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ACR1, a Yeast ATF/CREB Repressor

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Members of the mammalian ATF/CREB family of transcription factors, which are associated with regulation by cyclic AMP and viral oncogenes, bind common DNA sequences (consensus TGACGTCA) via a bZIP domain. In the yeast *Saccharomyces cerevisiae*, ATF/CREB-like sequences confer either repression or activation of transcription, depending on the promoter context. By isolating mutations that alleviate the repression mediated by ATF/CREB sites, we define a new yeast gene, *ACR1*, which encodes an ATF/CREB transcriptional repressor. ACR1 contains a bZIP domain that is necessary for homodimer formation and specific DNA binding to an ATF/CREB site. Within the bZIP domain, ACR1 most strongly resembles the mammalian cyclic AMP-responsive transcriptional regulators CREB and CREM; it is less similar to GCN4 and YAP1, two previously described yeast bZIP transcriptional activators that recognize the related AP-1 sequence (consensus TGACTCA). Interestingly, deletion of the *ACR1* gene causes increased transcription through ATF/CREB sites that does not depend on GCN4 or YAP1. Moreover, extracts from *acr1* deletion strains contain one or more ATF/CREB-like DNA-binding activities. These genetic and biochemical observations suggest that *S. cerevisiae* contains a family of ATF/CREB proteins that function as transcriptional repressors or activators.

ATF/CREB proteins are defined by their ability to bind specifically to DNA sequences (consensus TGACGTCA) found in numerous eukaryotic promoters (15, 17, 18, 22). These proteins bind as dimers to abutting half-sites by using a conserved bZIP structural motif, which consists of a leucine zipper dimerization element and an adjacent basic region that interacts directly with DNA (29). ATF/CREB proteins are structurally and functionally related to the AP-1 family of eukaryotic transcription factors, which includes the Jun and Fos oncoproteins and yeast GCN4 and YAP1 (2, 9, 10, 34, 50). ATF/CREB sequences contain the same half-sites as AP-1 sequences but differ in spacing by an additional base pair in the center of the sequence (42). In general, AP-1 proteins efficiently recognize ATF/CREB sites, whereas ATF/CREB proteins bind inefficiently to AP-1 sites (17, 42). Individual AP-1 and ATF/CREB proteins have distinct dimerization properties, and interactions within and between these families generate a large number of potential transcriptional regulatory proteins (16).

In mammalian cells, ATF/CREB sites are often associated with transcriptional regulation by cyclic AMP (cAMP) or by the adenovirus E1A transforming gene (17, 40). The mammalian ATF/CREB family contains at least 10 proteins that have well conserved but individually distinct bZIP domains (13, 15, 18, 22, 32, 57). Of these, CREB, CREM, and ATF-2 are the only ATF/CREB proteins with defined biological functions. Specifically, CREB stimulates transcription in response to cAMP and calcium in a phosphorylation-dependent manner (14, 43), CREM functionally counteracts the activity of CREB (12), and ATF-2 is responsible for mediating adenovirus E1A-dependent transactivation (31).

ATF/CREB DNA-binding activities distinct from those of GCN4 have been detected in cell extracts from the yeast *Saccharomyces cerevisiae*. First, a 66-kDa ATF/CREB binding protein, whose DNase I footprinting properties are similar to those of mammalian ATF proteins, can be purified by oligo-affinity chromotography (30). Second, a partially purified yeast ATF/CREB binding activity is phosphorylated by protein kinase A in vitro (26), suggesting a possible link between cAMP signaling and ATF/CREB protein function. Third, chromatographically separable activities that form electrophoretically distinct protein-DNA complexes have been described (42). Because these activities were defined by binding to nonidentical (although related) DNA sequences and were isolated by different experimental procedures, it is unclear whether they represent one or multiple ATF/CREB proteins. It is also unclear whether the chromatographically distinct DNA-binding activities represent multiple proteins or differentially modified or proteolytic products of a single factor.

When placed upstream of a *CYC1* or *GAL1* TATA element, ATF/CREB recognition sequences stimulate transcription in vivo (26, 30, 42). Although a single ATF/CREB site activates transcription very poorly, two or three sites can stimulate transcription to the level of a typical yeast promoter. In contrast, substitution of an ATF/CREB sequence in place of the GCN4 binding site in the wild-type *his3* promoter causes a significant decrease in basal *his3* transcription (42). Although GCN4 efficiently binds the ATF/CREB element in vitro and activates transcription through this sequence in vivo, the observed repression does not depend on GCN4 and hence presumably involves an ATF-CREB repressor. Moreover, the putative repressor appears to compete with GCN4 for binding to the ATF/CREB site in vivo (42).

If a single ATF/CREB protein exists in *S. cerevisiae*, then activation or repression could result from differences in the promoter context of the ATF/CREB recognition sequence, as has been observed for the transcriptional regulatory proteins RAP1 (44) and MCM1 (1, 27, 37). Alternatively, activation or repression could be mediated by distinct ATF/CREB proteins. To determine the role and activities of the yeast ATF/CREB protein(s), we have taken a genetic approach to obtaining mutations that eliminate repression

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TABLE 1. Strain list

Strain	Genotype
KY898	*MAT***a** *ura3-52 lys2-801 ade2-101 leu2-PET56 trp1-Δ1 his3-303*
KY899	*MATα ura3-52 lys2-801 ade2-101 his3-303 trp1-Δ1*
KY900	Isogenic to KY898 but *acr1-1*
KY901	Isogenic to KY898 but YCp87-Sc4906 (*his3-303 lacZ*)
KY902	Isogenic to KY898 but *acr1-Δ1*
KY903	Isogenic to KY899 but *acr1-Δ1*
KY904	Isogenic to KY898 but *gcn4::TRP1*
KY905	Isogenic to KY898 but *acr1-Δ1 gcn4::TRP1*
KY906	Isogenic to KY898 but *yap1::LEU2*
KY907	Isogenic to KY898 but *yap1::LEU2 acr1-Δ1*
KY908	Isogenic to KY898 but *acr1::URA3*
KY909	Isogenic to KY898 but *bcy1::URA3*
KY910	Diploid obtained by crossing KY899 and KY908

mediated through ATF/CREB sites. These mutations define a new gene, *ACR1*, which encodes a novel ATF/CREB repressor whose bZIP domain most strongly resembles that of the mammalian transcriptional regulatory proteins CREB and CREM. Furthermore, we present genetic and biochemical evidence for additional ATF/CREB proteins, including a transcriptional activator(s) that is distinct from GCN4 and YAP1, the two previously characterized yeast bZIP family members.

MATERIALS AND METHODS

Isolation of *acr1* mutants. YCp86-Sc4281, a *URA3* centromeric vector that contains the *his3-303* promoter region fused to the *lacZ* structural gene and that was constructed by substituting the 775-bp *Bam*HI-*Hin*dIII *his3* promoter fragment from pUC8-Sc4281 (42) for the equivalent fragment in YCp86-Sc3416 (47), was introduced into KY898 and KY899 (42) (Table 1). Twenty independent colonies (10 from each of the resulting strains) were plated (2×10^4 cells per plate) on glucose minimal medium supplemented with appropriate amino acids and 40 mM aminotriazole (AT). Colonies growing on AT were replica plated on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 mg/liter), and blue, AT-resistant colonies were selected for further analysis. In crosses between a total of 52 mutants and the appropriate starting strain, all of the diploids displayed a codominant phenotype, as determined by both AT sensitivity and *lacZ* activity. Nine mutant strains that appeared to be recessive had a very weak AT-resistant phenotype and were not studied further.

Cloning of *ACR1*. KY900 was transformed by YCp87-Sc4281, a molecule generated by inserting the *Bam*HI-*Hin*dIII fragment containing the *his3-303* promoter into a *LEU2* derivative of YCp86-Sc3416 (constructed by Joan Sellers). The resulting strain (KY901) was transformed to Ura^+ by a YCp50 library containing yeast genomic DNA fragments (obtained from Mark Rose), and approximately 20,000 colonies were replica plated on medium containing X-Gal. A single light blue colony was obtained and shown to confer a reduced level of resistance to 40 mM AT. Numerous attempts to rescue the YCp50 plasmid from this yeast strain were unsuccessful, presumably because of a mutation in either the ampicillin resistance gene or the bacterial replication origin. To circumvent this problem, we integrated YIp33, a *LEU2* plasmid obtained from Fred Winston, into the YCp50-*ACR1* plasmid by homologous recombination; the resulting molecule was then rescued in *Escherichia coli* and shown to contain a 22-kb insert. To prove that this DNA contained *ACR1*, we subcloned a 2.8-kb *Eco*RI-*Sal*I fragment (Sc5051) into *TRP1* integration vector pRS304 (46). The resulting molecule was cleaved with *Cla*I and integrated into KY899 through homologous recombination. The resulting strain was crossed with an *acr1* strain (KY900), and the diploids were subjected to tetrad analysis.

DNA manipulations. YCp315-*ACR1* contains a 9.3-kb *Pst*I-*Bam*HI fragment in pRS315 (46). The following molecules are derivatives of YCp315-*ACR1* (see Fig. 2): Sc4801, 1.4-kb *Hin*dIII-*Bam*HI fragment; Sc4802, 1.4-kb *Xba*I-*Pst*I fragment; Sc4803, a 6.2-kb *Xho*I-*Bam*HI fragment; Sc4804, a 3.8-kb *Hin*dIII fragment; Sc4805, a 3.1-kb *Hin*dIII fragment; Sc4806, a 4.1-kb *Xba*I fragment; Sc4807, a 7.8-kb *Pst*I-*Sac*I fragment; Sc4808, a 4.1-kb *Sal*I-*Bam*HI fragment; and Sc4809 and Sc4810, a 5.2-kb *Sal*I fragment cloned in opposite orientations. YCp88-Sc4918 contains a 4.8-kb *Eco*RV fragment from Sc4810 cloned into a filled-in *Xba*I site of YCp88 (24). YCp88-Sc4956 has a deletion of sequences in YCp88-Sc4918 between the polylinker *Sal*I site and the *ACR1 Asp*718 site resulting from filling in and blunt ligation. Sc5055 was generated by subcloning of the 4.8-kb *Eco*RV fragment from Sc4810 into the *Sma*I site of *URA3* integrating plasmid pRS306 (46) and deletion of a 1.8-kb region by partial *Xba*I digestion (*acr1-Δ1* allele). The *acr1::URA3* fragment (Sc4911) was generated by replacing the 1.8-kb *Xba*I fragment from Sc4891 with a 1.1-kb *Hin*dIII fragment containing *URA3*. The *yap1::LEU2* molecule was synthesized in two steps. First, a 2.4-kb *Apa*I-*Bst*EII *YAP1* fragment (34) was obtained by polymerase chain reaction amplification from genomic DNA and cloned between the *Apa*I and *Sma*I sites of pBSKS (after blunting of the *Bst*EII site). Second, a *Bam*HI-*Xho*I *LEU2* fragment was blunted and cloned in place of the 594-bp *Hin*cII fragment of *YAP1*. Deletions of Sc4809, Sc4810, and Sc4956 were constructed by double digestion with *Sac*I and *Bam*HI, exonuclease III treatment, and repair. Unidirectional deletions of YCp315-Sc4809, Sc4810, and Sc4956 were constructed by double digestion with *Sac*I and *Bam*HI, exonuclease III treatment, and repair. The resulting DNAs (representing a composite of overlapping clones on both strands) were sequenced by the dideoxy chain termination method and also tested for *ACR1* function.

Phenotypic analyses. To perform *his3* allele specificity experiments, we introduced YIp55 molecules containing mutant *his3* promoters (19, 33, 42, 49) into both the starting strain (KY898) and the *acr1* mutant strain (KY900) by gene replacement as described previously (5). The resulting strains were streaked on plates containing 40 mM AT and incubated for 4 to 5 days at 30°C, and phenotypes were scored as follows: +++, large colonies; ++, medium colonies; +, very small colonies; and ±, barely detectable growth. To assay for *ACR1* function, we introduced YCp315 DNAs containing various regions of *ACR1* into KY902 (*acr1-Δ1*) and assayed the resulting strains for growth on 40 mM AT. The *acr1-Δ1* strains were generated by first integrating *Sma*I-cleaved YIp55-Sc5055 DNA and then selecting for the desired gene replacement event by growth on 5-fluoroorotic acid. Strains containing the *yap1::LEU2*, *gcn4::TRP1*, *acr1::URA3*, and *bcy1::URA3* (plasmid kindly provided by Kunihiro Matsumoto) alleles were made by introducing appropriate linear DNA fragments into yeast cells and directly selecting for the relevant genetic marker; all such gene disruptions were confirmed by genomic hybridization. Measurements of β-galactosidase activity were per-

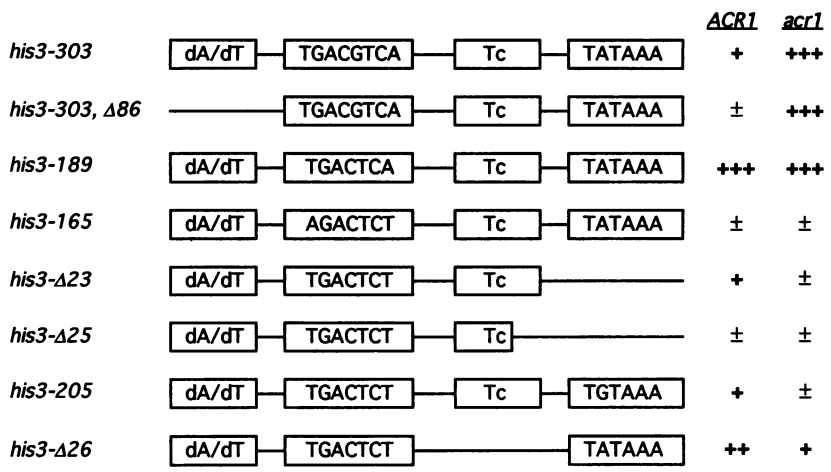

FIG. 1. *ACR1* functions through an ATF/CREB site. Shown is the growth of wild-type or *acr1* mutant strains containing the indicated *his3* promoters ([poly(dA) · poly(dT), the GCN4 or ATF/CREB binding sequence, T_C, and T_R TATA) in the presence of 40 mM AT. See the text for definitions of symbols.

formed with glucose-grown cells containing high-copy-number plasmids as described previously (42).

RNA analysis. ACR1 and TFIID RNA levels were determined by blotting of electrophoretically separated total nucleic acid by standard procedures. HIS3 and DED1 RNA levels were determined by hybridizing total nucleic acid to oligonucleotide probes and cleaving the products with S1 nuclease (6). The 5′ end of *ACR1* RNA was determined by hybridizing 1 μg of poly(A)-containing RNA to two probes (each generated by the polymerase chain reaction with an excess of a ^{32}P-labeled *ACR1* oligonucleotide as a primer) and cleaving the resulting products with S1 nuclease.

DNA-binding assays. ^{35}S-labeled ACR1 proteins were synthesized in vitro (23) with RNA transcribed from DNA templates linearized at *Eco*RI, *Xba*I, or *Afl*II sites. The resulting proteins were incubated with 1 μg of poly(dI) · poly(dC) and 2 ng of a 50-bp oligonucleotide containing a centrally positioned ATF/CREB site (ATGACGTCAT), and DNA-binding assays were performed as described previously (23). To assay for yeast ATF/CREB DNA-binding activities, we prepared a cell extract from an *acr1::URA3* strain (KY908) and fractionated it by heparin-agarose chromatography as described previously (42). DNA-binding assays were performed as described previously (42), except that the extract volume was reduced by half to avoid adverse effects of high KCl concentrations.

RESULTS

Isolation of mutations that alleviate repression through the ATF-CREB site. The wild-type *his3* promoter region contains the following elements (Fig. 1): a poly(dA) · poly(dT) sequence necessary for constitutive transcription (48); a GCN4 binding site required for transcriptional induction under conditions of amino acid starvation (23); T_C, an unusual TATA element that does not respond to upstream activator proteins but is necessary for basal transcription initiated from the +1 site (33); and T_R, a canonical TATA element that interacts with TFIID, responds to GCN4 and other acidic activators, and accounts for most of the transcription initiated from the +13 site (49, 56). Previously, we suggested that yeast cells contain an ATF/CREB repressor because the substitution of an ATF/CREB site for the GCN4 site (*his3-303*) markedly reduced the basal level of *his3* transcription (42). Moreover, the level of GCN4-activated transcription in *his3-303* strains was reduced, presumably because of competition between GCN4 and the putative repressor for the ATF/CREB sequence. Thus, mutations in the gene encoding the putative ATF/CREB repressor should increase transcription from the *his3-303* promoter under normal growth conditions and during amino acid starvation.

To isolate such mutations, we started with isogenic **a** and α strains that carry a chromosomal copy of the *his3-303* allele (KY898 and KY899) and a centromeric plasmid containing the identical promoter fused to a *his3-lacZ* structural gene (YCp86-Sc4906). Because of the relatively low activity of the *his3-303* promoter, these strains grew poorly in the presence of 40 mM AT, a competitive inhibitor of the *his3* gene product, and were white on X-Gal indicator plates. Thus, derivatives of the starting strains were selected for growth at high AT concentrations and then screened for the expression of the LacZ reporter (blue colonies). We obtained 59 independently derived strains that presumably contained mutations that were unlinked to *HIS3* and that increased transcription from the *his3-303* promoter.

When the mutant strains were crossed with the parental strain, the resulting heterozygous diploids displayed an intermediate sensitivity to AT and were faintly blue, suggesting that the mutations were not fully recessive. Crosses among the initial 59 mutants indicated that the mutations defined one complementation group. Tetrad analysis of two heterozygous diploids demonstrated that the AT-resistant phenotype segregated 2:2, indicating that a single genetic locus, termed *ACR1* (for ATF/CREB repressor), was involved.

To determine whether *ACR1* specifically affected repression through the ATF/CREB site, we analyzed *his3* derivatives with substitutions or deletions of the various promoter elements in *ACR1* and *acr1* strains (Fig. 1). In accordance with this hypothesis, only those promoters containing the ATF/CREB sequence displayed increased expression in the *acr1* mutant strain. In particular, the *ACR1* locus did not affect *his3* expression from promoters containing the wild-type or mutated GCN4 binding site, which contains the related AP-1 site. Sequences upstream of the ATF/CREB site (−103 to −447) are not important for *ACR1* action

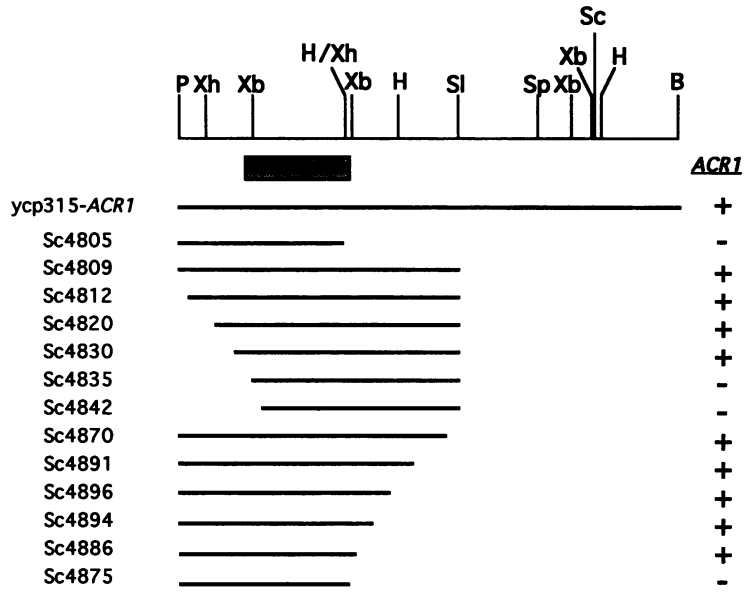

FIG. 2. Localization of *ACR1*. The structures and *ACR1* phenotypes (determined by complementation of the *acr1* mutation) of the indicated DNA fragments are shown below the restriction map of a 9.3-kb *Pst*I-*Bam*HI fragment containing the *ACR1* gene region (P, *Pst*I; Xh, *Xho*I; Xb, *Xba*I; H, *Hin*dIII; Sl, *Sal*I; Sp, *Sph*I; Sc, *Sca*I; B, *Bam*HI). The location of *ACR1* is indicated by the shaded box.

because the *his3-303,Δ86* promoter displays increased *his3* expression in the *acr1* mutant strain.

Cloning of the *ACR1* gene. To clone *ACR1*, we introduced a library of yeast genomic sequences into KY901, an *acr1 his3-303* strain containing the *his3-303 lacZ* plasmid. Of the 20,000 transformants screened, one grew poorly in 40 mM AT and was white, suggesting that it contained a plasmid that restored *ACR1* function. A plasmid containing 22 kb of yeast DNA was recovered from this transformant and shown to complement the *acr1* mutation. To verify that this DNA contained *ACR1*, we subcloned a 2.8-kb *Eco*RI-*Sal*I fragment (Sc5051) into a *TRP1* integration vector and introduced it into KY899 through homologous recombination. This integration event, which had no effect on the *ACR1* phenotype, was confirmed by genomic hybridization (data not shown). When this strain was crossed with an *acr1* strain (KY900), the resulting diploids (13 of 13) showed 2:2 segregation of the *TRP1* marker with the AT-sensitive phenotype, indicating that the cloned DNA contained the *ACR1* locus.

The *ACR1* gene was initially localized to a 9.3-kb *Pst*I-*Bam*HI fragment from the initial plasmid DNA. To define the *ACR1* gene boundaries more precisely, we generated a series of deletions and tested them in the complementation assay (Fig. 2). Deletions encroaching from one direction do not affect *ACR1* function until they reach the *Hin*dIII site, whereas deletions from the other direction do not affect *ACR1* activity until they reach the *Xba*I site. Thus, *ACR1* is localized within a 3.2-kb region of DNA.

Isolation and characterization of *acr1* deletion strains. The incompletely recessive nature of the *acr1* mutations raised the possibility that the initial mutant strains possessed residual or altered *ACR1* function. To clarify this issue and more directly assess the physiological role of *ACR1*, we generated DNA molecules in which a 1.8-kb region encompassing nearly the entire *ACR1* locus was either deleted or replaced by the *URA3* gene. These DNAs were introduced into yeast cells by gene replacement to generate strains lacking the *ACR1* gene (confirmed by genomic hybridization). The resulting *acr1* deletion strains grow at wild-type rates in rich or in supplemented minimal medium, indicating that *ACR1* is not an essential gene. Moreover, the deletion strains behave indistinguishably from the original *acr1* mutant strains with respect to both AT resistance and complementation by the cloned *ACR1* gene. Thus, the loss of *ACR1* function results in the failure to repress transcription through the ATF/CREB site.

Constitutive transcription of *ACR1*. To determine whether *ACR1* transcription is regulated, we assayed RNA levels under a variety of experimental conditions (Fig. 3). *ACR1* encodes a 2.7-kb RNA that is absent in a strain with a deletion of the gene. In rich medium containing glucose, galactose, ethanol, or glycerol as a source of carbon, the ACR1 transcript levels were equivalent to those of the TFIID internal control. In addition, deregulating the cAMP pathway by disruption of *BCY1* (which encodes the regulatory subunit of the cAMP-dependent protein kinases) or subjecting the cells to heat shock had no effect on *ACR1* transcription. Finally, *ACR1* does not appear to be autoregulated because similar RNA levels were observed in wild-type and *acr1* mutant strains.

ACR1 contains a bZIP domain homologous to that in mammalian ATF/CREB proteins. Sequence analysis of the *ACR1* gene region (Fig. 4) reveal a 647-amino-acid open reading frame beginning with a 5′-proximal AUG codon that lies within a context that favors efficient translation (8). Several considerations favor the idea that the 647-residue protein is the major (and possibly exclusive) translation product. First, the 5′ end of the major *ACR1* transcript (determined by S1 nuclease mapping) is located 49 nucleotides upstream of the putative AUG initiation codon (data not shown). Second, a DNA fragment containing only 7 codons upstream of the 5′-proximal AUG codon confers wild-type levels of *ACR1* function, as determined by the complementation assay. Third, a 4-bp insertion at the *Xba*I site, which disrupts the *ACR1* open reading frame 7 residues upstream of the 5′-proximal AUG codon, does not detect-

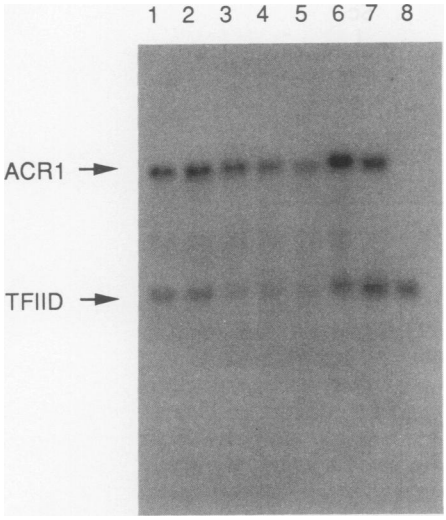

FIG. 3. *ACR1* transcription. RNA samples (25 μg) from strains grown in YP broth containing various carbon sources or under certain experimental conditions were electrophoretically separated and hybridized simultaneously to ACR1 and TFIID radiolabeled probes. Lanes: 1, KY898 in glucose; 2, KY900 (*acr1*-1) in glucose; 3, KY898 in galactose; 4, KY898 in glycerol; 5, KY898 in ethanol; 6, KY898 in glucose and heat shocked at 39°C for 15 min; 7, KY909 (*bcy1*) in glucose; 8, KY908 (*acr1*::*URA3*) in glucose.

ably affect *ACR1* function. However, conceptual translation of the *ACR1* locus actually reveals a potential open reading frame encoding a 741-amino-acid protein that includes an additional 94 amino acids prior to the 5′-proximal AUG codon. Although this 94-amino-acid region does not include any methionine residues, *ACR1* gene products larger than 647 residues could be generated in principle either by translational initiation at non-AUG codons or by initiation at a more upstream AUG codon that becomes fused to the *ACR1* open reading frame upon RNA splicing. For reasons described above, we doubt that such larger proteins contribute significantly to *ACR1* function; hence, the 647-residue protein will be considered the primary translation product, although the issue has not been resolved definitively.

The most noteworthy feature of the *ACR1* coding sequence is the region from residues 430 to 482, which possesses all the characteristics of a bZIP DNA-binding domain (Fig. 5) (29). The putative basic region of ACR1 contains the invariant asparagine and arginine residues, a pair of highly conserved alanines, and 5 of the 6 conserved positively charged residues (38). ACR1 also contains a 4-3 repeat of hydrophobic amino acids that strongly resembles the leucine zipper dimerization motif (29, 35, 36), even though only 2 leucine residues are found at position d of the coiled coil instead of the usual 4 or 5. Furthermore, the highly conserved and functionally critical spacing between the basic region and the leucine zipper (39, 54) is precisely maintained in ACR1.

When compared with other bZIP proteins (Fig. 5), ACR1 is most similar to the mammalian CREB and CREM proteins (52% sequence identity in the basic region), which are associated with the transcriptional response to cAMP. ACR1 is less similar to YAP1, GCN4, and CYS3, the known yeast bZIP family members. As expected from previous comparisons of bZIP proteins, ACR1 shows considerably more sequence similarity in the basic region than in the leucine zipper. With regard to previously characterized ATF/CREB family members, ACR1 is unique in having an isoleucine instead of a leucine at the first position of the zipper. However, at position d of the remaining heptad repeats, the amino acids found in ACR1 can be observed in other members of the ATF/CREB family. Finally, ACR1 contains a highly conserved lysine residue (Lys-451) that is found in 11 of 12 ATF/CREB proteins but is absent in AP-1 proteins, such as GCN4, YAP1, and the Jun family.

In addition to the highly conserved bZIP region, ACR1 contains the sequence KRRMS (residues 376 to 380), which conforms to both consensus sequences (KRRXS and RRXS) for phosphorylation by cAMP-dependent protein kinases (3, 28). Otherwise, the putative structural gene for ACR1 does not contain any other recognizable sequence motifs. In particular, the TPR (21, 45) and β-transducin (11) motifs found, respectively, in the yeast negative regulatory proteins SSN6 (41) and TUP1 (55) are not observed.

Functional mapping of ACR1. The region of ACR1 required for repressor function was defined by N- and C-terminal deletion analysis starting with a DNA molecule in which a 4.8-kb *Eco*RV *ACR1* fragment was cloned into a yeast expression vector between the *DED1* promoter and the *GCN4* termination sequences (Fig. 6A). A derivative that has a deletion of the 335 N-terminal residues of ACR1 and retains only 94 residues in front of the bZIP domain complements an *acr1* mutation and seems to have enhanced repressor activity with respect to the product of the wild-type gene (*acr1*-C312; Fig. 6B). This apparent increase in repressor function could be the result of the increased expression or stability of the truncated protein or the removal of an N-terminal region that counteracts repressor activity (see Discussion). In any event, this observation indicates that the C-terminal 312 residues are sufficient for ACR1 function.

In contrast, the deletion of only 13 amino acids from the C terminus of the *acr1*-C312 gene product abolishes repressor activity, as do more extensive C-terminal deletions. This result is surprising because the extreme C terminus of the protein is located far from the bZIP domain, and it suggests the possibility of a distinct functional domain necessary for transcriptional repression. However, in the absence of antibodies to ACR1, we cannot exclude the trivial possibility that proteins with C-terminal deletions are degraded in vivo.

ACR1 binds to an ATF/CREB site as a homodimer. To demonstrate that ACR1 binds DNA in a sequence-specific manner, we synthesized a variety of ^{35}S-labeled proteins in vitro by using a DNA template encoding a derivative lacking the N-terminal 335 residues that are dispensible for function in vivo (see above). By linearizing the template at several positions with respect to the C terminus, we generated proteins of 312, 247, and 152 amino acids (Fig. 7A). While the 152-amino-acid derivative does not bind DNA in vitro (data not shown), the two longer derivatives exhibit sequence-specific DNA binding (Fig. 7B). In particular, both ACR1 proteins bind efficiently to a conventional ATF/CREB site (TGACGTCA), extremely poorly to a conventional AP-1 site (TGACTCA), and nondetectably to a symmetric double mutant site (TGAGCTCA). Thus, ACR1 binding specificity strongly resembles that of mammalian ATF/CREB proteins.

To determine whether ACR1 binds DNA as a homodimer or as a heterodimer with a protein(s) in the wheat germ extract used for in vitro synthesis, we cotranslated the 312- and 247-residue ACR1 derivatives (Fig. 7B). When incubated independently with an ATF/CREB target site, each protein generates a protein-DNA complex of distinct electrophoretic mobility. When both ACR1 proteins are present,

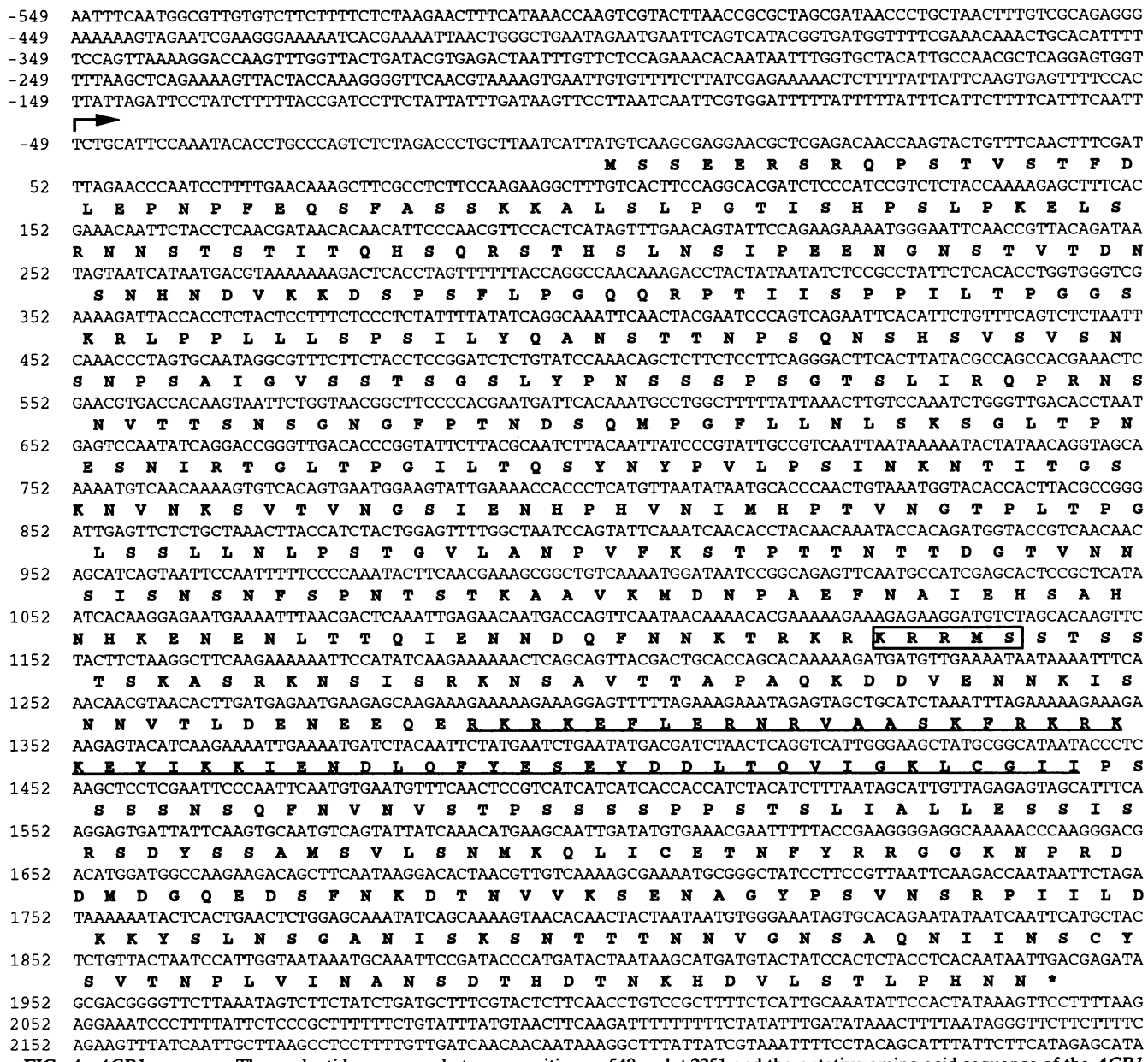

FIG. 4. *ACR1* sequence. The nucleotide sequence between positions −549 and +2251 and the putative amino acid sequence of the *ACR1* gene product (in boldface type; +1 is defined by the putative AUG initiation codon) are shown along with the 5′ end of the major *ACR1* transcript (arrow), a potential phosphorylation site for a cAMP-dependent protein kinase (box), and the bZIP domain (underlined).

a complex migrating with an intermediate mobility is observed, demonstrating that ACR1 binds DNA as a homodimer in vitro. Interestingly, as initially observed for GCN4 (25), the ACR1 heterodimer complex is not observed when the two proteins are synthesized independently and then mixed prior to the DNA-binding assay; this result indicates that ACR1 can form stable dimers in the absence of DNA.

An ATF/CREB-like activator(s) stimulates transcription through the ATF/CREB-like sequence. As expected from its defining phenotype, an *acr1-1* strain shows elevated levels of *his3* transcription from the *his3-303* promoter (Fig. 8). Moreover, *his3* RNA levels in strains with a deletion of the entire *ACR1* gene are elevated to the same extent, suggesting that proteins distinct from ACR1 activate transcription through ATF/CREB sites. To exclude the possibility that this increased transcription could be attributed to some feature of the *his3-303* promoter, we examined the effect of *acr1* on a heterologous promoter containing one or two copies of an identical ATF/CREB sequence upstream of the *gal1* TATA element and mRNA initiation region (42). When compared with a wild-type strain, *acr1-1* strains show a 5-fold increase in expression through a single ATF/CREB site and a 14-fold increase through two such sites (Table 2). Moreover, a control *gal1* promoter lacking ATF/CREB sites is inactive in both *ACR1* and *acr1-1* strains, indicating that the increased transcription observed in *acr1* strains is due to an ATF/CREB-like activator protein(s).

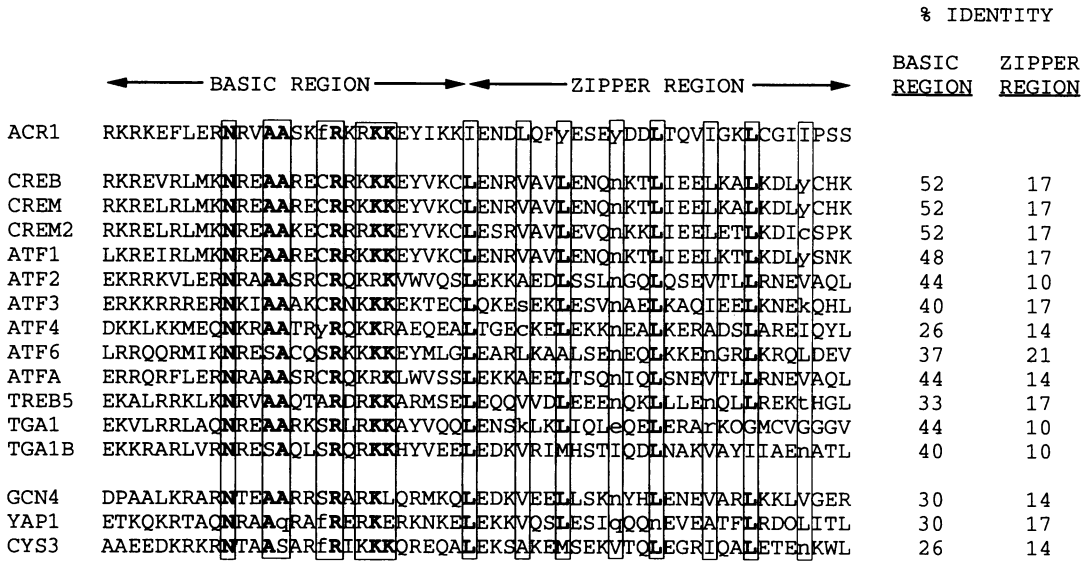

FIG. 5. *ACR1* bZIP domain. The sequence of the *ACR1* bZIP domain is compared (percent identity) with those of 12 previously identified ATF/CREB proteins from other organisms as well as yeast GCN4, YAP1, and CYS3. The boxed residues within the basic region include invariant asparagine and arginine residues as well as a highly conserved pair of alanines, cysteine, serine, and three positively charged residues; within the leucine zipper the boxed residues represent the 4-3 hydrophobic repeat including the conserved leucines.

One possibility for such an ATF/CREB-like activator protein is GCN4, which can stimulate transcription in vivo through an ATF/CREB site (42). However, in strains containing both *gcn4* and *acr1* deletion alleles, the level of *his3* transcription is similar to that observed in *acr1* deletion strains (Fig. 8). Similarly, activation through the ATF/CREB site in the context of the *gal1* TATA element is largely independent of GCN4 (Table 2). Finally, disruption of *YAP1*, which encodes a bZIP protein that binds the related AP-1 site (34), has no effect on *his3* levels in *ACR1* wild-type and mutant strains.

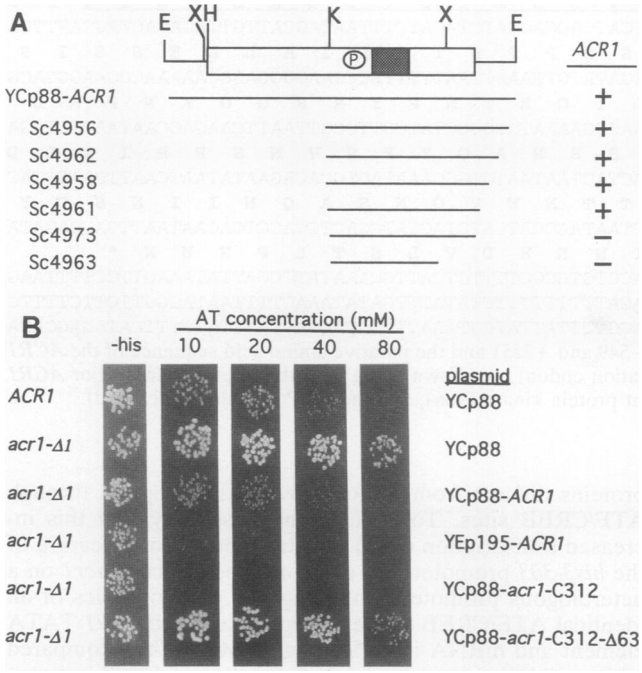

FIG. 6. Deletion analysis of *ACR1*. (A) Structures of the *acr1* deletions (the shaded box represents the bZIP domain, and a circled P represents the potential protein kinase A phosphorylation site) and their phenotypes as determined by the complementation assay. E, *Eco*RI; X, *Xba*I; H, *Hin*dIII; K, *Kpn*I. (B) Growth of *ACR1* or *acr1* strains containing the indicated YCp88 plasmid in the presence of various concentrations of AT.

cAMP-dependent protein kinases play a critical role in regulating the activities of some mammalian ATF/CREB proteins (14, 15, 40) as well as yeast ADR1 (7). To assess whether disruption of the cAMP signaling pathway in yeast cells leads to altered levels of transcription mediated through ATF/CREB sites, we inactivated *BCY1* (52), a gene encoding the regulatory subunit of the three cAMP-dependent protein kinases, TPK1, TPK2, and TPK3 (53). As shown in Fig. 8, transcription from the *his3-303* promoter appears to be very slightly elevated (twofold when normalized to the *DED1* internal control RNA) in a strain containing a *bcy1*::*URA3* disruption allele.

Biochemical identification of ATF/CREB-like proteins distinct from ACR1. We had demonstrated previously that yeast cell extracts contain multiple ATF/CREB-like DNA-binding activities that can be distinguished by chromatography on heparin-agarose (42). To determine whether these activities were distinct from that of ACR1, we examined chromatographic fractions from an *acr1* deletion strain for ATF/CREB activities by the electrophoretic mobility shift assay. Two major species that bind to an ATF/CREB site (Fig. 9) but not to the TGAGCTCA double mutant site (data not shown) were observed, providing biochemical evidence for additional yeast ATF/CREB-like proteins distinct from ACR1. A comparison of ATF/CREB binding activities in *acr1* extracts with those in isogenic *ACR1* wild-type extracts (42) reveals no significant differences with respect to the electrophoretic mobilities of the protein-DNA complexes and to the KCl concentrations necessary for elution from the heparin-agarose column. However, we cannot determine whether these *ACR1*-independent ATF/CREB activities are

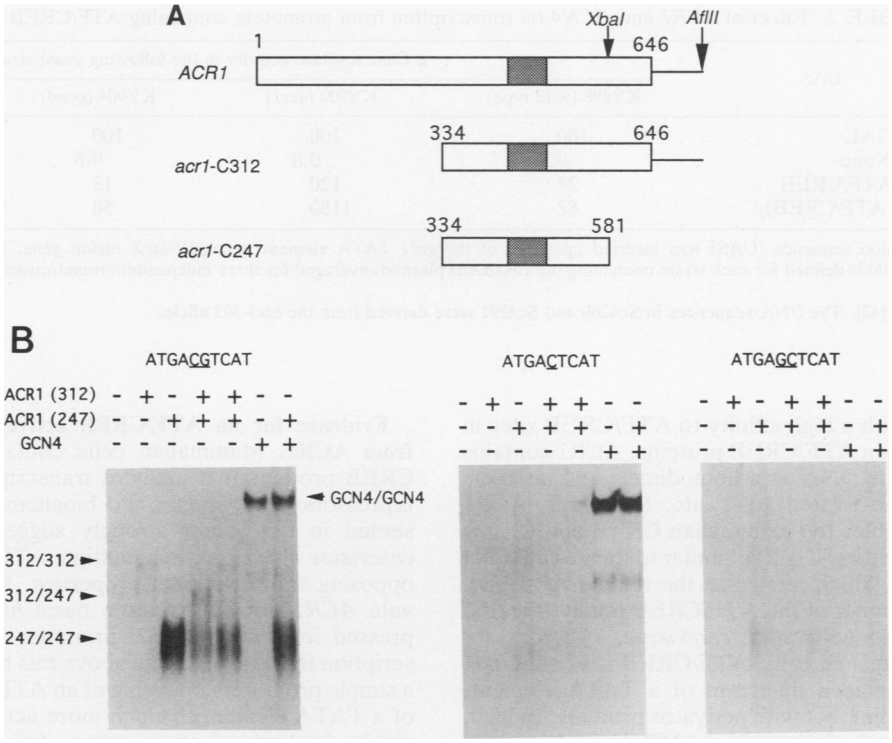

FIG. 7. ACR1 binds to ATF/CREB sites in vitro. (A) Structures of the ACR1 derivatives (amino acid residues are indicated, and the bZIP domain is shown by the shaded box) produced by transcription and translation in vitro (*Xba*I and *Afl*II sites were used to generate DNA templates). (B) The indicated ^{35}S-labeled proteins were incubated with ATF/CREB (ATGACGTCAT), AP-1 (ATGACTCAT), and double mutant (ATGAGCTCAT) sites to generate the indicated homodimeric or heterodimeric protein-DNA complexes. For the pairs of lanes containing both the 312- and 247-residue ACR1 proteins, the left lane represents cosynthesis, whereas the right lane represents mixing after independent synthesis.

encoded by one or more different genes or represent differentially modified or proteolytic products of a single gene.

DISCUSSION

ACR1, a yeast ATF/CREB repressor. Previously, we suggested that yeast cells contain an ATF/CREB repressor protein because an ATF/CREB site can reduce transcription in the context of the *his3* promoter (42). Using genetic selection based on this observation, we obtained mutations in a single gene, *ACR1*, that relieve the repression mediated specifically through ATF/CREB sites. This transcriptional effect is very likely to be direct because *ACR1* encodes a

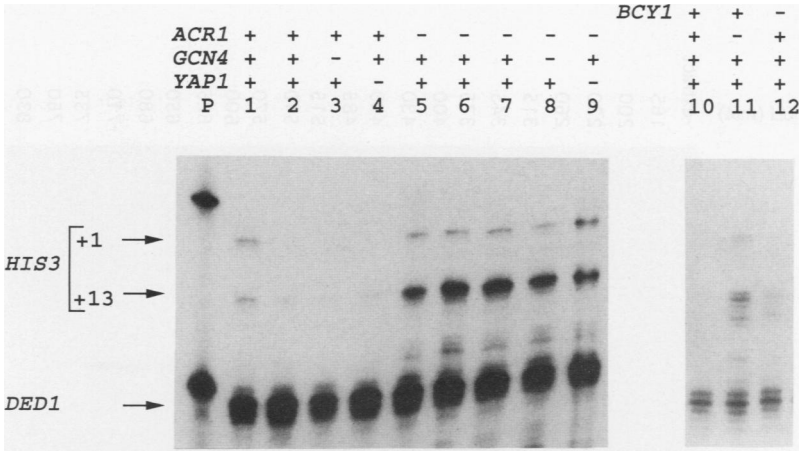

FIG. 8. Effect of *ACR1*, *GCN4*, *YAP1*, and *BCY1* on transcription from the *his3-303* promoter. Total RNA samples (25 μg) from the following strains were hybridized to completion with an excess of *HIS3* and *DED1* oligonucleotide probes (lane P), and the products were treated with S1 nuclease: 1, KY114 (*HIS3*); 2, KY898; 3, KY904; 4, KY906; 5, KY900; 6, KY902; 7, KY908; 8, KY905; 9, KY907; 10, KY898; 11, KY900; 12, KY909. The positions of the *DED1* and the *HIS3* +1 and +13 transcripts are indicated. Samples in lanes 10 to 12 were analyzed independently from samples in lanes 1 to 9, and the autoradiogram was exposed for a shorter time.

TABLE 2. Effect of *ACR1* and *GCN4* on transcription from promoters containing ATF/CREB sites[a]

Plasmid DNA[b]	UAS	β-Galactosidase activity in the following yeast strain			
		KY898 (wild type)	KY900 (*acr1*)	KY904 (*gcn4*)	KY905 (*acr1 gcn4*)
LR1Δ20B	GAL	100	100	100	100
ΔUAS/βG	None	0.3	0.8	0.6	0.3
Sc4289	ATF/CREB	24	120	13	90
Sc4291	$(ATF/CREB)_2$[c]	82	1180	50	510

[a] The upstream activation sequence (UAS) was inserted upstream of the *gal1* TATA element in a *gal1-lacZ* fusion gene. Data represent the relative β-galactosidase activity (100 is defined for each strain containing the LR1Δ20B plasmid) averaged for three independent transformants grown in glucose minimal medium lacking uracil.

[b] Described previously (42). The DNA sequences in Sc4289 and Sc4291 were derived from the *his3-303* allele.

[c] Two sites.

protein that binds with a high affinity to ATF/CREB sites in vitro. Like mammalian ATF/CREB proteins, ACR1 contains a bZIP domain, binds DNA as a homodimer, and interacts very poorly with the related AP-1 site. Moreover, ACR1 most strongly resembles the mammalian CREB and CREM transcriptional regulators; it is less similar to the yeast GCN4 and YAP1 proteins, which recognize the related AP-1 site. Thus, ACR1 is a member of the ATF/CREB family, the first such protein to be cloned from *S. cerevisiae*.

In yeast and mammalian cells, ATF/CREB sites stimulate transcription when placed upstream of a TATA element, suggesting that they interact with activator proteins. Indeed, CREB stimulates transcription in a cAMP-dependent manner (14), and ATF2 is necessary for transcriptional induction by the adenovirus E1A oncoprotein (31). In contrast, ACR1 is clearly a transcriptional repressor because deletion of the gene causes increased transcription from promoters containing ATF/CREB sites. In this sense, ACR1 appears to be functionally related to CREM, a recently identified mammalian protein that antagonizes activation through ATF/CREB sites mediated by CREB and perhaps other family members (12). It is important to stress, however, that we have only assayed ACR1 function on the *his3-303* promoter and the artificial promoter containing an ATF/CREB site(s) upstream of the *gal1* TATA element and initiation region. Thus, we cannot exclude the possibility that ACR1 may also function as a transcriptional activator in other promoter contexts.

Evidence for an ATF/CREB activator protein(s) distinct from ACR1. Mammalian cells contain a family of ATF/CREB proteins that includes transcriptional activators and repressors. The genetic and biochemical experiments presented in this paper strongly suggest that the yeast *S. cerevisiae* also expresses multiple ATF/CREB proteins with opposing transcriptional properties. Mutations that inactivate *ACR1* not only restore basal *his3* mRNA levels expressed from the *his3-303* promoter but also induce transcription from the +13 site above this basal level. Moreover, a simple promoter consisting of an ATF/CREB site upstream of a TATA element is much more active in an *acr1* mutant strain. In both of these cases, transcriptional activation through the ATF/CREB site is independent of GCN4 and YAP1. Thus, yeast cells must contain a transcriptional activator protein(s) that recognizes ATF/CREB sequences but is distinct from all previously characterized bZIP proteins.

This genetic argument is reinforced by the biochemical identification of two chromatographically distinct DNA-binding activities with ATF/CREB-like specificity. As these DNA-binding activities are observed in extracts prepared from *acr1* strains under conditions in which GCN4 expression is extremely low (20, 51), these activities represent as-yet-uncharacterized biochemical species. We cannot determine whether the two activities represent the products of different genes or whether either of them is equivalent to ATF/CREB-like DNA-binding activities that have been ob-

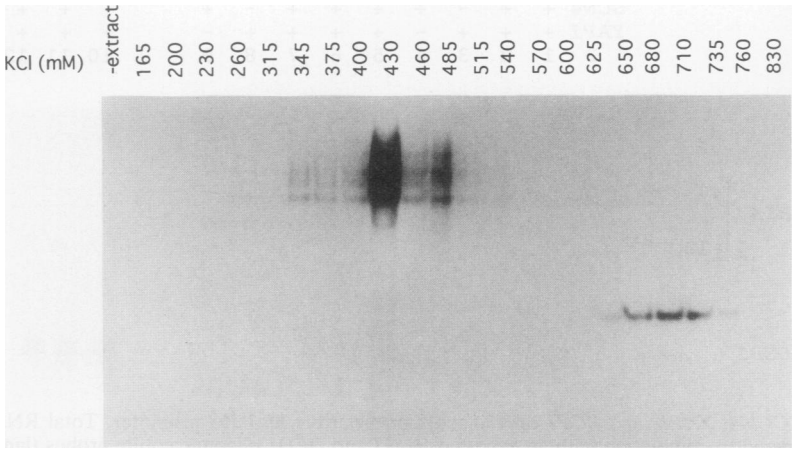

FIG. 9. ATF/CREB binding activities in an *acr1* mutant strain (KY900). Chromatographic fractions eluted from a phosphocellulose column at the indicated KCl concentrations were incubated with an oligonucleotide probe containing the ATF/CREB site from the *his3-303* promoter.

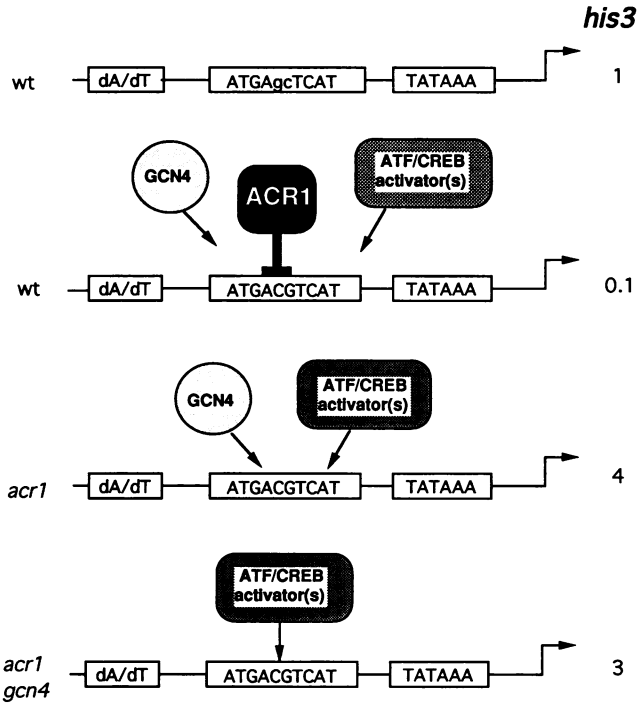

FIG. 10. Transcriptional regulatory proteins acting at ATF/CREB sites. ACR1 represses transcription both by inhibiting the binding of GCN4 and putative ATF/CREB activators and by interfering with the constitutive *his3* promoter, whose activity depends on the poly(dA) · poly(dT) element but not on a functional ATF/CREB sequence. wt, wild type. For each situation, the relative level of *his3* transcription is shown.

served by other laboratories (26, 30). Although we are hesistant to ascribe transcriptional properties to these activities, it seems extremely likely that at least one of them represents the ATF/CREB-like activator(s) that we have detected through genetic means.

The above considerations define DNA-binding and transcriptional activities that interact with ATF/CREB sites. By analogy with mammalian cells, it is likely that proteins possessing these activities are conventional members of the ATF/CREB family with highly conserved bZIP domains. If so, heterodimeric interactions among ACR1, GCN4, and these putative ATF/CREB proteins may increase the complexity of the transcriptional response through ATF/CREB sites. However, it is possible that the proteins defined by our genetic and biochemical experiments are structurally unrelated to conventional ATF/CREB proteins.

Nature of the repression by ACR1. ACR1 appears to repress transcription by at least two distinct mechanisms (Fig. 10). One mechanism involves competition between ACR1 and transcriptional activators for ATF/CREB sites. Competition between ACR1 and putative ATF/CREB proteins explains why ATF/CREB sites are poor upstream activating sequences, and competition between ACR1 and GCN4 accounts for the low levels of transcription from the *his3-303* promoter during amino acid starvation. As expected for a direct competition mechanism, transcriptional output is highly sensitive to changes in the intracellular concentration of repressor and/or activator proteins. For example, heterozygous *ACR1/acr1* diploids are less sensitive to AT than their homozygous *ACR1/ACR1* counterparts, indicating that a twofold reduction in ACR1 concentration reduces repression efficiency. This competition mechanism is analogous to the situation in mammalian cells, in which CREM antagonizes basal and cAMP-stimulated transcription mediated through ATF/CREB sites by CREB (12).

Alternatively, ACR1 can repress transcription mediated by heterologous promoter elements. For example, constitutive *his3* transcription is unaffected by mutations or deletions of the GCN4 binding site but is severely repressed by ACR1 when an ATF/CREB site is located between the poly(dA) · poly(dT) upstream promoter element and the TATA region (42). As initially described for the *Escherichia coli* LexA repressor (4), this repression may simply reflect promoter interference caused by ACR1 binding between the critical promoter elements for constitutive *his3* transcription. However, deletion of as few as 13 amino acids from the C terminus of a truncated ACR1 derivative destroys repressor activity in vivo without impairing DNA-binding activity in vitro. This result suggests that a domain outside the DNA-binding region may be required for repressor function, although we cannot exclude the trivial possibility that these protein derivatives with C-terminal deletions are unstable or not localized to the nucleus. Further analysis of the regulatory interactions at ATF/CREB sites in yeast cells will require biochemical and mutational analyses of ACR1 as well as identification and characterization of the gene(s) encoding the ATF/CREB-like transcriptional activator(s).

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