A carbon-source-responsive element is required for regulation of the hypoxic ADP/ATP carrier (AAC3) isoform in *Saccharomyces cerevisiae*

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The mitochondrial ADP/ATP carrier in *Saccharomyces cerevisiae* is encoded by three genes that are differentially expressed under different physiological conditions. We investigated the transcriptional control of *AAC3*, an oxygen-repressed isoform. By deletion analysis, DNA electrophoretic mobility-shift assays, DNase I footprinting and site-directed mutagenesis, we have identified a promoter region (upstream repressing sequence 1, URS₁) involved in a carbon-source-dependent repression of *AAC3*. It is different from the previously characterized oxygen-dependent ROX1 (regulation by oxygen 1) repressor-binding region (URS₂). The complex character of URS₁ includes the presence of two different *cis*-acting sequences: (i) a RAP1

(repressor activator protein 1)-binding site that is capable of binding the RAP1 protein *in vitro* and (ii) two putative ethanol-repression sequences, the modification of which derepresses the *AAC3* gene. These findings demonstrate that the hypoxic *AAC3* gene is regulated by two upstream repressor sites; one controlled by oxygen and haem, the other by the carbon source. Both sites function to completely switch off the expression of the *AAC3* isoform when ATP is made by oxidative phosphorylation, and they modulate *AAC3* expression when import of glycolytic ATP into mitochondria is required.

Key words: anaerobiosis, gene expression, hypoxic promoter.

INTRODUCTION

Genome-wide transcriptional analysis of aerobic and anaerobic cultures of *Saccharomyces cerevisiae* demonstrated the presence of more than 100 genes exhibiting several-fold higher expression under anaerobic than under aerobic conditions [1]. Transcriptional regulation of several such 'hypoxic' genes has been investigated intensively (reviewed in [2–4]), and two general pathways, one involving a ROX1 (regulation by oxygen 1) factor and the other ROX1-independent, were delineated [5–9]. Apart from ROX1, little is known about the other ROX1-independent factors participating in the hypoxic gene regulation. Previous studies have characterized the *AAC3* gene, which encodes an ADP/ATP carrier of mitochondria, as a hypoxic isogene expressed under anaerobic conditions [10,11]. A DNA sequence between –225 and –180 bp in the 5' flanking region of the *AAC3* gene (termed upstream repressing sequence 2, or URS₂), consisting of a ROX1 repressor-binding site (YYYATTGTTCTC) and a long poly-dT segment, was found to be essential for the repression of *AAC3* by oxygen [11]. The 28-fold higher expression of *AAC3* under anaerobiosis [1] most probably reflects the requirement of intramitochondrial ATP under these conditions [12]. Transcriptional regulation, similar to that exerted by ROX1, has been identified recently within the promoter of the human ADP/ATP carrier isoform, *ANT2* [13]. A new motif, termed GRBOX (glycolysis-regulated box) has been characterized and it was proposed that the GRBOX element may represent a link to the glycolytic metabolism of the highly proliferative cells.

The yeast *S. cerevisiae* possesses a high fermentative capacity even in the presence of oxygen. The variation in ATP production

in *S. cerevisiae* under different growth conditions, ranging from full respiratory competent aerobiosis to strict anaerobiosis, would require precise adjustment of the amount and type of ADP/ATP-carrier isoform. In this study we provide evidence that an additional upstream repressor sequence (URS₁) in the *AAC3* promoter is involved in carbon-source-dependent regulation. This URS₁ is different from the ROX1 site that has been implicated in the regulation by oxygen. The function of this new regulatory site is to completely exclude *AAC3* from mitochondria during oxidative phosphorylation and to modulate its level during high aerobic glycolysis.

MATERIALS AND METHODS

Strains and growth conditions

The following *S. cerevisiae* strains were used: RZ53-6 (*MATα*, *leu2-3*, *leu2-112*, *ura3-52*, *ade1-106*, *trp1-289*) and RZ53-b, which contains a *rox1::LEU2* allele, were provided by Dr Ch. Lowry (State University of New York at Albany, NY, U.S.A.). Yeast cells were grown aerobically at 30 °C in YP medium (1% yeast extract/2% bactopectone) supplemented with the indicated carbon sources, or under selective conditions in SM medium (minimal synthetic growth medium containing 0.67% yeast nitrogen base) containing the proper nutritional requirements. Anaerobic cultures were grown in glucose-rich (8%) or raffinose (2%) SM media, supplemented with 12 µg/ml ergosterol, 0.2% Tween 80 and nutritional requirements. The solid media contained 2% Bacto-Agar (Difco).

The construction of the YEPA4 plasmid containing 1150 bp of the *AAC3* promoter (*EcoRI/HindIII*, –1150/+269) fused to the *lacZ* gene was described previously [11]. This plasmid was

Abbreviations used: URS, upstream repressing sequence; ERA, ethanol-repression autoregulation; EMSA, electrophoretic mobility-shift assay; ROX1, regulation by oxygen 1; RAP1, repressor activator protein 1; SM medium, minimal synthetic growth medium.

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used to prepare all other deletion mutants used in this study, including the test plasmid, YEpXa. This plasmid contained the basal promoter of the *AAC3* gene (−158/+269) fused to the *lacZ* gene. Deletions in the *AAC3* promoter were made using unique restriction sites and *Bal31* nuclease. End points of all deletions and the mutated sequences were verified by sequencing using the T7 polymerase kit (Amersham Pharmacia Biotech).

Site-directed mutagenesis

The promoter fragment (*EcoRI/HindIII*, −1150/+269) from YEpA4 was cloned into the pGEM3Zf[−] vector and the resulting plasmid (p3ZA4) served as a template for mutagenesis. The following primers were used to introduce mutations into the RAP1 (repressor activator protein 1)-binding site (mutated nucleotides are in lower case and the respective binding sites are underlined): R_{US}, 5'-TGACAAAATGCAgGcTcTAAAAA-GGAAA-3', and R_{LS}, 5'-TTTCCTTTTTTAgAgCtcTGCATT-TTGTC-3', where US and LS refer to upper and lower strand, respectively.

To specifically alter the putative ethanol-repression auto-regulation (ERA) binding sequences at −301/−293 (ERA₁) and −324/−316 (ERA₂), the following pairs of primers were used: E1_{US}, 5'-GTCTGGATGACAgAgTcTTGGGTGTAAA-AAA-3', E1_{LS}, 5'-TTTTTACACCCAAGTAcTcTGTCATCC-AGAC-3', E2_{US}, 5'-CGGAccAaGgGTAATGTCTGGATGAC-3', and E2_{LS}, 5'-GTCATCCAGACATTACcCtTggTCCG-3'.

The p3ZA4 plasmids carrying the described mutations were constructed by overlap-extension PCR [14] in which the above-mentioned nucleotides and SP6 and T7 primers were used.

Electrophoretic mobility-shift assay (EMSA)

DNA EMSAs were performed as described in [15,16]. Protein extracts (5–20 µg of protein) were added to 10 µl of incubation mixture containing 20 mM Tris/HCl, pH 8.0, 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM CaCl₂, 0.1 mM EDTA, 1 ng of calf thymus carrier DNA (fragmented by sonication) and 0.2–0.5 ng (10 000 c.p.m.) of ³²P-end-labelled DNA fragments. The DNA–protein binding reactions were incubated on ice for 15 min and the resulting complexes were separated on 4% polyacrylamide gels in 0.5 × TBE (45 mM Tris/borate/1 mM EDTA, pH 8.0). The anti-RAP1 serum used in the EMSAs was provided by Dr S. Johnaton (University of Minnesota, St. Paul, MN, U.S.A.).

DNase I protection assay

For DNase I protection assays, the upper-strand (−378/−244) and lower-strand (−474/−158) fragments were labelled at their 5' ends. Reaction mixtures (50 µl) contained 50 mM KCl, 10 mM MgCl₂, 20 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 2.5–30 µg of protein extract and 1 ng of 5'-end-labelled probe. After 15 min incubation on ice, the samples were digested partly by DNase I (Boehringer Mannheim) and reactions were stopped by addition of 0.1 M EDTA/0.6 M ammonium acetate containing 20 µg/ml sheared single-stranded DNA from calf thymus. The labelled DNA was extracted by phenol/chloroform (1:1), precipitated, dissolved in loading buffer and separated on 6% polyacrylamide sequencing gels. Maxam–Gilbert sequencing reactions for purines (A + G) were performed on the same fragments.

Miscellaneous

Published procedures were used for isolation, manipulation, labelling and sequencing of DNA [17]. Yeast cells were trans-

formed by lithium acetate treatment [18]. Protein extracts were prepared as in [19]. For analysis of β -galactosidase expression, transformants were grown overnight in SM medium, diluted to the desired density in fresh medium and grown to D_{600} of approx. 1.0. For preparation of crude extracts, transformants were broken by vortex-mixing with glass beads and β -galactosidase activity was assayed as described in [20]. A minimum of five transformants was assayed in duplicate and each experiment was repeated three to five times. The values from each experiment were normalized for variation between experiments and the means of at least three experiments are reported.

RESULTS

Identification of carbon-source-dependent regulatory elements in the *AAC3* promoter

The *AAC3* gene was identified as a hypoxic gene that is regulated negatively by oxygen, haem and the ROX1 factor [10,11]. To determine other factors involved in its regulation, *AAC3* gene expression was analysed further in the Δ rox1 strain (RZ53-b). The β -galactosidase activity expressed from the YEpA4 plasmid carrying full-length promoter was very low in cells grown on either ethanol or lactate and was induced 3–20-fold in cells grown on raffinose or glucose (Table 1). Higher expression of the *AAC3* gene on glucose, as compared with the non-fermentable carbon sources, was also apparent in the wild-type (RZ53-6) strain in spite of the repression imposed by the ROX1 factor.

To delineate the promoter domains involved in the carbon-source-dependent regulation of the *AAC3* gene, a number of promoter deletion constructs were prepared. All constructs were fused to *lacZ*, transformed into the Δ rox1 strain and β -galactosidase activity expressed from each deletion construct was determined as the response to growth on glucose or lactate. As shown in Figure 1, deletion of the upstream sequences between −1150 and −474 bp did not affect the expression of β -galactosidase in cells grown on fermentable or non-fermentable carbon sources. Deletion of the sequences downstream of −474 bp, however, resulted in a several-fold increase of the reporter gene expression in cells grown on lactate. Under repressing conditions (8% glucose), none of the deletion constructs resulted in a more than 2-fold increase in reporter gene expression. The presence of a carbon-source-dependent negative regulatory element in the *AAC3* promoter was confirmed further by two internal deletions, −474/−244 bp and −314/−244 bp (Figure 1). Comparison of β -galactosidase expression from

Table 1 Expression of *AAC3-lacZ* reporter constructs in the Δ rox1 mutant and wild-type cells cultivated under different conditions

The YEpA4 plasmid used for transformation contains the *EcoRI/HindIII* (−1150/+269) fragment of *AAC3* fused to *lacZ*. Transformants were grown in SM media containing the indicated carbon sources under either aerobic (+O₂) or anaerobic (−O₂) conditions, as described in the Materials and methods section. ONPG, *o*-nitrophenyl β -D-galactopyranoside.

Strain	β -Galactosidase activity (nmol ONPG hydrolysed/min per mg of protein)					
	Carbon source					
	Glucose		Raffinose		Ethanol	Lactate
	+ O ₂	− O ₂	+ O ₂	− O ₂	+ O ₂	+ O ₂
RZ53-b (Δ rox1)	70.0	89.0	12.0	18.0	4.0	5.0
RZ53-6 (ROX1 ⁺)	2.0	34	2.0	12.0	0.1	0.1

		β -galactosidase activity (nmolONPG/min/mg protein)		
		Glucose	Lactate	glu/lac
-1150	//	114	16	7
-474		114	18	6.5
-318		148	82	1.8
-296		240	170	1.4
-291		203	140	1.4
-244		224	140	1.6
-474	-244	108	88	1.2
-314	-244	261	108	2.4
-322	-186	236	162	1.5
-244	-158	21	5	4.2

Figure 1 Deletion analysis of the *AAC3* upstream region

Deletions were constructed in the YE Δ 4 plasmid and then introduced into the Δ *rox1* (RZ53-b) deletion strain. Nucleotide positions refer to the translational start codon. Transformants were grown in synthetic media supplemented with 8% glucose or 2% lactate as a carbon source and the expression of β -galactosidase was measured. All values represent the means from at least three independent experiments. S.D. were less than 20% of the means. ONPG, *o*-nitrophenyl β -D-galactopyranoside.

constructs carrying these internal deletions also indicated that a part of the repression region may be located upstream of -314 bp. The expression from the remaining internal deletion constructs showed that the repression of *AAC3* did not require the presence of the previously characterized negative site (-201/-179 bp) responsible for oxygen repression [11]. The

data in Figure 1 thus indicate that the sequence between -322 and -244 bp might be involved in the repression of the *AAC3* gene by a respiratory carbon source.

The function of the carbon-source-dependent regulatory elements present in the -474/-244 bp region were determined by cloning of various fragments in front of the basal *AAC3* promoter (-158/+269 bp) fused to the *lacZ* gene (plasmid YE Δ Xa). The YE Δ Xa plasmid does not contain a ROX1-binding site and expresses β -galactosidase at a relatively high level. The ability of the individual fragments to repress the *AAC3* gene was tested in the wild-type strain (*ROX1*⁺) grown in either the presence or absence of oxygen (Figure 2). Two *AAC3* promoter sequences, -474/-244 bp and -323/-244 bp, were equally effective in suppressing *AAC3-lacZ* expression in cells grown both aerobically and anaerobically. The -474/-295 bp fragment resulted in less efficient repression only in aerobically grown cells, whereas under anaerobiosis a weak activation, rather than repression, was observed. These results suggest that distinct parts of the -474/-244 bp region may bind different regulatory proteins possessing different activities under either aerobic or anaerobic conditions.

Specific protein–DNA interactions within URS₁

To test for DNA–protein interactions within the URS₁ region of the *AAC3* promoter the -378/-244 bp fragment was isolated, end-labelled and used in DNA EMSA. Figure 3(A) shows protein binding to the -378/-244 bp fragment (lane 1) that was inhibited competitively by unlabelled probe (lanes 2–5). Figure 3(B) shows that the binding of protein or proteins is not subject to carbon-source regulation. The formation of a DNA–protein complex was observed with protein extracts from cells grown on different carbon sources (glucose, raffinose and lactate). These results suggest that the protein (or proteins) binding to the -378/-244 bp fragment of the *AAC3* promoter is not affected specifically by the carbon source.

To identify the *cis*-acting sequences on the -378/-244 bp fragment of the *AAC3* promoter, we employed DNase I footprinting analysis using cell extract from raffinose-grown wild-

		β -galactosidase activity (nmol ONPG hydrolysed/min/mg protein)		
		Lactate(+O ₂)	Glucose(-O ₂)	Raffinose(-O ₂)
-158		80	10	16
-474	-244	7	1.5	0.5
-474	-295	14	19	13
-323	-244	2	0.5	0.2

Figure 2 Repression induced by insertion of URS₁ fragments into the Δ URS₁ control plasmid

The indicated DNA sequences of the *AAC3* promoter were inserted in front of the basal promoter as specified in the Materials and methods section. Resulting plasmids were transformed into the wild type-strain (RZ53-6) and transformants were grown in media containing the indicated carbon sources under either aerobic (+O₂), or anaerobic (-O₂) conditions. ONPG, *o*-nitrophenyl β -D-galactopyranoside.

Figure 3 DNA mobility-shift analysis of the *AAC3* URS1 region

(A) The 134 bp 32 P-end-labelled DNA fragment (—378/—244) of the *AAC3* promoter was incubated with protein extracts (10 μ g) from the wild-type (RZ53-6) cells grown on 2% raffinose (lane 1). Lanes 2–5, 2, 10, 25 and 100 times molar excesses of unlabelled fragment were added to the binding reactions. (B) Protein extracts used (10 μ g) were prepared from cells grown on 2% raffinose (lane 2), 2% lactate (lane 3) and 8% glucose (lane 4). The control (lane 1) contains the labelled fragment incubated with 10 μ g of BSA. C, complex.

Figure 4 DNase I footprint protection assay

DNase I protection analysis was performed with both the upper (A) and lower (B) strands of the 32 P-end-labelled *AAC3* URS1 fragment. (A) Lane 1, Maxam–Gilbert sequencing reaction for A + G; lane 2, 10 μ g of BSA added to the binding reaction; lanes 3–9, increasing amounts of protein extract from the wild-type strain (RZ53-6) grown on raffinose were added to the binding reactions. (B) Lane 1, Maxam–Gilbert sequencing for A + G; lanes 2–5, increasing amounts of protein extract from the RZ 53-6 strain grown on raffinose were added to the binding reactions. The protected sequences are indicated. hs, hypersensitive site.

type cells (Figure 4). A relatively short sequence between —302 and —285 bp was protected on both strands (Figure 4A, lanes 3–9). In addition, a DNase I-hypersensitive site centred around —285 bp was identified on the upper strand (Figure 4B, lanes 3–5). These results indicate that the region between —302 and —285 bp is involved in specific DNA–protein interactions in the URS₁ region of the *AAC3* promoter.

Inspection of the protected DNA region and flanking sequences revealed the presence of *cis*-acting elements. One of them, complementary to the RAP1-protein-binding site (RMACCCANNCAYY) [21], is located between —300 and —288 bp. Two sequences, one from —301 to —293 bp (AAATGCATG) and the other from —324 to —316 bp (AAATGCATA), resemble the consensus sequence (AAATGCATA)

Figure 5 RAP1 protein binds *in vitro* to *AAC3* URS1

The 32 P-end-labelled $-378/-244$ *AAC3* fragment and the protein extract from the wild-type strain (RZ53-6) grown on raffinose were used in binding reactions. Lane 1, labelled fragment only; lane 2, 10 μ g of BSA; lanes 3 and 4, the fragment incubated with protein extract; lanes 5–7, increasing amounts of anti-RAP1 serum (0.5, 1 and 2 μ l) were added; lanes 8–10, increasing amounts of control serum (1, 2 and 4 μ l). C, complex.

Figure 6 Effect of mutations introduced into RAP1- and ERA-binding sites on *AAC3-lacZ* expression

Plasmids containing either unmodified or mutated RAP1- and ERA-binding sites were transformed into the wild-type (RZ53-6) and Δ *rox1* deletion (RZ53-b) strains. Transformants were grown in SM media containing the indicated carbon sources under aerobic or anaerobic conditions. The effect of mutations was evaluated as the ratio of β -galactosidase activities expressed from the mutated and the wild-type promoter constructs under identical growth conditions. glu, glucose; lac, lactate; raf, raffinose.

of the ERA element found in promoters of several genes involved in carbohydrate metabolism [22]. These regions are here referred to as ERA₁ and ERA₂, respectively. The ERA₁ sequence almost completely overlaps the consensus RAP1-binding site.

To confirm that RAP1 binds to the protected DNA region, we used a RAP1-specific antibody in EMSA experiments. As shown in Figure 5, the immune serum abolished the formation of specific protein–DNA complexes, whereas control serum did not, demonstrating the binding of the RAP1 protein to its cognate site in the *AAC3* promoter. These results suggest that RAP1 might participate in the transcriptional regulation of *AAC3*.

Promoter fragment $-378/-296$ bp, containing only the complete ERA₂ site from -324 to -316 bp, did not produce any specific DNA–protein complex with any of the protein extracts examined (results not shown).

Functionality of RAP1 and ERA sites

Results of the deletion analysis suggested that the sequences around the RAP1-binding site ($-300/-288$ bp) may be involved in the repression of the *AAC3* gene. To assess the functionality of the RAP1 and ERA sites, we introduced mutations in each consensus sequence and studied their effect on the expression of the reporter gene (Figure 6). It should be noted that the mutation of both the ERA₁ and the RAP1-binding site eliminated the binding of RAP1 protein *in vitro* (results not shown).

Data summarized in Figure 6 show that mutation of any of consensus binding sites affected *AAC3-lacZ* expression but that each mutation acted differently. In the presence of oxygen, mutation of any of the ERA sites de-repressed the *AAC3* gene in wild-type cells regardless of the carbon source. In Δ *rox1* cells grown on lactate or raffinose only mutation of the ERA₂ site resulted in de-repression of *AAC3*, whereas mutation of the ERA₁ site under these conditions increased *AAC3* expression only slightly. In Δ *rox1* cells grown on glucose, no significant alteration of *AAC3* expression was observed.

Under aerobiosis, RAP1 mutation had no effect in wild-type cells grown on lactate and caused a moderate de-repression of the *AAC3* expression in cells grown on glucose. In Δ *rox1* cells mutation of the RAP1 site had no effect on expression of the *AAC3* gene grown in the presence of oxygen.

In wild-type cells grown anaerobically, results showed that only mutations that abolished binding of the RAP1 protein (RAP1- and ERA₁-binding sites) de-repressed the *AAC3* expression when cells were grown on glucose or raffinose. The effect was higher in raffinose-grown cells (Figure 6). Mutation of the ERA₂ site increased the *AAC3* expression in both glucose- and raffinose-grown cells only slightly.

DISCUSSION

In the present study we demonstrated that separate negative DNA sequences, different from the ROX1-binding site, in the *AAC3* promoter might be linked to the glycolytic metabolism of yeast cells. Deletion analysis identified the presence of repressing sequences (URS₁) around -295 bp that appeared to be active under both aerobic and anaerobic conditions. Expression of the *AAC3* gene in the Δ *rox1* strain suggested that these sequences can function independently of the ROX1 repressor and respond to the carbon source.

Results obtained from EMSA using extracts from cells grown on glucose, raffinose or lactate indicated that a common *trans*-acting factor was bound to URS₁. A DNase I footprinting assay mapped the URS₁ to a 17 bp region ($-302/-285$) that appeared to be essential for DNA–protein interactions. The sequence of the protected region, the inhibition of DNA mobility shift by anti-RAP1 serum and the mutational analysis demonstrated that RAP1, a ubiquitous transcriptional regulator, binds to URS₁ and plays a role in the carbon-source-dependent regulation. Besides the RAP1 consensus sequence the repressing region identified in our study contained two *cis*-acting elements resembling the ethanol-repression sequence (AAATGCATA, termed ERA) [22] at $-324/-316$ and $-301/-293$ bp. This sequence was first identified as a *cis*-acting element controlling the expression of pyruvate decarboxylase, an enzyme that acts at the branch-point between fermentation and respiration. The

identity of protein(s) that bind the ERA element has remained unknown. Our results showed that the ERA sequences repressed the expression from the basal *AAC3* promoter. Although the DNA–protein binding assays used in this study failed to detect specific protein binding to the putative ERA sequences, the deletions spanning the ERA-containing region resulted in a significant increase in expression, indicating that these elements were essential for physiological expression of *AAC3*. This was corroborated further by site-directed mutagenesis of the ERA sites. The ratio between the activity of the mutated and the wild-type promoters revealed two important properties of these promoter elements.

First, modification of any of the putative ERA sites in the wild-type strain resulted in a several-fold de-repression of the *AAC3* gene in the presence of oxygen regardless of carbon source. In the ROX1-deleted strain, only mutation of the ERA₂ site caused de-repression on respiratory carbon sources, whereas the mutation of the ERA₁ site had very little effect on expression, indicating a possible co-operation between the two repressors, ERA and ROX1. Under aerobic conditions mutation of the RAP1-binding site had only a marginal effect on *AAC3* expression.

Second, the results of mutational analysis indicated that only the RAP1 factor was active under anaerobic conditions since only the mutation of RAP1 and ERA₁ sites de-repressed expression of the *AAC3* gene. We suppose that de-repression of the *AAC3* gene by mutated ERA₁ is due to the simultaneous abrogation of RAP1 binding. Mutation of the ERA₂ site had no effect.

Both RAP1- and ERA-binding sites participate in carbon-source-dependent regulation of the *AAC3* gene and the involvement of individual factors in this regulation is dependent on oxygen availability. It appears that under anaerobic conditions only RAP1 participates in the regulation of the *AAC3* gene, whereas both ERA and RAP1 participate in the repression under oxygen-proficient conditions.

In conclusion, our results demonstrate that the *AAC3* gene, encoding the hypoxic isoform of the mitochondrial ADP/ATP carrier, is regulated by two negative *cis*-acting DNA elements, URS₁ and URS₂. URS₂ binds ROX1 factor and confers sensitivity to oxygen, whereas URS₁ responds mainly to the nature of the carbon source used for cell growth. Through URS₁, a complex regulation, probably involving more than one regulatory protein (ERA, RAP1) is exerted. Under aerobic conditions, when respiratory carbon sources are present and the mitochondrial ADP/ATP-exchange transport is executed by the major AAC2 isoform, both negative sites completely switch off expression of *AAC3*. It is possible that the *AAC3* isoform is better adjusted for the inverse exchange of cytosolic ATP for mitochondrial ADP than the other isoforms, and this could be necessary for efficient oxidative phosphorylation under the conditions used. The carbon-source-dependent site in the *AAC3* promoter, described in this study, is most probably linked to the glycolytic metabolism of yeast cells and it could serve to co-ordinate the quantity of the carrier that catalyses inverse ADP/ATP exchange with the changing need for ADP/ATP exchange during shifts from oxidative to glycolytic metabolism.

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REFERENCES

- ter Linde, J. J. M., Liang, H., Davis, R. W., Steensma, H. Y., van Dijken, J. P. and Pronk, J. T. (1999) Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *J. Bacteriol.* **181**, 7409–7413
- Zitomer, R. S. and Lowry, C. V. (1992) Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **56**, 1–11
- Zitomer, R. S., Carrico, P. and Deckert, J. (1997) Regulation of hypoxic gene expression in yeast. *Kidney Int.* **51**, 507–513
- Kwast, K. E., Burke, P. V. and Poyton, R. O. (1998) Oxygen sensing and the transcriptional regulation of oxygen-responsive genes in yeast. *J. Exp. Biol.* **201**, 1171–1195
- Lowry, C. V., Cerdan, M. E. and Zitomer, R. S. (1990) A hypoxic consensus operator and a constitutive activation region regulate the ANB1 gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 5921–5926
- Sertil, O., Cohen, B. D., Davies, K. J. and Lowry, C. V. (1997) The DAN1 gene of *S. cerevisiae* is regulated in parallel with the hypoxic genes, but by a different mechanism. *Gene* **192**, 199–205
- Ansell, R., Granath, K., Hohmann, S., Thevelein, J. and Adler, J. M. (1997) The two isoenzymes for yeast NAD⁺-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. *EMBO J.* **16**, 2179–2187
- Donzeau, M., Bourdineaud, J. P. and Lauquin, G. J. (1996) Regulation by low temperature and anaerobiosis of a yeast gene specifying a putative GPI-anchored plasma membrane. *Mol. Microbiol.* **20**, 449–459
- Erratum (1996) *Mol. Microbiol.* **21**, 431
- Bourrot, S. and Karst, F. (1995) Isolation and characterization of the *Saccharomyces cerevisiae* SUT1 gene involved in sterol uptake. *Gene* **165**, 97–102
- Kolarov, J., Kolarova, N. and Nelson, N. (1990) A third ADP/ATP translocator gene in yeast. *J. Biol. Chem.* **265**, 14195–14201
- Šabová, L., Zeman, I., Supek, F. and Kolarov, J. (1993) Transcriptional control of *AAC3* gene encoding mitochondrial ADP/ATP translocator in *Saccharomyces cerevisiae* by oxygen, heme and ROX1 factor. *Eur. J. Biochem.* **213**, 547–553
- Šubík, J., Kolarov, J. and Kováč, L. (1972) Obligatory requirement of intramitochondrial ATP for normal functioning of the eucaryotic cell. *Biochem. Biophys. Res. Commun.* **49**, 192–198
- Giraud, S., Bonod-Bidaud, C., Wesolowski-Louvel, M. and Stepien, G. (1998) Expression of human ANT2 gene in highly proliferative cells: GRBOX, a new transcriptional element, is involved in the regulation of glycolytic ATP import into mitochondria. *J. Mol. Biol.* **281**, 409–418
- Ausubel, R. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1994) Mutagenesis by the polymerase chain reaction, in *Current Protocols in Molecular Biology*, chapter 8, section 8.5, John Wiley and Sons, New York
- Fried, M. and Crothers, D. M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* **9**, 6505–6525
- Garner, M. M. and Revzin, A. (1981) A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. *Nucleic Acids Res.* **9**, 3047–3060
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn, Cold Spring Harbor Press, Cold Spring Harbor
- Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163–168
- Arcangioli, B. and Lescure, B. (1985) Identification of proteins involved in the regulation of yeast iso-1-cytochrome C expression by oxygen. *EMBO J.* **4**, 2627–2633
- Guarente, L. (1983) Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.* **101**, 181–191
- Buchman, A. R., Kimmerly, W. J., Rine, J. and Kornberg, R. D. (1988) Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**, 210–225
- Liesen, T., Hollenberg, C. P. and Heinisch, J. J. (1996) ERA, a novel *cis*-acting element required for autoregulation and ethanol repression of PDC1 transcription in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **21**, 621–632