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# Characterization of the DNA Target Site for the Yeast ARGR Regulatory Complex, A Sequence Able to Mediate Repression or Induction by Arginine

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**We have determined the sequences and positions of the *cis* elements required for proper functioning of the *ARG3* promoter and proper arginine-specific control. A TATA box located 100 nucleotides upstream of the transcription start was shown to be essential for *ARG3* transcription. Two sequences involved in normal arginine-mediated repression lie immediately downstream of the TATA box: an essential one (arginine box 1 [AB1]) and a secondary one (arginine box 2 [AB2]). AB1 was defined by saturation mutagenesis and is an asymmetrical sequence. A stringently required CGPu motif in AB1 is conserved in all known target sites of $C_6$ zinc cluster DNA-binding proteins, leading us to propose that AB1 is the binding site of ARGRII, another member of the $C_6$ family. The palindromic AB2 sequence is suggested, on the basis of published data, to be the binding site of ARGRI, possibly in heterodimerization with MCM1. AB2 and AB1 correspond respectively to the 5′ and 3′ halves of two adjacent similar sequences of 29 bp that appear to constitute tandem operators. Indeed, mutations increasing the similarity of the other halves with AB1 and AB2 cause hyperrepression. To mediate repression, the operator must be located close to the transcription initiation region. It remains functional if the TATA box is moved downstream of it but becomes inoperative in repression when displaced to a far-upstream position where it mediates an arginine and *ARGR*-dependent induction of gene expression. The ability of the *ARG3* operator to act either as an operator or as an upstream activator sequence, depending on its location, and the functional organization of the anabolic and catabolic arginine genes suggest a simple model for arginine regulation in which an activator complex can turn into a repressor when able to interfere sterically with the process of transcription initiation.**

*Saccharomyces cerevisiae* can synthesize the amino acid arginine for protein synthesis; it can also use it as a nitrogen source. The corresponding metabolic fluxes are mutually exclusive: a common regulatory system, mediated by the ARGR proteins, responds to the presence of arginine by repressing arginine biosynthesis and by inducing its catabolism (16, 78, 79). Understanding the molecular basis of this ambivalent role of the ARGR proteins requires full characterization of the *cis*- and *trans*-acting elements involved in the regulation of the anabolic and catabolic genes.

Arginine-mediated repression is a negative regulation involving *cis*-acting elements (operators) linked to the structural genes (30, 48) and at least three *trans*-acting regulatory genes, *ARGRI*, *ARGRII*, and *ARGRIII* (2), which have been extensively characterized (5, 15, 17, 18, 50, 52, 66, 67). The cloning of *ARG3*, encoding ornithine carbamoyltransferase (OTCase; EC 2.1.3.3), and the characterization of its 5′ noncoding region allowed us to localize the *cis* elements involved in arginine repression between the presumptive TATA box and the start of transcription (7, 8, 10). In the *ARG5,6* gene, the repressor target is also located downstream of the presumptive TATA box (6); a recent analysis of the DNA-binding sequences of ARGR proteins (51) together with the results of this report, will be addressed in Discussion. So far, all of the data implicate a DNA-protein complex in arginine-specific regulation, but there is a controversy regarding the mechanism of repression: data of Messenguy and coworkers are interpreted to suggest a posttranscriptional regulation (6, 49), whereas our own data, obtained by another approach, support a fully transcriptional regulation (9).

In this work, we have dissected the *ARG3* operator and analyzed its functional relationships with other elements of the promoter by oligonucleotide-directed mutagenesis. The data lead to a precise identification of the elements constituting the basic operator module of the yeast arginine regulon and show that the operator must be located close to the transcriptional start in order to mediate repression; when displaced to an upstream position, the operator behaves as an upstream activator sequence (UAS). A model for ARGR-mediated regulation is presented.

## MATERIALS AND METHODS

**Strains.** Strains SS1 (α *ura3* Δ*arg3*) and SS2 (an $Arg^+$ derivative of SS1 obtained by transplacing into it the wild-type *ARG3* gene isolated from the FL100 strain of F. Lacroute) have been described elsewhere (11); the Δ*arg3* deletion removes the entire structural gene and more than 700 bp in front of it. 12S09 (**a** *ura3* Δ*arg3 argRI-2*) and 12S38b (**a** *ura3* Δ*arg3 argRII-10*) were obtained by crossing SS1 with isogenic strains derived from BJ102 (*argRI-2*) and BJ210 (*argRII-10*) from the collection of J. Béchet. Strain 12S22 (**a** Δ*arg3 gcn4*) derives from a cross of SS1 with strain L869 (**a** *gcn4-101 leu2*) from G. Fink. MG471 (α *ura3*) from M. Grenson, 02296b (*ura3 cargRI*) and 02013a (*ura3 cargRI argRII-10*) from E. Dubois, and 11S40c (*ura3 argRII*$^d$) all are strains isogenic to the wild-type Σ1278b. The *argRII*$^d$ mutation has been described elsewhere (16).

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**Media.** The basic minimal medium, containing 3% glucose, vitamins, and trace minerals, has been described previously (48). Mam is minimal medium supplemented with 0.02 M $(NH_4)_2SO_4$ as the nitrogen source. MamA is Mam supplemented with 1 mg of L-arginine per ml. A′ and O′ are minimal media with 1 mg of L-arginine per ml and 1 mg of L-ornithine per ml, respectively, as the sole nitrogen sources.

**Oligonucleotides.** Oligonucleotides were synthesized in our laboratory by using a New Brunswick Biosearch Cyclone DNA synthesizer.

**Oligonucleotide-directed in vitro mutagenesis and DNA sequencing.** We have used the Amersham mutagenesis system (RPN.1523), which is based on the method of Nakanaye and Eckstein (57). The single-strand antisense template used in mutagenesis was prepared from the pBP1 construction obtained by inserting in the Bluescript vector pBS(+SK) (Stratagene) a 4.2-kb *Cla*I-*Bam*HI fragment originating from plasmid pMC302 (11); this fragment bears the *URA3* gene and *ARG3* gene from the yeast strain FL100. After mutagenesis, the mutants were identified by one-track sequencing and confirmed by a local full-track sequencing using the dideoxynucleotide chain terminator method (71). The entire promoter region of a particular mutant was then sequenced (from nucleotide −420) to the beginning of the *ARG3* open reading frame (ORF) in order to detect any secondary mutations. This particular mutant and several independent ones from the same series were then further used in yeast transformation, the occurrence of transformants with equivalent phenotypic and enzymatic characteristics indicating the absence of secondary mutations in the rest of the *ARG3* ORF.

**Introduction of the mutated genes in the yeast genome.** Strain SS1 (*ura3* Δ*arg3*) was used for transformation, using the protocol of Ito et al. (29). The mutants expected to retain significant expression of *ARG3* and therefore to give an $Arg^+$ phenotype were introduced at the original locus by gene transplacement: the mutated pBP1 derivative was cut with restriction enzyme *Hin*dIII, and the restricted DNA was used to transform SS1 to arginine prototrophy on minimal medium supplemented only with uracil. As the Δ*arg3* present in strain SS1 deletes the ORF and more than 700 nucleotides of the 5′ noncoding region, the mutation brought in the genome can not be corrected by copying the wild-type template. In cases where an $Arg^-$ phenotype was found or could be expected, the mutated pBP1 derivative was looped into the genome by recombination using the *URA3* gene homology; the plasmid DNA was therefore linearized to completion by *Pst*I restriction (which cuts in the *URA3* DNA) prior to transformation of SS1 to uracil prototrophy on minimal medium supplemented only with arginine. The $Ura^+$ transformants were then tested for their arginine requirement. Tandem integration occurs occasionally with both strategies, gene transplacement and plasmid looping in, although more frequently in the latter case. This was readily detected by enzymatic assay of a series of analogous transformants, some individuals having activities being multiples of a basis value. In the looping-in approach, the copy number of multicopy integrated plasmids has been independently determined by assaying the activity of orotidine 5′-monophosphate decarboxylase (OMPdecase; EC 4.1.1.23), the product of the *URA3* gene.

**Construction of *argR* and *gcn4* derivatives.** To introduce the various promoter-operator mutations of *ARG3* in an *argR* background, the SS1 derivatives bearing the mutated gene either transplaced or looped into it (SS1 [α *ura3* MutX-*ARG3*] or SS1 [α *ura3*::pBP $URA3^+$ MutX-*ARG3* Δ*arg3*], respectively) were crossed with 12S09 (**a** *ura3* Δ*arg3* *argRI-2*) or with 12S38b (**a** *ura3* Δ*arg3* *argRII-10*): the MutX-*ARG3* genes were easy to detect either as the two $Arg^+$ spores of the tetrad or, in case in which MutX is $Arg^-$ itself, as the two $Ura^+$ spores. The *argR*-bearing spores were detected by their inability to grow normally on A′ and O′ media. The *gcn4* derivatives were similarly obtained from crosses with strain 12S22 (Δ*arg3* *gcn4*). The *gcn4-101* spores were identified by their sensitivity to 20 mM 3-aminotriazole.

**Enzyme assays.** All extracts were prepared by using the French press with cells harvested in the exponential phase of growth. OTCase was assayed as described in Ramos et al. (68). OMPdecase was assayed by the method of Wolcott and Ross (81) except that labeled $CO_2$ was collected on a filter paper containing 20 μl of hyamine 10× hydroxide (BDH Biochemicals). β-Galactosidase was measured according to Miller (53).

**Plasmid constructions.** pLG670-O ARG3-I and pLG670-O ARG3-II were obtained by cloning into pLG670 (cut by *Xho*I, filled in with T4 DNA polymerase and nucleoside triphosphates, and then dephosphorylated with calf intestine alkaline phosphatase) the 120-bp *Taq*I-*Hin*cII fragment bearing the *ARG3* operator isolated from plasmid pMC200 (7). This fragment was purified by electrophoresis on a 6% acrylamide gel of a filled-in *Taq*I-*Hin*cII restriction mixture. To confirm the nature of the cloned fragment and its orientation relative to plasmid pLG670, we sequenced the duplex DNA hybrid with an *ARG3* operator oligonucleotide as the primer and determined the mother plasmid nucleotides flanking the operator insertion.

**RNA primer extensions.** Three picomoles of 5′-end-labeled primer (CAGAAAGATCTTTTATAGAAATCAA, complementary to the *ARG3* ORF from positions +87 to +62) was mixed with 100 to 150 μg of total RNA and ethanol precipitated. The pellet was resuspended in 30 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 1 mM EDTA, 0.4 M NaCl, 80% formamide] for 10 min at 85°C and left overnight at an annealing temperature of 30°C. After ethanol precipitation, the pellet was resuspended and extension was performed by using Moloney murine leukemia virus RNase-free reverse transcriptase (Superscript; Bethesda Research Laboratories) as instructed by the supplier. The reaction was terminated by adding 4 μl of 0.25 M EDTA. A 30-min RNase (DNase-free) treatment was followed by phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in 4 μl of Tris-EDTA, and 6 μl of formamide loading dye was added. This mixture was heated for 5 min at 95°C, put on ice, and loaded on a 6% denaturing polyacrylamide gel together with the four dideoxynucleotide reaction mixtures of a M13 template extended with the same primer.

## RESULTS

**A single TATA box is required for *ARG3* expression.** The sequence TATATAAAT (abbreviated TATA), containing the consensus sequence for the transcription factor TFIID-binding site, is present 100 nucleotides 5′ to the transcription start of *ARG3* (Fig. 1) (8). To examine its function, we constructed a deletion (ΔT) as illustrated in Fig. 2a. Strain SS1 (*ura3* Δ*arg3*) was transformed with each of four independently constructed ΔT-$URA3^+$ plasmids. Uracil prototrophs, selected on minimal medium containing arginine, carried integrated plasmids, two categories of transformants being obtained in each case: $Arg^-$ (about 25%) and $Arg^{\pm}$.

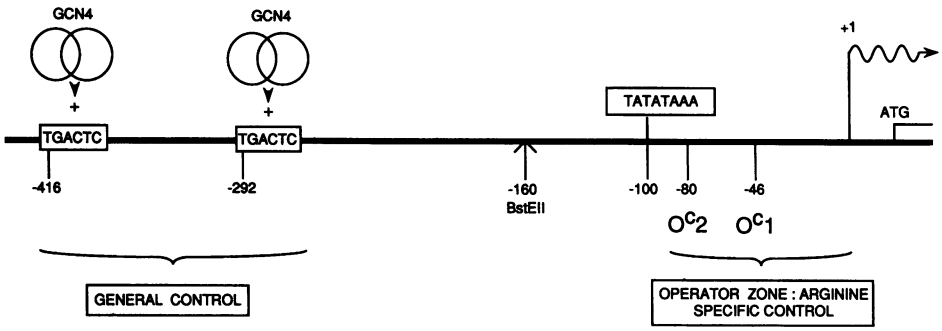



FIG. 1. Functional organization of the 5′ noncoding region of *ARG3*. The figure summarizes the data available when this work was started (8). The region involved in general control is located upstream and is distinct from the region involved in arginine-specific repression. The latter region is situated downstream of a TATA box candidate.

Assays of OMPdecase, the *URA3* gene product, showed that the $Arg^-$ strains had acquired a single copy of the ΔT plasmid whereas the $Arg^{\pm}$ transformants had integrated three or four copies. As the latter displayed 3 and 4%, respectively, of wild-type OTCase activity, we conclude that a single copy of the *ARG3* gene bearing a deletion of the TATA box produces only 1% of the wild-type level and that this amount is insufficient to sustain growth (Table 1). The relevant control strain has been used in these experiments; the wild-type level is the OTCase activity corresponding to one copy of plasmid pBP1 (see Materials and Methods) integrated in the *ura3* gene of strain SS1; this level is the same as in the wild-type strain Σ1278b. The residual *ARG3* expression measured in the arginine-leaky multicopy transformants is sensitive to repression by exogenous arginine (a close to normal, fivefold reduction in OTCase activity is observed); moreover, it is still sensitive to endogenous repression despite the limited production of arginine. Indeed, in *argRII* derivatives of such strains, OTCase activity is three times higher (Table 1). We suggest that ornithine, which should accumulate when the level of OTCase is low, acts as a corepressor. When the sequence TAATAA located about 40 nucleotides upstream of the transcription start was modified to TAATTG in a promoter already carrying ΔT, no further decrease of OTCase activity was observed (data not shown); this sequence therefore does not appear to be responsible for the residual OTCase activity of strains deprived of the essential TATA box. Also, the residual activity does not appear to depend on GCN4 (the activator of amino acid biosynthetic genes), since $Arg^{\pm}$ transformants were still obtained at comparable frequencies when the ΔT-bearing plasmid was introduced into a *gcn4* strain.

**Two arginine boxes, AB1 and AB2, contribute to *ARG3* repression; AB1 is essential.** The amplitude of arginine repression of the wild-type *ARG3* gene of strain Σ1278b is close to 13-fold; addition of arginine to the growth medium reduces OTCase activity 7-fold, whereas the loss of endogenous repression in *argR* or in operator-constitutive mutants almost doubles OTCase activity (Table 2, lines 1 to 4). The same is true for a Σ1278b-derived strain in which the original *ARG3* gene has been transplaced by the *ARG3* gene cloned from strain FL100 (Table 2, lines 5 and 6).

We showed previously by deletion analysis that the 170 nucleotides preceding the most proximal start point of transcription are sufficient to achieve normal arginine-specific repression (Fig. 1) (7); moreover, two operator-constitutive mutants isolated in vivo turned out to be single substitutions at positions −46 ($O^c1$, 1.5-fold residual repression) and −80 ($O^c2$, 2.4-fold residual repression) (7). We show here (Fig. 2b and c; Table 2, lines 7 and 8) that the deletion of 10-nucleotide stretches encompassing the $O^c1$ and $O^c2$ positions (ΔAB1 [from nucleotides −49 to −39] and ΔAB2 [from nucleotides −84 to −74], respectively) both result in constitutive expression. In a ΔAB2 strain, as in the $O^c2$ point mutant, repression is only partly reduced (a 2.5-fold repression remains), whereas a ΔAB1 strain is fully constitutive. The operator region therefore appears to subdivide functionally into two sequences that we will call arginine boxes 1 and 2 (AB1 and AB2), the limits of which will now be further defined.

The 3′ limit of the operator region, i.e., of AB1, should not extend beyond position −32. Indeed, neither deletion of the A/T-rich sequence extending from bp −31 to −12 nor insertion of a TATATAAAT sequence between bp −32 and −31 (Fig. 2d and e) has any effect on the level of *ARG3* expression or on the amplitude of repression (Table 2, lines 9 and 10).

The 5′ limit must not exceed bp −94 since, as discussed later, a fragment extending from −94 to +25 is able to mediate an *ARGR*-dependent response in a heterologous system.

**Sequence requirements of AB1.** Every nucleotide present 5′ or 3′ to position −46 (site of mutation of the in vivo-isolated $O^c1$ mutant) in the AB1 region was systematically mutated into the three other nucleotides, and the effect of each mutation was analyzed after transplacement into the yeast genome. Each transformant was assayed at least twice after growth on minimal medium and on minimal medium supplemented with L-arginine (1 mg/ml). Duplicate assays of independent cultures did not generally differ by more than 15%. As expected, loss of repression by externally added arginine correlated with increased OTCase activity on minimal medium due to reduced repression by endogenous arginine; in the fully constitutive mutants, a value close to 200 is reached. We classified the results (Fig. 3) by giving for each mutant the ratio between the fully derepressed (200) and the fully repressed OTCase activities; the mutants' residual repression factors are thus to be compared with 13, the repression coefficient characteristic of the wild-type strain. The data are summarized as a consensus sequence (Fig. 3b) in which each nucleotide is the one required to achieve at least twofold repression. Of the 22 mutagenized positions, 11 present specific requirements; the most exacting positions are CGPu-34 and PyAPu-44. No obvious symmetry can be recognized in the AB1 sequence. As discussed below, the 5′ extremity of AB1 overlaps the 3′ extremity of an adjacent AB2, some positions of which are important. Among the 66 mutations created, 7 are peculiar because they

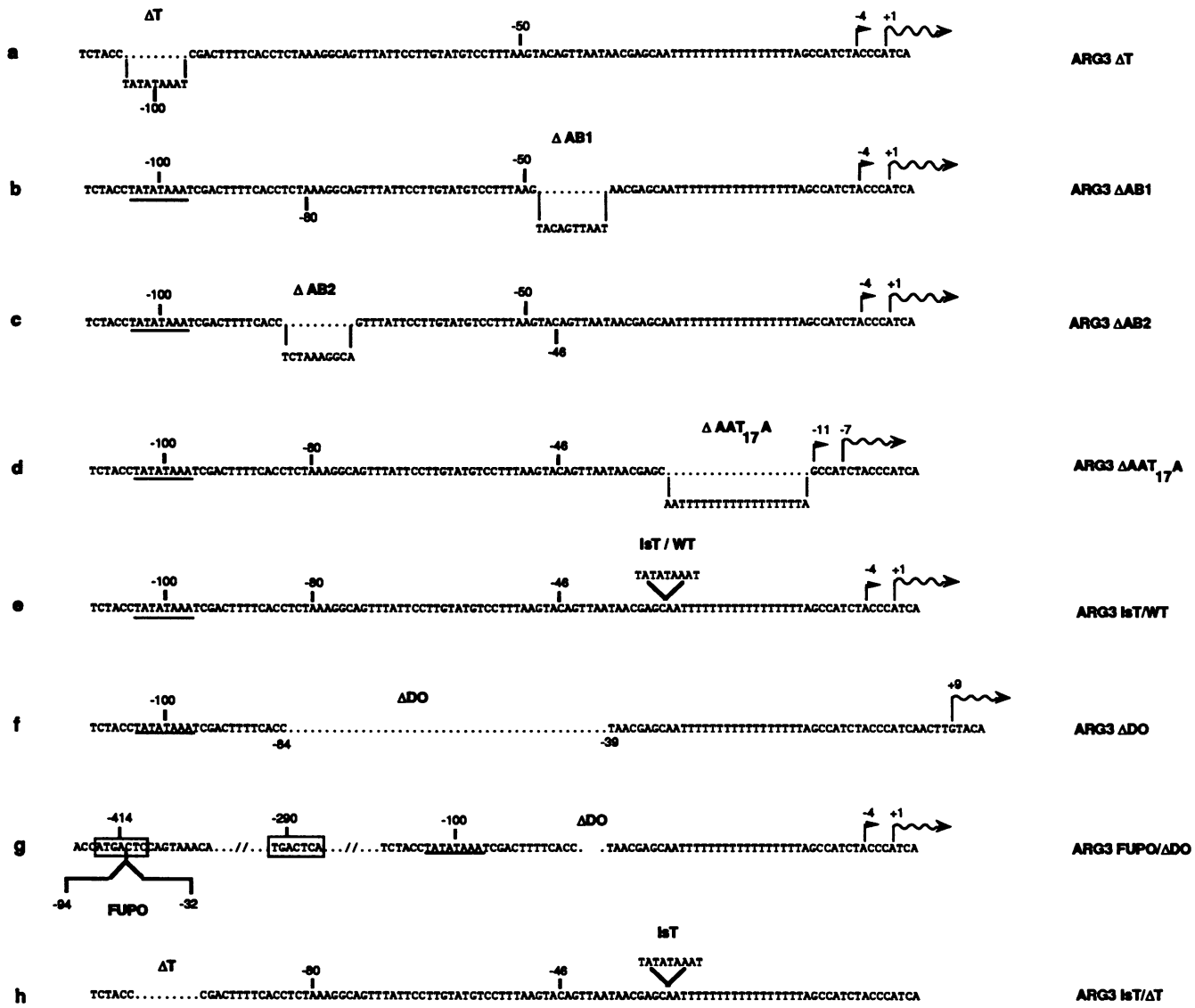



FIG. 2. Deletions and insertions created in the 5′ noncoding region of *ARG3*. Each line represents the construction named at the right. The deletions are indicated by dots, and the sequences deleted or inserted are written out of alignment. −80 and −46 are the positions that were previously found modified in the two operator-constitutive mutants that were isolated in vivo (see Fig. 1). The two target sites for GCN4 are boxed in line g. The TATA box is underlined. The FUPO sequence contains the two tandem operators of *ARG3* on a segment extending from positions −94 to −32.

give OTCase levels on Mam that are lower than that of the wild-type strain and moreover are more repressed by exogenous arginine; these mutations are indicated in bold type above the consensus sequence in Fig. 3b. As discussed below, these mutations can be interpreted in terms of hyperrepression and correspond to functional improvements of the original AB1 sequence. The results of this analysis enabled us to identify a functional AB1 box in the coregulated *ARG8* gene at a similar location, i.e., between the putative TATA box and the initiating codon (13a).

**Hyperrepressed mutants identifying an AB1-like sequence adjacent to AB2 and an AB2-like sequence adjacent to AB1 indicate the existence of two tandem operators of a composite AB2-AB1 structure.** Within the operator region (−94 to −32), one can recognize two contiguous sequences of 29 bp that have obvious similarities. As shown in Fig. 4, AB1 is located in the 3′ part of the second repeat and AB2 (which contains a near perfect palindrome, CCTCT/AAAGG, reminiscent of other elements; see Discussion) is located in the 5′ moiety of the first repeat. These similarities suggest that two operators, each containing partly conserved and overlapping AB2 and AB1 sequences, are present as tandem repeats in the control region of *ARG3*. The first repeat, containing the functional AB2 sequence, would be a weak operator; the second one, in which the AB1 box is essential, would be a strong operator. To test this hypothesis, we decided to improve the resemblance of the second half of the first repeat with AB1 (DAB1 mutation) and that of the first half of the second repeat with AB2 (DAB2 mutation). To create DAB2, we increased the internal symmetry of the AB2-like sequence by changing the TA-47 nucleotides to GG. To improve the AB1-like sequence, we could have changed TCCTT-62 to AACGA-62, thus reconstituting all of the critical positions of AB1. Instead, we modified the original

TABLE 1. OTCase activities in constructions bearing a deletion of the *ARG3* TATA box[a]

| Genotype of transformants | OTCase relative activity[b] | | Fold repression |
|---|---|---|---|
| | Mam | MamA | |
| Δ*arg3 ura3*::(pBluescript-URA3-ARG3)$_2$ | 200 | 30 | 6.6 |
| Δ*arg3 ura3*::(pBluescript-URA3-ARG3 ΔT)$_1$ | NG | 0 | |
| Δ*arg3 ura3*::(pBluescript-URA3-ARG3 ΔT)$_1$ *argRII-10* | NG | 0 | |
| Δ*arg3 ura3*::(pBluescript-URA3-ARG3 ΔT)$_4$ | 4[c] | 0.8 | 5 |
| Δ*arg3 ura3*::(pBluescript-URA3-ARG3 ΔT)$_4$ *argRII-10* | 13[c] | 11 | 1.2 |

[a] Plasmids bearing the wild-type *ARG3* gene or the *ARG3* gene deleted of its TATA box (Fig. 2a) were integrated in the chromosomal *ura3* gene by directed looping in. The number of integrated copies (indicated by the subscript numbers) was determined on the basis of OMPdecase (*URA3* gene product) assays.

[b] Expressed as percentage of the activity on minimal medium corresponding to one copy of the wild-type gene bearing plasmid. NG, no growth.

[c] The corresponding constructions grown on minimal medium have an arginine-leaky phenotype.

sequence to GACGA-62, which also fulfills the AB1 requirements (see AB1 consensus) in order to avoid the creation of a TATA box-like sequence. As shown in Fig. 4, both DAB1 and DAB2 are hyperrepressed by endogenous arginine; the OTCase levels on minimal medium are low, but in an *argR* genetic background (*argRII-10* and/or *argRI-2*), the activities are back to the normal wild-type derepressed level, indicating that the mutations do not affect promoter efficiency. In addition, external arginine represses more efficiently than in the wild type; therefore, the amplitude of repression is increased in these optimized arginine operator mutants: whereas the amplitude of repression is 13-fold in the wild-type strain, it becomes 35-fold in DAB2 and 175-fold in DAB1.

TABLE 2. OTCase activities in various constructions bearing mutations in the 5′ noncoding region of *ARG3*

| *ARG3* genotype of transformants[a] | OTCase relative activity (%)[b] | | | Fold repression[c] | Fold induction[d] |
|---|---|---|---|---|---|
| | Mam | MamA | A′ | | |
| 1. *ARG3*$_\Sigma$ | 100 | 15 | | 6.6 | |
| 2. *ARG3*$_\Sigma$ *argRII-10* | 200 | 182 | | 1.1 | |
| 3. *ARG3*$_\Sigma$-O$^c$1 | 159 | 107 | | 1.5 | |
| 4. *ARG3*$_\Sigma$-O$^c$2 | 165 | 69 | | 2.4 | |
| 5. *ARG3*$_{FL}$ | 100 | 15 | | 6.6 | |
| 6. *ARG3*$_{FL}$ *argRII-10* | 200 | 182 | | 1.1 | |
| 7. *ARG3*$_{FL}$-ΔAB1 | 209 | 189 | | 1.1 | |
| 8. *ARG3*$_{FL}$-ΔAB2 | 164 | 56 | | 2.9 | |
| 9. *ARG3*$_{FL}$-ΔAAT$_{17}$A | 100 | 15 | | 6.6 | |
| 10. *ARG3*$_{FL}$-IsT | 100 | 15 | | 6.6 | |
| 11. *ARG3*$_{FL}$-ΔDO | 85 | 76 | | 1.1 | |
| 12. *ARG3*$_{FL}$-FUPO/ΔDO | 89 | 86 | | 1 | |
| 13. *ARG3*$_{FL}$-ΔDO *gcn4* | 41 | 39 | 35 | 1 | |
| 14. *ARG3*$_{FL}$-FUPO/ΔDO *gcn4* | 28 | 64 | 97 | | 3.5 |
| 15. *ARG3*$_{FL}$-FUPO/ΔDO *gcn4 argRI-2* | 25 | 21 | NG[e] | 1 | |
| 16. *ARG3*$_{FL}$-IsT/ΔT | 8 | 1.4 | | 5.7 | |
| 17. *ARG3*$_{FL}$-IsT/ΔT *argRII-10* | 39 | 34 | | 1.1 | |

[a] Lines 1 to 4 give values corresponding to the Σ1278b *ARG3* gene (ARG3$_\Sigma$) and derepressed derivatives. All other constructions bear the wild-type *ARG3* gene of strain FL100 (ARG3$_{FL}$) or mutants thereof. For the correspondance between mutant gene denomination and structure, see Fig. 2.

[b] Activity of 100% corresponds to the activity of the wild-type *ARG3* gene (from Σ1278b or from FL100) on minimal medium.

[c] Ratio of activities in each construction on Mam and on MamA.

[d] Ratio of activities on A′ and on Mam.

[e] NG, no growth.

**The *ARG3* operator can mediate *ARGR*-dependent arginine induction in a heterologous *CYC1-lacZ* system.** The *ARGR* products respond to the presence of arginine by inducing the expression of arginine catabolic genes. Therefore, we determined what kind of effect the *ARG3* operator region exerts when it replaces the UAS of the *CYC1* promoter in a *CYC1-lacZ* reporter construction. Deleting plasmid pLG669-Z from a *Xho*I fragment bearing the two *CYC1* UASs leaves a *CYC1-lacZ* fusion which is expressed at only a low level from its minimal core promoter (25). We inserted the *Taq*I-*Hin*cII *ARG3* fragment extending from positions −93 to +25 (which bears the two *ARG3* operators) at the *Xho*I site of this UAS-free *CYC1-lacZ* fusion; clones with the operator region in the natural orientation (O-ARG3-I) or in the opposite orientation (O-ARG3-II) were identified by DNA sequencing. The three types of constructions (either with the UAS deletion only or with the *ARG3* operator in one of the two orientations) were introduced in strains with different genotypes (Table 3) and assayed in three media: Mam, MamA, and A′ (see Materials and Methods). The background levels of the UAS-less tester gene show some strain dependence, and the presence of the *ARG3* operator region further reduces this basal level; the cause of this effect is unknown but is independent of *ARGR* regulation (see behavior of strain 02013). However, addition of arginine clearly induces β-galactosidase activity in strains bearing the operator region in an *ARGR*$^+$ context (MG471 and 02296b). The arginine stimulation is also seen in strain 11S40c, which carries the dominant *argRII*$^d$ mutation. This mutation leads to a partially constitutive arginine catabolism (it presumably affects the interaction between the ARGR and cARGR products [16]), but it does not affect the repression of the anabolic genes. Significantly, arginine induction is totally absent in strain 02013a, in which anabolic repression is abolished because of the *argRII-10* allele. As in the case of arginine catabolic genes *CAR1* and *CAR2*, this heterologous *ARGR*-dependent transactivation is more pronounced when arginine is the nitrogen source and the arginine intracellular pool thus becomes higher (68).

**To mediate *ARG3* repression, the *ARG3* operator must be properly located; in *ARG3* also, the operator can behave as a UAS.** The *ARG3* operator region (−94 to −32) was inserted at position −414 in an *ARG3* promoter deleted of its normal operator; this construction, FUPO (far-upstream operators)/ΔDO, is drawn in Fig. 2g. In the control strain ΔDO, which bears only the deletion of the operator region (Fig. 2f), the OTCase activity is slightly below the wild-type level on minimal medium, an effect that we ascribe to the closer proximity of the TATA box to the transcriptional start (about 50 nucleotides). Addition of external arginine remains clearly without effect in the FUPO/ΔDO strain (Table 2, lines 11 and 12). However, in this construction, in which the newly inserted *ARG3* operator disrupts the more distal one of the two GCN4 targets of the *ARG3* promoter (8), the remaining site centered at position −289 is still intact. As the GCN4 transactivation contributes to the *ARG3* basal level on minimal medium (8) and might therefore interfere with a positive effect of arginine, we introduced a *gcn4* allele in the ΔDO and FUPO/ΔDO constructions. Arginine induction of OTCase synthesis linked to the presence of the newly inserted operator could now be detected (Table 2, lines 13 and 14). Moreover, the induction by arginine (twofold on

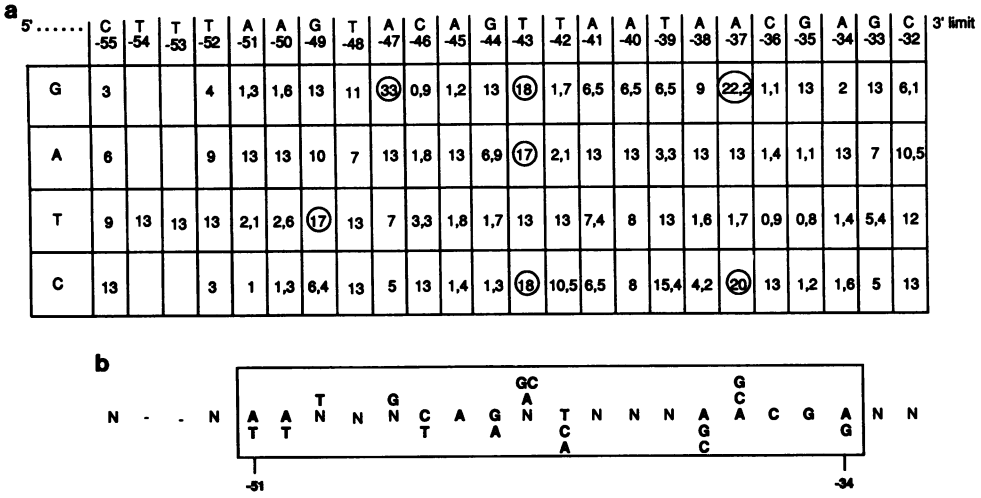
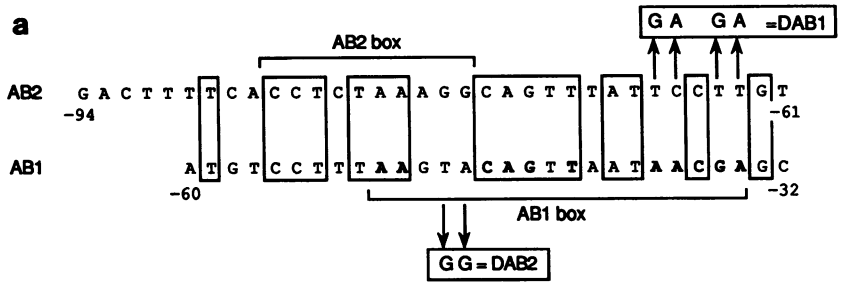



FIG. 3. Sequence requirements of AB1. (a) Wild-type sequence found in front of *ARG3* between positions −55 and −32 from the transcriptional start. In the grid, the repression coefficient of each mutation (as indicated at the left) is given as the ratio between 200 (derepressed OTCase in a fully constitutive strain) and the mutant OTCase level on MamA. (b) Consensus sequence required to obtain at least twofold repression. The nucleotides written above the consensus sequence are modifications that increased the repression mediated by this AB1.

Mam with 1 mg of L-arginine per ml and threefold when arginine is the sole nitrogen source) proved to be dependent on the integrity of the *ARGRI* gene product (Table 2, line 15). Thus, whether in its normal context or in a heterologous promoter (see above), the *ARG3* operator can mediate induction when it is moved to a far-upstream location. On the contrary, the operator remains fully functional as a negative control element when the TATA box is moved from its upstream position (relative to the operator) to an immediately downstream location (Fig. 2h; Table 2, lines 16 and 17). Again, this displacement correlates with a lower capacity of expression, as could be anticipated from the very short distance (30 bp) separating the displaced TATA box from the normal transcription initiation sites.

**RNA primer extension analysis.** We had previously identified two *ARG3* transcripts initiating 26 and 22 nucleotides upstream of the translational start codon; number 1 was assigned to the nucleotide initiating the latter transcript (8).




b

| | | OTCase relative activity /Mam | /MamA | Fold Repression* | Total Amplitude of repression** |
|---|---|---|---|---|---|
| $ARG3_{FL}$ | | 100 | 15 | 6,6 x | 13 x |
| $ARG3_{FL}$ | ,argRII-10 | 200 | 182 | 1,1 x | |
| $ARG3_{FL}DAB2$ | | 16 | 4,8 | 3,3 x | |
| $ARG3_{FL}DAB2$ | ,argRI-2 | 175 | 148 | 1,2 x | 35 x |
| $ARG3_{FL}DAB2$ | ,argRII-10 | 164 | 137 | 1,2 x | |
| $ARG3_{FL}DAB1$ | | 8 | 1 | 8 x | 175 x |
| $ARG3_{FL}DAB1$ | ,argRI-2 | 179 | 174 | 1,1 x | |

FIG. 4. Hyperrepressed mutants. (a) The *ARG3* regulatory region from nucleotides −94 to −32, drawn so as to highlight the similarity existing between the two repeated 29-bp-long sequences. The modifications made to create the DAB1 and the DAB2 mutants are shown (boxed) above the wild-type sequences. (b) OTCase activities measured in the DAB mutants and in their *argR* derivatives. The activities are expressed as percentages of the activity of wild-type strain SS2 grown on minimal medium. *, ratio of activities of each strain on Mam and on MamA; **, ratio of activities in *argR* derivatives on Mam and the correspondent $ARGR^+$ version on MamA.

TABLE 3. β-Galactosidase activities in a *CYC1-lacZ* heterologous system in which the *ARG3* operator replaces the natural *CYC1* UASs

| Strain | Genotype[a] | Plasmid[b] | β-Galactosidase activity (nmol/mg of protein/min) after growth on: | | |
|---|---|---|---|---|---|
| | | | Mam | MamA | A′ |
| MG471 | *ura3* | pLG669-ΔUAS$_{CYC1}$ | 7.7 | 7.3 | 8.1 |
| | | pLG669-O-ARG3-I | 3.1 | 5.2 | 21.1 |
| | | pLG669-O-ARG3-II | 3.9 | 13.4 | 124 |
| 02296b | *ura3 cargRI* | pLG669-ΔUAS$_{CYC1}$ | 23 | 31 | 28 |
| | | pLG669-O-ARG3-I | 14.1 | 31 | 34 |
| | | pLG669-O-ARG3-II | 8.4 | 54.3 | 116 |
| 02013a | *ura3 cargRI argRII-10* | pLG669-ΔUAS$_{CYC1}$ | 42 | 45 | 42 |
| | | pLG669-O-ARG3-I | 9.8 | 11 | 5 |
| | | pLG669-O-ARG3-II | 7 | 7 | 6 |
| 11S40c | *ura3 argRII*[d] | pLG669-ΔUAS$_{CYC1}$ | 30.5 | 27 | 35 |
| | | pLG669-O-ARG3-I | 9.2 | 23.3 | 82 |
| | | pLG669-O-ARG3-II | 15.3 | 66.6 | 232 |

[a] The *cargRI* mutation is necessary in the presence of the *argRII-10* mutation to allow growth on A′ medium. *argRII*[d] is a dominant mutation leading to a partially constitutive arginine catabolism. Repression of the biosynthetic genes is quasi-normal.
[b] See text.

These initiation sites had been determined by S1 and exonuclease VII nuclease mappings, using RNAs prepared from wild-type strain Σ1278b and isogenic derivatives. The mutants described in this work are in vitro modifications of the *ARG3* gene originating from strain FL100 and transplaced in Σ1278b-derived strains. We therefore determined whether the transcriptional starts were identical for both wild-type *ARG3* genes and had possibly been modified in the promoter mutants. RNA primer extension experiments (Fig. 5) confirmed our previous location of the *ARG3* messenger cap sites in strain Σ1278b and established the same 5′ ends for messengers initiated from the FL100 *ARG3* gene. As expected, no qualitative modification was observed in the operator-constitutive mutants except for the ΔDO and FUPO/ΔDO mutants. The ΔDO deletion indeed brings the TATA box about 50 nucleotides closer to the transcription starts; it causes the loss of the −4 transcript and the appearance of a shorter transcript initiating at position +9. Surprisingly, deletion of the TATA box (ΔT mutant, as observed in a *argRII* derivative) does not modify the transcription starts, whereas deletion of the T-rich region situated immediately upstream of the normal starts (ΔAAT17A mutant) creates new initiation sites closer to the TATA box (Table 2, line 9). Importantly, the relative amounts of mRNA present in the different strains and on the different media tested (Fig. 5B and C) are essentially in agreement with a mechanism of repression by arginine occurring at the transcriptional level, as we have already proposed on the basis of a detailed kinetic analysis (9).

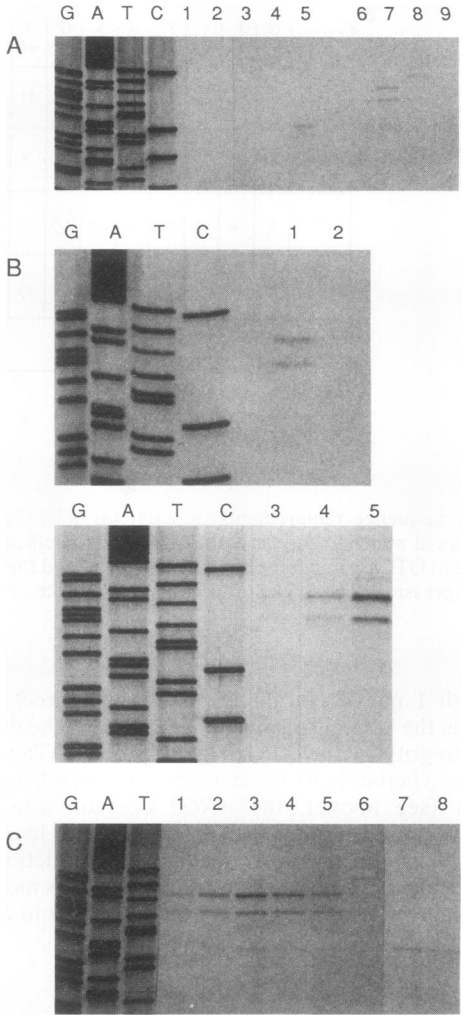



FIG. 5. RNA primer extension experiments. (A) Control and mutant strains grown on Mam (150 μg of total RNA was used in each lane). Lanes: 1, *ARG3*$_{\Sigma}$ (strain Σ1278b); 2, *ARG3*$_{FL}$ (strain SS2); 3, *ARG3*$_{FL}$-IsT; 4, *ARG3*$_{FL}$-IsTΔT; 5, *ARG3*$_{FL}$-ΔDO; 6, (::pBluescript-URA3-ARG3ΔT)$_4$ *argRII-10*; 7, (::pBluescript-URA3-ARG3)$_2$, 8, *ARG3*$_{FL}$-ΔAAT17A; 9, *ARG3*$_{FL}$-FUPO/ΔDO *gcn4*. (B) Two independent experiments showing ARG3$_{\Sigma}$ in different regulatory statuses (150 μg of total RNA was used in the first experiment [lanes 1 and 2] and 100 μg was used in the second experiment [lanes 3 to 5]). Lanes: 1, *ARG3*$_{\Sigma}$ (strain Σ1278b), Mam; 2, same strain, MamA; 3, *ARG3*$_{\Sigma}$, Mam; 4, *ARG3*$_{\Sigma}$ *argRII-10* (strain 10R27d), Mam; 5, *ARG3*$_{\Sigma}$ *arg11* (arginine-leaky strain MG409), Mam. MG409 is derepressed for both specific and general control, and its relative OTCase level is 500. (C) ARG3$_{FL}$ control and mutant strains. Total RNA amount is 150 μg except in lanes 2 to 5, which contain 100 μg. Lanes: 1, *ARG3*$_{FL}$ (strain SS2), Mam; 2, *ARG3*$_{FL}$-ΔAB1, Mam; 3, same strain, MamA; 4, *ARG3*$_{FL}$-ΔAB2, Mam; 5, same strain, MamA; 6, *ARG3*$_{FL}$-ΔAAT17A, Mam; 7, *ARG3*$_{FL}$-ΔDO, Mam; 8, same strain, MamA.

## DISCUSSION

Our primary goal in the study of arginine metabolism in *S. cerevisiae* is to understand the molecular mechanisms by which the three ARGR regulatory proteins repress arginine biosynthesis and induce arginine degradation.

In this work, we have analyzed functionally the *cis* elements involved in arginine-specific repression of gene *ARG3*. We have defined the sequence requirements for a functional operator and identified two types of operator elements, a stringent one (AB1) and a secondary one (AB2). On closer examination, it appears that the *ARG3* operator region consists of two tandem, imperfect repeats of 29 bp, each consisting of a sequence of the AB1 type and another one of the AB2 type (Fig. 4). Increasing the resemblance between the repeats at specific sites leads to hyperrepression. Finally, we have shown that the ARGR repressor

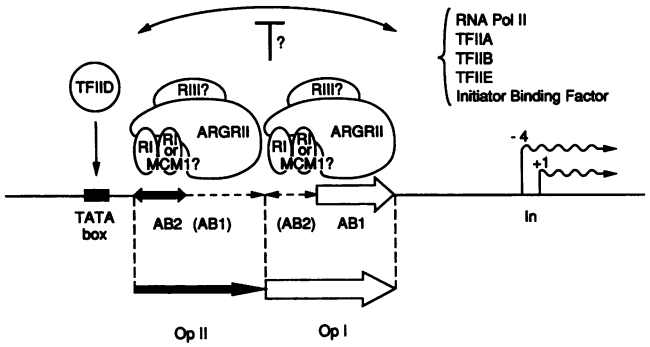



FIG. 6. Model for arginine repression in *ARG3*. The drawing represents to scale the two tandem operators identified between the TATA box and the transcriptional starts by two arrows whose thickness is proportional to their strength. A black symmetrical box indicates the functional AB2 palindrome in the weak operator II, and a white open arrow indicate the functional AB1 asymmetrical sequence in the strong operator I. The repressor complexes are drawn so as to illustrate the contacts between ARGRII and AB1 and between ARGRI dimers (or possibly ARGRI/MCM1 heterodimers) and AB2. A mechanism of repression by interference with the process of transcription initiation is indicated (T).

complex can actually stimulate transcription when the operator is translocated to an upstream position, either in a heterologous system or in the *ARG3* regulatory region itself. The results of this study, together with a considerable body of published data (see below), suggest the model outlined in Fig. 6: the arginine regulatory complex recognizes a basic operator module which is 29 bp long and composed of two subsites: the nonsymmetrical AB1, bound by the ARGRII component of the ARGR complex, and the palindromic AB2, bound by an ARGRI dimer or possibly by an ARGRI/MCM1 heterodimer. The strength of a particular operator depends on how similar each of its AB1 and AB2 boxes is to the optimal consensus sequence; a good consensus for at least one of the boxes is sufficient for function. The position of these operators relative to the transcription start sites determines whether the arginine regulatory complex will, in the presence of arginine, either stimulate transcription (induction of the catabolic genes) or inhibit transcription (repression of the anabolic genes).

Before a circumstantial discussion of this model, a few comments are needed to clarify the role played by the TATA box in the expression of *ARG3* and to discuss a previously unrecognized and endogenous regulatory effect.

**A unique TATA box is required for *ARG3* expression; endogenous repression, presumably by ornithine, occurs in *ARG3*-leaky mutants.** When the sequence TATATAAAT starting at position −104 is deleted, the residual activity of OTCase is so low (1% of the activity normally observed in minimal medium) that strains containing only one copy of the ΔT-*ARG3* gene are unable to grow without arginine supplement. We do not know which element is responsible for the residual activity; we have ruled out the participation of the TAATAA sequence located at position −42 as well as the contribution of the GCN4 general transactivator. The TATA sequence of *ARG3* is thus clearly essential. It is known that a minimal promoter containing only a TATA box and an initiation site can promote transcription in vitro (44, 45). What remains unclear, however, is to what extent such a basic combination of elements can contribute to transcription in vivo. In *ARG3*, the only UAS sequences identified up to now are the GCN4 protein-binding sites present at positions −417 and −292 (8); the GCN4 protein is known to be a TATA-dependent transactivator (60). The data available (8) show that in the absence of arginine repression, 60% of the OTCase level of a wild-type strain grown on minimal medium is still observed with a promoter conserving only 70 bp upstream of the TATA box and thus devoid of the GCN4 targets. The presence of an as yet unidentified UAS in this region is certainly not excluded. We have shown here that a stretch of 17 T's localized just upstream of the normal transcription initiation sites seems to play a role in defining those initiation sites, since its deletion causes transcription to start at a cluster of new starts closer to the TATA box.

The present analysis has brought to light a regulatory effect previously unrecognized in the arginine pathway: in strains in which arginine biosynthesis is limited, such as in ΔT mutants, the production of OTCase is still subject to an *ARGR*-dependent endogenous repression. As the intracellular level of arginine must be extremely low in these strains, we suggest that ornithine, which should preferentially accumulate when OTCase activity is very low, acts as a corepressor.

**The AB1 sequence: target site for the *ARGRII* product.** The amino terminus of the ARGRII protein shows similarities with the GAL4 and PPRI regulatory proteins in a cysteine-rich region (52) and has been shown to be required for the formation of a retarded complex in band shift assays (67). In the case of GAL4, the cysteine region is known to be part of the DNA-binding domain (35), and the arrangement of cysteines in the $C_6$ motif type is thought to determine the formation of a zinc cluster essential for binding to DNA (reviewed in references 20 and 74).

Our saturation mutagenesis of the AB1 sequence has detected a site with stringent nucleotide requirements, the CGPu sequence. Comparing known target sites of the yeast regulatory proteins of the $C_6$ family (Table 4), we realized that they all share such a conserved CGPu sequence that we propose to call CEZ (contributing element for binding of $C_6$ zine clusters). A recent study of DNA binding by the LAC9 protein has shown that the CGG nucleotides at both ends of the 17-mer UAS are essential (26). Moreover, it is possible that the CGPu motif is also involved in the recognition of the $C_2H_2$ type of zinc finger; indeed, it is present in the minor region in SWI5 that is protected against DNase, in the DNA region involved in the ADR1 response (but not in the 22-bp palindrome), in GC boxes recognized by Sp1, in the ICR control region governing TFIIIA binding, and in the MRE sequence bound by MTF1 (Table 4). In the case of the steroid receptors having a $C_4$ and a $C_5$ zinc motif, no CGPu motif is displayed in any of the three consensus elements that have been proposed as targets, whether they are positive or negative response elements (70). However, extensively repeated GC boxes are present ahead of steroid-regulated steroid receptor genes lacking the consensus elements (83).

In our model, therefore, AB1 is considered the site to which ARGRII binds through an amino-terminal $C_6$ zinc cluster. There is no apparent symmetry in AB1; it is thus possible that ARGRII binds to AB1 as a monomer. The HAP1 protein is the only other member of the $C_6$ zinc cluster family of proteins known to bind to apparently asymmetrical DNA sequences (the B region of *CYC1* UAS1 and the unrelated tandemly repeated 9-bp sequence found in the UAS in front of *CYC7*) (23, 24, 61).

**The AB2 palindrome: target for an ARGRI dimer or possibly an ARGRI/MCM1 heterodimer.** The ARGRI regula-

TABLE 4. Compilation of target sequences recognized by established or presumed Zinc finger DNA-binding proteins

| REGULATORY PROTEIN | $Zn^{2+}$ FINGER TYPE | TARGET GENES | TARGET ELEMENTS | | TARGET SEQUENCES |
|---|---|---|---|---|---|
| ARGRII (52) | 1x C6 | ARG3 (this work) | 1x | AB1 | AAGTACAGTTAATAACGA<br>AAGTACAGTTAATAACGG |
| | | ARG8[a] | 1x | AB1 | AAGTGCAAGTGACTGCGA |
| | | ARG5,6 (6) | 1x | box3 | AATGGAAATGGATAGCGA |
| GAL4 (34, 35, 41) | 1x C6 | GAL1,10 (34) | 4x | | CGGA(G/C)GACA<br>TCAG(G/C)AGGC |
| | | GAL7 (34) | 2x | $UAS_{GAL}$ | |
| | | GAL2 (34) | 2x | | |
| | | MEL1 (34) | 1x | | |
| LAC 9 (82) | 1x C6 | LAC4 (26, 82) | 1x | | CGGAAATTT<br>ACACCAGGC |
| PPR1 (38) | 1x C6 | URA3 (69) | 1x | | |
| | | URA1 (69) | 2x | $UAS_{URA}$ | TTCGGNRN TYNCCGAA<br>AAGCC GGCTT |
| | | URA4 (69) | 1x | | |
| PUT3 (46) | 1x C6 | PUT1 (72) | 2x | | CCGG(A/G)(A/T)AGC C(A/G)TTG(A/C)CCGA<br>GGC GGC |
| | | PUT2 (72) | 1x | | TCGGGAAGC CAACTCCGA<br>AGC GGC |
| HAP1 (62) | 1x C6 | CYC1 (61, 63) | 1x | $UAS1_{CYC1\ region\ B}$ | TGGCCGGGGTTTACGGACGA |
| | | CYC7 (61, 63) | 2x | $UAS_{CYC7}$ | TAATAGCGA<br>TAATAGCGA |
| LEU3 (21) | 1x C6 | LEU1 (21)<br>LEU2 (21)<br>LEU4 (21)<br>ILV1 (21)<br>ILV2 (21)<br>ILV5 (21) | 8x | | G<br>ACCGG<br>GGCCC<br>AT<br>A |
| SWI 5 (73) | 3x C2H2 | HO (56, 73) | minor binding site | | TGCCGGTGCCTGCGATGA<br>GGC |
| ADR1 (27) | 2x C2H2 | ADH2 (3) | -257 to -216 not 22 dyad symmetry | | CCTCTGCCGGAACACCGGGCA<br>GGC GGC |
| Sp1 (37) | 3x C2H2 | many (36) | GC box | | (G/T)GGGCGGRRY |
| TFIIIA (54) | 9x C2H2 | 5S rRNA genes (47, 64) | ICR | | 3x CGG 2x CGA |
| MTF-1 (80) | zinc responsive factor | metallothionein genes (80) | MRE | | CTNTGCRCNCGGCCC |

consensus → $C_{73}G_{73}\begin{matrix}G\\A\end{matrix}_{56}{}_{17}$ → CEZ element[b]

[a] Our data concerning *ARG8* will be published separately.
[b] The CEZ element (the sequence CGPu) is found in a total of 73 elements (asymmetrical elements or half palindromes).

tory protein consists of 177 amino acids (15), 80 of which are sufficient for function (19); this region (from residues 80 to 160) is similar to the DNA-binding/dimerization domain of two other specific DNA-binding proteins, the yeast MCM1 protein (55 identities [59]) and the mammalian serum-responsive factor (SRF; 40 identities [58]).

The MCM1 protein is essential for viability and has multiple functions (4, 39, 59). Moreover, it is now established that the *PRTF*, *GRM*, *FUN80*, and *MCM1* products are one and the same molecule (1, 14, 31, 32). *ARGRI* and *MCM1* were originally isolated on the same DNA fragment (15) and may derive from a common ancestor gene. The

16-bp consensus sequence (or P [palindrome] box) for binding of *MCM1* is TTTCCTAA/TTAGGAAA (32).

The nuclear protein SRF, involved in the transient transcriptional activation of genes responding to growth factors, binds as a dimer to the serum-responsive element, a sequence with dyad symmetry the core of which is related to the $CC(A/T)_6GG$ sequence found in front of the cardiac and skeletal muscle actin genes (55, 58). A recent selection by immunoprecipitation of SRF-bound binding sites from a pool of random sequence oligonucleotides confirms the requirement of the 10-bp-long palindromic core with two CC dinucleotides always present at positions −5 and −4 relative to the dyad and A or T at positions ±3, ±2, and ±1. The nucleotides external to the 10-bp core present less conservation (65). Thus, as ARGRI has a domain homologous to the DNA-binding domain of MCM1 and of SRF, and as both latter proteins recognize similar binding sites, it is logical to propose that the ARGRI protein binds to AB2, the core sequence of which, CCTCTAAAGG, is similar to the core of the serum-responsive element and of the P box.

The two yeast factors MCM1 and ARGRI are certainly not functionally equivalent, since *MCM1* is an essential gene and *ARGRI* is not (15). On the basis of the binding specificity of MCM1, it is not expected that the MCM1 factor would bind AB2 on its own. Nevertheless, MCM1 has been suggested as an additional regulatory element of arginine metabolism on the basis of increased retardation of *ARGR/ARG5,6* DNA complexes in bandshift assays in the presence of antibodies against a MCM1-specific peptide (18). ARGRI and MCM1 are perhaps able to form heterodimers differing in their binding specificity from MCM1 homodimers.

**The operator region of *ARG3* is composed of two tandem operators of composite AB2-AB1 structure.** Several lines of evidence indicate that the ARGR proteins must assemble in a complex in order to be functional; for example, whereas a mutation in any of the three regulatory genes (*argRI*, *argRII*, or *argRIII*) results in virtually total constitutivity, the deletion of AB2 affects repression only moderately, suggesting that ARGRI is not exclusively acting via the postulated binding to AB2. Also, in in vitro retardation assays, specific retarded bands can be observed only if the three proteins are present simultaneously; moreover, in vitro complementation is possible between different *argR* extracts (18).

Concerning the DNA-binding site, our data show that normal arginine repression involves essentially AB1 and secondarily AB2. Nevertheless, sequence conservation in the *ARG3* regulatory region suggests that the AB1 and AB2 sequences identified by mutagenesis are part of two tandemly repeated 29-bp-long sequences, AB2 being located at the 5′ end of the first repeat and AB1 being located at the 3′ end of the second repeat (Fig. 4). Two binding patterns can be proposed on this basis: either one regulatory complex binds the whole region (ARGRII contacting AB1, an ARGRI dimer contacting AB2, and the intervening DNA looping out) or, alternatively, two regulatory complexes are bound, one to each repeat. The first repeat, a weak operator, should bind an ARGR complex essentially through its ARGRI component, whereas the second repeat, a stronger operator, should bind the second complex essentially through its ARGRII component. We favor this latter model (Fig. 6) for the following reasons. First, we could create hyperrepressed mutants by increasing the similarity between the two repeats: in DAB1, a mutant in which the similarity with AB1 is increased at the 5′ end of the first repeat, and in DAB2, in which the similarity with AB2 is increased at the 3′ end of the second repeat. Second, recently published data of Messenguy et al. (51) fit very well with the existence of two operators, each consisting of a AB2 box and a AB1 box as defined in this work. Indeed, these authors observed two retarded bands for *ARG3* in band shift assays: a strong, fast-migrating band and a weaker, more slowly migrating band. These bands might respectively correspond to DNA with only one ARGR complex bound to the strong operator I and to DNA with two ARGR complexes, one on each operator, especially considering the fact that DNA bearing an operator-constitutive mutation in the equivalent of our functional AB2 box (thus in the weak operator) practically abolishes the slowly migrating band. Moreover, in DNase I protection experiments, these authors observed protection by the ARGR complex of a sequence longer than the functional regulatory element that they call box A; in fact, the protected area corresponds exactly to what we define as the strong operator I. Similar conclusions can be drawn from the results concerning the other genes that Messenguy et al. (51) and Kovari et al. (40) have studied; we have summarized in Fig. 7 and 8 a view synthesizing their results and ours. Thus, stretches of 29 nucleotides presenting homology to both AB2 and AB1 are found exactly where these authors have localized their functional box A and box B elements and identified sequences protected against DNase. These protections always extend in length and correspond in sequence to one (or two) strong operators as defined in this work. It can be seen in Fig. 8 that the stringently required CGPu motif of AB1, present in all strong operators I, is preceded by a G in all cases except *ARG3*, where a A is found instead. In fact, the phenotype of mutations in *ARG3*-AB1, where A-37 is changed in a G or a C, suggests that a G/CCGPu is preferred to achieve better binding: the cognate mutants indeed have lower OTCase activities on Mam and on MamA than does the wild-type strain, a phenotype expected for hyperrepressed mutants. The same phenotype was also observed for some other modifications at the 5′ extremity of AB1 which overlaps with AB2: they show that a T is preferred to a G in the A/T-rich internal core of AB2 and that a G is preferred to a A at position −47.

**The position of the arginine operators determines whether they mediate positive or negative effects on transcription.** The *ARG3* operator region can behave as a UAS when placed at an upstream position in a promoter. Our results indeed show that the ARGR system can function as a transactivator, since we observe an ARGRII-dependent induction of *CYC1-lacZ* expression when the *ARG3* operator (from positions −94 to +25) replaces the *CYC1* UASs in front of the *CYC1* core promoter. This capacity to function as an activator in a heterologous system containing a displaced *ARG3* operator is in keeping with the results of Qiu et al. (67) showing that the ARGRII protein can behave as an activator when fused to a *lexA* DNA-binding domain.

A threefold, *ARGR*-dependent induction by arginine of OTCase synthesis can also be observed when the *ARG3* operator is engineered at a far-upstream position in the *ARG3* promoter itself, the original operator sequence being deleted. However, this induction is detected only in a *gcn4* background, suggesting that the *GCN4* product, whose remaining target in that construction is located downstream from the operator, somehow masks the rather modest transactivating activity (threefold) of the ARGR complex. In fact, the *ARG3* promoter might not offer the optimal context to monitor transactivation; indeed, the presence of a *GAL4* expression plasmid in a Δ*gal4* strain with a transplaced *ARG3* gene bearing a GAL4 17-mer-binding site at position

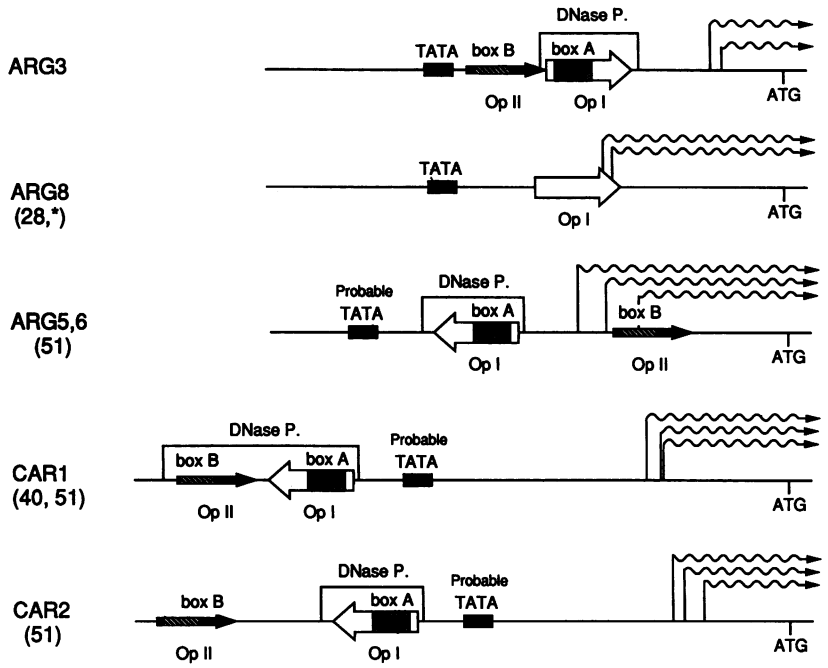



FIG. 7. Positions of the arginine operators in arginine anabolic and catabolic genes. The 5′ noncoding regions of several arginine genes are shown; arrows to scale represent the arginine operators as defined in Fig. 8. Strong operators I (Op I) are represented by thick open arrows, and weak operators II (Op II) are represented by thin black arrows. The sequences protected by ARGR complexes from DNase I digestion (DNase P.), as determined by Messenguy et al. (51), are indicated by brackets. *, our data concerning *ARG8* will be published separately.

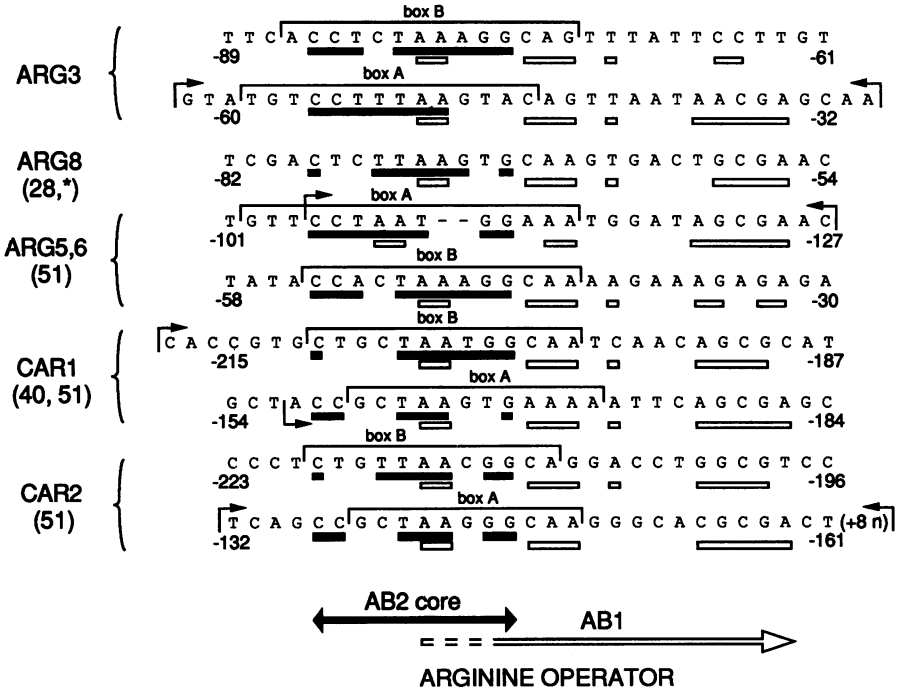



FIG. 8. The arginine operators. Arginine operator sequences identified in arginine anabolic and catabolic genes are aligned on the basis of the composite structure (AB2-AB1) revealed by this study. AB2-like sequences are underlined by black bars, and AB1-like sequences are underlined by open bars. Box A and box B as identified by Messenguy et al. (51) are overlined. The brackets delineate the sequences that are protected by ARGR complexes from DNase I digestion (51). Box A and box B in *CAR1* correspond to two of the three UAS-I elements revealed by the analysis of Kovari et al. (40). *, our data concerning *ARG8* will be presented elsewhere.

−160 allow only a modest threefold transactivation after growth on galactose medium (71a).

Thus, our data demonstrate that a given DNA sequence is able to mediate either stimulation or inhibition of transcription, depending on its position close to or far from the transcription initiation site. That arginine repression indeed is working at the level of transcription we inferred from the results of pulse-labeling and pulse-chase kinetic experiments in which we measured both the accumulation and the rate of degradation for the *ARG3* and *ARG1* mRNAs (9). The results of the primer extension experiments performed in the present study are essentially in agreement with a transcriptional mode of arginine regulation. The mechanism of repression suggested by the location of the ARGR targets in *ARG3* would thus consist in an interference exerted by the bound ARGR complex at the level of the assembly (or functioning) of the transcriptional preinitiation complex. When the ARGR target is located far enough from the initiation sites to be unable to exert steric hindrance, the inherent transactivating properties of the ARGR complex may be expressed. This view is in agreement with the large body of data that has been obtained by studying the functional organization of the 5′ noncoding regions of the biosynthetic (7, 8, 10, 30, 51) and catabolic (12, 13, 33, 40, 43, 51, 75–77) arginine genes. In the coregulated *ARG1* gene, however, we have not yet succeeded in identifying an arginine operator between the TATA and the translation start; indeed, the short sequence similarities with the *ARG3* regulatory region that had been noticed before the detailed analysis presented in this report was available (11) were shown by mutagenesis not to be functional (unpublished results). This situation thus needs to be investigated further. In *ARG8*, however, a functional AB1 sequence has been located between the TATA box of the gene and the codon initiating translation (13a).

A mechanism of transcriptional repression involving binding of the repressor in the vicinity of the transcriptional start is quite unusual. It nevertheless might not be unique to arginine biosynthetic genes, as Griggs and Johnston have recently localized two sites responsible for *GAL4* catabolic repression between positions −77 and −25 upstream from the *GAL4* messenger 5′ ends (22).

## ACKNOWLEDGMENTS

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