MOLECULAR AND CELLULAR BIOLOGY, Apr. 1994, p. 2378–2390
0270-7306/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 14, No. 4

A Segment of mRNA Encoding the Leader Peptide of the *CPA1* Gene Confers Repression by Arginine on a Heterologous Yeast Gene Transcript

PASCAL DELBECQ, MICHEL WERNER,† ANDRÉ FELLER, ROBERT K. FILIPKOWSKI,‡ FRANCINE MESSENGUY, AND ANDRÉ PIÉRARD*

Laboratoire de Microbiologie, Université Libre de Bruxelles, and Institut de Recherches du Centre d'Enseignement et de Recherches des Industries Alimentaires et Chimiques, B-1070 Brussels, Belgium

Received 26 July 1993/Returned for modification 10 September 1993/Accepted 4 January 1994

The expression of the yeast gene *CPA1*, which encodes the small subunit of the arginine pathway carbamoylphosphate synthetase, is repressed by arginine at a translational level. *CPA1* mRNA contains a 250-nucleotide-long leader which includes a 25-codon upstream open reading frame (uORF). Oligonucleotide site-directed mutagenesis of this uORF as well as sequencing of constitutive *cis*-dominant mutations has suggested that the leader peptide product of the *CPA1* uORF is an essential negative element for repression of the *CPA1* gene by arginine. In this work, a series of deletions affecting the regions 5′ and 3′ to the uORF in the leader sequence was constructed. The arginine-dependent repression of *CPA1* was little affected in these constructions, indicating that these regions are not essential for the regulatory response. This conclusion was further supported by the finding that inserting the mRNA segment encoding the leader peptide sequence of *CPA1* in the leader sequence of another gene, namely, *GCN4*, places this gene under arginine repression. Similarly, the behavior of fusions of the leader sequence of *CPA1* with those of *ARG4* or *GAL10* confirmed that the regions of this leader located upstream and downstream from the uORF are dispensable for the regulation by arginine. Finally, a set of substitution mutations which modify the uORF nucleotide sequence while leaving unchanged the corresponding amino acid sequence was constructed. The mutations did not affect the repression of *CPA1* by arginine. The data presented in this paper consequently agree with the conclusion that the leader peptide itself is the main element required for the translational repression of *CPA1*.

Whereas translation is considered an important target for the regulation of gene expression in eukaryotes, few translational regulations have been elucidated in detailed molecular terms. Therefore, the investigation of translational controls in yeasts which are accessible to detailed molecular studies is of particular interest. Two such mechanisms are especially well established. One affects the *GCN4* gene, which encodes the transcriptional activator of the general control of amino acid biosynthesis (for reviews, see references 11 and 12). The other concerns the *CPA1* gene, which codes for the subunit of an enzyme of arginine metabolism (35) and is the subject of this article. In both cases, the regulation mechanism appears to rely on a particular organization of the 5′ leader region of the corresponding mRNA.

The *CPA1* gene of *Saccharomyces cerevisiae*, which encodes the small glutaminase subunit of the arginine pathway carbamoylphosphate synthetase (CPSase) (27) is subject to dual regulation. Besides the general control of amino acid biosynthesis, the expression of *CPA1* obeys a specific control mechanism consisting of repression by arginine (15, 26). Whereas the general control is transcriptional (19), the evidence based on the limited reduction in the *CPA1* mRNA level accompanying the five- to sixfold repression of the synthesis of CPA1 protein has indicated that the latter mechanism operates primarily at a posttranscriptional level (20).

Sequencing of *CPA1* and investigation of the structure of its mRNA transcript has established that this transcript contains a 250-nucleotide-long leader, including a 25-codon upstream open reading frame (uORF), a feature which has been correlated with the posttranscriptional nature of this mechanism (34, 35). A set of missense, nonsense, and frameshift mutations altering this uORF has been constructed by site-directed mutagenesis. All resulted in a reduced repressibility of *CPA1* expression by arginine. In addition, several operator-constitutive (O^c)-type mutations obtained in vivo (33), the so-called *CPA1-O* (or *CPA80*) *cis*-dominant mutations, have been mapped within this uORF. A total of a dozen mutations affecting the coding sequence of the uORF are presently available; all result in a reduced repressibility of *CPA1* expression. Such a behavior favors a negative role of the leader peptide, the product of the *CPA1* uORF, in the specific repression of *CPA1* by arginine (35). On the basis of the features described above, there has been proposed a model for the translational regulation of *CPA1* in which the newly synthesized leader peptide, still bound to the ribosome, is recognized by arginine and the product of the *trans*-acting regulatory gene *CPAR* (33), or by another regulatory factor, to form a complex that prevents 40S ribosomal subunits from scanning further than this uORF. More recent measurements have shown the rate of synthesis of *CPA1* mRNA to be partially repressed by arginine, thus suggesting that a transcriptional component is involved in the arginine-specific repression of *CPA1* (7).

In the case of *GCN4*, multiple upstream AUG codons

* Corresponding author. Mailing address: Institut de Recherches du CERIA, 1, Avenue Emile Gryson, B-1070 Brussels, Belgium. Phone: 32-2-526.72.76. Fax: 32-2-526.72.73. Electronic mail address: ceriair@ulb.ac.be.

† Present address: Service de Biologie et de Génétique Moléculaire, Département de Biologie Cellulaire et Moléculaire, Centre d'Etudes de Saclay, F-91191 Gif-sur-Yvette Cedex, France.

‡ Present address: Zaklad Genetyki, Uniwersytet Warszawski, 00-478 Warsaw, Poland.

followed by short uORFs are present in the mRNA leader sequence and act in *cis* to regulate the number of scanning 40S ribosomal subunits able to reinitiate translation at the *GCN4* initiation codon (10, 24, 32). Despite the similarities existing in the leader sequences of these two genes, the control of *GCN4* appears different, since no evidence for a regulatory role of the short peptide products encoded by its uORFs exists.

In this report, we provide further evidence that the leader peptide itself functions in translational repression of *CPA1*. Deletions created either upstream or downstream from the uORF in the leader sequence had little effect on the arginine-dependent regulation of *CPA1* expression. We also showed that the mRNA segment encoding the leader peptide of CPA1 is sufficient to confer arginine regulation when inserted in the leader sequence of a heterologous yeast transcript. Finally, a set of substitution mutations which modify the uORF sequence while leaving unchanged the corresponding amino acid sequence was constructed and did not significantly affect the repression of *CPA1* by arginine.

(Preliminary accounts of some of the results presented here have appeared previously [34] or were presented at the Yeast Conference, The Hague, The Netherlands, July 1990 [7a], and the Yeast Conference, Vienna, Austria, August 1992 [7b].)

MATERIALS AND METHODS

Strains and media. The minimal medium, designated M, was that described by Ramos and Wiame (28), supplemented with 10 mM $(NH_4)_2SO_4$ as a nitrogen source (M.am) and, when indicated, with 25 μg of uracil per ml to repress CPSase P or to supplement uracil auxotrophs and with L-arginine (1 mg/ml) (M.am+arg) to achieve complete repression of *CPA1*. Strains were grown at 30°C.

The *CPA1*-deleted strain DELTA2 was constructed by a transplacement method as described previously (35), except that strain 12T7c, which is congenic to Σ1278b, was used instead of strain 10R34d and its derivative DELTA1, which flocculated.

Strains also used in this work were the following: 10W08d, *cpa1 ura3 leu2*; 10W51a, *ura2C-2 cpa1*Δ; 10W53d, *ura2C-2 ura3 cpa1*Δ; 12T7c, *ura3* (a gift from F. Ramos); and IBAF10-1-A, *ura3 arg4::LYS9 lys9*.

Oligonucleotides. Oligonucleotides were synthesized in a Cyclone synthesizer (New Brunswick Instruments) by the phosphoramidite method. Figure 1 lists the oligonucleotides used in this work. Oligonucleotide 7 was used as a sequencing primer to verify the sequence of the constructions. *Bam*HI linker CGGATCCG and *Bgl*II linker CAGATCTG were purchased from Pharmacia.

DNA procedures. Rapid isolation of plasmid DNA from *Escherichia coli* was done as described by Birnboim (3). Restriction analysis and gel electrophoresis were performed as described by Maniatis et al. (17). DNA sequencing was done by the dideoxynucleotide chain termination method (29) with M13mp phage (21) or Bluescript phagemids (Stratagene). Yeast cells were transformed by the lithium acetate method (30). Yeast strains were constructed by standard genetic techniques (30). Oligonucleotide-directed in vitro mutagenesis was performed by the Eckstein method (25, 31) as provided in the mutagenesis kit from Amersham.

Construction of the uORF cassette plasmids. To manipulate more easily the 5′ end of the *CPA1* gene, it was modified by in vitro mutagenesis with two oligonucleotides. One, oligonucleotide 5 (Fig. 1), allowed the changing of nucleotides −144 to −139 to a *Bam*HI site, leaving the four nucleotides upstream of the uORF initiation codon unchanged. Oligonucleotide 6 (Fig. 1) was used to change nucleotides −56 to −51, which are located just downstream from the uORF UAA stop codon, to a *Bam*HI site. Three different derivatives were constructed by mutagenizing a wild-type allele of *CPA1*: *CPA1-101*, with both *Bam*HI sites; *CPA1-102*, with only the upstream site; and *CPA1-103*, with only the downstream site (Fig. 2).

Plasmid pBMW1 was constructed by first inserting the 4.2-kb *Sal*I-*Bam*HI fragment bearing *CPA1* from plasmid pUCAF1 (35) into the homologous sites of Bluescript SK M13$^+$ (Stratagene). The *Bgl*II-*Bgl*II 1.1-kb *URA3* fragment from pFL44 (kindly provided by F. Lacroute [4]) was next inserted into the *Bam*HI site of pBMW1 to obtain plasmid pBMW2-2. The *URA3* gene is transcribed from *Sac*I to *Kpn*I in the direction opposite to that of *CPA1*. Plasmid pBMW2-2 was used for all the oligonucleotide-directed mutageneses.

Single-stranded pBMW2-2 DNA was obtained by cocultivating an XL1-Blue strain (*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* [F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*]) from Stratagene, transformed with the plasmid, with phage R408 as described by the manufacturer. The single-stranded DNA was extracted as described in the in vitro mutagenesis kit manual from Amersham and mutagenized as described previously with oligonucleotide 5 or 6 or both simultaneously. The plasmids were extracted and assayed for the presence of a *Bam*HI restriction site. Single-stranded DNA was prepared from the positive clones, and their sequences were determined. Plasmid pBP1 is the pBMW2-2 derivative which has been mutagenized with both oligonucleotides and has nucleotides −144 to −139 and −56 to −51 of *CPA1* changed to *Bam*HI sites. Plasmid pBP3 has only the −144 to −139 *Bam*HI site, while pBP5 has only the −56 to −51 one. Each of these mutations was introduced in strain 10W53d (*cpa1*Δ [in which nucleotides −640 to +875 have been deleted] *ura2C-2 ura3*), by transforming it with the 4.1-kb *Sal*I-*Sma*I fragment bearing the mutagenized *CPA1* gene from plasmid pBP1, pBP3, or pBP5, selecting for growth without added arginine. The derived strains were named IW53BP1 (*CPA1-101*), IW53BP3 (*CPA1-102*), and IW53BP5 (*CPA1-103*) (Fig. 2). As a control, the same strain was also transformed with the wild-type *Sal*I-*Sma*I fragment from pBMW2-2, yielding strain IW53MW2-2.

Creation of silent mutations in the *CPA1* uORF. Plasmid pBMW2-2 (35) was mutagenized in vitro with oligonucleotides 10, 11, and 12. Oligonucleotides 10 and 12 were degenerate (Fig. 1). The mutations were checked by DNA sequencing. Oligonucleotide 10 gave rise to mutants *cpa1-116* (plasmid pBMW20), *cpa1-117* (plasmid pBMW23), *cpa1-118* (plasmid pBMW24), *cpa1-119* (plasmid pBMW25), and *cpa1-120* (plasmid pBMW26). Oligonucleotide 11 gave rise to mutant *cpa1-121* (plasmid pBMW27). Oligonucleotide 12 generated mutants *cpa1-122* (plasmid pBMW29), *cpa1-123* (plasmid pBMW30), and *cpa1-124* (plasmid pBMW31). In order to avoid the presence of additional mutations in the *CPA1* coding sequence, a 1,354-bp DNA fragment (*Kpn*I-*Bst*EII) was isolated from each of the above plasmids and used to replace the equivalent fragment of plasmid pBP5. All these integrative plasmids were introduced into yeast strain 10W51a.

Construction of *CPA1-lacZ* fusions. Plasmid pBMWW2-2 was mutagenized in vitro with two synthetic oligonucleotides listed in Fig. 1. The first, called P2, modifies nucleotides −66 to −61. The second, P3, modifies nucleotides +9 to +14. The modification aimed at creating a *Bam*HI site located at amino acid 23 of the leader peptide (P2) and at amino acid 4 in the *CPA1* protein (P3), producing plasmids pBP8 (P2) and pBP9 (P3). These plasmids were opened with *Sal*I, blunt ended, and digested with *Bam*HI. The 1.1-kb fragment isolated from each

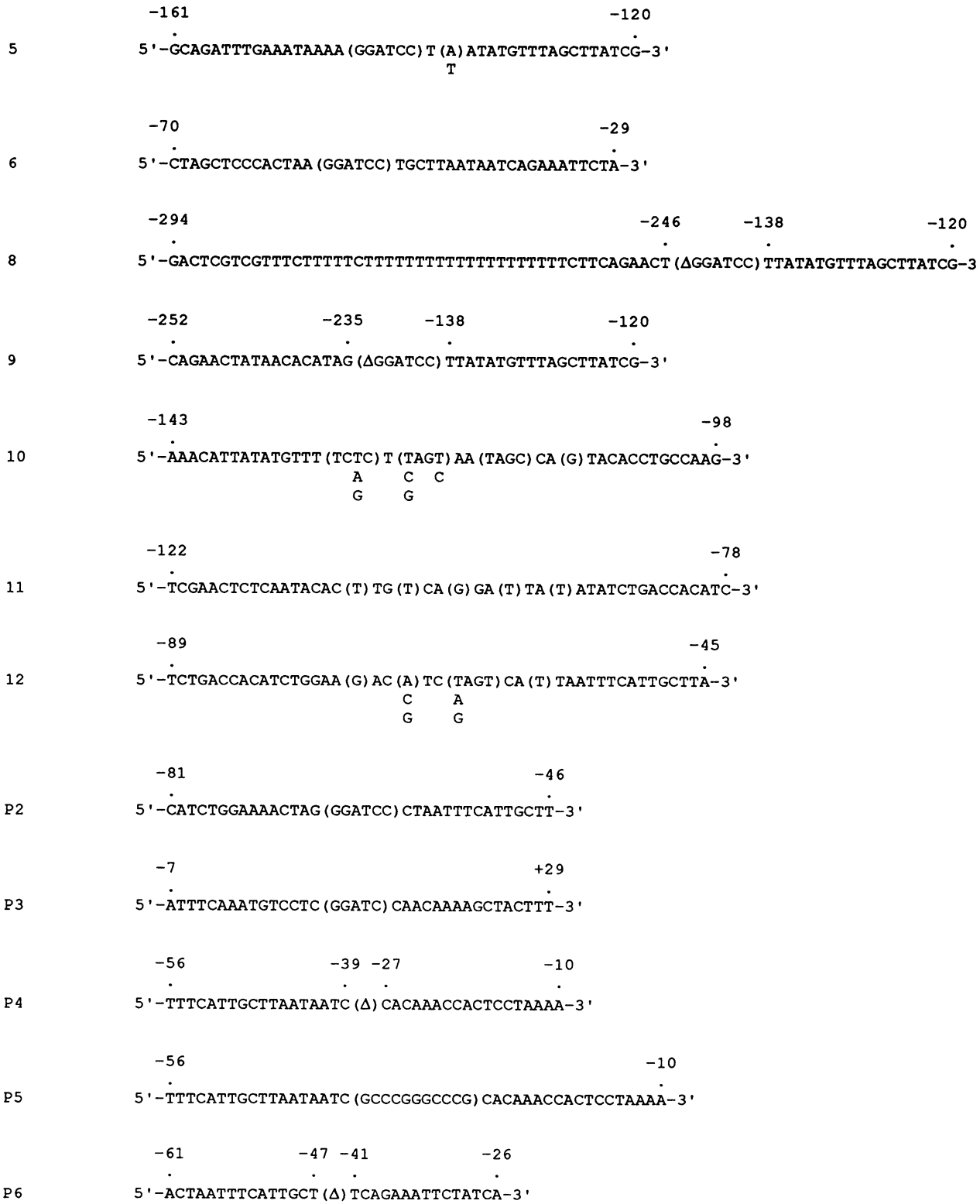


FIG. 1. Oligonucleotides used in this work. Nucleotides in parentheses are those which differ from the wild-type *CPA1* sequence. Δ, sequences that are deleted.

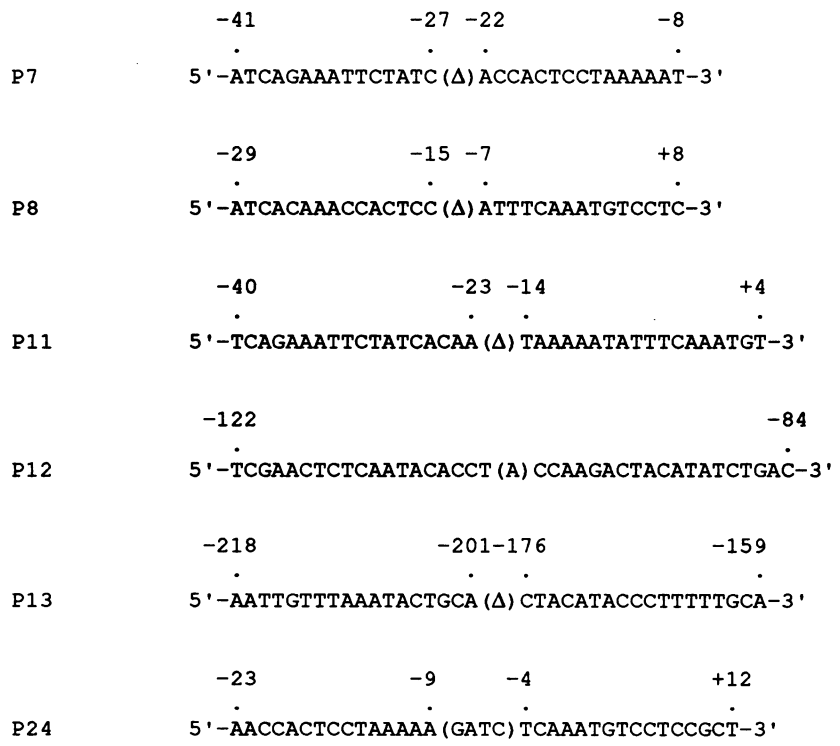


FIG. 1—*Continued.*

plasmid was then introduced into the pFL38 vector (pUC19 *ARS CEN6 URA3* [4]) opened by restriction with *Sma*I and *Bam*HI, leading to plasmids pCP5 (from pBP8) and pCP6 (from pBP9). These two plasmids were further restricted by *Bam*HI and *Sal*I and ligated with a 6.2-kb DNA fragment from plasmid pMC310 (pBR322 2μm *URA3* with the 6.2-kb *Bam*HI-*Sal*I fragment bearing the *lacZ* gene from pMC874 plasmid [5], a gift from M. Crabeel), digested by the same enzymes, leading to plasmids pCP3 and pCP4. Plasmid pCP3 thus contains the beginning of the *CPA1* leader including the uORF fused to *lacZ*, while pCP4 contains the entire *CPA1* leader and the sequence encoding the first three amino acids of CPA1 fused to *lacZ*.

Plasmids pBP8 and pBP9 were also mutagenized in vitro with oligonucleotide P12, which modifies nucleotide −130, leading to the creation of an O^c-type mutation in the uORF (changing cysteine 11 to tyrosine). These new plasmids were called pBP18 and pBP19 and were used in the same way as pBP8 and pBP9 to produce pCP18 and pCP19, respectively. They are thus equivalent to pCP3 and pCP4, but with an O^c-type mutation in the *CPA1* uORF.

Construction of the *CPA1-ARG4* fusion plasmids. The 1.1-kb *Bgl*II-*Bgl*II *URA3* fragment from plasmid pFL44 (pUC19 2μm *URA3* [4]) was inserted into the *Bam*HI site of Bluescript SK M13$^+$ (Stratagene) to obtain plasmid pBMW4. The unique *Sac*I site which is located in the polylinker was then filled in, yielding plasmid pBAF2. A 2.8-kb *Hin*dIII-*Hin*dIII fragment bearing the *ARG4* gene from plasmid pLG3 (pBR322 2μm *URA3 ARG4*, a gift from R. Loppes [16]) was inserted into the homologous site of pBAF2, yielding pBMW7. This plasmid was further modified by digestion with *Sac*I, which cuts 37 nucleotides upstream of the argininosuccinate lyase ORF (2), removing the overhanging ends by digestion with Klenow enzyme, and adding a CGGATCCG *Bam*HI linker to produce plasmid pBMW9.

Plasmid pBP5 was cut at its unique *Xho*I site and blunt ended. The linearized fragment was cut at the unique *Bam*HI site located just downstream of the uORF. The 1.25-kb fragment, bearing the 5′ end of *CPA1*, was purified and inserted in plasmid pBMW9 from which the *ARG4* promoter had been removed by first cutting at the *Eco*RI site, blunt ending with Klenow enzyme, cutting with *Bam*HI, and discarding the small fragment. From the resulting plasmid, called pBMW14, a 4.4-kb *Kpn*I-*Xba*I fragment containing the *CPA1-ARG4* fusion and the *URA3* gene was inserted into the homologous sites of the centromeric vector pCMW1 (pUC19 *ARS CEN6*), yielding pCAF4 (see Fig. 4B). These plasmids were used to transform strain IBAF10-1-A, selecting for uracil prototrophy.

Construction of a *GAL10-CPA1* fusion plasmid. Plasmid YEp52 (a gift from F. Lacroute), which harbors the 2μm replication origin, allows expression of an ORF from the *GAL10* promoter which also provides the start sites of transcription and the 5′ untranslated end of the mRNA. The transcription ends within the terminator of the *REP3* gene in the 2μm fragment.

A *Bam*HI CGGATCCG linker was inserted in the unique *Sma*I site of the plasmid pBP3, located at the 3′ end of the *CPA1* fragment. The 2.9-kb *Bam*HI-*Bam*HI fragment from the resulting plasmid, pBP4, was inserted into YEp52 to obtain pYAF3 (see Fig. 4A). Plasmid pYAF3 was transformed in strain 10W08d, selecting for the *LEU2* marker.

Increasing the distance between the *CPA1* uORF and the *CPA1* main coding sequence. Plasmid pBP5 bearing a *Bam*HI restriction site just after the *CPA1* uORF was mutagenized in vitro with oligonucleotide P24. This modification introduces a *Bgl*II site at positions −9 to −4, leading to plasmid pBP26.

Plasmid pBP26 was digested with *Bam*HI and *Bgl*II enzymes, and a 48-bp DNA fragment containing the region separating the two ORFs was isolated. It was introduced into the *Bam*HI site of the pBP5 plasmid. The number and orientation of insertions were determined by sequencing. We obtained plasmid pBP41, which contains two insertions in the same orientation as in the *CPA1* gene, thus increasing threefold the distance between the two ORFs. The 3.8-bp *Sal*I-*Hpa*I DNA fragment from plasmid pBP41 containing the *CPA1* gene was isolated and used to transform yeast strain 10W53d, leading to mutant IBP41.

Construction of deletions in the noncoding sequences of the *CPA1* leader. Plasmid pBP5 was digested with *Bam*HI, which cuts 3′ of the uORF, and digested at 30°C with 5 U of exonuclease III per μg of DNA. Aliquots were removed at regular intervals during 4 min, and the reaction was stopped. Under these conditions, about 125 nucleotides per min were digested from each end. The DNA was blunt ended with mung bean nuclease, and a *Bgl*II CAGATCTG linker was inserted. The deletion endpoints were determined by sequence analysis, and the small *Sal*I-*Bgl*II fragments of all the deleted plasmids were used to replace the homologous *Sal*I-*Bam*HI fragment of pCMW6 (pCMW1 in which the 5-kb *Xba*I-*Sal*I fragment from pBP3 containing *CPA1* and *URA3* genes was inserted and in which the polylinker *Bam*HI site had been destroyed), giving rise to the pCΔ plasmid series. The 4.0- to 4.2-kb *Sal*I-*Sma*I fragment from each pCΔ was transformed into strain 10W53d, selecting for the complementation of the *cpa1*Δ allele. The transformants formed the ICΔ strain series. Plasmid pBMW17 was constructed by site-directed mutagenesis of pBMW2-2 with oligonucleotide 8. Plasmid pBMW18 was obtained from oligonucleotide 9 mutagenesis of pBMW2-2. Since it contained unexpected mutations in the uORF, as revealed by sequencing, pBMW19 was constructed by replacing the 1.1-kb *Sal*I-*Bam*HI from pBP3 with that of pBMW18. The *Sal*I-*Sma*I fragments bearing the mutagenized fragment of *CPA1* from pBMW17, pBMW18, and pBMW19 were all transformed into strain 10W53d. For creation of the internal deletion from nucleotides −200 to −176, plasmid pBMW2-2 was mutagenized with oligonucleotide P13, leading to plasmid pBP21. The 3.7-kb *Sal*I-*Hpa*I DNA fragment from plasmid pBP21, containing the *CPA1* gene, was isolated and used to transform yeast strain 10W53d, leading to mutant IBP21.

To create deletions between the *CPA1* uORF and the *CPA1* main coding sequence, plasmid pBMW2-2 was mutagenized in vitro with oligonucleotides P4, P6, P7, P8, and P11 (Fig. 1) leading to, respectively, plasmids pBP10, pBP12, pBP13, pBP14, and pBP20. A 4-kb *Sal*I-*Sma*I DNA fragment from each of plasmids pBP10, pBP12, pBP13, and pBP14 and a 3.7-kb *Sal*I-*Hpa*I DNA fragment from plasmid PBP20, all containing the *CPA1* gene, were isolated and used to transform yeast strain 10W51a for arginine and uracil prototrophy. Mutated strains IBP10, IBP12, IBP13, IBP14, and IBP20 were obtained. The control strain IBMW2-2 was obtained by similarly taking the wild-type 4-kb *Sal*I-*Sma*I DNA fragment from pBMW2-2.

Insertion of the *CPA1* uORF cassette in the leader of *GCN4*. Plasmid p227 contains a *GCN4-lacZ* fusion in which the four upstream regulatory elements (uORFs) present in the leader of *GCN4* mRNA have been removed (24). The *CPA1* uORF was inserted either at the uORF1 or at the uORF4 position (see Fig. 6). These constructions were obtained as follows.

Plasmid pBP1 was digested by *Bam*HI enzyme, and the 88-bp DNA fragment containing the *CPA1* uORF was isolated and blunt ended. This fragment was then inserted in plasmid p227, opened with *Hin*dIII, and blunt ended. This *Hin*dIII site had been created in replacement of uORF1 in the leader of the *GCN4-lacZ* gene (24). The orientation of the *CPA1* uORF was determined by sequencing. We obtained plasmid pCP20, which contains the *CPA1* uORF in the same orientation as in the *CPA1* gene, and plasmid pCP21 (reverse orientation).

Plasmid p227 was digested with *Sal*I and *Bam*HI. The 900-bp fragment was purified, inserted into plasmid Bluescript SK, and opened with the same restriction enzymes, leading to plasmid pBP15. The 88-bp *Bam*HI-*Bam*HI DNA fragment from pBP1 described above was inserted into the *Bgl*II site of plasmid pBP15. The *Bgl*II site had been created in replacement of uORF4 in the *GCN4-lacZ* gene. The orientation of the *CPA1* uORF was determined by sequencing. We obtained plasmid pBP16, which contains the *CPA1* uORF in the same orientation as in the *CPA1* gene, and plasmid pBP17 (reverse orientation). Both pBP16 and pBP17 were digested by *Sal*I and *Bst*EII in order to generate an 800-bp DNA fragment. These two fragments were used to replace the equivalent regions present in plasmid p227, leading to plasmid pCP8 (from pBP16) and pCP9 (from pBP17).

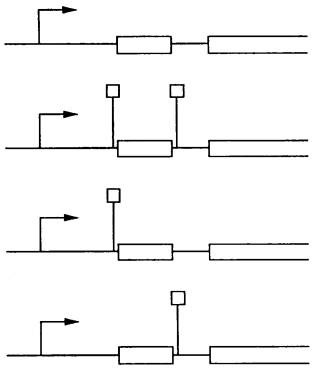
Plasmid pBP1 was also mutagenized in vitro with oligonucleotide P12, which modified nucleotide −130, leading to the creation of the *CPA1-O5* mutation (changing cysteine 11 to tyrosine [35]). The new plasmid was called pBP49 and was used in the same way as pBP1 to produce pCP31 and pCP32, which contain the uORF carrying a *CPA1-O5* mutation in the position of the *GCN4* uORF1 for pCP31 and in the *GCN4* uORF4 position for pCP32. In the two plasmids, the *CPA1* uORF is in the same orientation as in the *CPA1* gene.

All seven constructions (pCP8, pCP9, pCP20, pCP21, pCP31, pCP32, and p227) were transformed into yeast strain 12T7c.

Assay of CPSase. French press breakage of the cells and the assay of CPSase A (EC 6.3.5.5) were performed as described previously (15, 27) by coupling them with ornithine carbamoyltransferase and measuring the incorporation into citrulline of ^{14}C from labelled carbonate. Since the *CPA2* gene, which encodes the large subunit of CPSase A, is insensitive to repression by arginine (26), the assay of the overall CPSase A activity under the conditions used in this study represents a measurement of *CPA1* gene expression.

Assay of argininosuccinate lyase (EC 4.3.2.1). Cells were broken with a French pressure cell in the presence of 20 mM potassium phosphate, pH 7.5. The cell debris was removed by a 15-min centrifugation at 11,000 rpm in an SS34 rotor. The extracts were dialyzed twice at 4°C for 2 h against 5 liters of 2 mM potassium phosphate, pH 7.5. The enzyme was diluted in 0.5 ml of potassium phosphate (100 mM). The reaction was started with 0.5 ml of 25 mM argininosuccinate prepared in the following way. Eighty-five milligrams of argininosuccinate barium salt (Sigma Chemical Co.) was dissolved in 7 ml of water. After addition of 1 ml of 250 mM K_2SO_4, the barium sulfate precipitate was eliminated by centrifugation. The reaction was allowed to proceed for 1 h at 37°C. The reaction was stopped by boiling the sample for 5 min. The samples were centrifuged, and half of the supernatant (0.5 ml) was collected in a new tube, with 0.5 ml of water and 1 ml of 2 M sodium hydroxide being added. A blank without argininosuccinate was prepared for each sample. Enzyme activity was determined by measuring the amount of arginine formed (22).

Assay of β-galactosidase. Cells were broken with a French pressure cell in the presence of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 38 mM β-mercaptoethanol). The cell debris was removed by a 15-min centrifugation at 11,000 rpm in an SS34 rotor. The enzyme was diluted in 2 ml of Z buffer. The reaction was started with 0.4 ml of



Strain	Relevant Genotype	CPSase activity M.am*	CPSase activity M.am +arg*
IW53MW2-2	WT	0.336	0.055
IW53BP1	*CPA1-101*	0.219	0.040
IW53BP3	*CPA1-102*	0.196	0.037
IW53BP5	*CPA1-103*	0.308	0.045

FIG. 2. Effects of the introduction of *Bam*HI sites in the leader sequence on the arginine-specific repression of *CPA1*. The leftmost column represents a schematic of the structure of the 5′ end of the *CPA1* locus. The open rectangles represent the uORF and the *CPA1* ORF. The arrows above the sketches represent the direction of transcription, the start of which is represented by the vertical bar (see Fig. 3 for precise location). The *Bam*HI sites are indicated by the open squares. All strains are *ura2C-2* and *ura3* in addition to the indicated relevant genotype. The orientation of the uORF is always the same as in the wild-type *CPA1* gene. The uORF AUG is always located at −134. The AUG of the *CPA1* ORF is taken as nucleotide +1. Activities are specific activities of CPSase A measured in transformant strains grown on M.am and M.am+arg. Uracil (25 μg/ml) was added to the growth media.

o-nitrophenyl-β-D-galactopyranoside. The reaction was allowed to proceed at 28°C until a yellow coloration was obtained. The reaction was stopped by 1 ml of 1 M sodium carbonate. The amount of *o*-nitrophenol formed was determined by measuring the optical density at 420 nm.

Assay of protein and enzyme activities. Protein was assayed by the Folin method. Enzyme activities are reported as micromoles of product formed per hour per milligram of protein for CPSase and argininosuccinate lyase and as nanomoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per milligram of protein for β-galactosidase.

Expression of each construct was examined in at least two independent transformants of each strain, with each assay being repeated at least three times. The values reported throughout this work are averages calculated from these replicate determinations and had standard errors of 20% or less.

RESULTS

Deletion analysis of the leader sequences located upstream from the uORF. In a previous analysis, we showed that the uORF of the *CPA1* gene is essential for the regulation of its expression (35). In order to determine whether other regions of the *CPA1* leader play a role in this regulation as well, we decided to perform a functional analysis of the regions located 5′ and 3′ to this uORF.

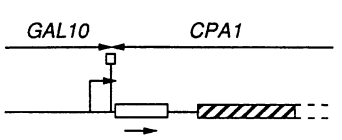
We began by creating *Bam*HI restriction sites upstream and downstream from the uORF (see Materials and Methods). As shown in Fig. 2, despite a slight inhibitory effect of the

		CPSase Activity	
	Strain	M.am*	M.am +arg*
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTGCAAAGAATACAAGGTAATCGACTCTTCTACATACCCTTTTTGCAGATTTGAAATAAAAAAAACATTAT **ATG TTT**	IW53MW2-2	0.336	0.055
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTGCAAAGAATACAAGGTAATCGACTCTTCTACATACCCTTTTTGCAGATTTGAAATAAAAGGATCCTTAT **ATG TTT**	IW53BP3	0.196	0.037
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTGCAAAGAATACAAGGTAATCGACTCTTCTACATACCCTTTTTGCAGATTTG.....CAGATCCTTAT **ATG TTT**	ICΔ31	0.231	0.049
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTGCAAAGAATACAAGGTAATCGACTCTTCTACATACCCTTTTTG.....CAGATCCTTAT **ATG TTT**	ICΔ16	0.224	0.040
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTGCAAAGAATACAAGGTAATCGACTCTT.....CAGATCCTTAT **ATG TTT**	ICΔ26	0.228	0.035
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTGCA.....CAGATCCTTAT **ATG TTT**	ICΔ28	0.381	0.197
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTG.....CAGATCCTTAT **ATG TTT**	ICΔ3	0.173	0.044
ACTATAACACATAGATACACTCGAACATCTAATTGTTT.....CAGATCCTTAT **ATG TTT**	ICΔ27	0.215	0.046
ACTATAACACATAGATACACTCGAACATCTAATTG.....CAGATCCTTAT **ATG TTT**	ICΔ30	0.197	0.038
ACTATAACACATAGATACACTCGAAC.....CAGATCCTTAT **ATG TTT**	ICΔ29	0.148	0.029
ACTATAACACATAG.....GGATCCTTAT **ATG TTT**	IBMW19	0.251	0.103
ACT.....GGATCCTTAT **ATG TTT**	IBMW17	0.267	0.117
ACTATAACACATAGATACACTCGAACATCTAATTGTTTAAATACTGCA.....CTACATACCCTTTTTGCAGATTTGAAATAAAAAAAACATTAT **ATG TTT**	IBP21	0.274	0.040

uORF start

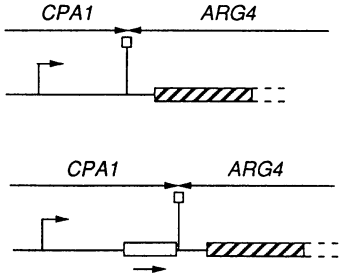
FIG. 3. Effects of deletions created in the *CPA1* leader upstream of the uORF. The upper line contains the nucleotide sequence of the *CPA1* leader from the transcription initiation start sites at −247, −246, −245, −235, and −234 (symbolized by asterisks) up to the two first codons of the uORF (presented in boldface). The underlined nucleotides are those which result from the constructions. Dots represent the deleted nucleotides. The different fragments containing the mutated *CPA1* gene were introduced into strain 10W53d (*ura2C-2 cpa1*Δ *ura3*). CPSase specific activities in the different transformant cells grown on M.am and M.am+arg were measured. Uracil (25 μg/ml) was added to the growth media.

A



		M.am	M.am +arg
pYAF3	glucose	0.175	0.046
	galactose	2.625	0.520

B



Plasmid	Argininosuccinate lyase activity	
	M.am	M.am +arg
pCAF12	5.0	2.9
pCAF4	0.5	0.1

FIG. 4. Effects of arginine on the expression of *GAL10-CPA1* (A) and *CPA1-ARG4* (B) fusions. The extent of each gene is indicated by the converging arrows above the open square, which represents the *Bam*HI joining site. The small open rectangle represents the *CPA1* uORF, oriented as in the wild-type *CPA1* allele. (A) The hatched rectangle represents the *CPA1* ORF. CPSase specific activities in the different transformed cells grown on M.am and on M.am+arg supplemented with either 3% glucose or 2% galactose were measured. (B) The hatched rectangle represents the *ARG4* coding sequence. Argininosuccinate lyase specific activities in transformant cells grown on M.am and M.am+arg were measured. The recipient strain is IBAF10-1-A (*ura3 arg4::LYS9 lys9*).

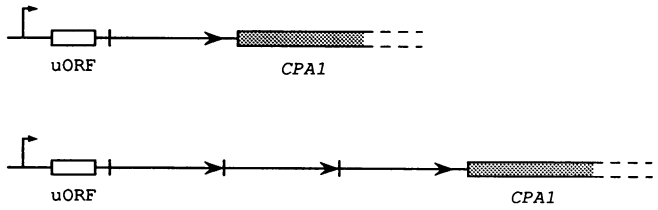
upstream *Bam*HI site on the basal level of *CPA1* expression (strains IW53BP1 and IW53BP3), such modifications had little effect on the repression by arginine. The upstream *Bam*HI site was used for creating a series of nested deletions in the region of *CPA1* mRNA located upstream from the uORF (Fig. 3). These deletions extended from nucleotide −139 from the *CPA1* AUG codon towards the 5′ end of the mRNA, thus leaving intact nucleotides −138 to −135, which constitute the immediate context of the uORF initiation codon. During the construction of the set, a *Bgl*II linker was inserted and ligated in the *Bam*HI site of pBP3 (see Materials and Methods). The nucleotides forming this site are underlined in Fig. 3. Allele *cpa1-102* (strain IW53BP3), as mentioned above, has a *Bam*HI site spanning from nucleotides −144 to −139, whereas in allele *cpa1-104* (strain ICΔ31), nucleotides −152 to −139 were removed and replaced by the CAGATCC sequence. The behavior of the strains carrying these alleles shows that the sequences immediately upstream of the uORF are dispensable and that their replacement by the CAGATCC sequence does not affect the repression. The results obtained for the various deleted strains are summarized in Fig. 3. All are still repressible to a certain extent despite significant variations in the levels of expression. The deletion in strain ICΔ28 (*cpa1-107*) extending to nucleotide −200 markedly increases the basal level and reduces the repression of *CPA1* expression compared with either of its neighbor deletions. This is particularly striking, since the removal of two more nucleotides, as in the *cpa1-108* allele (strain ICΔ3), allows it to regain a fourfold repression. This argues for the possibility that deletion *cpa1-107* creates a sequence at the junction of the deletion and the *Bgl*II linker that prevents the repression by the uORF from operating efficiently. We have therefore created an internal deletion between nucleotides −200 and −176 (see Materials and Methods), giving rise to strain IBP21, after integration of plasmid pBP21a in the genome. As shown in Fig. 3, this deletion has no effect on *CPA1* expression, confirming that the region between nucleotides −200 and −176 is not crucial for repression by arginine.

In order to confirm that the sequence located 5′ to the uORF is dispensable for repression by arginine, a fusion in which the *GAL10* gene provided the sequence 5′ to the uORF was constructed. This fusion was studied with the multicopy plasmid pYAF3, which is a derivative of YEp52-*Bam*HI, in strain 10W08d. As shown in Fig. 4A, the assay of the *CPA1* gene product of strain 10W08d/pYAF3 showed that arginine is able to repress the synthesis of CPA1 protein by a factor of 3.6

A

			Strain	CPSase Activity M.am	M.am +arg
TAA	TTTCATTGCTTAATAATCAGAAATTCTATCACAAACCACTCCTAAAAATATTTCAA	**ATG**	IBMW2-2	0.195	0.031
TAA	GGATCCTGCTTAATAATCAGAAATTCTATCACAAACCACTCCTAAAAATATTTCAA	**ATG**	IBP5	0.191	0.033
TAA	TTTCATTGCT.....TCAGAAATTCTATCACAAACCACTCCTAAAAATATTTCAA	**ATG**	IBP10	0.178	0.031
TAA	TTTCATTGCTTAATAATC.....CACAAACCACTCCTAAAAATATTTCAA	**ATG**	IBP12	0.174	0.027
TAA	TTTCATTGCTTAATAATCAGAAATTCTATC....ACCACTCCTAAAAATATTTCAA	**ATG**	IBP13	0.216	0.028
TAA	TTTCATTGCTTAATAATCAGAAATTCTATCACAA.....TAAAAATATTTCAA	**ATG**	IBP20	0.197	0.028
TAA	TTTCATTGCTTAATAATCAGAAATTCTATCACAAACCACTCC.....ATTTCAA	**ATG**	IBP14	0.224	0.029
uORF end		***CPA1* start**			

B



	CPSase activity M.am*	M.am +arg*
IW53BP5	0.308	0.045
IBP41	0.329	0.050

FIG. 5. (A) Effects of deletions and mutations created in the *CPA1* leader between the uORF and the *CPA1* main coding sequence. The end of the uORF (TAA) and the ATG of the *CPA1* gene are indicated in boldface. The upper line represents the wild-type sequence. The sequence that has changed is underlined. The dots represent the nucleotides that have been deleted. The different fragments bearing *CPA1* deletions were integrated into strain 10W51a (*ura2C-2 cpa1*Δ). CPSase specific activities in different transformant strains grown on M.am and M.am+arg were measured. (B) Effects of increasing the distance between the *CPA1* uORF and the *CPA1* main coding sequence. ⟼, sequence between the *CPA1* uORF and the *CPA1* main coding sequence that was triplicated in strain IBP41. The open box represents the *CPA1* uORF, and the dotted box represents the *CPA1* main coding sequence. The transcription start site is indicated by the vertical line. The different fragments bearing *CPA1* constructions were integrated into strain 10W53d (*ura2C-2 cpa1*Δ *ura3*). CPSase specific activities in different transformant strains grown on M.am and M.am+arg were measured. Uracil (25 μg/ml) was also added to the growth media.

to 5.1, depending on the growth conditions. Moreover, repression by arginine operates efficiently under conditions of galactose induction, thus showing that no factor is limiting for repression.

Analysis of the leader sequence located between the uORF and the *CPA1* coding sequence. We have also constructed five deletions (covering from 4 to 11 bp) at various locations in the mRNA sequence between the uORF and the *CPA1* main coding sequence (see Materials and Methods). We have also created a *Bam*HI site just downstream from the uORF (plasmid pBP5 [see Materials and Methods]) for the purpose of additional constructions. As shown in Fig. 5A, none of these mutations impaired the regulation of *CPA1* expression, suggesting that the leader sequence downstream from the uORF is not essential for this regulation.

To determine whether the distance between the two uORFs could be important, we have multiplied this distance by a factor of three by inserting two additional copies of the same sequence between the two uORFs (see Materials and Methods). As shown in Fig. 5B, this increase has no effect on repression or on the level of enzyme activity, suggesting that the spacing between the two uORFs is not critical for expression and regulation.

In order to confirm that the 3′ untranslated region of the *CPA1* leader plays no role in the arginine-mediated repression, we fused the *CPA1* leader including the uORF to the leader and the coding sequence of the *ARG4* gene (see Materials and Methods), leading to plasmid pCAF4. It is worth noting that *ARG4* is not regulated specifically by arginine and obeys only the general control of amino acid biosynthesis (8, 18). As shown in Fig. 4B, in this fusion argininosuccinate lyase synthesis was repressed fivefold when strain IBAF10-1-A, transformed with this centromeric plasmid, was grown in the presence of arginine. As a control, strain IBAF10-1-A was also transformed with plasmid pCAF12, which contains a fused *CPA1-ARG4* gene similar to that present in pCAF4 but devoid of the uORF. A residual repression by arginine of argininosuccinate lyase synthesis was, however, observed in this strain. This repression might be related to the effect observed by Crabeel et al. (7) concerning the effects of *argR* mutations on *CPA1* expression. Comparison of the argininosuccinate lyase levels of the strains harboring plasmids pCAF4 and pCAF12

does, however, indicate a strong inhibitory effect of the *CPA1* uORF on the basal level of expression of such constructions. Nevertheless, these results confirm that the sequences present in the *CPA1* leader downstream of the uORF are dispensable for repression by arginine.

Taken together, these data indicate that the uORF is the crucial element involved in the translational regulation of the *CPA1* gene by arginine. In contrast, the 5′ or 3′ sequences surrounding the uORF appear dispensable for the regulation but could play a role in the efficiency of repression, for example, by influencing the secondary structure of the mRNA around the uORF. On the basis of such findings, we attempted (see next section) to transfer the regulation characteristics of *CPA1* to a heterologous yeast gene by inserting its uORF into the leader sequence of gene *GCN4*.

Effect of insertion of the *CPA1* uORF in the leader sequence of *GCN4*. The GCN4 protein is a transcriptional activator of a large set of biosynthetic genes in response to amino acid starvation (13). The expression of the *GCN4* gene itself is regulated through a translational control mechanism involving four short uORFs (uORF1 to uORF4) in the leader of *GCN4* mRNA (10, 32). In order to examine the ability of the *CPA1* uORF to confer repression by arginine to other genes, we have inserted the *CPA1* uORF in the place of uORF1 or uORF4 in the leader sequence of a *GCN4-lacZ* fusion from which all four uORFs had been eliminated (24). As shown in Fig. 6, arginine had no noticeable effect on the expression of the original *GCN4-lacZ* fusion (plasmid p227), measured as β-galactosidase reporter activity. The results suggest that, in these constructions, the *CPA1* uORF acts in two ways.

First, the *CPA1* uORF, when inserted in the position of *GCN4* uORF1 or *GCN4* uORF4, conferred a significant repression by arginine to the expression of the *GCN4-lacZ* fusion (plasmids pCP20 and pCP8 in Fig. 6). This result was confirmed by the fact that the repression was abolished when a mutation known to limit the repression by arginine of *CPA1* expression (mutation *CPA1-O5*, which changes cysteine 11 to tyrosine [35]) was created in the uORF (plasmids pCP31 and pCP32 in Fig. 6).

On the other hand, the *CPA1* uORF carrying the *CPA1-O5* mutation, when inserted in the position of *GCN4* uORF1, reduced the expression of the *GCN4-lacZ* fusion by a factor of 2.1 (compare p227 and pCP31). The same uORF inserted in the place of uORF4 (plasmid pCP32) caused a strong inhibitory effect on *GCN4-lacZ* expression, which was reduced by a factor of 8.1 compared with that of the construction devoid of all four uORFs. These effects are similar to those of the *GCN4* uORFs in reducing the basal level of expression. The deleterious effect on expression is moderate at the position of uORF1, which is normally a leaky translational barrier; it is important in the uORF4 position, which, in *GCN4*, constitutes a highly efficient translation barrier. Such observations are in accord with the notion that it is the context of the uORF which is important in determining its effect on reinitiation at a downstream translation initiation codon (23). When the wild-type *CPA1* uORF is used in the same constructions, the decrease in *GCN4* expression is stronger, and this effect is more pronounced and is particularly drastic in the case of uORF4 position. This additional reduction probably results from repression by arginine, partial repression being already exerted by endogenous arginine formed during growth on M.am. However, this hypothesis does not account for the different effects observed for insertions in positions 1 and 4; the nucleotide context in the vicinity of the insertion points might be crucial for determining their effects. Indeed, the sequences surrounding position 1 seem to promote the translation of

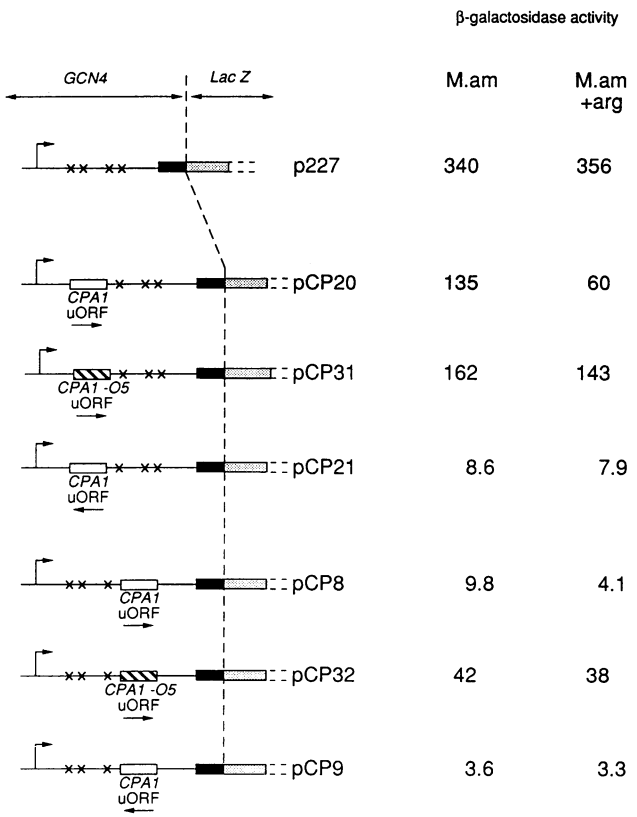


FIG. 6. Effects of the *CPA1* uORF on *GCN4* expression. The extent of each gene is indicated by converging arrows. The thin line represents the *GCN4* leader; X represents the sequences that replace uORF1, uORF2, uORF3, and uORF4; the black box represents the beginning of the *GCN4* ORF; the dotted box represents *lacZ* ORF; the open box represents the *CPA1* uORF; and the hatched box represents the *CPA1* uORF carrying a *CPA1-O5* mutation, inserted either at the uORF1 or the uORF4 position (24). The arrows under the uORF indicate the orientation of the *CPA1* leader peptide sequence. The transcription start site is indicated by the vertical line. All of these plasmids were introduced in strain 12T7c (*ura3*). β-Galactosidase specific activities in different transformant cells grown on M.am and M.am+arg were determined.

downstream coding sequences (23); thus, they might compensate for the partial repression achieved by endogenous arginine. The absence of such sequences in the vicinity of position 4 precludes a similar compensation effect and should consequently result in a further reduction of the expression of the *GCN4-lacZ* fusion gene.

For other controls for these experiments, we inserted the wild-type uORF in the reverse orientation at both positions (plasmids pCP21 and pCP9). Both insertions led to a considerable reduction of the basal level of *GCN4* expression but failed to confer repression by arginine. This effect of the insertion in the reverse orientation, particularly striking for insertion in the uORF4 position, might be related to the presence of two small uORFs, of 1 and 4 codons, in the reverse uORF orientation. The sequences surrounding these uORFs, which are shorter than and totally different from the original *CPA1* uORF, might have a particularly deleterious effect on reinitiation at a downstream translation initiation codon.

It is noteworthy that the amplitude of repression by arginine was significantly lower in the constructions described above

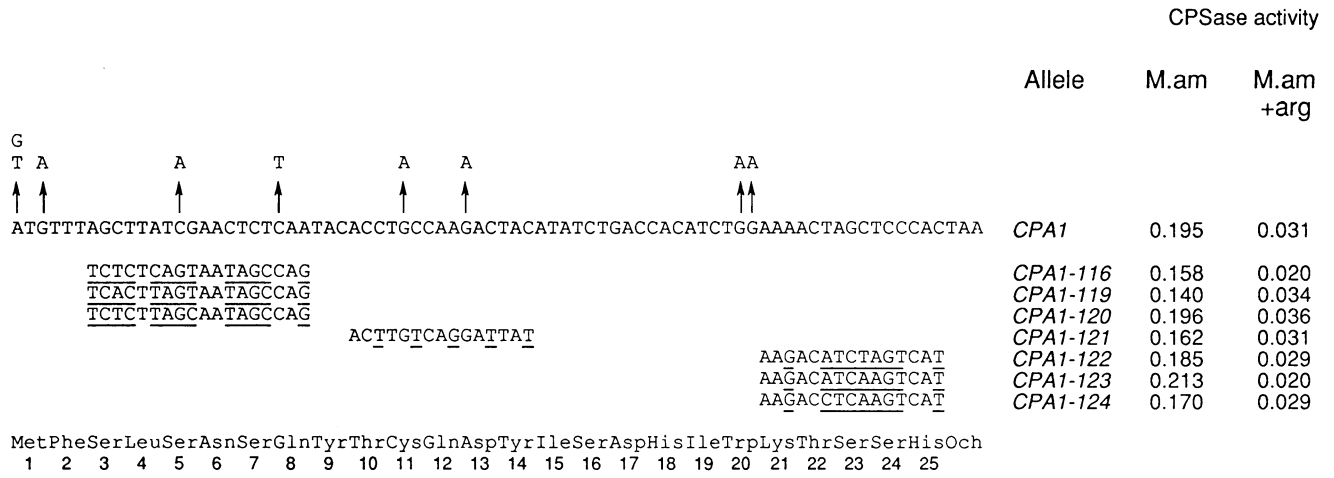


FIG. 7. Effects of silent mutations in the uORF on *CPA1* repression by arginine. The nucleotide sequence of the uORF is shown with, above, the nucleotide changes leading to the *CPA1-O* phenotypes described previously (35) and, below, the different nucleotide changes (underlined) leaving the amino acid sequence of the peptide unchanged. The amino acid sequence of the CPA1 leader peptide is also indicated. The different plasmids were integrated into the genome of strain 10W51a (*ura2C-2 cpa1*Δ).

(repression ratio, around 2.5) than that in the wild-type *CPA1* gene (repression ratio, 5 to 7). The reasons behind such a behavior remain to be determined but could be linked either to the modified sequence context surrounding the *CPA1* uORF in these constructions or to a difference in the structural or stability properties of *GCN4-lacZ* mRNA compared with those of the *CPA1* mRNA.

The observations described in this section are thus consistent with the view that the *CPA1* uORF has a specific function in the repression of *CPA1* by arginine and suffices to confer this repression on another gene.

Effect of mutations in the *CPA1* uORF that leave unchanged the amino acid sequence of the leader peptide. The results presented in the previous sections confirm the essential role played by the *CPA1* uORF in the repression of *CPA1* expression by arginine. We showed previously that a variety of mutations, of missense, nonsense, and frameshift types, located throughout the uORF sequence alter this regulation. On such a basis, it was proposed that within the uORF it is the coding sequence which is important for the repression mechanism. As an attempt to ascertain this possibility, we changed 16 of the 25 codons of the uORF by groups of 5 or 6 while taking advantage of the degeneracy of the genetic code to keep unchanged the sequence of the leader peptide. The results of this experiment are shown in Fig. 7. Despite the fact that in some of them as many as 13 nucleotides had been changed at the same time, all mutants obtained were still regulated like the wild-type strain.

Such results support the notion that the peptide encoded by the *CPA1* uORF is a negative element necessary for the regulation of the *CPA1* gene.

The synthesis of a hybrid protein comprising the CPA1 leader peptide fused to β-galactosidase is repressible by arginine. In order to investigate the translation of the *CPA1* uORF, we constructed a fusion of the 5′ end of the *CPA1* leader sequence including its uORF to the *lacZ* protein-coding sequence (plasmid pCP3). The resulting chimeric protein comprised the 23 first amino acids of the peptide fused directly to β-galactosidase. We also constructed a fusion of *lacZ* with the *CPA1* coding sequence (plasmid pCP4). As shown in Fig. 8, an assay of β-galactosidase activity in the strain transformed with pCP3 suggested that the uORF is translated at a relatively high level. In addition, the synthesis of β-galactosidase was repressed by arginine in both plasmids (by factors of 3 and 3.8). This result, which suggests that the synthesis of the CPA1 leader peptide itself is under arginine control, was confirmed by the observation that a mutation in the leader peptide (mutation *CPA1-O5*, changing cysteine 11 to a tyrosine [35]) known to limit the repression by arginine of *CPA1* expression also limited the repression of the synthesis of the fusion protein (compare plasmids pCP3 and pCP18 with plasmids

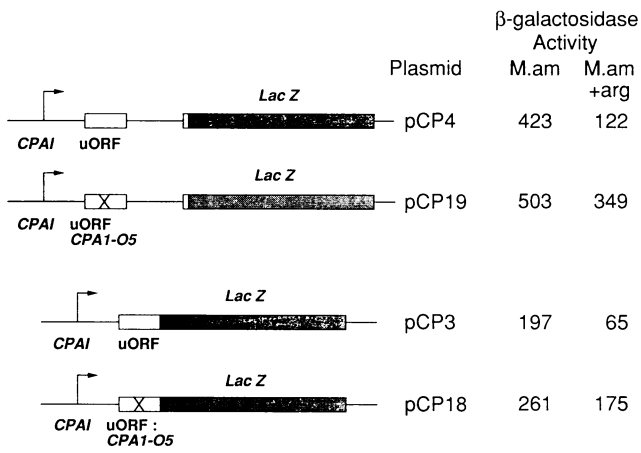


FIG. 8. Effects of arginine on the expression of *CPA1-lacZ* and *CPA1* uORF-*lacZ* fusions. The open box represents the part of the *CPA1* gene fused to the *lacZ* gene (dotted box). These constructions contain the *CPA1* promoter, the uORF, and part of the *CPA1* coding sequence (first 3 amino acids of pCP4 and pCP19) or the *CPA1* promoter and the first 23 codons of the uORF (pCP3 and pPC18). The vertical lines indicate the transcription start sites. Plasmids pCP18 and pCP19 contain a *CPA1-O5* mutation in the uORF, symbolized by X (cysteine 11 to tyrosine [35]). All of these plasmids were introduced into strain 12T7c (*ura3*). β-Galactosidase specific activities in different transformant cells grown on M.am and M.am+arg were determined and are expressed in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

pCP4 and pCP19). As already observed in a previous section, these fusions involving the β-galactosidase coding sequence resulted in reduced amplitude of repression by arginine compared with repression of *CPA1* expression.

DISCUSSION

The repression of the expression of the yeast *CPA1* gene by arginine is one of the well-documented cases of translational control in a eucaryotic organism (20, 35). Previous experiments have established the importance for this regulation of the leader sequence of *CPA1* mRNA and, in particular, of its 25-codon uORF. Most convincing in this regard was the finding that all the *CPA1-O* mutations selected in vivo for constitutivity of *CPA1* expression map within this uORF (35). By contrast, little was known of the role played in this regulation by the noncoding sequences present upstream and downstream from the uORF. The results reported here show that the upstream noncoding sequence can be considerably shortened without significantly affecting the repression of *CPA1* by arginine. Some of the deletions created in this region did result in a reduced amplitude of repression. This was the case for deletions resulting in the formation of extremely short leader sequences upstream to the uORF and for one central deletion. In the latter deletion, however, repression was restored at its normal level by removal of a few more nucleotides. It is suggested that these effects are due to a reduced efficiency of translation initiation at the AUG of the uORF and to a lowered synthesis of the leader peptide rather than to the elimination of regulation-specific sequences. Similarly, deletions within the noncoding sequence between the uORF and the *CPA1* main coding sequence had little effect on this regulation. Such findings suggest that the noncoding regions of *CPA1* leader contain no specific sequences required for the regulation. In agreement with this conclusion, we found that repression by arginine was preserved in constructions in which the 5′ and 3′ regions of the *CPA1* leader were replaced by the cognate regions of other genes that are not subject to repression by arginine.

Of the various regions of the *CPA1* leader, only one, namely, the uORF, consequently appears to be essential for the repression by arginine. This conclusion was further supported by the finding that this uORF, when inserted in a *GCN4* leader devoid of all its regulatory elements, was able to bring under arginine control this construction, which does not normally respond to this amino acid. The uORF is consequently sufficient to confer arginine repression to a heterologous gene. Nevertheless, the noncoding sequences of the leader may play a role in determining the amplitude of regulation.

The experiment comparing the expression of uORF-*lacZ* and *CPA1-lacZ* fusions indicates that the uORF is translated with a significantly higher frequency than expected on the basis of the model proposed previously (35). According to the scanning model (14), the ribosomes which have started translating at the AUG of the uORF are unable to reinitiate at a downstream AUG and, consequently, to translate the *CPA1* coding sequence. Therefore, eliminating the translation of the uORF by destruction of its initiator AUG (mutant *CPA1-OA* [35]) should allow all the ribosomes to achieve translation of the main *CPA1* ORF. The level of *CPA1* expression should therefore increase by a factor corresponding to the level of translation of the uORF. Nevertheless, comparison of the level of CPSase A activity of mutant *CPA1-OA* with that of a wild-type *CPA1* allele showed practically no difference in specific activity (35). Additional assumptions are therefore needed to reconcile this unusual behavior with the scanning hypothesis. We could, for example, assume that most ribosomes which have translated the uORF are able to reinitiate at the downstream AUG. In such a case, elimination of the uORF would not result in any significant increase in the translation of the *CPA1* main coding sequence.

There are cases, however, in which the region downstream from the uORF is provided by a heterologous gene (the *CPA1-ARG4* fusion or the insertion of the *CPA1* uORF in the leader of *GCN4*), and the elimination of the uORF results in a drastic increase in the level of expression of the downstream coding sequences. The behavior in such cases is in strict observance of the scanning hypothesis. These results lead us to suggest that the region between the two ORFs of gene *CPA1* contains a sequence which does somehow facilitate reinitiation at a downstream AUG, a point which is presently under investigation.

The inhibitory effect of the *CPA1* uORF inserted into the leader sequence of *GCN4* is less important when the insertion occurs in the position of uORF1 rather than in uORF4. Such a behavior is unlikely to result from a difference in AUG context, since the fragment carrying the *CPA1* uORF was chosen to be long enough to keep a constant initiation context. Alternative explanations can be proposed. The distance separating the two uORFs is longer for position 1 and probably improves the reinitiation capacity of the ribosomes. A relation between reinitiation ability and the distance of the two ORFs is the basis of a model proposed to account for the regulation of the expression of *GCN4* (1). However, in the case of *CPA1*, the increase by a factor of three in the distance between the uORF and the *CPA1* main coding sequence had no effect on the expression of this gene. In addition, Miller and Hinnebusch (23) have suggested that the nucleotides 3′ to the stop codons of uORF1 and uORF4 contribute to their distinct effects on reinitiation at the AUG of *GCN4*. In view of these findings, it may be proposed that little or no reinitiation is possible when the *CPA1* uORF is inserted in position 4, in accord with the scanning hypothesis. A strong inhibition of the synthesis of the protein encoded downstream results. By contrast, when the uORF lies in position 1, the mechanism deviates from the strict scanning hypothesis and some reinitiation is possible, resulting in a less marked inhibitory effect.

The precise mechanism of the translational repression of *CPA1* by arginine remains to be determined. On the basis of the degeneracy of the genetic code, we have achieved multiple changes in the nucleotide sequence of the uORF without modifying the sequence of the corresponding peptide. None of these changes results in any significant alteration of the repression of *CPA1* by arginine, in contrast to the previously described *CPA1-O* mutations, which were shown to affect the amino acid sequence of the resulting peptide (33, 35). These results do indicate that the peptide product of the uORF is the essential element involved in the regulation. Thus, neither the nucleotide sequence of the uORF nor its codon usage is important for its regulatory role.

Thuriaux et al. (33) showed that the *CPA1-O* mutations were expressed only in *cis*. Since it has been established that the leader peptide, the product of the uORF, is required for the translational control, it is clear that the regulatory peptide also operates in *cis*, close to the site of its synthesis, or, in other words, close to the place where translation occurs. The absence of a *trans* effect of the leader peptide suggests at least three explanations: first, the peptide is extremely unstable; second, its concentration is too low for efficient regulation except in the immediate vicinity of the site of its synthesis; and third, the peptide acts while still bound to the translation machinery. All

three possibilities imply that the site of action of the peptide is located close to its site of synthesis.

The observation that the synthesis of the uORF-*lacZ* fusion protein is repressed by arginine was unexpected. However, the repression of the hybrid protein was reduced when a *CPA1-O5* mutation, known to impair the repression of *CPA1* (33, 35), was introduced into the fusion. Since the regulation depends on the presence of the leader peptide, such a finding suggests that the peptide keeps its regulatory properties as part of a larger hybrid protein. Therefore, if the peptide, as postulated above, acts while still bound to the ribosome, it may cause repression before completion of the synthesis of its C-terminal end. Similarly, repression of the synthesis of the fusion protein would occur while the β-galactosidase region is not yet synthesized and does not disturb the regulatory mechanism. In any case, the interpretation of these experiments is difficult at present and requires more experimentation. It will in particular be of interest to know whether mutations affecting the initiation factor eIF-2, which have recently been shown to impair the translational control of *GCN4* (6, 9), also impair the regulation of *CPA1* expression. Such experiments are in progress.

ACKNOWLEDGMENTS

We are grateful to Alan Hinnebusch for the gift of *GCN4-lacZ* fusion plasmids and D. Gigot for supplying synthetic oligonucleotides.

This work was supported by grant 2.9003.88 from the Fonds de la Recherche Fondamentale Collective and by the Research Council of the Université Libre de Bruxelles. P. Delbecq is a Research Assistant of the Belgian National Fund for Scientific Research. R. K. Filipkowski was a Tempus grantee of the EEC.

REFERENCES

1. **Abastado, J. P., P. F. Miller, B. M. Jackson, and A. G. Hinnebusch.** 1991. Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for *GCN4* translational control. Mol. Cell. Biol. **11**:486–496.
2. **Beacham, I. R., B. W. Schweitzer, H. M. Warrick, and J. Carbon.** 1984. The nucleotide sequence of the yeast *ARG4* gene. J. Mol. Biol. **29**:271–279.
3. **Birnboim, H. C.** 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100**:243–255.
4. **Bonneaud, N., O. Ozier-Kalogeropoulos, G. Li, M. Labouesse, L. Minvielle-Sebastia, and F. Lacroute.** 1991. A family of low and high copy replicative, integrative and single stranded *S. cerevisiae/E. coli* shuttle vectors. Yeast **7**:609–615.
5. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143**:971–980.
6. **Cigan, A. M., E. K. Pabich, L. Feng, and T. F. Donahue.** 1989. Yeast translation initiation suppressor *sui2* encodes the α subunit of eukaryotic initiation factor 2 and shares identity with the human α subunit. Proc. Natl. Acad. Sci. USA **86**:2784–2788.
7. **Crabeel, M., R. Lavalle, and N. Glansdorff.** 1990. Arginine specific repression in *Saccharomyces cerevisiae*: kinetic data on *ARG1* and *ARG3* mRNA transcription and stability support a transcriptional control mechanism. Mol. Cell. Biol. **10**:1226–1233.

7a. **Delbecq, P., M. Werner, A. Feller, and A. Piérard.** 1990. Translational control by arginine of yeast gene *CPA1*. Yeast **6**:S395.

7b. **Delbecq, P., F. Messenguy, M. Werner, A. Feller, and A. Piérard.** 1992. Translational control of yeast gene CPA1: analysis of its leader sequence and effect of mutations in initiation factor eIF-2 on its expression. Yeast **8**:S60.

8. **Delforge, J., F. Messenguy, and J.-M. Wiame.** 1975. The regulation of arginine biosynthesis in *Saccharomyces cerevisiae*: the specificity of *argR* mutations and the general control of amino acid biosynthesis. Eur. J. Biochem. **57**:231–239.
9. **Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. F. Donahue, and A. G. Hinnebusch.** 1992. Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. Cell **68**:585–596.
10. **Hinnebusch, A. G.** 1984. Evidence for translational regulation of the activator of general amino acid control in yeast. Proc. Natl. Acad. Sci. USA **81**:6442–6446.
11. **Hinnebusch, A. G.** 1988. Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **52**:248–273.
12. **Hinnebusch, A. G.** 1990. Involvement of an initiator factor and protein phosphorylation in translational control of GCN4 mRNA. TIBS Trends Biochem. Sci. **15**:148–152.
13. **Hinnebusch, A. G., and G. R. Fink.** 1983. Positive regulation in the general amino acid control of *S. cerevisiae*. Proc. Natl. Acad. Sci. USA **80**:5374–5378.
14. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108**:229–241.
15. **Lacroute, F., A. Piérard, M. Grenson, and J.-M. Wiame.** 1965. The biosynthesis of carbamoylphosphate in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **40**:127–142.
16. **Loppes, R., and C. Denis.** 1983. Chloroplast and nuclear DNA fragments from *Chlamydomonas* promoting high frequency transformation of yeast. Curr. Genet. **7**:473–480.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Messenguy, F.** 1979. Concerted repression of the synthesis of the arginine biosynthetic pathway by amino acids: a comparison between the regulatory mechanism controlling amino acid biosynthesis in bacteria and yeast. Mol. Gen. Genet. **169**:85–95.
19. **Messenguy, F., and E. Dubois.** 1983. Participation of transcriptional and post-transcriptional regulatory mechanisms in the control of arginine metabolism in yeast. Mol. Gen. Genet. **189**:148–156.
20. **Messenguy, F., A. Feller, M. Crabeel, and A. Piérard.** 1983. Control mechanisms acting at the transcriptional and post-transcriptional levels are involved in the synthesis of the arginine pathway carbamoylphosphate synthase of yeast. EMBO J. **2**:1249–1254.
21. **Messing, J.** 1983. The new M13 vectors for cloning. Methods Enzymol. **101**:20–78.
22. **Micklus, M. J., and I. M. Stein.** 1973. The colorimetric determination of mono and disubstituted guanidines. Anal. Biochem. **54**:545–553.
23. **Miller, P. F., and A. G. Hinnebusch.** 1989. Sequences that surround the stop codons of upstream open reading frames in *GCN4* mRNA determine their distinct functions in translational control. Genes Dev. **3**:1217–1225.
24. **Mueller, P. P., and A. G. Hinnebusch.** 1986. Multiple upstream AUG codons mediate translational control of *GCN4*. Cell **45**:201–207.
25. **Nakamaye, K. L., and F. Eckstein.** 1986. Inhibition of restriction endonuclease *Nci*I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. Nucleic Acids Res. **14**:9679–9698.
26. **Piérard, A., F. Messenguy, A. Feller, and F. Hilger.** 1979. Dual regulation of the synthesis of the arginine pathway carbamoylphosphate synthase of *Saccharomyces cerevisiae* by specific and general controls of amino acid biosynthesis. Mol. Gen. Genet. **174**:163–171.
27. **Piérard, A., and B. Schröter.** 1978. Structure-function relationships in the arginine pathway carbamoylphosphate synthase of *Saccharomyces cerevisiae*. J. Bacteriol. **134**:167–176.
28. **Ramos, F., and J.-M. Wiame.** 1979. Synthesis and activation of asparaginase in asparagine auxotrophs of *Saccharomyces cerevisiae*. Eur. J. Biochem. **94**:409–417.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
30. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast

genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Taylor, J. W., and F. Eckstein.** 1985. The rapid generation of oligonunucleotide-directed mutations at high frequency using phophorothioate-modified DNA. Nucleic Acids Res. **13:**8765–8785.
32. **Thireos, G., M. Driscoll-Penn, and H. Greer.** 1984. 5′ untranslated sequences are required for translational control of a yeast regulatory gene. Proc. Natl. Acad. Sci. USA **81:**5096–5100.
33. **Thuriaux, P., F. Ramos, A. Piérard, M. Grenson, and J.-M. Wiame.** 1972. Regulation of the carbamoylphosphate synthetase belonging to the arginine biosynthetic pathway of *Saccharomyces cerevisiae*. J. Mol. Biol. **67:**277–287.
34. **Werner, M., A. Feller, P. Delbecq, and A. Piérard.** 1990. Translational control by arginine of yeast gene *CPA1*. NATO ASI Ser. Ser. H **49:**337–346.
35. **Werner, M., A. Feller, F. Messenguy, and A. Piérard.** 1987. The leader peptide of yeast gene *CPA1* is essential for the translational repression of its expression. Cell **49:**805–813.
36. **Werner, M., A. Feller, and A. Piérard.** 1985. Nucleotide sequence of yeast gene *CPA1* encoding the small subunit of arginine-pathway carbamoyl-phosphate synthetase: homology of the deduced amino acid sequence to other glutamine amidotransferases. Eur. J. Biochem. **146:**371–381.