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The Presence of a Defective *LEU2* Gene on 2μ DNA Recombinant Plasmids of *Saccharomyces cerevisiae* Is Responsible for Curing and High Copy Number

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The copy number of 2μ DNA-derived plasmids in *CIR*$^+$ *Saccharomyces cerevisiae* transformants is determined by its selective marker and is usually much lower than that of the endogenous plasmid. Only plasmids containing the *leu2* allele of pJDB219, designated as *leu2-d*, under selective conditions displayed a higher copy number than did endogenous 2μ DNA and by displacement generated cured cells. Spontaneous loss of 2μ DNA occurred with a frequency of about 0.02% per generation. Curing plasmids, like pMP78, have copy numbers of 35; noncuring plasmids, like pDB248 or YEp6, have copy numbers of 4 to 8. The 2μ DNA copy number in strains AH22 and YNN27 were determined to be 40 and 100, respectively. The high copy number of *leu2-d*-containing plasmids can be explained by its weak expression of less than 5% that of the wild-type *LEU2* gene. The *leu2-d* allele has a deletion of the 5′-end sequence starting from 29 base pairs before the ATG initiation codon, but surprisingly, its expression is still regulated. On YRp7, which contains the chromosomal autonomic replication sequence *ARS1*, the defective *leu2-d* allele could not complement a *leu2* host strain. This suggests a more stringent control of replication of *ARS1*-containing plasmids than of 2μ-containing plasmids.

Saccharomyces cerevisiae recombinant plasmids carrying 2μ DNA or its origin of replication use the same replication and propagation systems as the endogenous 2μ DNA. In transformed cells containing such a recombinant plasmid in addition to the endogenous plasmid, this generally leads to segregation and loss of the recombinant plasmid. Depending on the type of vector and the DNA inserted into it, the observed instability varies between 1 and 5% loss per generation (3, 13, 15, 23, 35, 36).

In a stability study of the 2μ DNA-derived vector pMP78, we found that transformants that lost pMP78 almost always lost the endogenous 2μ DNA as well and became *cir*^{0} (12). This very efficient curing of endogenous 2μ DNA by pMP78 was explained by the relatively high copy number of the latter. For stable maintenance in the cell, 2μ DNA and its derivatives require the *trans*-acting *REP* products of reading frames B and C (7). Since pMP78 lacks reading frames B and C, it is dependent on endogenous 2μ DNA for its stability. The high copy number of pMP78 leads to the loss of endogenous 2μ DNA; in such *cir*^{0} cells pMP78 is unstable and is lost at a frequency of 10% per generation. The 2μ DNA sequences of pMP78 (18) are derived from pJDB219 (3), which displays the same high copy number (3) and has been reported to cause occasional loss of endogenous 2μ DNA (9).

Tests of other 2μ DNA vectors, consisting of the same 2μ DNA fragment but carrying different selective markers, did not reveal any plasmid with the properties of pMP78 or pJDB219. We considered two alternative explanations for the high copy number of pMP78 and pJDB219 in transformants. (i) The integration of the *LEU2* gene fragment by AT tailing in pJDB219 (3) creates conditions that favor its replication and propagation over that of endogenous 2μ DNA, or (ii) the expression of the *LEU2* gene present on the vector is very low, and a high copy number of the plasmid is required for growth under conditions selective for leucine. The second hypothesis is favored by our recent report that curing does not take place under transformation conditions which are nonselective for the *LEU2* gene (33), indicating that this process is correlated with *LEU2* expression.

In this report we studied further the curing and replication properties of plasmids and found that the *LEU2* allele (designated as *leu2-d*) on pMP78 and pJDB219 allows only a very low

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level of gene expression. This reduced expression is probably a result of a deletion of regulatory sequences at the 5′ end of the structural gene. We also show that the replication behavior of yeast vectors like YRp7 carrying the *ARS1* origin is such that they are incapable of amplification to compensate for the poor *leu2-d* expression; in this respect they are different from 2μ DNA vectors, probably due to a different stringency of replication control during the cell cycle.

MATERIALS AND METHODS

Yeast strains, recombinant plasmids, and transformation. The yeast strains used and their genotypes are as follows: AH22, *leu2-3 leu2-112 his4-519 can1*; GRF18, *leu2-3 leu2-112 his3-11 his3-15* (a gift from G. Fink); YNN27, *ura3-52 trp1-289 gal2* (34); AY2a, *leu2-3 leu2-112 his4-519 trp1-289* (obtained from a cross between strains AH22 and YNN27). The following plasmids were used; pJDB219 (3), pMP78 (18), pADH040-2 (33), pYeleu10 (32), Yep6 (36), pDB248 (2), pMA56 (17). Plasmids constructed for this work were pHKB52, pL623, pL610, pL616, pL72, YRp7-*leu2-d*, and YRp7-*LEU2*. Yeast strains were transformed by the method of Beggs (3).

Yeast colony hybridization was performed as described by Hinnen et al. (16), and detection of β-lactamase was performed as described previously (33).

Plasmid construction. Plasmid constructions were performed in *Escherichia coli* JA221 *recA1 leuB6 ΔtrpE5 hsdR hsdM⁺ lacY* or JA300 *thr leuB6 thi thyA trpC1117 hsdR hsdM Str^r* (37). The procedures used for plasmid DNA isolation, restriction mapping, ligation, and transformation are general and have been described previously (5, 12).

Yeast minilysates were prepared by the method of Struhl et al. (36), and DNA sequencing was carried out according to Maxam and Gilbert (28).

β-Isopropylmalate dehydrogenase assay. Yeast cells were grown in minimal medium (2% glucose, 6.7 g of yeast nitrogen base, and 1 g of ammonium sulfate per liter) supplemented with amino acids (20 μg/ml) as required or in rich YEPD medium, (1% yeast extract, 2% peptone, 2% glucose).

The cell extract was prepared essentially as described by Kohlhaw et al. (25). Cells were collected in the late logarithmic phase, washed twice in cold 0.1 M potassium phosphate buffer (pH 6.9) containing 1.25 M ammonium sulfate, and finally suspended in 2 ml of phosphate buffer per 1 g (wet weight) of yeast cells. The cells were then mixed with 2 g of glass beads (0.45 mm diameter) and homogenized for 1 min in a Braun homogenizer at maximum speed. The homogenate was centrifuged at 12,000 × *g* for 30 min at 2°C. Portions of the resulting supernatant were used for enzyme assays. The activity of β-isopropylmalate dehydrogenase was assayed by the method of Parsons and Burns (30). The β-isopropylmalate substrate was synthesized by H. Dickopp, Institute for Organic Chemistry, University of Düsseldorf, by the method of Calvo et al. (8). Specific activity is defined as the formation of 1 nmol of NADH per min per mg of protein. Protein concentration was determined by the method of Lowry et al. (27) with bovine serum albumin used as a standard.

RESULTS

Curing is caused only by plasmids carrying the *leu2-d* allele derived from pJDB219. To investigate the cause of curing, we screened a number of 2μ DNA-derived recombinant plasmids differing in the selective marker, the 2μ DNA part, or the bacterial DNA part (for plasmid maps, see Fig. 1). Table 1 shows that the bacterial vector sequences are not involved in the curing process. For instance, the bacterial vector pBR322 can be found on yeast plasmids that cure, such as pMP78 and pADHO40-2, but also on those that do not cure, such as pDB248, pHKB52, and YEp6. The curing is also not the result of a specific part of the 2μ DNA on the vector. Except for the origin of replication fragment that is required for the replication of all 2μ DNA vectors in yeasts, no additional 2μ DNA sequences like the *REP* or *FLP* genes (6) are essential for curing. Only the selective gene used for transformation was found to be determinative. The *leu2-d* gene on pMP78 is contained in a 1.3-kilobase (kb) DNA fragment which was cloned from strain M127 into pJDB219 by Beggs (3). Using this allele, we found that after growth of AH22(pMP78) transformants on rich medium, two typical genotypes with respect to the plasmid content were obtained. First, cells which were still Leu⁺ and in most cases contained the original 2μ and pMP78 were obtained. Second, cells which became Leu⁻ arose with a frequency of 1.6% per generation (Table 1). They did not hybridize at all in the colony hybridization experiments and in all cases had lost 2μ DNA as well as pMP78. These cells were cured. The fact that we never found Leu⁻ descendants of pMP78 transformants which had retained endogenous 2μ DNA indicates that under those conditions, the 2μ DNA is lost first, followed by a loss of pMP78. Transformants containing recombinant plasmids carrying the *HIS3* or the *TRP1* gene did not lose the endogenous 2μ DNA (Table 1). Such plasmids were lost at the rate of about 1.4 to 3% of the transformants per generation, and the resulting cells all contained 2μ DNA.

To establish whether the curing was a general characteristic of the *LEU2* gene, we analyzed two additional independently cloned *LEU2* genes from pYeleu10 (32) and pJDB248 (3). pHKB52 (Fig. 1) carries the *LEU2* gene on a 2.2-kb *SalI-XhoI* fragment from pYeleu10 originally cloned from strain S288c (32). pDB248 (Fig. 1) (2) carries the *LEU2* gene on a 2.4-kb fragment cloned by Beggs (3) from strain M127. As can be seen in Table 2, these *LEU2* alleles on similar 2μ DNA vectors did not manifest the curing phenomenon. Curing is clearly restricted to plasmids carrying the *leu2-d* allele derived from pJDB219.

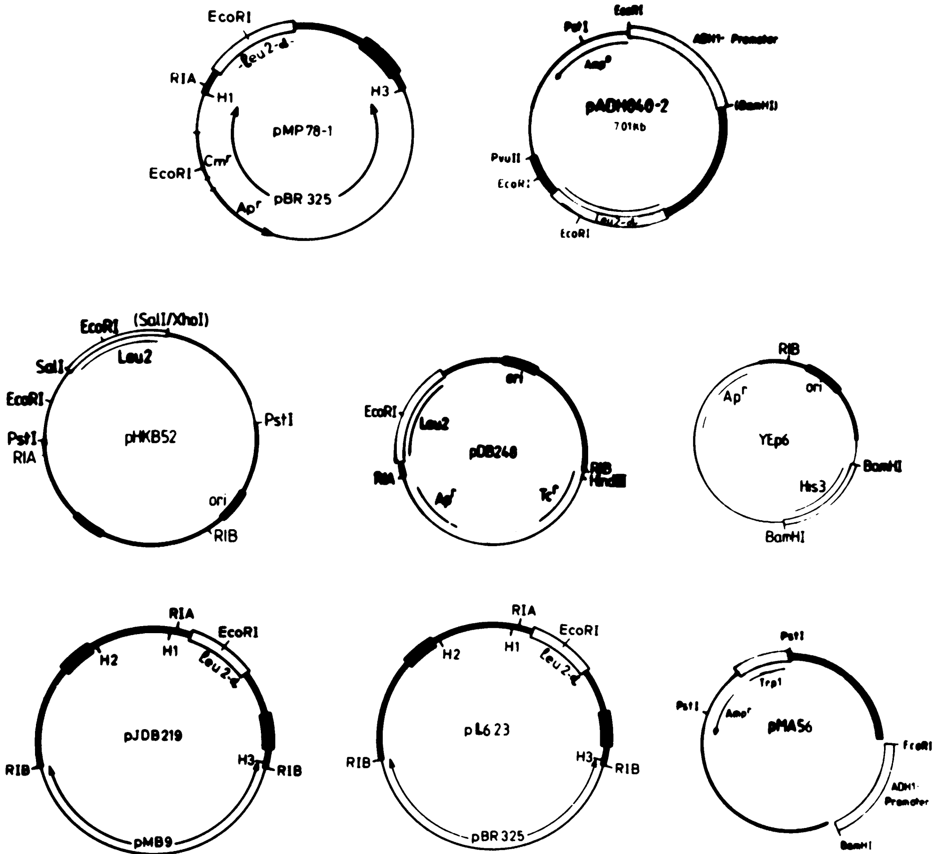

FIG. 1. Scheme of plasmids used for the curing studies. pMP78 (18) consists of pBR325 and fragment *Hin*dIII-3 of the 2μ DNA from pJDB219 containing *leu2-d*. pADHO40-2 (33) contains the *Bcl*I-*Pvu*II fragment from pJDB219 carrying *leu2-d*. The *Bcl*I-*Pvu*II fragment is very similar to the *Hin*dIII-3 fragment. pHKB52 contains the total 2μ DNA form 14 (B) inserted in pBR322 carrying *LEU2* from pYeleu10. pDB248 (2) contains the total 2μ DNA form 23 (A) and $LEU2^+$ from pJDB248 inserted in pBR322. YEp6 (36) contains a 2μ DNA fragment of form 14 (B) inserted in pBR322 carrying *HIS3*. pJDB219 (3) contains the total 2μ DNA form 14 (B) comprising *leu2-d* and pMB9. PL623 contains total 2μ DNA form 23 (A) comprising *leu2-d* and pBR325. pMA56 (17) contains a 2μ DNA fragment of form 14 (B), the *TRP1* gene, the *ADH1* promoter, and part of pBR322. H1, H2, and H3 refer to *Hin*dIII sites, and RIA and RIB refer to *Eco*RI sites on 2μ DNA. Cm^r, Ap^r, and Tc^r indicate resistance genes for chloramphenicol, ampicillin, and tetracycline, respectively.

In the case of noncuring plasmids, we found cir^0 cells at a low frequency (about 0.02 to 0.1% per generation) which, when compared with the 0.02% loss rate measured for nontransformed AH22, indicates that this reflects the spontaneous loss of 2μ DNA from this strain. This is slightly increased by pDB248 and might reflect a low curing activity.

Curing is correlated with high copy number. We previously proposed that the curing of 2μ DNA is due to displacement of the endogenous plasmid by the recombinant plasmid present in a higher copy number (12). To verify this proposal, we determined the copy number ratio of recombinant plasmid to 2μ DNA and the absolute number of plasmids per cell in a number of different transformants. The plasmid content of transformants containing curing or noncuring plasmids that differ in their selective marker was compared directly after transformation. Single transformant colonies were selectively grown overnight. The plasmid DNA was analyzed on Southern blots by hybridization with labeled pL623 (Fig. 1), a recombinant plasmid containing pBR325, whole 2μ DNA, and the *LEU2* gene. With this probe, all plasmid sequences hybridized so that the amount of recombinant DNA molecules and endogenous 2μ DNA could be compared.

The plasmid composition of typical AH22(pMP78) and AH22(pDB248) transformants after selection for *LEU2* expression is compared in Fig. 2. The copy number of pMP78 is much higher than that of endogenous 2μ

TABLE 1. Curing effect of different recombinant plasmids in *S. cerevisiae*

Host strain	Plasmid	Bacterial vector	2μ DNA fragment	Selective marker	% Loss of transformants/ generation on YEPD[a]	Curing[b]
AH22	pMP78	pBR325	*Hin*dIII-3	*leu2-d*	1.6	+
AH22	pADHO40-2	pBR322	Part of *Hin*dIII-3	*leu2-d*	1.4	+
GRF18	YEp6	pBR322	Part of *Hin*dIII-3	*HIS3*	3.2	−
YNN27	pMA56	pBR322	*Hin*dIII-3	*TRP1*	1.4	−
AH22	pJDB219	pMB9	Whole 2μ DNA	*leu2-d*	1.0	+

[a] Cells from single colonies were inoculated into rich medium (YEPD). After different periods up to 50 generations, portions of each culture were streaked on rich medium agar to obtain single colonies. These were examined for the presence of recombinant plasmids by testing for the presence of the selective marker. Plasmids containing pBR325 or pBR322 with an intact β-lactamase gene *(bla)* were identified by their β-lactamase activity as well.

[b] The presence of recombinant plasmids and natural 2μ DNA was also determined by colony hybridization with pL623, a plasmid containing pBR325, whole 2μ DNA, and the *LEU2* gene.

DNA, which has declined drastically. The two *Hin*dIII bands of pMP78 are very strong; the bands of endogenous 2μ DNA are very weak. Besides the pMP78 and 2μ DNA bands, an additional band is always present: the *Hin*dIII-1–*leu2* fragment of the 2μ plasmid which is created by intermolecular recombination between pMP78 and 2μ DNA (10, 12, 29). In some pMP78 transformants, only pMP78 is present, and 2μ DNA is lost completely. Figure 2 clearly shows that the copy number of the curing plasmid pMP78 is much higher than that of endogenous 2μ DNA, and the copy number of the noncuring plasmid pDB248 is much lower than that of the endogenous 2μ DNA. It can also be seen that individual colonies do not differ significantly in their plasmid composition. The slight variation in the total amount of plasmid DNA present in the lysates is due to variability in the efficiency of extractions. Since the transformants were grown selectively, the plasmid DNA analysis reflects the situation in cells that are still transformants and have not yet lost the recombinant plasmid.

The same correlation between high copy number and curing was observed when the curing pJDB219 and the noncuring pHKB52 or YEp6 (for plasmid maps, see Fig. 1) were compared. The copy number of the curing plasmid pJDB219 is very high, whereas pHKB52 and YEp6 have a low copy number (less than 10; data not shown).

To obtain a more accurate estimate of the relative number of *leu2-d* plasmids per cell, DNA from AH22(pMP78) transformants was digested with *Hin*dIII and fractionated on a gel in dilutions over a 50-fold range. After transfer to nitrocellulose, the DNA bands were hybridized to plasmid pL623 which contains all plasmid sequences and therefore should hybridize equally well to all fragments. As can be seen in Fig. 3A, the intensity of the *Hin*dIII-2 band of the 2μ DNA in the 1:2 dilution has about the same intensity as the *Hin*dIII-3–*leu2* band of pMP78 in the 1:30 dilution. Since fragment *Hin*dIII-2 occurs only in 50% of the 2μ DNA molecules, namely those of type B (type 14 in our nomenclature [20]), the transformants contain about seven times more pMP78 than 2μ DNA molecules. From the same type of comparison for AH22(pDB248) transformants (Fig. 2 and 3B), we estimate a ratio of 1:4 between pDB248 and 2μ DNA.

In summary, these comparative data show that a selective marker can influence the copy number and that only the *leu2-d* allele under selective conditions raises the copy number of its plasmid above that of the endogenous 2μ DNA. This difference is then amplified during successive replication and transmission at cell division and leads to segregation of cir^0 cells. The noncuring plasmids are present in a much lower copy number than the endogenous 2μ DNA and segregate out after cell division.

A comparison of the band intensities in Fig. 3, and in additional gels not shown here, indicate that the total number of plasmid molecules per transformed AH22 cell is constant and equal to the copy number of the 2μ DNA in untransformed AH22 cells. This conclusion is in agreement with a similar observation of Gerbaud and Guerineau (14) for other *S. cerevisiae* strains. To

TABLE 2. Comparison of the curing effect of recombinant plasmids carrying different *LEU2* alleles after 50 generations on complete medium (YEPD)

Plasmid[a]	Selective marker and its origin	% cir^0/ rec. plasmid[0b]	% CIR^+/ rec. plasmid[0]	% CIR^+/ rec. plasmid[+]
pMP78	*leu2-d*	80	0	20
pDB48	*LEU2* from pJDB248	5	78	17
pHKB	*LEU2* from pYeleu10	1	69	30

[a] Host strain AH22. About 250 colonies were tested during each experiment.

[b] rec. plasmid, Recombinant plasmid.

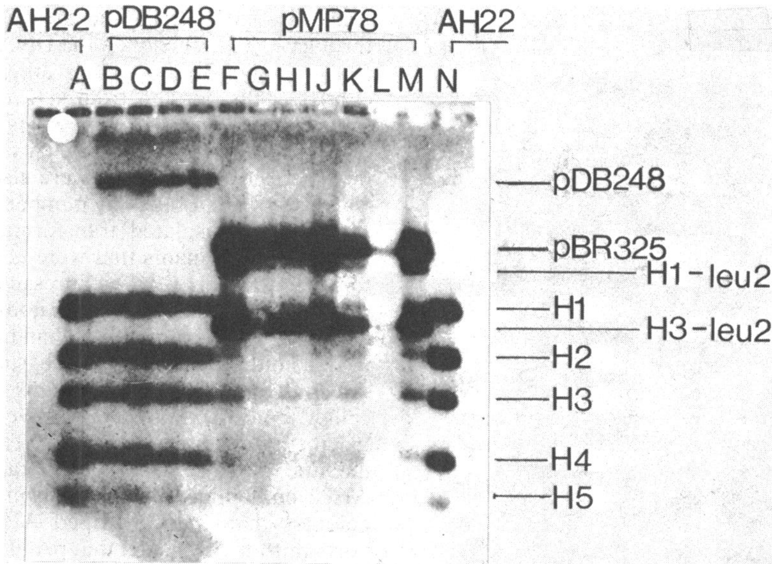

FIG. 2. Analysis of plasmid DNA from different transformants after selective growth. Minilysates from pDB248 and pMP78 transformants were digested with *Hin*dIII and hybridized with ^{32}P-labeled pL623. AH22 was the control. H1 through H5 indicate the *Hin*dIII fragments from 2μ DNA. Bands indicated as pBR325 and H3-*leu2* originate from pMP78. pDB248 is cut only once with *Hin*dIII. H1-*leu2* indicates a fragment that is derived from 2μ DNA-*leu2-d*, a recombination product in pMP78 transformants (10, 12).

determine the exact number of 2μ plasmids in strain AH22, total DNA was isolated, digested with *Hin*dIII, and after Southern transfer, hybridized to probe pL623 containing total 2μ DNA and the *leu2-d* DNA. This probe hybridizes equally to all 2μ DNA fragments and, in addition, to the chromosomal *LEU2* gene sequences used here as an internal marker. Chromosomal *LEU2* is located on a 10-kb *Hin*dIII fragment (11), from which only a 1.3-kb segment is present on the hybridization probe and thus can hybridize. Figure 4 shows that the band with the chromosomal *LEU2* fragment has about the same intensity as the *Hin*dIII-4 band of the 1:40 dilution. Since the size of the *Hin*dIII fragment is 1.3 kb as well and occurs in both types of 2μ DNA, the number of 2μ DNA molecules in AH22 is about 40 per cell. This is a maximal estimate, since we assume that the 10-kb chromosomal band is transferred to the hybridization filter with the same efficiency as the shorter fragments. On the basis of these copy numbers, we estimate there to be in AH22(pMP78) 5 2μ DNA and 35 pMP78 molecules per cell and in AH22(pDB248) about 32 μ DNA and 8 pDB248 molecules per cell.

A comparison of strains AH22 and YNN27 showed that YNN27 has at least 2.5 times as many plasmid molecules per cell as AH22 (data not shown). This means a copy number of approximately 100 for YNN27. This is similar to *S. cerevisiae* H1 that we found to contain the highest number, 100 copies per cell (19), among laboratory strains.

The spontaneous loss of 2μ DNA mentioned earlier was compared for AH22 and YNN27 by screening after 50 generations for the presence of 2μ DNA by colony hybridization. Among 800 colonies tested, about 1% of AH22 and 0.5% of YNN27 colonies were *cir*^{0}. The lower frequency of YNN27 *cir*^{0} cells could reflect the higher copy number of the 2μ DNA plasmid. DNA of spontaneous *cir*^{0} cells was analyzed on Southern blots, and no 2μ DNA sequences could be detected. This means that the *cir*^{0} cell lost all 2μ DNA information and that this loss is irreversible. To detect a possible difference in growth rate, we performed cocultivation experiments of equal numbers of *cir*^{0} and *CIR*$^+$ cells in complete and minimal medium. After every 100 generations, cells were plated out and the colonies were screened for 2μ DNA by hybridization. Over a period of 700 generations we found no significant change in the ratio of *CIR*$^+$ to *cir*^{0} cells.

***leu2-d* plasmids cure only under selective conditions.** We previously described a plasmid, pADHO40-2, that enables the detection of *S. cerevisiae* transformants under nonselective conditions (33). The plasmid contains as an indicator the bacterial β-lactamase gene (*bla*) under the control of the yeast *ADH1* promotor. Colonies of transformed cells can easily be differentiated from those of nontransformed cells on *bla* indicator plates (33) containing complete

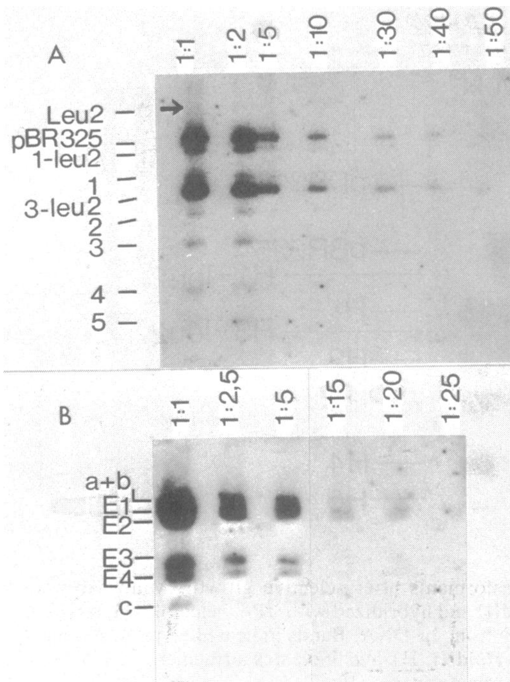

FIG. 3. Estimation of relative amounts of pMP78 and 2μ DNA (A) and pDB248 and 2μ DNA (B) in selectively grown transformants. (A) DNA isolated from AH22(pMP78) was digested with *Hin*dIII and applied to an agarose gel in decreasing amounts. pMP78 was cut in pBR325 and *Hin*dIII-3–*leu2*. The *Hin*dIII-1–*leu2* fragments originated from 2μ DNA-*leu2-d* generated by recombination (cf. reference 12). 1 to 5 are the *Hin*dIII fragments from endogenous 2μ DNA. The chromosomal *LEU2* fragment is barely visible and is indicated with an arrow. (B) DNA from AH22(pDB248) was digested with *Eco*RI before being applied in decreasing amounts to an agarose gel. E1 through E4 are the fragments from endogenous 2μ DNA. pDB248 (Fig. 1) consists of pBR322 and the *Eco*RI-2 fragment carrying the *LEU2* gene from pJDB248 (3). *Eco*RI generates bands a through c.

medium. pADHO40-2 (Fig. 1) contains the same *leu2-d* allele as pMP78 and has the same curing properties as pMP78. Transformants isolated on selective plates lacking leucine lose the 2μ DNA and the recombinant plasmid during subsequent growth with a frequency of about 1.5% per generation. On the other hand, no curing occurred if the transformants were isolated on complete medium by the use of the *bla* marker. The transformants had about the same stability, but cells that had lost pADHO40-2 still contained 2μ DNA. Figure 5 shows the results of Southern blots of DNA of different colonies. The initial transformant colonies were all grown on complete medium before analysis. Lane G (Fig. 5) contains DNA from a colony that was derived from a transformant that had become Leu$^-$ Bla$^-$. The cells still are *CIR*$^+$. Lanes D through F (Fig. 5) contain the DNA from a Leu$^+$ Bla$^+$ colony. Both plasmids are present. As a control, cells derived from selectively isolated transformants are shown in lanes A through C (Fig. 5). Lane C contains DNA from cells that became Leu$^\pm$; those cells were also *cir*^{0}. Figure 5 also shows that the copy number of pADHO40 in selectively isolated transformants is higher than in transformants that were never subjected to selection for *LEU2* expression. The copy number of pADHO40-2 under nonselective conditions is usually still higher than that of noncuring plasmids like pDB248. We assume that this is due to the fact that the conditions for a *leu2* host cell are not completely nonselective even if leucine is added to the medium. The fact that on medium supplied with leucine transformants of AH22 containing a *leu2-d* plasmid form larger colonies than untransformed AH22 cells supports this idea. The fact that pADHO40 can cure 2μ DNA only under selective conditions strongly suggests that its high copy number is not due to an inherent increase of replication efficiency compared with that of 2μ DNA but is due to the expression of the *leu2-d* allele.

The *leu2-d* allele is poorly expressed. To detect differences between the function of the various *LEU2* alleles compared in the curing experiments, the activity of β-isopropylmalate dehydrogenase, the enzyme encoded by *LEU2*, was determined in crude extracts of a wild-type strain and different transformants. Table 3 shows that under induced conditions the enzyme activity in AH22(pMP78) transformants is five to seven times lower than in transformants containing pDB248, although the copy number of pMP78 (35 per cell) is much higher than pDB248

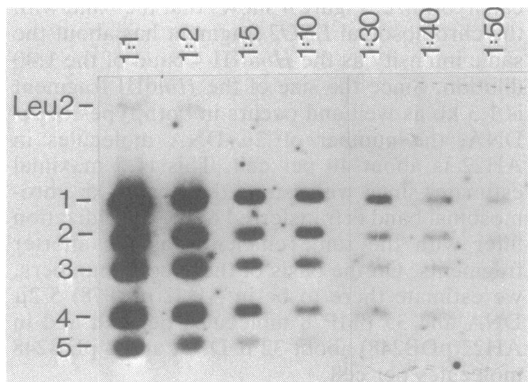

FIG. 4. Analysis of total AH22 DNA on a Southern blot hybridized to ^{32}P-labeled pL623 DNA. Decreasing amounts of *Hin*dIII-digested DNA are electrophoresed. The chromosomal *Hin*dIII fragment of 10 kb is visible only in the undiluted and the twofold diluted samples. See text for further details.

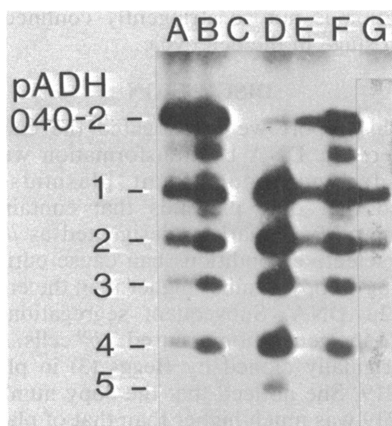

FIG. 5. Analysis of selectively and nonselectively isolated AH22(pADHO40-2) transformants. Transformants were all grown on complete medium for DNA isolation. Lanes A through C, selectively isolated transformants; lanes D through G, nonselectively isolated transformants. pADHO40-2 is cut only once by *Hin*dIII. The hybridization probe was pL623.

(six to eight per cell). Hsu and Kohlhaw (21) have shown that β-isopropylmalate dehydrogenase, if present on multicopy plasmids in the yeast cell, is overproduced in accordance with the higher gene dose. If we assume a similar gene dosage effect of the three *LEU2* alleles compared here, we calculate that, per gene copy, the *leu2-d* allele has less than 5% of the activity of the allele on pDB248. This clearly shows that the *leu2-d* allele is defective and suggests that a high copy number of this allele is required for growth in the absence of leucine.

This conclusion is supported by the following experiment. A plasmid, pL72 (Fig. 6), was constructed that contained, in addition to the *leu2-d* allele, the wild-type *LEU2* allele from pYeleu10. The DNA analysis of pL72 transformants (Fig. 7) shows that the copy number of pL72 is low under selective growth conditions. The enzyme activity, however, was about five times higher than in pMP78 transformants (Table 3), and the presence of pL72 did not lead to curing of 2μ DNA. Thus, the insertion of a wild-type *LEU2* gene in a curing plasmid reduced the copy number, prevented curing, and increased the β-isopropylmalate dehydrogenase activity.

The *leu2-d* allele is affected by a deletion. At least two reasons can be considered to explain the reduced activity of the *leu2-d* allele on 2μ DNA plasmids. (i) A position effect reduces the *leu2-d* allele expression or (ii) the *leu2-d* allele is not intact. To investigate the second possibility, we analyzed the ends of the *leu2-d* DNA fragment and compared the restriction map with that determined by Dobson et al. (11) for the *LEU2* region originating from the same strain (Fig. 8). At the 3′ end of the gene, the *leu2-d* fragment still contains the *Acc*I site and ends about 100 bp further, very close to the end of the fragment on pDB248. This suggests that the 3′ end is intact. At the 5′ end, *leu2-d* still contains the *Bst*EII site but not the next *Hin*cII site that is located within a short peptide coding region possibly involved in regulation of *LEU2* (1). The exact 5′ end of *leu2-d* was determined by sequencing of the small *Bst*EII-*Pvu*I fragment (Fig. 8). Since the cloning by Beggs (3) was done by deoxyribosyladenine-deoxyribosylthymine tailing, the end of the chromosomal fragment was directly visible by a long track of thymines. Comparison with the known sequence of this area (1) showed that the *leu2-d* allele ends at a position 29 bp before the ATG initiation codon. Except for a guanosine instead of a cytosine at −3, there were no other differences between the sequences. Most probably the deletion of sequences 5′ to the structural gene is responsible for the poor expression of *leu2-d*. The sequence coding for the small leucine-rich peptide (1) is completely lacking. It is of interest to note that *leu2-d* is still regulated (Table 3).

***ARS1* plasmids and 2μ DNA show a different regulation of replication.** The autonomous replication sequence *ARS1* (36) is considered to be

TABLE 3. Specific activity of β-isopropylmalate dehydrogenase in cell extracts of different yeast transformants under different growth conditions

Strain	*LEU2* allele on recombinant plasmid and its origin	Plasmid copy no.	Sp act in minimal medium with[a]:		Complete medium[b]
			− Leucine	+ 1 mM Leucine	
H1 (wild type)		0	2.0	1.0	0.3
AH22(pMP78)	*leu2-d*	35	7.0	1.1	0.8
AH22(pHKB52)	*LEU2* from pYeleu10	5–10	50.3	10.4	2.9
AH22(pDB248)	*LEU2* from pJDB248	8	39.8	ND[c]	0.9
AH22(pL72)	*LEU2* from pYeleu10 + *leu2-d*	5–10	32.0	ND	1.3

[a] Specific activity is defined as nanomoles of product formed per minute per milligram of protein.

[b] Cells were grown for 8 to 10 generations in complete medium. The loss of plasmid during this period was only 10 to 20% and was not responsible for the reduction in enzyme activity.

[c] ND, Not determined.

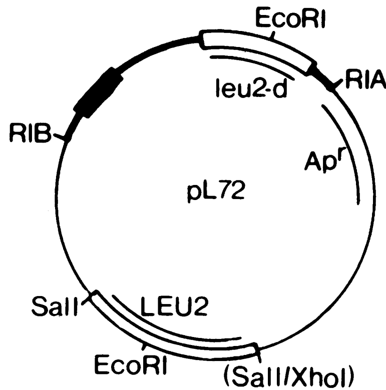

FIG. 6. Scheme of pL72. pL72 was derived from pMP80 (18) by insertion of the *Sal*I-*Xho*I fragment carrying the $LEU2^+$ gene from pYeleu10 (32). 2μ DNA sequences are indicated by a thick line. RIA and RIB refer to *Eco*RI sites on 2μ DNA. Ap^r indicates a resistance gene for ampicillin.

subjected to the same regulation of DNA replication as is the chromosomal DNA (38). Also, the replication of 2μ DNA is found to be regulated at least in part by the same genes (31, 26). YRp7, containing *ARS1* as a replication origin, is normally present in a low number per cell, but deletion of the bacterial part leads to a strong increase in copy number (38). To investigate whether the YRp7 copy number in AH22 could be increased by insertion of the *leu2-d* allele under appropriate selection, we constructed plasmids YRp7-*leu2-d* and YRp7-*LEU2* (Fig. 9). Strain AY2a *trp1 leu2* was transformed with each of both plasmids, and transformants were spread on medium lacking leucine and tryptophan. YRp7-*LEU2* gave transformants on both selective media, but surprisingly, YRp7-*leu2-d* transformants could be obtained only on medium without tryptophan, indicating that the *leu2-d* allele on YRp7-*leu2-d* cannot complement the *leu2* mutation during recovery from transformation. Apparently, the replication of YRp7 is subjected to a more stringent control than that of 2μ DNA leading to a copy number in the transformed protoplasts that is too low to allow synthesis of β-isopropylmalate dehydrogenase from the defective gene at high enough levels for growth. Presumably, a higher copy number can be obtained during successive cell divisions, since YRp7-*leu2-d* transformants selected on medium lacking tryptophan acquired the ability to grow on a medium lacking leucine. An alternative explanation assumes that regenerating protoplasts require a higher rate of leucine synthesis not compatible with the low copy number of YRp7-*leu-d*. We assume that 2μ DNA plasmids are able to attain a higher copy number in transformed protoplasts, possibly because their replication is not so stringently confined to a certain stage in the cell cycle.

DISCUSSION

In this report we investigated the cause of curing of 2μ DNA by transformation with 2μ DNA-derived recombinant plasmids. We showed that only plasmids that contain one particular *LEU2* marker, designated as *leu2-d*, under selective conditions can cause curing by attaining a copy number higher than the endogenous 2μ DNA. Subsequent segregation then leads to the generation of cured, cir^0 cells. *leu2-d* was originally cloned by Beggs (3) in plasmid pJDB219. She noticed that the copy number of pJDB219 was much higher than that of plasmids carrying other independently cloned *LEU2* markers, such as pJDB248, and presented evidence that the high copy number might be correlated with *LEU2* expression (4). We observed a strong curing effect of pMP78, carrying the *leu2-d* allele (12), and here we provided evidence which strongly suggests that curing is directly correlated with high copy number caused by the low gene activity of the partly defective *leu2-d* allele.

We used Southern blots to quantitate the copy number of recombinant plasmids and 2μ DNA

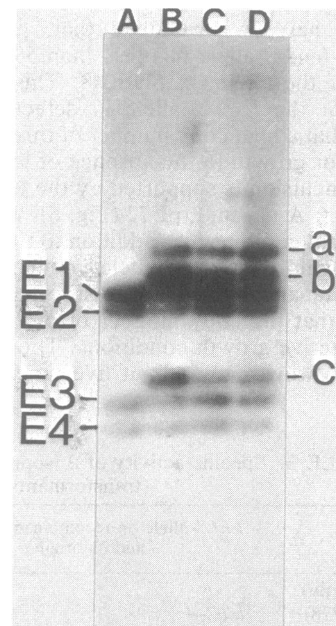

FIG. 7. Analysis of three AH22 (pL72) transformants (lanes B through D) isolated under selective growth conditions. Lane A, AH22 used as the control. All samples were digested with *Eco*RI and hybridized to ^{32}P-labeled pL623 DNA. pL72 is cut into four fragments (a through d) by *Eco*RI. The smallest fragment is not visible.

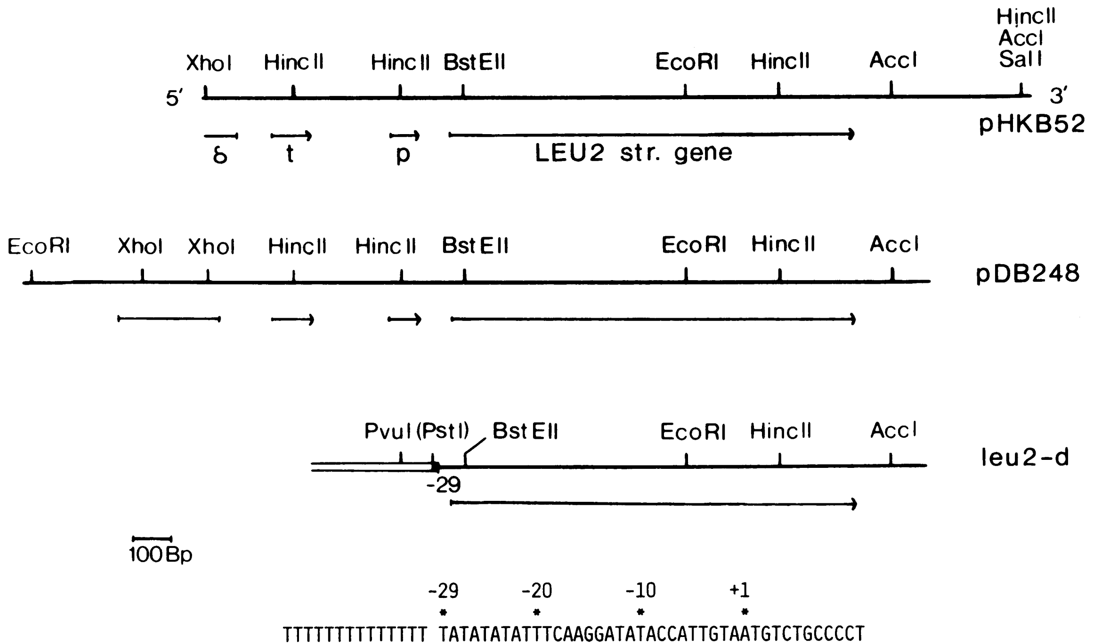

FIG. 8. Comparison of the *leu2-d* region of pMP78 with the *LEU2* regions of pHKB52 and pDB248. Bottom line presents the DNA sequence of the 5' end of *leu2-d* determined on the *PvuI-BstEII* fragment by the method of Maxam and Gilbert (28). t, $tRNA_3^{leu}$; p, peptide coding region; str. gene, structural gene; δ, part of Tyl-17.

and obtained consistent values over several determinations. Earlier (19) we determined the amount of supercoiled 2μ DNA to be as high as 4% of the total DNA in haploid strain H1, corresponding to about 100 copies per cell. Strain AH22 always seemed to contain a lower number of plasmid molecules and was estimated from the Southern blots to contain about 40 copies per cell. YNN27, however, has a higher content of about 100 copies per cell. Differences in 2μ DNA content have been described by Gerbaud and Guerineau (14), who observed that the plasmid copy number is strain specific and not changed in transformants. Our data agree with this observation, since we find roughly the same total number of plasmids per cell, whether they are recombinant plasmids or 2μ DNA. The number of noncuring recombinant plasmids in AH22 transformants is less than 10 per cell, whereas pMP78 can be as high as 35 per cell.

Since the gene activity of *leu2-d* is less than 5% that of the wild-type *LEU2* gene, we assume that the copy number of pMP78 increases immediately after transformation in the regenerating protoplast, but before the first cell division can occur. Under nonselective transformation con-

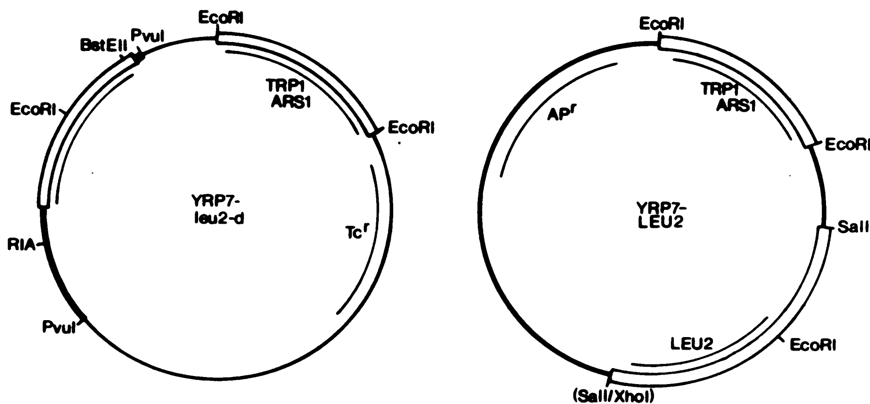

FIG. 9. Scheme of YRp7-*leu2-d* and YRp7-*LEU2*. RIA refers to the *EcoRI* site on 2μ DNA. Tc^r and AP^r indicate resistance genes for tetracycline and ampicillin, respectively.

ditions we found a higher frequency of transformant colonies (33) suggesting that amplification of this plasmid does not take place in all transformed protoplasts and may even be a function of the number of molecules taken up by the cell. An interesting difference was observed with YRp7 carrying *leu2-d*. This plasmid did not give transformants under conditions selective for leucine synthesis. Possibly the *ARS1* origin is not able to replicate up to its normal copy number in the absence of cell division. The normal copy number is high enough to give sufficient leucine synthesis for growth. If AY2a(YRp7-*leu2-d*) transformants are selected by use of the *TRP1* marker, they can grow on medium lacking leucine.

In general, vectors carrying the *leu2-d* allele as a selective marker can complement *leu2* mutants only if they are present in high copy number. This also explains the low transformation frequency that Beach and Nurse (2) obtained for *Schizosaccharomyces pombe* with pJDB219, in contrast to the high frequency with pDB248. A low copy number of 2μ DNA plasmids in the *cir*^{0} yeast *S. pombe* allows transformation only with wild-type selective markers.

By studying the generation of *cir*^{0} cells by noncuring plasmids, pHKB52 or YEp6, we found that the low amount of curing observed was due to the spontaneous loss characteristic of the host strain AH22. Since *cir*^{0} is an irreversible condition, even a low loss rate would result in an increase of *cir*^{0} cells, if they are not outgrown by *CIR*$^+$ cells. During 700 generations, no changes could be observed in a cocultivation experiment containing equal amounts of *cir*^{0} and *CIR*$^+$ cells. This means that the spontaneous generation of *cir*^{0} cells is balanced by a slightly slower growth rate. In a culture, about 1% of AH22 cells and 0.5% of YNN27 cells are *cir*^{0}; they arise with a frequency of at least 0.02 to 0.01% per generation.

The expression of the *LEU2* gene encoding the β-isopropylmalate dehydrogenase is under indirect specific control (22, 24). Yeast transformants that contained a wild-type *LEU2* gene on a high copy number vector contained up to 30 times more enzyme activity than the wild-type strain (21). The activity of the plasmid-encoded *LEU2* genes was regulated as in a wild-type strain, and the regulation is suggested to be pretranslational (24). Andreadis et al. (1) have sequenced the *LEU2* gene on pYeleu10 and found a small open reading frame from −151 to −80 that contains six codons for leucine. Those authors proposed that this leader peptide may play a role in the regulation of gene expression. We show in this paper that *leu2-d* is lacking all DNA sequences 5′ from 29 bp before the translation initiation codon, including the open reading frame. The expression of *leu2-d*, however, is still regulated. This suggests that the sequences involved in this regulation are still present in *leu2-d*. If the presumptive leader peptide plays a role in this regulation, then our data suggest that this effect is exerted in *trans* and that the regulatory target is located on the 3′ side of nucleotide −29. *leu2-d* presents an interesting deletion for further study of *LEU2* regulation.

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