Expression of the *Pseudomonas* Gene Coding for Carboxypeptidase G2 in *Saccharomyces cerevisiae*

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The *Pseudomonas* gene coding for carboxypeptidase G2 was introduced into *Saccharomyces cerevisiae* on an *Escherichia coli*/yeast shuttle vector pROG5. The level of enzyme activity obtained was independent of the orientation of the gene within the pBR322-derived tetracycline resistance gene of the vector, indicating that expression can occur from a *Pseudomonas* promoter in yeast.

INTRODUCTION

The enzyme carboxypeptidase G2 (CPG2) from *Pseudomonas* hydrolyses the C-terminal glutamate moiety from folic acid and its analogues. Carboxypeptidase G enzymes have been used for rescue therapy of patients suffering methotrexate toxicity during chemotherapy, and animal studies have shown that the enzymes themselves, by depleting the levels of circulating folate, have antitumour properties (Kalghatgi & Bertino, 1981). In order to increase yields of CPG2 so as to provide sufficient material for a full assessment in clinical trials, the gene coding for CPG2 has recently been cloned in *Escherichia coli* (Minton *et al.*, 1983), where it is expressed at 3–5% of the soluble protein. The nucleotide sequence has been determined (Minton *et al.*, 1984) and the coding region consists of 1·24 kb of DNA, including a coding region for a signal peptide of 22 amino acids at its N-terminus. The enzyme is located in the periplasmic space in both *Pseudomonas* and *E. coli* (Minton *et al.*, 1983).

Interest in yeast as an alternative host to *E. coli* has escalated in recent years and several commercially important products from eukaryotic sources have been cloned in *Saccharomyces cerevisiae* (Hitzeman *et al.*, 1981; Derynck *et al.*, 1983; Miyanojara *et al.*, 1983; Mellor *et al.*, 1983). In addition, there are now reports in the literature of both plasmid-derived (Hollenberg, 1979; Cohen *et al.*, 1980; Jimenez & Davies, 1980; Roggenkamp *et al.*, 1981; Cohen *et al.*, 1983; Gritz & Davies, 1983) and chromosomally-derived (Panthier *et al.*, 1980; Janowicz *et al.*, 1982; Fehor *et al.*, 1983) prokaryotic genes being functionally expressed in yeast. Studies on the expression of heterologous genes in alternative hosts are providing valuable information for interpreting the requirements for gene expression and the subsequent processing of the products.

In this report, we show that the CPG2 gene from *Pseudomonas* is functionally expressed in *S. cerevisiae*. This is significant both in considering yeast as a possible alternative host for the production of CPG2 and other proteins, and in facilitating further studies on the nature of gene expression and protein processing in yeast.

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Abbreviation: CPG2, carboxypeptidase G2.

METHODS

Bacterial and yeast strains. The strains used were *E. coli* JA221 (*recA1 leuB6 trpΔE5 hsdR M⁺ lacY C600*) and *S. cerevisiae* LL20 (*MATa leu2-112 leu2-3 his3-11 his3-15* [$2\mu\text{m}^+$]).

Plasmids. The structures of the plasmids used are shown in Fig. 1. The *S. cerevisiae*/*E. coli* shuttle vector, pROG5, was constructed by inserting the 3.2 kb *Hind*III fragment of pJDB219 (Beggs, 1978), containing the *leu2* gene of *S. cerevisiae* and the replication origin of the $2\mu\text{m}$ plasmid, into the *Hind*III site of pBR322 (Bolivar *et al.*, 1977).

The coding sequences for the promoter, signal peptide and complete structural gene of CPG2 are contained within a 2.034 kb *Bam*HI restriction fragment isolated from the recombinant plasmid pNM15 (Minton & Clarke, 1985). The recombinant plasmids pLEC3 and pLEC4 were constructed by inserting this fragment, in both orientations, into the *Bam*HI site within the tetracycline resistance gene (*tet*) of pROG5.

Media and culture conditions. Cultures of *E. coli* for plasmid isolation were grown at 37 °C in L-broth (1% tryptone (w/v), 0.5% yeast extract, 0.5% NaCl), to which Bactoagar (2%, w/v) was added for solid media. Minimal medium for *E. coli* was M9 (Miller, 1972) with 0.2% folic acid added where appropriate. Ampicillin (50 $\mu\text{g ml}^{-1}$) and tetracycline (15 $\mu\text{g ml}^{-1}$) were used for the selection of transformants.

Yeast cells were grown at 30 °C on complete YEPD medium [1% yeast extract (w/v), 2% peptone (w/v), 2% glucose (w/v)]. Solidified medium consisted either of YEPD or selective YNB medium with the addition of 3% (w/v) Bactoagar [YNB contains 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose (w/v) and the required amino acids to 20 $\mu\text{g ml}^{-1}$]. In order to screen for the *Fol*⁺ phenotype (ability to hydrolyse folic acid) the glucose concentration in YNB medium was reduced to 0.05% and 0.2% folic acid was added.

Preparation of plasmid DNA. Plasmids were isolated from chloramphenicol-amplified cultures of *E. coli* by Brij lysis (Clewell & Helinski, 1969) and caesium chloride/ethidium bromide density gradient centrifugation (Colman *et al.*, 1978). The small-scale plasmid isolation procedure of Holmes & Quigley (1981) was used for rapid screening of transformants.

Small-scale preparations of plasmids from *S. cerevisiae* were made by suspending an inoculating loop of cells in 100 μl spheroplasting buffer (1.0 M-sorbitol, 25 mM-EDTA, 67 mM-phosphate buffer pH 7.5 and 5 mg Zymolyase 60000 ml^{-1}). The spheroplasts were lysed by the procedure of Birnboim & Doly (1979). The final pellet was resuspended in 20 μl TE buffer (10 mM-Tris/HCl pH 8.0, 1 mM-EDTA) and emulsified with 20 μl chloroform/isoamyl alcohol (24:1, v/v). Following centrifugation, the plasmid-containing phase was used to transform competent *E. coli* cells. (This procedure was a personal communication from N. Urwin, Charing Cross Hospital, London, UK.)

Restriction, ligation and transformation procedures. Restriction enzymes and DNA ligase were purchased from Bethesda Research Laboratories, and were used according to the manufacturer's instructions. *E. coli* was transformed essentially as described by Cohen *et al.* (1972). Transformants were selected on ampicillin and screened for tetracycline sensitivity and the *Fol*⁺ phenotype, which is evident on folate-containing agar medium as concentric yellow haloes of precipitated pteric acid (Minton *et al.*, 1983). Transformation of *S. cerevisiae* was mediated by lithium acetate as described by Ito *et al.* (1983) and the transformants were screened for the *Leu*⁺ *Fol*⁺ phenotype.

Determination of CPG2 activity. Bacteria or yeast were grown in 500 ml batch cultures. Samples (100 ml) were centrifuged at 8000 *g* for 10 min and resuspended in 5 ml 0.1 M-Tris/HCl pH 7.3 containing 0.2 M-ZnSO₄. The cells were disrupted by two passages through a French press at 1250 lbf in⁻² (8.63 MPa). The cell debris was removed by centrifugation at 10000 *g* for 10 min and the supernatant was assayed for CPG2 activity by the method of McCollough *et al.* (1971) as described by Minton *et al.* (1983) (one unit represents the hydrolysis of 1 μmole of methotrexate per min at 37 °C). Protein was determined by the method of Bradford (1976).

RESULTS AND DISCUSSION

The shuttle vector pROG5 continues to express resistance to tetracycline at up to 60 $\mu\text{g ml}^{-1}$ despite the insertion of a fragment of yeast DNA into the *Hind*III site within the promoter of the gene (Backman & Boyer, 1983). This is almost certainly due to the reconstitution of a functional *tet* promoter as a result of the fortuitous presence of promoter-like sequences in the inserted $2\mu\text{m}$ DNA. Two conserved regions are known to be important for transcription initiation in prokaryotes: the sequences of the -35 and -10 regions relative to the start point of mRNA transcription, whose consensus sequences are TTGACA and TATAAT respectively (for review see Von Hippel *et al.*, 1982). The restriction enzyme *Hind*III cleaves immediately before the -10 region of the *tet* promoter (Fig. 2). The $2\mu\text{m}$ DNA inserted into the *Hind*III site, however, carries a pseudo -35 region TAGTCA, separated from the *tet* promoter -10 region by a spatially acceptable 16 nucleotides. The $2\mu\text{m}$ DNA sequence TTAGTC which is 17 bp from the

Fig. 1. Derivation of the *S. cerevisiae*/*E. coli* shuttle vector pROG5 and the recombinant plasmids pLEC3 and pLEC4. Arrows indicate the relative orientations of the tetracycline (Tc) and ampicillin (Ap) resistance genes within the plasmids. ■, Antibiotic resistance genes; ▨, yeast 2 μ m plasmid; ▤, CPG2 gene; ▩, yeast chromosomal *leu2* gene; □, pMB9 in pJDB219, pAT153 in pNM15, pBR322 elsewhere. H, HindIII cleavage site; B, BamHI cleavage site.

Fig. 2. Comparison of the nucleotide sequence of the tetracycline gene promoter from pBR322 and the same region in pROG5. The sequence of pBR322 was taken from Sutcliffe (1978) and that of the yeast 2 μ m plasmid from Hartley & Donelson (1980). Nucleotides derived from the yeast 2 μ m plasmid are underlined. The vertical arrows indicate the cleavage site of HindIII.

—10 region presents a second, though less likely, possibility. Similar —35 sequences have been observed on other prokaryotic promoters (Rosenberg & Court, 1979; Siebenlist *et al.*, 1980). The deviation of the possible pseudo —35 sequences from the known and consensus —35 sequences of the *tet* promoter (TTGACA) suggests that the promoter created would be less efficient than the natural promoter of the *tet* gene.

The CPG2 gene was introduced into pROG5 as described in Methods, and was characterized in *E. coli* and subsequently transformed into *S. cerevisiae*. In *E. coli* the level of expression of CPG2 was dependent on the orientation of the gene within the vector. Enzyme assays on cell extracts showed that pLEC3 directed the expression of up to 40-fold greater levels of CPG2 compared to pLEC4. The CPG2 gene in pLEC3 is in the same orientation as the *tet* gene and the higher level of expression obtained with this plasmid can probably be accounted for by transcriptional readthrough from the *tet* promoter. This phenomenon has been observed with previous constructs carrying CPG2 inserted into the *Bam*HI site of the pBR322 *tet* gene (Minton *et al.*, 1983). In that instance, the CPG2 gene was transcribed from an unimpaired *tet* promoter and the orientation-dependent effect resulted in a 1000-fold difference in the level of enzyme activity.

Transformation of the plasmids pLEC3 and pLEC4 into yeast resulted in Leu⁺ colonies, 90% of which turned yellow after 48 h in the presence of folate, indicating the Fol⁺ phenotype. Plasmids isolated from the Leu⁺ Fol⁺ colonies were transformed into *E. coli* and screened by restriction analysis for the presence of an intact CPG2 gene. No deviations from the original plasmids used to transform the *S. cerevisiae* cells were identified.

The ability of *S. cerevisiae* cells to grow on folate in the presence or absence of the CPG2 gene was examined in detail (Table 1). Cells carrying either pLEC3 or pLEC4 grew very poorly on folate as the sole carbon and nitrogen source and required 0.05% glucose for the demonstration of the Fol⁺ phenotype. At high concentrations of glucose (0.5%) and under conditions of limited nitrogen, yeast cells transformed with pROG5 alone became orange in the presence of folate. This was accompanied by the formation of a transparent halo in the medium around the colonies, indicating that folate had been taken up from the plates, but not hydrolysed. The orange coloration observed in such cells was distinct from the diffuse bright yellow coloration of cells carrying the CPG2 gene and hydrolysing folate to pterate. These results suggest that *S. cerevisiae* has an energy dependent uptake of folate and can accumulate folate. An energy requirement for the uptake of folate has previously been demonstrated in murine leukaemia cells and in lactobacilli (Huennekens *et al.*, 1978).

In contrast to *E. coli*, there was no orientation-dependent expression of CPG2 in *S. cerevisiae*, as cells carrying either pLEC3 or pLEC4 demonstrated similar levels of enzyme activity (Table 2). This suggests that CPG2 is expressed entirely from its own promoter in *S. cerevisiae*. Although the low specific activity of CPG2 in yeast cells prevented selection for the plasmid in minimal medium in liquid culture, replica plating of isolated colonies onto folate minimal medium demonstrated that at least 70% of the cells still had the Fol⁺ phenotype after 5 d continuous sub-culturing at 30 °C in shake flasks.

In *S. cerevisiae* the maximum levels of enzyme activity were observed after 48 h in liquid culture, compared to approximately 6 h in *E. coli* (Table 2) (Minton *et al.*, 1983). The highest specific activity observed was about 0.03 units (mg protein)⁻¹, representing approximately 0.005% of the soluble protein. This is of a similar order to the activity measured when the gene was expressed from its own promoter in *E. coli*, where it represented 0.01% of the soluble protein (Table 2) (Minton *et al.*, 1983).

This report contains the first published evidence of a gene isolated from *Pseudomonas* being expressed in *S. cerevisiae* and provides evidence that some expression can occur from a *Pseudomonas* promoter in yeast. Mapping of the transcripts of the *E. coli* β -lactamase gene in *S. cerevisiae* has indicated that *S. cerevisiae* RNA polymerase can recognize sequences at or near *E. coli* promoters (Breunig *et al.*, 1982). However, recent promoter mapping studies of the CPG2 gene have shown that CPG2 does not possess a typical *E. coli*-type promoter (Minton & Clarke, 1985). This may account, at least in part, for the low levels of expressions of this gene in *E. coli* and could contribute to the similarly low levels found in *S. cerevisiae*. Attention has also been drawn

Table 1. Phenotype of *S. cerevisiae* cells in the presence or absence of CPG2 when grown on folate and various concentrations of glucose as sole carbon and nitrogen sources

S. cerevisiae cells carrying the CPG2 gene were grown on solidified minimal medium in the presence of leucine, histidine and 0.2% folic acid, but in the absence of any other carbon or nitrogen source. A range of concentrations of glucose was evaluated for its effect on growth and demonstration of the Fol⁺ phenotype. +, Poor growth; ++, reasonable growth; +++, good growth; W, white; WY, weakly yellow; Y, yellow; O, orange.

Carbon and nitrogen source (% w/v)		Cells transformed with:		
Glucose	Folate	pROG5	pLEC3	pLEC4
0	0.20	++ (W)	+ (WY)	+ (WY)
0.05	0.20	+++ (W)	++ (Y)	++ (Y)
0.10	0.20	+++ (W)	+++ (Y)	+++ (Y)
0.50	0.20	+++ (O)	+++ (Y)	+++ (Y)
2.00	0.20	++ (O)	++ (Y)	++ (Y)

Table 2. Specific activity of CPG2 in cell extracts from *E. coli* and *S. cerevisiae* carrying either pLEC3 or pLEC4

E. coli cells were grown at 37 °C in L-broth, in the presence of ampicillin, in shake flasks. *S. cerevisiae* cells were similarly grown at 30 °C in YEPD medium. The cells were disrupted and CPG2 assayed as described in Methods. No CPG2 activity was detected in control cultures of cells carrying pROG5 alone.

Host	Time (h)	Specific activity [Units (mg protein) ⁻¹]	
		pLEC3	pLEC4
<i>E. coli</i>	6	0.177	0.003
	24	0.090	0.021
	48	0.040	0.004
<i>S. cerevisiae</i>	6	0.006	0.012
	24	0.009	0.010
	48	0.020	0.028
	72	0.008	0.026

to an interesting feature of the CPG2 promoter by the recent observation of Johnston & Downie (1984) that a region in the promoter of the *Pseudomonas xylABC* operon (Inouye *et al.*, 1984) conforms to the consensus sequence for the regulatory region in the promoters of the *nif* genes in *Rhizobium* and *Klebsiella*. A similar sequence occurs in the CPG2 promoter (Minton & Clarke, 1985), raising interesting possibilities concerning the control of this gene, both in *Pseudomonas* and in heterologous hosts.

It is not yet known whether CPG2 activity in yeast is due to enzymic activity of the preprotein or if the yeast cell has processed the preprotein to mature enzyme. The *E. coli* preproteins of β -lactamase (Roggenkamp *et al.*, 1981) and the outer-membrane protein Omp A (Janowicz *et al.*, 1982) can be processed in *S. cerevisiae*. We are working currently to increase the levels of expression of CPG2 in yeast by the use of high expression vectors containing yeast promoters and to examine the processing and localization of the enzyme in the yeast cell.

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