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Saccharomyces cerevisiae CDC8 Gene and Its Product

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The product of the *Saccharomyces cerevisiae CDC8* gene is essential for normal cellular DNA replication; the determination of the structure of the gene and the identification of its product would facilitate the examination of its role in this process. We have cloned a 1,000-base-pair fragment of the *S. cerevisiae* genome carrying the functional gene. The nucleotide sequence includes one long open reading frame; it is flanked by sequences typical of other *S. cerevisiae* genes. This sequence predicts a polypeptide chain product of 216 amino acids with a molecular weight of 24,600. A polyadenylated RNA transcript of this sequence was identified by hybridization; in vitro translation of RNA samples enriched for this transcript produced a specific polypeptide chain of apparent molecular weight between 24,000 and 25,000. Thus the reading frame identified represents the authentic *CDC8* gene, and the amino acid sequence of its product has been deduced. Our observations differ from two previous reports of the identification of the putative *CDC8* protein based upon in vitro complementation assays.

The effects of *cdc8* mutations on the *Saccharomyces cerevisiae* cell division cycle have been extensively studied. The *CDC8* gene product is required continuously during the S phase for normal DNA replication (8, 9). The mutant accumulates short replication bubbles at the restrictive temperature, suggesting that fork migration is defective (27). Short denaturation loops also appear under these conditions (11). Mitochondrial DNA synthesis (24, 34) and premeiotic DNA synthesis (28) are also defective in this mutant.

The isolation of the functional *CDC8* protein would allow a detailed study of its essential role in these DNA replication events. Several laboratories have developed cell-free DNA replication systems that appear to depend upon the *CDC8* gene product (4, 10, 13). Attempts have been made to identify and isolate the *CDC8* protein by using in vitro complementation assays based upon these cell-free systems. In one case the molecular weight of the putative *CDC8* gene product was estimated to be 37,000 to 40,000, whereas in another case the preliminary estimate was 10,000 to 20,000 (1, 14).

If the *CDC8* gene were cloned, one could examine its structure, identify its transcript, and characterize its protein product in a manner independent of these in vitro complementation assays. The cloning of a DNA segment carrying the functional *CDC8* gene was recently reported (15). We report the results of studies here which have led to the determination of the nucleotide sequence of the *CDC8* gene and the amino acid sequence of the authentic gene product. We discuss our findings relative to the earlier attempts to isolate the functional *CDC8* protein.

MATERIALS AND METHODS

Cells. *Escherichia coli* HB101 (F^- *thi leu pro hsdR hsdM recA endI*) was obtained from J. D. Engel. *S. cerevisiae* strains A364A (**a** *ade1 ade2 ura1 tyr1 his7 lys2 gal1*) and tsl98 (*cdc8-1* in same genetic background as A364A) were obtained from L. Hartwell. We note that the *S. cerevisiae* strain designated tsl98, sent to investigators by the Yeast Genetic Stock Center, Berkeley, before September 1982, was not the same tsl98 that we received from L. Hartwell and used in the experiments described in this report. The Yeast Genetic Stock Center strain contains two temperature-sensitive mutations based on genetic analysis. We believe that neither of these mutations is in the *cdc8* gene, which has been genetically linked to *ilv3* on chromosome X. First, the two temperature-sensitive markers in the Yeast Genetic Stock Center strain were complemented by the Hartwell strain containing the authentic *cdc8* mutation. Second, neither of the Tsm^- mutations in the Yeast Genetic Stock Center strain was linked to *ilv3* (R. Contopoulou, personal communication). *S. cerevisiae* strains LDY112 (**a** *leu2-1 leu2-112 cdc8-1*), LDY113 (α *ura3-52 cdc8-1 ade his7*), and LDY114 (**a** *ilv3 ura3-52 met his*) were constructed in this laboratory.

DNA molecules. A *Sau*3A partial digest *S. cerevisiae* genomic DNA library carried in plasmid vector CV13 was obtained from B. Hall. The genomic DNA was isolated from *S. cerevisiae* strain AB320 (*ade2 lys2 trp5 leu2 can1 met4 ura3* or *ura1* or both). CV13 consists of pBR322 carrying the *S. cerevisiae LEU2* gene and the 2μ plasmid autonomous replication sequence. Plasmid YIP5 was obtained from D. Botstein; it consists of pBR322 carrying the *S. cerevisiae URA3* gene. Plasmid YIP5/2μD was constructed by inserting the 2μ plasmid *Eco*RI fragment D (3) into YIP5. Fragment D carries the autonomous replication sequence of the *S. cerevisiae* plasmid.

Plasmid DNA molecules were extracted from small bacterial cultures by the method of Klein et al. (12). Large-scale purification was by the alkaline lysis method of Maniatis et al. (17). Equilibrium sedimentation in ethidium bromide-containing CsCl gradients, or RNase digestion and filtration through Sepharose 2B (25) was employed after the alkaline lysis. *S. cerevisiae* chromosomal DNA was isolated from 1-liter cultures by the method of Cryer et al. (5). Total nucleic acids were also extracted from small *S. cerevisiae* cultures by this method, but omitting α-amylase and RNase digestions and stopping after the first ethanol precipitation.

DNA structural analyses. Restriction endonucleases, T4 DNA ligase, DNA polymerases, and polynucleotide kinase were obtained from New England Biolabs and used as recommended by the supplier. Nick translation of DNA probes was carried out as described by Maniatis et al. (18). DNA was transferred to a nitrocellulose membrane by the

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procedures of Wahl et al. (32), and subsequent hybridization was performed as described by Davis et al. (6). The Maxam and Gilbert procedure (21) was used for nucleotide sequence determination, with the modification described by Smith and Calvo (30). Cleavage products were analyzed after electrophoresis on 5, 8, and 20% acrylamide gels and autoradiography at −80°C.

Genetic transformations. Transformation of *E. coli* cells was by the procedure of Dolan (Ph.D. thesis, Northwestern University, Ann Arbor, Mich., 1982). The procedure of Singh et al. (29) was used to transform *S. cerevisiae*.

RNA isolation. *S. cerevisiae* RNA was purified from 1-liter cultures of cells grown to a density of 2×10^7 to 5×10^7 cells per ml. Cells were harvested by centrifugation, washed with 100 ml of cold water, and suspended in 1 ml of fresh 0.1 M triethanolamine–0.1 M LiCl–1 mM EDTA–10 mM iodoacetate (pH 9). An equal volume of cold glass beads was added, and the mixture was vortexed for 2.5 min at 4°C. The beads were filtered out, and the debris was removed by centrifugation at 12,000 × *g* for 6 min at 2°C. The supernatant was extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) and two times with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated from the aqueous phase with ethanol at −20°C.

Two cycles of oligodeoxythymidylic acid-cellulose chromatography were used to enrich for polyadenylated RNA. Total RNA was ethanol precipitated and suspended in 20 mM triethanolamine–1% sodium dodecyl sulfate–2 mM EDTA (pH 7.4) to a final concentration of 5 mg/ml. The solution was heated for 10 min at 65°C, quenched on ice, and diluted twofold with an equal volume of 1 M LiCl. The sample was applied to an oligodeoxythymidylic acid-cellulose column equilibrated in 10 mM triethanolamine–0.5% sodium dodecyl sulfate–1 mM EDTA–0.5 M LiCl (pH 7.4). After extensive washing the absorbed RNA was eluted with 10 mM triethanolamine–0.05% sodium dodecyl sulfate–1 mM EDTA (pH 7.4) and precipitated with ethanol. This RNA sample was chromatographed once again.

RNA transcript identification. Formaldehyde denaturing agarose gel electrophoresis and transfer to nitrocellulose was by the method of Maniatis et al. (19), with some modifications; the electrophoresis running buffer contained 2.2 M formaldehyde, and the alkaline hydrolysis step before the transfer to nitrocellulose was omitted. After transfer the blot was washed with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and baked under vacuum. The nitrocellulose filters were prehybridized, hybridized, and washed by the method of Thomas (31), except that dextran sulfate was not included in the hybridization buffer. Autoradiography was carried out at −80°C with intensifying screens.

Hybrid selection and in vitro translation. Denatured linear DNA fragments were attached to nitrocellulose by the method of White and Bancroft (33). Hybridization and elution of polyadenylated RNA samples were carried out as described by Maniatis et al. (20). A rabbit reticulocyte lysate (New England Nuclear Corp.) was used to translate the hybrid selected mRNA in the presence of [^{35}S]methionine by the protocol suggested by the supplier. Translation products were separated by electrophoresis in denaturing 12.5% polyacrylamide gels (16). After electrophoresis the gels were impregnated with fluor, dried, and fluorographed at −80°C with intensifying screens.

RESULTS

Isolation of a DNA fragment able to complement the *cdc8-1* mutation. A genomic DNA fragment capable of complementing the *cdc8-1* temperature-sensitive mutation was cloned from a *Sau*3A random *S. cerevisiae* DNA fragment library carried in plasmid vector CV13. These library plasmids replicate autonomously in *S. cerevisiae* and express the *LEU2* gene. The library construction was similar to that described by Nasmyth and Reed (23). Complementing plasmids were cloned by selecting Leu$^+$ Tsm$^+$ transformants of *S. cerevisiae* LDY112 (*leu2-1 leu2-112 cdc8-1*). The spheroplasts were allowed to regenerate at room temperature for 12 h in agar medium lacking leucine; the desired transformants were then selected by 3 days of additional incubation at 35°C. Leu$^+$ Tsm$^+$ transformants were recovered at a frequency of 1 in 10^4 Leu$^+$ transformants.

Total nucleic acids were extracted from two Leu$^+$ Tsm$^+$ *S. cerevisiae* transformants and used to transform *E. coli* HB101 to ampicillin resistance. Isolation and characterization of the transforming plasmids demonstrated that both *S. cerevisiae* transformants had carried the same genomic library plasmid (data not shown). A restriction fragment map of the 4.7-kilobase-pair (kbp) yeast genomic DNA insert carried in this plasmid, pYeCDC8, is shown in Fig. 1. Restriction analysis showed that the distribution of *Eco*RV sites on the cloned DNA insert was the same as that at the homologous region in the *S. cerevisiae* genome (data not shown). No rearrangements were detected.

Recombinant DNA plasmid integration at the homologous *S. cerevisiae* locus. A segment of the *S. cerevisiae* DNA insert from pYeCDC8 was subcloned into plasmid YIP5 to produce a new recombinant plasmid, pDQ4 (Fig. 1). Because the vector lacked an *S. cerevisiae* autonomous replication sequence, this recombinant plasmid could only transform *S. cerevisiae* by integration at a site of homology in the genome. Plasmid pDQ4 was able to transform *S. cerevisiae* LDY113 (*ura3-52 cdc8-1*) to Ura$^+$ Tsm$^+$, indicating that its insert was still able to complement the *cdc8-1* mutation. One such transformant, LDY115, was chosen for further study.

Genomic DNA sequences homologous to the cloned insert were compared in *S. cerevisiae* LDY113 and LDY115. Figure 2 demonstrates that the transforming DNA in *S. cerevisiae* LDY115 integrated at the site of homology with the *cdc8*-complementing insert. The nick-translated 3.0-kbp *Hin*dIII-*Bam*HI insert DNA probe recognized one *S. cerevisiae* genomic DNA *Eco*RI fragment from the untransformed cells (Fig. 2, lane 5). The integrated plasmid introduced one new *Eco*RI site into the *S. cerevisiae* genome of the transformed cells. This new *Eco*RI site was carried within the transforming DNA vector sequences. If the transforming DNA plasmid had integrated within the *Eco*RI fragment observed in untransformed cells, the same DNA probe would recognize two different *Eco*RI fragments whose sizes would sum to that of the band in Fig. 2, lane 5, (approximately 16 kbp) plus pDQ4 (9 kbp). The hybridization in lane 6 shows two new fragments of approximately 18 and 6 kbp. Thus integration appears to have occurred within the homologous single-copy *Eco*RI fragment. The integrated plasmid also introduced one new *Bam*HI site into the *S. cerevisiae* genome. Since this site was at the vector-insert DNA junction in pDQ4, digestion with *Bam*HI should release a DNA fragment the same size as singly cut pDQ4 DNA plus a genomic fragment the same size as that from untransformed cells (Fig. 2, lane 8). The hybridization in Fig. 2, lane 9, shows that both fragments were observed. Thus the transforming DNA plasmid in LDY115 was inserted at the single genomic site homologous to the complementing DNA insert.

Map position of the locus of homology. The *cdc8* locus is

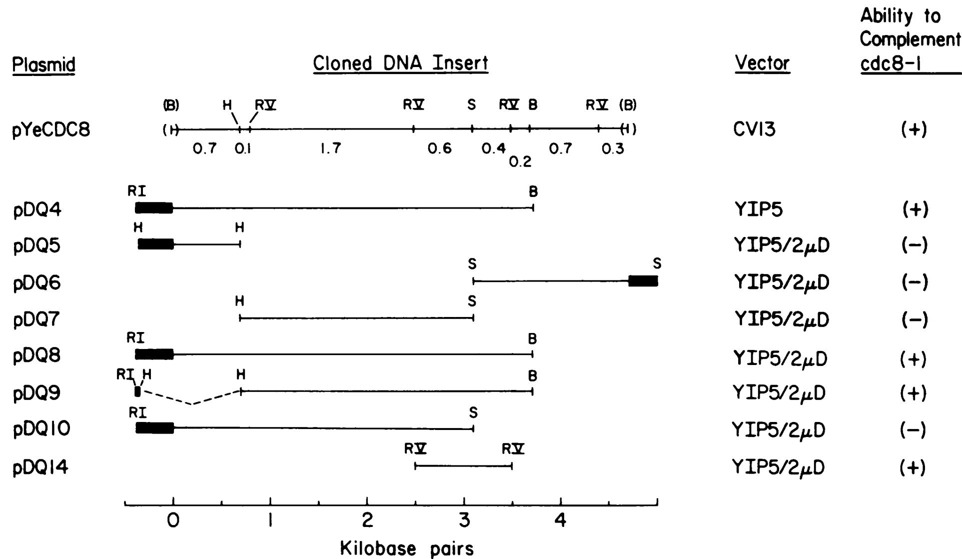

FIG. 1. *cdc8*-complementing DNA clone and its subcloned DNA fragments. Abbreviations: (+), able to complement the *cdc8-1* mutation (−), not able to complement; (B), cryptic vector *Bam*HI site; B, *Bam*HI site; H, *Hin*dIII site; RV, *Eco*RV site; RI, *Eco*RI site; S, *Sal*I site. Thin lines represent sequences from the original cloned DNA insert in pYeCDC8; thick lines represent vector DNA sequences.

linked to *ilv3* on chromosome X, distal to the centromere. The two loci are separated by 26 centimorgans (cM) (22) (Table 1). A standard genetic cross was used to demonstrate that the cloned DNA insert able to complement *cdc8-1* maps at the *cdc8* locus.

To first verify that the temperature-sensitive marker in LDY113 complemented by the cloned DNA insert mapped at the *cdc8* locus, we performed a two-factor cross between strains LDY113 (α *cdc8-1 ura3-52*) and LDY114 (**a** *ilv3 ura3-52*). Of 24 tetrads showing 2:2 segregation, half were parental ditypes and half were tetratypes (Table 1). These data place the temperature-sensitive marker in LDY113 at approximately 25 cM from *ilv3*, in good agreement with the previously determined position of the *CDC8* gene.

S. cerevisiae LDY115, carrying plasmid pDQ4 integrated at the genomic site homologous to the complementing DNA insert, was then crossed with LDY114. The *URA3* plasmid marker was used to assess the degree of linkage between the site of insertion and *ilv3*. Of 25 tetrads showing 2:2 segregation, 13 were parental ditype and 12 were tetratype (Table 1). These data demonstrate that the *URA3* plasmid marker was inserted at a position approximately 26 cM from the *ilv3* locus.

The same cross also allowed assessment of the linkage of the plasmid-borne *cdc8*-complementing marker in LDY115 (Tsm^+) to the *cdc8-1* marker (Tsm^-) in the same strain. All tetrads segregated Tsm^+:Tsm^-, 4:0. In no tetrad did the *cdc8-1* marker separate from the complementing marker.

These data demonstrate that the integrated pDQ4 plasmid in LDY115 is positioned 26 cM from *ilv3* on chromosome X, at the *cdc8* locus. Since the site of integration is homologous to the cloned DNA insert, we conclude that this insert carries the *CDC8* gene.

Location of *CDC8* within the cloned DNA fragment. A series of subclones of the genomic DNA insert in pYeCDC8 was used in genetic transformation experiments to localize the functional *CDC8* gene. Segments of the yeast DNA insert in pYeCDC8 were spliced into plasmid vector YIP5 carrying the *Eco*RI fragment D from the *S. cerevisiae* 2μ plasmid (3). This 2μ plasmid fragment carries an autonomous replication sequence. The recombinant plasmids generated are designated pDQ5 through pDQ8 (Fig. 1). All four of these autonomously replicating plasmids were able to transform LDY113 (*ura3-52 cdc8-1*) to Ura^+ (300 to 440

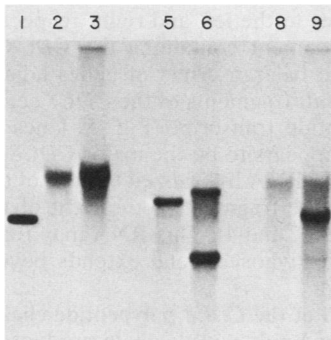

FIG. 2. Structural analyses of chromosomal DNA from *S. cerevisiae* LDY113 and LDY115 homologous to the *Hin*dIII-*Bam*HI insert DNA fragment isolated from pDQ4. Purified DNA samples were cut with the indicated restriction endonuclease, subjected to agarose gel electrophoresis, and transferred to nitrocellulose membrane. After hybridization with ^{32}P-labeled probe DNA, homologous DNA bands were identified by using autoradiography. Lanes: 1, *Eco*RI-cut pDQ4; 2, uncut LDY113 DNA; 3, uncut LDY115 DNA; 5, *Eco*RI-cut LDY113 DNA; 6, *Eco*RI-cut LDY115 DNA; 8, *Bam*HI-cut LDY113 DNA; 9, *Bam*HI-cut LDY115 DNA.

TABLE 1. Genetic data from two-factor crosses

Interval	Cross	Ascus type[a]			Map distance (cM)[b]
		PD	NPD	T	
ilv3-cdc8	—[c]	21	0	20	26.1
ilv3-cdc8	LDY114 × LDY113	12	0	12	25
ilv3-URA3[d]	LDY114 × LDY115	13	0	12	26

[a] PD, Parental ditype; NPD, nonparental ditype; T, tetraptype.
[b] Calculated by the method of Perkins (26).
[c] Data reproduced from Mortimer and Schild (22).
[d] *URA3* marker carried on integrated plasmid pDQ4.

transformants per μg of plasmid DNA), but only pDQ8 yielded Ura$^+$ Tsm$^+$ transformants able to form colonies at 35°C (270 per μg of DNA; Fig. 1).

The DNA insert in pDQ8 was further subcloned to yield pDQ9, pDQ10, and PDQ14 (Fig. 1). Both pDQ9 and pDQ14 were able to transform LDY113 to Ura$^+$ Tsm$^+$ (300 to 330 per μg of DNA), whereas pDQ10 was not. All produced Ura$^+$ transformants (320 to 420 per μg of DNA). These data demonstrate that the functional *CDC8* gene is carried on the 1-kbp *Eco*RV fragment cloned in pDQ14. Since pDQ10, pDQ6, and pDQ7 did not carry the functional gene, the *Sal*I site within this 1-kbp segment must be within the structural gene or its adjacent control elements.

Determination of the nucleotide sequence of the CDC8 gene. The entire sequence of both DNA strands of the 1-kbp *Eco*RV fragment carrying the functional *CDC8* gene was determined by the strategy shown in Fig. 3A. Only one of the six possible reading frames contains a long stretch uninterrupted by stop codons (Fig. 3B). The *Sal*I site interrupts this reading frame, consistent with the inability of cloned inserts terminating at this site to genetically complement the *cdc8-1* mutation (Fig. 1). These observations suggest that the reading frame we have identified specifies the *CDC8* gene product.

Figure 4 shows the nucleotide sequence of the entire 1,005-base-pair *Eco*RV fragment and the predicted amino acid sequence of the *CDC8* protein. The first in phase ATG codon is found at nucleotides 211 through 213. This start codon is preceded by at least two sequences common to other cloned *S. cerevisiae* genes (35): a postulated transcription initiation signal, TATAAAA at nucleotides 33 through 39, and a potential site of interaction with the 3′ terminus of the *S. cerevisiae* 18S rRNA molecule, GAAAGATC at nucleotides 192 through 199. A third sequence, CACCA at nucleotides 199 through 203, is somewhat similar to the sequence PuCACACA preceding the start codon in some *S. cerevisiae* genes (35).

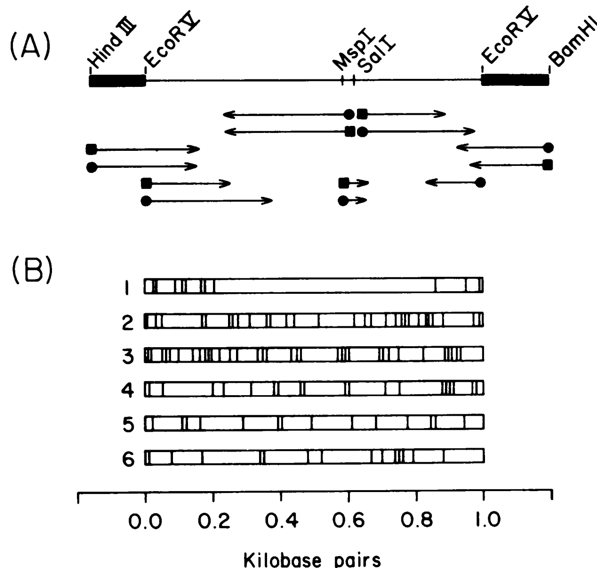

FIG. 3. Sequencing strategy and distribution of translational stop codons. (A) The thin line represents the 1-kbp *Eco*RV fragment; the thick lines represent vector DNA sequences. The arrows each represent the extent of useful sequence information derived from each end-labeled site. The *Msp*I site was used only to obtain sequences across the *Sal*I site; the *Sal*I site itself was used for the more extensive sequence determination of both strands in both directions from the middle of the DNA fragment. Restriction fragments were labeled at their 5′ ends (●) with [γ-^{32}P]ATP and polynucleotide kinase or at their 3′ ends (■) with an α-^{32}P-deoxynucleoside triphosphate and T4 DNA polymerase or the large fragment of *E. coli* DNA polyperase I. (B) The positions of translational stop codons are indicated in each of the six possible reading frames of the 1-kbp *Eco*Rv fragment by the short vertical lines.

The nucleotide sequence downstream from the single TAG stop codon at nucleotides 858 through 860 includes sites common to the 3′ untranslated regions of other *S. cerevisiae* genes (36). These include the TAG sequence at nucleotides 881 through 883 and 886 through 888, TAGT at nucleotides 904 through 907, and TTT at nucleotides 926 through 928, 933 through 935, and 942 through 944. These may facilitate transcriptional termination and polyadenylation. The indicated TAG sequences are actually portions of a larger hexanucleotide repeat, TAGTCT, at nucleotides 881 through 886, 886 through 891, and 904 through 909. The hexanucleotide sequence AATAAA required for mRNA polyadenylation in higher eucaryotes (7) is found at nucleotides 952 through 957. It may function as a polyadenylation signal in conjunction with the other sequences mentioned above. Thus, the open reading frame used to predict the amino acid sequence of the *CDC8* protein is flanked by sequences typical of other *S. cerevisiae* genes.

The nucleotide sequence predicts a polypeptide chain product of 216 amino acids. The predicted amino acid composition is shown in Table 2. The calculated molecular weight of the protein is 24,600. The pI of the predicted protein is near neutrality.

Identification of the *CDC8* RNA transcript. Polyadenylated mRNA was isolated from *S. cerevisiae* A364A and denatured. Four equal portions of the RNA sample were fractionated by agarose gel electrophoresis and blotted onto a nitrocellulose membrane. DNA fragment size markers were included in an adjacent lane. After baking under reduced pressure the membrane was cut into five strips representing each of the lanes of the gel. These strips were hybridized with nick-translated DNA probes as indicated in Fig. 5. Four different RNA species were identified as homologous to the cloned region encompassing the *CDC8* gene. These transcripts have lengths of approximately 1,200, 1,100, 890, and 670 nucleotides. The largest transcript (Fig. 5, lane 1) and the smallest (Fig. 5, lane 4) are homologous to *S. cerevisiae* DNA sequences to the left and right, respectively, of the 1-kbp *Eco*RV fragment containing the *CDC8* gene (Fig. 1). They appear to be transcripts of genes adjacent to *CDC8*. Both *Eco*RV-*Sal*I fragments of the *CDC8* gene hybridized to the 890-nucleotide transcript (Fig. 5, lanes 2 and 3). This RNA species appears to be the major *CDC8* transcript. The 1,100-nucleotide RNA hybridized to both of the *CDC8* DNA fragments and the fragment to the right of the *CDC8* gene (Fig. 5, lanes 2, 3, and 4). This RNA may represent a minor *CDC8* transcript whose 3′ end extends beyond that of the major transcript.

Identification of the *CDC8* polypeptide chain product. The identification of a polypeptide chain product translated from mRNA transcribed specifically from the *CDC8* gene required hybrid selection of polyadenylated RNA from *S. cerevisiae* cells carrying the *CDC8* gene on a multicopy plasmid. This allowed overproduction of the 890-nucleotide *CDC8* transcript by at least 10-fold (data not shown) such that a specific translation product could be detected (see below). No specific translation product was detectable when hybrid selection was carried out with polyadenylated RNA from cells carrying a single copy of the *CDC8* gene, presum-

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GATATCCTAA ACTAGTAGGT TACAGTTGAT AATATAAAAA GTTGGAAAAT AGCTTAGAAA TGAAGAAGAG AATAACGGCT TCTATGCGGT   90

TGTGACTTTT GACGCGTTAG GCGTGGTGCT AAAGATGGCG AAAAAATGTA ACGTCTTCCC AAGAAGTACG CTTCTAAACT AAATATGATT  180

CATAGTGGAC AGAAAGATCA CCATTTTGA ATG ATG GGT CGT GGC AAA TTA ATA CTG ATA GAA GGA TTG GAT AGG ACT GGT 260
                                Met Met Gly Arg Gly Lys Leu Ile Leu Ile Glu Gly Leu Asp Arg Thr Gly

AAA ACC ACG CAA TGT AAT ATT CTT TAC AAA AAA TTG CAA CCA AAC TGT AAA CTA TTG AAG TTC CCC GAA AGG TCT 335
Lys Thr Thr Gln Cys Asn Ile Leu Tyr Lys Lys Leu Gln Pro Asn Cys Lys Leu Leu Lys Phe Pro Glu Arg Ser

ACC CGA ATC GGA GGA CTC ATA AAC GAA TAT TTG ACG GAT GAT AGT TTC CAA TTA TCA GAT CAG GCA ATT CAC CTC 410
Thr Arg Ile Gly Gly Leu Ile Asn Glu Tyr Leu Thr Asp Asp Ser Phe Gln Leu Ser Asp Gln Ala Ile His Leu

TTG TTT TCG GCA AAT AGA TGG GAA ATA GTT GAC AAG ATA AAG AAA GAT TTA CTA GAA GGC AAG AAC ATT GTC ATG 485
Leu Phe Ser Ala Asn Arg Trp Glu Ile Val Asp Lys Ile Lys Lys Asp Leu Leu Glu Gly Lys Asn Ile Val Met

GAC AGA TAT GTT TAT TCT GGA GTG GCA TAT TCT GCC GCT AAG GGG ACA AAT GGA ATG GAT TTG GAT TGG TGC TTG 560
Asp Arg Tyr Val Tyr Ser Gly Val Ala Tyr Ser Ala Ala Lys Gly Thr Asn Gly Met Asp Leu Asp Trp Cys Leu

CAA CCC GAT GTA GGG TTG CTG AAA CCG GAT TTG ACA TTA TTT TTA AGC ACT CAA GAT GTC GAC AAT AAC GCC GAA 635
Gln Pro Asp Val Gly Leu Leu Lys Pro Asp Leu Thr Leu Phe Leu Ser Thr Gln Asp Val Asp Asn Asn Ala Glu

AAA TCT GGA TTT GGT GAC GAA AGA TAC GAA ACT GTT AAG TTT CAA GAA AAA GTG AAG CAA ACT TTT ATG AAG CTA 710
Lys Ser Gly Phe Gly Asp Glu Arg Tyr Glu Thr Val Lys Phe Gln Glu Lys Val Lys Gln Thr Phe Met Lys Leu

TTG GAT AAA GAG ATA AGG AAA GGC GAT GAG TCA ATC ACG ATT GTA GAC GTT ACT AAT AAG GGC ATT CAG GAA GTT 785
Leu Asp Lys Glu Ile Arg Lys Gly Asp Glu Ser Ile Thr Ile Val Asp Val Thr Asn Lys Gly Ile Gln Glu Val

GAA GCG CTT ATT TGG CAA ATC GTT GAG CCT GTT TTG AGT ACG CAT ATT GAT CAT GAT AAA TTT TCG TTC TTC TAG 860
Glu Ala Leu Ile Cys Gln Ile Val Glu Pro Val Leu Ser Thr His Ile Asp His Asp Lys Phe Ser Phe Phe ***

GAGGATCTGT ACATATATCC TAGTCTAGTC TATCTAAAAT ACTTAGTCTT TCTAACAGCA TATCCTTTGA AATTTCTTGT ATTTCCCCTC  950

CAATAAACAT CTCGTCCATT ATCATATAGA CTTTGTAAAA GTTGAATACG ATATC 1005
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FIG. 4. Nucleotide sequence and predicted amino acid sequence of the *S. cerevisiae CDC8* gene and its product. The underlined segments are explained in the text.

ably because the relative concentration of the *CDC8* transcript in the RNA samples was insufficient to allow the necessary enrichment.

Specific fragments of plasmid pDQ14 were fixed to nitrocellulose membranes and used to select polyadenylated RNA molecules isolated from *S. cerevisiae* LDY113 carrying the multicopy plasmid pDQ14. These hybrid-selected RNA species were then translated in vitro, and the resulting labeled polypeptide chains were separated by denaturing polyacrylamide gel electrophoresis. Figure 6 shows that the 0.4-kbp *Sal*I to *Eco*RV and 0.6-kbp *Eco*RV to *Sal*I DNA fragments of the *CDC8* gene specifically selected mRNA molecules able to be translated into a major polypeptide chain of apparent molecular weight between 24,000 and 25,000 (Fig. 6, lanes 5 and 6). These RNA molecules were not selected by hybridization with vector DNA sequences (Fig. 6, lane 4), and no comigrating polypeptide was observed upon translation of total *S. cerevisiae* polyadenylated RNA (Fig. 6, lane 3). Thus the selected RNA represents a minor transcript in the total polyadenylated RNA sample and is specifically homologous to the *CDC8* gene. The presence of a transcript specifically selected by *CDC8* DNA sequences that translates into a polypeptide chain whose size matches that predicted from the nucleotide sequence of the *CDC8* gene strongly supports our assignment of the structural gene to this particular DNA sequence. We conclude that the *CDC8* gene codes for the polypeptide chain shown in Fig. 4.

TABLE 2. Predicted amino acid composition of the *CDC8* protein

Amino acid	Residues
Ala	7
Arg	8
Asn	9
Asp	19
Cys	4
Gln	10
Glu	14
Gly	15
His	3
Ile	16
Leu	25
Lys	21
Met	5
Phe	10
Pro	5
Ser	11
Thr	13
Trp	2
Tyr	6
Val	13

DISCUSSION

A plasmid, pYeCDC8, isolated from a random *S. cerevisiae* genomic DNA library is capable of complementing the *cdc8-1* mutation. This plasmid carries a 4.7-kbp DNA insert. Southern blot hybridization showed that the fragment subcloned in pDQ4 hybridizes to a single genomic DNA fragment. The arrangement of *Eco*RV sites within the cloned

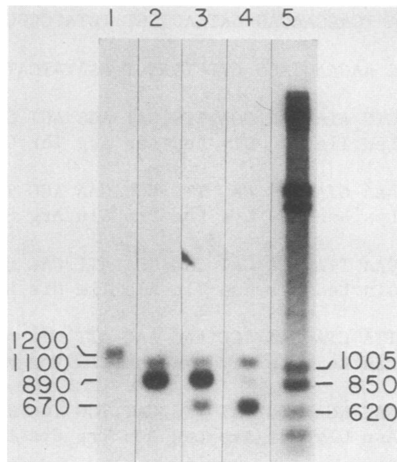

FIG. 5. Identification of the *CDC8* transcript. Four samples of polyadenylated RNA isolated from *S. cerevisiae* A364A and one of DNA size standards were subjected to agarose gel electrophoresis under denaturing conditions and transferred to nitrocellulose membrane. After cutting the membrane into strips, each sample was hybridized with a distinct ^{32}P-labeled DNA probe from the DNA insert in pYeCDC8 (Fig. 1) in lanes as follows: 1, 1.7-kbp *Eco*RV fragment; 2, 0.6-kbp *Eco*RV-*Sal*I fragment; 3, 0.4-kbp *Sal*I-*Eco*RV fragment; 4, 0.2-kbp *Eco*RV-*Bam*HI fragment. The DNA standards were probed with homologous DNA sequences. The specific activities of the DNA probes ranged from 3×10^7 to 5×10^7 cpm/μg of DNA. Equimolar amounts of each fragment were used. The chain lengths of the DNA standards (lane 5) are indicated at the right; the estimated chain lengths of the RNA transcripts are indicated at the left.

DNA element and the chromosome are the same. Transformation of an *S. cerevisiae cdc8-1* mutant with plasmid pDQ4 produced a Tsm$^+$ *S. cerevisiae* clone with the plasmid integrated into the genome at the site homologous to the cloned insert. The *URA3* marker carried in pDQ4 was used to map the site of integration of the plasmid. Tetrad analysis showed *URA3* to be approximately 26 cM from *ilv3* on chromosome X. The *cdc8* locus also maps 26 cM from *ilv3*. In these same analyses the *CDC8* marker carried on the integrated plasmid never segregated from the *cdc8-1* chromosome marker, indicating that the plasmid had integrated at the *cdc8* locus. Since it complements the *cdc8-1* mutation and integrates at a unique, homologous region of the genome that maps at the *cdc8* locus, we conclude that the cloned DNA insert carries the authentic *CDC8* gene.

Nucleotide sequence analysis shows that the cloned DNA fragment carrying the functional *CDC8* gene includes a long open reading frame of 216 codons surrounded by putative transcriptional and translational start and stop sequences typical of several *S. cerevisiae* genes. All five other reading frames have numerous translational stop codons. The long open reading frame includes the single *Sal*I site whose cleavage disrupts the functional gene. All of these criteria indicate that this particular reading frame specifies the *CDC8* gene product.

Our data imply that transcription of the *CDC8* gene occurs from left to right, as drawn in Fig. 1. RNA blot hybridization of polyadenylated yeast RNA allowed the detection of a transcript of approximately 890 nucleotides homologus with the *CDC8* gene. The size of this transcript is consistent with that expected of the *CDC8* gene. Assuming that transcription starts approximately 30 nucleotides upstream from the first ATG codon, 3′ to the next upstream ATG at nucleotides 175 through 177, and ends at least 100 nucleotides after the chain-terminating codon, we would predict a *CDC8* transcript of at least 780 nucleotides plus its polyadenylic acid tail. Recent S1 nuclease protection experiments designed to map the 5′ ends of *CDC8* transcripts relative to the DNA sequence presented in this report are consistent with transcription initiation occurring between nucleotides 184 and 201; the transcripts hybridize to the DNA strand complementary to the sequence shown in Fig. 4 (M. Brooks and L. Dumas, unpublished data).

S. cerevisiae mRNA enriched for molecules that hybridize to fragments of the *CDC8* gene translates into a specific polypeptide chain whose size matches that predicted from the nucleotide sequence of the gene. This corroborates our assignment of the *CDC8* gene coding region to the particular portion of the nucleotide sequence indicated in Fig. 4 and the *CDC8* gene product to the amino acid sequence shown in the same figure. The *CDC8* protein thus appears to be colinear with the nucleotide sequence. It consists of a polypeptide chain of 216 amino acids with a molecular weight of 24,600.

Our failure to detect the *CDC8* polypeptide chain after translation of mRNA selected from cells carrying a single copy of the *CDC8* gene is presumably due to its low abundance in such cells. Relative to *URA3* mRNA, which represents 1.3×10^{-4} of the total polyadenylated RNA in *S.*

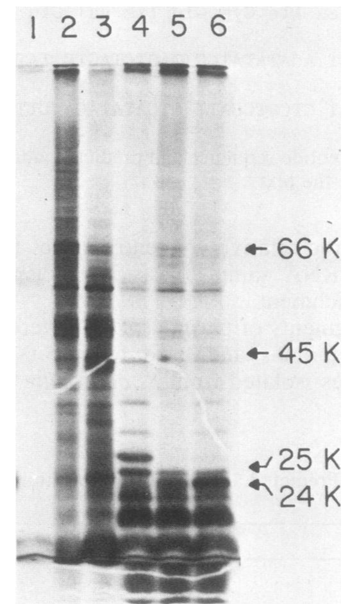

FIG. 6. In vitro translation products. [^{35}S]methionine-labeled translation products were fractionated on denaturing polyacrylamide gels and detected by fluorography. Lanes: 1, no exogenous RNA added to the translation reaction; 2, total polyadenylated yeast RNA (New England Nuclear Corp.); 3, total polyadenylated RNA from strain LDY113 carrying the multicopy plasmid pDQ4; 4, polyadenylated RNA selected by hybridization to YIP5/2μD vector DNA; 5, polyadenylated RNA selected by hybridization to the 0.4-kbp *Sal*I-*Eco*RV fragment of *CDC8*; 6, polyadenylated RNA selected by hybridization to the 0.6-kbp *Eco*RV-*Sal*I fragment of *CDC8*. Equal portions of each translation mixture were applied to lanes 1, 4, 5, and 6. Lesser portions were applied to lanes 2 and 3 so as to normalize the counts per minute added to that in lanes 4 through 6. The RNA translated in reactions 4 through 6 included tRNA carrier (20) not added to translation reactions 1 through 3. The positions of protein molecular weight markers were determined by staining (not shown). These positions and the corresponding molecular weights are indicated by the arrows and numbers at the right.

cerevisiae (2), we estimate the *CDC8* mRNA in A364A cells to represent approximately 1×10^{-5} of the total polyadenylated RNA (unpublished data). This RNA concentration was too low to produce a detectable translation product in our system, but the greater than 10-fold amplification in mRNA copy number achieved by carrying the *CDC8* gene on a multicopy plasmid allowed the detection of a specific translation product.

The protein identified by Arendes et al. (1) as the *CDC8* gene product has an apparent molecular weight of 37,000 to 40,000, whereas that identified by Kuo and Campbell (14) has an apparent molecular weight of 10,000 to 20,000. Neither corresponds closely to the size of the authentic *CDC8* gene product. Both groups used in vitro complementation assays to identify the putative *CDC8* protein. The higher molecular weight observed by Arendes et al. might be explained by assuming that the authentic *CDC8* protein acts as a positive regulator of the synthesis of the DNA-binding protein they isolated. If true, their protein should not exhibit temperature sensitivity in vitro even when isolated from *cdc8-1* cells; since they observed apparent temperature sensitivity, this explanation seems unlikely. Another possibility might be that the authentic *CDC8* polypeptide chain is modified post-translationally so as to increase its apparent molecular weight, for example, by the addition of sugar substituents. Our data do not exclude this possibility. However, data from our laboratory (unpublished observations) indicate that the in vitro complementation assay used by Arendes et al. is not specific for *cdc8* mutations or for the DNA-binding protein they isolated. We see no compelling reason, therefore, to associate a higher-molecular-weight protein with the *CDC8* gene.

The lower-molecular-weight complementing activity observed by Kuo and Campbell might be explained by assuming that the transcript of the *CDC8* gene is spliced so as to reduce the length of the translated segment below that predicted from the sequence of the gene. Our observation that the translation product of the *CDC8* transcript is the same size as that predicted from the nucleotide sequence negates this explanation. An alternative might be that the authentic gene product is proteolytically modified to yield a smaller protein still able to complement the genetic defect. Our data do not exclude this possibility. Since the size of the complementing activity detected by Kuo and Campbell is only an estimate based upon a crude protein fraction, it is also possible that further purification and characterization of their protein will remove the size discrepancy.

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