ENGINEERED YEASTS SIMULATING P450-DEPENDENT METABOLISMS: TRICKS, MYTHS AND REALITY

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INTRODUCTION

During the early development of the P450 field, an enormous amount of biochemical and biophysical data was accumulated on a limited number of purified forms, mainly originating from rat and rabbit. The first information on amino acid sequences appeared early in the nineteen-eighties /1/, followed by an explosive increase in the number of known P450 sequences, which had reached almost 200 by the end of 1992 /2/, including more than 30 human isoforms /2,3/. In contrast, the knowledge concerning the biochemistry of newly cloned isoforms has remained limited. A paradoxical situation arose of the accumulation of a lot of nucleotide sequences with almost no data on the corresponding enzymatic properties. The ascribing of a precise substrate specificity to newly cloned P450 sequences became a critical task, making the availability of a heterologous expression system crucial.

Soon after the successful production of human interferon in yeast /4/, Oeda *et al.*, working in Hideo Ohkawa's laboratory, reported in 1985 the first expression of a cDNA encoding a mammalian microsomal cytochrome P450 in *Saccharomyces cerevisiae* /5/. The recombinant rat P450 1A1 was found to be properly addressed to the yeast endoplasmic reticulum and inserted in the membrane with an orientation compatible with activity. Following this first result, expression of P450-encoding cDNAs in *Saccharomyces cerevisiae* rapidly became a very popular tool /6,7/. Progress in yeast molecular biology and development of new heterologous expression systems involving other hosts, such as insect and mammalian cells /8-10/, permitted the expression of a growing number of P450-encoding cDNAs. Bacteria-based expression systems, mainly in *Escherichia coli* /11,12/, were the last to be developed due to the difficulty of achieving a suitable folding of membrane-bound proteins in organisms devoid of the natural membrane target structures. Although very successful in some cases for large scale production of P450s /13/, bacterial expression systems are generally not self-sufficient. The expression of activity requires purification of the monooxygenase system components and reconstitution including addition of NADPH-cytochrome P450 reductase (P450 reductase) and cytochrome b_5 /14,15/. On the other hand, expression in mammalian cells, for example COS1, V79 or human B lymphoblastoid cell lines, gave rise to systems suitable for direct toxicologic or metabolic studies. Nevertheless, their

principal drawbacks are the high cost of cell culture and a rather low P450 specific content which renders this approach rather inapt for large scale expression or for systematic use in drug development or bioconversions.

The requirement for optimal P450 monooxygenase activity of adapted levels of P450 reductase, and in some instances of cytochrome b_5, is a severe constraint on the realization of an efficient self-sufficient expression system. In contrast to the great diversity of P450s, a single reductase and only one or two cytochromes b_5 are generally found in a given species /16/. Yeast microsomes contain some endogenous reductase activity and significant amounts of cytochrome b_5. A rather efficient redox coupling between human P450s and the yeast reductase was known to be possible although human and yeast reductases exhibit only 33% amino acid sequence identity /17,18/. The situation appeared to be quite different for the coupling with cytochrome b_5. Although yeast and human cytochromes b_5 share approximately 30% sequence identity /19/, no detectable coupling was observed when yeast cytochrome $b5$ was tested for supporting human P450 3A4 activity /20/. Fortunately, the intrinsic properties of recombination of yeast cells, and the avaibility of a wide range of molecular biological tools make clean gene substitutions easy, including the exchange of endogenous P450-associated redox proteins by their human equivalents. The purpose of this review is to provide for the reader a peregrination through the different generations of yeast expressing systems that succeeded one another over the last eight years to reach finally the concept of humanised cells (Figure 1).

1. YEAST ENDOGENOUS CYTOCHROMES P450

Saccharomyces cerevisiae contains a limited number of endogenous P450s. The first described was lanosterol-C14-demethylase or CYP51 /21,22/, a domestic enzyme involved in the ergosterol biosynthesis pathway. This P450, which is constitutively produced at low levels in aerobiosis, appears to be specific for C14-sterol demethylation and seems almost or totally inactive toward most xenobiotic compounds. The fortuitous discovery of a second yeast P450 was made during the study of sporulation mutants. CYP56, the product of the *DIT2* gene, is expressed only during sporulation /23/ and is involved in the production of the dityrosines involved in the spore wall reticulation.

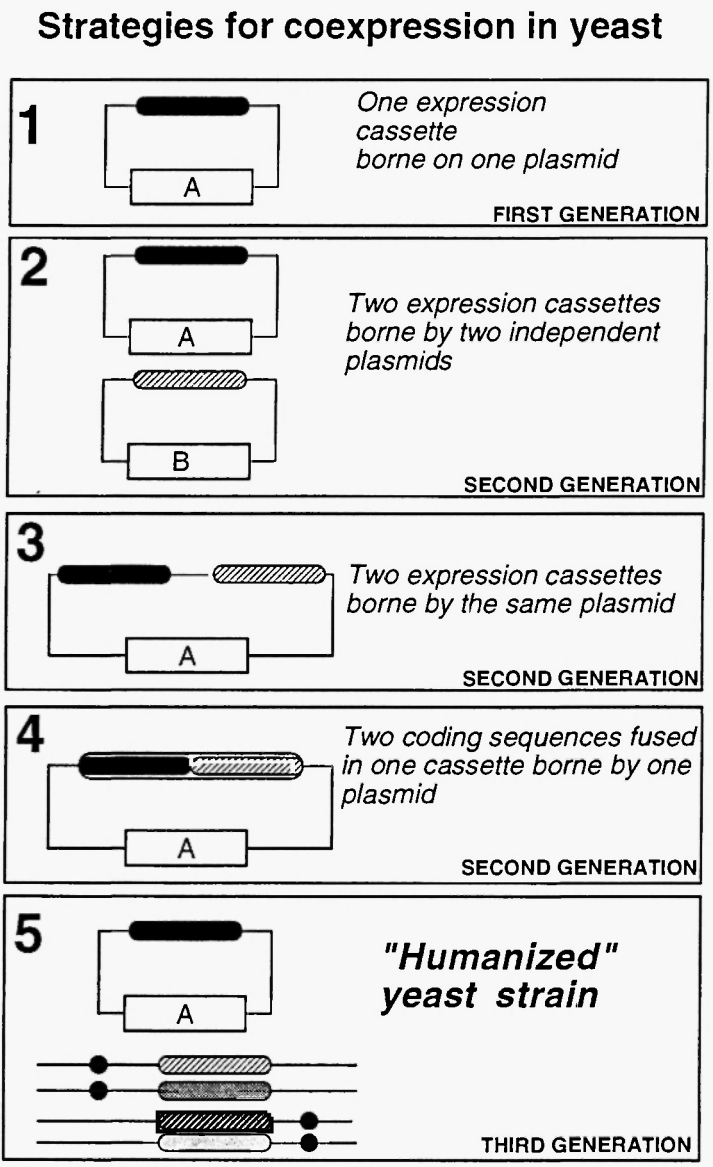

Fig. 1: **Evolution of P450-expressing yeast systems.** The first generation corresponds to production of a P450 in the unmodified yeast microsomal environment. The second generations are systems in which the activity of the produced P450 is enhanced by co-expression of the yeast or mammalian NADPH-P450 reductase. The last system corresponds to yeast strains with engineered chromosomal DNA (represented by solid lines with closed circles) to produce complex functions involving P450, and associated redox and phase II enzymes.

Nothing is known about its substrate specificity, but this P450 is absent under the vegetative conditions used for heterologous expression. A third one is only postulated to exist on the basis of biochemical studies such as carbon monoxide inhibition, but the gene has not yet been identified or cloned. The postulated activity is the Δ-22 desaturation of the side chain of the ergosta-5,7-dienol, an ergosterol precusor /24/. This isoform, or some other yet unidentified P450, seems to be highly induced at low dioxygen pressure, becoming spectrally detectable. This putative P450 might have some xenobiotic metabolic activity such as ethoxyresorufin-*O*-deethylation and benzo[*a*]pyrene-hydroxylation reactions /25/. These activities are nevertheless undetectable under aerobic conditions in the absence of catabolic repression /26/. Consistently, yeast cells grown in a galactose-containing medium have a very low amount of total endogenous P450s which remains undetectable both on the basis of spectral analysis and of the assay of xenobiotic-directed activity.

The microsomal levels of endogenous P450 reductase and cytochrome b_5 are independent of the culture conditions, being, respectively, 20 + 5 pmol/mg and 80 + 20 pmol/mg.

2. PRODUCTION OF HETEROLOGOUS P450 IN *S. CEREVISIAE*

General considerations

The production in *S. cerevisiae* of heterologous P450 is based on the construction of expression cassettes containing the coding phase of the P450 cDNA in question, sandwiched between yeast transcription promoter and terminator sequences. Efficient expression requires the deletion of all of the 5'-non coding region originally present in the cDNA before insertion of the open reading frame (ORF) in the yeast expression vector. A detailed study of the effects of 5'-non coding regions of various lengths on mouse *Cyp1a-1* expression in yeast illustrated this phenomenon /27/. In contrast, the presence of transcribed 3'-non coding region seems to be less deleterious for expression. Cloning and formatting are now easily performed in a single step by polymerase chain reaction (PCR) amplification using total cDNAs as template. Nevertheless, some caution should be taken to limit or correct potential amplification errors due to the limited fidelity of thermostable polymerases. The use of a DNA polymerase

having proof-reading activity, such as that of *Pyroccus furiosus*, short denaturation steps and low concentrations of dNTP and Mg^{++} ions considerably limit the misincorporation rate.

Constitutive versus induced expression

Depending on requirements, the yeast transcription promoter in the expression vector will be chosen to be of the inducible or constitutive type. In the former case, a response to a component in the culture broth (galactose, copper, phosphate, etc.) or to an external factor (heat-shock) can be involved. Alternatively, a promoter of a so-called constitutively expressed gene can be used, such as that of yeast phosphoglycerate kinase or glyceraldehyde-3-phosphate dehydrogenase genes. Constitutive promoters offer the advantage of direct expression in simple culture conditions, but the resulting permanent expression can affect plasmid copy number and cell viability. Genetic instabilities could result from the slower growth rate of yeast cells expressing heterologous P450s. Such effects can be associated with haemin pool depletion, anomalous membrane proliferation, depletion of tRNAs corresponding to low-usage yeast codons present in heterologous cDNAs, saturation of the endoplasmic reticulum targeting machinery, direct metabolic perturbation resulting from heterologous activities, P450 reductase or cytochrome b_5 mobilisations. Nevertheless, it is possible to limit genetic instabilities by careful culture and storage procedures.

A second predictable side effect of constitutive expression is the lowering of the plasmid copy number resulting from the negative selection against high-expressing cells. This favours formation of a bimodal population for plasmid copy number leading to a mixture of low- and high-expressing cells. This heterogeneity can impair interpretation of experiments involving multiple co-expressions, such as the association of phase I and phase II enzymes, or the study of the effect of the oxido-reduction (redox) environment on P450 activity. Although increasing genetic instability, such effects can be limited by the use of partially defective selection markers /28/.

Instabilities are dependent on cell growth. The use of inducible promoters can aid the resolution of these difficulties by separating the growth phase from the expression phase. Biomass is first generated with cells under repressed conditions, and expression of P450 and associated enzymes is subsequently induced under conditions of no or

limited cell growth. In practice, this procedure gives the best results but is time-consuming due to the shift in culture media required for induction. Intermediate protocols involving storage in the repressed state and full culture, including the growth phase, in induced conditions, can be conveniently carried out when optimal results are not required.

Pratical aspects

Total poly(A)-rich mRNAs are prepared and cDNAs are reverse-transcribed /29/. PCR amplification is performed using a pair of specific primers and total cDNAs as template. Each primer is composed of two parts, the 5'-end bearing a restriction site compatible with the expression vector and the 3'-end is identical (5'-primer) or complementary (3'-primer) to 18-25 base-pairs (bp) of the template sequence. When required, a hairpin structure surrounding the initiation codon, as is the case for the mRNA encoding human P450 reductase /18/, can be eliminated by introducing suitable mutations into the 5'-primer sequence. Transcription in yeast, as well as translation, is strongly inhibited by such hairpin structures /30/. PCR-amplified fragments are made flush and phosphorylated before cloning into an *E. coli* intermediate vector. After sequencing, the open reading frame (ORF)-containing fragment is transferred into a multicopy yeast expression vector bearing suitable auxotrophic complementation markers (Table 1). A particular feature of plasmids bearing the *URA3* marker (encoding orotate decarboxylase) is their full stability, even in the presence of exogenous uracil, provided that a recipient strain carrying a *ura3*, *fur1* double mutation is used /31/. Such a mutant strain which is unable to use extracellular uracil (*fur1* mutation) or to synthesise endogenous uracil (*ura3* mutation), is viable only upon plasmid complementation. Accordingly transformation is carried out by plasmid shuffling: a *fur1*, *ura3*, *ade2*, *trp1* quadruple mutant carrying a vector pYeDP80 bearing the *URA3* and *TRP1* markers, is transformed by a P450 expression vector including the *URA3* and *ADE2* markers. Transformants are selected on the basis of the Trp^+Ade^- to Trp^-Ade^+ phenotype shift. Such transformed strains can be cultivated to very high cell densities.

P450s produced in *S. cerevisiae* cells are generally obtained in the natural folded state without any particular precautions. For yeast subcellular fractionation, however, a critical step is the breaking of the

TABLE 1

Yeast expression vectors developed from the YeDP series

Name	Type	Characteristics		Selection(s)
		Promoter	Terminator	
pYeDP1/8-2	Multicopy	*GAL10-CYC1*	*PGK*	Uracil
pYeDP1/8-10	Multicopy			
pYeDP20	Multicopy			
pYeDPN	Multicopy			
pYeDP9	Multicopy	*GAL10-CYC1*	*ADH*	Uracil
pYeDP10	Multicopy	*PGK*	*PGK*	Uracil
pYeDP30	*CPR* locus directed integration	*GAL10-CYC1*	*PGK*	Uracil
pYeDP60	Multicopy	*GAL10-CYC1*	*PGK*	Uracil and Adenine
pYeDP70	Multicopy	*PGK*	*PGK*	Uracil and Adenine
pYeDP80	Multicopy	*GAL10-CYC1*	*PGK*	Uracil and Tryptophane
pYeDP100	Multicopy	*GAL10-CYC1*	*CPR*	Uracil
pYeDP110	*CPR* locus directed integration	*GAL10-CYC1*	*CPR*	Uracil

cellular wall without damaging P450 molecules. Being an aggregate of manno-proteins, complex sugars and chitin /32/, the cell wall is quite rigid. Two methods, based respectively on mechanical disruption and on enzymatic digestion of the cell wall, can be used. Mechanical breaking will be preferred for large-scale culture at high cell density, whereas the enzymic treatment is more suitable for small-scale culture, particularly when contamination by mitochondrial fractions has to be avoided.

3. CHARACTERIZATION OF HETEROLOGOUS P450 PRODUCED IN *S. CEREVISIAE*

Spectral studies

Depending on isoform, plasmid, and culture conditions, the P450 content ranges between 10 and 400 pmol of spectrally detectable P450 per mg of microsomal protein (Figure 2). Best results are generally obtained with inducible promoters, thus permitting the production of several hundred nmol of P450 per litre of culture at high cell density. This level is usually sufficient to enable the use of microsomal fractions without further purification as such, for spectroscopic studies like substrate binding /33/, since the yeast strains used are inherently devoid of spectrally detectable endogenous P450s when cultivated in aerobic conditions and in the absence of catabolic repression. An example of this is the determination by differential spectrophotometry of the binding constants of yeast-expressed human P450 3A4 for erythralosamine-2'-monobenzoate and dihydro-ergotamine /34/, or of yeast-expressed plant P450 73A1 for cinnamate /35/. The formation of Fe(II)-nitrosoalkane complex upon oxidation of N-hydroxy-amphetamine by yeast-expressed human P450 3A4 can similarly be observed /36/ without any purification step.

Activity studies

Yeast cells contain a moderate level of endogenous P450 reductase (about 25 pmol per mg of microsomal protein). This is enough to support more or less efficiently the enzymic activities of the heterologous P450s. Therefore, the basic yeast system is self-sufficient. A large number of activities can be tested by directly using whole living

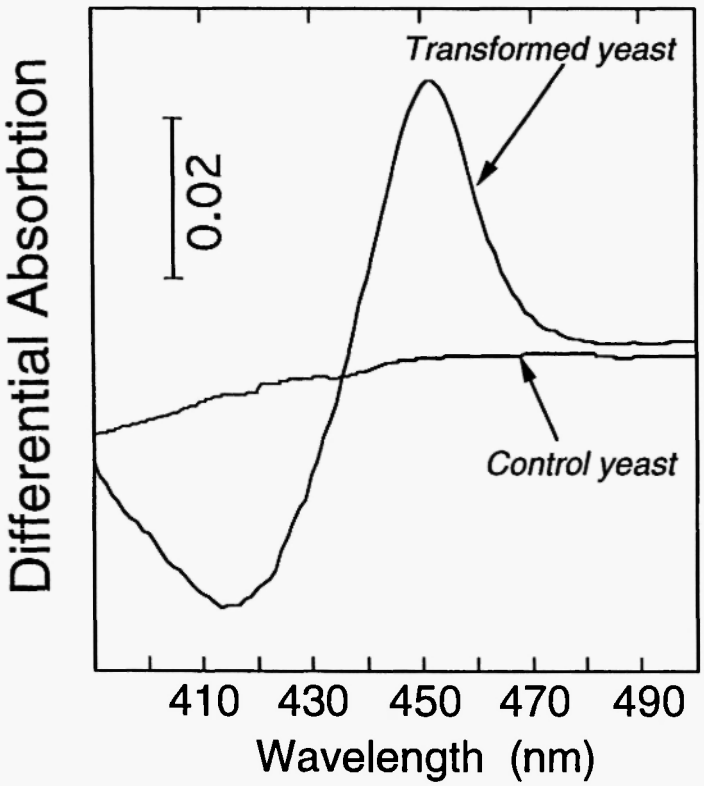

Fig. 2: Reduced carbon monoxide difference spectra of yeast microsomal fractions. Spectra were obtained with 0.4 mg of microsomal proteins from yeast cells transformed by a void pYeDP60 vector (Control yeast) or from cells producing a plant P450 (Transformed yeast).

cells as biocatalysts due to a rather efficient diffusion of apolar substrates within yeast cells. Thus a few ml of culture is frequently sufficient to achieve detectable bioconversions /20,35/. Nevertheless, the turnover rate of P450 in the wild-type yeast is often significantly lower than the value determined *in vitro* after purification and reconstitution with an excess of mammalian P450 reductase. An explanation for this is the possible limiting amount of yeast P450 reductase /6,37/. The turnover rate for mouse P450 1A1 ethoxyresorufin-*O*-deethylase activity was analysed in yeast microsomes during the course of P450 induction and was effectively found to progressively decrease with increasing P450 accumulation /6/.

At full induction, a 0.05 - 0.5 molar ratio (depending on P450 isoform) between P450 reductase and P450 was calculated. The lower value is similar to the ratio observed in human liver microsomes /38/, indicating that the heterologous nature of the P450 reductase, and not only its amount, might be responsible for the limiting effects. Despite these limitations, mammalian P450s produced in these "first generation" expression systems were used extensively for pharmacological studies /5,6,20,27,34,36,38-68/ and for biochemical characterization of both natural and mutant P450s /20,69-89/.

4. OPTIMIZING THE MONOOXYGENASE ACTIVITIES OF HETEROLOGOUS P450 PRODUCED IN *S. CEREVISIAE*

Electron transfers and catalytic efficiency

The P450 catalytic cycle involves two sequential electron transfer steps /90,91/. The first one initiates the cycle by shifting the P450 iron redox state from the (III) to the (II) oxidation numbers. This electron transfer, which occurs at a rather low redox potential (-150 to -320 mV), requires P450 reductase as the electron donor. The second electron transfer, which leads to the reduction of the ferrous-dioxygen complex, can be performed by either the reductase or cytochrome b_5 /92/ since it is characterized by a rather high redox potential (probably more than 0 V). A critical point for the coupling efficiency of the two subsequent electron transfers that yield productive catalytic cycles is the rate of the second electron transfer /93/. This fact is related to the short life-time of the ferrous-dioxygen intermediate (from 1 s to 100 s), whose formation is limited by the first electron transfer and productive utilisation by the second electron transfer. Uncoupling occurs when the second electron transfer is slow compared to the self-decay of the ferrous-dioxygen complex. Depending on the relative rates, the presence of cytochrome b_5 which potentiates the second electron transfer is required to reach optimal activity.

The yeast microsomal redox background

Few data are available on the relative affinities of yeast and human P450 reductases for P450s, or on the relative rates of first and second electron transfers. For cytochrome b_5, the yeast protein, which shares

30% of sequence identity with the human protein /19/, was found to be unsuitable as a substitute for human cytochrome b_5 for potentiating human P450 3A4 activity /20/. The yeast native microsomal background can thus be considered to be functionally cytochrome b_5-free for human P450 expression. Optimisation of the monooxygenase activity of any human P450 can thus be attempted either by over-expression of the yeast P450 reductase, and/or by coexpression of the human reductase and cytochrome b_5. Enhancement of monooxygenase activities either by over-expression of the yeast reductase ORF from a multicopy plasmid /6,37/ or by construction of an artificial gene expressed as a fusion protein between the heterologous P450 and yeast reductase /58,59/ have been reported. Such fusion constructs simulate the naturally occurring structures of P450 BM-3 in *Bacillus megaterium* /94/ and NO synthases in man /95-98/.

The fusion approaches were successful in the sense that improved turnover rates were systematically reported for monooxygenase activity catalysed by these engineered P450s. Nevertheless, it was never clearly demonstrated that true intra-molecular electron transfers really occurred in such complexes, or whether the observed effects could result from the associated overexpression of the reductase activity acting through intermolecular complexes. The main limitations to this approach are the difficulty of inserting a third redox component, such as cytochrome b_5, the intrinsic impossibility of modulating the component stoichiometries, the requirement of a special construction for each P450, and the possible perturbation of enzymatic properties of xenobiotic-metabolizing P450 in the fusion protein. An alternative solution was to improve recognition between P450 reductase and P450 by using yeast-expressed partners belonging to the same species as the expressed P450s. This approach offers the additional advantage of avoiding deleterious effects associated with the production of high levels of yeast P450 reductase (cell toxicity due to oxygen radical formation).

Designing a second generation of humanized yeast cells

Overproduction of yeast P450 reductase was first attempted by increasing the expression of the endogenous gene *CPR* /6/. The *CPR* coding sequence was amplified, reformatted by PCR and placed under the transcriptional control of *GAL10-CYC1* promoter by insertion into the pYeDP1/8-2 expression vector, yielding pYRedF/V8. A fragment

of the 5'-non coding region of the yeast *CPR* gene was inserted upstream of the fragment of the pYRedF/V8 vector containing the expression cassette and the *URA3* selection marker. The construction was used as a genomic integration cassette to substitute the natural, mild and non-inducible *CPR* promoter by the strong, galactose-inducible *GAL10-CYC1* promoter /20/ (Figure 3). The resulting engineered strain, named W(R), exhibits a mutant phenotype for endogenous *CPR* regulation. When grown on glucose, W(R) cells do not express detectable levels of P450 reductase activity, behaving as a *CPR*-disrupted strain. Such a strain is easily killed at low concentrations of ketoconazole, an inhibitor of the yeast P450 lanosterol 14-demethylase /99/. However, when grown in galactose-containing medium, the P450 reductase activity becomes detectable and increases more than three orders of magnitude, reaching a value 30-fold higher as compared to the value obtained in the wild-type strain (Table 2). In W(R) cells the level of endogenous yeast cytochrome b_5 remains similar when grown on glucose or in galactose. The direct over-expression of human cytochrome b_5 was preferred to the over-production of yeast cytochrome b_5 because of the poor (or absent) coupling of the latter with human P450s /20,36/. The ORF encoding human cytochrome b_5 was cloned by RT-PCR from total human liver mRNAs /6/. The expression cassette was placed under the control of the *GAL10-CYC1* promoter and was integrated into the yeast genome at the chromosomal yeast reductase locus, yielding the W(B) strain (Figure 3). In a galactose-containing culture medium, W(B) cells produce both yeast and human cytochromes b_5 (a total of 300 pmol/mg microsomal proteins from reduced minus oxidized differential spectra), but produce only yeast cytochrome b_5 (150 pmol/mg microsomal proteins) when grown on glucose. This suggests that human cytochrome b_5 is produced in W(B) cells at a level similar to that of the yeast protein. W(B) cells exhibit no detectable P450 reductase activity on either galactose or glucose since their *CPR* gene is disrupted. Mating of W(B), W(R) and W(N) (the wild type) in various combinations yielded a large set of isogenic diploid strains /20,100/, presenting various microsomal contents of both yeast P450 reductase and human cytochrome b_5 (Table 2).

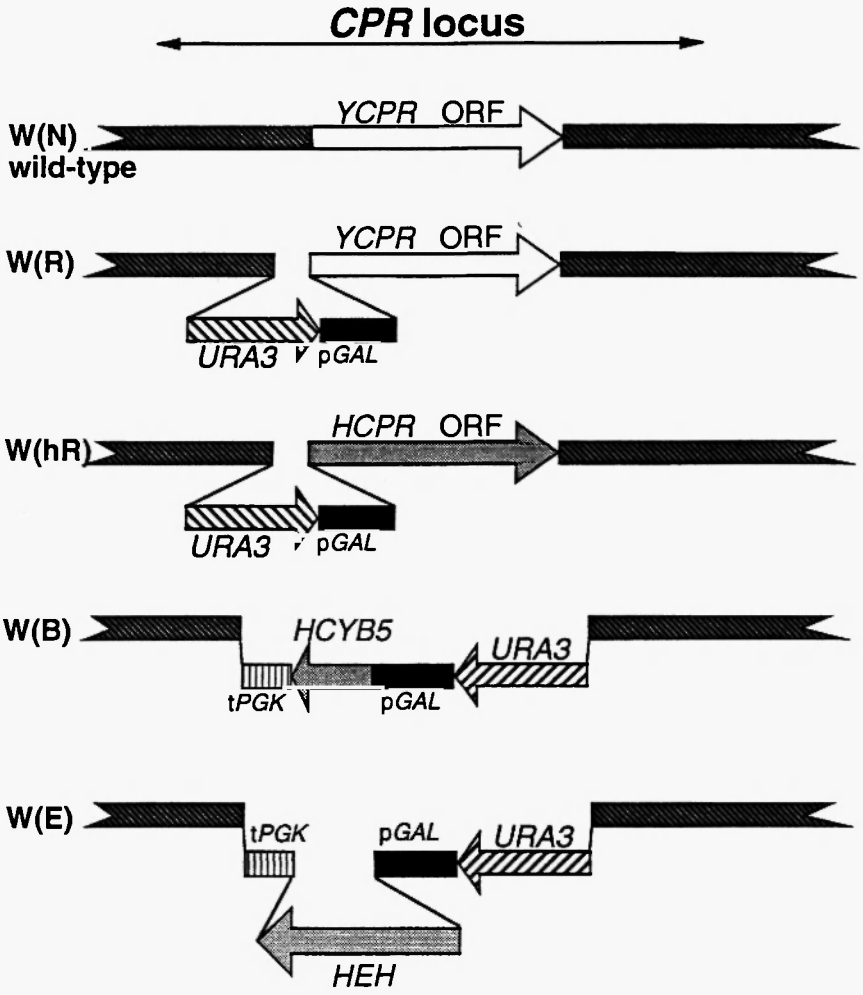

Fig. 3: Structures of the CPR locus in various haploid strains. ORF stands for open reading frame. *URA3*, p*GAL*, and t*PGK* stand, respectively, for orotate-decarboxylase encoding gene, *GAL10-CYC1* hybrid promoter, and phosphoglycerate kinase-encoding gene terminator. *YCPR*, *HCPR*, *HCYB5*, and *HEH* stand, respectively, for yeast P450 reductase, human P450 reductase, human cytochrome b_5, and human microsomal epoxide hydrolase.

TABLE 2

P450 reductase and cytochrome b_5 microsomal contents in modified yeast strains. Y and H stand respectively for yeast and human.

Strain	Yeast P450 reductase (nmol cyt *c* / min / mg protein)	Total cytochrome b5 (pmol / mg protein)	type
W (N) wild-type	80	90	Y
W (R)	2100	80	Y
W (B)	0	300	H + Y
W (R,N)	750	80	Y
W (B,N)	40	120	H + Y
W (B,R)	750	100	H + Y

Do changes in redox environment improve P450 turnover?

The functional expression of every P450-encoding cDNA could thus be achieved in these different redox environments simply by transforming these engineered strains by a plasmid-borne P450 expression cassette. The influence of P450 reductase and cytochrome b_5 levels was first tested using as a model the ethoxyresorufin-*O*-deethylation (EROD) activity catalysed by human P450 1A1 and the testosterone-6β-hydroxylation (THL) activity catalysed by human P450 3A4 /20/. The effects of the redox environment on P450 activities in yeast microsomes were significantly different for these two P450s (Table 3). A 20-fold increase in the yeast P450 reductase activity [compare W(N) and W(R)] resulted in a 4-fold increase of the EROD turnover number (P450 1A1), in contrast with a 63-fold increase of the THL turnover number (P450 3A4). Although illustrating the poor efficiency of wild-type amounts of yeast P450 reductase in supporting human P450 activity, these data indicate a strong isoform-dependent effect of yeast P450 reductase overproduction. Comparison of the P450 3A4-catalysed THL activity

TABLE 3

Effect of improved redox environment on human P450 monooxygenase activity in yeast microsomes

Strain	Human Cyt. b_5	P450 reductase Specific activity (nmol cyt c / min / mg protein)	Production levels (pmol P450 / mg protein)		Turnover numbers (mol / min / mol P450)	
			P450 1A1	P450 3A4	P450 1A1	P450 3A4
W (N)	No	80	42	140	3.2	0.03
W (R)	No	2100	52	60	14 0	1.9
W(B,N)	Yes	40	60	70	1.6	0 21

measured in W(B,N) cells with that determined in wild-type W(N) cells, allows one to address the specific effect of human cytochrome b_5. Although the P450 reductase level in W(B,N) is half that found in W(N), a 7-fold increase in the THL turnover number of 3A4 was observed. Since the two strains are isogenic and P450-expressing vectors identical, only the presence of human cytochrome b_5 in W(B,N) accounts for this difference. The same comparison for human P450 1A1 led to a different result. In this case, the 2-fold decrease in P450 reductase activity [W(N) compared to W(B,N)] resulted in a 2-fold decrease in the P450 1A1-catalysed EROD turnover rate. This revealed that although human cytochrome b_5 plays a determinant role in supporting the activity of human 3A4 in the presence of limiting amounts of yeast P450 reductase, the effect is strongly isoenzyme dependent. Complementary studies dealing with human 3A4 and several other substrates, including nifedipine and lidocaine, demonstrated that the effects of the coexpression of human cytochrome b_5 and of the over-expression of yeast P450 reductase are not only dependent on the P450 isoform but also on the substrate considered /36/. The possible reasons for this are currently under investigation and could be related to the difference of each isoform in the relative stabilities of the ferrous-dioxygen complex in the presence of different substrates.

Shifting the yeast P450 reductase to the human enzyme

The next stage in the analysis was to compare the efficiencies of human and yeast P450 reductases in supporting human P450 activity. This kind of study was first achieved in Eugster's laboratory /101/ by transforming a *CPR*-disrupted yeast strain with a plasmid bearing two expression cassettes, one encoding human P450 reductase and the other human P450 1A1. In our laboratory, we preferred to pursue the more versatile strategy involving genomic integration. Formally, this requires only to substitute the ORF of the yeast *CPR* gene by the human one in the W(R) strain. In practice, this was carried out in a way similar to that used in the construction of the W(R) strain but involving the sequence coding for human P450 reductase. This procedure resulted in the W(hR) strain which exhibits a gene structure at the *CPR* locus very similar to that of W(R) /102/. W(hR) microsomes do not present any P450 reductase activity when cultured in glucose-containing medium, but produce the human P450 reductase

and not the yeast enzyme when grown on galactose. Based on the NADPH-cytochrome *c* oxidoreduction assay, the specific P450 reductase activity in W(hR) microsomes was found to be rather low (about one third to one fifth that found in wild type yeast). However, upon transformation by a vector expressing human or mouse P450 1A1, the P450 EROD bioconversion rate was found to be slightly higher in living W(hR) cells than that observed with W(R) cells, although the latter strain exhibits a 100-fold higher level of cytochrome *c* reductase activity than W(hR). Preliminary measurements performed on microsomal fractions indicate that P450 1A1 in W(hR) exhibits a turnover rate which is slightly enhanced in comparison to that in the wild type yeast, and a clearly higher P450 content. Considering the low level of P450 reductase in W(hR) cells, these results suggest that for similar amounts, the human enzyme is much more efficient than the yeast enzyme in supporting human P450 activity. These results and the specific effect of human cytochrome b_5 on the catalytic activities of human P450s produced in yeast emphasize the importance of using a humanised redox environment reconstituted in yeast.

5. SIMULATION OF A HUMAN MULTI-STEP DRUG METABOLISM PATHWAY IN *S. CEREVISIAE*

The previous chapter discussed evidence that despite the fact that yeast contains endogenous P450-associated redox activities similar to those of mammalian cells, the co-expression of the human redox enzymes allows significant improvement of the activities of yeast-produced human P450s. The situation is even more dramatic for phase II enzymes which are, in most cases, naturally absent in *S. cerevisiae*. Consequently, simulation of phase I / phase II human liver metabolic activation cannot be achieved without providing yeast *de novo* with a human phase II context. The activation of polycyclic hydrocarbons was considered as a model. Benzo[*a*]pyrene is a pollutant mainly found in industrial and tobacco smokes acting as a procarcinogenic compound in lung cancers /103/. Studies on mouse skin tumour models led to the conclusion that a three-step activation occurs. Benzo[*a*]pyrene, which is highly lipophilic and chemically inactive, is first activated by a P450 1A1-catalysed epoxidation reaction yielding the 7,8-oxide derivative, among other products. This intermediate compound is subsequently hydrolysed into the corresponding 7,8-diol by microsomal epoxide

hydrolase. A second P450 1A1-dependent epoxidation reaction occurs, yielding 7,8-dihydro-7,8-dihydroxy-9,10-oxide-benzo[*a*]pyrene which is considered to be the ultimate mutagen /104-107/ .

Expressing a human phase II activity in *S. cerevisiae*

When microsomes prepared from W(R) cells transformed by an expression vector for human 1A1 are incubated with benzo[*a*]pyrene, production of 3-hydroxy-, 9-hydroxy- and quinone derivatives is easily detected /108/. Nevertheless, no formation of 7,8-diol was detected (the 7,8-oxide formed probably reacts with some components of the incubation mixture in the absence of any epoxide hydrolase activity). No metabolites were observed when benzo[*a*]pyrene was incubated with W(R) yeast cells transformed with expression vectors for human P450 1A2, P450 3A4, or microsomal epoxide hydrolase alone. This suggested that full reconstitution of the benzo[*a*]pyrene metabolism in yeast requires, in addition to the expression of a suitable P450, the co-expression of microsomal epoxide hydrolase /108,109/. The integration of the expression cassette coding for microsomal epoxide hydrolase was effected at the *CPR* locus under the control of *GAL10-CYC1* promoter, yielding the W(E) strain, in a similar way to that utilised for integration of the human cytochrome b_5 coding sequence /108/. After mating the W(E) strain with the W(R) strain, the resulting W(E,R) diploid strain expresses human epoxide hydrolase characterised by the typical styrene-oxide hydrolase activity and over-produces yeast P450 reductase when grown on galactose. After transformation of W(E,R) cells by the plasmid for human P450 1A1, incubation of microsomes with benzo[*a*]pyrene led to the efficient production of the expected products, and in particular the ultimate carcinogen 7,8-dihydro-7,8-dihydroxy-benzo[*a*]pyrene /108/. Interestingly, the formation of degradation products (7,8,9,10-tetrols) of the ultimate mutagen is also observed (Gautier *et al.*, unpublished results). The analysis of the kinetics of 7,8-diol production, in comparison with the formation of 3-hydroxy- and 9-hydroxy-benzo[*a*]pyrene, clearly indicates that no accumulation of the 7,8-oxide intermediate occurred in W(E,R) cells /108/. This means that a very efficient coupling had been established between the phase I and the phase II human enzymes in this engineered yeast. A full multistep activation involving a phase I - phase II - phase I sequence can thus be conveniently achieved.

6. USE OF THE PROPERTIES OF HOMEOLOGOUS RECOMBINATION OF *S. CEREVISIAE* TO GENERATE A LIBRARY OF CHIMAERIC P450S

Microsomal cytochromes P450 are intriguing enzymes because of their extraordinary ability to metabolise a wide variety of substrates /90,91,110/. For example, human P450 1A1 recognises small molecules such as acetanilide and a large polycyclic hydrocarbon such as benzo[*a*]pyrene, but, at the same time, differentiates between two alkoxyphenoxazines which differ only by a methylene group within a large dibenzopyrazine heterocyclic structure (7-ethoxyresorufin versus 7-methoxyresorufin). These paradoxical properties result from a multifactorial control of the substrate specificity involving sub-site recognition, molecular flexibility and subtle links between the substrate shape and the P450-dependent dioxygen activation process. This last factor controls, in turn, the macroscopic coupling efficiency between NADPH oxidation (through P450 reductase) and substrate oxidation. Therefore, the complex control of P450 catalysis makes the prediction of the change in the apparent substrate specificity caused by sequence alterations extremely difficult. In particular, understanding the molecular basis of the substrate-specificity differences between members of a given P450 family, or even for the same isoform originating from different species, is a puzzling problem. An experimental tool of choice is the construction and the biochemical study of chimaeric structures between P450 isoenzymes /46,69-71,73,82/. Although elegant, this approach is limited by the need for a large number of constructions to localise significant sequence divergences at the amino acid level. A very special property of yeast, homeologous recombination, i.e. recombination between similar but not identical nucleotide sequences, was utilised recently to simplify the chimaera-based approach /72,84/.

Homeologous recombination

Yeast DNA recombination machinery /111/ offers a unique opportunity to work efficiently with nucleotide sequences sharing only limited identity /72,84/. Routine work can be achieved with sequences exhibiting more than 60% identity without direct selection on shuffled functions. For lower sequence similarity (as low as 40% identity), a positive selection method is required on chimaera activities due to the increasing number of non-homologous recombination events /72,84/. The homeologous recombination events occurring *in vivo* respect the

optimal sequence alignment with a computer-like efficiency ... and sometimes more! In practice, to constitute a library of chimaeras between two P450 isoforms "A" and "B", yeast cells are transformed with a mixture of two linear DNA fragments (Figure 4). The first DNA fragment (the template) is an expression vector that bears the coding sequence of P450 "A". This fragment is linearised, with eventual deletions, at one extremity or within the P450 ORF. The second DNA fragment (the repair one) is a segment of the ORF encoding P450 "B" that has some sequence similarity and that overlaps the extremities of the gap introduced in the "A" nucleotide sequence. Gap repair events are selected only on the basis of the yeast transformation by the recircularised plasmid bearing the selection marker. This results in DNA exchanges that are aligned and located randomly but in frame. Part of the first P450 ORF is therefore substituted by the corresponding part of the second ORF /72,84/. A library of chimaeric P450 sequences is thus generated in a single step directly into the yeast expression vector. A few bp-long stretch of strict identity (equivalent to two or three amino acids), called the minimal efficient processing segment, is sufficient to allow most recombination events /84/. Provided that the parental P450s exhibit distinct enzymatic properties, the library can be screened for particular alterations in activity and shuffling. The selected chimaeric P450s are sequenced after plasmid rescue in *E. coli*. This simple technique can be used to obtain information on the relationship between structural determinants in P450s and function /72,84/. The strength of the so-called gap-repair approach is the possibility of selecting chimaeras with modified functions without any preconceived idea or model concerning the exact role of the exchanged segment. Although the method is of most interest for the analysis of substrate specificity, very surprising results were also obtained at the level of the P450 protein production.

Among a large collection of chimaeras between human P450s 1A1 and 1A2 obtained during the analysis of determinants of the substrate specificity, one, named $S_{1\text{-}2}$, was selected on the basis of its strongly enhanced EROD bioconversion in yeast cells /112/. A more detailed biochemical analysis performed on the isolated microcrosomal fractions indicated that this chimaera exhibits substrate specificity and turnover rate very similar to those of authentic P450 1A2, but differs by an amazingly enhanced production level. Marked accumulation of the S12 protein in yeast, as compared to that of parental P450s, accounted for the enhancement of the EROD bioconversion activity initially

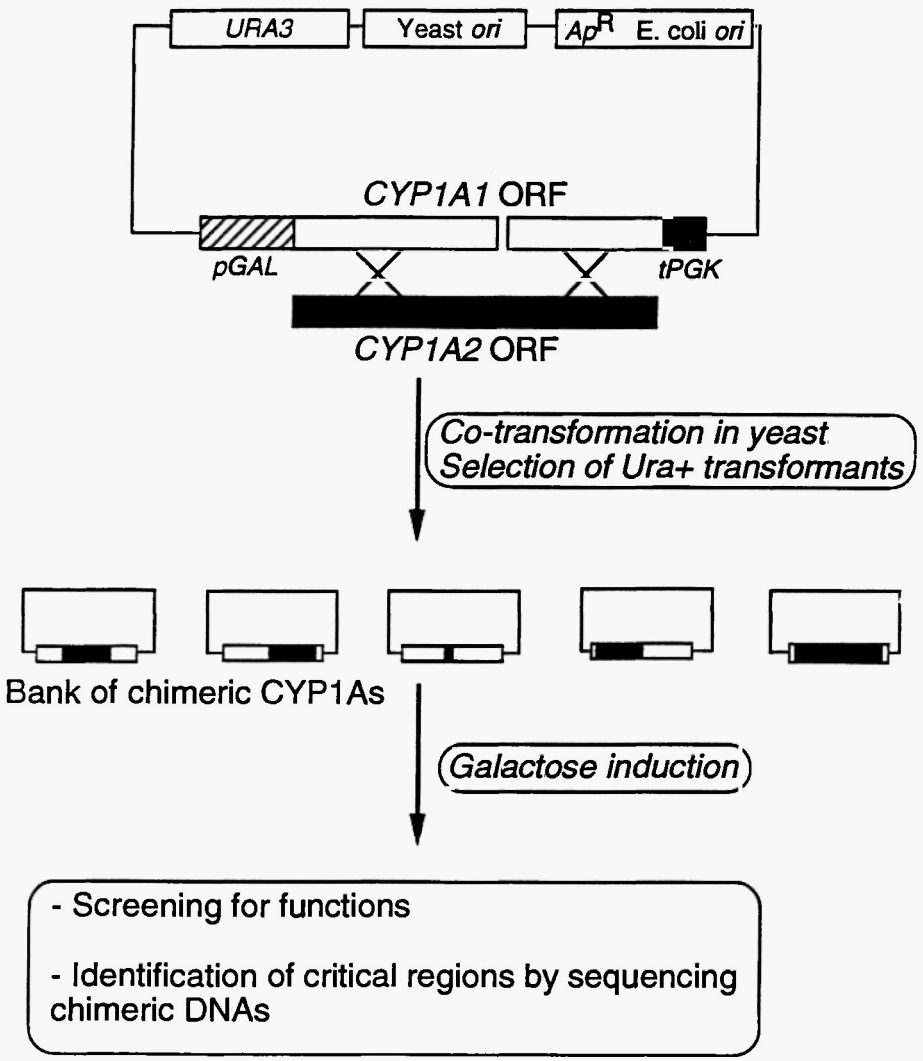

Fig. 4: **Chimaera construction by homeologous recombination.** The expression vector for P450 1A1 is linearised at a unique site situated in the CYP1A1 coding sequence; this refers to the template fragment. The entire sequence coding P450 1A2 serves as the repair fragment. Yeast reconstitutes a circularized vector from both fragments by two recombination events.

observed. Sequence analysis identified the human chimaeric S12 structure as composed of the 88 N-terminal amino acid residues of P450 1A1 and the remaining sequence of P450 1A2 /112/. The molecular determinants responsible for the unusually strong accumulation of this protein are under investigation. In another series of chimaeric structures obtained between mouse P450 1A1 and rabbit P450 1A2, 14 different chimaeras have been analysed for their methoxyresorufin- and phenacetine-O-deethylase relative activities, two substrates having very different molecular skeletons. All the chimaeras except two were found to have detectable activities, but the activity ratios were observed to vary in a large range around that of the parental enzymes /84/.

Homeologous recombination can thus generate chimaeric DNAs encoding recombinant proteins that exhibit a wide variety of modified activities. This approach associated with yeast expression is a powerful tool for protein engineering, making possible the creation of new activities or the improvement of P450 expression without any preconceived ideas on which amino acid(s) should be changed.

7. CONCLUDING REMARKS

Mechanistic and structural knowledge of eukaryotic cytochromes P450 was, and continues to be, more and more dependent on their expression in heterologous hosts. Expression in *Saccharomyces cerevisiae* combines the advantages of a robust organism with fast growth which is also characterised by typical eukaryotic features associated with extraordinary recombination properties.

The concept of "humanised" yeast strains supersedes approaches using protein fusion or multiple expression based on plasmids, since it allows the expression of virtually any human P450 in a large range of redox / phase II environments without requiring specific construction or protein modification. The only requirement is to construct an expression vector for the P450 of interest and to transform the humanised yeast strain which is the best adapted to the particular use considered. The multi-integration strategy can be very easily extended to the coexpression in yeast of a large number of heterologous proteins. Such an extension opens the field for the third generation of humanised yeast strains catalysing complex heterologous metabolic pathways. Among different applications, the simulation of human drug

and pollutant metabolism, environmental depollution and the full biosynthesis of molecules of industrial interest can be considered.

Two apparently unconnected events occurred in 1985, the publication by Thomas Poulos of the first three-dimensional structure of a cytochrome P450, that of *Pseudomonas putida* P450cam /113/, and the first heterologous expression of a mammalian cytochrome P450 /5/. Nine years later, two additional 3D-structures, the haemoprotein domain of *Bacillus megaterium* P450BM-3 /114,115/ and *Pseudomonas* spp. P450terp /115/, have been resolved and several dozens of P450 have been cloned and expressed. The coming years promise resolution of two major challenges, the determination of the first three-dimensional structure of a membrane-bound P450, and the use of P450-based biocatalysts in industry /116/. It is likely that one day, structural and biotechnological approaches will join to enable the design of a "core P450" structure, a functional catalytic block whose substrate specificity could be assigned at will by assembling it to substrate-specific recognition structures. Practical design of artificial proteins with customised activities by assembling a catalytic core with recognition modules could represent a useful future extension from the heterologous multi-expression systems derived from the humanised cell concept.

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