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Aca1 and Aca2, ATF/CREB Activators in *Saccharomyces cerevisiae*, Are Important for Carbon Source Utilization but Not the Response to Stress

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In *Saccharomyces cerevisiae*, the family of ATF/CREB transcriptional regulators consists of a repressor, Acr1 (Sko1), and two activators, Aca1 and Aca2. The AP-1 factor Gcn4 does not activate transcription through ATF/CREB sites in vivo even though it binds these sites in vitro. Unlike ATF/CREB activators in other species, Aca1- and Aca2-dependent transcription is not affected by protein kinase A or by stress, and Aca1 and Aca2 are not required for Hog1-dependent salt induction of transcription through an optimal ATF/CREB site. Aca2 is important for a variety of biological functions including growth on nonoptimal carbon sources, and Aca2-dependent activation is modestly regulated by carbon source. Strains lacking Aca1 are phenotypically normal, but overexpression of Aca1 suppresses some defects associated with the loss of Aca2, indicating a functional overlap between Aca1 and Aca2. Acr1 represses transcription both by recruiting the Cyc8-Tup1 corepressor and by directly competing with Aca1 and Aca2 for target sites. Acr1 does not fully account for osmotic regulation through ATF/CREB sites, and a novel Hog1-dependent activator(s) that is not a bZIP protein is required for ATF/CREB site activation in response to high salt. In addition, Acr1 does not affect a number of phenotypes that arise from loss of Aca2. Thus, members of the *S. cerevisiae* ATF/CREB family have overlapping, but distinct, biological functions and target genes.

Eukaryotic organisms from yeast to human contain multiple ATF/CREB family proteins that activate or repress the expression of specific genes (14, 18, 19, 21). ATF/CREB proteins bind as homodimers or heterodimers to specific DNA sequences (consensus TGACGTCA) via a bZIP structural motif, which consists of a leucine zipper that mediates dimerization and an adjacent basic region that contacts DNA. ATF/CREB proteins can also form DNA-binding heterodimers with AP-1 proteins (17), members of a structurally related family that bind similar half-sites but differ in the requirement for half-site spacing (29, 50).

ATF/CREB proteins often serve as the ultimate targets of signal transduction pathways, and they play critical roles in many biological processes. In multicellular organisms, CREB stimulates transcription in response to cyclic AMP and calcium in a phosphorylation-dependent manner (41), and ATF-2 is the target of stress-activated mitogen-activated (MAP) kinases (16, 35, 63). ATF/CREB proteins control diverse biological functions such as memory (1, 53), opiate tolerance (37), spermatogenesis (10), circadian rhythms (9), and skeletal and neural development (47).

In the fission yeast *Schizosaccharomyces pombe*, ATF/CREB proteins are important for sexual development, entry into stationary phase, response to osmotic and oxidative stress, and activation of a hot spot for meiotic recombination (32, 51, 57, 66, 67). Atf1 is a direct target of the Spc1(Sty1) stress-activated MAP kinase (51, 67), an observation that is remarkably similar to the situation in mammalian cells. Although Atf1 is sufficient to mediate this stress response, activation of meiotic recombination requires an Atf1-Pcr1 heterodimer, whose activity also depends on Spc1 kinase (32, 33). Pcr1 is required for the nuclear localization of Atf1, and phosphorylation and association of Atf1 with Spc1 kinase is required for nuclear retention of Spc1 (12).

In the baker's yeast *Saccharomyces cerevisiae*, two ATF/CREB proteins have been identified. Acr1 (Sko1) is a repressor (43, 65), although the DNA-binding domain is not sufficient for repression (65). Overexpression of Acr1 suppresses the toxicity caused by high levels of protein kinase A (PKA) (43) or high levels of Rap1, a transcriptional regulator that also binds to telomeres (11), but the molecular bases for these effects are unknown. More recently, Acr1 has been implicated as a downstream effector of the HOG signal transduction pathway that responds to osmotic stress (46). Hac1 (44) is a transcriptional activator that induces a variety of genes in response to unfolded proteins in the endoplasmic reticulum (4, 42); for reasons discussed below, we believe that Hac1 should not be classified as an ATF/CREB protein.

Previously, we provided genetic and biochemical evidence for an ATF/CREB activator(s) in *S. cerevisiae* (65). Specifically, strains lacking the Acr1 repressor confer transcriptional activation through ATF/CREB sites in vivo, and they possess ATF/CREB-binding activities in vitro. Hac1 is not the putative ATF/CREB activator(s), because these transcriptional and DNA-binding activities are observed under normal growth conditions where Hac1 is not produced (52). Here, we show that two previously uncharacterized proteins, Aca1 and Aca2, are the ATF/CREB activators in *S. cerevisiae*. Phenotypic analysis indicates that Aca2 is important for growth on nonoptimal carbon sources as well as resistance to a variety of drugs, and that the individual ATF/CREB proteins play distinct biological roles. In addition, we show that Acr1 does not fully account for osmotic regulation through ATF/CREB sites, and that a novel activator(s) distinct from Aca1 and Aca2 supports ATF/CREB site-dependent activation in response to high salt.

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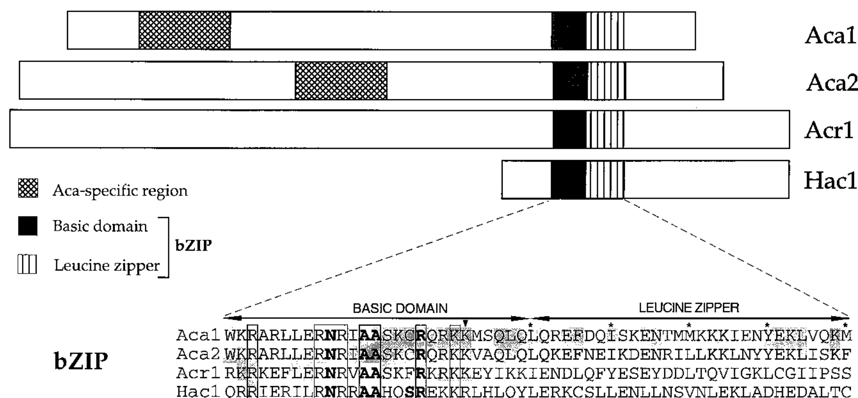

FIG. 1. Structures of *S. cerevisiae* ATF/CREB proteins. For each protein, the relative size, locations of the bZIP domains (striped and shaded boxes), and the Aca-specific region (hatched boxes) are indicated. Sequences of the bZIP domains of the ATF/CREB proteins and Hac1 are compared; residues in darker boxes are identical in Aca1 and Aca2, residues in lighter boxes are identical in Aca1, Aca2, and Acr1, and open boxes indicate residues common to all four proteins. Conserved residues in the basic region that contact DNA (bold), the lysine characteristic of ATF/CREB proteins (arrowhead), and the leucines or other residues defining position **d** of the leucine zipper (asterisks) are indicated.

MATERIALS AND METHODS

DNA molecules. Plasmids used for synthesizing ^{35}S-proteins in vitro were created by cloning PCR-generated fragments encoding the bZIP domains of Aca1 (C-terminal 300 or 138 residues), Aca2 (C-terminal 334 or 167 residues), or Hac1 (full-length 231 or N-terminal 108 residues) downstream of the SP6 promoter of Ycp88 (23). The comparable plasmids used to produce Gcn4 (23) or Acr1 (65) have been described previously. The plasmids expressing LexA fusion proteins were obtained by cloning *Sma*I-*Nco*I fragments encoding full-length ATF/CREB proteins into the YCp91-LexA vector (61); LexA-Acr1 has been previously described (26). Plasmids overexpressing the various proteins contain the following chromosomal fragments cloned into Yeplac195: Aca1, *Pvu*II-*Xho*I; Aca2, *Sph*I-*Spe*I; Acr1, *Nhe*I-*Xma*I; Hac1, *Pvu*II-*Hin*dIII. YIp56-Sc3674, which contains a *gcn4* deletion allele (7), and plasmids containing *bcy1*::*URA3* (59), *hog1*::*TRP1* (obtained from Haruo Saito), *tup1*::*LEU2* (obtained from Joe Geisberg), and *cyc8*::*LEU2* (5) were used to introduce null mutations into KY898 derivatives.

DNA-binding assays. ^{35}S-labeled proteins were synthesized by transcription and translation in vitro using SP6 RNA polymerase and wheat germ extract (Promega), and the translation products were analyzed on denaturing polyacrylamide gels as described previously (55). For experiments to determine whether proteins bind as homodimers or heterodimers, proteins of different sizes were cotranslated. Equimolar amounts of the resultant proteins were incubated at 25°C for 30 min in buffer containing 20 mM Tris-HCl (pH 7.4), 0.1 mg of gelatin/ml 1 mM EDTA, 12.5% glycerol, 3 mM $MgCl_2$, 50 mM KCl, 500 ng of poly(dI)-poly(dC), and 2 ng of a ^{32}P-labeled 50-bp oligonucleotide probe containing a centrally positioned DNA-binding site. Protein-DNA complexes were analyzed on a 5% native polyacrylamide gel as described previously (55). Under the conditions used in these experiments, the intensities of the bands representing the protein-DNA complexes are roughly proportional to the binding constants (20, 22, 55).

Yeast strains. All yeast strains were derived from KY898 (**a** *ura3-52 lys2-801 ade2-101 leu2*::*PET56 trp1-Δ1 his3-303*) (65), with the exception of FT4/pLF98 (8) and L9FT4 (61), which were used to analyze *his3* transcription dependent on an optimal Yap site and a LexA operator, respectively. Deletions of the various ATF/CREB and other proteins were generated by standard two-step gene replacement. Structures of the deletion alleles are as follows: Δ*aca1*, which lacks a *Bgl*II-*Hpa*I fragment, removes nearly the entire protein-coding region but retains 16 amino acids (aa) at the C terminus; Δ*aca2*, which lacks an *Msc*I-*Xho*I fragment, removes most of the protein-coding region but retains 163 aa at the N terminus; Δ*acr1*, which lacks an *Nhe*I-*Afl*II fragment, is deleted for the entire protein-coding region as well as 482 bp upstream and 235 bp downstream; Δ*hac1*, which lacks an *Spe*I-*Bss*HII fragment, is deleted for the entire coding region as well as 28 bp upstream and 442 bp downstream. *bcy1* disruption strains were generated by one-step gene replacement and were used immediately after construction to prevent accumulation of suppressor mutations (30).

Phenotypic analyses. Growth phenotypes of the various strains were determined by spotting 10^5, 10^4, 10^3, and 10^2 cells on appropriate media and scored as follows: +++, grows better than wild type; ++, grows comparably to wild type; +, grows more poorly than wild type; ±, barely detectable growth; −, no growth. Transcriptional activation by LexA fusion proteins was assayed in cells containing JK103, a multicopy *URA3* plasmid containing a *lacZ* reporter driven by a promoter with four LexA operators upstream of the *GAL1* TATA and initiator elements (27). Transcriptional repression by LexA-Acr1 was assayed on reporters that either contain four (JK1621) or zero (pLGΔ312S) LexA operators upstream of the intact *CYC1* promoter (28). β-Galactosidase assays were performed on permeabilized cells as described previously (61). Values were normalized to A_{600} and represent the average of at least four independent transformants; they are accurate to ±20%. To measure RNA levels, total RNA (40 μg as quantitated by A_{260}) was hybridized to completion with an excess of the appropriate ^{32}P-labeled oligonucleotide probes and treated with S1 nuclease as described previously (25). RNA levels were quantitated with respect to *DED1* or *tRNAw* internal controls by PhosphorImager (Molecular Dynamics) analysis.

RESULTS

Identification of Aca1 and Aca2 as new members of the *S. cerevisiae* ATF/CREB family. Previously, we identified the complete set of 14 bZIP proteins in *S. cerevisiae* by searching the complete genome with a degenerate motif based on the sequences of a large number of basic regions within bZIP domain (8). Twelve of these bZIP proteins have been characterized: the AP-1 factor Gcn4 (22); Met28 (34); Yap1 through 8, a novel and fungus-specific family of bZIP proteins (8); Hac1 (44); and the ATF/CREB repressor Acr1 (Sko1) (43, 65). Here, we describe the remaining two bZIP proteins, which for reasons discussed below are termed Aca1 and Aca2 (for ATF/CREB activators).

Aca1 (489 residues) and Aca2 (587 residues) have identical basic regions over the critical 17-aa region (Fig. 1), and they contain a lysine residue characteristic of ATF/CREB but not AP-1 proteins (29). The basic regions of Aca1 and Aca2 are most closely related to Acr1 and many ATF/CREB proteins in other eukaryotic species (averaging 80% identity and 90% similarity); their similarity to basic regions of AP-1 and other types of bZIP proteins is less pronounced. The Hac1 basic region is very divergent from those of ATF/CREB proteins, and we believe that Hac1 should not be categorized as an ATF/CREB protein even though it can bind the consensus ATF/CREB site in vitro (44) (see below). The Aca1 and Aca2 leucine zippers are similar to each other (54% identical, 75% similar) but are essentially unrelated in sequence to other leucine zippers. Aca1 and Aca2 contain a stretch of 80 aa with 85% similarity that is not present in known proteins. Thus, Aca1 and Aca2 are highly related proteins with an ATF/CREB-like bZIP domain.

Aca1 and Aca2 bind ATF/CREB sites as homodimers and heterodimers. ^{35}S-labeled derivatives of Aca1 and Aca2 bind efficiently to the ATF/CREB consensus site, with affinities that are roughly comparable to those of Acr1, Hac1, and the AP-1

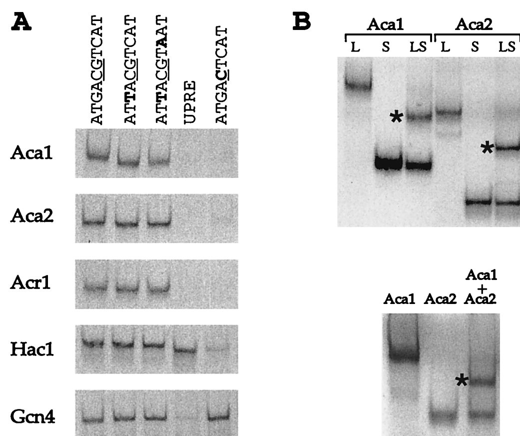

FIG. 2. Aca1 and Aca2 bind ATF/CREB sites as homodimers and heterodimers. (A) The indicated ^{35}S-labeled proteins were incubated with an optimal ATF/CREB site (TGACGTCA), derivatives with one or two substitutions (bold) at the ±2position, the UPRE (GGAACTGGACAGCGTGTCGAAA), and the optimal AP-1 site (TGACTCA). (B) ^{35}S-labeled derivatives of the indicated proteins (L, large; S, short; LS, cosynthesized mixture) were incubated with the ATF/CREB optimal binding site. The intermediate-sized products indicative of large-small and Aca1-Aca2 heterodimers are marked with asterisks.

factor Gcn4 (Fig. 2A). However, Aca1 and Aca2, like other ATF/CREB proteins, do not bind the AP-1 consensus, in contrast to Gcn4, which prefers this site over the ATF/CREB site (50). The Aca proteins bind with comparable affinity to related ATF/CREB sites with single or double substitutions at positions ±2 that are tolerated by other ATF/CREB proteins. Thus, Aca1 and Aca2 display the DNA-binding specificity of ATF/CREB proteins.

bZIP proteins bind their DNA targets as dimers, and certain combinations of bZIP domains can interact with each other and bind as heterodimers. A mixture of two differently sized versions of Aca1 or Aca2 yields a protein-DNA complex of intermediate mobility (Fig. 2B), indicating that these proteins bind as homodimers. Examination of all pairwise combinations of ATF/CREB proteins indicates that Aca1 and Aca2 can bind ATF/CREB sites as a heterodimer with an affinity comparable to that of the corresponding homodimers. However, DNA-binding heterodimers were not observed for any other combination (data not shown).

In addition to binding ATF/CREB sites (44), Hac1 binds the UPRE (unfolded protein response element), a 22-bp sequence in *KAR2* and other promoters that are activated in response to unfolded proteins in the endoplasmic reticulum (4, 42). Levels of Hac1 binding to the ATF/CREB and UPRE sequences are roughly comparable, whereas Aca1, Aca2, and Acr1 do not bind the UPRE (Fig. 2A). Thus, ATF/CREB proteins are not involved in the unfolded protein response, and Hac1 and ATF/CREB proteins have distinct DNA-binding specificities.

Aca1 and Aca2 are transcriptional activators. As an initial test to determine whether Aca1 and Aca2 are transcriptional activators, we fused their full-length coding sequences to the LexA DNA-binding domain. When assayed on a *GAL1* promoter derivative with four LexA operators, activation by LexA-Aca2 occurs at a level comparable to that achieved by LexA-Gcn4 or LexA-Gal4, whereas activation by LexA-Aca1 is approximately fivefold more efficient (Fig. 3A). As expected from the fact that LexA-Acr1 functions as a repressor (26), no activation was observed in strains containing LexA-Acr1. LexA-Aca1 and LexA-Aca2 also stimulate transcription from a *his3* promoter derivative with LexA sites in a manner that is unaffected by a *bcy1* mutation and hence high levels of PKA (Fig. 3B). Thus, Aca1 and Aca2 contain functional activation domains.

Using a modified *his3* promoter (*his3-303*) in which the AP-1 site is converted to an ATF/CREB site, we previously showed that ATF/CREB sites can stimulate transcription in a manner repressed by Acr1 (65). To address whether Aca1 and/or Aca2 are responsible for ATF/CREB site-dependent transcription from the *his3-303* promoter, we analyzed *his3* mRNA levels in *aca1*, *aca2*, or double-mutant strains. When Acr1 is present, *his3* transcription occurs at a low level (Fig. 4, lanes 1 to 4) that appears to be independent of Aca1 and Aca2. In the absence of Acr1, *his3* transcription occurs a high level that is essentially unaffected by loss of Aca1 and is only slightly reduced in the absence of Aca2 (lanes 5 to 7). However, the loss of both Aca1 and Aca2 reduced *his3* transcription in the *acr1* deletion strain to the background level (lane 8), demonstrating that Aca1 and Aca2 are the activators responsible for transcription through the ATF/CREB site. Both proteins contribute to ATF/CREB site-dependent activation, with Aca2 appearing to be more important.

Gcn4 does not activate transcription from ATF/CREB sites in vivo. In vitro, the AP-1 factor Gcn4 binds with comparable affinity to its natural target sites and the consensus ATF/CREB site (50). It has been suggested that Gcn4 might not activate transcription through ATF/CREB sites (56), but this study was complicated by the presence of Aca1 and Aca2. Treatment of the strains described above with aminotriazole (AT), which

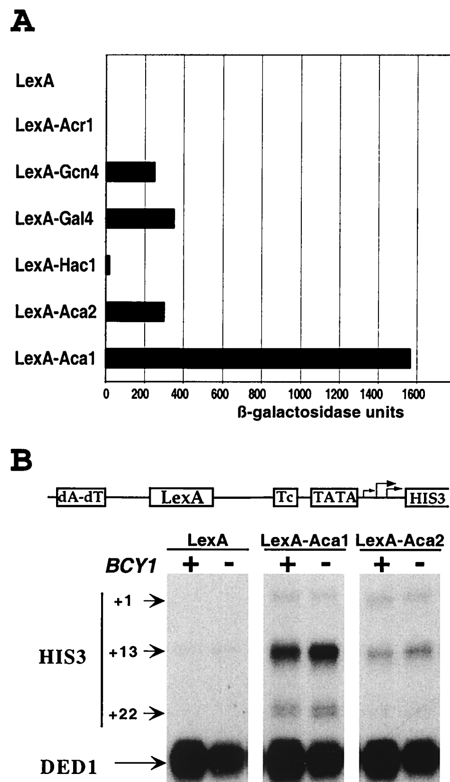

FIG. 3. Aca1 and Aca2 contain transcriptional activation domains. (A) β-Galactosidase activities (average from six independent transformants; values accurate to ±15%) of cells carrying the indicated LexA fusion proteins and a *lacZ* reporter with four LexA operators upstream of the *GAL1* TATA element. (B) RNAs from strain XY and its *bcy1* derivatives containing the indicated LexA fusion protein were analyzed for levels of *his3* (+1, +13, and +22 transcripts) and *ded1* (internal control) RNAs by quantitative S1 analysis.

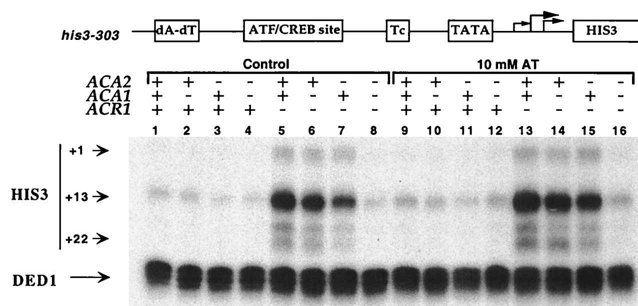

FIG. 4. Aca1 and Aca2 activate transcription through ATF/CREB sites. RNAs from KY898 derivatives (contain the *his3-303* promoter; functional elements indicated) with the indicated genotypes were analyzed for levels of *his3* (+1, +13, and +22 transcripts) and *ded1* (internal control) RNAs by quantitative S1 analysis. Strains were grown in minimal medium lacking histidine in the absence (lanes 1 to 8) or presence (lanes 9 to 16) of 10 mM AT.

induces Gcn4-dependent activation, yields results similar to those obtained under normal growth conditions (Fig. 4, lanes 9 to 16). In particular, AT does not induce *his3* transcription in the *acr1 aca1 aca2* strain, conditions in which there should be no other ATF/CREB proteins to compete for binding the ATF/CREB site. As the Gcn4 activation domain functions when fused to heterologous DNA-binding domains (24, 54), it is likely that the transcriptional defect reflects the inability of Gcn4 to bind ATF/CREB sites in vivo. This striking discrepancy between in vitro and in vivo activity might involve some feature of chromatin structure (e.g., rotational positioning of the target site on nucleosomes) or the interaction of proteins such as Mbf1 that increase Gcn4-dependent binding in vitro and transcription in vivo (58).

Physiological functions of Aca1 and Aca2. The ATF/CREB family is not essential for cell growth, because strains lacking any combination of Aca1, Aca2, Acr1, Hac1, and Gcn4 are viable. However, Aca2 is important for a variety of physiological functions. Strains lacking Aca2 have no apparent defects in mating, sporulation, or cell wall function (assayed by flocculence and sensitivity to calcofluor white), but their growth is slightly reduced in standard rich and minimal media and severely affected at 15°C. In addition, *aca2* deletion strains are sensitive to a variety of unrelated drugs such as cycloheximide, hygromycin B, formamide, rapamycin, and oligomycin (Table 1), although they behave normally in response to staurosporine, canavanine, tunicamycin, and 5-fluoro-orotic acid (data not shown). Finally, *aca2* strains grow extremely poorly or not at all on a variety of alternative carbon sources (Table 2), whereas they grow normally on medium containing poor nitrogen sources (1% proline or glutamate).

In contrast, Aca1 plays a minor physiological role. Loss of Aca1 does not cause or exacerbate any of the phenotypes associated with the loss of Aca2. However, overexpression of Aca1 can suppress the cold sensitivity and poor growth in nonoptimal carbon sources caused by an *aca2* deletion. This suggests that the Aca1 proteins are functionally related and that, in many respects, Aca1 is a less active or less abundant version of Aca2. Curiously, loss of Aca1 results in increased growth of an *acr1 aca2* double-mutant strain on all nonoptimal carbon sources tested (Table 2), indicating that Aca1 and Aca2 functions do not completely overlap.

Consistent with the result that Acr1 and the Aca proteins have opposing transcriptional effects at the *his3-303* promoter, the caffeine sensitivity conferred by an *acr1* mutation is partially suppressed by either an *aca1* or *aca2* mutation and completely suppressed by the combination of *aca1* and *aca2* (Table 1). However, an *acr1* mutation does not suppress any of the phenotypes conferred by the *aca2* deletion. Conversely, overexpression of Acr1 does not cause or exacerbate any of the phenotypes associated with *aca2* deletions (data not shown). These results suggest that Acr1 and Aca2 affect distinct biological functions and target genes.

In contrast to the ATF/CREB activators in the fission yeast *S. pombe* (51, 67), Aca1 and Aca2 do not seem to be involved in the response to osmotic or oxidative stress. Specifically, the various *aca* mutant strains are comparable to the wild-type strain when grown in the presence of hydrogen peroxide, NaCl, KCl, LiCl, or sorbitol. Aca1 and Aca2 are functionally distinct from Hac1. Unlike *hac1* strains, *aca1* or *aca2* strains are insensitive to tunicamycin (an inducer of the unfolded protein response), capable of growing in medium without inositol, and unable to activate UPRE-dependent transcription of *KAR2* (Table 1 and data not shown).

Aca2-dependent transcription is modestly regulated by carbon source but not by PKA. Because *aca2* mutant strains grow poorly in nonoptimal carbon sources, we examined whether ATF/CREB site-dependent transcription is regulated by carbon source (Fig. 5A). In the wild-type strain, levels of transcription from the *his3-303* promoter are comparably low in all media tested, suggesting that repression by Acr1 is unaffected by carbon source. However, in the *acr1* deletion strain, transcription from the *his3-303* promoter is reduced when cells are grown in glycerol (3.5-fold), ethanol (2-fold), and galactose (1.5-fold) but not in fructose or raffinose. Indistinguishable results are observed in the *acr1 aca1* double-mutant strain, indicating that Aca2-dependent transcription is modestly reg-

TABLE 1. Temperature and drug sensitivities of wild-type and mutant strains

Strain or genotype	Growth: YPD 16°C	YPD 30°C	YPD 37°C	Cycloheximide, 0.4 μg/μl	Hygromycin B, 25 μg/μl	Caffeine, 7.5 mM	Formamide, 3%	Rapamycin, 5 μg/μl	Oligomycin, 1.5 μg/μl
KY898	++	++	++	++	++	++	++	++	++
Δ*aca1*	++	++	++	++	++	++	++	++	++
Δ*aca2*	−	+	+	−	±	++	−	+	±
Δ*aca1* Δ*aca2*	−	+	+	−	±	++	−	+	±
Δ*acr1*	++	++	++	++	++	−	+	++	++
Δ*acr1* Δ*aca1*	++	++	++	++	++	+	+	++	++
Δ*acr1* Δ*aca2*	−	+	+	−	±	+	−	+	±
Δ*acr1* Δ*aca1* Δ*aca2*	−	+	+	−	++	++	−	+	±
2μm-Aca1 in Δ*aca2*	++	++	ND[a]	ND	ND	ND	ND	ND	ND

[a] ND, not determined.

TABLE 2. Effects of carbon source on growth of wild-type and mutant strains

Strain or genotype	Growth						
	Glucose, 2%	Glycerol, 3%	Ethanol, 3%	Maltose, 2%	Galactose, 2%	Sucinate + 2-diacyl-glycerol 2% + 0.2 mg/ml	Raffinose, 2%
KY898	++	++	++	++	++	++	++
Δ*aca1*	++	+++	++	++	++	++	++
Δ*aca2*	+	−	−	−	±	−	±
Δ*aca1* Δ*aca2*	+	−	−	−	±	−	±
Δ*acr1*	++	+++	++	++	++	+++	++
Δ*acr1* Δ*aca1*	++	+++	++	++	++	+++	++
Δ*acr1* Δ*aca2*	+	−	−	−	±	−	±
Δ*acr1* Δ*aca1* Δ*aca2*	+	±	±	±	+	±	+
2μm-Aca1 in Δ*aca2*	++	+	+	++	+	++	ND[a]

[a] ND, not determined.

ulated by carbon source. These results also suggest that Aca1-dependent transcription is not significantly regulated by carbon source, but this cannot be examined directly because the growth of *aca2* strains is severely impaired in nonoptimal carbon sources. The modest reduction in Aca2-dependent transcription in certain nonoptimal carbon sources is unexpected given the importance of Aca2 for growth in these conditions. While growth in nonoptimal carbon sources could conceivably result in Aca2 being a more efficient activator or repressor of the relevant natural target genes, we suspect that the reduction in Aca2-dependent transcription is simply too modest to have an appreciable effect on the growth phenotype. In this regard, diploids heterozygous for *ACA2* grow normally in nonoptimal carbon sources.

CREB proteins in various species activate transcription in a manner dependent on phosphorylation by PKA (41). However, in strains lacking various combinations of ATF/CREB proteins, transcription from the *his3-303* promoter is essentially unaffected by loss of Bcy1, which causes very high PKA levels (Fig. 5B). This observation suggests that Aca1 and Aca2, unlike CREB, are not activated by cyclic AMP and PKA.

Transcriptional activation through ATF/CREB sites in response to high salt is independent of Aca1 and Aca2. ATF/CREB activators in *S. pombe* and mammalian cells are targets of stress-inducible MAP kinases and play an important role in the response to several kind of stress (16, 35, 51, 63, 67). Although *aca1* and *aca2* mutant strains grow well in response to all stress agents tested, we directly examined ATF/CREB site-dependent transcription in response to stress (Fig. 6A). Transcription from the *his3-303* promoter is unaffected by heat shock and oxygen stress (0.4 mM hydrogen peroxide), but it is strongly induced by high salt (15- to 20-fold in 0.8 M NaCl or KCl). Transcription from this promoter is also significantly induced by 250 mM LiCl (10-fold) or 200 mM $CaCl_2$ (5-fold) but is only slightly induced by 0.3 M NaCl or 1.2 M sorbitol (2-fold). This pattern of induction resembles that of the osmosensing HOG signal transduction pathway kinase (2, 36), although it is unclear if the response of the *his3-303* promoter is related to ionic strength, osmolarity, or the distinct effects of particular ions. For this reason, we will use the term "salt induction" to describe the behavior of the *his3-303* promoter.

Salt induction depends on the ATF/CREB site in the *his3-303* promoter, because comparable promoters containing the natural AP-1 site or an optimal Yap site (8) are uninducible by NaCl (Fig. 6B), and ATF/CREB sites upstream of the *cyc1* TATA element are sufficient to confer salt regulation of a *lacZ* reporter construct (data not shown). Induction of *his3-303* transcription is detectable after 5 min of treatment with 0.8 M NaCl (data not shown) and is completely eliminated in a *hog1* deletion strain (Fig. 6C), indicating the involvement of the HOG pathway and the Hog1 MAP kinase (2, 36). The complete Hog1 dependence of the *his3-303* promoter on high salt differs from the situation with native genes (e.g., *GPD1*) whose salt induction is only partially Hog1 dependent. Salt induction of ATF/CREB site-dependent transcription via the HOG pathway has been observed previously, and it has been suggested that it reflects inactivation of Acr1 in response to osmotic stress (46).

Whether or not high salt inactivates Acr1, there must be an activator(s) that binds the ATF/CREB site to account for the high level of transcription from the *his3-303* promoter under these conditions. Surprisingly, Aca1 and Aca2 are not required for activation in response to high salt, because the *aca1 aca2 acr1* triple-mutant strain is indistinguishable from the wild-type strain for transcription from the *his3-303* promoter (Fig. 7). Moreover, salt induction of the *his3-303* promoter in the triple-mutant strain depends on Hog1 (Fig. 6C). Thus, there must be a salt-regulated activator(s) that stimulates transcription

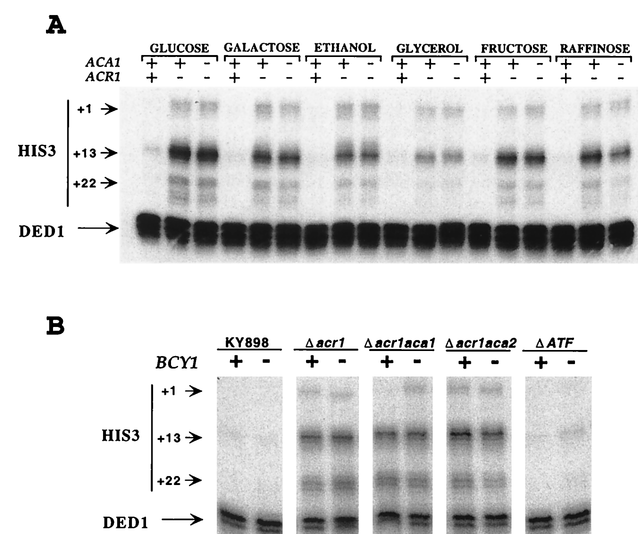

FIG. 5. Aca2-dependent transcription is regulated by carbon source but not PKA. (A) RNAs from KY898 derivatives with the indicated genotypes were grown in various carbon sources and analyzed for levels of *his3* (+1, +13, and +22 transcripts) and *ded1* (internal control) RNAs by quantitative S1 analysis. (B) Effect of *bcy1* mutation, which causes high levels of PKA, on transcription from the *his3-303* promoter.

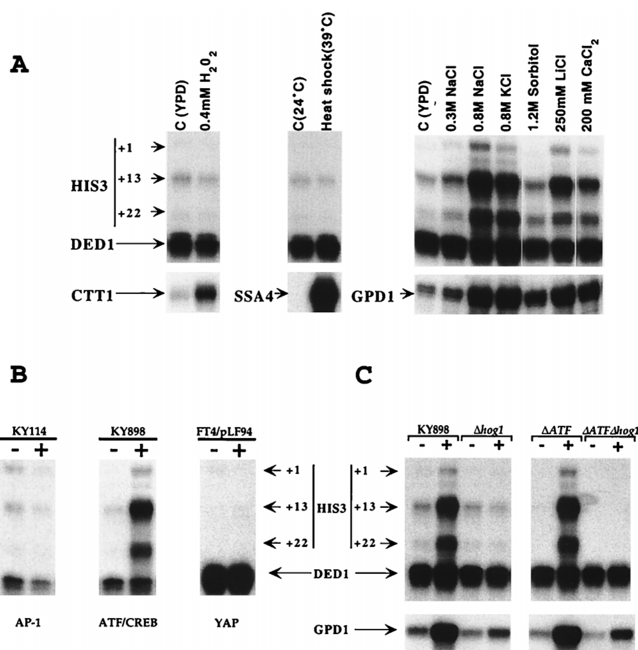

FIG. 6. ATF/CREB site-dependent transcription is induced by salt in a Hog1-dependent manner. (A) RNAs from KY898 cells (contains ATF/CREB site) grown in YPD medium that were subjected to the indicated compounds for 1 h. (B) RNAs from KY898, KY114 (contains AP-1 site of the natural *his3* promoter), and FT4/pLF94 (contains the optimal Yap-binding site) cells that were (+) or were not (−) treated for 1 h with 0.8 M NaCl. (C) RNAs from KY898 and the *acr1 aca1 aca2* (ΔATF) derivative as well as their isogenic *hog1* deletion derivatives were (+) or were not (−) treated for 1 h with 0.8 M NaCl. In all cases, levels of *his3* (+1, +13, and +22 transcripts) and *ded1* (internal control) RNAs were analyzed by quantitative S1 analysis.

through an optimal ATF/CREB site but is not a member of the ATF/CREB family. Analysis of mutant strains indicates that Hac1 and Gcn4 are not responsible for salt regulation of the *his3-303* promoter, and Gcn4- and Hac1-regulated genes are not activated in response to salt (data not shown). Similarly, transcription dependent on the optimal Yap-binding site is unaffected by salt stress (Fig. 6B), suggesting that the eight Yap proteins are not involved. Although overexpression of Yap4 (Hal6) or Yap6 (Hal7) causes increased resistance to high salt (40), a strain lacking Yap4, Yap6, and all ATF/CREB proteins is fully capable of induction of the *his3-303* promoter in response to 0.8 M NaCl (data not shown). Finally, the *his3-303* promoter is fully salt inducible in strains lacking all ATF/CREB proteins as well as Met28 or Hot1, a nuclear protein involved in osmotic stress-inducible regulation (48).

Curiously, *acr1* mutants actually have threefold-lower levels of transcription (Fig. 7), indicating that Acr1 contributes positively to transcription under these conditions. Decreased transcription in the *acr1* mutant strain is reversed when both Aca1 and Aca2 are also eliminated, suggesting that the ATF/CREB activators function negatively. The simplest explanation for these observations is that the ATF/CREB proteins regulate the transcription of an inhibitor of the salt-regulated activator(s) in a manner similar to the *his3-303* promoter.

The ATF/CREB family regulates *GRE2* transcription but is not responsible for salt regulation of native yeast promoters containing ATF/CREB sites. We also examined whether the ATF/CREB proteins affect transcription of *ENA1*, *YPR1*, and *GRE2* (Fig. 8), genes that are induced by osmotic stress (13, 38, 45) and that contain ATF/CREB sites in their promoters. Regulation of *ENA1* by high salt is mediated through the ATF/

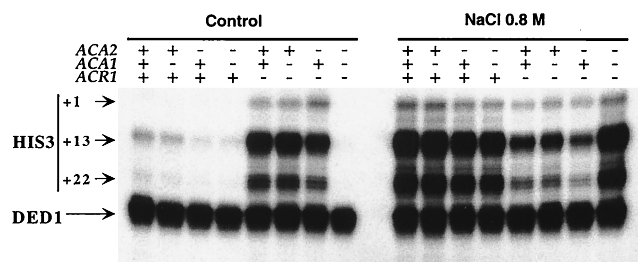

FIG. 7. Aca1 and Aca2 are not involved in the salt response of the *his3-303* promoter. RNAs from KY898 derivatives with the indicated genotypes were or were not treated for 1 h with 0.8 M NaCl and analyzed for levels of *his3* (+1, +13, and +22 transcripts) and *ded1* (internal control) RNAs by quantitative S1 analysis.

CREB site in the promoter (46), whereas this has not been demonstrated for *GRE2* and *YPR1*. In contrast to a previous study using an *ENA1-LacZ* reporter (46), the very low level of *ENA1* expression in YPD medium is minimally affected by loss of Acr1 function. Similarly, *YPR1* transcription is essentially unaffected by the ATF/CREB proteins under these growth conditions. However, *GRE2* transcription is increased approximately threefold in an *acr1* deletion strain and is virtually eliminated in the *acr1 aca1 aca2* triple-mutant strain. The transcriptional profile of *GRE2* resembles (but is not identical to) that of the *his3-303* promoter, indicating that *GRE2* is a physiological target of Acr1, Aca1, and Aca2.

As observed with the artificial *his3-303* promoter, transcriptional induction of *ENA1* (approximately 100-fold), *GRE2* (6-fold), and *YPR1* (2-fold) in response to salt stress is observed in strains lacking Acr1, Aca1, and Aca2 (Fig. 8). Furthermore, *GRE2* and *YPR1* resemble the *his3-303* promoter in that osmotic induction is reduced in strains lacking Acr1 except in the situation where Aca1 and Aca2 are also absent. In the case of *ENA1*, transcription in 0.8 M NaCl is reduced approximately 1.5-fold in *aca2* strains, suggesting that Aca2 makes a minor contribution to expression of this gene. Thus, salt regulation of *ENA1*, and perhaps *GRE2* and *YPR1*, is mediated primarily by an activator(s) that binds ATF/CREB sites but is not a member of the ATF/CREB family.

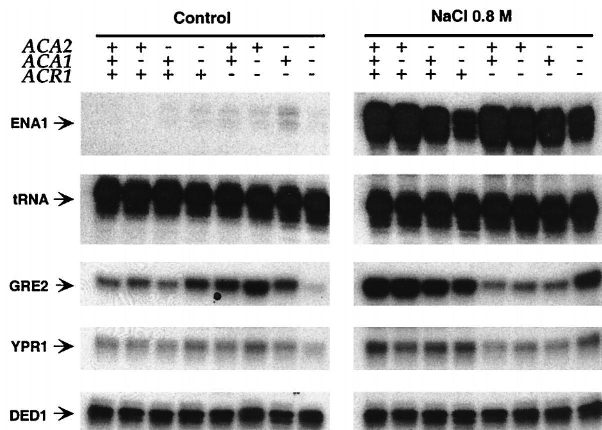

FIG. 8. ATF/CREB proteins are not significantly involved in salt regulation of native yeast promoters containing ATF/CREB sites. RNAs from KY898 derivatives with the indicated genotypes were or were not treated for 1 h with 0.8 M NaCl and analyzed for levels of the indicated RNAs by quantitative S1 analysis.

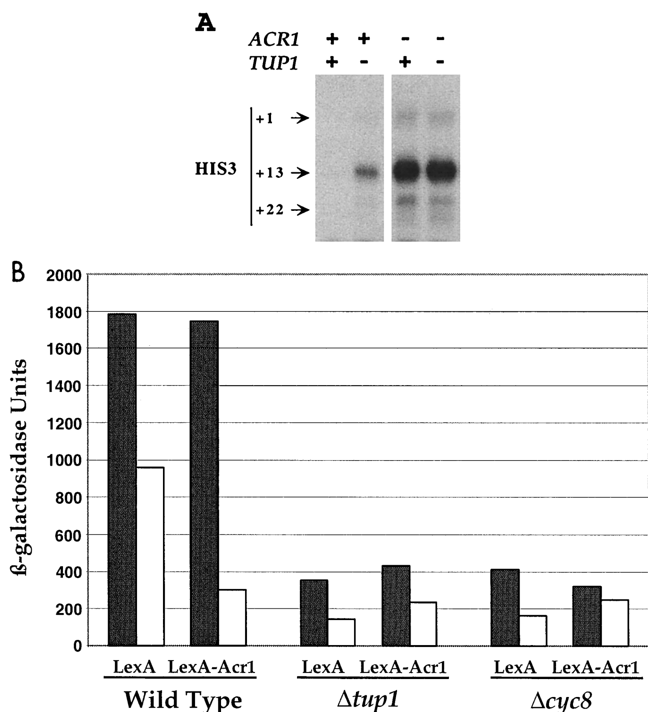

FIG. 9. Acr1 repression is mediated by the Cyc8-Tup1 corepressor complex. (A) RNAs (40 μg) from KY898 derivatives with the indicated genotypes were analyzed for levels of *his3* (+1, +13, and +22 transcripts) and *ded1* (internal control) RNAs by quantitative S1 analysis. (B) β-Galactosidase activities (average from three to four independent transformants) of wild-type and indicated mutant strains expressing LexA or LexA-Acr1 and *lacZ* reporter plasmids that either lack (shaded boxes) or contain (open bars) four LexA operators upstream of the *CYC1* promoter. Values are accurate to ±15%.

Acr1 represses through the Cyc8-Tup1 corepressor complex. Several lines of evidence suggest that Acr1 does not repress transcription solely by competing with Aca1 and Aca2 for ATF/CREB sites. First, Acr1 represses the *his3-303* promoter below the level observed in the corresponding promoter lacking the ATF/CREB site (50). Second, deletion of the C-terminal 15 residues of Acr1 abolishes repression in vivo but does not affect ATF/CREB binding in vitro (65). Third, Acr1 represses transcription even when tethered upstream of a heterologous promoter via the LexA DNA-binding domain (26). These observations suggest that Acr1 may repress transcription by recruiting a corepressor, although repression by Acr1 does not involve the Sin3-Rpd3 histone deacetylase corepressor complex (26).

The Cyc8-Tup1 corepressor complex is required for repression by a variety of pathway-specific DNA-binding proteins such as α2, Mig1, and Rox1 (6, 28, 31, 60, 61, 62). To examine whether Acr1-dependent repression requires Cyc8-Tup1, we examined transcription from the *his3-303* promoter in a *tup1* deletion strain. As shown in Fig. 9A, *his3* transcription is significantly increased in a *tup1* strain, although the level is approximately threefold less than observed in the isogenic *acr1* strain. Furthermore, repression of a heterologous promoter by LexA-Acr1 is abolished by *cyc8* and *tup1* strains (Fig. 9B). These observations suggest that Acr1-dependent repression is mediated by the Cyc8-Tup1 corepressor complex. However, the difference in transcription from the *his3-303* promoter in *tup1* and *acr1* strains suggests that part of the repression involves ATF/CREB site competition between Acr1 and the Aca proteins.

DISCUSSION

The ATF/CREB family of *S. cerevisiae* consists of two activators and a repressor. ATF/CREB proteins are defined by three criteria: DNA-binding specificity, amino acid sequence of the basic region within the bZIP domain, and transcriptional regulation of promoters containing ATF/CREB sites. By these criteria, Aca1 and Aca2 and the previously described Acr1 (Sko1) represent the complete set of ATF/CREB proteins in *S. cerevisiae*. Although Hac1 can bind the consensus ATF/CREB site and was originally designated an ATF/CREB protein (44), we believe that Hac1 should not be classified as an ATF/CREB protein because its basic region is highly diverged from those of other members of the family. Moreover, unlike other ATF/CREB proteins, Hac1 efficiently binds an unrelated sequence (UPRE) through which it mediates the response to unfolded proteins, its major physiological role (4, 42). Within the *S. cerevisiae* ATF/CREB family, Aca1 and Aca2 are more closely related, as they share a conserved domain and can form a heterodimeric complex that binds ATF/CREB sites.

Aca1 and Aca2 can independently stimulate transcription through ATF/CREB sites, and each protein contains a functionally autonomous activation domain. Conversely, Acr1 represses transcription through ATF/CREB sites, and it presumably contains a repression domain at the extreme C terminus (65) that recruits the Cyc8-Tup1 complex to target promoters. Taken together, these observations suggest that Aca1 homodimers, Aca2 homodimers, and Aca1-Aca2 heterodimers function as activators, whereas the Acr1 homodimer functions as a repressor. However, it remains possible that these ATF/CREB proteins might associate with other proteins (either by heterodimerization through the leucine zipper or by some other interaction) and hence affect transcription in more complex ways.

Aca1 and Aca2 have different biological functions than ATF/CREB activators in other eukaryotic organisms. ATF/CREB activator proteins in fission yeast and mammalian cells play an important role in mediating the response to activation of PKA and to a wide variety of environmental stresses (16, 51, 67). The molecular basis for the stress response is remarkably conserved in that these ATF/CREB proteins are directly phosphorylated by stress-responsive MAP kinases, whereupon they activate target genes containing ATF/CREB sites. In contrast, the ATF/CREB family in *S. cerevisiae* does not appear to mediate the responses to PKA or to stress. In response to thermal, osmotic, or oxidative stress, *S. cerevisiae* strains lacking any combination of ATF/CREB protein are essentially normal. Furthermore, transcriptional activity through ATF/CREB sites is not induced by PKA, heat shock, or oxidative stress, and the strong response to high salt is independent of Aca1 and Aca2. These observations are consistent with the idea that the biological functions of homologous transcriptional regulators can differ considerably between budding and fission yeasts (64). In this regard, the general stress response in *S. cerevisiae* is mediated primarily by the Msn2 and Msn4 activators (15, 39, 49), whose activity is negatively regulated by PKA. Thus, these results provide another example supporting the view that mammals and many other multicellular eukaryotes are more evolutionarily related to *S. pombe* than to *S. cerevisiae*.

Aca2 is important for carbon source utilization and regulation. Although the ATF/CREB proteins do not appear to be involved in stress responses, Aca2 is important for a variety of physiological functions, suggesting that Aca2 affects distinct classes of target genes. Most interestingly, Aca2 is important for cells to grow on nonoptimal carbon sources. It is unclear

whether the poor growth on nonoptimal carbon sources reflects a common function related to glucose repression or a set of analogous functions that are more specific to individual or particular types of carbon sources. Although *aca1* mutant strains are phenotypically normal, Aca1 probably contributes to carbon source regulation, because overexpression of Aca1 suppresses the inability of *aca2* mutant strains to grow on nonoptimal carbon sources.

Some growth defects of *aca2* deletion strains are similar to those observed in strains lacking Snf1 kinase, which plays a key role in glucose repression and utilization of alternative carbon sources (3). This suggests the possibility that Aca2 and Snf1 act in a common pathway of glucose repression and that Aca2 might be a substrate or transcriptional regulator of Snf1. Poor growth in nonoptimal carbon sources is also observed in strains lacking Bcy1, the regulatory subunit of PKA (59), although our results do not suggest a connection between PKA and the ATF/CREB family.

A salt-inducible activator(s) that functions through ATF/CREB sites but is distinct from the ATF/CREB family and is not a bZIP protein. The ATF/CREB site in the *ENA1* promoter is a physiological target for Acr1, and it is responsible for salt regulation via the HOG pathway (46). In addition, repression by artificial recruitment of Acr1 (via the Gal4 DNA-binding domain) is alleviated by high salt, suggesting that Acr1 is functionally inactivated in response to salt stress (46). However, our results indicate that salt regulation through ATF/CREB sites is not simply due to inactivation of Acr1. First, *ENA1* transcription is minimally affected by loss of Acr1, whereas it is dramatically stimulated by treatment with 0.8 M NaCl. Second, transcription of *GRE2*, *YPR1*, and the artificial *his3-303* promoter under conditions of high salt is actually reduced in the *acr1* deletion strain. Third, and most important, salt induction of the *his3-303*, *ENA1*, *GRE2*, and *YPR1* promoters occurs in the *acr1 aca1 aca2* strain, which lacks all members of the ATF/CREB family. In the case of the *his3-303* promoter, transcriptional activation is completely dependent on the ATF/CREB site, thereby defining a novel, Hog1-dependent activator(s) that functions through an optimal ATF/CREB site in response to high salt. This novel activator(s) is likely to contribute to salt regulation of the native *ENA1*, *GRE2*, and *YPR1* promoters, but this remains to be demonstrated. Although this salt-inducible activator(s) has yet to be identified, our results have essentially excluded all bZIP proteins and Hot1.

Evidence that the ATF/CREB activators and the repressor have related but distinct biological functions and target genes. The simplest model for transcriptional regulation by the *S. cerevisiae* ATF/CREB family is that Acr1 opposes the action of the functionally redundant activators Aca1 and Aca2 at a common set of promoters. Acr1 inhibits transcription both by competing with Aca1 and Aca2 for ATF/CREB sites and by an active repression mechanism involving recruitment of the Cyc8-Tup1 corepressor. This model accounts for the activity of the artificial *his3-303* and natural *GRE2* promoters, and it can explain why the caffeine sensitivity conferred by an *acr1* mutation is partially suppressed by either an *aca1* or *aca2* mutation and completely suppressed by the combination of *aca1* and *aca2*. In this view, caffeine sensitivity is caused by high Aca1- and Aca2-dependent activation through ATF/CREB sites, and the natural target promoters responsible for this phenotype are functionally analogous to the *his3-303* promoter. For promoters subject to this competition model, transcriptional regulation in response to a signal could occur by affecting the activities or levels of one or more of the ATF/CREB proteins.

However, several observations suggest that individual ATF/CREB proteins have distinct biological functions. First, many phenotypes of an *aca2* strain are not suppressed by loss of Acr1, nor are they caused or enhanced by overexpression of Acr1. This suggests that Acr1 does not play a significant role in many biological processes affected by the ATF/CREB activators. Second, although Aca1 and Aca2 activate the *his3-303* and *GRE2* promoters to near comparable levels, *aca1* mutations do not cause or enhance various *aca2* phenotypes, indicating that Aca1 and Aca2 make unequal contributions to a variety of biological functions. This inequality could be explained by proposing that Aca1 is a weakened version of Aca2 and that the overall level of ATF/CREB activation differentially affects specific biological functions, but this suggestion does not account for why loss of Aca1 increases growth of an *acr1 aca2* strain on nonoptimal carbon sources. Thus, despite the shared properties of Aca1 and Aca2, these ATF/CREB activators may not be functionally redundant in all respects.

The distinct biological functions of individual ATF/CREB proteins imply that these proteins have distinct promoter specificities and target genes. Although the basic regions of Aca1 and Aca2 are identical, the Acr1 basic region has a few differences, including one at a residue that makes base-specific contacts with the ATF/CREB site. Hence, differential recognition of native yeast promoters might be involved in the functional distinction between Acr1 and the ATF/CREB activators. In addition, differences in protein-protein interactions that mediate cooperative binding to promoters or modulate transcriptional activity are likely to contribute to promoter specificity. Finally, the various ATF/CREB proteins may be differentially affected by signal transduction pathways, thereby resulting in distinct transcriptional outputs depending on the physiological conditions.

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