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A Carbon Source-Responsive Promoter Element Necessary for Activation of the Isocitrate Lyase Gene *ICL1* Is Common to Genes of the Gluconeogenic Pathway in the Yeast *Saccharomyces cerevisiae*

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The expression of yeast genes encoding gluconeogenic enzymes depends strictly on the carbon source available in the growth medium. We have characterized the control region of the isocitrate lyase gene *ICL1*, which is derepressed more than 200-fold after transfer of cells from fermentative to nonfermentative growth conditions. Deletion analysis of the *ICL1* promoter led to the identification of an upstream activating sequence element, UAS_{ICL1} (5′ CATTCATCCG 3′), necessary and sufficient for conferring carbon source-dependent regulation on a heterologous reporter gene. Similar sequence motifs were also found in the upstream regions of coregulated genes involved in gluconeogenesis. This carbon source-responsive element (CSRE) interacts with a protein factor, designated Ang1 (activator of nonfermentative growth), detectable only in extracts derived from derepressed cells. Gene activation mediated by the CSRE requires the positively acting derepression genes *CAT1* (=*SNF1* and *CCR1*) and *CAT3* (=*SNF4*). In the respective mutants, Ang1-CSRE interaction was no longer observed under repressing or derepressing conditions. Since binding of Ang1 factor to the CSRE could be competed for by an upstream sequence derived from the fructose-1,6-bisphosphatase gene *FBP1*, we propose that the CSRE functions as a UAS element common to genes of the gluconeogenic pathway.

The utilization of various carbon sources by the yeast *Saccharomyces cerevisiae* requires a complex pattern of gene regulation. In general, genes necessary for the utilization of a certain carbon source are repressed when a more favorable substrate becomes available. Since fermentable substrates such as glucose or fructose are metabolized more easily than alternative sugars (sucrose, maltose, or galactose) or nonfermentable compounds (glycerol, lactate, ethanol, or acetate), a hierarchy of successively used carbon sources can be observed. The glucose repression system (reviewed in references 20, 26, and 63) affects enzymes of mono- and disaccharide metabolism (encoded by *SUC*, *MAL*, or *GAL* genes), respiration (e.g., cytochromes), peroxisomal function (e.g., enzymes of β oxidation), and gluconeogenesis (e.g., isocitrate lyase, fructose-1,6-bisphosphatase) as well as additional enzymes involved in the utilization of nonfermentable carbon sources (e.g., alcohol dehydrogenase II and acetyl coenzyme A synthetase). This regulation by the carbon source of the growth medium is exerted mainly at the level of transcription (e.g., for *CYC1* [70], *SUC2* [3], *GAL1* and *GAL10* [59], *FBP1* [56], and *ICL1* [51]), although the control of mRNA turnover may act synergistically in some cases (described for the $SDHI_P$ gene encoding the iron-protein subunit of the succinate dehydrogenase complex [33]).

cis-acting control elements mediating the activation of carbon source-regulated genes have been described for *SUC* genes (24, 49), *MAL* genes (32, 43), *GAL* genes (reviewed in reference 25), respiratory genes (19), the glucose-repressible alcohol dehydrogenase gene *ADH2* (12, 68), and genes of β oxidation and peroxisomal biogenesis (10, 11, 17). Pathway-specific DNA-binding proteins (e.g., Gal4p, Mal63p, Hap2p/Hap3p/Hap4p, and Adr1p) interact with these UAS (upstream activation site) elements, thus leading to the expression of the respective structural genes under appropriate conditions of glucose derepression and/or substrate induction.

In addition to transcription factors acting specifically on a subset of carbon source-controlled genes, several pleiotropic regulators have been identified. Regulatory genes *HEX1* (=*HXK2*; encodes hexokinase isoenzyme PII [13, 47]), *HEX2* (=*REG1* and *SRN1* [14, 34, 44, 64]), *CAT80* (=*GRR1* [2, 14, 18]), *CID1* (=*GLC7*; encodes a protein phosphatase; cited in references 26 and 42), and *MIG1* (=*CAT4*; encodes a zinc finger-containing repressor protein that binds to the *SUC2* promoter and to the *GAL1-GAL10* intergenic region [38, 40, 54]) are necessary for glucose repression of *SUC*, *MAL*, *GAL*, and *CYC1* genes but do not affect genes necessary for the utilization of nonfermentable substrates such as *ADH2*, *ICL1*, and *FBP1* (the exception is that a *hex2* mutation leads to a partial derepression of *ADH2* even on glucose medium [9]). *CYC8/SSN6* encodes a nuclear phosphoprotein containing 10 copies of the tetratricopeptide repeat motif (57) that appear to be functionally important. Cyc8p is physically associated with the *TUP1* gene product (for allelic gene designations, see reference 26) that acts on numerous cellular processes such as glucose repression, flocculation, mating, sporulation, and minichromosome maintenance (31, 37, 50, 60). The Tup1p-Cyc8p complex functions as a general negative regulator presumably facilitating the action of various DNA-binding repressor proteins (27).

In contrast to mutations leading to glucose-insensitive gene

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TABLE 1. Strains of *S. cerevisiae* used in this work

Strain	Genotype	Reference or source
JS89.27-3	**a** *ura3 leu2 trp1 can1 MAL3 SUC3*	51
JS87.11-17C	α *ura3 leu2 cat1::HIS3 MAL3 SUC3*	54
JS87.15-7D	**a** *ura3 trp1 cat3::LEU2 MAL3 SUC3*	53
JS92.23-1	**a** *ura3 leu2 trp1 can1 mig1-Δ2::LEU2 MAL3 SUC3*	51
JS92.24-1	**a** *ura3 leu2 trp1 can1 ICL1::ICL1-lacZ::TRP1 MAL3 SUC3*	51
JS92.25-1	**a** *ura3 leu2 trp1 can1 ICL1::ICL1-lacZ::TRP1 mig1-Δ2::LEU2 MAL3 SUC3*	This work
JS92.36-1	α *ura3 his3 can1 ICL1::ICL1-lacZ::TRP1 cat1::HIS3 mig1-Δ2::LEU2 MAL3 SUC3*	This work
JS92.37-1	**a** *ura3 leu2 his3 can1 ICL1::ICL1-lacZ::TRP1 cat1::HIS3 MAL3 SUC3*	This work
JS92.39-1	**a** *ura3 his3 trp1 cat1::HIS3 mig1-Δ2::LEU2 MAL3 SUC3*	51
JS93.10-1	α *ura3 leu2 his3 can1 ICL1::ICL1-lacZ::TRP1 cat3::LEU2*	This work
JS93.17-4	α *ura3 trp1 cyc8-Δ1::LEU2 MAL3 SUC3*	This work
WAY.6-2B	α *ura3 hxk2::LEU2 MAL3 SUC3*	47
GA1-8C	**a** *ura3 leu2 his3 trp1 ctt1*	58
GA1-8Cadr	**a** *ura3 leu2 his3 trp1 ctt1 adr1::HIS3*	58
YHH45	α *ura3 leu2 his3 pra1::URA3*	D. Wolf
ASH4	α *ura3 leu2 his3 pra1::URA3 cat1::HIS3*	This work
ASH5	α *ura3 leu2 his3 pra1::URA3 cat3::LEU2*	This work
ASH7	**a** *ura3::ΔUAS-FAS1-lacZ::URA3 leu2 trp1 can1 MAL3 SUC3*	This work
ASH8	**a** *ura3::CSRE-FAS1-lacZ::URA3 leu2 trp1 can1 MAL3 SUC3*	This work

expression, derepression mutants are constitutively repressed and, consequently, unable to activate structural genes necessary for utilization of alternative carbon sources. The gene products of *CAT1/SNF1/CCR1/HAF3* and *CAT3/SNF4* encode components of a protein kinase complex mediating the release of a large number of genes from glucose repression (4, 6, 7, 15, 28, 41, 52, 69). Thus, the respective mutants show pleiotropic growth defects on media containing sucrose, maltose, galactose, glycerol, or ethanol. Regulators *ADR6/SWI1*, *SNF2/SWI2/GAM1/TYE3*, *SWI3*, *SNF5/TYE4*, and *SNF6* act in a more general manner to overcome chromatin repression affecting a diverse set of highly regulated genes (8, 30, 46, 65, 67).

Enzymes of the glyoxylate cycle and of the gluconeogenic pathway are necessary for growth of yeast cells on C_2 or C_3 substrates. The respective structural genes are mainly controlled by the positively acting derepression genes. Even a strong selection system failed to identify mutants showing constitutive expression of gluconeogenic enzymes (35). Although both the fructose bisphosphatase gene *FBP1* and the isocitrate lyase gene *ICL1* contain a presumable Mig1p/Cat4p repressor binding site in their respective upstream regions, glucose repression of these genes remains unaffected in a Δ*mig1* null mutant (36, 51). *cis*-acting sequence motifs mediating the positive control of genes of the gluconeogenic pathway have not yet been identified. We thus used the previously cloned isocitrate lyase gene *ICL1* (16, 51) for a deletion analysis of the respective promoter region. In this work, we describe the identification and characterization of a UAS element (carbon source-responsive element [CSRE]) necessary and sufficient for carbon source-dependent control of gluconeogenic genes.

MATERIALS AND METHODS

Yeast strains and media. Strains of *S. cerevisiae* used in this work are listed in Table 1. Auxotrophic and regulatory gene symbols have been explained previously (52, 54). Mutations *hxk2* (47), *mig1* (40, 54), and *cyc8* (62) lead to constitutive expression of invertase and maltase. Strains carrying a *ctt1* mutation are defective for catalase T. The *adr1* mutants lack a functional alcohol dehydrogenase regulator (58). The vacuolar proteinase yscA is absent in *pra1* strains (1). The compositions of yeast rich medium (YEP based) and synthetic complete (SC) medium have been described elsewhere (52). For growth of transformants under selective conditions, an appropriate omission medium was used. To ensure glucose repression of reporter genes, cells were grown in SC medium in the presence of 2% glucose (SCD_2). Conversely, derepression conditions were achieved by growth in SC medium plus 0.2% glucose and 3% ethanol ($SCD_{0.2}E_3$).

Oligonucleotides. Oligonucleotides used for insertion studies, gel retardation experiments, and site-directed mutagenesis were purchased from MWG Biotech (Ebersberg, Germany). To obtain synthetic double-stranded DNA fragments, single-stranded oligonucleotides of reverse complementary sequence were mixed in equimolar amounts. The following synthetic DNA fragments were used (nucleotides corresponding to an original upstream sequence are shown in capital letters):

OAS12 (*ICL1* upstream region, positions −402 to −383), 5′ tcgaGTTTCCATTCATCCGAGCGAgatct 3′
3′ CAAAGGTAAGTAGGCTCGCTctagaagct 5′

OAS34 (*MLS1* upstream region, positions −411 to −425), 5′ tcgaagatctAAAATTTATCCGAAC 3′
3′ tctagaTTTTAAATAGGCTTGagct 5′

OAS56 (*FBP1* upstream region, positions −489 to −504), 5′ tcgaagatctGCGATTCCATCCGTCC 3′
3′ tctagaCGCTTAAGGTAGGCAGGagct 5′

OAS78 (*FBP1* upstream region, positions −495 to −507), 5′ tcgaagatctCCATCCGTCCGGAc 3′
3′ tctagaGGTAGGCAGGCCTgagct 5′

Recombinant DNA methods. Restriction enzymes were handled as recommended by the manufacturer. Cloning procedures and additional recombinant DNA techniques were performed according to established protocols (48).

Deletion analysis of the *ICL1* promoter. The *ICL1-lacZ* fusion construct pJS310 carrying about 2.1 kb of the *ICL1* upstream sequence in an episomal *URA3* vector has been described elsewhere (51). A single *Xba*I restriction site at position −1180 (with respect to the translational start codon) was removed by a fill-in reaction to give plasmid pAS23. This construct was linearized at the unique *Xho*I site (−527) and subsequently digested with exonuclease *Bal* 31 for various times. After gel purification of the shortened linear plasmids, phosphorylated *Xba*I linkers were ligated to the ends thus generated. The positions of linker insertions were determined by DNA sequencing. Sequences upstream of position −527 were reintroduced by transfer of a 1.6-kb *Hin*dIII-*Xba*I fragment from plasmid pAS25 (which contains an *Xba*I site at −527) into the shortened constructs obtained. The resulting plasmids vary only with respect to the extent of deletion downstream of position −527.

Plasmid constructions. To obtain a deletion construct lacking the complete upstream activation region of the *ICL1* gene (−527/−164), a 0.8-kb *Eco*RI-*Bam*HI fragment from pJS310 was inserted into pBluescript II KS(+) (Stratagene, Heidelberg, Germany). Subsequently, a 0.8-kb *Xho*I-*Bam*HI fragment from the resulting plasmid pAS26 was reinserted into *Xho*I-*Bam*HI-cleaved pJS310, leading to the upstream deletion construct pAS27 devoid of the *Xho*I-*Eco*RI fragment that was able to confer *ICL1*-specific gene control on a heterologous promoter (51). For insertion of synthetic double-stranded DNA (OAS12, OAS34, OAS56, and OAS78; see above) into a UAS-less promoter test construct, plasmid pJS286 (ΔUAS-*FAS1-lacZ URA3* 2μm [51]) was used. Plasmid pJS286 was linearized at the single *Xho*I site (159 bp upstream of the start codon of the *FAS1-lacZ* fusion gene) and religated together with 5′-phosphorylated synthetic DNA fragments. Orientation and copy number of oligonucleotide inserts were determined by DNA sequencing. Similarly, OAS12 was also inserted into the *Xho*I sites of plasmids pAS27 (ΔUAS-*ICL1-lacZ*; see above) and pAS39 (*ICL1*[$CSRE_{Mut}$]-*lacZ*; see below), giving plasmids pAS40 and pAS52, respectively. Episomal plasmids pJS286 and pAS35R (contains a single OAS12 oligonucleotide insertion within the pJS286 context) were converted into the respective integrative plasmids pAS42 (ΔUAS-*FAS1-lacZ*) and pAS43R ([$OAS12]_R$-*FAS1-lacZ*) by transfer of 4.7-kb *Hin*dIII-*Nco*I fragments containing the reporter gene cassettes into YIp352 (23), cleaved by the appropriate enzymes. The *ADH2-lacZ* fusion plasmid pJS154 (*ADH2-lacZ URA3* 2μm) was derived from plasmid pHH70 (kindly provided by C. Denis, Durham, N.H.). It contains about 1 kb of the *ADH2* upstream region within a plasmid backbone similar to pJS310.

Site-directed mutagenesis. To alter specifically the core sequence of the UAS_{ICL1} motif (CSRE), the Kunkel strategy for site-directed mutagenesis was used (29). A 2.8-kb *Xba*I-*Bam*HI fragment containing the *ICL1* control region together with the N-terminal 205 codons was subcloned into double-stranded phage M13mp19, giving M13/AS36. After transfection into the *dut ung Escherichia coli* host RZ1032 (29), U nucleotide-containing single-stranded template DNA was prepared. Following annealing of the mutagenic primer Mut1 (5′ GATAAGTGATCGCGTAAGCTTCTGGAAACCTGGG 3′), in vitro elongation of the primer was achieved by using Sequenase 2.0 (U.S. Biochemical). The ligated double-stranded heteroduplex was subsequently transfected into the dut^+ ung^+ host strain BMH71-18 (48) necessary for selection against wild-type phages. Constructs containing the desired mutation (M13/AS37) were confirmed by DNA sequencing and subsequently used for reverse transfer of the mutated 2.8-kb *Xba*I-*Bam*HI fragment into the pJS310 context, resulting finally in plasmid pAS39 (*ICL1*[$CSRE_{Mut}$]-*lacZ*). Plasmid pAS39 is identical to the *ICL1-lacZ* wild-type fusion with the exception of 9 bp altered in the core of UAS_{ICL1} (underlined):

wild type, 5′ GTTTCCATTCATCCGAGCGATC 3′
mutated UAS_{ICL1}, 5′ GTTTCCAGAAGCTTACGCGATC 3′

Yeast transformation. Yeast transformation was performed as previously described (55). To direct integrative constructs to the *URA3* locus, plasmids were linearized at the unique *Nco*I restriction site prior to transformation. For the construction of a *mig1-Δ2::LEU2* null allele, plasmid pJN22 (40) (kindly provided by Hans Ronne, Uppsala, Sweden) was used. Similarly, plasmid pDSB (62) (kindly provided by Robert Trumbly, Toledo, Ohio) containing a *cyc8-Δ1::LEU2* disruption construct was used to obtain the respective null mutant.

β-Galactosidase assay. β-Galactosidase specific activities in yeast transformants were determined as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute per milligram of protein as described elsewhere (55). Data presented are mean values from at least two transformation experiments, each assaying eight independent transformants. Standard deviation was below 25% of the respective mean value.

Electrophoretic mobility shift assay (EMSA). Gel retardation experiments were carried out as described previously (55), with minor modifications. Strains defective for the vacuolar proteinase yscA (YHH45 or derivatives) were grown under repressing (SCD_2 medium) or derepressing ($SCD_{0.2}E_3$ medium) conditions until late log phase (about 5×10^7 cells per ml). Protein extracts used for the binding reaction represented total cellular protein of the 10 to 70% $(NH_4)_2SO_4$ fraction. Routinely, synthetic DNA fragments (see above) were end labelled by a fill-in reaction using [^{32}P]dATP. The labelled probe (5,000 cpm; about 0.5 ng) was incubated with 30 μg of total cellular protein and subsequently separated on a 4% native polyacrylamide gel.

RESULTS

Identification of a CSRE in the *ICL1* control region. We have previously identified a 364-bp restriction fragment derived from the *ICL1* promoter (−527/−164; all positions are given with respect to the translational start) that was able to confer the regulatory properties of the isocitrate lyase gene on a heterologous reporter gene (51). For a more precise localization of sequence elements responsible for the carbon source-dependent regulation of *ICL1*, we performed a deletion analysis of the upstream region of a *ICL1-lacZ* fusion gene. Deletion of sequences up to −398 did not lead to a significant alteration of repressed or derepressed β-galactosidase activities measured in the respective transformants (not shown). However, a dramatic drop in reporter gene expression under derepressing conditions was observed after removing the 10 bp between −398 and −388 (Fig. 1). No deletion constructs leading to an increased reporter gene activity were obtained, arguing for the absence of repression sites 5′ to the positively acting *cis* element.

Insertion of the synthetic oligonucleotide OAS12 corresponding to the UAS_{ICL1} interval −403/−383 into a UAS-less reporter plasmid (pJS286; ΔUAS-*FAS1-lacZ* [51]) led to an orientation-independent stimulation of basal gene expression by a factor of about 40 (Table 2). Insertion of multiple copies

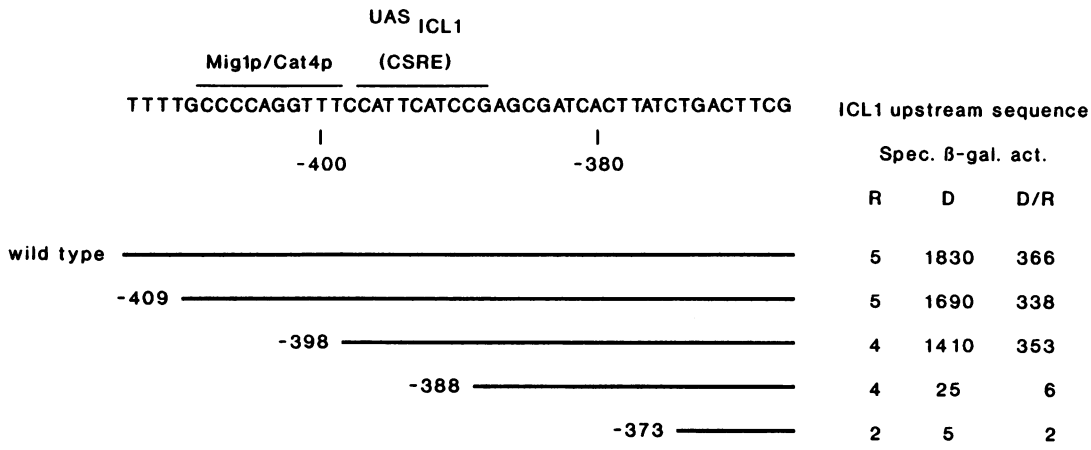

FIG. 1. Deletion analysis of the *ICL1* upstream region. Deletion constructs presented were derived from plasmid pAS23 (*ICL1-lacZ URA3* 2μm) by exonuclease digestion, starting from the *Xho*I site at −527. Nucleotide positions refer to the translational start codon. Deletion constructs were transformed into the wild-type strain JS89.27-3. β-Galactosidase specific activities are given in nanomoles of ONPG converted per minute per milligram of protein. R, repressed conditions (growth in SCD_2 lacking uracil); D, derepressed conditions (growth in $SCD_{0.2}E_3$ lacking uracil); D/R, derepression factor.

of UAS_{ICL1} into the test plasmid further enhanced the reporter gene activity, but the influence was less than additive (plasmids pAS35RN and pAS35NNRR in Table 2). Similar results were obtained with either episomal plasmids or integrative constructs targeted to the *URA3* locus (plasmid pAS43R in Table 2). Maximum activation could be observed with transformants grown under derepressing conditions (limiting amounts of a fermentable sugar, e.g., 0.2% glucose, or the presence of a nonfermentative carbon source), while substrates easily utilized by fermentation did not cause any stimulation of basal gene expression (Fig. 2). An intermediary effect was obtained upon growth with raffinose, a fermentative substrate leading to a limited intracellular sugar supply to the cells as a result of its slow cleavage by external invertase. This result is in support of the view considering carbon source-regulated gene expression linked to the metabolic turnover of sugar substrates. No induction of UAS_{ICL1}-dependent reporter gene activity above the derepressed level was found in the presence of oleic acid, indicating that it is distinct from the previously characterized β-oxidation box (10, 11, 17). In contrast, a four- to fivefold induction by oleic acid with respect to derepression

TABLE 2. Insertion of CSRE-related oligonucleotides into a ΔUAS test plasmid

Plasmid[a]	Construct[b]	β-Galactosidase sp act (nmol of ONPG hydrolyzed/min/mg of protein)	
		R[c] (activation factor[d])	D[e] (activation factor)
pJS286	ΔUAS-*FAS1-lacZ*	75 (1)	40 (1)
pAS35N	$OAS12_N$-*FAS1-lacZ*	90 (1.2)	1,720 (43)
pAS35R	$OAS12_R$-*FAS1-lacZ*	95 (1.3)	1,600 (40)
pAS35RN	$OAS12_R$-$OAS12_N$-*FAS1-lacZ*	110 (1.5)	1,990 (50)
pAS35NNRR	$OAS12_{NN}$-$OAS12_{RR}$-*FAS1-lacZ*	150 (2)	2,590 (65)
pAS38R	$OAS34_R$-*FAS1-lacZ*	85 (1.1)	30 (0.8)
pAS53R	$OAS56_R$-*FAS1-lacZ*	70 (0.9)	45 (1.1)
pAS54R	$OAS78_R$-*FAS1-lacZ*	90 (1.2)	1,560 (39)
pAS42[f]	ΔUAS-*FAS1-lacZ*	26 (1)	9 (1)
pAS43R[f]	$OAS12_R$-*FAS1-lacZ*	40 (1.5)	420 (47)

[a] Plasmids were transformed into wild type-strain JS89.27-3.

[b] Subscript N or R indicates normal or reverse orientation of the oligonucleotide insert. Origins of upstream sequences: OAS12, *ICL1* (−402/−383); OAS34, *MLS1* (−411/−425); OAS56, *FBP1* (−489/−504); OAS78, *FBP1* (−495/−507).

[c] R, repressed growth conditions of wild-type transformants (SCD_2 lacking uracil).

[d] Ratio of β-galactosidase specific activities in transformants with respect to the ΔUAS reference (pJS286 or pAS42).

[e] D, derepressed growth conditions of wild-type transformants ($SCD_{0.2}E_3$ lacking uracil).

[f] Integrative plasmid targeted to the *URA3* locus of wild-type strain JS89.27-3. The resulting strains were designated ASH7 (transformed with pAS42) and ASH8 (transformed with pAS43R).

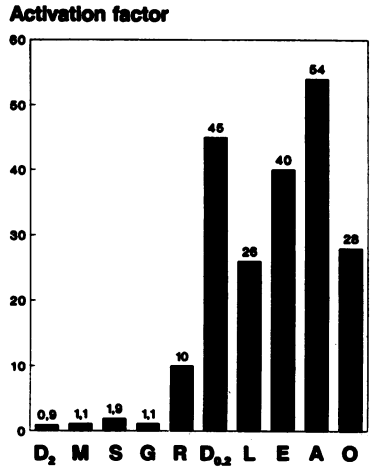

FIG. 2. Carbon source-dependent reporter gene activation mediated by UAS_{ICL1}. Transformants of the wild-type strain JS89.27-3 were grown in synthetic complete media under selective conditions (lacking uracil). Carbon sources added: D_2, 2% glucose; M, 2% maltose; S, 2% sucrose; G, 2% galactose; R, 2% raffinose; $D_{0.2}$, 0.2% glucose; L, 0.2% glucose plus 2% lactate; E, 0.2% glucose plus 3% ethanol; A, 0.2% glucose plus 1% acetate; O, 0.2% glucose plus 0.2% oleic acid. The activation factor was calculated as the ratio of β-galactosidase specific activities in transformants of pAS35N (UAS_{ICL1}-*FAS1-lacZ*) and pJS286 (ΔUAS-*FAS1-lacZ*).

TABLE 3. Site-directed mutagenesis of the CSRE in the *ICL1* upstream region

Plasmid[a]	Construct	β-Galactosidase sp act (nmol of ONPG hydrolyzed/min/mg of protein)	
		R[b]	D[c]
pJS310	*ICL1-lacZ*	5	1,830
pAS39	*ICL1*($CSRE_{Mut}$)-*lacZ*	1	1
pAS27	ΔUAS(−527/−164)-*ICL1-lacZ*	1	2
pAS40[d]	CSRE-ΔUAS(−527/−164)-*ICL1-lacZ*	2	1,110
pAS52[d]	CSRE-*ICL1*($CSRE_{Mut}$)-*lacZ*	2	33

[a] Plasmids were transformed into wild-type strain JS89.27-3.
[b] R, repressed growth conditions of wild-type transformants (SCD_2 lacking uracil).
[c] D, derepressed growth conditions of wild-type transformants ($SCD_{0.2}E_3$ lacking uracil).
[d] For reinsertion of a CSRE sequence, oligonucleotide OAS12 (UAS_{ICL1}[−402/−383]) was used.

by ethanol was observed in cells transformed with a reporter gene put under the control of the peroxisomal thiolase gene *POT1/FOX3* (data not shown). Since gene activation mediated by UAS_{ICL1} strictly depends on the nature of the carbon source available in the growth medium, the motif is characterized as a CSRE. Site-directed mutagenesis of the CSRE in the *ICL1* upstream region resulted in a complete loss of gene activation (plasmid pAS39 in Table 3), indicating that all stimulatory influence of the promoter acts via the CSRE. Reinsertion of the CSRE-containing oligonucleotide OAS12 into a ΔUAS(−527/−164)-*ICL1-lacZ* deletion construct (plasmid pAS27) restored β-galactosidase activity under derepressing conditions to about 60% of the wild-type level (plasmid pAS40 in Table 3). In a similar experiment, plasmid pAS39 containing the full-length *ICL1* promoter with a mutated CSRE (*ICL1*[$CSRE_{Mut}$]-*lacZ*) was used for insertion of oligonucleotide OAS12 at position −527, about 140 bp further upstream than its natural position (pAS52; CSRE-*ICL1* [$CSRE_{Mut}$]-*lacZ*). Interestingly, such transformants exhibited less than 2% of derepressed wild-type activity, indicating that the relative arrangement of regulatory important elements within the *ICL1* control region significantly influences its stimulatory properties.

CSRE-like sequence motifs upstream of additional genes of the gluconeogenic pathway. Since genes specifically necessary for gluconeogenesis become coordinately derepressed under nonfermentative conditions, we looked for CSRE-related motifs in the respective upstream regions by a comparative data base search. Similar elements were identified in the promoters of the fructose-1,6-bisphosphatase gene *FBP1*, the malate synthase gene *MLS1*, the phosphoenolpyruvate carboxykinase gene *PCK1*, and the peroxisomal citrate synthase gene *CIT2* (Table 4). Two sequence motifs of the *FBP1* promoter and one element of the *PCK1* promoter map to regions that have been shown previously to be critical for transcriptional activation of these genes (36, 45). A sequence element distantly related to the CSRE from the *ICL1* promoter was also found upstream of the glucose-repressible alcohol dehydrogenase gene *ADH2* (Table 4). However, the significance of this motif for *ADH2* regulation remains questionable since its position does not coincide with previously identified controlling elements (68). Glucose-repressible genes involved in alternative routes of sugar fermentation (such as *SUC*, *MAL*, and *GAL* genes) lack CSRE-related elements. This result is not unexpected, since CSRE-dependent gene activation could not be observed under various fermentative conditions (Fig. 2). The functional importance of CSRE-like sequences in the upstream regions of permease genes *GAL2* (encoding galactose permease) and *HXT1* (encoding a hexose transporter) is at present unclear. Table 4 also lists some sequences closely related to the CSRE found upstream of genes not particularly known to be subject to regulation by carbon source.

TABLE 4. Compilation of sequence motifs similar to the CSRE[a]

Type of sequence motif	Gene	Function	Sequence (5′→3′)	Position
Closely related to CSRE	*ICL1*(A)	Isocitrate lyase	CATTCATCCG	−397/−388
	FBP1(A)	Fructose-1,6-bisphosphatase	CATCCGTCCG	−495/−504
	PCK1(A)	Phosphoenolpyruvate carboxykinase	CTTTCATCCG	−480/−471
	MLS1(A)	Malate synthase	CTTCTATCCG	−465/−474
	CIT2	Citrate synthase	CATTTATCCG	−380/−371
	HXT1	Hexose permease	CTTTTATCCG	−233/−224
	GAL2	Galactose permease	CTTTCGTCCG	−408/−399
	ILV2	Acetolactate synthase	CTTTCATCCG	−494/−503
	DAF1	Cell division	CATCCATCCG	−174/−165
	CLB3	Cyclin B	CATTTGTCCG	−152/−143
	BEM1	Cell polarity	CATTTGTCCG	−166/−175
	TIF1	CAP-binding protein	CATCCATCCG	−172/−181
	SRP1	Serine-rich protein	CATTTATCCG	−663/−672
	SLY2	Suppressor of *YPT1* function	CATCCATCCG	−568/−559
	Consensus		CATYCRTCCG	
Distantly related to CSRE	*ICL1*(B)	Isocitrate lyase	ATTTCATCCG	−355/−346
	ICL1(C)	Isocitrate lyase	TCGTCATCCG	−441/−432
	ICL1(D)	Isocitrate lyase	AAAGCATCCG	−462/−471
	FBP1(B)	Fructose-1,6-bisphosphatase	ATTCCATCCG	−491/−500
	PCK1(B)	Phosphoenolpyruvate carboxykinase	CCTTTATCCG	−353/−362
	MLS1(B)	Malate synthase	AATTTATCCG	−413/−422
	ADH2	Alcohol dehydrogenase II	CGTCTCTCCG	−476/−467

[a] A data base search was performed with the current releases of GenBank (release 77.0, June 1993) and EMBL (release 35.0, June 1993). The list of sequences distantly related to the CSRE contains only motifs present in the upstream regions of gluconeogenic genes.

TABLE 5. Influence of regulatory mutations on expression of an *ICL1-lacZ* reporter gene

Strain[a]	Genotype	β-Galactosidase sp act (nmol of ONPG hydrolyzed/min/mg of protein)	
		R[b]	D[c]
JS89.27-3	Wild type	5	1,830
JS87.11-17C	*cat1*	3	22
JS87.15-7D	*cat3*	2	65
JS92.23-1	*cat4*	6	1,780
JS92.39-1	*cat1 cat4*	3	280
GA1-8C	Wild type	10	2,630
GA1-8Cadr	*adr1*	12	2,480

[a] The episomal reporter plasmid pJS310 (*ICL1-lacZ URA3* 2μm) was used for transformation.
[b] R, repressed growth conditions (SCD_2 lacking uracil)
[c] D, derepressed growth conditions ($SCD_{0.2}E_3$ lacking uracil).

TABLE 6. CSRE-dependent gene activation in regulatory mutants

Strain	Genotype	β-Galactosidase sp act (nmol of ONPG hydrolyzed/min/mg of protein)			
		Repressed conditions[a]		Derepressed conditions[b]	
		pJS286[c]	pAS35N[d]	pJS286	pAS35N
JS89.27-3	Wild type	75	90	40	1,720
JS87.11-17C	*cat1*	100	110	30	50
JS87.15-7D	*cat3*	80	70	30	55
JS92.23-1	*cat4*	80	100	40	1,620
JS92.39-1	*cat1 cat4*	80	60	35	40
GA1-8Cadr	*adr1*	45	55	50	1,800
WAY.6-2B	*hxk2*	30	40	40	1,810
JS93.17-4	*cyc8*	25	25	20	420

[a] Growth in SCD_2 lacking uracil.
[b] Growth in $SCD_{0.2}E_3$ lacking uracil.
[c] ΔUAS-*FAS1-lacZ* reporter gene.
[d] CSRE-*FAS1-lacZ* reporter gene.

Not all sequences listed in Table 4 appear to function as activating elements. A deletion construct of the *ICL1-lacZ* reporter gene retaining motifs *ICL1*(B), *ICL1*(C), and *ICL1*(D) did not exhibit any significant derepression under nonfermentative conditions (Fig. 1; −388 deletion). Similarly, insertion of the oligonucleotide OAS34 containing motif *MLS1*(B) into the promoter test plasmid pJS286 did not lead to any activation above the basal expression level (plasmid pAS38R in Table 2). In contrast, insertion of motif *FBP1*(A) represented in oligonucleotide OAS78 into the same UAS-free test system caused a carbon source-dependent stimulation of β-galactosidase activity (plasmid pAS54R), while motif *FBP1*(B) turned out to be nonfunctional (OAS56, contained within plasmid pAS53R). The preliminary consensus sequence 5′ CATYCRTCCG 3′ for a functional CSRE may be derived from these comparisons. Since the functional CSRE of the *FBP1* gene present in OAS78 (5′ CATCCGTCCG 3′) is incomplete in OAS56, the essential character of the 3′-terminal G nucleotide missing in OAS56 is apparent from the comparison of these partially overlapping oligonucleotides.

Influence of *trans*-acting factors on CSRE-dependent gene activation. Among regulatory factors affecting carbon source utilization, the derepression genes *CAT1* (*SNF1*, *CCR1*, *HAF3*) and *CAT3* (*SNF4*) are necessary for the expression of most glucose-repressible structural genes. Derepression of an episomal *ICL1-lacZ* reporter gene has an absolute requirement for functional *CAT1* and *CAT3* genes (Table 5). Identical results were obtained by using integrative reporter plasmids targeted to the *ICL1* locus (data not shown). Interestingly, the failure of the *ICL1-lacZ* fusion gene to be derepressed in *cat1* mutants is partially overcome in a *cat1 cat4* double mutant. In addition to the influence of *CAT1* and *CAT3* on complex promoters, we subsequently investigated the effects of these regulatory genes on CSRE-controlled synthetic promoters. Expression of reporter genes in plasmids pJS286 (ΔUAS-*FAS1-lacZ*) and pAS35N (CSRE-*FAS1-lacZ*) was assayed in various regulatory mutants under repressing or derepressing conditions. As shown in Table 6, the CSRE-dependent gene activation is completely abolished in either *cat1* or *cat3* mutants, indicating a positive regulatory influence of the respective wild-type gene products on the CSRE. In contrast to the complex *ICL1* promoter, no epistatic effect is observed in the *cat1 cat4* double mutant. This finding argues for a *CAT4*-independent expression of the transcription factor mediating the CSRE-dependent gene activation. Similarly, the *ADR1* regulator involved in expression of the glucose-repressible *ADH2* gene, the catalase A gene *CTA1*, and the peroxisomal thiolase gene *FOX3/POT1* (10, 58) is not necessary for CSRE-controlled derepression of the reporter gene. With the *ADH2-lacZ* reporter plasmid pJS154 used as a control, β-galactosidase activity in the *adr1* mutant decreased to about 27% of the wild-type reference level (data not shown).

Besides positive regulators involved in the metabolic control of carbon source utilization, several negative factors necessary for repressed gene activities under appropriate conditions have been described. Mutations in the hexokinase PII structural gene *HXK2* (13) and the *CYC8/SSN6* gene (5, 61) led to a glucose-insensitive expression of invertase and maltase. However, no elevated expression of the CSRE-dependent reporter gene in glucose-grown *hxk2* or *cyc8* transformants could be observed. The derepressed β-galactosidase activity measured in the *cyc8* mutant reached only 25% of the level found in the isogenic wild-type strain. This partial derepression defect with respect to gluconeogenic genes may be a possible explanation for the growth deficiencies of Δ*cyc8* mutants on nonfermentable carbon sources.

Protein factors interacting with the CSRE. The oligonucleotide fragment OAS12 used for the insertion studies described above was also examined in EMSAs. With protein extracts prepared from derepressed (ethanol-grown) wild-type cells, four protein-DNA complexes (CI, CII, CIII, and CIV in Fig. 3a, lane 3) could be observed. Specificity of these interactions was demonstrated by competition with an excess of unlabelled OAS12 (lane 4). Interestingly, CI (and also the diffuse signal representing CII) was absent when protein extracts from glucose-grown cells were used (Fig. 3a, lane 2). This result strongly indicates a specific function of the protein factors involved in CI and CII for the CSRE-mediated gene activation under derepressing conditions. We thus designate the respective protein factors as Ang1 (activator of nonfermentative growth 1, forming CI) and Ang2 (CII). The relationship of Ang1 to Ang2 is presently unclear. Proteolytic degradation of Ang1, which would lead to faster-migrating protein-DNA complexes, may be taken into consideration. However, this is not a plausible explanation, since the ratios of CI to CII signal intensities are similar for extracts derived from a wild-type strain and a mutant deficient for vacuolar proteinases. Phosphorylation as a means for a possible CI-CII transition appears unlikely, since treatment of the protein extract with calf intestinal phosphatase did not alter the observed retardation

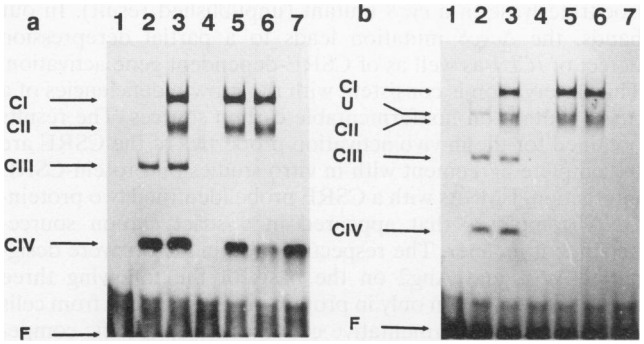

FIG. 3. Gel retardation experiments with CSRE probes OAS12 (a; ICL1[A], −402/−383) and OAS78 (b; FBP1[A], −495/−507). For each experiment, 5,000 cpm of ^{32}P-labelled oligonucleotide probe (about 0.2 ng) was used. Binding reactions contained 30 μg of cellular protein. For competition experiments, a 200-fold molar excess of unlabelled oligonucleotide was used. Lanes: 1, no protein extract added; 2, protein extract from repressed cells; 3 to 7, protein extract from derepressed cells; 4, competition with OAS12 (ICL1[A], −402/−383); 5, competition with OAS34 (MLS1[B], −411/−425); 6, competition with OAS56 (FBP1[B], −489/−504); 7, competition with OAS78 (FBP1[A], −495/−507). CI, CII, CIII, and CIV, protein-DNA complexes; F, free DNA; U, unspecific binding.

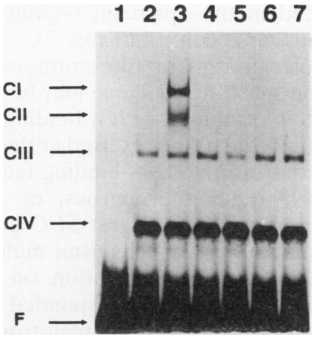

FIG. 4. Gel retardation experiments with CSRE probe OAS12 (ICL1[A], −402/−383). Experimental conditions and abbreviations are as defined in the legend to Fig. 3. Lanes: 1, no protein extract added; 2, 4, and 6, protein extract from repressed cells (SCD_2); 3, 5, and 7, protein extract from derepressed cells ($SCD_{0.2}E_3$); 2 and 3, extract from wild-type strain YHH45; 4 and 5, extract from *cat1* mutant strain ASH4; 6 and 7: extract from *cat3* mutant strain ASH5.

pattern. Alternatively, factors involved in CI and CII may, at least in part, be unrelated.

Besides OAS12, representing the CSRE from the *ICL1* upstream region, three additional oligonucleotide probes from other genes of the gluconeogenic pathway were also tested in EMSAs. No derepression-specific retardation signal was observed with oligonucleotide probe OAS34 containing the CSRE-related sequence *MLS1*(B) (not shown). Consequently, OAS34 was nonfunctional as a competitor for the carbon source-dependent Ang-OAS12 complexes (Fig. 3a, lane 5). In contrast, CIII, which is unaffected by the carbon substrate of the growth medium, is competed for by OAS34. Identical results were obtained with the *FBP1*(B) sequence motif contained within OAS56 (lane 6). Finally, oligonucleotide OAS78, representing the functional CSRE *FBP1*(A) as defined by its gene-activating competence, efficiently competed for the carbon source-dependent complexes CI and CII (Fig. 3a, lane 7). The use of OAS78 as a probe led to a gel retardation pattern almost identical to that observed with OAS12 (Fig. 3b). Carbon source-dependent protein-DNA complexes containing either OAS12 or OAS78 were competitive with each other. Thus, functional CSREs lead to the formation of protein-DNA complexes under conditions coincidental with their gene-activating properties.

Gene activation mediated by a CSRE did not occur in the pleiotropic derepression mutants *cat1* and *cat3*. Hence, protein extracts prepared from repressed as well as derepressed *cat* mutants were used in EMSAs. As shown in Fig. 4, the Ang-OAS12 complexes are absent in mutant extracts under both conditions (lanes 4 to 7). Identical results were obtained with OAS78 as a probe (not shown). These results strongly suggest a crucial role of Ang1 and/or Ang2 for the control of genes encoding enzymes of the gluconeogenic pathway. At least for *ICL1* and *FBP1*, the functional identity of two CSREs appearing in the respective control regions is apparent from these gel retardation studies.

DISCUSSION

This work presents the characterization of a UAS element necessary and sufficient for the activation of the gluconeogenic isocitrate lyase gene *ICL1* from *S. cerevisiae*. Since the transition from fermentative to nonfermentative conditions is a regular process after consumption of the sugar provided in a growth medium (diauxic growth), this sequence motif may play a central role in the regulation of yeast carbon metabolism. This view is further supported by the demonstration of an equivalent UAS element upstream of the fructose-1,6-bisphosphatase gene *FBP1*. The identification of similar sequences in the promoter regions of additional gluconeogenic genes by a data base search suggests its general importance for nonfermentative growth. Gene activation mediated by UAS_{ICL1} strongly depends on the carbon source available in the medium, thus leading to the designation CSRE. Regulatory properties as well as sequence requirements of the CSRE are different from those of previously characterized *cis*-acting

TABLE 7. Compilation of *cis*-acting sequence elements involved in activation of carbon source-regulated genes

Designation	Consensus binding sequence (5′→3′)	Binding factor(s)	Target genes	Reference(s) or source
UAS_{SUC}	A/CA/GGAAAT	?	*SUC1–5*, *SUC7*	24, 49
UAS_{MAL}	GAAAA/TTTTCGC	Mal63p	*MAL61*, *MAL62*	32, 43
UAS_{GAL}	CGGAG/CGACA/TGTCG/CTCCG	Gal4p	*GAL1*, *GAL7*, *GAL10*	21
UAS_{ADH2}	TCTCCAACTTATAAGTTGGAGA	Adr1p	*ADH2*, *CTA1*, *POT1*	58, 68
UAS_{CYC}	TNATTGGT	Hap2p, Hap3p, Hap4p	*CYC1*, *COX4*, *HEM1*	19
ORE	TNTNAA/TA/CNCCG	?	*FOX1–3*, *PAS1*	10, 11, 17
CSRE	CATC/TCA/GTCCG	Ang1, Ang2	*ICL1*, *FBP1*	This work

elements identified in the upstream regions of other genes subject to carbon source control (Table 7).

Two general mechanisms of transcriptional control mediated by the carbon substrate available may be considered: (i) a constitutive UAS is combined with a binding site for a regulated repressor; (ii) regulation is exerted at the UAS itself, e.g., by biosynthetic control of a DNA-binding factor or by modulation of its DNA-binding properties, or at the level of transcriptional transactivation. Yeast *SUC* genes may represent an example of the first mechanism: multiple copies of a heptamer element conferred activation on a heterologous reporter gene that subsequently responded only weakly to glucose repression (49). In addition, pleiotropic *snf* derepression mutations leading to a complete loss of *SUC* gene expression affected UAS_{SUC}-driven gene activation only partially. Thus, carbon source control of *SUC* genes may be mediated to a considerable extent by the action of regulated repressor proteins, one of which is encoded by the *CAT4/MIG1* gene (38, 40, 54). The derepression deficiency of *SUC* genes in a *cat1/snf1* mutant is suppressed in a *cat1 cat4* double mutant (54), arguing for a deactivation of the Cat4p/Mig1p repressor protein by the (direct or indirect) influence of the Cat1p/Snf1p protein kinase. However, constitutive *SUC* gene expression as a consequence of a *cat4/mig1* mutation is dependent on the genetic background of the strain used, indicating the existence of redundant mechanisms of repression. A possible candidate for a repressor acting in a pathway separate from Cat4p/Mig1p is the leucine zipper protein encoded by the *SKO1* gene that interacts with a cyclic AMP-responsive element-like site in the *SUC2* promoter (39).

In contrast to *SUC* gene regulation, gluconeogenic genes follow a different mechanism of transcription control. The CSRE as an activating element of gluconeogenic genes is a representative of the second mechanism of carbon source control as defined above. Gene activation mediated by the CSRE requires derepressing growth conditions, while high concentrations of an easily fermentable sugar render it inactive. The derepression deficiency of a CSRE-controlled synthetic promoter in a *cat1* mutant is not suppressed in a *cat1 cat4* double mutant, as is observed for *SUC* gene expression. In addition, CSRE-dependent gene control is still completely sugar repressible in a *cat4* null mutant, indicating that a putative transcription factor interacting with the CSRE (Ang1 or Ang2) is not biosynthetically affected by *CAT4/MIG1* as has been described for the *GAL4* activator (22, 38). A similar mechanism may influence transcriptional activators Mal63p and Hap4p, since the upstream regions of the respective genes also contain Cat4p/Mig1p binding sites (33a). This would be an explanation for the glucose-insensitive expression of maltase and iso-1-cytochrome *c* in the previously characterized *cat4* mutants (54). Interestingly, a Mig1p/Cat4p binding site is also present in the *ICL1* promoter immediately upstream of the CSRE. This motif might be responsible for the partial suppression of the derepression defect due to a *cat1* mutation in the *cat1 cat4* double mutant. However, this suppression presumably requires a pathway distinct from CSRE-dependent *ICL1* activation.

The mechanistic difference of carbon source control affecting either genes of alternative sugar utilization (*SUC*, *MAL*, and *GAL* genes) or gluconeogenic genes (*ICL1* and *FBP1*) is further emphasized by a comparison of the influence of regulatory mutations *hxk2* (13) and *cyc8/ssn6* (5, 61) on both systems. While biosynthesis of invertase, maltase, and GAL enzymes becomes glucose insensitive in these mutants, CSRE repression remains unaffected. In contrast to a previous report (61), we could not confirm a lack of glucose repression for isocitrate lyase in a *cyc8* mutant (unpublished result). In our hands, the Δ*cyc8* mutation leads to a partial derepression defect of *ICL1* as well as of CSRE-dependent gene activation. This observation is consistent with the growth deficiencies of a Δ*cyc8* mutant on nonfermentable carbon sources. The results obtained for the in vivo activation properties of the CSRE are in complete agreement with in vitro studies on protein-CSRE interaction. EMSAs with a CSRE probe identified two protein-DNA complexes that appeared in a strict carbon source-dependent manner. The respective protein factors were designated Ang1 and Ang2 on the basis of the following three criteria: (i) detection only in protein extracts derived from cells grown under nonfermentative conditions; (ii) specific competition of Ang-CSRE complexes in EMSAs only by functional, not by nonfunctional, even though sequence-related, CSRE sequences; and (iii) absence in protein extracts prepared from *cat1* or *cat3* mutants cultivated under either condition, while carbon source-independent protein-CSRE complexes still can be detected. Since a direct interaction of the Cat1p/Snf1p protein kinase complex with the CSRE appears unlikely, biosynthetic control of Ang1/Ang2 by *CAT1/SNF1* may be taken into consideration. Alternatively, a posttranslational modification of Ang1/Ang2 may occur. In a previous study on activating elements of the *FBP1* promoter, a 30-bp sequence (designated UAS_2) was identified, deletion of which led to a considerable decrease in gene activation (45). The CSRE from the *FBP1* control region as defined by our experiments is contained completely within this UAS_2 region. However, the gel retardation pattern observed by these authors with a UAS_2 probe differs significantly from our results (Fig. 3b). Since no data on the carbon source dependence of the single protein-UAS_2 complex were presented, the relation to the four complexes CI to CIV identified in our studies is unclear. Recently, in the course of the promoter analysis of the *POT1/FOX3* gene encoding the peroxisomal 3-oxoacyl-coenzyme A thiolase, another *cis*-acting element of nonfermentative metabolic control has been characterized (10, 11). These studies defined a 23-bp imperfect inverted repeat motif responsible for oleate induction of genes necessary for peroxisomal biogenesis and β oxidation (oleate response element [ORE]; the consensus sequence of a half-site of the palindrome is shown in Table 7). In EMSAs with an ORE probe, a carbon source-dependent protein-DNA complex could be detected. Although this result may appear similar to our studies on Ang-CSRE complexes, three lines of evidence argue against an identity of ORE and CSRE: (i) with the exception of the 3′-terminal CCG nucleotides, the consensus sequences for ORE and CSRE differ completely (Table 7); (ii) one half-site of the ORE inverted repeat functions only as a weak activating element compared with the intact palindrome, while the CSRE-mediated activation is not significantly stimulated after addition of a second copy of the element in inverted orientation (compare plasmid pAS35N with plasmids pAS35RN and pAS35NNRR in Table 1); (iii) a synthetic CSRE-containing promoter does not show oleate induction (Fig. 2).

The characterization of various control regions upstream of carbon source-regulated genes led to the identification of several *cis*-acting sequence elements defining pathway-specific activation systems (Table 6). Intensive efforts concentrated on regulatory mechanisms of alternative sugar utilization which is apparently regulated in a different manner from the nonfermentative metabolism. Although some *trans*-acting factors are shared among all systems considered (e.g., the pleiotropic derepression genes *CAT1/SNF1* and *CAT3/SNF4*), the signal transduction steps subsequent to these global effectors of the glucose repression-derepression system presumably diverge.

The recent application of the two-hybrid system designed for the detection of in vivo protein-protein interactions to the Cat1p/Snf1p protein kinase identified a putative kinase substrate (encoded by the *SIP1* gene [66]) which may specifically transduce a derepression signal to *SUC* genes but not to other genes also regulated by the carbon source. Distinct substrates of the Cat1p/Snf1p protein kinase complex might trigger (directly or indirectly) the activation of the remaining subpathways under appropriate conditions of derepression and/or induction by specific carbon sources. The identification of the CSRE as an important activating sequence motif of the yeast nonfermentative carbon metabolism should help to close remaining gaps in our understanding of the components of the signal transduction pathway involved.

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