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Effect of presence and expression of the genomic DNA of the lignin peroxidase gene *LiPH8* from *Phanerochaete chrysosporium* in the yeast *Saccharomyces cerevisiae*.

Thesis Submitted for the Degree of

Magister Philosophiae

Candidate:

Simone Ugolini

Supervisor:

Prof. Carlo Bruschi

Academic Year 1991/1992

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In Memory of my Brother Giacomo

CONTENTS

1. INTRODUCTION

1.1 The lignin structure

Lignin is an abundant aromatic polymer synthesized by plant cells from phenylpropanoid monomers, p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol; these alcohols polymerize in a three-dimensional structure by formation of ether bonds mainly between the phenolic group on the C_4 carbon of the aromatic ring, and the ß carbon of the side chain (Kirk and Farrell, 1987), Fig. 1. Lignin is interwoven with hemycellulose and cellulose to form a lignocellulose matrix, a very robust structure rather resistant to degradation. Lignocellulose is the most abundant renewable organic compound in nature, and there is a developing interest in industry for the utilization of this resource. There are many potential utilizations of the bioconversion products derived from lignocellulosics, given an appropriate degrading system, either using the original ligninolytic organism, or different microbes producing recombinant enzymes. Potential applications of biological degrading systems have been considered, such as delignification of wood for the production of cellulose, utilization of byproducts in the animal food industry, and treatment of industrial wastes (Kirk and Chang, 1981; Dale, 1987).

1.2 Lignin-degrading enzyme systems

The white rot basidiomycete *Phanerochaete chrysosporium* has a lignin-degrading enzyme system composed of lignin peroxidases (LIPs) (Tien and Kirk, 1983) and manganese-dependent peroxidases (MNPs) (Huynh and Crawford, 1985), along with glyoxal oxidase, a copper oxidase responsible for the production of extracellular H_2O_2 (Kersten and Kirk, 1987). Over ten lignin peroxidase isozymes have been isolated, from the extracellular fluid of this filamentous fungus, and they have been named H1 to H10, where "H" represents the hemeprotein and the number indicates the order of elution from a HPLC Mono Q column (Pharmacia/LKB, Uppsala, Sweden) (Kirk, *et al.*, 1986).

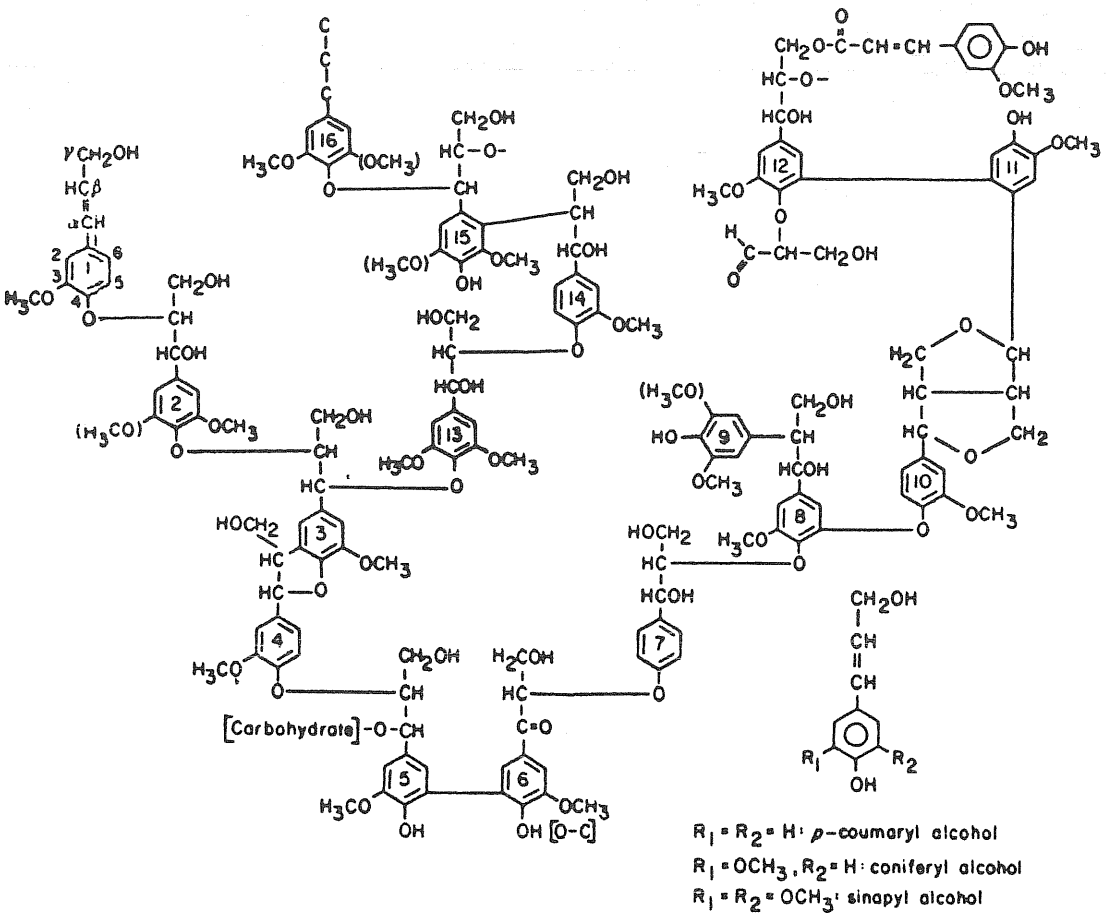


Figure 1 Shematic representation of the structure of lignin. Numbers within the aromatic rings indicate different types of interphenylpropane linkages between monomers. The major type of linkage is the ß-O-4 type, shown between units 1 and 2. The phenylpropanoid monomers are shown at the lower right (Kirk and Farrell, 1987).

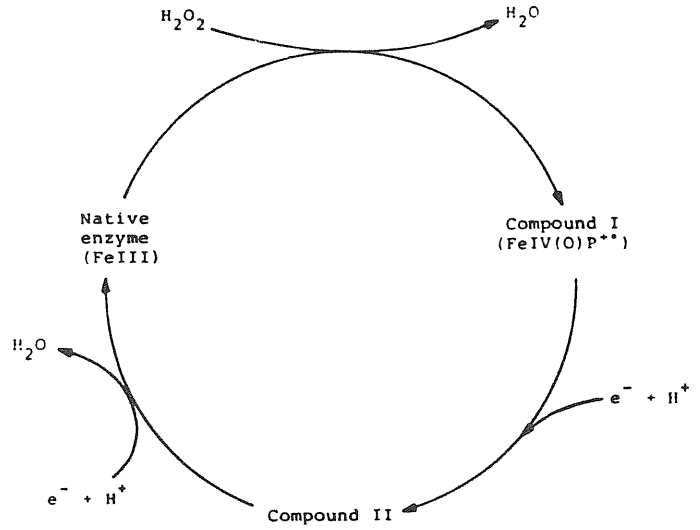


Figure 2 Catalytic cycle of a peroxidase. The resting enzyme, containing ferric iron Fe3+, undergoes a two electron oxidation by reacting with hydrogen peroxide, with the production of Compound I, an intermediate lacking two electrons. Compound I extracts one electron from the substrate, resulting in the formation of Compound II which removes one more electron from the substrate, thus completing the catalytic cycle.

These isozymes are of two different types:

- Lignin peroxidases: H1 (pI=4.7), H2 (pI=4.4), H6 (pI=3.7), H7 (pI=3.6), H8 (pI=3.5), and H10 (pI=3.3).

- Mn-dependent peroxidases: H3 (pI=4.9), H4 (pI=4.55), H5 (pI=4.2), and H9.

These extracellular glycosylated enzymes, with a protoporphyrin IX prosthetic group, and a molecular weight ranging from 37000 to 42000, allow the depolymerization of the lignin polymer, during secondary metabolism under carbon, nitrogen, or sulfur starvation and at high O_2 tension (Kirk and Farrell, 1987; Kirk, *et al.*, 1986). The production of lignin peroxidases under these circumstances is correlated to low levels of Mn^{2+} in the growth medium, while Mn-dependent peroxidases behave in the opposite way (Bonnarme and Jeffries, 1990).

Lignin peroxidase and manganese-dependent peroxidase possess a different oxidative mechanisms of action on their substrates. The first, is found free extracellularly, and exhibits catalytic activity by abstracting single electrons from aromatic rings of the lignin polymer using hydrogen peroxide as the oxidant with formation of a cation radical, Fig. 2, followed by ring fission (Gilardi, *et al.*, 1990; Harvey *et al.*, 1985; Harvey, 1986, Schoemaker, *et al.*, 1985). The second, is closely associated with the fungal mycelium, and acts by oxidizing Mn^{2+} to the higher oxidation state Mn^{3+}, a very active diffusible catalyst that, once stabilized by chelators, can attack and oxidize the phenolic structures in lignin (Paszczynski, *et al.*, 1986).

Lignin peroxidases have a wide degradative activity on different compounds, either part of the fungus metabolism, or numerous xenobiotics. For example, this enzymatic system can decarboxylate oxalic acid, a secondary metabolite characteristic of woodrotting basidiomycetes, to carbon dioxide (Akamatsu, *et al.*, 1990). Another secondary

metabolite of the fungus *P. chrysosporium*, veratryl alcohol (3,4-dimethoxybenzyl alcohol), can be oxidize to veratraldehyde (3,4-dimethoxybenzaldehyde) through formation of a cation radical, as suggested by different experiences, and summarized by Schoemaker and Leisola, (1990), Fig. 3. This molecule is responsible for the maintenance of the catalytic cycle in lignin degradation, by actively reducing the oxidized ligninase therefore regenerating the native enzyme (Schoemaker and Leisola 1990), Fig. 4.

Lignin peroxidases catalyze depolymerization of natural lignin, as well as synthetic lignin *in vitro*, provided H_2O_2 and veratryl alcohol (Hammel and Moen, 1991); These ligninases oxidize also different aromatic substrates by a one-electron transfer mechanism (Harvey, *et al.*, 1985; Millis, *et al.*, 1989), and have been shown to degrade a wide variety of environmentally persistent organopollutants (Bumpus, *et al.*, 1985), such as:

- Chlorophenols: 2,4-Dichlorophenol (Valli, and Gold, 1991), DDT (Bumpus, and Aust, 1987), and pentachlorophenol (Mileski, *et al.*, 1988).

- Nitroaromatics such as 2,4-Dinitrotoluene (Valli, *et al.*, 1992), and TNT (Fernando, *et al.*, 1990).

- Others: i. e. a large number of poorly degradable dyes used in the textile and dyestuff industries (Bumpus, and Brock, 1988; Cripps, *et al.*, 1990; Paszczynski, *et al.*, 1991; Field, *et al.*, 1992).

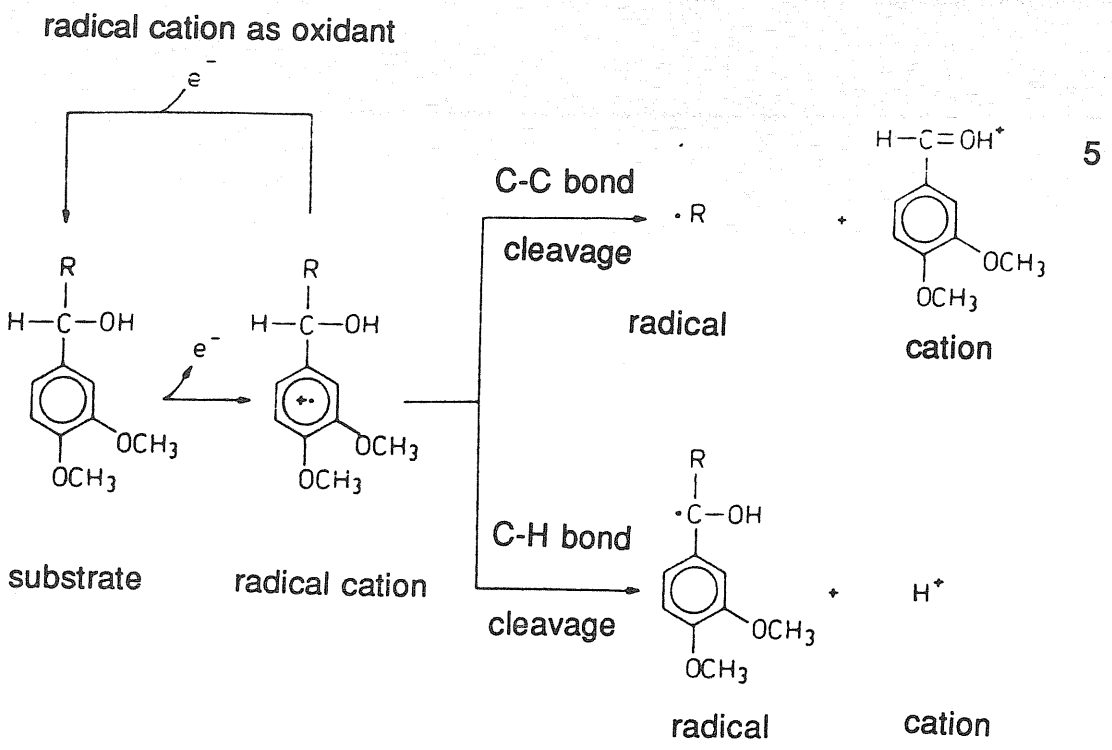


Figure 3 Formation of a radical cation by the action of ligninase. When R is primary, as in veratryl alcohol, the radical cation breaks at the C-H bond. When R is secondary, as in the lignin polymer, the radical cation breaks at the C-C bond (Harvey, 1986).

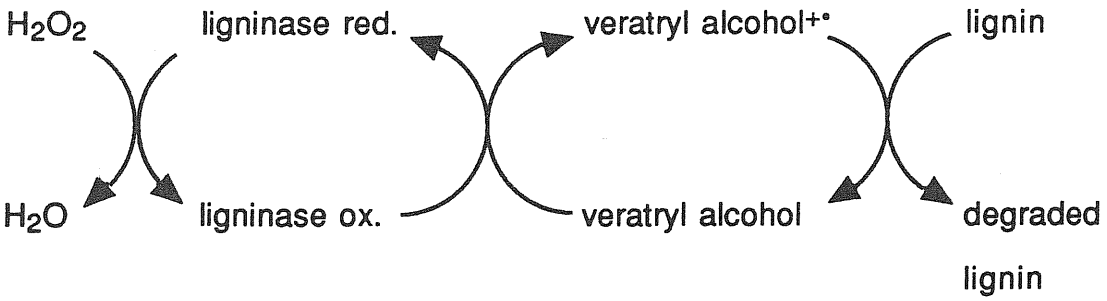


Figure 4 Proposed role of veratryl alcohol as mediator in the peroxidatic cycle of lignin peroxidase. Veratryl alcohol radical cation produced by ligninase removes an electron from a second oxidizable compound (lignin), functioning as one-electron oxidant. Thus veratryl alcohol is regenerated, and the substrate oxidized. In the absence of a second oxidizable compound, veratryl alcohol is readily oxidized by ligninase to veratraldehyde (Harvey, 1986).

Interestingly, compounds recalcitrant to biodegradation, such as some synthetic azo dyes, can be rendered more susceptible to degradation by adding to the original molecule some additional chemical group. This operation makes possible for the enzymatic system of the organism to attack the modified molecule with higher efficiency; a bright example of this approach comes from the experiments of Paszczynski and collaborators (1991), that obtained a highly degradable compound by linking a guaiacol (2-methoxyphenol) group into the poorly degradable synthetic azo dye structure of acid yellow 9 (4-amino-1,1'-azobenzene-3,4'-disulfonic acid). These observations are important for the design of new synthetic dyes that maintain the same important properties for the industry, but with an improved biodegradability.

The described wide catalytic activity of these particular enzymes can be utilized for the bioremediation of polluted environments, and some can also find applications in the paper and chemical industry (Kirk and Chang, 1981). In fact, the use of lignin peroxidases and manganese-dependent peroxidases has been suggested for the treatment of pulp bleach plant effluent (BPE), which contains largely high-molecular weight, modified and chlorinated lignin, released into the environment by the pulp and paper industry (Michel, *et al.*, 1991; Lackner, *et al.*, 1991). White-rot fungi, such as *P. chrysosporium* and *Trametes versicolor* have been shown to efficiently decolorize the stable high-molecular weight chromophores released by kraft mill bleacheries (Archibald, *et al.*, 1990; Messner, *et al.*, 1990).

The growing interest in the field of biomass conversion has lead to the molecular characterization of several organisms able to degrade lignocellulose. Genes encoding for enzymes responsible for the oxidation of lignin have been cloned in different fungi and bacteria by a number of laboratories. However, these organisms are generally producers also of cellulases, and the two enzymatic activities are carried out under similar growth conditions, resulting in unwanted product degradations. This is the case, for example, of the paper industry, for which the degradation of cellulose is detrimental to

the process. To overcome this problem, the expression of ligninolytic enzymes, such as lignin peroxidase isozyme H8 (LiPH8), in non cellulolytic organisms, such as *E. coli* and the yeast *S. cerevisiae*, represents an interesting alternative.

1.3 Expression of lignin peroxidases in heterologous hosts

Lignin peroxidase isozyme H8 (LiPH8), encoded by the cDNA clone λML-1 (Tien and Tu, 1987), the allelic variant λML-4 (Andrawis, *et al.*, 1989), or the corresponding genomic clone (Schalch, *et al.*, 1989; Smith, *et al.*, 1988), is the principal extracellular peroxidase of *P. chrysosporium* (strain BKM-1767 corresponding to ATCC 24725) when grown under nitrogen starvation; this enzyme, involved in the biodegradation of lignin (Kirk, *et al.*, 1986), has a main peak of appearance of its mRNA in ligninolytic cultures, after about 6 days (Holzbaur, *et al.*, 1988; Andrawis, *et al.*, 1989). LiPH8 is a glycoprotein with a molecular weight of 41000 D. The sequence of the genomic clones coding for the LiPH8 gene, corresponding to the cDNA clone λML-4 (Andrawis, *et al.*, 1989) and the one isolated by Smith, *et al.*, (1988), shows that the gene is interrupted by 8 intervening sequences (IVS) of an approximate length of 55 base pairs each, Fig. 5.

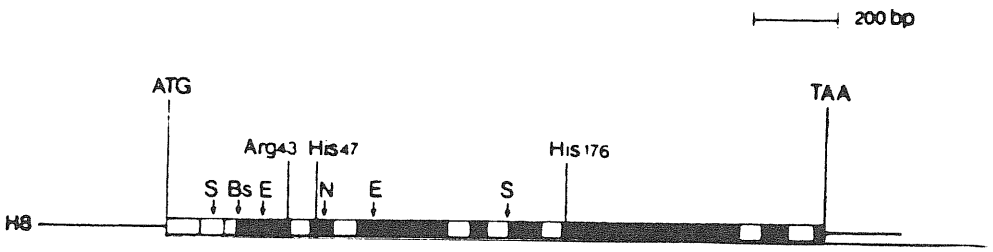


Figure 5 Schematic representation of the genomic DNA coding for the LiPH8 gene from *Phanerochaete chrysosporyum*. Signal sequences and introns are shown as open boxes, exons as closed boxes. Positions of the histidine and arginine residues important for the enzymatic activity are shown according to the cDNA positions. Restriction endonuclease recognition sites are abbreviated as follows: S: SphI; Bs: BssHII; E: EcoRV; N: NcoI; H: HindIII; X: XhoI; K: KpnI; (Schalch, *et al.*, 1989)).

The expression in *E. coli* of the cDNA coding for ligninase H8 resulted, as expected, in the lack of glycosylation. On the other hand experiments on the activity of ligninases with partial N-deglycosylation, showed that the degree of glycosylation appears not to affect the specific activity of this recombinant ligninase (Farrell, *et al.*, 1988). This situation cannot be generalized to other enzymes, and different eukaryotic proteins largely rely on posttranslational modifications necessary for their enzymatic activity. This is the case for example of a xylanase from the yeast *Cryptococcus albidus*, which shows enzymatic activity only when glycosylated (Morosoli, *et al.*, 1988). When the cDNA encoding this xylanase was expressed in *S. cerevisiae,* enzymatically active protein was secreted in the culture medium (Moreau, *et al.*, 1992). The heterogeneous pattern of glycosylation of *S. cerevisiae,* (Romanos, *et al.*, 1992; Ballou, 1982) can even confer new characteristic to the expressed protein, as it is the case of the endoglucanase I from the ascomycete *Trichoderma reesei* that when produced in yeast appears to be more resistant to thermal inactivation (Van Arsdell, *et al.*, 1987).

The expression in *E. coli* of the cDNA for LiPH8 resulted also in a poor incorporation of the prosthetic group, protoheme IX, in the rH8 recombinant protein, so that heme addition *in vitro* was required for recombinant ligninase production (Farrell, *et al.*, 1988). Expression in *E. coli* of the NADPH-cytochrome-P-450 (P-450) reductase from liver, a peroxidase very similar in structure to lignin peroxidases, resulted mostly in the production of apoprotein, and only a small percentage of the recombinant P-450 incorporated the prosthetic group (Unger, *et al.*, 1986). However, when expressed in yeast, NADPH-cytochrome-P-450 reductase exhibited a higher proportion of correctly assembled recombinant protein. This can be explained by the fact that the yeast *S. cerevisiae* is able to accurately incorporate heme groups into their corresponding proteins, as demonstrated by the existence of the yeast P-450, and other several imported proteins from mitochondria responsible for the biosynthesis of heme, i. e. the well characterized cytochrome *c* peroxidase (Ccp) (Finzel, *et al.*, 1984). Moreover, diverse peroxidases from different organisms, such as turnip (Welinder and Mazza, 1977), horseradish (HRP) (*Armoracia rusticana*) (Welinder, 1976), and cytochrome *c*

(Ccp) from yeast (Kaput, *et al.*, 1982), are very similar in sequence to ligninase H8 near the proximal and distal conserved histidine residues, which are considered essential for the activity of the protein, as pointed out by Tien and Tu, (1987), Fig. 6. Additionally, hydrophobicity plots of the yeast Ccp and the ligninase LIII from the white-rot fungus *Phlebia radiata* show extensive homology, suggesting similar tertiary structure (Saloheimo, *et al.*, 1989).

Figure 6 Protein sequence alignment of peroxidases from different organisms at regions adjacent to the the proximal and distal conserved histidine residues. The conserved arginine residue can also be identified. Peroxidases are from turnip (Welinder and Mazza, 1977), yeast cytochrome *c* (Ccp) (Kaput, *et al.*, 1982), horseradish (HRP) (Welinder, 1976) and LIPH8 from *P. chrysosporyum*, (Tien and Tu, 1987). For LIPH8 peroxidase the conserved residues are His 175, His 47, Arg 43.

	distal histidine	proximal histidine
Turnip	^{31}RMGASILRLFFHDCFVNGCD^{50}...^{153}AVGLSTRDMVALSGAHTIGQSR^{174}	
Ccp	^{41}GYGPVLVRLAWHTSGTWDKH^{61}...^{159}QRLNMDREVVALMGAHALGKTH^{180}	
HRP	^{31}RIAASIIRLHFHDCFVNGCD^{50}...^{155}VGLNRSSDLVALSGGHTFGKNQ^{176}	
LiPH8	^{36}AEAHESIRLVFHDSIAISPA^{55}...^{161}AGEFDELELVWMLSAHSVAAVN^{182}	

1.4 The challenge of splicing of foreign genes in yeast

This reports suggest that the yeast *Saccharomyces cerevisiae* is a possible candidate organism in which to express foreign lignin peroxidases from white-rot fungi. Moreover, budding yeast has been widely used as well as a model system in which to study the mechanisms of nuclear pre-mRNA splicing, and important information on the ability of this yeast to correctly splice foreign genes from other fungi can be made by expressing foreign genomic DNAs. There are a number of reports on the expression in yeast of different cDNAs from other fungi, as shown above, but only a few authors investigated the requirements for a correct splicing in yeast of nuclear encoded interrupted genes from different eukaryotes (Moreau, *et al.*, 1992). In the present study we tested the ability of *S. cerevisiae* to recognize the splicing consensus sequences of the genomic DNA encoding LiPH8, with the aim to examine possible correlation between the phylogenetic distance among these fungi and the different organization of evolutionary related genes, i. e. peroxidases. It is interesting that genes coding for enzymes with evolutionary conserved functions, can be differently organized and regulated in different fungi at the level of splicing. This kind of research can help delineate how splicing can be regulated to control gene expression of related genes in diverse organisms. The sequence requirements for pre-mRNA splicing in the yeast *S. cerevisiae* are similar to those of other eukaryotes, though particularly strict for the intron-contained TACTAAC sequence, which has been shown to be essential for splicing (Langford and Gallwitz, 1983). This sequence, located near the 3' end of the intron, determines the site of binding of one of the components of the spliceosome complex, the U2 small nuclear ribonucleoprotein (snRNP), with subsequent definition of the site of RNA branch formation (the branchpoint) and formation of the lariat (Green, 1991), Fig. 7a and 7b.

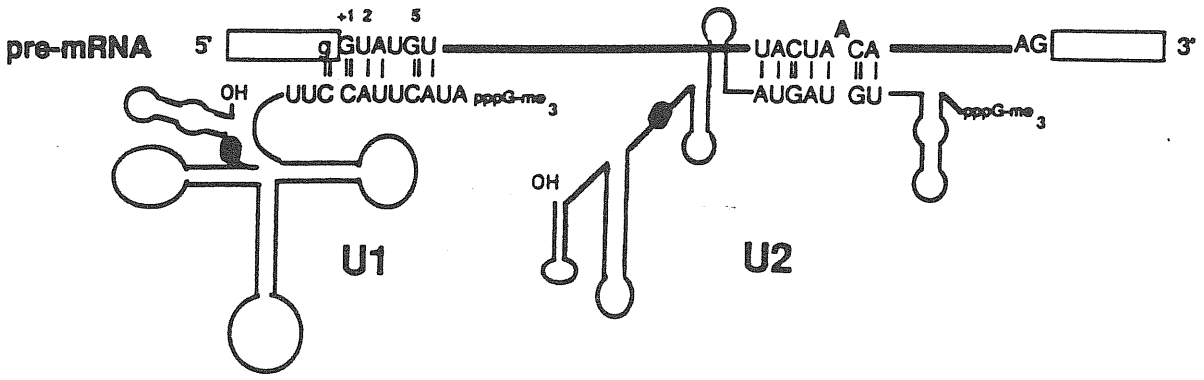


Figure 7a Schematic drawing of the mechanism of nuclear pre-mRNA splicing, with the interaction of U1 and U2 snRNPs with the 5' splice site and the branchpoint, (the site of RNA branch formation), respectively (Green, 1991).

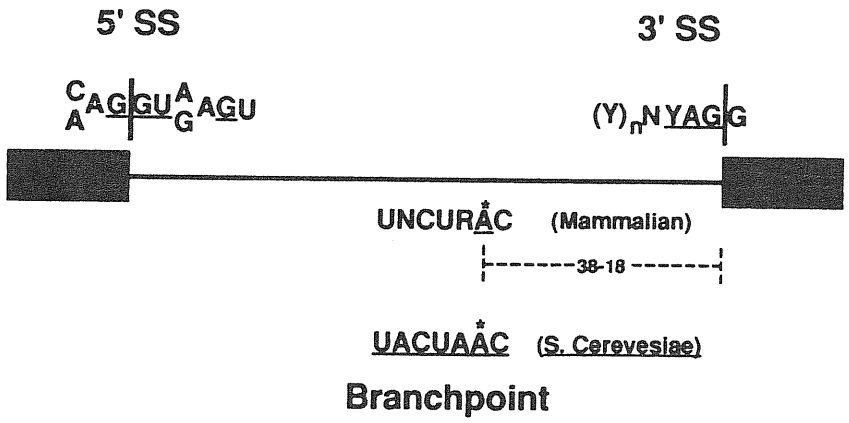


Figure 7b Pre-mRNA consensus splicing signals in yeast and in mammalian cells. Highly conserved residues are underlined. Abbreviations are as follows: R: purine; Y: pyrimidine; N: any base.

A point mutation, i. e. A—>C transversion, at the first or third adenosine of the conserved TACTAAC box completely abolishes splicing in yeast (Langford, *et al.*, 1984). This situation is different from that of higher eukaryotes, i. e. mammalian cells, for which mutations of the branchpoint result in the selection of alternative branchpoints, comprised within 18-38 nucleotides upstream the 3' splice site, still allowing splicing to occur (Green, 1991). In this case the position of the branchpoint seems predominant on the sequence itself. Furthermore, mammalian introns have a conserved polypyrimidine tract, distinctive of all vertebrates, next to the 3' splice site and important for the spliceosome assembly. Introns of non-vertebrate eukaryotes are quite similar to those of yeast, and lack the polypyrimidine tract distinctive of vertebrate intervening sequences, although short T-runs are common. Intron consensus sequence comparisons reveal that the 5' and 3' splice sites are highly conserved in distantly related organisms, respectively for an invariant GT dinucleotide and an AG dinucleotide (Csank, *et al.*, 1990), Fig. 8. Comparison of the intron consensus sequences form the *LiPH8* gene from *P. chrysosporium* with the sequences from *N. crassa* and *S. cerevisiae* indicates a wider consensus at the 5' and 3' splice sites (Holzbaur, *et al.*, 1988). However, the intron-contained consensus varies form one organism to another. In *P. chrysosporium* we can find a putative consensus sequence CTGAAC, located at approximately 11 bp from the 3' splice site, Fig. 9. This situation is different from that of *S. cerevisiae* introns, in which the internal consensus is located more upstream, between 18-53 bp from the splice site, Fig. 9. Thus, these differences in position and sequence of the internal consensus could represent a potential way to evolutionarly differentiate lignin peroxidase gene expression and thus be a challenge for the recognition of the splicing signals in yeast.

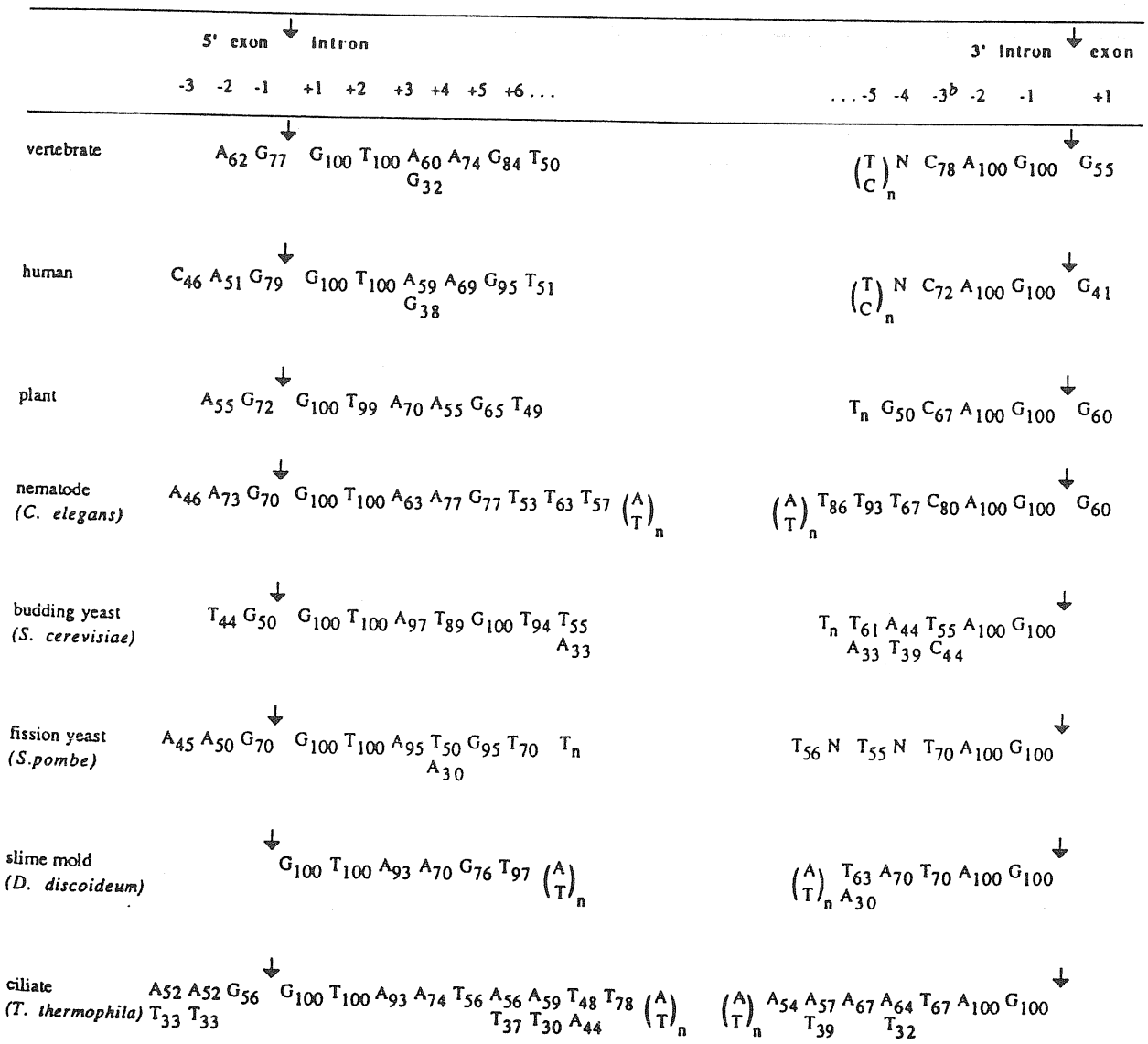


Figure 8 Consensus sequence comparison of intron junctions from distantly related organisms. Nucleotide frequencies are subscripted (Csank, *et al.*, 1990).

	intron #	5' site	internal consensus	3' site
Lignin peroxidase H8	1	GTATGC	CTGAAC – 12 bp –	CAG
	2	GTGAGT	TTGAAC – 9 bp –	CAG
	3	GTAAGT	CTGATC – 9 bp –	CAG
	4	GTATAC	CTAACT – 9 bp –	TAG
	5	GTAAGT	CTGACT – 7 bp –	CAG
	6	GTAAGT	CTGAGAC – 6 bp –	CAG
	7	GTGCGT	CTGAAC – 10 bp –	CAG
	8	GTGAGT	CTGACA – 12 bp –	CAG
P. chrysosporium		GTA/GAGT	CTGAAC 7-12 bp	C/TAG
N. crassa		GTAA/CGT	A/GCTA/GA 7-19 bp	C/TAG
S. cerevisiae		GTATGT	TACTAAC 18-53 bp	C/TAG

Figure 9 Comparison of the intron consensus sequences found in the *LiPH8* gene of *Phanerochaete chrysosporyum* with those from *S. cerevisiae* and *N. crassa* (Holzbaur, *et al.*, 1988).

1.5 Effect of presence and expression of *LiPH8* genomic DNA in yeast

To address the basic issue of the mechanism of splicing of heterologous genes and its potential biotechnological applications, we have carried out preliminary experiments assessing the feasibility of the expression of the lignin peroxidase LiPH8 from *Phanerochaete chrysosporium* in yeast. *Saccharomyces cerevisiae* has been in fact extensively used in the biotechnological industry, either in the traditional way as a fermentator agent or as an organism in which to express foreign genes although with alternating fortunes (Romanos, *et al.*, 1992). The reason of such popularity is due to several interesting features, such as its powerful classical and molecular genetics, rapid growth to high cell density and possibility of industrial scale-up, lack of toxins, high nutritional content, and ease of manipulation. In this view, the absence of cellulase activity in yeast could represent an advantage for its utilization for example in the process of delignification occurring during the production of paper. In this process the cellulose that will be used to produce the paper must be separated from lignin, and this cannot be achieved with cultures of *P. chrysosporium*, which produce cellulases and hemicellulases, as multiple isoenzymes (Eriksson and Pettersson, 1975), or other ligninolytic fungi, and therefore could decrease the cellulose content of the pulp.

We tested the effect of the heterologous expression of the cloned genomic *LiPH8* gene from *P. chrysosporium* in yeast, and its effect on plasmid stability under non selective conditions. The 2μ-based expression plasmid pBLITZ2μ-C (Ludwig, D. L., 1991) was the vector selected for this study in which to clone and test the *LiPH8* gene, Fig. 10.

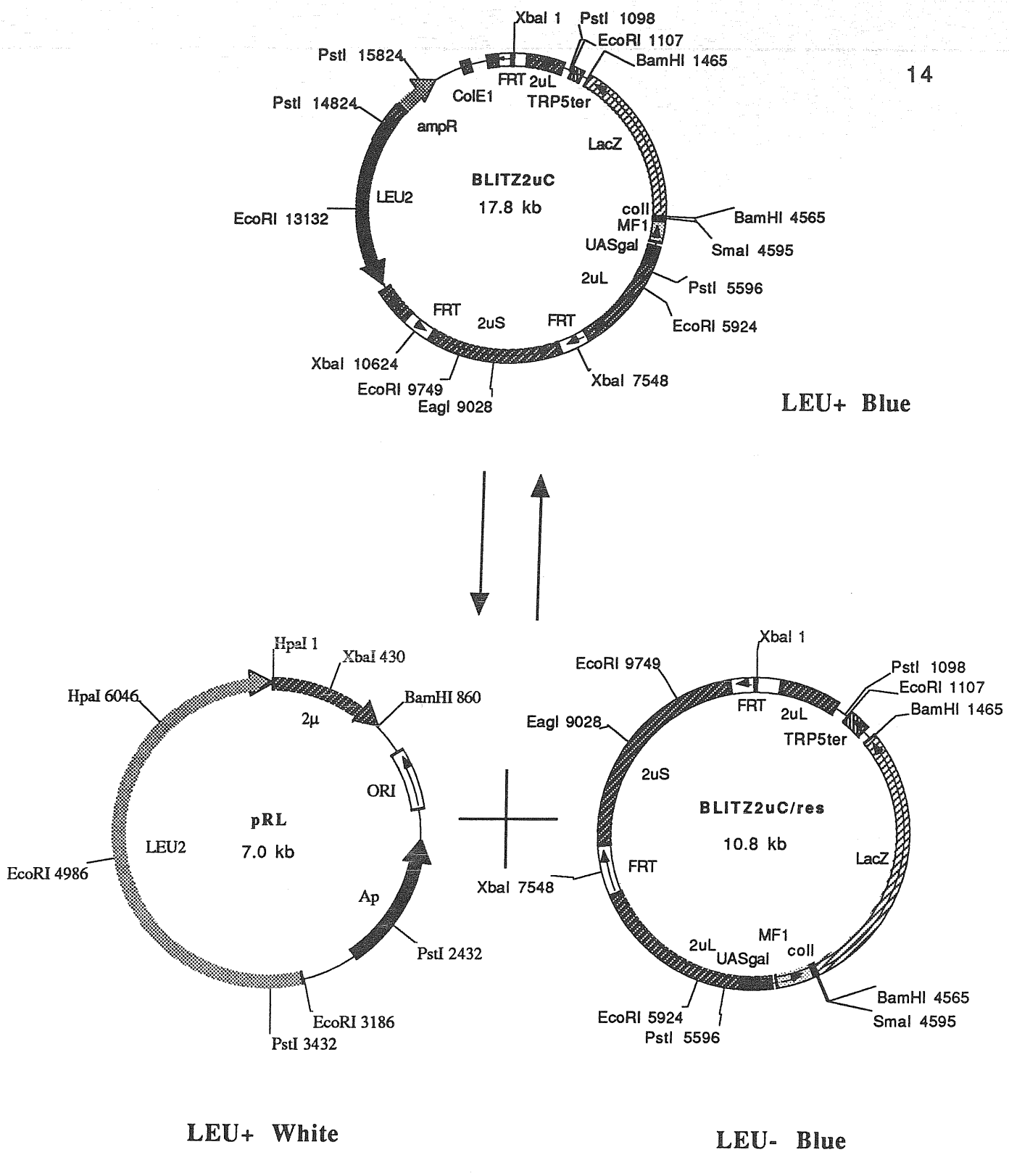


Figure 10 Representation of the pBLITZ2μ-C DNA molecule resolution, occurring via *in vivo* recombination between the two 599 bp direct repeat sequences resulting in the resolution of the plasmid into two circular DNA molecules, pRL and pBLITZ2μ-C/res (Bruschi and Howe, 1988; Bruschi and Ludwig, 1989; Ludwig, 1991). The phenotypes of the cells harboring these plasmids is indicated. Abbreviations are as follows: LEU: leucine prototroph (+) or auxotroph (-); B: blue colony; W: white colony.

This is a plasmid based on the *E. coli*-yeast shuttle vector pBL-1S, a FLP-mediated site specific recombination product between homologous FLP target sites present on the native yeast endogenous plasmid 2-µm and on pRL, a 2-µm YEp derivative; The pRL plasmid contains the yeast selectable marker *LEU2*, coding for the enzyme ß-isopropylmalate dehydrogenase, the 2-µm origin of replication with a 599 bp repeat sequence containing the FLP recombination target (FRT) required for the site-specific recombination, and the pBR322 DNA sequences necessary for selection and maintenance in *E. coli* (Bruschi and Howe, 1988). The plasmid pBL-1S has the 2-µm HpaI restriction endonuclease site converted to a unique SmaI site, in which the BLITZ expression cassette was cloned (Ludwig and Bruschi,1991). The BLITZ expression cassette is composed of following different elements, shown in Fig. 10:

a) The yeast *GAL10* upstream activator site (UAS).
b) The truncated promoter, signal sequence and leader coding region of the yeast alfa mating factor *MFα1*.
c) A synthetic DNA sequence encoding a SmaI cloning site and three collagenase recognition target sequences (coll).
d) Downstream the SmaI cloning site, there is the *E. coli lacZ* reporter gene, encoding the enzyme ß-galactosidase.
e) The yeast *TRP5* transcriptional terminator sequence (Ludwig, D. L., 1991).

This cassette allows the cloning, expression, and secretion of a foreign gene, in our case *LiPH8*, fused to the *lacZ* bacterial reporter gene, Fig. 11. The entire 2-µm sequence present in the pBLITZ2µ-C plasmid is responsible for the high stability of this vector under nonselective conditions. In fact, this plasmid molecule can encode for all the 2µ circle functions, that is the site-specific FLP recombinase responsible for the flipping activity at the FRT sites, encoded by the *FLP* gene; the REP1 and REP2 gene products from their corresponding genes which are responsible for efficient segregation, and the

D protein. Additionally, all 2μ circle cis-elements are also present; these are the *ORI* origin of replication, the *STB* locus necessary for stability, and the two 599 base pair inverted repeats, containing the FLP recombination targets (FRT) required for the site-specific recombination occurring at these sequences (Broach, 1981).

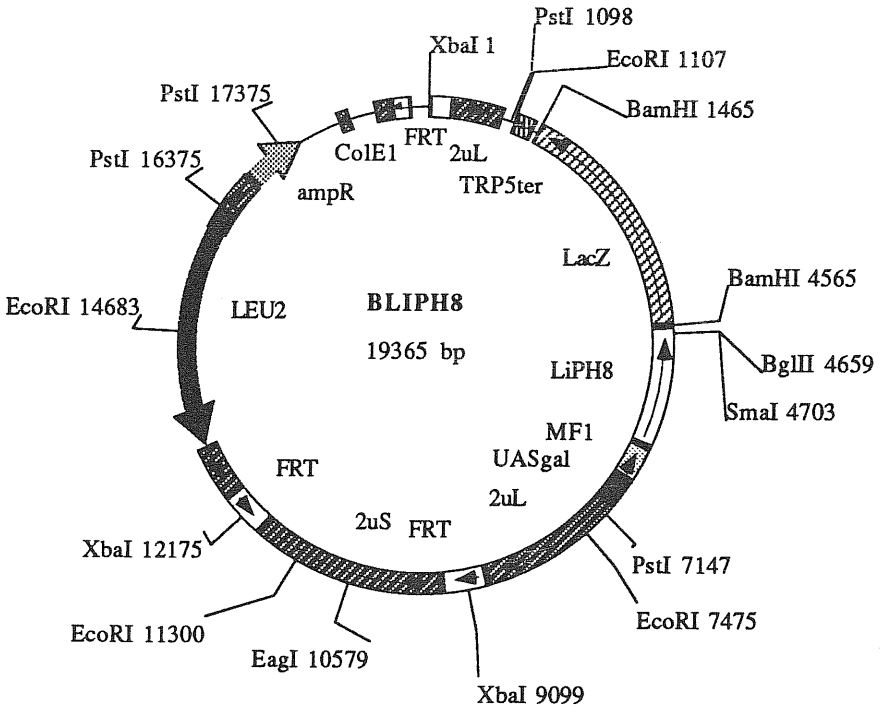


Figure 11 The pBLIPH8 vector, carrying the LiPH8 ORF cloned into the SmaI restriction endonuclease site of the pBLITZ2μ-C plasmid.

2. MATERIALS AND METHODS

Strains *E. coli* bacterial strain used for routine work was DH5alfa (*endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, deltalacU169 (phi80lacZdeltaM15)*, (Hanahan, 1983) or HB101 (*hsdS20, supE44, ara14, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA13, mcrB, mcrA, mrr)*, (Boyer and Roulland-Dussoix, 1969).
Saccharomyces cerevisiae yeast strain was CBL1-20 (*alfa, [cir^0], ura3-52, leu2-3,112, trp1-289, pep4-3*), constructed in our laboratory by D. L. Ludwig (1991).

Media and General techniques Standard Luria-Bertani (LB) medium was used for bacteria cultivation, according to Sambrook, *et al.* (1989):

LB

1% Bacto-tryptone

1% NaCl

0.5% Bacto-yeast extract

pH 7.0

Medium was sterilized by autoclaving for 20 minutes at 1.0 bar.
Stock solution of ampicillin was prepared at 100 mg/ml in redistilled water, sterilized by filtration through a 0.22 µm filter, and stored at -20°C. Ampicillin was added to warm medium (50°C) after autoclaving a final concentration of 100 µg/ml for selection of recombinant plasmids.

Yeast media, semi-defined yeast extract peptone dextrose (YPD), yeast extract peptone galactose (YPGAL) and leucine-less (LEU), were as described in Sherman, *et al.* (1986):

YPD

1% Bacto-yeast extract

2% Bacto-peptone

2% Dextrose.

YPGAL

1% Bacto-yeast extract

2% Bacto-peptone

2% Galactose.

LEU medium was made with synthetic minimal medium described below and amino acids supplement (w/o leucine).

Synthetic minimal medium

2% Glucose

0.5% Ammonium sulfate

0.17% Yeast nitrogen base, w/o amino acids and ammonium sulfate

pH 5.8

100X amino acids supplement A (500 ml)

			1X Final [ ], mg/liter
0.5	g	Adenine Sulfate	10
2.5	g	L-Arginine HCl	50
1.0	g	L-Histidine HCl	20
5.0	g	L-Leucine	100
2.5	g	L-Lysine HCl	50
1.0	g	L-Methionine	20
2.5	g	L-Phenylalanine	50
15.0	g	L-Threonine	300
2.5	g	L-Tryptophan	50

100X amino acids supplement B (500 ml)

2.5	g	L-Tyrosine	50
1.0	g	Uracil	20

Amino acids were dissolved in 400 ml of distilled water by adding 2-3 ml of 10 N NaOH. Volume was brought to 500 ml and filter sterilize. For Drop-out medium the specific amino acid was omitted. To make a Drop-out medium 10 ml of the sterile Drop-out supplement was aseptically added to 1 liter of autoclaved warm (50°C) synthetic minimal medium .

For solid media 1.5% Bacto-agar was added prior to autoclaving.

Counting yeast cells

Yeast cells were counted with a Tiefe hemocytometer, Bürker type. Typically, 8 µl of cell suspension were placed in the chamber, viewed under 400X magnification and counted within 12 of the 144 major squares. The average number represents the number of cells in a volume of $4X10^{-6}$ ml, and was multiplied by $2.5x10^{6}$ to determine cell density in 1 ml.

DNA techniques

Bacterial plasmid DNA minipreps

Bacterial plasmid DNA minipreps were prepared by a modification of the alkaline lysis procedure, as described by Zhou *et al.* (1990).

Bacteria were inoculated with a sterile loop (approximately 10 µl) into 5 ml of liquid LB medium with the appropriate antibiotic, and grown overnight at 37°C in a rotary shaker. Cells from 1.5 ml of the culture were pelleted by centrifugation in a microcentrifuge at top speed for 20 seconds. The medium was almost completely removed, leaving approximately only 100 µl of supernatant in which cells were resuspend by vortexing;

300 µl of TENS solution (TE buffer containing 0.1 N NaOH and 0.5% sodium dodecyl sulfate) were added, and the mixture vortexed for a few seconds. After adding 150 µl of 3.0 M sodium acetate, pH 5.2, the mixture was vortexed again to mix completely, and then spinned for 2 minutes in a microcentrifuge to pellet cell debris and chromosomal DNA. Supernatant was transferred to another tube, and mixed with 0.9 ml of cold (-20°C) 100% ethanol. Plasmid DNA (along with RNA) was pelleted by 2 minutes centrifugation as above. The supernatant was discarded the pellet washed with 70% ethanol and dried under vacuum. The pellet was resuspended in 40 µl of TE buffer (10 mM Tris-HCl, pH 7.6 and 1 mM EDTA, pH 8.0) or distilled water, and stored at 4°C until needed. RNA was degraded during incubation with restriction endonucleases by addition to the mixture of 25 µg/ml RNase.

Large scale plasmid DNA purification

Large scale plasmid DNA purification was carried out as outline by Garger *et al.*, (1983). Bacteria were first extracted with alkali according to a modified procedure of Birnboim and Doly, (1979). Plasmid containing cells were grown overnight at 37°C with shaking in a 2 liter flask containing 500 ml LB medium supplemented with 100 µg/ml ampicillin. Culture was sedimented in a Sorvall® RC-5B centrifuge (Du Pont, Wilmington, Delaware) in 250 ml bottles using a GSA rotor at 4000Xg at 4°C for 10 minutes. Supernatant was discarded and the pellet washed with 25 ml of cold STE (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0). The mixture was transferred to 40 ml Oak Ridge tubes and cells pelleted in a SS34 rotor as above. Again, the supernatant was poured off, and 5 ml of glucose buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) containing 5 mg/ml of lysozyme (Sigma, St. Louis, MO, USA) (freshly prepared) were mixed to the pellet, and the solution incubated at room temperature for 20 minutes. Lysis was obtained by addition of 10 ml of lysis buffer (1% SDS (w/v), 0.2 N NaOH), gentle mixing and incubation on ice for 10 minutes. Precipitation of cell debris and high molecular weight nucleic acids from the aqueous phase was achieved by mixing well the solution with 7.5 ml of potassium acetate (3 M potassium, 5 M acetate)

pH 4.8, followed by incubation on ice for at least 45 minutes. The mixture was pelleted in a Sorvall® SS34 rotor at 15000Xg for 1 hour at 4°C. The clear lysate was divided into four portions of approximately 11 ml in fresh Oak Ridge tubes, mixed with 0.6 volumes of isopropanol, and left at room temperature for 15 minutes. DNA was recovered at 12000Xg for 15 minutes at room temperature. The supernatant was then discarded, and the pellet dried under vacuum. For the DNA purification each pellet was dissolved in 1.0 ml TE, the four samples pooled, and one more ml was used to rinse the tubes; 4.8 ml of this solution were dispensed in a 50 ml tube (Sarstedt, Germany) , combined and mixed with exactly 8.4 g CsCl until complete dissolution. At this point 0.8 ml of ethidium bromide (10 mg/ml in water) were added, to give a density of 1.80 g/ml, and the mixture centrifuged at low speed in a Sorvall® RT6000B centrifuge, so that proteins would float at the top of the tube. A light solution of CsCl (density of 1.470 g/ml) was made by dissolving 60 g of CsCl in 80 ml TE; 8 ml of this solution were dispensed in each one of two polyallomer ultracentrifuge tubes (Quick-Seal®, 16X76 mm, Beckman, Palo Alto, CA, USA), and 4.0 ml of the dense nucleic acid-containing CsCl solution (underneath the protein layer) was carefully layered with a Pasteur pipette underneath the light CsCl solution into the ultracentrifuge tubes. Tubes were then filled and balanced with the light CsCl solution, sealed and run in a Beckman VTi65 rotor at 50000 rpm for 4-5 hours at 20°C.

Closed circular plasmid DNA was visualized under a long wave UV lamp, and recovered by puncturing the tube with a syringe bearing a 18-gauge needle, as described by Sambrook, *et al.*, (1989). Ethidium bromide was removed by extraction with organic solvents. One volume of 1-butanol (saturated with water) was added to the recovered DNA solution, thoroughly mixed, and then centrifuged at 1500 rpm in a microfuge for 3 minutes at room temperature. The lower aqueous phase was recovered and the extraction repeated until the solution would become clear. CsCl was removed by dialysis for 48 hours against several changes of 1 liter TE (pH 8.0) at 4°C in dialysis bags (GIBCO/BRL, Gaithersburg, MD, USA). DNA was recovered by addition of 0.1 volumes of

3 M sodium acetate (pH 5.2), and precipitation with 2.5 volumes of anhydrous ethanol at -20°C. DNA was pelleted in a microfuge at top speed for 15 minutes. Pellet was rinsed with 70% ethanol and spinned again. DNA was finally resuspended in approximately 1 ml of TE (pH 8.0) and concentration and purity were calculated spectrophotometrically by reading the absorbance at 260 and 280 nm, and assuming that 1 $OD_{260} \cong 50$ µg/ml of double-stranded DNA. Purity of the sample was determined by the ratio OD_{260}/OD_{280}, considering a value of 1.8 as a pure DNA preparation (Sambrook, *et al.*, 1989). DNA was stored at 4°C until needed.

Yeast plasmid DNA minipreps

Yeast plasmid minipreps were carried out as outlined in Sherman *et al.*, (1986). Cells were grown overnight in 5 ml of YPD, at 30°C in a rotary shaker. The culture was pelleted by spinning in a Sorvall® RT6000B centrifuge for 10 minutes at 2000 rpm. Cells were then resuspended in 0.5 ml of 1 M sorbitol, 0.1 M EDTA pH 7.5, and the mixture was transferred to a microfuge tube. In order to digest the cell wall, 75 µl of lyticase (Sigma, 900 units/ml, 67 mg/ml) were added, and the reaction tube incubated at 37°C for 60 minutes. Following incubation, spheroplasts were pelleted in a microfuge for 1 minute, and resuspended in 0,5 ml 50 mM Tris pH 7.4, 20 mM EDTA. Cells were lysed by addition of 50 µl of 10% SDS, followed by mixing and incubation at 65°C for 30 minutes. Proteins, chromosomal DNA, and high molecular weight RNA were precipitated by addition of 0.2 ml of 5 M potassium acetate and incubation in ice for 60 minutes. The mixture was spinned in a microfuge at top speed for 5 minutes, and the supernatant transferred to a fresh tube. DNA (along with RNA) was precipitated by addition of 1 volume of isopropanol at room temperature. The solution was mixed and incubated for 5 minutes at room temperature. After brief spinning in a microfuge, the supernatant was poured off and the pellet was dried under vacuum. The pellet was resuspended in 0.3 ml TE or distilled water. RNase treatment was carried out as described above.

Agarose gel electrophoresis of DNA

Usually DNA samples were run on 0.8% agarose gels, prepared by adding the amount of agarose to a 0.5X TBE buffer (5X TBE: 0.45 M Tris-borate, 0.01 M EDTA pH 8.0), and melting in a microwave oven. After the solution reached approximately 60°C, the mixture was poured and let solidify. DNA samples were added 1X of DNA sample buffer (6X: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and loaded on the wells. The gel was run in 0.5X TBE at approximately 8 V/cm. Gels were stained by immersion in a solution of 0.5 µg/ml of ethidium bromide, and visualized under UV light.

Purification of synthetic oligonucleotides

Oligonucleotides were synthesized by the ICGEB oligonucleotide service, and delivered, already deblocked, in solution with ammonium hydroxide. Oligonucleotides were purified according to Sambrook, *et al.*, (1989). The oligonucleotide solution was freeze-dried in a centrifugal evaporator (Heto, Birkerød, Denmark), resuspended in 1 ml of sterile, filtered water, and centrifuged at 12000Xg for 5 minutes. The supernatant was transferred to a fresh tube, and extracted three times with 400 µl of 1-butanol (*n*-butyl alcohol), discarding the upper organic phase. The solution was evaporated again as above, and the pellet redissolved into 200 µl of water. Concentration and purity of the solution were calculated spectrophotometrically by reading the absorbance at 260 and 280 nm, and assuming that 1 $OD_{260} \cong 33$ µg/ml of oligonucleotide solution, in a 1 cm path-length cuvette. Concentration of the oligonucleotide was also determined using the equation: total OD_{260} /10 x length of oligonucleotide = µmoles of oligonucleotide present in the solution (Sambrook, *et al.*, 1989). Oligonucleotides were further purified by denaturing polyacrylamide gel electrophoresis, through a 19% polyacrylamide gel for 20mers used for PCR, and 15% polyacrylamide for the 30mer used for the Northern analysis. Gels were prepared with the following final concentration of reagents: 19% or 15% acrylamide from 40% acrylamide stock (38 % acrylamide, 2% *N,N'*-methylenebisacrylamide), filtered through a 0.45 µm pore size filter; 1X TBE from 5X TBE (0.45 M Tris-borate, 0.01 M EDTA pH 8.0); 7 M urea, dissolved by heating the solution to

37°C; 0.06% ammonium persulfate from a freshly prepared 10% stock, and 1/2000 of TEMED (N,N,N', N' -tetramethylethylenediamine). Gels were pre-run in 1X TBE at 400 V for 15 minutes, while 20 µl (out of 200 µl) of the oligonucleotide solution were thoroughly mixed to an equal volume of formamide, heated to 55°C for 5 minutes, and finally loaded on each lane. To follow the migration of buffer front, 0.2% of the dye orange G was included. After the run, oligonucleotides were visualized under long wave UV light by placing the gel over a silica gel F_{254} thin-layer chromatography plate (Merck, Darmstadt, Germany) excised with a scalpel, and submerged with 100 mM ammonium acetate in a microfuge tube. Following overnight incubation at room temperature, oligonucleotides were centrifuged at 12000Xg at room temperature for 5 minutes, and the supernatant was passed through a NAP™-5 column prepacked with Sephadex® G-25 (Pharmacia/LKB). Oligonucleotides were quantitated spectrophotometrically as already described.

Plasmid constructions

The genomic clone of H8 (*LiPH8*) was a gift of Dr. D. Cullen (USDA Forest Products Lab, Madison, WI 53705, USA), Fig. 5. The ORF of *LiPH8* was amplified by the polymerase chain reaction (PCR), starting at the first nucleotide following the ATG, to the last nucleotide preceding the TAA stop codon. The PCR reaction was performed using a PREM Series III instrument (LEP Scientific Limited, Milton Keynes, UK). DNA amplification was carried out in 1X Vent™ NEBuffer (New England Biolabs, Beverly, MA, USA) (10 mM KCl, 20 mM Tris-Cl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 100 µg/ml bovine serum albumin, with 200 µM each dNTP, 100 pmol each primer, 100 ng template DNA, with 2.5 U Vent™ DNA polymerase (Biolabs), in a final volume of 50 µl. The mixture was subjected to 30 cycles constituted by a denaturing step of 1 minute at 94°C, an annealing step of 2 minutes at 50°C, and an extension step of 3 minutes at 72°C.

The PCR product was purified by 0.8% agarose gel electrophoresis, and the band excised under a long wave UV lamp. The DNA was recovered by means of the Prep-A-Gene™ DNA purification kit (Bio-Rad, Richmond, CA, USA) and cloned into the SmaI restriction site of the expression cassette of the Shuttle vector pBLITZ2μ-C (Ludwig, 1991) as shown in Fig. 11. The ligation mixture was used transform bacterial cells.

The resulting plasmid pBLIPH8 was recovered in *E. coli*, identified by colony hybridization, checked by restriction analysis, and the construct carrying the insert in the same orientation of *lacZ* was used to transform the yeast strain CBL1-20; Transformant cells were selected for leucine prototrophy onto LEU minimal medium. Plasmid stability of pBLIPH8 was then determined according to Ludwig and Bruschi (1991), as outlined in Fig. 12.

DNA transformations

E. coli competent cells were prepared following a modified $CaCl_2$-heat shock method based on the experiments of Mandel and Higa (1970), as recently described by Sambrook, *et al.*, (1989). The recipient strain was inoculated in 5 ml LB medium and grown to stationary phase as described above. An aliquot of 0.1 ml of overnight culture was used to inoculate 25 ml of fresh LB, and cells were grown for approximately 3 hours to an OD_{600} of 0.4. The culture was transferred to an Oak Ridge tube, cooled on ice for 15 minutes, and centrifuged at 10000 rpm in a Sorvall® SS34 rotor for 10 minutes at 4°C. Medium was discarded, cells resuspended in 25 ml of ice cold 100 mM $MgCl_2$. Cells were spinned as above, pellet was resuspended in 25 ml of 100 mM $CaCl_2$ and left overnight on ice. Competent cells were pelleted again, and resuspended in 1.2 ml of 100 mM $CaCl_2$; 0.2 ml aliquots of this suspension were thoroughly mixed with glycerol (in a final 85%:15% ratio), quickly frozen in isopropanol-dry ice bath, and dispensed at -80°C until needed. Transforming DNA was added to thawed *E. coli* competent cells, and tubes were incubated on ice for 30 minutes. Cells were then heat shocked by incubation in a 42°C water-bath for 2 minutes, after which were grown in 2 ml LB at 37°C for 2

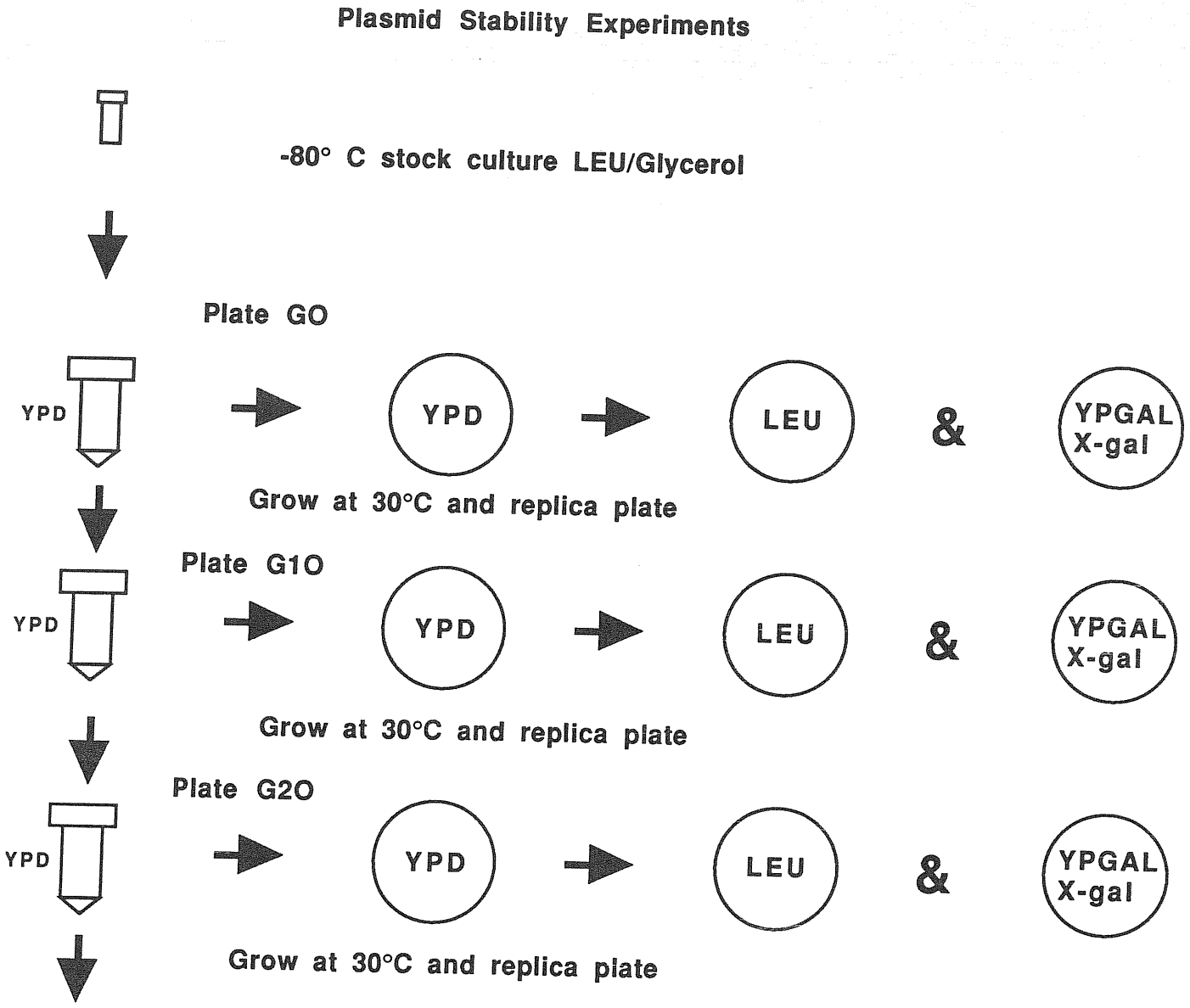


Figure 12 Flow chart representing the methodology used for the determination of plasmid stability. Aliquots from the -80°C stock cultures were grown to stationary phase by a round of growth in selective LEU medium, diluted and plated onto rich non selective medium (YPD), incubated at 30°C for 3 days to yield 100-300 CFU/plate (G∅), big enough to be replica-plated onto selective medium (LEU/Glucose) and nonselective (YPGAL) medium (pH 7.0, with 100 µg/ml of X-gal). Colonies were then scored for growth and color as an indication of the presence and type of plasmid. Continuous cultures were maintained by inoculating 10 µl of the grown culture into 10 ml of fresh YPD. This procedure was carried out for at least fifty generations of growth in liquid medium.

hours. Dilutions were plated on selective medium and grown overnight at 37°C. Transformant colonies were screen by colony hybridization, as discussed below, or used for plasmid DNA minipreps, and recombinant plasmids checked by restriction endonuclease analysis as already described.

Yeast transformation

Yeast cells were transformed according to the method of Bruschi, *et al.*, (1987). Cells were inoculated at a density of $1X10^5$ cells/ml in 100 ml of YPD and grown at 30°C with shaking to a density of $2X10^7$ cells/ml. The culture was divided into four 25 ml aliquots in 50 ml tubes (Sarstedt, 114 X 29 mm Ø) to give $5X10^8$ cells per experimental tube. Tubes were centrifuged at 2000 rpm in a Sorvall® RT6000B centrifuge for 10 minutes, and the medium discarded. Cells were washed once with 10 ml TE, the washes pooled together in one 50 ml tube, and pelleted as above. Cells were then resuspended in 10 mM CaTE (10 mM $CaCl_2$ in TE) to a density of $1.5X10^8$ cells/ml, transferred to a 50 ml tube, and gently shaken on a rocking platform at 24°C for 30 minutes. Cells were pelleted again as above, and resuspended in TE at a density of $5X10^8$ cell/100 µl and a volume of 400 µl. Aliquots of 100 µl were made in microfuge tubes, 1 µg of transforming DNA was added, and tubes were put on ice for 30 minutes. Cells were heat shocked by incubation for 5 minutes in a 42°C water-bath, and then returned to ice for 10 minutes. Cells were gently mixed to 1 ml of filter sterilized PEG solution (40% PEG 4000 made in TE, pH 7.7), and left at room temperature for 45 minutes. The mixture was pelleted by centrifugation in a Sorvall® RT6000B centrifuge at 2000 rpm for 6 minutes, and the solution was discarded and the tubes inverted to dry off the excess of liquid. Cells were gently rescued with 0.5 ml of SOS medium (10 ml 1 M sorbitol, 6.7 ml YPD, 1.3 ml 100 mM $CaCl_2$, 2.0 ml water), and incubated at room temperature for 1 hour with gentle shaking. Aliquots of 100 µl were plated onto selective medium, and 10^{-5} dilutions onto complete plates. Plates were incubated at 30°C for 3 days.

Colony hybridization

Colony hybridization was used to identify *E. coli* recombinant clones harboring the pBLIPH8 plasmid. This technique was carried out essentially as described by Sambrook, *et al.*, (1989). Bacterial cells were transformed and plated onto selective medium as described above. Individual colonies were picked with a sterile toothpick and transferred onto selective plates using a numbered colony grid as a template. These plates were replica plated onto a nylon membrane (Hybond™-N+, Amersham, UK), and carefully marked with a pencil to ensure correct orientation of colonies. Cells were lysed as outlined by Grunstein and Hogness, (1975), with some modifications suggested by the manufacturer of the filter. Nylon membranes were placed with the colony side up onto Whatman 3 MM paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH), and exposed for 7 minutes. Filters were then transferred to 3MM paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA), and left there for 3 minutes. This neutralizing step was repeated, and filters were washed with 2X SSPE (20X SSPE: 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA pH 7.7) or 2X SSC (20X SSC: 3 M NaCl, 0.3 M sodium citrate). DNA was fixed by the alkaline fixation procedure as described for Southern blots.

Filter hybridization was carried out as recommended by the membrane manufacturer. Reagents for hybridization were prepared as specified by Sambrook, *et al.*, (1989). Denhardt's solution (Denhardt, D. T., 1966), was made as 50X, containing 1% [w/v] Ficoll™ (type 400, Pharmacia/LKB), 1% [w/v] polyvinylpyrrolidone (PVP), 1% [w/v] bovine serum albumin (fraction V, Sigma), and stored at -20°C until needed. 1X Pre-hybridization solution (5X SSPE, 5X Denhardt's solution, 0.5% [w/v] SDS) was used in a ratio of 0.2 ml/cm^2 of filter; denatured (5 minutes at 100°C, then chilled on ice) sonicated non-homologous (salmon sperm) DNA was added to a final concentration of 100 µg/ml. The pre-hybridization was carried out in a sealed plastic bag submerged in a shaking water-bath at 65°C for 1 hour. Hybridization was carried out adding denatured labeled

probe (see below for labeling) to the pre-hybridization bag, in an amount not exceeding a concentration of 20 ng/ml. The hybridization bag was sealed inside a second plastic bag and incubated for at least 12 hours at 65°C. Filters where washed by incubating them with 2X SSPE, 0.1% [w/v] SDS, at room temperature for 10 minutes. This washing was repeated, and the the membrane was incubated in 1X SSPE, 0.1% [w/v] SDS, at 65°C for 15 minutes. Since the probe had 100% homology with the target sequence, an additional high stringency wash was done with 0.1X SSPE, 0.1% [w/v] SDS, at 65°C for 1 minutes. Filter was then removed and hybridization was detected by autoradiography at -80°C using Kodak XAR-2 X-ray film (Eastman Kodak, Rochester, NY, USA).

Southern blot transfer

DNA samples were run on an agarose gel (0.8%) in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA), and transferred by capillary blotting to a nylon membrane (Hybond™-N+, Amersham) by the transfer technique described by Southern, (1975). The gel was placed on a platform covered by three sheets of 3MM filter paper (Whatman, Clifton NJ, USA) with their ends into a reservoir containing blotting buffer, either 20X SSC (3 M NaCl, 0.3 M sodium citrate) or 20X SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA pH 7.7). The stack of filter paper was also saturated with blotting buffer, and the nylon membrane of the size of the gel was placed over it, being careful to exclude air bubbles by rolling with a pipette. Three more sheets of 3MM paper were placed over the membrane, and a final stack of absorbent paper towels was placed on the top. A weight of approximately 500 g was placed over the construction, and transfer was allowed to run overnight.

DNA labeling

After blotting, filters were fixed with alkali by placing them on filter paper (Whatman No.1) soaked in 0.4 M NaOH for a period of 20 minutes. Filters were then rinsed by brief immersion in 5X SSPE or 5X SSC, and hybridized as outlined by Sambrook, *et al*, (1989). The DNA probe (25 ng) was radioactively labeled by the random primed DNA labeling method (Boehringer Mannheim, Mannheim, Germany) according to Feinberg and Vogelstein, (1983). DNA was denatured by heating at 100°C for 10 minutes, and then chilled on ice. The following was added to the denatured DNA: 3 µl of dNTPs mixture (dATP + dGTP + dTTP) at a final concentration of 25 µM each, 2 µl random hexanucleotides (3.1 mg/ml final) in 10X concentrated reaction buffer (500 mM Tris-HCl pH 7.2, 100 mM $MgCl_2$, 1.0 mM dithioerythritol, 2.0 mg/ml bovine serum albumin), 5 µl (50 µCi) of [α-^{32}P] dCTP (specific activity at reference 3000 Ci/mmol), 1 µl Klenow enzyme (2 units) labeling grade. Final volume was adjusted to 20 µl with sterile redistilled H_2O and the reaction tube was incubated at 37°C for 30 minutes. The reaction was stopped by adding 2 µl EDTA, 0.2 mM pH 8.0. The radiolabeled DNA probe was purified from unincorporated nucleotides by gel-filtration chromatography through Sephadex® G50 (Fine) (Pharmacia/LKB) with a Quick Spin™ column (Boehringer Mannheim), as specified by the manufacturer. Hybridization and autoradiography were done as described above.

Growth of yeast cultures for total RNA isolation

CBL1-20 yeast cells transformed with pBLITZ2µC (Cont), or with pBLIPH8 (T1) plasmid DNAs were inoculated into 400 ml YPD at $4x10^5$ cells/ml, and grown overnight to stationary phase. Cells were then pelleted in a Sorvall® RC-5B centrifuge in 250 ml bottles using a GSA rotor at 5000 rpm at 4°C for 10 minutes. Supernatant was discarded the pellet washed with sterile YPGAL medium for the induction of the GAL1/10 promoter and pelleted again. Cells were resuspended in YPGAL, counted with an hemocytometer, and inoculated into liquid YPGAL at $2x10^7$ cells/ml, at 30° C with vigorous shaking. Two parallel 400 ml cultures were prepared for each transformant cell, CBL1-20 harboring

the control plasmid pBLITZ2μC, cultures ContI and ContII, and CBL1-20 harboring the recombinant vector pBLIPH8, designed T1I and T1II. Cultures were grown until they reached respectively $4x10^7$ cells/ml (ContI), $8.8x10^7$ cells/ml (T1I) and $4.3x10^7$ cells/ml (ContII), and $7,2x10^7$ cells/ml (T1II).

Yeast cultures grown in YPGAL medium were poured into 250 ml centrifuge bottles containing an equal volume of crushed ice with 50 mM sodium azide, and centrifuged immediately in a Sorvall® GSA rotor at 5000 rpm for 10 minutes. Cell were washed once with RNA buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA pH 8.0), transferred to 2 ml microfuge tubes and pelleted; supernatant was discarded and pellets were frozen at -80°C until needed. Total RNA was isolated as described below.

RNA techniques

Total RNA was extracted from the YPGAL cultures with the glass beads protocol, according to Sherman, *et al.*, (1986). Frozen yeast cells were thawed and resuspended 0.25 ml of LETS buffer (0.1 M LiCl, 0.01 M EDTA pH 8.0, 0.01 M Tris-HCl pH 7.4, 0.2% SDS). The suspension, kept on ice, was then added 1 g of acid-washed glass beads (B.Braun Melsungen AG, 0.45-0.50 mm Ø), and approximately 300 μl of phenol equilibrated with LETS buffer, so that meniscus would be just above surface of glass beads, for optimal cell disruption. Cells were lysed by vortexing at maximum speed each tube for at least 10 times for 30 second periods, alternated to 30 seconds intervals on ice. Cell disruption was monitored microscopically under phase contrast, looking for broken cells that appear as non-refractile ghosts. When ghosts represented at least the 90% of the population, some additional 0.25 ml of ice cold LETS buffer were added, thoroughly mixed, and centrifuged at 5000Xg for 5 minutes to separate the two phases. The aqueous phase, just above the surface of the beads, was recovered, transferred to a fresh tube, and extracted twice with phenol (equilibrated with TE, pH 7.6)/ chloroform/ isoamyl alcohol (25:24:1). Nucleic acids were precipitated by addition of 0.1 volumes of 10 M LiCl, 2.5 volumes of 100% ethanol, and incubation at -20°C for 3 hours. Following

centrifugation at 14000Xg for 15 at 4°C, the pellet was first washed with 80% ethanol, then once with ice cold 3 M sodium acetate to remove DNA, and again with 80% ethanol. RNA was quantitated spectrophotometrically, by reading the absorbance at 260 and 280 nm, and assuming that 1 $OD_{260} \cong 40$ µg/ml of RNA. Purity of the sample was determined by the ratio OD_{260}/OD_{280}, considering a value of 2.0 as a pure RNA preparation (Sambrook, *et al.*, 1989).

Isolation of Poly (A)+ RNA

Isolation of Poly (A)+ RNA was done by affinity chromatography through an oligo (dT)-cellulose column (Aviv and Leder, 1972) prepared following the manufacturer's specifications (Boehringer Mannheim). Column was prepared by suspending 1 g of the dry powder in RNase-free 1X elution buffer (10 mM Tris-HCl, 1 mM EDTA, 0.05% [w/v] SDS; pH 7.5), to a final volume of 1 ml, and by pouring it into a 2.5 ml syringe, plugged with glass wool previously sterilized by overnight incubation at 250°C.

Successive operations were conducted according to the protocol given by Sambrook, *et al.*, (1989). Column was prepared by washing with sterile RNase-free 1X binding buffer (10 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 0.1% [w/v] SDS; pH 7.5) until the pH of the effluent would be less than 8.0. Total RNA, in sterile RNase-free water, was heated at 65°C. for 5 minutes, to disrupt region of secondary structure, then quickly cooled to room temperature and mixed with an equal volume of 2X binding buffer. This solution was carefully applied to the column, and 1 ml fraction were collected. Column was then added 1 column volume of 1X binding buffer, and the eluate collected as above. The eluted solution was then heated again, and reapplied to the column as above, and the eluate collected. The column was then washed with 10 column volumes of 1X binding buffer, again collecting 1 ml fractions. For each fraction the decreasing OD_{260} was monitored, following the elution of non-polyadenilated RNA. Poly(A)+ RNA was eluted from the column with 3 column volumes of 1X elution buffer, collecting 300 µl fractions. The absorbance of each fraction was read as above, using quartz cuvettes treated with

concentrated HCl-methanol (1:1), and then profusely rinsed with sterile RNase-free water.

Fractions containing poly(A)+ RNA were pooled, the concentration of NaCl adjusted to 0.5 M, and the solution was further purified by a second round of chromatography on the same column, as described above. The purified poly(A)+ RNA was finally eluted, mixed to 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of 100% ethanol precooled at -20°C, and incubated at -20°C. Poly(A)+ RNA was recovered by centrifugation in a microcentrifuge at 14000Xg for 15 minutes at 4°C. The pellet was washed with 70% ethanol and centrifuged again. Once dried, the poly(A)+ RNA was resuspended in sterile RNase-free water, and absorbance read as already described above. From 10 mg of total RNA, approximately 2 µg of poly (A)+ RNA, in 100 µl volume, were recovered for the control culture ContI, and approximately 3 µg, again in 100 µl volume, for the recombinant culture TM1.

RNA gel electrophoresis

The quality of the total RNA and purified poly(A)+ RNA was checked by formaldehyde agarose gel electrophoresis, using RNase-free reagents, as outlined by Sambrook, *et al.*, (1989). Typically, for a final gel volume of 100 ml, agarose gel was prepared by melting in a microwave oven 1% agarose in 62 ml of water, cooling it at 60°C, and adding 20 ml of 5X formaldehyde gel-running buffer (5X FGRB: 0.1 M MOPS [3-(N-morpholino) propanesulfonic acid] pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0) for a final concentration of 1X, and 18 ml of filtered 12.3 M formaldehyde (pH > 4.0) to give a final concentration of 2.2 M. Pure RNA samples, or aqueous RNA solutions, were mixed in a final concentration of approximately 25% with 1X formaldehyde gel sample buffer (1X FGSB: 0.5X FGRB, 2.2 M formaldehyde, 50% deionized formamide) heated to 65°C for 15 minutes, and then chilled on ice. The mixture was added 0.1 volume of 1X formaldehyde gel-loading buffer (1X FGLB: 50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF). Gel was prerun for 5 minutes at 5 V/cm, the

samples loaded and run in 1X FGRB at 5 V/cm. Following brief staining with 0.5 µg/ml ethidium bromide in 1X FGRB, RNA was visualized under UV light.

Northern analysis

Capillary blot was set up as described earlier for the Southern blotting. Northern blots were fixed by the alkali procedure, using 0.05 M NaOH. Filters were rinsed by immersion in 2X SSPE (or 2X SSC), with gentle agitation. The DNA probe used in the Northern analysis is a synthetic oligonucleotide provided by the ICGEB oligonucleotide service, and consists of a 30 nucleotide DNA sequence, called I1-30, entirely comprised within the first intron of the *LiPH8* gene, complementary to the corresponding mRNA.

Labeling of the DNA probe

The gel purified probe was radiolabeled at its 5'OH terminus with [γ-^{32}P] ATP using the enzyme bacteriophage T4 polynucleotide kinase (Boehringer Mannheim), in the supplier's incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM dithiothreitol; pH 7.5 at 37°C). The protocol was that suggested by Sambrook, *et al.*, (1989). Typically, to label the probe at high specific activity, 3 pmoles of oligonucleotide (in 1 µl volume) were used, by adding 1 µl of water, 2 µl of 10X kinase buffer, 1 µl (10 units) of T4 polynucleotide kinase, and 15 µl of [γ-^{32}P] ATP (5000 Ci/mmol; 10 µCi/µl, 5 µCi/pmole), resulting in 20 µl of final volume. The reaction tube was incubated at 37°C for 45 minutes, and stopped by heating at 68°C to inactivate the enzyme. The radiolabeled oligonucleotide was purified from unincorporated radioactivity by addition of 40 µl of water, 240 µl of 5 M ammonium acetate, 750 µl of precooled 100% ethanol and then precipitated by incubation at -20°C. The radiolabeled oligonucleotide was recovered by centrifugation in a microfuge at 12000Xg for 20 minutes at 4°C. The supernatant was carefully removed, 500 µl of 80% ethanol added, and the mixture centrifuged again. The supernatant was removed and the pellet was finally resuspended into 100 µl of TE (pH 7.6).

Filter hybridization was carried out as per the Southern blot, except that a final concentration of 50% formamide was included in the hybridization bag, and the reaction took place at 50°C overnight. Autoradiography was performed as for the Southern hybridization.

Plasmid stability experiments

Following transformation, single colonies were picked from LEU plates and used to inoculate 10 ml of selective LEU medium, to maintain the pRL portion of the pBLITZ2μ-C plasmid. These cultures were grown to stationary phase, and were stored in 15% glycerol at -80°C for subsequent analysis. Aliquots from the -80°C were then grown to stationary phase by another round of growth in selective LEU medium, diluted and plated onto rich non selective medium (YPD), incubated at 30°C for 3 days to yield 100-300 CFU/plate (G0), big enough to be replica-plated as shown in Fig. 12 and described below.

The presence of the 2μm-*LiPH8* portion of the pBLIPH8 plasmid in cells grown in LEU medium was checked by Southern hybridization, using the [α-^{32}P] labeled 700 bp SphI fragment of the *LiPH8* gene as a probe, as shown in Fig. 13. In order to determine plasmid stability, cells from the LEU cultures were used to inoculate 10 ml of YPD to a density of 2 x 10^5 cells/ml, and grown for 10 generations to a density of 2 x 10^8 cells/ml at 30°C on a rotary shaker. Cultures were then diluted and plated onto rich non selective medium (YPD) to yield 100-300 CFU/plate, and incubated at 30°C for 3 days (G10).
Colonies were both replica-plated onto selective medium (LEU/Glucose) and nonselective (YPGAL) medium, previously buffered at pH 7.0, (Sherman *et al.*, 1986) and containing 100 μg/ml of X-gal (5-bromo-4-chloro-3-indoxyl-ß-D-galactoside), as shown in Fig. 12. The galactose in the YPGAL plates induces the expression of the cassette containing the *lacZ* reporter gene (or the *LiPH8/LacZ* fusion in the case of pBLIPH8). Colonies were then scored for growth and color as an indication of the presence and type of plasmid. Cells harboring the pBLITZ2μ-C plasmid were used as a

control. Continuous cultures were maintained by inoculating 10 µl of the grown culture into 10 ml of fresh YPD. This procedure was carried out for at least fifty generations of growth in liquid medium.

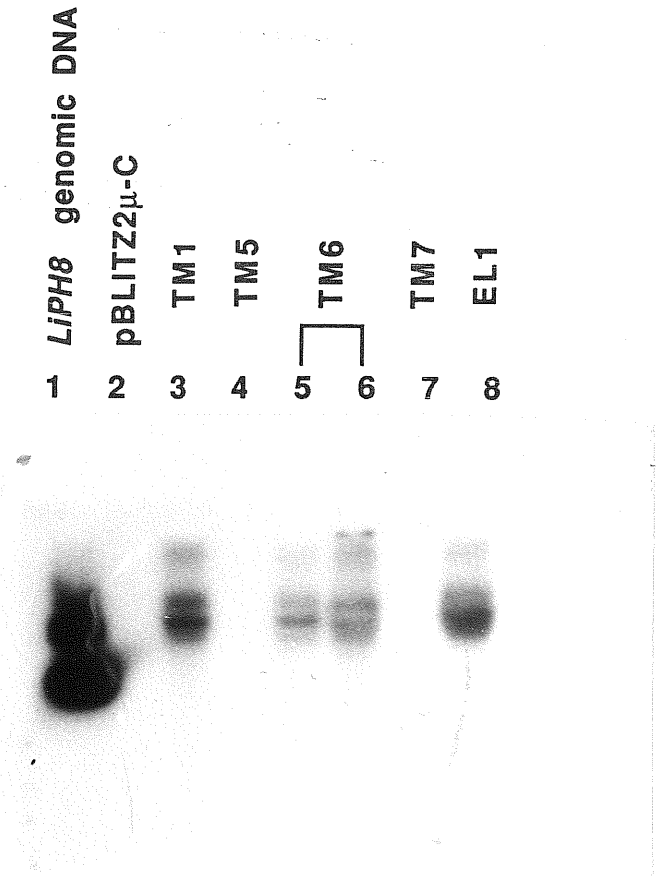


Figure 13 Southern analysis of some yeast recombinant clones transformed with pBLIPH8 plasmid DNA, carrying the *LiPH8* ORF. Aliquots of transformant cells previously stored at -80°C were grown to stationary phase by a round of growth in selective LEU medium, and aliquots from these cultures were used for small scale DNA preparation used for the Southern analysis. Filters were probed with the 700 bp SphI fragment of the *LiPH8* gene, as described in the text.

Lane 1: positive control, uncut pBluescript II ks plasmid carrying the genomic *LiPH8* DNA

Lane 2: negative control, uncut pBLITZ2µ-C plasmid DNA

Lane 3 and 4: uncut pBLIPH8 plasmid DNA form recombinant clones TM1 and TM5

Lanes 5 and 6: uncut pBLIPH8 plasmid DNA form recombinant clone TM6

Lane 7 and 8: uncut pBLIPH8 plasmid DNA form recombinant clone TM7 and EL1

3. RESULTS

3.1 Plasmid stability

Following the growth period of ten generations in liquid YPD, cells were plated in YPD medium, and plasmid resolution for each single colony was monitored by replica-plating it onto LEU and YPGAL/X-gal plates and subsequent recording of the following phenotypes:

a) the ability of growth onto LEU medium, due to the expression of the *LEU* gene.

b) the blue color (on YPGAL/X-gal plates), due to the expression of the *lacZ* gene.

The pBLITZ2μ-C plasmid undergoes *in vivo* recombination between the two 599 bp direct repeat sequences, resulting in the resolution of the plasmid into two circular DNA molecules (Bruschi and Howe, 1988; Bruschi and Ludwig, 1989; Ludwig, 1991), Fig. 10. The colonies harboring the original plasmids pBLIPH8 or pBLITZ2μ-C, have a phenotype LEU+ Blue; the *LEU2* gene is present in the pRL portion of the plasmid, while the ß-galactosidase is encoded by the *lacZ* gene in the 2μm portion of the plasmid. The presence of the resolved plasmid pRL alone confers a LEU+ White phenotype, while the recombinant resolved plasmid pBLIPH8/res or the control plasmid pBLITZ2μ-C/res, that have lost the pRL, result in a LEU- Blue phenotype, Fig. 10. Finally, cells that have lost either the unresolved original plasmid pBLITZ2μ-C or the pBLIPH8, or both the resolved forms pBLITZ2μ-C/res or pBLIPH8, have a LEU- White phenotype.

3.2 Stability of pBLITZ2μ-C vector

This analytical strategy in the use of color/auxotrophic markers allows the study of the resolution of the pBLITZ2μ-C based plasmids, and the effect of the presence and expression of a cloned foreign DNA sequence on plasmid stability. Fig. 14 shows the fate of the control plasmid pBLITZ2μ-C during continuous growth onto liquid non selective medium for at least 50 generations. In the beginning (G0), the majority of the population 60%, is represented by cells harboring either the unresolved plasmid

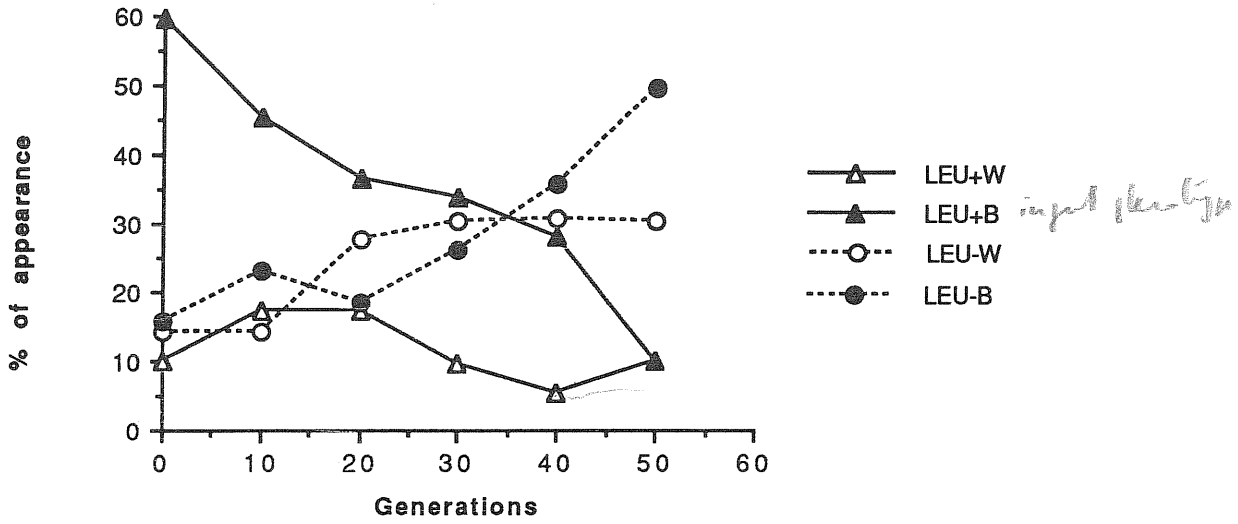


Figure 14 Analysis of the fate of pBLITZ2μ-C plasmid during 50 generations of growth in liquid YPD. Phenotypes of colonies are as follows: LEU: leucine prototroph (+) or auxotroph (-); B: blue colony; W: white colony.

pBLITZ2μ-C, or both the resolved forms pRL and pBLITZ2μ-C/res. Since cells were first grown onto LEU medium and then plated onto rich YPD medium, this growth period on the YPD plate, i. e. approximately 25 generations with no selective pressure, could account for the percentage of the population that has lost one of the two plasmid types, likely pRL that lacks a complete FLP-recognition target system and the cis-acting STB DNA sequence of the 2μm circle necessary for its maintenance (Broach, 1981).

Following cell division, the number of cells with the original vector decreases almost linearly, representing 10% of the population after 50 generations as shown in Fig. 15. This is expected because of the effect of the the activity of the site-specific FLP recombinase (Broach and Hicks, 1980) that results in the generation of two distinct plasmid DNA molecules, pBLITZ2μ-C/res and pRL. The reverse cointegration reaction can also take place with regeneration of the input plasmid, but the frequency of this event is very low, as reported by Bruschi and Howe, (1988). These authors used a tester plasmid carrying two direct 599 bp 2μ repeat sequences allowing intramolecular recombinational events to occur, and leading to the formation of two DNA molecules that were detected by a shift from red to white in the color of the yeast colony, due to the mitotic loss of one of the resolved DNA molecules carrying the *ADE8* gene responsible for the white phenotype. These experiments showed that the resolution of the tester plasmid DNA molecule into two different molecules is an event that occurs much more frequently than the cointegration reaction due to intermolecular recombination events. Thus, the intramolecular recombination seems to be much more efficient than the intermolecular recombination. The less stable pRL portion of the input plasmid is then mitotically lost, and the intermolecular recombination event cannot take place anymore.

In the absence of selective pressure, the pRL plasmid undergoes mitotic loss, with the consequent increase of the LEU$^-$ Blue phenotype due to the presence of the 2μm portion carrying the *lacZ* gene. This is in agreement with previous experiments, were the 2μm portion lacking a selectable marker is maintained at high level in cells grown in rich

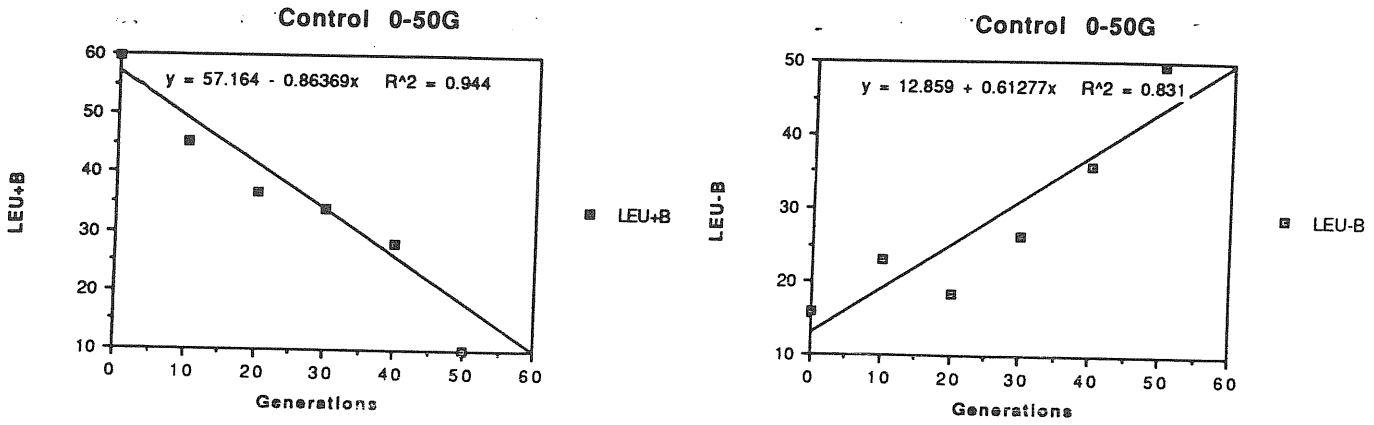


Figure 15 Scatter diagram showing the regression equation and the coefficient of correlation for different species of plasmids derived from the resolution of the pBLITZ2μ-C vector. Numbers on the y-axis represent the percentage of the indicated phenotype, numbers on the x-axis represent generations of cell growth under non-selective pressure. The coefficient of correlation is highly significant for the LEU+ B (< 2%) and significant for LEU- B (< 10%) phenotypes. The coefficient of correlation for LEU+ W and LEU- W, (0.240 and 0.767, respectively) were too low to represent the data points by linear regression.

medium (Ludwig, 1991). As the LEU+ Blue phenotype decreases, so the LEU- Blue phenotype due to 2µm, increases. However, some cells lose both plasmids, and the number of LEU- White phenotypes increases, reaching a steady level of 30% around the 30th generation (G30). This phenomenon could be explained by the fact that after a few generations the percentage of the cells that have lost all kind of plasmids becomes very low, compared to the cells that still maintain at least the 2µm-based plasmid. Furthermore, pRL alone is maintained at low level, around 10%, during the course of 50 generations (G50). This pBR322-based plasmid contains an yeast origin of replication, the *LEU2* gene, and one 2µm repeat sequence, but it lacks any other 2µm sequence. The 2µm origin of replication would explain the maintenance, even if at low levels, of this vector under nonselective conditions. When present, however, pRL appears to be incompatible with the 2µm portion of the starting plasmid, both carrying the same origin of replication.

3.3 Stability of pBLIPH8 recombinant plasmid

The effect of the presence and expression of a foreign DNA sequence, such as LiPH8, is shown in Fig. 16. In this case the presence of the LiPH8 ORF seems to lower the overall efficiency of the 2µm replication during growth in LEU medium (G0). However, the dynamics of resolution and partitioning of pBLIPH8 becomes similar to that of the control plasmid pBLITZ2µ-C, after 10 generations (G10) of growth in liquid YPD. This fluctuation in the data can be due to the technical difficulty of discriminating the actual number of blue colonies, replica plated onto YPGAL/X-gal medium. Usually, when grown on these plates, colonies harboring the control plasmid pBLITZ2µ-C show a dark blue center with poor cell growth, surrounded after several cell divisions, by a proliferation of white cells that have lost the plasmid fragment encoding ß-galactosidase. Typically, cells harboring the recombinant plasmid pBLIPH8 show the blue color with a considerable delay compared to cells with the control plasmid pBLITZ2µ-C. In fact, the control colonies become blue within 24 hours from plating, while the recombinant ones show this phenotype after one week or more of growth. Although scoring of the phenotype was

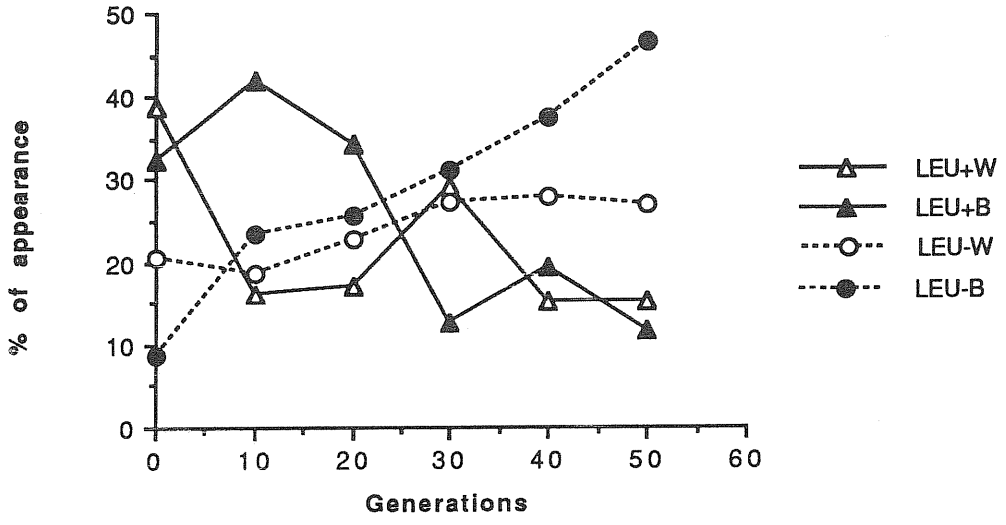


Figure 16 Analysis of the fate of pBLIPH8 plasmid during 50 generations of growth in liquid YPD. Phenotypes of colonies are as follows: LEU: leucine prototroph (+) or auxotroph (-); B: blue colony; W: white colony.

checked several times, "delayed" phenotypes could have been incorrectly assigned to one phenotype or the other, because single colonies that were originally separated on the plate had grown so much to render difficult the associating of the phenotype to the right colony. This situation could therefore explain the fluctuation of the data concerning the experiments with the TM1 recombinant clone, Fig. 17.

The dynamics of the disappearance of the phenotype LEU+ Blue is similar to that of pBLITZ2μ-C, reaching the same level at G50. Similarly, the percentage of the population harboring the resolved plasmids pBLIPH8/res (LEU-Blue) increases during the 50 generation period. This would account for a non-toxic effect of the *LiPH8* ORF expression in this system, even if the *LiPH8* gene is not properly spliced, as shown by Northern analysis using the sequence of the first intron (IVS1) as a probe with the poly(A)+ RNA isolated from cultures grown in the presence of galactose, Fig. 18. This shows that *S. cerevisiae* is unable to recognize the splicing signals of this foreign gene, though the 5' and 3' consensus sequences are the same, except for the position and the sequence of the internal consensus which differs just for a few nucleotides, which can completely abolish any biological activity of the LiPH8 peroxidase. This situation could be analogous to that of the *Cryptococcus albidus* xylanase gene expressed in yeast, where the the seven introns interrupting the gene are not spliced out by *S. cerevisiae* and no enzyme is produced (Moreau, *et al.*, 1992).

3.4 Lack of splicing of *LiPH8* pre-mRNA in *S. cerevisiae*

The ability to correctly splice genes with intervening sequences, depends upon the recognition of specific splicing signals, present at the splicing junctions and within the intron (Green, 1991; Ruby and Abelson, 1991). The yeast *S. cerevisiae* has a highly conserved sequence element, the TACTAAC box located 20-53 nucleotides upstream of the 3' splice site within the intron, deletion or mutation of which generally abolish splicing (Langford and Gallwitz, 1983; Langford, *et al.*, 1984). This consensus sequence is not present in *LiPH8*, where a putative consensus sequence CTGAAC is found,

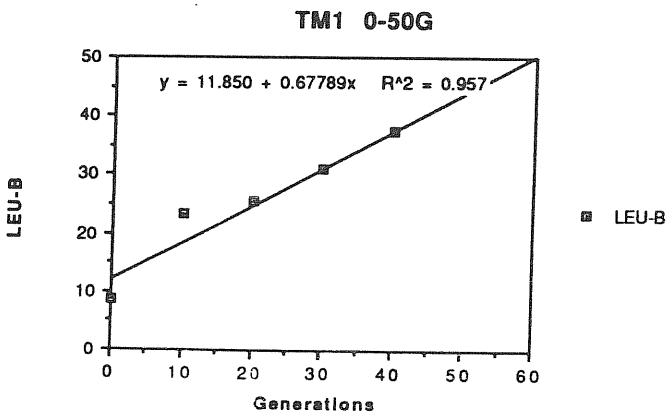


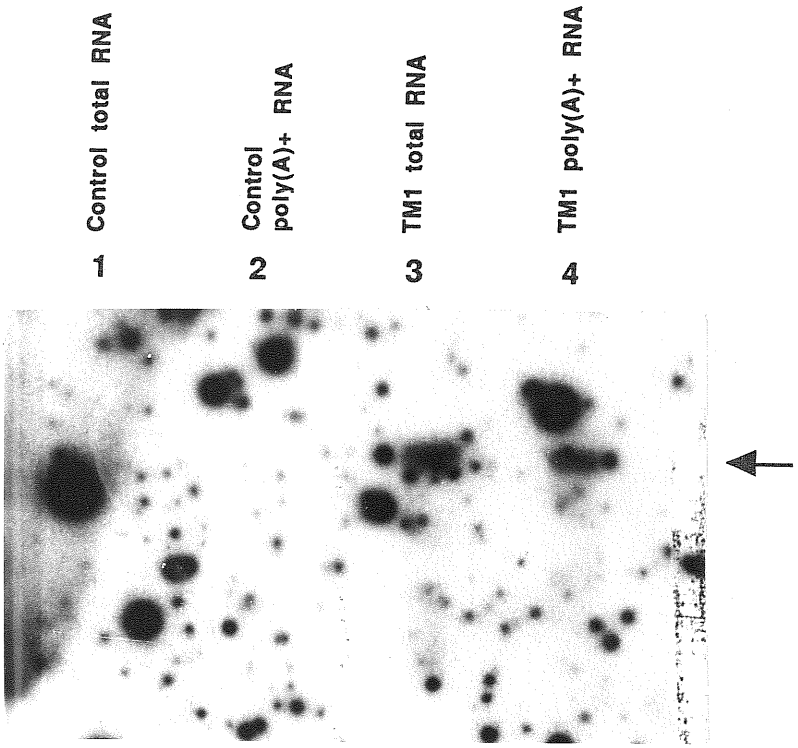
Figure 17 Scatter diagram showing the regression equation and the coefficient of correlation for the 2μ-derived portion from the resolution of the pBLIPH8 vector. Numbers on the y-axis represent the percentage of the indicated phenotype, numbers on the x-axis represent generations of cell growth under non-selective pressure. The coefficient of correlation is highly significant for the LEU- B (< 1%) phenotype. The coefficient of correlation for LEU+ B, LEU+ W and LEU- W (0.671, 0.344 and 0.770) are too low to represent the data points by linear regression.

located approximately 12 nucleotides from the 3' end of the intron (Holzbaur, *et al.*, 1988). The difference in this essential intra-intronic sequence could explain the lack of splicing when the *LiPH8* gene is expressed in yeast.

For this reason in the present study we assess the effect of the presence and expression of the heterologous *LiPH8* DNA regardless to the original biological activity of its product, but focusing on the effect of the DNA sequence itself. There is evidence that plasmid stability can be sequence dependent, as noticed for the insertion into 2-µm of two homologous genes of the same size, the *ADE8* gene (3.8 kb) versus the *LEU2* gene (3.9 kb) (Ludwig and Bruschi, 1991).

The situation for the LEU- White phenotype (complete loss of plasmid) parallels that of the control experiment. The population with no plasmids at all increases marginally during growth in nonselective medium, becoming stabilized at approximately the 25% of the entire population at G50. The cells harboring the pRL plasmid alone (LEU+ white) decrease to a fraction of the total population fluctuating around 20%, Fig. 16.

Figure 18 Northern analysis of the trascripts from cultures expressing the *LiPH8* ORF/*lacZ* hybrid DNA.The probe used in this assay was a synthetic oligonucleotide of 30 residues, designated I1-30, entirely comprised within the first intron (IVS1) of the *LiPH8* gene. The *LiPH8* gene is not properly spliced, as shown by the hybridization of the probe with the poly(A)+ RNA isolated from yeast cultures grown in the presence of galactose and expressing the pBLIPH8 plasmid (lane 4). This analysis demonstrates transcription of the *LiPH8* gene, but lack of splicing of IVS1.



Lane 1: Total RNA isolated from CBL1-20 yeast cultures harboring and expressing the control pBLITZ2μ-C plasmid DNA. Approximately 10 μg of total RNA were loaded.

Lane 2: poly(A)+ RNA isolated from total RNA of control cultures of lane 1. Approximately 2 μg of poly (A)+ RNA were loaded.

Lane 3: Total RNA isolated from CBL1-20 yeast cultures harboring and expressing the recombinant pBLIPH8 plasmid DNA, used as positive control. Approximately 10 μg of total RNA were loaded.

Lane 4: poly(A)+ RNA isolated from total RNA of recombinant cultures of lane 3. Approximately 3 μg of poly (A)+ RNA were loaded.

4. DISCUSSION

Our data on plasmid stability suggest that 2μm-based plasmids could be used for the cloning and eventual expression of foreign genes in yeast, since their stability allows cell growth in nonselective medium with no dramatic loss of the vector after 50 generations. The dynamics of plasmid resolution and partitioning during a period of 50 generations shows that, at least for the presence of the LiPH8 ORF DNA, 45% of the population still retains the pBLIPH8 recombinant plasmid. This could represent an interesting characteristic for large-scale systems in which plasmid loss must be minimized. From the graphs showing the dynamic of resolution of pBLIPH8, appears that the percentage of cells harboring pRL is higher than the control, suggesting some instability of the 2-μm counterpart. This datum is consistent with the fact that in cells that have grown for two rounds at stationary phase in selective LEU medium, the 2-μm portion of pBLIPH8 is lost with a frequency of 1 to 4 as noticed by Southern analysis. This could be due to incompatibility between the resolved plasmid forms, when, under selective pressure, (LEU) pRL is maintained, and pBLIPH8/res is lost. On the other hand, in rich medium pBLIPH8/res has the advantage of possessing all the 2-μm sequences, feature that allows its maintenance during cell division.

From an industrial perspective, it must be considered that this particular foreign DNA, split by eight introns lacking the conserved yeast TACTAAC box, might be particularly "difficult" for the yeast cell to express. A clear example of this problem is the expression in yeast of the Cryptococcus *albidus* xylanase gene (Moreau, *et al.*, 1992). When the cDNA encoding this enzyme was expressed, active extracellular xylanase was produced, while in the case of the genomic DNA, there was transcription, but the seven introns were not spliced out. This seems to be the case of the expression of the genomic DNA of the LiPH8 gene, in which Northern analysis has shown transcription of the gene, but lack of splicing, (Fig. 18). Successful expression and secretion in yeast of bacterial genes coding for enzymes involved in biomass conversion have been reported, i. e.

endoglucanases and exoglucanases (Wong, *et al.*, 1988). Expression and secretion in yeast of a cDNA encoding endoglucanase I from the ascomycete *Trichoderma reesei* also proved to be successful (Van Arsdell, *et al.*, 1987). However, the expression in yeast of foreign fungal interrupted genes seems arduous, and this situation can be viewed as the result of the evolutionary divergence between different eukaryotes. Splicing is in fact a powerful way to control gene expression in multicellular organisms, as well as in unicellular ones. There are several examples of how the same genomic DNA sequence can be functionally "recycled" depending on the cellular needs, so that multiple proteins are produced from a single gene. Thus, the complex spliceosome apparatus necessary to carry out splicing correctly offers the evolutionary advantage of an increase genome flexibility, by expanding the number of possible proteins still maintaining the same amount of DNA. A system that would allow the yeast splicing machinery to recognize and splice genes from other fungi is now under study in our laboratory

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