Selection by genetic complementation and characterization of the gene coding for the yeast porphobilinogen deaminase

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The porphobilinogen deaminase (PBG-D) gene of *Saccharomyces cerevisiae* has been isolated by genetic complementation of a mutant GL7 (α *hem* 3) strain, previously shown to be defective in this haem-biosynthetic enzyme [Gollub, Liu, Dayan, Adlersberg & Sprinson (1977) *J. Biol. Chem.* **252**, 2846–2854]. The gene was selected from a yeast wild-type genomic DNA library ligated into the shuttle vector YEp13. The complementing gene restored growth of the *hem* 3 (PBG-D) mutant strain on media in the absence of exogenous haem or fatty acid and sterol supplements. The recombinant plasmid was retained in the Hem⁺ transformant provided that selective pressure for plasmid-dependent growth was maintained. Transformation of the mutant strain (*hem* 3) restored the PBG-D activity to levels up to 10-fold those of the parental strain. The mutant strain GL7 does not show any measurable enzymic activity. Analysis of the plasmid designated YEpPBG-D (containing the PBG-D gene) by hybrid-selected translation revealed that it contained the coding information for a single protein of apparent M_r 43000. The coding region was localized on an 1.5 kb endonuclease-*Eco*RI fragment (E4), within the 5.5 kb genomic insert in YEpPBG-D.

INTRODUCTION

Haem is synthesized in a sequential process involving several enzymes located both in the cytosol and in the mitochondrial matrix. The biological significance of this dual compartmentalization as well as the overall regulation of haem biosynthesis is still poorly understood. The third step in this biosynthetic pathway is catalysed by porphobilinogen deaminase (PBG-D; EC 4.3.1.8). The reaction involves the condensation of four molecules of porphobilinogen, forming one molecule of uroporphyrinogen-1 [1]. PBG-D has been isolated to homogeneity from several sources, such as *Rhodospirillum rubrum* [2], spinach [3] and human [4]. The M_r values for the bacterial, plant and human enzymes have been estimated to be 36000 [2], 40000 [3] and 37000 [4] respectively. The subunit composition of the human enzyme has been suggested to be monomeric [4]. However, no data is yet available on the primary structure of this enzyme. Cloning and sequencing of the *hem* 3 locus from a genomic library would, for the first time, provide information not only on the primary sequence of the protein but also data on the 5'- and 3'-terminal sequences; this would open up the possibility of studying the co-ordinate regulation and expression of the enzyme in the haem-biosynthetic pathway. Genetic complementation of yeast mutants defective in a defined gene with a wild-type genomic DNA library ligated into an *Escherichia coli* shuttle vector has proved to be a convenient tool for isolating several yeast genes [5–8]. In the present report we have used the *hem* 3 mutant isolated by Gollub *et al.* [9] for this purpose. This *hem* mutant strain, when grown on media supplemented with ergosterol and Tween 80, contains no detectable

cytochromes, cannot respire and lacks PBG-D activity. Yeast double mutants containing the non-reverting *leu* 2 mutation [10] and *hem* 3 have been used to select, by co-transformation of the *leu hem* alleles, the PBG-D gene. Analysis of the product encoded in the plasmid YEpPBG-D indicates that the PBG-D gene codes for a 43 kDa product. This molecular mass is similar to that of PBG-D isolated from plants [3] and human erythrocytes [4].

MATERIAL AND METHODS

Strains and cell growth

The PBG-D-deficient mutant strain GL7 (α *hem* 3) was kindly provided by Dr. Edith Gollub, Barnard College, New York, NY, U.S.A. This strain was derived in previous studies [9] from strain X2180 (α *gal* 2), which was obtained for the present analysis from the Yeast Genetic Stock Center, Berkeley, CA, U.S.A. The non-reverting *leu* 2 mutation [10] was obtained from yeast strain 6657-4D (*a leu* 2-3, *leu* 2-112, *his* 3-11, *his* 3-15, *can* 1-11) kindly provided by Dr. G. Fink, Massachusetts Institute of Technology, Cambridge, MA, U.S.A. Yeast mutant XJ6 (*a hem* 3, *leu* 2-3, *leu* 2-112, *can* 1-11) was obtained by standard yeast methods [11,12]. *E. coli* strain RRI described previously [13] was used for the propagation of selected plasmids in a procaryotic host.

Yeast *hem* mutants were maintained and grown on solid (2% agar) or liquid media containing 1% yeast extract/2% bacto-peptone/2% glucose supplemented with Tween 80 (2.5 mg/ml) and ergosterol (12 µg/ml) (YPDTE). Selection of Hem⁺ Leu⁺ transformants was performed on the leucine-minus regeneration agar

Abbreviations used: YEpPBG-D, plasmid containing the porphobilinogen deaminase gene; SSC, 0.15 M-NaCl/0.015 M-sodium citrate; kb, kilobase pair(s); PBG-D, uroporphyrinogen deaminase.

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described in previous studies [13,14]. The Hem⁺ Leu⁺ yeast transformant was grown on either solid or liquid SD-leucine or YPD media as previously described [13,14].

E. coli were grown on LB media [15] or on plates containing 1.5% agar with or without ampicillin (50 µg/ml) (LB amp) or tetracycline (25 µg/ml) (LB tet) as required. M9 salts medium supplemented as described previously [13,15] was used for growth of *E. coli* for plasmid production.

Mitotic segregation of Leu⁺ and Hem⁺ in transformants

Hem⁺ transformants were grown for 30 generations on liquid YPDTE media. At the end of this growth period, cells were spread for single colonies on YPDTE plates. After 2 days at 30 °C the YPDTE master plates were replicate-plated on to solid medium selecting for either Hem⁺ (YPD) or Leu⁺ (SDTE-leucine). After 5 days the replicate plates were scored. The percentage retention is expressed as Hem⁺ or Leu⁺ clones divided by the total number of colonies scored. The co-segregation frequency is expressed as the number of Hem⁺ colonies divided by the number of Leu⁺ colonies. In control experiments monitoring growth of TJ6 on either YPD (Hem⁺ selection only) or SDTE-leucine (Leu⁺ selection only), all colonies examined as described above retained both the Hem⁺ and Leu⁺ alleles.

Yeast transformation

Selection of Hem⁺ Leu⁺ co-transformants was performed essentially as described previously [13]. Transformation of *hem leu* yeast mutants utilized the method of Beggs [16]. Hem⁺ transformants could be maintained on YPD media without loss of transforming plasmid. The pool of yeast wild-type genomic DNA carried in the restriction-endonuclease-*Bam*HI site of the LEU2 replicating vector YEp13 used in this study, was provided by Dr. Benjamin Hall, University of Washington, Seattle, WA, U.S.A.

Plasmid isolation and restriction analysis

Isolation of plasmid DNA from yeast utilized a small-scale procedure [17], modified from a method for the preparation of bacteriophage DNA [18]. Large-scale and small-scale preparation of plasmid DNA from *E. coli* was performed with modifications described in previous studies [13]. Restriction-endonuclease digestion of isolated plasmids was performed in either medium- or high-salt buffer as described, followed by analysis on agarose mini-gels [13].

Hybrid-selected translation

Total LiCl-precipitable yeast RNA was prepared from yeast Hem⁺ Leu⁺ transformants grown to 5 × 10⁷ cells/ml on YPD media as previously described [19]. The following procedure for hybridization translation represents a significant modification of our previous procedure [13,17] and will be described in detail. A 10 µg portion of plasmid DNA was digested overnight with endonuclease *Eco*RI, precipitated, and then dissolved in water to 1 µg/µl. The linearized DNA was then heated to 100 °C for 10 min, quickly cooled on solid CO₂/ethanol and incubated in 1 vol. of 1 M-NaOH at room temperature for 20 min. After neutralization with

0.5 vol. of 1 M-NaCl/0.3 M-sodium citrate/0.5 M-Tris/HCl (pH 8.0)/1 M-HCl, the DNA was rapidly cooled on ice and spotted on a 4 mm × 4 mm nitrocellulose filter paper. The filter was placed on top of a Whatman 3 M filter paper, wetted with 4 × SSC [15] to allow slow passage of the liquid through the nitrocellulose paper. After spotting, the nitrocellulose filter was air-dried for 1 h, washed three times in 1 ml of 6 × SSC at room temperature and then air-dried again for 1 h. The nitrocellulose filter was finally baked in a vacuum oven at 80 °C for 2.5 h. The filters could be stored at room temperature until ready to use.

Before hybridization, the nitrocellulose filter was washed once in 1 ml of sterile water at 100 °C and once with 1 ml of water at room temperature to remove loosely attached DNA. The hybridization reaction (500 µl total volume) contained 10 µg of bound DNA, 65% deionized formamide, 10 mM-Pipes, pH 6.4, 0.4 M-NaCl and 1.25 mg of total RNA/ml or 12.5 µg of poly(A)⁺ RNA/ml, essentially as described by Ricciardi *et al.* [20]. The reaction mixture was heated to 70 °C for 10 min before the addition of the nitrocellulose filter. The hybridization was carried out for 24 h at 50 °C with continuous shaking. After the hybridization, the nitrocellulose filter was washed ten times in 1 ml of 1 × SSC/0.5% SDS and five times in 2 mM-EDTA, pH 8.0.

The washed nitrocellulose filter was boiled for 1 min in 300 µl of water containing 20 µg of carrier calf liver tRNA (Boehringer) to release hybridized RNA. The RNA was then precipitated, washed in 70% (v/v) ethanol, dried, and finally dissolved in 20 µl of 5 mM-Hepes, pH 7.4.

A 10 µl portion of the hybrid-selected mRNA was translated for 40 min at 29 °C in a reticulocyte-lysate system (total volume 50 µl) in the presence of [³⁵S]methionine (1.2 mCi/ml), essentially as described by Pelham & Jackson [21]. After completion of the translation *in vitro*, 10 µl samples were withdrawn, mixed with 2 vol. of 2% SDS/10 mM-Tris/HCl, pH 6.8, and treated with 1/10 vol. of pancreatic ribonuclease (1 mg/ml) for 20 min and 30 °C. This procedure removes much of the background label in the gel patterns. The digested samples were finally boiled in the presence of 10% (v/v) β-mercaptoethanol and loaded on to an SDS/10% (w/v) polyacrylamide gel. The buffer system used was as described by Laemmli [22]. After electrophoresis, gels were fixed in boiling 5% (w/v) trichloroacetic acid for 10 min, soaked in water for 30 min and then an additional 30 min in 1 M-sodium salicylate and finally dried and exposed to preflashed RPI X-ray film [23].

Miscellaneous

PBG-D activity was measured exactly as described previously [11]. Protein determinations were performed as described by Lowry *et al.* [24], with minor modifications.

RESULTS

Selection of genetic complementation of the gene coding for PBG-D

The *hem 3* mutant strain of *Saccharomyces cerevisiae*, defective in the structural gene coding for PBG-D, was used to select clones from a genomic library of yeast

DNA sequences carried in the yeast LEU2 vector YEp 13 [8]. The *hem 3* (α GL7) mutant was crossed with strain a 6657-4D and haploid XJ6 *hem 3 leu 2-3 leu 2-12 can^R* strains were selected by random spore analysis.

Genetic complementation of the *hem leu 2* mutants was performed by using a partial endonuclease-*Sau3A*-digested pool of yeast DNA as previously described [13,14]. After transformation and selection of transformants on SD-leucine agar the transformants were subsequently plated on YPD. On this medium lacking added Tween 80 and ergosterol, no growth was observed for the XJ6 (*hem 3*) mutant.

To confirm that the observed growth was due to complementation and not reversion of the *hem* allele, a co-loss and co-retention experiment of the *leu 2* gene (vector marker) and the selected gene was performed. As discussed previously [8], plasmids like YEp 13 containing 2 μ circle DNA are unstably maintained in yeast transformants. When grown under non-selective conditions (media containing leucine), the YEp 13 plasmid will be lost at a rate of 1% per generation. This property of mitotic segregation of the vehicle and of its inserted sequences provides a convenient test to confirm the location of the transforming gene on the plasmid [13,14]. Growth of TJ6 transformants (*hem 3 leu 2-3 leu 2-112 can^R*) for approx. 20–30 generations under non-selective conditions for both *leu* and *hem* alleles caused the co-ordinate loss of 76% of both alleles, resulting in a co-segregation frequency of 100%. This strongly suggests that the *hem⁺* phenotype is plasmid-derived. In control studies growth of transformants on medium selecting for either *Leu⁺* or *Hem⁺* resulted in no loss of either marker during growth.

Analysis of Hem⁺ transformants

A Hem⁺ transformant of *hem 3* (TJ6-1) was assayed for PBG-D activity (Table 1). As previously reported [9,27], no measurable PBG-D activity was detected in the *hem 3* mutant XJ6. The PBG-D activity of the Hem⁺ transformant of XJ6 (TJ6-1) was 10-fold higher than the activity of the parental strain (X2180). The increased PBG-D activity of the transformant above that of the parental strain may reflect the elevated copy number of the gene on the multicopy-number 2 μ vector [8,28]. Additional studies will be required to determine the mechanism for this increase.

Restriction analysis of the plasmid isolated from the Hem⁺ transformant (TJ6-1)

In order to characterize the plasmid which complements the *hem 3* mutant strain, DNA prepared from Hem⁺ transformants was amplified by transformation

Table 1. Specific activity of PBG-D found in mutant and transformant strains

Yeast strain	Phenotype	PBG-D activity (nmol/h per mg of protein)
X2180	Hem ⁺	3.69
GL7	Hem ⁻	0
XJ6	Hem ⁻	0
TJ6-1	Hem ⁺	38.6

Fig. 1. Restriction analysis of YEpPBG-D

(a) Plasmid DNA prepared from yeast transformants was propagated in *E. coli* (RR1) and isolated as described in the Materials and methods section. Plasmid YEpPBG-D as well as vector plasmid YEp13 were digested by the following restriction enzymes: lane 1, λ -DNA/*HindIII*; lane 2, YEpPBG-D/*EcoRI*; lane 3, YEpPBG-D/*HindIII* plus *EcoRI*; lane 4, YEpPBG-D/*HindIII*; lane 5, YEp13/*EcoRI*; lane 6, YEp13/*HindIII*; lane 7, YEp13/*HindIII* plus *EcoRI*; lane 8, λ -DNA/*HindIII* (standard DNA having the following fragment lengths: 23.3, 9.5, 6.4, 4.2, 2.2, 1.8, and 0.53 kb), and analysed by agarose (0.9%)-gel electrophoresis. On the basis of this analysis, a restriction map was constructed (b). The wavy lines represent the 2 μ circle DNA of the vector. E, *EcoRI*; P, *PstI*; B, *BamHI*; S, *Sall*; H, *HindIII*; The numbers refer to fragment lengths in kb. *leu 2* and *amp* are the yeast isopropylmalate dehydrogenase and the pBr322 ampicillin genes respectively.

into *E. coli* strain RR1. Amp^R transformants were picked and plasmid YEpPBG-D isolated. The plasmid has a total size of 16.3 kb. On the basis of restriction-enzyme digestions of YEpPBG-D and YEp13 (Fig. 1a), a restriction map for plasmid YEpPBG-D was constructed (Fig. 1b). The size of the genomic insert was estimated to 5.5 kb, and was shown to contain several endonuclease-*Hind*III sites, but no *Pst*I or *Sal*I sites. Furthermore, one *Bam*HI site is lost at one end of the insert.

Characterization of plasmid-encoded proteins

Determination of the proteins encoded by YEpPBG-D utilized translation *in vitro* of plasmid-selected mRNA obtained from wild-type yeast. Analysis of plasmid-dependent gene products (Fig. 2) revealed that a unique translation product could be assigned to plasmid YEpPBG-D. Translation *in vitro* of mRNA selected by YEpPBG-D gave two products of apparent M_r 43 000 and 45 000 (lane 3). The 43 000- M_r product is assigned to the PBG-D protein. This assignment is based on: (1) similar M_r to that reported for UPG-D isolated from human erythrocytes [4], plants [3] and rat liver (M_r

Fig. 2. Translation *in vitro* of mRNA previously hybrid-selected by YEpPBG-D

Translatable mRNA was hybrid-selected by YEpPBG-D as described in the Materials and methods section. A 10 μ g portion of immobilized YEp13 or YEpPBG-D plasmid DNA was respectively hybridized with 0.4 mg of total RNA isolated from TJ6. The hybrid-selected mRNA species were translated in a rabbit reticulocyte-lysate system and were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Lane 1, no plasmid; lane 2, YEp13; lane 3, YEpPBG-D. The $10^{-3} \times M_r$ values shown were determined relative to known standards.

Fig. 3. The PBG-D gene is located on the 1.5 kb *Eco*RI fragment in YEpPBG-D

Plasmid YEpPBG-D was digested with *Eco*RI and each of the six fragments generated were individually hybridized to total RNA. Hybrid-selected mRNA species were translated in a rabbit reticulocyte-lysate system and translational products were subsequently analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Translational products obtained from hybrid-selected mRNA to *Eco*RI fragment: lane 1, 5.8 kb (E1); lane 2, 4.2 kb (E2); lane 3, 2.0 kb (E3); lane 4, 1.5 kb (E4); lane 5, 1.3 kb (E5); lane 6, 1.0 kb (E6); lane 7, no fragment (control). For fragment identification, see Fig. 1(b).

42 000) [28]. (2) Only one gene product is encoded in plasmid YEpPBG-D, not found in the vector YEp13. (3) Plasmid YEpPBG-D and not YEp13 restores PBG-D activity in the GL7 mutant strain. The 45 kDa gene product encoded in YEpPBG-D as well as in the vector YEp13 (lane 2) has previously been described as the *leu* 2 gene product isopropylmalate dehydrogenase [13,19].

Localization of the coding region for PBG-D within the genomic insert in YEpPBG-D

In order to locate the coding region for PBG-D within the genomic insert, plasmid YEpPBG-D was digested with *Eco*RI, generating six fragments (E1–E6). Each fragment was isolated and used to select mRNA essentially as outlined above (see the Materials and methods section). Selected mRNA species were subsequently translated *in vitro* and the gene products analysed by gel electrophoresis and autoradiography (Fig. 3). The 1.5 kb *Eco*RI fragment (E4) was the only fragment found that hybridized a specific mRNA, which upon translation gave rise to a gene product of M_r 43 000. The *leu* 2 gene was as expected found on the 2.0 kb *Eco*RI fragment (E3). The protein band seen in all lanes, including the control (lane 7), is an endogenous product of the reticulocyte-lysate system.

DISCUSSION

In the present paper transformation of defined yeast Hem⁻ mutant to Hem⁺ has been utilized to select the gene that restores PBG-D activity. This gene was selected from a pool of yeast genomic DNA molecules carried in the shuttle vector YEp13 [8,13]. Plasmid YEpPBG-D co-transformed with high efficiency the *leu* 2 and the Hem⁺ allele and restored activity to levels exceeding what is observed in the wild-type strain. The *hem* 3 mutant lacked any detectable PBG-D activity. Upon transforma-

tion of *hem 3* with YEpPBG-D the specific cellular activity of the PBG-D was increased 10-fold.

Previous studies from this laboratory and elsewhere have shown that the plasmid copy number of YEp13 derivatives is approx. 10–15 copies/cell [13,29]. These data would suggest that the expression of PBG-D is gene-dose-dependent. Additional studies indicate that the 10-fold increase in the specific activity of the PBG-D is due to an overproduction of this enzyme and not due to the biosynthesis of an altered protein. Thus, yeast transformants containing the multicopy-number plasmid YEpPBG-D should provide a convenient source for the isolation of this enzyme. Translation of mRNA selected by plasmid YEpPBG-D revealed the presence of a protein of M_r 43000. A similar M_r was found for the human PBG-D by Desnick *et al.* [4] as well as for the rat liver enzyme (M_r 42000) [28]. Interestingly, preliminary hybridization experiments using the yeast PBG-D gene as a hybridization probe to screen a human liver cDNA library have given positive signals, indicating homology between the yeast and human genes. Work is now needed to determine the DNA sequence for the yeast enzyme.

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