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Control mechanisms acting at the transcriptional and post-transcriptional levels are involved in the synthesis of the arginine pathway carbamoylphosphate synthase of yeast

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In *Saccharomyces cerevisiae*, the synthesis of the arginine pathway enzyme carbamoylphosphate synthase (CPSase A) is subject to two control mechanisms. One mechanism, the general control of amino acid biosynthesis, influences the expression of both *CPA1* and *CPA2* genes, the structural genes for the two subunits of the enzyme. The second mechanism, the specific control of arginine biosynthesis, only affects the expression of *CPA1*. To study these mechanisms in more detail, we have cloned the *CPA1* and *CPA2* genes and used their DNA to measure the *CPA1* and *CPA2* mRNA content of cells grown under various conditions. A close coordination was observed in the variation of the levels of *CPA1* and *CPA2* mRNAs and polypeptide products under conditions where the general control of amino acid biosynthesis operates. In contrast, little correlation was found between the levels of *CPA1* mRNA and the corresponding protein for conditions affecting repression by arginine: the total amplitude of variation was 6-fold higher for the *CPA1* protein than for the *CPA1* messenger transcript. Such findings are consistent with the conclusion that the general control operates at the transcriptional level and that the specific arginine control acts primarily at a post-transcriptional level.
Key words: arginine biosynthesis/carbamoylphosphate/transcriptional control/post-transcriptional control/*Saccharomyces cerevisiae*

Introduction

The mechanisms by which the expression of eukaryotic genes is regulated have remained poorly understood as compared with similar mechanisms in prokaryotes. Although in eukaryotes regulatory mechanisms operate at many different levels (for a review, see Darnell, 1982), few if any of them are known in detail at the molecular level. Fungi, in particular, have been the subject of much attention because they are amenable to genetic analysis which has been invaluable for identifying the regulatory circuits that control enzyme synthesis in fungal metabolism. Such genetic analysis has distinguished the specific and general regulatory mechanisms which participate in the control of the arginine pathway of *Saccharomyces cerevisiae* (Béchet *et al.*, 1970; Delforge *et al.*, 1975; Messenguy, 1976, 1979). Genetic analysis does not, however, provide direct information about the level at which such regulatory mechanisms operate. Additional approaches, including 'Northern blot' analysis, have been necessary to show that the control of gene expression in the arginine metabolism of *S. cerevisiae* may occur at levels other than transcription (Messenguy and Dubois, 1983). We report here the results of a study of the control of the arginine pathway carbamoylphosphate synthase in *S. cerevisiae*.

As a general rule, carbamoylphosphate (CP) is required for two major biosynthetic pathways, those of arginine and pyrimidine. Considerable variation occurs in the manner in which the biosynthesis of this metabolite is regulated (Piérard *et al.*, 1973; Makoff and Radford, 1978; Piérard, 1983). Most bacteria possess a single glutamine-dependent carbamoylphosphate synthase (CPSase; EC 6.3.5.5) endowed with appropriate controls. In contrast, fungi usually rely on two strictly compartmentalized and independently regulated enzymes for CP production. The yeast *S. cerevisiae* occupies an intermediate position (Figure 1), with two independently regulated CPSases feeding interchangeable cellular CP pools (Lacroute *et al.*, 1965). One enzyme, CPSase P, is repressed and feedback inhibited by the pyrimidines; it is encoded, with aspartate carbamoyltransferase, by the complex *URA2* locus. The second enzyme, CPSase A, is encoded by the unlinked *CPA1* and *CPA2* genes and is repressed by arginine. CPSase A comprises two subunits (Piérard and Schröter, 1978). A large subunit (mol. wt. 130 000), the product of gene *CPA2*, is responsible for the synthesis of CP from ammonia, bicarbonate and ATP; a small subunit (mol. wt. 36 000), the product of gene *CPA1*, transfers the amide nitrogen atom of glutamine, the physiological nitrogen substrate for CP synthesis, to the large subunit.

Two types of regulatory mechanisms have been found to control the synthesis of CPSase A. One mechanism is specific and is responsible for the repression of CPSase A during growth in the presence of arginine (Lacroute *et al.*, 1965). An interesting feature of this 'specific control', revealed by using a sensitive *in vitro* complementation assay of the *CPA1* and *CPA2* proteins, is that the expression of gene *CPA1* alone is regulated (Piérard *et al.*, 1979). Free *CPA2* subunit consequently over-accumulates under conditions where the overall glutamine-dependent activity is repressed by arginine. Lack of CPSase P, in a *ura2C*$^-$ mutant, results in sensitivity to

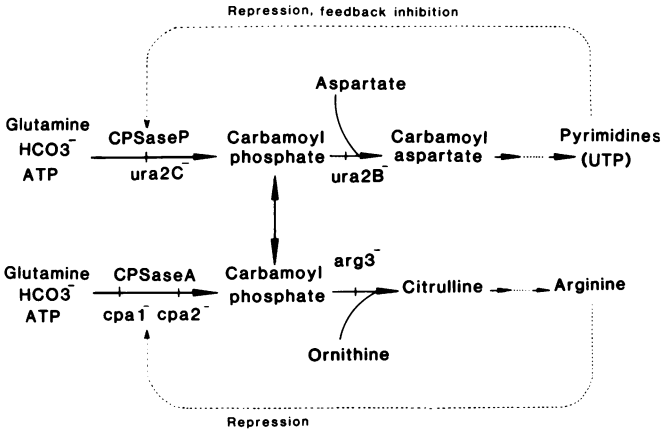

Fig. 1. Schema of the organization of carbamoylphosphate synthesis in *S. cerevisiae* (modified from Lacroute *et al.*, 1965).

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Table I. List of strains used in this work

Strains	Genotype
Σ1278b	wild-type
1C1082a	*ura2C*$^-$ *cpa1*$^-$ *ura3*$^-$: strain bearing a *ura2C*$^-$ (previous denomination *cpu*$^-$) mutation affecting the CPSase P, a *cpa1*$^-$ mutation affecting the small subunit of CPSase A and a *ura3*$^-$ mutation affecting oritidine 5'-phosphate decarboxylase.
1C1652b	*ura2C*$^-$ *cpa2*$^-$ *ura3*$^-$: strain bearing a *ura2C*$^-$ mutation affecting CPSase P, a *cpa2*$^-$ mutation affecting the large subunit of CPSase A and a *ura3*$^-$ mutation affecting orotidine 5'-phosphate decarboxylase.
MG409	*arg11*$^\pm$: a leaky arginine auxotrophic mutant. This mutation affects an unknown metabolic step and complements all known arginine auxotrophic mutants.
MG583	*ilv*$^\pm$: isoleucine-valine leaky auxotrophic mutant.
18337d	*ilv*$^\pm$ *cpa1*$^+$*0*$^-$: strain bearing a leaky isoleucine-valine auxotrophic mutation and a *cis*-dominant constitutive mutation affecting gene *CPA1*.
7305d	*lys*$^\pm$: lysine leaky auxotrophic mutant.
18400c	*lys*$^\pm$ *cpa1*$^+$*0*$^-$: strain bearing a leaky lysine auxotrophic mutation and a *cis*-dominant constitutive mutation affecting gene *CPA1*.
9177b	*ura2C*$^-$ *cpa1*$^+$*0*$^-$: strain bearing a *ura2C*$^-$ mutation affecting CPSase P and a *cis*-dominant mutation affecting gene *CPA1*.
1C1230a	*ura2C*$^-$ *cpaR*$^-$: strain bearing a *ura2C*$^-$ mutation and a *cpaR*$^-$ recessive mutation affecting gene *CPA1* and unlinked to this gene.

arginine caused by arginine repression (Lacroute *et al.*, 1965). Advantage has been taken of this sensitivity to select two classes of mutations affecting the specific arginine control: *cpa1*$^+$*0*$^-$ mutations, *cis*-dominant and closely linked to gene *CPA1*, and *cpaR*$^-$, recessive and unlinked to the structural gene (Thuriaux *et al.*, 1972). The mechanism defined by these mutations is entirely independent of that delineated by the *argR*$^-$ mutations which control the other arginine enzymes (Béchet *et al.*, 1970; Delforge *et al.*, 1975). Such mutations have suggested a regulatory mechanism basically similar to those controlling the bacterial operons.

The second mechanism regulating CPSase A synthesis is the general control which has been shown to regulate enzyme synthesis in the arginine and several other amino acid biosynthetic pathways (Schürch *et al.*, 1974; Delforge *et al.*, 1975; Wolfner *et al.*, 1975; Messenguy, 1979). The general control has been shown to modulate the expression of both *CPA1* and *CPA2* genes over a wide range extending from derepression occurring during starvation for any single amino acid to full repression observed during growth on amino acid-rich medium (Piérard *et al.*, 1979).

To obtain a deeper insight into the mechanisms involved in the control of expression of the *CPA1* and *CPA2* genes, we have compared the steady-state levels of the *CPA1* and *CPA2* mRNAs and polypeptides following growth of *S. cerevisiae* under various physiological conditions. Using this approach, we hoped to answer the question of whether or not these mechanisms act at the transcriptional level.

Results

Cloning of the yeast CPA1 and CPA2 genes

Genes *CPA1* and *CPA2* were cloned from a pool of hybrid plasmids constructed by inserting a partial *Sau*3A digest of total nuclear DNA from *S. cerevisiae* FL100 into a unique *Bam*HI site of the chimeric plasmid pFL1 (pBR322-2μD3-ura3) (Chevalier *et al.*, 1980). This pool, a gift of F.Lacroute, was used to complement the yeast strains 1C1082a (*cpa1*$^-$ *ura2C*$^-$ *ura3*$^-$) and 1C1652b (*cpa2*$^-$ *ura2C*$^-$ *ura3*$^-$) (see Table I). Several co-transformant clones capable of growing on minimal (M.am) medium were obtained. Two of them, one for each recipient strain, were analyzed in more detail. They were found to contain plasmids pYe11-2 carrying gene *CPA1* on a 4.1-kb yeast DNA insert and pYe35 carrying gene *CPA2* on a 18-kb insert, respectively. The restriction maps of these inserts are shown in Figure 2. The map for pYe35 is similar to that obtained after cloning gene *CPA2* from a different genetic background (Lusty and Lu, 1982).

The Cpa1$^+$ and Cpa2$^+$ phenotypes associated with the presence of these plasmids were verified by assaying CPSase A activity in strains carrying these plasmids. As shown in Table II, the presence of the plasmids confers wild-type overall CPSase A specific activity to the appropriate recipient strains.

The *in vitro* complementation assay of the *CPA* proteins does however show that cells harbouring plasmid pYe11-2 contain a 4-fold excess of the *CPA1* protein whereas cells carrying plasmid pYe35 synthesize a 24-fold excess of the *CPA2* protein. High levels of the *CPA* proteins are expected because of the high plasmid copy number. The lower level of *CPA1* protein as compared with *CPA2* protein agrees with the earlier observation that free *CPA1* protein is less stable than free *CPA2* protein (Piérard and Schröter, 1978). The expression of the cloned *CPA1* gene was normally repressible by arginine (Table II).

A strain lacking CPSase P is inhibited by arginine because of the repression of *CPA1* protein by arginine (Lacroute *et al.*, 1965; Piérard *et al.*, 1979). The introduction of plasmid pYe35 into such a background results in reduced sensitivity towards arginine. This indicates that the product of gene *CPA2*, the larger subunit of CPSase A, which in its free form only synthesizes CP from ammonia (Piérard and Schröter, 1978), may be functional *in vivo* if present in a sufficient number of copies.

We conclude from these various experiments that the yeast DNA inserts of plasmids pYe11-2 and pYe35, respectively, contain the structural genes *CPA1* and *CPA2*. In subsequent experiments they were used as probes for detecting the corresponding mRNAs.

Identification of CPA1 and CPA2 mRNAs

The DNA fragments described above were used as probes for detecting *CPA1*- and *CPA2*-specific mRNAs by the 'Northern' RNA-DNA hybridization technique (Alwine *et al.*, 1977). After being separated by gel electrophoresis and blot-transferred onto diazobenzyloxymethyl (DBM) paper, total yeast RNA was hybridized with the radiolabelled yeast DNA inserts of these plasmids. The label from the total pYe11-2 insert which carries gene *CPA1* was found to bind only to a 1.2 ± 0.1 kb RNA transcript whereas the label from the total pYe35 insert carrying gene *CPA2* bound to a single 4.0 ± 0.5 kb transcript. Such sizes are consistent with the known mol. wts. of the *CPA1* and *CPA2* proteins (Piérard and Schröter, 1978). Further experiments established that the hybridizing capacity of the pYe11-2 DNA insert was equally distributed between a 2.4-kb *Bam*HI-*Eco*RI fragment and a 2.0-kb *Eco*RI-*Sal*I fragment (see the restriction map in Figure 2), thus indicating that gene *CPA1* overlaps the *Eco*RI site. These DNA fragments were subsequently used as probes for measuring the *CPA1* mRNA. Similarly, a 3.2-kb *Bam*HI-

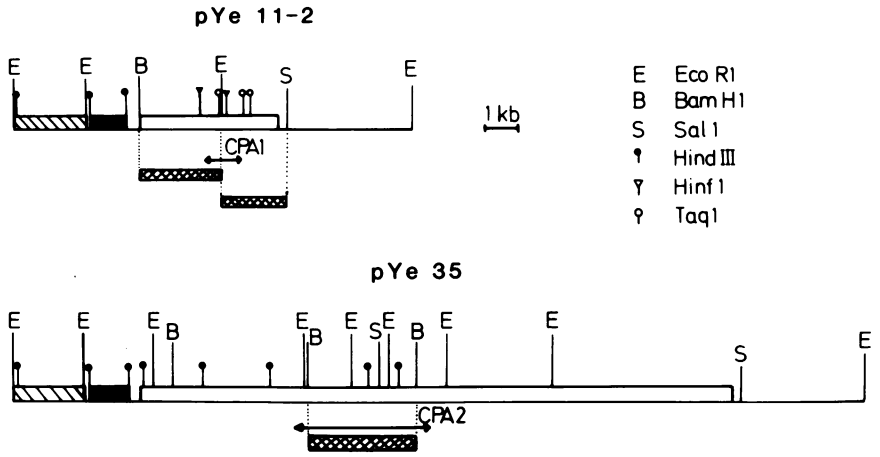

Fig. 2. Restriction maps of recombinant plasmids pYe11-2 and pYe35 carrying the yeast genes *CPA1* and *CPA2*. Thin lines: the pBR322 DNA of plasmid pFL1 (Chevalier *et al.*, 1980). Hatched bars: the 2.2-kb *Eco*RI fragment of yeast 2μ DNA. Solid bars: the 1.1.-kb *Hind*III fragment of the yeast *URA3* gene. Open bars: the yeast DNA fragments that are inserted at the *Bam*HI site of pFL1. Cross-hatched bars: the yeast DNA segments which were used as probes for measuring *CPA1* and *CPA2* mRNAs. Arrows indicate the approximate positions of *CPA1* and *CPA2* genes in the yeast DNA inserts.

Table II. CPSase A activity and level of *CPA1* and *CPA2* proteins in wild-type yeast and in plasmid-harbouring derivatives

Strain	Genotype	Growth medium	Overall CPSase A activity	*CPA1* protein	*CPA2* protein
Σ1278b	wild-type	M.am	0.31	0.31	0.33
		M.am + arginine	0.06	0.07	0.34
1C1082a/pYE11-2	(*cpa1*$^-$ *ura2C*$^-$ *ura3*$^-$/p*CPA1 URA3*)	M.am	0.45	1.36	n.d.[a]
		M.am + arginine	0.36	0.27	n.d.[a]
1C1652a/pYe35	(*cpa2*$^-$ *ura2C*$^-$ *ura3*$^-$/p*CPA2 URA3*)	M.am	0.66	0.85	7.90

[a]n.d. = not determined.

*Bam*HI fragment of the pYe35 insert was found to contain 80% of the *CPA2* structural gene and was used to assay the *CPA2* messenger.

Measurement of CPA2 mRNA

Gene *CPA2* escapes the specific control of arginine biosynthesis and is solely regulated as a function of the general control of amino acid biosynthesis (Piérard *et al.*, 1979). Repression of *CPA2* expression is achieved by growing the yeast cells on amino acid-rich medium; derepression occurs during slow growth of leaky amino acid auxotrophic mutants (for arginine or for any other amino acid) on minimal medium. The levels of *CPA2* mRNA were compared with those of the *CPA2* protein in yeast cells grown under these various conditions. As shown in Table III, derepression of the synthesis of the *CPA2* protein in arginine, lysine or isoleucine-valine leaky auxotrophs was accompanied by a parallel increase in *CPA2* mRNA. Similarly, growth on the amino acid-rich YEPD medium, while severely repressing the synthesis of *CPA2* protein, resulted in the lowering of the *CPA2* mRNA level to a barely measurable value. These data show a very good correlation, over a 100-fold range, between the levels of *CPA2* messenger transcript and polypeptide product indicating that the expression of this gene is primarily regulated at the level of transcription.

Measurement of CPA1 mRNA

The expression of gene *CPA1* was measured in similar experiments. Two sets of conditions had to be investigated since the expression of gene *CPA1* is known to respond to both the general and the specific controls of amino acid biosynthesis (Piérard *et al.*, 1979). Conditions which set the general control into operation are first discussed. As shown in Table IV, growth of a wild-type yeast on YEPD resulted in lowered levels of both *CPA1* mRNA and *CPA1* protein. Likewise, the marked increase in *CPA1* protein which follows growth of a leaky arginine auxotroph (strain MG409) on minimal medium was accompanied by an increase in *CPA1* mRNA. A similar correlation was observed in leaky lysine and iso-

Table III. Comparison of *CPA2* protein levels and *CPA2* mRNA levels in yeast cells grown under various conditions

Strain	Genotype	Growth medium	Level of *CPA2* protein[a]	Relative level of *CPA2* protein[a,b]	Relative level of *CPA2* mRNA[a,b]
Σ1278b	wild-type	M.am	0.34	1	1
		M.am + arginine	0.34	1	1
		YEPD	0.03	0.1	≤0.1
MG409	*arg11*$^\pm$	M.am	3.30	9.7	10.0
MG583	*ilv*$^\pm$	M.am	1.29	3.8	5.0
18337d	*ilv*$^\pm$ *cpa1*$^+$*0*$^-$	M.am	1.45	4.25	4.0
7305d	*lys*$^\pm$	M.am	1.26	3.7	4.8
18400c	*lys*$^\pm$ *cpa1*$^+$*0*$^-$	M.am	1.23	3.6	4.0

[a]The values given are mean values of at least three independent measurements.
[b]Calculated by taking the level measured for wild-type cells grown on M.am medium as equal to 1. The method used for quantifying the mRNA level is described in Materials and methods.

Table IV. Comparison of *CPA1* protein levels and *CPA1* mRNA levels in yeast cells grown under various conditions

Strain	Genotype	Growth medium	Level of *CPA1* protein[a]	Relative level of *CPA1* protein[a,b]	Relative level of *CPA1* mRNA[a,b]
Σ1278b	wild-type	M.am	0.31	1	1
		M.am + arginine	0.06	0.2	0.7 (1.1[c])
		M.arginine	0.03	0.1	0.75
		YEPD	0.03	0.1	<0.1
9177b	*cpa1*$^+$*0*$^-$*ura2C*$^-$	M.am + arginine + uracil[d]	0.24	0.65	1.55
1C1230a	*cpaR*$^-$*ura2C*$^-$	M.am + arginine + uracil[d]	0.14	0.45	3.0
MG409	*arg11*$^\pm$	M.am	3.10	10.0	3.95
MG583	*ilv*$^\pm$	M.am	0.26	0.85	4.55
18337d	*ilv*$^\pm$*cpa1*$^+$*0*$^-$	M.am	1.30	4.2	5.15
7305d	*lys*$^\pm$	M.am	0.105	0.35	5.30
18400c	*lys*$^\pm$*cpa1*$^+$*0*$^-$	M.am	0.70	2.25	5.00

[a]The values given are mean values of at least three independent measurements.
[b]Calculated by taking the levels measured for wild-type cells grown on M.am medium as equal to 1. The method used for quantifying the mRNA level is described in Materials and methods.
[c]Values obtained by extracting mRNA from yeast spheroplasts. This value is the average of values obtained for three separate extractions (1.1, 1.2 and 1.1). The value given for extraction with glass beads (0.7) is the average from four extractions (0.8, 0.65, 0.45 and 0.95).
[d]Most strains used carried the wild-type allele of gene *URA2*. These strains, containing a mutated (*ura2C*$^-$) allele, were grown in the presence of uracil (25 μg/ml) in order to avoid any interference from the pyrimidines pathway.

leucine-valine auxotrophs provided that a *cpa1*$^+$*0*$^-$ mutation affecting the specific control was present simultaneously (in Table IV, see strains 18337d *versus* MG583 and 18400c *versus* 7305d). The presence of a *cpa1*$^+$*0*$^-$ mutation was required to overcome the arginine pools existing in such bradytrophic strains (Delforge *et al.*, 1975; Piérard *et al.*, 1979). Such behavior was of course not observed in the case of *CPA2* expression (Table III). In summary, as far as the general control mechanism is concerned, a significant correlation was observed between the variations of *CPA1* messenger and protein.

Markedly different results were obtained with regard to conditions affecting the specific control of arginine biosynthesis. Whereas an appreciable repression of the synthesis of the *CPA1* polypeptide took place during growth of a wild-type strain in the presence of arginine (M.am + arginine or M. arginine), only a limited reduction in *CPA1* mRNA level occurred under these conditions. Interestingly, growth on M. arginine medium resulted in the same low level of *CPA1* protein as growth on YEPD medium; yet, the mRNA level remained 7- to 8-fold higher. In this respect, the behavior of the leaky auxotrophs, already mentioned above, is worth emphasizing. Such mutants, when grown on minimal medium, display an elevated level of *CPA1* mRNA but synthesize limited amounts of *CPA1* protein (Table IV). The introduction of a *cpa1*$^+$*0*$^-$ mutation in this context, without markedly modifying the mRNA content resulted in a 5- to 6-fold increase of *CPA1* protein. A striking lack of correlation consequently existed between the *CPA1* mRNA content and the level of *CPA1* polypeptide under conditions where arginine-specific repression of *CPA1* gene occurs. We have also estimated the *CPA1* mRNA content of strains harbouring the *cis*-dominant *cpa1*$^+$*0*$^-$ and recessive *cpaR*$^-$ mutations. These mutations have been shown to reduce the repressibility by arginine of gene *CPA1* expression, without appreciably changing the level of expression on minimal medium (Thuriaux *et al.*, 1972; Piérard *et al.*, 1979). Both mutations were nevertheless observed to increase significantly the *CPA1* mRNA content of the cells (Table IV). This was peculiarly striking for mutation *cpaR*$^-$ which allows a 55% repression of the *CPA1* protein synthesis but at the same time results in a 3-fold increase of the *CPA1* mRNA content.

Most mRNA measurements were achieved using the glass bead extraction method (see Materials and methods). A few extractions were nevertheless performed using the spheroplast technique. They provided significantly higher values than the glass bead method for the *CPA1* mRNA content of cells grown on M.am + arginine medium (see Table IV). Since no such discrepancy was observed for cells grown on M.am medium, this observation might be an indication of a different location of *CPA1* mRNA in the cell whether arginine is present or not.

Discussion

The 'Northern' DNA-RNA hybridization technique has been used to measure the *CPA1* and *CPA2* mRNA contents of yeast cells grown under various conditions and to compare them with the levels of the corresponding polypeptide products, the two subunits of the arginine pathway carbamoylphosphate synthase. Two main conclusions have emerged from such measurements. On the one hand, a close coordination is observed in the variations of the levels of messenger and polypeptide product for all the conditions where the general control of amino acid biosynthesis is known to operate. This finding strongly suggests that the general control acts primarily at the transcriptional level by regulating the amount of specific mRNA molecules synthesized. The possibility that this control mechanism occurs at the level of mRNA stability rather than synthesis may not be entirely excluded. However, the study of β-galactosidase synthesis from *HIS4-lacZ* (Silverman *et al.*, 1982) and *ARG3-lacZ* (Crabeel *et al.*, 1983) fusions has suggested that the information from the general control response is located at the 5′ end of the *HIS4* and *ARG3* regions. On the other hand, the same methodology, when applied to conditions affecting the specific control of *CPA1* expression by arginine, yielded very poor correlation between the levels of *CPA1* messenger and polypeptide product. Growth in the presence of arginine resulted in large variations of the level of *CPA1* protein but produced only limited changes of the *CPA1* mRNA content. The total amplitude of variations was 6-fold higher for the *CPA1* polypeptide product than for the corresponding mRNA transcript. At a time when genetic evidence alone was available, a negative transcriptional control similar to those known in prokaryotes was proposed to account for the control of *CPA1* polypeptide synthesis (Thuriaux *et al.*, 1972). The observations presented here are, however, difficult to reconcile with a purely transcriptional control and call for a fundamental reappraisal of the mode and level of action of specific arginine regulation on the expression of gene *CPA1*.

Two post-transcriptional models have been proposed to account for similar observations made concerning the specific regulation of other enzymes of arginine metabolism in yeast (Messenguy and Dubois, 1983). They might also apply to the control of *CPA1* expression. The first model consists of a control of the mRNA flux out of the nucleus; this control

would be exerted by arginine and the products of the *ARGR* genes which regulate the entire arginine pathway except CPSase A. The second model involves a modulation, by the same elements, of the translation frequency of the arginine mRNAs. Marked differences in stability of the mRNAs of *ARG3* (structural gene for ornithine carbamoyltransferase) and *CAR1* (gene for arginase) have been observed irrespective of whether or not arginine is present in the growth medium (Messenguy and Dubois, 1983). Such differential stabilities of mRNAs, depending for instance on their localization (in the nucleus or in the cytoplasm) or state (being translated or not) can be taken as consequences of the above models. The observation that the regulation by arginine is preserved after fusion of the *Escherichia coli lacZ* gene with the proximal part of the *ARG3* gene (Crabeel *et al.*, 1983) supports the view that such destabilization effects are consequences rather than intrinsic elements of the controls. They might however play an essential role during the establishment of repressive conditions. We have looked for a decrease in *CPA1* mRNA stability under conditions of arginine repression, especially in the presence of the transcription inhibitor 8-hydroxyquinoline, but found no change in stability (data not shown). The increased levels of *CPA1* mRNA detected in the presence of $cpa1^{+}0^{-}$ and $cpaR^{-}$ mutations might at least partly result from such stabilization-destabilization phenomena.

Alternatives to purely post-transcriptional mechanisms might, however, be presented. In a way similar to the yeast invertase gene (Carlson and Botstein, 1982), the arginine genes, including gene *CPA1*, might possess separate transcription initiation sites leading to two mRNAs differing in their regulation at the transcriptional level and in their capacity for translation. Such a model is however difficult to reconcile with all the features of the control of *CPA1* expression and with the fact that no indication for more than one *ARG3* or *CPA1* mRNA species has been obtained. In addition, S1 nuclease mapping experiments have shown no difference in *ARG3* mRNA 5′ ends whether or not arginine was present in the growth medium (R.Huygen *et al.*, in preparation).

Finally, the lack of proportionality between the levels of *CPA1* mRNA and *CPA1* protein might be due to an increase in the degradation rate of *CPA1* protein in conditions of repression by arginine. Yet, this appears unlikely since the $cpa1^{+}0^{-}$ and $cpaR^{-}$ mutations, which reduce the repressibility of *CPA1* protein by arginine, result in increased *CPA1* mRNA levels (Table IV).

Experiments aimed at more precisely defining effects of the general and specific controls of amino acid biosynthesis on the expression of genes *CPA1* and *CPA2* are presently in progress. They include identification and sequencing of the control region of these genes in mutated as well as in wild-type strains. *In vitro* mutagenesis will be useful to complete the set of available site mutations affecting these controls. Measurements of *CPA1* mRNA present in isolated nuclei and on polysomes are also being carried out.

Materials and methods

Organisms

All strains used are derived from the wild-type strain Σ1278b and are listed in Table I with their genotypes, comments concerning their phenotypes, and references.

Media and growth of cells

The minimal medium was medium M described by Ramos and Wiame (1979). It was supplemented with 0.01 M $(NH_4)_2SO_4$ as a nitrogen source and denoted M.am. When mentioned, this medium was supplemented with arginine (1 mg/ml) and (or) uracil (25 μg/ml). YEPD medium contained Difco yeast extract (10 g/l), Difco bacto-peptone (10 g/l) and glucose (20 g/l). Growth at 29°C, harvesting and breakage of cells for enzyme assays was as described previously (Piérard and Schröter, 1978; Piérard *et al.*, 1979).

DNA recombination procedures and transformation of yeast cells

Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc., USA, or Boehringer, Mannheim, FRG, and used according to the specifications of the manufacturers.

Yeast cells were transformed using the procedure of Hinnen *et al.* (1978).

RNA isolation

Total RNA was extracted from log-phase growing yeast cells ($0.5-1 \times 10^6$ cells/ml) either by the glass beads method of Waldron and Lacroute (1975) as modified by Messenguy and Dubois (1983), or from spheroplasts by the high salt extraction technique described by St-John and Davis (1979). Spheroplasts were prepared with zymolyase. In the hybridization experiments, total RNA was used instead of poly(A)-containing RNA. This allows a better quantification of the mRNA level as a function of the growth conditions (Messenguy and Dubois, 1983). In addition, since poly(A)-enriched RNA results from a post-transcriptional event, its amount might not be proportional to the amount of the primary transcript.

Agarose gel electrophoresis and transfer of RNA to DBM paper

Agarose gel (1.4%) electrophoresis of glyoxal-treated RNA was performed as described by Mac Master and Carmichael (1977). For each experiment, 100 μg of total RNA, as measured by absorbance at 260 nm, were put on the gel. The transfer of RNA from the gel to diazotized paper was done as described by Alwine *et al.* (1977).

DNA-RNA hybridization

Pretreatment and hybridization of diazotized paper sheets were performed as described by Alwine *et al.* (1977). Washing of the papers and X-ray film exposure were as described by Messenguy and Dubois (1983).

Nick-translation

Nick-translation of appropriate DNA probes was performed following the procedure of Rigby *et al.* (1977).

Preparation of DNA probes carrying the yeast genes CPA1 and CPA2

The DNA probes used for measuring the levels of *CPA1* and *CPA2* mRNAs are described in Results. These probes were prepared by restriction of the pYe11-2 and pYe35 plasmids and purified on a low melting agarose gel, following the conditions described by BRL Inc., USA who provide this special agar.

Quantification and normalization of RNA content on DBM paper

Each DBM paper was hybridized to the *CPA1* and *CPA2* DNA probes and to a 2.2-kb *Eco*RI DNA fragment that contained the *CYC7* structural gene and another gene that hybridized to a 1.45-kb transcript. The latter transcript was extremely abundant in log-phase cells and, like the *CYC7* transcript, did not vary according to the growth conditions used. These transcripts were chosen as internal controls and referred to as 'control transcripts'. In many cases, the DBM papers were also hybridized to probes containing the URA3 gene. The autoradiograms were scanned and quantitated by densitometry. For each RNA preparation, the surface of the peaks obtained for the *CPA1* and *CPA2* gene transcripts were then normalized to the values obtained for the control transcripts.

Preparation of cell extracts and assay of carbamoylphosphate synthase activity

The preparation of cell extracts and the assay of glutamine-dependent CPSase A activity by coupling with ornithine carbamoyltransferase (EC 2.1.3.3) in the presence of ornithine were as described previously (Hilger *et al.*, 1973; Piérard and Schröter, 1978). No significant CPSase P activity was detected under the conditions used (Piérard and Schröter, 1978). Protein was estimated using the method of Lowry *et al.* (1951). Specific enzyme activities are expressed as μmol of product formed per hour per mg protein.

Assay of CPA1 and CPA2 gene products by in vitro complementation

The assay of *CPA1* and *CPA2* gene products by reconstitution of glutamine-dependent CPSase A activity in the presence of excesses of extracts of strains 6987a ($cpa1^{-}$ $arg11^{\pm}$) and 9040a ($cpa2^{-}$ $arg11^{\pm}$) was as described previously (Piérard and Schröter, 1978).

Reagents and chemicals

[(*m*-nitrobenzyloxy)methyl]pyridinium chloride used in the preparation of DBM paper was from Pierce Chemical Co., USA. Zymolyase 60 000 was a product from Kirin Brewery Co., Ltd., Tokyo, Japan and diethylpyro-

carbonate a product from Sigma, USA. Agarose was purchased from Miles Co.. Slough, UK. DNA polymerase 1 and deoxyribonuclease used in the nick-translation of the DNA probes were products of Boehringer, Mannheim, FGR. [^{32}P]dATP was purchased from the Radiochemical Center, Amersham, UK.

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