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Transcriptional regulation of the isocitrate lyase encoding gene in *Saccharomyces cerevisiae*

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In this work, we studied the transcriptional regulation of isocitrate lyase synthesis. In Northern blot analyses we first showed that the steady-state *ICL1* mRNA levels depend on the carbon source used for growth. In addition, we determined the kinetics of transcriptional repression upon a shift of ethanol-grown cells to glucose and of the induction when cells were transferred from glucose to ethanol. By deletion analyses as well as by studying the influence on expression of different fragments cloned into the heterologous *CYC1* promoter lacking its own *UAS* sequences, we defined *UAS* and *URS* elements in the *ICL1* promoter. A region mediating the control by *CAT3*, a gene also involved in the control of expression of other genes subject to carbon catabolite repression, was found to overlap with one of these *UAS* elements.

Isocitrate lyase; Glyoxylate pathway; Promoter element; Regulation; Yeast

1. INTRODUCTION

Growth of *Saccharomyces cerevisiae* on ethanol as a sole carbon source requires the glyoxylate pathway that operates as an anaplerotic route for replenishing C_4 compounds to the tricarboxylic acid cycle. Isocitrate lyase is one of the key enzymes of the glyoxylate pathway [1]. In yeast, the enzyme is composed of four identical subunits [2] with a molecular weight of 62.5 kDa each, as deduced from the nucleotide sequence of the encoding gene [3]. Isocitrate lyase does not contain any known peroxisomal targeting signals at its C-terminal end, suggesting that the enzyme is not located in these organelles. Deletion mutants lack detectable isocitrate lyase activity and do not grow on ethanol medium demonstrating that the *ICL1* gene is essential for the utilization of this compound [3].

In previous works, synthesis of isocitrate lyase in *S. cerevisiae* was shown to be induced by ethanol and repressed by glucose [4,5,6]. In addition, several genes including those involved in carbon catabolite derepression such as *CAT1*(= *SNF1*) and *CAT3*(= *SNF4*) [7,8,9] have been shown to regulate expression of this enzyme. In this paper we studied the kinetics of transcriptional regulation of expression of the *ICL1* gene under different metabolic conditions. We also provide data on the identification of the promoter elements responsible for this regulation.

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2. MATERIALS AND METHODS

2.1. *Strains*

S. cerevisiae strains 10.7-11A (*MATa leu1 MAL3 suc3-3*) described in [10], JS87.11-1A (*MALα trp1-289 cat1::HIS3 MAL2-8^c MAL3 SUC3*) and JS87.15-1B (*MATα ura3-52 trp1-289 cat3::LEU2 MAL2-8^c MAL3 SUC3*) kindly provided by K.-D. Entian (Frankfurt, Germany) and icl1d (*MATα icl1::LEU2*) [3] were used in expression studies. Strain AMW-13C$^+$ (*MATa trp1*(fs) *ura3*(fs) *leu2-3,112 his3-11,15 can1*; where 'fs' stands for frameshift mutation) was used as a recipient in transformation experiments.

2.2. *Media, growth conditions and enzymatic analysis*

Rich media were based on 1% yeast extract and 2% peptone (YEP). 2% glucose (D), 3% potassium acetate (A), 3% ethanol (E) or 3% glycerol (G), were added as carbon sources. Synthetic media, consisting of 0.67% yeast nitrogen base w/o amino acids supplemented with amino acids as required and 2% glucose or 3% ethanol, were used to select for transformants of plasmids carrying a *URA3* marker. β-galactosidase activity was assayed according to [11].

2.3. *Nucleic acid preparations and hybridization experiments*

Total RNA (10 μg) isolated from yeast cells according to [12] was separated on 1.5% agarose/MOPS/formaldehyde gels [13]. Northern blot analysis was performed by standard procedures [14]. All other DNA manipulations were as described previously [3].

2.4. ICL1-lacZ *fusions*

A *Bam*HI-*Hin*dIII fragment containing about 1000 nucleotides from the 5′ noncoding region and 171 bp of the *ICL1* coding sequence was cloned in frame to *lacZ* into YIp356 [15]. Promoter deletions were constructed by using available restriction sites in conjunction with the *Exo*III nested-deletion kit of Pharmacia. The resulting plasmids were integrated into the *URA3* locus by digestion with *Stu*I prior to transformation of the yeast strain AMW-13C$^+$ and single copy integration was confirmed by Southern analysis of genomic DNA digested with *Bgl*II and probing with a 1.1 kb *Hin*dIII fragment containing *URA3*.

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2.5. *Subcloning of* ICL1 *promoter fragments into the heterologous* CYC1 *promoter*

pLGi, a derivative of plasmid pLG-669Z [16] lacking 2μ sequences, was used to study the function of putative UAS elements. Four plasmids were constructed by substituting the *Xho*I fragment of pLGi containing the two UAS sequences of the *CYC1* promoter for *ICL1* promoter fragments flanked by *Xho*I sites. pLGICL-1 contains the 365 bp *Xho*I-*Eco*RI fragment of the *ICL1* promoter (−528/−164 relative to the ATG translation start codon; to obtain compatible ends, a *Xho*I-*Eco*RI linker was added to the *Eco*RI end). pLGICL-2 carries the DNA fragment from −528 to −295 and was obtained by PCR using the oligonucleotides 5′-ATCTCGACGTCAGTAATATGTCTCGAGA-3′ and 5′-ATCCTCGAGGATGACGTTGAGTGTTAA3-′ (underlined sequences designate the *Xho*I sites used for subcloning). As a template, plasmid pICL1.1 [3] containing the entire *ICL1* gene was used. pLGICL-3 carries the DNA fragment from −331 to −164 and pLGICL-4 from −386 to −247. They were constructed using PCR as described for pLGICL-2 using the oligonucleotide pairs 5′- ATCCTCGAGAAGCCAATCACCACA3-′/5′ -ATCCTCGAGAATTCCGATGTGCCTGG3-′ and 5′- ATCCTCGAGGCGATCACTTATCTGACT3-′/5′- ATCCTCGAGATGCTAGTCCGGACTATG3-′, respectively. Each of the four plasmids was integrated into the strain AMW-13C$^+$ and analyzed as described above for the *ICL1-lacZ* fusions. As controls, the original vector (pLGi) containing the *CYC1* activating sequences and a plasmid where the latter have been deleted (pLG-delta) were also tested. pLGICL-1 and pLGICL-2 were also integrated into the strain JS87.15-1B (*cat3*) at the *URA3* locus.

3. RESULTS AND DISCUSSION

3.1. *Regulation of* ICL1 *gene expression*

The steady-state levels of isocitrate lyase mRNA isolated from cells grown on different carbon sources were determined by Northern blot analysis (Fig. 1A). A strong hybridization band was obtained when cells were grown on ethanol or acetate but not on glucose media. Only a weak band was detected in the preparation from glycerol. A similar regulation was reported for the transcription of the gene encoding the malate synthase isoenzyme functioning in the glyoxylate pathway [17,18].

To study the effects of carbon catabolite repression the decrease in mRNA levels was followed after shifting ethanol grown cells to glucose medium. As shown in Fig. 1B, 5 min after the shift the intensity of the band hybridizing to the *ICL1* probe was reduced to about half as compared to the one from ethanol grown cells (time 0 min). After 30 min no signal was detected, anymore. Presumably, the decrease of mRNA can be attributed to both a block of transcription and a change in the half-life of the mRNA on glucose. The latter mechanism has been reported for other genes subject to glucose repression such as *CYC1* [19] and *SDHip* [20].

In a complementary experiment the release from repression was studied upon a shift of glucose-grown cells to ethanol medium. A progressive accumulation of the *ICL1* mRNA starting after 60 min was detected up to 165 min (Fig. 1C). After this time a constant steady-state mRNA level was reached (data not shown).

3.2. *Mapping of* cis*-acting regulatory elements*

To identify *cis*-acting regulatory elements controlling the transcription of the *ICL1* gene we first made a series of deletions in the 5′-noncoding region (the complete sequence is available from the EMBL database under the accession number X61271). To assess their influence, we used a fusion to *lacZ* as a reporter gene (see section 2). Single copy transformants with the constructs were grown either on ethanol or on glucose media and specific β-galactosidase activities were determined (Fig. 2). A fusion containing about 1000 bp of 5′-noncoding sequences (YIpICL-1) showed a high level of expression when grown on ethanol that was repressed more than 500-fold on glucose media. This indicates that all essential promoter elements are located within this region. For deletions starting from the 5′-end

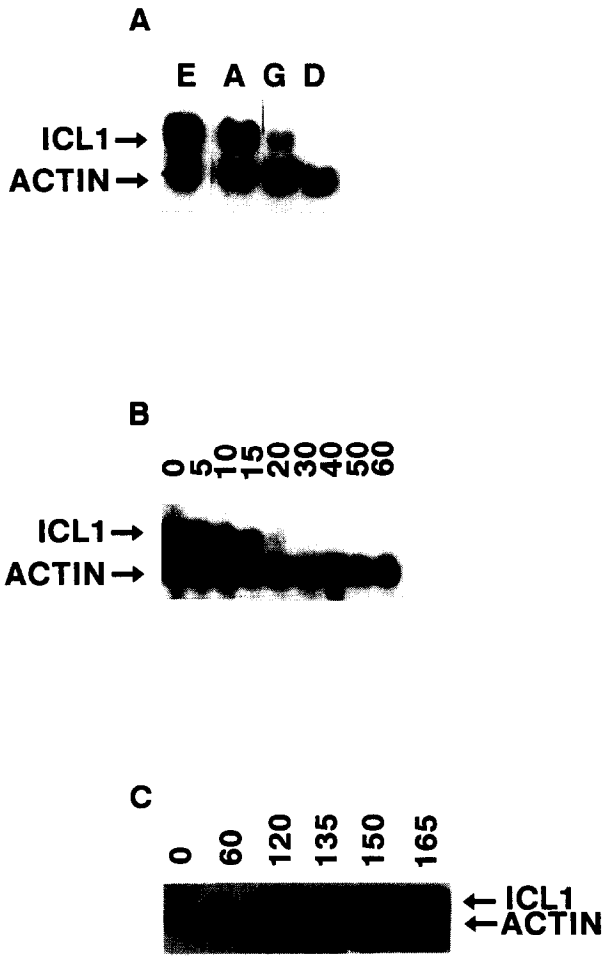


Fig. 1. Northern blot analyses. As probes a mixture of DNA fragments was used, containing the *ICL1*- and the yeast actin-encoding genes. (A) Effect of carbon sources on *ICL1* transcription. Total RNA was prepared from cells of strain 10.7-11A grown on rich media with ethanol (E), potassium acetate (A), glycerol (G) and glucose (D) as carbon sources. (B) Effect of glucose on *ICL1* transcription. Cells of strain 10.7-11A grown overnight in YEPE were harvested, washed and resuspended in YEPD medium. Total RNA was isolated from cells taken at various times as indicated, blotted and hybridized. (C) *ICL1* transcription during ethanol induction. Strain 10.7-11A was grown on YEPD. After 12 h cells were harvested, washed and resuspended in YEPE. At the times indicated samples were removed from which total RNA was prepared.

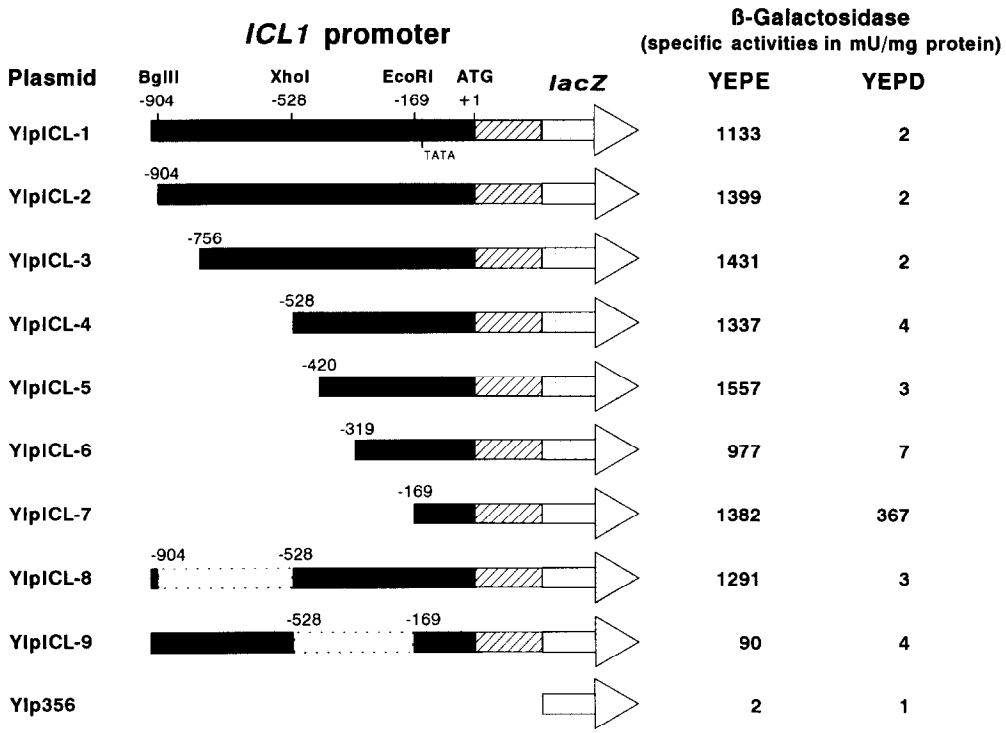
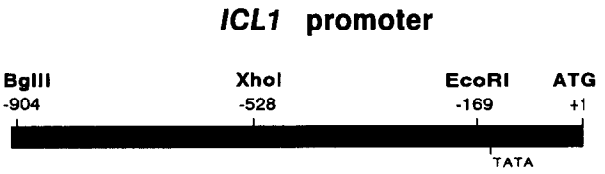


Fig. 2. Effect of promoter deletions on reporter gene transcription. A fusion of the *ICL1* 5'-noncoding sequence to the bacterial *lacZ* reporter gene was constructed and used as a basis for deletion analysis as described in section 2. Dark bars indicate the promoter fragments still present in the deletion constructs and numbers above each bar refer to the last bp retained. Cells were grown on the media indicated to late logarithmic phase prior to the preparation of crude extracts.

(YIpICL-2 to YIpICL-7), only the one lacking promoter sequences upstream of −319 (YIpICL-6) showed a slight reduction in expression when grown on ethanol medium. Therefore, one cannot directly deduce the location of regulatory elements from this series. In internal deletions, removing the DNA fragment from −904 to −528 (YIpICL-8) does not affect promoter activity. In agreement with other data [6] no regulatory functions seem to be located within this region. However, deleting an internal promoter fragment from −528 to −169 (YIpICL-9) led to a more than 10-fold reduction in β-galactosidase activities indicating that an important *UAS* function lies within this region. The activity still observed in the latter construct (YIpICL-9) suggests the presence of a second activating function exerted from sequences located between −169 and +171 (the fragment from −904 to −528 also being retained in this construct does not contain any activating sequences as discussed above). The fact that YIpICL-7, only containing the DNA fragment from −169 to +171, still shows β-galactosidase activity partially repressed by glucose also supports this idea. The high levels of expression found in YIpICL-7 as compared with YIpICL-9 may be due to transcriptional activation caused by flanking vector sequences. Such an activating effect was previously observed for fusions of the yeast phosphoglycerate mutase promoter in analogous expression vectors [21].

The data described above suggest that a major positive *cis*-acting element of the *ICL1* promoter is located between −528 and −169 relative to the ATG translation start codon. Recently, this region has also been identified as important for regulation of *ICL1* expression by others [6]. Therefore, we further investigated these sequences by subcloning the entire fragment as well as parts of it into the heterologous *CYC1* promoter (Fig. 3; see section 2 for details). The complete fragment (pLGICL-1) led to β-galactosidase expression when ethanol was used as a carbon source, but not on glucose media. Of the three subfragments tested only the one containing the region from −528 to −295 (pLGICL-2) conferred expression to the reporter gene in ethanol-grown cells. Moreover, the levels of β-galactosidase obtained for the former construct (pLGICL-2) were about 10-fold higher than the ones corresponding to the complete fragment (−528/−164; pLGICL-1). Thus, an *URS* function has to be located between −295 and −164. As neither pLGICL-3 nor pLGICL-4 conferred expression to the reporter gene, an *UAS* element must be located between −528 and −386. Furthermore, additional *UAS* functions could be located in the fragments contained in the latter plasmids (pLGICL-3 and pLGICL-4) that are repressed by the *URS* element. The presence of multiple *UAS* elements and their interaction with the *URS* element could explain why deletions of most of the up-



		ß-Galactosidase (specific activities in mU/mg protein)		
		CAT3		*cat3*
		YEPE	YEPD	YEPE
pLGICL-1	-528 -169	52	1	14
pLGICL-2	-528 -295	593	2	39
pLGICL-3	-331 -169	12	1	nd
pLGICL-4	-389 -247	15	1	nd
pLGi	CYC1 UAS1/UAS2	53	4	nd
pLGi-Δ	without insertion	7	1	nd

Fig. 3. Effect of *ICL1* promoter fragments on transcription in the heterologous *CYC1* promoter. Fragments substituting the UAS sequences of the *CYC1* promoter were obtained as described in section 2. Dark bars indicate the promoter fragment tested with the numbers above giving their endpoints in relation to the *ICL1* translation start codon. AMW-13C$^+$-integrants were tested after grown on ethanol (YEPE) or glucose (YEPD) media. JS87.15-1B (*cat3*)-integrants were grown on YEPD for 12 h and transferred to YEPE for 6 h to allow for induction. nd = not determined.

stream promoter sequences (Fig. 2) did not result in significant decreases in expression (e.g. the *UAS* element located between −169 and +171 could counteract the negative effect of the *URS* function).

From the data discussed above, the strongest activating function seems to be located between −528 and −295. This region contains two sequences (5′-TGTTCCCTTTTGCCCCAGG3-′ at −420/−401 and 5′-GGGTTTTGCTACTCGTCAT3-′ at −453/−434) that we proposed earlier to function in the transcriptional control of genes involved in ethanol metabolism [3]. They are quite well conserved in the promoters of the genes encoding alcohol dehydrogenase II, fructose- 1,6-bisphosphatase and the malate synthase isoenzyme functional in the glyoxylate pathway. Recently, the promoter of the fructose-1,6-bisphosphatase gene has been studied in detail [22,23]. Both groups reported on the presence of an *UAS* element (around −443/−416), that does not coincide with the sequences mentioned but shows a weak homology with the region −483/−457 in the *ICL1* promoter. Moreover, a second *UAS* element located further upstream (−506/−477) in the fructose-1,6-bisphosphatase promoter partially overlaps with the sequence −420/−401 from *ICL1* [23].

3.3. *Influence of* cat1/cat3 *mutants on* ICL1 *gene expression*

CAT1 and *CAT3* encode a protein kinase and a subunit necessary for its activity, respectively [24,25]. The complex is needed for derepression of certain genes, including isocitrate lyase, after a shift of cells to non-fermentable carbon sources. Therefore, we examined RNA prepared from *cat1* as well as from *cat3* mutants for the presence of the *ICL1* transcript by Northern blot analysis. No signals were detected for either mutant (Fig. 4). In addition plasmids pLGICL-1 and pLGICL-

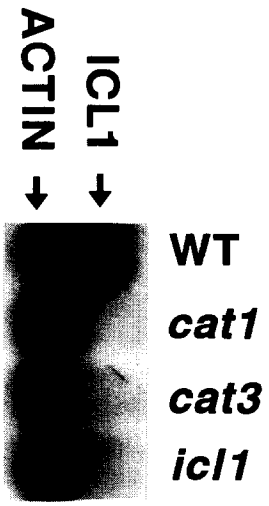


Fig. 4. Expression of the *ICL1* gene in *cat1* and *cat3* deletion mutants. Total RNA was prepared from cells of the strains 10.7-11A (wt), JS87.11-1A (*cat1*), JS87.15-1B (*cat3*) and icl1d (*icl1*) pregrown on YEPD and transferred to YEPE for 6 h prior to preparation.

2 were integrated into the genome of a strain containing a *cat3* deletion. A drastic reduction of β-galactosidase activity was observed for both constructs on ethanol media (Fig. 3). This result demonstrates that Cat1p/Cat3p acts on sequences contained within the region of the *ICL1* promoter carrying the *UAS* element between −528 and −295.

The data presented above show that expression of the yeast *ICL1* gene is subject to a tight transcriptional control depending on the carbon source used for growth. Transcription seems to be controlled mainly by two sequence elements (a *UAS* and a *URS* element) that both reside within a 365 bp fragment defined by our studies. General control mechanisms as the one exerted by the Cat1p/Cat3p-complex also act on *ICL1*-transcription via this promoter region. Investigations to identify DNA/protein interactions within these sequences are in progress.

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REFERENCES

[1] Vanni, P., Giachetti, E., Pinzauti, G. and McFadden, B.A. (1990) Comp. Biochem. Physiol. 95B, 431–458.
[2] Lopez-Boado, Y., Herrero, P., Fernández, M.T., Fernández, R. and Moreno, F. (1988) Yeast 4, 41–46.
[3] Fernández, E., Moreno, F. and Rodicio, R. (1992) Eur. J. Biochem. 204, 983–990.
[4] Polakis, E.S., Bartley, W. and Meek, G.A. (1965) Biochem. J. 97, 298–302.
[5] Herrero, P., Fernández, R. and Moreno, F. (1985) Arch. Microbiol. 143, 216–219.
[6] Schöler, A. and Schüller, H.-J. (1993) Curr. Genet. 23, 375–381.
[7] Zimmermann, F. K., Kaufmann, I., Rasenberger, H. and Hausmann, P. (1977) Mol. Gen. Genet. 151, 95–103.
[8] Ciriacy, M. (1977) Mol. Gen. Genet. 154, 213–220.
[9] Gancedo, J.M. (1992) Eur. J. Biochem. 206, 297–313.
[10] Rodicio, R. and Zimmermann, F.K. (1985) Curr. Genet. 9, 539–545.
[11] Miller, J.H. (1972) Experiments in Molecular Genetics, p. 403, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[12] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[13] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[14] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[15] Myers, A.M., Tzagoloff, A., Kinney, D.M. and Lusty, C.J. (1986) Gene 45, 299–310.
[16] Guarente, L. (1983) Methods Enzymol. 101, 181–191.
[17] Hartig, A., Simon, M.M., Schuster, T., Daugherty, J.R., Yoo, H.S. and Cooper, T.G. (1992) Nucleic Acids Res. 20, 5677–5686.
[18] Fernández, E., Fernández, M. and Rodicio, R. (1993) FEBS Lett. 320, 271–275.
[19] Zitomer, R.S. and Nichols, D.L. (1978) J. Bacteriol. 135, 39–44.
[20] Lombardo, A., Cereghino, G.P. and Scheffler, I.E. (1992) Mol. Cell. Biol. 12, 2941–2948.
[21] Rodicio, R., Heinisch, J.J. and Hollenberg, C.P. (1993) Gene 125, 125–133.
[22] Mercado, J.J. and Gancedo, J.M. (1992) FEBS Lett. 311, 110–114.
[23] Niederacher, D., Schüller, H.J., Grzesitza, D., Gütlich, H., Hauser, H.P., Wagner, T. and Entian, K.D. (1992) Curr. Genet. 22, 363–370.
[24] Celenza, J.L. and Carlson, M. (1986) Science 233, 1175–1180.
[25] Celenza, J.L., Eng, F.J. and Carlson, M. (1989) Mol. Cell. Biol. 9, 5045–5054.