Spontaneous Amplification of the *ADH4* gene in *Saccharomyces cerevisiae*

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ABSTRACT

Five spontaneous amplifications of the *ADH4* gene were identified among 1,894 antimycin A-resistant mutants isolated from a diploid strain after growth at 15°. Four of these amplifications are ~40-kb linear extrachromosomal palindromes carrying telomere homologous sequences at each end similar to a previously isolated amplification. *ADH4* is located at the extreme left end of chromosome *VII*, and the extrachromosomal fragments appear to be the fusion of two copies of the end of this chromosome. The fifth amplification is a chromosomal amplification carrying an extra copy of *ADH4* on both homologs of chromosome *VII*. These results suggest that the ADH system can be used to study amplification in *Saccharomyces cerevisiae*.

PRIMARY gene amplification, the change from one copy of a gene per genome to two or more copies per genome, is an important but poorly understood type of mutation. Primary gene amplification is the first step in the formation of gene families and the development of new functions (MAEDA and SMITHIES 1989; KIMURA and OHTA 1974). One copy of a gene can maintain the normal gene function while the other copies diverge to perform new functions when several gene copies are present in a genome. Gene amplifications have been reported in many different organisms (for reviews see ANDERSON and ROTH 1977; HAMLIN *et al.* 1984; STARK *et al.* 1989).

Amplifications appear to be a rare type of mutation in normal eukaryotic cells. The high rates of amplification reported in mammalian tissue culture cells are apparently related to the immortalization of the cell lines since several groups have been unable to detect amplification events in normal diploid mammalian cells (WRIGHT *et al.*, 1990; TLSTY, MARGOLIN and LUM 1989; TLSTY 1990). Amplifications of only four genes, *acp1*, *CUP1*, *ADH2* and *ADH4* have been observed in *Saccharomyces cerevisiae*. Translocated duplications of the acid phosphatase gene, *acp1*, were selected in phosphate-limited chemostats (HANSCHE, BERES and LANGE 1978), and *CUP1* is present in tandem, direct, chromosomal amplifications in many yeast strains (KARIN *et al.* 1984; FOGEL and WELCH 1982). However, a primary amplification event in which one copy of *CUP1* is amplified to two or more copies has never been reported. The isolation of a chromosomal amplification of *ADH2* (PAQUIN *et al.* 1992) and an extrachromosomal amplification of *ADH4* (WALTON *et al.* 1986) among 208 antimycin A-resistant mutants suggested that this system could be used to study primary amplification.

The structural genes coding for the four yeast alcohol dehydrogenase (ADH) isozymes are *ADH1*, the classic fermentative isozyme (CIRIACY 1975a; WILLIAMSON *et al.* 1980); *ADH2*, the glucose-repressed isozyme (CIRIACY 1975a,b; RUSSELL *et al.* 1983); *ADH3*, the mitochondrial isozyme (CIRIACY 1975a; YOUNG and PILGRIM 1985) and *ADH4*, a newly discovered isozyme of unknown function (WILLIAMSON and PAQUIN 1987). The nucleic acid sequences of *ADH1* and *ADH2* are 88% similar and *ADH3* is approximately 70% similar to *ADH1* and *ADH2*. The *ADH4* gene, however, appears to be unrelated to *ADH1*, *ADH2* or *ADH3* and its predicted amino acid sequence is most similar to the *ADH2* gene of the fermentative bacterium, *Zymomonas mobilis*. The antimycin A-resistant mutants carrying amplifications of *ADH2* and *ADH4* (PAQUIN *et al.* 1992; WALTON *et al.* 1986) were selected from strains carrying a deletion of the 5′ end of *ADH1*. Strains lacking *ADH1* cannot grow on medium containing glucose and antimycin A because this antibiotic blocks respiration and ADH activity is required for fermentation. Normally, *ADH2* and *ADH4* are not expressed in the presence of glucose and *ADH3* is confined to the mitochondria. Thus, any mutation that allows the expression of sufficient ADH activity for fermentation will permit growth in the presence of antimycin A. The ADH system has been useful in selecting rare events such as Ty transposition because the mutation rate to antimycin A resistance is low (1×10^{-7}–10^{-9} mutations per cell per generation, PAQUIN and WILLIAMSON 1984, 1986) and a single antimycin A-resistant mutant can be selected from a background of 10^9 antimycin A-sensitive cells on a plate. We have not succeeded in isolating any additional *ADH2* amplifications (PAQUIN *et al.* 1992). However, we report here the isolation and preliminary characterization of five additional *ADH4* amplifications.

MATERIALS AND METHODS

Yeast strains and media: The yeast strains used are shown in Table 1. Yeast strains carrying amplifications are

TABLE 1

Yeast strains

Strain	Genotype	Reference or source
315-1D	*MATα adh1-Δ1 ADH2 ADH3 ADH4 ADR1 trp1 ura1*	PAQUIN and WILLIAMSON (1984)
JL18C	*MATα adh1-Δ1 ADH2 ADH3 ADH4 adr1-Δ1*	PAQUIN *et al.* (1992)
CP2AB	*MATa/MATα adh1-Δ1/adh1-Δ1 ADH2/ADH2 ADH3/ADH3 ADH4/ADH4 ADR1/ADR1 trp2/trp2 ura1/ura1*	PAQUIN and WILLIAMSON (1984)
15-3-18	*MATa/MATα adh1-Δ1/adh1-Δ1 ADH2/ADH2 ADH3/ADH3 ADH4/ADH4 ADR1/ADR1 trp2/trp2 ura1/ura1 ADH4-EA1* (Extrachromosomal Amplification 1)	WALTON *et al.* (1986)
3-2-9	*MATa/MATα adh1-Δ1/adh1-Δ1 ADH2/ADH2 ADH3/ADH3 ADH4/ADH4 ADR1/ADR1 trp2/trp2 ura1/ura1 ADH4-EA2*	This study
3-8-14	*MATa/MATα adh1-Δ1/adh1-Δ1 ADH2/ADH2 ADH3/ADH3 ADH4/ADH4 ADR1/ADR1 trp2/trp2 ura1/ura1 ADH4-EA3*	This study
5-3-6	*MATa/MATα adh1-Δ1/adh1-Δ1 ADH2/ADH2 ADH3/ADH3 ADH4/ADH4 ADR1/ADR1 trp2/trp2 ura1/ura1 ADH4-EA4*	This study
7-2-1	*MATa/MATα adh1-Δ1/adh1-Δ1 ADH2/ADH2 ADH3/ADH3 ADH4/ADH4 ADR1/ADR1 trp2/trp2 ura1/ura1 ADH4-CA1* (Chromosomal Amplification 1)	This study
16-4-16	*MATa/MATα adh1-Δ1/adh1-Δ1 ADH2/ADH2 ADH3/ADH3 ADH4/ADH4 ADR1/ADR1 trp2/trp2 ura1/ura1 ADH4-EA5*	This study

named on the basis of how they were isolated. For example 3-2-9 is the ninth antimycin A-resistant colony from the second flask in the third mutation rate experiment. Thus 3-2-9 and 3-8-14 are independent mutations because they were isolated from different flasks during experiment 3. The media used were YEP (CIRIACY 1979) containing 5% wt/vol glucose or 5% (vol/vol) ethanol for liquid cultures and 2% (wt/vol) glucose for solid media (CIRIACY 1979). Selection for antimycin A resistance was done on YEP medium containing 5% (wt/vol) glucose and 1 μg of antimycin A per ml at 30° (CIRIACY 1979). Sporulation and tetrad analysis was carried out as described by SHERMAN and HICKS (1991) using a Lawrence Precision Machine micromanipulator.

Mutation rates: Mutation rate experiments were performed as previously described (PAQUIN and WILLIAMSON 1984, 1986). The strains were grown at various temperatures before selection but all antimycin A-resistant mutants were selected at 30° so that any differences in mutation rates are due to differences in the rate at which mutations occur at a specific temperature and not to differences in selection conditions. Mutation rates were calculated by the P_0 method (LEA and COULSON 1949). The P_0 method is based on the number of cultures without mutants rather than the number of mutants per culture and thus eliminates any bias due to differences in the growth rates of the parental and the mutant strains. This method was used because antimycin A-resistant mutants are able to ferment and thus grow faster than the antimycin A-sensitive parent strain. Amplification rates cannot be calculated from any single experiment due to the low frequency of amplifications. However an estimate of amplification rate can be obtained by combining data from all the experiments.

Amplification screen: Antimycin A-resistant strains were screened by colony hybridization to detect colonies which hybridized to an *ADH4* probe more intensely than the parent strain using the previously isolated amplification strain 15-3-18 as a positive control. This eliminated approximately 95% of the antimycin A-resistant mutants from consideration. The remaining 5% were screened by Southern blot analysis of genomic DNA restricted with *Eco*RI for the appearance of new DNA fragments which hybridized to an *ADH4* probe or increased hybridization intensity. New DNA fragments would be detected for amplifications with novel joints (the joint formed between two previously separated DNA sequences) within 1 kb upstream and 5.5 kb downstream of the open reading frame of *ADH4*.

Southern blots and colony hybridizations: *S. cerevisiae* genomic DNA was prepared by the method of DENIS and YOUNG (1983). "Genomic DNA" describes DNA isolated in solution and thus exposed to shearing forces. "Snapback" DNA was prepared by the method of DEVENISH and NEWLON (1982). Southern blots were carried out as described in KLESSIG and BERRY (1983). Colony hybridizations were carried out by a modification of an *E. coli* colony hybridization protocol (TAUB and THOMPSON 1982) as described below. The yeast strains were streaked on sterile discs of #541 Whatman paper, placed on YEPD plates and incubated at 30° for 12 hr. The discs were then removed, washed twice in 500 ml 0.5 N NaOH at room temperature for 3 min, washed twice in 500 ml 0.5 M Tris at pH 8.0 at room temperature for 3 min and then washed in 200 ml SCE (1 M sorbitol, 0.1 M sodium citrate and 0.06 M EDTA at pH 7.0) for 3 min at 37°. The discs were then wet with 1 mg/ml zymolyase (20 units/mg, ICN Biomedicals) and 8 μl/ml β-mercaptoethanol in SCE and incubated for 2–3 hr at 37°. The discs were washed twice in 400 ml 1 × SSC (0.15 M NaCl, 0.015 M Na citrate at pH 7) at 37° for 1 min. Next, the discs were wet with 500 μg/ml pronase in 1 × SSC (Calbiochem) and incubated at 37° for 30 min (a 5 mg/ml pronase stock solution is prepared in 1 × SSC, incubated for 1 hr at 37° for self-digestion to eliminate nucleases, and stored at −20°). Finally the discs were washed twice in 400 ml of 1 × SSC at room temperature for 1 min and twice in 400 ml 95% EtOH at room temperature for 2 min. The discs were air dried at room temperature on Saran wrap and then hybridized as described in WILLIAMSON *et al.* (1980).

Pulsed field gel electrophoresis: Pulsed field gels were run on a Bio-Rad CHEF (CHU, VOLLRATH and DAVIS 1986) apparatus. Chromosomal DNA was prepared as described by CARLE and OLSON (1985). "Chromosomal DNA" describes DNA isolated from cells embedded in agarose to minimize the fragmentation of chromosomes. Positions of chromosomes were determined by hybridization with DNA probes and by comparison with published patterns (CARLE and OLSON 1985; LINK and OLSON 1991).

Amplification copy number: Extrachromosomal amplification copy number was estimated from Southern blots of chromosomal DNA separated on CHEF gels to minimize the possibility of differential loss of genomic and amplified

TABLE 2

Antimycin A-resistant mutants

Yeast strain	Mutation rate to antimycin A resistance[a] ±SD	No. of independent cultures	No. of independent cultures with antimycin A-resistant colonies	No. of antimycin A-resistant colonies screened	No. of amplifications of the *ADH4* gene isolated	Approximate amplification rate[b]
Growth at 30°						
JL1-18C[c]	$1 \pm 1 \times 10^{-9}$	544	311	311[d]	0	$<2 \times 10^{-12}$
CP2AB[e]	$3 \pm 2 \times 10^{-9}$	66	42	199[f]	0	$<5 \times 10^{-11}$
Growth at 15°						
CP2AB[g]	$7 \pm 4 \times 10^{-8}$	54	23	23[d]	1	2×10^{-10}
CP2AB[h]	$6 \pm 3 \times 10^{-8}$	295	255	1,894[f]	5	5×10^{-10}

[a] Mutation rates were calculated by the P_0 method of LEA and COULSON (1949) and are reported as the average and standard deviation of several different mutation rate estimates.

[b] Approximate amplification rates were calculated combining the data from several experiments and then calculating the amplification rate by the P_0 method of LEA and COULSON (1949).

[c] Data from twenty-three independent experiments.

[d] Colonies were screened by Southern blot analysis for amplifications (see MATERIALS AND METHODS).

[e] Data from two independent experiments.

[f] Colonies were screened by colony hybridization for amplifications (see MATERIALS AND METHODS)

[g] Data from three independent experiments (PAQUIN and WILLIAMSON 1986).

[h] Data from thirteen independent experiments. Mutation rates could be calculated for only six of the experiments because every culture had antimycin A-resistant mutants in the other seven experiments.

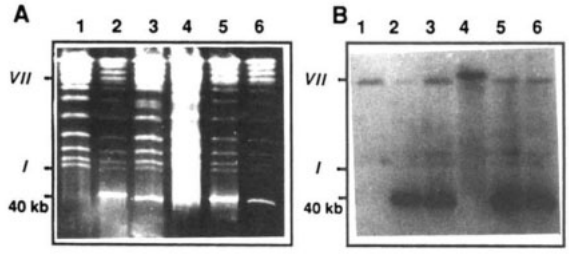

FIGURE 1.—Chromosomal DNAs from the parental strain CP2AB (lane 1), amplification strain 3-2-9 (lane 2), amplification strain 3-8-14 (lane 3), amplification 7-2-1 (lane 4), amplification strain 5-3-6 (lane 5) and amplification strain 15-3-18 (lane 6) separated on CHEF pulsed field electrophoresis gels. The locations of chromosomes *I* and *VII* as well as the location of the 42-kb extrachromosomal DNA found in 15-3-18 are indicated. Panel A shows a picture of the ethidium bromide stained gel and panel B shows a Southern blot of this gel probed with *ADH4* DNA.

copies of *ADH4* during DNA preparation. Chromosomal amplification copy number was estimated from Southern blots of genomic DNA restricted with *Eco*RI. Southern blots were quantified on a Hoefer Scientific Instruments GS300 Transmittance/Reflectance scanning densitometer using the GS360 Data System.

RESULTS

Isolation of *ADH4* amplifications: Five independent amplifications of *ADH4* were isolated from diploid strain CP2AB grown at 15° by screening 1,894 spontaneous antimycin A-resistant colonies from 255 independent cultures by colony hybridization (Table 2 and Materials and Methods). The previously reported amplification of *ADH4* was also isolated from this strain after growth at 15° (WALTON *et al.* 1986). In contrast, no amplifications of *ADH4* were detected among 311 independent antimycin A-resistant mutants isolated from haploid strain JL18C grown at 30°, or 199 antimycin A-resistant colonies from 42 independent cultures of CP2AB grown at 30° (Table 2). This is particularly surprising because the mutation rate to antimycin A resistance is about ten fold higher at 15° than at 30° so that the background of antimycin A-resistant mutants which are not amplifications was expected to be 10-fold higher at 15°. If the amplification rates at the two temperatures were the same, approximately 10 independent amplifications should have been isolated from the 42 independent cultures of strain CP2AB grown at 30°, suggesting that amplification is more frequent at 15° than at 30°.

Identification of amplifications: Antimycin A-resistant mutants isolated from strain CP2AB after growth at 15° or 30° which appeared to hybridize strongly to an *ADH4* probe during colony hybridization were screened for amplifications by Southern blot analysis. Four of the 99 antimycin A-resistant mutants that showed strong hybridization had extra bands in *Eco*RI restricted genomic DNA (DNA prepared for restriction digestion) probed with an *ADH4* probe and 18 had intensely hybridizing bands at the normal location for the *ADH4* gene. Chromosomal DNA (DNA prepared for pulsed field gel electrophoresis) from these 22 strains was prepared and run on pulsed field gels. Southern blot analysis of chromosomal DNA from these 22 mutants separated on pulsed field gels showed that the four strains that had extra bands and one of the strains with an intensely hybridizing band carried amplifications of *ADH4* (Figure 1, strain 16-4-16 is not shown). Strains 3-2-9, 3-8-14, 5-3-6 and 16-4-16 show new extrachromosomal bands of approximately 40 kb, similar to the 42-kb extrachromosomal band in the original *ADH4* amplification strain, 15-3-18 (WALTON *et al.* 1986). In the fifth strain (7-2-1) the band which hybridizes to *ADH4* is

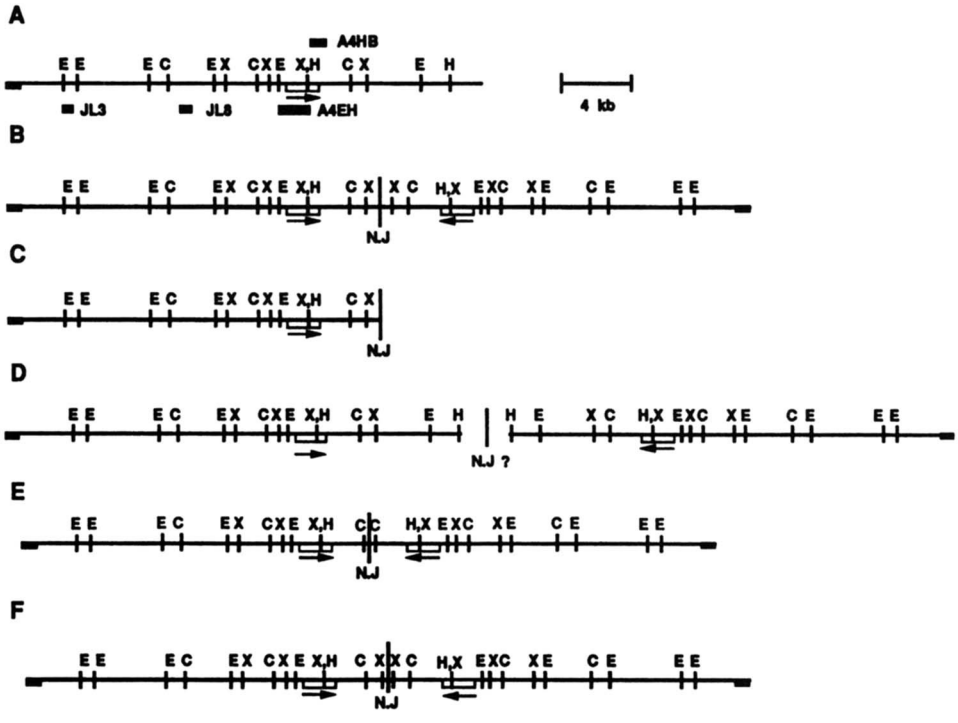

FIGURE 2.—Restriction maps of: (A) the left end of chromosome *VII*, (B) amplifications carried by strains 15-3-18 and 3-8-14, (C) "snapback" DNA from strains 15-3-18 and 3-8-14, (D) the amplification carried by strain 3-2-9, (E) the amplification carried by strain 5-3-6, (F) the amplification carried by strain 16-4-16. Broken lines indicate regions not yet mapped. Open boxes indicate the *ADH4* open reading frame. The direction of transcription is indicated by an arrow. Black boxes on the restriction maps indicate telomere homology. These maps are compiled from Southern blots of genomic and snapback DNA probed with the four probes A4EH, A4HB, JL3 and JL8 shown as black boxes above and below the map of chromosome *VII*. Not all restriction sites are shown. Abbreviations for restriction enzymes are E, *Eco*RI; H, *Hin*dIII; C, *Cla*I; X, *Xba*I. The restriction maps of chromosome *VII* and the amplification carried by strain 15-3-18 are from WALTON *et al.* (1986).

larger than the normal size of chromosome *VII* on which *ADH4* is located (Figure 1). The presence of a chromosomal amplification in 7-2-1 was confirmed by the segregation analysis discussed below.

Restriction maps of the amplifications: Southern blots of genomic DNAs were used to compile restriction maps of the four new extrachromosomal amplifications (Figure 2). For example, genomic DNA restricted with *Hin*dIII from all of the strains including the parent strain has a 6.0 kb band which hybridizes to the *ADH4* probe representing the normal copy of *ADH4* (Figure 3). DNA from the extrachromosomal amplification strain 15-3-18 (carrying amplification *ADH4-EA1* described by WALTON *et al.* 1986) and three of the new extrachromosomal amplification strains (3-8-14, 5-3-6, and 16-4-16) also have intensely hybridizing extra bands of 8.0, 8.0, 5.1 and 6.2. kb, respectively (Figure 3). These bands represent the amplified copies of *ADH4*. The chromosomal amplification strain, 7-2-1, no longer shows an extra band when the genomic DNA is cut with *Hin*dIII rather than *Eco*RI (Figure 3) suggesting that the novel joint in this strain is located between the *Eco*RI site approximately 1.0 kb upstream of the *ADH4* open reading frame and the *Hin*dIII site 1.0 kb inside the *ADH4* open reading frame. Analysis of Southern blots of genomic DNAs result in restriction maps for the extrachromosomal amplifications which are identical to the left end of chromosome *VII* in the parent strain except at the novel joints.

The restriction maps of the four new extrachromosomal *ADH4* amplifications derived from genomic restriction mapping suggested that these amplifications were palindromes similar to the previously isolated *ADH4* amplification, *ADH4-EA1* (WALTON *et al.* 1986). A characteristic of this palindromic amplification is that when the DNA is melted or denatured, each single strand of DNA can renature to form a hairpin by base pairing with itself, resulting in a dou-

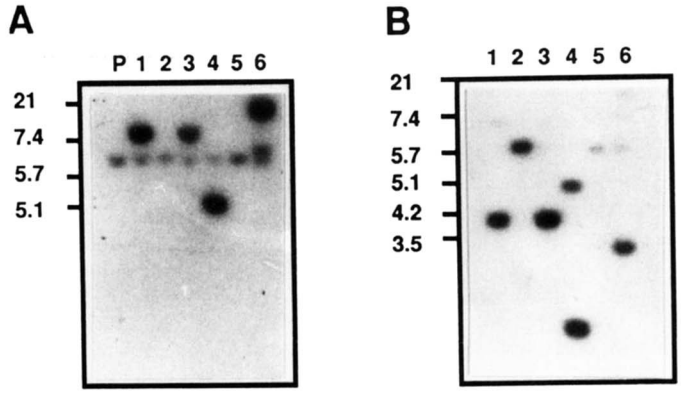

FIGURE 3.—Comparison of genomic and snapback DNA from the *ADH4* amplifications. Both genomic and snapback DNAs were digested with *Hin*dIII, electrophoresed on agarose gels, transferred to nitrocellulose and probed with A4HB (Figure 2). The size (in kb) of lambda standards are shown. Panel A shows genomic DNA from: CP2AB (lane P, parental strain), 15-3-18 (lane 1), 3-2-9 (lane 2) (less DNA was loaded in this lane than in the other lanes so the 6.0-kb *ADH4* band does not appear to hybridize intensely), 3-8-14 (lane 3), 5-3-6 (lane 4), 7-2-1 (lane 5), 16-4-16 (lane 6). Less DNA was loaded in lane 2 than in the other lanes. The DNA in lane 6 is not digested to completion but on other Southern blots performed with this DNA sample the largest band is completely digested to the smaller two bands. Panel B shows "snapback" DNA from: 15-3-18 (lane 1), 3-2-9 (lane 2), 3-8-14 (lane 3), 5-3-6 (lane 4), 7-2-1 (lane 5), 16-4-16 (lane 6). The DNA in lane 4 is not digested to completion but on other Southern blots performed with this DNA sample the largest band is completely digested to the smaller band.

ble stranded "snapback" molecule that is half the size of the normal amplification (Figure 2C). Figure 3 shows a comparison between genomic and "snapback" DNA from the amplification strains digested with *Hin*dIII and analyzed on Southern blots. The "snapback" DNA from strains 15-3-18, 3-8-14, 5-3-6 and 16-4-16 show hybridization to bands of 4.1, 4.1, 2.5 and 3.2 kb, respectively (Figure 3). Thus, the "snapback" DNA from these strains which hybridizes to the *ADH4* probe is approximately half the size of the genomic DNA fragment. *Eco*RI cut "snapback" DNA which hybridizes to the *ADH4* probe is also half the size of *Eco*RI genomic DNA fragments (data not shown). The decrease in size of the "snapback" DNA fragments is expected if the amplifications are palindromic since the "snapback" *Eco*RI and *Hin*dIII DNA fragments that hybridize to the *ADH4* probe are half the size of the normal amplification DNA fragments (see Figure 2C). The fifth extrachromosomal amplification strain, 3-2-9, does not show an extra band in genomic or "snapback" DNA restricted with *Hin*dIII but does show an intensely hybridizing band at the normal size (6.0 kb) for the *ADH4* gene and an extrachromosomal band which hybridizes to *ADH4* on pulsed field gels (Figures 1 and 3; In panel A of Figure 3 less genomic DNA from strain 3-2-9 was loaded on the gel so it does not appear to have an intensely hybridizing band but intense hybridization is seen to the snapback DNA from strain 3-2-9 in Figure 3 panel B and to an extrachromosomal band in chromosomal DNA from strain 3–2–9 in Figure 1). This amplification is probably also a palindrome because it is concentrated by the snapback procedure but the joint between the two palindromic arms is too far away to be detected by the *ADH4* probes. This strain also has a slightly larger extrachromosomal band on pulsed field gels suggesting that it carries the largest extrachromosomal amplification (Figure 1). "Snapback" DNA from the five extrachromosomal amplification strains show identical patterns of hybridization to telomeric DNA probes (data not shown) suggesting that four new extrachromosomal amplifications carry telomeres at each end as was previously shown for amplification *A4-EA1* (WALTON *et al.* 1986). "Snapback" DNA from the chromosomal amplification strain, 7-2-1, does not show intense hybridization to an *ADH4* probe suggesting that it is not a palindrome (Figure 3).

Analysis of Southern blots of genomic and "snapback" DNAs result in restriction maps for the extrachromosomal amplifications which are identical to the left end of chromosome *VII* in the parent strain except at the novel joints (Figure 2). Two of the independent extrachromosomal amplification strains, 15-3-18 and 3-8-14, have amplifications with identical restriction maps suggesting that the novel joints, where two previously separated DNA sequences are joined in the

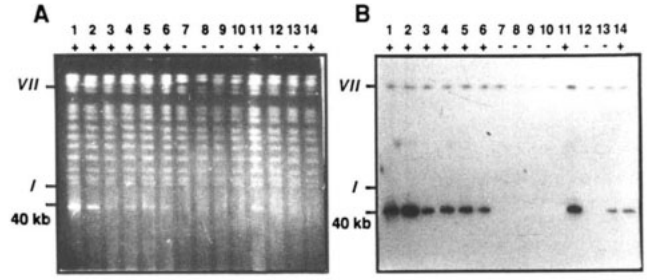

FIGURE 4.—Analysis of the segregation of the amplification in strain 3-8-14. Chromosomal DNA from 3-8-14 is in lane 1, lane 2 shows chromosomal DNA from amplification 15-3-18 and the remaining lanes contain DNA from spore colonies derived from 3-8-14. Lanes 3–6 are chromosomal DNAs from the four spore colonies from a single tetrad, lanes 7–10 contain DNAs from a second tetrad and lanes 11–14 DNAs from a third tetrad. Antimycin A resistance of the spore colonies is indicated by a "+" and antimycin A sensitivity by a "−." Panel A shows an ethidium bromide stained gel of chromosomal DNAs separated on a pulsed field electrophoresis gel. Panel B shows a Southern blot of the gel shown in panel A probed with *ADH4* DNA. The locations of chromosomes *I* and *VII* and the 42-kb amplification found in 15-3-18 are indicated.

amplification, may be identical. These results show that there are at least five locations adjacent to *ADH4* at which novel joints can be formed, one upstream of *ADH4* (the chromosomal amplification strain 7-2-1) and four downstream of *ADH4*.

Amplification segregation: In order to determine whether the antimycin A resistance segregated with the presence of the amplifications, amplification strains were sporulated and the resultant tetrads dissected. The spore colonies were tested for antimycin A resistance and the presence or absence of the amplification was determined by Southern blot analysis of chromosomal DNA (Figures 4 and 5). Tetrads from strains 5-3-6 and 3-2-9, contain at most two viable spores (Table 3), suggesting that amplification was accompanied by the formation of recessive lethal mutations in these two strains. All four spores were viable in the majority of tetrads from the other four amplification strains (Table 3) and eight antimycin A-resistant mutants isolated from the same parent strain that do not carry amplifications.

All antimycin A-resistant spore colonies from the extrachromosomal amplification strains carried amplifications when tested by Southern blot analysis of pulsed field gels (see Table 4 and Figure 4 for an example of the Southern blot analysis) confirming that the antimycin A resistance is due to the presence of the amplifications. Extrachromosomal amplifications could also be detected at a low level (one to two extra copies of *ADH4* per cell or less than one amplification per cell) in three of the spore colonies that were antimycin A sensitive. Some antimycin A-resistant spore colonies have only two to three extra copies of *ADH4* per cell (one to two copies of the amplification per cell). These results suggest that two copies of the amplification (or four extra copies of *ADH4*) are sufficient to confer antimycin A resistance.

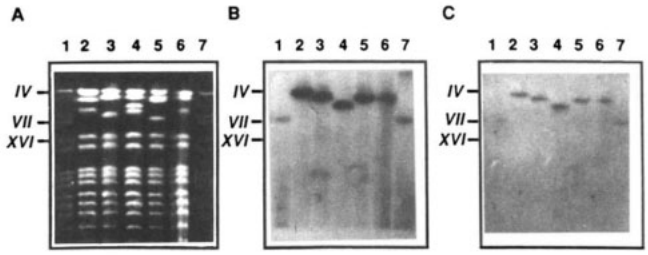

FIGURE 5.—Analysis of the segregation of the amplification in strain 7-2-1. Chromosomal DNA from strains 3-8-14 and 15-3-18 are in lanes 1 and 7, respectively. Lane 6 contains chromosomal DNA from amplification strain 7-2-1 and lanes 2–5 contain chromosomal DNA from spore colonies derived from a single tetrad of 7-2-1. The smaller yeast chromosomes have been run off the gel to achieve good separation of the larger chromosomes. Panel A is a photograph of the ethidium bromide stained pulsed field electrophoresis gel (CHEF). Panel B is a Southern blot of this gel hybridized to *ADH4* DNA and panel C is an identical blot hybridized to DNA from the centromere of chromosome *VII*. The locations of chromosomes *IV*, *VII* and *XVI* are indicated. Changes in the size of chromosomes other than chromosome *VII* occurred in some spores from amplification strains 3-2-9, 3-8-14, 5-3-6 and 7-2-1. This was unexpected since CP2AB, the parent strain, is an isogenic diploid and spores derived from CP2AB have chromosomes identical in size to CP2AB. However, similar differences in chromosome size are seen in tetrads from two of four antimycin A-resistant mutants isolated during these experiments that do not carry amplifications, suggesting that these variations occur in strains grown at 15° and are not directly related to amplification formation.

TABLE 3

Viability of spores from amplification strains

Strain	No. of tetrads with 0, 1, 2, 3 or 4 viable spores					Percent viable spores
	0	1	2	3	4	
15-3-18[a]					10	100 (40/40)
3-2-9	3	4	52			46 (108/236)
3-8-14				1	9	98 (39/40)
5-3-6	6	4	44			43 (92/216)
7-2-1					8	100 (32/32)
16-4-16		1	1	1	13	91 (58/64)

[a] Data from WALTON *et al.* (1986).

The chromosomal amplification also shows segregation of antimycin A resistance with the amplification (Table 3 and Figure 5). This amplification strain, 7-2-1, although diploid, shows only a single chromosomal band hybridizing to *ADH4* and the centromere of chromosome *VII*, suggesting that the same amplification is carried on both homologs of chromosome *VII*. Alternatively, one homolog of chromosome *VII* may have been lost, but this is unlikely since all eight tetrads dissected from this strain had four viable spores. Twenty seven of the spores were antimycin A resistant and contained larger than normal chromosomal bands which hybridized to *ADH4* and the centromere of chromosome *VII* (Figure 5). The remaining five antimycin A-sensitive spores had normal size chromosome *VII* bands that hybridized to both *ADH4* and the centromere of chromosome *VII*. Among the antimycin A-resistant spores the size of the band hybridizing to *ADH4* and the centromere of chromosome *VII* varies (Figure 5). The largest band is about the size of chromosome *IV* which is 1,650 kb. This is an increase of about 500 kb from the normal size of chromosome *VII*, 1120 kb, or 3% of the yeast genome. These results confirm that strain 7-2-1 carries a chromosomal amplification of *ADH4* which results in antimycin A resistance.

TABLE 4

Meiotic segregation of antimycin A resistance in *ADH4* amplification strains

Strain	Segregation of antimycin A resistance in 4-spored tetrads					Segregation of antimycin A resistance in 2-spored tetrads			Percent of spores in which an amplification can be detected by Southern blot analysis
	4:0	3:1	2:2	1:3	0:4	2:0	1:1	0:2	
15-3-18[a]	7	1			2				78 (31/40)
3-2-9						7	3	2	71 (17/24)
3-8-14	2		1	1	5				30 (12/36)[b]
5-3-6						2	2	5	44 (8/18)[c]
7-2-1	4	3	1						84 (27/32)
16-4-16	2				4				33 (8/24)

[a] Data from WALTON *et al.* (1986).

[b] Includes one antimycin A-sensitive spore colony in which the amplification was detected by Southern blot analysis.

[c] Includes two antimycin A-sensitive spore colonies in which the amplification was detected by Southern blot analysis.

Amplification copy number: The number of copies of an amplification can be estimated by comparing the intensity of hybridization of the genomic copy of *ADH4* with the intensity of hybridization of the amplified DNA on Southern blots of either genomic or chromosomal DNA. The amplification strains are diploid and the extrachromosomal amplifications each carry two copies of *ADH4* so a single copy of the amplification should result in extrachromosomal and chromosomal bands of equal intensity. The chromosomal amplification is present on both copies of chromosome *VII* so a single extra copy of *ADH4* on each homolog would also result in bands of equal intensity. Estimates of the number of copies of the extrachromosomal amplifications vary from 4 to 29 copies per cell while the chromosomal amplification was present in approximately 2 copies per cell, presumably one copy on each homolog of chromosome *VII* (Table 5).

DISCUSSION

The low amplification rates for *ADH4*, 10^{-10} amplifications per cell per generation, are consistent with the low amplification rates for *acp1*, 10^{-11}-10^{-12} duplications per mitosis (HANSCHE, BERES and LANGE 1978), the lack of amplifications in normal diploid mammalian cells (WRIGHT *et al.* 1990; TLSTY 1990) and the infrequent isolation of amplifications in other eukaryotic systems. It is interesting that amplifications

TABLE 5

Amplification copy number

Strain	Ratio of amplified copies of *ADH4* to genomic copies of *ADH4*[a]
15-3-18	28.8, 19.8, 13.4
3-2-9	8.0, 7.1
3-8-14	8.9, 7.0
5-3-6	7.5, 4.5, 7.5
7-2-1	1.1, 0.89, 1.3
16-4-16	17.2

[a] Each number represents an independent estimate of copy number from a separate lane on a Southern blot.

were only detected after the cells were grown at 15°. Ty transposition events are also more frequent at 15°(PAQUIN and WILLIAMSON 1984) suggesting that the types and frequencies of mutations that occur at 15° may be completely different than those that occur at 30°.

The isolation of five extrachromosomal linear palindromes demonstrates that this type of amplification is a rare but reproducible type of mutation in *S. cerevisiae*. The identification of both chromosomal and extrachromosomal amplifications demonstrates that, as in mammalian cells, amplification of a particular gene can be either chromosomal or extrachromosomal. All of the types of amplifications identified in mammalian tissue culture cells except extrachromosomal circular amplifications have been identified in *S. cerevisiae* [extrachromosomal amplifications of *ADH4* (WALTON *et al.* 1986; this paper); chromosomal amplifications of *ADH4* (this paper) and of *CUP1* (FOGEL and WELCH 1982); translocated chromosomal amplifications of *acp1* (HANSCHE, BERES and LANGE 1978) and of *ADH2* (PAQUIN *et al.* 1992); tandemly repeated *CUP1* genes (FOGEL and WELCH 1982); and inverted repeats of *ADH4* (WALTON *et al.* 1986; this paper)].

The isolation of two of six independent amplifications with identical restriction maps suggests that there may be preferential sites for novel joint formation. However since all of the remaining amplifications have unique novel joints, there must be many possible locations for novel joints adjacent to *ADH4*. Comparison of the DNA sequences at the novel joints of the extrachromosomal *ADH4* amplifications should suggest whether specific types of DNA sequences are preferred sites for amplification formation. The novel joint of the chromosomal amplification is located upstream of the *ADH4* open reading frame while the novel joints of all the extrachromosomal amplifications are downstream. The low copy number of the chromosomal amplification, two copies per cell or one copy per haploid genome and the presence of the novel joint upstream from the open reading frame of *ADH4* suggests the possibility that the antimycin A resistance of this strain may be due to increased expression of the amplified copies of *ADH4* rather than simply multiple copies of *ADH4*. The extrachromosomal amplification strains carry many extra copies of *ADH4* per cell [8–58 extra copies (see Table 5) and WALTON *et al* (1986) estimated that the original amplification strain 15-3-18 carried 64–128 extra copies of *ADH4*]. However, spore colonies with as few as two to three extra copies of *ADH4* per cell (or one to two copies of the amplification) are antimycin A resistant. Thus although it is unclear exactly how many copies of *ADH4* are required to confer antimycin A resistance upon a single cell, it appears that cells with as few as two copies of an extrachromosomal amplification (or 4 extra copies of *ADH4*) are antimycin A resistant and would be detected by this system.

The presence of recessive lethals in two of the amplification strains suggests that amplification may be accompanied by other changes in the genome. WINDLE *et al.* (1991) present evidence that chromosome breakage is involved in amplification formation in mammalian cells and that amplification is often accompanied by deletion of the amplified loci. It will be interesting to determine if the recessive lethals present in these amplification strains are due to deletions on chromosome *VII* that include the *ADH4* gene. In addition, if amplification is often accompanied by lethal recessive mutations amplification rates should be lower in haploids than in diploids.

The presence of the chromosomal amplification on both homologs of chromosome *VII* is surprising. The second copy of the amplification could be due to a secondary event rather than a concerted amplification of both copies of *ADH4*. Once an amplification is present on one copy of chromosome *VII*, a gene conversion event could result in the presence of the amplification on both homologs. Gene conversion events that result in changes in the number of copies of the tandemly repeated *CUP1* gene have been reported (WELCH, MALONEY and FOGEL 1990). In addition, loss of the amplification in five of 32 spores and variability in the size of the chromosome carrying the amplification suggests that recombination events that change the number of copies of the amplification occur meiotically. Cells carrying amplifications on both homologs might be selected for because of their increased production of *ADH4* and thus faster growth rate. An alternative hypothesis is that a broken chromosome *VII* was formed during the amplification event as suggested by the model of WINDLE *et al.* (1991). Then DNA repair of the broken chromosome *VII* from the intact chromosome *VII* carrying the amplification of *ADH4* resulted in both chromosomes carrying identical amplifications.

The ADH system was successfully used to isolate amplifications of *ADH4* even though less than 1% of the antimycin A-resistant mutants carry amplifica-

tions. The addition of a second dosage dependent gene adjacent to *ADH4* so that amplifications could be identified by their phenotype could improve this system. However, the system, as it is currently designed, is adequate to screen for environmental or genotypic factors that substantially increase amplification rates. For example, it should be possible to test the hypothesis that chromosome breakage increases amplification (WINDLE *et al.* 1991) by determining whether agents that result in chromosome breakage increase amplification rates. In addition, the characterization of the amplifications themselves may provide information about specific DNA sequences involved in amplification.

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