YPD at 25°C were shifted to 35°C and incubated for 6 h, after which the population of unbudded cells was determined.

[c] CB103 (α *cdc33-1 bcy1::URA3 leu1 ura3 trp1*) was generated by transforming CB101 (α *cdc33-1 leu1 ura3 trp1*) with a *Bam*HI DNA fragment containing *BCY1* disrupted with *URA3* (19).

for temperature sensitivity and 2+:2− segregation for Ura. In addition, the diploid formed by mating CB102 with KMY65-1D (a *cdc33-1 ura3 his3 leu2 trp1*) was analyzed. The temperature sensitivity and Ura segregations were 2+:2− in 16 tetrads; all non-temperature-sensitive segregants were Ura$^+$ and all temperature-sensitive segregants were Ura$^-$. These results indicate that plasmid YIp5 carrying the *Cla*I-*Hin*dIII fragment had integrated at the *cdc33-1* locus and that we had cloned the wild-type *CDC33* gene.

Transcription of the *CDC33* gene was analyzed by using the 1.3-kb *Cla*I-*Eco*RI fragment (Fig. 1) as a radioactively labeled hybridization probe. Yeast poly(A)$^+$ RNA was separated by gel electrophoresis and blotted to nitrocellulose filters. A single band of about 900 bases was detected (Fig. 2).

The nucleotide sequence of the 2-kb *Cla*I-*Hin*dIII fragment (Fig. 1) that contains the *CDC33* gene was determined (Fig. 3), revealing that the *CDC33* gene contains a 639-base-pair open reading frame. This open reading frame would encode a 24-kilodalton polypeptide of 213 amino acid residues. Recently, Altmann et al. (1) purified protein synthesis initiation factor eIF-4E from *S. cerevisiae*. They subsequently cloned the gene that encodes this protein (2). The predicted *CDC33* protein sequence was found to be identical to that of the yeast eIF-4E.

In the present study, we show that *CDC33* is identical to the gene encoding the yeast mRNA cap-binding protein eIF-4E (2). This result indicates that the regulation of start may involve translational initiation. Since protein synthesis is required not only for the G1-to-S transition but also through the cell cycle, it is interesting to ask why *cdc33* mutants arrest at the G1 phase. Iida and Yahara (6) have compared protein synthesis among *cdc25*, *cdc33*, *cdc35*, and wild-type cells and have shown that at least nine proteins are synthesized specifically in the resting state. Six of these proteins have been identified as heat shock proteins. In support of the idea that certain proteins may be required for G1 arrest, we have recently demonstrated that the polyubiquitin gene (*UBI4*) is required for G1 arrest because *ubi4* disruption mutants are defective in the processes of the G1 phase: sporulation, resistance to starvation, and morphological G1 arrest (18). To test the effect of *cdc33-1* on the level of *UBI4* expression, β-galactosidase activities produced by *CDC33*$^+$ and *cdc33-1* cells carrying *GAL7-lacZ* or *UBI4-lacZ* fusion plasmids were compared. These fusion genes contain the promoters and sequences coding for the first 133 and 79 amino acids of *GAL7* and *UBI4*, respectively, linked to *Escherichia coli* β-galactosidase (17, 18). *GAL7* encodes galactose-1-phosphate uridyltransferase and is induced in the presence of galactose (17). This gene was chosen as a control. Since the *cdc33-1* mutation affected the G1 phase at the permissive temperature, the enzyme activity from cultures growing at 25°C was measured. *CDC33*$^+$ cells expressed a twofold higher activity from the *GAL7-lacZ* fusion than did *cdc33-1* cells in the presence of galactose (*CDC33*$^+$ cells, 960 U per optical density of cells at 660 nm [OD_{660}]; *cdc33-1* cells, 460 U/OD_{660}). On the other hand, *cdc33-1* cells expressed a 1.8-fold higher activity from the *UBI4-lacZ* fusion than did *CDC33*$^+$ cells (*CDC33*$^+$ cells, 80 U/OD_{660}; *cdc33-1* cells, 140 U/OD_{660}). These results indicate that the *cdc33-1* mutation did not cause the uniform reduction of all protein synthesis but may have led to preferential synthesis of certain proteins, such as polyubiquitin, required for G1 arrest. The result that *bcy1::URA3* suppressed G1 arrest caused by *cdc33-1* is consistent with the facts that expression of *UBI4* is repressed by cAMP-dependent protein phosphorylation and that *UBI4* expression is required for G1 arrest (18).

On the basis of the result that *CDC33* encodes eIF-4E, it is possible that in *cdc33-1* mutants a distinct and differential effect on the production of particular polypeptides required for G1 arrest could occur if the 5′ termini of a class(es) of

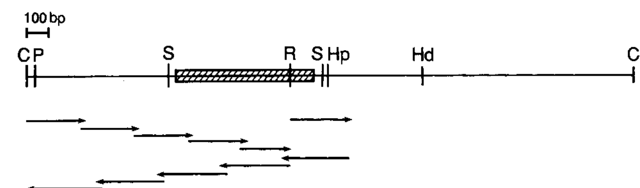

FIG. 1. Restriction enzyme map of and sequencing strategy for *CDC33*. C, *Cla*I; P, *Pst*I; S, *Spe*I; R, *Eco*RI; Hp, *Hpa*I; Hd, *Hin*dIII. ▨, Position of the open reading frame. Transcription proceeds from left to right.

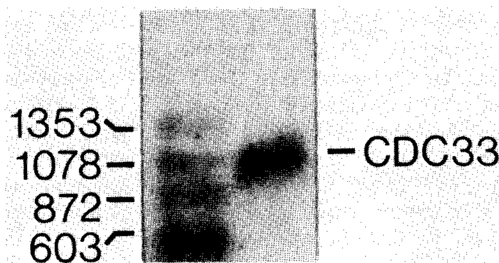

FIG. 2. Northern (RNA) blot of *CDC33*. Lanes: left, end-labeled, denatured *Hae*III digest of φX174 RF DNA; right, 5 μg of poly(A)$^+$ RNA from KMY125 (a/α wild type). The blot was probed with the 1.3-kb *Cla*I-*Eco*RI fragment of *CDC33*. Size markers are indicated in nucleotides.

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ATCGATACGAACTCTTCGGCATTGCTGCAGACGTCTTAAAATACTGCCCATTCGAAGATATCATGGGTTCTGAGGGTGACCAATCTTCCATTCGGTTGTTTTGCGAACGCTGTGGTGAGT  120
                       ↑
TAATCACTAACGAAAGTTCTAAAGAAAAGCTCAGAGCGGAAGCTCAGCAGACGGGCAATAAGAAGATCATGGACAAGTTTGGATACTGGTATTGTGACTCTTGTAAGAAGAAGAATACAT  240

CTTGTGTTCTATGTGAAAGACCATTAAAGAAACTACCATGGTCATCCTCCCCTGTGGACACGAAGGTCCTTCCAGTGCATACAAGAATGGTTTCTCGATGAGAATGAACAAGAATGTCCC  360
                                         ↑                              ↑         ↑
GGCGGTTGCCCCGGTGTTGCATTCATCTAGGTTCTCCACATAATGTATAGTTTAACATATCATCACCATTGTTTAGTTAAATCGTTTAGAGTAATATTACCCGTCAAAAAGGTCGGGTAA  480

AATTTTATTACCCTCTCCGAAAAGAAAATTTTTTTCGTCGTCAATAGAGTTTAATGCAATACCTGATAAAGAGAGTTTTACATTGCAAGAGGTAGTGTTAATTCTGGATTTATATTGTAC  600

ATATGTGTTTGTGTTAGTGCTTGAGTACTTCCTAGGAGTTTTACGAAAAATAAAAGCATTTTTGTCTGAAAACTAGTGAAAGGAAGAAAAATGTCCGTTGAAGAAGTTAGCAAGAAGTTT  720
                                                                                           MetSerValGluGluValSerLysLysPhe   10

GAAGAAAACGTTTCAGTCGATGATACCACAGCTACTCCAAAGACTGTTTTAAGTGACAGTGCTCACTTCGATGTCAAGCACCCATTGAACACCAAATGGACTTTATGGTACACAAAGCCA  840
GluGluAsnValSerValAspAspThrThrAlaThrProLysThrValLeuSerAspSerAlaHisPheAspValLysHisProLeuAsnThrLysTrpThrLeuTrpTyrThrLysPro   50

GCCGTCGATAAATCTGAGTCGTGGTCTGATCTATTACGTCCCGTCACTTCATTCCAAACTGTTGAAGAATTTTGGGCTATCATTCAAAATATTCCTGAGCCACACGAACTACCATTGAAA  960
AlaValAspLysSerGluSerTrpSerAspLeuLeuArgProValThrSerPheGlnThrValGluGluPheTrpAlaIleIleGlnAsnIleProGluProHisGluLeuProLeuLys   90
             *
TCAGATTACCACGTCTTCCGTAATGACGTTAGACCTGAATGGGAAGATGAAGCCAATGCTAAAGGTGGTAAATGGTCTTTCCAACTTAGAGGAAAAGGTGCTGATATTGATGAATTATGG 1080
SerAspTyrHisValPheArgAsnAspValArgProGluTrpGluAspGluAlaAsnAlaLysGlyGlyLysTrpSerPheGlnLeuArgGlyLysGlyAlaAspIleAspGluLeuTrp  130

CTAAGAACTTTACTAGCAGTTATTGGTGAAACAATTGATGAAGACGACTCCCAAATTAACGGTGTCGTTTTAAGCATTAGAAAAGGTGGTAACAAGTTTGCCTTATGGACTAAATCTGAA 1200
LeuArgThrLeuLeuAlaValIleGlyGluThrIleAspGluAspAspSerGlnIleAsnGlyValValLeuSerIleArgLysGlyGlyAsnLysPheAlaLeuTrpThrLysSerGlu  170

GACAAAGAACCACTATTGAGAATTGGTGGTAAATTCAAGCAAGTTTTAAAATTAACCGATGACGGGCATTTGGAATTCTTTCCACATTCCAGTGCCAATGGTAGACACCCTCAACCATCA 1320
AspLysGluProLeuLeuArgIleGlyGlyLysPheLysGlnValLeuLysLeuThrAspAspGlyHisLeuGluPhePheProHisSerSerAlaAsnGlyArgHisProGlnProSer  210

ATCACCTTGTAAGATAGTCTGAATTTTTCTTAAGATAATTGTTATTTTAATCAAAATTATATATATAATCATAATATAATATATATACATTCTACTAGTTAGGATGCACGTATAAACTCA 1440
IleThrLeu •                                                                                                              213

TCATGTTAACACAGAAGGAAAAGTCAAAATGAAGAAGAAAAAAAAAAAAAAAAAAAAAAA                                                             1500
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FIG. 3. Sequence of *CDC33* genomic DNA (1,500 nucleotides of the coding strand are shown). Restriction sites shown in Fig. 1 are underlined. Differences between this sequence and that of Altmann et al. (2) are indicated by arrows. The TATA box is boxed. An asterisk indicates the serine at position 55 which may correspond to serine 53 of human eIF-4E, which has been shown to be phosphorylated (16).

mRNAs have altered affinity for mutated eIF-4E or if these mRNAs are uncapped. Translational discrimination has been reported in the case of mammalian eIF-4F, a cap-binding protein complex composed of p220, eIF-4A, and eIF-4E. It has been reported that inactivation of eIF-4F in lysates of heat-shocked cells is responsible for a decreased rate of protein synthesis and for the preferential translation of heat shock mRNAs (11). In addition, eIF-4E is a phosphorylated protein and is dephosphorylated upon heat shock (5). These findings suggest that eIF-4E phosphorylation may play a role in the regulation of initiation. Since the sequence surrounding the phosphorylation site of human eIF-4E (15, 16) is highly conserved in yeast eIF-4E (Fig. 3), yeast eIF-4E may also be phosphorylated. It will be interesting to investigate the phosphorylation state of yeast eIF-4E and the functional significance of this with regard to heat shock, translation, and cell cycle control.

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