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 *cl* 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 (434^{P22}) 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

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Abbreviation: UAS, upstream activator sequence.

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 *cI* gene product has been altered both by a single amino acid change, 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 *cI* gene-product by the amino acids in the corresponding positions in the DNA-binding helix of the P22 repressor, a hybrid 434 repressor (434^{P22}) 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^{P22} 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 adjacently 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 *cI* 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^{P22} 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 2 μ m⁺*.

E. coli: DH5 α (Hanahan, 1983); F⁻ *endA1 hsd(r⁻ m⁺) supE44 thi-1 λ^- recA1 gyrA96 relA1 Δ (argF-lacZYA)U169 ϕ 80d*lacZ* Δ M15. X11-blue (Bullock *et al.*, 1987); *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F' [proAB⁺ lacI^q lacZ* Δ M15 Tn10]. RZ1032 (Kunkel *et al.*, 1987); HfrKL16 PO/45 [*lysA*(61-62)] *dut-1 ung-1 thi-1 relA1 supE44 λ bd-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* DH5 α unless otherwise stated.

Site-directed mutagenesis. This was carried out by a variation of the method of Kunkel *et al.* (1987). An overnight culture of *E. coli* RZ1032 was inoculated with M13mp19 phage containing the DNA to be mutated inserted in the polylinker and the culture was left at room temperature for 30 min. This was diluted 100-fold into 2 $\times$ YT (g l⁻¹: Bacto-tryptone 16, Bacto yeast extract 10, NaCl 5) with 7.5 μ g 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 μ l sterile, deionized water containing 1.2 μ l 20 $\times$ SSC. The synthesis of the complementary strand, primed from the mutagenic oligonucleotide, was carried out in a 100 μ l reaction containing 500 μ M 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 μ l 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 *Hind*III fragment and cloned into the *Hind*III site in the polylinker of M13mp19. Site-directed mutagenesis (Kunkel *et al.*, 1987) was used to introduce a *Sma*I 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 as 1 kb *Sma*I-*Hind*III fragments and ligated into the polylinker of a variant of pUC19, pUC19EN, which contains a *Nar*I site in place of the *Eco*RI site in the polylinker 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 *Eco*RV and *Xho*I and annealed oligonucleotides used to

Fig. 1. The basic reporter plasmid. The reporter plasmid contains the origin of replication and the ampicillin-resistance gene from pUC19 for maintenance in *E. coli*, the *URA3* gene for homologous recombination and prototrophic selection in *S. cerevisiae* and the β -galactosidase gene from *E. coli* under the control of the yeast *PGK* promoter and terminator. The promoter in the constructs pYCW37–41 contain 434 operators within the *PGK* promoter region. The location of these operators does not affect the levels of expression of β -galactosidase in yeast in the absence of repressor.

replace the DNA between the two sites, retaining existing sequences bound by yeast transcription factors but also introducing a 434 operator, to give plasmid pYCW9. Plasmid pYCW7 was cut with *Bgl*II and *Stu*I and annealed oligonucleotides used to replace DNA between the two sites with original sequence containing a 14 bp replacement downstream of the mRNA initiation point to introduce the 434 operator, to give plasmid pYCW12.

Part of the *lacZ* gene was cut from pAH193-41A (van Gorcom *et al.*, 1986) on a *Hind*III–*Eco*RI fragment and cloned between the *Hind*III and *Eco*RI sites in pBR328 (Soberon *et al.*, 1980). This plasmid was cut with *Hind*III and *Sa*I and the first 11 codons of the *lacZ* gene introduced by ligation of annealed complementary oligonucleotides containing a *Bam*HI site 5' of the initiation codon, to give the plasmid pCW7. Neither the *Sa*I nor the *Hind*III site was retained in the ligation.

The plasmid pYCWPGK was cut at the unique *Hind*III site 3' of the *PGK* terminator and a *Hind*III 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 *Eco*RI-compatible overhang at the 5' end and a *Bgl*II-compatible overhang at the 3' end. This fragment was ligated between the *Eco*RI and *Bgl*II sites in pYCW19 and the product checked by sequencing. This plasmid was opened at the *Nar*I and *Eco*RI sites and the fragment containing the remainder of the *lacZ* gene, isolated from pCW7 on an *Eco*RI–*Nar*I fragment, introduced by ligation. The resulting plasmid, pCW31, was cut at the unique *Bam*HI site immediately 5' of the start codon of the *lacZ* gene and at the unique *Nar*I site further upstream of the *lacZ* gene. The series of *PGK* promoters containing operators, isolated on *Bgl*II–*Nar*I fragments from pYCWPGK, 8, 9, 10, 11 and 12, could then be ligated in to form a series of reporter constructs (Fig. 1). These

Fig. 2. Plasmid designed to express the 434 repressor. The plasmid pYCW36 is based on pYCDE-2 (Hadfield *et al.*, 1986). It contains an origin of replication and the ampicillin-resistance gene for maintenance and selection in *E. coli*, and the 2 μ m origin and the *TRP1* gene for maintenance and selection in yeast. The 434 gene is under the control of a truncated version of the *ADH1* promoter which confers constitutive expression (Beier & Young, 1982) plus the *CYC1* terminator.

could be linearized at the unique *Nco*I 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 2 μ m origin and most of the *TRP1* gene were removed from pYCDE-2 (Hadfield *et al.*, 1986) by digestion with *Xba*I and *Hind*III, followed by religating the remainder of the plasmid with annealed oligonucleotides used to create a small linker joining the *Xba*I and *Hind*III ends. The *Xba*I site was destroyed in the ligation and an *Xba*I site introduced. The resulting plasmid, pCDE1, was opened at the unique *Eco*RI cloning site between the *ADH1* promoter and the *CYC1* terminator, and a small linker inserted to introduce unique *Xba*I and *Bam*HI sites to give the plasmid pACXB1. The *Eco*RI site was not retained in the ligation. The 434 *cI* gene was cut from pPLRT1 (Webster *et al.*, 1992) on an *Xba*I–*Sau*3AI fragment and ligated into pACXB1 opened at its *Xba*I and *Bam*HI sites to give pYCW34. Both pYCW34 and pYCDE-2 were cut with *Sa*I and *Mlu*I and the 434 expression cassette ligated into the 2 μ m plasmid to give pYCW36 (Fig. 2).

Construction of plasmids expressing the 434^{P22} repressor. This construction was similar to the one described above except that the 434^{P22} gene was substituted for the wild-type 434 gene. This involved isolating the 434^{P22} coding region from pPLRT2 (Webster *et al.*, 1992) on a *Xba*I–*Sau*3AI fragment and substituting it for the *Xba*I–*Sau*3AI fragment containing the wild-type 434 *cI* gene to give pYCW28 (Fig. 3a).

A second expression vector was also constructed for the 434^{P22} repressor. This was designed to integrate into the yeast genome maintaining single copy number. The promoter-repressor-terminator cassette made by ligating the *Xba*I–*Sau*3AI fragment containing the 434^{P22} repressor coding region into pACXB1 was removed on a *Sa*I–*Xba*I fragment and cloned into the unique *Sa*I site in pYRG12 (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 434^{P22} 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 434^{P22} repressor was also designed, based on the centromere plasmid pBM150 (Johnston & Davis, 1984). The 434^{P22} repressor gene was cut from pPLRT2 on an *XbaI*–*Sau3AI* fragment and cloned into the polylinker of pIC19H (Marsh *et al.*, 1984) opened at its *Bam*HI and *XbaI* sites, to give pCW37B. This plasmid was then cut with *Bgl*II and *Sal*I and the 434^{P22} repressor gene cloned into pBM150 opened at its *Sal*I and *Bam*HI sites to give the plasmid pYCW42 (Fig. 3c). This places the 434^{P22} repressor gene under the control of the yeast *GAL1* 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 2.5×10^6 cells ml⁻¹ in YPD (1%, w/v, yeast extract, 2%, w/v, peptone and 2%, w/v, glucose) and grown to 10^7 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

Fig. 3. Plasmids designed to express the 434^{P22} repressor. (a) The plasmid pYCW28 is identical to pYCW36 (Fig. 2) except that it is designed to express the 434^{P22} repressor. (b) The plasmid pYCW20 is designed to express the 434^{P22} repressor from the same expression system as pYCW28 and pYCW36, except that it does not contain the 2µm origin of replication and cannot be maintained in the yeast nucleus without integration into the host genome. This is achieved by linearization of the plasmid within the *HIS3* locus at the unique *XhoI* site, which increases the frequency of homologous recombination up to 1000-fold (Orr-Weaver *et al.*, 1981). (c) The plasmid pYCW42 is based on the plasmid pBM150 (Johnston & Davis, 1984) and is designed for the inducible expression of 434^{P22} from the *GAL1* promoter. The plasmid contains the origin of replication and ampicillin-resistance gene for maintenance and selection in *E. coli*, the *ARS1* and *CEN4* elements for autonomous replication in yeast and the *URA3* gene for prototrophic selection. The 434^{P22} repressor gene is under the control of the *GAL1* promoter: during growth on a glucose-based media the *GAL1* promoter is silent whereas during growth on galactose-based media the *GAL1* promoter drives expression of the 434^{P22} gene.

assayed were grown overnight in quadruplicate in semi-defined minimal selective medium (6.7%, 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 × Z buffer (1 × 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 × Z buffer. To 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 pYcDE-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 *cl* 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. pYCW39 contains an operator 3' of the CTTC 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 closest 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 TFIID 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

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

* 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^{-13} M (Riggs *et al.*, 1970), compared with a K_D of 10^{-9} 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

Searches were carried out using the GCG program Findpatterns (Devereux *et al.*, 1984) with the pattern ACAA(G/A/T)(A/T)(A/T)(A/T)(A/T)(A/T/C)TTGT. This pattern is based on the influence of the central bases on the affinity of the operator for the 434 repressor. The position of the operator-like sequence within the database entry is indicated, together with the sequence of the operator and flanking DNA.

<i>S. cerevisiae</i> DNA sequence	Accession no.	Operator-like sequence
<i>CDC68</i> gene	M73533	1 628 : TCGTT ACAATAATTATTGT TCTAA
Chromosome III complete DNA sequence	X59720	30 435 : TAAAT ACAATAAATTTTGT AAATA 148 350 : TCTTA ACAATATTTCTTGT AAGAT 291 040 : CGTGT ACAAATAATTTTGT AGTAT
<i>DCG1</i> gene	M64719	287 : TGGAA ACAAGAATACTTGT TGTGA
<i>HOM2</i> gene for aspartic β -semialdehyde dehydrogenase (ASAD)	X15649	637 : CACAT ACAAATTAAATTGT AGAAA
Gene <i>RPO21</i> for RNA polymerase II largest subunit	X03128	6 175 : AGCAA ACAATATTTATTGT TTATG
29.7 kb of right arm of chromosome II	X75891	11 193 : CGAAA ACAAAAAATCTTGT GTGTT
Cosmid 9998	V00030	17 547 : AAGCT ACAAATATAATTGT AAAAT
Chromosome XI reading frame ORF YKL006W	Z28006	1 741 : GTATA ACAAATTTAATTGT TTGTC
Chromosome XI reading frame ORF YKR016W	Z28241	2 152 : CTTCA ACAAATTTAATTGT TTGTC
<i>SNM1</i> (PSO2) gene	X64004	1 570 : TGAAA ACAAAATTATTTGT TATGC

lexA repressor has been used to repress expression from a *GAL1* promoter containing *lexA* operators (Brent & Ptashne, 1984). Here the level of repression was considerably higher than either the 434 repressor in this study or the *lexA* 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^{P22} repressor in yeast

Attempts to transform yeast with the 2 μ m plasmid (pYCW28) or the integrating plasmids (pYCW20A or B) designed to express the 434^{P22} repressor were consistently unsuccessful. Control plasmids similar to the expression plasmids except for lacking the 434^{P22} expression cassette transformed *S. cerevisiae* S150 2B at high frequency under identical conditions. This information implicates the 434^{P22} repressor as an inhibitor of yeast cell growth.

To investigate this phenomenon further, the plasmid pYCW42 (Fig. 3c) was constructed to allow galactose-inducible expression of the 434^{P22} repressor in yeast. *S. cerevisiae* S150-2B was transformed with the vectors pYCW42 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 glucose plates. The control plasmid pBM150 also produced a large number of transformants on the galactose plates. However, the plasmid pYCW42 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 ANTNAAG(G/A/T)(A/T)(A/T)(A/T)(A/T)(A/T/C)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^{P22} repressor 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.

<i>S. cerevisiae</i> DNA sequence	Sequence no.	Operator-like sequence
Argininosuccinate lyase (<i>ARG4</i>) gene	K01813	199 : CAGGC ATTTAATTTTTCTTTACT CTTCC
Nuclear <i>ATP2</i> gene encoding mitochondrial F ₁ ATPase	M12082	1817 : TATTC ACTAAAAAAATTTTATT CTATA
<i>ATR1</i> gene conferring aminotriazole resistance	M20319	2853 : CAAAA AATTAAATTAAATTAAT TAATA 2864 : ATTAA ATTAAATTAATATTAAT GTCAT
Gene for aspartyl-tRNA synthase	X06665	323 : GAAGA AATGAAAAATTATTTAAT GTCAT
<i>RIM</i> , <i>MSI1</i> , <i>PGI</i> , and ribosomal protein L21 genes	Z21487	22050 : CAGAC AGTAAAAATTTTTTCATT CATT
Chromosome III complete DNA sequence	X59720	100789 : GAGAA ACTGAAATTTTATTGAGT GTTTT
Kinesin-related protein (<i>CIN8</i>) gene and protease B (<i>PR81</i>) 3' end	M90522	81 : GATAA ATTGAATATTATTTAATT CTTCA
Chromosome segregation (<i>CSE1</i>) gene	L14838	3490 : AAGAA AATCAAGTAAAAATTAAT CAATT 2203 : TTAAT ATTGAAAAATTTTGACT ATTAG
<i>EMP70</i> gene for p24a protein	X67316	946 : ATGAT AATGAAGTTTATTTTACT TATTC
<i>ENO1</i> gene for enolase 1	D14474	622 : ATATT AATCAAATTTATTTTACT TCTTT
<i>ESP1</i> gene	L07289	1353 : GACCT AATAAAATATATTTAAAT TCATT
Genes for histone H3 and H4 (copy-I genes from PMS191)	X00724	1714 : AGGGT ATTTAATTTAATTTTAAAT TGGGT
Genes <i>HSS1</i> , <i>NPL4</i> and <i>HSP</i>	X72224	358 : AATTC AATTAATTTAACTTCAGT TTCAA
Chromosomes IX and X telomere, X-Y' junction	M63935	670 : TTATT ATTCAAAATATTTTAAAT ACAAA
<i>SceL12eIA</i> gene encoding L12eIA protein	M26505	786 : TTGTG ATTTAAAAATAATTAATT ACTGT
<i>MEF1</i> gene for mitochondrial elongation factor G	X58378	2629 : AACTT AATTAAGTAAATTTAAAT AAAAA
Nuclear class I differentially regulated mRNA clone mit78	M15163	80 : TTGTG ATTTAAAAATAATTAATT ACTGT
Gene for mitochondrial ribosomal protein L6	X69480	2744 : ATATC AGTGAATTTTTCCTACT GGAAG
<i>NME1</i> gene encoding RNA component of RNase MRP	Z14231	318 : AATGG AGTAAAAATTTTTCCTACT CAGTA
<i>PHO81</i> gene	D13228	5067 : ATTTG ATTAATTTATCTTTAAT TCTTA
Protease B (<i>PRB1</i>) gene	M18097	3836 : GATAA ATTGAATATTATTTAATT CTTCA
<i>PSE-1</i> gene	Z11538	216 : CAGAA ACTGAAAAATTTTCATT TTCTA
Protein-tyrosine phosphatase gene	M82872	19 : ACATC ACTCAAATTAATTTGATT TGCCT
<i>PUP1</i> gene	X61189	306 : ACTAT ACTAAAATATACTTAAGT TCTAT
<i>RADH</i> gene for putative helicase	X15665	4521 : ATAGG AGTTAATTTTATTTTACT GGTAG
<i>REC114</i> gene	Z14315	83 : TAGGA AATAAAATATAATTCAGT TTACC
Tyrosine phosphatase <i>PTP2</i> gene	M38723	3157 : AGGCA AATCAATTAATTTTGAGT GATGT
<i>REV1</i> gene required for normal induction of mutation	M22222	3433 : TGGAC AATTAATAAAATTAAGT AAAAA
Ribosomal protein L45 gene	J03761	870 : TTGTG ATTTAAAAATAATTAATT ACTGT
<i>RPC53</i> gene for RNA polymerase C subunit 4	X63501	543 : TCATT ATTGAATTTTTCCTACT GCAGA
<i>SDH1</i> gene for L-serine dehydratase	X52657	2609 : AGAGT AGTAAAGAAAATTTTATT TACAT
tRNA-Glu(3) gene and flanking regions (CYH82)	X08049	1054 : GATAT ATTTAATATTTTATTTAAT TTGAC
Vanadate-resistance protein (<i>VAN1</i>) gene	M33957	1980 : TATTA ATTTAATTTAATTTAATT TTTTG 1969 : ATGAC ATTTAATATTAATTTAAT TTAAT
Casein kinase I (<i>YCK2</i>) gene	M74453	278 : TGAGT AATGAAAAATTTTAAAT TTTTC 2407 : CTATT ATTAAAAAATATTAAGT ACTTT
Chromosome III complete DNA sequence	X59720	100789 : GAGAA ACTGAAATTTTATTGAGT GTTTT
<i>SMY2</i> gene	X74437	2543 : TTGAA ACTGAAGTTAAATTAATT GAATT
Genes for histone H2A and H2B, trehalase, and hexapren388	Z26494	6948 : TATTT ATTTAATTTATTAAGT AAAAA
Sec66p (<i>SEC66</i>) gene	U00797	550 : TTGAA ACTGAAGTTAAATTAATT GAATT
CKI1 mRNA for casein kinase-1	X60326	1890 : CTATT ATTAAAAAATATTAAGT ACTTT
Chromosome XI (28.6 kb) DNA for F206/YKL331, B969/YKL3	X75781	9685 : TCAAA AATCAATAATAATTAATT TAATA
Genes <i>HSS1</i> , <i>NPL4</i> and <i>HSP</i>	X72224	358 : AATTC AATTAATTTAACTTCAGT TTCAA
Gene for rab geranylgeranyl transferase	D26441	92 : TAAAC ATTTAAGTTTAAATTTATT ACTGT
<i>FET3</i> gene	L25090	2457 : TCTCT AGTAAAAATAATTTACT CTATT
Chromosome X left telomere 40.1 kb DNA fragment	Z34098	4303 : TTTGT ATTTAAAAATTTTGAAT AATAA

Table 3. (cont.)

<i>S. cerevisiae</i> DNA sequence	Sequence no.	Operator-like sequence
Sec66p (<i>SEC66</i>) gene, complete cds	V00797	550 : TTGAA ACTGAAGTTAAATTAATT GAATT
<i>MR56</i> gene	X70339	575 : TAAAC ATTTAAGTTTAATTTATT ACTGT
Multicopper oxidase (<i>FET3</i>) gene, complete cds	L25090	2 457 : TCTCT AGTAAAAATAATTTACT CTATT
Chromosome XI reading frame ORF YKL061w	Z28061	380 : TCAAA AATCAATAATAATTAATT TAATA
Chromosome XI reading frame ORF YKL062w	Z28062	3 982 : TCAAA AATCAATAATAATTAATT TAATA
Chromosome XI reading frame ORF YKL116c	Z28115	129 : GAATT AATAAAAAATATTTTTATT CGTAA
Chromosome XI reading frame ORF YKL117w	Z28117	1 712 : GAATT AATAAAAAATATTTTTATT CGTAA
Chromosome XI reading frame ORF YKR027w	Z28252	4 336 : TATCA AGTCAAGAATTTTTTATT CTTGC
Chromosome XI reading frame ORF YKR028w	Z28253	330 : TATCA AGTCAAGAATTTTTTATT CTTGC
Genes for histone H2A and H2B, trehalase, and hexaprenyl pyrophosphate synthetase	Z26494	6 948 : TATTT ATTTAATTATTATTAAGT AAAAA

containing the plasmid pYCW42 were unable to grow on the galactose media. These observations support the notion that the 434^{P22} repressor is inhibitory to the growth of the yeast: cells having the 434^{P22} repressor gene under the control of the *GAL1* promoter have a galactose-sensitive phenotype.

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^{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^{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^{P22} repressor could be informative. Repression studies in yeast using either the 434^{P22} homodimer or the 434/434^{P22} heterodimer have obviously been made impractical by these findings.

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