Molecular Cloning of Chromosome I DNA from *Saccharomyces cerevisiae*: Isolation and Characterization of the *CDC24* Gene and Adjacent Regions of the Chromosome

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Molecular cloning techniques were used to isolate and characterize the DNA including and surrounding the *CDC24* and *PYK1* genes on the left arm of chromosome I of the yeast *Saccharomyces cerevisiae*. A plasmid that complemented a temperature-sensitive *cdc24* mutation was isolated from a yeast genomic DNA library in a shuttle vector. Plasmids containing *pyk1*-complementing DNA were obtained from other investigators. Several lines of evidence (including one-step gene replacement experiments) demonstrated that the complementing plasmids contained the bona fide *CDC24* and *PYK1* genes. These sequences were then used to isolate additional DNA from chromosome I by probing a yeast genomic DNA library in a lambda vector. A total of 28 kilobases (kb) of contiguous DNA surrounding the *CDC24* and *PYK1* genes was isolated, and a restriction map was determined. Electron microscopy of R-loop-containing DNA and RNA blot hybridization analyses indicated that an 18-kb segment contained at least seven transcribed regions, only three of which corresponded to previously known genes (*CDC24*, *PYK1*, and *CYC3*). Southern blot hybridization experiments suggested that none of the genes in this region was duplicated elsewhere in the yeast genome. The centers of *CDC24* and *PYK1* were only ~7.5 kb apart, although the genetic map distance between them is ~13 centimorgans. As previous studies with *S. cerevisiae* have indicated that 1 centimorgan generally corresponds to ~3 kb, the region between *CDC24* and *PYK1* appears to undergo meiotic recombination at an unusually high frequency.

The yeast cell cycle provides an attractive system in which to study how cell shape and spatial organization are generated in eucaryotic cells (53, 72). In this context, the *Saccharomyces cerevisiae* *CDC24* gene is of great interest; *cdc24* mutants have pleiotropic phenotypes suggesting that the gene product plays a central role in the morphogenetic processes of the cell cycle (64). Isolation of the *CDC24* gene by molecular cloning should facilitate identification of its product and elucidation of its function at the molecular level.

The cloning of *CDC24* acquired additional interest when this gene was mapped to the left arm of chromosome I (29), as this chromosome has recently provided a vivid example of the "gene number paradox" (i.e., the general discrepancy between genetic and molecular estimates of the numbers of genes [29, 52]). Genetic and physical evidence indicates that chromosome I of *S. cerevisiae* contains 260 to 300 kilobase pairs (kb) of DNA (10, 35, 42, 59). Given this size and the typical spacing of one transcribed region per 2 to 3 kb of DNA observed in *S. cerevisiae* (see Discussion), this chromosome is expected to contain at least 100 genes. However, 32 independently isolated temperature-sensitive lethal mutations that mapped to chromosome I all fell into the three previously known genes *CDC15*, *CDC24*, and *PYK1* (or *CDC19*) (29). Other genetic studies have identified only 10 to 11 additional genes on chromosome I (42).

Several factors may be contributing to the large discrepancy between the expected and observed numbers of genes on chromosome I. (i) Chromosome I may contain fewer

genes than expected from its length of DNA. (ii) Only a small fraction of the genes on chromosome I may encode products essential for aerobic growth on rich medium (the conditions used in seeking temperature-sensitive lethal mutants [29]) and hence be capable of giving rise to mutations lethal under these conditions. (iii) Some of the genes on chromosome I may encode essential products but be difficult to identify from temperature-sensitive mutations because they are duplicated in the genome or are otherwise functionally redundant. (iv) Some of the nonredundant, essential genes on chromosome I may encode products that are not easily mutable to temperature sensitivity with the temperature ranges and mutagens used. (v) There may be some remarkable hot spots for the induction of mutations on chromosome I by the mutagens used. These possibilities have been discussed in detail previously (29).

To begin assessing the relative contributions of these factors to the gene number paradox in this system, we have chosen several segments of chromosome I for detailed molecular analyses. Our intentions are to identify all transcribed regions in these segments, to determine which of these regions correspond to the genes already known from *in vivo* mutational analyses, to determine (by gene disruption procedures) which of the newly identified genes are essential, and to determine which of the "nonessential" genes may be so because they are duplicated. We report here the isolation and partial characterization of the *CDC24* gene and of 28 kb of DNA surrounding this gene on the left arm of the chromosome.

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MATERIALS AND METHODS

Organisms, plasmids, and bacteriophages. The *S. cerevisiae* strains used are listed in Table 1. *Escherichia coli* SF8

TABLE 1. *S. cerevisiae* strains used in this study^a

Strain	Genotype ^b	Source or reference
C276-4B	α (prototrophic)	75
SK-1	a/α <i>HO/HO</i>	R. Roth (28)
JPT19 α	α <i>cdc24-4^c</i> (prototrophic)	64
DC5	a <i>his3 leu2-3,112 can1-11</i>	J. Hicks (8)
S2072A	a <i>arg4 leu1 trp1 gal2</i>	YGSC ^d
TD4	a <i>ura3-52 his4-519 leu2-3,112 trp1-289</i>	G. Fink
KGC24-1	α <i>cdc24-4 his3 leu2-3,112</i>	This study ^e
KGC24-2	α <i>cdc24-4 trp1</i>	This study ^e
KGC24-3	α <i>cdc24-4 ura3-52 his4 leu2 trp1</i>	This study ^e
X1221a-7C ^f	a/α <i>ade1-1/0 adeX/+ arg4/+ hisX/+ leu1/+ met1/+ thrX/+ trp1/+</i>	J. Bruenn (29)
X3402-15C ^g	a <i>ADE1/ADE1 ade5 ura3 arg1 arg4 his5 leu1 met2 gal1 pet17</i>	R. Mortimer (29)
DK8 ^h	a/α <i>ade1/ade1 arg4/+ leu1/+ trp1/+</i>	27
PWO24-16C	a <i>cdc19-1ⁱ cdc24-4 trp1</i>	This study ^j
KGC24-D1	a/α <i>cdc24-4/+ his3/his3 leu2-3,112/leu2-3,112</i>	This study ^k
DK337	a/α <i>ade1/+ ade2/+ ura3/+ his3-11,15/his3-11,15 leu2/leu2 trp1/trp1 can1/+</i>	This study ^l
DK144-9D	α <i>pyk1-101^m ade1</i>	29
DK144-22D	a <i>pyk1-101^m ade1</i>	29
POD18	a/α <i>cdc19-1ⁱ cdc24-4^c/+ ade1/+ ura1/+ cys1/+</i>	J. Pringle ⁿ
POD18-71C	a <i>cdc19-1ⁱ cdc24-4^c</i>	29
RW1770	α <i>mak16-1 ade1 trp1 leu2</i>	R. Wickner (29)

^a Other strains generated in the course of this study are described in the text.

^b Some strains may carry additional nutritional markers.

^c A tight temperature-sensitive lethal allele of gene *CDC24*; *cdc24-4* strains grow well at 24°C and arrest in the first cell cycle at 36°C (64).

^d Yeast Genetics Stock Center, Berkeley, Calif.

^e KGC24-1, KGC24-2, and KGC24-3 are segregants from the cross of JPT19 α with DC5, S2072A, and TD4, respectively.

^f Monosomic for chromosome I; i.e., haploid for this chromosome and diploid for all other chromosomes.

^g Disomic for chromosome I; i.e., diploid for this chromosome and haploid for most or all other chromosomes.

^h This strain appears to be a normal diploid.

ⁱ This temperature-sensitive allele of *PYK1* is still referred to by its original *cdc19-1* designation (29).

^j A segregant from the cross of RW1770 with POD18-71C (29).

^k Diploid obtained by mating strains KGC24-1 and DC5.

^l Diploid obtained by mating strain D139-11B (obtained from J. Szostak) with strain W301-1A (obtained from R. Rothstein).

^m This temperature-sensitive allele of *PYK1* is from the mutant *tsb35* (29).

ⁿ Diploid obtained by mating strain POD17-5A with strain LH395BD1-1A (29).

(49), HB101 (40), and C600 (40) were used to maintain and amplify plasmids and recombinant lambda bacteriophages. The genomic DNA library in the autonomously replicating shuttle plasmid YRp7 (71) was a gift from K. Nasmyth and contained fragments produced by partial *Sau3A* digestion of DNA from *S. cerevisiae* AB320 (44). The genomic DNA library in phage λ MG14 was a gift from M. Olson and was constructed (M. Y. Graham and M. V. Olson, personal communication) by ligating a partial *Sau3A* digest of DNA from *S. cerevisiae* AB972 (58) to the right arm of lambda Charon 30 (55) and the left arm of lambda 1059 (30). Plasmid YEp13-PYK1 (a gift from G. Kawasaki and D. Fraenkel) was one of several *pyk1*-complementing plasmids with overlapping but nonidentical inserts isolated from an *S. cerevisiae* (strain AB320) genomic DNA library in vector YEp13 (31, 45; G. Kawasaki and D. Olsen, personal communications). Plasmid pBR322-PYK1 (a gift from P. Sinha) was

derived from another of these plasmids (P. Sinha and D. Olsen, personal communications); it contains a 7.0-kb *HindIII* fragment, carrying the *pyk1*-complementing region from the parent plasmid plus some vector sequences, subcloned into the *HindIII* site of pBR322. Plasmids pBR322 (40), pBR325 (5), YIp5 (71), YEp13 (8), and pWJ12 (48) have been described previously. Plasmid YEp103 is YIp5 in which the small *EcoRI-HindIII* fragment has been replaced by the 2.0-kb *EcoRI-HindIII* fragment containing the *S. cerevisiae* 2 μ m plasmid origin of replication (S. Lillie, personal communication).

Media and growth conditions. Yeast strains were grown routinely in the rich, glucose-containing media YEPD (61) or YM-P (37). When required, yeast were grown in YEPLG (same as YEPD except that 2% [wt/vol] sodium lactate and 0.1% [vol/vol] glycerol were substituted for glucose) or on the glucose-containing minimal medium SD (61). SD was used with appropriate supplements (61) for auxotrophic strains; the single missing supplement is identified in each case. Yeast cultures were grown aerobically at 23, 30, or 37°C as appropriate. *E. coli* strains and recombinant lambda bacteriophages were grown by standard procedures (40).

Isolation of DNA and RNA. Plasmid and bacteriophage DNAs were isolated from *E. coli* by standard procedures (40). Yeast DNA used for transformation of *E. coli* was prepared by a yeast DNA miniprep procedure (61). Yeast DNA used for genomic Southern blots was prepared either as described by Holm et al. (23) or from isolated nuclei (H. Y. Steensma, J. Crowley, and D. B. Kaback, manuscript in preparation). Total RNA was prepared from strain SK-1 or C276-4B (Table 1), growing exponentially in YEPD or YM-P medium, either as described previously (28) or essentially as described by Maccacchini et al. (39), with standard precautions against RNases (40). Poly(A)-containing [poly(A)⁺] RNA was then isolated by chromatography on either poly(U)-Sephadex (Bethesda Research Laboratories, Gaithersburg, Md.) or oligo(dT)-cellulose (28).

Genetic and recombinant DNA manipulations. Routine genetic manipulations and transformations were carried out by standard procedures (22, 40, 61). Yeast transformants were tested for mitotic stability of their transformed phenotypes essentially as described previously (24). Digestion with restriction endonucleases, agarose gel electrophoresis, ligation of DNA fragments, and ³²P labeling of DNA by nick translation were done by standard procedures (40, 54). Restriction fragments were isolated by electroelution (40), followed by passage of the eluate through a column of AG50W-X8 resin (Bio-Rad Laboratories, Richmond, Calif.) that had been equilibrated with TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0), and precipitation of the eluted DNA with ethanol. Screening of recombinant phage by plaque filter hybridization was performed as described by Benton and Davis (3). Formation, stabilization, and analysis of R-loop-containing DNA were performed as described previously (26).

Blot hybridizations. DNA blot hybridizations and determination of fragment sizes were performed by standard procedures (40, 43, 61, 65). Electrophoresis of RNA and Northern blotting were done by either of two procedures. In one, RNA was electrophoresed in CH₃HgOH-containing agarose (2) and then blotted and analyzed as described previously (1, 74) with Transa-Bind aminobenzyloxymethyl paper (Schleicher & Schuell, Keene, N.H.). Alternatively, glyoxal-treated RNA was electrophoresed, blotted to nitrocellulose (Schleicher & Schuell, type BA85), and analyzed essentially as described by Thomas (73). To determine the sizes of the

FIG. 1. (A) Physical map of the *CDC24-PYK1* region of the left arm of *S. cerevisiae* chromosome I. The orientation of this region in the chromosome was determined from the relative positions of the *CDC24* and *PYK1* genes, which were identified as described in the text; the actual distances to the telomere (T) and centromere (C) are not known. Restriction endonucleases: R, *EcoRI*; H, *HindIII*; B, *BglII*; S, *Sall*; X, *XhoI*; Bm, *BamHI*. The circle indicates a pair of sites whose order is uncertain. The sites shown are consistent with those determined by Burke et al. (9; P. Tekamp-Olson, personal communication) on an independently derived *pyk1*-complementing plasmid. The lines above the map represent the segments cloned in the indicated recombinant plasmids and lambda phages. l and s, Long and short arms of the λ MG14 vector, respectively (see the text). The precise location of the terminus of the λ C1a insert with respect to the circled pair of sites is uncertain. Shown below the map (wavy lines) are the approximate locations and sizes (in kilobases) of the transcribed regions identified by R loop and Northern blot analyses and (double-headed arrows) the segments used as radiolabeled probes for the Northern blot experiments. (B) Autoradiograms of Northern blot hybridizations that allowed the identification and partial localization of transcribed regions in the portion of chromosome I represented by the inserts in YRp7(*CDC24*)1 and λ C1a. Poly(A)⁺ RNA from strain SK-1 (lanes 4 to 6) or C276-4B (lanes 1 to 3 and 7 to 13) was analyzed as described in the text. Transcript sizes (in kilobases) are indicated. As all probes were labeled to approximately the same total radioactivity, the intensities of the bands provide a rough measure of the abundance of the various RNA species, if allowance is made for (i) the different exposure times (given below) of the various autoradiograms and (ii) the variable extents of homology between the probes and the transcripts identified. Most of the segments used as probes are shown in panel A with numbers that correspond to the lanes shown here. These segments were either purified electrophoretically from YRp7(*CDC24*)1, λ C1a, or appropriate subclones or subcloned first into pBR322 or pBR325. In addition, lane 1 was hybridized with the *TRP1*-containing 1.45-kb *EcoRI* fragment isolated from YRp7(*CDC24*)1 DNA. The probe for lane 3 was actually the entire plasmid YRp7(*CDC24*)1; thus, the *TRP1* mRNA is visualized as well as the transcripts homologous to the cloned segment of chromosome I DNA (probe 3, panel A). The probe for lane 6 was actually a 5.2-kb *EcoRI* fragment isolated from YRp7(*CDC24*)1; thus, it contained pBR322-derived sequences as well as the segment of chromosome I shown in panel A. Exposure times for the autoradiograms shown were: lane 1, 28 h; lanes 2 and 3, 12 h; lane 4, 18 h; lane 5, 12 h; lane 6, 16 h; lane 7, 20 h; lane 8, 2 h; lane 9, 22 h; lane 10, 18 h; lane 11, 22 h; lane 12, 18 h; and lane 13, 24 h.

transcripts identified, a lane containing total RNA was cut from each gel prior to blotting and stained with ethidium bromide to visualize the 18S and 25S rRNA bands, whose sizes were taken to be 1.7 and 3.4 kb, respectively (51). As only the two standards were used, the transcript sizes given below should be regarded as rough estimates only. Bands in blots derived from different gels were aligned by (i) the size estimates just described; (ii) comparisons between blots of the distances migrated by a particular RNA species (as determined by hybridization to the same radiolabeled probe); and (iii) rehybridization of particular blots with additional radioactive probes.

RESULTS

Isolation of a *cdc24*-complementing plasmid. The *trp1 cdc24* strain KGC24-2 (Table 1) was transformed with DNA from a yeast genomic library in the *TRP1*-containing shuttle vector

YRp7 (see Materials and Methods). Selection on plates lacking tryptophan at the restrictive temperature (37°C) yielded four independent *Trp*⁺ *Cdc*⁺ transformants. In each case, both the *Trp*⁺ and *Cdc*⁺ phenotypes were unstably maintained during growth under nonselective conditions. The plasmids were recovered in *E. coli* and subjected to restriction endonuclease analysis. Digestion with five enzymes gave indistinguishable patterns for the four plasmids (Fig. 1A), suggesting that they contained similar if not identical inserts. Retransformation of strain KGC24-2 with the purified plasmids and selection for *Trp*⁺ at 24°C yielded transformants that were uniformly *Cdc*⁺ when tested at 37°C. One of the isolated plasmids was designated YRp7(*CDC24*)1 and studied further.

Homology to chromosome I DNA of sequences complementing *cdc24* and *pyk1* mutations. The *PYK1* gene, which encodes pyruvate kinase (9, 15), is located ~13 centimorgans (cM) centromere proximal to *CDC24* (29, 42). Recombinant

FIG. 2. DNA blot hybridization experiments. (A) Homology of *pyk1*-complementing DNA to chromosome I. 32 P-labeled pBR322-PYK1 (lanes 1 to 3) or *HIS3* (lanes 4 to 6) DNA was hybridized to *Eco*RI-digested nuclear DNA ($\sim 10 \mu\text{g}$ per lane) from strains with different doses of chromosome I (see Table 1). Lanes: 1 and 4, strain X1221a-7C (monosomic for chromosome I); 2 and 5, strain DK8 (diploid); 3 and 6, strain X3402-15C (disomic for chromosome I). The *HIS3* DNA was the 1.7-kb *Bam*HI fragment from plasmid pWJ12 (48). Fragment sizes are indicated in kilobases (cf. Fig. 1A and reference 70). (B) One-step gene replacement in the *CDC24* region. 32 P-labeled YIp5(*CDC24*) DNA (see text and Fig. 1A) was hybridized to blots of *Hind*III-digested total DNA ($\sim 1 \mu\text{g}$ per lane) from diploid strain KGC24-D1 (Table 1) and its *Leu*⁺ *Cdc*⁻ transformant KGC24-D1T1 (see text). The KGC24-D1 DNA (lane 1) shows the expected 4.0-kb *Hind*III fragment corresponding to that cloned in YIp5(*CDC24*) as well as the 1.1-kb *Hind*III fragment of chromosome V DNA that carries the *URA3* gene (71). The KGC24-D1T1 DNA (lane 2) shows these same fragments plus the 7.0-kb fragment predicted to be present if one chromosomal copy of the 4.0-kb *Hind*III segment has been replaced by the 7.0-kb *Hind*III segment present in the transforming DNA (see text). (C) One-step gene replacement in the *PYK1* region. 32 P-labeled pBR322-PYK1 DNA (lanes 1 and 2) or *HIS3* DNA (as in panel A; lanes 3 and 4) was hybridized to *Eco*RI-digested nuclear DNA ($\sim 7 \mu\text{g}$ per lane) from diploid strain DK337 (Table 1; lanes 1 and 3) and its *His*⁺ transformant 337/56 (see text; lanes 2 and 4). The DK337 DNA showed the expected fragments of 1.9, 2.2, and 5.0 kb (see Fig. 1A) when probed with pBR322-PYK1 and a single fragment of 10.1 kb (derived from chromosome XV [70]) when probed with the *HIS3* fragment. The 337/56 DNA showed these same fragments plus the 3.6-kb fragment, homologous to both probes, predicted to be present if one chromosomal copy of the 1.9-kb *Eco*RI segment has been replaced by the 3.6-kb *Eco*RI segment present in the transforming DNA (see text).

plasmids YEp13-PYK1 and pBR322-PYK1, which contain DNA capable of complementing *pyk1* mutations (see Materials and Methods), were obtained from other workers to serve as a potential second starting point for the isolation of DNA from the left arm of chromosome I. Restriction sites were determined in these plasmids (Fig. 1A). To determine whether the sequences responsible for complementation of *cdc24* and *pyk1* mutations were derived from chromosome I, probes derived from plasmids YRp7(*CDC24*)1 and pBR322-PYK1 were hybridized to Southern blots of DNA isolated from three strains with different doses of this chromosome. A strain monosomic for chromosome I, a diploid strain, and a strain disomic for chromosome I were compared. The relative intensities in the three strains of each *Eco*RI band that hybridized to pBR322-PYK1 DNA varied in the expected way (Fig. 2A). Similar results were obtained with the 4.0-kb *Hind*III fragment from YRp7(*CDC24*)1 (Fig. 1A) as the radioactive probe (not shown). In contrast, a fragment

containing the *HIS3* gene from chromosome XV hybridized with nearly equal intensity to a single 10.1-kb *Eco*RI band (70) in all three strains (Fig. 2A).

Integration of the *cdc24*-complementing plasmid at the *CDC24* map position. Strain KGC24-2 harboring plasmid YRp7(*CDC24*)1 was subjected to repeated cycles of nonselective and selective growth until mitotically stable *Trp*⁺ *Cdc*⁺ clones were obtained. Such clones generally arise when the complementing plasmid integrates into a yeast chromosome by homologous recombination (6, 14, 44). Three such clones (putative genotype *CDC24::TRP1 cdc24 PYK1 trp1*) were crossed with strain PWO24-16C (genotype *cdc24 pyk1 trp1*; Table 1). Tetrad analysis of these diploids showed that *TRP1* (a gene normally on chromosome IV) and *CDC24* both showed 2:2 segregation and were tightly linked in each case, as expected if the only *TRP1* and *CDC24* markers were those on an integrated plasmid. In one case, *TRP1* and *CDC24* showed no linkage to *PYK1*, indicating that integration had occurred at some site other than the *CDC24* locus. However, in the other two cases, *TRP1* and *CDC24* showed linkage to *PYK1* (8 to 11 cM) that was similar to the previously determined map distance between *CDC24* and *PYK1* (9 to 16 cM [29]). Thus, it appeared likely that the *cdc24*-complementing plasmid contained the *CDC24* gene itself, rather than some kind of suppressor.

Identification of the *CDC24* and *PYK1* genes by subcloning and gene disruption. The analyses described below showed that the inserts in YRp7(*CDC24*)1 and pBR322-PYK1 each contained all or part of at least three transcribed regions (see summary in Fig. 1A). To determine which transcribed region was responsible for the *cdc24*-complementing activity, the central 4.0-kb *Hind*III fragment from YRp7(*CDC24*)1 was subcloned into the *Hind*III site of the *URA3*-containing shuttle vector YEp103 (see Materials and Methods) to form plasmid YEp103(*CDC24*) (Fig. 1A). Transformation of the *ura3 cdc24* strain KGC24-3 (Table 1) with this plasmid and selection on medium lacking uracil at 24°C yielded transformants that were all *Cdc*⁺ when tested at 37°C. Thus, the central transcribed region of YRp7(*CDC24*)1 appeared to be the *CDC24* gene.

This hypothesis was confirmed by a one-step gene replacement experiment (56) in which the putative *CDC24* coding region was disrupted in vitro and transformed into yeast so as to replace the resident wild-type copy of this region with the disrupted copy. The 4.0-kb *Hind*III fragment from YRp7(*CDC24*)1 was subcloned into the *Hind*III site of plasmid YIp5 (71) to form plasmid YIp5(*CDC24*) (Fig. 1A). A 3.0-kb *Bgl*III fragment containing the yeast *LEU2* gene was then isolated from plasmid YEp13 (8, 56) and inserted into YIp5(*CDC24*) at its single *Bgl*III site. The resulting plasmid was cleaved with *Hind*III into two fragments, one of which was the desired 7.0-kb fragment containing *LEU2* inserted into the putative *CDC24* coding region (Fig. 1A). The mixture of fragments was used to transform the *leu2/leu2 CDC24/cdc24* (phenotypically *Leu*⁻ *Cdc*⁺) diploid strain KGC24-D1 (Table 1), selecting transformants on medium lacking leucine at 24°C. Two mitotically stable *Leu*⁺ transformants were obtained; one was still *Cdc*⁺ and was not analyzed further. The other transformant (KGC24-D1T1) had become *Cdc*⁻: it was unable to proliferate at 37°C and developed the abnormal cell morphology characteristic of *cdc24* mutants. Southern blots of DNA from KGC24-D1T1 and its untransformed parent (Fig. 2B) indicated that, as expected (6, 56), transformation had occurred by homologous recombination leading to replacement of one chromosomal copy of the 4.0-kb *Hind*III segment by the 7.0-kb

*Hind*III segment containing *LEU2* DNA inserted at the *Bgl*II site.

When KGC24-D1T1 was sporulated, all 35 tetrads dissected yielded two viable and two nonviable spores at the permissive temperature. All 70 viable spores were *Leu*⁻, and 69 of these were temperature-sensitive *Cdc*⁻. Thus, it appeared that the insertion of *LEU2* DNA had produced a recessive lethal mutation that was tightly linked to *CDC24* and failed to complement the temperature-sensitive *cdc24* mutation. Thus, the transcribed region that spans the *Bgl*II site at which the insertion occurred (Fig. 1A) must be the *CDC24* gene.

Similarly, the *PYK1* transcribed region was identified by showing that disruption of this region in the yeast genome produced a *pyk1* mutation. As pyruvate kinase is an abundant enzyme (15), the region of pBR322-PYK1 that gave rise to the most abundant transcript (Fig. 2B, lanes 8 and 9) seemed likely to be the *PYK1* gene. Thus, a 1.7-kb *Bam*HI fragment containing the yeast *HIS3* gene from plasmid pWJ12 (48) was inserted into pBR322-PYK1 at the *Bgl*II site that appeared to be within this transcribed region (Fig. 1A). The resulting plasmid was cleaved with *Eco*RI into four fragments, including the desired 3.6-kb fragment containing the putative *PYK1* gene disrupted by the *HIS3* gene, and used to transform the *PYK1/PYK1 his3/his3* diploid strain DK337 (Table 1). A mitotically stable *His*⁺ transformant, 337/56, was isolated and analyzed. Southern blots of DNA from 337/56 and its untransformed parent (Fig. 2C) indicated that transformation had occurred by homologous recombination, leading to replacement of one chromosomal copy of the 1.9-kb *Eco*RI segment by the 3.6-kb *Eco*RI segment containing *HIS3* DNA inserted at the *Bgl*II site.

Analysis of tetrads from transformant 337/56 showed an apparent 2:2 segregation of a microcolony phenotype when segregants were grown on YEPD medium. In contrast, all viable segregants grew equally well when replica plated to lactate-glycerol medium (see Materials and Methods). The glucose-negative mutation was apparently caused by insertion of the *HIS3* DNA into the yeast chromosome (37 of 38 segregants producing microcolonies were *His*⁺, whereas 42 of 43 segregants producing normal-sized colonies were *His*⁻) and was apparently a *pyk1* mutation, as it failed to complement the temperature-sensitive *pyk1-101* allele at the restrictive temperature. (That is, mating the glucose-negative segregants from 337/56 to DK144-9D or DK144-22D [Table 1] yielded exclusively diploids that could grow well on glucose at 23 but not at 37°C.) Thus, the transcribed region spanning the *Bgl*II site in pBR322-PYK1 appeared to be the *PYK1* gene.

Isolation of additional DNA from chromosome I. To isolate additional DNA from the *CDC24-PYK1* region, pBR322-PYK1 and the 4.0-kb *Hind*III fragment from YRp7(*CDC24*)1 (Fig. 1A) were used as probes to screen a lambda library of yeast genomic DNA by plaque filter hybridization. Several recombinant phages were selected with each probe; all of these contained inserts that were overlapping. Two of these inserts (phages λ C1a and λ H9a) were characterized further and are shown in Fig. 1A. In total, 28 kb of chromosome I DNA appears to lie between the centromere-distal end of the insert in YRp7(*CDC24*)1 and the centromere-proximal end of the insert in λ H9a.

Phage λ C1a hybridized to both plasmid-derived probes. Approximate localization of the *CDC24* and *PYK1* transcribed regions on the insert from λ C1a (see preceding and following sections; results summarized in Fig. 1A) indicated that the distance between the centers of these two genes is

FIG. 3. DNA blot-hybridization experiments comparing the structure of DNA from the *CDC24-PYK1* region in λ C1a (see text and Fig. 1A) with that in the yeast strains used previously to estimate the genetic map distance between *CDC24* and *PYK1* (29). DNA from λ C1a (lanes 3 and 7) and total DNAs isolated from strains RW1770 (lanes 1 and 5), POD18-71C (lanes 2 and 6), and POD18 (lanes 4 and 8) were digested with *Eco*RI (lanes 1 to 4) or *Hind*III (lanes 5 to 8), electrophoresed, and hybridized to ³²P-labeled λ C1a DNA. Shown for lanes 5 through 8 is a composite of 7-h (upper bands) and 24-h (lower bands) exposures during autoradiography. Lane 3 reveals the expected bands due to the λ MG14 arms (which contain no *Eco*RI sites) plus the expected bands of about 5.0, 2.7, 2.2, 1.9, 1.7, 1.2, and 0.35 kb. Lanes 1, 2, and 4 display all of these bands except those due to the lambda arms and the 0.35-kb fragment (which is presumably just too faint to be visible on this autoradiographic exposure). In addition, the bands at 5 kb appear more intense than expected for a single fragment, presumably because they reflect hybridization to the 5.0-kb *Eco*RI fragment contained in λ C1a (cf. lane 3) and to the 4.9-kb *Eco*RI fragment in which lies the centromere-proximal terminus of the insert in λ C1a (cf. Fig. 1A). Finally, lanes 1, 2, and 4 also reveal very faint bands at 1.3 kb that are presumably the *Eco*RI fragment in which lies the centromere-distal terminus of the insert in λ C1a (cf. Fig. 1A; note that there is only a short stretch of homology between this *Eco*RI fragment and the λ C1a probe). Lane 7 reveals the expected bands due to the λ MG14 arms (which contain no *Hind*III sites) plus the expected bands at 8.5 (not fully resolved from the shorter lambda arm), 2.5, and 1.0 kb. Lanes 5, 6, and 8 display all of these bands except those due to the lambda arms, plus two additional bands at 4.0 and 0.7 kb due to the *Hind*III fragments in which lie, respectively, the centromere-distal and centromere-proximal termini of the insert in λ C1a (cf. Fig. 1A).

~7.5 kb. This was much less than expected given the genetic map distances of 9.4 and 16 cM obtained in two different crosses (29) (see Discussion). This discrepancy was apparently not due either to rearrangements during cloning or to gross polymorphisms between the strains used for genetic mapping and that used for construction of the λ MG14 library. First, λ C1a was one of four independently isolated phages with nonidentical inserts that hybridized to both of the plasmid-derived probes. Second, Southern blot experiments in which DNA from λ C1a and the strains used in mapping were digested with *Eco*RI (Fig. 3, lanes 1 to 4), *Hind*III (Fig. 3, lanes 5 to 8), and *Bgl*II (not shown) and hybridized to ³²P-labeled λ C1a DNA revealed the same fragments in all cases (except for the fragments involved in the junctions to the lambda arms; see figure legend for details).

The Southern blot experiments also indicated that none of

the sequences in the 17-kb region cloned in λ C1a was closely duplicated elsewhere in the yeast genome.

Identification of additional transcribed regions in the vicinity of *CDC24* and *PYK1*. Electron microscopy of R-loop-containing YRp7(*CDC24*)1 DNA (data not shown) suggested that the segment cloned in this plasmid contained one centrally located and two flanking transcribed regions. Both of the latter appeared to extend beyond the ends of the cloned segment, as indicated by the presence of "tails" (representing unhybridized portions of the mRNA molecules [26]) on the corresponding R loops. These results were then confirmed and extended by RNA blot hybridization (Northern blot) experiments with probes derived from YRp7(*CDC24*)1 and λ C1a (Fig. 1B, results summarized in Fig. 1A).

When YRp7(*CDC24*)1 DNA was used as the radiolabeled probe, four RNA species of 0.7, 1.2, 2.0, and 2.2 kb were detected (Fig. 1B, lane 3). The 0.7-kb species appeared to be the *TRP1* transcript, because a single species of this molecular size was observed when the probe was either YRp7 DNA (not shown) or a purified fragment containing the *TRP1* gene (Fig. 1B, lane 1). The 1.2-kb RNA species appeared to be derived from the centromere-distal portion of the segment cloned in YRp7(*CDC24*)1, as a species of this molecular size was observed when the probe was the 1.3-kb *EcoRI* fragment from YRp7(*CDC24*)1 (Fig. 1B, lane 2), but not when the other probes derived from this plasmid were used (Fig. 1B, lanes 4 to 6). Both the 2.0- and 2.2-kb RNA species were observed when the probe was the 2.7-kb *EcoRI* fragment from YRp7(*CDC24*)1 or λ C1a (Fig. 1B, lane 5) or the 1.2-kb *BglII-EcoRI* fragment from YRp7(*CDC24*)1 (Fig. 1A, probe 5'; data not shown). In contrast, only the 2.2-kb RNA species was seen when the probe was the 1.5-kb *EcoRI-BglII* fragment from YRp7(*CDC24*)1 (Fig. 1B, lane 4), and only the 2.0-kb species was seen when the probe was either the centromere-proximal portion of the insert from YRp7(*CDC24*)1 (Fig. 1B, lane 6) or the 5.0-kb *EcoRI* fragment from λ C1a (Fig. 1B, lane 7). Thus, the 2.2-kb and 2.0-kb RNA species appear to be derived from the central (spanning the *BglII* site) and centromere-proximal portions, respectively, of the segment cloned in YRp7(*CDC24*)1.

The experiments described above demonstrated that the transcribed region giving rise to the 2.2-kb RNA species is the *CDC24* gene. As the flanking transcribed regions do not correspond to known genes (42; see below), they have been given the temporary names *FUN9* (function unknown now; centromere-distal region) and *FUN10* (centromere-proximal region). The positioning of these transcribed regions in Fig. 1A is based on both the R loop and Northern blot results.

When the 5.0-kb *EcoRI* fragment from λ C1a was used as the radiolabeled probe, two additional RNA species of 0.8 and 1.6 kb were observed (Fig. 1B, lane 7). The 0.8-kb species must be derived from a transcribed region that lies between *FUN10* and *PYK1*. This transcribed region has been further localized (as shown in Fig. 1A) and identified as *CYC3* (shown previously to map in this region [41, 57]) by R. Rothstein and his co-workers and by M. Dumont and F. Sherman (personal communications).

A 1.6-kb RNA species also gave a much stronger hybridization signal when the 1.9-kb *EcoRI* fragment from λ C1a was used as the probe (Fig. 1B, lane 8; note the short exposure used to make this autoradiogram) and a moderately strong signal when the adjacent 2.2-kb *EcoRI* fragment was used as the probe (Fig. 1B, lane 9). The experiments described above, as well as the nucleotide sequencing and associated experiments reported by Burke et al. (9), demon-

strate that the 1.6-kb RNA species arises from the *PYK1* gene, which spans the *BglII* site within the 1.9-kb *EcoRI* segment. The evidence that the *PYK1* transcribed region extends centromere-proximally a short distance (thus accounting for the reduced hybridization signal) into the 2.2-kb *EcoRI* segment (Fig. 1B, lane 9) is also consistent with the data of Burke et al. (9). However, their data imply that the *PYK1* transcribed region does not extend into the centromere-distal 5.0-kb *EcoRI* segment (Fig. 1A, probe 7); thus, the 1.6-kb RNA species visualized in Fig. 1B, lane 7, may be derived from a distinct transcribed region. However, Southern blots revealed that the gel-purified 5.0-kb *EcoRI* fragment that was used as the probe for Fig. 1B, lane 7, contained trace amounts of DNA from other portions of λ C1a, including the *PYK1* region. Given the abundance of the *PYK1* mRNA, this trace contamination of the probe may have sufficed to give the weak band at 1.6 kb that is seen in Fig. 1B, lane 7.

When the 2.2-kb *EcoRI* fragment was used as the radiolabeled probe, a 1.3-kb RNA species was also observed (Fig. 1B, lane 9). This species had the same mobility as the only RNA species detected when the adjacent 1.2-kb *EcoRI* fragment was used as the probe (Fig. 1B, lane 10). Thus, the 1.3-kb RNA species presumably is derived from a single transcribed region that spans the *EcoRI* site at the junction between the 2.2-kb and 1.2-kb *EcoRI* segments. As this transcribed region does not correspond to a known gene (42), it has been given the temporary designation *FUN11* (Fig. 1A).

When the radiolabeled probe was the 1.7-kb *EcoRI* fragment from λ C1a (Fig. 1A, probe 11), its 1.1-kb *EcoRI-BglII* subfragment (Fig. 1A, probe 11'), its 0.6-kb *BglII-EcoRI* subfragment (Fig. 1A, probe 12), or the short arm of *EcoRI*-digested λ C1a (which contains 1.3 kb of yeast DNA; Fig. 1A, probe 13), a 3.0-kb RNA species gave the only prominent hybridization signal (Fig. 1B, lanes 11 to 13). As this species was not observed when the 1.2-kb *EcoRI* fragment was used as the probe (Fig. 1B, lane 10), the corresponding transcribed region can be positioned approximately as shown in Fig. 1A. As it does not correspond to a known gene (42), it has been given the temporary designation *FUN12*. With probe 11, but not with probes 11', 12, or 13, a weak signal was also observed at 1.6 kb (Fig. 1B, lane 11). The significance of this band is uncertain. It could conceivably represent a distinct transcript, a processing or breakdown product of the 3.0-kb RNA species, or an artifact related to the abundant *PYK1* mRNA that was present in the blot.

DISCUSSION

Isolation of the *CDC24* gene. Studies of *cdc24* mutants have suggested that the *CDC24* product interacts with Ca^{2+} ions (46) and plays a central role in the morphogenetic processes of the yeast cell cycle (64). Thus, it will be of great interest to identify this product and elucidate its functions at the molecular level. Isolation of the *CDC24* gene is a step toward this goal. Sequencing the cloned gene may allow inferences about the structure and possible functions of the *CDC24* gene product, and the availability of the cloned gene should allow the generation of antisera with which this product can be identified and localized in cells (53). The cloning of *CDC24* has also recently been reported by Ohya et al. (46), whose results appear to be consistent with ours.

In the course of our studies, we encountered difficulties in maintaining active *CDC24* genes on multicopy plasmids. For example, we repeatedly recovered plasmids that had lost

cdc24-complementing activity, although their restriction maps appeared to be unaltered. These observations suggest that there is selection for *CDC24*-inactivating mutations during propagation of such plasmids in *S. cerevisiae* or *E. coli* and raises the possibility that the isolated gene may not be fully normal, despite its retention of at least partial biological activity. This possibility may affect the use of the cloned gene for certain types of studies.

Isolation and partial characterization of a segment of chromosome I. Kawasaki and co-workers had isolated several *pyk1*-complementing plasmids (31) (see Materials and Methods). Our results, together with those of Burke et al. (9), establish that the *pyk1*-complementing activity in YEp13-PYK1 and in pBR322-PYK1 is in fact due to the bona fide *PYK1* gene. Using pBR322-PYK1 and a fragment from YRp7(*CDC24*)1 as probes, we were able to isolate several λ MG14 clones containing inserts of chromosome I DNA. Together, the inserts in YRp7(*CDC24*)1, λ C1a, and λ H9a define a 28-kb segment in the distal portion of the left arm of the chromosome (Fig. 1A). Using similar methods, we have also isolated substantial segments of DNA from the right arm and the proximal portion of the left arm of chromosome I (13; H. Y. Steensma, J. C. Crowley, and D. B. Kaback, manuscript in preparation). The availability of these cloned DNAs makes it possible to begin a systematic molecular analysis of the reasons for the gene number paradox in this system (see Introduction).

Of the 28-kb segment described in this report, the centromere-distal portion has been characterized more thoroughly than the centromere-proximal portion. In particular, we have obtained good evidence that the 18-kb segment cloned in YRp7(*CDC24*)1 and λ C1a is equivalent to the corresponding segment in the chromosomes of normal strains (Fig. 1A and 3), and we have mapped at least some of the transcribed regions in this segment. The transcribed regions corresponding to the three known genes *CDC24*, *CYC3*, and *PYK1* have been identified (see Results and Fig. 1A). In addition, four other distinct transcribed regions were found that do not correspond to any known genes; these have been given the temporary designations *FUN9*, *FUN10*, *FUN11*, and *FUN12* (Fig. 1A). For several reasons, the seven transcribed regions found represent a minimal number for the segment investigated. First, we worked only with the poly(A)⁺ RNA fraction from cells growing exponentially in rich, glucose-containing medium under aerobic conditions. Thus, any regions producing only nonpolyadenylated transcripts (20, 36), including any tRNA genes, probably would have been overlooked, as would any genes that are transcribed appreciably only in other cell types or under other growth conditions (11, 38, 50, 68, 76). Second, there are some residual uncertainties in our results that might reflect the presence of additional transcribed regions (see Results) (see also Fig. 2 in reference 9).

Taking our results at face value, the transcribed regions identified occupy roughly 11.5 of the 18 kb, or about 64% of the DNA, and there is, on average, one 1.7-kb transcript per 2.7 kb of DNA. Similar numbers have been obtained with other cloned yeast chromosome segments in which the transcribed regions have been mapped. Taken together, the *CDC24*-*PYK1* region (this study), the histone H2A and H2B gene regions (21), several ribosomal protein gene regions (33, 77), the *HIS3* region (70), the *GAL7,10,1* region (68), the *CYC1*-*CDC8* region (4, 16, 32, 38, 60, 62; L. Melnick, personal communication), the *CDC36* and *CDC37* regions (7), the *ACT1*-*TUB2* region (17, 72), the *RNA3* and *RNA11* regions (34), the *DUR1,2*-*MET8* region (11, 18), the *DAL*

gene region (79), the *CDC10*-*CEN3* region (28, 78), the *SIR2*, *SIR3*, and *SIR4* regions (25, 63), and the *MET14*-*CEN11* region (78) encompass ~220 kb of DNA and contain 98 identified transcribed regions (including tRNA genes) producing a total of ~125 kb of transcript. Thus, for these regions, ~57% of the DNA is transcribed, and there is, on average, one 1.3-kb transcript per 2.2 kb of DNA. These appear to be minimal estimates, as most of the studies cited could have overlooked transcribed regions for one or more of the reasons noted above. It might be argued that the regions studied are atypical, in that all were characterized because they were already known to contain at least one gene of interest. However, in total genomic R-looping studies (26), no evidence was found either for extensive clustering of transcribed regions or for long segments of nontranscribed DNA. Moreover, it is striking that in sets of fragments cloned with considerable bias against the presence of regions transcribed in nonsporulating cells, such regions were nonetheless found to be present in rather high frequency (19, 50). If the regions studied to date are indeed representative of the genome as a whole, the ~14,000 kb of the yeast haploid genome (35) should contain $\sim 14,000 \div 2.2$, or ~6,400 transcribed regions. From the limited evidence available, it appears that most of these regions are transcribed detectably in cells of a given mating type growing vegetatively in rich, aerobic medium.

These estimates are in reasonable agreement with the estimates for the density (one per 3 kb) and total number (~5,000) of transcribed regions obtained by total genomic R-loop mapping experiments in which genomic DNA was hybridized to saturation with poly(A)⁺ RNA from cells growing exponentially in aerobic YEPD medium (26). However, in these R loop studies, only ~33% of the DNA appeared to be transcribed, in part because the average size of the R loops observed was only 1.05 kb. We now believe that these relatively low values reflect displacement of the R loops by the complementary DNA strands, which makes the transcribed regions appear smaller than they really are (26). Similarly, experiments measuring the kinetics of RNA-cDNA hybridization and the proportion of genomic DNA rendered double stranded by hybridization to poly(A)⁺ RNA suggested that only 30 to 40% of the genome was transcribed in cells growing in aerobic YEPD medium (20). On the assumption that the average *S. cerevisiae* transcript is 1.5 kb, this yielded an estimate of 3,000 to 4,000 different transcribed regions (20). We suggest that these figures are underestimates resulting from the failure of some sequences to hybridize efficiently. Clearly, careful studies on additional cloned sequences will improve our estimates of the total number of genes expressed in yeast under various conditions.

In the case of chromosome I, its size of 260 to 300 kb (10, 35, 42, 59) suggests that it should contain ≥ 100 transcribed regions. However, classical genetic studies, including an intensive search for genes identifiable by temperature-sensitive lethal mutations (29), have identified only 13 to 14 genes on this chromosome (42). It seemed possible that this discrepancy might be due in part to an atypically sparse packing of genes on chromosome I. However, the available data do not support this interpretation. The densities of transcribed regions both in the *CDC24*-*PYK1* region (see above) and in the *ADE1*-*CDC15* region of the right arm of the chromosome (Steensma et al., manuscript in preparation) are clearly within the normal range. It is also possible that some genes on chromosome I are difficult to identify by in vivo mutational analysis because they are duplicated in the

genome (reference 29 and references cited therein). This possibility does not appear to apply to the *FUN9*, *FUN10*, *FUN11*, and *FUN12* transcribed regions described in this report, as no additional bands were seen when Southern blots of genomic DNA were hybridized to probes containing all or portions of these genes (Fig. 2 and 3). However, it should be noted that (i) functionally significant gene duplications might be detected only by hybridizing Southern blots at reduced stringency and (ii) functional redundancy might in some cases involve alternative pathways rather than a duplication of the genes involved in a single pathway. It is also possible that some of the transcribed regions on chromosome I have been difficult to detect in genetic analyses because they serve no important function, at least in vegetatively growing cells. However, it may be noted that most, if not all, of the species of poly(A)⁺ RNA detectable in total yeast RNA are also detectable in the polysomal RNA fraction (20, 26) and thus are presumably being translated into protein. We are presently conducting gene disruption experiments to explore the functions of the previously unidentified transcribed regions on chromosome I.

A hot spot for recombination in the *CDC24-PYK1* interval. When the *CDC24* and *PYK1* genes were located on the cloned DNA from chromosome I, they proved to be surprisingly close together. Genetic mapping studies (29) had shown that these genes were separated by ~13 cM (9.4 and 16 cM in two separate crosses). Studies of the genome as a whole (35, 42) and of a large circular derivative of chromosome III (69) had indicated that on average, 1 kb of physical distance corresponds to ~0.34 cM of genetic map distance in *S. cerevisiae*. Thus, it was anticipated that *CDC24* and *PYK1* would be 35 to 40 kb apart. Instead, the centers of the corresponding transcribed regions were only ~7.5 kb apart (Fig. 1A). As the precise locations of the *cdc24-4* and *cdc19-1* (an allele of *PYK1*; see Table 1) mutations used in the genetic mapping are not known, these sites could actually be anywhere from 6 to 9.5 kb apart (Fig. 1A). Thus, over this short interval, 1 kb appears to correspond to 1.4 to 2.1 cM. This appears to be the highest value yet observed in yeast, although other regions with higher-than-average recombination frequencies have also been noted (e.g., the 17.4 cM [41, 42] for the ~20-kb [C. Newlon, personal communication] *LEU2-HIS4* interval). Southern blot experiments (Fig. 3) indicated that the apparent high recombinational activity in the *CDC24-PYK1* interval was not due simply to a cloning artifact or to a difference in the length of this interval between the strains used for genetic mapping and the strain from which the recombinant DNA library was constructed. Moreover, the high rate of reciprocal recombination between the *cdc24-4* and *cdc19-1* markers is paralleled by high rates of meiotic gene conversion for various *pyk1* (29, 66; G. Kawasaki, personal communication; D. Olsen and B. Hall, personal communication) and *cyc3* (R. Rothstein and B. Gallay, personal communication) alleles.

As the overall genetic map length (~100 cM [41, 42]) and physical length (260 to 300 kb [10, 59]) for chromosome I yield a value for recombination per unit physical length (~0.38 cM/kb) that is similar to that for the genome as a whole, the recombinational hot spot in the *CDC24-PYK1* interval is presumably balanced by regions of lower-than-average recombinational activity elsewhere on the chromosome. Such recombinational cold spots (with recombination rates as low as 0.05 cM/kb) have been observed previously on chromosomes IV (67), X (60), and III (12, 33, 41, 42; C. Newlon, personal communication).

The reasons for the pronounced region-to-region variability

in the rate of meiotic recombination per unit physical length are not understood. They could be related to DNA polymorphisms that somehow induce or suppress recombination. No such polymorphisms were detected in the *CDC24-PYK1* region by Southern blot experiments (Fig. 3), but these experiments do not rule out the possibility of small polymorphisms that would be detectable only by DNA sequence analyses. Further studies of the *CDC24-PYK1* region should help to distinguish among this and the various other factors (47) that may affect the recombinational activity of particular regions.

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