A Spontaneous Chromosomal Amplification of the ADH2 Gene in Saccharomyces cerevisiae

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ABSTRACT

A spontaneous anticin A-resistant mutant carrying approximately four extra copies of ADH2 on chromosome XII was isolated from yeast strain 315-1D which lacks a functional copy of ADH1 and thus is anticin A-sensitive. The additional copies of the normally glucose-repressed ADH2 are expressed during growth on glucose accounting for the anticin A resistance. These extra copies are inserted into nonadjacent ribosomal DNA sequences (rDNA) near the recombination stimulating sequence H0T1. Each extra copy of the ADH2 gene (1548 bp) replaces most of the 37S transcript (approximately 7400 bp) in one of the approximately 200 copies of the rDNA present in the yeast genome. All four extra copies of ADH2 are lost at a rate of approximately 1 × 10⁻¹⁰ deletions per cell per generation. One of the joints between the rDNA and ADH2 DNA is located 7 nucleotides downstream from 20 adenine residues in the normal copy of ADH2. This joint occurs at the end of a stretch of 16–29 thymidines in the rDNA which has been expanded to 57–59 thymidines. The other novel joint is located in a short region of sequence similarity between ADH2 and the rDNA. These observations suggest that amplification of ADH2 was a step two process: first the ADH2 gene was inserted into the rDNA, then multiple copies were generated by unequal crossing over or gene conversion within the rDNA.

PRIMAR Y amplification, the change from one copy of a gene per genome to two or more copies, is an important but poorly understood type of mutation. The large number of gene families attests to the importance of duplication and divergence of genes in the evolution of new functions (MAEDA and SMITHEES 1989). An understanding of primary amplification is fundamental to the understanding of the evolution of new functions. In addition, amplification is a mechanism by which genes can be overexpressed.

The first well documented amplification event was the Bar mutation in Drosophila (BRIDGES 1936). Since then, amplifications have been characterized in phage, bacteria, lower eukaryotes, plants and cultured mammalian cells (for reviews see ANDERSON and ROTH 1977; HAMLIN et al. 1984; STARK et al. 1989). The amplified DNA can be located on a chromosome or on circular or linear extrachromosomal DNA. Chromosomal amplifications may occur at the normal location for the amplified sequence or be translocated to other locations in the genome. Both tandem copies and inverted repeats of amplified DNA sequences have been reported.

Only a few amplifications have been reported in the yeast, Saccharomyces cerevisiae. Some naturally occurring strains of S. cerevisiae have high levels of copper resistance due to tandem direct chromosomal repeats of CUP1, the gene encoding copper chelatin (FOGEL and WELCH 1982). HANSCHE, BERES and LANGE (1978) report the duplication and translocation of the acid phosphatase locus at very low rates (10⁻¹¹–10⁻¹² duplications per mitosis), and GREER and FINK (1979) observed duplication of the his4 locus. A third type of amplification, extrachromosomal, linear palindromes containing two copies of the ADH4 locus (WALTON et al. 1986; C. PAQUIN, unpublished data) is also rare (10⁻¹⁰ amplifications/cell/generation at 15°C, M. DORSEY, C. PETERSON, K. BRAY and C. PAQUIN, unpublished data). The only amplification in S. cerevisiae that has been sequenced is the tandemly repeated CUP1 gene. The novel joint, the location where two previously separated DNA sequences are joined in the amplification, of the CUP1 repeat is 3 base pairs (bp) from a stretch of 15 adenines (KARIN et al. 1984). Seven of the nine sequenced novel joints of mammalian amplifications are in A-T rich regions (Reviewed in STARK et al. 1989). However, the significance of the poly(A) region near the novel joint of CUP1 is in question because this amplification is not a de novo mutation.

The ADH system has been useful for studying mutations that alter gene expression because compounds are available to select both for and against ADH expression. The structural genes for four yeast alcohol
dehydrogenase (ADH) isozymes have been identified, cloned and sequenced. These are ADH1, the classic fermentative isozyme (CIRIACY 1975a; WILLIAMSON et al. 1980); ADH2, the glucose-repressed isozyme (CIRIACY 1975a,b; RUSSELL et al. 1983b); 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. A strain lacking ADH1 cannot grow on medium containing glucose and antymycin A because this antibiotic blocks respiration and ADH activity is required for fermentation. In the presence of glucose, ADH2 and ADH4 are not normally expressed and ADH3 is confined to mitochondria. A mutation that allows the expression of either ADH2 or ADH4 in the presence of glucose will permit growth on medium containing glucose and antymycin A. In addition to this positive selection for ADH activity by antymycin A, it is possible to select against the ADH activity produced by ADH1, ADH2 and ADH3 using allyl alcohol which is converted by these ADH isozymes to the toxic compound acrolein.

Four types of mutations which allow ADH expression on glucose have been described: (1) Ty (Transposition yeast) insertions upstream from ADH2 (WILLIAMSON, YOUNG and CIRIACY 1981) and ADH4 (PAQUIN and WILLIAMSON 1986); (2) up promoter mutations at ADH2 (RUSSELL et al. 1983a); (3) mutations in the regulatory genes for ADH2, ADR1 (CIRIACY 1976), cre1 and cre2 (DENIS 1984); and (4) a chromosomal amplification and five independent extrachromosomal amplifications of ADH4 (WALTON et al. 1986; C. PAQUIN, unpublished data). The ADH1 system has been useful in selecting rare events such as Ty transposition and amplification because mutations to antymycin A resistance are rare (1 × 10^-7^-10^-9 mutations per cell per generation depending on the temperature at which the cells are grown; PAQUIN and WILLIAMSON 1984, 1986) and a single antymycin A-resistant mutant due specifically to increased ADH1 expression can be selected from a background of 10^9 antymycin A-sensitive cells on a plate.

An antymycin A-resistant mutant that contained multiple copies of ADH2 was identified among mutants screened for Ty insertions at ADH2. This mutant was analyzed in detail because of the very limited information on spontaneous, primary gene amplification events in S. cerevisiae. In addition, the DNA sequence of the novel joints of this de novo amplification were sequenced in order to gain insight into the mechanism of amplification.

MATERIALS AND METHODS

Yeast strains and media: Yeast strains used were 315-1D (MATa adh1-1 ADH2 ADH3 ADH4 ADR1 trpl ura), CP2A (MATa adh1-1 ADH2 ADH3 ADH4 ADR1 trpl ura), 2-4A (MATa adh1-1 ADH2 ADH3 ADH4 ADR1 ura 3-52) constructed by the authors and 161-2b (MATa adh1-11 ADH2 ADH3 ADH4 adr1-1 trpl ura) was obtained from CLYDE DENIS. CP30-20, an antymycin A-resistant mutant strain derived from strain 315-1D (from culture 20 grown at 30°), carries an amplification of ADH2 (ADH2-CA1, Chromosomal Amplification 1). CA1-1A and CA1-1D are haploids derived from a cross of 161-2b and CP30-20 which carry both an adr1-1 allele and ADH2-CA1. CA1-1A-AA^a-1 through CA1-1A-AA^a+3 are allyl alcohol-resistant mutants derived from CA1-1A. 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 antymycin A resistance was done on YEP medium containing 2% (wt/vol) glucose and 1 µg of antymycin A per ml at 30° (CIRIACY 1979). Selection for allyl alcohol resistance was done on YEP medium containing 2% (wt/vol) glucose and 0.6 µl of allyl alcohol per ml at 30° (CIRIACY 1979).

ADH assays: Extracts of yeast cells were prepared as described by CIRIACY (1975a) and ADH assays were carried out as described by WILLIAMSON, YOUNG and CIRIACY (1981).

Mutation rates: Mutation rates were estimated as previously described (PAQUIN and WILLIAMSON 1984, 1986). Mutation rates were calculated by the P_n method of LEA and COULSON (1949).

Southern blots, colony hybridizations and yeast transformations: Yeast genomic DNA was isolated by the method of DENIS and YOUNG (1983). Southern blots were carried out as described in KESSIG and BERRY (1983). Estimations of copy number were determined on a Helena Laboratories Quick Scan Densitometer. Colony hybridizations were done as described in TAUB and THOMPSON (1982). Yeast transformations were done by the method of KRAMER et al. (1989).

DNA sequencing and analysis: DNA sequence was determined by the method of SANGER, NICKLEN and COULSON (1977). M13 mp18 and 19 were used for M13 cloning (MESSING 1983). Plasmid sequencing was by the method of TEBOR and RICHARDSON (1987). DNA sequences were analyzed with Microgenie (Beckman) or PC Gene (Intelligentics). PC Gene's Hairpin program was used to find potential hairpin loops in DNA sequences (TINOCO et al. 1973).

Pulsed field gel electrophoresis: Pulsed field gels were run on a Bio-Rad CHEF (CHI, VOLLATH and DAVIS 1986) apparatus. Chromosomal DNA was prepared as described by CARLE and OLSON (1985). Positions of chromosomes were determined by hybridization with yeast probes and by comparison with published patterns (CARLE and OLSON 1985).

RESULTS

ADH2 is present in multiple copies in strain CP30-20: The amplification of ADH2 was one of two amplifications (the other amplification is described in WALTON et al. 1986) detected as extra bands in Southern blots of DNA from 208 spontaneous antymycin A-
resistant mutants screened for Ty insertions at ADH2 or ADH4 (Paquin and Williamson 1984, 1986). CP30-20 is one of 35 spontaneous antimycin A-resistant mutant strains selected from the haploid yeast strain 315-1D, which carries a deletion of the 5' region of ADH1 (Williamson, Beier and Young 1983), and is thus antimycin A-sensitive (Paquin and Williamson 1984). Mutants were obtained during growth on glucose at 30 °C, conditions under which the mutation rate to antimycin A resistance is about 5 × 10−6 mutations per cell per generation. Genomic DNA was prepared from 315-1D and CP30-20, digested with EcoRI, subjected to electrophoresis, blotted to nitrocellulose and probed with DNA containing the ADH2 open reading frame (Figure 1, panel A). Strain 315-1D shows only a single EcoRI band which contains both the 4.5-kb band representing the remaining 3' region of ADH1 and a 4.8-kb band representing the normal copy of ADH2, but CP30-20 shows both this band and an additional 3.2-kb band with homology to the ADH2 probe. An extra band is also seen when CP30-20 DNA is digested with HindIII (data not shown). The additional band is more intense than the normal band suggesting that multiple copies are present. To determine copy number, a Southern blot of a dilution series of CP30-20 DNA was probed with ADH2 DNA (Figure 1, panel D). In this Southern blot the 4.5-kb DNA fragment representing the 3' region of ADH1 is resolved from the 4.8-kb fragment of ADH2. The hybridization to the new band is at least four times as intense as hybridization to the single copy of ADH2 suggesting that there are approximately four new copies of ADH2 present in CP30-20. These results are confirmed by using densitometer tracings of lane 3 of Figure 1D. However, the densitometer was not able to resolve the 4.5-kb ADH1 band and the 4.8-kb ADH2 band. The relative absorbance of the 4.5-kb ADH1 and the 4.8-kb ADH2 bands is 35% vs. 65% for the 3.2-kb new band, a ratio of 1.9, again suggesting that there are approximately four extra copies of ADH2. Southern blots of CP30-20 DNA digested with Asp7181, EcoRI, HindIII, XbaI and EcoRV (data not shown) show no differences between the extra copies of ADH2 indicating that the size and insertion site of the four copies are very similar if not identical.

**Characterization of the amplification:** The extra 3.2-kb EcoRI band with homology to ADH2 segregated 2:2 as expected for a single nuclear gene (Figure 1, panel E) when CP30-20 was crossed to strain CP2A, which lacks ADH1, indicating that the new copies were linked genetically. The 3.2-kb band segregated with the antimycin A-resistant phenotype, confirming that the resistance was due to the extra copies of ADH2.

To determine if the new copies of ADH2 are regulated by ADRI, the positive regulator of ADH2, strain 161-2b, carrying a normal copy of ADH2, a null point mutation in ADH1 and a deletion of ADRI, was crossed to CP30-20. Strain 161-2b is antimycin A sensitive and allyl alcohol resistant even when grown without glucose because it is unable to express ADH2 due to the deletion of the positive regulator ADRI. The progeny of this cross segregated 2:2 for antimycin A resistance suggesting that the extra copies of ADH2...
are not under the control of ADRI. Segregants CAI-1A and CAI-1D from a single tetrad were shown to carry deletions in ADH1, a normal copy of ADH2 and the amplification ADH2-CAI by Southern blot analysis. Analysis of the other two segregants in the tetrad allowed us to conclude that CAI-1A and CAI-1D carried deletions of ADRI (the other two spores in the tetrad are antimycin A resistant and allyl alcohol sensitive when grown in the absence of glucose and so must carry the two functional copies of ADRI segregating in the tetrad). Both CAI-1A and CAI-1D are antimycin A resistant and allyl alcohol sensitive when grown on glucose as expected if ADRI function is not required for expression of the amplified copies.

The rate of appearance of allyl alcohol-resistant cells in these two strains was $1 \times 10^{-5}$ antimycin A-resistant cells per generation (Table 1). Southern blots of DNA digested with EcoRI from 13 of the allyl alcohol-resistant colonies (CAI-1A- AA$^R$-1 through CAI-1A- AA$^R$-13) showed loss of the new 3.2-kb band in every case. These results confirm that the antimycin A-resistant, allyl alcohol-sensitive phenotype is due to the extra copies of ADH2 and indicate that deletion of all four amplified copies of ADH2 is a frequent event compared to the rate at which genes are normally lost.

ADH assays were performed and specific activities calculated for yeast strains 315-1D, CP30-20, CAI-1A, CAI-1A- AA$^R$-5 and CAI-1A- AA$^R$-6 after growth on both ethanol and glucose (Table 2) to directly assess ADH activity in these strains. 315-1D, the parental strain, shows the expected repression of ADH activity when grown on glucose and derepression when grown on ethanol. CP30-20, carrying the extra copies of ADH2, has a high level of activity after growth on glucose and activity is induced to an even higher level on ethanol. CAI-1A, which carries the extra copies of ADH2 and a deletion in ADRI, has essentially the same levels of activity on both glucose and ethanol as CP30-20 indicating that most of the observed activity in CP30-20 is ADRI independent. CAI-1A- AA$^R$-5 and CAI-1A- AA$^R$-6 both show almost no activity under either growth condition as expected for strains carrying only a normal copy of ADH2 and deletions of ADH1 and ADRI.

**Table 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of appearance of allyl alcohol-resistant cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAI-1A</td>
<td>7.3 ± 6 × 10^{-6}</td>
</tr>
<tr>
<td></td>
<td>14 ± 10 × 10^{-6}</td>
</tr>
<tr>
<td></td>
<td>14 ± 10 × 10^{-6}</td>
</tr>
<tr>
<td>CAI-1D</td>
<td>6 ± 4 × 10^{-6}</td>
</tr>
<tr>
<td></td>
<td>6 ± 4 × 10^{-6}</td>
</tr>
</tbody>
</table>

* Events/cell/generation calculated by the P, method of LEA and COULSON (1949).

**Table 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>ADRI</th>
<th>ADH2</th>
<th>ADH2-CAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol</td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>315-1D</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CP30-20</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CAI-1A</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CAI-1A- AA$^R$-5</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CAI-1A- AA$^R$-6</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* All of these strains carry a deletion of the 5' region of ADH1.

**Attempt to isolate additional ADH2 amplifications:** The isolation of an amplification of ADH2 from among 35 antimycin A-resistant mutants selected from strain 315-1D suggested that such amplifications were rare but obtainable. In addition, evidence that expression of ADH2-CAI is independent of ADRI suggested that the background of antimycin A-resistant mutants that are not amplifications could be decreased by using a strain carrying a deletion of ADRI to eliminate ADRI+ mutations. A haploid strain, JL18C, carrying a deletion of ADRI has, as expected, a lower spontaneous mutation rate to antimycin A resistance ($I \pm 1 \times 10^{-9}$ mutations/cell/generation, mean and standard deviation of 18 mutation rate estimates) than related strains with an intact copy of ADRI ($6 \pm 1 \times 10^{-9}$ mutations/cell/generation, mean and standard deviation of four mutation rate estimates, PAQUIN and WILLIAMSON 1984). No amplifications of ADH2 were detected among 311 independent antimycin A-resistant mutants from 23 separate mutation rate experiments screened by Southern blot analysis. The rate of amplification in strain JL18C grown at 30° is $<5 \times 10^{-12}$ amplifications/cell/generation, if the data from all 23 experiments is combined and considered as a single amplification rate estimate.

**Cloning the novel ADH2 insertion:** DNA from CP30-20 was isolated, cut with EcoRI and subjected to electrophoresis on an agarose gel. DNA fragments of approximately 3.2 kb were electroeluted and ligated into pUC8. This DNA was used to transform *Escherichia coli* strain JM83 and the resultant colonies were screened by colony hybridization with the ADH2 probe. One colony was identified which showed hybridization to ADH2. The plasmid carried by this colony was designated ADH2-D. The restriction map of this insert is shown in Figure 2A. Southern blots probed with an EcoRI/HindIII fragment of ADH2-D (probe B, Figure 2) show hybridization to both the normal 4.8-kb ADH2 DNA and a 2.4-kb band in DNA.
Amplification of ADH2

A

Probes C and D hybridize to the 3.2-kb band and the 2.4-kb band, respectively. Probe A hybridizes to the 4.8-kb ADH2 band.

B

Probe A hybridizes only to the 2.4-kb band. The intensity of hybridization indicates that it corresponds to a repeated sequence. Probe B shows a lesser degree of hybridization to the 2.4-kb band because it contains only a short sequence from the insertion site. Therefore, ADH2-D includes both the 3' and 5' joints between the ADH2 DNA and the sequence into which each of the extra copies are inserted.

Sequence of the novel joints: The sequences of the joints of the ADH2 amplification were determined (Figure 3) and found to correspond to ribosomal DNA (rDNA) sequences indicating that ADH2 has inserted into ribosomal DNA. Comparison to rDNA sequences indicates that 1584 bp of ADH2 DNA replaces about 7400 bp of the 9060-bp rDNA repeat as shown in Figure 2B. The ADH2 gene replaces most of the 37S transcript and is flanked on both sides by a 5S coding region (Figure 2). The translocated copies of ADH2 do not include the upstream activator sequence (UAS) regulatory elements located upstream of ADH2 (Yu, Doniel, and Young 1989). However, both the "TATA" sequence and the normal mRNA initiation and termination sequences are present on the translocated DNA.

The restriction map of the extra copies of ADH2 obtained by Southern blot analysis combined with the known restriction map of the rDNA suggests that the copies of ADH2 are dispersed rather than tandemly repeated within the rDNA (Figure 2). Each rDNA repeat containing an extra copy of ADH2 carries a single EcoRI site located in the 37S transcript and a single HindIII site located within the open reading frame of ADH2. The 3.2-kb EcoRI fragment represents one complete rDNA-ADH2 repeat. Digestion with HindIII would result in a DNA fragment identical in size to the 3.2-kb EcoRI fragment if any of the rDNA repeats carrying ADH2 are tandemly repeated. However, when CP30-20 genomic DNA digested with HindIII or EcoRI, subjected to electrophoresis, blotted to nitrocellulose and probed with ADH2 DNA the 3.2-kb band is seen in the EcoRI digested DNA but only a smaller 2.9-kb band is seen in the HindIII digested DNA (data not shown). Only a 2.9-kb fragment is expected if all the extra copies of ADH2 are flanked by rDNA (see Figure 2C).

The novel joint 5' of ADH2 is located 215 bp upstream from the start of the open reading frame, about 148 bp upstream from the start of the normal ADH2 transcript (Figure 3A). This junction is 7 bp downstream of a string of 20 adenine residues. The
ADH2

size of the poly(T) tract in the rDNA is reported to vary from 16-29

transcript sequences for by an A or a T with the number

(b) is aligned with (a) the sequence of the 3' region of the

rDNA from CP30-20. This rDNA fragment had 28 Ts but the

rDNA had 28 Ts but the

number

of T's is expanded to 57-59 at this junction. It has not been possible to determine the exact number of T's

The novel joint 3' of ADH2 is located 325 bp downstream of the open reading frame, about 220 bp from the end of the normal ADH2 transcript (Figure 3B). This joint in the rDNA is 264 bp downstream from the 5' end of the 37S transcript in the external transcribed spacer region. The exact nucleotide of the novel joint is ambiguous because the ADH2 DNA and the rDNA share identical 2 bp at the novel joint (Figure 3B).

**Genomic location of ADH2-CA1:** To determine the chromosomal location of the amplification, CP30-20 and 315-1D chromosomal DNA was separated on pulsed field gels (CHEF; CHU, VOLLRATH and DAVIS 1986) blotted to nitrocellulose and probed with the ADH2 open reading frame (data not shown). Hybridization was seen to chromosomes XIII and XV, the endogenous locations of ADH2 and ADH1, respectively, in both strains. CP30-20 shows hybridization to chromosome XII as well. Identical Southern blots probed with rDNA show hybridization to chromosome XII in both strains 315-1D and CP30-20. These results indicate that, as expected, the extra homology to ADH2 is located on chromosome XII, the chromosome on which the rDNA is located (PETES 1983).

**Insertion of a single copy of the ADH2 amplification into the rDNA:** A single copy of the amplified ADH2 locus was inserted into the rDNA using recombinant DNA techniques to determine whether all four copies of ADH2 are necessary for antimycin A resistance. The EcoRI fragment of the ADH2-D clone containing the ADH2 gene flanked by rDNA sequences was subcloned into the EcoRI site of the yeast integrating plasmid YIp5. This plasmid was partially cut with BstEII and transformed into yeast strain 2-4A which carries a deletion of the ADH1 gene and the *ura3-52* allele. Transformants were selected for growth on minimal media lacking uracil to select for integration of the YIp5 plasmid which contains the *URA3* gene. Three of the transformants were shown by Southern blot analysis to carry a single copy of the ADH2 gene inserted into the rDNA. All three transformants were antimycin A-resistant and had ADH activities of 785, 805 and 975 milliunits/mg protein when grown on glucose. These strains have approximately a quarter of the ADH activity of the original amplification strain which carries four copies of ADH2.
Amplification of ADH2

DISCUSSION

The amplification of ADH2 is a rare event, occurring in only one of 35 antimycin A-resistant mutants under conditions where the mutation rate to antimycin A resistance is approximately 10^{-9} (Paquin and Williamson 1986). A diligent search for similar events has been unsuccessful and thus their occurrence is estimated to be <5 \times 10^{-12} amplifications/cell/generation. Our detection of amplifications of ADH2 may be restricted by a requirement for one of the endpoints to remove the upstream regulatory regions of ADH2. Thus it may be that some amplifications were not detected because not enough ADH activity was produced. Our low estimate of amplification rates in yeast cells is in agreement with recently published reports that amplification frequencies are less than 10^{-8} in normal diploid human and rodent cells (Wright et al. 1990; Tilsty 1990). Amplifications were detected at frequencies as high as 10^{-3} in transformed cell lines but no amplifications were detected in normal human or rodent cells.

The extra copies of ADH2 are highly expressed even during growth on glucose which normally suppresses ADH2 expression. Even a single copy of the ADH2 gene is sufficient to confer antimycin A resistance. The 37S rRNA and the ADH2 gene are transcribed from complementary DNA strands so the increased expression of ADH2 can not be due to read through from the promoter of the 37S RNA transcript. The 5S gene is transcribed in the same direction as the ADH2 gene but the normal termination of the transcript is before the junction between ADH2 DNA and rDNA. The amplified copies of ADH2 are no longer under the control of ADR1, presumably because the UAS to which ADR1 binds (Yu, Doniél and Young 1989) is not present in the amplified copies. Several examples of Ty insertion mutants between the poly(A) sequence and the transcription start of ADH2, separating the poly(A) stretch and the UAS from the transcription start, have been characterized (Williamson, Young and Ciriacy 1981; Williamson et al. 1983a). In these mutants, ADH2 is expressed in the presence of glucose and the transcription start sites are identical to those in derepressed cells. Thus, it is likely that the transcription start site of ADH2-D is the same as wild type. Other changes in the DNA sequence of the extra copies of ADH2 could affect their regulation and expression. The 5' junction creates a sequence similar to two previously described antimycin A-resistant up-promoter mutations at ADH2 that still carry the ADR1 binding site (Russell et al. 1983a). These up promoter mutations are deleted for the identical 4 bp downstream of the poly(A) tract and have an increase in the length of the poly(A) tract to 54 or 55 adenines. In addition the levels of ADH in these promoter-up mutants are high on glucose (516 and 410 milliunits/mg) and further derepressed on ethanol (4440 and 4680 milliunits/mg; Williamson, Young and Ciriacy 1981; Russell et al. 1983a), similar to the ADH2 amplification strain. Struhl (1985) proposes that upstream poly(A) sequences serve as promoter elements for constitutive expression of yeast genes and that the longer poly(A) sequences are more effective promoter elements.

The results presented above demonstrate that the extra copies of ADH2 are located in the rDNA. The amplification of ADH2 was most likely a two-step process in which ADH2 was first inserted within the rDNA followed by an increase in the number of copies of ADH2 due to unequal crossing over or gene conversion within the rDNA repeats. The amplification of ADH2 is unlikely to be due to insertion of a cDNA copy of ADH2 mRNA because the amplified DNA includes both DNA upstream of the mRNA initiation site and DNA downstream of the mRNA termination site. A model can be proposed for the mechanism of the original insertion into the rDNA based on the results obtained. The initial event was likely to have been similar to a gene conversion since the parent strain is a haploid and the original ADH2 gene is still present at its normal location. In this model, a single strand of ADH2 invades the ribosomal DNA because of the weak sequence similarity (70-75%, see Figure 3B) at the 3' novel joint. The region of maximum similarity is a 12-bp region of 75% similarity extending from 6 bp upstream of the novel joint through the identical 2 bp and ending 4 bp downstream from the novel joint (nucleotides 1349-1372 in Figure 3B). The 5' novel joint is near a poly(A) region and the mechanism may involve the ease with which poly(A) stretches are denatured. Poly(A) regions have been found near the novel joints in mammalian amplifications (reviewed in Stark et al. 1989) and the CUP1 novel joint in yeast (Karlin et al. 1984). However, the poly(A) region upstream of ADH2 is on the opposite strand of the poly(A) region in the rDNA suggesting that the mechanism by which this joint is formed is more complicated than recombination between poly(A) stretches. It may be significant that the recombination stimulating sequence, HOT1, is located adjacent to the insertion site of ADH2 in the rDNA (Stewart and Roeder 1989) and in the proper orientation to promote recombination (see Figure 2). In addition, the chromosome containing HOT1 preferentially receives information in gene conversion events promoted by HOT1 (Voelkel-Meiman and Roeder 1990).

In this model, the second step in the amplification of ADH2 is the generation of multiple copies within the rDNA. A single copy of the ADH2 gene in the rDNA is sufficient to confer antimycin A resistance but four copies result in a level of ADH activity close...
to the 4908 milliunits/mg protein found in strains with an intact copy of \textit{ADH1}. Cells carrying multiple copies of the \textit{ADH2} gene in the rDNA are thus likely to have faster growth rates due to higher levels of fermentation. Petes (1980) and Szostak and Wu (1980) investigated meiotic and mitotic unequal crossing over, respectively, in the tandemly repeated rDNA of \textit{S. cerevisiae} using a \textit{LEU2} gene inserted into the rDNA with recombinant DNA techniques. They detected both increases and decreases in the copy number of the \textit{LEU2} gene. Welch, Maloney and Fogel (1990) have shown that both gene conversion and unequal crossing over contribute to variation in copy number of the tandemly repeated \textit{CUP1} gene in \textit{S. cerevisiae}. The high rate of loss of all of the extra copies of \textit{ADH2}, $1 \times 10^{-5}$ deletions per cell per generation, suggests that gene conversion or unequal crossing over could account for both gain and loss of \textit{ADH2} sequences within the rDNA. Szostak and Wu (1980) reported that the \textit{LEU2} gene inserted into the rDNA by recombinant DNA techniques was lost during mitotic growth at a frequency of $5 \times 10^{-4}$ deletions per generation. In addition, duplications of \textit{LEU2} were separated by one to five rDNA repeats and the extra copies of \textit{ADH2} are separated by at least one rDNA repeat. Thus the results reported here are completely consistent with insertion of \textit{ADH2} into the rDNA followed by amplification of \textit{ADH2} by gene conversion or unequal crossing over.

Amplifications involving rDNA genes have been reported in both bacteria and mammalian cells. Anderson and Roth (1981) showed, that in one region of the \textit{Salmonella typhimurium} genome a common amplification type has endpoints in the rDNA genes which are highly iterated. Wahl, Vrtto and Rubintz 1983 report coamplification of rDNA with the CAD gene in Syrian hamster cells. However, the mutant described here is the first report of amplification by insertion into a repeated sequence. Once a unique sequence is inserted into a repeated sequence, unequal crossing over or gene conversion among the repeated sequences can result in a high rate of variation in the copy number of the amplified gene. In addition, our results suggest that recombination between short regions of similarity and interactions between poly(A) regions are involved in chromosomal primary amplification in \textit{S. cerevisiae}.

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LITERATURE CITED


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