

Amplification of large artificial chromosomes

(yeast/cloning vectors/conditional centromere/thymidine kinase)

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ABSTRACT Yeast artificial chromosome cloning is an attractive technology for genomic mapping studies because very large DNA segments can be readily propagated. However, detailed analyses often require the extensive application of blotting-hybridization techniques because artificial chromosomes are normally present at only one copy per haploid genome. We have developed a cloning vector and host strain that alleviate this problem by permitting copy number amplification of artificial chromosomes. The vector includes a conditional centromere that can be turned on or off by changing the carbon source. Strong selective pressure for extra copies of the artificial chromosome can be applied by selecting for the expression of a heterologous thymidine kinase gene. When this system was used, artificial chromosomes ranging from about 100 to 600 kilobases in size were readily amplified 10- to 20-fold. The selective conditions did not induce obvious rearrangements in any of the clones tested. Reactivation of the centromere in amplified artificial chromosome clones resulted in stable maintenance of an elevated copy number for 20 generations. Applications of copy number control to various aspects of artificial chromosome analysis are addressed.

An efficient system for cloning large DNA segments as artificial chromosomes in *Saccharomyces cerevisiae* (YACs) was described about 3 years ago (1). Since that time, several groups have reported the construction and screening of YAC genomic libraries (2–8), and a number of modified vectors have been constructed (8–10). Most YACs that have been studied appear to contain single, contiguous, faithfully propagated DNA fragments (11, 12). Unstable sequences have been encountered but are relatively rare (5, 13). These results have encouraged the development of methods for constructing physical maps made up of overlapping YAC molecules (5, 6, 14–16). However the sensitivity and convenience of such methods are limited by the single-copy nature of artificial chromosomes and the relative complexity of the yeast genome compared with that of *Escherichia coli*. The analyses could be simplified if an effective amplification system for YACs were available.

In *S. cerevisiae*, DNA replication initiates only once at each origin per cell cycle (17). DNA molecules with centromeres usually partition equally at each cell division (18–20). Centromere function can be disrupted, however, by high-level transcription toward conserved centromeric elements (21–23). Circular plasmids containing such conditional centromeres have been described (22, 23). When the centromere is active, the plasmids are present at 1–2 copies per cell. When the centromere is inactivated by induced transcription from a *GAL1* or *ADH2* promoter, selection for plasmid markers results in a substantial increase in copy number (22, 23). This increase is thought to reflect a segregation bias of the plasmid for the mother cell and the loss of daughter cells without plasmids from the population (24).

Certain selectable markers can be used to produce high copy numbers (≥ 100 copies per cell) of circular or linear plasmids. These include a poorly expressed *LEU2* gene (*leu-2d*) (25, 26), a heterologous thymidine kinase (TK) marker (27, 28), and a heterologous dihydrofolate reductase gene (29).

As part of our efforts to develop improved YAC vectors, we incorporated a conditional centromere to determine whether large artificial chromosomes could be amplified. We chose TK as the selectable marker because it is responsive to a wide concentration range of selective agents (28). Furthermore, yeast does not contain a TK gene, so the isolation of host mutants during the selective procedure is unlikely. Selection for the TK gene can be accomplished by adding exogenous thymidine in the presence of methotrexate and sulfanilamide (27). The latter two compounds inhibit enzymes involved in the recycling or *de novo* synthesis (respectively) of folate cofactors required for the synthesis of deoxythymidilic acid, dTMP (30). Other vector modifications designed to facilitate the isolation and analysis of YACs are described.

MATERIALS AND METHODS

Strains and Media. *E. coli* strain XL1-blue [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac* (F' *proAB lacI^q lacZ*ΔM15 Tn10, (tetracycline resistant); Stratagene] was used for all vector construction experiments. Bacterial cells were transformed by electroporation (Bio-Rad Gene Pulser) according to the manufacturer's recommendations. Selection for ampicillin (Amp) or kanamycin (Kan) resistance was done as described (ref. 31, p. 1.4.2). *S. cerevisiae* strains included AB1380 (*MATa ura3-52 trp1 ade2-1 lys2-1 his5 can1-100*; ref. 1) and CGY2516 (*MATa GAL⁺ ura3-52 trp1-Δ63 leu2-Δ1 lys2-Δ202 his3-Δ200*). The latter strain was isolated from genetic crosses of strains FY86, FY17, and FY105 (F. Winston and C. Dollard, unpublished results). Yeast media (components from Difco) included YPD and YPgal (ref. 32; p. 163), S-glc (0.67% yeast nitrogen base, 1% casamino acids, 3% glucose, with histidine, methionine, and adenine at 50 μg/ml), S-gal (S-glc with 3% galactose instead of glucose), ST-gal (S-gal with thymidine at 0.8 mg/ml), and MST-gal (ST-gal with sulfanilamide at 2 mg/ml and methotrexate at 50 μg/ml). Solid media contained the same components with 2% agar added. For plating yeast transformants, selective media were supplemented with 1 M sorbitol (ref. 32, pp. 117–119).

Enzymes, Probes, and DNA Preparation. All enzymes were obtained from New England Biolabs and were used under the conditions suggested by the supplier unless otherwise noted. Molecular probes included a 456-base-pair (bp) *Stu I/Pst I* fragment from the yeast *URA3* gene of pCGS908 (see below), a 0.3-kilobase (kb) human *Alu*-repeat probe (blu α - β), and a 2.6-kb *EcoRI/Xho I* fragment isolated by plasmid rescue (1) from the end of a human DNA segment contained in an uncharacterized YAC clone.

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Abbreviations: YAC, yeast artificial chromosome; TK, thymidine kinase.

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Plasmid minipreparations and large-scale DNA preparations were performed as described (ref. 31, pp. 1.6.1–1.6.2). Fragments were purified from low-melt gels by melting, extraction with phenol, and precipitation with isopropyl alcohol (ref. 31, pp. 2.6.4–2.6.5). Human DNA was derived from the normal (male) fibroblast cell line GM6167; agarose blocks containing approximately 10 μ g of DNA each were prepared as described (33). Yeast chromosomal DNA was prepared essentially as described (34). Oligonucleotides were purchased from Operon Technologies (San Pablo, CA).

Construction of YAC Vectors. In each cloning step, component fragments were gel purified and vector fragments containing the selected marker (e.g., ampicillin resistance) were treated with calf intestinal alkaline phosphatase to reduce background.

First, missing *TRP1* promoter elements were added to pYAC3. A tryptophan prototroph of strain AB1380 was isolated by transforming with a library of plasmids containing yeast DNA inserts (35). The plasmid carried by one of the resulting clones was found to contain an 8.0-kb genomic fragment from the *TRP1* region inserted into the *Bam*HI site of YCp50. A 1.3-kb junction fragment extending from the *Aat* II site in pBR322-derived sequences to the *Xba* I site in *TRP1* was used to replace a similar fragment in pYAC3. The resulting plasmid was similar to pYAC3 except it contained approximately 900 bp of yeast DNA upstream of the *Eco*RI site in the *TRP1* promoter. The two *Eco*RI sites added by this procedure were removed by religating two subfragments of the plasmid: the 10-kb *Eco*RI fragment (ends filled in) and the 700-bp *Eco*RI (filled in)/*Eco*RV fragment containing upstream promoter elements. A resulting YAC vector (pCGS908) contained a *Sna*BI site at the expected location relative to the *TRP1* gene (36), indicating that the upstream promoter elements were inserted in the natural orientation.

Next, a cloning site containing T7 and T3 bacteriophage RNA polymerase promoters and a second *ARS1* element were added. A 2.4-kb *Aat* II/*Dra* III (*TRP1*–*ARS1*) fragment from pCGS908 was ligated to two synthetic DNA fragments simultaneously:

5'–pCGTCGACGCTGTGTGGATCCCC
TGCAGCAGCTGCACACACCTAGGGCCp–5'
(*Aat* II end/*Sal* I/*Bam*HI/*CC* end)

and

5'–pCCCCATCGATTAATACGACTCACTATAGGGATACGTATCCCTTTAGT–
GGTAGCTAATATGCTGAGTGATCCCTATGCATAGGAAATCA–

GAGGGTTAATTCACACA
CTCCCAATTAAGTgp–5'

(*CC* end/*Cla* I/T7 promoter/*Sna*BI/T3 promoter/*Dra* III end).

The products of the reaction were digested with *Bam*HI and *Cla* I and were ligated to the 6.5-kb *Bam*HI/*Cla* I fragment from pCGS908. The resulting vector (pCGS879) contained both synthetic fragments and an inverted duplication of the *TRP1*–*ARS1* region. A 5.4-kb *Aat* II fragment from pCGS879 was treated with mung bean nuclease (New England Biolabs) and ligated to the 4.0-kb partial *Sna*BI fragment from the same vector. A resulting plasmid (pCGS922) had the same overall orientation of fragments as pCGS879, contained a single *Sna*BI site, and lacked promoter elements upstream of the former *Sna*BI sites in the *TRP1* genes. One *TRP1* gene was inactivated by partial digestion of pCGS922 with *Eco*RV followed by digestion with *Sal* I, filling in the ends, and ligation. The resulting plasmid (pCGS929) retained sequences necessary for *ARS1* function (37) while lacking coding sequences from one of the *TRP1* genes.

A conditional centromere was added, and a complete YAC vector was reassembled as follows. Plasmid pCGS929 was digested with *Hpa* I and ligated to a *Sal* I (filled in)/*Rsa* I

fragment from pCGS231. The fragment thus inserted contains *GAL1* promoter elements extending from position 364 in figure 1 of ref. 38 to position 815 and terminating in the linker sequence CCCGGGTCGAC. A resulting plasmid (pCGS931) contained the *GAL1* promoter in the desired orientation and was digested with *Bam*HI, partially filled in with dA and dG, and digested again with *Xho* I. The 6.5-kb fragment thus generated was ligated to a 9.7-kb fragment from pYACneo (pNN415; ref. 8) that was digested with *Sal* I, partially filled in with dT and dC, and partially digested with *Xho* I. A product of this construction was the YAC vector pCGS934.

An *Eco*RI cloning site with flanking T3 and T7 promoters and *Sna*BI sites was then added. The synthetic fragment shown below was ligated to a 16.1-kb fragment produced by partial *Dra* III and complete *Cla* I digestion of pCGS934.

5'–pCGCTACGTAATAACCCCTCACTAAAGGGAATTCCTATAGTGAGTCGTAT–
GATGCATTTAATTTGGGAGTGATTTCCCTTAAGGGATATCACTCAGCATA–

TATTACGTAACA

ATAATGCATp–5'

(*Cla* I end/*Sna*BI/T3 promoter/*Eco*RI/T7 promoter/*Sna*BI/*Dra* III end).

After *Eco*RI cutting, religation, and transformation, a new plasmid, pCGS957, was isolated.

Finally, a heterologous TK gene and upstream *TRP1* promoter elements (removed during the construction) were added. This was done by ligating a 14.6-kb fragment, produced by partial digestion of pCGS957 with both *Bsu*36I and *Pvu* I, to a 5-kb *Bsu*36I/*Pvu* I fragment from pCGS963. Plasmid pCGS963 was constructed by inserting a 3.1-kb *Eco*RI fragment with the TK gene from pAYE56 (ref. 28; treated with mung bean nuclease and bacteriophage T4 DNA polymerase) into linearized pCGS908 cut with *Aat* II and treated with T4 DNA polymerase. One *Eco*RI site that was regenerated during pCGS963 construction was removed by digestion with *Eco*RI, filling in the ends, and religation.

Gel Electrophoresis and Nucleic Acid Hybridization. Analytical and preparative gels were run in 50 mM Tris borate/2.5 mM EDTA buffer as described (ref. 31, pp. 2.5.1–2.5.5). Pulsed-field gel electrophoresis employing contour-clamped homogeneous electric fields (CHEF; ref. 39) was performed as described (33), using 0.8% SeaKem GTG agarose (FMC) at 6.7 V/cm and 12°C with a 50-sec switch time.

Southern blots of CHEF gels were prepared as described (33) with capillary transfer in 1.5 M NaCl/0.5 M NaOH using Duralon membranes and UV fixation (Stratagene). DNA probes were prepared, and hybridizations were carried out essentially as described (33). Autoradiograms were developed at –70°C and the bands were quantitated by using a Shimadzu (Kyoto) scanning densitometer.

Artificial Chromosome Construction. Artificial chromosomes were generated from gel-fractionated ligation products. Vector DNA was digested with *Eco*RI and *Bam*HI and treated with 5 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) per 500 μ g of DNA. Agarose blocks of human target DNA (approximately 100 μ g) were partially digested with *Eco*RI in a competition reaction with *Eco*RI methylase (4 units of *Eco*RI, 200 units of *Eco*RI methylase in 2 ml of 0.1 M NaCl/0.1 M Tris-HCl, pH 7.5/2 mM MgCl₂/5 mM 2-mercaptoethanol at 37°C for 16 hr). The blocks were equilibrated in 70 mM Tris acetate, pH 7.6/10 mM MgCl₂/100 mM sodium acetate and melted at 68°C for 15 min. An equal volume of vector DNA (100 μ g) in the same buffer, including 100 μ M ATP, 10 mM dithiothreitol, no sodium acetate, and 4000 units of T4 DNA ligase, was added at 37°C. After ligation for 16 hr at 37°C, the ligase was destroyed by digestion for 6 hr at 50°C with proteinase K (Boehringer Mannheim) at 1 mg/ml after adding SDS to 0.1% and EDTA to 20 mM.

Ligation products were fractionated on a 0.8% SeaPlaque GTG agarose (FMC) gel in 50 mM Tris borate/2.5 mM EDTA

at 12°C and 3.3 V/cm with a 20-sec switch time for 48 hr. The focused band was removed as described (34), equilibrated in 10 mM Tris-HCl, pH 7.5/1 mM EDTA/30 mM sodium acetate, and melted at 68°C for 10 min. Agarase (Calbiochem; 50 units/ml) was added at 37°C, and the mixture was used directly for transformation experiments after digestion for 1 hr. Transformation was done essentially as described (40, 41). Cells were plated in 3 ml of 2% SeaPlaque top agarose.

Colony Screening. The preparation of nylon membranes (0.45 μ m pores, Schleicher & Schuell) containing DNA from yeast colonies was done as described (4) with the following exception. Yeast cell walls were digested by floating the membranes on 2 ml of 0.8 M sorbitol/100 mM EDTA, pH 8/1% 2-mercaptoethanol containing yeast lytic enzyme (ICN no. 190123) at 1 mg/ml. Specific culture conditions are described in *Results*.

RESULTS

Vector Modifications. The vector pCGS966 (Fig. 1) contains major elements from pYAC2 (1), pYACneo (8), and pAYE56 (28). The vector incorporates two advantages of pYACneo: the ability to rescue an *E. coli* plasmid from either end of an artificial chromosome and the presence of a selectable marker and replication origin capable of functioning in mammalian cells. A yeast replication origin (*ARS1*) was added to the *URA3* arm and a conditional centromere was added to the *TRP1* arm. The function of these elements and the heterologous TK gene in pCGS966 is discussed below. Upstream *TRP1* promoter elements were included in pCGS966 to improve *TRP1* expression and allow simultaneous selection for both the *URA3* and *TRP1* markers.

The region surrounding the cloning site (*EcoRI*) is described in *Materials and Methods*. The synthetic fragment introduced contains bacteriophage T7 and T3 RNA polymerase promoter sequences (identical to those contained in Stratagene Bluescript vectors and *SnaBI* restriction sites for digestion of circular ligation products during the isolation of fragments from the ends of cloned inserts by inverse polymerase chain reaction (PCR) (14). The integrity of these

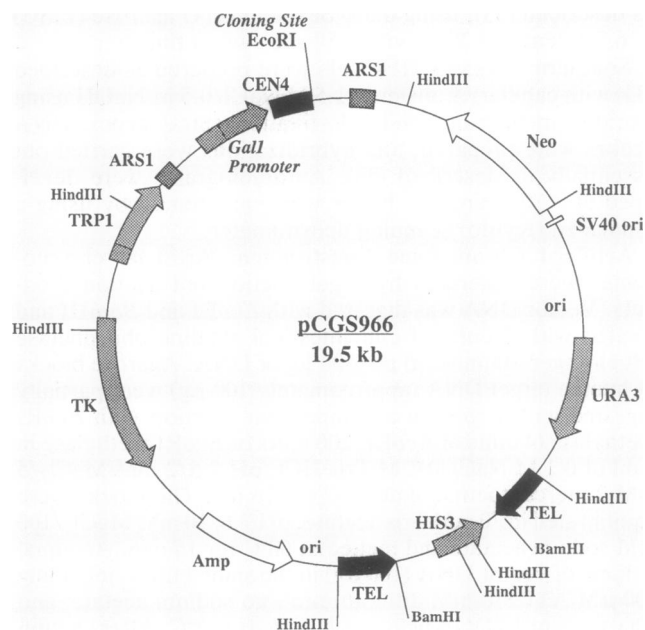


FIG. 1. YAC vector pCGS966. The arrangement of genes and functional elements is indicated. The DNA sequence of the region surrounding the cloning site (*EcoRI*) with T-bacteriophage promoters and other restriction sites is described in *Materials and Methods*. *CEN4*, centromere; *TEL*, telomere; *ori*, origin.

sequences was verified by DNA sequencing of pCGS966. Restriction sites available for inverse PCR rescue include *Mbo* I (flanking *EcoRI* by 107 and 77 bp), *Rsa* I (flanking *EcoRI* by 297 and 352 bp), and *Taq* I (flanking *EcoRI* by 508 and 869 bp).

Amplification of Artificial Chromosomes. Using vector pCGS966, we sought to determine whether YACs could be "amplified" by selection for a subpopulation of cells in which random mis-segregation of the YAC results in a higher copy number. Several clones with YACs spanning a broad size range in the *GAL*⁺ host CGY2516 were grown under a variety of conditions to assess the effects on YAC copy number (Fig. 2). In S-glc medium, the YACs were present at about 1 copy per haploid genome. However, when either methotrexate alone or methotrexate plus sulfanilamide was added to ST-gal medium, substantial increases in copy number were observed. The copy numbers or artificial chromosomes were measured by densitometry. Autoradiograms of Southern blots containing separated chromosomal DNAs were hybridized to a *URA3* probe (Fig. 2D) and scanned to reveal the number of YAC chromosomes relative to natural chromosome V. The results of this analysis for each of the growth conditions tested are given in Table 1.

All of the clones grew very slowly in media containing methotrexate and sulfanilamide (about 4 days to reach saturation from a starting density of 10⁵ cells per ml). Cells subjected to selective conditions displayed striking morphological changes such that many doublets, chains, clusters, and elongated cells were present. The walls of these cells were relatively resistant to digestion with lytic enzymes; this resulted in reduced yields of chromosomal DNA in agarose block preparations.

Stability of Amplified Chromosomes. In several separate amplification experiments, selective growth did not appear to induce any obvious insertions or deletions in any of the YACs studied. Early experiments consistently revealed low levels of an extraneous 130-kb molecule in clone 2 (400-kb YAC; see Fig. 2B). However, when the original unamplified isolate of the clone was colony purified and two subclones then amplified, only one contained the 130-kb molecule. In subsequent experiments, the subclone lacking this molecule continued to reveal only the 400-kb YAC (see Fig. 2C). Thus, it is most probable that the 130-kb molecule represents a contaminant harbored by some cells in the original isolate of clone 2 and was not produced as a result of the selective conditions used for amplification.

When amplified YAC clones were subcultured back into S-glc media, the cells began to divide faster and reverted to a nearly normal morphology. An elevated YAC chromosome level of about 4–10 copies per cell was maintained in the subcultured cells after 20 cell doublings (Fig. 2 and Table 1).

Colony Screening. Primary YAC transformants plated in low-melting agarose were replicated onto YPgal and MST-gal plates (the top agarose layer dried down to a thin film after several days so that most colonies were growing on the surface). After growing for 1 day (YPgal) or 4 days (MST-gal), colonies were lifted onto nylon filters and regrown for 1 day on YPgal plates, and colony blots were prepared. Regrowth of amplified cells on rich medium was found to be essential for efficient cell wall digestion. The blots were probed with a fragment derived from one of the clones on the original plate. The hybridization signal on the MST-gal replica (Fig. 3B) was considerably stronger than that from the YPgal plate, presumably reflecting a higher YAC copy number. Similar results were obtained with a probe from an *Alu* repetitive element (Fig. 3D) and also after regrowth of the cells on YPD plates (not shown).

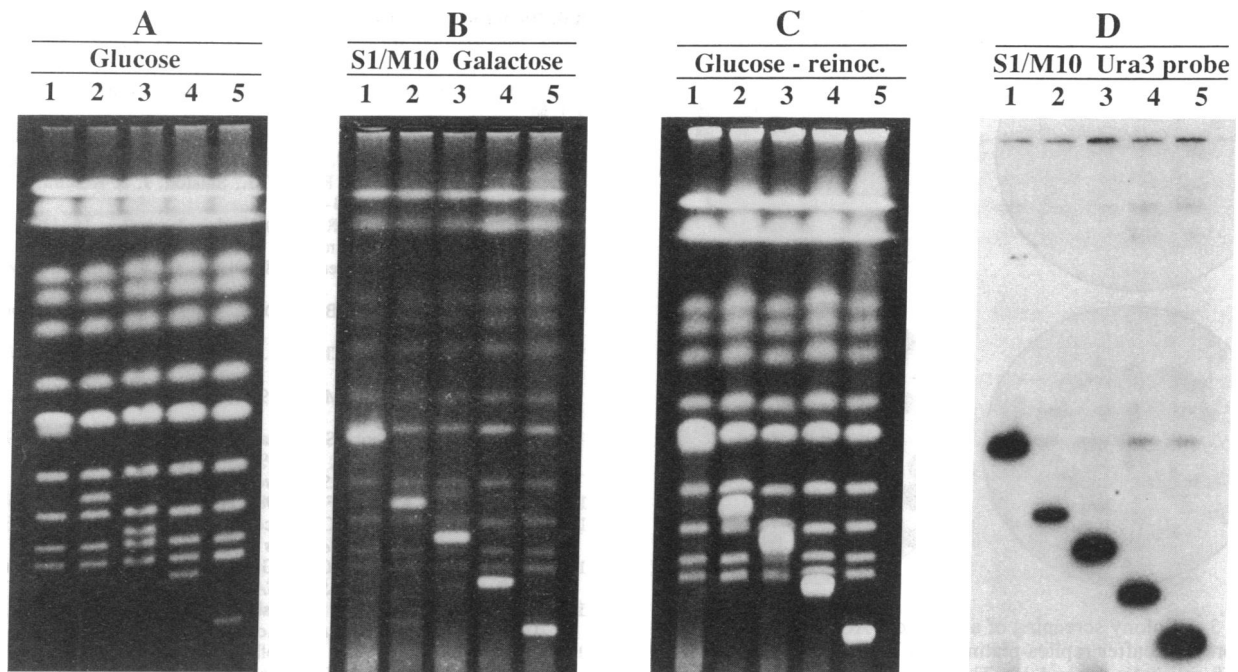


FIG. 2. Amplification of artificial chromosomes. Each panel displays chromosomal DNAs from a set of five clones with YACs ranging from 120 to 560 kb fractionated on pulsed-field gels (sizes given in Table 1). (A–C) Ethidium bromide-staining gels. (D) Southern blot (of the gel in B) hybridized with a *URA3* probe to allow the copy number to be determined (see text). The cells were grown in the following media: S-glc (A); sulfanilamide at 1 mg/ml and methotrexate at 10 μ g/ml in ST-gal (S1/M10) (B and D); and second 1:1000 S-glc subculture of S1/M10-selected cells (derived from a different S1/M10-selected culture than the one shown in B) (C).

DISCUSSION

YAC Amplification. Previous studies have shown that linear plasmids (without a centromere) can be amplified to high copy number in *S. cerevisiae* by applying strong selective pressure (42). The present study extends these observations by demonstrating that artificial chromosomes up to at least 560 kb in size with a conditional centromere can be amplified 10- to 20-fold.

We have not fully investigated the limits to which large YACs can be amplified with this system. However, several observations were made. The presence of both selective agents for the TK gene, methotrexate and sulfanilamide, was required for high amplification. Clones that were subcultured for a longer time in selective medium (6 days) had higher artificial chromosome copy numbers, based on ethidium bromide staining (not shown). When the concentration of thymidine was varied in the presence of methotrexate (alone) at 100 μ g/ml in S-gal, less amplification was observed with thymidine at 80 μ g/ml than at 800 μ g/ml. Thus, the extent of amplification appears to be related to the number of cell divisions and may be limited by the intracellular level of thymidylates. Inadequate thymidylate levels could limit the rate of DNA replication. It should be noted that, in highly amplified cells, the artificial chromosome may constitute over 20% of the total DNA (calculated from data in Table 1). The morphological changes observed in the cells appear to be similar to those previously noted in cells selected for amplification of a 2 μ vector containing a TK gene (28). They also resemble changes observed in multiple-*CEN*-containing strains (43). It is possible that faster growth rates and greater amplification could be achieved in a host strain with increased permeability for thymidine.

There exists some controversy in the literature over the effects of excess centromere sequences in *S. cerevisiae*. Some workers have observed that the excess cloned centromeres are deleterious and are rapidly lost from cells (23, 43). Others have reported that plasmids with a conditional centromere can be amplified to high copy number and the

centromere reactivated without obvious effects, although not all centromeres were fully active (22). Our present studies with large, heterologous, artificial chromosomes demonstrate that yeast cells will tolerate the presence of at least 10 extra *CEN4* copies. These centromeres appear to be functional when amplified cells are regrown on glucose, as judged by the maintenance of a high YAC copy number over 20 generations. However, there may be an initial decrease in YAC copy number when the cells are shifted from MST-gal medium back to glucose. Cells with amplified YACs grow somewhat slowly on S-glc medium—about half the normal rate, judging by the size of cell pellets. This may be more a consequence of an extra burden of the DNA replicative machinery than of excess centromeres. Consider, for in-

Table 1. YAC copy numbers under various growth conditions

Size of artificial chromosome, kb	Copy number of artificial chromosome*			
	S2	M100	S1/M10	glc-reinoc.
560 [†]	3.2	3.2	13	8.2
400	1.1	3.7	11	3.7
300	3.4	7.4	16	7.9
220	3.1	8.1	13	9.5
120	4.0	8.8	20	11.3

*Growth conditions are as follows: S2, sulfanilamide at 2 mg/ml in ST-gal; M100, methotrexate at 100 μ g/ml in ST-gal; S1/M10, sulfanilamide at 1 mg/ml and methotrexate at 10 μ g/ml in ST-gal (data derived from Fig. 2D); and glc-reinoc., second 1:1000 subculture of S1/M10-selected cells in S-glc (data derived from gel shown in Fig. 2C).

[†]The 560-kb YAC and 580-kb endogenous chromosome V were recorded as a single peak. The chromosome V level in these samples was estimated by averaging the values for two or more other samples in the same set with similar ethidium bromide staining intensities but smaller YACs. This value was then subtracted from the total to give an estimate of the 560-kb YAC signal; the ratio was then calculated.

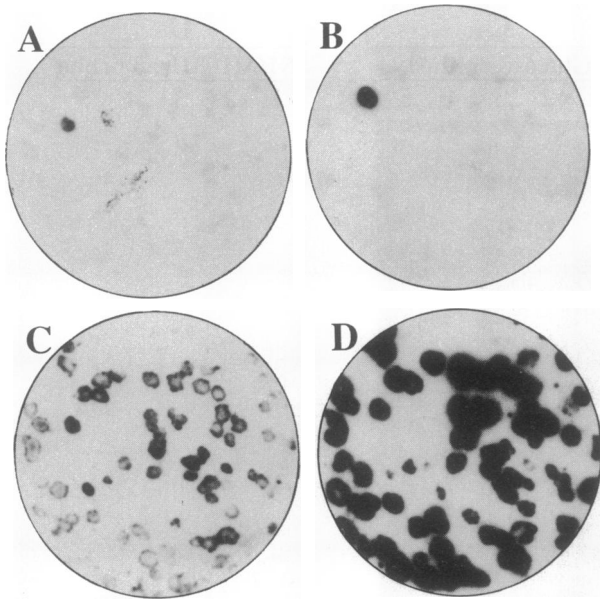


FIG. 3. Colony screening of amplified YAC clones. Colony lifts were prepared after replica-plating cells on YPGal plates (A and C) or MST-gal plates (B and D). The blots were first hybridized to a single copy probe derived from one of the clones on the original plate (A and B). After this, the blots were stripped and hybridized to the human *Alu*-repeat probe blur-8 (C and D).

stance, that clone 1 contained the smallest YAC (120 kb) and grew noticeably faster than all of the other clones, but it had the highest copy number when regrown on glucose (Table 1). Clones 1 and 2, with the largest YACs, grew slowest.

Applications of an Amplifiable Vector. Many existing analytical techniques will benefit from the availability of a YAC amplification system. One such application is the enhancement of signal strength in colony screening techniques. Yeast colony screening by hybridization is an important method for identifying YAC clones that contain specific DNA sequences (4, 8). However, such methods often yield poor signals and high background. The results presented here (Fig. 3) demonstrate that significant signal enhancement can be attained when YACs are amplified prior to the preparation of colony blots. Probes derived from human repetitive elements can be used to identify clones with YACs containing segments of specific human chromosomes derived from hybrid cell line DNA (41).

Other useful applications may include the following: signal enhancement on Southern blots, direct restriction mapping of amplified chromosomes (without the need for blotting), direct fingerprinting, and direct probe preparation from the ends of cloned inserts by RNA polymerase transcription. The ability to generate large amounts of a particular artificial chromosome would also aid in gel (or other) purification methods.

If YAC amplification is to be employed in genomic mapping studies, one would like to be certain that structural changes will not be introduced. Although we have not surveyed a large number of clones, none of those we did study appear to have undergone rearrangement as a result of the selective conditions used. Nevertheless, caution should be exercised until more individual clones have been studied in detail. The host strain CGY2516 and YAC vector pCGS966 can be obtained from the American Type Culture Collection (accession numbers 74013 and 68371, respectively).

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