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The General Control Activator Protein GCN4 Is Essential for a Basal Level of ARO3 Gene Expression in Saccharomyces cerevisiae

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The ARO3 gene encodes one of two 3-deoxy-D-arabino-heptulosonate-7-phosphate isoenzymes catalyzing the first step in the biosynthesis of aromatic amino acids. The ARO3-encoded 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (EC 4.1.2.15) is feedback inhibited by phenylalanine; its isoenzyme, the ARO4 gene product, is inhibited by tyrosine. Both genes ARO3 and ARO4 are strongly regulated under the general control regulatory system. Cells carrying only one intact isogene are phenotypically indistinguishable from a wild-type strain when grown on minimal medium. The complete functional ARO3 promoter comprises 231 base pairs and contains only one TGACTA binding site for the general control activator protein GCN4. Mutating this element to TTACTA inhibits binding of GCN4 and results in a decreased basal level of ARO3 gene product and slow growth of a strain defective in its isogene ARO4. In addition, ARO3 gene expression cannot be elevated under amino acid starvation conditions. An ARO3 ar04 strain with gcn4 genetic background has the same phenotype of low ARO3 gene expression and slow growth. The amount of GCN4 protein present in repressed wild-type cells therefore seems to contribute to a basal level of ARO3 gene expression. The general control activator GCN4 has thus two functions: (i) to maintain a basal level of ARO3 transcription (basal control) in the presence of amino acids and (ii) to derepress the ARO3 gene to a higher transcription rate under amino acid starvation (general control).

There are two different regulatory mechanisms of the living cell to adjust the transcription of amino acid biosynthetic genes according to environmental signals. (i) In bacteria, the availability of an amino acid in the growth medium shuts down the transcriptional activity of the genes that are specifically involved in the cognate biosynthetic pathway. These genes, like the trp operon of Escherichia coli, are often organized in clusters that facilitate the coordinate regulation from a common promoter by site-specific DNA-binding proteins (30). (ii) In contrast, a eukaryotic cell, like the yeast Saccharomyces cerevisiae, maintains in the tryptophan biosynthetic pathway a basal level of gene expression independent of the presence or absence of an amino acid in the cellular environment. The genes that encode the enzymes of a biosynthetic pathway are spread over the whole genome and need their own promoters to be regulated (14). Starvation for a certain amino acid results in an increase of transcription not only of the cognate biosynthetic genes but of many genes of unrelated amino acid biosynthetic pathways. This regulation is known as the general control regulatory system (11, 24).

In the promoters of S. cerevisiae structural genes for amino acid biosynthesis, one can distinguish between elements necessary for the basal level of transcription in the presence of amino acids and elements necessary for an additional stimulation of transcription in the absence of amino acids.

A basal level of transcription depends on the presence of upstream promoter elements and elements involved in the "basal level control," beside TATA elements and initiation sites of transcription. In the HIS3 gene, the constitutive upstream elements are stretches of poly(dA-dT) located upstream of the TATA element (27). In the HIS4 promoter, additional elements were shown to be necessary to maintain a basal level of transcription by binding the trans-acting regulators BAS1 and BAS2 (2). BAS2, which is identical to PHO2, is also involved in the regulation of the TRP4 gene (6; G. Braus, H. U. Mösch, K. Vogel, A. Hinnen, and R. Hütter, submitted for publication) and of the acid phosphatase gene PHO5 (1). To shut down even a basal level of gene expression in an intact yeast cell, the complex interplay of several additional cis- and trans-acting factors is necessary, as is the case for the silencer sequences at the mating-type-specific genes HMR and HML (20).

In the absence of biosynthetic end products, the basal level of transcription can be increased by the interaction of additional regulatory promoter elements with trans-acting activator proteins; e.g., for the at least 30 amino acid biosynthetic genes subject to the general control regulatory network, the starvation response is mediated by the GCN4 activator protein. This protein binds specifically to 5'-TGACTC-3' upstream activation sequences (UASs) usually present in multiple copies in the promoters of these genes. Removing the UAS sites in such genes or introducing a gcn4 mutation into the yeast cell prevents activation of the corresponding genes upon amino acid starvation (10, 12).

In this paper we show that GCN4 is involved not only in the activation but also in the basal control of the aromatic amino acid biosynthetic gene ARO3. In S. cerevisiae, the genes ARO3 and ARO4 encode two isoenzymes for 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, one gene product being feedback inhibited by phenylalanine (AR03), the other one by tyrosine (AR04) (29). Each isoenzyme contributes approximately equal amounts of enzyme activity to the total level of DAHP synthase in the wild-type yeast cell. In the ARO3 promoter, a TGACTA element in inverse orientation is the only GCN4-binding site in vitro, although the ARO3 gene is very well regulated under the general control. Mutating this sequence inhibits binding of the GCN4 protein and mimics, when reintroduced into the genome, the phenotype of a gcn4 mutant strain: the basal level of ARO3 gene expression is reduced and a strain
TABLE 1. ARO3 gene expression at the URA3 locus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>ARO3-encoded DAHP synthase activitya (nmol min⁻¹ mg of protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH1313</td>
<td>ARO3 aro4-1</td>
<td>17</td>
</tr>
<tr>
<td>RH1396</td>
<td>aro3-2 aro4-1 (Δura3 ARO3)</td>
<td>18</td>
</tr>
<tr>
<td>RH1335</td>
<td>ARO3 aro4-1 gcdc2-1</td>
<td>60</td>
</tr>
<tr>
<td>RH1397</td>
<td>aro3-2 aro4-1 (ΔURA3 ARO3) gcdc2-1</td>
<td>62</td>
</tr>
</tbody>
</table>

a Activities were determined in ARO3 aro4 genetic background as total DAHP synthase activity. Values are averages of at least two independent cultivations, each measured twice (standard deviation, <25%).

defective in the isoenzyme ARO4 has a slow growth phenotype. Cells in the inverse genetic situation (aro3 ARO4 gcn4) do not show a drop in the enzyme level and grow at wild-type speed. Together with the fact that derepression of ARO3 under amino acid starvation conditions also acts through GCN4, the data suggest that transcription of ARO3 is activated through one cis-acting GCN4-binding site from a low level without binding of GCN4 protein, to a basal level in wild-type background, and to a high transcription rate under amino acid starvation.

MATERIALS AND METHODS

Strains, plasmids, and media. All yeast strains used are derivatives of the S. cerevisiae laboratory strains X2180-1A (MATα gal2 SUC2 mal CUP1) and X2180-1B (MATα gal2 SUC2 mal CUP1). The strains appear in Tables 1 and 2 with their relevant genotypes, except for RH1319 (MATα aro3-2 aro4-1) and RH1326 (MATα aro3-2 aro4-1 leu2-2 gcdc2-1).

Plasmid p164, carrying the GCN4 gene, was obtained from A. Hinnebusch (National Institutes of Health, Bethesda, Md.); pAB 100, with the GCN4 gene fused to the lambda P1 promoter in E. coli AR68, was a gift from K. Arndt and G. Fink (Massachusetts Institute of Technology, Cambridge); pUC19 was described by Norrander et al. (21). YE. complete medium and MV minimal medium, used for cultivation of S. cerevisiae, were described by Miozzari et al. (18). Amino acids and amino acid analogs were added to MV medium as indicated in the text. For cultivation of gcn4 mutant strains, all media were supplemented with 40 μg of arginine per ml.

Genetic techniques. Yeast genetic crosses were performed as described by Sherman et al. (25).

Yeast transformations. Transformation of S. cerevisiae was performed by the method of Ito et al. (15). Typically, 1 μg of plasmid DNA was added to 100 μl of competent cells. For integrative transformations, 200 ng of linear DNA fragment was applied together with 10 μg of circular pUC19 used as carrier DNA.

Southern analysis. Chromosomal DNA from S. cerevisiae was isolated as described by Braus et al. (3), and Southern blot analysis was carried out (26). As probes, DNA fragments labeled according to the “oligo-labeling” technique described by Feinberg and Vogelstein (4) were applied.

Gel retardation assays. The gel retardation method was described earlier (5, 8). For the DNA-binding assay with GCN4 protein, radiolabeled DNA fragments (10,000 to 20,000 cpm) were incubated with 1 μg of E. coli extract enriched for GCN4 protein (1) for 25 min at 25°C in 20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; pH 7.9), 0.16 mM EDTA, 5 mM MgCl2, 40 mM KCl, 8% glycerol, and 100 μg of poly(dI-dC) per ml in a 25-μl assay. Samples were separated on a native 6% polyacrylamide gel. Afterwards the gel was fixed, dried, and autoradiographed.

TABLE 2. ARO3 gene expression with relation to the GCN4-TGACTC system

<table>
<thead>
<tr>
<th>Strain</th>
<th>UAS sequence</th>
<th>Genotype</th>
<th>Amino acid starvationa</th>
<th>DAHP synthase activitya (nmol min⁻¹ mg of protein⁻¹)</th>
<th>Growth rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X2180-1A</td>
<td>TGACTA (wt)c</td>
<td>ARO3 ARO4</td>
<td>–</td>
<td>11/10</td>
<td>0.32</td>
</tr>
<tr>
<td>RH1370</td>
<td>TGACTA (wt)</td>
<td>ARO3 aro4-1 Δura3</td>
<td>–</td>
<td>+</td>
<td>0.32</td>
</tr>
<tr>
<td>RH1394</td>
<td>TTACTA</td>
<td>ARO3 aro4-1 Δura3</td>
<td>–</td>
<td>+</td>
<td>0.16</td>
</tr>
<tr>
<td>RH1395</td>
<td>TGACTC</td>
<td>ARO3 aro4-1 Δura3</td>
<td>–</td>
<td>+</td>
<td>0.34</td>
</tr>
<tr>
<td>RH1380</td>
<td>wt</td>
<td>ARO3 aro4-1 gcn4-101 ure3-52</td>
<td>–</td>
<td>+</td>
<td>0.15</td>
</tr>
<tr>
<td>RH1380(p164)</td>
<td>(GCN4 URA3)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>0.30</td>
</tr>
<tr>
<td>RH1398</td>
<td>wt</td>
<td>ARO3 aro4-1 gcn4-201</td>
<td>–</td>
<td>15</td>
<td>0.18</td>
</tr>
<tr>
<td>RH1316</td>
<td>wt</td>
<td>aro3-2 ARO4</td>
<td>–</td>
<td>4</td>
<td>0.33</td>
</tr>
<tr>
<td>RH1401</td>
<td>wt</td>
<td>aro3-2 ARO4</td>
<td>–</td>
<td>3</td>
<td>0.30</td>
</tr>
</tbody>
</table>

a Cells were grown overnight in MV medium at 30°C (–) or cultivated for another 8 h in the presence of 10 mM of the histidine analog 3-amino triazole (+) before being harvested.

b Values of at least two independent cultivations, each measured twice (standard deviation, <25%).

c The tyrosine- (ARO4/phenylalanine-(ARO3) sensitive DAHP synthase activities were determined in the presence of L-tyrosine (1 mM) and L-phenylalanine (3 mM), respectively, and are separated by a slash.

d aro3 aro4 double mutant strains completely lack DAHP synthase activities (data not shown). Activities were determined in ARO3 aro4 background as total DAHP synthase activity, thus corresponding to only ARO3 gene expression.

*e activities determined in aro3 ARO4 background as total DAHP synthase activity.

f Wild type.
DNase I footprint analysis. DNase I protection analysis was performed as described by Galas and Schmitz (7). 3'-End-labeled AR03 promoter DNA fragments (10,000 to 20,000 cpm) and 6 to 12 µg of E. coli extract containing partially purified GCN4 protein (1) were incubated in binding buffer as described above for the gel retardation assay, except that the poly(dI-dC) concentration was lowered to 40 µg/ml. After 20 min of incubation on ice, DNase I was added to a final concentration of 20 ng/ml. DNase I treatment was terminated after 90 s by adding 125 µl of 0.12% 12 mM EDTA-0.36 M sodium acetate containing 5 µg of yeast tRNA. Samples were separated on a standard sequencing gel and autoradiographed. A G/A sequencing ladder was used as a size marker (17).

Mutagenesis. Point mutations were generated using the Muta-Gene in vitro mutagenesis kit from Bio-Rad (Richmond, Calif.), based on a method described by Kunkel (16). The DNA to be mutated was cloned into the M13-based cloning vector M13mp18 (21), and single-stranded DNA was isolated from an E. coli host with defective dUTPase and uracil-N-glycosylase (dut ung), which occasionally incorporates dUTP instead of dTTP into the nascent DNA. This uracil-containing strand was used as a template for the in vitro synthesis of an oligonucleotide-primed non-uracil-containing mutant strand. The resulting double-stranded DNA was transformed into a cell with intact dUTPase and uracil-N-glycosylase, which inactivated the uracil-containing strand with high efficiency, leaving the non-uracil-containing mutant strand survivor to replicate. The sequence of the mutated strand was verified by the dideoxy method of Sanger et al. (23).

Enzyme assays. For determination of DAHP synthase, exponentially growing cells were broken by three passages through a French pressure cell at 4 × 10⁷ Pa and centrifuged at 4,000 × g for 10 min (4°C). The crude extracts were adjusted to 1 mM MgSO₄, and nucleic acids were digested with DNase I (10 µg/ml) and RNase A (10 µg/ml) for 1 h on ice. DAHP synthase was assayed by the method of Takahashi and Chan (28) with the modifications described by Teshiba et al. (29). Total protein concentrations were determined by the method of Herbert et al. (9), and specific enzyme activities are expressed in nanomoles of product formed per minute per milligram of protein.

Growth rates. Yeast cells were grown at 30°C in 5-ml liquid cultures on a rotary shaker. Growth was followed turbidimetrically at 546 nm. The specific growth rate is given as µ and is defined as µ = (lnx₂ - lnx₁)/(t₂ - t₁), where x is the optical density at 546 nm at the corresponding time.

RESULTS

A 231-bp fragment of AR03 promoter containing one GCN4-binding site is sufficient for full and regulated expression. The AR03 gene was cloned on a 1.7-kilobase (kb) HindIII-XbaI fragment (29), and the sequence was determined (21a). In the 231-base-pair (bp) promoter region starting at the HindIII site (Fig. 1) there are putative promoter elements, but as a possible binding site for the general control activator GCN4 only a TGACTA in inverse orientation was found. To check whether this fragment contains all promoter elements necessary for full and regulated expression, it was integrated at the chromosomal URA3 locus in the aro3-2 aro4-1 double mutant strain RH1319, which completely lacks DAHP synthase activity. The HindIII-XbaI fragment was cut out as a HindIII-Smal fragment from the polylinker of pUC19 and cloned after filling in the ends into the URA3 gene, replacing an internal EcoRV-Stul fragment (22). The hybrid construct, located on a 2.6-kb HindIII fragment, was transformed as linear DNA into strain RH1319. Transformants were selected for their Aro⁺ Ura⁻ phenotype, and in one strain (RH1396) integration of the AR03 gene at the URA3 locus was confirmed by Southern blot analysis (Fig. 2). Strain RH1396 was crossed with RH1326 to combine a gcc2 mutation with the integrated copy of the AR03 gene, yielding strain RH1397. The gcc2 mutation confers constitutively derepressed enzyme levels of genes coregulated under the general control system (18). Extracts of integrants with wild-type and gcc2 backgrounds were assayed for the AR03 gene product DAHP synthase. The data of Table 1 demonstrate that the AR03 promoter up to the HindIII site at position −231 is sufficient to provide (i) the same basal level of expression at the URA3 position as at the AR03 locus and (ii) full derepression in strain RH1397.

To test whether the putative UAS site in AR03 is able to bind the general control regulator protein GCN4 in vitro, we used the gel retardation method (5, 8). A set of promoter fragments were incubated with GCN4 protein and run on a native polyacrylamide gel. Both fragments B and C (Fig. 3) which showed retarded migration contained the UAS in question, whereas the fragments with unaltered mobility did not, suggesting a specific protein-DNA complex between the UAS and the GCN4 protein.

The GCN4 binding site was analyzed in more detail by DNase I protection analysis (7) using E. coli-produced.

![FIG. 1. Restriction map of the AR03 region. The black bar indicates the AR03 coding sequence. The translational start codon ATG is marked with an arrow (21a).](http://mcb.asm.org/)

![FIG. 2. Integration of the AR03 gene at the URA3 locus. Chromosomal DNA from the wild-type strain X2180-1A (lane A) and from strain RH1396 ([Δaro3 AR03]; lane B) was cut with XbaI and hybridized to the radiotactively labeled 1.1-kb HindIII URA3 fragment from YEp24 (22).](http://mcb.asm.org/)
binding of GCN4. Even with high amounts of GCN4 protein, no second protected region could be detected. Figure 4A thus correlates with the results obtained in the gel retardation experiments showing that the proposed UAS is the only site in the ARO3 promoter that is able to bind GCN4 protein in vitro.

As a control a second DNase I footprint analysis with the same GCN4 preparation was carried out for the TRP4 promoter (Fig. 4B). In the TRP4 5'-flanking region are found UASs, (containing a single GCN4 recognition element) and UASs, (two adjacent elements) (6). UASs is the major regulation site in vivo and has the highest affinity to GCN4 in vitro, and one of the two repeats of UASs has the lowest. In Figure 4B the high amount of GCN4 applied even largely protects the repeat with the lowest affinity (Braus et al., submitted). Figure 4 demonstrates that similar amounts of GCN4 protein are necessary to protect the two UASs of the TRP4 promoter and the single UAS of the ARO3 promoter, suggesting similar affinities of these elements to GCN4 in vitro.

A point mutation in the TGACTA element results in reduced ARO3 gene expression. To analyze the role of the TGACTA element in vivo, this element was mutated to TTACTA and tested for function integrated at its original locus on the chromosome. The TTACTA sequence was reported to have only low affinity to GCN4 protein (1, 10).

First, we constructed a strain with a deleted ARO3 gene. The deletion comprised the complete coding sequence of the ARO3 gene and the promoter region up to the HindIII site. A HindIII linker was cloned into the Stul site of the ARO3 gene (Fig. 1), and the 1.3-kb HindIII-(Stul)HindIII fragment was replaced by the 1.1-kb HindIII URA3 fragment (22). The hybrid construct was transformed as a linear 2.2-kb Sphl-XbaI fragment into strain RH1370 (ARO3, aror-1 Δura3). Transformants were selected by their Ura+ Aro- phenotype and checked by Southern blot analysis. Figure 5 shows that the 5.5-kb HindIII wild-type fragment is reduced to a 4.2-kb HindIII band in strain RH1393 with the deleted ARO3 sequence, due to the inserted HindIII linker at the Stul site.

In a second step, the complete ARO3 gene with the TGACTA sequence in the promoter mutated to TTACTA as described in Materials and Methods was reintroduced onto its original locus on the chromosome as a 2.4-kb Sphl-XbaI fragment into strain RH1393, this time replacing the previously introduced URA3 gene. Selection was for Aro+ Ura- phenotype, and integration was confirmed by Southern blot analysis (Fig. 5). Thus the wild-type situation at the ARO3 locus was restored with the exception of a single point mutation, yielding strain RH1394. As expected, the mutated sequence was unable to form a complex with GCN4 protein when assayed in a gel retardation experiment (Fig. 5). Furthermore, the mutation had a clear effect in vivo: the growth rate of strain RH1394 was 0.16 h⁻¹ in MV medium. This is a decrease of 50% compared with strain RH1370, which grows at the wild-type rate of 0.32 h⁻¹. The DAHP synthase activity conferred by the mutated promoter dropped from 17 nmol min⁻¹ mg⁻¹ to 4 nmol min⁻¹ mg⁻¹ and could not be elevated by cultivating the cells in the presence of 10 mM of the histidine analog 3-aminotriazol, indicating that the ARO3 gene was no longer able to respond to amino acid starvation (Table 2).

A second mutation, TGACTA to TGACTC, representing the consensus sequence for GCN4 binding, was generated and introduced as described above (strain RH1395; Fig. 5). As expected, the sequence showed a clear band shift when assayed in a gel retardation experiment (Fig. 5). Further-

FIG. 3. Gel retardation assays of ARO3 promoter fragments. The tested DNA fragments and their localization on the ARO3 promoter are shown below. The DNA was end labeled and analyzed for complex formation with E. coli-produced GCN4 protein (+). In lanes marked by (--), E. coli extracts without GCN4 protein were used. Products were run on a native polyacrylamide gel. Relevant restriction sites and the putative UAS element −181 TGACTA −186 are deduced from the nucleotide sequence (21a).
FIG. 4. DNase I footprints. (A) Analysis of the ARO3 promoter-GCN4 complex. The coding strand of the 314-bp HindIII-BglII ARO3 promoter fragment was 5' end labeled at the HindIII site and the DNA probes were incubated with 0.1 to 10 μg of E. coli extracts containing GCN4 protein (lanes 3 through 10) or without any protein (lane 2). After treatment with DNase I the samples were separated on a standard sequencing gel. An A/G ladder (17) was used as a size marker (lane 1). Protected sequences are bracketed and shown below. ←, Single inverted UAS element of the ARO3 promoter. (B) TRP4 promoter footprint. The same GCN4 preparation as in panel A was used: (lane 1) A/G ladder; (lanes 2 and 5) no protein added; (lane 3) 5 and (lane 4) 10 μg of E. coli extract added, containing GCN4 protein. At 10 μg, the low-affinity repeat of UAS2 ← is also largely protected. →, UAS elements of the TRP4 promoter.

more, growth, enzyme level, and derepression of this strain were indistinguishable from strain RH1370 with a wild-type ARO3 gene (Table 2), suggesting that on the one hand a C-to-A mutation in the consensus sequence is tolerable for the ARO3 promoter, but on the other hand a perfect consensus sequence represents no improvement over the wild-type ARO3 situation.

GCN4 mediates basal control and general control. Wild-type yeast cells have two genes coding for isoenzymes of DAHP synthase, ARO3 and ARO4. Either one of these isoenzymes, however, is sufficient to confer enough DAHP for normal growth of the cells. Introducing an additional gcn4 mutation (gcn4-101 or gcn4-201) has different effects on each gene. Cells with an aro3 ARO4 gcn4 genotype show
wild-type levels of ARO4 gene expression and therefore grow at wild-type speed. ARO3 ara4 gcna4 mutant strains, however, drop their DAHP synthase activity from 17 to 4 nmol min⁻¹ mg⁻¹ and show slow growth on MV medium (Table 2).

Growth and enzyme level can be restored by transforming ARO3 ara4 gcna4 cells with the GCN4 gene on the low-copy-number plasmid YCp50 (p164), imitating a wild-type situation (Table 2). Normal growth alone can be restored by supplementing the minimal growth medium with phenylalanine, tyrosine, and tryptophan (data not shown).

Slow growth and low DAHP synthase enzyme levels were also observed in strain RH1394, described above, carrying a mutated UAS element which inhibits binding of GCN4 protein to the ARO3 promoter. Thus, introducing a gcna4 genetical background or a point mutation destroying the GCN4 target sequence in the ARO3 promoter leads to the same weak ARO3 gene expression. To establish the transcriptional nature of the observed effects, we carried out a Northern (RNA) blot analysis. Poly(A)⁺RNA from the wild-type strain, a gcna4 strain, and a gcdn2 mutant strain (the gcdn2 mutation causes constitutively derepressed enzyme levels) was cohybridized against radiolabeled ARO3 and URA3 DNA. The URA3 hybridization signal served as an internal standard for the amount of RNA present in each lane. Figure 6 shows that, compared to the wild type, the ARO3 mRNA levels were decreased in the gcna4 mutant strain and increased in the gcdn2 strain. Thus, the effects observed at the enzyme level in various regulatory backgrounds were correspondingly paralleled by a change in transcript levels. This indicates that the presence of active GCN4 protein is indispensable to ARO3, not only to respond to the general control system but also for a basal level of transcription without amino acid limitation.

Therefore the range of phenylalanine-sensitive DAHP synthase activity provided by GCN4 can be characterized by three different levels, as follows (Fig. 7). (i) Low levels of ARO3 gene product are expressed in strains without a functional GCN4 protein (gcna4-101 gcna4-201) (Fig. 7A). Strains with an additional ara4 mutation grow slowly on MV medium. (ii) Basal levels of the phenylalanine-sensitive DAHP synthase (AR03) are expressed in wild-type cells, in an ara4 single mutant strain, and also in ara4 gcna4 transformed with the GCN4 gene on low-copy-number plasmid YCp50 (p164; Fig. 7B). (iii) Finally, derepressed levels are obtained either by starving the cells for amino acids (Fig. 7C) or by introducing a gcdn2 mutation causing constitutively derepressed enzyme levels (Fig. 7D). These data suggest that in the case of the ARO3 gene the activation conferred by the GCN4 protein is mediated through a single binding site as follows: (i) in the presence of amino acids, it is mediated from a low-level gene expression without any binding of GCN4 to the ARO3 promoter to basal expression in wild-type regulatory conditions (basal control), similar to the BAS genes for the HIS4 promoter (2), and (ii) in the absence of amino acids, activation is mediated from wild-type level to a derepressed state of gene expression as the activator protein in response to the general control.

**DISCUSSION**

The main finding of this report is that the GCN4 gene product, previously identified as the general control activa-
tor, in the case of ARO3 is involved not only in the general control regulation, but in addition in maintaining a basal level of ARO3 transcription. Yeast cells defective only in one of the DAHP synthase isoenzymes, ARO3 or ARO4, still show normal growth on MV medium. Introducing an additional gcn4 mutation, however, leads to slow growth of a strain with only an intact ARO3 gene, whereas cells with only the functional ARO4 gene remain unaffected. Normal growth of cells with the ARO3 aro4 gcn4 genotype could be restored by adding the aromatic amino acids phenylalanine, tyrosine, and tryptophan to the growth medium, and both growth and enzyme level were restored by transforming the cells with the GCN4 gene on a low-copy-number plasmid.

A relatively short promoter fragment of 231 bp was shown to contain all sequences necessary for full ARO3 expression and regulated derepression under the general control. Band shift experiments and a subsequent DNase I footprint analysis revealed only a single binding site for the general control activator protein GCN4. In vivo the functionality of 3'-TGACTA-5' in inverse orientation as the only GCN4-binding site was demonstrated by mutating this sequence to TTACTA and expressing it at its chromosomal origin in a regulatory wild-type background. Surprisingly, a drop of DAHP synthase levels was observed, and the strain with the promoter mutation together with a defective ARO4 isogene had a slow growing phenotype, although only a failure in the general control response had been expected.

The ARO3 enzyme levels obtained in our study without GCN4 activation were lowered in comparison to the wild-type levels, but were not completely repressed. Long stretches of adenosines and thymines have been postulated to be responsible for a constitutive level of HIS3 transcription (27). In the ARO3 promoter there is a 18-bp stretch of (dT-dA) nucleotides 5' of the UAS element. Whether this stretch is mediating the residual ARO3 activity is subject to further experiments. In reference to the HIS4 gene, which has several GCN4 recognition elements, Arndt et al. (2) showed that a gcn4 mutant strain had a decreased HIS4 expression compared to the wild type, based on data obtained with HIS4-lacZ fusion genes. The gcn4 strain was not auxotrophic for histidine, but no data were presented on the growth rate in the absence of a histidine supplement. A gcn4 bas1 (or bas2) strain had virtually no more HIS4-lacZ gene expression and required histidine for growth. Thus the BAS genes were essential for a basal level of HIS4 expression (2). At least BAS2 protein, which is identical to PHO2, does not bind to the ARO3 promoter, as determined in gel retardation experiments, and a bas2 gcn4 ar0 strain is not auxotrophic for aromatic amino acids (unpublished data). Besides GCN4, additional transcription factors might be involved in the basal control of the ARO3 gene, but any assumed trans-acting factor is not sufficient for wild-type ARO3 gene activation and is unable to compensate for defects in the GCN4-TGACTA activating system of the ARO3 gene.

In the GCN4-binding site, the nucleotides flanking the core sequence TGACTC also show considerable sequence conservation (10). Optimal binding was observed with the palindromic sequence ATGAC/GTCAT, and it was shown that GCN4 binds as a dimer to target DNA (13). None of the native promoter sequences known up to now, however, is perfectly symmetrical, and it is not known precisely which nucleotides are contacted by each GCN4 monomer (13). The TGACTA in the wild-type ARO3 promoter indicates strong regulation under the general control system, although such a sequence was reported to have only low affinity to GCN4 (1) and, when introduced into the HIS3 promoter, to confer only weak response to starvation conditions (10). Three of six nucleotides flanking the TGACTA core match the sequence suggested by Hill et al. (10), and the symmetry in this sequence is very low. We assume that in the ARO3 promoter the nucleotide of the core UAS is not directly contacted by GCN4 and therefore is of less importance for the activation process. This is also supported by the fact that a TGACTC sequence does not improve activity. A mutation, however, located more 5' in the core sequence, TTACTA, completely inhibits binding of GCN4 and results in reduced ARO3 gene expression. In the ARO3 promoter, a TGTCCT sequence was observed at a distance of 10 nucleotides upstream of the TGACTA core (21a), and it was speculated that these nucleotides might be involved in the activation process, as they are located on the same side of the DNA helix as the UAS site. This sequence was not protected in vitro by GCN4 protein, however, and we therefore do not assign it any cooperation with the adjacent TGACTA.

GCN4-mediated activation of transcription is thought to be controlled by the levels of GCN4 protein available in the cell. Interestingly, the amount of GCN4 protein present in wild-type cells, repressed by translational regulation to a low level in the presence of amino acids (19), seems to be sufficient for binding the ARO3 promoter, whereas expression of all the other genes coregulated under the general control remains unaltered under these conditions. At least in vitro, however, the affinity of the ARO3 UAS for GCN4 protein is similar to that of the UASs of the TRP4 promoter (6), elements which stimulate TRP4 transcription only under starvation conditions (6; Braus et al., submitted). In addition, the gene ARO4, coding for a DAHP synthase isozymic to the ARO3 gene product (but feedback inhibited by tyrosine), can serve as a direct reference: it is also subject to the general control and carries a single TGACTC sequence in its 5' region (G. Paravicini and K. Kunzler, manuscript in preparation). Strains with the genotype aro3 ARO4 gcn4 (with each gcn4 allele), however, are completely unaffected in their basal enzyme level. In the cell, more

FIG. 7. DAHP synthase levels (phenylalanine sensitive) under various regulatory conditions. (A) Low level of ARO3 gene product; (B) basal level, (C and D) derepressed levels of DAHP synthase activity. See text for details.
subtle differences than can be detected in DNaseI footprints may play a role in GCN4-binding affinities. Also, stronger binding of the activator does not necessarily mean stronger stimulation of transcription (2). Still, the simplest idea to explain these data would be that in the cell the flanking sequences of the AR03 UAS make it a high-affinity site for GCN4. At the low GCN4 concentrations that prevail in repressing conditions, GCN4 protein would be only partially bound and would interact more strongly at the higher GCN4 levels in derepressing conditions. As GCN4 protein binds as a dimer to target DNA (13), there is another model that in a wild-type cell low levels of GCN4 could exist mainly in a monomeric or otherwise modified form, which is better suitable for the AR03 promoter than for other general control promoters. Only with increased amounts of GCN4 present in the cell would the protein exist as a dimer and mediate its well-known function as general control activator protein. It remains to be investigated how a TGACTA sequence confers stimulation of a gene by GCN4 under wild-type regulatory conditions, while a TGA(TC) in the gene coding for its isoenzyme, and in other genes subject to the general control system, is only necessary for activation under conditions of amino acid starvation.

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LITERATURE CITED


