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Physical mapping of large DNA by chromosome fragmentation

(*Saccharomyces cerevisiae*/homologous recombination/contour-clamped homogeneous electric fields)

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ABSTRACT **A technique is described for physically positioning any cloned DNA on a native or artificial *Saccharomyces cerevisiae* chromosome. The technique involves splitting a chromosome at a specific site by transformation with short linear molecules containing the cloned DNA at one end and telomeric sequences at the other. Recombination between the end of the linear molecules and homologous chromosomal sequences gives rise to chromosome fragments comprising all sequences distal or proximal to the mapping site depending on the orientation of the cloned DNA. The recombinant products are recovered by screening for stabilization of a suppressor tRNA on the linear molecules using a colony color assay. The cloned DNA is positioned relative to the chromosome ends by sizing the chromosomal fragments using alternating contour-clamped homogeneous electric field gel electrophoresis. Application of this technique to organisms other than *S. cerevisiae* and to the analysis of exogenous DNA cloned in yeast is discussed.**

The advent of alternating field gel electrophoresis techniques (1–3) has made possible the resolution of linear DNA molecules >10 megabases (4). This has allowed the separation of chromosome-sized DNA molecules from a diversity of organisms, many of which are not accessible by classical genetic mapping techniques (5–9). By hybridization of probes to these resolved DNA molecules, chromosomal assignment of cloned sequences can now be accomplished. The next step toward a more complete understanding of the genomic organization of these organisms is the construction of a map that provides information about the position and order of DNA segments along each individual chromosome.

In eukaryotes, genetic mapping techniques describe the position of a DNA segment in terms of the frequency of recombination between it and its respective centromere or between it and other previously mapped loci on the same chromosome. In contrast, this paper describes a direct physical mapping technique that positions a cloned DNA segment relative to the ends of a linear chromosomal molecule. The technique involves breaking a chromosome at the position of a cloned DNA segment and determining the size of the two resulting fragments with alternating contour-clamped homogeneous electric field (CHEF) gel electrophoresis. The position of the mapped segment is thus described in terms of kilobases (kb) of DNA from each end.

The yeast *Saccharomyces cerevisiae* has been used here as an initial application of the technique because simple methods exist for breaking a yeast chromosome at the site of a cloned DNA segment (refs. 10 and 11; P.H., C.C., and D.V., unpublished data). The method takes advantage of the fact that free ends of DNA are recombinogenic in yeast, stimulating "targeted" recombination with homologous genomic sequences (12). A diploid yeast cell is transformed with a small linear molecule, one end of which contains the DNA segment to be mapped and the other end of which contains a yeast telomere-adjacent sequence (13). If the mapping segment is in the correct orientation, the recombination event that occurs between it and homologous sequences on the chromosome will yield a stable shortened molecule. A telomere is formed near the site of the recombination event and all chromosomal sequences distal to the site are lost (Fig. 1*C Upper*). The chromosome is thus broken at the location of the DNA segment and one of the two fragments (i.e., the proximal fragment) is recovered. To recover the reciprocal fragment, a second small linear molecule is introduced into the yeast cell in a separate transformation. This second molecule also contains a telomere-adjacent sequence at one end and the mapping segment at the other. However, the mapping segment is in the opposite orientation compared to the first molecule and the second molecule contains a yeast centromere. Recombination between this centromere-containing molecule and the chromosome results in addition to the small linear molecule of all sequences distal to the mapping segment (Fig. 1*C Lower*). The sizes of the two chromosome fragments can now be measured and the DNA segment can be positioned relative to the chromosome ends.

To illustrate the utility of this technique in yeast, three genes on chromosome II have been physically mapped by chromosome fragmentation. Two of these genes had been previously mapped genetically, while the third had not.

MATERIALS AND METHODS

Media and Strains. Selective (SD) and rich (YPD) media and plates were prepared as described (14). All selective plates contained a reduced amount of adenine (6 μg/ml) to enhance colony color development. Yeast transformation was performed by the LiOAc procedure (15). YNN290 (16) (**a**/α, *ura3-52/ura3-52, ade2-101/ade2-101, lys2-801/lys2-801, trp1-Δ1/trp1-Δ1*) is isogenic to S288c. YNN337 (**a**/α, *ura3-52/ura3-52, ade2-101/ade2-101, MEL1/MEL1*) is derived from D585-11c and YNN282. YPH149 (**a**, *ura3-52, lys2-801, his7, trp1-Δ1*, ade$^-$) is a derivative of strain YNN295 (3) with chromosome VII split at *RAD2* by the method described here.

Vector Construction. The yeast chromosome *f*ragmentation (YCF) vectors, YCF3 and YCF4, are derivatives of vectors YCF1 and YCF2, respectively (P.H., C.C., and D.V., unpublished data). YCF3 is a YRp14 derivative (16) in which the 346-base-pair (bp) *Hin*dIII/*Bam*HI pBR322 fragment is replaced by a 1.5-kb *Hin*dIII/*Bgl* II fragment from the telomere-adjacent sequence Y′ (13) and a polylinker made by annealing synthesized single-stranded oligonucleotides

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5'-GATCTGCGGCCGCGAATTCAAGCTTG-3'
    3'-ACGCCGGCGCTTAAGTTCGAACCTAG-5'
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Abbreviation: CHEF, contour-clamped homogeneous electric field.

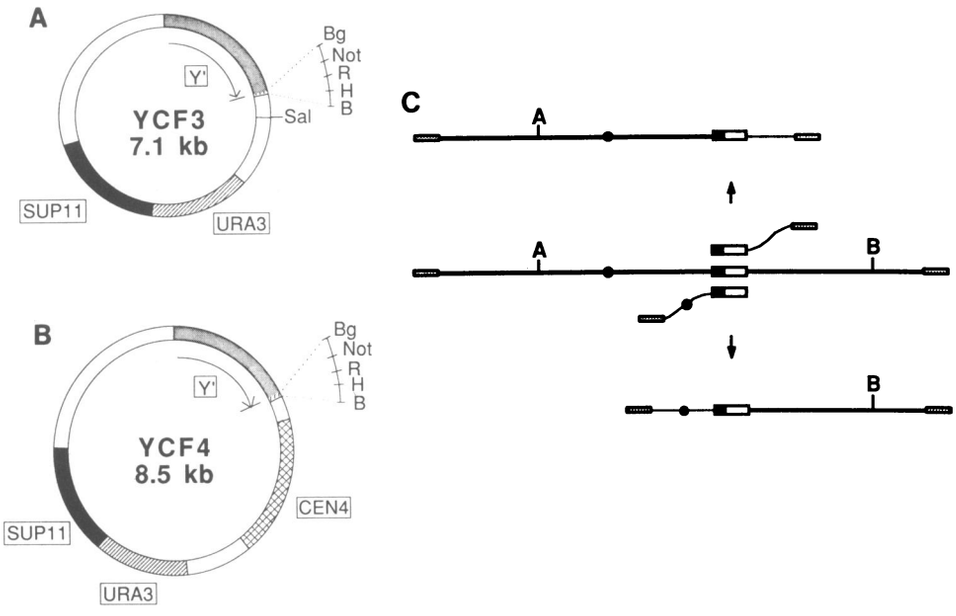

FIG. 1. YCF vectors. (*A*) The acentric vector YCF3 is derived from pBR322 (unshaded areas) and contains sequences from the yeast telomere-adjacent repeat (Y') as well as an ochre-suppressing tRNA gene (*SUP11*) and a yeast selectable marker (*URA3*). The polylinker contains sites for the restriction enzymes *Bgl* II (Bg), *Not* I (Not), *Eco*RI (R), *Hin*dIII (H), and *Bam*HI (Bam). (*B*) The centric vector YCF4 is identical to YCF3 except for the addition of a 1.6-kb *Xho* I fragment containing yeast centromere sequences (CEN4) at the *Sal* I site (Sal) of YCF3. (*C*) Chromosome fragments produced when a mapping sequence (box) is added to YCF3 and YCF4. Chromosomal sequences proximal to the mapping sequence are recovered with the YCF3 derivative (*Upper*), while those sequences distal to the mapping sequence are recovered with the YCF4-derived plasmid (*Lower*). Circles represent centromeres; stippled boxes at each end represent telomeres. Diagram is not drawn to scale.

inserted in the orientation shown in Fig. 1. The pBR322-derived *Eco*RI and *Hin*dIII sites were eliminated by removing a 104-bp *Aat* II/*Hin*dIII fragment, filling in the overhangs with T4 DNA polymerase, and blunt-end ligation. YCF4 is a YRp14/CEN4 derivative (16) modified in the same manner as YCF3 except a 1.6-kb CEN4-containing *Xho* I fragment was cloned into the *Sal* I site. The *Bgl* II site normally present in this CEN4 fragment was removed by filling in with DNA polymerase (Klenow fragment) and blunt-end ligation. pBR322/TRP1 was constructed by blunt-end ligation of the 0.8-kb *Eco*RI/*Bgl* II fragment containing the *TRP1* gene (17) into the *Pvu* II site of pBR322.

Preparation of Chromosomal DNA. Chromosome-length DNA molecules of *S. cerevisiae* were prepared essentially as described (1). Yeast were grown to stationary phase in 8 ml of minimal glucose medium with selection for chromosome fragments where appropriate. The cells were washed once with 1 ml of LET buffer, resuspended to a density of $\approx 5 \times 10^9$ cells per ml and mixed with 2 μl of Zymolyase 5000 (20 mg/ml) (Seikagaku Kogyo, Tokyo). The cells were then mixed with 5/3 vol of 1% low melting point agarose in 125 mM EDTA (pH 8.0) drawn into a 1-ml plastic syringe and solidified by placing the syringe on ice. The end of the syringe was cut off and the sample was extruded and placed in an equal volume of LET buffer with 0.5% 2-mercaptoethanol. The sample was incubated for 18–24 hr at 37°C. The LET buffer was replaced by an equal volume of NDS buffer plus proteinase K (2 mg/ml) and the sample was incubated at 50°C for 18–24 hr. The agarose plug was washed extensively with 50 mM EDTA (pH 8.0) at 50°C and stored at 4°C.

Gel Electrophoresis. Alternating field gel electrophoresis was carried out with a modification of the CHEF apparatus described (3). The distance between driving electrodes was 27 cm. Sixty milliliters of molten 1% agarose in 0.5× TBE buffer (18) was poured directly onto the sand-blasted bottom of the buffer tank using a 12- × 12-cm frame. Samples were either loaded as solid plugs or heated for 2 min at 65°C and loaded as a molten mixture into wells 1 mm thick. Electrophoresis was carried out for 24–27 hr in 0.5× TBE buffer at 200 V and 9°C with the switching interval given in the figure legends. Concatamers of bacteriophage λ were prepared as described (4).

Transfer and Hybridization. After staining in ethidium bromide (0.5 μg/ml), the gel was irradiated for 1 min with 254-nm ultraviolet light and placed in 250 ml of denaturing solution (0.5 M NaOH/1.5 M NaCl) for 20 min with gentle shaking. This step was repeated and followed by a 30-min incubation in 250 ml of 1 M NH_4Ac/0.1 M NaOH, again with gentle shaking. The DNA was transferred to a Genatran 45 nylon filter (Plasco, Woburn, MA) using a reservoir of 500 ml of 1 M NH_4Ac/0.1 M NaOH for 20–24 hr. Hybridization was carried out as described (5).

RESULTS

The first step in physically mapping a cloned sequence is its assignment to a particular chromosome. In the case of *S. cerevisiae*, a strain has been constructed that allows resolution of all 16 chromosomal DNAs as 17 distinct bands by alternating CHEF gel electrophoresis (Fig. 2*A*) (C.C. and P.H., unpublished data). (Chromosome VII has been split at *RAD2* by the method described here to allow its resolution from chromosome XV.) Cloned yeast sequences can be unambiguously mapped to one of the 16 chromosomes by a single DNA hybridization.

The next step, positioning a sequence along its resident chromosome, requires construction of the two small linear molecules described in the introduction. Fig. 1 *A* and *B* diagrams two vectors, YCF3 and YCF4, designed to facilitate the construction of these molecules. Since the centromere/telomere orientation of the segment to be mapped is usually not known, four molecules must be constructed, with both orientations of the mapping segment inserted into each of the two vectors. This can be conveniently accomplished by cutting each vector at a single site in the polylinker and introducing a mapping segment with compatible ends. Only

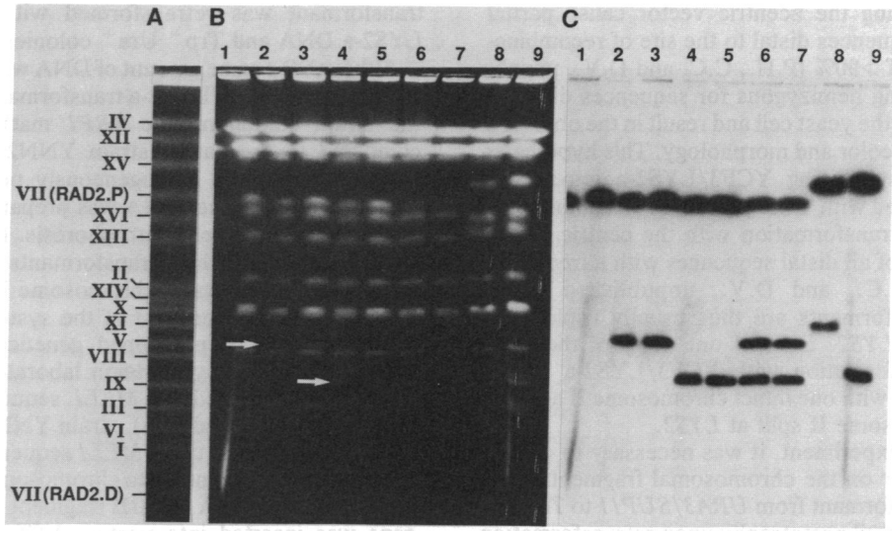

FIG. 2. Physical mapping of *LYS2* in two strains. (*A*) YPH149 allows unambiguous assignment of cloned sequences to 1 of the 16 yeast chromosomes. Chromosome VII is split at *RAD2* into proximal (RAD2.P) and distal (RAD2.D) fragments. The chromosomal DNA molecules were separated in a 1% agarose gel at 9°C, 200 V, and a switching interval of 80 sec by an alternating CHEF gel apparatus. The relative mobility of the two largest molecules, chromosomes IV and XII, is variable. (*B*) Intact chromosomal DNA was prepared from transformants described in Table 1 and separated by alternating CHEF gel electrophoresis for 27 hr with a switching interval of 65 sec. Samples 1–7 are YNN290 transformed with the following plasmids: lane 1, none; lanes 2 and 3, YCF3/LYS2-a; lane 4, YCF4/LYS2-b; lane 5, YCF4/LYS2-b and pBR322/TRP1; lanes 6 and 7, YCF4/LYS2-b, pBR322/TRP1, and YCF3/LYS2-a. Samples in lanes 8 and 9 are YNN337 transformed with YCF3/LYS2-a and YCF4/LYS2-b, respectively. Arrows, chromosomal fragments. (*C*) Autoradiograph of the gel in *B* was made after hybridization with a 5-kb *Bgl* II fragment containing *LYS2* coding sequences (19). As a probe for sequences distal to *LYS2*, a 1.4-kb *Bgl* II fragment homologous to *suc3*^{0} was used (20). It hybridizes to the smaller chromosome fragment. The larger fragment hybridizes to *Gal11,10*, a probe for sequences proximal to *LYS2* (data not shown) (21, 22).

two ligations are required to generate the four molecules. To stimulate homologous recombination with the chromosome, the four molecules must be linearized, leaving the mapping segment at one end and the telomere-adjacent repeat at the other. The infrequently occurring *Not* I site has been placed next to the telomere-adjacent (Y') sequences for this purpose.

Once the linear plasmids have recombined with the chromosome, the stability of the resulting molecules can be rapidly assessed by a colony color assay (16). This assay exploits the fact that *ade2* auxotrophs are red and *ADE2* prototrophs are white. If one copy of the *SUP11* gene is introduced into a diploid cell homozygous for the *ade2-101* ochre mutation, the cell is pink. Loss of a *SUP11*-containing molecule can be monitored in such a diploid by the appearance of a red sector in the midst of a pink colony.

As a demonstration of the mapping system, a gene, *LYS2*, whose genetic map position is known, was physically mapped. A *Bgl* II fragment containing *LYS2* coding sequences was inserted in both orientations into the *Bam*HI site of vectors YCF3 and YCF4. The resulting plasmids (YCF3/LYS2-a and -b, YCF4/LYS2-a and -b) were linearized by cleavage at the unique *Bgl* II site in the polylinker and introduced into the diploid strain YNN290 by selection for uracil prototrophy. It was expected that *LYS2* inserted in one orientation into YCF3 and in the opposite orientation into YCF4 would yield productive chromosome fragments corresponding to all sequences proximal or distal to *LYS2*, respectively. Insertion of *LYS2* in the wrong orientation should yield acentric distal sequences (YCF3) or dicentric proximal sequences (YCF4), both of which would be highly unstable. Transformants obtained by transformation of each of the four plasmids were streaked onto YPD plates for colony color development. The results are compiled in Table 1.

Plasmids YCF3/LYS2-a and YCF4/LYS2-b gave rise to significantly more transformants than plasmids YCF3/LYS2-b and YCF4/LYS2-a; also, plasmids YCF3/LYS2-a and YCF4-LYS2-b do indeed contain the *LYS2* sequences in opposite orientations as expected. In addition, a large proportion of the transformants arising from plasmid YCF4/LYS2-b were pink. This is consistent with the stabilization of the small molecule containing the *SUP11* gene by addition of a large piece of a chromosome. Intact chromosomal DNA was prepared from several transformants for each of the four plasmids. The DNA samples were subjected to alternating CHEF gel electrophoresis, transferred to a nylon membrane, and hybridized to a *LYS2* probe. The same 475-kb band was present in seven of eight YCF4/LYS2-b samples. A 365-kb band was present in seven of eight YCF3/LYS2-a samples. Examples of the two bands are shown in Fig. 2 *B* and *C* (lanes 2–5). Hybridizations with probes from both sides of *LYS2* demonstrate that these bands are fragments of chromosome II broken at *LYS2* (see legend to Fig. 2).

The 365-kb chromosomal fragment arising from transformation with YCF3/LYS2-a should be very stable, resulting in pink colonies. However, the predominant phenotype of the colonies was pink with multiple red sectors. These transformants also showed wide variation in colony size. Recombi-

Table 1. Transformation of yeast with *LYS2* mapping plasmids

Plasmid	Total	Homogeneously pink colonies
YCF3/LYS2-a*	51	0/36†
YCF3/LYS2-b*	11	1/8
YCF4/LYS2-a*	28	0/16
YCF4/LYS2-b*	101	28/36
YCF3/LYS2-a‡	>700	20/20
YCF3/LYS2-b‡	14	2/10

*Three micrograms of each of the four *LYS2* mapping plasmids was introduced into YNN290 by transformation. The total number of transformants and proportion of homogeneously pink colonies was determined for each.

†30/36 were variegated in colony size and color.

‡Three micrograms of each of the two acentric *LYS2* mapping plasmids was introduced into YNN290 after transformation with pBR322/TRP1.

nation events involving the acentric vector cause partial monosomy for all sequences distal to the site of recombination at a frequency of >90% (P.H., C.C, and D.V., unpublished data). Becoming hemizygous for sequences distal to *LYS2* may be toxic to the yeast cell and result in the observed variegation in colony color and morphology. This hypothesis can be tested by introducing YCF3/LYS2-a into a cell previously transformed with YCF4/LYS2-b. Recombination events arising from transformation with the centric vector result in duplication of all distal sequences with a frequency of >90% (P.H., C.C., and D.V., unpublished data). YCF4/LYS2-b transformants are thus usually triploid for sequences distal to *LYS2*. Loss of one copy of these sequences upon transformation with YCF3/LYS2-a should result in a diploid cell with one intact chromosome II and two fragments of chromosome II split at *LYS2*.

To carry out this experiment, it was necessary to change the selectable marker on the chromosomal fragment in the YCF4/LYS2-b transformant from *URA3/SUP11* to *TRP1* to allow selection for uracil prototrophy upon retransformation with YCF3/LYS2-a. Plasmid pBR322/TRP1, containing the *TRP1* gene, was cleaved at the *Sal* I and *Cla* I sites and introduced into a YCF4/LYS2-b transformant. Stable Trp^+ transformants should arise via a one-step gene replacement (23) in which the *URA3/SUP11* region on the short (pBR322) arm of the chromosome fragment is replaced by the *TRP1* gene. Trp^+ colonies were selected and screened for uracil auxotrophy and the absence of the *SUPII* gene. One such transformant was retransformed with linearized YCF3/LYS2-a DNA and Trp^+ Ura^+ colonies were selected.

Although the same amount of DNA was used in both cases, 15-fold more YCF3/LYS2-a transformants were produced in the strain containing the *TRP1* marked *LYS2* fragment compared to the parent strain YNN290 (Table 1). These transformants were homogeneously pink as originally expected. Chromosomal DNA was prepared and separated by alternating CHEF gel electrophoresis. Fig. 2 *B* and *C* (lanes 6 and 7) shows that these transformants contain both the 475- and 365-kb fragments of chromosome II.

As a more stringent test of the system, the gene *MEL1*, which has not been mapped genetically, was physically mapped. Since many common laboratory strains including YNN290 are deleted for *MEL1* sequences (C. Mann and R.W.D., unpublished data), strain YNN337 (which contains the *MEL1* gene) was used. *MEL1* sequences hybridized to the DNA band corresponding to chromosome II in YNN337 (Fig. 3 *A* and *B*, lane 1). A *Hin*dIII fragment containing the *MEL1* gene was inserted into vectors YCF3 and YCF4 in both orientations and the four plasmids were cleaved with *Not* I. Two of the four plasmids (YCF3/MEL1-a and YCF4/MEL1-b) gave rise to significantly more transformants, many of which exhibited stable pink phenotypes when streaked onto nonselective plates. The size of the resulting chromosomal fragments was assessed by alternating CHEF gel electrophoresis. Examples of the two types of fragments observed are shown in Fig. 3 *A* and *B* (lanes 2–5).

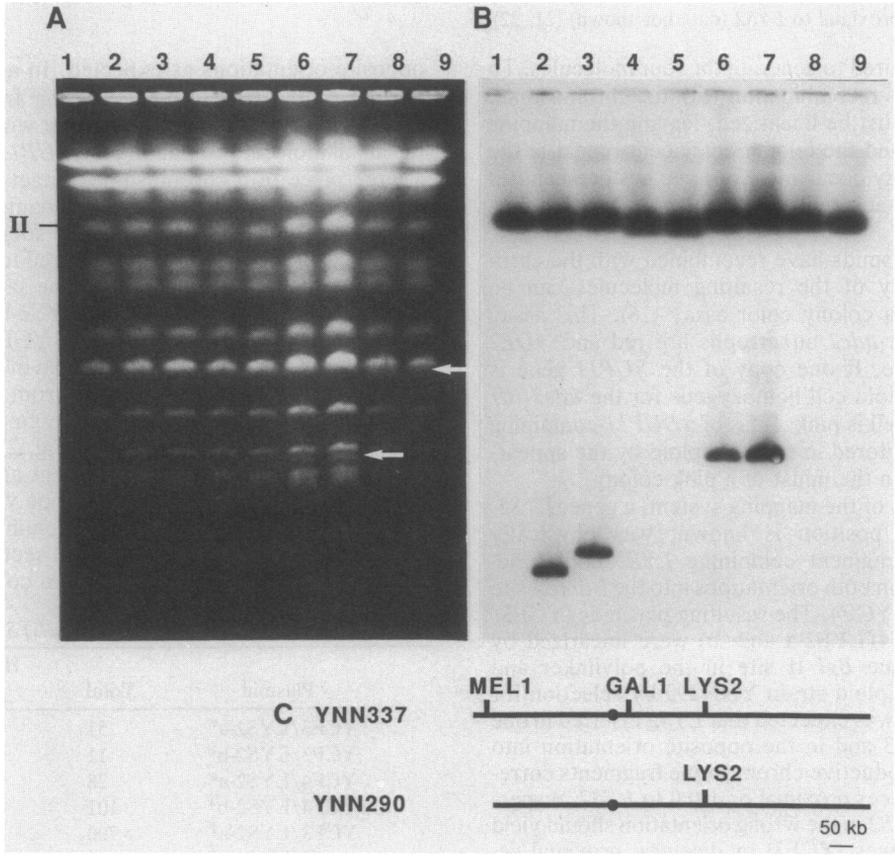

FIG. 3. Physical mapping of *MEL1* and *GAL1*. (*A*) Intact chromosomal DNA was separated as described in Fig. 2*B*. The samples consisted of YNN337 transformed with the following plasmids: lane 1, none; lanes 2 and 3, YCF4/MEL1-b; lanes 4 and 5, YCF3/MEL1-a; lanes 6 and 7, YCF3/GAL1-a; lanes 8 and 9, YCF4/GAL1-b. Arrows, chromosomal fragments. (*B*) Autoradiograph of the gel in *A* was made after hybridization with a 7.5-kb *Hin*dIII fragment containing *MEL1* and surrounding sequences (D.V. and R.W.D., unpublished data). A *LYS2* probe hybridizes to the chromosome fragment in lanes 8 and 9. Note the slightly higher mobility of chromosome II in lanes 4 and 5. The small difference in size between the fragments in lanes 2 and 3 may be due to variability in the number of telomere-adjacent Y′ repeats added during recombination (24). (*C*) A physical map of chromosome II in two strains was constructed by compiling data from Fig. 2 and *A* and *B*. ●, Centromere, which has been placed to the left of *GAL1* based on genetic data (22).

Fragments arising from transformation with YCF4/MEL-b are 30–40 kb (≈15 kb of which are vector sequences), while those from YCF3/MEL1-a are slightly smaller than intact chromosome II DNA. These results indicate that *MEL1* is very close to one of the ends of chromosome II. To determine which end, chromosome II was split at the *GAL1,10* locus in a manner similar to that used for *LYS2* and *MEL1* (Fig. 3 *A* and *B*, lanes 6–9). The identity of the resulting 320- and 560-kb fragments was confirmed by stripping the membrane and reprobing with *LYS2* sequences. *MEL1* hybridizes to the 320-kb fragment, which corresponds to sequences from *GAL1,10* through the centromere to the left end of chromosome II. Therefore, *MEL1* is located ≈25 kb from the left end of chromosome II (Fig. 3*C*).

DISCUSSION

A simple and rapid method for physically mapping any cloned sequence in yeast has been developed and used to map three genes. The technique consists of the following steps. (*i*) The mapping sequence is assigned to a chromosome by hybridization to 1 of the 17 chromosomal DNA molecules of strain YPH149. (*ii*) Four small linear molecules containing the sequence are constructed and used to transform strain YNN290, which contains the useful genetic markers *ura3-52*, *ade2-101*, and *trp1-Δ1*. (*iii*) Twenty-four independent transformants arising from each of the four plasmids are streaked onto rich media plates and grown for 3 days to allow the colony color to develop. Two of the four constructions are expected to yield homogeneously pink colonies. These two molecules should contain the mapping sequences in opposite orientations in the YCF3 and YCF4 vectors. (*iv*) Intact chromosomal DNA is prepared from 5–10 pink colonies for each of the two plasmids, separated by alternating CHEF gel electrophoresis, transferred to a nylon membrane, and hybridized with the mapping sequence. In each of the two cases, a band appears that is identical in size in 80–100% of the samples. The two bands represent fragments of the chromosome split at the mapping sequence. These fragments are sized by using concatamers of phage λ DNA or the chromosomes of strain YPH149. (*v*) The mapping sequence is unambiguously positioned with respect to the chromosome ends by rehybridizing the membrane with one or more previously mapped probes from the same chromosome.

A potential limitation of the above technique arises from the fact that fragmentation with the acentric vector (YCF3) usually results in a diploid cell hemizygous for certain sequences, while fragmentation with the centric vector (YCF4) usually causes triploidy of a chromosomal region. Such changes in gene dosage could occasionally be deleterious to the yeast cell. This seems to be true for loss of sequences distal to *LYS2*. Although transformants were obtained with plasmid YCF3/LYS2-a, they arose at a low frequency and did not display the stability expected for molecules of their size. Despite this caveat, in 26 different fragmentation events (11 YCF3 and 15 YCF4 events) at 15 different loci on eight different chromosomes, the desired chromosome fragment has been obtained.

The technique described here is attractive for application to the growing number of unmapped organisms whose chromosomal DNA molecules can be separated by alternating field gel electrophoresis. The major barrier to its extension is the requirement for a functional centromere, telomere, and origin of replication from the organism of interest. This problem might be overcome by modification of the chromosome fragmentation procedure. The ≈15-bp recognition site for the HO restriction endonuclease could be integrated at the location of the mapping sequence with subsequent cleavage of chromosomal DNA *in vitro* (25). This approach has been successfully used to cleave *S. cerevisiae* chromosome VI at an introduced HO site with a crude preparation of the HO enzyme (D.V., F. Hefron, and R.W.D., unpublished data).

Recently, a technique has been described for cloning large segments of exogenous (e.g., human) DNA as yeast artificial chromosomes (YACs) (26). The method reported here complements the YAC technique by allowing the physical mapping of any restriction fragment length polymorphism probe, cDNA, or other cloned sequence along an artificial chromosome without requiring the preparation of a detailed restriction map. The YAC technique will likely find wide application in the cloning of genes through complementation of mutant mammalian cells with large segments of human DNA. Because these segments could be >1 megabase, the fragmentation method described here should also be useful for the rapid deletion mapping of the complementing clones.

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