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The Upstream Region of the *FOX3* Gene Encoding Peroxisomal 3-Oxoacyl-Coenzyme A Thiolase in *Saccharomyces cerevisiae* Contains ABF1- and Replication Protein A-Binding Sites That Participate in Its Regulation by Glucose Repression

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Expression of the *FOX3* gene, which encodes yeast peroxisomal 3-oxoacyl-coenzyme A thiolase, can be induced by oleate and repressed by glucose. Previously, we have shown that induction was mediated by an oleate response element. Just upstream of this element a negatively acting control region that mediated glucose repression was found. In order to study this negative control region, we carried out DNA-binding assays and analyzed phenotypes of mutations in this region and in the *trans*-acting factor CAR80, which is identical to UME6. DNA-binding assays showed that two multifunctional yeast proteins, ABF1 and RP-A, interacted with the negative control element independently of the transcriptional activity of the *FOX3* gene. ABF1 and RP-A, the latter being identical to BUF, were able to bind to DNA independently of one another but also simultaneously. The phenotypes of mutations in either DNA-binding sites of ABF1, RP-A, or both, which affected the DNA binding of these factors in vitro, indicated that these sites and the proteins that interact with them participate in glucose repression. The involvement of the RP-A site in glucose repression was further supported by our observation that the CAR80 gene product, which is required for repression mediated by the RP-A site, was essential for maintenance of glucose repression. In addition to the RP-A site in the *FOX3* promoter, similar sequences were observed in other genes involved in peroxisomal function. RP-A proved to bind to all of these sequences, albeit with various affinities. From these results it is concluded that the ABF1 and RP-A sites are being required in concert to mediate glucose repression of the *FOX3* gene. In addition, coordinated regulation of expression of genes involved in peroxisomal function in response to glucose is mediated by proteins associated with the RP-A site, probably RP-A and CAR80.

The enzyme 3-oxoacyl-coenzyme A thiolase (abbreviated here to thiolase) participates in the β-oxidation of fatty acids, a process that in *Saccharomyces cerevisiae* is exclusively present in peroxisomes (29). Expression of the *FOX3* gene encoding thiolase is strictly controlled in response to cellular demands. In the presence of an inducer of peroxisome proliferation (oleic acid or one of its metabolites), thiolase is produced at high levels (17, 47). In the absence of inducer and glucose, thiolase production is about 10-fold lower, and in the presence of glucose even lower levels of the enzyme were found (17, 47). Induction of thiolase proved to be accompanied by a large increase in the steady-state levels of thiolase-specific mRNA (17). This observation, and those indicating that sequences controlling regulation are situated upstream of the transcribed region, led to the suggestion that induction of thiolase production is primarily regulated at the level of transcription (16, 17).

Transcriptional induction in response to oleate of the *FOX3* gene is mediated by the oleate response element (ORE), consisting of an imperfect invertedly repeated sequence (15). Similar OREs are also present upstream of several other oleate-inducible genes (15, 19). By deletion analysis, a negatively acting region required for glucose repression just upstream of the ORE was identified. Gel retardation experiments have demonstrated that the multifunctional yeast regulatory factor ABF1 (autonomously replicating sequence [ARS] binding factor 1) binds in vitro to a DNA fragment containing this region (16, 17).

ABF1 is an abundant yeast phosphoprotein binding to the consensus DNA sequence 5′-RTCRYNNNNNACG-3′, where R represents purine, Y represents pyrimidine, and N represents any nucleotide. This sequence, first noted to be included in protein-binding DNA segments at different ARSs, has also been identified at many other sites within the yeast genome, in many cases coinciding with upstream activating sequences (UASs; for a review, see reference 9). ABF1 is an essential multifunctional protein; depending on the context of the binding site, it acts as a repressor or an activator, or it is involved in DNA replication (2, 4–6, 8, 10, 13, 21, 22, 27, 28, 34, 35, 43, 46). In order to understand the function of the ABF1 site in regulation of the thiolase gene (*FOX3*), we analyzed this DNA region in more detail.

Here we describe a protein-binding site upstream of *FOX3* which partially overlaps the ABF1 site and shares sequence similarity to the upstream repressing sequence (URS1) in the 5′ region of the *CAR1* gene coding for arginase (33, 45). The *CAR1* URS1 site has been shown to bind to a protein desig-

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nated binding URS1 factor (BUF) (31). The CAR80 protein, which is identical to UME6, is required for URS1-mediated repression of the *CAR1* gene, but CAR80 does not influence DNA-binding activity of BUF (40). BUF is an essential protein binding to positively acting UAS elements as well as negatively acting regulatory sequences upstream of a wide variety of genes in *S. cerevisiae* (32, 33, 45). BUF is a hetero-oligomer, and two of the genes encoding proteins in this complex (BUF1 and BUF2) have been cloned and sequenced (31, 32). The deduced protein sequences were identical to the hetero-oligomer replication protein A (RP-A) (also designated replication factor A [RF-A]) studied by Heyer and coworkers (25) and Brill and Stillman (3) as a component of the DNA replication apparatus.

By DNase I footprint analysis and gel retardation assays, we now show that the RP-A (BUF) and ABF1 proteins bind to the region involved in glucose repression of the *FOX3* gene. Sequence comparison revealed the presence of URS1-homologous sites in several other 5′-flanking regions of genes encoding peroxisomal proteins. These sites were also studied for their abilities to bind RP-A. Finally, we describe the effects of mutating either the ABF1 or the RP-A or both binding sites in the *FOX3* gene and demonstrate that the CAR80 gene product is required for the negative regulation of *FOX3.*

MATERIALS AND METHODS

Strains and culture conditions. In this study *S. cerevisiae* BJ1991 (α *leu2 trp1 ura3-52 prb1-1122 pep4-3*) was used routinely unless stated otherwise. Furthermore, *S. cerevisiae* TCY15 (**a** *lys5 ura3*) and HCY12 (**a** *car80 ura3*) were used (40), as were *S. cerevisiae* W303-1A (**a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and H174 (**a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-d1*::*LEU2*), which were kindly provided by H. Ronne (38). *Escherichia coli* DH5α (*recA hsdR supE endA gyrA96 thi-1 relA1 lacZ*) and yeast cells were transformed and cultured as described before (17).

Plasmids. Plasmids yTL985, yTL203, pTL985, pBT87, and pBT42 were described earlier (15, 17). Site-directed mutagenesis of either the ABF1 site or the RP-A site in the *FOX3* promoter region was carried out with the P-select mutagenesis system (Promega) in a manner similar to that previously described for other *FOX3* promoter mutants (17), with oligonucleotide M3 (5′-GTGTA AGGCAAGCTATCAAAG-3′), oligonucleotide M4 (5′-GTAATGATGTGGTG GGCCCGTGTAAGGC-3′), or M5 (5′-CCCTTTGATAGCTGCCTTACCCG GGCCCACCACATCATTAC-3′). This resulted finally in the construction of centromeric shuttle vectors yTLabf985, yTLrpa985, and yTLdm985 containing, respectively, 985 nucleotides of the *FOX3* promoter sequence in which either the ABF1 site or the RP-A site or both sites are mutated. To be able to assay promoter activity, these mutated promoter sequences are hooked up to a luciferase reporter as described for the wild-type promoter construct yTL985 (17). The mutations contained in these plasmids were confirmed by sequence analysis.

Plasmids pBT42abf (with a mutant ABF1 site) and pBT42rpa (with a mutant RP-A site) were constructed as described for the wild-type pBT42 (17); DNA fragments containing either the mutant ABF1 or the RP-A site were amplified by PCR with plasmids yTLabf985 and yTLrpa985 as templates and cloned into plasmid Bluescript M13 (Stratagene). The sequences of the DNA fragments after PCR amplification were verified by sequence analysis.

DNA-binding assays. For the gel retardation assays, DNA fragment SE1 (−238 to −196 [see Fig. 2]), containing the wild-type ABF1- and RP-A-binding sites, was isolated after digestion of plasmid pBT42 with endonucleases *Eco*RI and *Xho*I. In a similar manner, DNA fragments containing either the mutant ABF1 site or mutant URS1-homologous site were isolated from plasmids pBT42abf and pBT42rpa, respectively. For the DNase I footprint, the 127-bp *Xho*I-*Eco*RI DNA fragment from plasmid pBT87, which was end labeled at the *Eco*RI site, was used. Labeling of the DNA fragments and preparation of the crude protein extracts have previously been described (17). ABF1, produced in *E. coli*, was kindly provided by H. Halfter (21).

DNA-binding assays were performed under a variety of conditions. Typically, 1 to 10 μg of yeast crude protein extract or 0.25 μg of extract from a bacterial culture producing ABF1 was preincubated with 0.4 μg of pEMBL9 and 0.1 μg of poly(dI-dC) (Sigma) for 10 min at 30°C in a 40-μl volume containing 4 mM Tris-HCl (pH 8.0), 40 mM NaCl, 4 mM $MgCl_2$, 4% glycerol, and 1 mM phenylmethylsulfonyl fluoride. Next, a total of 0.1 to 1 ng of a labeled DNA fragment was added and further incubated at 30°C for 10 min. Samples were subsequently loaded onto a 4% vertical polyacrylamide gel and electrophoresed at 135 V for 2 to 4 h in Tris-borate-EDTA buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA) at 4°C. Gels were treated for 10 min with 10% acetic acid and dried, after which autoradiographs were prepared.

For competition assays, a 10- to 100-fold molar excess of unlabeled competitor DNA over probe was added prior to the addition of crude protein extract. The total amount of DNA present in the reaction mixture was kept constant, at 0.5 μg. As competitors, the double-stranded oligonucleotides 40-1/40-2 and 40-3/40-4, containing the wild-type and mutant ABF1-binding sites of the *QCR2* gene, respectively, were used, as described by Dorsman and colleagues (14).

DNA fragments containing ABF1 sites of the *S33*, *QCR2*, and *CYC1* genes or of the *TRP1-ARS1* sequence were isolated from plasmids pUCS33-Taq-Taq, pEMBL9-Δ24, p9-CYC1-II, and YRp7, respectively, and were used as competitors in the gel retardation analysis, as described by Dorsman and colleagues (14 [and references therein]).

Gel retardation analyses with purified RP-A (BUF) were carried out as follows. Purified RP-A was obtained as previously described (31). The reaction mixtures contained approximately 50 ng of the DNA fragment, which served as the insert for plasmid pRL58 (33). The fragment was labeled by filling in with Klenow fragment and [^{32}P]dCTP. The reaction mixtures also contained (at final concentrations) 4 mM $MgCl_2$, 150 mM KCl, 5% glycerol, 100 mM NaCl, 4 mM Tris-HCl (pH 7.9), 1 mM dithiothreitol, and a 150-fold excess of sonicated calf thymus DNA. Protein-containing lanes contained 2 μl of purified RP-A. Reaction volumes were kept constant at 22 μl, regardless of the amount of competitor DNA present. For competition analyses, up to a 20-fold molar excess of unlabeled double-stranded oligonucleotides (Table 1) was used. The binding reactions were performed at room temperature for 20 min. The samples were then loaded onto a 4% preelectrophoresed polyacrylamide gel (in Tris-borate-EDTA buffer) and electrophoresed at 10 V/cm at room temperature for 3.5 h. The gels were then dried, and autoradiographs were prepared.

DNase I footprint analysis. The standard reaction mixtures as described for the gel retardation assay were scaled up fourfold. Purified ABF1, which was kindly provided by H. Halfter, was produced in and purified from *E. coli* as described by Halfter and coworkers (21). Purified ABF1 (1 to 2 μg) was incubated with the 127-bp *Xho*I-*Eco*RI DNA fragment from plasmid pBT87 which had been labeled at the *Eco*RI site with Klenow large fragment and [α-^{32}P]dATP. After the binding reaction had been completed, the DNA was digested with empirically determined amounts of DNase I for 1 to 2 min at 30°C. The reaction was stopped by the addition of EDTA (final concentration, 10 mM). Samples were phenol extracted, ethanol precipitated, and analyzed by electrophoresis on a 6% polyacrylamide gel containing 6 M urea in Tris-borate-EDTA buffer. Gels were treated for 10 min with 10% acetic acid and dried, after which autoradiographs were prepared. As a marker, the sequence of the thiolase promoter was determined with pTL985 as a template.

Miscellaneous. Luciferase assays were performed as previously described (17). Activities are given (in percentages) as relative light units (RLU) per microgram of protein extract mediated by the wild-type promoter induced by oleate or in arbitrary units relative to the glucose-repressed wild-type promoter activity. Protein concentrations were measured by the method of Bradford (1), with bovine serum albumin as a standard. All cloning strategies were based on standard methods (42). Oligonucleotides M3, M4, and M5 were obtained from Pharmacia. Restriction enzymes were obtained from Bethesda Research Laboratories, Boehringer Mannheim, and New England Biolabs. DNase I, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were from Pharmacia. DNA polymerase I was from Bethesda Research Laboratories. [α-^{32}P]dATP (800 Ci/mmol) and [γ-^{32}P]ATP (3,000 Ci/mmol) were from New England Nuclear. Sequence analysis was carried out by the dideoxy chain termination method (42) with the Sequenase kit from United States Biochemicals.

RESULTS

Two factors interact with the negatively acting control region upstream of the *FOX3* gene that participates in glucose repression. In order to identify the *trans*-acting factors involved in glucose repression of *FOX3* gene expression, a gel retardation analysis (Fig. 1A) was performed with a small DNA fragment, SE1 (−238 to −196), containing the putative upstream repressing sequence (URS) that had been identified previously (15). Two complexes, C1 and C2, were observed when crude extracts from cells grown on glucose-containing medium were used (Fig. 1A, lanes 2 and 3). We have previously shown that ABF1 protein specifically binds to the DNA fragment SE1 (17), and to ascertain which of the two complexes was ABF1 specific, we performed a series of competition experiments.

It has been shown by Dorsman and coworkers (14) that in *S. cerevisiae*, binding sites for ABF1 are present in the 5′ region of the *QCR2* gene which encodes the 40-kDa subunit of the QH2:cytochrome *c* oxidoreductase, in the ribosomal protein *S33* gene, in the *CYC1* gene, and in the *TRP1-ARS1* region. DNA sequences from these four genes were therefore used as

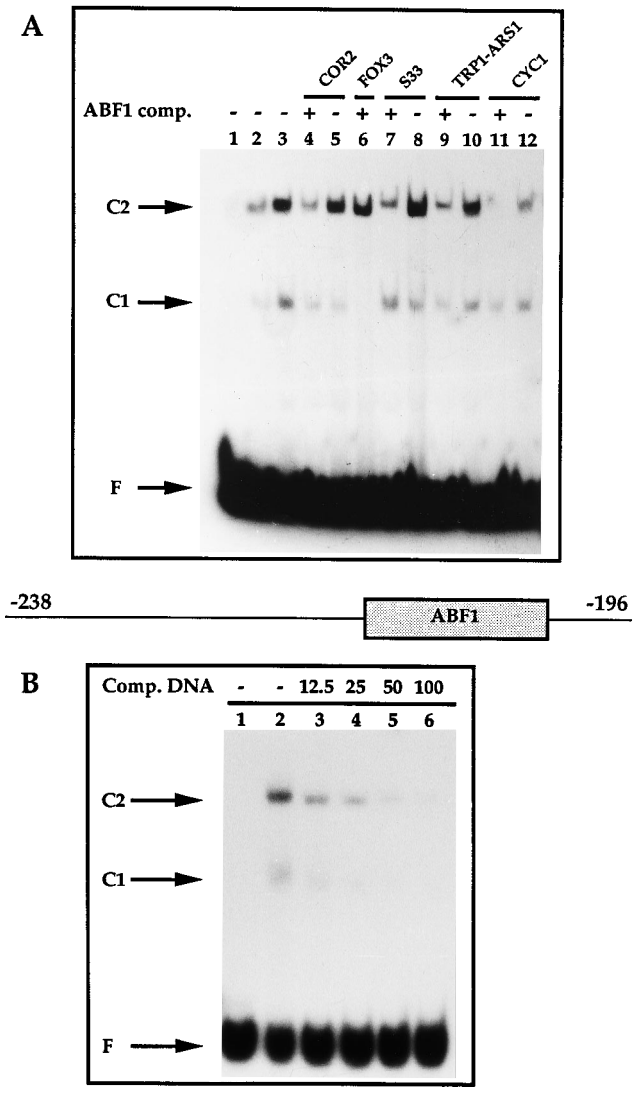

FIG. 1. Gel retardation competition studies identifying two protein-DNA complexes (C1 and C2), including the ABF1-DNA complex. (A) DNA fragment SE1 (−238 to −196) is illustrated at the bottom. The reaction mixture resolved in lane 1 was devoid of cell extract. The reaction mixtures resolved in lanes 2 and 3 contained, respectively, 1 and 2 μg of cell extract obtained from *S. cerevisiae* BJ1991 cells grown in the presence of 5% glucose as described earlier (16, 17). Lanes 1 to 3 did not contain competitor DNA. For the remaining lanes, 2 μg of the same cell extract and the following competitors were used: 40-1/40-2 wild-type and 40-3/40-4 mutant ABF1 double-stranded oligonucleotides derived from the *QCR2* gene (lanes 4 and 5, respectively); unlabeled *FOX3* fragment SE1 (lane 6); unlabeled *S33* gene sequences, either intact or cleaved in the ABF1 site with *Xba*I (lanes 7 and 8); unlabeled sequences of the *TRP1-ARS1* region, either intact or cleaved in the ABF1 site by *Dde*I (lanes 9 and 10); and unlabeled *CYC1* sequences, either intact or cleaved in the ABF1 site by *Tth*111I and *Hin*fI (lanes 11 and 12). Indicated is whether ABF1 competitor DNA was intact (+) or mutated or digested (−) in the ABF1 site. These competitors, added in a 12.5-fold molar excess, should be able (+) or unable (−) to compete for ABF1-DNA complex formation. C1 and C2, protein-DNA complexes 1 and 2, respectively; F, free DNA. (B) DNA fragment SE1 was used as for panel A. The reaction mixture resolved in lane 1 was devoid of cell extract. The remaining reaction mixtures contained 1 μg cell of extract with no competitor DNA (lane 2) or with a 12.5-, 25-, 50-, or 100-fold molar excess of unlabeled *FOX3* fragment SE1 added (lanes 3, 4, 5, or 6, respectively). C1 and C2, protein-DNA complexes 1 and 2, respectively; F, free DNA.

specific competitors for ABF1 binding (Fig. 1A). Double-stranded oligonucleotides containing the wild-type and mutant ABF1-binding sites of the *QCR2* gene were able and unable, respectively, to compete for formation of the C2 complex (Fig. 1A, lanes 4 and 5). DNA fragments containing the ABF1-binding sites in the *S33* gene, in the *TRP1-ARS1* region, or in the *CYC1* gene were all able to compete for C2 complex formation (Fig. 1A, lanes 7, 9, and 11). Binding was specific for ABF1, as indicated by the fact that in each case competition was eliminated by restriction cleavage within the various ABF1 sites (Fig. 1A, lanes 8, 10, and 12). However, of all these yeast competitors, none significantly competed for complex C1, suggesting that C1 is not a degradation product of C2. Only a 12.5-fold molar excess of nonradioactive *FOX3* DNA fragment SE1 (−238 to −196) was able to compete for C1, indicating that the interaction was specific (Fig. 1A, lane 6). Remarkably, this amount of *FOX3* competitor DNA did not compete for the formation of complex C2. However, competition with increasing amounts of *FOX3* competitor DNA drastically reduced both C1 and C2 complex formation (Fig. 1B). The order of competition for the formation of C1 and C2 complexes indicated either that the protein(s) giving rise to complex C1 is present in a lower amount than ABF1 or that ABF1 has a lower affinity for the DNA fragment. Taken together, these results suggested that at least two factors, one of which is ABF1, bind to *FOX3* DNA fragment SE1 if extracts from cells grown on glucose are used.

Two *URS*-binding factors simultaneously interact with *FOX3* DNA and do so whether or not the gene is transcriptionally active. To locate the exact position of the ABF1-binding site, a DNase I footprint analysis with purified ABF1 protein from *E. coli* was carried out (Fig. 2). ABF1 bound to a region covering the sequence 5′-AGCGCCTTACACG-3′ on the noncoding strand, which resembles the 5′-RTCRYYYNN NACG-3′ consensus sequence of a strong ABF1 site (14).

To determine whether growth conditions affected the abilities of the two factors to interact with the DNA fragment SE1, a gel retardation analysis was performed with crude extracts taken from cells grown on glucose (repression), glycerol (derepression), and oleate (induction) (Fig. 3). When a large amount (10 μg) of crude extract taken from cells grown on glucose was used, a third complex (C3) which migrated more slowly than complex C2 appeared (Fig. 3, lane 1). Similar gel retardation patterns were detected with extracts taken from cells grown on glycerol or oleate (Fig. 3, lanes 2 and 3). These results suggested that the observed DNA-binding activities are present under all conditions tested, independent of the transcriptional activity of the *FOX3* gene.

Competition with a 12.5-fold molar excess of nonradioactive fragment SE1, with extracts obtained from cells grown on glucose, glycerol, or oleate, caused the disappearance not only of complex C1 but also of complex C3 (Fig. 3, lanes 4 to 6). If the factors giving rise to complexes C1 and C2 bind in a mutually exclusive fashion, this result would suggest that the appearance of complex C3 when large amounts of crude extracts are used is due to oligomerization of the factor giving rise to complex C1. However, since competition for complex C1 does not increase the formation of complex C2 and vice versa (Fig. 3, lanes 4 to 6, and Fig. 1, lanes 4, 7, 9, and 11), it is more likely that the factors giving rise to complexes C1 and C2 do not bind in a mutually exclusive manner but instead bind simultaneously to one DNA molecule, leading to the formation of complex C3.

RP-A binds to the *FOX3* region involved in glucose repression. To identify the protein(s) giving rise to complex C1, the SE1 fragment sequence was searched for protein-binding sites

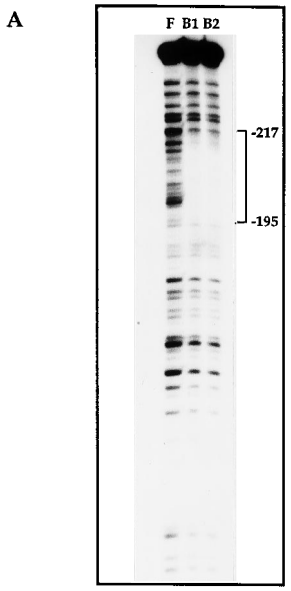

FIG. 2. Characterization of the ABF1-binding site. (A) The *Xho*I-*Eco*RI *FOX3* DNA fragment, 3′ end labeled at the *Eco*RI site, was used for the DNase I protection analysis. Lane F, DNase I digestion of free DNA; lanes B1 and B2, DNase I digestion of DNA bound to the ABF1 activity (1 and 2 μg, respectively, of bacterially produced ABF1 was used). As a marker, the sequence of the *FOX3* promoter was determined (data not shown). Protected nucleotides between −217 and −195 of the upper strand are indicated by the bracket. (B) Diagram representing ABF1 protected sequences of the upper strand of the DNA sequence located upstream of the *FOX3* gene. The numbering of the nucleotides is relative to the translational start codon. The sequence motif resembling the ABF1 consensus site (14) is located between −213 and −201. Nucleotides protected by ABF1 against DNase I digestion are indicated by the bracket. A schematic representation of the DNA fragment SE1 (−238 to −196) used in the gel retardation analyses is shown underneath the sequence.

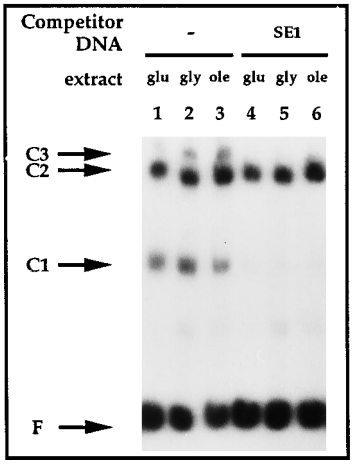

FIG. 3. Factors interacting with *FOX3* DNA fragment SE1 are present in crude extracts from yeast cells grown on media containing glucose, glycerol, or oleate. ^{32}P-labeled DNA fragment SE1 (Fig. 1A) was used. The reaction mixtures resolved in all lanes contained 10 μg of cell extract obtained from cells grown on glucose (lanes 1 and 4), glycerol (lanes 2 and 5), or oleate (lanes 3 and 6). Three specific complexes (C1, C2, and C3) are apparent when incubated without the addition of nonradioactive fragment SE1 (lanes 1 to 3). Competition for C1 and C3 occurred when unlabeled fragment SE1 (12.5-fold molar excess) was added to the reaction mixtures (lanes 4 to 6). F, free labeled DNA.

which had been reported in the literature. The putative MIG1-binding site located upstream of and partially overlapping the ABF1 site which we found (Fig. 4A) proved to resemble the consensus MIG1-binding site described by Nehlin and coworkers (38). In *S. cerevisiae*, MIG1 (multicopy inhibitor of GAL gene expression) is a DNA-binding repressor protein which is at least in part responsible for glucose repression of the *SUC2* and the *GAL1-GAL10* genes (39). GC-rich binding sites for MIG1 have been found upstream of the *SUC2* gene (39), the *GAL1-10* genes (38), and the *GAL4* gene (20, 38). Because MIG1 is a DNA-binding protein involved in glucose repression of several yeast genes, we reasoned that MIG1 might be involved in glucose repression of the *FOX3* gene by binding to DNA near the ABF1-binding site. To test whether MIG1 might be involved in glucose repression, we compared expression in a *mig1*$^-$ strain (H174) and its isogenic wild-type strain (W303-1A) mediated by the wild-type (yTL985) thiolase promoter with the firefly luciferase cDNA as a reporter on centromeric plasmids as described earlier (17) (Fig. 4B).

Using the luciferase gene as a reporter, we set up a sensitive assay system to measure promoter activity of the *FOX3* gene. In crude extracts of *S. cerevisiae*, we were able to measure the activity of as low as 3×10^5 molecules (5×10^{-19} mol) of luciferase. We assayed the luciferase activities in crude extracts prepared from transformed cells grown on media containing glucose, glycerol, or oleate. Activity is given relative to the activity measured in oleate-induced wild-type W303-1A cells transformed with construct yTL985 containing the wild-type upstream region. Luciferase activity dropped to 9.1 and 0.1% when these transformants were grown on glycerol and glucose, respectively. Even activities under glucose-repressed conditions (46,000 RLU/μg of protein) are more than 400-fold above the background levels, making this assay system very suitable to study expression under glucose-repressed conditions. As illustrated in Fig. 4B, the expression mediated by the intact *FOX3* promoter fused to the luciferase reporter in a *mig1*$^-$ strain (H174) did not significantly differ from that observed with the isogenic wild-type strain (W303-1A), indicating that MIG1 is not involved in the regulation of *FOX3* transcription.

Further comparison of the nucleotide sequences within this region of the *FOX3* gene with previously identified protein-binding sites revealed a resemblance to the GC-rich RP-A (BUF)-binding site (URS1) present in the 5′ region of the *CAR1* gene encoding arginase, as is shown in Fig. 5 (33, 45). To ascertain whether RP-A can bind to the *FOX3* sequence, the *FOX3* DNA fragment SE1 containing the URS1-homologous site was used as a probe in a gel retardation analysis. Purified RP-A gave a strong signal in this assay (Fig. 6A, lane 6), and no other complexes were detectable (data not shown). Competition analyses revealed that the RP-A-binding site in the *CAR1* URS1 was able to compete for binding of RP-A to the *FOX3* DNA fragment SE1 (Fig. 6A, lanes 1 to 5), further supporting the argument that RP-A binds specifically to the *CAR1* URS1-homologous site on that DNA fragment. The reverse experiment in which the *CAR1* URS1 was used as probe and a *FOX3* double-stranded oligonucleotide containing the URS1-homol-

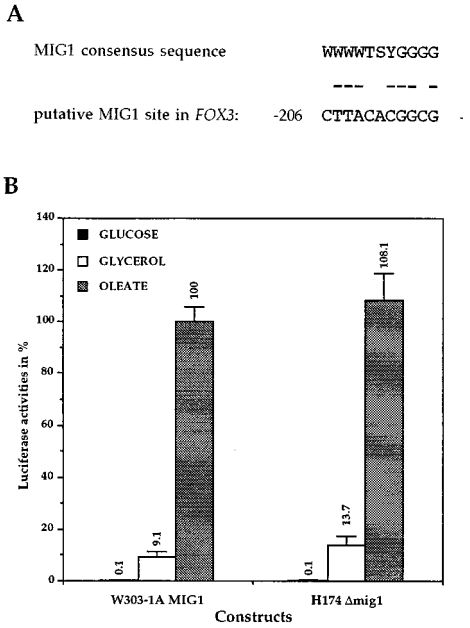

FIG. 4. Expression mediated by the *FOX3* promoter in MIG1 wild-type and mutant yeast strains. (A) Comparison between the MIG1 consensus sequence (38) and a site between −206 and −216 on the noncoding strand of the *FOX3* gene. W = A or T, S = G or C, and Y = C or T. Homologous nucleotides are indicated by a dash. (B) Luciferase activities in crude extracts of MIG1 wild-type cells (W303-1A MIG1) and *mig1* mutant cells (H174 Δmig1) transformed with construct yTL985. Cells were cultured in media containing glucose, glycerol, or oleate as the sole carbon source. The luciferase activity measured in crude extracts of the yTL985 transformants shifted to oleate was taken as 100%. Even expression levels under glucose-repressed conditions (46,000 RLU/μg of crude extract) were more than 400-fold higher than background levels (100 RLU/μg of crude protein extract).

ogous site was used as a competitor (see *FOX3* in Table 1) demonstrated that RP-A binding to the *CAR1* URS1 was decreased by the addition of increasing amounts of the *FOX3* sequence (Fig. 6D, lanes 8 to 13). Even a similar *FOX3* sequence with mutations in the ABF1 site (see *FOX3** in Table 1) was as effective a competitor as the *FOX3* wild-type sequence (Fig. 6F, lanes 8 to 13), indicating that the ABF1 site does not influence RP-A binding. A mutant competitor DNA fragment derived from the *CAR1* UAS_{C2} region of the gene (fragment BS52/53 [32]) was not an effective competitor, attesting to the specificity of the assay (Fig. 6A, lanes 8 to 13).

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CAR1         TAG CCGCC GRRR
             --- ----   - -
FOX1  -260 TAG CCGCT AATG -271
             --  ----- ----
FOX2  -223 TAA CCGCC GAGA -234
             ---  ---- - -
FOX3  -219 TAG -CGCC GTGT -209
             --- ----   ---
CTA1  -250 TAG CCGCG CAAG -239
             --- ----   -
PAS1  -108 TAG CCGCA ATAT  -96
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FIG. 5. Homology between the URS1 of the *CAR1* gene and upstream sequences of various yeast genes involved in peroxisomal function. The *CAR1* URS1 sequence was described by Sumrada and Cooper (45). R = purine. The palindromic sequence 5′-AGCCGCCGA-3′ has been shown by mutational analysis to be important for *CAR1* URS1 function (33). Dashes indicate homologous nucleotides. Sequences are *FOX1* encoding the yeast acyl-coenzyme A oxidase (12), *FOX2* encoding the yeast multifunctional enzyme (26), *FOX3* encoding the yeast thiolase (17), *CTA1* encoding the peroxisomal catalase A (7), and *PAS1* encoding a protein involved in peroxisome assembly (18).

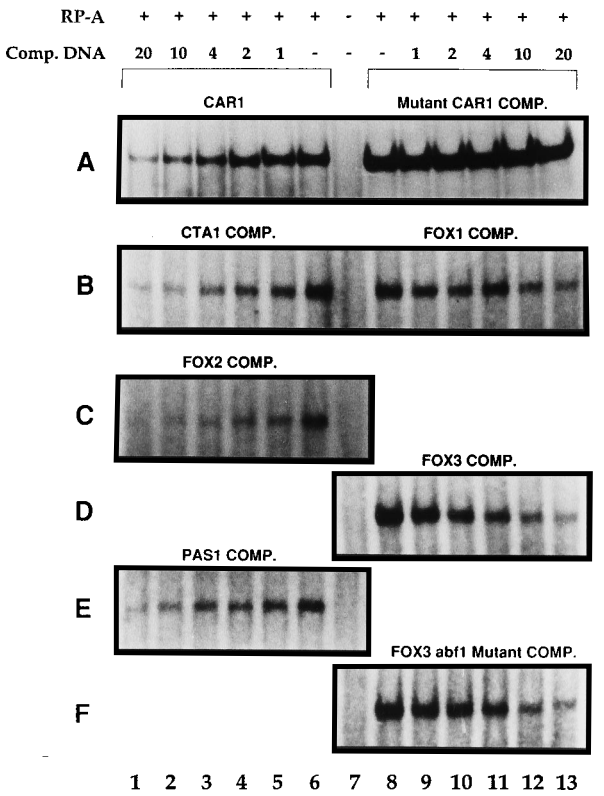

FIG. 6. Gel retardation assays with purified RP-A. Shown are competitions with various DNA fragments derived from genes involved in peroxisomal biogenesis with a DNA fragment carrying the *CAR1* URS1 element. In each lane, 50 ng of the insert of plasmid pRL58 containing the *CAR1* URS1 element (33) was used as a probe, except in panel A, lanes 1 to 7, in which 50 ng of end-labeled *FOX3* fragment was used as the probe. Affinity-purified RP-A was the source of protein in these competition reactions (+). Lanes 7 contained no RP-A (−), while lanes 6 to 8 contained no competitor DNA (−). The genes from which the competitor oligonucleotides were derived are indicated, and their sequences are shown in Table 1. Lanes 5 to 1 and 9 to 13 contained increasing amounts (in micrograms) of competitor DNA, as indicated. The mutant *CAR1* competitor assayed in panel A, lanes 9 to 13 (BS52/53), has previously been described (32).

RP-A binds to multiple yeast genes involved in peroxisomal function. *CAR1* URS1-homologous sequences (with consensus 5′-TAGCCGCCGRRR-3′) have been reported in the upstream region of the yeast peroxisomal catalase A gene and 13 other unrelated genes (45). This prompted us to search for similar sequence motifs in other genes involved in peroxisomal function. Comparison of promoter sequences of yeast genes encoding either peroxisomal proteins or a protein involved in peroxisome assembly revealed that the URS1-homologous sequence motif was commonly found in genes involved in the biogenesis of peroxisomes (Fig. 5). Gel retardation assays with purified RP-A protein demonstrated that double-stranded oligonucleotides containing each of these sites (Table 1) were able to compete for the binding of purified RP-A to a DNA fragment containing the *CAR1* URS1 site, albeit with differing efficiencies (Fig. 6B, C, and E).

Mutations in the ABF1 or RP-A site affect *FOX3* gene expression. To determine whether the two protein-binding sites mediated negative control of *FOX3* expression, we mutated either the ABF1- or the RP-A-binding site or both sites (Fig. 7). The mutation in the ABF1 site increased the spacing between the two conserved elements in the ABF1-binding site. Such a mutation has been shown by Kimmerly and coworkers to result in a drastic reduction of the capacity to bind ABF1

TABLE 1. Oligonucleotides used in band shift assays with purified RP-A

Gene	Sequence[a]
Consensus *CAR1* URS1	TAGCCGCCGRRR
CTA1	TCGAGCGGAATTAGCCGCGCAAGTTGGTGGTCGA
	AGCTCGCCTTAATCGGCGCGTTCAACCACCAGCT
FOX1	TCGAGGGCTATTAGCCGCTAATGACCGAAGTCGA
	AGCTCCCGATAATCGGCGATTACTGGCTTCAGCT
FOX2	TCGAGGGCAAATAACCGCCGAGAGTTCTAGTCGA
	AGCTCCCGTTTATTGGCGGCTCTCAAGATCAGCT
FOX3	TCGAGATGTGGTAGCGCCGTGTAAGGCGCGTCGA
	AGCTCTACACCATCGCGGCACATTCCGCGCAGCT
*FOX3**[b]	TCGAGATGTGGTAGCGCCGTGTAAGcgatGTCGA
	AGCTCTACACCATCGCGGCACATTCgctaCAGCT
PAS1	TCGAGTGTGCGTAGCCGCAATTATAAAGGGTCGA
	AGCTCACACGCATCGGCGTTAATATTTCCCAGCT

[a] Underlining indicates the consensus sequence.
[b] *FOX3**, *FOX3* sequence with four mutations (in lowercase letters) in the ABF1 site.

(27). In the URS1-homologous or RP-A site, three nucleotides were substituted by nucleotides known to affect RP-A binding drastically (33). These mutant DNA-binding sites were then tested for their abilities to bind ABF1 and RP-A, respectively. As shown in Fig. 7A, binding of purified ABF1 to the ABF1 mutant fragment was drastically reduced compared with the binding of ABF1 to the wild-type fragment (Fig. 7A, lanes 1 and 2). The use of crude extracts of cells grown on glucose indicates that RP-A binding was drastically reduced because of the three mutations in the RP-A site (Fig. 7A, lanes 3 and 4). Surprisingly, ABF1 binding was slightly affected by these mutations, which flank the ABF1 consensus binding site. However, it is not without precedent that mutations or deletions flanking this consensus sequence reduce ABF1 binding (23, 36).

To test whether the aforementioned mutations affected expression in vivo after growth of the cells on different carbon sources, we compared expression mediated by the wild-type and mutated thiolase promoters with the firefly luciferase cDNA as a reporter. As illustrated in Fig. 7B, we assayed the luciferase activities in crude extracts of BJ1991 yeast cells transformed with yTL985, yTLrpa985, yTLabf985, or yTLdm985 centromeric plasmid carrying the *FOX3* promoter with, respectively, wild-type RP-A and ABF1 sites, a mutant RP-A site, a mutant ABF1 site, or both mutant sites. Because of the sensitivity of the luciferase assay, the expression levels under glucose-repressed conditions are more than 400-fold above background levels. Mutation of the ABF1 site increased expression on glucose-containing medium 4- to 5-fold; a minor increase (1.5- to 1.8-fold) was observed in the levels of expression on media containing glycerol or oleate (yTLabf985 [Fig. 7B]). A similar result was obtained when the mutation in the RP-A site was analyzed (yTLrpa985), indicating that both sites independently of one another are able to mediate glucose repression. A more drastic relief of glucose repression (18-fold) is obtained when both sites are inactivated (yTLdm985). Thus, both the ABF1 site and the RP-A site act in concert to mediate glucose repression of the *FOX3* gene. Furthermore, the expression mediated by the mutant promoters under derepressed growth conditions (on glycerol or oleate) also increased about twofold, suggesting that the ABF1 and RP-A sites also moderately affect the basal level of expression.

The CAR80 gene product is required for repression mediated by the negatively acting control region of the *FOX3* upstream region. The participation of *CAR1* URS1-homologous sequences and RP-A in the negatively acting control region of *FOX3* predicted that the CAR80 gene product would be required for repression as well. This hypothesis was tested by growing wild-type and *car80* null mutant strains in glucose- and glycerol-containing media and monitoring expression supported by wild-type and *FOX3* mutant reporter plasmids. As shown in Fig. 8, a wild-type *FOX3* reporter plasmid supported very low levels of reporter gene expression under glucose-repressed versus glucose-derepressed conditions when carried in a wild-type strain (plasmid yTL985 in strain TCY15). Reporter gene expression in glucose-containing media increased sixfold when plasmid yTL985 was assayed in the *car80* null mutant strain (HPY12), whereas under glycerol growth conditions, expression increased not more than twofold. When the experiment was repeated with a reporter plasmid with a mutation in the GC-rich sequences forming the RP-A site and carried in wild-type strain TCY15, values were observed to be roughly equivalent to those observed for the wild-type plasmid carried in the *car80* null mutant strain (plasmid yTLrpa985 in strain TCY15). The values increased less than twofold when the experiment was repeated with the *car80* null mutant strain.

To assess the contribution of the ABF1 site to the repression observed, a mutant reporter plasmid with a defective ABF1-binding site was assayed with the wild-type and mutant strains. This plasmid in strain TCY15 supported nearly the same levels of reporter gene expression as did plasmid yTLrpa985 in strain TCY15, which indicates that the ABF1 site played a similar role in the repression process. When mutant plasmid yTLabf985 was assayed with the *car80* null mutant strain, a strong increase (52-fold) in expression of the reporter gene under glucose-growth conditions was observed compared with the level obtained with the wild-type plasmid assayed in the wild-type strain, whereas expression under glycerol growth conditions increased slightly (3-fold). A similar pattern of expression was observed when both sites were mutated or deleted from the *FOX3* promoter, and this pattern of expression was independent of CAR80 function (yTLdm985 and yTL203, respectively, in the wild-type and *car80* mutant strain). Together, these data support the notion that both the ABF1- and RP-A-binding sites are required for the repression observed in glucose medium and that CAR80 protein function was essential for repression mediated via the RP-A site of the *FOX3* gene.

DISCUSSION

From previous deletion mapping it was concluded that the region (−238 to −203) of the *FOX3* gene containing the motif resembling the *CAR1* URS1- and ABF1-binding sites is involved in the repression of transcription in response to glucose (15). By a series of gel retardation analyses and DNase I footprinting (Fig. 1 to 3 and 6), it is shown here that two multifunctional yeast proteins, ABF1 and RP-A (BUF), bind to overlapping sites in the upstream region of the *FOX3* gene. Although the binding sites overlapped, these factors do not seem to bind in a mutually exclusive manner, since competition for RP-A (Fig. 3, complex C1) or mutations in the RP-A site did not increase ABF1 binding, and vice versa (Fig. 1). In contrast, as concluded from Fig. 3, both factors were able to bind simultaneously to one DNA molecule, but they could also

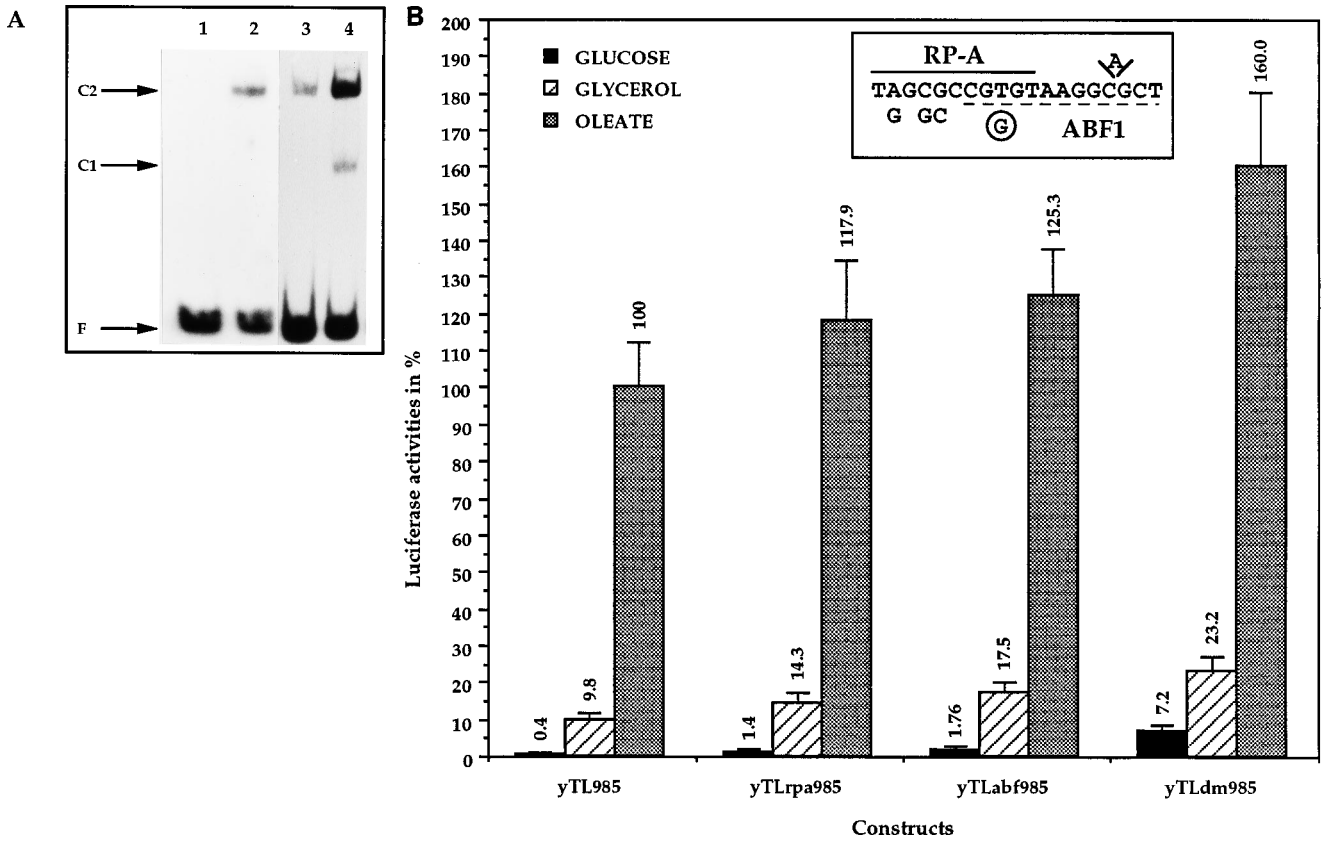

FIG. 7. (A) Gel retardation analysis of *FOX3* DNA fragments containing a mutant RP-A site, a mutant ABF1 site, or wild-type binding sites. *FOX3* DNA fragments containing either the mutant ABF1 site (lane 1) or the wild-type ABF1-binding site (lane 2) were end labeled and incubated with equal amounts of ABF1 produced in bacteria. In lanes 3 and 4, respectively, radioactively labeled *FOX3* DNA fragments containing either the mutant or wild-type RP-A-binding site were incubated with crude extracts obtained from BJ1991 cells grown on 5% glucose. For a detailed description of the mutations, see below. (B) Effects of mutations in either the ABF1 or the RP-A site on expression of the thiolase-luciferase fusion gene. Luciferase activities were assayed in crude extracts of BJ1991 yeast cells transformed with yTL985, yTLrpa985, yTLabf985, or yTLdm985 shuttle vectors carrying, respectively, the wild-type RP-A and ABF1 sites, the mutant RP-A site, the mutant ABF1 site, or both mutant sites. The sequences of the overlapping RP-A and ABF1 sites are shown. These sites are marked by a bar and a dashed line, respectively. The mutations in the RP-A site are indicated underneath its sequence, and the position of the insertion mutation in the ABF1 site is drawn above the sequence. In the double-mutant construct yTLdm985, the above-mentioned mutations in both sites and an additional mutation (indicated by the circle) in the overlapping sequence were made. Transformants were grown on media containing 5% glucose, 2% glycerol, or 0.1% oleate. Luciferase activity measured in extracts of the yTL985 transformants after growth on oleate-containing media was taken as 100%.

bind independently of one another. Furthermore, binding was not significantly altered when *FOX3* transcriptional activity was switched on or off by oleate or glucose, respectively. However, mutation analysis of their respective binding sites demonstrated that both these factors participated in the transcriptional regulation of this gene in response to glucose (Fig. 7). It is conceivable that phosphorylation of both ABF1 and RP-A might determine whether they participate in glucose repression, since ABF1 as well as RP-A has been shown to be phosphorylated (11, 41, 44). However, the widespread occurrence of both the ABF1- and the RP-A-binding sites (9, 32, 45) and the fact that additional proteins such as CAR80 are required for their function (40) indicate that more than just these two factors are involved. In this regard, the transcription activator proteins VP16 and GAL4 have been shown to bind to RP-A and stimulate replication of DNA (24, 30). For ABF1, such proteins have yet to be identified. However, it is interesting to note that ABF1 can induce large DNA bends (37), which could facilitate the formation of specific nucleoprotein complexes.

Data obtained in this study with wild-type or *car80* mutant yeast strains (Fig. 8) carrying wild-type and mutant reporter gene plasmids strongly suggest that both the ABF1 and the RP-A sites and the CAR80 gene product play an important role in the transcriptional repression mediated by this region of the *FOX3* gene promoter. Inactivating either the ABF1 or the RP-A site affects glucose expression levels in the wild-type strain TCY15 moderately (8- to 10-fold), indicating that both sites independently of one another are able to repress to some extent transcription in response to glucose. Therefore, ABF1 and RP-A seem to affect different aspects of glucose repression of the *FOX3* gene. If both sites are inactivated, the drastic increase (61-fold) observed is roughly equivalent to the sum of the effects of both these factors. A similar conclusion can be drawn from Fig. 7B, although the effects in this wild-type strain (BJ1991) are less pronounced. This is the first report of these two sites being required in concert to mediate repression. Furthermore, CAR80 protein function proved to be essential for glucose repression of the *FOX3* gene mediated by RP-A, indicating that CAR80 and RP-A act via the same pathway.

It is noteworthy that the ABF1 and RP-A sites and the CAR80 gene product most likely also affect the basal level of expression of the *FOX3* gene, because mutational analysis revealed a two- to threefold increase in expression under oleate as well as glycerol growth conditions (Fig. 7 and 8). It also has to be remarked that even if the ABF1 and RP-A sites are inactivated or deleted and the CAR80 gene product is inacti-

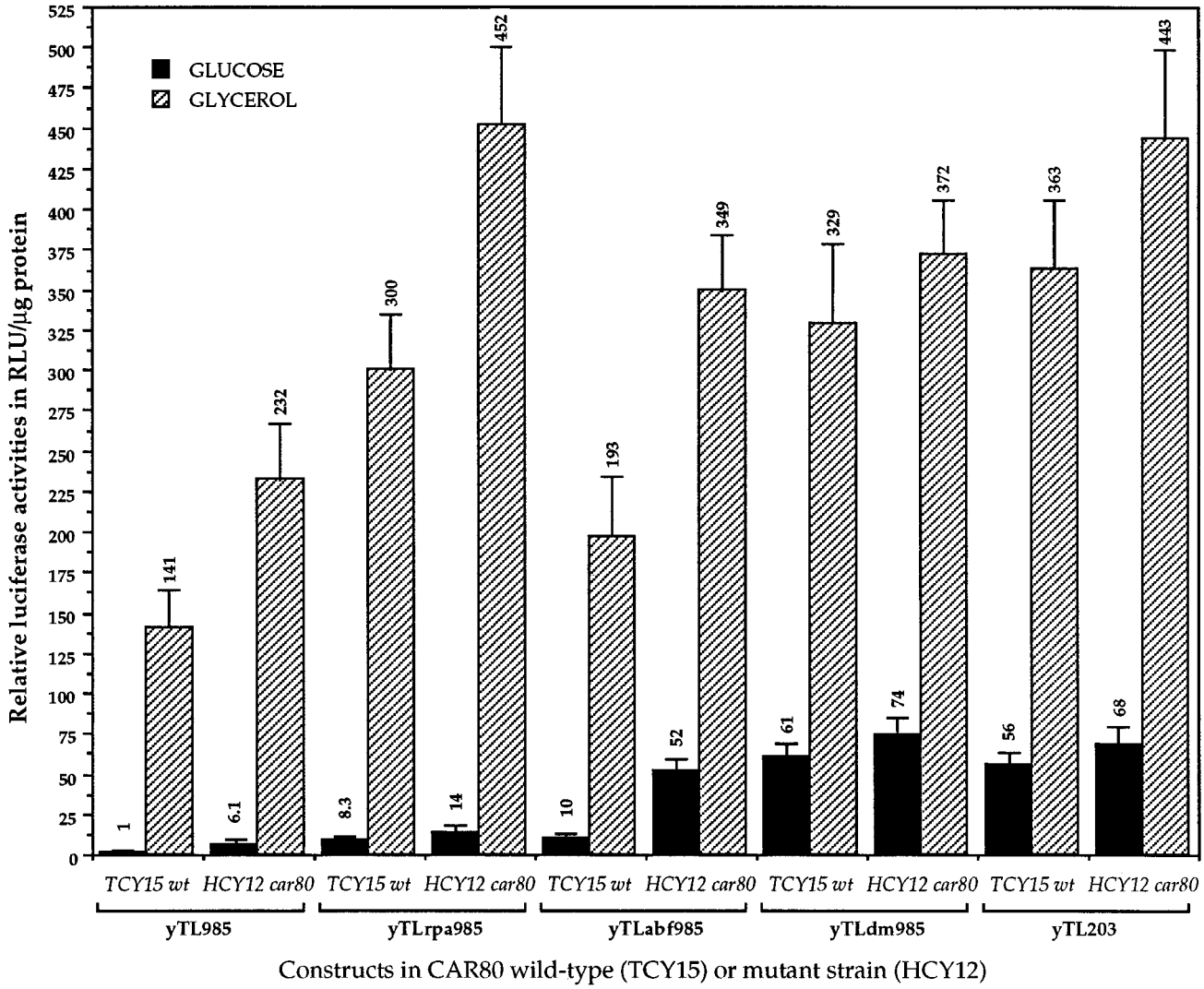

FIG. 8. Involvement of CAR80, ABF1, and RP-A in glucose repression of the *FOX3* gene. Relative luciferase activities were assayed in crude extracts of glucose-grown wild-type TCY15 and *car80* mutant HCY12 yeast cells transformed with yTL985, yTLrpa985, yTLabf985, yTLdm985, or yTL203 containing, respectively, the wild-type sites, the mutant RP-A site, the mutant ABF1 site, both mutated sites, or the truncated *FOX3* promoter. In yTL203 the region containing the RP-A and ABF1 sites is deleted. Luciferase activity measured in extracts of the yTL985 transformed to the wild-type TCY15 strain after growth on glucose-containing media is set to 1.

vated, a significant amount of repression (fivefold) is still detectable (Fig. 8, yTLdm985 or yTL203 in HCY12). This indicates that at least one other *cis*-acting element is involved in glucose repression. This element is located more towards the transcriptional start site, since in construct yTL203 the ABF1 and RP-A sites and all upstream promoter sequences are deleted.

Recently, in the *FOX1* (*POX1*) gene encoding the yeast peroxisomal acyl-coenzyme A oxidase a *cis*-acting element mediating glucose repression with the consensus sequence 5′-AGGGTAAT-3′ has been identified (48). An identical sequence is located just upstream of the overlapping RP-A and ABF1 sites in the *FOX3* gene (positions −234 to −226). However, the data presented here rule out the possibility that the 5′-AGGGTAAT-3′ sequence in the *FOX3* promoter context contributes to glucose repression, since a *FOX3* promoter with mutant ABF1 and RP-A sites in construct yTLdm985 mediates an expression pattern similar to that of a promoter construct in which the whole DNA region, including the 5′-AGGGTAAT-3′ site, the RP-A site, and the ABF1 site, is deleted, as is the case with yTL203 (Fig. 8).

In summary, it is clear from the data obtained so far that the ABF1 and RP-A (URS1) *cis*-acting sites as well as the CAR80 gene product are required for the down regulation of the *FOX3* gene in response to glucose. Sequence analysis of promoter regions of yeast genes encoding other peroxisomal proteins (*FOX1,2* and *CTA1*) or a protein involved in peroxisomal assembly (*PAS1*) revealed that the *CAR1* URS1 consensus sequence motif is found in many genes sequenced to date (Fig. 5). We have demonstrated that all these sites were able to bind RP-A specifically (Fig. 6), although with various affinities. This raises the possibility of a common element participating in the coordination of transcriptional repression of these genes. This is substantiated by the finding that CAR80 function is required for glucose repression of the peroxisomal *CTA1* gene, because in a *car80* mutant strain grown on glucose, catalase activity is increased fivefold compared with wild-type levels (data not shown). In view of these findings, it is reasonable to

assume that coordinate glucose repression of genes involved in peroxisomal function is at least in part mediated by the RP-A site and the proteins such as RP-A and CAR80 associated with it.

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