Proc. Natl. Acad. Sci. USA
Vol. 78, No. 8, pp. 5026–5030, August 1981
Genetics

Cloning *arg3*, the gene for ornithine carbamoyltransferase from *Saccharomyces cerevisiae*: Expression in *Escherichia coli* requires secondary mutations; production of plasmid β-lactamase in yeast

(transgenotic expression/eukaryotic regulation/arginine biosynthesis/penicillinase/arginine constitutive mutant)

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Communicated by Adrian M. Srb, March 30, 1981

ABSTRACT **The yeast *arg3* gene, coding for ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3), has been cloned on a hybrid pBR322-2-μm plasmid. The cloned gene gives a normal regulatory response in yeast. It is not expressed at 35°C when a mutation preventing mRNA export from the nucleus at this temperature is included in the genetic make-up of the carrier strain. In *Escherichia coli*, no functional expression can be observed from the native yeast *arg3* gene. The study of a mutant plasmid (M1) producing low levels of yeast carbamoyltransferase in *E. coli* has permitted the localization and orientation of *arg3* on the plasmid. The mutation involved is a deletion that alters the regulatory response of *arg3* in yeast. The plasmid *bla* gene produces detectable amounts of β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) in yeast: the data provide an estimate of the β-lactamase activity associated with one exemplar of the plasmid expressing *arg3* (0.6 units).**

Several features of arginine biosynthesis in *Saccharomyces cerevisiae* make it particularly useful for the analysis of eukaryotic gene regulation at the molecular level. Two types of regulatory mechanism are found in yeast for the control of anabolic enzyme synthesis. One type of regulation is specific for the arginine biosynthetic pathway. Such control is negative, of the type found in bacteria, and defined by the isolation of recessive derepressed (*arg3*) mutants (1, 2). The second type of regulation is general and involves many (if not all) amino acids. It is dependent upon the level of tRNA aminoacylation (3).

A selection (4) based on overproduction of ornithine carbamoyltransferase (OrnCarbTase; carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3) has permitted the isolation of *cis*-dominant *arg3* regulatory mutants in which the specific regulation is abolished (5). Mutations affecting the general control have been isolated also (3). Cloning *arg3* therefore appeared a useful step toward understanding the regulatory mechanism operative in the arginine pathway. We have cloned this gene on a pBR322 plasmid and have addressed the following questions: (*i*) Can the cloned genes be expressed in the prokaryotic as well as in the eukaryotic background? (*ii*) Does the cloned gene remain susceptible to regulation in yeast? (*iii*) Is expression of the cloned gene sensitive to a conditional mutation preventing RNA export from the nucleus? (*iv*) Can the prokaryotic pBR322 *bla* gene, which renders *Escherichia coli* ampicillin (amp)-resistant (amp^r) be expressed in *S. cerevisiae*? In this paper, we provide answers to these questions.

For clarity, we use the genetic symbols *argF* and *pyrF* (pyr, pyrimidine) for the *E. coli* genes and *arg3* and *ura3* for the corresponding yeast genes. A preliminary report has been published in abstract form (6).

MATERIALS AND METHODS

Strains and Media. An *arg3* mutant of *S. cerevisiae* strain Σ1278b (α mating type) and the nonisogenic *ura3* mutant FL100 (*a* type) were crossed to produce the 1C1064C *arg3 ura3* recombinant. An *argF pyrF E. coli* strain was constructed by transducing a tryptophan (*trp*) mutant of *E. coli* C600 r^-m^- deletion (*pro-lac-argF*)*argI* (7) to Trp$^+$ *pyrF*$^-$ with *E. coli* phage 363 grown on *pyrF* strain 3050AD36 from F. Jacob. The minimal media used for *E. coli* (7) and yeast (5) have been described.

Plasmid DNA. Plasmid DNA was recovered from *E. coli* as described (7). Plasmid transfer from yeast to *E. coli* was carried out as follows: A loopful of yeast cells freshly grown on a minimal agar plate was suspended in 0.3 ml of 25% (wt/vol) sucrose in 50 mM Tris base, pH 7.5/0.2 M NaCl in an Eppendorff reaction tube (1.5 ml). Glass beads (≈0.5-mm diameter), washed in 1.0 M HCl and rinsed in water, were added so as to be covered by 2 mm of cell suspension, and the mixture was treated on a Vortex for 3 min; 0.3 ml of redistilled phenol saturated with 10 mM Tris base/1 mM EDTA, pH 7.5 (TE buffer) was added, and the mixture was treated on a Vortex for 2 min. After centrifugation, aqueous phase was pipetted out and extracted three times with water-saturated ether. DNA was precipitated from the aqueous phase with ethanol and suspended in 0.2 ml of TE buffer. After overnight dialysis against a 2000-fold excess of the same Tris buffer, the DNA was used for transformation.

Construction of Yeast DNA Hybrids. Total yeast DNA (8) from *S. cerevisiae* FL100 and DNA of plasmid pBR322-ura3 (clone 8)-2-mμD3 (designated pYe *ura3*) (9) were treated with *Bam*HI endonuclease, the 5′-terminal phosphates of the "linear" plasmid DNA were removed with alkaline phosphatase and then the mixture of plasmid and DNA fragments was treated with DNA ligase. Enzyme treatments were as prescribed by Boehringer Mannheim for *Bam*HI and by New England BioLabs for the phosphatase and the ligase. Hybrid plasmids so created were amplified in *E. coli* by selecting for amp^r transformants. About 45,000 colonies obtained after overnight growth on plates containing 25 μg of amp per ml were pooled and used to prepare plasmid DNA (7) used in transformation.

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Abbreviations: amp, ampicillin; amp^r and amp^s, ampicillin-resistant and -sensitive, respectively; pyr, pyrimidine; OrnCarbTase, ornithine carbamoyltransferase; kb, kilobase(s).

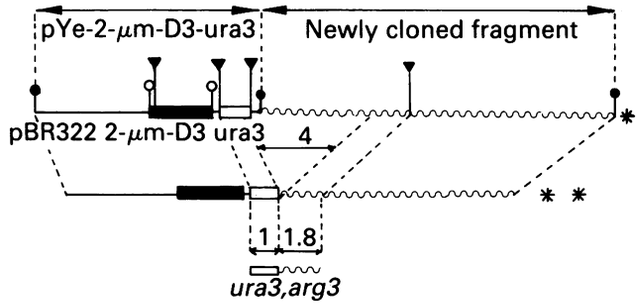

FIG. 1. Anatomy of plasmids used. Cleavages were at *Bam*HI (●), *Eco*RI (○), and *Hin*dIII (▼). Other *Hin*dIII and *Eco*RI sites are present in the newly cloned fragment. The *ura3* gene, coding for orotidine monophosphate decarboxylase is carried on a 1.1-kb *Hin*dIII fragment (9); 2-μm D3 is the smallest *Eco*RI fragment on the B form of the 2-μm yeast plasmid (11). *, pYe *ura3 arg3*; **, pYe *ura3 arg3*MI. Dimensions are in kb. The *ura3*- and *arg3*-bearing 2.8 kb *Hin*dIII fragment from pYe *ura3 arg3* MI was functional in *E. coli* on further cloning in pBR322 in either orientation. To subclone that fragment, the relevant band was sliced from a 0.7% agarose gel and cut in small pieces before immersion in TE buffer. The mixture was made 0.5–1.0 M in NaOAc and an equal volume of redistilled TE buffer-saturated phenol was added. The homogenized mixture was brought to 70°C and treated on a Vortex for 2 min; this operation was repeated five times. After centrifugation, two-thirds of the aqueous phase was recovered and dialyzed against TE buffer. The fragment was then cloned into *Hin*dIII, alkaline phosphatase-treated pBR322 DNA. Hybrid molecules were selected by transforming *E. coli* C600 r^-m^- deletion (*prolac argF*) *argI pyrF* to Pyr^+ in the presence of arginine. All Pyr^+ clones (3000 for 1 μg of DNA) had become Arg^+.

Transformation Procedures. The method of Petes *et al.* (8) was used for *E. coli* with one modification: transformants were not allowed to grow before plating.

For yeast, the procedure of Hinnen *et al.* (10) was used with the following modifications. Zymolyase (from Kirin Brewery, 0.02 mg/ml of cell suspension) was used instead of Glusulase. Agar for regeneration of yeast protoplasts was Difco yeast nitrogen base (6.7 g/liter) supplemented with 1.0 M sorbitol/2% (wt/vol) glucose/3% (wt/vol) agar/0.01% Difco yeast extract.

Gel-Paper Hybridization. Southern blots were done as described (12); nick translation of the plasmid DNA probe was as described by Rigby *et al.* (13).

Restriction and Electrophoresis. These were performed as described (7).

Enzyme Assays. OrnCarbTase and arginase (EC 3.5.3.1) were assayed as described (2). Inhibition of OrnCarbTase by arginase was monitored as described (14). Acetylglutamate kinase (EC 2.7.2.8) and argininosuccinate lyase (EC 4.3.2.1) were assayed according to published methods (3, 15). β-Lactamase (EC 3.5.2.6) was assayed as described (16).

The results of all enzyme assays are expressed as $\mu M\ hr^{-1}\ mg^{-1}$ of protein.

Containment. The experiments were carried out under P2/EK1 conditions as specified by the National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Cloning the Yeast *arg3* Gene. Our early attempts to clone the *arg3* gene from yeast by complementing *E. coli argF* mutants were unsuccessful. Accordingly, we resorted to a yeast transformation procedure that took advantage of the following.

We used the DNA of a pBR322/2-mμD3 plasmid carrying the yeast *ura3* locus (9) as vector and the *ura3 arg3* yeast strain 1C1064C as recipient, so as to check independently for transfer of *ura3* or *arg3*. Purified DNA from the Arg^+ strain FL100 and plasmid DNA were treated with endonuclease *Bam*HI to insert

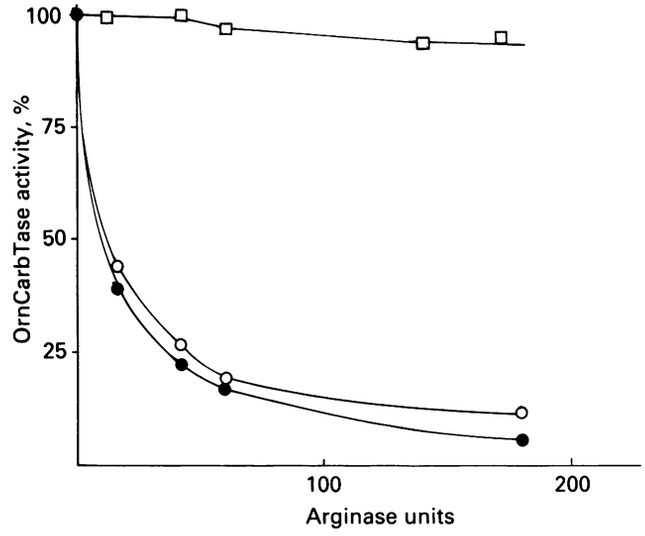

FIG. 2. Sensitivity to inhibition by arginase of OrnCarbTase in cell-free extracts of wild-type yeast (●), carriers of the cloned *arg3* gene (○), and *E. coli* (□).

fragments at the site shown on Fig. 1. This mixture was treated and used to transform strain 1C1064C to Pyr^+ or Pyr^+ Arg^+ phenotypes; ≈5 μg of DNA gave ≈1 Pyr^+Arg^+ transformant per 2000 Pyr^+ transformants.

Cells from one such Pyr^+Arg^+ colony were assayed for OrnCarbTase after growth on minimal medium and found to synthesize 30 times as much enzyme as wild-type yeast cells (Table 1). The following approaches provided proof that this yeast strain harbored plasmids carrying both the *arg3* and the *ura3* genes (further designated as pYe *ura3 arg3* plasmids).

(*i*) Total DNA extracted from the above yeast transformant was found to transform *argF* ampicillin-sensitive (amp^s) *E. coli* to amp^r. Plasmid DNA prepared from such bacteria transformed 1C1064C (*arg3 ura3*) to Pyr^+ and Pyr^+Arg^+ at similar frequencies. All Pyr^+ transformants tested were Arg^+ also.

(*ii*) The OrnCarbTase synthesized in the transformants was found to display a property (Fig. 2) that is specific for this enzyme in *S. cerevisiae* and a few other yeasts: yeast OrnCarbTase can be inhibited reversibly by arginase from the same source in the presence of arginine and ornithine (14).

(*iii*) A Southern hybridization test was performed between the hybrid plasmid as probe and the *Bam*HI-restricted DNAs listed in Fig. 3. In total yeast DNA, an 11.2-kilobase (kb) fragment was found to correspond to the chromosomal *Bam*HI insert of the pYe *ura3 arg3* plasmid; the shortest fragment (5.4 kb) is the *Bam*HI-delimited yeast *ura3* chromosomal segment (unpublished results).

By using DNA from pBR322 bearing the *E. coli argF* gene (7), it was found that yeast and *E. coli argF* DNAs do not form molecular hybrids despite the great structural similarity between the corresponding enzymes (17).

Stability and Genetic Behavior of the Plasmid Carrying the *arg3* Gene. Overnight cultures of transformants grown in the presence of arginine contain 3–5% of Arg^-Pyr^- segregants. The Arg^+Pyr^+ phenotype was found to be linked to an extrachromosomal factor. To observe enough tetrads to support this conclusion, it proved necessary to check spores on minimal medium immediately after dissection of the asci. From a cross between the pYe *ura3 arg3* carrier and *ura3 arg3* strain 1C1064C, 13 tetrads were analyzed; 7 were complete (4/0), 3 contained three spores (Arg^+Pyr^+) and 3 contained two spores (Arg^+Pyr^+, as well).

Table 1. Specific activities of arginine pathway enzymes in wild-type yeast and in plasmid-harboring derivatives

Strain	Genotype *arg*	Genotype *ura*	Plasmid present	Activity, $\mu M\ hr^{-1}\ mg^{-1}$ of protein: OrnCarbTase Medium only	OrnCarbTase Plus Arg	OrnCarbTase Plus Arg and Orn	Argininosuccinate lyase*	Acetylglutamate kinase Medium only	Acetylglutamate kinase Plus Arg
Σ1278b	+	+	—	29	4	4	3.0	0.25	0.06
1C1064C	*arg3*	*ura3*	pYe *ura3 arg3*	615	265	40	3.2	0.54	0.05
1C1064C	*arg3*	*ura3*	pYe *ura3 arg3* M1	356	416	238	1.4	0.17	0.01

Enzyme activity was assayed in minimal medium plus additions at 10 mg/ml as indicated.
* Not subject to arginine-specific repression.

Regulatory Response of the Plasmid-Borne Gene in Yeast. Cultures of the plasmid carrier growing on minimal medium synthesize 20–30 times as much enzyme as the wild-type Σ1278b (Table 1). This plasmid-dependent OrnCarbTase synthesis is repressed 2- to 3-fold by arginine alone. This reduction of specific activity is due to repression and not to plasmid loss in absence of selection for maintainance of the Arg^+ character, as shown by the following. (*i*) The differential rate of OrnCarbTase synthesis in pYe *ura3 arg3* carriers underwent an immediate reduction to one-third on addition of arginine (Fig. 4). (*ii*) Cultures were grown without uracil, the plasmid-borne *ura3* gene being required for growth. (*iii*) The level of prokaryotic β-lactamase detectable in yeast carriers of pBR322-2mμD (see below) did not significantly change after arginine was added to the medium. (*iv*) In an *argR*$^-$ (genetically derepressed) plasmid carrier, addition of arginine did not modify the differential rate of OrnCarbTase synthesis (Fig. 4). Moreover, in such an *argR*$^-$ strain, the enzyme level was 50% higher (data not shown) than in the $argR^+$ background (Table 1).

On minimal medium, *N*-α-acetylglutamate kinase was derepressed in the plasmid-bearing strain, suggesting that titration of the *arg* repressor by supernumerary *arg3* genes occurred in those conditions; kinase synthesis remained fully repressible by arginine, while full repression of OrnCarbTase synthesis required the simultaneous addition of ornithine and arginine, the most severe repression conditions currently known (18).

The ratio of OrnCarbTase activities found in Σ1278b and plasmid carriers grown under maximal repression conditions (40/4; see Table 1) gives an estimate of the number of expressed plasmid copies present in yeast carrier cells.

Response of Plasmid-Borne *arg3* to a Mutation Affecting Chromosome-to-Cytoplasm Information Transfer. In the yeast mutant *rna-1* (or *ts136*) (19), transfer of genetic information to the cytoplasm can be blocked by raising the temperature to 35°C. We have studied OrnCarbTase synthesis in an *arg3* strain harboring both the pYe *ura3 arg3* plasmid and the *ts136* mutation (1C1228C); Arg^+ *ts136* strain 7561b was taken as control. Fig. 5 shows that the shift to nonpermissive temperature had similar effects in both organisms, the coding capacity for OrnCarbTase decayed with a 5- to 6-min half-life, in contrast with a 32-min half-life for the bulk of the proteins.

A New Mutation Is Needed for Expression of Yeast *arg3* in *E. coli*. *ArgF* amp^s *E. coli* cells transformed to amp^r by DNA from cloned yeast *arg3* remain strictly Arg^- and display no measurable OrnCarbTase activity. Nevertheless, plasmid DNA prepared from these amp^{r}Arg$^-$ *E. coli* cells transform *arg3 ura3* yeast cells to prototrophy with 100% linkage between the Arg^+ and Pyr^+ phenotypes.

We obtained *E. coli* Arg^+ derivatives from amp^r Arg^- transformants (1 per 10^7 cells), while the parent *argF E. coli* mutant did not revert at all ($\leq 10^{-11}$). From two such amp^r Arg^+ derivatives (called M1 and M2), plasmid DNA was recovered and found able to transform either *argF E. coli* cells to Arg^+ amp^r or the yeast *arg3 ura3* strain to Arg^+ Pyr^+. The mutation activating *arg3* in the bacterial background of M1 and M2 is thus plasmid borne and compatible with expression of the *arg3* gene in yeast. This was confirmed by comparing OrnCarbTase activ-

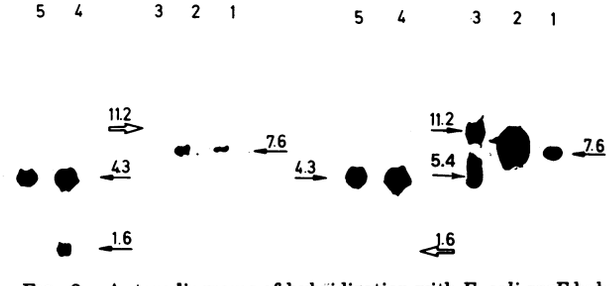

FIG. 3. Autoradiograms of hybridization with *E. coli argF* hybrid plasmid (*Left*) and yeast *ura3 arg3* hybrid plasmid (*Right*) on Southern blots bearing a series of *Bam*HI-restricted DNAs. Lanes: 1, pBR322-2-μmD3-*ura3*; 2, pBR322-2-μmD3-*ura3 arg3*; 3, total yeast DNA; 4, pBR322; 5, pBR322-*E. coli argF* (pMC20 in ref. 7). Fragment lengths are in kb. Open arrows indicate levels at which crosshybridization between *E. coli* and yeast *arg3* DNA would have been detected.

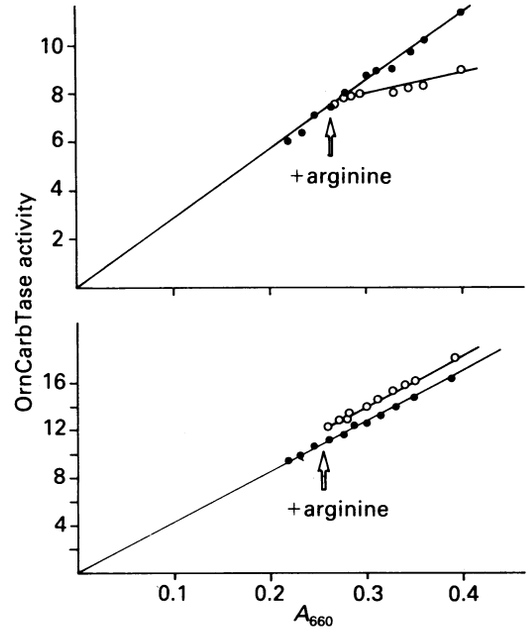

FIG. 4. Differential rate of synthesis of OrnCarbTase in *argR*$^+$ (*Upper*) and *argR*$^-$ (*Lower*) hosts of pYe *ura3 arg3*. Cultures growing in the absence of arginine (●) were divided to give two subcultures, one of which received arginine (100 μg/ml) (○) Absorption was measured in 1-cm cuvettes in a Beckman model B spectrophotometer. Activity is expressed in μmol of citrulline per hr/ml of culture.

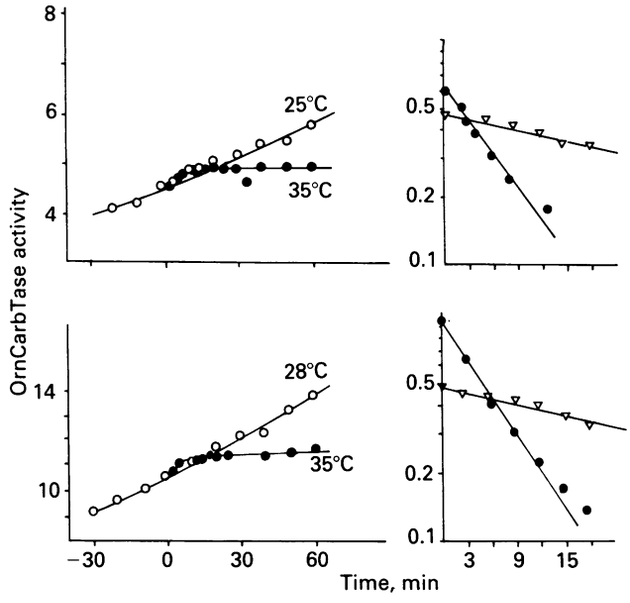

FIG. 5. Effect of a shift from 25°C to 35°C on the synthesis of OrnCarbTase in an *rna1* (*ts136*)*argR* strain (*Upper*) and in *argR* carriers of the cloned *arg3* gene (*Lower*). (*Left*) Evolution as a function of time. (*Right*) Decay of synthetic capacity for OrnCarbTase (●) and total protein (▽). $E_\infty - E_t$ was plotted versus time as in ref. 20; $E_{1/2}$ is 32 min for total protein and 5.5 min for OrnCarbTase.

ities in the original transformants and in yeast cells carrying the activated gene (Table 1; see below).

ArgF pyrF E. coli cells transformed with DNA from the M1 or M2 plasmids become Arg^+ and Pyr^+ simultaneously. The yeast *ura3* gene previously cloned on pBR322-2mμD is indeed known to be expressed in *E. coli* although it is not clear which promoter is involved (9).

The OrnCarbTase synthesized in M1 and M2 carriers is clearly the yeast enzyme, as shown by the results of the arginase inhibition test (13). This suggests that the structural part of the *arg3* gene carried by the M1 and M2 plasmids has remained intact.

E. coli M1 and M2 carriers, respectively, synthesize 7 and 14 units of OrnCarbTase specific activity. Considering a probable number of 15 plasmids per *E. coli* cell, the activity expressed by a single activated gene is 0.2–0.5% of that found in a genetically derepressed Arg^+ yeast cell (≈200). The specific activity of OrnCarbTase in *E. coli* carriers of M1 and M2 was not influenced by the addition of arginine to the growth medium.

Comparison of Structures of the pYe *ura3 arg3* and M1 Plasmids. When the M1 plasmid was transferred back to yeast, it determined an OrnCarbTase specific activity that was no longer repressible by arginine; even in the simultaneous presence of ornithine and arginine, only 35% repression was observed (Table 1).

M1 was found to differ from the parent pYe *ura3 arg3* plasmid by deletion of ≈4.0 kb of the newly cloned 11.2-kb fragment; the 4.0-kb segment is situated between *ura3* and *arg3* (Fig. 1). The deletion enters the *ura3* region by ≈100 nucleotides but does not suppress *ura3* expression; it could come close enough to the *argF* control region to account for the altered regulatory response observed in yeast. Indeed, we have been able to recover a functional *arg3* gene by cloning, from the M1 plasmid into pBR322 and pBR322-2-mμD3, the segment between the *Hin*dIII sites indicated in Fig. 1. Thus, ≈1.8 kb is left to accommodate *arg3* and at least part of the control region; the minimum of genetic information necessary for the subunit of the trimeric OrnCarbTase is ≈1.0 kb (17).

Expression of the Prokaryotic *amp* (*bla*) Gene in the Eukaryotic Background of *S. cerevisiae*. Knowing whether the *amp* (or *bla*) gene carried on pBR322 becomes expressed in yeast is not only of basic interest but also may offer a means of monitoring the number of plasmids present in a yeast cell. It was, therefore, useful to be able to detect a significant β-lactamase activity in plasmid carriers (≈6 units); this value is 1/50 that in *E. coli* cells harboring the same vector. Wild-type yeast cells exhibited no detectable activity (≤0.1 units). Assuming 10 plasmids to be present and expressed (see above), a β-lactamase specific activity of 0.6 units would represent one copy of plasmid.

This finding has been reported (6) and used to work out a colorimetric test allowing recognition of plasmid carriers on plates (21).

DISCUSSION

Cloning of *arg3* has required the yeast cell as host. Indeed, native *arg3* from *S. cerevisiae* is unable to produce OrnCarbTase in *E. coli*. Thus, the present observation provides a warning against the idea (22) that there may be no barrier to the expression of yeast genes in *E. coli*. One may wonder whether some yeast genes that have been cloned successfully in *E. coli* were not silent in this foreign background, becoming expressed only as the result of secondary mutations acquired during transformant selection or expressed from other promoters than their own. Recent experiments (unpublished data) suggest that functional expression of yeast genes in *E. coli* may be the exception rather than the rule.

It is important to know whether the signals for expression of the cloned gene have been cloned also. In the case of *arg3*, the dissection of the hybrid plasmid indicates that a complete control region is present; the structural part of *arg3* is situated at least 4.0 kb from the proximal end of the 11.2-kb cloned fragment (Fig. 1). Besides, the cloned gene displays full repressibility by a mixture of ornithine and arginine. This is of great significance for the isolation of the arginine repressor and for comparison of the DNA sequences of wild-type *arg3* with those of derivatives carrying *cis*-dominant regulatory mutations (5).

The fact that *arg3* becomes both expressed in *E. coli* and little repressible in yeast after deletion of the 4.0-kb proximal segment in the mutant plasmid M1 is a strong indication that *arg3* is polarized from left to right (Fig. 1). The opposite orientation would require the deletion to have damaged the gene for a diffusible molecule of the repressor type. However, the only known genes of that kind are unlinked to *arg3* and *ura3* (1). Moreover, this by itself would not explain why the parent plasmid of M1 does not express *arg3* in *E. coli*. The lack of expression of the native *arg3* gene in *E. coli* is probably due to defective DNA transcription. Indeed, the properties of several vectors in which *arg3* and *ura3* have been joined in different relative orientations (unpublished data) shows that the "novel-joint" brought about by the M1 deletion functions as a weak promoter for *arg3* in *E. coli*.

The deletion of plasmid M1 abolishes *arg3* repressibility in yeast. The physical mapping data (Fig. 1 and *Results*) suggest that the deletion may have altered the control region of *arg3* and be directly responsible for the observed modification in regulatory behavior.

Two observations suggest that OrnCarbTase synthesis in yeast plasmid carriers is determined by genes residing in the nucleus: (*i*) the fact that expression of the cloned gene gives a normal regulatory response and (*ii*) the fact that the *ts136* mu-

tation, known to block nucleus-to-cytoplasm information transfer at high temperatures (19), produces the same effect on OrnCarbTase synthesis in pYe *ura3 arg3* carriers and in wild-type yeast. No straightforward control of the effect of the *ts136* mutation on nonnuclear genes is currently available to prove this interpretation.

That the prokaryotic β-lactamase becomes expressed, albeit poorly, in *S. cerevisiae* may be due to some intrinsic difficulties in the process of information transfer or decoding of this bacterial gene in a eukaryotic background. It has been shown *in vitro* (23) that the extent of 5-methylguanine "cap" formation limits the translation of prokaryotic mRNA by eukaryotic ribosomes. The nascent enzyme itself is probably active without further processing although this has not been proved (for discussion, see ref. 24).

Expression of prokaryotic genes in yeast is a potentially useful phenomenon. Provided that selection could be applied, high-expression variants of prokaryotic genes could be obtained in yeast before further transfer to higher eukaryotes.

Note Added in Proof. While this manuscript was being reviewed, it came to our knowledge that expression of β-lactamase in yeast, which we had reported at a 1979 meeting of the Belgian Society for Biochemistry (6, 21), had also been observed by Hollenberg (25).

We thank H. Lemmers for his help with the Southern technique and G. O. Donavan and T. Cooper for their assistance regarding English usage. This work was supported by the FRFC–FKFO Fonds National de la Recherche Scientifique (Belgium) and the Centre National de la Recherche Scientifique (France; ATP Microbiologie 3070). M.C. acknowledges a short-term European Molecular Biology Organization Fellowship.

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