The bacteriophage 434 operator/repressor system in yeast

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The ability of the bacteriophage 434 operator/repressor system to function in a eukaryotic cell has been explored. An idealized 434 operator was placed at various positions in the PGK promoter of Saccharomyces cerevisiae: within the upstream activator sequence, close to the TATA box, and downstream of the transcription-initiation site. Expression of the 434 ci gene from a 2 μm-based plasmid resulted in significant repression of gene expression from constructs containing the altered promoters linked to a β-galactosidase reporter gene. Attempts to use a variant of the 434 repressor that has the binding specificity of the P22 repressor (434P22) were unsuccessful, due to a severely inhibitory effect of this gene-product on the growth of the yeast cells.

Keywords: bacteriophage 434 operator/repressor, P22 repressor, Saccharomyces cerevisiae, regulation of gene expression

INTRODUCTION

Repressor proteins effect the negative control of gene expression by binding to their cognate operator sequences with high affinity, thereby preventing access of RNA polymerase to the promoter. For the best-studied examples of repressors derived from bacteria and bacteriophages high-resolution structures of repressor–operator complexes are available.

Several repressors that normally function in prokaryotic cells have been used to repress expression from suitably modified promoters in a number of different eukaryotic cells. In mouse cells expressing the Escherichia coli lexA repressor gene the insertion of lexA operators into the thymidine kinase promoter resulted in repression of a chloramphenicol acetyltransferase reporter gene by up to 10-fold (Smith et al., 1988). The lac operator/repressor system has been shown to function in both mammalian and plant cells. Hu & Davidson (1987) showed that in mouse cells the insertion of a lac operator between the initiation codon and the transcription-start site, between the transcription-start point and the TATA box, or upstream of the TATA box, could block transcription in the presence of the lac repressor and that this block was relieved in the presence of the inducer IPTG. Similar findings have also been reported by Brown et al. (1987) and Figge et al. (1988). The lac repressor has further been shown to repress expression when the operator is located some distance downstream of the transcription-initiation site (Deuschle et al., 1990), thus demonstrating that a bacterial repressor can be an effective block to transcriptional elongation by RNA polymerase II. Wilde et al. (1992) demonstrated that the lac operator/repressor system functioned in tobacco cells, where expression of both stably transformed and transient reporter constructs were efficiently repressed, and that this repression could be relieved by IPTG. The tet repressor is also able to mediate efficient repression of expression from a plant promoter, the effect being relieved by tetracycline (Gatz & Quail, 1988; Gatz et al., 1991).

In yeast, RNA polymerase II-directed transcription has been repressed by the E. coli lexA repressor when lexA operators have been inserted into the GAL1 promoter between the upstream activator sequence (UAS) and the TATA box (Brent & Ptashne, 1984). Also in yeast, the tet repressor has been shown to block RNA polymerase III-dependent transcription when operators were inserted near to the transcription-start site of a tRNA gene (Dingermann et al., 1992).

The bacteriophage 434 operator/repressor system has been extensively characterized both in vivo and in vitro, but its ability to function in eukaryotic cells has not previously been investigated. In common with many other bacterial DNA-binding proteins, the 434 repressor utilizes the helix-turn-helix motif for DNA recognition (Pabo & Sauer, 1984). X-ray crystallographic analyses have shown that the third α-helix within the N-terminal DNA-binding domain of the protein makes contact with the operator DNA in the major groove (Anderson et al., 1985), with
sequence-specific contacts being made by three glutamine residues within this α-helix, at positions 28, 29 and 33 of the protein (Anderson et al., 1987; Aggarwal et al., 1988). The DNA-binding specificity of the 434 cl gene product has been altered both by a single amino acid change, residues within this α-helix, at positions 28, 29 and complementing single base-pair changes in the operator (Wharton & Ptashne, 1987), and by more substantial alterations to the α-helix (Wharton et al., 1984; Wharton & Ptashne, 1985). By substituting just five amino acids in the α-3 helix of the 434 cl gene-product by the amino acids in the corresponding positions in the DNA-binding helix of the P22 repressor, a hybrid 434 repressor (434 Pa) was created which retained all the properties of the 434 repressor except for the DNA-binding specificity, which was switched to that of the P22 repressor (Wharton & Ptashne, 1985). Hollis et al. (1988) subsequently demonstrated that by combining purified 434 and 434 Pa repressor proteins in vitro, a footprint could be produced on hybrid operator DNA consisting of half-sites from each of the 434 and the P22 operators, indicating the in vitro formation of a heterodimeric repressor of hybrid specificity. Recently it has been demonstrated that this heterodimeric 434 repressor is able to repress gene expression in E. coli at a level similar to those achieved with either homodimer (Webster et al., 1992).

We wish to be able to control gene expression in eukaryotes by the binding of a repressor to an operator sequence within the promoter of a target gene. In order to be able to do this without prior manipulation of the promoter sequence, the 434 repressor was seen as advantageous because of the existence of well-understood variants of this repressor with altered DNA-binding specificities (Wharton & Ptashne, 1985, 1987), and the established efficacy of the heterodimeric repressor (Webster et al., 1992). In addition, we have developed a genetic selection system that allows selection of altered specificity repressors from a mini-library of 434 repressor genes containing randomized codons for the amino acids that confer DNA-binding specificity (R. J. Wilde and others, unpublished).

To determine whether the 434 repressor is able to function efficiently in the environment of a eukaryotic nucleus, initial repression studies have been undertaken in Saccharomyces cerevisiae. Although 434 operators normally occur in tandem in the phage immunity region, where co-operative interactions between adjacent bound repressor-dimers increase the degree of repression, in this study single operators were used to assess the efficiency of the simple operator-repressor interaction. The results demonstrate that the 434 cl gene-product is able to function in S. cerevisiae by repression of gene expression from a PGK promoter–lacZ reporter; gene containing introduced 434 operators within the PGK promoter. The ability of the 434 Pa repressor to function in yeast was also investigated, but its expression in S. cerevisiae severely inhibited the growth of the host cells. This observation suggests the caution that heterologous repressor proteins may bind to sequences within a eukaryotic genome and have a major influence on the continued growth of the host cell.

### METHODS

#### Strains

Saccharomyces cerevisiae: S150-2B; MATa ura3-52 trp1-289 his3-D1 leu2-3 leu2-112 2μm.

E. coli: DH5α (Hanahan, 1983); F' endA1 hsdR17(rK' mK') supE44 thi-1 recA1 gyrA96 relA1 (argU–lacZΔM15)U169 φ80diazam15. X11-blue (Bullock et al., 1987); supE44 hsdR17 recA1 endA1 gyrA46 thi-1 relA1 lacY1 [proAB lacIq ZM15 Tn10], RZ1032 (Künkel et al., 1987); HfrK16 PO/45 [Jr: A(61–62) dút-1 ung-1 thi-1 relA1 supE44 sbd-279]: Tn10.

#### Standard molecular biology techniques

All general molecular biological techniques were carried out according to Sambrook et al. (1989). All plasmids were maintained in E. coli DH5α unless otherwise stated.

#### Site-directed mutagenesis

This was carried out by a variation of the method of Künkel et al. (1987). An overnight culture of E. coli RZ1032 was inoculated with M13mp19 phage containing the DNA to be mutated inserted in the polyclinker and the culture was left at room temperature for 30 min. This was diluted 100-fold into 2 x YT (g 1-l: Bacto-tryptone 16, Bacto yeast extract 10, NaCl 5) with 7.5 μg tetracycline ml-1 and grown for 6 h at 37 °C with vigorous agitation. The cells were removed by centrifugation at 8000 g for 20 min and the phage precipitated from the supernatant by the addition of NaCl and polyethylene glycol (PEG) 6000 to 30 mg ml-1 and 40 mg ml-1 respectively. The phage particles, collected by centrifugation at 8000 g for 20 min, were resuspended in 10 ml TE (10 mM Tris/HCl, 0.1 mM EDTA; pH 8.0) by vigorous vortexing. The protein coats were removed by phenol extraction and the DNA precipitated by ethanol precipitation and resuspended in 0.5 ml sterile deionized water. This DNA contains U in place of T residues at a small number of positions, making it unstable in strains of E. coli that do not carry the ung mutation.

Approximately 1 pmol of the template and 10 pmol of 5' phosphorylated mutagenic oligonucleotide were annealed in 20 μl sterile, deionized water containing 1:2 μl 20 × SSC. The synthesis of the complementary strand, primed from the mutagenic oligonucleotide, was carried out in a 100 μl reaction containing 500 μM of each dNTP, 1 mM ATP, 10 mM MgCl₂, 100 mM Tris pH 7.5, 2 mM DTT, 20 units of T4 DNA polymerase and 20 units of T4 ligase. The reaction mixture was incubated on ice for 5 min, at room temperature for 5 min and at 37 °C for 2 h, after which the reaction was terminated by the addition of 5 μl 500 mM EDTA, pH 8.0. An aliquot of this reaction was used to transform E. coli strain XL1-blue and mutated phages were identified by restriction enzyme digestion of the RF DNA or sequencing of the single-stranded phage DNA.

#### Construction of reporter plasmids

The PGK promoter and terminator were cut from pCH137 (Hadfield et al., 1990) on a HindIII fragment and cloned into the HindIII site in the polylinker of M13mp19. Site-directed mutagenesis (Künkel et al., 1987) was used to introduce a Smal site at position −605, relative to the RNA initiation point, in the PGK promoter. Further site-directed mutagenesis was used to create unique restriction sites at various positions in the PGK promoter: The six different PGK promoter-terminator constructs were removed from M13 RF DNA at 1 kb Smal–HindIII fragments and ligated into the polyclinker of a variant of pUC19, pUC19EN, which contains a NarI site in place of the EcoRI site in the polyclinker region, to give the plasmids in Table 1. The plasmids pYCW3, pYCW5 and pYCW6 were opened at the unique, introduced restriction sites and 434 operators inserted as annealed oligonucleotides, to give the plasmids pYCW8, pYCW10 and pYCW11 respectively. Plasmid pYCW4 was cut with EcoRV and XhoI and annealed oligonucleotides used to
ligated in to form a series of reporter constructs (Fig. 1). These unique NarI site further upstream of the immediately 5' of the start codon of the pYCW19 and the product checked by sequencing. This plasmid PGK on an EcoRI-NarI fragment, introduced by ligation. The resulting plasmid, pCW31, was cut at the unique BamHI site and a BglII-compatible overhang at the 3' end. This fragment was ligated between the EcoRI and BglII sites in pBR328 (Soberon et al., 1980). This plasmid was cut with HindIII and SalI and annealed oligonucleotides containing a BamHI site 5' of the initiation codon, to give the plasmid pCW7. Neither the SalI nor the HindIII site was retained in the ligation.

The plasmid pYCPGK was cut at the unique HindIII site 3' of the PGK terminator and a HindIII fragment containing the URA3 gene from YIp30 inserted to give pYCW19. The last 17 codons of the lacZ gene were synthesized as complementary oligonucleotides with an EcoRI-compatible overhang at the 5' end and a BglII-compatible overhang at the 3' end. This fragment was ligated between the EcoRI and BglII sites in pYCW19 and the product checked by sequencing. This plasmid was opened at the NarI and EcoRI sites and the fragment containing the remainder of the lacZ gene, isolated from pCW7 on an EcoRI-NarI fragment, introduced by ligation. The resulting plasmid, pCW31, was cut at the unique BamHI site immediately 5' of the start codon of the lacZ gene and at the unique NarI site further upstream of the lacZ gene. The series of PGK promoters containing operators, isolated on BglII-NarI fragments from pYCPGK, 8, 9, 10, 11 and 12, could then be ligated in to form a series of reporter constructs (Fig. 1). These could be linearized at the unique SalI restriction site within the URA3 gene to aid homologous recombination into the ura3 locus of the S. cerevisiae host.

**Construction of a plasmid expressing the 434 repressor.** The 2µm origin and most of the TRP1 gene were removed from pYcDE-2 (Hadfield et al., 1986) by digestion with XbaI and HindIII, followed by religating the remainder of the plasmid with annealed oligonucleotides used to create a small linker joining the XbaI and HindIII ends. The XbaI site was destroyed in the ligation and an XbaI site introduced. The resulting plasmid, pDE1, was opened at the unique EcoRI cloning site between the ADH1 promoter and the CYC1 terminator, and a small linker inserted to introduce unique XbaI and BamHI sites to give the plasmid pACXB1. The EcoRI site was not retained in the ligation. The 434 cl gene was cut from pPLRT1 (Webster et al., 1992) on an XbaI-Sau3AI fragment and ligated into pACXB1 opened at its XbaI and BamHI sites to give pYCW34. Both pYCW34 and pYcDE-2 were cut with SalI and MluI and the 434 expression cassette ligated into the 2µm plasmid to give pYCW36 (Fig. 2).

**Construction of plasmids expressing the 434P22 repressor.** This construction was similar to the one described above except that the 434P22 gene was substituted for the wild-type 434 gene. This involved isolating the 434P22 coding region from pPLRT2 (Webster et al., 1992) on a XbaI-Sau3AI fragment and substituting it for the XbaI-Sau3AI fragment containing the wild-type 434 cl gene to give pYCW28 (Fig. 3a). A second expression vector was also constructed for the 434P22 repressor. This was designed to integrate into the yeast genome maintaining single copy number. The promoter-repressor-terminator cassette made by ligating the XbaI-Sau3AI fragment containing the 434P22 repressor coding region into pACXB1 was removed on a SalI-XbaI fragment and cloned into the unique SalI site in YRG12 (Hadfield et al., 1987). Two
clones were picked, each having the repressor cassette but in different orientations: these were pYCW20A and B (Fig. 3b). These plasmids should express the 434p22 repressor constitutively from a truncated version of the ADH1 promoter and carry the HIS3 gene. Linearization of the plasmid with XhoI, which cuts uniquely within the HIS3 gene, improves the frequency of homologous recombination into the HIS3 locus.

A third plasmid to express the 434p22 repressor was also designed, based on the centromere plasmid pBM150 (Johnston & Davis, 1984). The 434p22 repressor gene was cut from pPLRT2 on an XbaI-Sau3A1 fragment and cloned into the polylinker of pIC19H (Marsh et al., 1984) opened at its BamHI and XbaI sites, to give pCW37B. This plasmid was then cut with BglII and SalI and the 434p22 repressor gene cloned into pBM150 opened at its SalI and BamHI sites to give the plasmid pYCW42 (Fig. 3c). This places the 434p22 repressor gene under the control of the yeast GAL1.1 promoter. During growth on glucose-based media the repressor gene should be silent, whereas during growth on galactose in the absence of glucose it should be expressed.

DNA sequencing. Both single-stranded M13 phage DNA and double-stranded plasmid DNA were sequenced by the dideoxy-termination method of Sanger et al. (1974), using a Pharmacia T7 sequencing kit according to the manufacturer's instructions.

Transformation of yeast. An overnight culture of S. cerevisiae was diluted to $2.5 \times 10^8$ cells ml$^{-1}$ in YPD (1%, w/v, yeast extract, 2%, w/v, peptone and 2%, w/v, glucose) and grown to $10^9$ cells ml$^{-1}$. The cells were collected by centrifugation, washed in TE (pH 7.5) several times and resuspended in 1/10 the original culture volume in lithium acetate solution (0.1 M lithium acetate in TE, pH 7.5). These suspensions were incubated at 30 °C for 1 h with light agitation and divided into 300 µl aliquots. DNA was added to the 300 µl aliquots at 1 µg for closed circular plasmids and 10 µg for linearized plasmids. Then 700 µl of a 50% (w/v) solution of PEG 4000 in sterile deionized water was added and incubated at 30 °C for 30 min without agitation. The cells were heat-shocked at 42 °C for 5 min and plated onto selective media. Transformants appeared as individual colonies after 3–7 d at 30 °C.

Assay of β-galactosidase activity. The assay method used was a variation of the procedure of Miller (1972). The strains to be
assayed were grown overnight in quadruplicate in semi-defined minimal selective medium (65.7% w/v, Yeast Nitrogen Base without amino acids, 2%, w/v, glucose, 40 μg leucine ml⁻¹, 40 μg histidine ml⁻¹) at 30 °C with vigorous shaking. The OD₆₀₀ of each culture was determined immediately prior to determining the β-galactosidase activity. A sample of 440 μl was taken and mixed with 110 μl 5 X Z buffer (1 X Z buffer is 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol, adjusted to pH 7.0 and filter-sterilized); a 10 μl sample of this was diluted 1/10 in 1 X Z buffer. Each sample was added 5 μl 0.1% SDS and 25 μl chloroform followed by vortexing for 10 s. To start the reaction 90 μl of 4 mg ONPG ml⁻¹ was added; the samples were incubated at 30 °C for 30 min then the reaction stopped by the addition of 250 μl 1 M Na₂CO₃. Cells were cleared from the solution by a 30 s spin in a microfuge at full speed and the A₄₂₀ determined. Results were treated as follows: the A₄₂₀ was corrected for dilutions, multiplied by 1000 and divided by 30 (min) and the OD₆₀₀ of the culture; this gives the results in β-galactosidase units min⁻¹ per OD₆₀₀ unit. Data from control and repressed cultures were statistically treated using Student's t-test to check the hypothesis that they were different data-sets to greater than 95% certainty.

**RESULTS AND DISCUSSION**

**Experimental approach**

In order to measure the repression achieved by the 434 repressor in yeast, strains carrying the reporter plasmids were transformed with pYCW36 expressing the 434 repressor, and with pYeCDE-2 as a negative control. The difference in the levels of β-galactosidase expression from the reporter gene between the two strains would then give the level of repression. A control was also included which measured the repression of the reporter construct when no artificially introduced operators were present.

**Analysis of 434 c expression in yeast**

The yield of the 434 repressor in strains carrying the plasmid pYCW36 was determined by Western analysis, using a polyclonal antiserum against the 434 repressor. The results (not shown) demonstrated that the 434 repressor was expressed at 0.25-0.5% of cell protein. This is less than the 1-2% of cellular protein produced by expressing the E. coli chloramphenicol acetyltransferase gene from a similar plasmid (Hadfield et al., 1987). The reasons for this difference in yield of the two gene-products are unclear.

**Location of operators in the PGK promoter**

For studies of repression of gene expression in yeast by the 434 operator/repressor system the PGK promoter of *S. cerevisiae* was chosen. This choice was based on the promoter's ability to direct high-level expression of foreign genes in *S. cerevisiae* (Derynck et al., 1983), facilitating detection of the reporter gene-product even at repressed levels. In addition, much useful information is available about the location of regions within the PGK promoter responsible for the high levels of transcription associated with this gene.

**Fig. 4. Repression of PGK promoters containing 434 operators by the 434 repressor.** pYCWPGK is the construct containing the wild-type PGK promoter. pYCW37 contains an operator 5' of the UAS. pYCW38 contains an operator 5' of the RAP1-binding site within the UAS. pYCW93 contains an operator 3' of the CTTCC boxes within the UAS. pYCW40 contains an operator 5' of the TATA box. pYCW41 contains an operator 3' of the transcription start-point.

The PGK promoter contains two functional TATA boxes, at positions −152 and −113, although the one at position −152 is preferred (Rathjen & Mellor, 1990). It also contains a CT-rich region close to the transcription start-point which is common to other yeast genes, although this has been shown by deletion analysis not to be required for efficient transcription (Rathjen & Mellor, 1990). A short sequence of 7 bases, ACAGATC, appears to influence the initiation-site, being sufficient for the discrete determination of the latter when located at the correct distance from the TATA box (Rathjen & Mellor, 1990). In this region two operators have been positioned, one inserted immediately 5' to the TATA box at −152 and one by replacement of DNA immediately downstream of the transcription-initiation point. It was hoped that neither insertion of the operator, locating the operator upstream of the TATA box, nor replacement of sequences near the initiation point with operator sequences would alter the transcription-startpoint.

Measurement of β-galactosidase activities expressed from the reporter constructs containing these altered promoters, in the absence of the 434 repressor, revealed expression at levels very similar to those obtained with the wild-type promoter. In the presence of the 434 repressor, the operator positioned closed to the TATA box gave the greatest repression in this study, reducing expression by 65% (Fig. 4, Table 1). This repression is likely to be due to steric interference with the formation of the TFIIID complex at the TATA box, with consequent inhibition of the initiation of transcription.
Table 1. Repression of β-galactosidase gene expression from PGK promoters containing 434 operators

<table>
<thead>
<tr>
<th>Parent plasmid*</th>
<th>Location of operator</th>
<th>Reporter plasmid</th>
<th>Percentage repression†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYCWPGK</td>
<td>Wild-type promoter</td>
<td>pYCWWT</td>
<td>0 (3.0)</td>
</tr>
<tr>
<td>pYCW3</td>
<td>5' of ABF1 binding site</td>
<td>pYCW37</td>
<td>0 (3.3)</td>
</tr>
<tr>
<td>pYCW4</td>
<td>5' of RAP1 binding site</td>
<td>pYCW38</td>
<td>41 (2.8)</td>
</tr>
<tr>
<td>pYCW5</td>
<td>3' of last CTTCC box</td>
<td>pYCW39</td>
<td>31 (2.2)</td>
</tr>
<tr>
<td>pYCW6</td>
<td>5' to TATA box</td>
<td>pYCW40</td>
<td>65 (1.8)</td>
</tr>
<tr>
<td>pYCW7</td>
<td>Downstream of RNA start-point</td>
<td>pYCW41</td>
<td>22 (9.8)</td>
</tr>
</tbody>
</table>

* Refers to the parent plasmid containing the mutated PGK promoter prior to the introduction of the operator.
† Using Student's t-test, repression results for pYCWWT and pYCW37 conform to the null hypothesis, that is there is no significant difference between the control and the experimental data. All other repression results show that the control and repressed data are from different populations to better than 95% certainty. The figures in parentheses represent the standard deviation of the percentage repression, calculated using formulae for the propagation of error.

An operator positioned downstream of the transcription-startpoint allowed repression at approximately 22%. This relatively low level is likely to reflect the fact that preinitiated transcription is only moderately impeded by the presence of the 434 repressor bound to its operator. Deuschle et al. (1990) showed that the lac repressor could act as an efficient block to transcript-elongation by RNA polymerase II in mammalian cells. The difference between the findings with the 434 repressor and the lac repressor in this instance are probably due to the differences in the dissociation constants, $K_D$, of the two repressors. The lac repressor has an estimated $K_D$ of $10^{-19}$ M (Riggs et al., 1970), compared with a $K_D$ of $10^{-8}$ M for the 434 repressor bound to the operator used in this study (Koudelka et al., 1988); thus the lac repressor would be less likely to be displaced by the passage of RNA polymerase II.

The PGK promoter has been shown to be dependent on a UAS for its high activity (Ogden et al., 1986), with only sequences 3' of position -538 being necessary for efficient transcription (Stanway et al., 1989). This UAS has been studied in detail and the transcription factors which bind it well characterized (Stanway et al., 1987, 1989; Chambers et al., 1988, 1989, 1990). Binding sites for the transcription factors RAP1 and ABF1 have been located. In addition, the UAS contains three copies of the pentamer sequence CTTCC which are important for high levels of transcription, but the associated transcription factors have not yet been identified.

Since the arrangement of the UAS is important for the high levels of expression seen from this promoter, the 434 operators were introduced at sites least likely to disrupt the function of the promoter, but where the binding of the 434 repressor would be likely to inhibit the protein–DNA interactions between yeast transcription factors and the UAS. One operator was placed immediately 5' of the ABF1-binding site, a second immediately downstream of the 3' CTTCC box and a third introduced as a replacement of DNA between the ABF1-binding site and the RAP1-binding site such that the binding of a repressor would be likely to interfere with the binding of RAP1.

The operator 5' of the UAS (pYCW37) did not yield detectable repression. This observation is consistent with the finding that deletion of the ABF1-binding site has little effect on the rate of transcription from the PGK promoter (Stanway et al., 1987), though this element is essential for correct initiation in the absence of the RAP1 binding site (Stanway et al., 1989).

The operators positioned adjacent to the RAP1-binding site (pYCW38) or immediately downstream of the CTTCC boxes (pYCW39) within the UAS give similar levels of repression in the presence of the 434 repressor: 41% for pYCW38 and 31% for pYCW39. In addition to direct competition for access to the UAS between the 434 repressor and the transcription factors, it is also possible that, by bending the operator DNA on binding, the 434 repressor alters the conformation of the UAS sufficiently to alter binding of the transcription factors.

The results presented here for the repression of a PGK promoter–β-galactosidase fusion reporter system in yeast by the 434 repressor are remarkably similar to those obtained by Smith et al. (1988) for repression of a thymidine kinase promoter–chloramphenicol acetyltransferase reporter system by the E. coli $lexA$ repressor in mammalian cells. The $lexA$ repressor was able to produce repression levels between 16 and 61% when single operators were introduced into the promoter of the reporter construct. The best repression of 61% was seen when the operator was located 5 bp upstream of the TATA box, an almost identical position to that of the 434 operator in pYCW40 which produced a very similar level of repression (65%). The 434 and the $lexA$ repressors also have similar dissociation constants of $10^{-9}$ M (Koudelka et al., 1988; Brent & Ptashne, 1984). However, in yeast the
Table 2. 434 operator sites within the S. cerevisiae DNA sequences present in the EMBL database

<table>
<thead>
<tr>
<th>S. cerevisiae DNA sequence</th>
<th>Accession no.</th>
<th>Operator-like sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC68 gene</td>
<td>M73533</td>
<td>1628: TGGTT ACAATAATTAATGT TCTAA</td>
</tr>
<tr>
<td>Chromosome III complete DNA sequence</td>
<td>X59720</td>
<td>30435: TAAAT ACAATAAAATTTGT AAAAA</td>
</tr>
<tr>
<td>DGC1 gene</td>
<td>M64719</td>
<td>287: TGGAA ACAAGAATATCTTGT TGTGAA</td>
</tr>
<tr>
<td>HOM2 gene for aspartic β-</td>
<td>X15649</td>
<td>637: CACAT ACAAAATATTGT AGAA</td>
</tr>
<tr>
<td>semialdehyde dehydrogenase (ASAD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene RPO21 for RNA polymerase II largest subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.7 kb of right arm of chromosome II</td>
<td>X03128</td>
<td>6175: AGCAA ACAATATTTATGT TTATG</td>
</tr>
<tr>
<td>Cosmid 9998</td>
<td>X75891</td>
<td>11193: CGAAA ACAAAATACCTTGT GTGTT</td>
</tr>
<tr>
<td>Chromosome XI reading frame ORF</td>
<td>V00030</td>
<td>17547: AAGCT ACAATAATTTG TAAAT</td>
</tr>
<tr>
<td>YKL006W</td>
<td>Z28006</td>
<td>1741: GTATA ACAAAATATTGT TGTGTC</td>
</tr>
<tr>
<td>Chromosome XI reading frame ORF</td>
<td>Z28241</td>
<td>2152: CTTCA ACAAAATTATTGT TGTGCA</td>
</tr>
<tr>
<td>YKR016W</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNM1 (PSO2) gene</td>
<td>X64004</td>
<td>1570: TGGAA ACAAAATATTGT TATGC</td>
</tr>
</tbody>
</table>

*lecA* repressor has been used to repress expression from a *GAL1* promoter containing *lecA* operators (Brent & Ptashne, 1984). Here the level of repression was considerably higher than either the 434 repressor in this study or the *lecA* study of Smith *et al.* (1988).

Other bacterial repressors have also produced higher levels of repression in eukaryotic cells than the 434 repressor in this study. In yeast the *tet* repressor was able to repress RNA polymerase III-based transcription of a tRNA gene by up to 50-fold (Dingermann *et al.*, 1992), substantially higher repression than achieved with any other bacterial repressor used in yeast. In plant cells, repression levels of 10-fold have been reported for the *tet* repressor in transient assay in tobacco protoplasts (Gatz & Quail, 1988) and in stably transformed lines (Gatz *et al.*, 1991). Repression could be increased to 80-fold in the stably transformed lines by the use of tandem operators. Using the *lac* repressor in tobacco cells, repression levels of up to 10-fold were observed by transient assay and in stably transformed lines (Wilde *et al.*, 1992). In mammalian cell culture, repression levels of between 8- and 12.5-fold have been reported for single operator-INSERTIONS and up to 50-fold for double operator-INSERTIONS (Hu & Davidson, 1987; Brown *et al.*, 1987). As the *lac* repressor has a much lower *K*_D* than other bacterial repressors, higher repression levels might have been expected. The reasons for large variations in repression levels are unclear, but may reflect differing sensitivities of eukaryotic promoters to repression, as has been observed with bacterial promoters (Lanzer & Bujard, 1988). Direct comparisons of the efficiency of bacterial repressors in eukaryotic cells can only be made if identical hosts, promoters and positions of operator insertion are used for the analysis.

**Growth-inhibitory effect of the 434*P* repressor in yeast**

Attempts to transform yeast with the 2µm plasmid (pYCW28) or the integrating plasmids (pYC20A or B) designed to express the 434*P* repressor were consistently unsuccessful. Control plasmids similar to the expression plasmids except for lacking the 434*P* expression cassette transformed *S. cerevisiae* S150 2B at high frequency under identical conditions. This information implicates the 434*P* repressor as an inhibitor of yeast cell growth.

To investigate this phenomenon further, the plasmid pYC202 (Fig. 3c) was constructed to allow galactose-inducible expression of the 434*P* repressor in yeast. *S. cerevisiae* S150-2B was transformed with the vectors pYC202 and pBM150 (Johnston & Davis, 1984) and the transformation mixtures divided into two: one half was plated onto semi-defined minimal medium containing glucose, leucine, tryptophan and histidine and the other half onto identical medium containing galactose in place of the glucose. The plates were incubated at 30 °C until colonies appeared. Both plasmids produced high-efficiency transformation, as judged by the number of colonies appearing on the galactose plates. The control plasmid pBM150 also produced a large number of transformants on the galactose plates. However, the plasmid pYC202 failed to produce colonies on the galactose plates. When colonies from the glucose plates were streaked onto galactose plates, those containing the plasmid pBM150 were able to grow, whereas cells...
Table 3. P22 operator sites found in the S. cerevisiae DNA sequences present in the EMBL database

Searches were carried out using the GCG program Findpatterns (Devereux et al., 1984) with the pattern ANTNAA(G/A/T)(A/T)(A/T)(A/T) (A/T)/(A/T)/(A/T)/(C/T)TTNANT, where N = G, A, T or C. This pattern is based on the influence of the central bases on the affinity of the operator for the P22 repressor. The 434 operator is identical to the 434 repressor apart from four amino acids within the DNA-binding helix, which have been altered to switch the DNA-binding specificity to that of the P22 repressor. The position of each operator-like sequence within the database entry is indicated, together with the sequence of the operator and flanking DNA.

<table>
<thead>
<tr>
<th>S. cerevisiae DNA sequence</th>
<th>Sequence no.</th>
<th>Operator-like sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argininosuccinate lyase (ARG4) gene</td>
<td>K01813</td>
<td>199: CAGGC ATTTAAATTTTCTTT TACT CTTC</td>
</tr>
<tr>
<td>Nuclear ATP2 gene encoding mitochondrial F1 ATPase</td>
<td>M12082</td>
<td>1817: ATATTC ACTAATAAAAAATATT ATTACTA TAAT</td>
</tr>
<tr>
<td>ATR1 gene conferring aminotriazole resistance</td>
<td>M20319</td>
<td>2853: CAAAAtATTAAAATAAAAAT TATA</td>
</tr>
<tr>
<td>Gene for aspartyl-tRNA synthase</td>
<td>X06665</td>
<td>323: GAGGA AAATGAAATATTTTAT TTAT</td>
</tr>
<tr>
<td>RIM, MS1, PGI, and ribosomal protein L21 genes</td>
<td>Z21487</td>
<td>22050: CAGAC AGTAAATTTTTTCTT CATTA</td>
</tr>
<tr>
<td>Chromosome III complete DNA sequence</td>
<td>X59720</td>
<td>100789: GAGCA AGTGAATTTTTTATTGAG GTTTT</td>
</tr>
<tr>
<td>Kinesin-related protein (CIN8) gene and protease B (PRB1)</td>
<td>M50522</td>
<td>81: GATAA ATGGAAATTTTTTAT TCTTA</td>
</tr>
</tbody>
</table>

Argininosuccinate lyase (ARG4) gene

Nuclear ATP2 gene encoding mitochondrial F1 ATPase

ATR1 gene conferring aminotriazole resistance

Gene for aspartyl-tRNA synthase

RIM, MS1, PGI, and ribosomal protein L21 genes

Chromosome III complete DNA sequence

Kinesin-related protein (CIN8) gene and protease B (PRB1) 3' end

Chromosome segregation (CSET) gene

EMP70 gene for p24a protein

ENO1 gene for enolase 1

EIP1 gene

Genes for histone H3 and H4 (copy-I genes from PMS191)

Genes HSS1, NPL4 and HSP

Chromosomes IX and X telomere, X-Y' junction

Genes for histone H3 and H4 (copy-I genes from PMS191)

Genes

EMP70 gene for p24a protein

ENO1 gene for enolase 1

EIP1 gene

Genes for histone H3 and H4 (copy-I genes from PMS191)

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whether the growth-inhibiting effect of the repression and analysis of galactose-resistant derivatives of the galactose-sensitive yeast containing the galactose-repressor gene. Therefore, it is impossible to determine at present whether the growth-inhibiting effect of the repressor is inhibitory to the growth of the yeast: cells having the repressor could be informative. Re-examination of yeast sequences in the EMBL database for both 434 and P22 operator-like sequences revealed potential repressor-binding sites in yeast genomic DNA (Tables 2 and 3). Despite the identical number of conserved bases used for the search pattern for each operator sequence, there are twelve 434 operator-like sequences compared to 59 for the P22 operator. The conserved bases of the P22 operator are exclusively A/T base-pairs whereas the 434 operator contains two G/C base-pairs. The S. cerevisiae genome is approximately 64% A/T (Strickberger, 1976), which would favour the existence of P22 operators over 434 operators in the ratio of approximately 3:1. This suggests that more yeast genes could be repressed by the 434\(^{\text{P22}}\) repressor than the wild-type 434 repressor.

The yeast genes containing P22 operator sequences include several encoding essential proteins, including three ribosomal proteins, a subunit of RNA polymerase III, an aminoacyl tRNA synthase and two histone proteins. However, it is impossible to determine at present whether the growth-inhibiting effect of the 434\(^{\text{P22}}\) repressor is due to repression of a single gene or of two or more of those containing operator-like sequences. Selection and analysis of galactose-resistant derivatives of the galactose-sensitive yeast containing the galactose-inducible 434\(^{\text{P22}}\) repressor could be informative. Repression studies in yeast using either the 434\(^{\text{P22}}\) homodimer or the 434/434\(^{\text{P22}}\) heterodimer have obviously been made impractical by these findings.

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