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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 in *Saccharomyces cerevisiae* 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 aro4* 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 eucaryotic 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 (*ARO3*), the other one by tyrosine (*ARO4*) (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

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TABLE 1. *ARO3* gene expression at the *URA3* locus

Strain	Genotype	*ARO3*-encoded DAHP synthase activity[a] (nmol min^{-1} mg of $protein^{-1}$)
RH1313	*ARO3 aro4-1*	17
RH1396	*aro3-2 aro4-1 (Δura3 ARO3)*	18
RH1335	*ARO3 aro4-1 gcd2-1*	60
RH1397	*aro3-2 aro4-1 (Δura3 ARO3) gcd2-1*	62

[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 (*MAT***a** *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 (*MAT***a** *aro3-2 aro4-1 leu2-2 gcd2-1*).

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 p_L 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). YEPD 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 $MgCl_2$, 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

Strain	UAS sequence	Genotype	Amino acid starvation[a]	DAHP synthase activity[b] (nmol min^{-1} mg^{-1})			Growth rate (h^{-1})
				ARO3 ARO4[c]	*ARO3 aro4*[d]	aro3 ARO4[e]	
X2180-1A	TGACTA (wt)[f]	*ARO3 ARO4*	−	11/10			0.32
			+	36/38			
RH1370	TGACTA (wt)	*ARO3 aro4-1 Δura3*	−		17		0.32
			+		39		
RH1394	TTACTA	*ARO3 aro4-1 Δura3*	−		4		0.16
			+		5		
RH1395	TGACTC	*ARO3 aro4-1 Δura3*	−		18		0.34
			+		41		
RH1380	wt	*ARO3 aro4-1 gcn4-101 ura3-52*	−		4		0.15
			+		3		
RH1380(p164)		(*GCN4 URA3*)	−		15		0.30
			+		37		
RH1398	wt	*ARO3 aro4-1 gcn4-201*	−		5		0.18
			+		4		
RH1316	wt	*aro3-2 ARO4*	−			10	0.33
			+			33	
RH1401	wt	*aro3-2 ARO4 gcn4-201*	−			9	0.30
			+			8	

[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-aminotriazole (+) 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] wt, Wild type.

DNase I footprint analysis. DNase I protection analysis was performed as described by Galas and Schmitz (7). 3'-End-labeled *ARO3* 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 4×10^7 Pa and centrifuged at 4,000 × *g* for 10 min (4°C). The crude extracts were adjusted to 1 mM $MgSO_4$, 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 $\mu = (\ln x_2 - \ln x_1)/(t_2 - t_1)$, where x is the optical density at 546 nm at the corresponding time.

RESULTS

A 231-bp fragment of *ARO3* promoter containing one GCN4-binding site is sufficient for full and regulated expression. The *ARO3* gene was cloned on a 1.7-kilobase (kb) *Hin*dIII-*Xba*I fragment (29), and the sequence was determined (21a). In the 231-base-pair (bp) promoter region starting at the *Hin*dIII 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 *Hin*dIII-*Xba*I fragment was cut out as a *Hin*dIII-*Sma*I fragment from the polylinker of pUC19 and cloned after filling in the ends

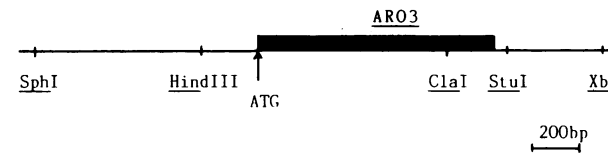

FIG. 1. Restriction map of the *ARO3* region. The black bar indicates the *ARO3* coding sequence. The translational start codon ATG is marked with an arrow (21a).

into the *URA3* gene, replacing an internal *Eco*RV-*Stu*I fragment (22). The hybrid construct, located on a 2.6-kb *Hin*dIII 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 *ARO3* gene at the *URA3* locus was confirmed by Southern blot analysis (Fig. 2). Strain RH1396 was crossed with RH1326 to combine a *gcd2* mutation with the integrated copy of the *ARO3* gene, yielding strain RH1397. The *gcd2* mutation confers constitutively derepressed enzyme levels of genes coregulated under the general control system (18). Extracts of integrants with wild-type and *gcd2* backgrounds were assayed for the *ARO3* gene product DAHP synthase. The data of Table 1 demonstrate that the *ARO3* promoter up to the *Hin*dIII site at position −231 is sufficient to provide (i) the same basal level of expression at the *URA3* position as at the *ARO3* locus and (ii) full derepression in strain RH1397.

To test whether the putative UAS site in *ARO3* 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,

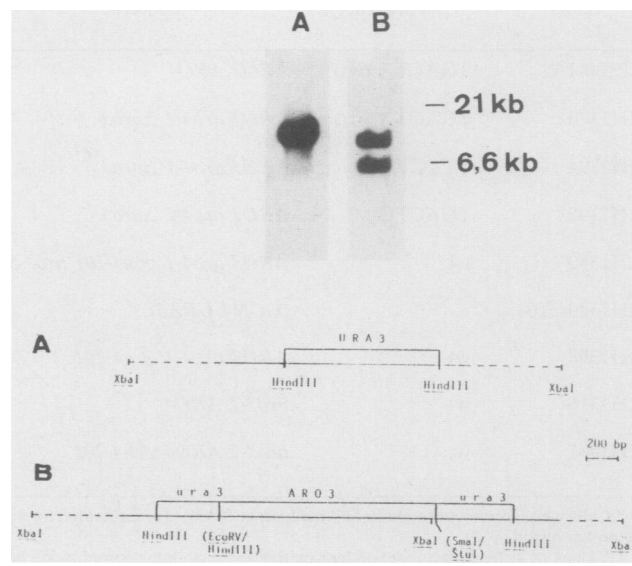

FIG. 2. Integration of the *ARO3* gene at the *URA3* locus. Chromosomal DNA from the wild-type strain X2180-1A (lane A) and from strain RH1396 [(Δ*ura3 ARO3*); lane B] was cut with *Xba*I and hybridized against the radioactively labeled 1.1-kb *Hin*dIII *URA3* fragment from YEp24 (22).

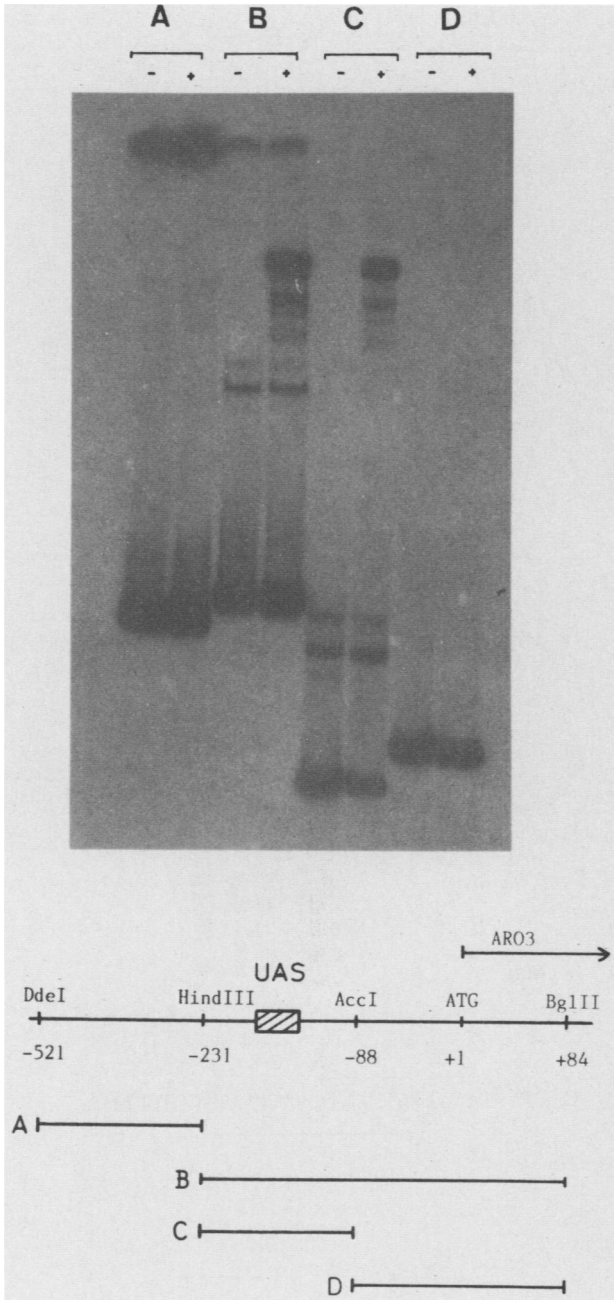

FIG. 3. Gel retardation assays of *ARO3* promoter fragments. The tested DNA fragments and their localization on the *ARO3* promoter are shown below. The DNA was end labeled and analyzed for complex formation with *E. coli*-produced GCN4 protein (+). In lanes marked by (−), *E. coli* extracts without 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).

partially purified GCN4 protein. The *Hin*dIII site of the *ARO3* promoter was labeled at the 5′ end, and the 314-bp *Hin*dIII-*Bgl*II fragment was incubated with increasing amounts of GCN4 protein, treated with DNase I, and analyzed on a sequencing gel as described in Materials and Methods. Figure 4A indicates the nucleotides in the region between −175 and −191 relative to the translational start site that are protected from DNase I cleavage because of the 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 UAS_1 (containing a single GCN4 recognition element) and UAS_2 (two adjacent elements) (6). UAS_1 is the major regulation site in vivo and has the highest affinity to GCN4 in vitro, and one of the two repeats of UAS_2 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 *Hin*dIII site. A *Hin*dIII linker was cloned into the *Stu*I site of the *ARO3* gene (Fig. 1), and the 1.3-kb *Hin*dIII-(*Stu*I)*Hin*dIII fragment was replaced by the 1.1-kb *Hin*dIII *URA3* fragment (22). The hybrid construct was transformed as a linear 2.2-kb *Sph*I-*Xba*I fragment into strain RH1370 (*ARO3*, *aro4-1* Δ*ura3*). Transformants were selected by their Ura^+ Aro^- phenotype and checked by Southern blot analysis. Figure 5 shows that the 5.5-kb *Hin*dIII wild-type fragment is reduced to a 4.2-kb *Hin*dIII band in strain RH1393 with the deleted *ARO3* sequence, due to the inserted *Hin*dIII linker at the *Stu*I 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 *Sph*I-*Xba*I 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-aminotriazole, 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-

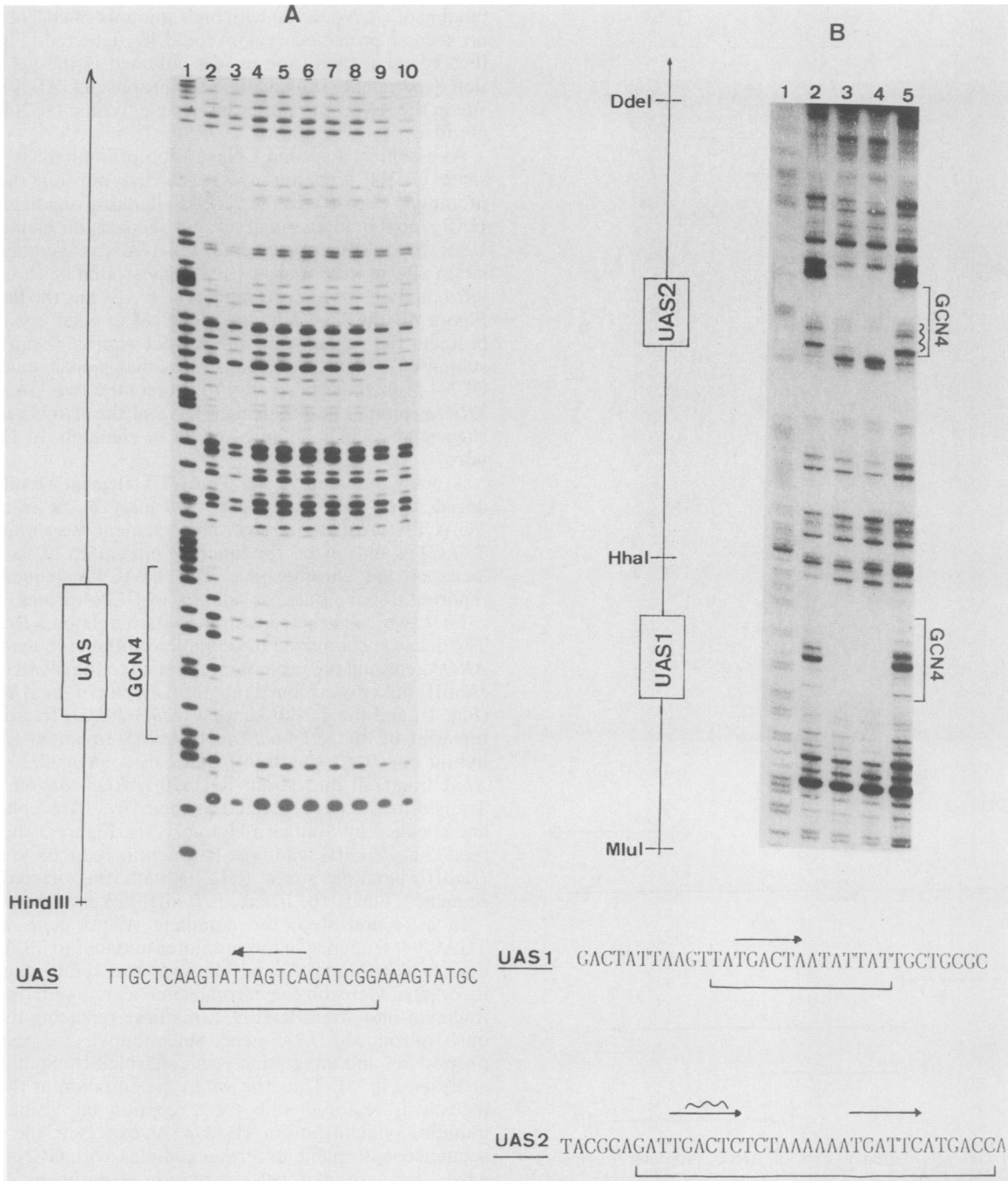

FIG. 4. DNase I footprints. (A) Analysis of the *ARO3* promoter-GCN4 complex. The coding strand of the 314-bp *Hin*dIII-*Bgl*II *ARO3* promoter fragment was 5′ end labeled at the *Hin*dIII site and the DNA probes were incubated with 0.1 to 10 μg of *E. coli* extracts containing GCN4 protein (lanes 3 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 UAS_2 ~ 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

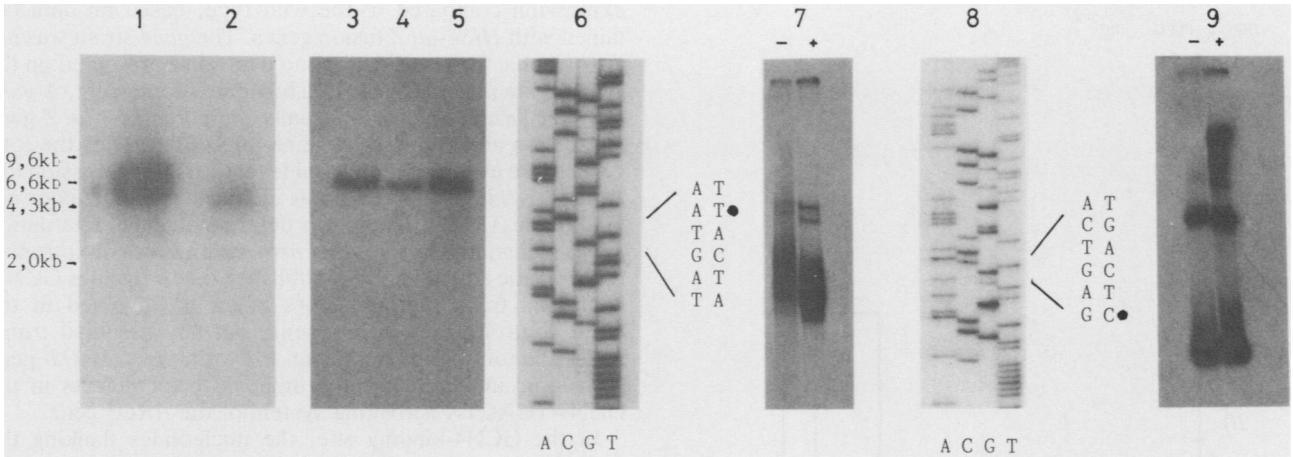

FIG. 5. Deletion of the *ARO3* gene and subsequent integration and analysis of *ARO3* promoter mutations. Chromosomal DNA from strain X2180-1A with an intact *ARO3* gene (lanes 1 and 3), from strains with the deleted *ARO3* gene (RH1393; lane 2), and from the strains with the reintegrated *ARO3* gene containing the point mutations described in the text (RH1394, lane 4; RH1395, lane 5) were cut with *Hin*dIII and hybridized against the radiolabeled 1.7-kb *Hin*dIII-*Xba*I *ARO3* fragment. The point mutations were verified by sequencing (lanes 6 and 8). The dot (•) indicates the mutated nucleotide as compared with the wild-type sequence. The mutated 314-bp *Hin*dIII-*Bgl*II *ARO3* promoter fragments were 3′ end labeled and incubated with *E. coli* extracts with (+) or without (−) GCN4 protein (lanes 7 and 9).

wild-type levels of *ARO4* gene expression and therefore grow at wild-type speed. *ARO3 aro4 gcn4* 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 aro4 gcn4* 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 *gcn4* 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 *gcn4* strain, and a *gcd2* mutant strain (the *gcd2* 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 *gcn4* mutant strain and increased in the *gcd2* 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 (*gcn4-101 gcn4-201*) (Fig. 7A). Strains with an additional *aro4* mutation grow slowly on MV medium. (ii) Basal levels of the phenylalanine-sensitive DAHP synthase (*ARO3*) are expressed in wild-type cells, in an *aro4* single mutant strain, and also in *aro4 gcn4* strains 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 *gcd2* 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-

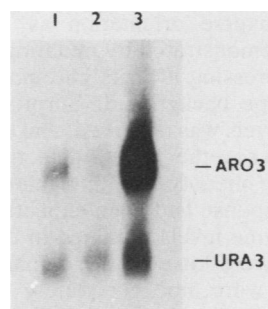

FIG. 6. *ARO3* transcript levels. Samples (25 μg) of poly(A)$^+$ RNA of *S. cerevisiae* strains RH1398 (*aro4-1 gcn4-201*; lane 1), X2180-1A (lane 2), and RH558-1 (*gcd2-1*; lane 3) were cohybridized against the radioactively labeled 1.7-kb *Hin*dIII-*Xba*I *ARO3* gene and the 1.1-kb *Hin*dIII *URA3* gene (22). The *URA3* gene serves as an internal standard for the amount of RNA loaded onto each lane. Transcript size of *ARO3* mRNA was 1.3 kb, and that of *URA3* mRNA was 0.9 kb.

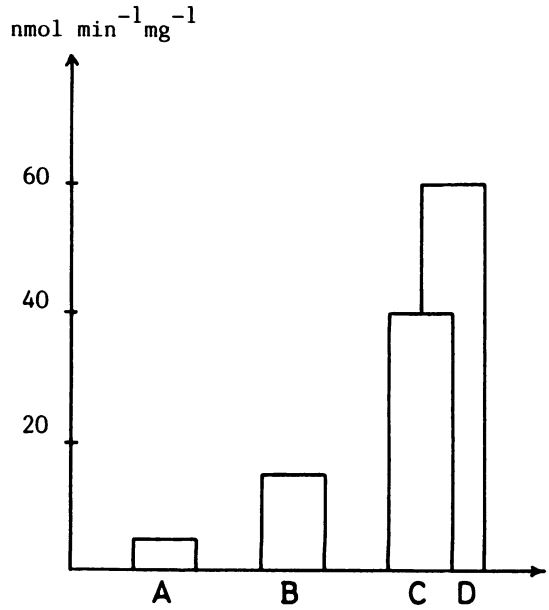

FIG. 7. DAHP synthase levels (phenylalanine sensitive) under various regulatory conditions. (A) Low level of *ARO3* gene product; (B) basal level, (C and D) derepressed levels of DAHP synthase activity. See text for details.

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 thymidines 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 aro4* 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 ATGA(C/G)TCAT, 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 TGCTC 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, M. Künzler, and G. Braus, manuscript in preparation). Strains with the genotype *aro3 ARO4 gcn4* (with either *gcn4* allele), however, are completely unaffected in their basal enzyme level. In the cell, more

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 *ARO3* 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 *ARO3* 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 TGACTC 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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