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Genetics

Isolation of the centromere-linked *CDC10* gene by complementation in yeast

(*Saccharomyces cerevesiae*/yeast transformation/yeast cloning vector/recombinant DNA)

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ABSTRACT **A hybrid plasmid colony bank was constructed in *Escherichia coli* using the *E. coli–Saccharomyces cerevisiae* shuttle vector pLC544 and randomly sheared segments of yeast DNA. By transformation with a hybrid plasmid DNA pool from this collection and complementation of a temperature-sensitive *cdc10* mutation in yeast, a plasmid was isolated that carries 8 kilobase pairs of DNA around the chromosome III centromere-linked *CDC10* locus. This DNA segment overlaps a larger region of DNA (40 kilobase pairs) previously identified to be around the *LEU2* locus on chromosome III [Chinault, A. C. & Carbon, J. (1979) *Gene* 5, 111–126] and physically establishes the directionality of the cloned DNA sequences with respect to the genetic map and the centromere. In the *leu2–cdc10* interval, the relationship between physical distance on the DNA and genetic distance as measured by recombinational frequencies is about 3 kilobase pairs per centimorgan.**

Interest in the structure of centromeres and in the nature of centromeric DNA has prompted us to isolate and study several segments of cloned *Saccharomyces cerevesiae* DNA that carry genes known to map close to yeast centromeres. Among these cloned segments are those carrying the *LEU2* region on the left arm of chromosome III (1), the *ARG4* region on the right arm of chromosome VIII (2), and, as described below, the *TRP1* region from the right arm of chromosome IV. The segments of DNA listed above were isolated on ColE1 hybrid plasmids and were originally identified by complementation of appropriate auxotrophic markers in *Escherichia coli*. All have subsequently been shown to complement appropriate mutations in yeast (3, 4). In addition, immunological screening of *E. coli* clones, each carrying a different ColE1–DNA (yeast) hybrid plasmid, with radioactive antibody directed against yeast 3-phosphoglycerate kinase (PGK) has made possible the isolation of a region of DNA containing the *PGK* locus (5), which maps very close to the centromere on the right arm of chromosome III (ref. 6; R. Mortimer, personal communication). Thus, by using the *LEU2* and *PGK* cloned DNA segments as landmarks on either side of a centromere, coupled with overlap hybridization screening techniques (chromosome "walking") (7), two large regions of DNA around the centromere of chromosome III have now been identified, mapped, and studied, with the eventual goal of tying together these two regions to include centromeric DNA.

So that these segments might eventually be joined, it is imperative to identify DNA of at least one other genetically defined locus in this area in order to establish directionality of the characterized regions of DNA around either *LEU2* or *PGK*. An obvious third marker on chromosome III is the *cdc10* locus for which complementable, temperature-sensitive mutations have been described (8, 9), and which maps extremely close to the centromere on the left arm of chromosome III (ref. 9; R. Mortimer, personal communication). In the host at 37°C the mutant *cdc10* allele results in cell cycle arrest at cytokinesis. For the isolation of this gene, the development of a transformation system in yeast (3) and of plasmid vectors that transform and replicate efficiently in both yeast and *E. coli* (10–12) is extremely useful, because identification of specific DNA segments through complementation of yeast auxotrophic markers by hybrid plasmids introduced into yeast is now possible. This paper describes the construction and use of an *E. coli*–yeast shuttle vector to establish a pool of hybrid plasmids from which a plasmid carrying the *CDC10* region was identified by transformation and complementation in yeast. In addition, the plasmid DNA has been purified, mapped, and found to contain DNA in common with the previously identified large segment around *LEU2* (7), as well as additional DNA in what is now known to be the direction of the centromere. Structural aspects of this region of DNA, including the genetic versus physical distances between the *LEU2* and *CDC10* loci, are discussed.

MATERIALS AND METHODS

Strains and Media. *E. coli* strains used are the following: W3110(*trpC1117*) (13) obtained from R. Mosteller and SF8 (C600 *hsdM hsdR recBC lop11*) (14). *S. cerevesiae* strains used are: RH218 (*a trp1 gal2 SUC2 mal CUP1*) (15) obtained from D. Stinchcomb, 6204-18A (*α leu2-3,112 cdc10 thr can1*) obtained from G. Fink, XSB52-23C (*α leu2 cdc10 trp1 gal*) obtained from R. Mortimer and constructed by crossing RH218 with 6204-18A, and X2180-1A (*a SUC2 mal gal2 CUP1*) from the Yeast Genetics Stock Center, University of California, Berkeley. M9 (minimal medium) and LB (rich medium) for *E. coli* (16) and YPD (rich medium) and SD (minimal medium) for yeast (17) were used throughout.

DNA Preparations, Establishment of Colony Banks, and Transformations. Yeast DNA for construction of hybrid plasmid colony banks was prepared as described (2) from strain X2180-1A. Purification of covalently closed circular plasmid DNAs from *E. coli* has been described (18). Crude plasmid DNA preparations from yeast, subsequently used for *E. coli* transformations, were prepared according to Hsiao and Carbon (4). The hybrid plasmid colony bank from which ColE1–DNA (yeast) plasmids complementing the *E. coli trpC117* mutation were isolated was that used by Chinault and Carbon (7). The new colony bank employing the shuttle vector pLC544 described below under *Results* was constructed in a similar way as our previous collections (2, 7, 18) by using randomly sheared segments of yeast DNA joined at the *Bam*HI site of the vector by poly(dA-dT) connectors (18). Transformations of *E. coli* (19) and yeast (4) were carried out as described.

Agarose Gel Electrophoresis, Southern Blot Hybridizations, and Enzymes. Fractionation of DNAs and of restriction enzyme digests of plasmid DNAs were carried out in 1.2%

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Abbreviations: kbp, kilobase pair; cM, centimorgan; PGK, 3-phosphoglycerate kinase.

agarose gels by using a horizontal gel apparatus according to Kaplan *et al.* (20). DNA blot hybridizations were carried out according to the methods of Southern (21) by using plasmid DNA labeled *in vitro* by nick translation (22) as described by Chinault and Carbon (7). *Eco*RI endonuclease and λ 5′-exonuclease were isolated as described (18). Deoxynucleotidyl terminal transferase was from R. Ratliff. Other enzymes were purchased from BioLabs (Beverly, MA).

Biosafety Considerations. This work was carried out under containment conditions specified by the National Institutes of Health Guidelines for Recombinant DNA Research (December 1978) and the National Institutes of Health memorandum of March 28, 1978, entitled "Recombinant DNA Experiments Involving Yeast Hosts."

RESULTS

Isolation of Hybrid Plasmids Complementing *E. coli trpC1117* Mutations and Construction of the Shuttle Vector. The *TRP1* locus on chromosome IV of *S. cerevesiae* was originally of particular interest to us because it maps approximately 1 centimorgan (cM) (map unit) from its centromere (23). The *TRP1* gene specifies the production of the enzyme, *N*-(5′-phosphoribosyl)anthranilate isomerase. In *E. coli* this isomerase and indolyl-3-glycerol phosphate synthetase are part of the same 45,000-dalton polypeptide chain specified by the *trpC* gene (13). However, *E. coli* mutants carrying the *trpC1117* allele lack isomerase activity, but retain normal levels of synthetase (13). One such mutant, W3110(*trpC1117*), was transformed with a pool of ColE1–DNA (yeast) hybrid plasmid DNAs from our colony collection (7). The colicin E1-resistant Trp^+ transformants obtained carried either one of two hybrid plasmids with large regions of cloned yeast DNA. Restriction maps of the two plasmids, pYe(*TRP1*)54 and pYe(*TRP1*)74 are shown in Fig. 1 *Upper*. Plasmid pYe(*TRP1*)54 was restriction digested to completion with *Eco*RI and mixed with *Eco*RI-cut ampicillin-resistant pBR313 DNA (24), and the DNAs were ligated. When this mixture was used to transform *E. coli* W3110(*trpC1117*), selecting for Trp^+ Amp^R, 5 of 12 transformants screened contained plasmids consisting of pBR313 and a single 1.45-kilobase pair (kbp) *Eco*RI fragment from pYe(*TRP1*)54. This small fragment was inserted into pBR313 DNA in both orientations and the structure of these plasmids, pLC544 and pLC548, are shown in Fig. 1 *Lower*. Although enzyme levels have not been measured, functional expression of the *TRP1* gene in *E. coli* is quite adequate, because strain W3110(*trpC1117*) has the same doubling time (42 min) in M9 minimal medium with casamino acids and added tryptophan as when it harbors pYe(*TRP1*)54, pYe(*TRP1*)74, pLC544, or pLC548 and is grown in the absence of tryptophan.

The 1.45-kbp DNA fragment is of special interest because, as first pointed out by Struhl *et al.* (11), its presence in hybrid DNA molecules leads to high-frequency transformation of yeast and allows hybrid DNAs to replicate autonomously and to express both *TRP1* and other structural genes in the absence of recombination into the host genome, presumably because of a replication origin included on the fragment. In other work involving replication of pLC544 (pBR313-*TRP1*) in yeast, we have shown that yeast strains transformed by pLC544 are unstable for the Trp^+ character (see below), that pLC544 replicates autonomously in the yeast nucleus, and that the plasmid can be transmitted in matings and remains structurally unaltered by passage in yeast and by the processes of mating, meiosis, sporulation, and germination (12). The above properties make pLC544 particularly suitable as a vector for the introduction of specific DNA segments into yeast. In addition, plasmid pLC544 is a useful shuttle vector, because it has a selectable marker in yeast (*TRP1*), four selectable markers in *E. coli* (colicin $E1^R$, $TrpC^+$, amp^R, tet^R), and single *Bam*HI and *Sal* I restriction sites into which foreign DNA may be introduced. Thus, the plasmid or its derivatives may easily be recovered from yeast by transformation of *E. coli* with yeast transformant DNA, as is shown below. A similar *TRP1* vector (YRp7) has been described by Struhl *et al.* (11).

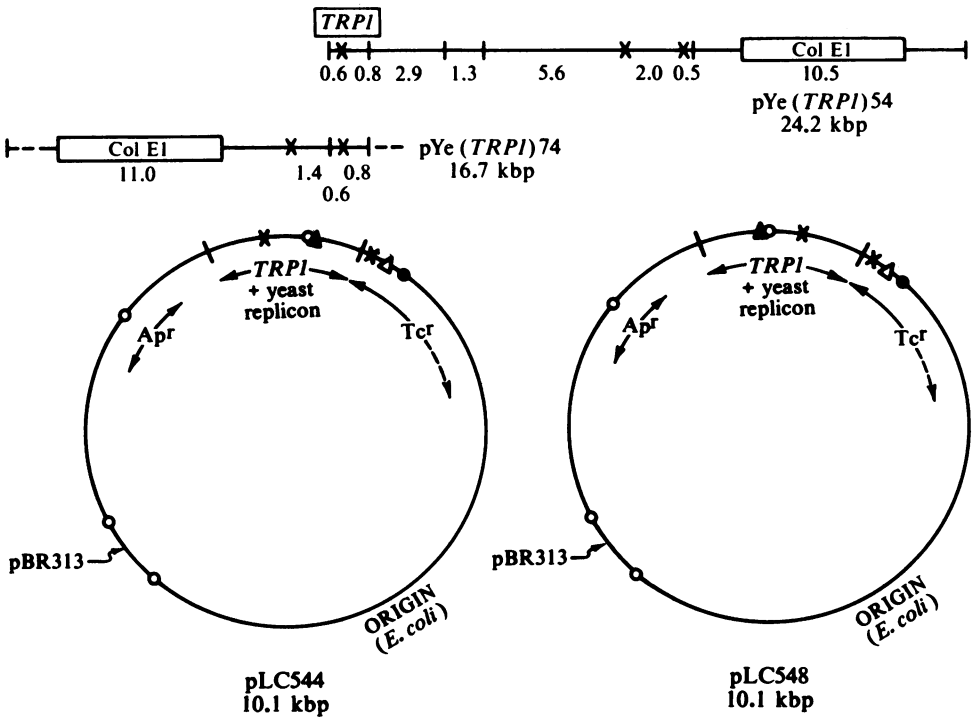

FIG. 1. (*Upper*) Restriction maps of ColE1–(*TRP1*) plasmid DNAs showing location of *Eco*RI (—|—) and *Hin*dIII (—✕—) sites. (*Lower*) Restriction maps of vector DNAs pLC544 and pLC548 constructed of pBR313 DNA and a 1.4-kbp *Eco*RI fragment from pYe(*TRP1*)54. Sites are indicated as follows: *Eco*RI (—|—); *Hin*dIII (—✕—); *Sal* I (—●—); *Bam*HI (—△—); *Bgl* II (—▲—); *Pst* I (—○—).

Construction of a Colony Bank in *E. coli* by Using Vector pLC544 and Randomly Sheared Segments of Yeast DNA. Randomly sheared segments of yeast DNA (8–12 kbp in length) were annealed to pLC544 DNA at the *Bam*HI site by using poly(dA-dT) connectors, and the DNA preparation was used to transform *E. coli* strain *SF8*, selecting for ampicillin resistance. After incubation for 24 hr at 37°C, approximately 9000 small colonies were picked from the transformation plates, transferred to fresh plates in a grid array, and ultimately transferred to microtiter dishes for permanent storage (18). Small colonies were picked from the original plates, because preliminary screening revealed they had the larger inserts. A hybrid plasmid DNA preparation was then purified from a pool of colonies in this collection (18).

Isolation of a Yeast DNA Segment that Complements the *cdc10* Temperature-Sensitive Mutation in Yeast. Approximately 15 μg of the hybrid plasmid DNA pool described above was used to transform yeast strain XSB52-23C (*leu2 cdc10 trp1*). Spheroplasts were plated in minimal SD agar with casamino acids (no tryptophan) and allowed to incubate at room temperature for 8 hr and then at the restrictive temperature (37°C). After 48 hr 1 colony (of a total of 15,000 Trp^+ transformants) appeared and upon restreaking it continued to grow at 37°C in the absence of tryptophan. Microscopic observation of individual cells after a repeated restreaking revealed that at the restrictive temperature most cells looked normal, but about 1 in 500 expressed the mutant $Cdc10^-$ phenotype. Cells with a *cdc10* mutation are defective in cytokinesis and are easily distinguished, because they develop multiple elongated buds that do not separate from the parent cell (8). The presence of a high percentage of cells (20–50%) with the mutant phenotype among the putative transformants would be expected because pLC544 and its derivatives are unstable and readily segregated (11, 12). The unusual mitotic stability of yeast transformants carrying pYe(*CDC10*)1 could be a result of stabilizing centromeric DNA sequences on the plasmid. Because of the genetic proximity of the *cdc10* locus to *leu2*, the Trp^+ $Cdc10^+$ clone, which was originally established in a *leu2* strain, was plated onto minimal selective medium lacking leucine, but the clone was found to be phenotypically Leu^-.

A crude preparation of total nucleic acid was isolated (4) from the Trp^+ $Cdc10^+$ putative transformant and used to transform *E. coli* strain *SF8*, selecting for ampicillin resistance. Four transformants were thus obtained by using 4 A_{260} units of the crude preparation and each contained the hybrid plasmid pYe(*CDC10*)1, whose restriction map is presented in Fig. 2 *Upper*. From this map, it is evident that the gross structure of the vector has remained unchanged in the passage from *E. coli* to yeast and back to *E. coli*. The insert of yeast DNA is approximately 8 kbp in length. That the *Eco*RI fragments in the insert are the same size as those in genomic DNA, and thus that the integrity of the yeast DNA insert has been maintained, was confirmed by Southern blot hybridization (ref. 21; see Fig. 3) with *Eco*RI-cut total yeast DNA and [^{32}P]-labeled pYe(*CDC10*)1 DNA. The failure of this probe to hybridize with other fragments, even weakly, in the autoradiogram indicates the absence of extensive repeated sequences in the cloned DNA.

A comparison of the restriction map of pYe(*CDC10*)1 with that of the 40-kbp region we have now identified around *LEU2*, coupled with Southern hybridizations, has confirmed that the insert of yeast DNA in pYe(*CDC10*)1 "overlaps" or has DNA in common with the ColE1 hybrid plasmid pYe46B2 described by Chinault and Carbon (ref. 7; Figs. 2 and 3). Plasmid pYe(*CDC10*)1 DNA has both a 1.6-kbp *Eco*RI–*Hin*dIII fragment and part of a 3.5-kbp *Eco*RI–*Hin*dIII fragment in common with DNA from pYe46B2 (Fig. 3). Thus, the map obtained from pYe(*CDC10*)1 extends our identification of the region around *LEU2* by approximately 3 kbp and does so in the direction of the centromere, because *LEU2* maps about 8 cM from the centromere and *CDC10* maps <1 cM from the centromere (refs. 9, 25, and 26; R. Mortimer, personal communication).

Location of *CDC10* on pYe(*CDC10*)1 DNA. The location of the *CDC10* gene on pYe(*CDC10*)1 DNA was investigated by restriction of the DNA with *Bam*HI, religation, transformation into *E. coli* selecting for ampicillin resistance, and isolation of a new plasmid pYe(*CDC10*)1-1 that contains all the DNA of pYe(*CDC10*)1, except the 3.5-kbp *Bam*HI fragment (see Fig. 2 *Upper*). In a second experiment the 3.5-kbp fragment was recloned at the *Bam*HI site of pLC544 to give plasmid pYe(*CDC10*)1-2. These two recloned plasmid DNAs and that of the original pYe(*CDC10*)1 were utilized to transform yeast strain XSB52-23C (*cdc10 trp1*), selecting in each case for

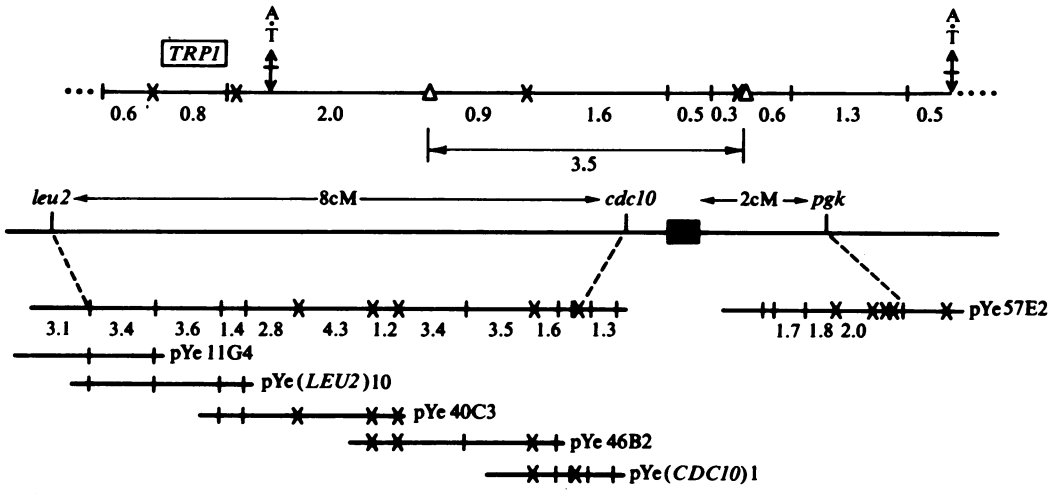

FIG. 2. (*Upper*) Restriction map of yeast DNA insert in pYe(*CDC10*)1 showing location of *Eco*RI (—|—), *Hin*dIII (—x—), and *Bam*HI (—Δ—) sites and the orientation of the insert with respect to the 1.4-kbp *TRP1* fragment in pLC544. (*Lower*) Physical and genetic maps of the region around the centromere of chromosome III. The restriction map shows the location of *Eco*RI (—|—) and *Hin*dIII (—x—) sites. Numbers refer to kbp. A map of plasmid pYe57E2 which carries the *PGK* locus (5) is also shown, although the orientation of pYe57E2 DNA with respect to centromere (■) is not known. The order of inserts in overlapping plasmids previously identified (7) and in pYe(*CDC10*)1 is indicated below the restriction map.

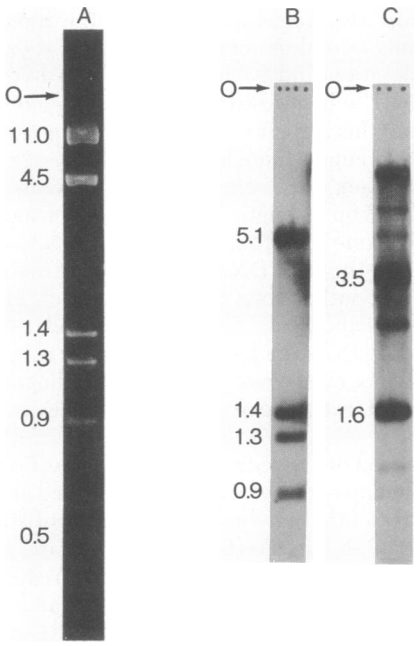

FIG. 3. (*A*) Electrophoretic pattern of *Eco*RI-digested pYe(*CDC10*)1 DNA in 1.2% agarose. (*B*) Southern blot hybridization autoradiograph of *Eco*RI-digested total yeast DNA with pYe(*CDC10*)1 [^{32}P]DNA as probe. The 5.1-kbp band is the overlapping (centromere distal) *Eco*RI fragment in genomic DNA (see Fig. 2, pYe46B2). The centromere-proximal genomic *Eco*RI fragment is either coincident with one of the labeled bands or is too small to be seen on this gel. The 0.5-kbp region was run off the gel. (*C*) Southern blot autoradiograph of a combined *Eco*RI–*Hin*dIII digest of pYe46B2 DNA with pYe(*CDC10*)1 [^{32}P]DNA as probe. The large band at the top is a result of hybridization between vector sequences. The faint bands in this lane are attributed to hybridization of probe with *Hin*dIII partial digestion products. All fragment sizes (numbers in kbp) were determined relative to sizes of λ *Hin*dIII–*Eco*RI fragments on the same gel. O, origin.

Trp$^+$ and for Trp$^+$ Cdc10$^+$ transformants (Table 1). As expected, pYe(*CDC10*)1 DNA transforms yeast with high frequency to both Trp$^+$ and Trp$^+$ Cdc10$^+$, and of 20 Trp$^+$ transformants tested all were also Cdc10$^+$. DNAs of pYe(*CDC10*)1-1 and pYe(*CDC10*)1-2 also transform XSB52-23C with high efficiency to Trp$^+$, but not to Cdc10$^+$, and none of the Trp$^+$ transformants screened were Cdc10$^+$. These data indicate that *CDC10* may be located at or near one of the two *Bam*HI sites in pYe(*CDC10*)1 DNA. The most likely location for the gene is around the centromere-proximal *Bam*HI site, because attempts to transform yeast strain 6204-18A (*cdc10*) with pYe46B2 DNA, which has an extensive amount of DNA around the centromere-distal *Bam*HI site, have been unsuccessful.

Table 1. Transformation of yeast with pYe(*CDC10*)1 DNA and its derivatives

Plasmid DNA	Trp$^+$ transformants/ μg DNA	Trp$^+$ Cdc10$^+$ transformants/ μg DNA	% of Trp$^+$ transformants that are Cdc10$^+$
pYe(*CDC10*)1	400	480	100
pYe(*CDC10*)1-1	680	0	0
pYe(*CDC10*)1-2	390	0	0

The yeast strain used as recipient was XSB52-23C. Trp$^+$ Cdc10$^+$ selection plates were incubated at room temperature for 48 hr before being placed at the restrictive temperature (37°C) for Cdc10$^+$ selection.

DISCUSSION

We have described the use of a shuttle vector, pLC544, that replicates autonomously in both yeast and *E. coli*, for cloning the *CDC10* region of yeast DNA by complementation of a temperature-sensitive *cdc10* mutation in yeast. By using a related vector, YRp7, which also incorporates the *TRP1* 1.4-kbp *Eco*RI fragment (11), Nasmyth and Reed have cloned and identified yeast DNA segments carrying *CDC28* and neighboring *TYR1* (25). It is expected that such vectors, which are characterized by their ability to transform yeast with high efficiency, will be valuable for cloning many specific segments of yeast DNA as well as other eukaryotic or prokaryotic DNAs in yeast.

In the original yeast transformation experiments outlined here, with 15 μg of pooled hybrid plasmid DNA, one Trp$^+$ Cdc10$^+$ colony was obtained from a total of about 15,000 Trp$^+$ transformants. If the hybrid DNA pool was representative of the yeast genome, we would have expected two to three more (18). This apparent low frequency may be a result of underrepresentation of that segment in the DNA pool. On the other hand, subsequent transformations of yeast strain XSB52-23C (*cdc10 trp1*) with purified pYe(*CDC10*)1 DNA have indicated that the frequency of transformation to Cdc10$^+$ is markedly dependent on the length of time the transformation plates are kept at the permissive temperature before shift-up to 37°C. For example, plates kept at room temperature for only 6 hr yielded no transformants when placed at 37°C, whereas those left for 2 days at room temperature gave the expected high number of Cdc10$^+$ Trp$^+$ transformants upon subsequent incubation at 37°C (see Table 1). This result probably stems from properties pertaining to the *cdc10* mutant that may require time for spheroplast regeneration at room temperature or adequate expression from the plasmid *CDC10* gene before being placed at the restrictive temperature at which the defect in cytokinesis is manifested. In the original transformation with the hybrid DNA pool, transformation plates were incubated at room temperature only 8 hr before being placed at 37°C. A longer preincubation at room temperature may have increased the number of Cdc10$^+$ Trp$^+$ clones.

The major aim of preparing a colony collection and hybrid plasmid pool with pLC544 and of cloning the *CDC10* region was to identify more DNA near the centromere on the left arm of chromosome III. Very little, if any, genetic recombination has been observed between the *cdc10* locus and its centromere, placing it, at least genetically, directly next to the centromere (refs. 9 and 26; R. Mortimer, personal communication). Plasmid pYe(*CDC10*)1 has been very useful in extending our information about this region, because it both overlaps previously identified DNA segments and establishes directionality of the 40-kbp region we have characterized around *LEU2*. Assuming *CDC10* is located at or near the centromere-proximal *Bam*HI site in pYe(*CDC10*)1, there are approximately 25 kbp of DNA between this locus and *LEU2* (Fig. 2). The map distance between these loci is approximately 8 cM (refs. 26 and 27; R. Mortimer, personal communication), for an average of about 3 kbp/cM. This number is slightly larger than the 1.75 kbp/cM cited by Nasmyth and Reed (25) for the distance between *CDC28* and *TYR1*. It is not surprising that the relationship between physical and genetic distances should vary in different regions of the yeast genome. We have evidence that the physical distance between *PGK*, which maps about 2 cM from the centromere on the right arm of chromosome III (R. Mortimer, personal communication), and *CDC10* is considerably larger than the genetic parameter of 2–3 kbp/cM would dictate, although the DNA of these loci have not yet been physically linked (5), indicating that recombination frequencies are

markedly suppressed in the centromere region. Similarly, genetic analysis has established in *Drosophila*, for example, that little crossing over occurs within centromeric heterochromatin, and genes on either side of a centromere tend to remain linked together (28). An accurate determination of the physical distance between *CDC10* and *PGK* across the centromere of chromosome III will soon be possible, because the pertinent DNA segments carrying these loci have now been isolated. Restriction mapping and Southern blot hybridizations have so far revealed no homology between these DNA segments on chromosome III and those carried by pYe(*TRP1*)54 and pYe(*TRP1*)74 from the region near the centromere on chromosome IV.

Note Added in Proof. Recent genetic and biochemical experiments indicate that unintegrated plasmid pYe(*CDC10*)1 in yeast undergoes meiotic segregation in a manner expected for a minichromosome carrying a functional centromere.

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