Multiple regulatory elements control the expression of the yeast *ACR1* gene

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Abstract The *ACR1* gene, encoding a succinate-fumarate transporter, is required by the yeast *Saccharomyces cerevisiae* for ethanol utilization. Accordingly, gene expression is induced by ethanol and repressed by glucose. Here, we investigated three carbon source response elements present in its promoter region. Specific deletions as well as functional analysis of the elements in a heterologous promoter confirmed their role in transcriptional regulation. Protein binding to carbon source response elements of the *ICL1* promoter was competed by all three elements to various extents by the respective *ACR1* sequences. In addition, two putative stress response promoter elements present in the *ACR1* promoter were investigated in deletion analyses and shown to contribute to gene expression.

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Key words: *ACR1* gene; Succinate-fumarate transporter; Ethanol metabolism; Carbon source response element; Stress response promoter element

1. Introduction

Carbon catabolite repression is a general control mechanism that allows yeast cells to adapt their metabolism to the availability of hexoses. Thus, in the presence of glucose or other related sugars, such as fructose or galactose, enzymes involved in other anabolic or catabolic pathways are synthesized at very low levels. These include key enzymes of gluconeogenesis and the glyoxylate cycle, which are required for sugar phosphate formation when cells are growing on ethanol as a sole carbon source (see [1] and [2] for recent reviews).

The strategies used to study glucose repression involve both the characterization of promoter elements of genes that are regulated by carbon catabolite repression and the identification of the *trans* acting factors that regulate transcription from these promoter elements either directly (by binding to the DNA) or indirectly (e.g. by influencing the binding or activation capacity of transcription factors).

Among the genes encoding enzymes involved in ethanol utilization, the 5′ non-coding sequences of *ICL1* (encoding the unique functional isocitrate lyase) constitute one of the most thoroughly characterized promoters. After initial studies defining certain regulatory regions [3], an element that is both necessary and sufficient for controlled gene expression was identified [4]. This element, carbon source response element (CSRE), is also present in the promoter of other genes whose products are required under gluconeogenic growth conditions,

like *FBP1* (encoding fructose-1,6-bisphosphatase [5,6]), *PCK1* (encoding pyruvate carboxykinase [7]), *ACS1* (encoding an isoenzyme of acetyl-coA-synthetase [8]), and *MLS1* (encoding the glyoxylate cycle enzyme malate synthase [9]). In addition, several other positive as well as negative regulatory elements could be identified in the promoters of these genes [8–11].

Regarding *trans* acting factors, transcription of the genes mentioned above is dependent on *CAT1* (= *SNF1*), a gene encoding a protein kinase that plays a central role in derepression after glucose depletion [12]. Cat1 is closely associated with Cat3 (= Snf4) [13]. Other proteins that may participate in this multiprotein complex are Sip1, Sip2 and Gal83 [14,15]. The exact mechanism by which this complex controls gene expression is not yet understood. However, the product of the *CAT8* gene, likely to bind directly to the DNA in the promoters of gluconeogenic genes, activates transcription in a more direct fashion [16,17]. Cat8 acts through CSRE elements and requires Cat1 for its activity [18].

ACR1 was first identified in a screen for mutants unable to grow on ethanol as a sole carbon source [19]. Deletion mutants confirmed that the gene product is essential for ethanol utilization. Although a relationship between *ACR1* and the expression of acetyl-CoA synthetase has been suggested by our previous work, more detailed analyses did not confirm this phenotype (this laboratory, unpublished results). Sequence analysis defined the *ACR1* gene as encoding a putative mitochondrial carrier [19]. Heterologous expression studies in *Escherichia coli* demonstrated that the protein is indeed a succinate-fumarate carrier. This leads to a model where Acr1p transports succinate produced by the glyoxylate pathway into mitochondria in an exchange for fumarate that can then be used as precursor for gluconeogenesis [20].

Preliminary studies on transcriptional regulation revealed that the gene is expressed on ethanol media, but repressed in the presence of glucose, as shown for other genes required for ethanol utilization [19]. In this paper we describe a detailed analysis of the *ACR1* promoter and its controlling elements.

2. Materials and methods

2.1. Strains

Saccharomyces cerevisiae strains used in this work were: VW1B (*MATa ura3-52 leu2-3,112 his3Δ1 trp1-289 MAL2-8ᶜ SUC2 GAL*), a strain kindly provided by Michael Ciriacy (Düsseldorf, Germany) and its isogenic deletion derivative CEN.PK-7A (*cat1::HIS3*), a gift from Peter Kötter (Frankfurt, Germany) and W303-1A (*MATa SUC2 ade2 can1 his3 leu2 trp1 ura3*) and its isogenic strain Wmsn2msn4 (*msn2-Δ3::HIS3 msn4-1::TRP1*), kindly provided by F. Estruch (Valencia, Spain).

Bacterial transformation and large scale preparation of plasmid DNA were performed in *E. coli* TG1 (*rec0 (K12 supE thi Δ(lac-proAB) hsdΔ5 F′(traD36 proA⁺B⁺ lacIq lacZ ΔM15)*) [21].

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2.2. Media, growth conditions and enzymatic analysis

Rich media were based on 1% yeast extract and 2% peptone (YEP) supplemented with 4% glucose (D) or 3% ethanol (E) as carbon sources. To select for transformants, synthetic media consisting of 0.67% yeast nitrogen base w/o amino acids supplemented with amino acids as required and 2% glucose was used.

For preparation of crude extracts, cells were grown on YEPD, collected when glucose concentrations were still higher than 0.5% and then transferred to YEPE for a period of 6 h to allow induction of enzyme synthesis. β-Galactosidase activity was assayed according to [22]. The method of [23] was followed to determine the protein concentrations, using bovine serum albumin as a standard.

2.3. Nucleic acid preparations, hybridization experiments and sequencing

Competent E. coli cells were obtained according to [24]. Amplified plasmid DNA was isolated by the method of [25] or using Quiagen columns (Quiagen, Germany) following the instructions provided by the supplier.

Yeast cells were transformed by the method of [26] and yeast chromosomal DNA was prepared according to [27]. Southern analyses and other DNA manipulations were performed by the standard methods described in [28].

DNA sequences were obtained using the sequenase kit from USB according to the method of [29].

2.4. Oligonucleotides used in this work and PCR conditions

All oligonucleotides used in this work were purchased from Pharmacia. Sequences are shown in Table 1.

DNA was amplified using Taq DNA polymerase from Pharmacia with 25 cycles of 15 s at 94°C, 20 s at 55°C, 3 min at 72°C or, alternatively, 40 cycles of 30 s at 94°C, 1 min at 55°C and 1.5 min at 72°C (dependent on the size of the expected fragments).

2.5. ACR1-lacZ fusions

A SalI–EcoRI fragment containing 960 nucleotides from the 5′ non-coding region and 275 bp of the ACR1 coding sequence was cloned in frame to lacz into YIp356R [30], resulting in plasmid YIpACR1 (Fig. 1). Within this plasmid, deletions were introduced in the ACR1 promoter by inverse PCR following the method of [31]. By using suitable inside out oligonucleotides (see Table 1), all plasmid sequences but the ones to be deleted could be amplified. Linear fragments obtained from this amplification were then subjected to restriction digestions with the enzymes indicated by the recognition sequences in the oligonucleotides, re-ligated and used to transform E. coli selecting for ampicillin resistance. OLI-19 and OLI-20 for D1, OLI-25 and OLI-30 for D2, OLI-28/OLI-31 for D3, OLI-17 and OLI-18 for D4, OLI-17 and OLI-19 for D5, OLI-19/OLI-30 for D6 and OLI-19/OLI-31 for D7. Other constructions were obtained employing restriction endonucleases. Thus D8 was performed by using AvaI (which has two recognition sites in the ACR1 promoter at −360 and −643 relative to the ATG translational start codon) followed by treatment with Bal31 and religation and D9 was constructed by using XhoI (at position −360) followed by Bal31 and religation. All constructs were analyzed by sequencing the respective promoter region.

The resulting plasmids were integrated into the URA3 locus by digestion with StuI prior to transformation of the yeast strain and single copy integration was confirmed by Southern analysis of genomic DNA digested with BglII and probing with a 1.1 kb HindIII fragment containing URA3.

2.6. Subcloning of ACR1 promoter fragments into the heterologous CYC1 promoter

pNI17, a derivative of pNG22 [32] lacking 2μ sequences and the UASs of the CYC1 promoter, was used to study the function of putative UAS elements. All the inserts studied were obtained by PCR using suitable oligonucleotides (Table 1) and plasmid P-ACR1 (a plasmid containing the same SalI–EcoRI DNA fragment as YIpACR1 but in a pUK1921 vector: see Section 2.5) as a template. PCR-A was obtained with OLI-18/OLI-24, PCR-B with OLI-17/OLI-28, PCR-C with OLI-29/OLI-30, PCR-0 with pUC19-R/OLI-19, PCR-22 with pUC19-R/OLI-22, PCR-18 with pUC19-R/OLI-18 and PCR-18M with pUC19-R/OLI-18M.

PCR products were cloned into available restriction sites of the pNI17 to give plasmids termed pNI followed by the number or letter of the PCR product.

Each of the plasmids was integrated into the yeast strains indicated and analyzed as described above for the ACR1-lacZ fusions. As a control, the pNI17 vector without insertion was also tested.

PCR products were also used in gel retardation experiments as indicated.

2.7. Gel retardation assays

Yeast protein extracts were prepared as follows: yeast cells were grown in 10–20 ml of rich medium (YEPE) at 28°C to an A_{600} of 2.0. Cells were then collected, washed with 1 ml of 1 M sorbitol and suspended in 100 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, containing 40 mM PMSF and 0.42 mM NaCl). The cells were broken with glass beads (diameter of 0.5 mm with 0.5 g/tube) using an IKA-VIBRAX for 10 minutes at 4°C, and 400 μl of lysis buffer were added to the suspension. After centrifugation at 14 000 rpm for 30 min at 4°C, the supernatant was used as a crude protein extract.

Binding reaction mixtures contained 10 mM HEPES, pH 7.5, 1 mM DTT, 1–5 μg of poly(dI-dC) and about 1 ng of end-labelled DNA in a volume of 50 μl. The $ICL1_{CSRE}$ probe was obtained by annealing of oligonucleotides OLI-49 and OLI-50 (Table 1), corresponding to $OL18_{ICL1sense}/OL18_{ICL1antisense}$ used in [10], following the procedure described there. Unlabelled competitor DNA was added in the amounts indicated. The binding reaction mixtures included 50 μg of protein extract. After 30 min of incubation at room temperature, reactions were loaded onto a 4% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 10 V/cm of gel for 60–90 minutes in 0.5×TBE buffer (45 mM Tris/borate, 1 mM EDTA). Gels were dried and subjected to autoradiography using Kodak X-Omat S film at −70°C with an intensifying screen.

3. Results and discussion

3.1. Three CSRE-like elements are present in the ACR1 promoter

Sequence analysis of the ACR1 promoter revealed that it possesses three motives matching the CSRE consensus sequence [4]. We called them CSRE-A (CGGTTTAATGGA), CSRE-B (CGGTTGAATGGA) and CSRE-C (CGGAGAAATGGA) corresponding to the positions −678–−667, −617–606 and −579–−568, respectively. To investigate whether these elements are of functional significance, we prepared YIpACR1, containing the ACR1 promoter fused to the lacZ gene from E. coli. Starting from this, plasmids carrying different parts of the ACR1 promoter were prepared (see Section 2). The constructions were used to transform the VW1B wild-type strain and its isogenic cat1 deletion mutant, affected

Table 1
Oligonucleotides used in this work

Designation	Sequence
pUC19-R	5′-TTGTGAGCGGATAACAATTTC-3′
OLI-17	5′-acagctagcAGACTAGAGAATCATC-3′
OLI-18	5′-acagctagCTTTTCCATTAAACCG-3′
OLI-19	5′-acagctagCTAAATCACGTGACA-3′
OLI-20	5′-acagctagGAGGGGCGTTTTTCGA-3′
OLI-22	5′-acagctagcAAACCGAGGGTGCTGT-3′
OLI-24	5′-acagtcGACAGCACCCTCGGTT-3′
OLI-25	5′-acagctagCTAGTCTCCCCTCGGGTT-3′
OLI-28	5′-acagctaGCTCCATTCAACCGA-3′
OLI-29	5′-acagctagGCCAACGTCCATTTC-3′
OLI-30	5′-acagctaGCCCGGAGAAAT-3′
OLI-31	5′-acagctaGCGAATGAGCCAT-3′
OLI-49	5′-tcgaGTTTCCATTCATCCGAGC-3′
OLI-50	5′-tcgaGCTCGGATGAATGGAAAC-3′

Capital letters represent the sequences present either in the ACR1 promoter or in the ICL1 promoter (OLI-49 and OLI-50 are ICL1 specific oligonucleotides corresponding to $OL18_{ICL1sense}/OL18_{ICL1antisense}$ [10]). Small letters show nucleotides added to the original sequences where bases underlined were introduced as synthetic linkers.
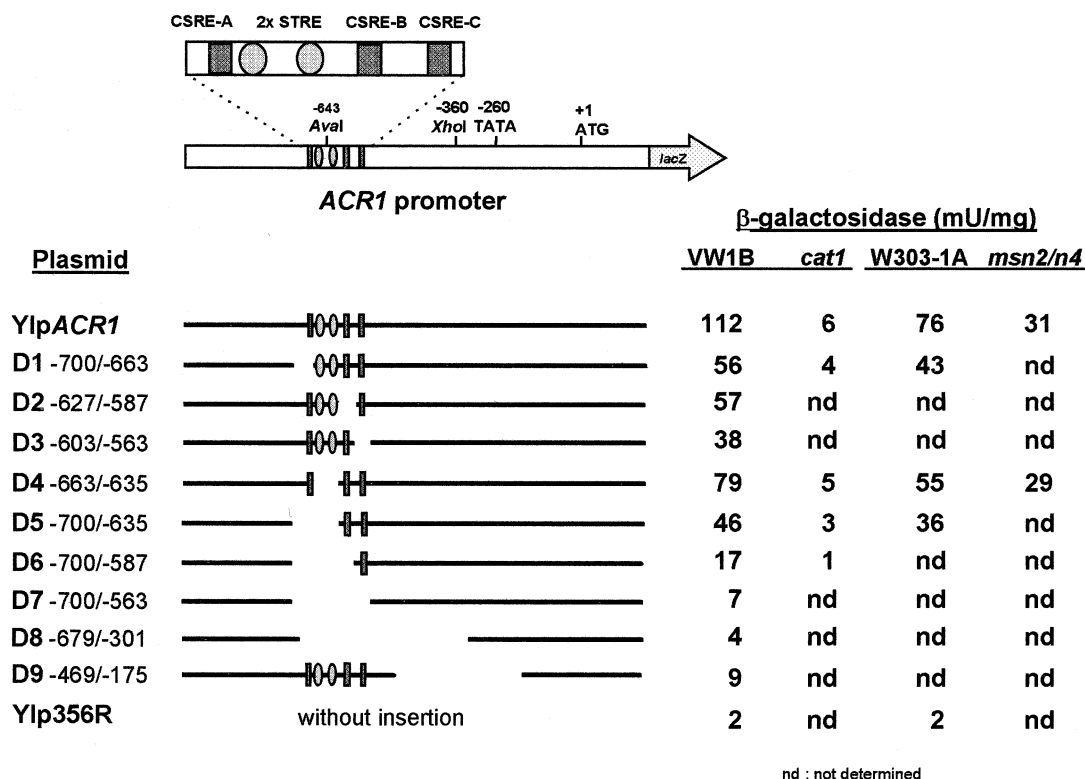
Fig. 1. Composition of the *ACR1* promoter and internal deletion constructs used to determine the function of consensus regulatory elements (see text for details). Positions are numbered relative to the ATG translational start codon. The *ACR1* promoter and part of the coding sequence were fused to the bacterial *lacZ* gene in an integrative vector and interal deletions were obtained by PCR (see Section 2). As recipients, either the wild-type strain VW1B and an isogenic *cat1* deletion mutant or the wild-type strain W303-1A and a *msn2 msn4* double deletion mutant (*msn2/4*) were used. Single copy integrants were tested for the expression of the reporter β-galactosidase after growth on rich medium containing 3% ethanol as a carbon source. β-galactosidase activities are mean values of at least two independent determinations, that did not vary by more than 15% from each other.

in catabolite repression. Single copy integrants were selected after Southern analysis (data not shown) for further investigation. The β-galactosidase activities generated by these constructs are given in Fig. 1.

Deletion of each individual CSRE element led to a 50–70% reduction in specific β-galactosidase activity (constructs D1, D2 and D3). A deletion of a DNA fragment carrying CSRE-A and CSRE-B (D6) maintained only 20% of the original activity. Further deletion of the CSRE-C as well (D7, D8) decreased activities to the basal levels measured in the absence of Cat1p. In addition, deletion of sequences between −469 and −175 also resulted in a loss of activity even though all three CSRE elements are still present (D9). Since a putative TATA box is located at position −260 [19], the lack of activity in the latter construct may be attributed to the absence of the element and hence a lack of correct transcription initiation.

We proceeded by further delimiting functional promoter elements by checking their ability to function in a heterologous promoter. Thus, DNA fragments of the *ACR1* 5′ noncoding sequence were tested for their ability to activate transcription from a *CYC1* promoter lacking its original UAS elements. The results are shown in Fig. 2. Constructs containing the CSRE-A (pNI-A, pNI-18) conferred a high level expression that is completely lost upon eliminating part of the consensus sequence (pNI-22). The CSRE-B element activated the heterologous promoter in a similar way as CSRE-A (pNI-

B, pNI-A). In addition, sequences upstream from CSRE-A do not display any positive regulatory function in the constructs tested (pNI-0, pNI-22).

Neither of the *lacZ* reporter constructs showed activity above basal levels when integrated in the *cat1* deletion strain (Fig. 1) or when transformants were grown on glucose media (data not shown). This clearly demonstrates that glucose repression is still conferred by the DNA sequences not deleted from the different constructs tested.

Thus, the data reported above (Figs. 1 and 2) are in line with previous reports from other gluconeogenic gene promoters, where the function of CSRE elements has been shown to be dependent on the presence of a functional Cat1 protein [1,2].

3.2. Two stress response promoter elements (STRE) are located between the CSRE-A and the CSRE-B elements

The STRE confers increased transcription to a set of genes in response to environmental or metabolic stress [33] and is mainly dependent on the two zinc finger proteins Msn2 and its homolog Msn4 [34,35].

To investigate whether the two STRE sequences (consensus AGGGG) present in the *ACR1* promoter at positions −662–−658 and −639–−635 in fact contribute to transcriptional regulation, a strategy similar to the one described above for the characterization of the CSRE elements was followed. In addition to the yeast strains employed in the characterization

of the CSRE elements, we included a double deletion mutant (*msn2 msn4*) and its isogenic wild-type (see Section 2).

Independent of the wild-type strain used, deletion of a fragment containing both STRE consensus sequences led to a decrease in the specific β-galactosidase activities of about 30% (D4, Fig. 1). When the region containing the STRE elements is eliminated in a construction already lacking CSRE-A, a further decrease of 10% is observed (compared D1 with D5, Fig. 1). For the *msn2 msn4* strain the integrant carrying the complete *ACR1* promoter (YIp*ACR1*) shows half the activity of the corresponding wild-type. Thus, these results confirm that the STRE sequences present in the *ACR1* promoter are functional.

3.3. *ACR1*$_{CSRE}$ elements compete for protein interaction with the *ICL1*$_{CSRE}$

Previous work showed that CSRE elements from the promoters of a variety of gluconeogenic genes compete with each other for protein binding, substantiating that they indeed interact with the same regulatory factors [4,8,9]. The element located in the promoter of the isocitrate lyase encoding gene (*ICL1*$_{CSRE}$) has been well characterized in this respect and was shown to form four complexes when incubated with protein extracts from yeast grown on ethanol as a carbon source [4]. The pattern of competition for each of these complexes differed, depending on the source of the CSRE element used as unlabelled competitor DNA (e.g. *MLS1*$_{CSRE-B}$ and *FBP1*$_{CSRE-B}$ only compete in complex III formation but *FBP1*$_{CSRE-A}$ competes with complexes I, II and III [4]).

We also used the *ICL1*$_{CSRE}$ element in competition experiments (Fig. 3). As stated above, the formation of four complexes was observed upon incubation of the labelled *ICL1*$_{CSRE}$ with extracts from ethanol grown yeast (lanes 2 and 9: note that complexes I and II only appeared as very faint bands, according to published data [4]). Complex formation (complexes I, II and III) was reduced by addition of excess unlabelled specific *ICL1*$_{CSRE}$ DNA (lane 3) but not by the addition of either calf thymus DNA (lane 8) or a fragment containing the STRE elements (lane 7), indicating specificity
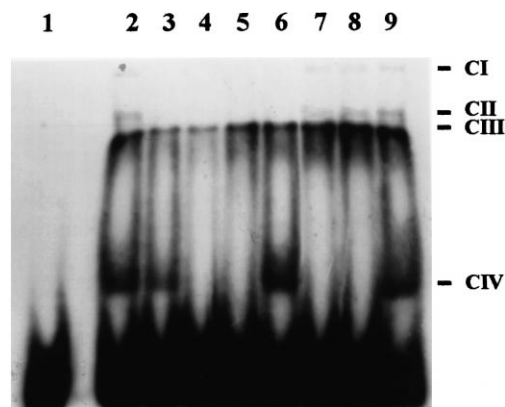

Fig. 3. Gel retardation assays. The CSRE element from the *ICL1* promoter was radioactively labelled (see Section 2) and used for protein-binding assays. Protein extracts were obtained from strain VW1B grown on rich medium with ethanol as a carbon source. Lane 1: no protein extract added; lanes 2 and 9: probe with protein extract, no competitor DNA added; lane 3: probe with protein extract and 100-fold molar excess of non-labelled *ICL1* fragment; lane 4: probe with protein extract and 100-fold molar excess of non-labelled PCR-A fragment; lane 5: probe with protein extract and 100-fold molar excess of non-labelled PCR-B fragment; lane 6: probe with protein extract and 100-fold molar excess of non-labelled PCR-C fragment; lane 7: probe with protein extract and 100-fold molar excess of non-labelled PCR-STRE fragment (obtained with OLI-20 and OLI-25: see Table 1); lane 8: probe with protein extract and calf thymus DNA as non-specific competitor.

of binding. It should be noted that the band representing complex IV disappeared when we added either of the unspecific DNAs (lanes 7 and 8). Yet, all three CSRE elements from the *ACR1* promoter described above competed for complex formation, when added as unlabelled DNA (Fig. 3: lanes 4–6), although the different elements competed to different extents (in the order CSRE-A > CSRE-B ≫ CSRE-C). This indicates that the proteins binding to the three *ACR1*$_{CSRE}$ elements are the same as those binding to CSRE elements in other gluconeogenic promoters.

4. Conclusions

Transcriptional regulation of structural genes required for growth on ethanol depend on the presence of CSRE elements in their promoters. These elements have been shown to be controlled by the Cat8 protein, whose activity in turn is regulated by the Cat1/Cat3 protein kinase complex [1,2]. Elements other than CSRE contribute to transcriptional regulation. In this context, various other protein-DNA interactions apart from CSRE binding have also been reported for a number of promoters, such as *ICL1, FBP1, MLS1* and *ACS1* [8–11].

The data presented here clearly demonstrate that this is also true for the carbon source dependent regulation of *ACR1* gene expression. Thus, the Acr1 protein, a mitochondrial succinate-fumarate carrier [20], is synthesized in coordination with gluconeogenic enzymes and exerts its function in the utilization of non-fermentable carbon sources, such as ethanol. Similar to those, regulation is conferred by CSRE elements, three of which have been characterized in this work. In addition, our data suggest that transcriptional regulation at the *ACR1* promoter is also controlled by two stress responsive elements.
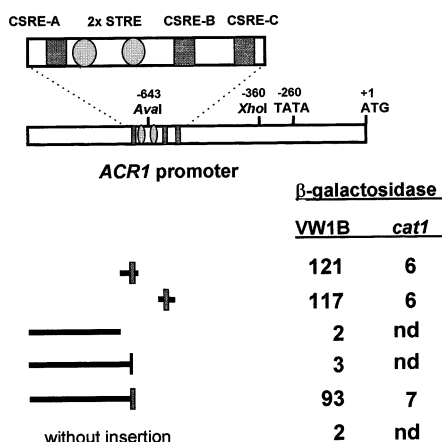

Fig. 2. Influence of *ACR1* promoter sequences on the transcription of a heterologous promoter devoid of its own UASs. Different fragments of the *ACR1* promoter were subcloned into the heterologous *CYC1* promoter as indicated and specified in Section 2. Transcriptional regulation was followed using β-galactosidase activities as a reporter (compare Fig. 1). Single copy integrants were tested in a wild-type (VW1B) and an isogenic *cat1* strain.

Plasmid		β-galactosidase VW1B	*cat1*
pNI-A (-690/-663)		121	6
pNI-B (-635/-604)		117	6
pNI-0 (-956/-701)		2	nd
pNI-22 (-956/-673)		3	nd
pNI-18 (-956/-663)		93	7
pNI17	without insertion	2	nd

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