Functional analysis of upstream regulating regions from the *Yarrowia lipolytica* XPR2 promoter

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The *XPR2* gene from *Yarrowia lipolytica* encodes an inducible alkaline extracellular protease. Its complex regulation involves pH, carbon, nitrogen and peptones. Two previously identified upstream activating sequence (UAS) regions were analysed in a reporter system, outside the *XPR2* context. Fragments from the UAS regions were inserted upstream of a minimal *LEU2* promoter directing the expression of a reporter gene. The activity of the hybrid promoters was assessed following integration into the *Y. lipolytica* genome. This study confirmed the presence of two UASs composed of several interacting elements. Within the distal UAS (UAS1), a TUF/RAP1 binding site exhibited a UAS activity, which was enhanced by the presence of two adjacent repeats, overlapping sites similar to the *CAR1* upstream repressing sequence from *Saccharomyces cerevisiae*. Within the proximal UAS (UAS2), the UAS activity required the interaction of both an ABF1-like binding site and a decameric repeat, containing *Aspergillus nidulans* PacC site consensus sequences. This decameric repeat was able to mediate repression due to carbon and/or nitrogen sources as well as pH-dependent activation. A study in the context of *trans*-regulatory mutations in the *Y. lipolytica* *RIM101* gene showed that the PacC-like sites, potential binding sites for YIRim101p, were implicated in the derepression of UAS2-driven expression at neutral-alkaline pH. The *in vivo* response of the PacC-like decamers to external pH was dependent on the status of the pH-regulated activator YIRim101p, which is homologous to the *A. nidulans* PacC regulator. The carbon/nitrogen regulation imposed on the decamers was shown to be independent of YIRim101p and to override its effects.

Keywords: *Yarrowia*, XPR2, UAS, regulation, promoter

INTRODUCTION

The initiation and regulation of transcription in eukaryotes has been extensively studied, especially in yeast (reviewed by Struhl, 1987; Guarente & Bermingham-McDonogh, 1992; Ptashne & Gann, 1997). The *cis*-acting elements involved in the regulation of gene expression often consist of an association of multiple sites, binding different interacting transcription factors (reviewed, for *Saccharomyces cerevisiae*, by

Svetlov & Cooper, 1995). In yeast cells, upstream activation sequences (UASs) are necessary for transcription. They function in either orientation and at a variable distance with respect to the TATA box and transcription start site. In contrast to enhancers in higher eukaryotes, they are found essentially upstream from these basal elements, although several exceptions have been reported (Fantino *et al.*, 1992; Sinclair *et al.*, 1994). UASs are targets for transcriptional activators, whose binding can be either induced by physiological conditions, or constitutive. In the latter case, their activity can be regulated by another protein (for example, via phosphorylation, as was shown for the *S. cerevisiae* general transcription factor ABF1 by Trawick

Abbreviations: AEP, alkaline extracellular protease; DMS, dimethylsulphate; UAS, upstream activator sequence; URS, upstream repression sequence.

et al., 1992). Although most cases of repression in yeast seem to result from inactivation or absence of transcription factors, some negative regulatory sites, or upstream repression sequences (URSs), have been identified (Luche *et al.*, 1990; Ronne, 1995; Gailus-Durner *et al.*, 1997). The repressors binding these sites could exert their effect by interacting either directly with basal transcription factors or with activator proteins (Hanna-Rose & Hansen, 1996). In some cases, a single protein may, in different contexts, display either activating or repressing activity (Ozcan *et al.*, 1996; Rubin-Bejerano *et al.*, 1996). This is in particular the case for the *S. cerevisiae* general transcription factor RAP1 (Drazinic *et al.*, 1996), for which the two activities have been separated in some mutants (Sussel & Shore, 1991). The existence of co-activator and co-repressor proteins, that bridge the interaction of an activator or of a repressor with its ultimate target, has also been reported (Guarente, 1995; Hanna-Rose & Hansen, 1996; Stargell & Struhl, 1996).

Yarrowia lipolytica is a dimorphic yeast with high secreting capacities (Barth & Gaillardin, 1996). The XPR2 gene from *Y. lipolytica* encodes an inducible alkaline extracellular protease (AEP) which is the major protein secreted by this yeast (Ogrydziak *et al.*, 1977; Davidow *et al.*, 1987). The XPR2 promoter (pXPR2) has been used to direct heterologous protein production in *Y. lipolytica* (Franke *et al.*, 1988; Nicaud *et al.*, 1989a, 1991; Tharaud *et al.*, 1992; Hamsa & Chattoo, 1994; Park *et al.*, 1997) but its complex regulation hinders its industrial use. Derepression of AEP occurs at pH above 5.5 on media lacking preferred carbon and nitrogen sources; full induction of the pXPR2 requires high levels of peptones in the culture medium, although the exact nature of the inducer is unknown (Ogrydziak *et al.*, 1977). The functional pXPR2 encompasses roughly 800 bp (Davidow *et al.*, 1987). Thus, for both fundamental and practical reasons, we undertook to identify some of the *cis*-acting elements which participate in the complex regulation of pXPR2.

Deletion analysis of pXPR2 evidenced two major UASs essential for promoter activity (Blanchin-Roland *et al.*, 1994). *In vivo* genomic DMS footprints demonstrated that several sequences within each UAS interacted with proteins. No difference was found between binding patterns under inducing or repressing conditions, suggesting that the activity of the bound transcription factors was modulated by other regulatory proteins, or that different factors bound the same sequence under different conditions (Blanchin-Roland *et al.*, 1994).

Further studies of regulatory mutants affecting the synthesis of *Y. lipolytica* extracellular proteases (AEP and the acidic protease AXP) led to the identification of YIRim101p (Lambert *et al.*, 1997). This protein of the His₂ Cys₂ zinc finger family is homologous to the transcriptional activators Rim101p of *S. cerevisiae*, required for the entry into meiosis, and PacC of *Aspergillus nidulans*, *Aspergillus niger* and *Penicillium chrysogenum*, which mediate regulation by ambient pH.

YIRim101p is essential for mating and sporulation, and for AEP derepression. In *A. nidulans*, the model for pH regulation assumes that, at ambient neutral-alkaline pH (which is sensed by a cascade of PAL genes; Arst *et al.*, 1994), the PacC protein becomes a substrate for an unidentified protease that removes 60% of the polypeptide from its C-terminus (Orejas *et al.*, 1995). This proteolytic event converts PacC into a functional form able to activate the transcription of genes expressed at alkaline pH, and to repress genes expressed at acidic pH. This model seems to apply also to YIRim101p (Lambert *et al.*, 1997). Indeed, a potential proteolytic site is present in the protein; a C-terminally truncated version of YIRim101p acts as a dominant suppressor of *pal* mutations in *Y. lipolytica* and it activates XPR2 expression independently of ambient pH (Lambert *et al.*, 1997). Furthermore, two highly conserved PacC binding sites were found in one of the XPR2 UASs, and were shown to be bound by protein(s) *in vivo* (Blanchin-Roland *et al.*, 1994).

In this study, we wished to define more precisely the contribution of each UAS to the activity of the pXPR2 and to specify the contribution of the putative PacC/Rim101p targets to its regulation. In order to avoid potential compensation or position effects which may occur with the whole promoter, we developed a new reporter system. Several DNA fragments from each UAS region were used to construct hybrid promoters, in order to analyse their physiological activity outside the XPR2 context. They were inserted in front of a minimal *LEU2* promoter directing the expression of the *lacZ* gene. The activity of the hybrid promoters was assessed, following integration into *Y. lipolytica* genome, under different physiological conditions. We also checked the activity of some of our hybrid promoters, in strains carrying different mutations in *YIRIM101* (namely a null mutation or an activating C-terminal truncation), in order to separate the different regulations that act upon each UAS.

METHODS

Plasmids. Plasmids used are described in Table 1. New constructs and mutated plasmids were screened by restriction analysis or PCR, then confirmed by DNA sequencing.

Plasmids with hybrid promoters. The pINA781 reporter plasmid (see Fig. 2) was constructed by replacing the *SphI*-*Bam*HI fragment from pINA354 with a DNA fragment obtained by *SphI*/*Bam*HI digestion of a PCR product synthesized from pINA354 and the following two mutagenic oligonucleotides: 5' TCTTGGgaTcCTTAGTTTCGGGTTCCAT 3' and 5' GGTTTAGGgCaTgCACtgAtCACGGGC-AAAAGTGCCT 3' (lower-case letters indicate mutations creating the underlined restriction sites, respectively: *Bam*HI, *Sph*I and *Bcl*I).

The derivatives of pINA781 were constructed by insertion at the *Sph*I site of the following DNA fragments. 'UAS1A' was obtained by hybridization of the two following oligonucleotides: 5' CCGAGGTGTCTCACAAGTGCCGTGC-AGTCCGCATG 3' and 5' CGGACTGCACGGCACTTGTGAGACACCTCAGGCATG 3' (*Sph*I cohesive ends under-

Table 1. Plasmids used

The plasmids with hybrid promoters, carrying various fragments of pXPR2 (described in Methods) inserted at the *Sph*I site of pINA781, are described in Table 2. In some of these plasmids, the *LEU*2 marker gene was replaced by the *URA*3 marker gene, as explained for pINA1272: they are described in Table 3.

Plasmid	Relevant features	Reference or source
pINA303	<i>Cl</i> aI– <i>X</i> baI fragment of <i>Y. lipolytica</i> XPR2 gene (XPR2 promoter and prepro region) in pBSKS+	Fabre <i>et al.</i> (1991)
pINA918	Replacement into pINA303 of the <i>A</i> paI– <i>E</i> coRV fragment by an <i>A</i> paI– <i>E</i> coRV fragment synthesized from pXPR2, using PCR-directed mutagenesis to change the pXPR2 TATA box into a <i>S</i> phI site	This work
The following plasmids are pBR322-based:		
pINA354	Integrative plasmid bearing <i>Y. lipolytica</i> <i>LEU</i> 2 gene; translational fusion [pXPR2:: <i>lacZ</i>]; fully functional XPR2 promoter, downstream from <i>S</i> phI site; creation of a <i>S</i> maI site instead of the <i>E</i> coRV site	Blanchin-Roland <i>et al.</i> (1994)
pINA404	Integrative plasmid bearing <i>Y. lipolytica</i> <i>LEU</i> 2 gene; translational fusion [pXPR2:: <i>lacZ</i>]; fully functional XPR2 promoter, downstream from <i>M</i> luI site; deletion of the <i>E</i> coRV site	Blanchin-Roland <i>et al.</i> (1994)
pINA781	Reporter plasmid with translational fusion [minimal p <i>LEU</i> 2:: <i>lacZ</i>]; <i>S</i> phI– <i>B</i> amHI fragment carrying minimal <i>LEU</i> 2 promoter (TATA box and ATG region from nucleotide –94 to +18; restriction sites created by PCR-directed mutagenesis) replacing the same fragment carrying pXPR2 into pINA354; the <i>S</i> phI site allows insertion of DNA fragments upstream of minimal p <i>LEU</i> 2 (Fig. 2)	This work
pINA919 and 920	Insertion into pINA781 of the <i>S</i> phI fragment from pINA918, carrying the whole functional XPR2 promoter devoid of its TATA box, in direct and inverted orientation, respectively	This work
pINA311	Replicative plasmid bearing <i>ARS</i> 18, <i>URA</i> 3 and XPR2 genes from <i>Y. lipolytica</i>	Cordero Otero & Gaillardin (1996)
pINA1272	Derivative of pINA781, in which the <i>K</i> pnI– <i>A</i> seI fragment carrying the <i>LEU</i> 2 marker gene was replaced by the corresponding fragment from pINA311, carrying the <i>URA</i> 3 marker gene	This work

lined). ‘UAS1B’ was obtained by *S*phI digestion of a PCR product synthesized from pINA354 and the following two mutagenic oligonucleotides: 5′ ATCAGGTgCATgCTGAGG-TGTCTCACAAGTGC 3′ and 5′ ATGCATAGcAtgCCGG-ATCGAGGTGGGCGG 3′ (lower-case letters indicate mutations creating the underlined *S*phI restriction sites). ‘UAS2A’, ‘UAS2B’ and ‘UAS2C’ were obtained respectively by hybridization of the following pairs of oligonucleotides: 5′ CGGCCAAGCTTCATCGTCTTGGCGCGTCTTGGCG-AGAAGCTTGCATG 3′ and 5′ CAAGCTTCTCGCCAAG-ACGCGCCAAGACGATGAAGCTTGGCCCGCATG 3′; 5′

CATCGTCTTGGCGCGTCTTGGCGAGAGCCGTGTT-TCGTGACGCATG 3′ and 5′ CGTCACGAAACACGGC-TCTCGCCAAGACGCGCCAAGACGATGCATG 3′; 5′ CAGAGCCGTGTTTCGTGACGCATG 3′ and 5′-CGTC-ACGAAACACGGCTCTGCATG 3′ (*S*phI cohesive ends underlined). ‘MutGCN’ and ‘MutABF’ were obtained by *S*phI digestion of two PCR products synthesized from pINA354 with the same upstream mutagenic oligonucleotide: 5′ GATAATGcatGCATCGTCTTGGCGC 3′ (lower-case letters indicate mutations creating the underlined *S*phI restriction site) and two downstream mutagenic oligonucleotides,

respectively: 5' TGCTCTGcaTGCCTCACGAAACCGTG-CTCTCGCCAAG 3' (mutations indicated in bold-face type destroy the homology to the consensus ABF1 binding site of *S. cerevisiae*: 5' GTCACGAAACACG 3') or 5' GAAACTG-gcaTGCtTAAGGTCACGAAACACGGCTC 3' (mutations indicated in bold-face type destroy the homology to the consensus GCN4 binding site of *S. cerevisiae*: 5'-TTGCGTCAC).

The plasmid pINA918, providing the *SphI* fragment carrying the whole pXPR2 devoid of its TATA box, was obtained by replacing the *ApaI*-*EcoRV* fragment from pINA303 with a DNA fragment obtained by *ApaI*-*EcoRV* digestion of a PCR product synthesized from pINA303 with the following two oligonucleotides: 5' TCAACGGGCCCCGTCGATGGGGT-gcATgcCGTGTTCCTGGAC 3' (lower-case letters indicate mutations creating the underlined *SphI* restriction site replacing the TATA box; the *ApaI* restriction site is also underlined) and 5' ACCATGTTTCAGCGCAATCCGACT-TCCAACCC 3' (located downstream of the *EcoRV* restriction site).

Some of the plasmids with hybrid promoters (pINA781 and derivatives with two direct copies of respectively 'UAS1B', 'UAS2A' and 'UAS2B' fragments) were modified for use with the LAM100 and AM319ΔC strains, by replacing the *LEU2* marker gene by the *URA3* one (resulting plasmids: pINA1272 and respectively pINA1273, 1274 and 1275): the *KpnI*-*AseI* fragment from these plasmids (carrying the XPR2 terminator, *LEU2* gene and pBR sequence) was replaced by the corresponding fragment from pINA311 (carrying the XPR2 terminator, *URA3* gene and pBR sequence).

Plasmids carrying mutations within pXPR2. Deletions and/or mutations in pXPR2 were constructed by the method of Kunkel *et al.* (1987), using pINA303 as a template.

To generate deletions of GCN4-like sites, the following oligomers were used: 5' CTGCACGGCACTGATCAGAT-TGTACC 3' (deletion from -800 to -792 within UAS1: ΔGCN4-1), introducing a *BclI* site (underlined); 5' AACTGCTCTGATAAGGTCACGAAACAC 3' (mutations indicated in bold-face type destroy the site within UAS2: MutGCN4-2 - same mutations as in the 'MutGCN' fragment) and 5' TGGTGTGTGAAGCTTCAAAGTCGGTTC 3' (deletion from -288 to -280 between the two UASs: ΔGCN4-3), introducing a *HindIII* site (underlined).

To generate deletions of each of the imperfect repeats of the UAS1 region, oligomers 5' CAAAGAGAAGCAAGGACT-GCACGGCAC 3' and 5' CCCCAGATCGGAACAACGGTC 3' were used to delete from position -775 to -767 (ΔIR1) and -717 to -708 (ΔIR2), respectively.

All these deletions were then transferred to pINA404 as follows: the [*NheI*-*BsmI*] fragment carrying ΔGCN4-1 or ΔIR1 or ΔIR2, and [*NheI*-*PstI*] and [*PstI*-*BsmI*] fragments, both from pINA404, were recombined; the [*ApaI*-*PvuI*] fragment carrying MutGCN4-2 or ΔGCN4-3, and [*ApaI* (partial)-*PstI*] and [*PstI*-*PvuI*] fragments, both from pINA404, were recombined.

To delete the three GCN4-like sites all together, first the [*NheI*-*EcoRV*] fragment carrying ΔGCN4-3 was ligated into the plasmid carrying MutGCN4-2 cleaved by the same enzymes. Then the [*PvuI*-*ApaI*] fragment carrying both mutations was recombined with the [*PvuI*-*PstI*] fragment carrying ΔGCN4-1 and with the [*PstI*-*ApaI* (partial)] fragment from pINA404.

To delete both imperfect repeats of the UAS1 region, the [*MluI*-*SnaBI* (partial)] fragment carrying ΔIR1 and the [*SnaBI*-*BsmI*] fragment carrying ΔIR2 were recombined into

pINA303. Then the [*NheI*-*BsmI*] fragment carrying both deletions, and [*BsmI*-*PstI*] and [*PstI*-*NheI*] fragments, both from pINA404, were recombined.

Strains and transformation procedures. The bacterial strains used were *Escherichia coli* HB101 (Boyer & Roulland-Dussoix, 1969) and DH5αF' (Stratagene). *E. coli* was transformed as described by Hanahan (1985).

The *Y. lipolytica* strain used was JM23SB, previously obtained (Blanchin-Roland *et al.*, 1994) by integrative transformation of JM23 (*MatB leu2-35 lys5-12 ura3-18 xpr2::LYS5*) with plasmid pINA300', providing a wild-type *URA3* gene and a pBR322 platform useful for plasmid targeting. Plasmids pINA404, pINA781 and their derivatives were targeted to this docking platform by *NotI* digestion.

Two *Y. lipolytica* strains mutated in the *trans*-acting regulation pathway of XPR2 expression were also used. Strain LAM100 (*MatA ura3 lys11 rim101-1018*) carries a *YIRIM101* null mutation (deletion of the zinc finger region, from codon 157 to 914, and replacement by the *LEU2* marker gene). This strain was obtained from the previously described LAM57 strain, carrying the null mutation *Yirim101-1018* and a *URA3* marker at the XPR2 locus (see Fig. 1A of Lambert *et al.*, 1997): *Ura*⁻ clones were selected on 5-fluoroorotic acid medium and recombination events reconstituting the wild-type XPR2 locus were selected (loss of *URA3* and pXPR2::*lacZ* fusion; Blanchin-Roland, unpublished). Plasmids pINA1272 to 1275 were targeted by *KpnI* digestion to the terminator of the wild-type XPR2 locus of the LAM100 strain. Strain AM319ΔC (*MatA ura3 lys11 RIM101-1119*) carries a C-terminally truncated form of *YIRim101p*, which mimics the putative *in vivo* truncation by abutting codon 330 to the natural *YIRIM101* terminator and is able to activate XPR2 expression regardless of ambient pH (Lambert *et al.*, 1997). Strain AM319ΔC already carries the pINA404 plasmid inserted at the XPR2 locus. The transformation of strain AM319ΔC by plasmids pINA1272 to 1275 was performed by a double crossing-over: a *KpnI*/*DraIII* digestion of the plasmids made it possible to direct the replacement of the genomic pINA404 region (XPR2 terminator, *LEU2* gene, pBR sequence and pXPR2::*lacZ*) by the corresponding one from the pINA1272 (to 1275) plasmid (XPR2 terminator, *URA3* gene, pBR sequence and hybrid promoter::*lacZ*).

Y. lipolytica integrative transformation and Southern blot checking of yeast transformants were done as previously reported (Hoffman & Winston, 1987; Xuan *et al.*, 1988).

Culture media, growth conditions and enzyme assays. The fully inducing medium (YPDm) contained 0.2% (w/v) yeast extract, 0.1% glucose and 5% proteose peptone in 50 mM sodium phosphate buffer (pH 6.8; Nicaud *et al.*, 1989b). When the effect of acidic pH was assayed, YPDm was buffered with 200 mM sodium citrate buffer (pH 4.0). The peptone-free medium YEg (Blanchin-Roland *et al.*, 1994) contained 1% yeast extract and 0.1% glucose in 50 mM sodium phosphate buffer (pH 6.8). The peptone-free medium MMAM (Blanchin-Roland *et al.*, 1994) contained 200 mM sodium phosphate buffer (pH 6.8), glycerol (10 g l⁻¹) as a carbon source and ammonium sulfate (2 g l⁻¹) as a nitrogen source. For β-galactosidase assays, cultures were grown at 23 °C and harvested at the beginning of the stationary phase, corresponding to an OD₆₀₀ of about 14 in YPDm, and about 8 in MMAM and YEg media. The β-galactosidase activity was measured as previously described (Blanchin-Roland *et al.*, 1994) and was normalized for the OD₆₀₀ of the culture broth.

For each construction, two or three independent transformants (checked for correct integration by Southern blotting) were assayed, at least in duplicate. Reproducibility was in the range of $\pm 5\%$ between the duplicates and in the range of $\pm 10\%$ between repeated experiments.

RESULTS

Activity of hybrid promoters containing fragments from pXPR2, in different media

In order to dissect functionally the UAS1 and UAS2 regions outside their original pXPR2 context, several DNA fragments were inserted into the control plasmid pINA781, upstream from a minimal *LEU2* promoter directing the expression of the *lacZ* gene (Figs 1 and 2). The resulting plasmids were integrated into the genome of *Y. lipolytica* JM23SB, at the pBR322 platform; transformants were checked for being the result of a single integration event. The β -galactosidase activity of the integrants was measured in three media: YPDm, YEG and MMAM, all buffered at pH 6.8. The native pXPR2 activity was maximum on peptone-containing YPDm; it was intermediate on the peptone-free YEG medium, and minimal on MMAM, which contains preferred carbon and nitrogen sources. Some constructions were also checked for a pH effect, on YPDm medium buffered at pH 4, where native pXPR2 exhibited very low activity. The results are shown in Table 2.

Test of the reporter system. In order to validate our system, we inserted into pINA781 the whole pXPR2, devoid of its TATA box, in direct (pINA919) or inverted

(pINA920) orientation. The regulation pattern and range of activity exhibited by the strain carrying pINA919 were similar to those observed for the native pXPR2 (Table 2, lines 3 and 1). The slight quantitative differences observed may reflect a contribution of the *LEU2* TATA box. The results obtained with pINA920 (line 4) were similar except that the maximum activity, on YPDm, was twofold lower, probably reflecting the fact that the UAS2 region was less efficient in inverted orientation (see below). These results validate our reporter system since the pXPR2 devoid of its TATA box was able to impose an XPR2-like regulation upon the minimal *LEU2* promoter.

Analysis of the UAS1 region. The DNA fragment 'UAS1A' corresponds to position -805 to -776 of pXPR2; it carries a GCN4-like site and a sequence similar to the UAS_{RPG} from *S. cerevisiae* (TUF/RAP1 binding site: see Vignais *et al.*, 1990; Blanchin-Roland *et al.*, 1994). The 'UAS1B' fragment corresponds to position -805 to -701 of pXPR2 and carries, in addition to 'UAS1A', an adjacent region encompassing two imperfect repeats, overlapping sites homologous to the URS_{CAR1} from *S. cerevisiae*. These four sequences (GCN4-like, UAS_{RPG}-like and repeats) were protected from DMS *in vivo* (Blanchin-Roland *et al.*, 1994, and Fig. 1). Both fragments were able to promote an enhancement of activity on YPDm (and also on YEG and MMAM media for 'UAS1B') which was orientation-independent and increased with the number of copies. The 'UAS1A' fragment alone exhibited the properties of an UAS under non-repressing conditions (Table 2, lines 5 to 8), suggesting that the GCN4-like and/or the UAS_{RPG}-like

Fig. 1. Scheme of the UAS regions of pXPR2. The two regions of the distal UAS1 and the proximal UAS2 are represented. The sequences protected during DMS footprinting *in vivo* (Blanchin-Roland *et al.*, 1994) are underlined with bold lines (only GC base pairs are tested by this method). The sequences homologous to known regulatory sites from *S. cerevisiae* or *A. nidulans* are underlined. The repeated sequences are underlined with dashed arrows. The DNA fragments used in the construction of the hybrid promoters are indicated.

Fig. 2. The integrative pINA781 reporter plasmid. (a) Map of pINA781. The unique *Sph*I restriction site was used to introduce various DNA fragments from pXPR2, upstream of the minimal *LEU2* promoter directing the expression of the *lacZ* gene. The unique *Not*I restriction site was used to direct the integration of the plasmids obtained into the genome of *Y. lipolytica* strain JM235B, at the pBR322 platform. (b) Fragment of sequence from pINA781 showing the minimal *LEU2* promoter and the translational fusion with the *lacZ* reporter gene. The sequence conserved from the *LEU2* promoter encompasses position -94 to +18 (in the numeration of the *LEU2* gene). The TATA box and the ATG sequence are underlined in bold (Gaillardin & Ribet, 1987). 'GTC' is the first conserved codon from the β -galactosidase sequence and corresponds to the tenth codon of the *lacZ* gene.

sequence were implicated in the UAS activity. This fragment did not mediate any sensitivity to pH. The enhancement of activity was higher with 'UAS1B' (lines 9 to 12) than with 'UAS1A', showing that the added region also participates in the UAS effect. For the constructions carrying the 'UAS1B' fragment, activities on YPDm and YEG media were similar, showing that no peptone-sensitive element was present. The activities were at most threefold lower on MMAM and less than twofold lower on YPDm at pH 4, but still well above those exhibited by the control plasmid pINA781 (line 2). Hybrid promoters containing this fragment are therefore poorly affected by environmental conditions (either carbon and/or nitrogen sources or acidic pH), and appear to drive a quasi-constitutive expression.

Analysis of the UAS2 region. The DNA fragment 'UAS2A' corresponds to position -149 to -124 of pXPR2 and carries a decameric repeat, previously found to be protected from DMS *in vivo* (Blanchin-Roland *et al.*, 1994). This repeat overlaps two sites identical (in

inverted orientation) to the *A. nidulans* consensus PacC site: 5' GCCARG 3' (Tilburn *et al.*, 1995; Suarez & Peñalva, 1996). The fragment 'UAS2B' corresponds to position -150 to -106 of pXPR2; it carries, in addition to 'UAS2A', a site matching (in inverted orientation) the *S. cerevisiae* ABF1 site consensus (5' RTCRYBNNN-NACG 3'; Della Seta *et al.*, 1990a), and a partially overlapping GCN4-like site. The fragment 'UAS2C' corresponds to position -126 to -106 of pXPR2 and carries only these two last sites.

We first analysed the effects of these fragments on peptone (inducing) medium. The 'UAS2A' fragment was unable to activate the expression significantly in YPDm medium (Table 2, lines 13 to 16): the decameric repeat alone does not constitute a UAS. The 'UAS2B' fragment promoted a fivefold increase of activity in YPDm medium (line 17), and only a twofold one in the opposite orientation (line 18). A similar twofold activation, in inverted orientation, was reported for an ABF1 binding site from the *S. cerevisiae* *TDH3* gene

Table 2. Analysis of UAS1 and UAS2 regions: β -galactosidase activity of *Yarrowia lipolytica* strains carrying integrated plasmids with hybrid promoters, in different media

The results shown are the means of several experiments: for each construction, two or three independent transformants were assayed, at least in duplicate. β -Galactosidase activity is shown as units $\text{ml}^{-1} \text{min}^{-1}$ per OD_{600} unit. The overall standard deviation was in the range of $\pm 10\%$ between independent measurements. ND, Not determined.

	Plasmid	Promoter	β -Galactosidase activity in:			
			Inducing YPDm medium	Peptone-free YEg medium	MMAm medium	Acidic (pH 4) YPDm medium
1	pINA404	XPR2 native promoter	303	70	1.6	5.5
2	pINA781	Minimal pLEU2	11	20	20	11
Hybrid promoters with whole pXPR2, devoid of its TATA box, inserted into pINA781, upstream of minimal pLEU2:						
3	pINA919	'pXPR2' →	326	40	7.4	26
4	pINA920	← 'pXPR2'	146	31	14	ND
Hybrid promoters containing fragments from UAS1 region (inserted into pINA781, upstream of minimal pLEU2):						
5	pINA791	'UAS1A' →	17	19	11	21
6	pINA792	← 'UAS1A'	22	ND	ND	ND
7	pINA793	'UAS1A' → 'UAS1A' →	52	ND	11	65
8	pINA794	← 'UAS1A' ← 'UAS1A'	62	45	10	ND
9	pINA795	'UAS1B' →	32	37	21	26
10	pINA796	← 'UAS1B'	44	57	28	ND
11	pINA797	'UAS1B' → 'UAS1B' →	133	116	45	78
12	pINA798	← 'UAS1B' ← 'UAS1B'	150	137	46	ND
Hybrid promoters containing fragments from UAS2 region (inserted into pINA781, upstream of minimal pLEU2):						
13	pINA783	'UAS2A' →	11	5.3	3.2	7.7
14	pINA784	← 'UAS2A'	9.6	5.3	3.4	ND
15	pINA785	'UAS2A' → 'UAS2A' →	19	7.4	3.0	5.2
16	pINA786	← 'UAS2A' ← 'UAS2A'	17	6.2	2.9	ND
17	pINA787	'UAS2B' →	53	24	6	11
18	pINA788	← 'UAS2B'	19	ND	ND	ND
19	pINA789	'UAS2B' → 'UAS2B' →	129	39	5.4	9
20	pINA790	← 'UAS2B' ← 'UAS2B'	72	24	3.4	12
21	pINA913	'MutGCN' →	57	15	5.9	ND
22	pINA915	'MutABF' →	13	9.2	7.4	ND
23	pINA1047	'UAS2C' →	23	ND	14	17
24	pINA1048	← 'UAS2C'	21	ND	15	ND
25	pINA1050	← 'UAS2C' ← 'UAS2C'	21	ND	9.4	29
Hybrid promoters containing fragments from both UAS1 and UAS2 regions (inserted as above):						
26	pINA910	'UAS1B' → 'UAS2B' →	126	79	16	45
27	pINA907	← 'UAS1B' ← 'UAS2B' → 'UAS2B' →	341	104	9.6	ND

(Jung *et al.*, 1995). The increase was more than additive with two copies of 'UAS2B' (lines 19 and 20). The GCN4-like and/or the ABF1-like sites are therefore necessary for UAS activity. However, the 'UAS2C' fragment alone was unable to exhibit a significant UAS activity (lines 23 to 25), showing that the presence of the decameric repeat is also necessary for the UAS activity of the UAS2 region.

In order to define more precisely the role of GCN4-like and ABF1-like sites, we tested DNA fragments similar to 'UAS2B' in which either the GCN4-like site ('MutGCN') or the ABF1-like site ('MutABF') were modified using directed mutagenesis (cf. Methods and Fig. 1). The construction carrying the 'MutGCN' fragment (Table 2, line 21) exhibited activities similar to those of 'UAS2B' in the three media: the GCN4-like site

is apparently not implicated in the UAS activity nor in its regulation. In contrast, the activity of the construction carrying the 'MutABF' fragment (line 22) was similar to that of the control plasmid on YPDm medium, suggesting that the ABF1-like site is necessary for UAS activity.

We then turned to peptone-free media. The constructions carrying the 'UAS2A' fragment exhibited a lower activity than the control plasmid on MMAM and YEG media (lines 13 to 16). This effect was reproducibly observed, but to a lesser extent, with the 'UAS2B' fragment (and with both 'MutGCN' and 'MutABF'), but not with the 'UAS2C' fragment (lines 23 to 25). This repressive effect can therefore be attributed to the sole decameric repeat.

Concerning pH (comparison of YPDm at pH 6.8 and pH 4), a slight diminution of activity was observed with 'UAS2A' under acidic conditions (Table 2, line 15), which was more pronounced with 'UAS2B' (line 17), and especially with two copies (lines 19 and 20), but was absent with 'UAS2C' (lines 23 and 25). This suggests that the decameric repeat, a supposed target of the RIM101/PacC regulator (Lambert *et al.*, 1997), is indeed important for pH control. Note that the decameric repeat does not promote significant repression of the basal level of expression driven by the minimal *LEU2* promoter (compare line 2 with lines 13–20 on YPDm pH 4), but seems to be required for its induction at pH 6.8 on YPDm (see also below).

Reconstitution experiments. In order to test if the presence of both UAS1 and UAS2 upstream from the *LEU2* basal promoter was sufficient to restore a regulatory pattern similar to that of the native *XPR2* promoter, we inserted both 'UAS1B' and 'UAS2B' fragments into pINA781. As shown with pINA910 (Table 2, line 26), such a response was indeed observed since the lowest activity was measured on MMAM medium, whereas the highest was obtained on YPDm at pH 6.8, as was the case for pINA919 (line 3: *pXPR2* inserted into pINA781).

We fortuitously obtained a construct carrying one inverted copy of 'UAS1B' followed by two copies of 'UAS2B' (the first one inverted and the second one direct). A strain carrying this promoter exhibited, on YPDm and YEG media, activities comparable to those of the native *pXPR2* (pINA907, line 27, compared to line 1). The exact role of the intermediate copy of 'UAS2B' is not clear: it could bind itself to regulatory proteins, allowing their interaction with those bound to other fragments, or play essentially the role of a spacer sequence, allowing only a better interaction between regulatory proteins bound to 'UAS1B' and the direct copy of 'UAS2B'.

The activity on MMAM medium of the construction carrying pINA907 was lower than that of pINA910, and only slightly above those of constructions carrying two copies of the 'UAS2B' fragment (lines 19 and 20). This indicates that the decameric repeat from UAS2 region was able to shut down the expression driven by the UAS1 region.

Activity of *pXPR2* carrying internal deletions or modifications, in different media

In order to highlight the precise role of some potential sites within the whole *pXPR2*, several internal deletions or modifications were performed in the *pXPR2* region of pINA354, directing the expression of the *lacZ* gene. Some of the strains carrying these constructions have already been described by Blanchin-Roland *et al.* (1994) and were analysed further.

Deletion of the imperfect repeats of the UAS1 region. These sequences were identified previously (Blanchin-Roland *et al.*, 1994) as protected from DMS *in vivo* and overlap sequences matching the consensus for the URS_{CARI} (5' WVYCGCBND 3', determined by saturation mutagenesis by Luche *et al.*, 1990), except that the upstream one exhibits a C at its 3' border. Other sequences similar to URS_{CARI} in the whole *pXPR2* are all in the inverted orientation and are more degenerate (except one, in the decamer region, as discussed below: see Discussion and Fig. 3). Each or both of the imperfect repeats of the UAS1 region were deleted from the whole *pXPR2* (nucleotides –775 to –767 and/or –717 to –708: cf. Methods). The strains carrying these constructions exhibited levels of expression similar to those of the strain carrying pINA404 (wild-type *pXPR2*) on both YPDm and MMAM media (data not shown). This result contrasts with those obtained in the reporter system, where the region carrying the imperfect repeats (in the 'UAS1B' fragment) enhanced the activity of the core UAS1 (constituted by the 'UAS1A' fragment). It suggests that other regions, in the whole *pXPR2*, could be additionally involved in the regulation of the UAS1 activity and compensate for the loss of the imperfect repeats.

Deletion of the three GCN4-like sites of *pXPR2*. Three DNA sequences resembling the GCN4 binding site of *S. cerevisiae* (5' ATGACTCAT 3', core sequence underlined; Hill *et al.*, 1986) are present in the *pXPR2*: in the UAS1 region (from nucleotide –800 to –792: 5' gTGtCTCAc 3'), in the region between the two UASs (from nucleotide –288 to –280: 5' gaGACTCAc 3') and in the UAS2 region (from nucleotide –113 to –105: 5' gTGACgCAa 3'). These sites were deleted or mutated, either separately or all together, in the whole *pXPR2* directing the expression of the *lacZ* gene (cf. Methods). Strains carrying these constructs exhibited levels of expression similar to those of the wild-type *pXPR2*, in all three media (data not shown). None of the three sites therefore seems to be implicated in either the expression or the regulation of the *XPR2* gene, under the conditions tested. This correlates with the reporter analysis, which showed that the GCN4-like site in the UAS2 region did not participate in the expression. It also indicates that, in the UAS1 region, the UAS_{RPG} alone should constitute the core UAS.

Deletions in the UAS2 region: effect at different pHs. Although the results from Table 2 implicated the decameric repeat in the activation by neutral-alkaline pH, it remained unclear if the low UAS2 activity at pH 4

Table 3. Effect of *YIRIM101* mutations on β -galactosidase expression directed by hybrid promoters, in different media

For each kind of hybrid promoter tested (minimal pLEU2 alone or with pXPR2 fragments inserted at the *SphI* site), two plasmids are indicated: pINA781, 797, 785 and 789 carry the *LEU2* gene and were integrated in strain JM23SB (same constructions as in Table 2); pINA1272–1275 are their derivatives carrying the *URA3* gene and were integrated in strain LAM100 or AM319 Δ C2. The genotype and status of the strains, concerning *YIRIM101*, are the following: JM23SB is *RIM101*⁺ (wild-type); LAM100 carries a null mutation *rim101-1018* impairing the expression of *XPR2* gene; AM319 Δ C2 carries a C-terminal truncation leading to the activation of *XPR2* expression, independently of ambient pH (Lambert *et al.*, 1997). β -Galactosidase activity is shown as units ml⁻¹ min⁻¹ per OD₆₀₀ unit. The results are the means of at least three independent experiments and at least two transformants were assayed for each construction. The overall standard deviation was in the range of $\pm 10\%$ between independent measurements.

Promoter (plasmid)	β -Galactosidase activity in:									
		Inducing YPDm medium pH 6.8			Acidic YPDm medium pH 4.0			Peptone-free MMAM medium		
	Strain ...	JM23SB	LAM100	AM319AC2	JM23SB	LAM100	AM319AC2	JM23SB	LAM100	AM319AC2
	Genotype ...	<i>RIM</i> ⁺	<i>rim-1018</i>	<i>RIM-1119</i>	<i>RIM</i> ⁺	<i>rim-1018</i>	<i>RIM-1119</i>	<i>RIM</i> ⁺	<i>rim-1018</i>	<i>RIM-1119</i>
Status ...	wt	<i>rimΔZf</i>	<i>RIMΔC2</i>	wt	<i>rimΔZf</i>	<i>RIMΔC2</i>	wt	<i>rimΔZf</i>	<i>RIMΔC2</i>	
Minimal pLEU2 (pINA781 or pINA1272)		11	12	13	11	11	10	20	18	17
+ 'UAS1B' → 'UAS1B' → (pINA797 or pINA1273)		133	110	129	78	87	93	45	31	31
+ 'UAS2A' → 'UAS2A' → (pINA785 or pINA1274)		19	3.7	27	5.2	3.5	19	3.0	2.9	3.9
+ 'UAS2B' → 'UAS2B' → (pINA789 or pINA1275)		129	11	98	9	11	71	5.4	5.5	7.4

reflected an absence of activation or a repression effect, the values being close to the activity of the control promoter. In order to clarify this point, we tested some strains carrying deletions in the pXPR2 at different pHs.

A deletion of the decameric repeat (from position -154 to -124) was previously shown to lead to a residual activity of 13–15% in the three media, at pH 6.8 (Blanchin-Roland *et al.*, 1994). The additional deletion of the ABF1-like site (and overlapping the GCN4-like site) did not decrease the activity further (from position -154 to -104: residual activity of 14% in YPDm medium), showing that the deletion of the decamers was sufficient to inactivate totally the UAS2 (Blanchin-Roland *et al.*, 1994). This result correlates with those of the reporter system.

These two deleted strains were tested for a pH effect. The strains carrying the deletion of the decamers, alone or together with adjacent sites, exhibited an activity on YPDm at pH 4 similar to that of the wild-type pXPR2 (4 units, instead of 3 units for pINA404). If the decamers were the target for a repressor under acidic conditions, an increase of activity at pH 4 should have been obtained for the deleted strains, compared to the wild-type. The fact that no difference was observed is in favour of the hypothesis that decamers mediate only activation under neutral-alkaline conditions on YPDm.

Effect of *YIRIM101* mutations on the activity of hybrid promoters

The results in Table 2 suggested that the decamers were able to mediate both activation in response to neutral-alkaline pH, and repression in response to the preferred carbon and nitrogen sources. In an attempt to separate

these effects, we analysed some of the hybrid promoters in strains carrying mutated versions (inactive or constitutively active) of the pH-responsive transcriptional activator YIRim101p. In order to allow their selection in the *YIRIM101*-mutated strains, we exchanged the *LEU2* for the *URA3* marker in the plasmids pINA781 (control minimal pLEU2), pINA797 (two direct copies of 'UAS1B'), pINA785 (two direct copies of 'UAS2A') and pINA789 (two direct copies of 'UAS2B'). This modification preserved the overall structure of the resulting plasmids, respectively pINA1272 to 1275. These plasmids were integrated into the genome of strain LAM100 carrying a null mutation of YIRim101p (deletion of the zinc finger region, denoted *rim Δ Zf*), and of the strain AM319 Δ C carrying a C-terminal truncation (*RIM Δ C2*) which renders the YIRim101p constitutively functional, independently of ambient pH (Lambert *et al.*, 1997). The results observed in different media are shown in Table 3.

The levels of β -galactosidase expression driven by the minimal pLEU2 or by the hybrid promoter with 'UAS1B' fragments were independent of the *YIRIM101* context, on all the media tested (YPDm medium at pH 6.8, YPDm medium at pH 4 and MMAm medium). Thus, the *YIRIM101* context does not modify the UAS1-driven expression, neither at different pHs, nor in the presence of preferred carbon and nitrogen sources.

In contrast, the hybrid promoters with 'UAS2A' fragments (decamers containing PacC-like sites) or with 'UAS2B' fragments (decamers and ABF1/GCN4-like sites) exhibited, in the YPDm medium buffered at pH 6.8 or at pH 4, activities strongly dependent on the *YIRIM101* context. In the *rim Δ Zf* context (LAM100), the activities were, for both ambient pHs, in the same

range as those exhibited at pH 4 in the wild-type context (and markedly reduced compared to those observed at pH 6.8 in the wild-type context). In the C-terminally truncated *RIM4C2* context (AM319ΔC), the activities were, for both ambient pHs, in the same range as those exhibited at pH 6.8 in the wild-type context, and much higher than those observed at pH 4 in the wild-type context. Thus, in the absence of a functional YIRim101p, no derepression of UAS2-driven expression occurred at pH 6.8; in the presence of an active version of YIRim101p, the UAS2-driven expression was activated, at pH 4, at levels similar to those observed under inducing conditions.

On MMAM, irrespective of the status of *YIRIM101*, the activity of hybrid promoters with 'UAS2A' or 'UAS2B' fragments was very low, actually lower than that of the control promoter (minimal pLEU2). Thus, the repression imposed on the minimal pLEU2 by the UAS2 on MMAM, where the preferred carbon and nitrogen sources are available, is insensitive to the *YIRIM101* status and appeared to be dominant over the inducing effect exerted by a constitutively active form of YIRim101p.

DISCUSSION

The use of a reporter system allowed us to assess the transcriptional role of specific DNA sequences from the pXPR2, outside their normal promoter context. Our data partly confirm and partly invalidate previous results (Blanchin-Roland *et al.*, 1994). The presence of two UASs, presumably composed of several interacting elements, inferred from both deletion and footprinting experiments (Blanchin-Roland *et al.*, 1994) was confirmed by the characteristics of the fragments analysed in the reporter system and by the reconstitution experiments.

In the UAS1 region, the 'UAS1A' (UAS_{RPG}-like) fragment alone exhibited the properties of a UAS, but the activity was enhanced by the presence of the adjacent region carrying imperfect repeats (URS_{CAR1}-like sequences). However, the deletion of these repeats in the pXPR2 did not modify expression. The possibility remains that, in the whole pXPR2, other sequences may compensate for the loss of the imperfect repeats by interacting with the core UAS1. Another possibility would be that the enhancement of UAS1 activity in the reporter system was not due to the imperfect repeats but to another sequence in the 'UAS1B' fragment, which remained undetected by DMS footprinting.

Concerning the UAS2, the interaction of both 'UAS2A' (decameric repeat) and 'UAS2C' (ABF1-like site) fragments appeared necessary for activity. These results essentially agree with those previously observed when performing deletions in pXPR2 (Blanchin-Roland *et al.*, 1994): the deletion of the decameric repeat gave only 13–15% of residual activity in the three media. Thus, both reporter and deletion analyses showed that decamers were necessary for UAS activity. In contrast,

the deletion of the overlapping ABF1- and GCN4-like sites in pXPR2 gave 50% residual activity (Blanchin-Roland *et al.*, 1994), whereas we found here that the corresponding 'UAS2C' fragment was also necessary: it is possible that another sequence present in the pXPR2 is able to interact with the decamers, and compensates in part for the loss of 'UAS2C'.

None of the GCN4-like sites of pXPR2 seem to be implicated in its activity or regulation: their deletion, separately or together, led to no detectable change under the conditions tested. Moreover, the GCN4-like site of the UAS2 region had no effect in the reporter system, as shown by directed mutagenesis experiments. These results correlate with the fact that none of these sites are identical to the consensus of *S. cerevisiae*, and that their sequences are also different from each other. In the previous footprinting analysis (Blanchin-Roland *et al.*, 1994), only one of these sites (in UAS1) was found to be clearly protected from DMS.

The reconstitution experiments confirmed that UAS1 and UAS2 were major determinants of the promoter activity. However, these experiments also showed that these UASs alone reconstituted grossly the regulation of the whole pXPR2. This result was unexpected since previous deletion experiments led to the conclusion that none of the UAS regions were implicated in the regulation (the expression was greatly reduced, but still regulated by medium composition: Blanchin-Roland *et al.*, 1994). This discrepancy can be explained by compensation effects from other sequences still present in the deleted promoter; it exemplifies the necessity of comparing the results obtained by different methods.

The UAS1 region was able to drive an enhancement of activity in all media (this effect was, however, low on MMAM, but increased with the fragment copy number). Thus, the UAS1 region is poorly sensitive to repressing conditions. It appears to drive a quasi-constitutive expression, especially when the number of 'UAS1B' fragments in the hybrid promoter was increased to 3 or 4 (Madzak *et al.*, 1995). Such hybrid promoters, driving a high level of expression in various media, are interesting for heterologous protein expression and have been patented (Madzak *et al.*, 1995).

In contrast, the repression by carbon and nitrogen sources and the pH regulation appeared to be linked to the UAS2 region. The 'UAS2A' fragment, associated with both of these regulations, encompasses two kinds of overlapping sequences: the decameric repeat, matching *A. nidulans* PacC binding sites, and two adjacent sequences similar to the URS_{CAR1} from *S. cerevisiae*. However, as shown in Fig. 3, only one copy of these latter sequences is identical to the URS_{CAR1} consensus and the other contains a mutation known to inactivate the site in *S. cerevisiae* (Luche *et al.*, 1990). These two sequences are also in inverted orientation, like seven other homologous sequences found in the pXPR2 for which there is no evidence of possible implication in transcriptional regulation (data not shown, based on sequence homologies and deletion/

Fig. 3. Comparison of previous *in vivo* footprinting data for the UAS2 region with putative binding sites for known regulatory proteins. The sequence of the coding strand of pXPR2, from position -146 to -124, is shown. This sequence was included into both 'UAS2A' and 'UAS2B' fragments. The data obtained previously in DMS *in vivo* footprinting experiments were similar for cells grown on YPDm or MMAm media (Blanchin-Roland *et al.*, 1994) and are summarized as follows: full and partial protections of specific guanines are indicated respectively by solid and open squares, above the sequence for the coding strand and below for the non-coding one; one hyperreactive site is indicated by the arrowhead. The decameric repeat is boxed. The homologies to binding sites for known regulatory factors from *S. cerevisiae* (URS_{CAR1}) or *A. nidulans* (PacC) are indicated by the arrows. A mismatch with the consensus URS_{CAR1} sequence is indicated by the cross.

footprinting experiments from Blanchin-Roland *et al.*, 1994). In contrast, the two sequences homologous to the URS_{CAR1} in the UAS1 region, which were partially protected in DMS footprinting (Blanchin-Roland *et al.*, 1994) and presumably implicated in the UAS activity (data from reporter system) are identical to the consensus and in direct orientation. The results from DMS footprinting experiments performed on UAS2 (Blanchin-Roland *et al.*, 1994; see Fig. 3) have shown that: (i) the protection pattern is identical on the two decamers and covers them almost entirely; (ii) the G nucleotide located between the PacC-like sequences corresponds to a hyperreactive site, as frequently found in the immediate vicinity of (and not inside) binding sites; (iii) the 5' part of the first decamer is clearly protected, when it does not correspond to an URS_{CAR1} homology; (iv) the 3' part of the second URS_{CAR1} homology is clearly not protected, although it is identical to the consensus. The observed pattern was identical under inducing or repressing conditions.

On the basis of the above arguments, the implication of PacC-like sites in the different regulations mediated by the UAS2 appears probable, and that of URS_{CAR1}-like sequences unlikely. Unless the DMS footprints were unable to distinguish between occupation of close, but distinct, sites, it seems possible that PacC-like sites are the target for different regulatory proteins, under different physiological conditions.

Results obtained from hybrid promoters, and with native pXPR2 carrying deletions in UAS2, strongly suggest that the repeated decamers are the targets of the UAS2 pH response. Since previous results (Lambert *et al.*, 1997) suggested that they were bound by YIRim101p, a PacC homologue, we tested some of our constructions

with hybrid promoters carrying 'UAS1B', 'UAS2A' or 'UAS2B' fragments, in different media, in different YIRIM101 contexts. Our results confirmed that the UAS1 activity was independent of the pH-responsive regulation driven by YIRIM101 gene. The expression driven by UAS2 fragments appeared to be strongly dependent on the YIRIM101 context: the absence of a functional YIRim101p impaired UAS2-driven expression at pH 6.8, and a constitutively functional C-terminally truncated YIRim101p allowed us to obtain, regardless of ambient pH, an activity similar to that observed under inducing conditions. These effects were observed with 'UAS2A' as well as with 'UAS2B', clearly implicating the decameric repeat (carrying PacC-like sites) in the YIRIM101-driven pH-sensitive regulation. These results confirm a model of pH regulation in *Y. lipolytica* similar to that described in *A. nidulans*, where YIRim101p is activated by a C-terminal truncation at neutral-alkaline pH, then binds to PacC-like sites, and thus activates the expression of the pXPR2 UAS2. The fact that, in the presence of preferred carbon and nitrogen sources, the UAS2-driven expression cannot be turned on by the C-terminally truncated YIRim101p, and even slightly represses basal promoter activity, suggests that a still unidentified regulatory protein is able to bind the decamer region under these conditions and to impair YIRim101p activation. This would agree with the footprinting data, indicating constant occupation of the PacC sites. Thus, in *Y. lipolytica*, in contrast to the situation in *A. nidulans*, and in agreement with that in *P. chrysogenum* (Suarez & Peñalva, 1996), alkaline pH does not override the negative effect of a repressing carbon (and/or nitrogen) source.

The structure of pXPR2 UASs recalls what seems to be a general situation in *S. cerevisiae*, namely the association of a general ubiquitous transcriptional factor (ABF1, RAP1) and of factors specific for a small number of co-regulated promoters (Della Seta *et al.*, 1990b; Kovari & Cooper, 1991; Drazinic *et al.*, 1996; Gailus-Durner *et al.*, 1997; Devlin *et al.*, 1991, and references therein).

Given the large evolutionary distance which separates *Y. lipolytica* from other yeasts (Barns *et al.*, 1991), one would not necessarily predict that regulating sites (and transcriptional factors) would be conserved. Our work, however, suggests that those sequences and the corresponding binding factors may have been strongly conserved among ascomycetous yeasts. The cloning of YIRIM101, encoding a transcriptional factor with putative binding sites in UAS2 (PacC-like sites), which is implicated in a regulatory pathway similar to that described for *A. nidulans*, constitutes a strong argument in favour of this hypothesis. Another one is the homology found between all sequences from UAS regions implicated in transcriptional activity and consensus sites for known regulatory factors from other yeasts. The isolation of genes encoding these putative homologous factors will be pursued in our laboratory, permitting the testing of predictions from *cis*-acting sequences analysis.

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