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Plasmid DNA Maintenance as an Indication of Metabolic Stress in *Saccharomyces cerevisiae*: Fitness of Different Selectable Markers

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1. INTRODUCTION

1.1 Starvation and Cellular Metabolism in *Saccharomyces cerevisiae*: the General Amino Acid Control

Cell growth and division depends on the availability of nutrients in the environment. The presence of an appropriate carbon, phosphate and nitrogen source is fundamental for the completion of these events. Carbon is needed for energy and biosynthesis; phosphate is necessary primarily for nucleic acids and phospholipids biosynthesis (Jonhnston and Carlson, 1992); nitrogen is fundamental for the synthesis of amino acids. Yeast can synthesise all 20 amino acids utilising ammonia as the sole nitrogen source, uptaken with the help of specific permeases. Yeast can also make use of the amino acid pool present in the environment, by internalisation using the general amino acid permease (Magasanik, 1992). Generally, the utilisation of all these nutrients is highly regulated, in a way that only the best sources are utilised. A well studied example is the preferential utilisation of glucose over galactose in the yeast *Saccharomyces cerevisiae.* When both are present, only the genes for the utilisation of glucose are turned on, while those for galactose are tightly transcriptionally repressed. A similar type of regulation occurs for the utilisation of inorganic phosphate over organic phosphate (Oshima, 1982), When the carbon source is exhausted or missing, the cell fails to divide and enters sporulation, provided the presence of the other two nutrients.

It is known that the regulation of amino acids and nucleotide biosynthesis in yeast is complex, involving the existence of a cross-pathway regulatory system, known as general amino acid control, that increases the expression of genes involved in the amino acid biosynthesis under condition of amino acid starvation (Hinnebusch, 1992; Jones and Fink, 1982).

The expression of at least thirty-five genes encoding enzymes in twelve different amino acids biosynthetic pathways is co-regulated in *S. cerevisiae* (Hinnebusch, 1992). Typically, starvation for a given amino acid not only induces an increase in the level of the enzymes necessary for its biosynthesis, but results also in elevated levels of enzymes for the biosynthesis of the other co-regulated amino acids (Delforge et al., 1975; Wolfner et al., 1975). Starvation for any one of eleven amino acids (histidine, arginine, lysine, isoleucine, valine, leucine, serine,

phenylalanine, trpyptophan, methionine, and proline) increases the transcription of genes - under the general control system - from two- to tenfold. A good example of genes whose products are derepressed in response to amino acid starvation are all the genes of arginine biosynthesis (*ARG1-ARG7*), including the *ARG4* gene (Delforge et al., 1975; Messenguy, 1979). We focused our attention on the effect of this regulation on some of the most commonly used markers in yeast, that is genes in the pathways of histidine, leucine, tryptophan and uracil, using episomal costructs of these genes. All the gene for the histidine pathway (*HIS1-HIS5*), including *HIS3* (Hill et al., 1986; Struhl and Davis, 1981; Struhl, 1982; Kinney and Lusty, 1989), are derepressed in response to amino acid starvation. For the leucine pathway (*LEU1-LEU4*), the *LEU2* gene is the only gene coding for an enzyme that is not derepressed in response to amino acid starvation (Hsu et al., 1982), while for the tryptophan biosynthesis (*TRP1-TRP5*) the *TRP1* gene is the only one that does not exhibit this type of regulation (Miozzari et al., 1978, Niederberger et al., 1983). The key positive regulator element of this system is the product of the *GCN4* gene, Gcn4p. Under starvation, Gcn4p derepresses the genes under its control, allowing transcription from their promoters. However, many amino acid biosynthetic genes are also under the control of a pathway-specific regulation, that can override the Gcn4p-mediated general amino acid control. Interestingly, Gcn4 has been shown to control at least two genes involved in the purine biosynthesis, *ADE2* (encoding phosphoribosyl-aminoimidazole-carboxylase, EC 4.1.1.21, Stotz and Linder, 1990; Stotz et al., 1993) and *ADE4* (encoding amido-phosphoribosyl-transferase, EC 2.4.2.14, Mösch et al., 1991). The promoters of the genes involved in adenine biosynthesis contain the hexanucleotide sequence TGACTC (Stotz and Linder, 1990; Myasnikov et al., 1991; Daignan-Fornier and Fink, 1992), shown to be essential for proper regulation of gene expression. This sequence has been shown to bind the transcription factors Gcn4, Bas1, Bas2, and Yap1 (Arndt et al., 1987; Harshman et al., 1988). Bas1 and Bas2 factors are basal activators of transcription in the absence of Gcn4 (Arndt et al., 1987). The target gene for Yap1 still remains to be identified (Harshman et al., 1988). The biosynthesis of some amino acids and nucleotides are linked. In fact, the adenine and histidine biosynthetic pathways share a common intermediate, phospho-ribosyl-aminoimmidazole carboximide, (ACAIR, Jones and Fink, 1982). The presence of similar promoter elements suggests a common regulation for adenine and amino acid biosynthesis. Furthermore, Gcn4 binds *in vitro* and regulates *in vivo* the expression of the *ADE4* gene (Mösch

et al., 1991). Stotz et al. (1993) reported stimulation of *ADE2* expression upon histidine starvation. However, this effect was observed only during adenine repression. The biosynthesis of the pirimidine uracil seems not to be under Gcn4 regulation.

Given the type of regulation described above, it becomes clear that growth on minimal medium *per se* cannot produce gene derepression by the general control response, rather the ability to synthesise one of the amino acids must be impaired, either by:

- growth in the presence of amino acid analogs; that is growing wild-type cells in the presence of an inhibitor of an amino acid biosynthetic enzyme (i.e. 3-aminotriazole, a competitive inhibitor of the enzyme in the histidine pathway encoded by the *HIS3* gene).

- growth on minimal medium a strain containing a leaky mutation in an amino acid biosynthetic gene.

- growth in minimal medium containing amino acid imbalance: Leu w/o Ile and Val, or Phe and Tyr in the absence of Trp. Derepression occurs in these instances because the amino acids being provided reduce the biosynthesis or utilisation of those amino acids that are missing from the medium (Niederberger et al., 1981).

Hence, starvation for one or more amino acids or nucleotides may dramatically alter the anabolic metabolism, with possible reflections onto the transcription, replication and maintenance in the cell of plasmid species carrying genes encoding amino acids or nucleotides generally used as selectable markers.

1.2 Regulatory Mutants in General Amino Acid Control

Two classes of mutants have been identified that alter the expression of the enzymes under the general amino acid control. In the first class, nine recessive genes have been identified, mutations of which result in constitutive enzyme repression upon starvation. These genes have been named General Control Nonderepressible (*GCN1-GCN9*). All these genes were shown to

regulate enzyme synthesis at the level of mRNA abundance (Hinnebusch, 1988), and their phenotype suggests that they are positive regulators of gene expression.. The second class of mutants has a constitutive derepressed phenotype, and includes twelve General Control Derepressed (GCD) genes (*GCD1-GCD12*). Their phenotype suggests that they are negative regulators of gene expression, and they act at the transcriptional level (Hinnebusch, 1988).

1.3 Role of Gcn4 in General Amino Acid Control

The *GCN4* gene encodes the major regulator of amino acid biosynthesis, the Gcn4 protein. This protein is the proximal positive regulator in this type of control. Most of the *gcn4* and *gcd* double mutants show a Gcn- phenotype, since, when under starvation, they are unable to derepress enzymes under the general amino acid control. This indicates that the positive control of Gcn4 is necessary even in the absence of the negative action of the Gcd factors. it was shown that Gcd factors act epistatically as negative effectors of the Gcn4 protein (Niederberger et al., 1986). Based on genetic evidence from different laboratories, a model was proposed for regulation of the amino acid biosynthesis. Amino acid starvation would positively induce the *GCN1*, *GCN2*, and *GCN3* genes, which act as negative regulators on the *GCD* genes. This repression releases the *GCD*-mediate repression of the *GCN4* gene, resulting in the increased expression of the genes coding for amino acids controlled by this regulator (Hinnebusch, 1992). The Gcn4 protein was shown to bind general control promoters at all 5' TGACTC 3' sequences of genes for the biosynthesis of amino acids, and to stimulate their transcription (Hope and Struhl, 1985; Arndt and Fink, 1986).

The regulation of the Gcn4 protein is quite complex, and takes place at the translational levels. In fact, there are four short open reading frames (uORF1-uORF4) in the leader of *GCN4* mRNA at which translation initiation can occur. This initiation event prevents initiation at the *GCN4* open reading frame, thus negatively regulating production of Gcn4. In fact, according to the scanning model for translation in eukaryotes, the reinitiation event at the *GCN4* start site is very inefficient (Kozak, 1989). Experimental evidence suggests a model for GCN4 translational regulation in which amino acid starvation conditions increase the translation of *GCN4* mRNA via the mediation of *GCN*-factors that reduce the function of the eIF-2 general translation factor.

Reducing the level of the eIF-2 factor would allow ribosomes to scan past uORFs 2, 3, and 4 without initiating translation and then reinitiate at the GCN4 instead (Williams et al., 1989). The functionality of eIF-2α is controlled by phosphorylation by the protein kinase Gcn2p (Dever et al., 1992). In amino acid-starved cells the level of Gcn4 is elevated, thus increasing transcription of structural genes under its control.

Gcn4 binds as a dimer to double-stranded DNA at a specific short nucleotide sequence upstream of the start sites of transcription at some of the genes for amino acids and nucleotides. This sequence is approximately twelve base pairs in length, and contains the highly conserved hexanucleotide core 5'-TGACTC-3' (Hope and Struhl, 1985; Arndt and Fink, 1986). Binding of Gcn4 to this sequence transcriptionally activates the gene which is downstream of it. The HIS3 gene is a typical example of this type of regulation, containing multiple TGACT-related sequences at its 5'-noncoding region (Struhl, 1982).

1.4 Pathway-Specific Regulation of Amino Acid Biosynthesis

The biosynthesis of histidine

Histidine biosynthesis starts with the condensation of phosphoribosyl-ATP (PR-ATP) from phosphoribosyl-pyrophosphate (PRPP) and ATP. The production of histidine requires ten enzymatic steps. We focused our attention on the *HIS3* gene, encoding the seventh step in the pathway. This gene codes for imidazoleglycerolphosphatedehydratase that catalyses the formation of imidazoleacetolphosphate by dehydration of imidazoleglycerolphosphate (Fink, 1964).

There is no known histidine-specific repression of enzymes involved in the biosynthesis of this amino acid. It is interesting to note, that a metabolite produced by the histidine pathway is utilised in purine nucleotide biosynthesis. The interplay between these two pathways is coordinated by the Bas1 and Bas2 transcription factors (Arndt et al., 1987).

Pyruvate family: the biosynthesis of leucine

The pyruvate family includes the formation of alanine, valine, and leucine. Isoleucine also shares some reactions with this pathway. The biosynthesis of leucine starts with the deamination of threonine to yield alfa-ketobutyrate. The formation of leucine requires a total of eight enzymatic

reactions, of which corresponding genetic blocks have been isolated. We were interested in analysing the *LEU2* gene, coding for beta-isopropylmalate dehydrogenase, the product of the seventh reaction in the pathway. This enzyme catalyses the formation of alfa-ketoisocapronate by dehydrogenation of beta-isopropylmalate. This compound is then converted to leucine by transamination.

In addition to the general amino acid control, some of the genes in the leucine biosynthetic pathway are under the regulation of pathway-specific enzymes. This is the case of the *LEU1*, *LEU2*, and *LEU4* genes, which require a positive regulator encoded by the *LEU3* gene for normal expression of their mRNA products. The Leu3 protein that contains a intact amino-terminal DNA-binding moiety and a functional carboxy-terminal activation domain stimulates *LEU2* expression to the same extent in the presence or absence of leucine (Zhou et al., 1987). The *LEU2* gene is not subject to cross-pathway control (Hsu et al., 1982).

Aromatic family: the biosynthesis of tryptophan

The aromatic family includes phenylalanine, tyrosine, and tryptophan. The synthesis of these three amino acids proceeds via a common pathway up to chorismate, at which point the pathway branches. The synthesis of tryptophan, starting from erythrose-4-phosphate, requires twelve enzymatic reactions. steps. The product of our gene of interest, *TRP1* (Tschumper and Carbon, 1980), catalyses the eleventh reaction in the pathway. This is the isomerization of 5'-phosphoribosyl-anthranilate to 1'-(O-carboxyphenylamino)-1'-deoxyribulose-5-phosphate. This isomerase is the only enzyme in the pathway not to respond to cross-pathway control. All the other enzymes are under this type of control and can be derepressed by starvation for histidine, leucine, arginine, or tryptophan.

As per the synthesis of histidine, also for this biosynthetic pathway there is no known tryptophan-specific repression of enzymes.

Pyrimidine nucleotides: the biosynthesis of uracil

This pathway, leads to the synthesis of uridine triphosphate starting from glutamine, CO_2, and ATP. The actions of eight enzymes are required for the making of cytidine triphosphate, the final product. All the corresponding genetic blocks have been isolated, and of these, we

decided to analyse the effect of the *ura3* mutation on the cellular metabolism. The wild-type *URA3* allele codes for the sixth enzyme in the pathway, orotidine-5'-phosphate decarboxylase (EC 4.1.1.23, Lacroute, 1968), which leads to the formation of uridine-5'-phosphate by decarboxylation of uridine-5'-phosphate. Three more steps are necessary for the formation of the final product. Furthermore, this genetic block is the last one before the integration with utilisation of exogenous pyrimidines and pyrimidine nucleosides, after uptake by specific permeases (Cooper, 1982).

It is intriguing to note the existence of a metabolic linkage between the biosynthetic pathways for arginine and uracil, since both have the common precursor carbamoylphosphate, compound synthesised by the enzyme carbamoylphosphate synthetase, that, in the case of arginine biosynthesis, is a dimeric enzyme composed of two subunits that are the gene products of the two arginine-specific unlinked genes, *CPA1* and *CPA2*; additionally, carbamoylphosphate can be synthesised by the pyrimidine-specific carbamoylphosphate synthetase, encoded by the *URA2C (cpu)* gene (Lacroute, 1968). One species of this enzyme, the *CPA1*-gene product, is repressed by growth in arginine-containing medium, while the *CPA2*-gene product is not (Piérard et al., 1979). Selective pressure for either arginine or uracil could interfere with the biosynthesis of the intracellular pool of carbamoylphosphate, with possible repercussions on the regulation of the linked pathway.

1.5 The endogenous 2μm circle molecule as a molecular fitness model: study on the curing process

The cryptic 2μm circle confers no overt phenotype on its host yeast, but it is kept in the cell with high fidelity (0.01-0.02% per generation) during mitotic and meiotic cell division, hence representing a clear example of a selfish DNA molecule (Broach, 1981; Mead et al., 1986; Futcher et al., 1988). It has been noted by several authors that the 2μm plasmid confers to the host cell a selective disadvantage of about 1-3% in term of growth rate, when compared to plasmid-free cells (Futcher and Cox, 1983; Mead et al., 1987). This stable plasmid has been exploited for the construction of derivative molecules carrying a selectable marker, useful for the maintenance of these constructs also in selective conditions (Parent et al., 1985; Romanos et al., 1992). Once

these artificial constructs are introduced into a yeast strain harboring the 2μm circle, they can undergo intermolecular recombination with the 2μm itself (Dobson et al., 1980_a), or, under certain circumstances, they can out compete the native 2μm, hence curing the host cell from its endogenous plasmid (Dobson et al., 1980_b; Toh-e and Wickner, 1981; Erhart and Hollenberg, 1983; Harford and Peeters, 1987; Xiao and Rank, 1990). This phenomenon occurs when selective pressure is maintained for the selectable marker present on the artificial episomal plasmid. However, the efficiency of this event varies according to the genetic background of the host cell, since different strains carrying the same type of plasmid are cured with different degrees of efficacy.

Several factors encoded by the host cell can be considered affecting the maintenance of a 2μm-based plasmid. First of all, it has been reported in the literature that the chromosomal mutation *mcm2* can influence the replication of the 2μm plasmid (Sinha et al., 1986; Maiti and Sinha, 1992), probably by impairing the 2μm *ORI* function, leading to plasmid loss. In this case, the segregation and amplification of the 2μm plasmid were shown to be unaffected, while a strong reduction in the copy number - seven to ten times less DNA than that of the wild-type - appeared to be due to impaired replication. These same authors found that it was extremely easy to cure the 2μm plasmid in the *mcm2* background, the curing frequency being much higher in the mutant than in the wild type.

Second, the *map1* mutation was identified, that decreases the stability of the *STB*-stabilised 2μm derivative plasmid (Kikuchi and Toh-e, 1986). This mutation is also able to destabilise plasmids carrying *ARS1* and *CEN4*, such as YCp50 and YCp19, but without any sequence from the 2μm plasmid. Plasmids carrying increasing number of copies of *ARS1* showed proportionally increasing stability, hence suggesting the Map1 protein acts through the *ARS*.

Third, another chromosomal gene was identified which regulates the copy number of the 2μm plasmid, the *NIB1* gene (Holm, 1982). A mutation in this gene, *nib1*, over-amplifies the 2μm plasmid leading to cell death depending upon the cell background (Sweeny and Zakian, 1989). It has been proposed that the *NIB* allele normally represses the amplification of 2μm copy number, the mutant *nib* allele being defective in this function.

Fourth, it has been found that 2μm plasmid replication was blocked in *cdc* mutants, as per the genomic DNA, hence demonstrating the dependence of 2μm on the host replication

machinery (Livingston and Kupfer, 1977). Furthermore, as reported by several authors, in a *cdc* background 2μm-based vectors are lost at higher frequency than in the wild-type cells (Kouprina et al., 1988: Storici et al., 1995).

Finally, both 2μm-encoded and host-encoded *STB*-binding activities have been recently identified (Hadfield et al., 1995). The cis-acting *STB* locus (Murray and Cesareni, 1986) has been shown to be a multiple protein binding site for factors involved in the partitioning mechanism. Interestingly, the host-encoded sequence-specific *STB*-binding factor was found only in urea-solubilized fractions, indicating that it is normally insoluble, and, therefore, presumably associated with a subcellular structure.

1.6 Aim of the project

Given the above considerations on the effect of amino acid starvation, the aim of our project is then to investigate the effect of different metabolic selective pressures on the cellular metabolism. In this respect, it is interesting to test whether starvation for the nucleotide uracil would be less stressing for the cell than that for the purine adenine. Our hypothesis is that under selective condition (deprivation of an amino acid, or nucleotide) for a marker under the general amino acid control (*HIS3*, or *ADE2*, *ADE4*) the cell undergoes metabolic stress due to the parallel induction of a large number of genes. In the case of the an amino acid or nucleotide not under Gcn4 regulation (*LEU2*, *TRP1*, *URA3*) the number of genes turned on would be limited only to those of the pathways under stress, without involving other genes. It would be extremely difficult to quantify the metabolic stress by ATP consumption or gene expression. Therefore, as signal of overall stress, final maintenance, and replication (copy number) of the episomal marker have been used as a measure, employing the naturally stable, metabolic stress-tolerant 2μm plasmid as a carrier of those markers. To this end, we wanted to test different markers, either genes coding for enzymes involved in the biosynthesis of amino acids, such as the following:

- the *HIS3* gene coding for imidazoleglycerol-P-dehydratase (EC 4.2.1.19, Fink, 1964), for the synthesis of histidine. Derepressed by the general amino acid control (Hill, et al., 1986; Struhl, 1982).

- the *LEU2* gene coding for beta-isopropylmalate dehydrogenase (EC 1.1.1.85, Ratzkin and Carbon, 1977; Hinnen et al., 1978), for the synthesis of leucine. Not derepressed by the general amino acid control (Hsu et al., 1982).
- the *TRP1* gene coding for phosphoribosylanthranilate isomerase (EC 2.4.2.18, De Moss, 1965; Doy and Cooper, 1966), for the synthesis of tryptophan. Not derepressed by the general amino acid control (Miozzari et al., 1978; Niederberger et al., 1983).

or genes encoding enzymes involved in the biosynthesis of nucleotides, such as:

- the *URA3* gene coding for orotidine-5'-phosphate decarboxylase (EC 4.1.1.23, Lacroute, 1968), for the synthesis of uracil. Not derepressed by the general amino acid control.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

E. coli bacterial strains used for routine work were:

- DH5alfa (*endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, deltalacU169 (phi80lacZdeltaM15)*), (Hanahan, 1983)
- HB101 (*hsdS20, supE44, ara14, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA13, mcrB, mcrA, mrr*), (Boyer and Roulland-Dussoix, 1969)
- KC8 (*hsdR17, hisB463, leuB6, pyrF::Tn5 KmR, trpC9830, Δ(lacZYA), strA, galU, GalK*) (K. Struhl).

2.2 Yeast Strains and Strain Constructions

Saccharomyces cerevisiae yeast strains FAS20 (*Mat alfa [cir$^+$] ade1 ade2 ade8-18 leu2-3,112 trp1-289$_a$ ura3-52 can1^R*), and the isogenic *[cir^0]* FAS21 are described in Bruschi and Howe, 1988. The strain CBU1-31 (*Mat alfa [cir$^+$] ade8-18 his3-Δ1 leu2-3,112 trp1-289$_a$ ura3-52 can1^R ARG4*) was constructed as follows. Strain DBY746 (*Mat alfa [cir$^+$] his3-Δ1 leu2-3,112 trp1-289$_a$ ura3-52 gal^S can1^R CUP1 ADE8 ARG4*) (D. Botstein) was crossed with strain 31-1-17B (*Mat a [cir$^+$] ade8-18 arg4 trp1-289$_a$ ura3-52 CUP1 LEU2 HIS3*) (S. Fogel) and the resulting diploid was selected onto ARG-LEU- double drop-out medium. Then, twenty colonies were streaked again onto ARG-LEU- drop-out, grown for two days at 30°C, and replicated onto ADE-, HIS-, URA-, TRP- and SPIII sporulation medium. The phenotype of the diploid strain was Ade$^+$, His$^+$, Ura$^-$, Trp$^-$ and Sporulation$^+$. Each of the sporulated diploid was streaked onto canavanine containing plates (ARG- drop-out with 60 μg/ml canavanine) for isolation of single colonies. After growth, canavanine resistant colonies were streaked onto CAN plates a second time, in an array of forty. Finally, the master plate was replicated onto diagnostic drop-outs ADE-, ARG-, CAN-, HIS-, LEU-, TRP-, and URA-. Clones #3, #8, #10, #11, and #15 that were Ade+, Arg+, Can^R, His-, Leu-, Trp-, and Ura-, were picked and named CBU1. These CBU1 clones were streaked for single isolated colonies and twenty more colonies were used to prepared a

YPD master plate. After growth, this plate was replicated onto the above mentioned drop-out media, to check strain homogeneity. In all cases, all the isolated colonies showed the same CBU1 phenotype, indicating that each strain was homogeneous.

2.3 Testing Mating Type of CBU1 Clones

Mating type of the CBU1 strains was determined by crossing with tester strains and auxotrophy complementation. Testers were as indicated in the following table 2.1:

CG379	*Mat alfa ade5 his7-2 leu2-3,112 trp1-289$_a$ ura3-52 [Kil-O]*	(C. Giroux)
CG397	*Mat alfa ade3 his5-2 leu1-c trp5-20 ura1*	(C. Giroux)
Z-136-1-7D	*Mat alfa arg4-2 leu1-1 ade1 trp-1-1 gal1*	(S. Fogel)
CG396	*Mat a ade3 his5-2 leu1-c trp5-20 ura1*	(C. Giroux)
Z-136-1-13C	*Mat a arg4-2 leu1-1 ade1 trp-1-1 gal1*	(S. Fogel)
X3163-4C	*Mat a arg1 met1 trp3 ade5 ura3 leu1 mal gal2*	(R. K. Mortimer)

Table. 2.1 . Tester strains used for assignment of mating type

CBU1 clones were crossed with each of these testers by mixing and plating an aliquot of the mixture onto YPD. After three days of incubation at 30°C, plates were replicated onto diagnostic double drop-out media, and colonies were scored for growth. In all cases, CBU1 clones mated efficiently only with *Mat a* testers, and never with the *Mat alfa* testers, indicating that the mating type was in all cases *Mat alfa*.. This marked bias can be due to the fact that *Mat alfa* was on the same chromosome III as well as *leu2-3,112* mutation (on DBY746) for which we selected for in the haploid CBU1-31. CBU1-3 was further purified for single colonies, and subclone #1 was chosen as working strain.

2.4 Reversion Rate of the Yeast Strain CBU1-31

Fundamental for the proper interpretation of the stability and competition experiments was the determination of the reversion rate of the auxotrophic markers present in the working strain. This was done by plating increasing amounts of CBU1-31 cells onto appropriate selective plates and counting the number of cells grown after three days of incubation at 30°C. As expected, some markers did not revert at all, according to the type of mutation. In fact, the *ade8-18* is a non-revertible 38-base pair deletion (White et al., 1985); the *his3-Δ1* mutation is again a deletion; the *leu2-3,112* is a double mutation, while *ura3-52* is a Ty insertion within the coding sequence of the gene (Rose and Winston, 1984). The $trp1\text{-}289_a$ is a revertible amber mutation, and indeed it gave a relative high reversion rate. Figures are reported in table. 2.2:

CBU1-31: [cir^+] *Mat alfa ade8-18 his3-Δ1 leu2-3,112* $trp1\text{-}289_a$ *ura3-52* can^R *ARG4*

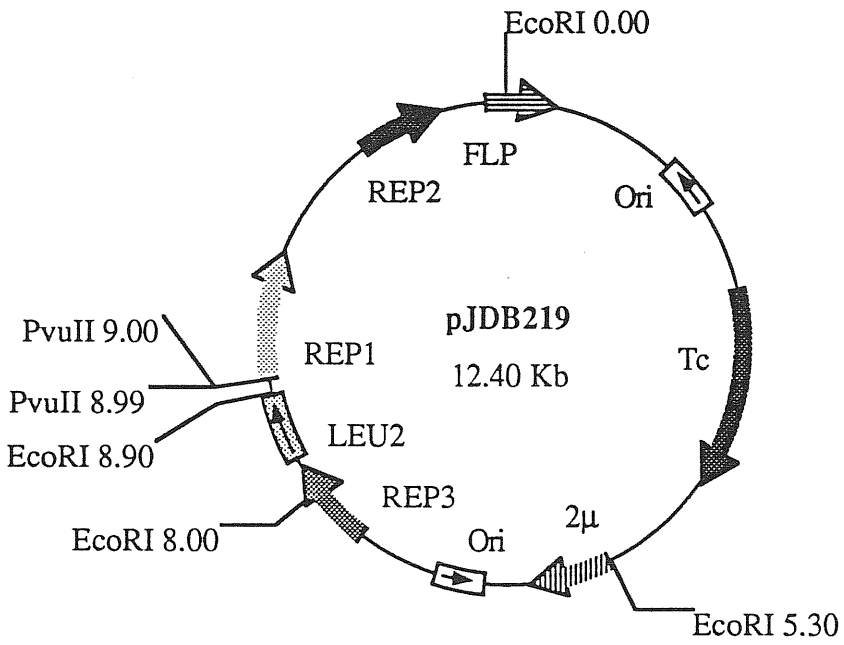
Number of cfus plated onto drop out media (vital count)	$1.43x10^6$	$7.13x10^6$	$2.85x10^7$	$1.07x10^8$	Mutation tested
ADE-	---	---	---	---	*ade8-18*
HIS-	---	---	---	---	*his3-Δ1*
LEU-	---	---	---	---	*leu2-3,112*
TRP-	---	2 cfus	13 cfus	38 cfus	$trp1\text{-}289_a$
URA-	---	---	---	---	*ura3-52*

Table 2.2 . Reversion rate of the selectable markers present in the CBU1-31 yeast strain.

2.5 Attempts to Cure CBU1-31 from the Endogenous 2μm Plasmid

We were interested in having a [cir^0] strain completely isogenic with CBU1-31, to avoid intramolecular recombination between the 2μm DNA and the episomal plasmids used in our experiments (Broach, J. R., 1981). We tried two protocols for curing CBU1-31 from the endogenous 2μm using other competitor plasmids carrying the entire 2μm DNA, such as pJDB219 (Beggs, 1978; Hinnen et al., 1978; Dobson et al., 1980b; Erhart and Hollenberg, 1981; Toh-e and Wickner, 1981) and pBLU-D (Ludwig and Bruschi, 1991), Fig. 2.1.

Fig. 2.1



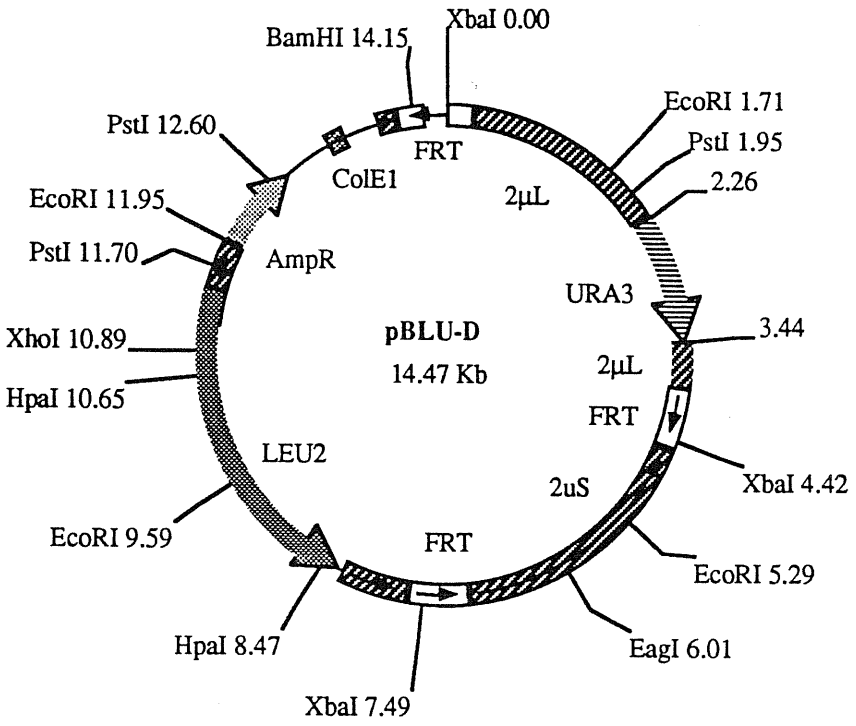


Fig. 2.1. Detailed maps of plasmids pJDB219 and pBLU-D. pJDB219 is the whole 2μm plasmid (B form) in pMB9 (5.3kb EcoRI fragment). The yeast d-leu2 gene has been cloned into the 2μm portion. pBLUD is the whole 2μm plasmid recombined with pRL. The URA3 gene was cloned into the HpaI site in the large portion of 2μm.

Both attempts were unsuccessfully with our strain, while they proved very efficient with other yeast strains such as CBL1-20 (Ludwig and Bruschi, 1991) and FAS20 (Bruschi and Howe, 1988) and YPH250 (J. Oberto, personal communication). The strategy used in our attempt is outlined below. CBU1-31 was transformed with plasmid pJDB219, with the protocol of Bruschi et al. (1987), and cells were plated onto LEU- medium. After colony growth, two independent transformants were inoculated into 10 ml liquid LEU- medium and grown in two parallel cultures (A and B) for ten generation up to mid-log phase. Then, a new culture was started in the same way, and the procedure was repeated for a total of seventy generations (G70) of growth in selective conditions. This constant selection was necessary to allow copy amplification of the pJDB219 plasmid carrying the defective *leu2-d* gene, with consequent competition with the

endogenous 2μm for the replication and partitioning machinery (Dobson et al., 1980b; Erhart and Hollenberg, 1981; Toh-e and Wickner, 1981). Then, a cycle of growth in non selective rich medium was started - to allow mitotic loss of the pJDB219 plasmid - by inoculating 10 ml YPD with 10μl of the G70 LEU- culture. This procedure was repeated for hundred and ten generations (G110) and finally cells were plated onto YPD plates, incubated for three days at 30°C and replicated onto LEU- medium to score for loss of pJBD219. The number of Leu+ colonies was still high, indicating that at least the *leu2-d* part of pJDB219 was still present as replicative plasmid. This phenomenon was already known in the literature, and it has been explained by intramolecular recombination between pJDB219 and the endogenous 2μm plasmid (Toh-e and Wickner, 1981). However, Leu- colonies were present, and were streaked again onto YPD and later replicated onto LEU- medium for further confirmation of their phenotype. Six Leu- clones, B7-1, B51-5, B52-8, B4-13, A7-16, and A5-20, were inoculated into 10 ml YPD and grown to stationary phase at 30°C. Total yeast DNA was prepared from 5 ml culture with the protocol of Rose et al. (1990) as described later in this section (Yeast plasmid DNA minipreps). DNA was quantitated Spectrophotometrically as described later, and readings of (1/20 dilution rate) samples were as follows:

	OD_{260}	OD_{280}	OD_{260}/OD_{280}	mg/ml (1/1 dilution)
B7-1	1.17	0.67	1.75	1170
B51-5	1.93	1.05	1.84	1930
B52-8	1.11	0.61	1.82	1110
B4-13	2.19	1.15	1.90	2190
A7-16	0.77	0.42	1.83	770
A5-20	1.39	0.74	1.88	1390

10 μl (of 1/1 dilution) DNA were loaded on a 0.7% agarose gel together with FAS20 and FAS21 control DNAs. DNA was visualized by ethidium bromide staining (Fig. 2.2) and the gel was subsequently transferred to a nylon membrane for Southern blotting.

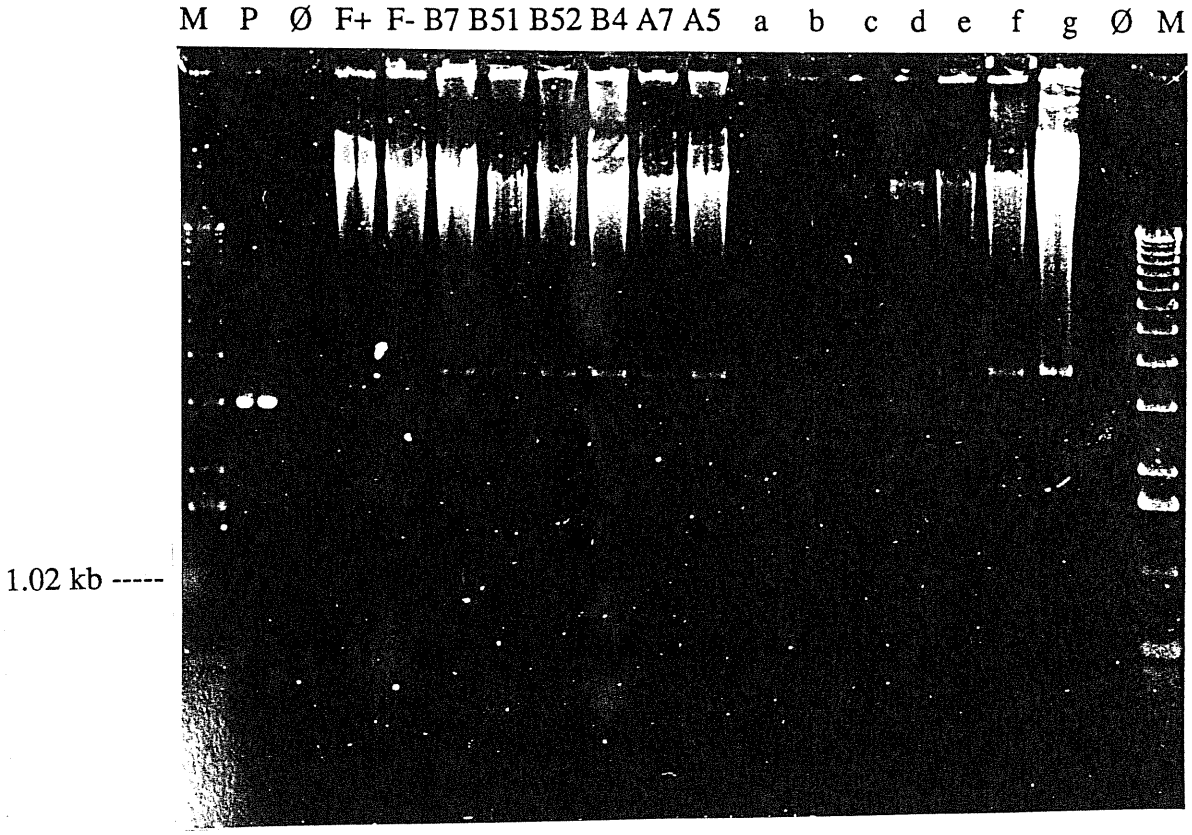


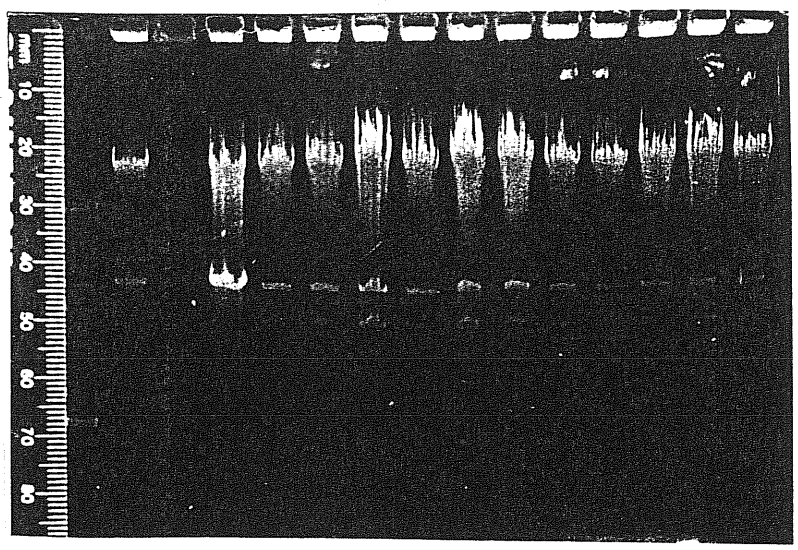
Fig. 2.2. Picture of the agarose gel of the yeast minipreps coming from the curing experiment using plasmid pJDB219. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075; Gibco BRL-Life Technologies Ltd, Paisley, UK; Hartley and Donelson, 1980). **P**: the ≈ 3.1 kb DNA purified from pBLU-D/XbaI, used as DNA probe after radioactive labeling. **Ø**: empty lane. **F+**: Fas20 DNA, [*cir*$^{+}$] strain. **F-**: Fas21 DNA, [*cir*^{0}] strain. **Lanes B7 - A5**: DNA samples from yeast minipreps after the curing experiment. **a - g**: 10 µl of a dilution of a A5 Leu+ DNA (a=1/80; b=1/40; c=1/20; d=1/10; e=1/2; f=1/1; g=1/1, 30 µl) used as calibration for the copy number determination experiment.

After probing with the ≈ 3.1 kb 2μm-specific DNA probe from pBLU-D/XbaI, it was clear that all the six "cured" Leu- CBU1-31 clones were indeed still [cir^+]. Southern blot of the "putative cured" samples is shown in the section 3.1.

Since the pJDB219 plasmid proved to be inadequate, we decided to use a different competitor plasmid, pBLU-D (Ludwig and Bruschi, 1991) and to repeat the curing experiment. In this case, we started three parallel cultures in URA- medium of two CBU1 clones, #3 and #8, transformed with plasmid pBLU-D. Selection in URA- was kept for ninety generations (G90), after which cells underwent a round of non selective growth for seventy generations (G70). These G70 cells were plated onto YPD, and then replicated onto URA- plates to score for cells having lost pBLU-D. Ura- cells were used to prepared plasmid DNA which was run on a 0.7% agarose gel (Fig. 2.3), and subsequently used in the Southern blot analysis with the labeled 3.1 kb fragment from pBLU-D as probe.

Fig. 2.3 M C+ Ø F- 1 2 3 4 5 6 7 8 9 10 11

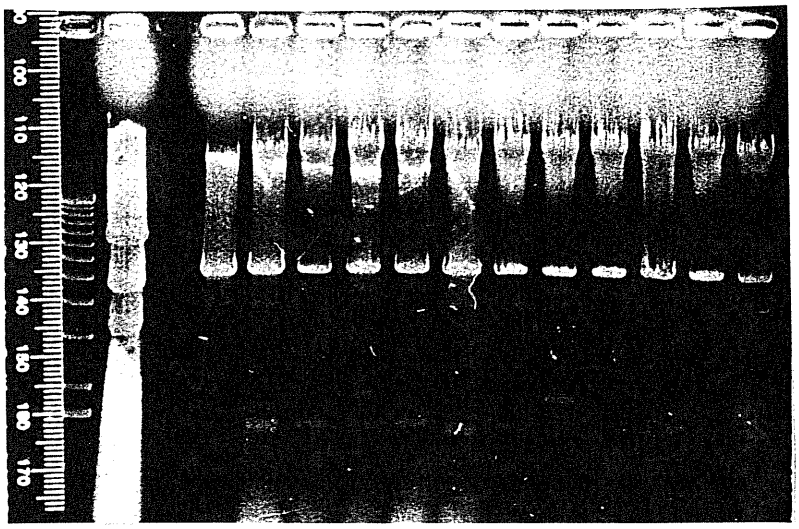
a)



1.02 kb -----

Fig. 2.3

b)


M P Ø 12 13 14 15 16 17 18 19 20 21 22 23
1.02 kb -----

c)

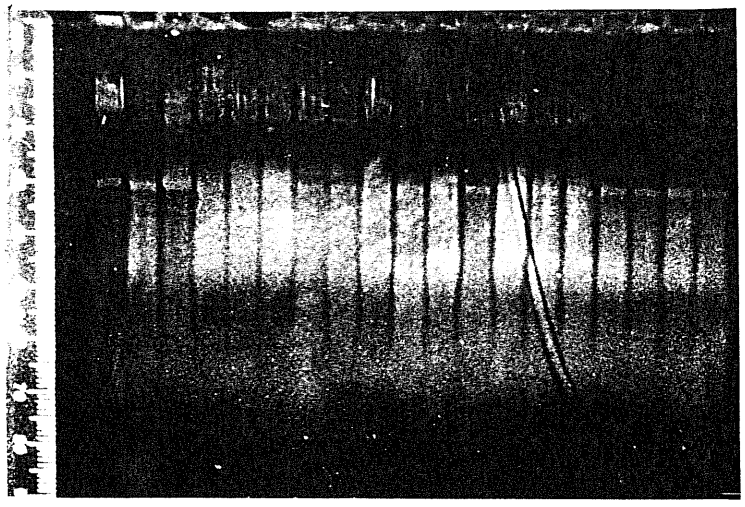
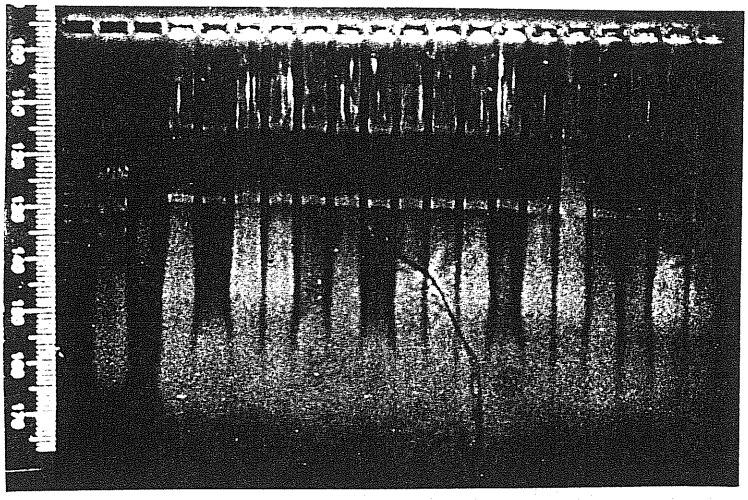

M C+ F+ F- 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
1.02 kb -----

Fig. 2.3

d)

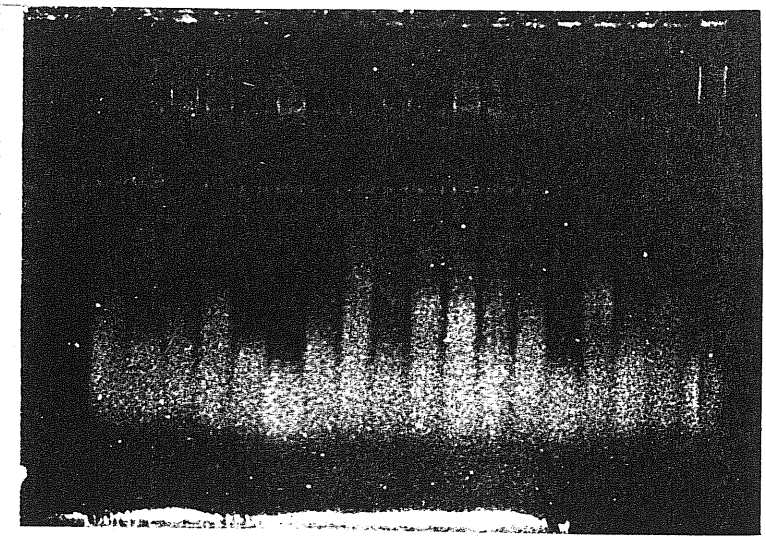
M P Ø40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56



1.02 kb -----

e)

M Ø F+ F- 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72



1.02 kb -----

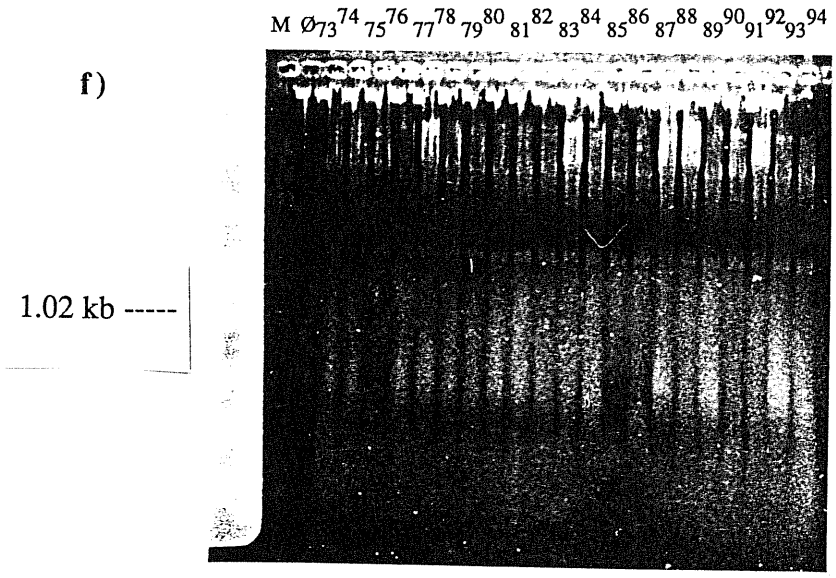


Fig. 2.3. Pictures of agarose gels of the yeast minipreps coming from the curing experiment using plasmid pBLU-D. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **C+**: CBU1-31 DNA, [cir^+] strain. **Ø**: empty lane. **F+**: Fas20 DNA, [cir^+] strain. **F-**: Fas21 DNA, [cir^0] strain. **P**: pBLU-D/XbaI DNA, showing three DNA bands of approximately ≈ 3.1 kb (used as DNA probe after radioactive labeling), ≈ 4.42 kb, and ≈ 7.0 kb. **Lanes 1-94**: DNA samples from yeast minipreps after the curing experiment (**a**, **b**, **c**, **d**, **e**, **f**).

As usual, FAS20 and FAS21 DNA were used as controls for detection of 2μm DNA. In this experiment, ninety four Ura- cells were analyzed, none of which resulted free of 2μm DNA, see section 3.1 for the Southern blots. The CBU1-31 strain proved to be exceptionally recalcitrant to curing.

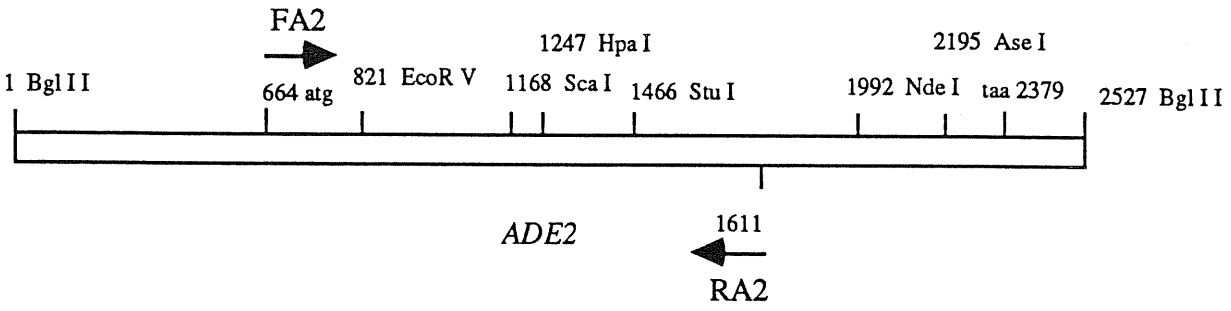
2.6 Construction of CBU1-31 *ade2::LEU2* and CBU1-31 *ade2::URA3* Strains

Necessary for the competition experiments in double drop-out media was the construction of appropriate strains carrying a disrupted *ADE2* gene (encoding phosphoribosyl-aminoimidazole carboxylase, EC 4.1.1.21; Silver and Eaton, 1969; Woods, 1969). To this purpose, a series of integrative vectors was constructed to carry out gene disruptions as described by Rothstein, 1991. The YIpA2L2 and YIpA2U3 vectors were restricted at the BglII sites flanking the integrative construction, the fragments carrying *Δade2::LEU2* and *Δade2::URA3* were gel purified and used to transform CBU1-31 harboring either YCpLA or YCpUA, two centromeric plasmids with a functional *ADE8* gene (see later for details on plasmid constructions). Integration events at the *ADE2* locus, in an *ADE8* background, would results in a white to red shift in the cell pigmentation (Roman, 1956; 1957), thus allowing visual screening of the recombinant colonies. Transformant colonies were selected onto double drop-out LEU-URA- medium, and red clones were further analyzed (2 red/8 white colonies for CBU1-31 [YCpUA] *Δade2::LEU2*; 4 red/94 white colonies for CBU1-31 [YCpLA] *Δade2::URA3*). These putative disruptant cells were purified again for single isolated colonies, and were replicated onto LEU- and URA- media. As expected, the red CBU1-31 [YCpLA] *Δade2::URA3* gave rise to white colonies when plated onto URA- medium (loss of YCpLA), and remained red when on LEU- medium (stable integration). Conversely, CBU1-31 [YCpUA] *Δade2::LEU2* gave rise to white colonies only when plated onto LEU- medium (loss of YCpUA), and remained red when kept onto URA- medium, indicating a stable integration at the *ADE2* locus. This behaviour is in excellent agreement with the indication that the starting red transformants were indeed real disruptant. This evidence was further confirmed by the PCR analysis described below. The strain carrying the *Δade2::LEU2* construction was named CBU1-31 A2L2, while that one carrying *Δade2::URA3* was called CBU1-31 A2U3.

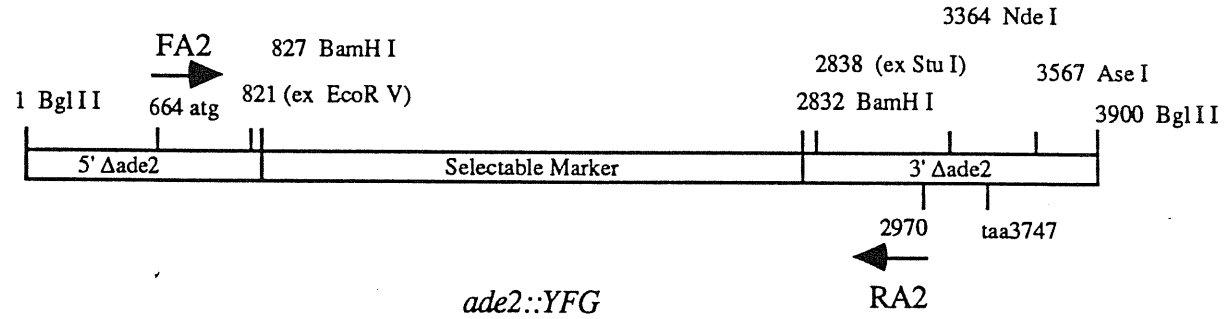
2.7 Checking the Disruptions at the *ADE2* Locus Using the Polymerase Chain Reaction

To check the genomic constructions in the yeast strains CBU1-31 A2L2 and CBU1-31 CBU1-31 A2U3, we used a PCR approach according to the elegant protocol of Sathe et al.

(1991). Briefly, oligonucleotides (20mers) were chosen to amplify the 5' upstream region outside the disrupting gene (oligo FA2) and at its 3' (oligo RA2) in a PCR reaction (Mullis and Faloona, 1987). The approach, both for the wild type *ADE2* allele and the disrupted *ade2::YFG* allele, is outlined below:



(0.95 kb amplified DNA in the wild type gene)



(approximately ≈ 2.3-2.5 kb amplified DNA, depending on the insert size)

FA2 5′ ATGGATTCTAGAACAGTTGG 3′

RA2 5′ GGCGTTCGTTGTAATGGTGG 3′

Fig. 2.4. Schematic representation of the amplifications at the *ADE2* and *ade2::YFG* loci using the two convergent primers FA2 and RA2. (YFG: Your Favorite Gene)

The PCR reaction was prepared according to Sambrook et al. (1989), with modifications in the reaction buffer (1X Vent™ NEBuffer: 10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100) as recommended by the DNA polymerase supplier (New England Biolabs, Beverly, MA, USA):

63 µl H_2O

10 µl 10X Vent™ NEBuffer (NEB)

16 µl dNTPs mixture (from 2 mM each stock)

5 µl FA2 oligo (≈ 170 pmol)

5 µl RA2 oligo (≈ 170 pmol)

1 µl Vent™ DNA polymerase (2 units, NEB)

100 µl total volume.

Using a sterile toothpick part of a yeast colony was taken directly from a plate and dispersed into the reaction mixture. Finally 50 µl of mineral oil (Sigma Chemical Company, St. Louis, Missouri) were overlaid on top. The polymerase chain reaction was run on a PREM™ III thermal cycler (LEP Scientific, UK) programmed as follows:

	Denaturation	Annealing	Extension
First cycle	94°C for 5 min.	50°C for 2 min.	72°C for 3 min.
25 cycles	94°C for 1 min.	50°C for 2 min.	72°C for 3 min.
Last cycle	94°C for 1 min.	50°C for 2 min.	72°C for 10 min.

After the reaction, samples were loaded and run onto a 0.7% agarose gel. Amplification of genomic DNA from non transformed CBU1-31 control yeast cells resulted in the appearance of a ≈ 1 kb band, corresponding to the amplification of the wild type *ADE2* gene. In the disrupted CBU1-31 A2L2 and CBU1-31 A2U3 cells a higher band appeared, at ≈ 2.55 kb for the *ade2::LEU2* construct, and at ≈2.30 kb for *ade2::URA3* construct, confirming the site of insertion of the integrative DNA fragments, Fig. 2.5.

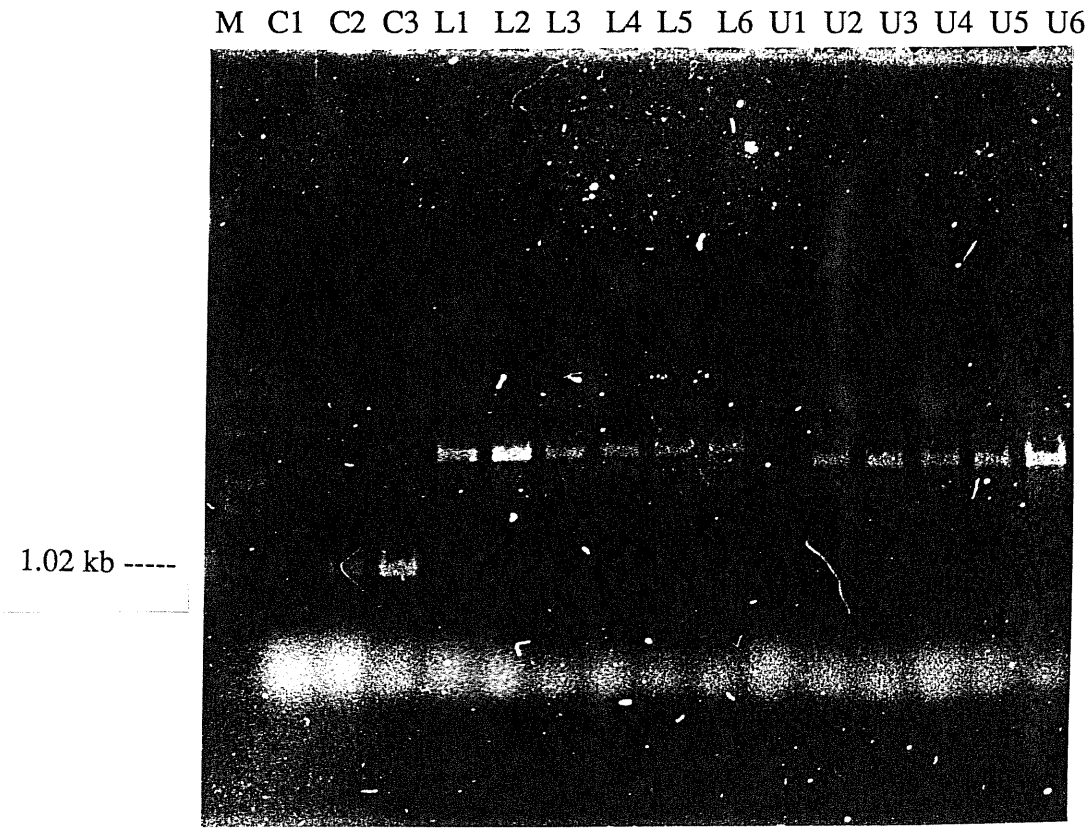


Fig. 2.5. Picture of agarose gel of PCR of yeast minipreps DNA coming from the integration experiment . **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **C1-C3**: DNA from non transformed CBU1-31 strain, showing the amplified 1 kb wild-type *ADE2* gene. **L1-L6**: DNA from transformed CBU1-31 A2L2, showing amplification of the ≈2.5 kb *ade2::LEU2* construct. **U1-U6**: DNA from transformed CBU1-31 A2U3, showing amplification of the ≈2.3 kb *ade2::URA3* construct.

2.8 Media

Media for Bacteria

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

LB (1X g/litre)

Bacto-tryptone	10 g
NaCl	10 g
Bacto-yeast extract	5 g

pH 7.0 (with 5N NaOH)

1.5% Agar for plates

Bacto-tryptone is a pancreatic digest of casein used as nitrogen source; Bacto-yeast extract is the water soluble portion of autolyzed yeast (Difco laboratories, Michigan, USA; Difco manual, 1994). Medium was sterilised by autoclaving for 20 minutes at 1.0 bar. Stock solution of ampicillin was prepared at 100 mg/ml in redistilled water, sterilised 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.

Drop-out media for selection of prototrophs in KC8:

Minimal "A" medium (10X g/litre) (Miller, 1992)

K_2HPO_4	105 g
KH_2PO_4	45 g
$(NH_4)_2SO_4$	10 g
Sodium Citrate · $2H_2O$	5 g

1.5% Agar was added to 1X "A" medium when necessary. Medium (10X) was sterilised by autoclaving for 20 minutes at 1.0 bar. After autoclaving the following was added (to 1X):

$MgSO4 \cdot 7H_2O$	1 ml from 1M stock solution per litre
B1 (thiamine hydrochloride)	0.5 ml from 1% stock solution per litre final [1μ/ml]
Glucose	10 ml of a 20% solution per litre
L-amino acids as required	4 ml from a 10 mg/ml stock solution per litre final [40μ/ml]

Stock solution of ampicillin was prepared at 100 mg/ml in redistilled water, sterilised 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.

Media for Yeast

Semi-defined yeast extract peptone dextrose (YPD), selective drop-outs, sporulation and drug-containing media, were as described in Rose et al. (1990):

YPD

1% Bacto-yeast extract

2% Bacto-peptone (Difco laboratories, Michigan, USA; Difco manual, 1994)

2% Glucose (dextrose)

Synthetic minimal medium

2% Glucose (dextrose)

0.5% Ammonium sulfate

0.17% Yeast nitrogen base, w/o amino acids and ammonium sulfate (Difco laboratories, Michigan, USA; Difco manual, 1994)

pH 5.8

100X amino acids supplement A (500 ml)

	1X Final [ ], mg/litre
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 sterilise. 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 litre of autoclaved warm (50°C) synthetic minimal medium. For solid media 1.5% Bacto-agar was added prior to autoclaving.

Sporulation medium

SPIII sporulation medium is composed as follows:

1% Potassium acetate

0.1% Bacto-yeast extract

0.05% Glucose (dextrose)

2% Agar

To increase sporulation of auxotrophic strains 25% of the amino acid supplement was added.

2.9 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 $2.5x10^5$ conversion factor to determine cell density in 1 ml.

2.10 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 [10 mM Tris-HCl (pH 7.6) and 1 mM EDTA, pH 8.0] 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.

2.11 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 litre flask containing 500 ml LB medium supplemented with 100 µg/ml ampicillin. Culture was sedimented in a Sorvall® RC-5B centrifuge (Du Pont, Wilmington, DE, USA) in 250 ml bottles using a GSA rotor at 4,000Xg 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 15,000Xg 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 12,000Xg 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 buffer, 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 polypropylene tube, 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 buffer; 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 50,000 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 litre TE buffer (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 buffer (pH 8.0) and concentration and purity were calculated spectrophotometrically by reading the absorbance at 260 and 280 nm in a quartz cuvette, and assuming that 1 $OD_{260} \div 50$ 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.

2.12 Yeast Plasmid and Genomic DNA Minipreps for Southern Blot Analysis

Yeast plasmid and genomic DNA minipreps were carried out according to the protocol reported by Hoffman and Winston (1987), as outlined in Rose et al. (1990). Cells were grown overnight in 10 ml of medium, at 30°C in a rotary shaker. The culture was pelleted by spinning in a Sorvall® RT6000B centrifuge for 5 minutes at 3000 rpm. The supernatant was drained, the cells resuspended in 0.5 ml of sterile distilled water, and the mixture was transferred to a 1.5 ml microfuge tube. The centrifugation was repeated and water poured off. The pellet was

resuspended in the residual liquid and the suspension was mixed with 0.2 ml of the following buffer:

2% Triton X-100

1% SDS

100 mM NaCl

10 mM Tris-Cl (pH 8)

1 mM Na_2 EDTA

Then, 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) mixture were added together with 0.3 g of acid-washed glass beads (0.45-0.50 µm Ø, B. Braun Melsungen AG, Melsungen, Germany). The mixture was vortexed at maximum speed for 4 minutes, and, finally, 0.2 ml of TE buffer (pH 8) were added to the combination. The tube was centrifuged in a microfuge at maximum speed (14000 rpm) for 5 minutes, and the aqueous layer transferred to a fresh tube. This solution was mixed with 1 ml 100% ethanol pre-cooled at -20°C, and centrifuged as above to precipitate plasmid and chromosomal DNA (as well as RNA). The supernatant was discarded, and the pellet resuspended in 0.4 ml of TE buffer (pH 8) plus a 10 mg/ml solution of RNase A. Following incubation at 37°C for 5 minutes, 10 µl of 4 M ammonium acetate were added, together with 1 ml of 100% ethanol. DNA was pelleted by centrifugation as specified above, the supernatant discarded and the pellet dried under vacuum. DNA was resuspended in 50 µl of TE buffer, 10 µl of which were used for Southern blot analysis.

2.13 Agarose Gel Electrophoresis of DNA

Usually DNA samples were run on 0.7-0.8% 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.

2.14 Recovery of DNA from Agarose Gels

Generally, all DNA fragments used in ligation reactions were purified by electrophoresis on a 0.7-0.8% agarose gel. After ethidium bromide staining, the part of the gel containing the DNA of interest was excised under UV light, and DNA was recovered from the slice using the QIAEX gel extraction kit (distributed by DIAGEN GmbH, Hilden, Germany). In brief, the agarose slice was weighted, finely minced with a clean scalpel in a 2.0 ml test tube, and 100 µl QX1 solubilization buffer (containing sodium perclorate, $NaClO_4$) were added per every 100 mg of gel. The tube was incubated at 50°C until complete solubilization of the agarose. Then, 10 µl of freshly vortexed QIAEX suspension (3 µm silicagel particles) were added, and the incubation repeated for ten minutes. The mixture was then pelleted in a microfuge at maximum speed for 30 seconds, the supernatant discarded and the matrix resuspended in 500 µl of QX2 high salt buffer to remove agarose and contaminants. The pelleting and the wash with QX2 were repeated a second time. The pellet was resuspended by vortexing in 500 µl of QX3 wash buffer to remove salts, and pelleted as above. This wash was repeated a second time. Finally, the pellet was air-dried and the DNA resuspended in 20 µl of TE buffer, ready for use in a ligation reaction.

2.15 Purification of Synthetic Oligonucleotides

Oligonucleotides were synthesized by the ICGEB oligonucleotide service, and delivered already deblocked as 0.2 µmol scale in solution with ammonium hydroxide. Oligos 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 $OD_{260} \div 33$ µg/ml of oligonucleotide solution, in a 1 cm path-length quartz 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).

2.16 Construction of a Non-functional Allele of the *ADE8* Gene

To detect the different plasmid forms present in the population, we constructed a non-functional version of the *ADE8* gene, coding for phosphoribosylglycinamide formyl transferase (EC 2.1.2.2; Woods and Jackson, 1973), for the synthesis of adenine, Fig. 2.6. This non-functional allele was named *ade8-xp*, Fig. 2.7. CBU1-31 *ade2::YFG* cells harbouring this gene would remain white as compared to the red ones carrying the wild type *ADE8* allele. To make this mutation, we restricted the pBRADE plasmid (Fig. 2.8 and 2.9) with XhoI (C˘TCGAG), thus cutting the *ADE8* gene within its coding sequence. The linearized plasmid was then purified by gel electrophoresis, and the overhangs filled-in by PolIK (the Klenow fragment). After religation, this treatment generated a 4-base pair frameshift (CTCGATCGAG) and a new PvuI recognition site (XhoI C˘TCGAG—>PvuI CGAT˘CG, briefly noted as XP), allowing easy identification of recombinant clones. This frameshift occurs in a region of the *ADE8* coding sequence whose integrity is fundamental for the functionality of the protein, as demonstrated by White et al. (1985). The protocol used is the following (Sambrook et al., 1989):

8 μl H_2O

5 μl pBRADE/XhoI DNA

2 μl 10X nick-translation buffer

4 μl dNTPs mixture (2 mM each)

1 μl PolIK, 2 units/μl labeling grade (Boehringer Mannheim, Germany)

20 μl total volume.

(10X nick-translation buffer is: 0.5 M Tris-CL [pH 7.5], 0.1 M MgSO4, 1 mM dithiothreitol, 500 μg/ml bovine serum albumin [fraction V; Sigma]) The mixture was incubated at 37°C for 30 minutes. Then, the combination was again restricted with an excess of XhoI, and was finally religated as follows:

3.0 µl H_2O

15 µl pBRADE/XhoI, filled-in DNA

0.5 µl 10X concentrated OPA^+ reaction buffer (Pharmacia, Uppsala, Sweden)

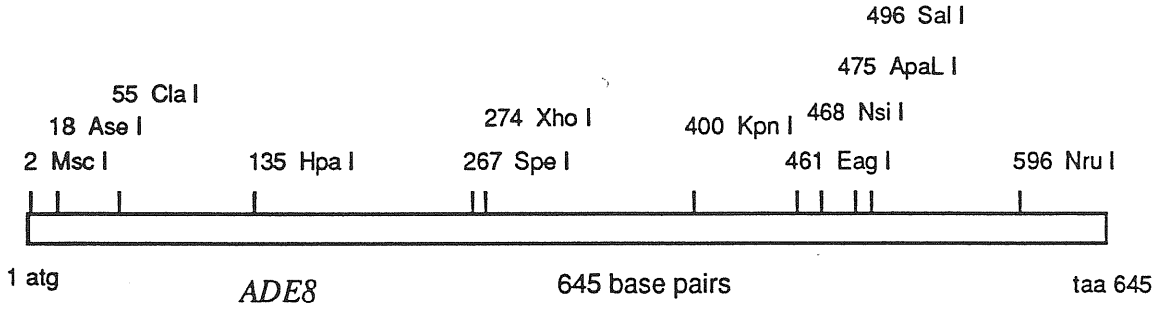
0.5 µl ATP (50 mM in Tris buffer)

1.0 µl T4 DNA ligase, 8 units/µl (Pharmacia)

20 µl total volume.

(OPA^+ reaction buffer is: 100 mM Tris acetate [pH 7.5], 100 mM magnesium acetate, and 500 mM potassium acetate). The reaction was incubated overnight at 16°C. Transformation of DH5alfa was achieved as described, and transformants were selected onto LB Ampicillin plates. Identification of recombinant clones was done by restriction analysis with PvuI restriction enzyme. In fact, the recombinant pBRADE-XP/PvuI clones exhibited two bands migrating at approximately 3.7 kb and 4.5 kb, as compared to the pBRADE/PvuI plasmid with only one single 8.2 kb band (Fig. 2.9). The ≈ 1.7 kb BglII/EcoRI fragments from either pBRADE or pBRADE-XP (carrying *ADE8* and *ade8-xp* respectively), were gel purified, and recovered from the agarose slice with the QIAEX gel extraction kit as specified above. The DNA was resuspended in 20 µl of TE buffer, and an aliquot was used in a ligation reaction with the recipient plasmid vectors (see later).

ADE8 plot

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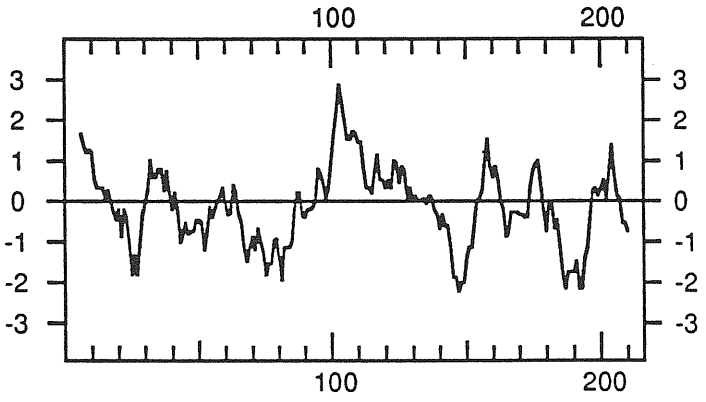
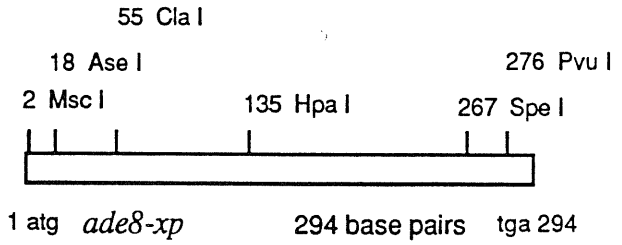
 41 KKAYGLTRAADNNIPTKVCSLYPYTKGIAKEDKAARAKAR   80
 81 SQFENDLAKLVLEEKPDVIICAGWLLILGSTFLSQLQSVP  120
121 ILNLHPALPGCFDGTTHAIEMAWRKCQDENKPLTAGCMVH  160
161 YVIEEVDKGEPLVVKKLEIIPGEETLEQYEQRVHDAEHIA  200
201 IVEATYKVLQQLHKZ                          215
```



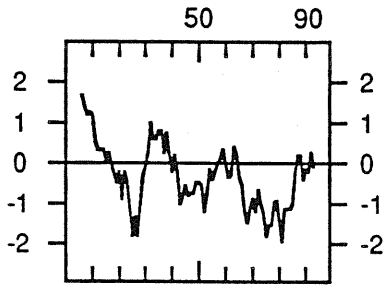
ADE8 protein Hydrophaty Plot (Kyte Doolittle)

Fig. 2.6. Schematic restriction map of the *ADE8* gene (top), its protein sequence (center), and the protein hydropathy plot (bottom).

ade8-xp plot




```
  1 MARIVVLISGSGSNLQALIDAQKQGQLGEDAHIVSVISSS   40
 41 KKAYGLTRAADNNIPTKVCSLYPYTKGIAKEDKAARAKAR   80
 81 SQFENDLAKLVLDRGKAZ                         98
```



ade8-xp protein Hydrophaty Plot (Kyte Doolittle)

Fig. 2.7. Schematic restriction map of the *ade8-xp* gene (top), its truncated protein sequence (center), and the protein hydropathy plot (bottom).

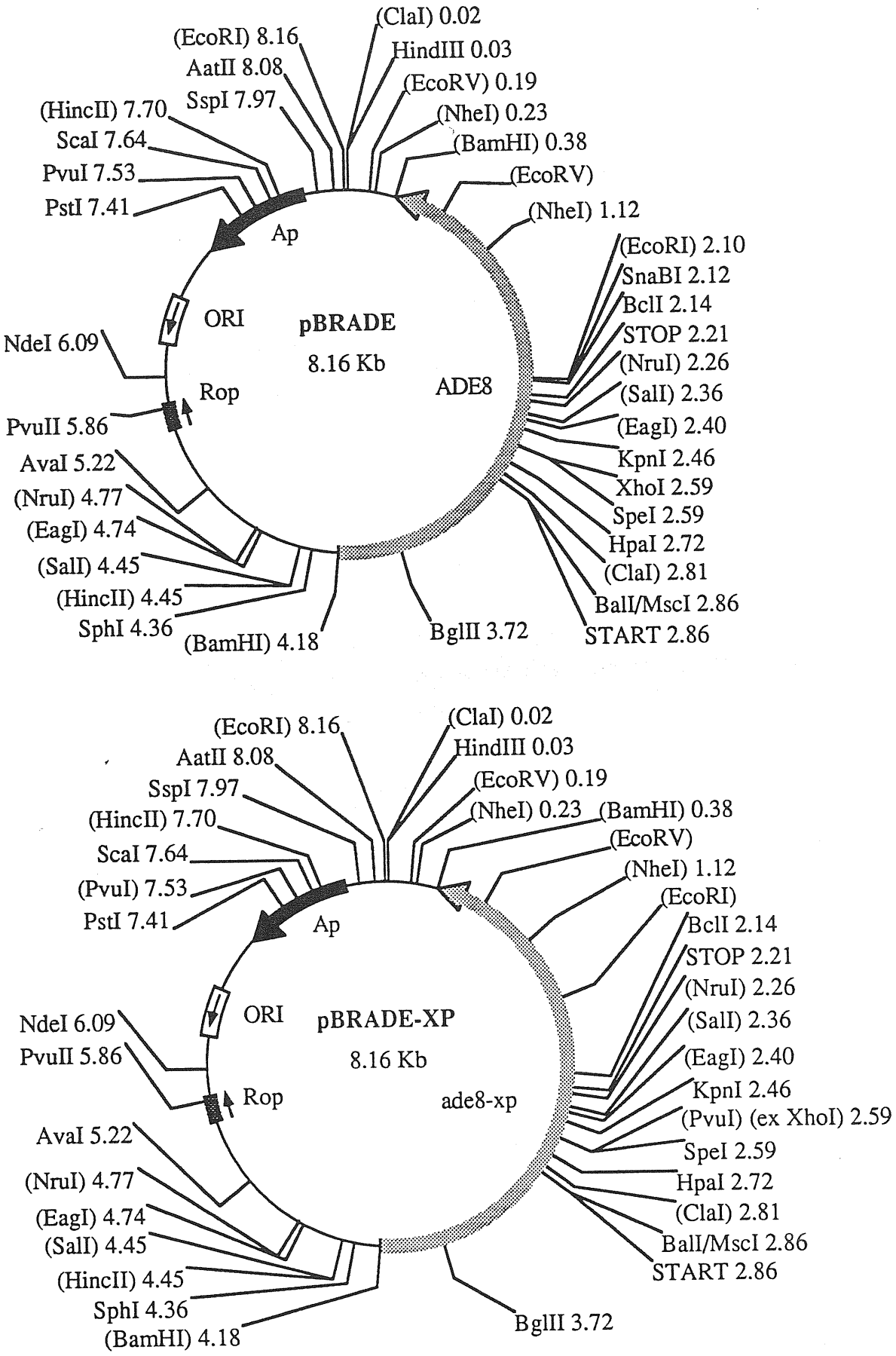


Fig. 2.8 . Detailed restriction maps of plasmids pBRADE and pBRADE-XP.

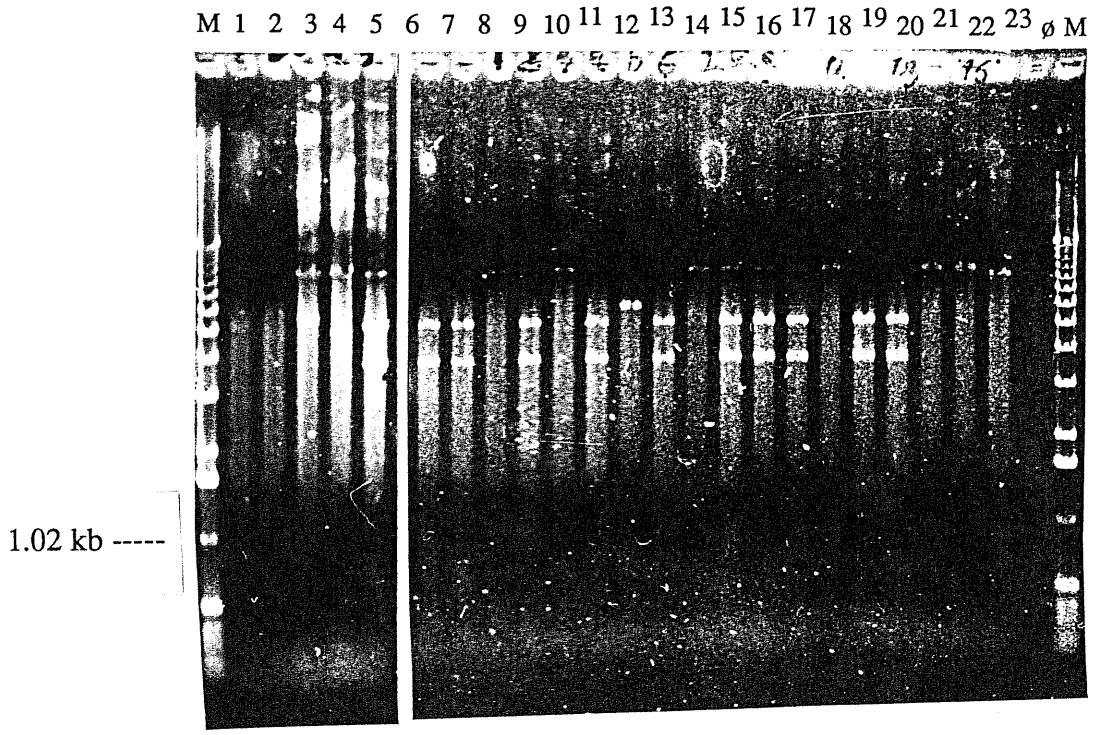


Fig. 2.9. Agarose gel showing the restriction pattern of the pBRADE and pBRADE-XP plasmids. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Ø**: empty lane. **Lanes 1 and 3**: pBRADE/PvuI, linear ≈ 8.2 kb plasmid. **Lanes 2 and 5**: pBRADE/PvuI/XhoI, ≈4.5 kb and 3.7 kb. **Lane 4**: pBRADE/XhoI, linear ≈ 8.2 kb plasmid. **Lanes 6 - 23**: putative pBRADE-XP/PvuI DNAs; clones presenting the expected ≈ 4.5 kb and ≈ 3.7 kb bands are true recombinant pBRADE-XP.

2.17 Plasmid Constructions

The pRAP Family of Yeast Episomal Plasmids

To study the effect on general cellular metabolism of selective pressure for multicopy selectable markers, we constructed a series of basic yeast episomal plasmids carrying the *HIS3*, *LEU2*, *TRP1*, and *URA3* genes. To do so, the 7.5 kb pBH15 plasmid (Bruschi and Howe, 1988) was restricted with HpaI and NruI, the reaction mixture was diluted and then religated. After transformation, the recombinant clone lacking the whole *LEU2* gene was identified by restriction analysis, and the new 4.37 kb construct was named YPA. This plasmid was cut with XbaI and PvuI and the 1.76 kb portion carrying the 2μm origin of replication - together with the FRT - was gel purified and used later for ligation.

In a different reaction, the 12.5 kb pBHD plasmid (Bruschi and Howe, 1988) was restricted to completion with PvuI and only partially with XbaI. The 7.5 kb *ADE8*-2μm-FRT portion deriving from this reaction was recovered and ligated to the previously isolated 1.76 kb YPA/XbaI/PvuI DNA. The resulting 9.26 kb vector was named pBUD. This construct is basically equivalent to pBHD, but it lacks the complete 3.3 kb HpaI-NruI *LEU2* gene and its genomic flanking sequences. This plasmid also carries the two FRTs cloned in the direct orientation, and upon activity of the FLP recombinase the plasmid undergoes site-specific resolution, generating an integrative (pIA) and an episomal (pRAP) plasmid without any yeast selectable marker and with a unique BamHI cloning site. To recover this episomal form, pBUD was resolved *in vitro* by cutting with XbaI and religation of the purified 3.73 kb episomal fragment. This pRAP construct was the recipient carrier for the cloning of different yeast markers.

First, the pRAP plasmid was utilized to construct the 5.49 kb pRAP-HIS3, an episomal vector that carries the *HIS3* gene, coding for the enzyme imidazoleglycerol-P-dehydratase (EC 4.2.1.19; Fink, 1964), for the synthesis of histidine. This was achieved by cutting pRAP with BamHI, purification of the vector DNA, and ligation to the 1.76 kb *HIS3*/BamHI fragment from plasmid pYAC3 (Burke et al., 1987).

Second, we cloned in unique BamHI site of pRAP a gene coding for a dominant marker the *APH* gene from Tn903, encoding the enzyme aminoglycoside 3'-phosphotransferase. This

gene, conferring kanamycin, neomycin and G418 resistance (Oka et al., 1981; Taylor and Rose, 1988), was taken from plasmid pUC-4K (Pharmacia) as 1.26 kb BamHI fragment. The resulting 4.99 kb construct, called pRK, was restricted with SalI, the 3.74 kb vector DNA purified and ligated to the 2.2 kb *LEU2*/SalI/XhoI DNA from pBH15. The *LEU2* gene codes for ß-isopropylmalate dehydrogenase (EC 1.1.1.85; Ratzkin and Carbon, 1977; Hinnen et al., 1978) for the synthesis of leucine. The 5.93 kb pRAP-LEU2 recombinant clone carrying the *LEU2* gene in the same orientation as the unique FRT was identified by restriction analysis.

The pRAP-LEU2 vector was used for the construction of the last two episomal plasmids, pRAP-TRP1 and pRAP-URA3; the first one carries the *TRP1* gene coding for phosphoribosylanthranilate isomerase (EC 2.4.2.18; De Moss, 1965; Doy and Cooper, 1966) for the synthesis of tryptophan, while the latter carries the *URA3* gene coding for orotidine-5'-phosphate decarboxylase (EC 4.1.1.23; Lacroute, 1968) for the synthesis of uracil.

The *TRP1* gene was taken as 1.3 kb FspI/SspI blunt-ended fragment from plasmid YIplac204 (Gietz and Sugino, 1988), cloned into the SmaI site of plasmid pUC19 (Yanisch-Perron et al., 1985) and recombinant white clones - with the insert in the opposite orientation to *lacZ* - were identified as described. This plasmid was named pUC-T. Then, the *TRP1* gene was moved from pUC-T as 1.42 kb PvuII/SalI fragment and cloned into pRAP-LEU2/HpaI/SalI. The resulting 5.41 kb final construct was named pRAP-TRP1.

The same strategy was exploited for the making of pRAP-URA3. In fact, the *URA3* gene was isolated as 1.6 kb *URA3*/SspI/PvuII blunt-ended fragment from plasmid YIplac211 (Gietz and Sugino, 1988). This fragment was inserted into the SmaI cloning site of pUC19, thus generating plasmid pUC-U. The marker was then moved from this plasmid as 1.72 kb *URA3*/PvuII/SalI fragment and finally cloned into pRAP-LEU2/HpaI/SalI, thus generating the 5.71 kb pRAP-URA3.

A summary of the whole cloning strategy for the pRAP family of vectors is given below:

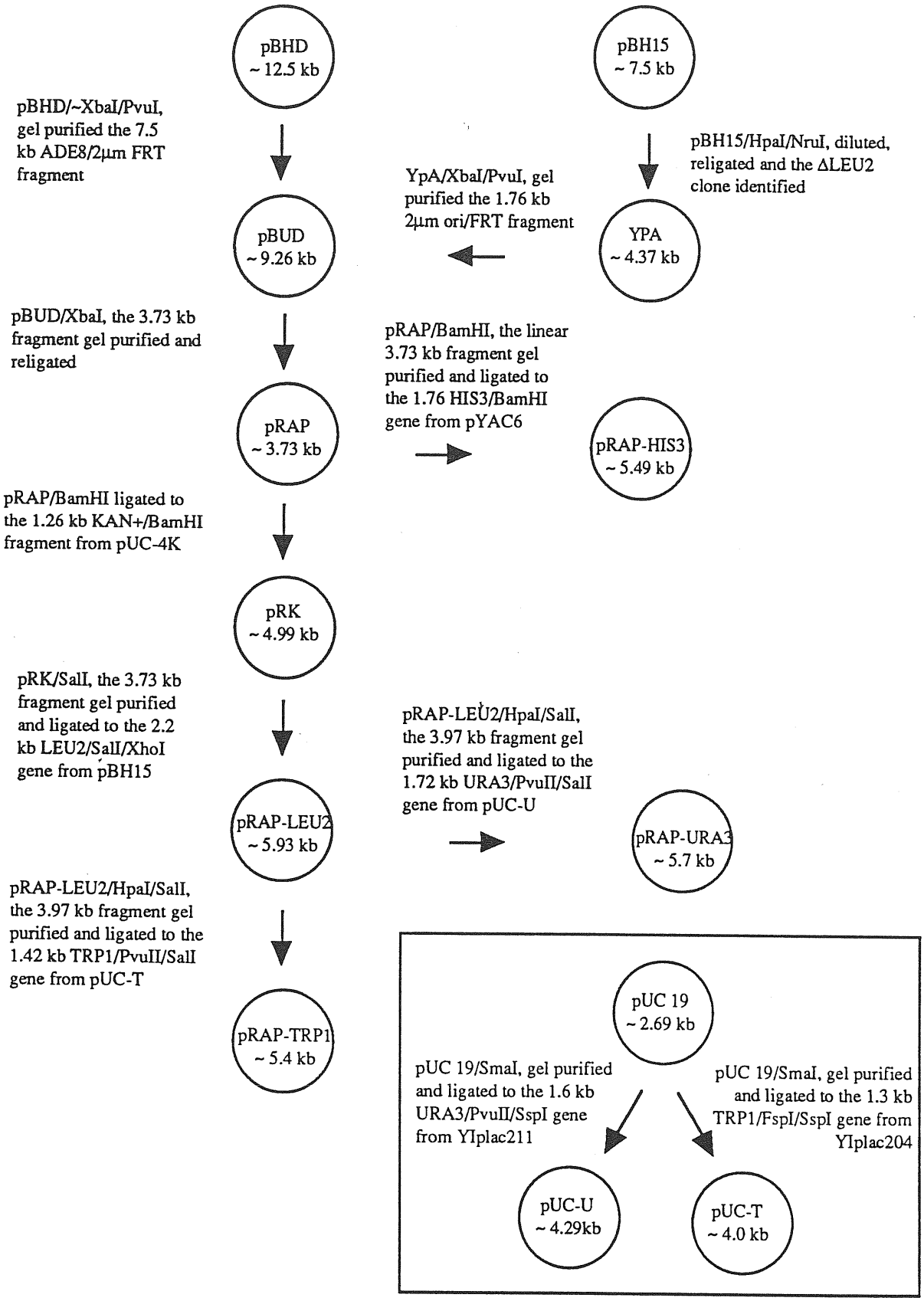


Fig. 2.10 . Construction of the pRAP family of yeast episomal vectors used in the growth curve, plasmid stability and copy number experiments.

Detailed maps of these plasmids are shown below:

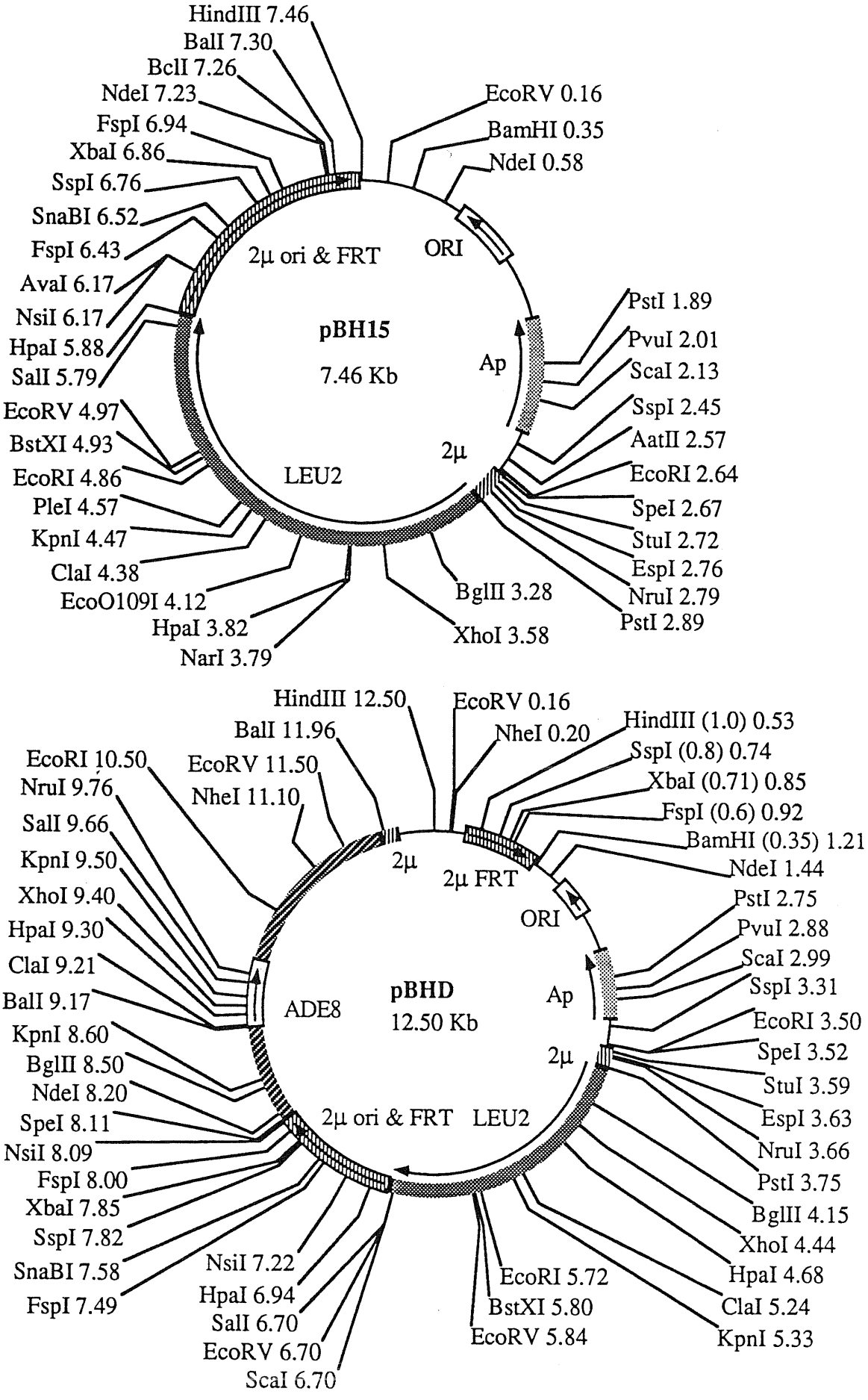


Fig. 2.11 . Detailed restriction maps of plasmids pBH15 and pBHD.

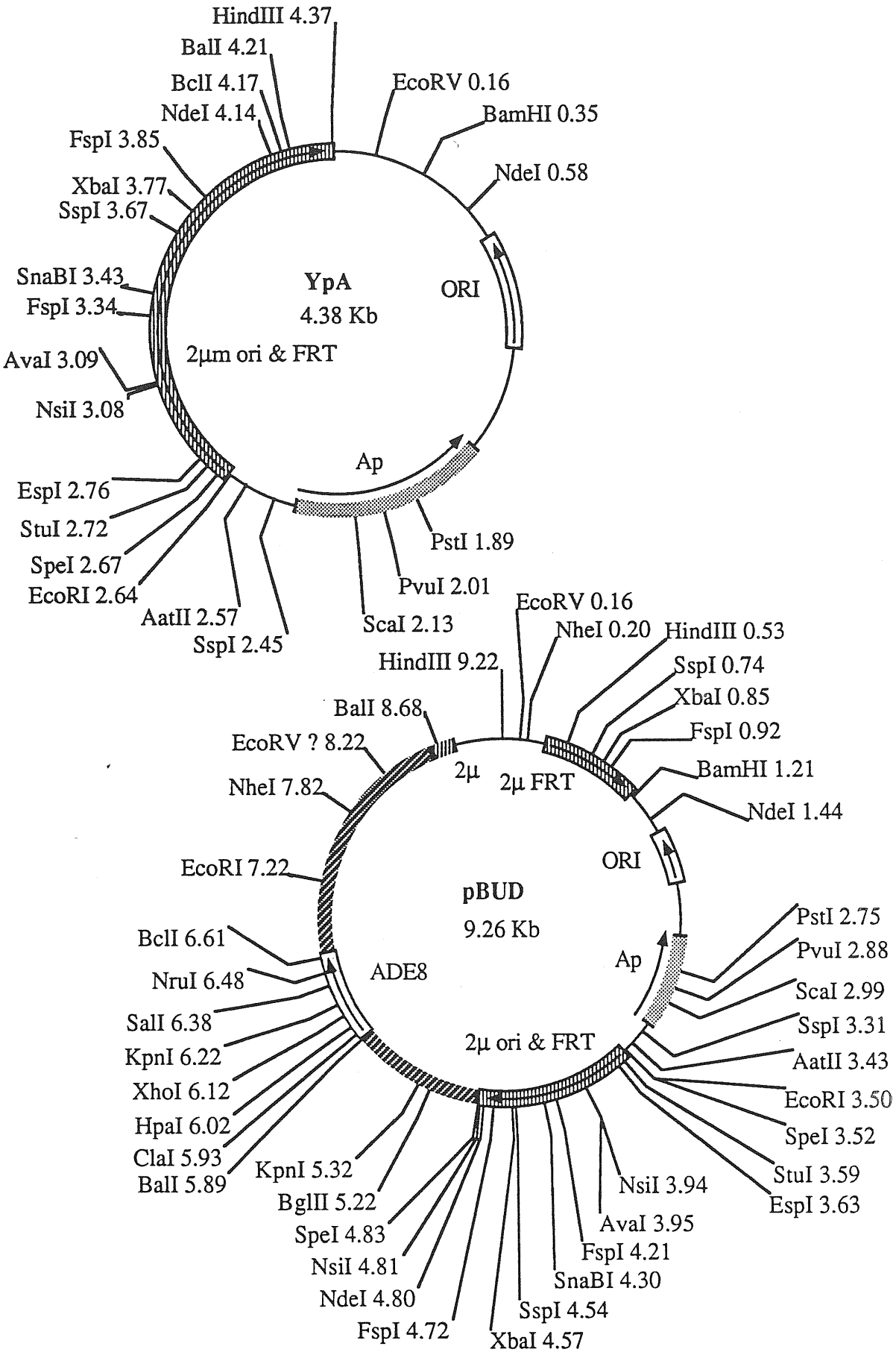


Fig. 2.12. Detailed restriction maps of plasmids YpA and pBUD.

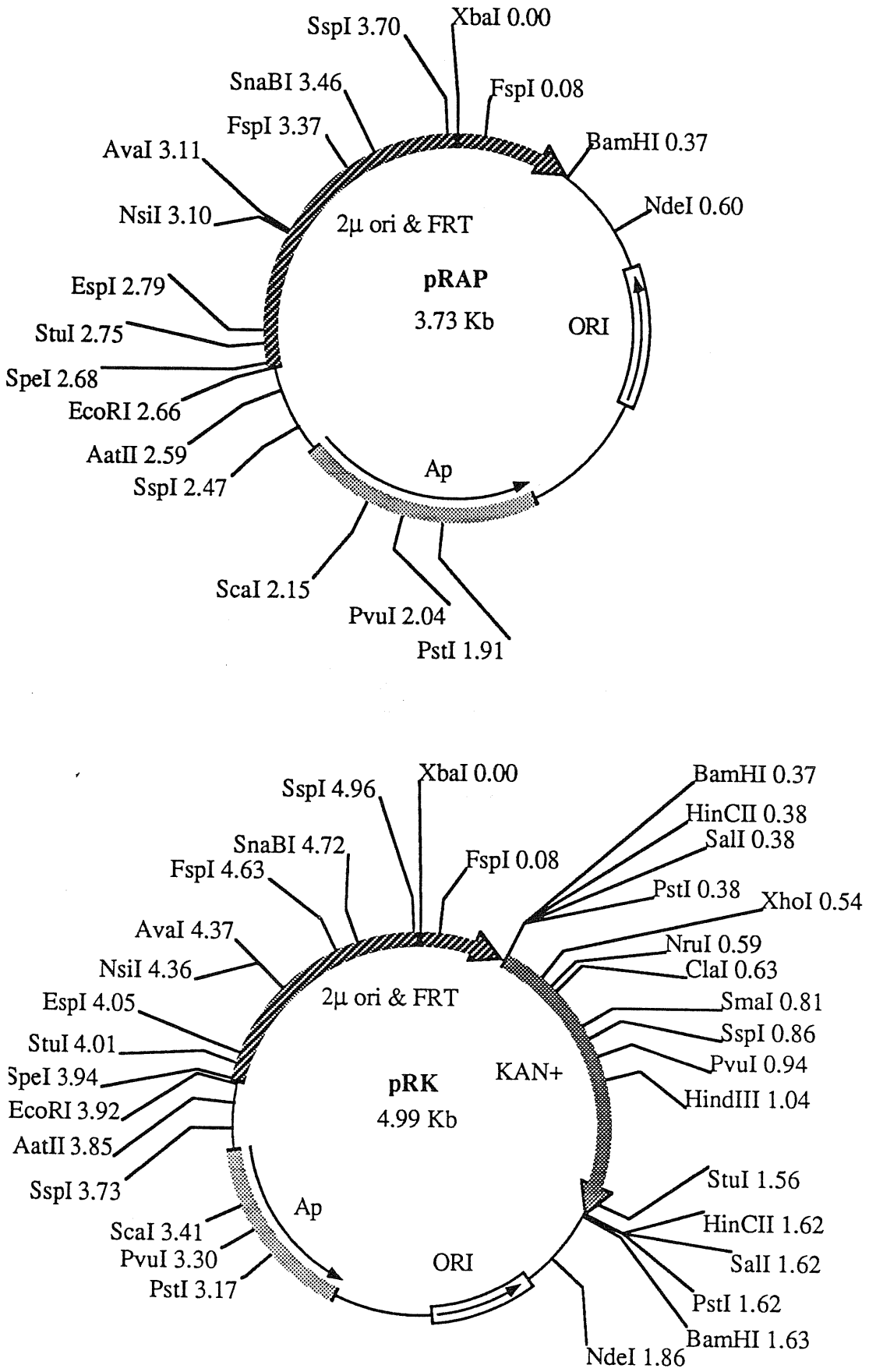


Fig. 2.13. Detailed restriction maps of plasmids pRAP and pRK.

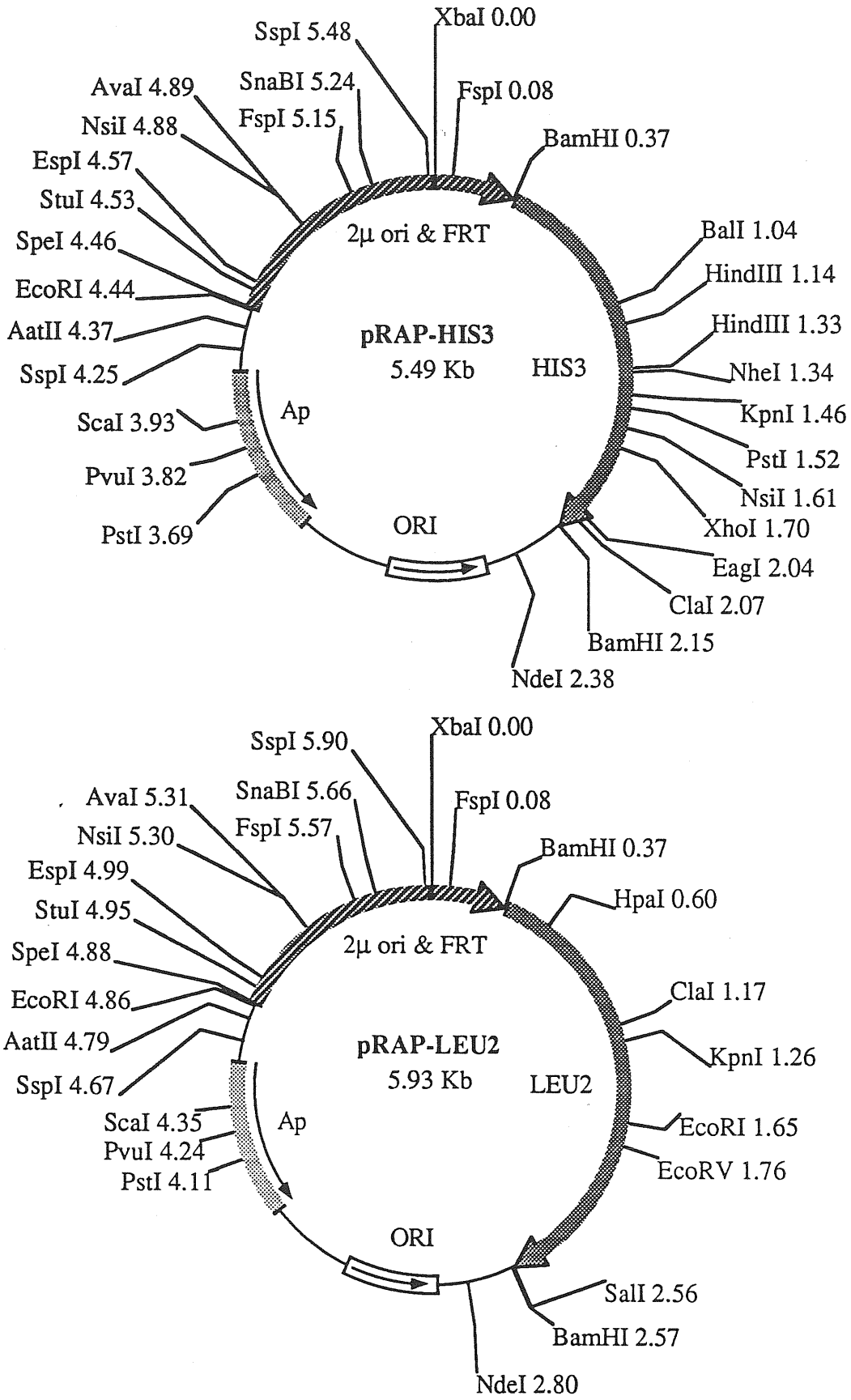


Fig. 2.14 . Detailed restriction maps of plasmids pRAP-HIS3 and pRAP-LEU2.

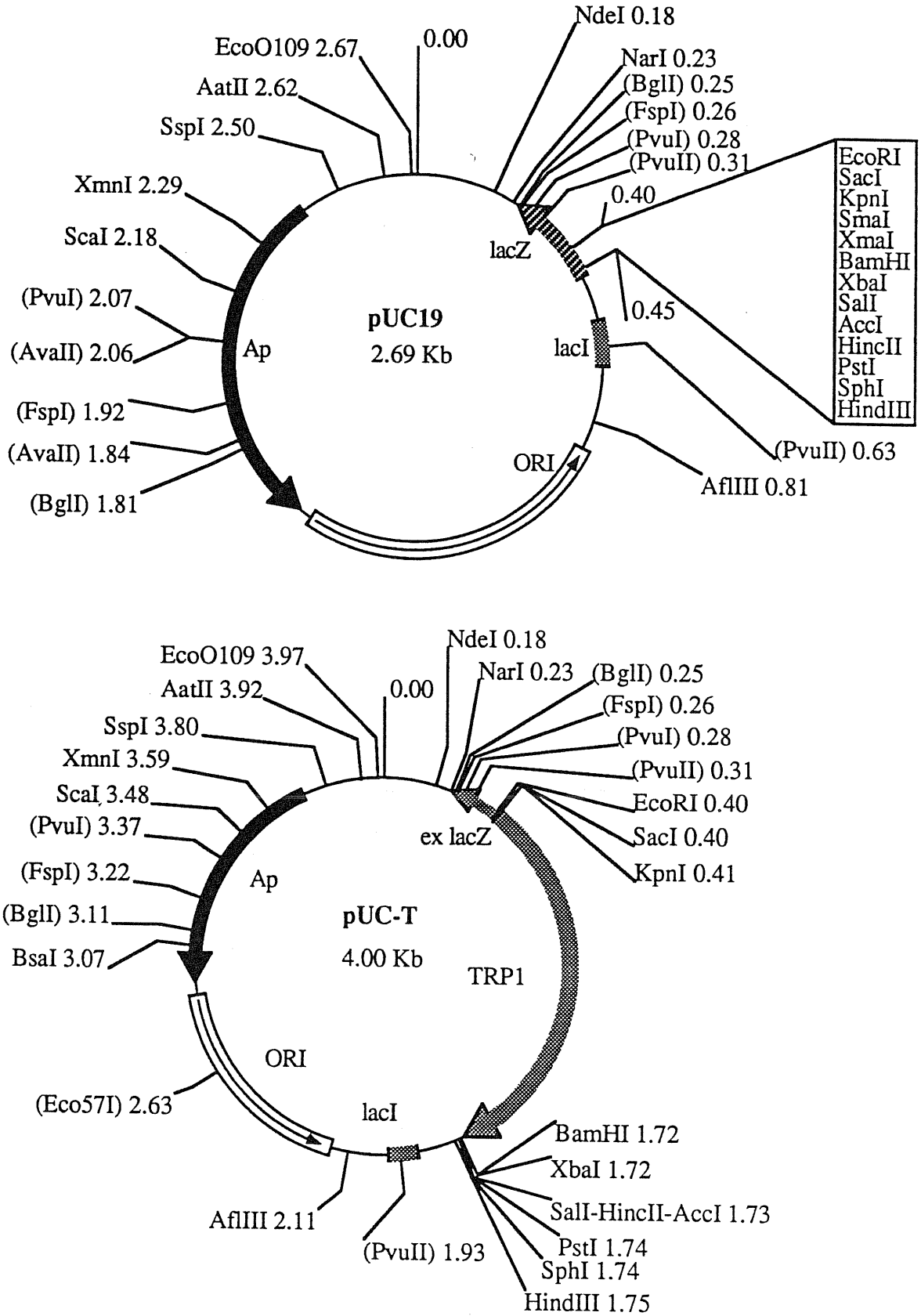


Fig. 2.15 . Detailed restriction maps of plasmids pUC19 and pUC-T.

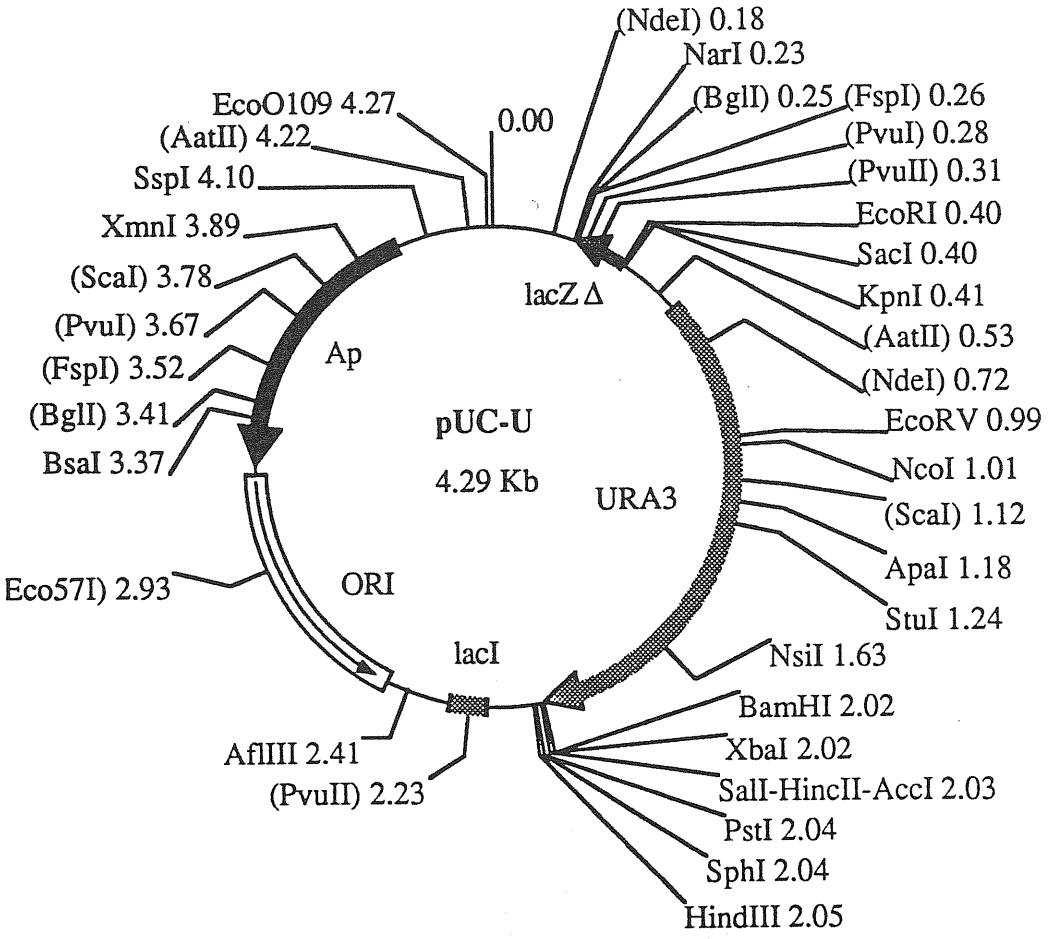


Fig. 2.16 . Detailed restriction map of plasmid pUC-U.

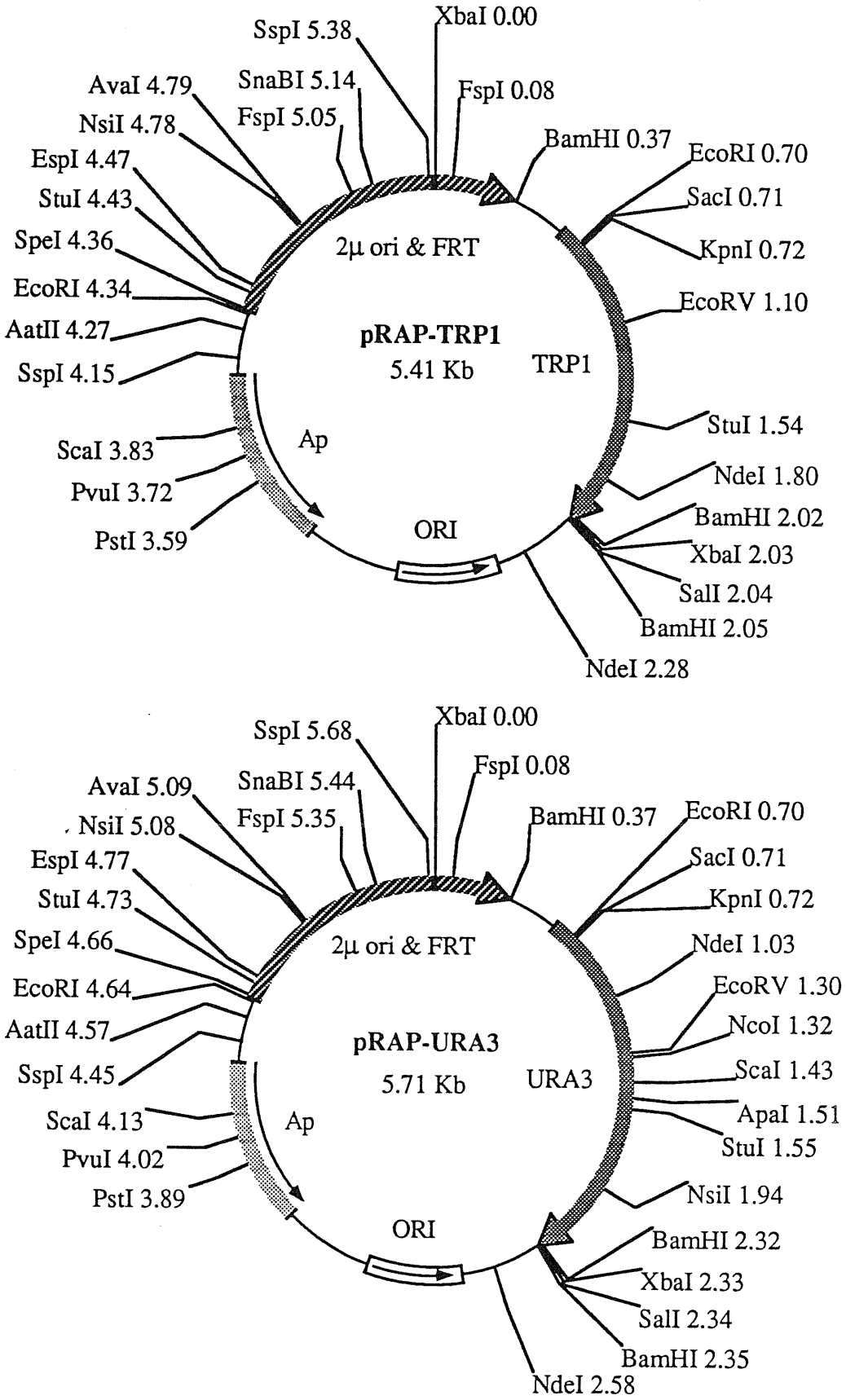


Fig. 2.17 . Detailed restriction maps of plasmids pRAP-HIS3 and pRAP-LEU2.

The YCp and YEp Families of Plasmids with two Yeast Markers

The purified *ADE8*/BglII/EcoRI and *ade8-xp*/BglII/EcoRI DNA fragments were used in the construction of the plasmids necessary for our experiments. The recipient vectors for the cloning of these reporter genes were those constructed by Gietz and Sugino (1988).These authors reported the construction of a series of versatile centromeric (YCplac), episomal (YEplac), and integrative (YIplac) yeast-*E. coli* shuttle vectors containing the pUC19 multicloning site and a convenient *lacZ* system for blue/white selection of recombinant clones (Sambrook et al., 1989). Furthermore, these vectors contain *in vitro* modified yeast markers lacking six-base pair restriction sites, and allowing the use of the full set of pUC19 restriction sites for further cloning activities. In detail, the *LEU2* gene lacks the EcoRI and KpnI restriction sites (within the open reading frame [ORF]); the *TRP1* gene lacks the HindIII, PstI, and XbaI sites (the HindIII and XbaI sites being in the ORF, and the PstI site downstream the ORF); the *URA3* gene lacks the PstI site located upstream the ORF (Gietz and Sugino, 1988). These modifications were shown not to affect the properties of these selectable markers. The centromeric and episomal series of vectors (YCplac111, YCplac22, and YCplac33; YEplac181, YEplac112, and YEplac195 respectively) were restricted with BamHI and EcoRI restriction enzymes, the fragments gel purified, the DNAs extracted and ligated to the *ADE8*/BglII/EcoRI and *ade8-xp*/BglII/EcoRI DNAs. The ligation mixture was incubated overnight at 16°C, and subsequently used to transform the bacterial strain DH5alfa. Recombinant white clones were analyzed by restriction analysis utilizing the XbaI and EcoRI restriction enzymes. Recombinant plasmids were named as follows:

for the centromeric series of two-marker vectors:
YCpLA and YCpLA*: YCplac111 (*LEU2*) with either the *ADE8* or *ade8-xp* gene
YCpTA and YCpTA*: YCplac22 (*TRP1*) with either the *ADE8* or *ade8-xp* gene
YCpUA and YCpUA*: YCplac33 (*URA3*) with either the *ADE8* or *ade8-xp* gene

and for the episomal series of two-marker vectors:
YEpLA and YEpLA*: YEplac 181 (*LEU2*) with either the *ADE8* or *ade8-xp* gene
YEpTA and YEpTA*: YEplac112 (*TRP1*) with either the *ADE8* or *ade8-xp* gene

YEpUA and YEpUA*: YEplac195 (*URA3*) with either the *ADE8* or *ade8-xp* gene

A summary of the whole cloning strategy for the two types of vectors is given below:

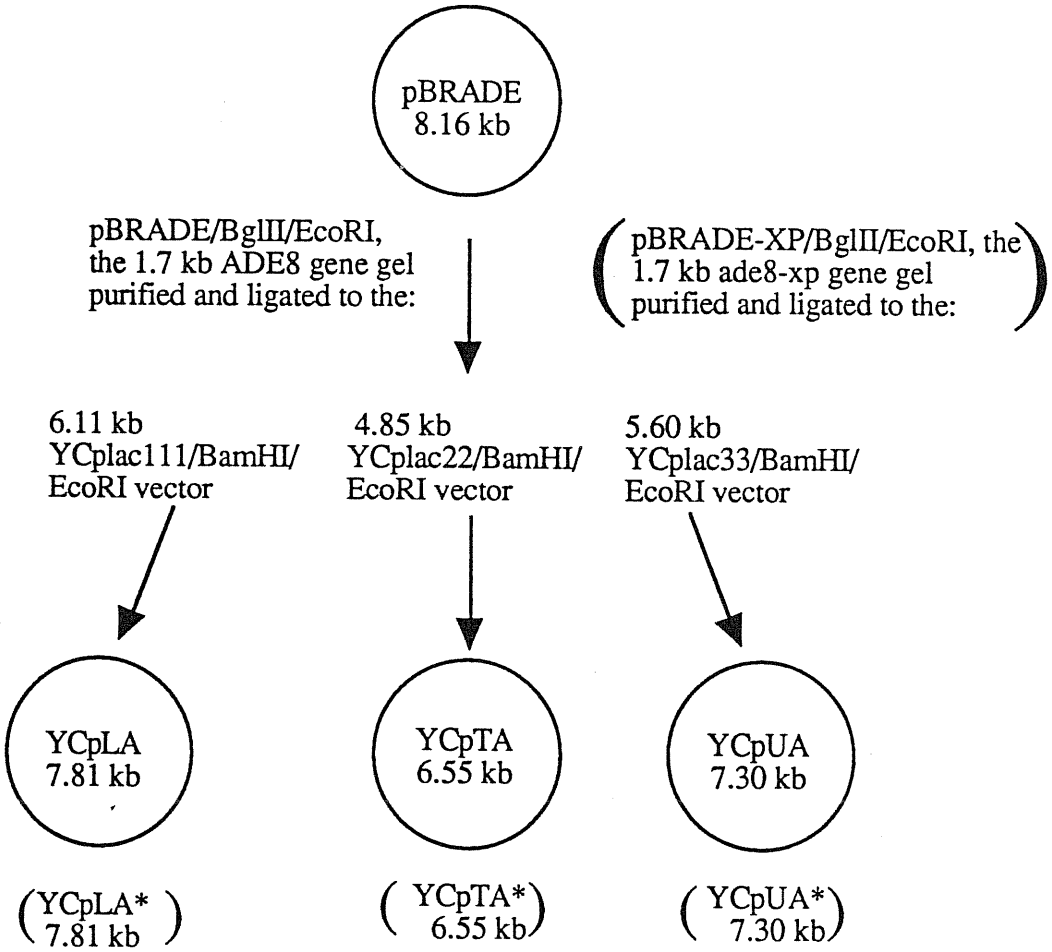


Fig. 2.18 . Construction of the YCp family of yeast centromeric plasmids carrying two selectable markers (*LEU2*, *TRP1*, or *URA3*) and the *ADE8* gene or its frame-shifted version *ade8-xp* (shown in brackets)

Detailed restriction maps of these constructions are as follows:

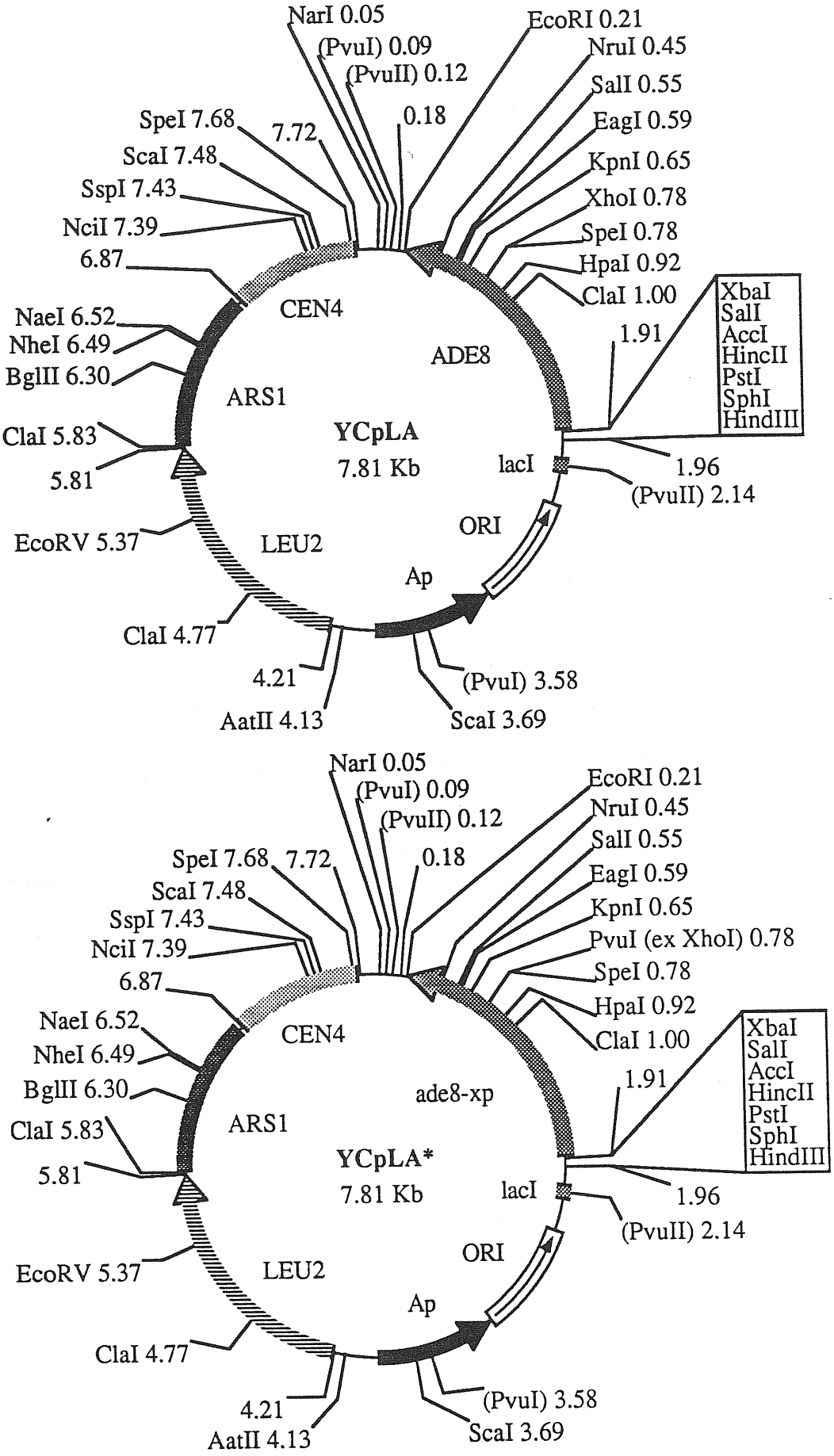


Fig. 2.19 . Detailed restriction maps of plasmids YCpLA and YCpLA*.

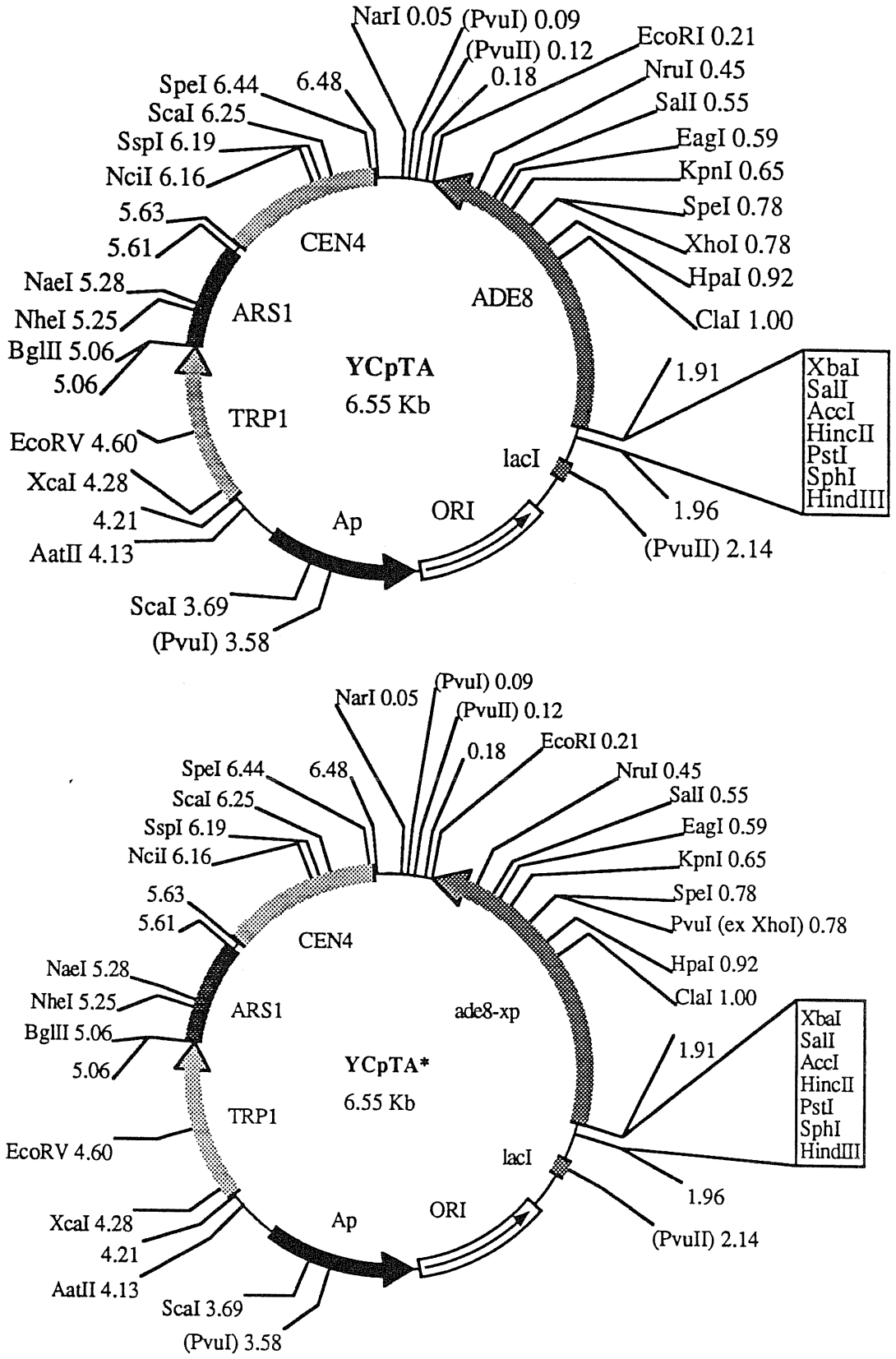
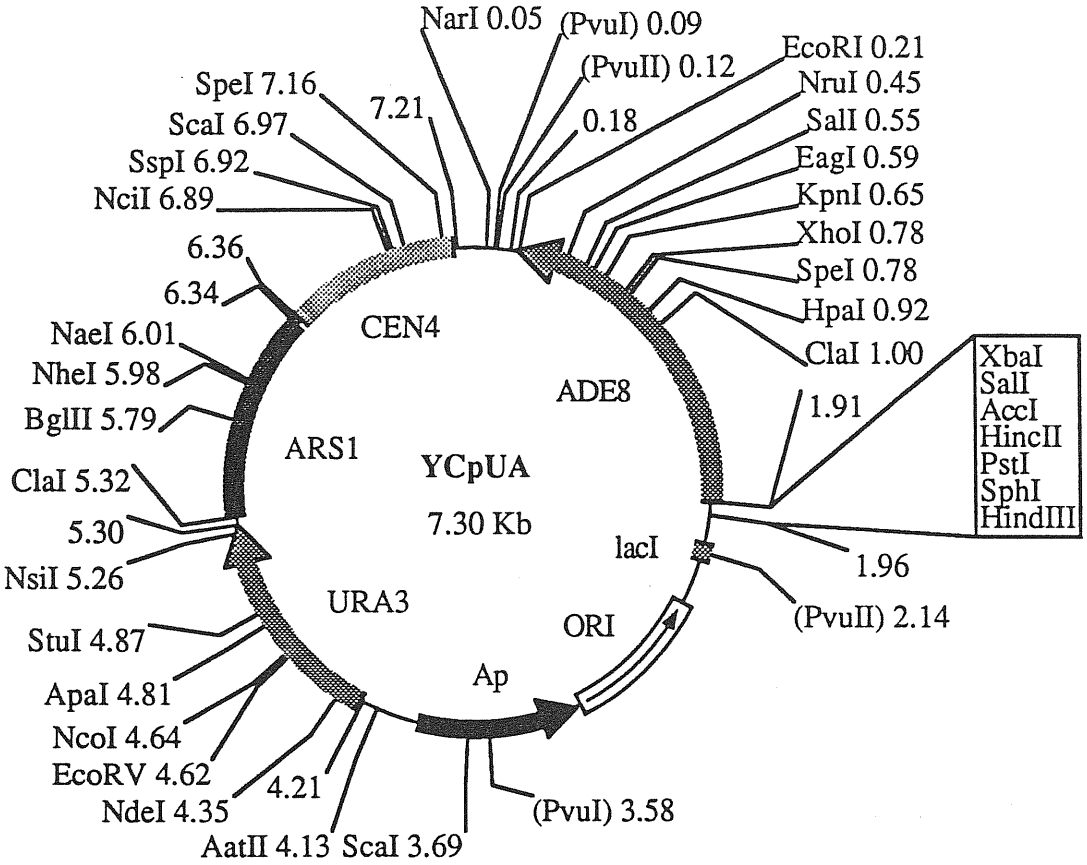


Fig. 2.20 . Detailed restriction maps of plasmids YCpTA and YCpTA*.



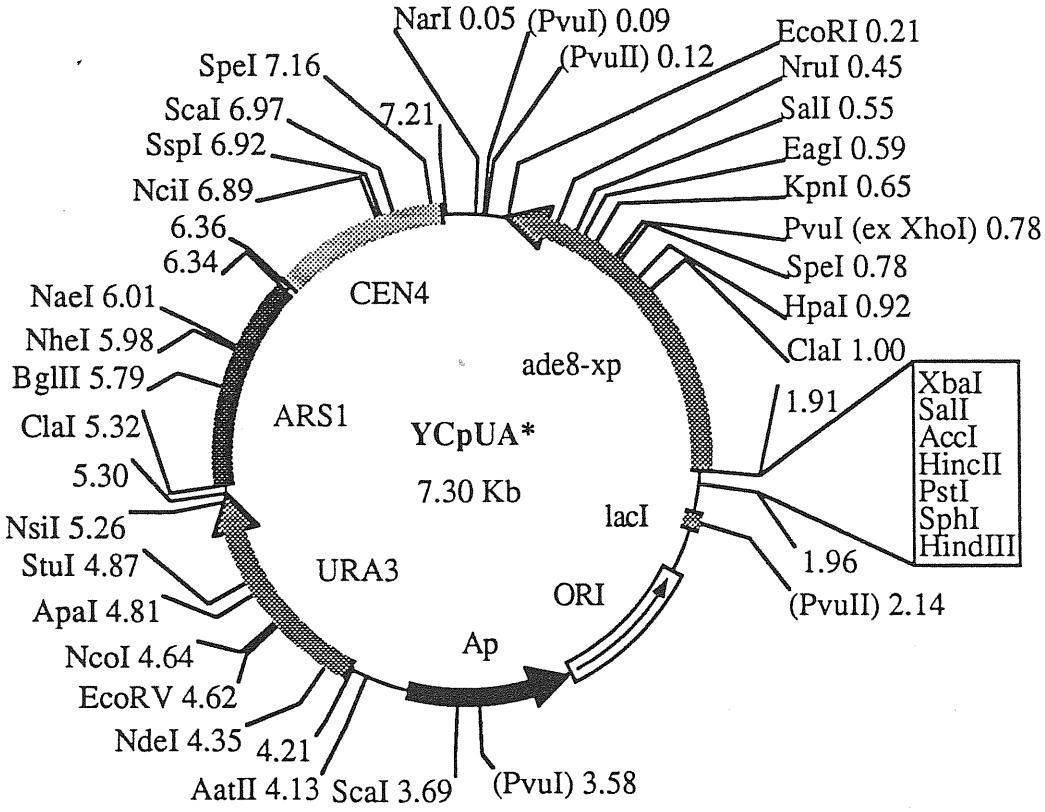
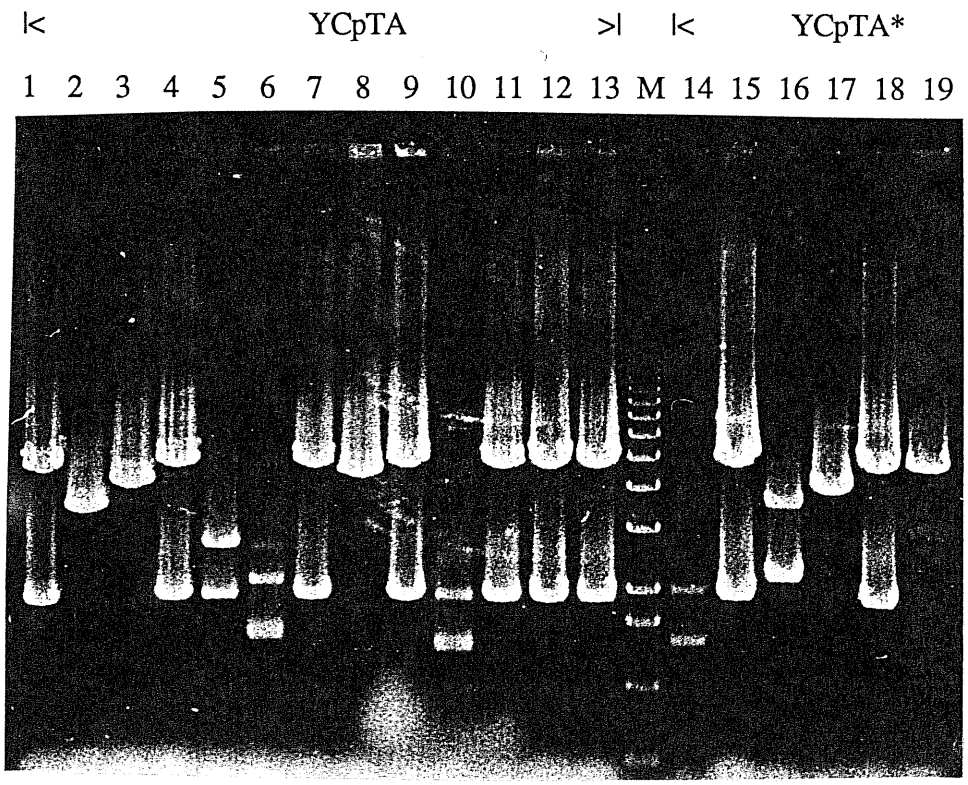


Fig. 2.21 . Detailed restriction maps of plasmids YCpUA and YCpUA*.

Fig. 2.22


|<
YCpTA
>|
|<
YCpTA*
1 2 3 4 5 6 7 8 9 10 11 12 13 M 14 15 16 17 18 19
----- 1.02 kb

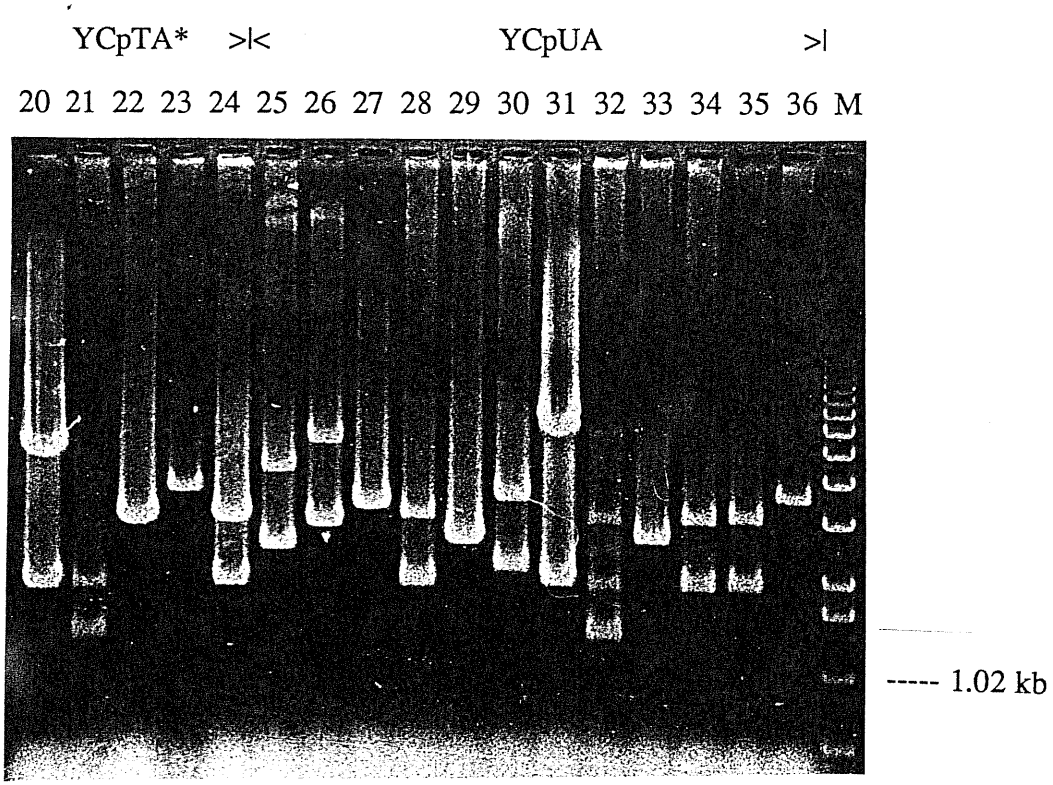
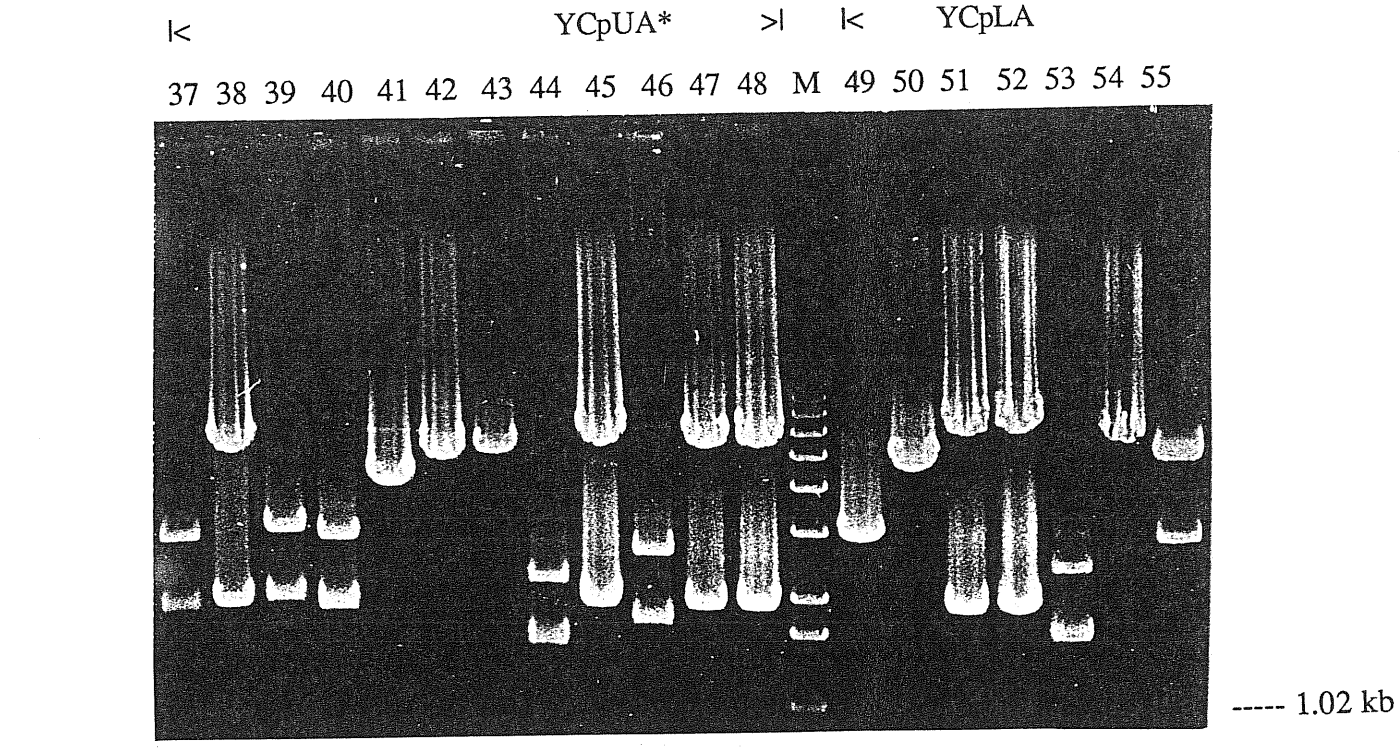

YCpTA*
>|<
YCpUA
>|
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 M
----- 1.02 kb

Fig. 2.22


|<
YCpUA*
>|
|<
YCpLA
37 38 39 40 41 42 43 44 45 46 47 48 M 49 50 51 52 53 54 55
----- 1.02 kb

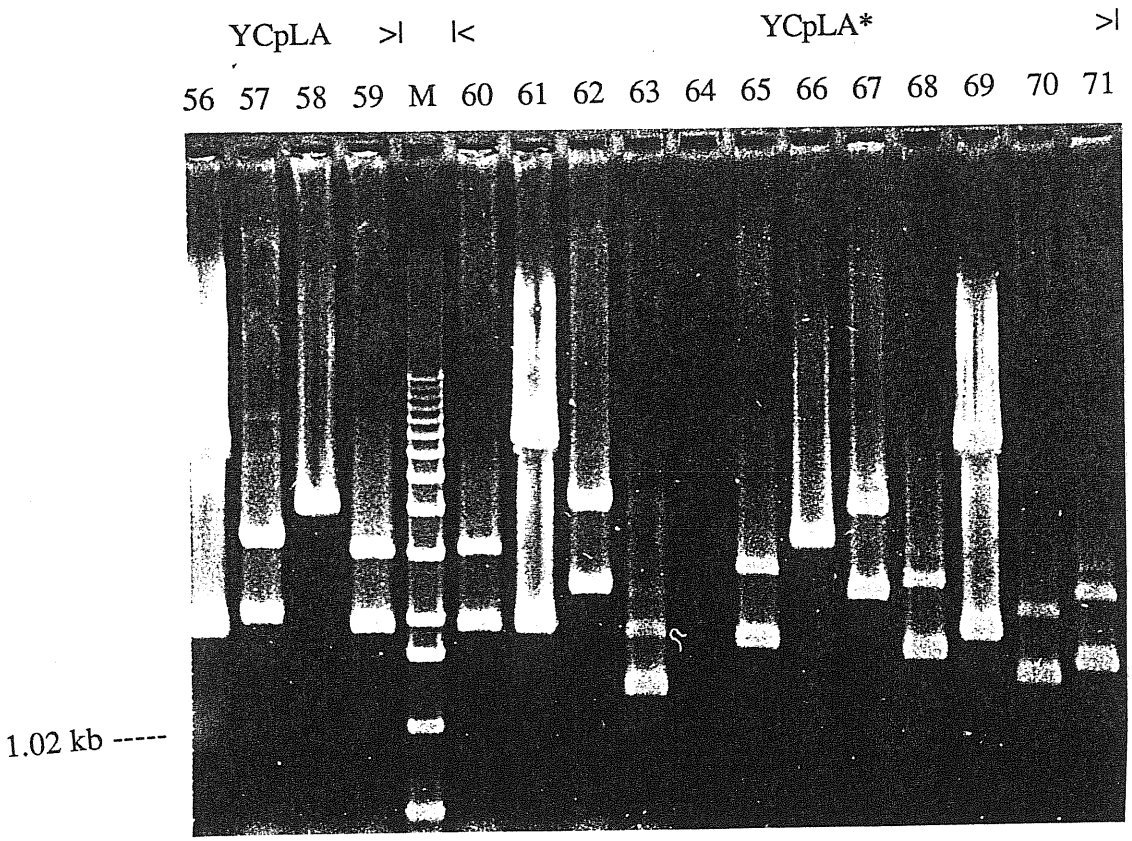

YCpLA
>|
|<
YCpLA*
>|
56 57 58 59 M 60 61 62 63 64 65 66 67 68 69 70 71
1.02 kb -----

Fig. 2.22 . Pictures of agarose gels of bacterial miniprep DNAs of the centromeric family of plasmids. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Lanes 1-13**: YCpTA/EcoRI/XbaI, true recombinants present the expected 4.85 kb and 1.7 kb (*ADE8* gene) bands; **Lanes 14-24**: YCpTA*/EcoRI/XbaI, 4.85 kb and 1.7 kb (*ade8-xp* gene); **Lanes 25-36**: YCpUA/EcoRI/XbaI, true recombinants show the expected 5.6 kb and 1.7 kb (*ADE8* gene) bands; **Lanes 37-48**: YCpUA*/EcoRI/XbaI, with the 5.6 kb and 1.7 kb (*ade8-xp* gene) bands; **Lanes 49-59**: YCpLA/EcoRI/XbaI, 6.11 kb and 1.7 kb (*ADE8* gene) bands; **Lanes 60-71**: YCpLA*/EcoRI/XbaI, with the 6.11 kb and 1.7 kb (*ade8-xp* gene) bands.

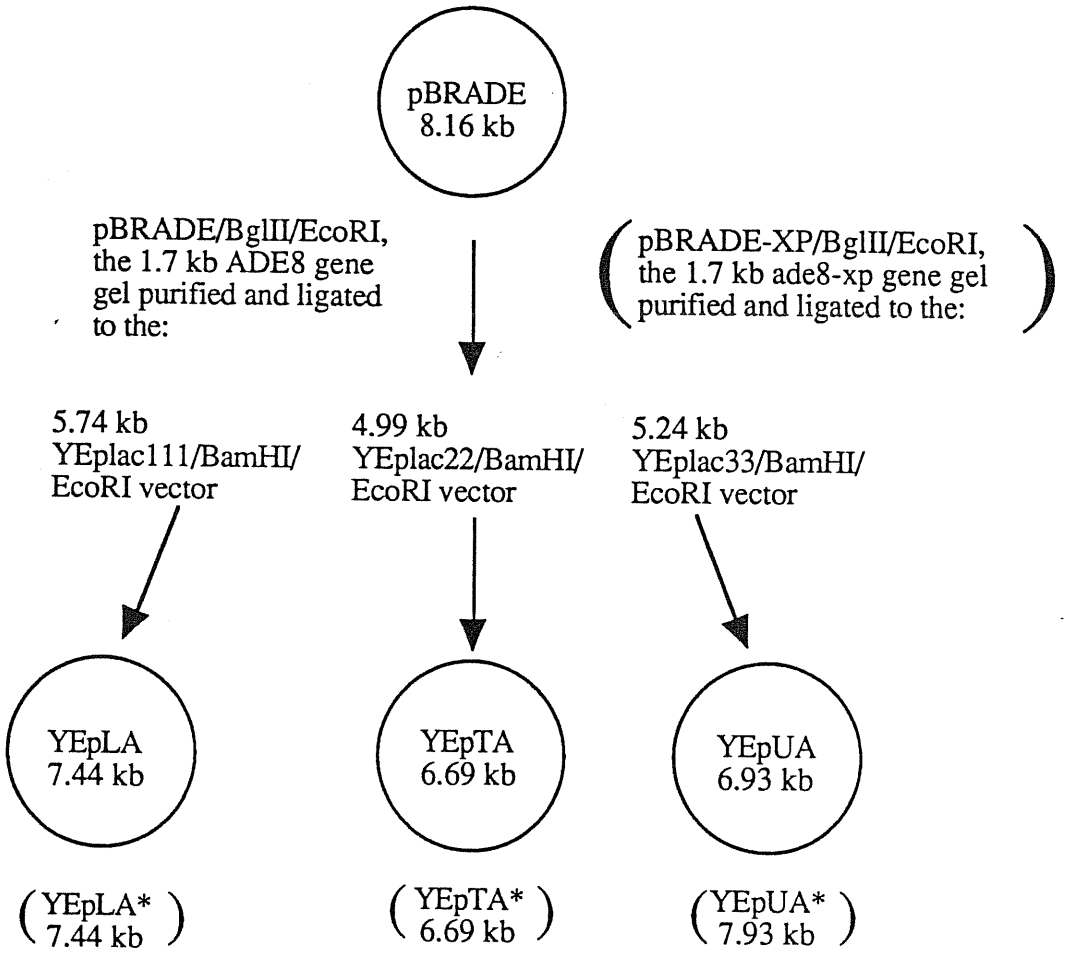


Fig. 2.23 . Construction of the YEp family of yeast episomal plasmids carrying two selectable markers (*LEU2*, *TRP1*, or *URA3*) and the *ADE8* gene or its frame-shifted version *ade8-xp* (shown in brackets)

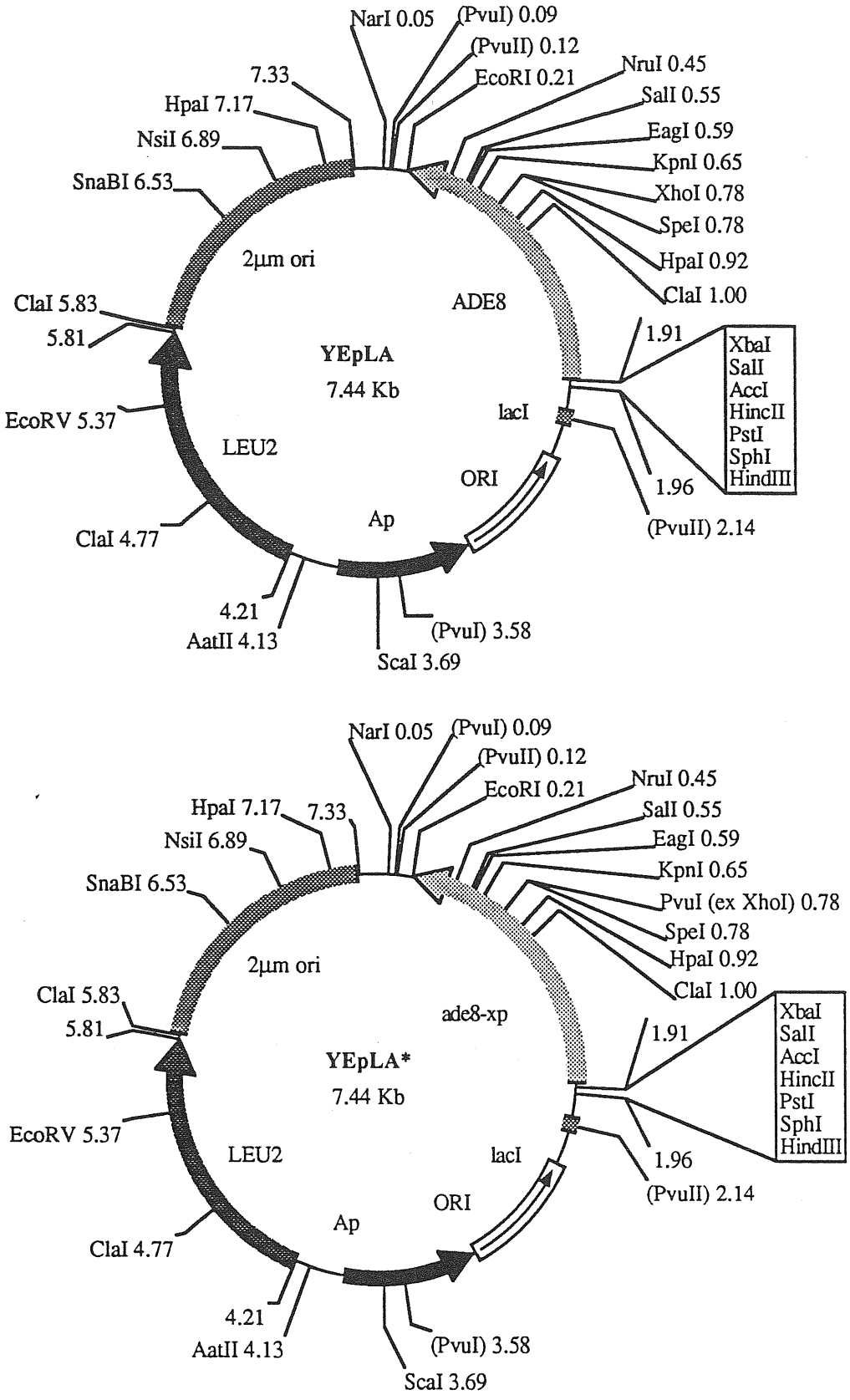


Fig. 2.24 . Detailed restriction maps of plasmids YEpLA and YEpLA*.

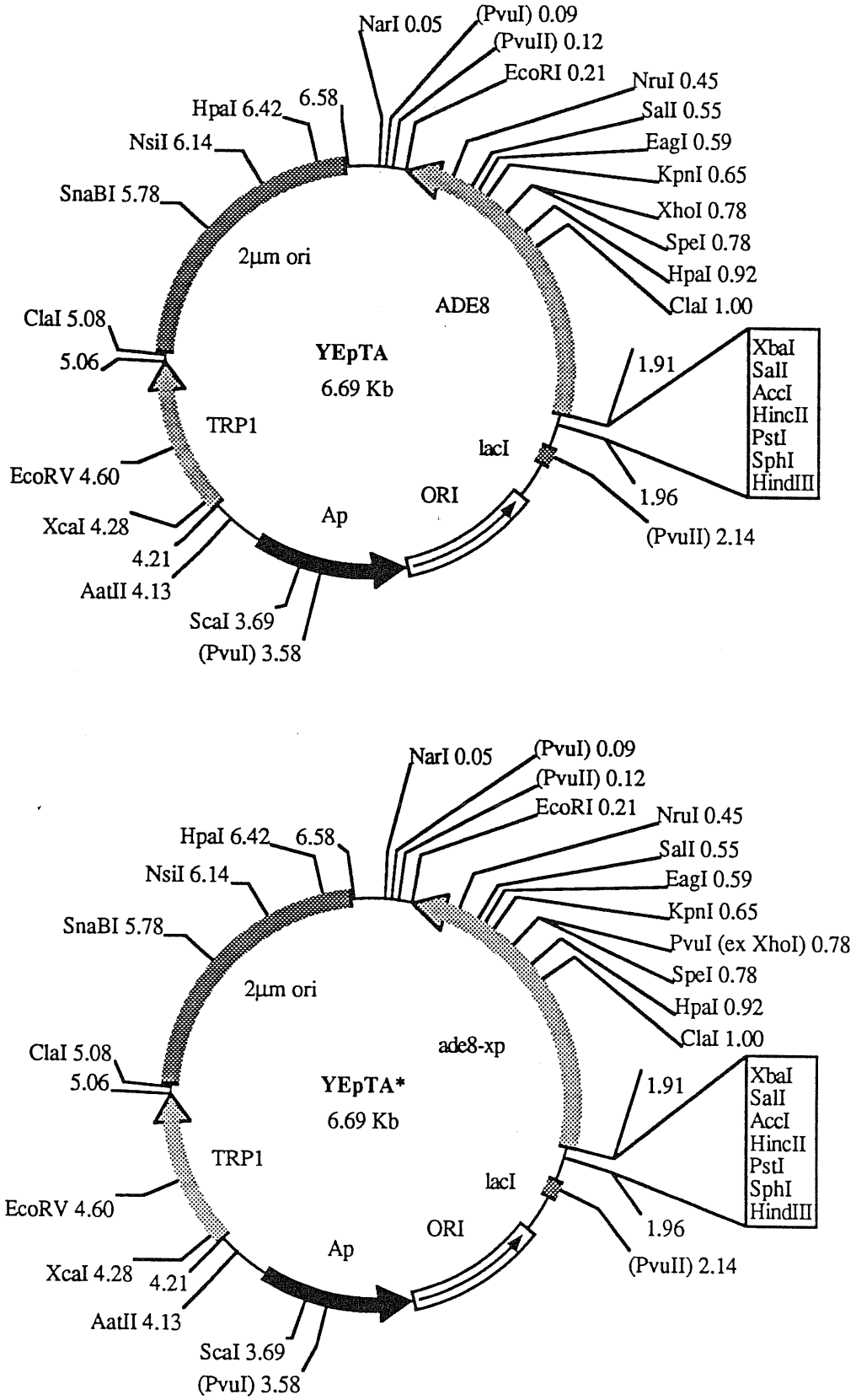


Fig. 2.25 . Detailed restriction maps of plasmids YEpTA and YEpTA*.

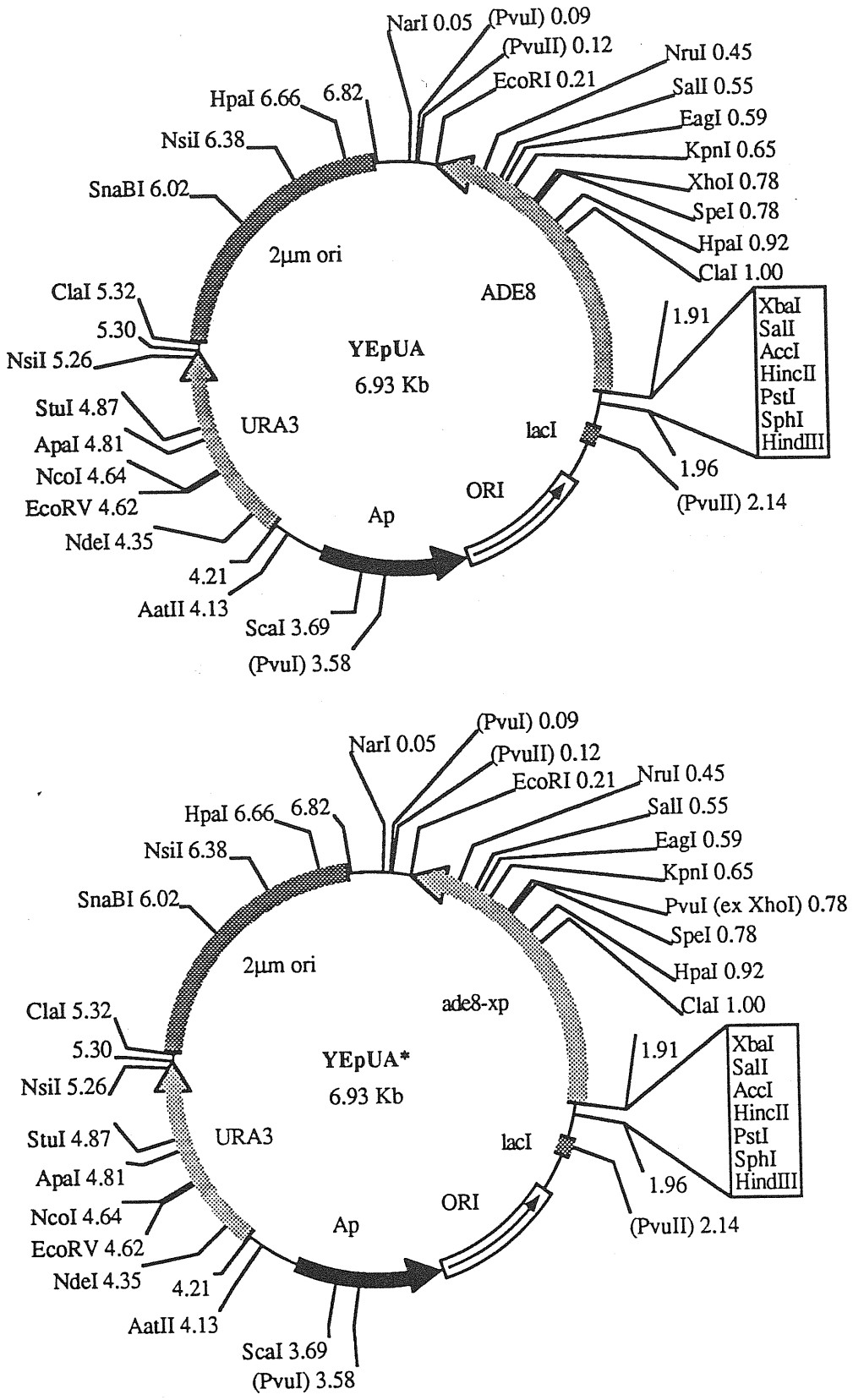
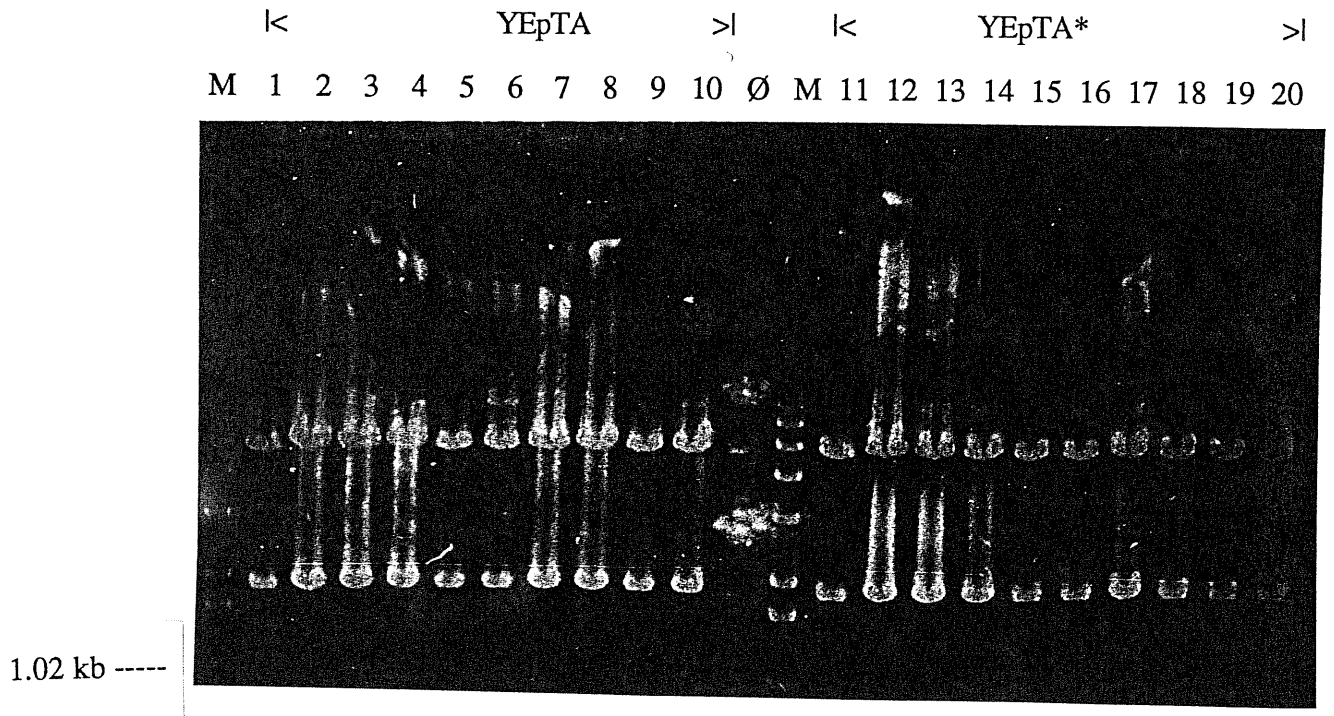
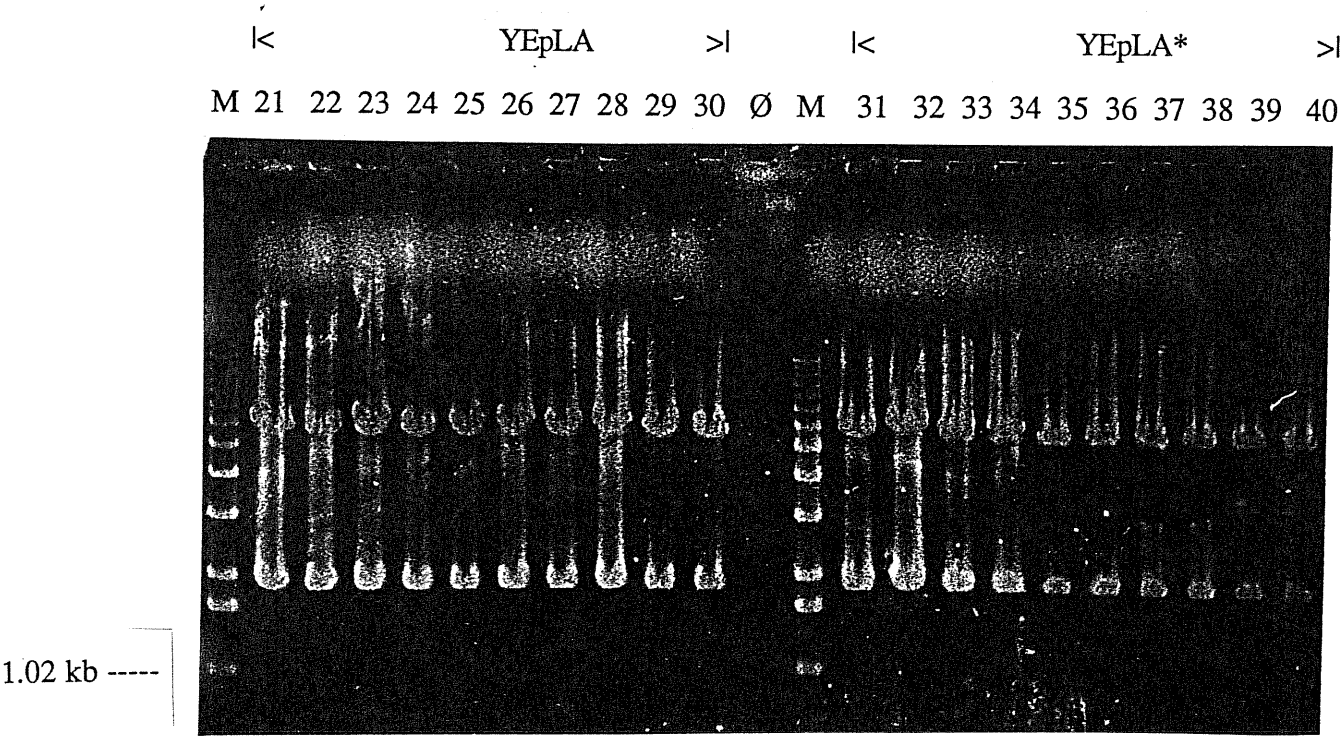


Fig. 2.26 . Detailed restriction maps of plasmids YEpUA and YEpUA*.

Fig. 2.27


|<
YEpTA
>|
|<
YEpTA*
>|
M 1 2 3 4 5 6 7 8 9 10 Ø M 11 12 13 14 15 16 17 18 19 20
1.02 kb -----


|<
YEpLA
>|
|<
YEpLA*
>|
M 21 22 23 24 25 26 27 28 29 30 Ø M 31 32 33 34 35 36 37 38 39 40
1.02 kb -----

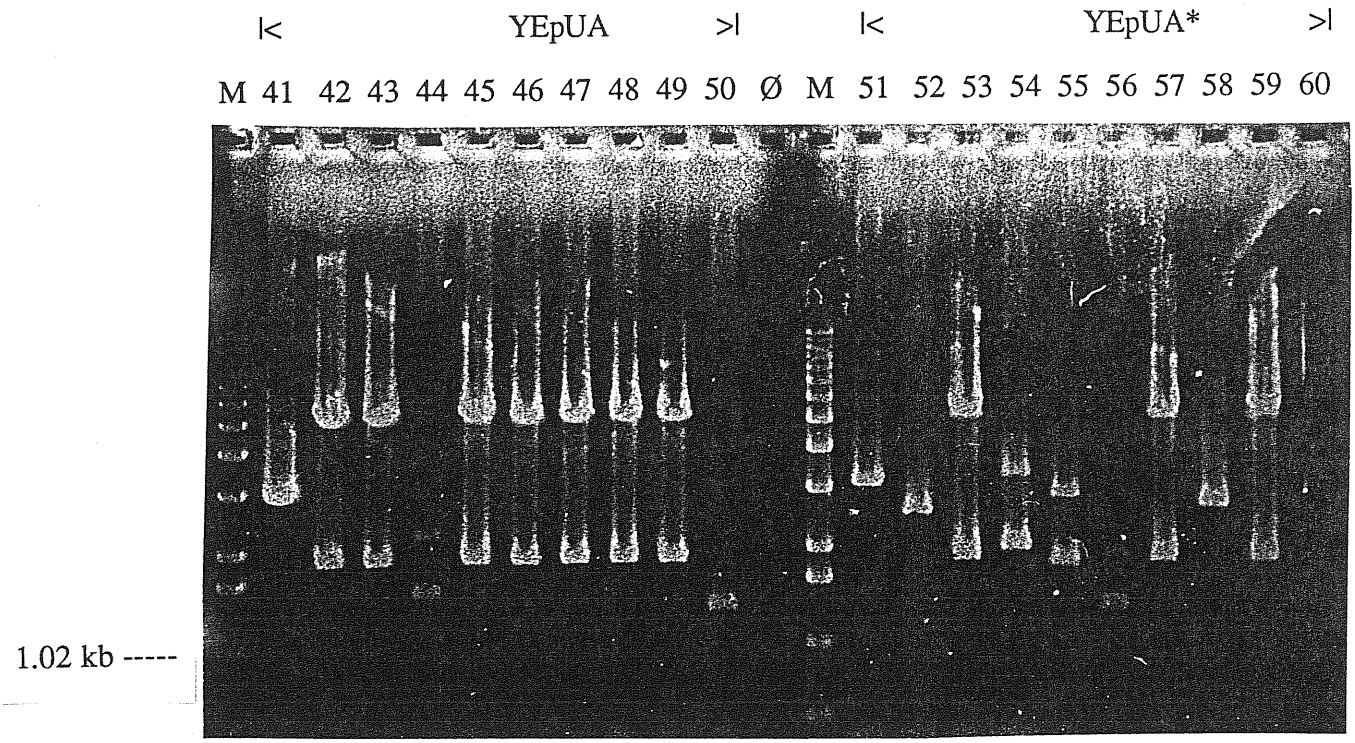


Fig. 2.27 . Pictures of agarose gels of bacterial miniprep DNAs of the episomal family of plasmids. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Lanes 1-10**: YEpTA/EcoRI/XbaI, true recombinants present the expected 5 kb and 1.7 kb (*ADE8* gene) bands; **Lanes 11-20**: YEpTA*/EcoRI/XbaI, with the 5 kb and 1.7 kb (*ade8-xp* gene); **Lanes 21-30**: YEpLA/EcoRI/XbaI, with the 5.74 kb and 1.7 kb (*ADE8* gene) bands; **Lanes 31-40**: YEpLA*/EcoRI/XbaI, with the 5.74 kb and 1.7 kb (*ade8-xp* gene) bands. **Lanes 41-50**: YEpUA/EcoRI/XbaI, true recombinants show the expected 5.23 kb and 1.7 kb (*ADE8* gene) bands; **Lanes 51-60**: YEpUA*/EcoRI/XbaI, with the 5.23 kb and 1.7 kb (*ade8-xp* gene) bands;

YIpA2 Family of Yeast Integrative Plasmids

The starting plasmid for the construction of the CBU1-31-disrupted strains, was the integrative pASZ10, kindly provided by A. Stotz (Biozentrum, Basel, CH; Stotz and Linder, 1990). This 5.4 kb plasmid was restricted with SmaI, gel purified and ligated to the 1.27 kb HincII/StuI fragment from another copy of the same plasmid. A recombinant vector carrying the insert in the same orientation as the original one was identified by restriction analysis. Thus, this 6.67 kb construct named pASZ-HS carries a partial repeat of the *ADE2* gene in the multicloning site of pASZ10. The 6.67 kb pASZ-HS plasmid was then restricted with EcoRV and HincII restriction enzymes (1.92 kb deletion), the 4.75 kb fragment was gel purified and religated. The resulting plasmid was named pASZ-Δade2, carrying a non functional *Δade2* gene with a 645 bp EcoRV-StuI deletion in its coding sequence, corresponding to approximately 37% deletion in the open reading frame. The genes under investigation were cloned in the unique BamHI site of pASZ-Δade2. The *HIS3*/BamHI, *LEU2*/BamHI, *TRP1*/BamHI, and *URA3*/BamHI genes were cloned in the opposite orientation of the partially deleted *Δade2* gene. This choice was based on the idea to have a terminator between the selectable marker and the *Δade2* gene. Clones with recombinant integrative plasmids were selected for onto appropriate Amp drop-out plates in the bacterial strain KC8, in which the yeast markers can complement the corresponding bacterial mutations (Bach et al., 1979; Struhl and Davis 1980; Rose et al., 1984). The final integrative plasmids were named YIpA2H3, YIpA2L2, YIpA2T1, and YIpA2U3 (see following figures), carrying in the order: the 1.76 kb *HIS3* gene from pRAP-HIS3, the 2.2 kb *LEU2* gene from pRAP-LEU2, the 1.65 kb *TRP1* gene from pRAP-TRP1, and the 1.95 kb *URA3* gene from pRAP-URA3, Fig. 2.33. These integrative vectors share a 0.82 kb sequence homology with the 5' portion and part of the coding region (up to the EcoRV site) with the *ADE2* gene, and also, a 1.05 kb from the StuI site downstream (Stotz and Linder, 1990).

A summary of the construction of the integrative vectors is shown in figure below.

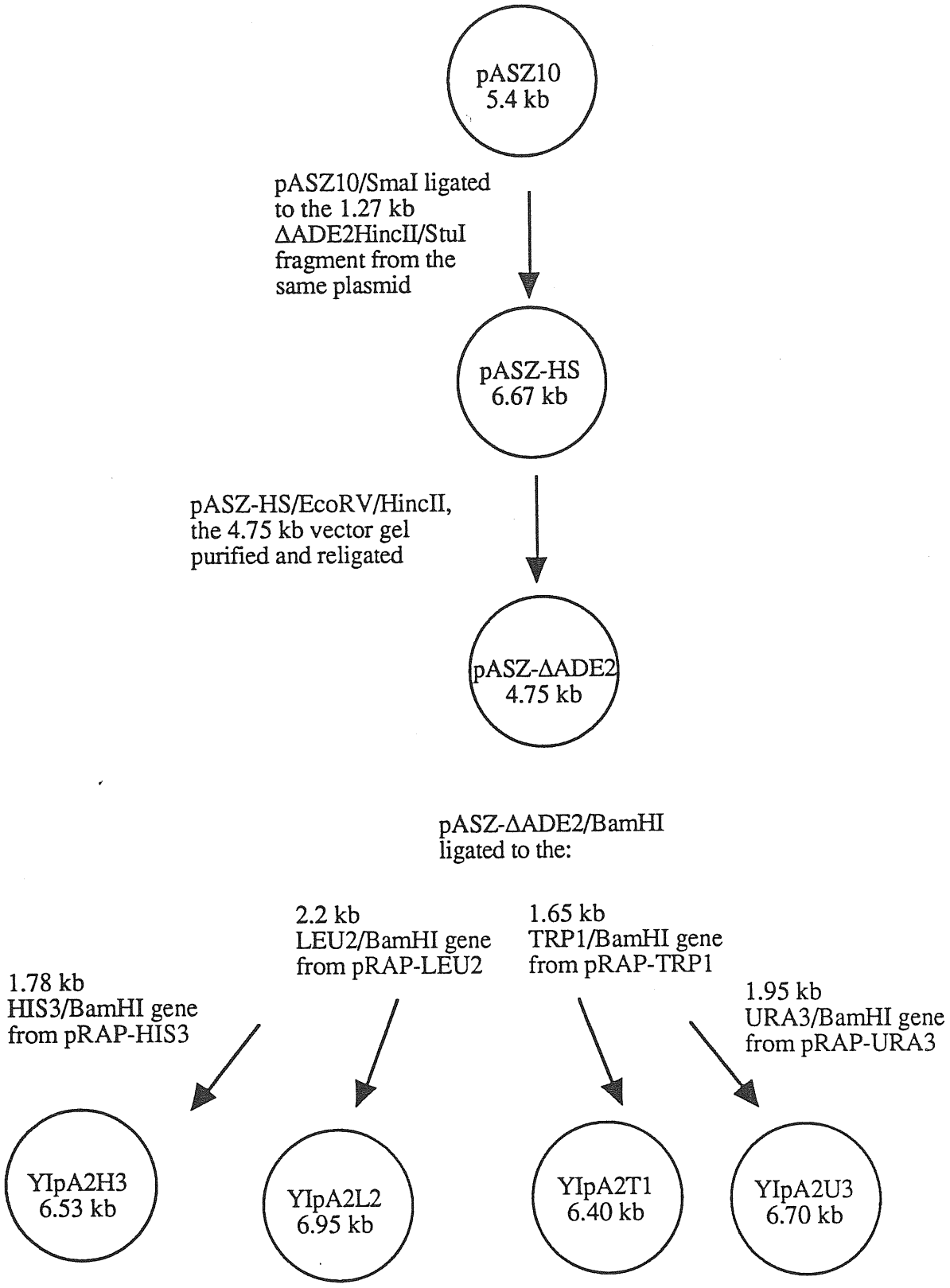


Fig 2.28 . Construction of the YIpA2 family of yeast integrative vectors for disruption of the *ADE2* gene

Detailed restriction maps of these constructions are as follows:

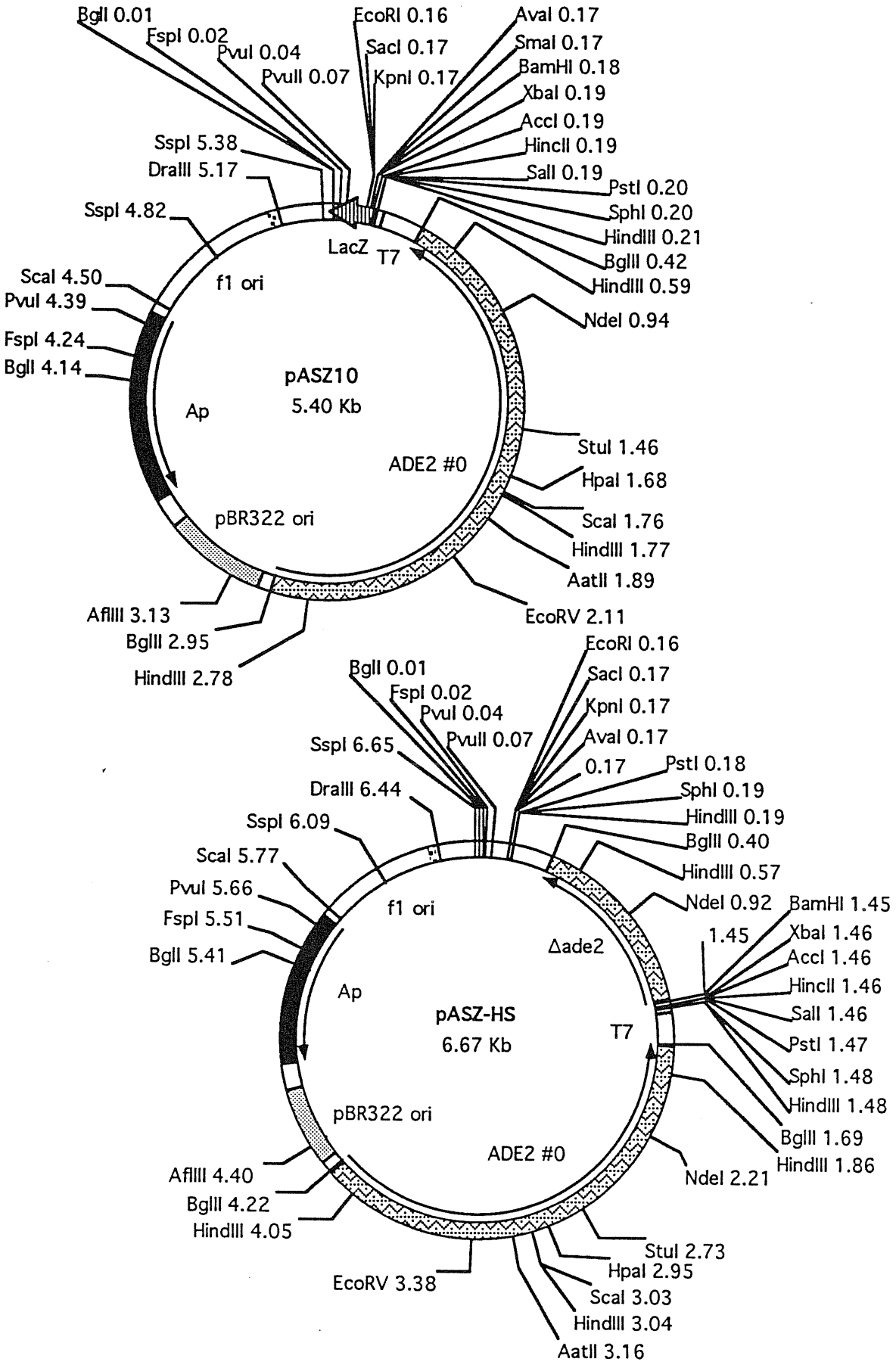


Fig. 2.29 . Detailed restriction maps of plasmids pASZ10 and pASZ-HS.

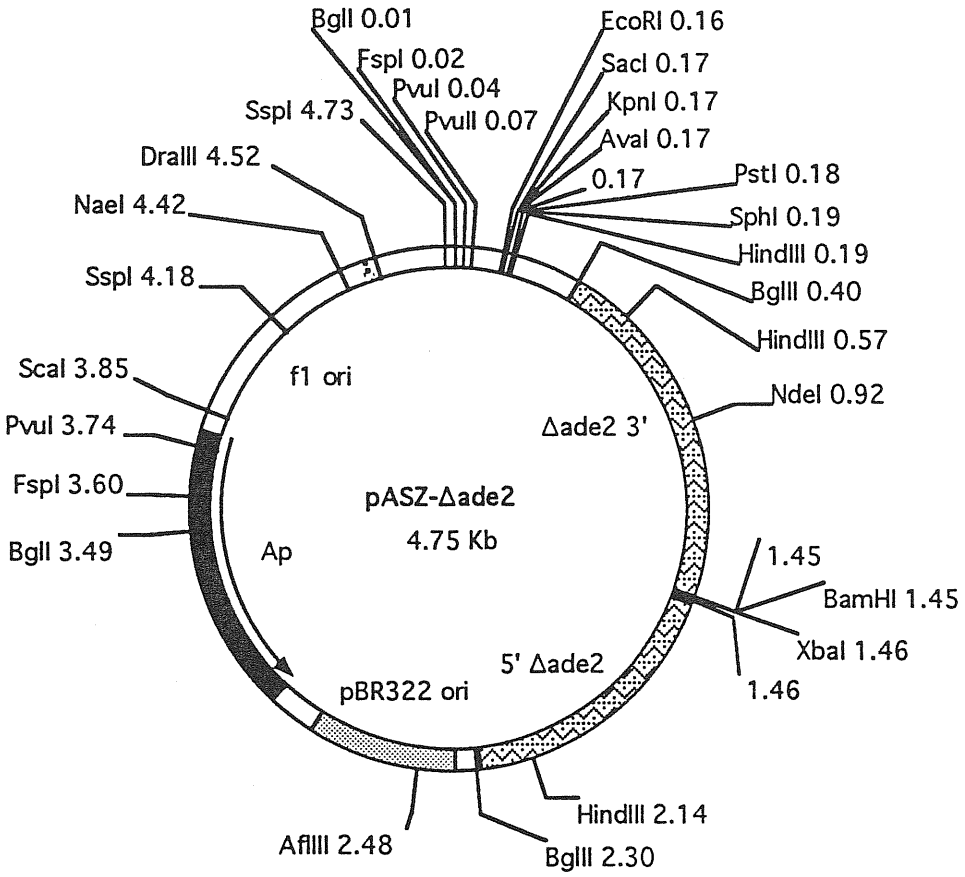


Fig. 2.30 . Detailed restriction map of plasmid pASZ-Δade2.

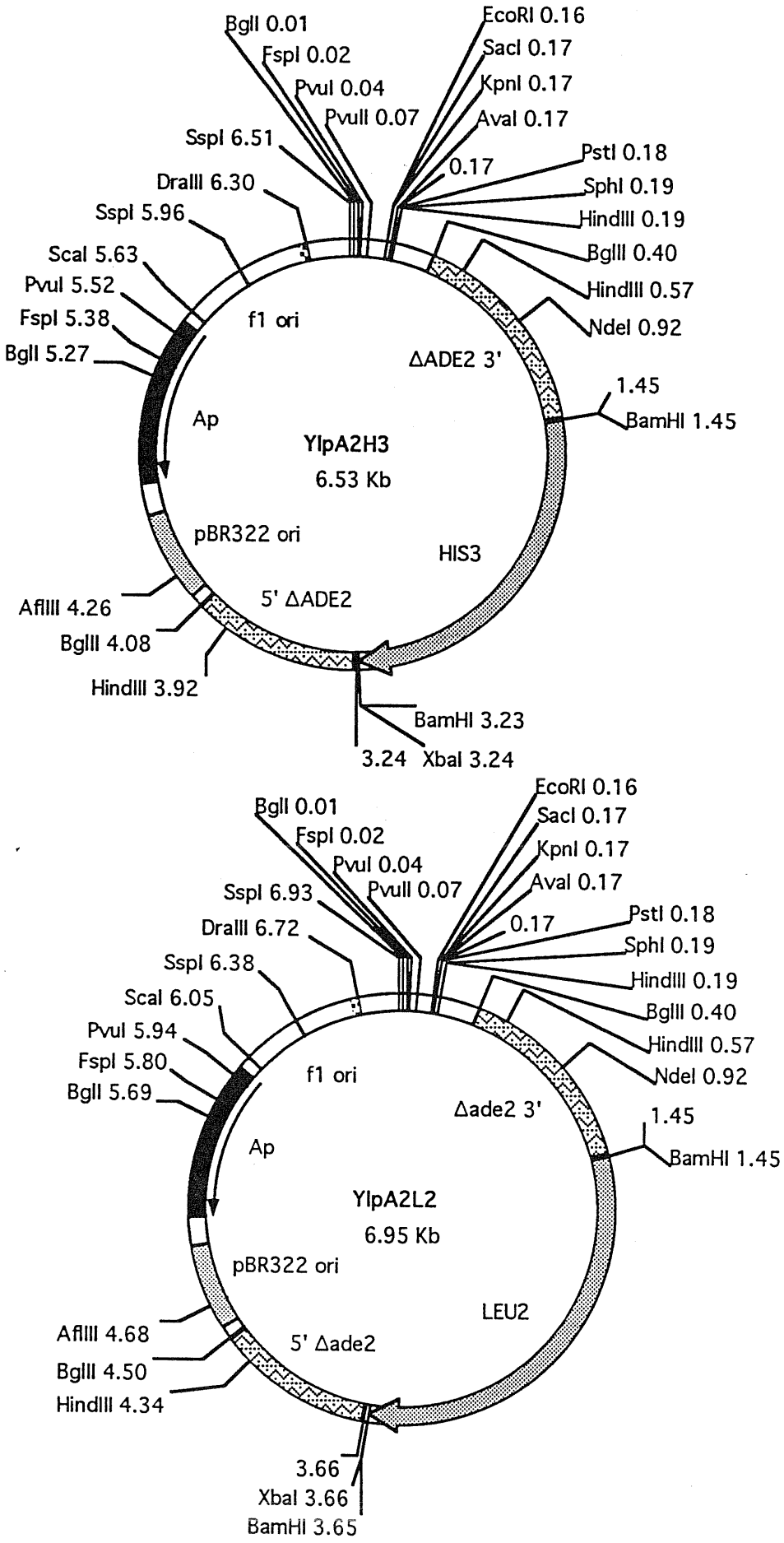


Fig. 2.31 . Detailed restriction maps of plasmids YIpA2H3 andYIpA2L2.

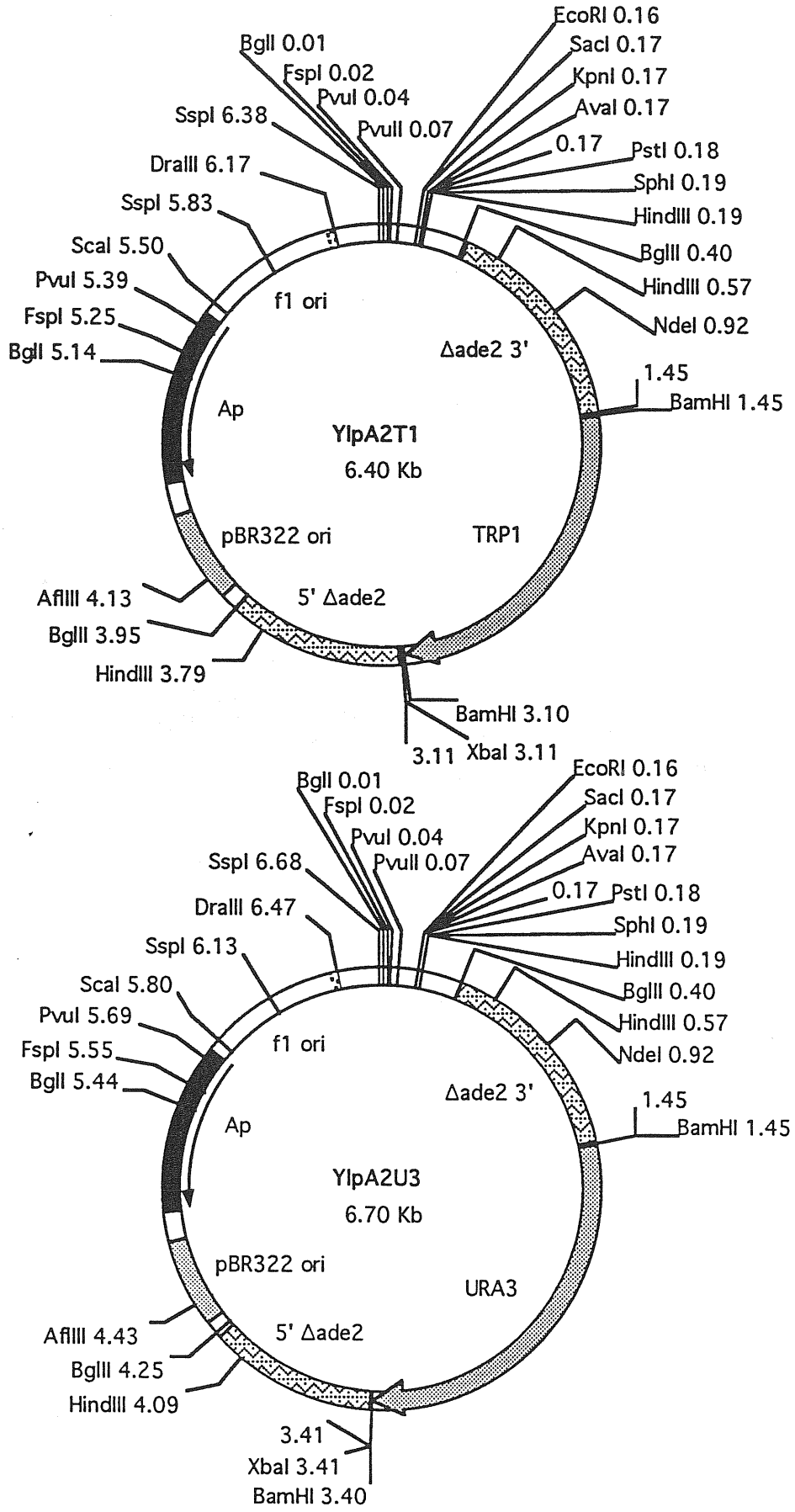


Fig. 2.32 . Detailed restriction maps of plasmids YIpA2T1 andYIpA2U3.

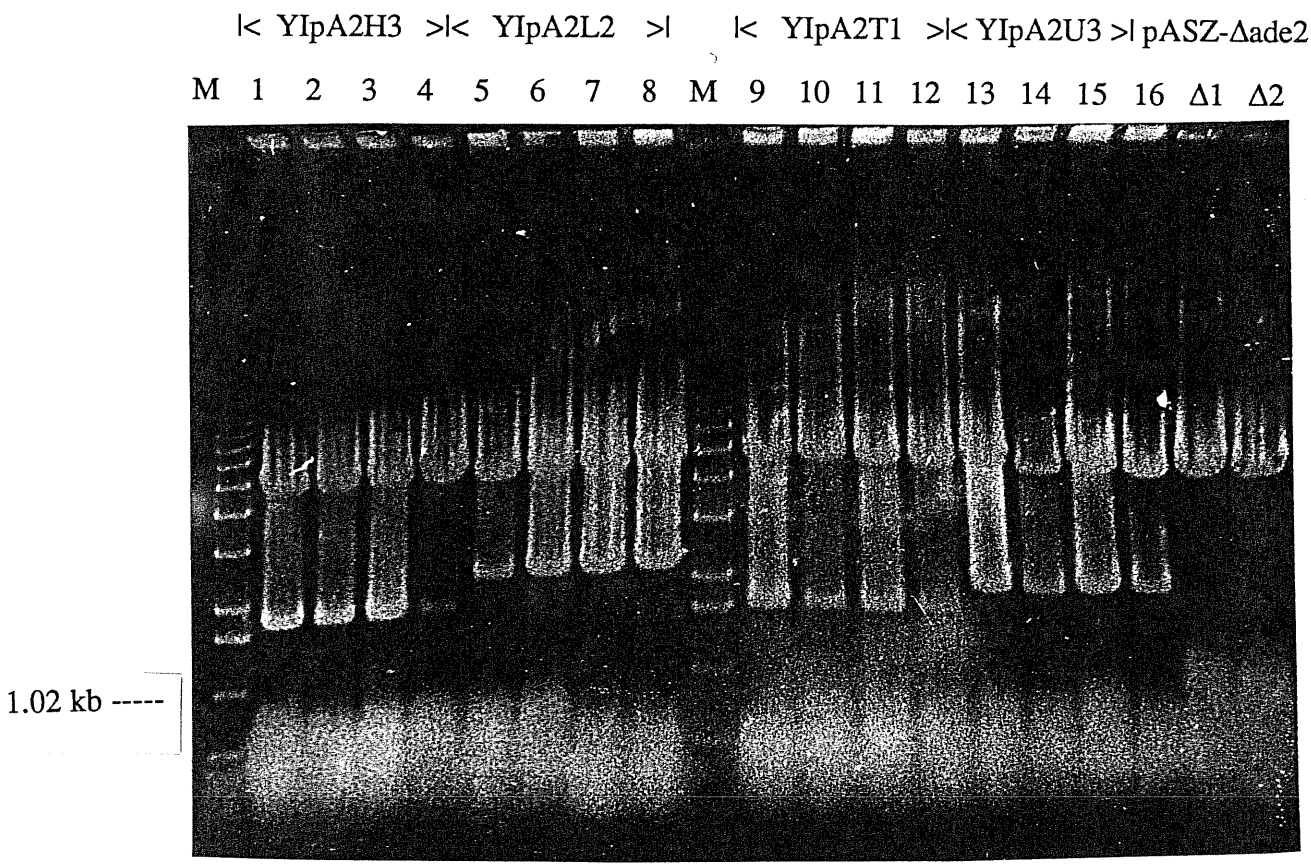


Fig. 2.33 . Pictures of an agarose gel showing the restriction pattern of the YIp family of plasmids. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Lanes 1-4**: YIpA2H3/BamHI, showing the expected 4.75 kb and 1.76 kb (*HIS3* gene) bands. **Lanes 5-8**: YIpA2L2/BamHI, showing the expected 4.75 kb and 2.2 kb (*LEU2* gene) bands. **Lanes 9-12**: YIpA2T1/BamHI, showing the expected 4.75 kb and 1.65 kb (*TRP1* gene) bands. **Lanes 13-16**: YIpA2U3/BamHI, showing the expected 4.75 kb and 1.95 kb (*URA3* gene) bands. **Lanes Δ1 and Δ2**: pASZ-Δade2/BamHI (clones #1 and #2), resulting in the expected linear 4.75 kb band.

2.18 Checking the Functionality of *ADE8* and *ade8-xp* Genes by Transformation in FAS20

The centromeric and episomal constructions were tested in the yeast strain FAS20, in which functionality of the *ADE8* gene and the non-functionality of the *ade8-xp* gene could be readily seen by the colour of the transformed colonies. As expected, the YCpLA, YCpTA, and YCpUA plasmids, as well as their episomal equivalents YEpLA, YEpTA, and YEpUA, carrying a wild type *ADE8* gene, conferred a red phenotype to the host FAS20 cells. The red color was much stronger in cells with the episomal plasmids compared to those with the centromeric ones. Accordingly, FAS20 cells transformed with YCpLA*, YCpTA*, or YCpUA*, as well as YEpLA*, YEpTA*, or YEpUA*, carrying a frameshifted *ade8-xp* gene, remained white.

2.19 *E. coli* Transformation

E. coli competent cells were prepared following the RbCl method based on the experiments of Hanahan (1983). The recipient strain was plated for single isolated colonies onto LB medium and incubated overnight at 37°C. The following day, a liquid culture was started with one colony per 10 ml of LB medium, and cells were grown at 37°C with moderate agitation up to an optical absorbance at 550 nm of 0.5-0.6. The culture was collected in 50 ml polypropylene tubes and chilled on ice for 10 minutes. Then, cells were pelleted at 4°C by spinning at 3,000 rpm for 10 minutes in a refrigerated Sorvall® RT6000B centrifuge. The supernatant was discarded, the pellet resuspended by gentle vortexing into RF1 buffer to 1/3 of the original culture volume, and the mixture was incubated on ice 1 hour. Then, cells were pelleted as above, drained, resuspended in RF2 buffer to 12.5 of the original volume, and incubated on ice for 15 minutes. As result of this manipulations, each 2.5 ml of culture were concentrated into a transformation mixture of 200 µl; this quantity was distributed into aliquots in 2 ml test tubes and quickly frozen in isopropanol-dry ice bath. Tubes were stored 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 90 minutes, after which they were grown in 2 ml LB at 37°C for 30 minutes. Dilutions were plated on selective medium and grown overnight at 37°C. Transformant colonies were used to inoculate 4 ml of LB-antibiotic medium, grown as usual, and

used for plasmid DNA minipreps. Recombinant plasmids were identified by restriction endonuclease analysis as already described. Buffers were as follows:

RF1 buffer:

100 mM RbCl
50 mM $MnCl_2 \cdot 4H_2O$
30 mM K acetate
10 mM $CaCl_2 \cdot 2H_2O$
15% glycerol (v/v)

pH was adjusted to 5.8 with 0.2 M acetic acid. The solution was sterilized by filtration through a 0.22 µm disposable filter.

RF2 buffer:

10 mM RbCl
10 mM MOPS
75 mM $CaCl_2 \cdot 2H_2O$
15% glycerol

pH was adjusted to 6.8 with 1 N NaOH. The solution was sterilized by filtration through a 0.22 µm disposable filter.

2.20 Yeast Transformation

Whole Cell Transformation

For routine work, 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 polypropylene tubes to give $5X10^8$ cells per experimental tube. Tubes were centrifuged at 2,000 rpm in a Sorvall® RT6000B centrifuge for 10 minutes, and the medium discarded. Cells were washed once with 10 ml TE buffer, the washes pooled together in one 50 ml polypropylene tube, and pelleted as above. Cells were then resuspended in 10 mM CaTE (10 mM $CaCl_2$ in TE buffer) to a density of $1.5X10^8$ cells/ml, transferred to a 50 ml polypropylene tube, and gently shaken on a rocking platform at 24°C for 30 minutes. Cells were pelleted again as above, and resuspended in TE buffer 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 sterilised PEG solution (40% PEG 4000 made in TE buffer, pH 7.7), and left at room temperature for 45 minutes. The mixture was pelleted by centrifugation in a Sorvall® RT6000B centrifuge at 2,000 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.

Electroporation Protocol

For the disruption of the *ADE2* gene we used the electroporation transformation protocol, giving a very high frequency of transformation (Becker and Guarente, 1991). Typically, the method is the following: yeast cells were grown in 100 ml rich YPD medium to an optical density OD_{600} = 1.2 -1.5, harvested by centrifugation in a Sorvall® RT6000B centrifuge at 3,000 rpm for 10 minutes, the supernatant discarded, and the pellet washed with 50 ml ice-cold sterile H_2O. Cells where pelleted again as above and the wash repeated. After new pelleting, cells were washed with 4 ml 1M sorbitol, and centrifuged as above. Again, supernatant was discarded and cells where finally resuspended in 50 µl 1M sorbitol. At this stage, 40 µl of cell suspension were mixed with approximately 100 ng transforming DNA (in TE buffer) in a 0.2 cm electrode gap cuvette (Bio-Rad cat# 165-2086), and incubated on ice for 5 minutes. Electroporation was done with a Bio-Rad Gene Pulser apparatus (Bio-Rad, Richmond, CA, USA) set to 1.5 kV and 25 µF. Pulse Controller was set at 200 Ω. Time constant was around 4.5-5.0 msec. After one electric pulse, 1 ml of 1M sorbitol was immediately added, and the suspension was gently pelleted, the supernatant poured off, and the cells resuspended in the remaining sorbitol solution. These were finally plated onto selective drop-out media containing 1M sorbitol and incubated at 30°C for growth.

2.21 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, UK) 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.

2.22 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 hybridised as outlined by Sambrook et al (1989). The DNA probe (25 ng) was radioactively labeled with the random primed DNA labeling kit (Boehringer Mannheim, Germany) according to the method by Feinberg and Vogelstein, (1983). DNA was denatured by heating at 100°C in boiling water for 10 minutes, and then quickly chilled on ice. The following was added to the denatured DNA (in H_2O up to 20 µl):

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 [alfa-^{32}P] dCTP (specific activity at reference 3000 Ci/mmol)

1 µl Klenow enzyme (2 units) labeling grade (Boehringer Mannheim)

20 µl final volume

The reaction was incubated at 37°C for 30 minutes, then 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) with a Quick Spin™ column (Boehringer Mannheim), as specified by the manufacturer. Hybridization and autoradiography were carried out as described below.

2.23 Filter Hybridization

Filter hybridization was carried out as recommended by the manufacturer of the positively charged nylon membrane used in the experiment (Hybond™-N+ version 2.0, Amersham International plc, Amersham, UK). Reagents for hybridization were prepared as specified by Sambrook et al. (1989):

100X Denhardt's solution (Denhardt, D. T., 1966):

2% [w/v] Ficoll™ (type 400, Pharmacia)

2% [w/v] polyvinylpyrrolidone (PVP)

2% [w/v] bovine serum albumin (fraction V, Sigma)

The solution was 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 (herring or 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 by 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 membrane was then incubated in 1X SSPE, 0.1% [w/v] SDS, at 65°C for 15 minutes. Since the probe had always 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 10 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).

2.24 Growth Curves Under Different Experimental Conditions

CBU1-31 yeast cells were transformed with the pRAP series of episomal vectors (pRAP-HIS3, pRAP-LEU2, pRAP-TRP1, and pRAP-URA3), the cells plated onto selective media and incubated at 30°C for three days. Three independent transformant colonies - #1, #2, and #3 - (for each plasmid type) were picked from the drop-out plates and purified by streaking for single isolated colonies onto selective plates. Single isolated colonies were used to inoculate 10 ml parallel cultures of selective (HIS3-, LEU2-, TRP1-, and URA3-), complete medium (COM), and rich (YPD) medium, at a cell density of $1X10^5$ cells/ml (two 20 ml glass vials in parallel [A and B] per each independent transformant [#1, #2, and #3] per each type of medium [selective, complete, and rich]). The resulting eighty-four cultures (72 + 12 non transformed control cultures) were grown in a rotary shaker at 30°C up to stationary phase, and cell growth was measured as absorbance at OD_{600} after 9, 15, 21, 32, 38 and 44 hours. Average of the data from each type of cultures are reported in Table 3.1. Results. A graphic representation of this data is also shown.

2.25 Plasmid Stability Experiments

As per the growth curve experiments, the CBU1-31 yeast strain was transformed with the different episomal plasmids, the cells from three independent transformants (#1, #2, and #3) plated onto selective media and incubated at 30°C for three days. After the usual round of purification, single isolated colonies were used to inoculate twelve 10 ml parallel cultures (three for each type of marker) into selective (SEL) medium. Cells were grown overnight to stationary phase (G0), and stability experiments were carried out as previously described (Ludwig et al., 1993). Briefly, aliquots from these initial drop-out cultures were plated onto rich YPD medium, incubated at 30°C for three days, and replicated onto diagnostic media for plasmid stability determination (G0). An aliquot from these twelve G0 cultures was used to new cultures into selective (SEL), complete (COM), and rich (YPD) medium, for a total of start thirty-six parallel cultures. Again, cells were grown for ten generations (G10), and processed as above. At the end of each round of ten generations (from G0 to G50), the remaining stationary-phase culture was combined with 1 ml 1M sodium azide and stored at -80°C, for later analysis of plasmid copy number. Aliquots from the G10 overnight cultures were then grown again to stationary phase by another round of ten generations of growth (G20), and the process repeated. This methodology, shown in Fig.(???), was repeated for the usual fifty generations (Ludwig et al., 1993). Plasmid stability was determined as the number of prototrophs over the number of auxotrophs. A total of 62000 single colonies were analyzed (15500 for each marker, approximately 2580 per marker per ten generation); the result of this analysis is reported in Table 3.3, and a graphic representation is shown in Fig.. 3.4 .

Plasmid Stability Experiments

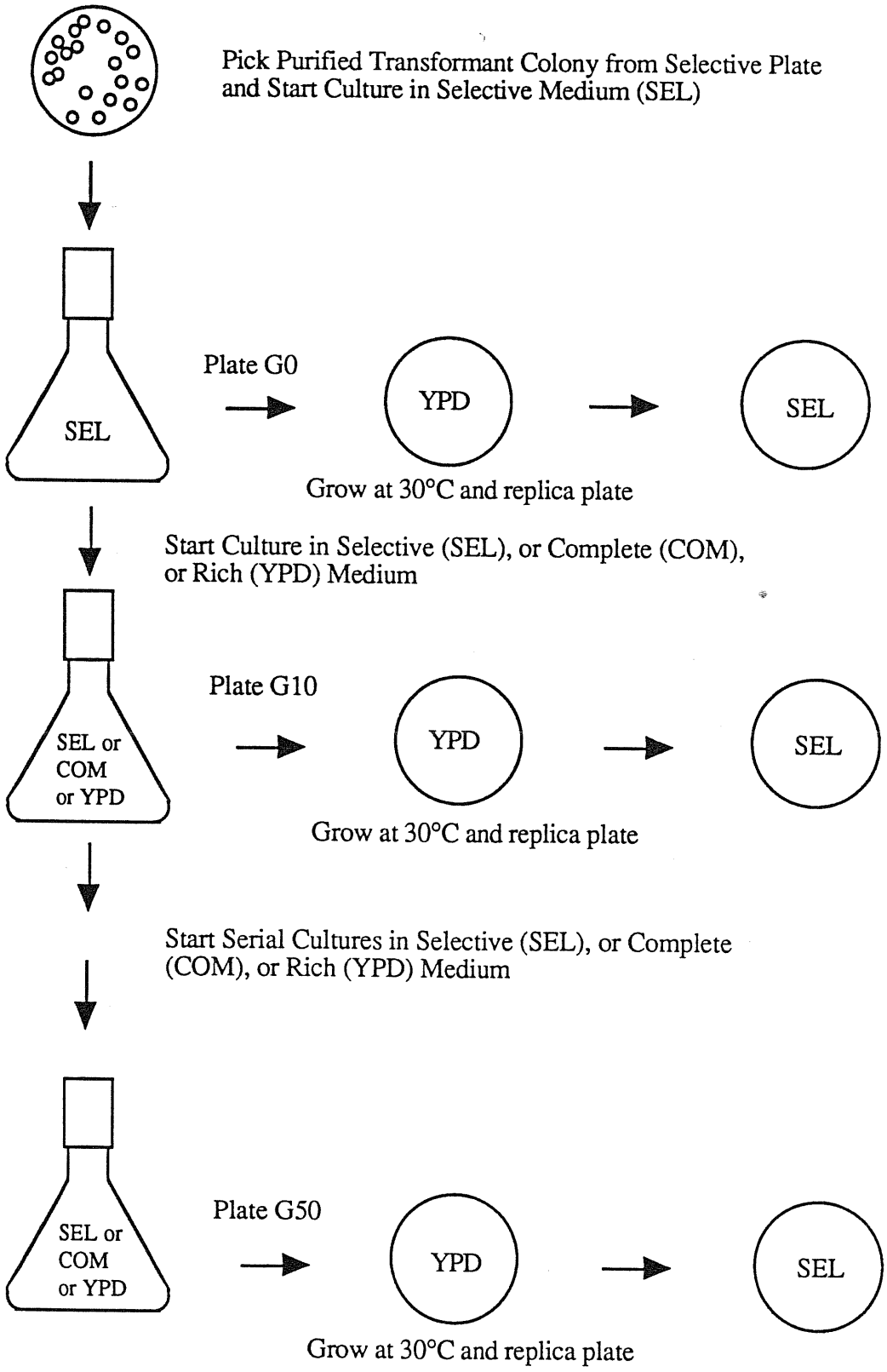


Fig. 2.34 . Diagrammatic representation of the metodology used for the plasmid stability experiments.

2.26 Determination of Plasmid Copy Number

The frozen cells from the end of each round of growth of the plasmid stability experiment, were thawed, and 3.3 ml aliquots from each one of the three cultures (#1, #2, and #3 independent transformants) were pooled. Yeast total DNA was prepared from the pooled mixture as described above in the text. Aliquots of this DNA were restricted overnight with an excess of a specific restriction enzyme, according to the site available on each plasmid type. Plasmid pRAP-HIS3 was restricted with an excess of XbaI, which cuts only once in the plasmid, thus generating a 5.49 kb band corresponding to the linear vector. We could not restrict pRAP-HIS3 with BamHI because this enzyme cuts exactly in the same place in the corresponding chromosomal *HIS3* gene, thus generating two indistinguishable 1.78 kb fragments. Plasmids pRAP-LEU2, pRAP-TRP1, and pRAP-URA3, were digested with an excess of BamHI, which cuts in all cases at the end of the yeast gene. This restriction generated a 2.2 kb *LEU2*, 1.65 kb *TRP1*, and a 1.95 kb *URA3* band respectively. In the chromosomal *LEU2* and *URA3* genes, BamHI digestion originated fragments much longer than those coming from the episomal plasmids, and the two bands were well separated on the agarose gel. In the case of chromosomal *TRP1*, the BamHI restriction originated a shorter fragment as compared to the plasmidic one, thus migrating at a faster rate on the agarose gel. Dilutions were run on a 0.7% agarose gels, which after ethidium bromide staining and visualization under UV light, were transferred to a nylon membrane as already described. Hybridizations were carried out with specific probes for each one of the yeast markers. In particular, the same 2.2 kb *LEU2*, 1.65 kb *TRP1*, and a 1.95 kb *URA3* fragments were used as probes against their respective targets in the yeast minipreps. The 1.76 kb *HIS3*/BamHI fragment was used as probe against the linear 5.49 kb pRAP-HIS3 DNA. Densitometric quantification of the autoradiographs was done with the GelScan™ XL evaluation software (version 2.1) in conjunction with the UltroScan™ XL laser densitometer (both from Pharmacia). Results from these analysis are reported in section 3.4 (Results).

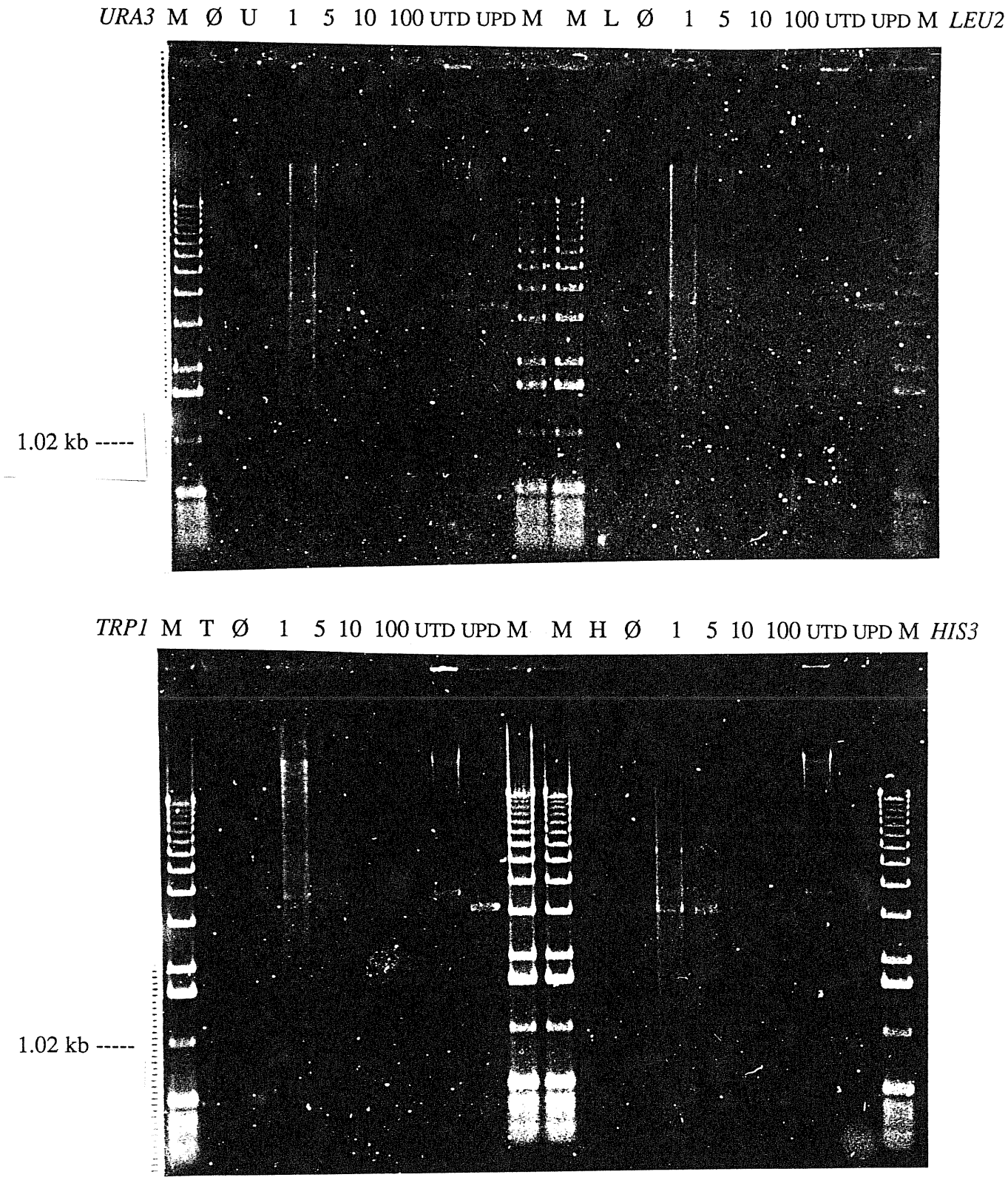


Fig. 2.35 . Pictures of agarose gels loaded with DNAs from non transformed yeast cultures and plasmid-transformed bacterial cultures DNA. M: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-

0.075). **Ø**: empty lane. **1, 5, 10, and 100**: different dilutions of restricted total yeast DNA, 1/1, 1/5, 1/10, and 1/100 respectively; DNA was restricted overnight with an excess of either BamHI (*URA3*, *LEU2*, and *TRP1*) or XbaI (*HIS3*) **H**: 1.76 kb *HIS3*/BamHI gene from pRAP-HIS3, radiolabeled and used as probe for the chromosomal *HIS3* gene. **L**: 2.2 kb *LEU2*/BamHI gene from pRAP-LEU2, radiolabeled and used as probe for the chromosomal *LEU2* gene. **T**: 1.65 kb *TRP1*/BamHI gene from pRAP-TRP1, radiolabeled and used as probe for the chromosomal *TRP1* gene. **U**: 1.95 kb *URA3*/BamHI gene from pRAP-URA3, radiolabeled and used as probe for the chromosomal *URA3* gene. **UTP**: uncut total DNA from non transformed yeast cultures. **UDP**: uncut plasmid DNA from bacterial minipreps.

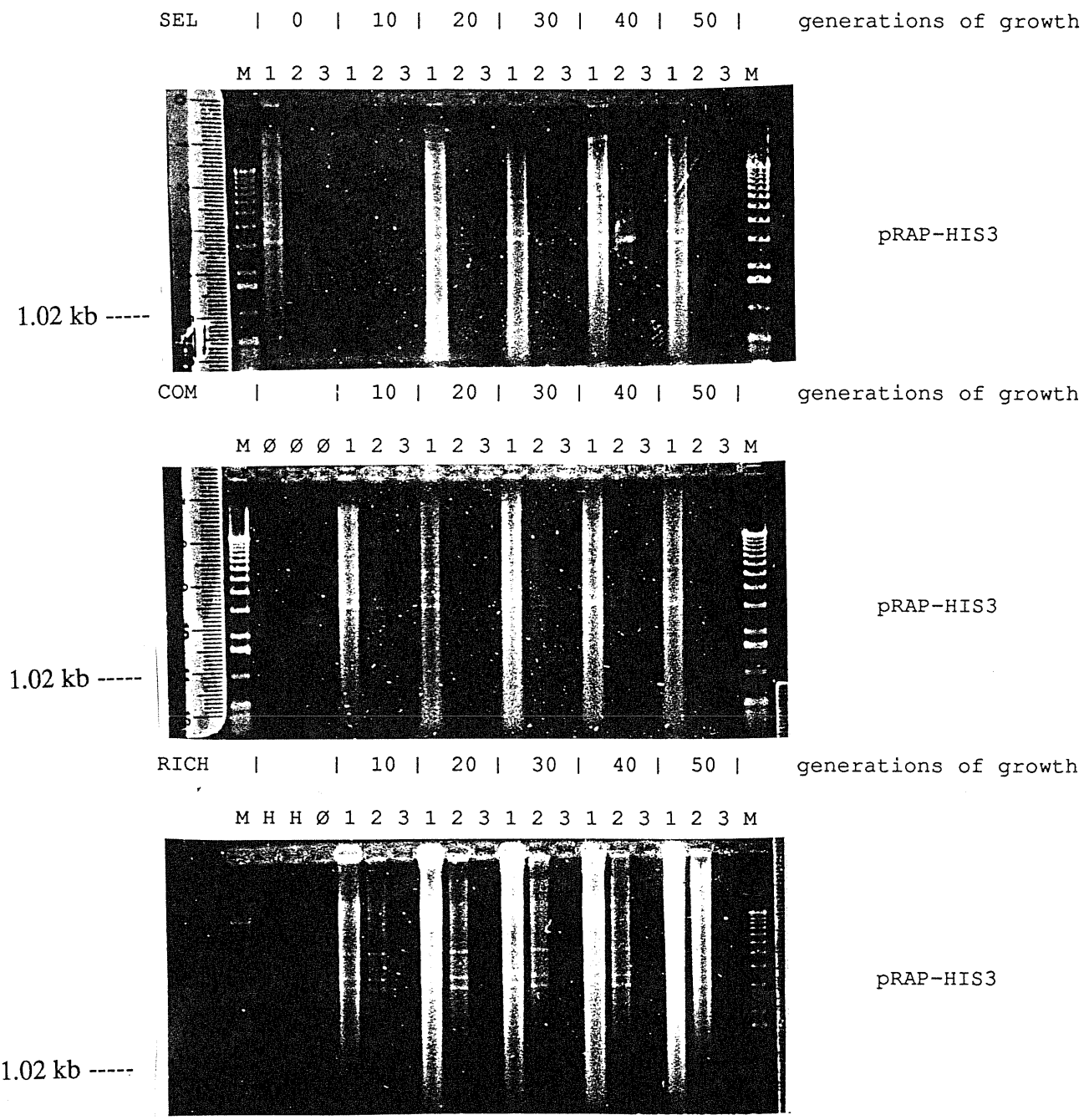


Fig. 2.36 . Gels with total yeast DNA from cultures transformed with the pRAP-HIS3 plasmid digested overnight with XbaI or BamHI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Ø**: empty lane. **1,2, and 3**: different dilutions of restricted total yeast DNA, 1/1, 1/10, and 1/100 respectively. **H**: 1.76 kb *HIS3*/BamHI gene from pRAP-HIS3, radiolabeled and used as probe for pRAP-HIS3. SEL, COM, and RICH, are different media as described in text.

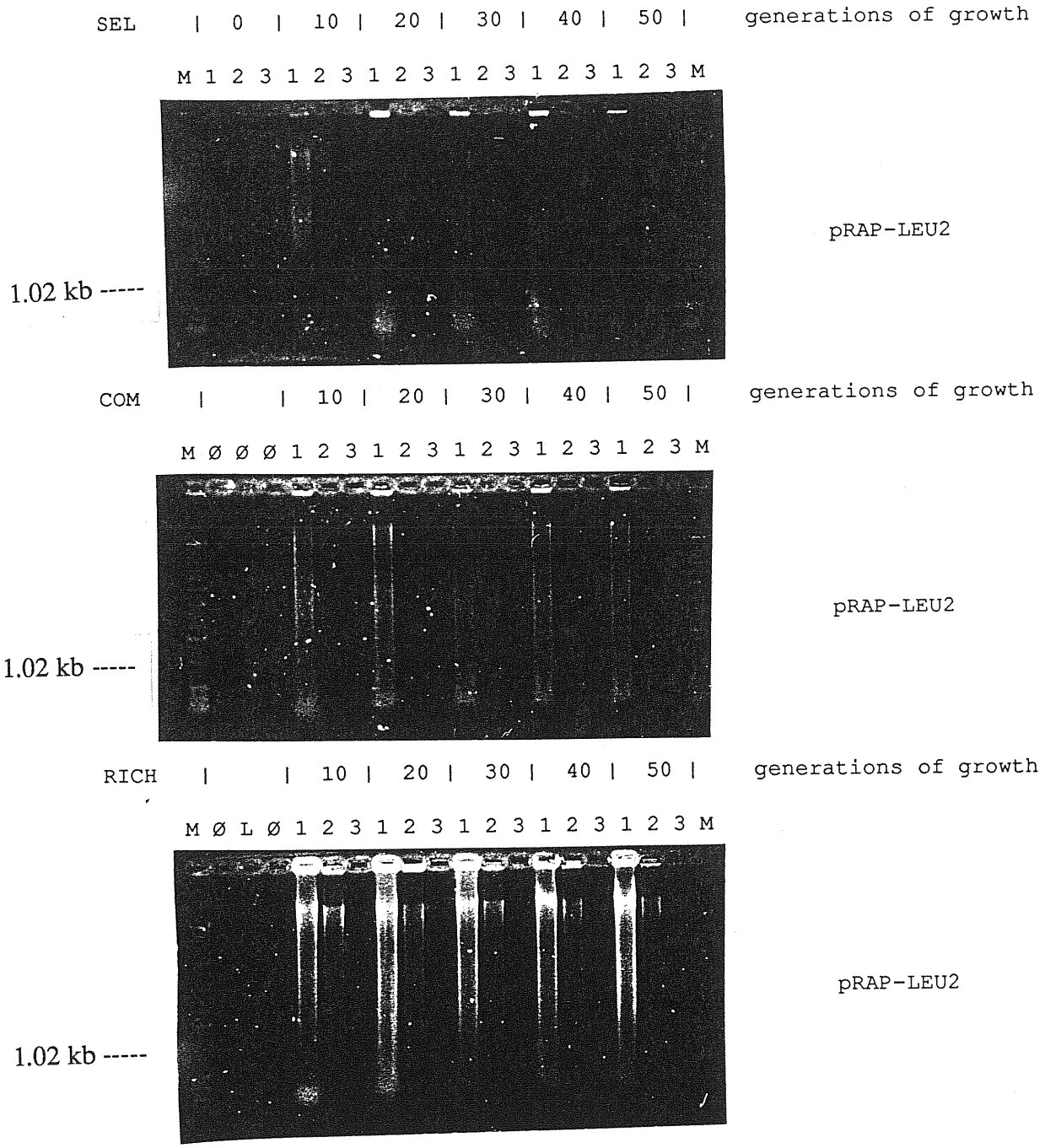


Fig. 2.37 . Gels with total yeast DNA from cultures transformed with the pRAP-LEU2 plasmid digested overnight with BamHI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Ø**: empty lane. **1,2, and 3**: different dilutions of restricted total yeast DNA, 1/1, 1/10, and 1/100 respectively. **L**: 2.2 kb *LEU2*/BamHI gene from pRAP-LEU2, radiolabeled and used as probe for pRAP-LEU2. SEL, COM, and RICH, are different media as described in text.

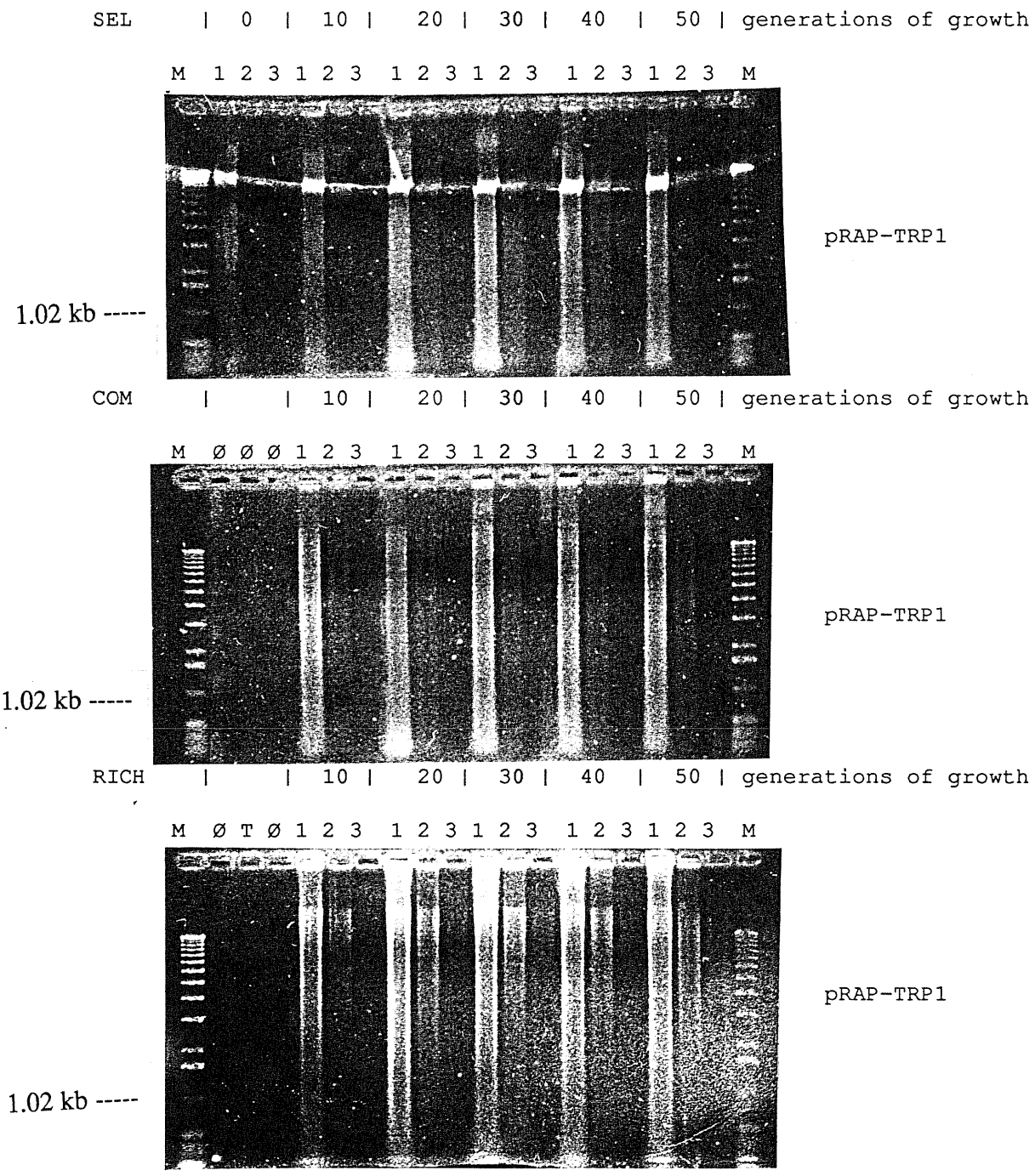


Fig. 2.38 . Gels with total yeast DNA from cultures transformed with the pRAP-TRP1 plasmid digested overnight with BamHI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Ø**: empty lane. **1,2, and 3**: different dilutions of restricted total yeast DNA, 1/1, 1/10, and 1/100 respectively. **T**: 1.65 kb *TRP1*/BamHI gene from pRAP-TRP1, radiolabeled and used as probe for pRAP-TRP1. SEL, COM, and RICH, are different media as described in text.

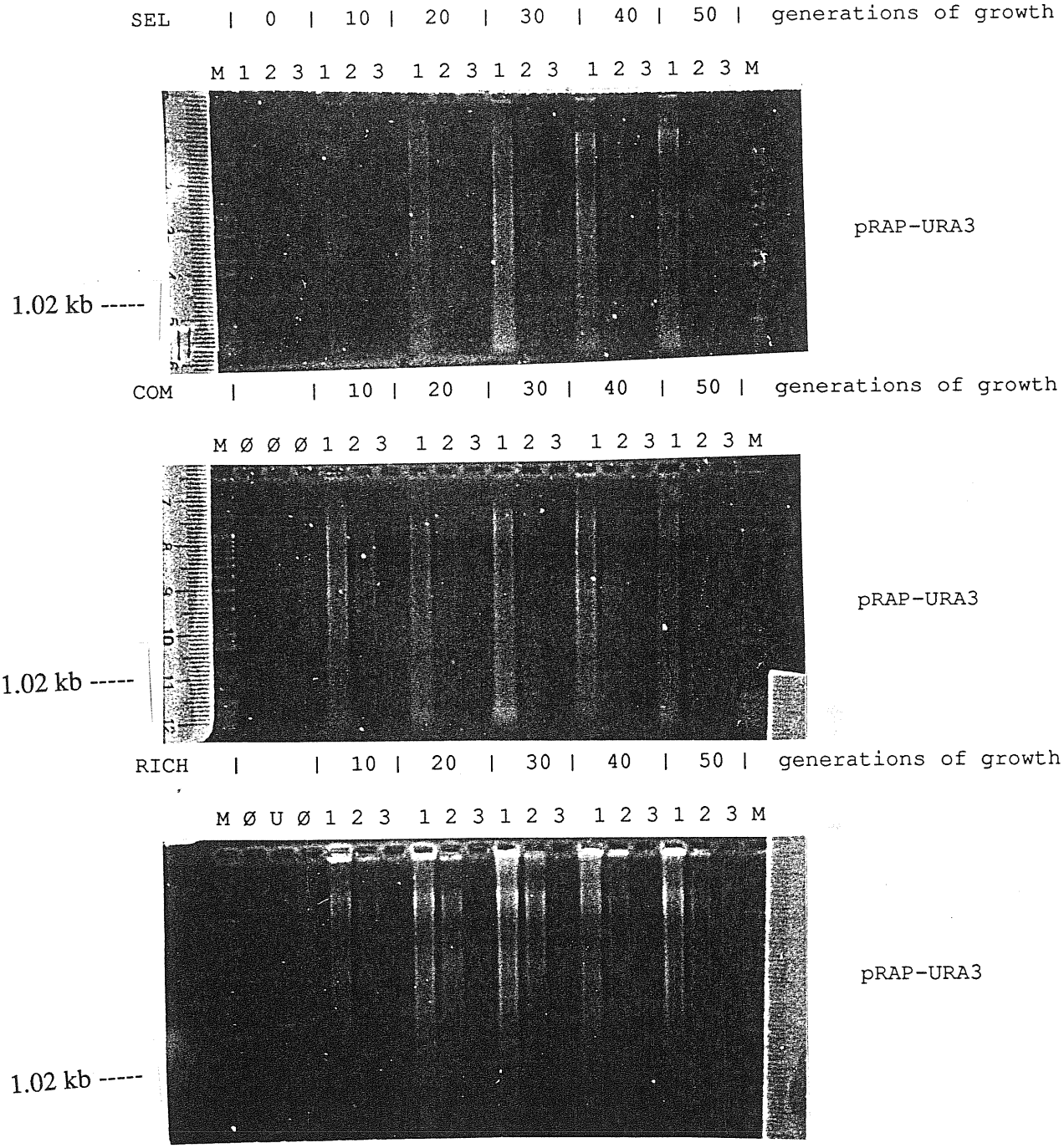


Fig. 2.39 . Gels with total yeast DNA from cultures transformed with the pRAP-URA3 plasmid digested overnight with BamHI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Ø**: empty lane. **1,2, and 3**: different dilutions of restricted total yeast DNA, 1/1, 1/10, and 1/100 respectively. **U**: 1.95 kb *URA3*/BamHI gene from pRAP-URA3, radiolabeled and used as probe for pRAP-URA3. SEL, COM, and RICH, are different media as described in text.

2.27 Competition Experiments

Competition experiments in non selective complete and rich media (COM and YPD)

To confirm the results obtained with the plasmid stability experiments - namely the poor stability of the pRAP-LEU2 plasmid compared to that of the pRAP-URA3, pRAP-TRP1, and pRAP-HIS3 - we devised a co-culture experiment in which all this forms were present under the same conditions. The expectation from this experiment was that, once given an appropriate lag of time, the yeast strain harbouring the most stable plasmid form would compete out all other less stable plasmid species. To set up this experiment we transformed independently the CBU1-31 yeast strain with all the different plasmid species, thus obtaining four different transformant clones, CBU1-31 [pRAP-HIS3], CBU1-31 [pRAP-LEU2], CBU1-31 [pRAP-TRP1], and CBU1-31 [pRAP-URA3]. Three independent transformant clones from each type of transformation were further purified, and then grown to stationary phase in the 5 ml of the proper liquid selective media. Finally, three mixtures, #1, #2, and #3, were prepared by combining equal volumes of the four stationary phase cultures with the different plasmids. In all cases, these three mixtures were prepared with purified independent transformants. These three combinations (G0) were used to inoculated three parallel culture in 5 ml of complete (COM) and rich media (YPD) at a cell density of $1x10^5$ cells/ml ($2.5x10^4$ cells/ml per each type of species). These cultures were grown for ten generations, and new cultures were started by serial inocula, as per the plasmid stability experiments. The process was repeated up to fifty generations(G50). At the end of each round of growth a dilution of the cell suspensions was plated in YPD medium. After three days of growth these plates were replicated onto His-, Leu-, Trp-, and Ura- plates, and the number of prototrophs was determined. The frequency of appearance of each plasmid species was determined, by taking as 100% the total number of prototrophs. A diagrammatic representation of these experiments in shown in Fig. 3.40. Results of these experiments are reported in section 3.5.

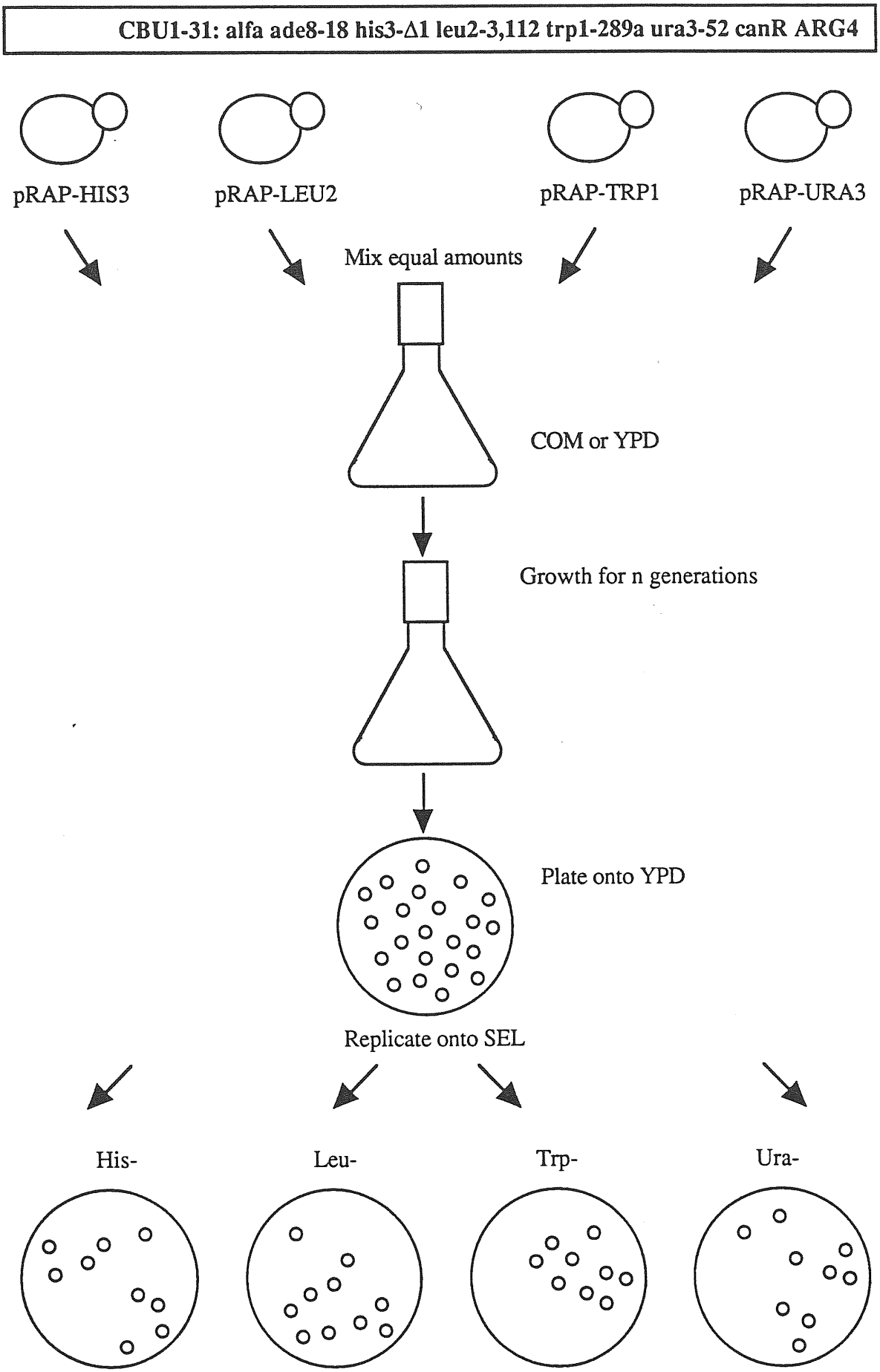


Fig. 2.40. Diagrammatic representation of the competition experiments in non selective conditions

Competition experiments with a reporter gene

These experiments were carried out to test the effect on the cellular metabolism of selective pressure for a particular marker, carried either on a low, or high copy vector. To do so, the yeast strain CBU1-31 A2L2 was transformed with the centromeric YCpUA or YCpUA* plasmids, and, in a parallel experiment, with the episomal YEpUA or YEpUA* constructs. In a similar transformation experiment, the CBU1-31 A2U3 strain was used as recipient for the centromeric YCpLA or YCpLA* plasmids, and for the episomal YEpLA or YEpLA* constructs. Purified recombinant clones were then used to start competition experiments (Futcher and Cox, 1983), which consisted in starting a yeast culture with the same number of cells from two different strains, CBU1-31 A2L2 transformed with YEpUA and CBU1-31 A2U3 carrying YEpLA*. After purification, the two strains were individually grown to stationary phase in selective drop out media, in order to synchronize the two cultures. Then, an amount of $5x10^4$ cells/ml was inoculated for each one of the two strains, thus resulting in a total of $1x10^5$ cells/ml inoculum in 5 ml of selective medium. The freshly mixed culture was immediately plated onto rich YPD medium, to confirm the 1/1 ratio of the two strains at the inoculum (G0). The mixed culture was then grown for ten generations, up to stationary phase (overnight at 30°C with shaking), and an aliquot was used for starting a new culture in the usual way. The process was repeated up to fifty generations, and a second plating was done at this time (G50). The presence of the different plasmid forms was assessed as the red/white ratio in the YPD plate. The process is outlined in the flowchart below.

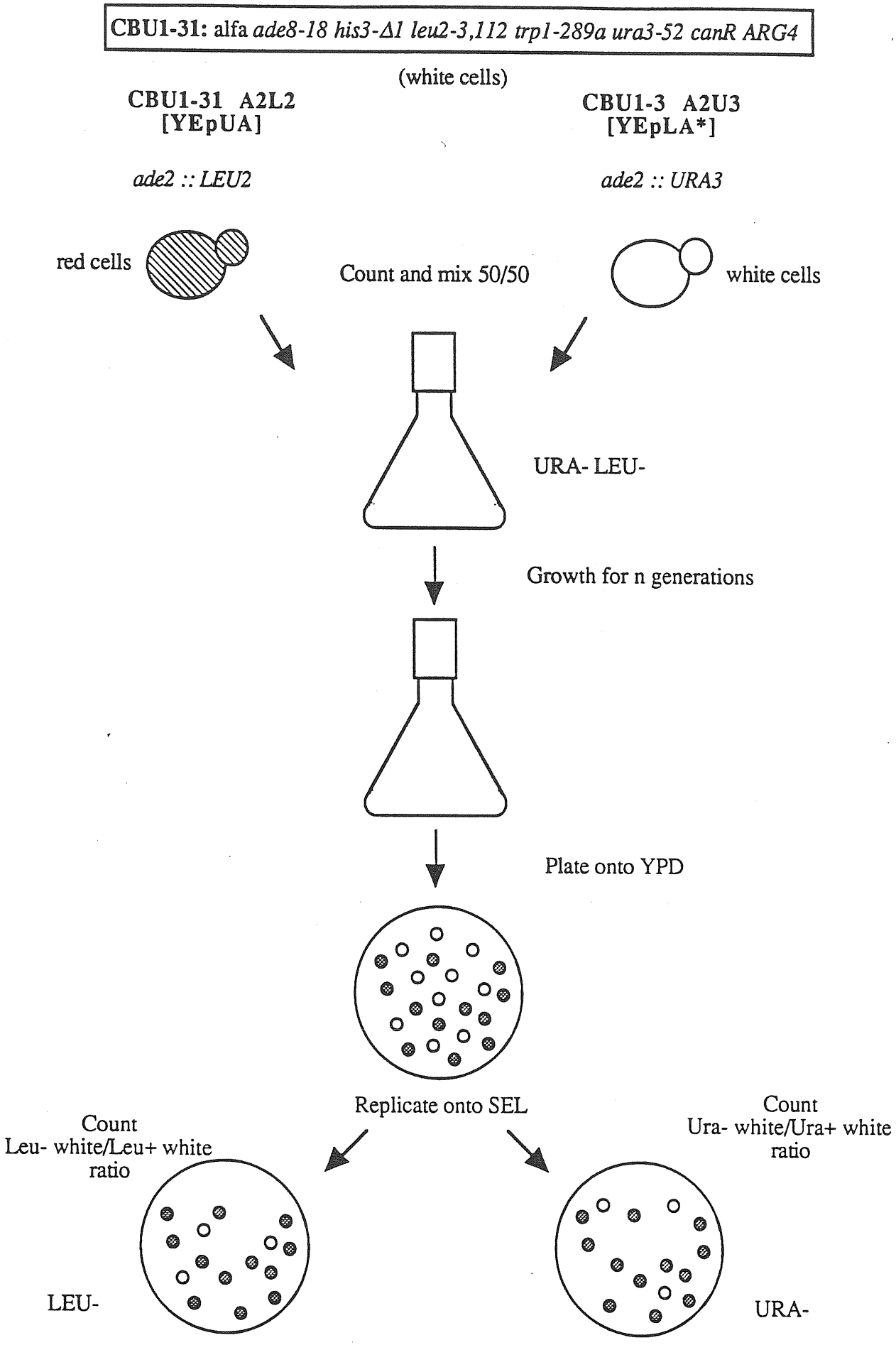


Fig. 2.41. Diagrammatic representation of the competition experiments with a reporter gene

3. RESULTS

3.1 Results from Curing Experiments

The maintenance of plasmids within the cell is a phenomenon that requires the presence of genes and DNA sequences, either on the plasmid itself, or in the chromosome of the host cell. In fact, yeast plasmids containing an autonomously replicating sequence (ARS) of DNA, of chromosomal origin, can undergo replication together with chromosomes, but they are lost at quite a frequent rate during the mitotic divisions (Murray and Szostak, 1983). Plasmids bearing a centromeric DNA sequence (CEN) can be maintained at much higher frequency than ARS-based plasmids, though their copy number is maintained at the level of one or two (Clarke and Carbon, 1980). Finally, plasmids containing the 2μm origin of replication and functions for partitioning are maintained at a rate similar to that of the chromosomal genes (Futcher and Cox, 1983). This is due to the fact that the native 2μm plasmid encodes the genes necessary for its maintenance, together with a well functioning system for transmission to the daughter cell (Hadfield et al., 1995; Jayaram et al., 1983; Broach, 1981).

The results presented hereafter- together with further evidence from our laboratory (Storici et al., 1995) - support the idea that the process of curing the cell from the endogenous 2μm plasmid, as well as the maintenance of artificial plasmids, could be a phenomenon dependent also on the genetic background of the host strain, thus involving chromosomal genes. For example, the association of the 2μm plasmid with folded chromosomes might indicate the existence of chromosomally-encoded functions, necessary for the proper maintenance of the plasmid (Taketo et al., 1980), and these functions can be more or less efficient depending of the strain. For instance, the yeast strains FAS20 and FAS21 loose 2μm-based plasmids at an higher rate than other laboratory strains tested (Vladimir Jong, personal communication), and they are also easier to cure from the 2μm plasmid than other strains. In fact, with the same methodology used to cure strain FAS20 (Bruschi and Howe, 1988; Toh-e and Wickner, 1981), we could not cure strain CBU1-31. We then tried the approach used by Ludwig and Bruschi (1991), but we could not detect any 2μm-free cell. In spite of all our attempts, strain CBU1-31 proved recalcitrant to curing, hence suggesting the existence of strain-dependent factors involved in the maintenance of this plasmid.

Figure 3.1 shows the result of the Southern hybridisation on the yeast minipreps coming from the curing experiment in which plasmid pJDB219 was used. In this experiment the DNA probe used was the purified and labelled ≈3.1 kb fragment from the restriction of plasmid pBLU-D with the restriction enzyme XbaI (plasmid map is shown in Fig. 2.1.). This DNA fragment contains only 2μm sequence, namely the small portion of the 2μm plasmid (2μS). Yeast DNA was probed as described, and, as expected, a strong signal was present in the lane containing FAS20 DNA, the positive control of this experiment (a [cir^+] strain). In the case of the negative control, DNA from the [cir^0] FAS21 strain, no signal could be detected. Strong signals were also evident in the case of DNA preparations coming from the samples from the curing process (Fig. 3.1., lanes B7 to A5), hence indicating persistence of the native 2μm circle in all the samples tested. Lanes a through g represent DNA hybridisation on dilutions of a DNA sample coming from the treated strain A5, which, when tested, gave Leu+ colonies, an indication of the presence of the pJDB219 plasmid. This provides evidence for the presence of 2μm sequences in this strain, the hybridisation of which resembles the pattern presented by that of the 2μm circle itself. The appearance of multiple hybridisation bands reflects the ability of 2μm to form concatemers of increasing size, an important step in the copy number amplification of this plasmid (Futcher, 1986). The fact that the reference marker (1kb DNA ladder) hybridises to the probe is due to the fact that DNA standard is a mixture of concatemers of DNA sequences from the 2μm plasmid (Gibco BRL-Life Technologies Ltd, Paisley, UK; Hartley and Donelson, 1980).

Since the curing experiment with plasmid pJDB219 was unsuccessful, we tried a different competitor plasmid, pBLU-D (Fig. 2.1.), previously used for the curing of 2μm in a different yeast strain (Ludwig and Bruschi, 1991). Figure 3.2. shows Southern hybridisation on yeast minipreps coming from the curing experiment using plasmid pBLU-D. In this experiment we analysed a wider number of samples, but we could not detect any 2μm-free cells (lanes 1-94). Overexposure of the film showed that a signal was present also in lanes 28, and 88-94. Thus, curing the 2μm circle from CBU1-31 proved to be particularly difficult, this strain being impossible to cure with standard approaches.

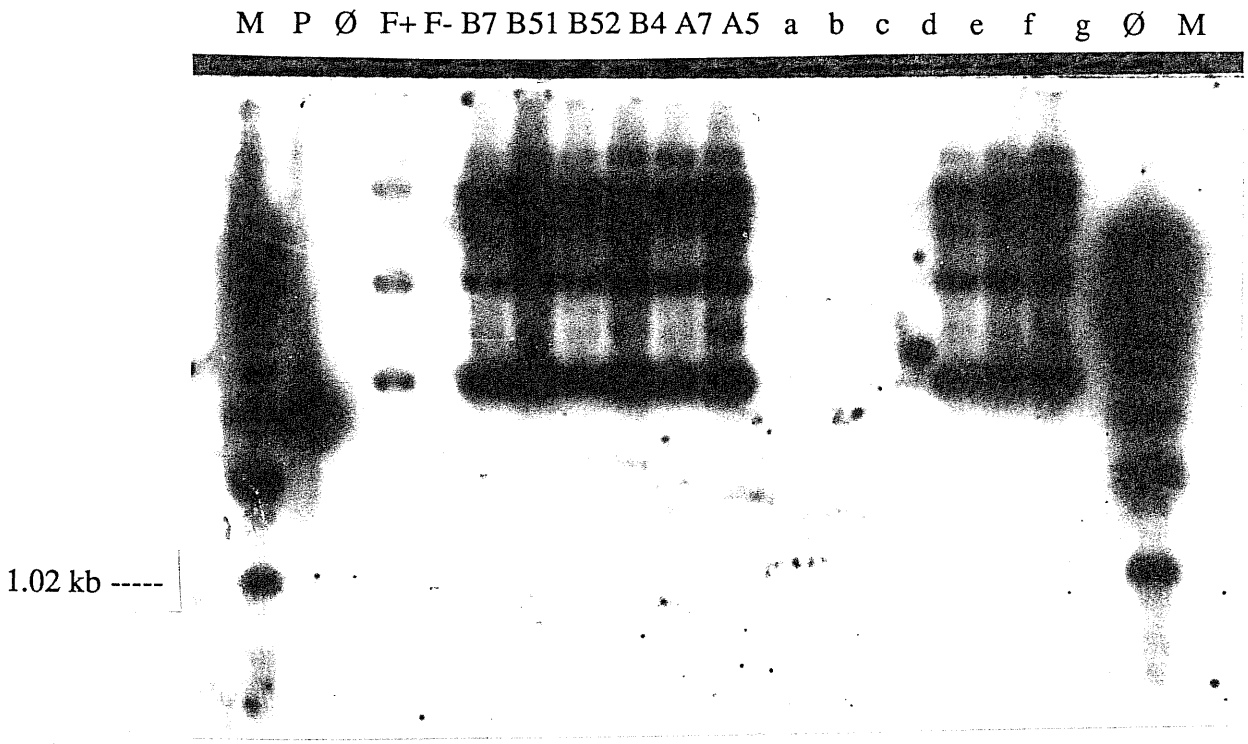


Fig. 3.1. Southern hybridisation of yeast minipreps from the curing experiment using plasmid pJDB219. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075; Gibco BRL-Life Technologies Ltd, Paisley, UK; Hartley and Donelson, 1980). **P**: the ≈ 3.1 kb DNA purified from pBLU-D/XbaI, used as DNA probe after radioactive labelling. **Ø**: empty lane. **F+**: Fas20 DNA, [*cir*$^{+}$] strain. **F-**: Fas21 DNA, [*cir*^{0}] strain. **Lanes B7 - A5**: DNA samples from yeast minipreps after the curing experiment. **a - g**: 10 µl of a dilution of a A5 Leu+ DNA (a=1/80; b=1/40; c=1/20; d=1/10; e=1/2; f=1/1; g=1/1, 30 µl) used as calibration for the copy number determination experiment.

Fig. 3.2

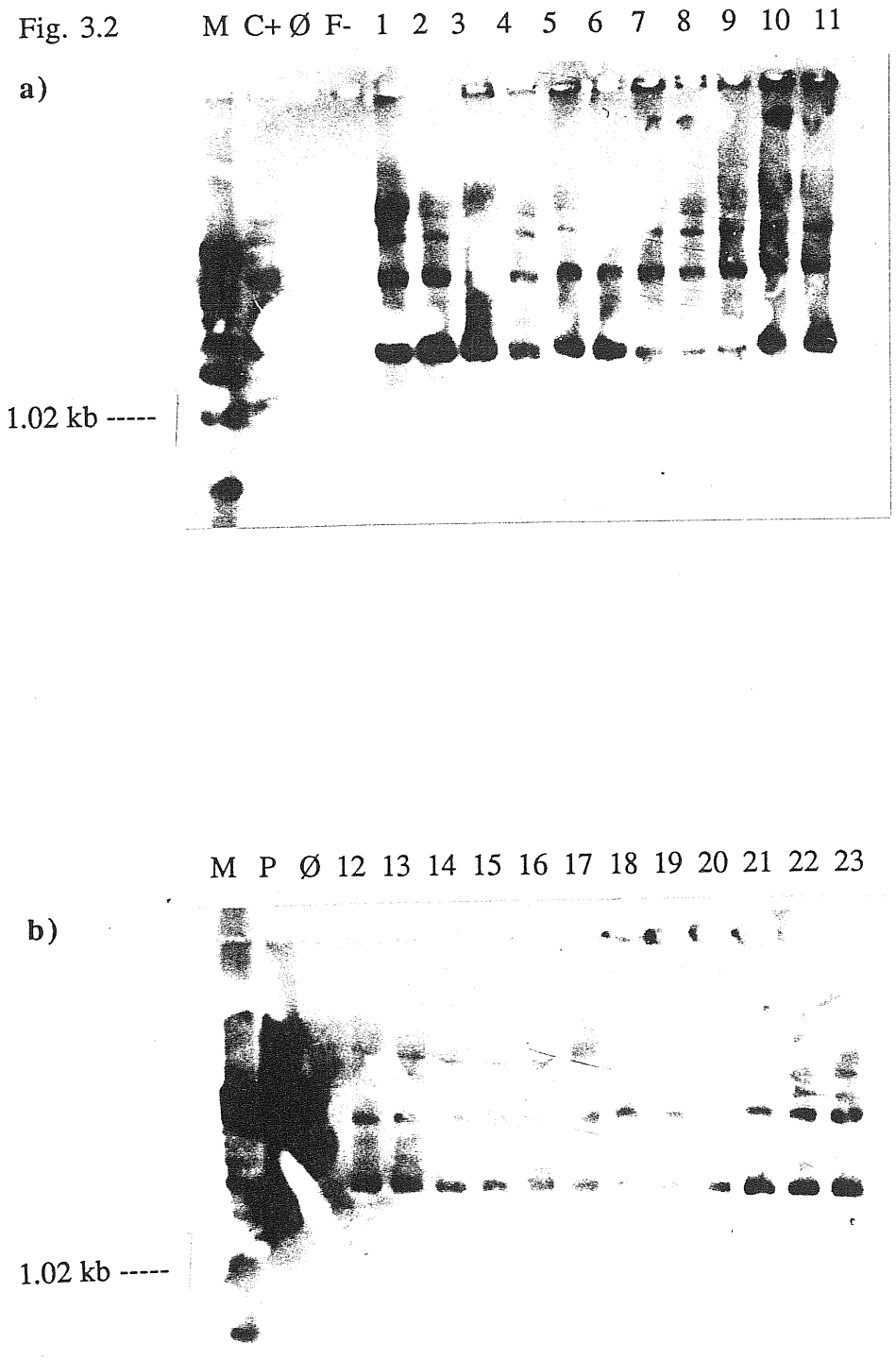
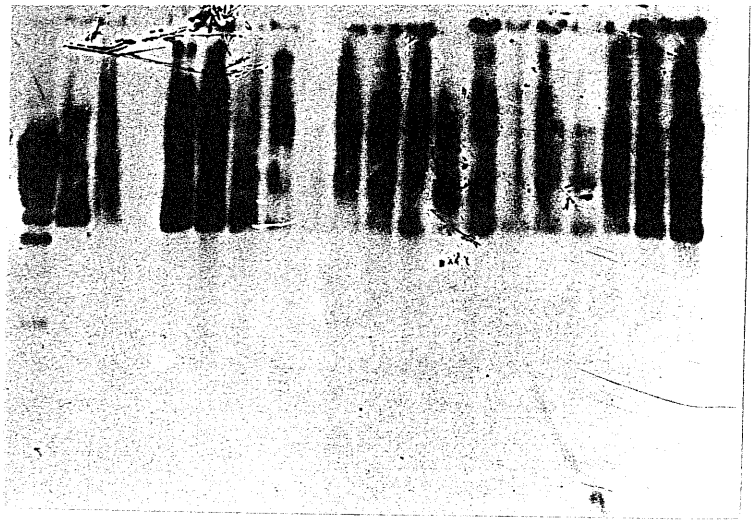

M C+ Ø F- 1 2 3 4 5 6 7 8 9 10 11
a)
1.02 kb -----
M P Ø 12 13 14 15 16 17 18 19 20 21 22 23
b)
1.02 kb -----

Fig. 3.2 $M^{C+}{}_{F+}{}^{F-}\ {}_{24}{}^{25}{}_{26}{}^{27}{}_{28}\ {}^{29}{}_{30}{}^{31}{}_{32}{}^{33}{}_{34}\ {}^{35}{}_{36}{}^{37}{}_{38}{}^{39}$

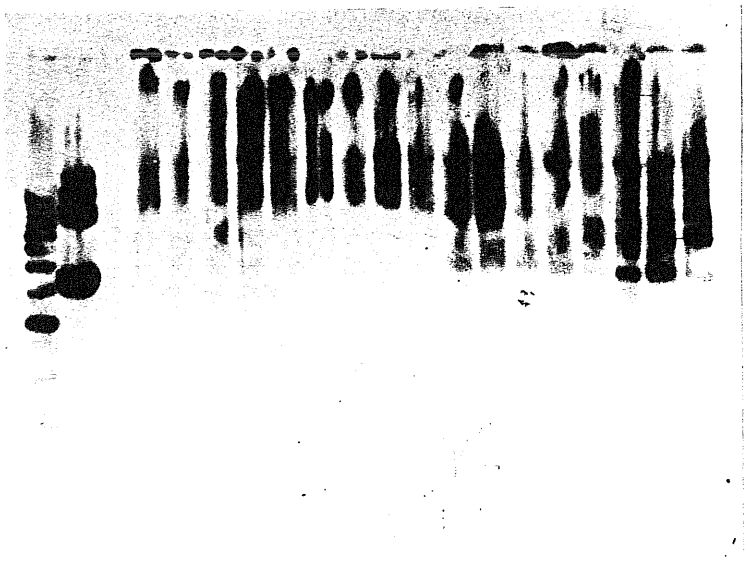
c)



1.02 kb -----

$M\ {}^{P}\ \emptyset\ {}_{40}{}^{41}{}_{42}{}^{43}{}_{44}\ {}^{45}{}_{46}{}^{47}{}_{48}{}^{49}\ {}_{50}{}^{51}{}_{52}{}^{53}\ {}_{54}{}^{55}{}_{56}$

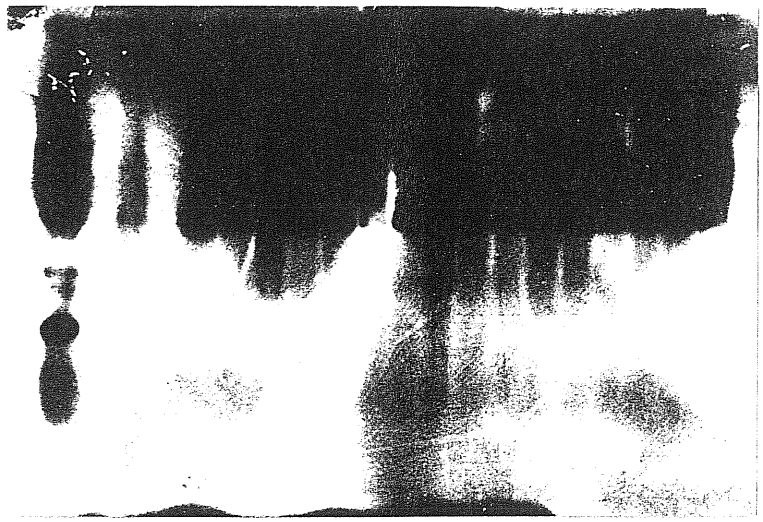
d)



1.02 kb -----

Fig. 3.2 M Ø F+ F- 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72

e)



M Ø 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94

f)

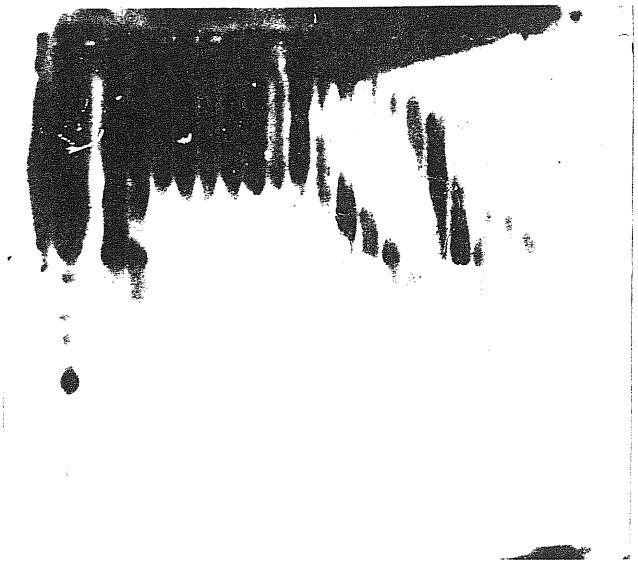


Fig. 3.2. Southern hybridisation of yeast minipreps from the curing experiment using plasmid pBLU-D. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **C+**: CBU1-31 DNA, [*cir*$^+$] strain. **Ø**: empty lane. **F+**: Fas20 DNA, [*cir*$^+$] strain. **F-**: Fas21 DNA, [*cir*^{0}] strain. **P**: pBLU-D/XbaI DNA, showing three DNA bands of approximately ≈ 3.1 kb (used as DNA probe after radioactive labelling), ≈ 4.42 kb, and ≈ 7.0 kb. **Lanes 1-94**: DNA samples from yeast minipreps after the curing experiment (**a**, **b**, **c**, **d**, **e**, **f**).

3.2 Results from Selective and Non-selective Growth Experiments

As already described, we constructed a series of 2μm-based plasmids carrying genes for the synthesis of either amino acids (histidine, leucine or tryptophan) or a nucleotide (uracil). These plasmids were individually introduced in a recipient yeast strain, named CBU1-31 ([*cir*$^+$] *Mat alfa ade8-18 his3-Δ1 leu2-3,112 trp1-289 ura3-52 can1*^{R}) and, parallel cultures were grown both in selective and non-selective media as described in section 2.24. Growth was measured as absorbance at OD_{600}. Data are shown in Table 3.1 together with standard deviation (σ_n)

Table 3.1.

Growth (OD_{600}) of non transformed CBU1-31 in complete (COM) medium			Growth (OD_{600}) of non transformed CBU1-31 in rich (YPD) medium		
Hours	X-average	σ_n (±)	Hours	X-average	σ_n (±)
9	0.24	$\pm 9.4 \times 10^{-4}$	9	0.15	±0.03
15	1.44	$\pm 8.2 \times 10^{-3}$	15	1.47	±0.01
21	1.93	±0	21	2.51	$\pm 4.7 \times 10^{-3}$
32	2.23	±0.01	32	2.74	±0.01
38	2.47	$\pm 4.7 \times 10^{-3}$	38	2.82	±0

T1	(+9 hours)	X-average (OD_{600})	σ_n (±)	T1	(+9 hours)	X-average (OD_{600})	σ_n (±)
SEL	*HIS3*	0.096	0.016	SEL	*LEU2*	0.126	0.017
SEL	*URA3*	0.131	0.015	SEL	*TRP1*	0.093	0.010
COM	*HIS3*	0.141	0.020	COM	*LEU2*	0.164	0.024
COM	*URA3*	0.189	0.034	COM	*TRP1*	0.131	0.012
YPD	*HIS3*	0.141	0.026	YPD	*LEU2*	0.195	0.025
YPD	*URA3*	0.186	0.030	YPD	*TRP1*	0.149	0.023

Table 3.1.

T2	(+15 hours)	X-average (OD_{600})	σ_n (±)
SEL	*HIS3*	0.473	0.073
SEL	*URA3*	0.690	0.042
COM	*HIS3*	0.687	0.094
COM	*URA3*	0.926	0.167
YPD	*HIS3*	0.712	0.116
YPD	*URA3*	1.035	0.198

T2	(+15 hours)	X-average (OD_{600})	σ_n (±)
SEL	*LEU2*	0.558	0.084
SEL	*TRP1*	0.364	0.031
COM	*LEU2*	0.930	0.081
COM	*TRP1*	0.802	0.119
YPD	*LEU2*	1.006	0.072
YPD	*TRP1*	0.943	0.133

T3	(+21 hours)	X-average (OD_{600})	σ_n (±)
SEL	*HIS3*	1.524	0.057
SEL	*URA3*	1.682	2.9×10^{-3}
COM	*HIS3*	1.7	0.039
COM	*URA3*	1.692	0.092
YPD	*HIS3*	1.892	0.138
YPD	*URA3*	2.191	0.095

T3	(+20 hours)	X-average (OD_{600})	σ_n (±)
SEL	*LEU2*	1.491	0.066
SEL	*TRP1*	1.029	0.037
COM	*LEU2*	1.668	0.012
COM	*TRP1*	1.667	0.045
YPD	*LEU2*	1.941	0.037
YPD	*TRP1*	2.052	0.063

T4	(+32 hours)	X-average (OD_{600})	σ_n (±)	T4	(+32 hours)	X-average (OD_{600})	σ_n (±)
SEL	*HIS3*	1.983	0.062	SEL	*LEU2*	2.048	0.013
SEL	*URA3*	2.022	1.1×10^{-3}	SEL	*TRP1*	2.013	0.016
COM	*HIS3*	2.168	0.047	COM	*LEU2*	2.113	5.0×10^{-3}
COM	*URA3*	2.003	0.058	COM	*TRP1*	2.058	0.016
YPD	*HIS3*	2.504	0.018	YPD	*LEU2*	2.468	7.6×10^{-3}
YPD	*URA3*	2.467	0.015	YPD	*TRP1*	2.421	2.9×10^{-3}

T5	(+38 hours)	X-average (OD_{600})	σ_n (±)	T5	(+38 hours)	X-average (OD_{600})	σ_n (±)
SEL	*HIS3*	2.216	0.059	SEL	*LEU2*	2.261	0.012
SEL	*URA3*	2.166	0.028	SEL	*TRP1*	2.165	0.021
COM	*HIS3*	2.323	0.028	COM	*LEU2*	2.275	8.8×10^{-3}
COM	*URA3*	2.234	0.125	COM	*TRP1*	2.144	6.7×10^{-3}
YPD	*HIS3*	2.613	0.018	YPD	*LEU2*	2.650	0.019
YPD	*URA3*	2.544	0.025	YPD	*TRP1*	2.544	8.6×10^{-3}

Table 3.1 . Optical density (OD_{600}) of the growth rate experiments.

"X-average" represents the arithmetic average, and σ_n (+/-) the standard deviation. Data are from parallel cultures of three independent transformants (#1, #2, and #3). SEL represents selective medium, either HIS-, LEU-, TRP-, and URA- drop-out media, according to the type of plasmid present in the strain. COM and YPD are rich non-selective media, as described in section 2.8 of Materials and Methods. Plasmids carrying selectable marker genes are indicated by the name of the corresponding gene (*HIS3*, *LEU2*, *TRP1*, and *URA3*).

A summary of the data from the growth curve experiments is shown in the following Table 3.2:

Time Hours	*HIS3** SEL	*HIS3** COM	*HIS3** YPD	*URA3** SEL	*URA3** COM	*URA3** YPD
9	0.096	0.141	0.141	0.131	0.189	0.186
15	0.473	0.687	0.712	0.69	0.926	1.035
21	1.524	1.7	1.892	1.682	1.692	2.191
32	1.983	2.168	2.504	2.022	2.003	2.467
38	2.216	2.323	2.613	2.166	2.234	2.544

Time Hours	*LEU2** SEL	*LEU2** COM	*LEU2** YPD	*TRP1** SEL	*TRP1** COM	*TRP1** YPD
9	0.126	0.164	0.195	0.093	0.131	0.149
15	0.558	0.93	1.006	0.364	0.802	0.943
20	1.491	1.668	1.941	1.029	1.667	2.052
32	2.048	2.113	2.468	2.013	2.058	2.421
38	2.261	2.275	2.65	2.165	2.144	2.544

Table. 3.2 . Summary table of the OD 600 of yeast cultures growing in selective (SEL) and non-selective (COM or YPD) conditions

* represents the average of the data of three independent transformants [#1, #2, and #3] (two parallel cultures for each one [A and B]). Plasmids carrying selectable marker genes are indicated by the name of the corresponding gene (*HIS3*, *LEU2*, *TRP1*, and *URA3*).

A graphic representation of the data shown above is presented in the Fig. 3.3.

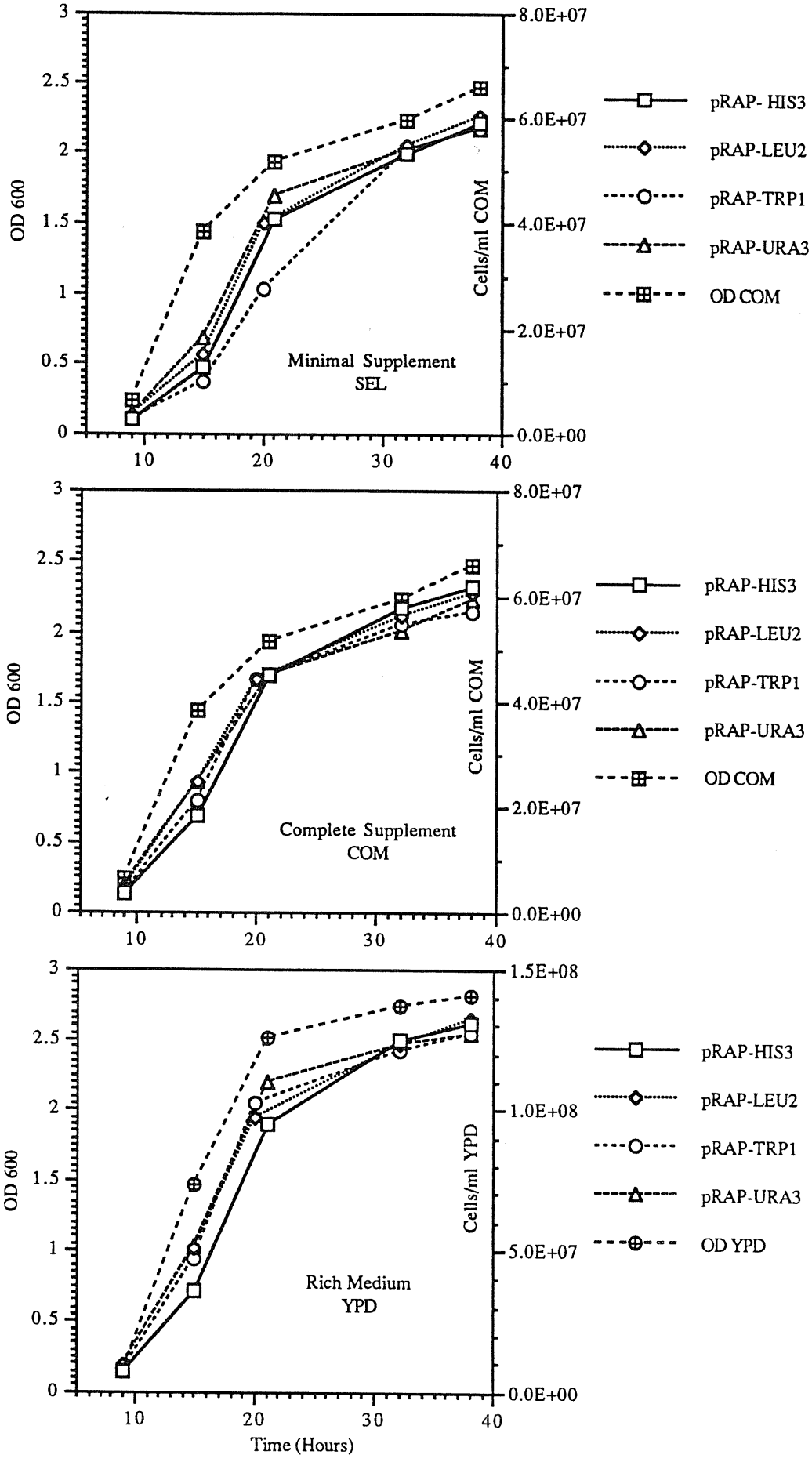


Fig. 3.3 . Growth profile of the CBU1-31 strain transformed with different episomal plasmids.

Figure 3.3 shows that there is no appreciable difference, at least at this level of investigation, in the growth rate of the transformed cultures both in selective, synthetic and rich media. In fact, all the cultures grown in selective (SEL) medium present the same profile, though the strain carrying the pRAP-TRP1 plasmid seems to display a slower growth rate after twenty hours. However, the remaining data points are similar to those of the other cultures, and the general behaviour appears to be the same. The growth profile in complete (COM) medium is matching that one in selective, where cultures reach the maximum optical density of 2. Again all the transformed strains behave in a homogeneous fashion, with no relevant differences. This conduct is presented also in the case of growth in rich (YPD) medium. However, the optical density reached in this case in higher than that attained in the other two media, with a value higher than 2.5. Furthermore, cell density in this medium, about 1.3 $x10^8$ cells/ml, is more than twofold that in selective and complete synthetic media, about $6.0x10^7$ cells/ml, a phenomenon well described in literature. In fact, in the rich YPD medium stationary phase is reached much later than in the synthetic media, probably because of the lack of still undefined nutrients (Werner-Washburne et al., 1993). It is interesting to note that both in complete and rich media the transformed cultures show a lower growth profile than the non transformed controls, hence suggesting that plasmid maintenance is an energy-expensive task. This phenomenon has been described also for the cryptic 2μm circle, whose maintenance confers to the host cell a selective disadvantage of about 1% in term of growth rate, when compared to plasmid-free cells (Futcher and Cox, 1983; Walmsley et al., 1983; Mead et al., 1986).

3.3 Results from Plasmid Stability Experiments

These experiments were designed to assess differences, in terms of plasmid maintenance, due to the metabolic stress induced by the selective pressure. Plasmid maintenance was monitored during the course of fifty generations, in selective and non-selective conditions. Cells harbouring these plasmids showed a different level of maintenance when kept under selection. The plasmid carrying the *LEU2* gene was clearly maintained at the lowest level of all, on average 51.3% (σ_n=±4.2%) of the population, while that with the *URA3* gene was kept at the highest level, average of 67.4% (σ_n=±3.5%). Vectors with *HIS3* and *TRP1* genes were maintained at an

intermediate level, in average 59.2% (σ_n=±3.0%) and 62.9% (σ_n=±3.9%), respectively. When cells were grown without selective pressure in synthetic medium (COM) plasmid loss was lower for the genes encoding tryptophan and uracil (11.5% and 4.6%) of prototrophs after fifty generations), and higher for the other two markers histidine and leucine (2.2% and 1.7%) of maintenance), as shown in Table 3.3. A similar pattern of plasmid loss after fifty generation of growth was observed for cells growing in rich (YPD) medium, again with *TRP1* and *URA3* genes being maintained at a higher level (14.9% and 8.8% respectively) than the other two genes *HIS3* (1.8%) and *LEU2* (2.5%). Data from these experiments are reported in Table 3.3 below:

Table 3.3

Gen.	S-*HIS* (%)		S-*LEU* (%)		S-*TRP* (%)		S-*URA* (%)	
	X-av.	σ_n (±)	X-av.	σ_n (±)	X-av.	σ_n (±)	X-av.	σ_n (±)
0	55.99	±2.27	51.84	±5.50	62.23	±1.69	70.28	±4.49
10	61.57	±2.52	45.19	±3.0	61.73	±2.11	69.17	±1.46
20	59.12	±4.80	49.57	±4.16	60.91	±4.21	69.96	±3.11
30	60.40	±3.99	49.72	±3.81	60.62	±2.63	66.22	±5.75
40	63.33	±1.61	59.16	±3.84	71.66	±1.26	68.53	±4.15
50	54.63	±1.36	52.29	±6.57	60.46	±5.32	60.18	±4.67
X-av.	59.17	± 3.04	51.29	± 4.20	62.93	± 3.95	67.39	± 3.48

Gen.	C-*HIS* (%)		C-*LEU* (%)		C-*TRP* (%)		C-*URA* (%)	
	X-av.	σ_n (±)	X-av.	σ_n (±)	X-av.	σ_n (±)	X-av.	σ_n (±)
0	55.99	±2.27	51.84	±5.50	62.23	±1.69	70.28	±4.49
10	33.44	±3.06	29.22	±2.75	41.57	±1.95	41.45	±3.96
20	20.38	±3.36	17.66	±3.16	35.54	±6.12	32.34	±0.77
30	9.57	±1.98	9.18	±1.82	25.38	±1.23	14.51	±3.82
40	4.71	±1.51	4.58	±1.54	19.71	±2.26	10.73	±1.19
50	2.17	±1.49	1.70	±0.66	11.48	±2.74	4.60	±0.32

Gen.	R-*HIS* (%)		R-*LEU* (%)		R-*TRP* (%)		R-*URA* (%)	
	X-av.	σ_n (±)	X-av.	σ_n (±)	X-av.	σ_n (±)	X-av.	σ_n (±)
0	55.99	±2.27	51.84	±5.50	62.23	±1.69	70.28	±4.49
10	32.57	±4.03	25.35	±3.53	36.53	±3.79	39.08	±1.46
20	16.44	±2.52	13.77	±1.90	28.81	±0.48	28.99	±0.67
30	8.90	±2.05	5.59	±1.61	25.96	±1.79	16.72	±1.37
40	5.91	±1.17	1.54	±0.46	17.73	±1.45	10.16	±1.81
50	1.83	±0.07	2.54	±1.10	14.92	±2.19	8.81	±1.79

Table 3.3 . Data from the plasmid stability experiments. Values represent percentage of prototrophs out of the total number of cells plated.

Gen. is the number of generations, S, C, and R, represent selective, complete and rich media respectively. "X-av." represents the arithmetic average, and σ_n (±) the standard deviation.

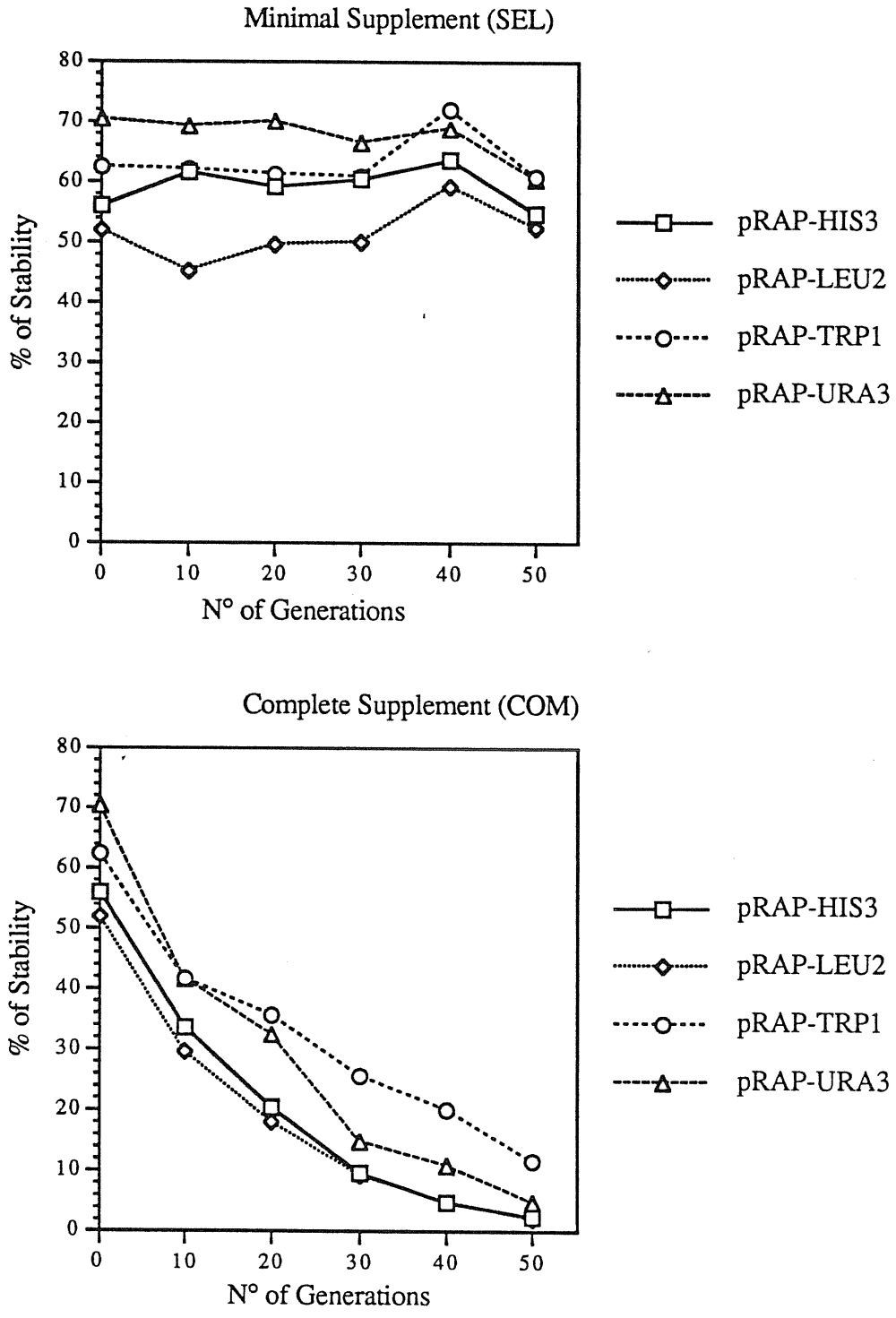
All the data presented in Table 3.3 are summarised in the following Table 3.4:

Gen.	S-*HIS*	S-*LEU*	S-*TRP*	S-*URA*	C-*HIS*	C-*LEU*	C-*TRP*	C-*URA*	R-*HIS*	R-*LEU*	R-*TRP*	R-*URA*
0	55.99	51.84	62.23	70.28	55.99	51.84	62.23	70.28	55.99	51.84	62.23	70.28
10	61.57	45.19	61.73	69.17	33.44	29.22	41.57	41.45	32.57	25.35	36.53	39.08
20	59.12	49.57	60.91	69.96	20.38	17.66	35.54	32.34	16.44	13.77	28.81	28.99
30	60.40	49.72	60.62	66.22	9.57	9.18	25.38	14.51	8.90	5.59	25.96	16.72
40	63.33	59.16	71.66	68.53	4.71	4.58	19.71	10.73	5.91	1.54	17.73	10.16
50	54.63	52.29	63.85	60.18	2.17	1.70	11.48	4.60	1.83	2.54	14.92	8.81

Table 3.4 . Summary of the data from the plasmid stability experiments.

A graphic presentation of the data contained in Table 3.4 is shown in the following Fig. 3.4:

Fig. 3.4



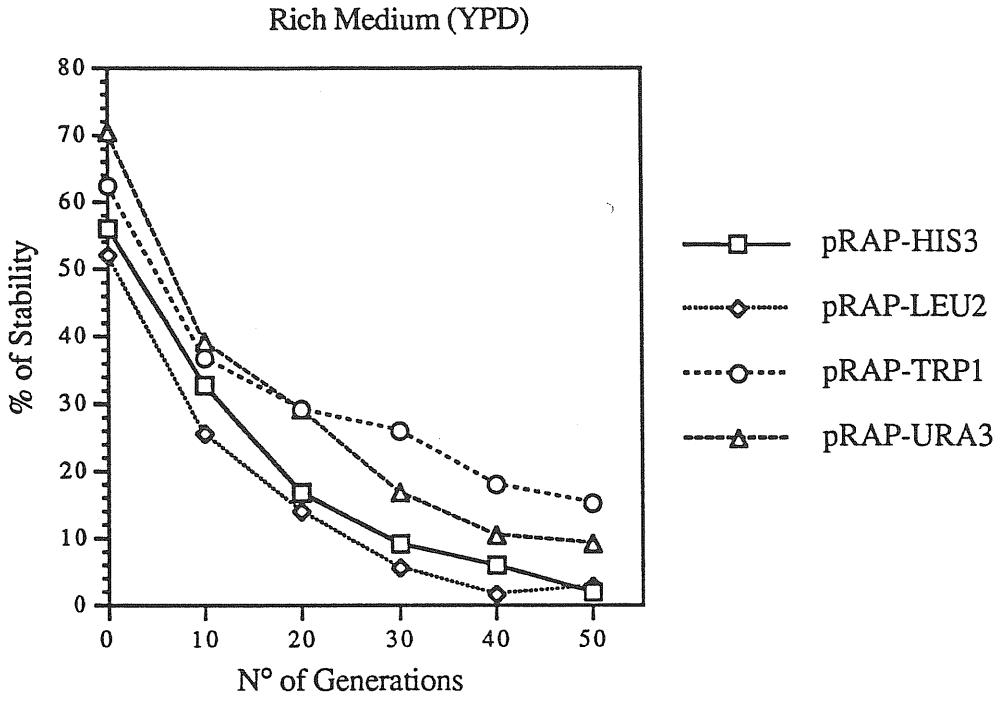


Fig. 3.4 . Stability of different episomal plasmids in the yeast strain CBU1-31. Plasmid stability was monitored during the course of fifty generations of growth, in selective (SEL) and non-selective (COM and YPD) conditions.

3.4 Results from Plasmid Copy Number Experiments

A sample of cells was taken at the end of each round of growth during the experiments for the determination of plasmid maintenance, and total DNA was extracted to determine plasmid copy number as detailed in section 2.12 of Materials and Methods. Pictures of the Southern hybridisations of these filters are shown below.

Figure 3.5 shows the Southern hybridisation of yeast total DNA from non transformed CBU1-31 cultures, together with hybridisation of plasmid DNA from transformed bacterial cultures with the same plasmids (pRAP-HIS3, pRAP-LEU2, pRAP-TRP1, and pRAP-URA3). Different dilutions of restricted total yeast DNA, 1/1, 1/5, 1/10, and 1/100 respectively, were loaded on the gel, together with uncut total DNA from non transformed yeast cultures (**UTD**), as negative control. Uncut plasmid DNA from bacterial minipreps was also loaded for each plasmid species, **UPU**, **UPL**, **UPT**, and **UPH**, representing pRAP-URA3, pRAP-LEU2, pRAP-TRP1, and pRAP-HIS3, respectively. Lanes **U**, **L**, **T**, and **H**, represent DNA fragments that have been used as probe for the chromosomal reference genes, the 1.95 kb *URA3*/BamHI gene from pRAP-URA3, the 2.2 kb *LEU2*/BamHI gene from pRAP-LEU2, the 1.65 kb *TRP1*/BamHI gene from pRAP-TRP1, and the 1.76 kb *HIS3*/BamHI gene from pRAP-HIS3. In lane **1** of the *URA3* section (top left panel) is visible a band of approximately 12 kb, indicating the size of the chromosomal fragment deriving from the restriction with BamHI, containing the URA3 gene. Hybridisation with *LEU2*/BamHI of the restricted yeast chromosomal DNA (lane **1**, *LEU2* section, top right panel) identifies of a main ≈7.1 kb band, together with some fainter bands probably deriving from partial digestion. Hybridisation with *TRP1*/BamHI gene, identifies the chromosomal counterpart as a band of more than 12 kb, and a smaller band of approximately 10 kb (lane **1**, *TRP1* section, bottom left panel), only visible after longer exposure of the film. Hybridisation with the *HIS3*/BamHI fragment detects a band of more than 12 kb in size, together with a fainter band of roughly 3.1 kb, the minimum fragment containing that gene (lane **1**, *HIS3* section, bottom right panel). These hybridisations represent the controls of blots of the transformed yeast cultures, shown in Fig. 3.6, 3.7, 3.8, and 3.9, to determine the size of the chromosomal genes.

Figure 3.6 shows the hybridisation pattern of the *HIS3* gene in the CBU1-31 strain transformed with the pRAP-HIS3 plasmid. DNA was restricted overnight with XbaI, and probed with the 1.76 kb *HIS3*/BamHI fragment. This detects the *HIS3* gene located in the linearised 5.49 kb plasmid (Pl., plasmid map is shown in Fig. 2.14), as well as the chromosomal gene, appearing as a fainter 3.1 kb band (Ch., SEL panel, top). The plasmid band is well manifest along the course of the fifty generations, which is expected for the growth in selective condition. This indicates that the plasmid is well maintained under selective conditions. The panel in the middle (COM) represents hybridisation of the sample coming from cultures grown in complete medium. Again, the plasmid band roughly migrates as 5.5 kb band (Pl.), while the chromosomal gene migrates at the expected 3.1 kb (Ch.). It is evident that plasmid pRAP-HIS3 is lost, the signal fading away during the course of the usual fifty generations, while the chromosomal band is still visible. Finally, the bottom panel (RICH) shows hybridisation to DNA extracts from cultures grown in rich YPD medium. The amount of DNA restricted was in excess with the restriction enzyme. This resulted in the appearance of the >12 kb band (Ch.), which was described in the control Southern shown in Fig. 3.5. Thus, hybridisation to the chromosomal *HIS3* gene results in two signals, the usual 3.1 kb and the >12 kb bands, while the gene on the plasmid is detected as a 5.5 kb band (Pl.). This plasmid band still gives quite a strong signal even after the fiftieth generation, in good agreement with the plasmid stability data.

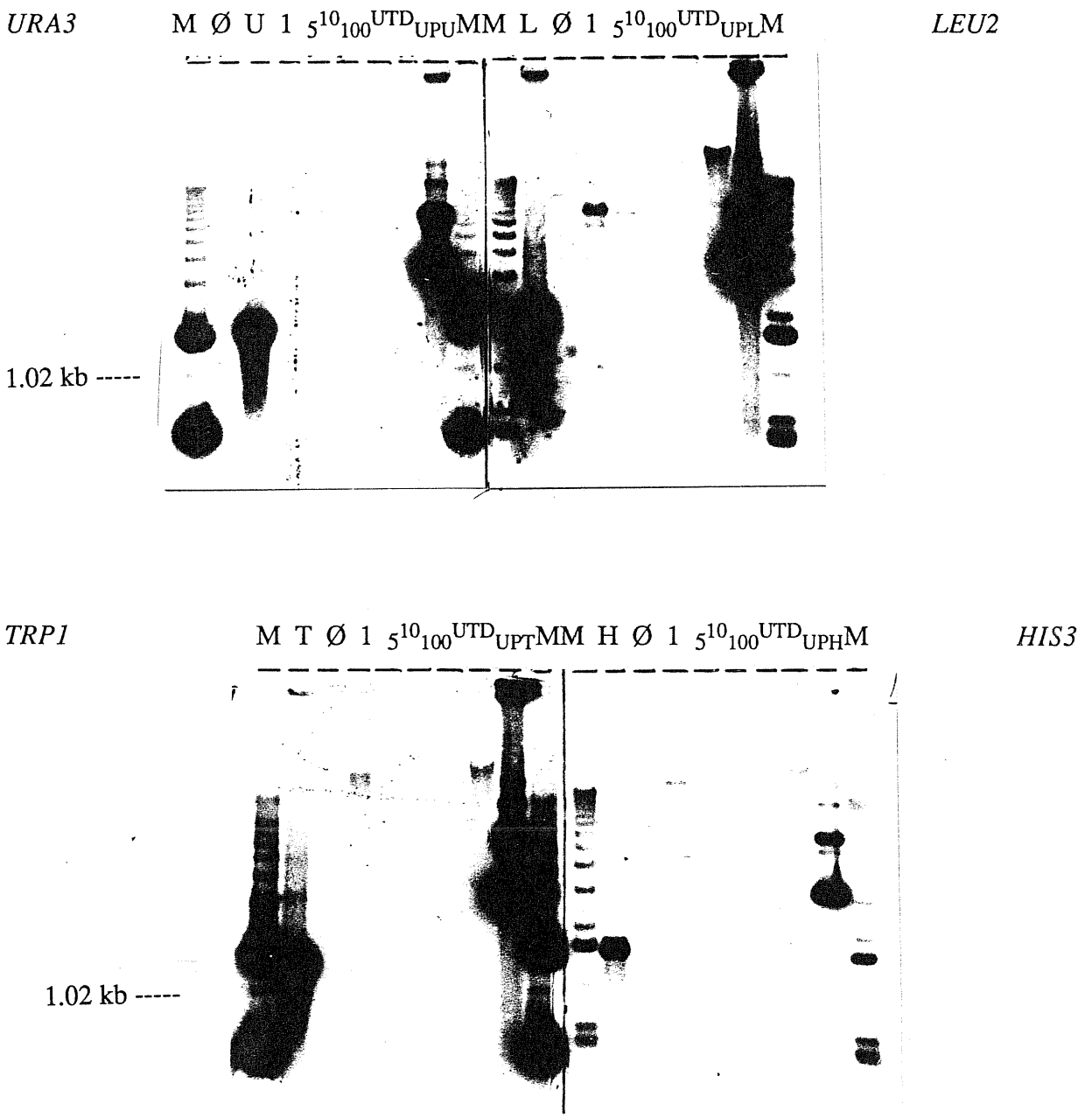


Fig. 3.5 . Southern hybridisation of yeast total DNA from non transformed yeast cultures and plasmid-transformed bacterial cultures DNA. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Ø**: empty lane. **1, 5, 10, and 100**: different dilutions of restricted total yeast DNA, 1/1, 1/5, 1/10, and 1/100 respectively; DNA was restricted overnight with an excess of either BamHI (*URA3*, *LEU2*, and *TRP1*) or XbaI (*HIS3*). **U**: 1.95 kb *URA3*/BamHI gene from pRAP-URA3, radiolabelled and used as probe for the chromosomal *URA3* gene. **L**: 2.2 kb *LEU2*/BamHI gene from pRAP-LEU2, radiolabelled and used as probe for the chromosomal *LEU2* gene. **T**: 1.65 kb *TRP1*/BamHI gene from pRAP-TRP1, radiolabelled and used as probe for the chromosomal *TRP1* gene. **H**: 1.76 kb *HIS3*/BamHI gene from pRAP-HIS3, radiolabelled and used as probe for the chromosomal *HIS3* gene. **UTD**: uncut total DNA from non transformed yeast cultures. **UPU, UPL, UPT, and UPH**: uncut plasmid DNA, pRAP-URA3, pRAP-LEU2, pRAP-TRP1, and pRAP-HIS3 respectively, from bacterial minipreps. **Ch.**: Chromosomal band. **Pl.**: Plasmid band.

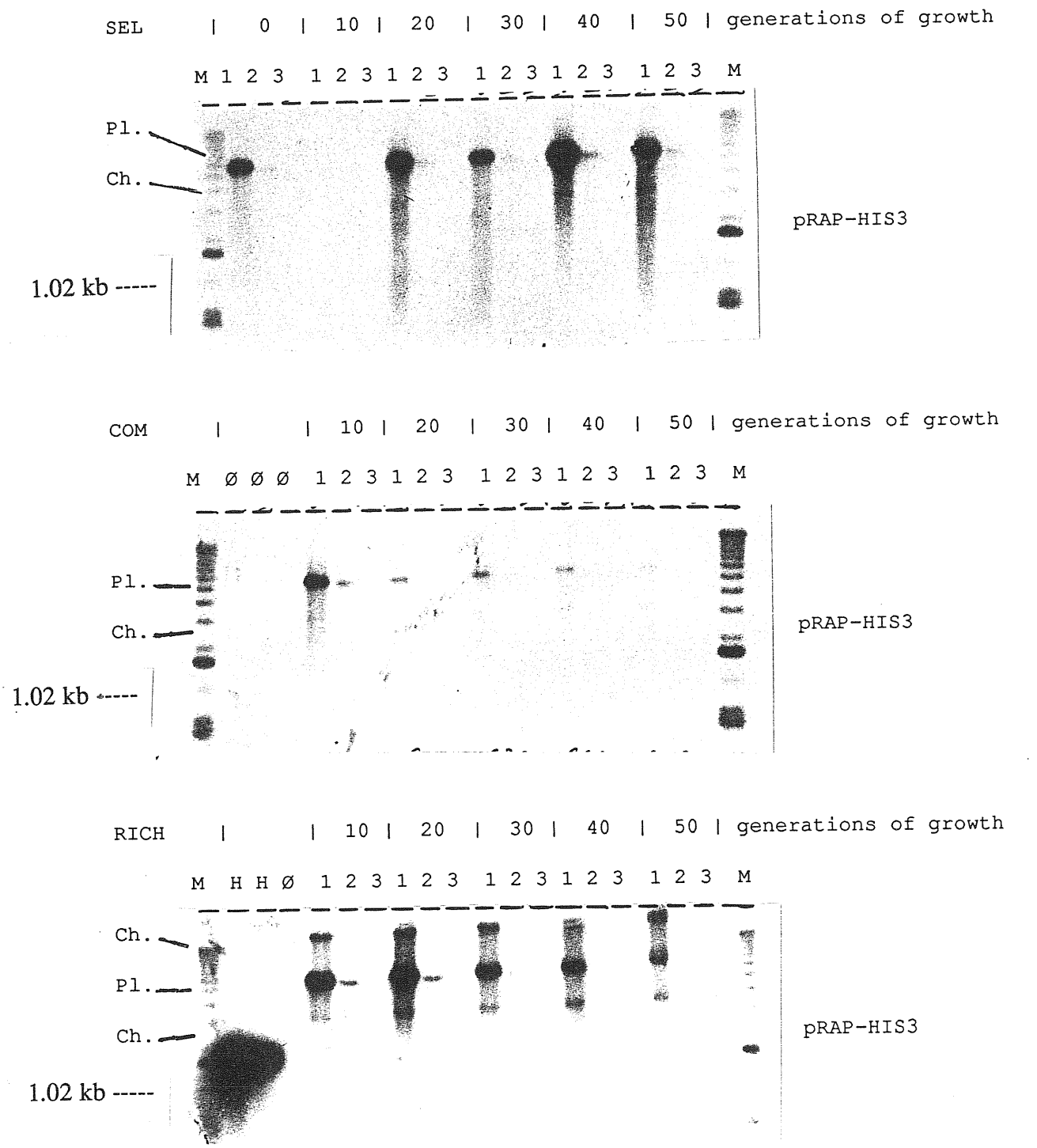


Fig. 3.6 . Southern hybridisation of total yeast DNA from cultures transformed with the pRAP-HIS3 plasmid digested overnight with XbaI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075); the 1.64 kb fragment gives a strong hybridisation signal. **Ø**: empty lane. **1, 2, and 3**: different dilutions of restricted (XbaI) total yeast DNA, 1/1, 1/10, and 1/100 respectively. **0-50**: number of generations grown in the specific medium. **H**: 1.76 kb *HIS3*/BamHI gene from pRAP-HIS3, radiolabelled and used as probe for pRAP-HIS3. SEL, COM, and RICH, are different media as described in text. **Ch.**: Chromosomal band. **Pl.**: Plasmid band.

Hybridisation with the *LEU2*/BamHI probe is shown in Fig. 3.7 , reporting the Southern hybridisation on yeast total DNA digested with BamHI from cultures transformed with the pRAP-LEU2 plasmid. Hybridisation to the plasmid *LEU2* gene results in the appearance of a sharp band with the same size of the probe, 2.2 kb as expected, and one of higher size, roughly 6 kb, corresponding in size to the linear pRAP-LEU2 plasmid (Pl.).The chromosomal *LEU2* gene gives rise to a signal of approximately 7.1 kb (Ch.), as shown in the control hybridisation reported in Fig. 3.5. Both signals are strong during the course of the experiment in selective conditions, SEL panel (top), as expected for the maintenance of the plasmid in such situation. When cells are grown in non selective complete medium, COM panel (centre), the plasmid is lost at a quite high rate, again in accordance with the poor stability of this construct resulting from the plasmid stability experiments. In rich YPD medium, bottom panel (RICH), the plasmid bands of 2.2 kb and 6 kb, corresponding in size to the totally restricted and to partially digested linear pRAP-LEU2 plasmid, are quickly disappearing, being almost completely invisible after the fortieth generation (Pl.). This phenomenon perfectly matches the situation observed in the plasmid stability experiments in rich medium, described in Fig. 3.4.

The situation with the *TRP1* marker is reported in Fig. 3.8, with the organisation of the preceding figures. The top panel (SEL) shows the hybridisation signals of DNA preparations from cultures grown in selective conditions. Here, the plasmid DNA band has the same size of the probe, 1.65 kb (Pl.), while the chromosomal one migrates at roughly 4.1 kb and, in some cases, at >12 kb (Ch.), as shown in Fig. 3.5. In these selective conditions the plasmid is maintained, as expected, and the corresponding signal is present in all the lanes. In the case of lack of selection in complete medium, centre panel (COM), the band corresponding to the plasmid DNA is still quite intense even at the end of the experiment, hence indicating good stability of this construct even in the absence of selective pressure. This condition is also evident in the case of growth in rich medium, bottom panel (RICH), where the plasmid band is well detectable at the end of the experiment, and its intensity is still strong compared to that of the chromosomal reference. The presence of such intense band corresponding to plasmid DNA confirms that the high number of Trp+ cells from the plating of the CBU1-31 culture, harbouring pRAP-TRP1, might be due to real plasmid maintenance and not to reversion to the chromosomal gene.

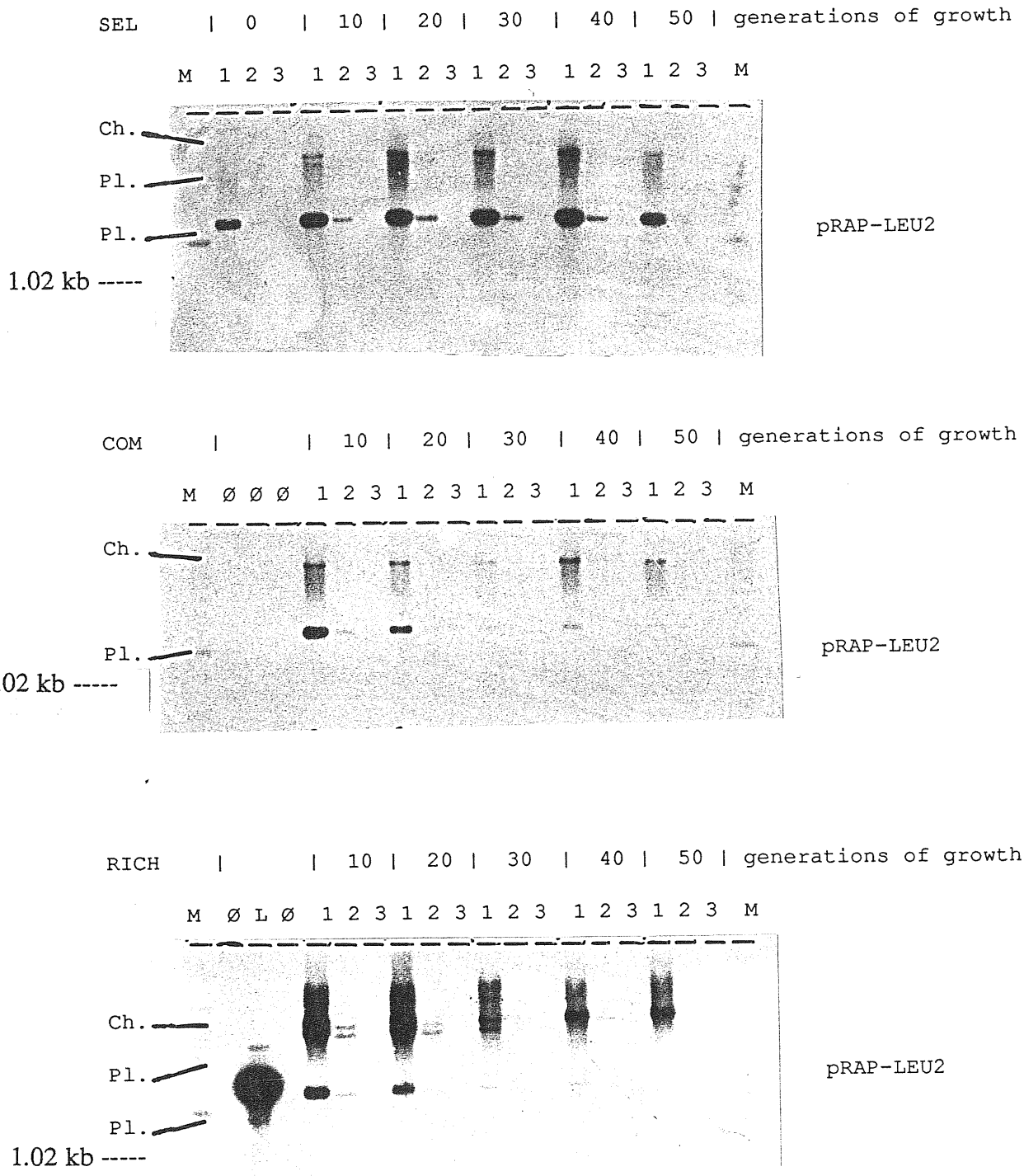


Fig. 3.7 . Southern hybridisation of total yeast DNA from cultures transformed with the pRAP-LEU2 plasmid digested overnight with BamHI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075); the 1.64 kb fragment gives a strong hybridisation signal. **Ø**: empty lane. **1,2, and 3**: different dilutions of restricted total yeast DNA, 1/1, 1/10, and 1/100 respectively. **0-50**: number of generations grown in the specific medium. **L**: 2.2 kb *LEU2*/BamHI gene from pRAP-LEU2, radiolabelled and used as probe for pRAP-LEU2. SEL, COM, and RICH, are different media as described in text. **Ch.**: Chromosomal band. **Pl.**: Plasmid band.

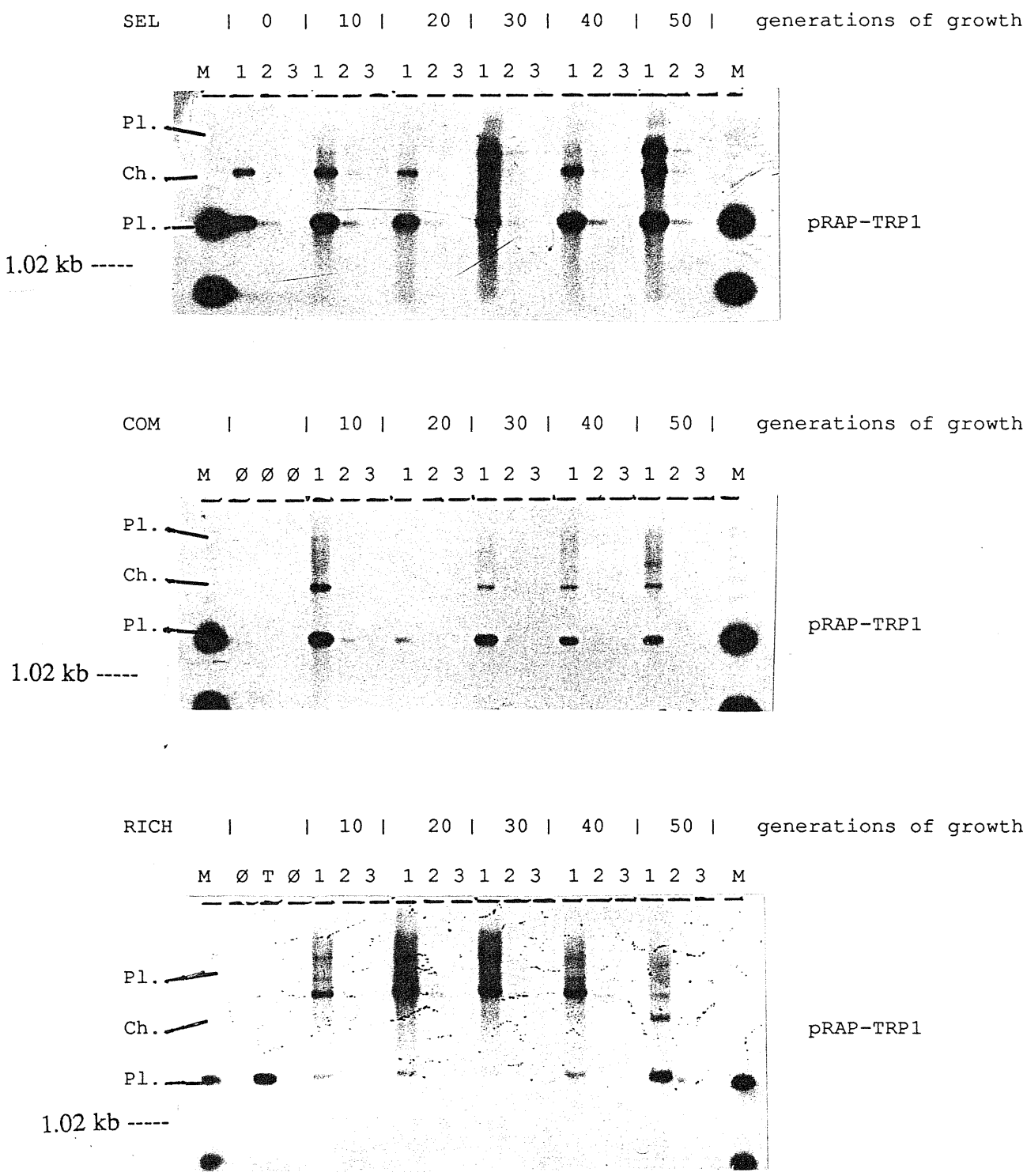


Fig. 3.8 . Southern hybridisation of total yeast DNA from cultures transformed with the pRAP-TRP1 plasmid digested overnight with BamHI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075); the 1.6 kb and 0.51 kb fragments gives strong hybridisation signals. **Ø**: empty lane. **1,2, and 3**: different dilutions of restricted total yeast DNA, 1/1, 1/10, and 1/100 respectively. **0-50**: number of generations grown in the specific medium. **T**: 1.65 kb *TRP1*/BamHI gene from pRAP-TRP1, radiolabelled and used as probe for pRAP-TRP1. SEL, COM, and RICH, are different media as described in text. **Ch.**: Chromosomal band. **Pl.**: Plasmid band.

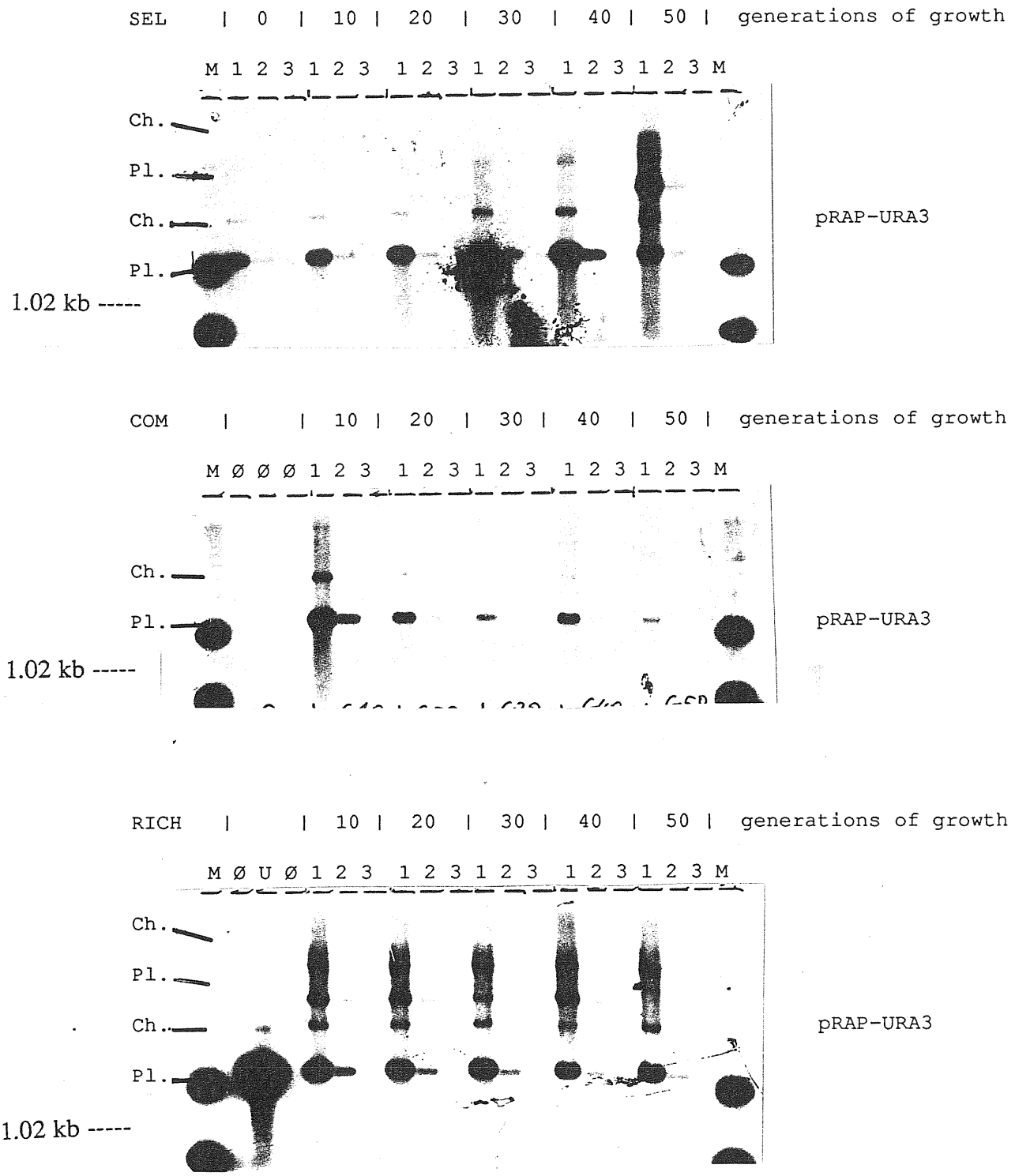


Fig. 3.9 . Southern hybridisation of total yeast DNA from cultures transformed with the pRAP-URA3 plasmid digested overnight with BamHI. **M**: 1kb DNA ladder (kb: 12.22; 11.2; 10.2; 9.16; 8.14; 7.13; 6.11; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.51; 0.4; 0.34; 0.3; 0.22-0.075). **Ø**: empty lane. **1,2, and 3**: different dilutions of restricted total yeast DNA, 1/1, 1/10, and 1/100 respectively. **0-50**: number of generations grown in the specific medium. **U**: 1.95 kb *URA3*/BamHI gene from pRAP-URA3, radiolabelled and used as probe for pRAP-URA3. SEL, COM, and RICH, are different media as described in text. **Ch.**: Chromosomal band. **Pl.**: Plasmid band.

The experiment with the *URA3* marker is shown in Fig. 3.9. Under selective conditions (top panel, SEL) the plasmid, 1.95 kb and 5.7 kb bands (Pl.) corresponding to completely digested and linear plasmid DNAs respectively, is maintained at high level, and the corresponding bands are well evident. When cells are cultured in complete medium (centre panel, COM) the intensity of the plasmid band is still stronger than the chromosomal reference band, thus supporting the data from the plasmid stability experiments. Finally, in rich medium (bottom panel, RICH) the signal from hybridisation to the plasmid-derived DNA is very strong even at the fiftieth generation, thus supporting data from previous experiments.

In summary, qualitative analysis of the Southern hybridisations carried out on the same cultures used for the plasmid stability assays seems to support the results obtained from these experiments.

We were then interested in quantifying data from the these Southern analysis, by means of laser densitometry, as described in section 2.26. Briefly, the value of each area of each band in the Southern hybridisations was determined, and the ratio between the area of the plasmid band(s) over that of the chromosomal band(s) was assumed to reflect the copy number of that plasmid in the haploid genome. However, this assumption is true only when the chromosomal gene and that on the plasmid contribute equally to the intensity of the signal, that is when all the cells harbour their plasmid (stability 100%). In our case, some (or most) of the cells have lost their plasmid, hence the ratio plasmid/chromosomal band underestimates the real copy number of the plasmid species. To overcome this effect, the copy number was normalised to the real stability of the plasmid dividing this ratio by the percentage of plasmid stability.

ps = plasmid stability

p = plasmid copy number intensity value

c = chromosomal normalization number intensity value

$$\mathbf{pn = p/c \cdot 1/ps}$$

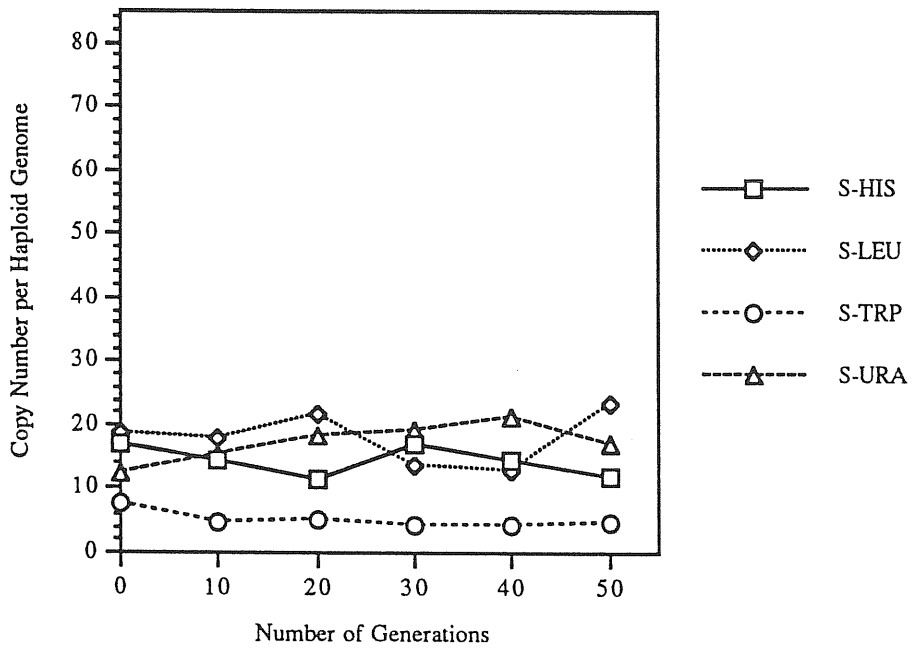
This number reflects the real copy number of that plasmid. Results from this analysis are reported in the Table 3.5, shown below.

Gen.	S-HIS	C-HIS	R-HIS	S-LEU	C-LEU	R-LEU	S-TRP	C-TRP	R-TRP	S-URA	C-URA	R-URA
0	17.18	---	---	18.74	---	---	7.6	---	---	12.42	---	---
10	14.45	50.98	16	17.83	23.96	7.32	4.47	14.79	20.11	15.11	54.86	17.71
20	11.62	42.3	15.51	21.5	14.82	10.63	5.13	22.31	29.87	18.29	56.57	20.3
30	17	38.56	14.61	13.54	27.67	11.81	4.25	20.34	27.93	19.12	75	22.82
40	14.48	39.28	15.17	12.78	20.43	16	4.23	15.64	32.26	21.05	82.31	30.02
50	12	45.91	29.44	23.46	15.29	10.4	4.6	16.2	37.47	16.82	83.26	47.02
X-av.	14.45			17.97			5.05			17.13		
σn	±2.16			±3.87			±1.18			±2.80		

Table 3.5 . Figures from the densitometric analysis of the copy number experiments.

Different graphic displays of these data are presented in the following Fig. 3.10 and Fig. 3.11.

Fig. 3.10



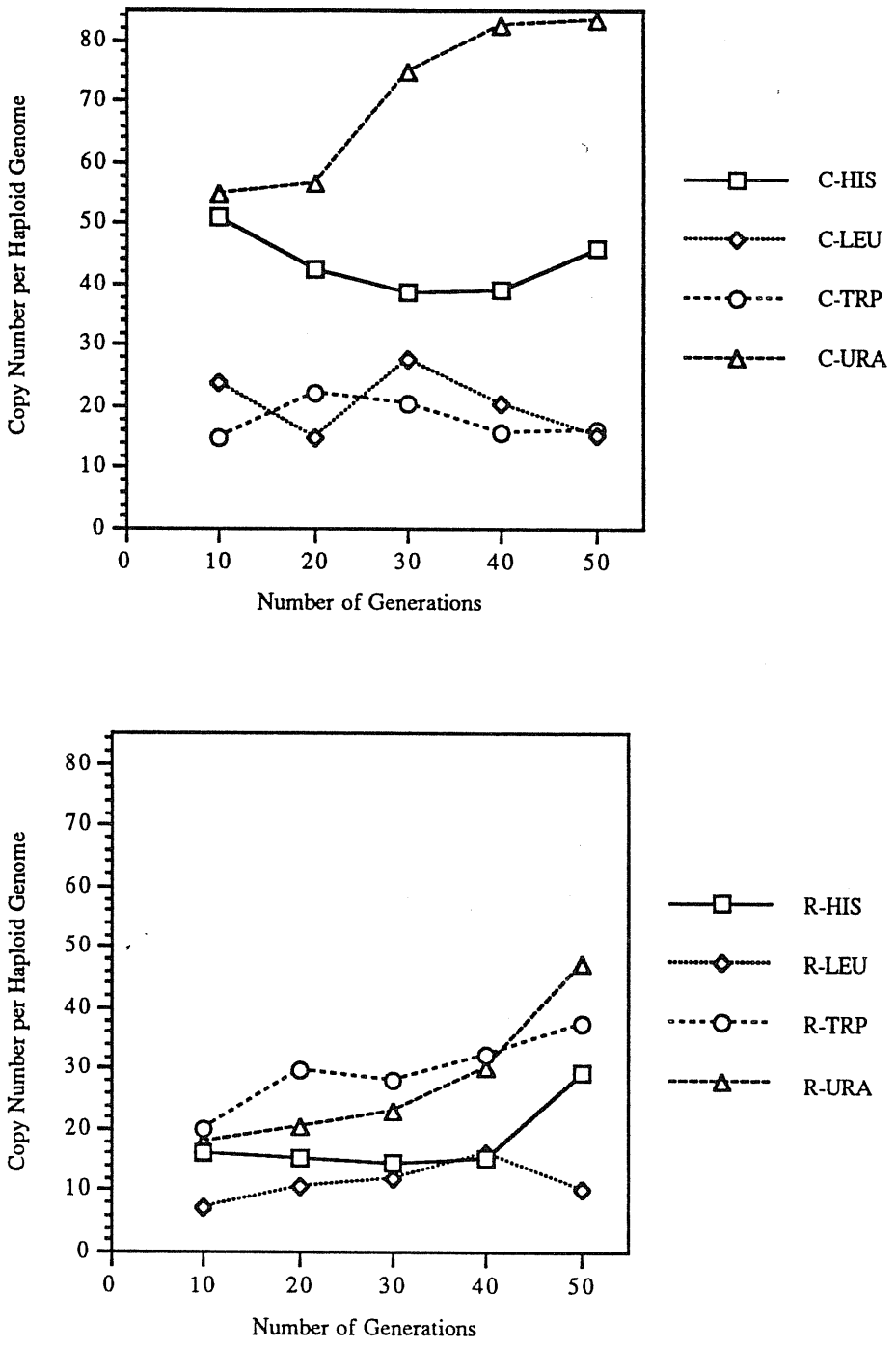
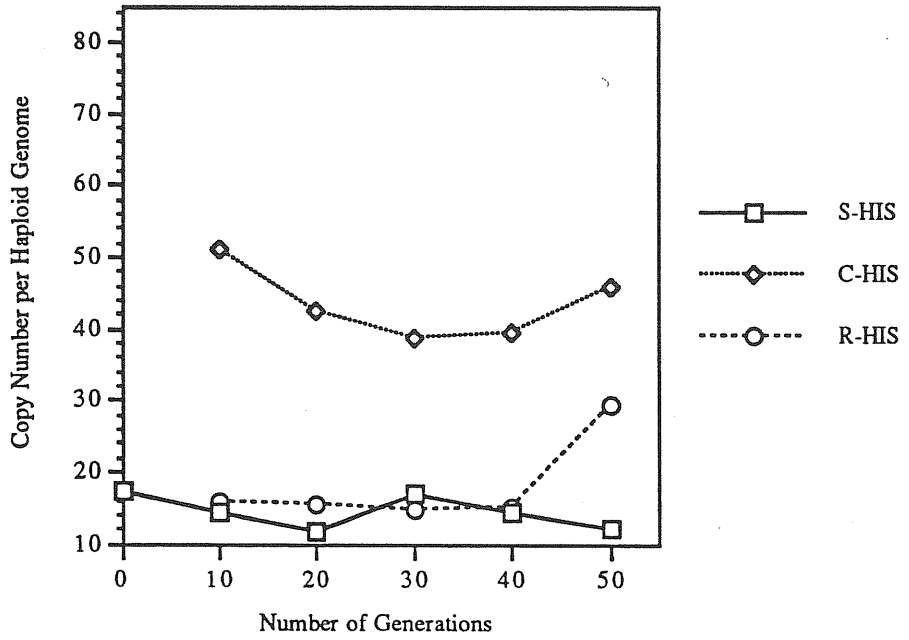
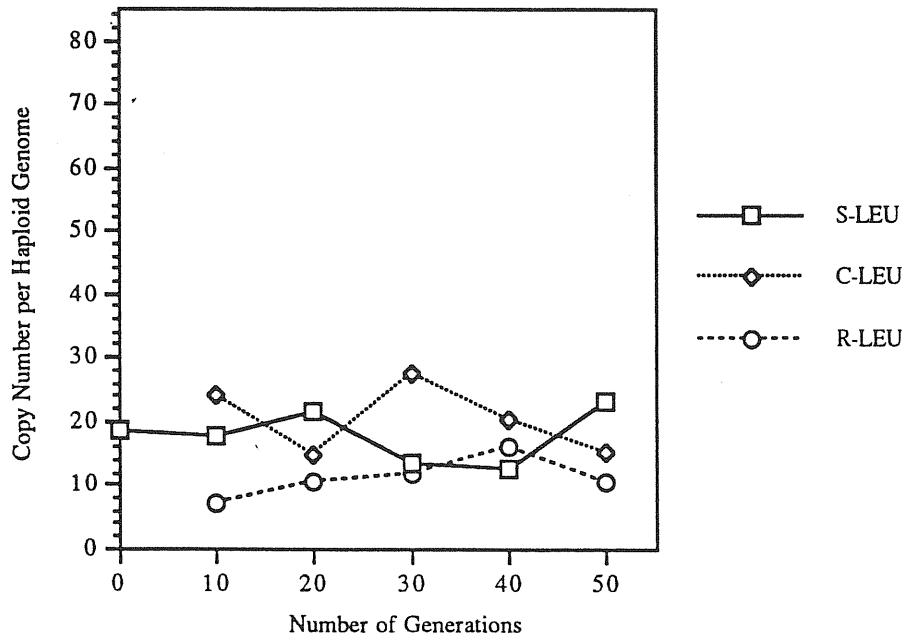


Fig. 3.10 . Results from the copy number determination, grouped according to the type of medium. Data points represent number of copies of plasmid per haploid genome. S, C, and R, are Selective, Complete, and Rich medium respectively. HIS, LEU, TRP, and URA, represent plasmids pRAP-HIS3, pRAP-LEU2, pRAP-TRP1, and pRAP-URA3 respectively.

Fig. 3.11


Copy Number per Haploid Genome
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Number of Generations
S-HIS
C-HIS
R-HIS


Copy Number per Haploid Genome
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Number of Generations
S-LEU
C-LEU
R-LEU

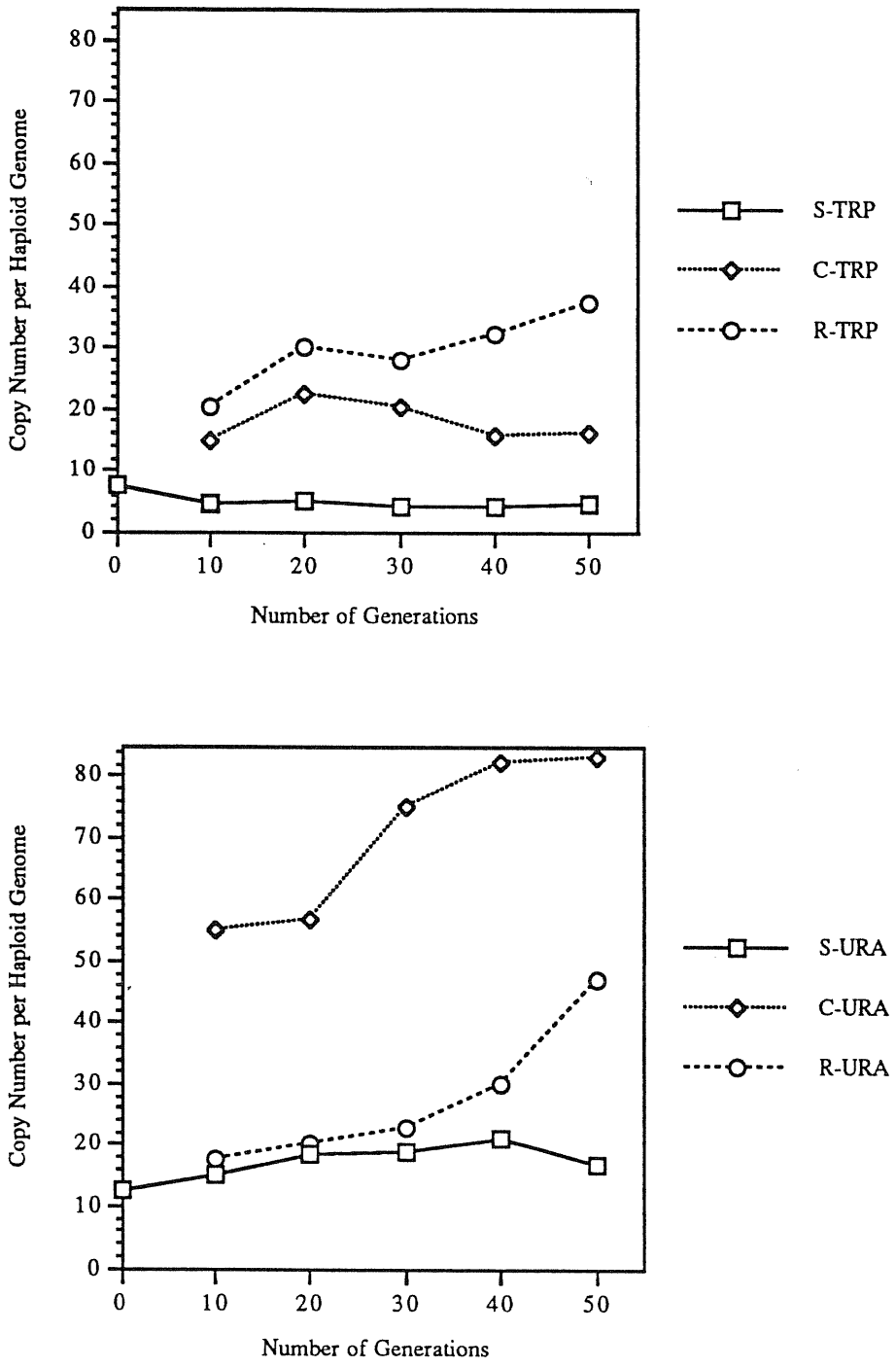


Fig. 3.11 . Results from the copy number determination, grouped according to the type of plasmid. Data points represent number of copies of plasmid per haploid genome. S, C, and R, are Selective, Complete, and Rich medium respectively. HIS, LEU, TRP, and URA, represent plasmids pRAP-HIS3, pRAP-LEU2, pRAP-TRP1, and pRAP-URA3 respectively.

Graphic representations of the densitometric analysis of the Southern hybridisation presented above have been grouped in different ways to better reveal similarities and differences among the various plasmid species. In Fig. 3.10 is shown the result of the determination of the copy number in selective conditions (S panel). It is evident that all type all plasmids are maintained in a quite low copy number, with averages of 14.4 for pRAP-HIS3, 18.0 for pRAP-LEU2, 5.0 for pRAP-TRP1, and 17.1 for pRAP-URA3. Interesting to note, the copy number for the pRAP-TRP1 plasmid seems particularly low compared to that of the other vectors, while its stability is relatively high (Table 3.3, X-av. = 62.9). This situation could be clarified by the fact that the marker located on the chromosome can revert, a particularly favourable event under selective conditions, hence accounting for the high prototrophic values and the very low copy number.

The situation in complete medium (C panel) is quite different, where two distinct groups, low and high copy number, are defined. In fact, The pRAP-TRP1 and pRAP-LEU2 constructs show a similar behaviour as in selective medium, with an average copy number of roughly 20. The pRAP-HIS3 and pRAP-URA3 vectors show a considerably higher copy number, of approximately 40 and 70 respectively. This high level plasmid copy number, for these episomal vectors, favours the propagation of the plasmid to the daughter cell, thus increasing the probability to be transmitted to the progeny.

In rich medium (R panel), the pRAP-TRP1 and pRAP-URA3 constructs exhibit the highest copy number, which correlates well with their highest stability in this medium (Table 3.3). Conversely, the pRAP-HIS3 and the pRAP-LEU2 vectors show a lower copy number, again in accordance with their lower stability. The quite low copy number of episomal plasmids in a 2μm-containing host cell has been reported in the literature, and seems to be due to competition among the two types of molecules (Futcher and Cox, 1984). The results from these experiments confirm the notion that plasmids that are maintained at high copy number, are more stable than those kept at lower copy (Futcher and Cox, 1984), therefore indicating that loss is a random phenomenon due to fluctuation in copy number, rather than a discrete, genetically determined event. In this case, in fact, copy number would not matter and loss would occur regardless to it.

3.5 Results from Competition Experiments

Results from competition experiments in non selective complete and rich media (COM and YPD)

Competition experiments in non selective complete (COM) and rich (YPD) media were carried out as described in section 2.27 of Materials and Methods. After replica-plating onto selective media, the number of prototrophs was determined, and the sum of these was taken as reference (100%) for the calculation of the relative percentage of appearance of the each possible phenotype. Results from this analysis are reported in Table 3.6.

Plasmid Species After Co-culture experiment in Complete (COM) Medium:

N° of Generations	His+ (%)	Leu+ (%)	Trp+ (%)	Ura+ (%)	Total N° of Prototrophs (100%)	Total N° of Cells plated
0	27.15	22.11	22.11	28.63	674	921
10	26.00	17.54	28.92	27.54	799	1334
20	25.0	6.13	27.83	41.04	212	996
30	15.58	0.97	30.87	52.58	206	1384
40	8.53	1.28	28.57	61.62	469	4573
50	3.55	0	27.55	68.89	225	3387

Plasmid Species After Co-culture experiment in Rich (YPD) Medium:

N° of Generations	His+ (%)	Leu+ (%)	Trp+ (%)	Ura+ (%)	Total N° of Prototrophs (100%)	Total N° of Cells plated
0	27.15	22.11	22.11	28.63	674	921
10	28.53	16.43	25.65	29.39	347	608
20	26.14	16.34	32.68	24.84	681	965
30	26.96	12.35	35.30	25.39	575	1884
40	24.38	11.23	36.72	27.67	365	2800
50	16.97	7.34	48.62	27.07	218	3580

Table 3.6 . Figures from the competition experiments in non selective conditions.

At the beginning of the experiment (Table 3.6, G0), all plasmid species are represented in roughly the same proportions, although plasmids pRAP-HIS3 and pRAP-URA3 are slightly above the expected 25%. Right after the first round of growth, the relative proportions have changed, and the pRAP-LEU2 species appears to drop quite below the level of the other constructs, both in complete and YPD medium. This decline persists for the rest of the experiments, with the plasmid completely disappearing in complete medium, while reaching the lowest level of 7.3% in the case rich medium. The pRAP-HIS3 plasmid is also competed out in complete medium, though at a much slower rate, dropping to 3.5% at the end of the serial growth (G50). However, in rich medium this construct proves to be more stable, still representing the 17.0% of the total population of prototrophs.

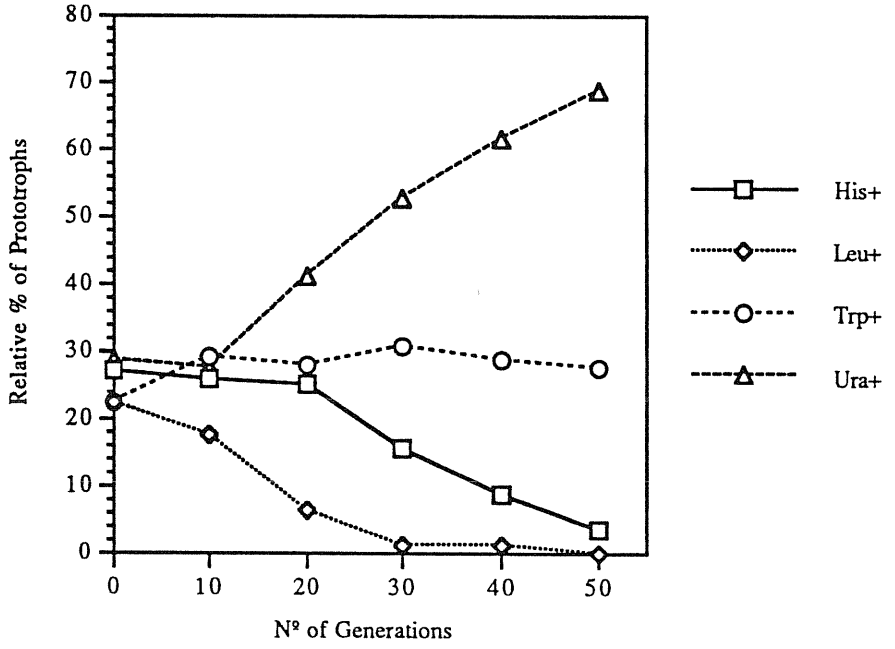
The other two plasmid species, pRAP-TRP1 and pRAP-URA3, are very stable in both type of media, never dropping below the input level. In fact, plasmid pRAP-TRP1 remains around 25%, slightly increasing in the last two rounds of growth in complete medium (G40 and G50). In rich medium this construct represents the majority of the population, attaining at the end of the experiment the 48.6% of all prototrophs. The pRAP-URA3 plasmid is extremely stable in complete medium largely competing out all the other forms. In rich YPD medium this species is also very persistent, being represented at the same level of the initial culture. It is important to stress the fact that these values do not represent absolute stability values, which instead are reported in Fig. 3.4. This fact becomes clear if we observe that to score for the same number of prototrops, an increasing number of cells had to be plated at each successive round of growth. Furthermore, the total number of cells plated is increasingly higher with the progression of the experiment, hence indicating the consistent plasmid loss in non selective conditions.

The above results clearly indicate that after the co-culture experiments, both in complete and YPD medium, the CBU1-31 strain carrying the pRAP-LEU2 plasmid is underrepresented in the population, hence strongly authenticating the results obtained with the stability experiments. The high permanence of the pRAP-TRP1 and pRAP-URA3 plasmids in long-term cultures is also a valid confirmation that these two constructs are preferentially maintained in the strain CBU1-31. It is quite interesting to note that, as previously observed for the plasmid stability and copy number experiments, there is a clear different behaviour in the two non selective media. It appears, in fact, that while the release of selection in COM medium results in the progressive loss of the plasmid, in

rich YPD medium it does not, at least with the same rapidity. This is indicating that selection for the marker is not the only physiological conditions affecting its episomal stability. These results are presented in the graphic display shown in Fig. 3.12.

Fig. 3.12 .

a)



b)

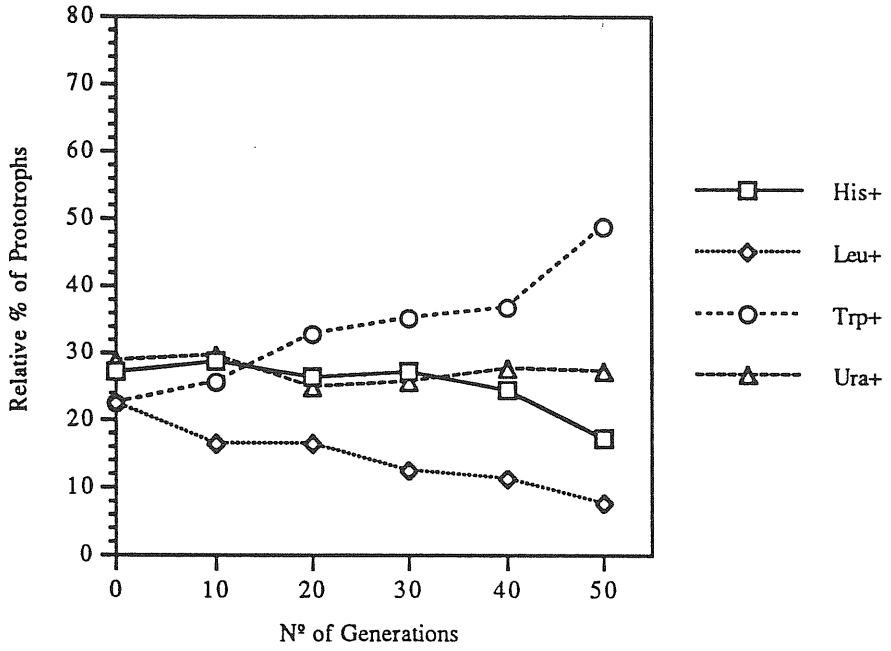


Fig. 3.12 . Graphic representation of the data presented in Table 3.6. Percentage of the phenotypes (His+, Leu+, Trp+, and Ura+) are calculated on the total number of prototrophs (100%). Relative % of plasmid species after co-culture experiments in complete (COM) medium (a), and in rich (YPD) medium (b).

Competition experiments in selective medium (SEL), using *ADE8* as reporter gene

These competition experiments in selective medium were carried out to test the feasibility of the use of a non selective reporter gene to detect differences in plasmid maintenance and the effect of selection on the cellular metabolism. We decided to use *ADE8* as reporter gene, because it allows the phenotipic expression of a red-pigment color in a cell carrying a mutation in the *ADE2* gene, in this case an *ad hoc* gene disruption generated in this work (*ade2::LEU2* or *ade2::URA3*; Fischer, 1969). As described in section 2.6 of Materials and Methods, we constructed a non functional ADE8 gene, *ade8-xp*, which leaves the cells white in the *ade2* yeast strain. The markers under study, the *LEU2* gene and the *URA3* gene, were associated both to the functional *ADE8* gene, and to the non functional *ade8-xp* gene, in centromeric or episomal plasmids. Following transformation in appropriate strains (CBU1-31 *ade2::LEU2* or CBU1-31 *ade2::URA3*), these associations of genes confers to the host cell a unique phenotype, depending on the particular combination of genes, therefore making it detectable among the others. Table 3.7 summarises all the possible phenotypes deriving from these transformations.

	Markers on plasmid	phenotype in plasmid + cells	phenotype in plasmid - cells
YCpLA (and YEpLA)	*LEU2 ADE8*	Leu$^+$ Red Ura$^+$	Leu$^-$ White Ura$^+$
YCpUA* (and YEpUA*)	*URA3 ade8-xp*	Leu$^+$ Ura$^+$ White	Leu$^+$ Ura$^-$ White
YCpUA (and YEpUA)	*URA3 ADE8*	Leu$^+$ Ura$^+$ Red	Leu$^+$ Ura$^-$ White
YCpLA* (and YEpLA*)	*LEU2 ade8-xp*	Leu$^+$ White Ura$^+$	Leu$^-$ White Ura$^+$

Table 3.7 . Scheme of the possible phenotypes deriving from the presence or loss of the different plasmid species in the appropriate host cells. Underlining indicates phenotypes deriving from genes located on plasmid.

The competition experiment were designed as specified in section 2.27. The combinations of strains and plasmids used are reported in the Table below:

C/LU*: RED CBU1-31 *ade2::URA3* [YCpLA] in co-culture with the WHITE CBU1-31 *ade2::LEU2* [YCpUA*].

C/UL*: WHITE CBU1-31 *ade2::URA3* [YCpLA*] in co-culture with the RED CBU1-31 *ade2::LEU2* [YCpUA].

E/LU*: RED CBU1-31 *ade2::URA3* [YEpLA] in co-culture with the WHITE CBU1-31 *ade2::LEU2* [YEpUA*].

E/UL*: WHITE CBU1-31 *ade2::URA3* [YEpLA*] in co-culture with the RED CBU1-31 *ade2::LEU2* [YEpUA].

Table 3.8 . Scheme of the combinations of the co-culture experiments. A* indicates the presence on the plasmid of the non functional *ade8-xp* gene.

Three independent transformants for each type of plasmid (YCpLA, YCpLA*, YEpLA, YEpLA*, YCpUA, YCpUA*, YEpUA, and YEpUA*) were purified by streaking onto selective medium for several rounds, and purified single colonies were then amplified by growing into 5 ml of selective medium up to stationary phase. Equal amounts of cells were mixed in the combinations described above and this mixture was used to inoculate three parallel cultures (G0) in selective medium (SEL). The initial density was $5x10^4$ cells/ml; aliquots of this initial cultures (G0) were immediately plated onto YPD plates. Following growth at 30°C, these plates were replicated onto selective Leu- and Ura- plates to determine the stability at time zero (G0). Cultures were grown as already described, and values of plasmid stability were calculated at the fiftieth generation (G50). Results from these competition (co-culture) experiments are reported in the Table 3.9 below. Control experiments were prepared by growing individual cultures of the same transformed strains.

Phenotypes resulting from the co-culture experiments

	Leu$^+$ R Ura$^+$ (%)		Leu$^-$ W Ura$^+$ (%)		Leu$^+$ Ura$^+$ W (%)		Leu$^+$ Ura$^-$ W (%)	
	G0	G50	G0	G50	G0	G50	G0	G50
C/LU*	26.07	3.63	21.98	20.17	43.36	66.24	8.59	9.96
	(±6.45	±3.54)	(±6.29	±6.44)	(±2.13	±5.96)	(±0.64	±3.42)
E/LU*	28.08	1.54	14.04	0	48.02	83.96	9.86	14.50
	(±4.07	± - - -)	(±7.24	± - - -)	(±6.27	±7.58)	(±0.11	±8.35)
	Leu$^+$ Ura$^+$ R (%)		Leu$^+$ Ura$^-$ W (%)		Leu$^+$ W Ura$^+$ (%)		Leu$^-$ W Ura$^+$ (%)	
	G0	G50	G0	G50	G0	G50	G0	G50
C/UL*	33.49	2.29	12.27	14.15	45.33	76.98	8.91	6.58
	(±9.94	±1.24)	(±5.01	±3.56)	(±4.90	±3.02)	(±1.39	±0.70)
E/UL*	41.15	6.50	2.41	6.05	46.34	80.89	10.10	6.56
	(±5.10	±4.05)	(±0.62	±3.32)	(±4.50	±4.40)	(±1.43	±4.0)

Table 3.9 . Plasmid stability resulting from the competition experiments. C/LU* represents the co-culture experiment of CBU1-31 A2U3 [YCpLA] with CBU1-31 A2L2 [YCPUA*]. C/UL* is the opposite experiment in which the *LEU2* gene is associated with the non-functional frameshifted *ade8-xp* gene. E/LU* and E/UL* are the same experiments with the episomal versions of the plasmids. Underlining indicates phenotypes deriving from genes located on plasmid. Figures represent the percentage of appearance of the given phenotype. Numbers in brackets represent standard deviation σ_n.

Individual cultures were also processed in the same way, to determine the rate of plasmid loss without competition. Results are reported in the table below.

	Phenotypes							
	Leu$^+$ R Ura$^+$ (%)		Leu$^-$ W Ura$^+$ (%)		Leu$^+$ Ura$^+$ W (%)		Leu$^+$ Ura$^-$ W (%)	
	G0	G50	G0	G50	G0	G50	G0	G50
YCpLA	83.22	84.29	16.78	15.71	---	---	---	---
YEpLA	77.71	86.40	22.29	13.60	---	---	---	---
YCpUA*	---	---	---	---	72.58	76.22	27.42	23.78
YEpUA*	---	---	---	---	80.62	89.65	19.38	10.35
	Leu$^+$ Ura$^+$ R (%)		Leu$^+$ Ura$^-$ W (%)		Leu$^+$ W Ura$^+$ (%)		Leu$^-$ W Ura$^+$ (%)	
	G0	G50	G0	G50	G0	G50	G0	G50
YCpUA	82.39	75.84	17.61	24.16	---	---	---	---
YEpUA	77.30	92.50	22.70	7.50	---	---	---	---
YCpLA*	---	---	---	---	84.39	86.30	15.61	13.70
YEpLA*	---	---	---	---	79.87	86.36	20.13	13.64

Table 3.10 . Plasmid stability from individual cultures transformed with the plasmid indicated.

At the beginning of the C/LU* co-culture experiment - Table 3.9 - the relative percentage of cells with phenotype indicative of the presence of the initially red strain, with or without the centromeric plasmid (Leu$^+$ R Ura$^+$ and Leu$^-$ W Ura$^+$), accounts for roughly the half of the total population 48.0% (G0; 26.07% + 21.98%), as expected for this initial step. The other two possible phenotypes in this experiment, deriving from the initially white strain (Leu$^+$ Ura$^+$ W and Leu$^+$ Ura$^-$ W), represent the other half, roughly 51.9% (G0; 43.36% + 8.59%). Thus, the initial inocula were about equal, though plasmid loss was already very different at this stage. In fact,

within the red strain only roughly 54.3% of cells (Leu$^+$ R Ura$^+$) still harbor the plasmid (G0; 26.07% out of 48.05%), while in the white strain most of cells carry the plasmid (Leu$^+$ Ura$^+$ W), roughly 83.5% (G0; 43.36% out of 51.95%). At the second plating (G50) most of the population, 76.2%, is represented by cells coming from the white strain (66.24% + 9.96%). The number of cells from the red transformants, either with or without plasmid, only represent the 23.8% of the population (3.63% + 20.17%). Therefore, these have lost the centromeric plasmid, maintaining it only in the 3.6% of the population (G50; Leu$^+$ R Ura$^+$), while the number of white transformants with plasmid (Leu$^+$ Ura$^+$ W) represents the majority of the population, reaching the value of 66.2%. Thus, while cells from the red transformants loose their plasmid - and their relative proportion decreases - those from the white transformants that carry it show a selective advantage, outgrowing their competitors. The percentage of cells without plasmid from both strains remains constant (G0-G50; Leu$^-$ W Ura$^+$ and Leu$^+$ Ura$^-$ W). This behaviour is also evident for the other experiments shown below.

In the experiment with the reverse combination of markers (C/UL*), inocula values for the red and white strains are more distant from the expected value of 50%, i. e. 45.8% and 54.2%, respectively. Somewhat higher initial values are found in the percentage of plasmid-containing cells in the red transformants (Leu$^+$ Ura$^+$ R), approximately 73.2% (G0; 33.49% out of 45.76%). Plasmid-containing cells in the white transformants (Leu$^+$ W Ura$^+$) represent the 83.6% of the population (G0; 45.33% out of 54.24%), a very close value to that found in the previous competition experiment with the opposite combination of markers. At G50, the majority of cells in the population - 76.98% (Leu$^+$ W Ura$^+$) - are represented by white transformants containing the YCpLA* plasmid, which together with the 6.58% that have lost it (Leu$^-$ W Ura$^+$), account for the 83% of the population. Again, red cells carrying the YCpUA plasmid (Leu$^+$ Ura$^+$ R) are competed out by white cells, dropping from 33.49% to 2.29%. As noted above, the number of cells from both strains without plasmid varies very little (G0-G50; Leu$^+$ Ura$^-$ W and Leu$^-$ W Ura$^+$).

In the case of the E/LU* competition experiment involving episomal plasmids, initial inocula values are the most divergent, 42.1% for the red and 57.9% for the white transformants. The situation of plasmid maintenance is similar to that of the centromeric experiments, with the 66.7% (G0; 28.08% out of 42.12%) of the red transformants carrying the plasmid (Leu$^+$ R

Ura$^+$), and the 83% (G0; 48.02% out of 57.88%) of the white transformants (Leu$^+$ Ura$^+$ W). The situation after fifty generations (G50) is similar to what previously observed. In fact, 83.96% of the population is formed by white transformants carrying the YEpUA* plasmid (Leu$^+$ Ura$^+$ W), while red cells have almost disappeared (1.54%). Interestingly, in this case the number of white cells (G50; Leu$^-$ W Ura$^+$) deriving from the red transformants does not remain constant, but drops to zero. White cells that have lost their plasmid slightly increase in number (Leu$^+$ Ura$^-$ W).

In the last competition experiment, E/UL*, the percentage of cells after the initial inoculum is 43.6% for the red transformants and 56.4% for the white one. However, in contrast to the previous results, the input plasmid is present in 94.4% of the cells (Leu$^+$ Ura$^+$ R) coming from the red transformants (G0; 41.1% out of 43.6%), and in 82.2% of those from the white transformants (G0; Leu$^+$ W Ura$^+$; 46.3% out of 56.4%). The value of plasmid stability for the white strain is in agreement with our previous results, in average 83% at G0 for the four experiments. In this last example, the percentage of plasmid-containing red transformants is much higher than observed for the previous cases, on average about 72% at G0 for the four experiments. Finally, at G50 the portion of plasmid-containing white transformants is about 80.9%, while that of red cells is only 6.5%. The behaviour of the other phenotypes is very similar to that described above.

Thus, in all cases, cells carrying the *ADE8* gene on the plasmid tend to loose this construct at an higher rate than cells carrying the *ade8-xp* gene, independently from the association of markers (*LEU2* or *URA3*).

One possible explanation for this behaviour could be that the *ADE8* gene allows the accumulation in *ade2* (and *ade1*) cells of the intermediate phospho-ribosylamino imidazole (AIR), substrate for the activity of the *ADE2* gene product, whose gene has been disrupted in our strain (*ade2::LEU2* or *ade2::URA3*). The red pigment that accumulates in *ade2* (and *ade1*) mutant derives from an oxidation of this intermediate (Woods, 1969). This compound contains an imidazole ring, which, upon accumulation, could have toxic effects on the cellular metabolism. Red cells that loose the *ADE8*-containing plasmid or white cells that carry the non functional *ade8-xp* allele, would have a growth advantage over cells accumulating the red pigment. This advantage of white cells over red cells can be also noted in cells carrying both mutations in the chromosomes. A second possibility could be that cells carrying the *ADE8* gene on a centromeric plasmid do not

become clearly deep red as those with that gene on an episomal vector, remaining most of the time pale pink, even after storage at +4°C in the presence of oxygen, condition that develops a full colour. This situation, together with the fact that real prototrophs cannot always be readily distinguished from outgrown auxotrophs in the replicated plate, could have led to an overestimate of the white cells over red cells, thus providing a simpler interpretation of these data.

Apart from these considerations, it is evident that in all cases in which red cells whose selectable marker on the plasmid, *LEU2* or *URA3*, is associated with the *ADE8* gene, are outgrown by white cells, which may not be impaired by accumulation of the red pigment. In fact, this becomes evident analysing the increased proportion of Leu^+ $\underline{Ura}^+$ $\underline{W}$ cells at the end of the experiments in the cases of C/LU* (66.2%) and E/LU* (84.0%) competitions, and the number of $\underline{Leu}^+$ $\underline{W}$ Ura^+ in the case of the C/UL* (77.0%) and E/UL* (80.9%) control experiments.

The results shown above indicate that the choice of the *ADE8* gene, in a *ade2* background, did not help to clarify whether the presence on a multicopy plasmid of a selectable marker could affect the cellular metabolism depending on the nature of the marker. On the other hand, we demonstrated that, independently from the marker used for the selection, the accumulation of the pigment intermediate strongly impairs cell growth, thus indicating that the use of the red/white scoring system is not neutral, in the sense that red cells may resent from the accumulation of the red compound in the vacuole (Fischer, 1969), while white cells can growth at the usual growth rate. This result has very important implications for the choice made by several authors of the red/white colony color assay as an impartial system for detection of genetic events leading to a change in such phenotype, as discussed later.

4. DISCUSSION

4.1 Dependence of the curing process on the genetic background of the host cell.

The existing evidence on host-encoded factors that influence the maintenance of 2μm and 2μm-derived vectors, is in agreement with our results regarding the dependence of the curing process on the genetic background of the host cell. In fact, different yeast strains cured with the same methodology in our laboratory gave very different curing efficiency. Some strains could be cured in a very short time (Bruschi and Howe, 1988; Ludwig and Bruschi, 1991), while in the case of this study we could not obtain 2μm-free cells even after serial passages on selective media for many generations, as described in the literature for different strains (Futcher and Cox, 1984). Thus, the CBU1-31 strain could present alleles in the genome that stabilise the endogenous 2μm plasmid. A wide comparative study on the ability of different strains to be cured of the endogenous 2μm plasmid would be of great help for the quantitation of this phenomenon. The identification of the host-encoded factor(s) regulating the process of curing - together with the availability of mathematical models (Anderson and Lustbader, 1975; Summers, 1991; Van der Sand et al., 1995) - would provide new tools for the prediction and control of plasmid maintenance in *Saccharomyces cerevisiae.*

4.2 Fitness of different selectable markers

Analysis of the growth curve indicates that there are no detectable differences in the growth rate of the transformed cultures with the four different plasmid types neither when selective pressure is applied (SEL), nor in the absence of selection (COM and YPD). Thus, the presence, replication and expression of either the *HIS3*, *LEU2*, *TRP1*, or *URA3* genes in a multicopy plasmid seems not confer any particular growth advantage or disadvantage to the host cell. This uniformity in the growth rate of the different transformant cultures is very important for plasmid maintenance. In fact, it has been shown that - in the absence of selective pressure - a strong reduction in the growth rate leads to rapid plasmid loss (Kleinman et al., 1986; Bugeja et al.,

1989). The fact that the non transformed cultures grow faster than the transformed ones supports the idea that the maintenance of plasmids inside the cell, in addition to the nuclear genome, is an event requiring more energy than the maintenance of the genome alone. Hence, the importance of optimising the construction of artificial plasmids, to avoid sequestering cellular resources and interfering heavily with the normal host growth rate and metabolism (Godwin and Slater, 1979; Walmsley et al., 1983; Caulcott, 1984; Summers, 1991).

Significant differences are observed instead for the level of plasmid stability when these different genes are used in the same genetic context (Fig. 3.4). Plasmids pRAP-URA3 and pRAP-TRP1 appear to be the most stable under all conditions (Fig. 3.4), while pRAP-HIS3 and pRAP-LEU2 are consistently maintained at a lower level. In particular, plasmid pRAP-LEU2 represents the worst example of this series, being lost at the highest rate. Under selective conditions, the difference between the percentage of maintenance of pRAP-URA3 and pRAP-TRP1 at different generation times compared to pRAP-LEU2 - the best and the worst performers - is quite consistent, reaching the values of approximately 16% and 12% respectively. This difference is also evident in complete medium, pRAP-URA3 being roughly 10% more stable than pRAP-LEU2; the best performer, pRAP-TRP1, is even more stable, of a value of about 14% in average. Such situation holds for the stability in rich medium, where pRAP-URA3 and pRAP-TRP1 are more stable than pRAP-LEU2 of about 12% and 14% respectively. Clearly, the *LEU2* gene is not a good candidate for the construction of episomal plasmids, i.e. plasmids to be used for the construction of DNA libraries. The pRAP-HIS3 vector behaves quite well only under selective conditions, showing a stability level - roughly 59% - similar to that of pRAP-TRP1. However, in complete and rich medium its percentage of loss corresponds to that of the pRAP-LEU2 plasmid.

These results are reinforced by the data obtained with the competition experiments in non selective conditions, clearly demonstrating that the plasmid carrying the *LEU2* gene is quickly competed out from the population. Again, the *URA3* and *TRP1* genes are the most stable DNA sequences in complete medium and perform well in rich medium, together with the *HIS3* gene (Fig. 3.12). The high stability of the pRAP-TRP1 and pRAP-URA3 plasmids in long-term cultures is a further confirmation that these two constructs are preferentially maintained in the strain CBU1-31. It is interesting to note that the complete supplement (COM) appears to be a type of medium more "stressing" than YPD, being able to evidentiate subtle differences in the fitness

between plasmids carrying various genes. In fact, the *HIS3* gene is more stable in COM than in YPD. This may be due to the fact that complete supplement is missing some still undefined growth-promoting substances which are available in YPD medium (Rose et al., 1990; Difco manual, 1994), and are fundamental for optimal growth rates. This phenomenon is clearly shown in the growth curve experiments (Fig. 3.3).

How to explain the difference in maintenance of these genes? One possible explanation can be that the behaviour of these genes may reflect a different degree of molecular evolution of codon usage, resulting from different levels of selective constraints (Sharp and Li, 1987). The idea is then that of an inverse relationship between the rate of evolution and the degree of selective pressure, as originally proposed in the neutral theory of molecular evolution by Kimura (1983). Thus, genes that are under a strong selective pressure adapt codon usage for a "optimal" translation efficiency, using codons that are recognised by the most represented tRNA species available in the organism (Sharp and Cowe, 1991). To this regard, there is evidence that synonymous substitution in protein-coding genes occurs at a slower rate than substitution in pseudogenes (Li, et al., 1981). Thus, assuming that evolutionary differences between genes are reflected in the codon usage bias, genes which have less constraint present higher variability in the use of synonymous substitutions. A clear positive correlation between the degree of codon bias - expressed as Codon Adaptation Index value (CAI, Sharp and Li, 1987) and/or Codon Bias Index value (CBI, Bennetzen and Hall, 1982)- and the level of gene expression has been well established, showing that genes that are transcribed at an high rate show a strong bias for those codons highly homologous to the anticodons of the major yeast isoacceptor tRNA species (Bennetzen and Hall, 1982; Sharp and Li, 1986; Sharp et al., 1986; Sharp and Cowe, 1991; Lloyd and Sharp, 1993). A mRNA presenting this type of bias may present particularly favourable properties at the level of mRNA translation efficiency, and/or mRNA secondary structure and stability, a possibility suggested by several authors (Bennetzen and Hall, 1982; Ikemura, 1982). The CAI and CBI values for the genes examined in this study is the following (Sharp and Cowe, 1991):

	CAI	CBI
HIS3	0.09	0.01
LEU2	0.44	0.60
TRP1	0.12	0.05
URA3	0.18	0.21

For comparison, the CAI value of the highly expressed yeast ribosomal protein genes is 0.53-0.91, while the lowly expressed regulatory gene *GAL4* has a CAI value of 0.12, and, interestingly, the CAI values for the genes on the very stable 2μm plasmid is 0.99-0.11 (Sharp and Li, 1987). The CAI and CBI values of our genes of interest correlates with their observed low level of transcription, typical of amino acid- or nucleotide-biosynthesising enzymes (*HIS3*, Struhl, and Davis 1981;*LEU2*, Andreadis et al., 1984; *TRP1*, Kim et al., 1986; *URA3*, Rose and Botstein, 1983). Interestingly, different authors have documented the association of actively transcribed genes with the nuclear matrix (Robinson et al., 1983; Hutchinson and Weintraub, 1985; Jackson and Cook, 1985), suggesting that transcription may lead to unequal partitioning of a gene located on plasmid molecule due to its association with the mother nucleus. To this regard, it is relevant to note that a lowly transcribed TRP1-*ARS1* plasmid can be destabilized by the insertion of pBR322 sequences (Stinchcomb et al., 1979), which in yeast are heavily transcribed (Marczynski and Jaehning, 1985). A similar effect has been also reported for 2μm derivatives containing pBR322 sequences (Dobson et al., 1980b; Toh-e et al., 1980; Futcher and Cox, 1984;). In this regard, it is important to note that lower CAI values of these genes correlate with higher plasmid stability values (Fig. 3.4). The lower value of the CAI of the gene (*URA3* and *TRP1*), the highest the stability of the plasmid carrying that same gene (pRAP-URA3 and pRAP-TRP1), both in selective conditions, and in non selective media. The highest CAI value is that of the *LEU2* gene, suggesting that this gene has been subject to higher selective constraint in the evolutionary process. The other genes present a remarkably lower bias in the use of the codon, indicating an higher degree of freedom. Our hypothesis is that a strong bias for the codon usage may represent a disadvantage in conditions of high metabolic stress, when the pool of amino acids is low and tRNA uncharged, a situation typical of starvation. Thus, genes which are generally expressed at lower rate and with higher versatility for the codon usage may be favoured when the pool of charged tRNAs is decreasing. In these conditions highly expressed genes - such as the

approximately 140 genes for ribosomal proteins - may titrate out the pool of one species of tRNA, thus stalling the translation of our most codon-biased gene of interest, *LEU2*. Therefore, under selective conditions a gene with higher versatility for the codon use (low CAI, CBI) - i.e. *URA3* - may still be efficiently translated by using a wider set of tRNAs than *LEU2*. One way to test this hypothesis could be to set up similar stability experiments as above, but with genes - in the respective biochemical pathway - with different CAI indexes, and see whether they differ in plasmid stability values.

In addition to what discussed above, the difference in stability observed in non selective conditions (COM and YPD) may simply reflect the natural stability resulting from the phase of culture amplification in selective medium, condition in which there was coupling between maintenance of the marker and its transcription and translation for the production of the necessary gene product. In fact as indicated above, plasmids are lost in the same proportions relative to each other, in every type of media, as it appears evident by comparing the difference in the level of plasmid maintenance between plasmids pRAP-URA3 and /or pRAP-TRP1 with pRAP-LEU2.

However, other factors may be accountable for the difference in stability of these genes, presumably involving some features intrinsic in the nature of the DNA sequence of the marker, rather than with the gene product itself. A different interpretation of the different stabilities of these genes may result from a selective process occurring at the DNA level, i.e. in terms of optimisation of the binding of the replication machinery, activity of RNA polymerase, or chromatin assembly and nucleosome phasing (Sakonju et al., 1980; Bogenhagen et al., 1980; Bernardi et al., 1992; Bell and Stillman, 1992). These fundamental cellular processes may be influenced by the DNA sequence present in some genes. For example, it has been shown that sequences distinct from the origin of replication may exert a profound influence on the function of the *ARS1* origin of replication, and, interestingly, one of the sequences responsible for promoting the origin preference has been identified at the 3' two-thirds of the *URA3* gene (Brewer and Fangman, 1994). This same gene has also been shown to affect the positioning of nucleosome (Bernardi et al., 1992) - an important process for the regulation of DNA transcription and replication (Grunstein, 1990; Simpson, 1990; Straka and Hörz, 1991). The *URA3* gene was in fact shown to represent a chromatin unit - characterised by six precisely positioned nucleosomes flanked by nuclease sensitive regions at the 5'-end and 3'-end - determined by species specific protein-DNA

interactions on both ends (Bernardi et al., 1992). These considerations on nucleosome assembly have been proposed also for the *TRP1* (containing four unstable nucleosomes), and *HIS3* genes (with nine nucleosomes on the *pet56-HIS3-del* region), presenting positioned nucleosomes flanked by nuclease sensitive regions at the 5'-end and 3'-end (Thoma, 1986; Losa et al., 1990; Bernardi et al., 1992).These global structures appear to be independent of the chromosomal location or episomal localisation (Thoma, 1986; Pérez-Ortín et al., 1987; Lohr and Torchia, 1988; Pérez-Ortín et al., 1989; Bernardi et al., 1992) and independent of their orientation in plasmids (Thoma, 1986; Thoma and Zatchej, 1988). In this context, it is important to point out that the 2μm plasmid (and its derivatives) also possesses a nucleosomal organisation (Livingston and Hahne, 1979; Nelson and Fangman, 1979; Seligy et al., 1980). The chromatin structure of *LEU2* gene has been reported to be independent of either the transcriptional state of the gene or the chromosomal or episomal location, but the absence of hypersensitive sites within this gene appears to be an exception with respect to the rest of the yeast genes studied (Martínez- García et al., 1989). In the light of what is known so far, other factors, either than nucleosome positioning, may be responsible for the different level of plasmid maintenance observed in our experiments. Protein-DNA interactions may play a central role in nucleosome positioning (Thoma and Simpson, 1985), and, in addition, DNA sequence of a given gene may contain also binding sites for some cellular factors, again, as it has been proposed for the case of the *URA3* gene, carrying potential binding sites for some transcription factors (Brewer and Fangman, 1994) and still unidentified factors (Roy et al., 1990). Some transcription factors have been identified that bind to the specific TGACTC DNA sequence present in the promoters of many amino acid biosynthesis genes (Hinnebusch, 1988). Among the genes used in this work, this sequence is present in the *HIS3*, *ADE2*, and *ADE8* genes (Stotz et al., 1993). The transcription factors identified as binding to this DNA stretch comprehend Gcn4, Bas1, Bas2, and Yap1 (Arndt et al., 1987; Harshman et al., 1988; Moye-Rowley et al., 1989; Daignan-Fornier and Fink, 1992). To test the above hypotheses on the effect of the DNA structure of these genes and its effect on the protein-DNA interaction, more detailed information should be gained on the behaviour of all the genes tested in this study. In addition to the above considerations, it must be pointed out that the artificial plasmid location of a chromosomal gene may interfere with the normal level of gene expression and transcription, as

reported for the selectable marker genes *TRP1* and *URA3*, whose transcripts were present at increased levels relative to chromosomal copies of the genes (Marczynski and Jaehning, 1985).

Finally, copy number values determined by densitometric analysis of the chromosomal and plasmid DNA bands - carrying the selectable markers - are in line with those obtained in previous studies (10 to 40 in a [cir^+] strain, Gerbaud and Guérineau 1980; Harford and Peeters, 1987). The lower copy number of the pRAP-TRP1 plasmid in selective medium could be explained by the fact that the chromosomal marker may have reverted in one or more of the three independent transformants pooled for this type of analysis. In addition, it has been reported in the literature that different transformant clones may show a wide variation in plasmid copy number, a phenomenon which is still unexplained (Purvis et al., 1987; Loison et al., 1989). This clonal fluctuation in copy number may reflect in a wide variability in plasmid maintenance, a phenomenon reported by Bruschi and Ludwig (1989). As an alternative explanation, it has been observed that some competition can occur between artificial episomal vectors and the 2µm plasmid, resulting in a depressed copy number for both types of plasmids (Futcher and Cox, 1984). To test this hypothesis, copy number of the 2µm plasmid should be determined in this combination. The increase in copy number observed in the case of the pRAP-URA3, remarkable in complete medium and still evident in rich, may be the result a stable recombinational event occured between the episomal vector - or part of it including the *URA3* gene- and the endogenous 2µm plasmid, a circumstance that may lead to the amplification of the number of copies of the selectable marker, and loss of the remaining vector. This kind of phenomenon has been well described for the recombination of the *leu2-d* gene from the pJDB219 plasmid onto 2µm DNA (Erhart and Hollenberg, 1981; Bruschi and Howe, 1988). In addition, it has been shown that in glucose-limited non selective cultures the distribution of plasmid in the population was such that a small fraction of the population contained a very high level of molecules, the most of the cells being plasmid-free (Bugeja et al., 1989). This phenomenon of segregational breakdown has been also demonstrated with other plasmids (Murray and Szostak, 1983).

4.3 The red/white system: white *ade8-18* cells have a selective advantage over red *ade2* cells.

Mutations in the *ade2* and/or *ade1* genes confer adenine auxotrophy together with a cell-limited red pigment - due to accumulation of precursors in the vacuole - while wild-type *ADE2* and /or *ADE1* cells are white (Roman, 1956; Fischer, 1969; Silver and Eaton, 1969; Woods, 1969). Expression of other auxotrophic mutations upstream in the adenine biosynthetic pathway, namely *ade4*, *ade5*, *ade8*, *ade6*, and *ade7*, are epistatic to *ade2* and *ade1* mutations, blocking adenine biosynthesis before the formation of the red pigment and resulting in a white cell phenotype (Roman, 1956; 1957). The use in appropriate combinations of wild-type and mutant alleles of these markers allowed the detection of several different genetic events, by monitoring the shift from red to white in the cell phenotype. This white/red colony color assay has been widely used as a neutral scoring system for the detection of various types of genetic situations, such as haploidization or recombination in diploids (Zimmermann and Vig, 1975, Parry and Zimmerman, 1976), reciprocal recombination (Kelly et al., 1983), chromosome loss in disomic strains (Esposito et al., 1982), plasmid stability (Hieter et al., 1985; Koshland et al., 1985; Bruschi and Howe, 1988), and isolation of mutants (Riles and Olson, 1988). Furthermore, the primary fluorescence exhibited by *ade2* and/or *ade1* mutants has been used as a tool to for the nonselective enrichment for yeast adenine mutants by flow cytometry (Bruschi and Chuba, 1988). The assumption was that red cells would exhibit the same characteristics of white cells, and their ratio would indicate the frequency of the genetic events under investigation. However, it was noted by Futcher and Cox (1983) that the presence of the *ade2* mutation was conferring to the red cell a growth disadvantage compared to the wild-type Ade^+ white cell. These authors used competition experiments to determine the growth rate of 2μm-containing [cir^+] cells versus [cir^0] cells using either wild-type Ade^+ host strain, or a strain carrying the *ade2* mutation. Results from experiments with the reciprocal combination of markers, showed that Ade^+ had a selective advantage over *ade2* cells of approximately 2.5%. However, this advantage could have been due to other mutations induced by the treatment with the mutagen ethyl methane sulfonate, a necessary step in the construction of the *ade2* strain. Our experiments using isogenic strains - except for the nature of the marker disrupting the *ADE2* gene - indicate that mutant white *ade8-18*, *ade2* cells show a remarkable selective advantage over red *ade2* cells. Thus, the accumulation of the red pigment in *ade2* cells, and not other mutations in the genome, is likely to be the cause for impaired growth in these cells.

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