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* hydrolys 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; Miyanohara 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; Jiminez & 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.
METHODS

**Bacterial and yeast strains.** The strains used were E. coli JA221 (recA1 leuB6 trpDE5 hsdR M+ lac Y C600) and S. cerevisiae LL20 (MATa leu2-112 leu2-3 his3-11 his3-15 [2 μm*]).

**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 HindIII fragment of pJDB219 (Beggs, 1978), containing the leu2 gene of S. cerevisiae and the replication origin of the 2 μm plasmid, into the HindIII 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 BamHI 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 BamHI 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 μg ml⁻¹) and tetracycline (15 μg ml⁻¹) were used for the selection of transformants.

Yeasts 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 μg 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 chromaphenicol-amplified cultures of E. coli by Brij lysis (Clewell & Helsinki, 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 pteroic 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/HC1 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 μg ml⁻¹ despite the insertion of a fragment of yeast DNA into the HindIII 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 μm 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 HindIII cleaves immediately before the −10 region of the tet promoter (Fig. 2). The 2 μm DNA inserted into the HindIII site, however, carries a pseudo −35 region TAGTCA, separated from the tet promoter −10 region by a spatially acceptable 16 nucleotides. The 2 μm DNA sequence TTAGTC which is 17 bp from the
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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 2μm plasmid; ■■, CPG2 gene; □□□□, yeast chromosomal *leu2* gene; □□□□, pMB9 in pJDB219, pAT153 in pNM15, pBR322 elsewhere. H, HindIII cleavage site; B, BamHI cleavage site.

![Diagram of plasmid derivations](image)

pBR322

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TGT[TTGACAGCTTATCATCGATAAGC]TTAAT3CG
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17 bp

pROG5

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A[ACTTACAGCTCGTACATTTAAAGC]TTAAT3CG
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16 bp

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 2μm plasmid from Hartley & Donelson (1980). Nucleotides derived from the yeast 2μm 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 BamHI 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 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 pteroate. 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 expression of this gene in E. coli and could contribute to the similarly low levels found in S. cerevisiae. Attention has also been drawn
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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.

<table>
<thead>
<tr>
<th>Carbon and nitrogen source (% w/v)</th>
<th>Cells transformed with:</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>Folate</td>
</tr>
<tr>
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<td>0:50</td>
<td>0:20</td>
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<tr>
<td>2:00</td>
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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.

<table>
<thead>
<tr>
<th>Host</th>
<th>Time (h)</th>
<th>Specific activity [Units (mg protein)-1]</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>pLEC3</td>
</tr>
<tr>
<td>E. coli</td>
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<tr>
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<tr>
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<td></td>
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<td>0:008</td>
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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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REFERENCES


