Nuclear Localization of Aspartate Transcarbamoylase in Saccharomyces cerevisiae

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ABSTRACT   The cytochemical technique using the in situ precipitation of orthophosphate ions liberated specifically by the aspartate carbamoyltransferase (ATCase) (EC 2.1.3.2) reaction indicated that in Saccharomyces cerevisiae this enzyme is confined to the nucleus. This observation is in accordance with the result reported by Bernhardt and Davis (1972), Proc. Natl. Acad. Sci. U. S. A. 69:1868–1872) on Neurospora crassa. The nuclear compartmentation was also observed in a mutant strain lacking proteinase B activity. This finding indicates that this proteinase is not involved in the nuclear accumulation of ATCase, and that the activity observed in the nucleus corresponds to the multifunctional form associated with the uracil path-specific carbamoylphosphate synthetase and sensitive to feedback inhibition by UTP. In a ura2 strain transformed by nonintegrated pFL1 plasmids bearing the URA2-ATCase activity encoding gene, the lead phosphate precipitate was observed predominantly in the cytoplasm. This finding enhances the reliability of the technique used by eliminating the possibility of an artifactual displacement of an originally cytoplasmic reaction product during the preparation of the material for electron microscopy. On the other hand, nuclei isolated under hypoosmotic conditions do not exhibit the ATCase activity that is recovered in the cytosolic fractions after differential centrifugation of the lysate in Percoll gradient. A release of the protein from the nuclei during the lysis step, consistent with its nucleoplasmic localization, is postulated.

Although most metabolic reactions are known and in a number of cases well-described in vitro, much less information is available concerning the conditions under which these reactions proceed in the cell. Subcellular localization of the enzymes, compartmentation, channeling, interactions with the cellular environment, relative concentration of enzymes and intermediary metabolites have to be considered (1–4).

Reported studies on the intracellular compartmentation of enzymes have centered on organelles such as vacuoles and mitochondria, whose role as "compartment" is ensured by their semipermeable membrane functioning as an effective permeability barrier between their interior and the cytosol (3–7). As far as the nucleus is concerned, its definition as a cellular compartment is, at present, more ambiguous. A conceptual difficulty lies in the presence of the pore complex in the nuclear envelope, rendering its analogy with a true semipermeable membrane rather questionable (8, 9). Furthermore, because of the well-known leak-out of the soluble nuclear material during the procedure of cell fractionation, very little is known about the nuclear localization of enzymes other than those firmly bound to the chromatin.

Enzymes catalyzing the reactions that liberate orthophos-phate ions can be localized through the in situ precipitation of lead phosphate, detectable by electron microscopy. In rat and mouse hepatocytes, this method was used to localize ornithine transcarbamoylase (OTCase: carbamoylphosphate : ornithine carbamoyltransferase, EC 2.1.3.1) in mitochondria (10, 11) and aspartate transcarbamoylase (ATCase: carbamoylphosphate : aspartate carbamoyltransferase, EC 2.1.3.2) in rough endoplasmic reticulum (12). In Neurospora crassa the method was used to demonstrate the mitochondrial localization of OTCase and the nuclear compartmentation of ATCase (13). In the work reported here, this method was adapted to Saccharomyces cerevisiae and the localizations of ATCase in several strains of this organism were compared.

MATERIALS AND METHODS

Medium and Cultures

The yeasts were grown at 28°C, except for the thermosensitive strain HP.232-2B (grown at 23°C), in a minimum medium (YNB) containing 0.67% of yeast nitrogen base (Difco Laboratories, Detroit, MI) and 2% of glucose supplemented as described for each strain. Cells were collected during the first half of the logarithmic growth (OD = 0.2 at 546 nm in an Eppendorf photometer) and washed with distilled water.

Strains

MD.171-1C (fur4, ura3, cpa2), derived (but not isogenic) from the wild-type FL-100, was provided by Dr. M. Denis-Duphil, this laboratory. This strain lacks the arginine path-specific carbamoylphosphate synthetase (CPSase, EC 2.7.29) coded for by the CPA2 gene, is auxotrophic for uracil (lacking OMP decarboxylase coded for by the URA3 gene), and lacks uracil permease (FUR4 gene). It was grown on YNB supplemented by 40 mg/liter of both arginine and uracil. Because of the lack of permease, this uracil concentration was limiting and ensured the derepressed biosynthesis of ATCase and uracil path-specific CPSase.

FL-100 ura2-19 and FL-100 ura2-20, both lacking the ATCase and uracil path-specific CPSase activities which are coded for by the URA2 gene. They were grown on YNB medium supplemented with 40 mg/liter of both arginine and uracil (14).

HP.232-2B (a, ade1, his7, lys2, prb1, ura1) was a kind gift from Dr. D. Wolf (15). This strain, deficient in protease B activity, was grown in YNB medium supplemented with 40 mg/liter each of adenine, histidine, and lysine, under uracil-limiting (8 mg/liter) conditions.

FL-100, cpa1, cpa2, ura2-15,30,9 (triple nonsense), transformed by nonintegrated pFL1 bearing a sal × h01 fragment of 15 kilobase pairs corresponding to the URA2 locus, was kindly sent to us by Dr. J. L. Souciet, IBMC, Strasbourg. It was grown on YNB medium without any supplementation.

Preparation of Spheroplasts

1 g of freshly harvested cells was suspended in 2 ml of 0.1 M 2-mercaptoethanol, 30 mM EDTA, and 30 mM Tris-HCl at pH 8 and incubated at 30°C for 10 min. The suspension was then diluted with ~20 ml of 1 M sorbitol, 20 mM potassium phosphate buffer pH 6.8, 0.5 mM $CaCl_2$ (buffer A), and the cells were spun down at 3,000 g for 5 min in a Sorvall RC2-B centrifuge (Du Pont Co., Wilmington, DE). The pellet was resuspended in 20 ml of buffer A and spun down as described above. 5 ml of buffer A containing 15% of glusulase (Endo Laboratories Inc., New York) were then added and the suspension was incubated at 30°C with occasional shaking for ~40–50 min. When the spheroplast formation appeared complete under microscope observation, the suspension was diluted by 20 ml of buffer A and spun down for 5 min at 3,000 g. The pellet was washed with 20 ml of buffer A, and the spheroplasts were centrifuged as described above.

Fixation of Spheroplasts

The method used was adapted from Karnovsky (16). Its advantage, for our purpose, was the strong inactivating effect of paraformaldehyde on the phosphatase activities. The pellet of protoplasts was suspended in 2 ml of 1 M sorbitol, 20 mM HEPES/Tris buffer at pH 7.8. 2 ml of a solution containing 1 M saccharose, 0.1 M cacodylate buffer, pH 7.2, 2% glutaraldehyde, and 2% of freshly dissolved paraformaldehyde were added dropwise at 0°C with gentle shaking. After 2 h at 0°C, the fixed spheroplasts were spun down at 3,000 g for 5 min and then washed three times with 10 ml of 0.6 M sorbitol, 0.1 M HEPES/Tris, pH 7.8.

Formation of the Lead Phosphate Precipitate through ATCase Activity and Preparation of Samples for Electron Microscopy

About 0.1 g of fixed pelleted spheroplasts was suspended in 5 ml of the incubation medium containing 0.6 M sorbitol, 6 mM Pb acetate, 50 mM Tris/acetate buffer at pH 7.5, 50 mM L-aspartate, 10 mM Li_2-carbamoylphosphate and incubated at 30°C for 15–30 min. Protoplasts were pelleted as described above and washed three times with 0.05 M HEPES/Tris buffer at pH 7.8.

The cells were postfixed in 1% osmium tetroxide at 4°C overnight, rinsed with distilled water, dehydrated in a graded series of acetone solutions, and embedded in Araldite. Blocks were sectioned on an LKB ultratome and examined with an Itachi model HU11B electron microscope without any staining.

Isolation of Nuclei and Purification on Percoll Gradient

Protoplasts, prepared from 2 g of cells as described above, were suspended in 6 ml of 18% Ficoll containing 20 mM potassium phosphate buffer, pH 6.5, 0.5 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was homogenized and diluted twice with buffer A as described by Lohr and Ide (17). 12 ml of the lysate were loaded on 34 ml of a preformed 0–30% Percoll gradient containing 1 M sorbitol, 1 mM PMSF, 0.5 mM $CaCl_2$, pH 6.5, and spun for 15 min at 9,000 g in a HS4 swinging bucket rotor. The gradient was divided into 1-ml fractions with a disposable pipette.

RESULTS

Cytochemical Evidence for the Nuclear Localization of ATCase Activity in Spheroplasts of S. cerevisiae

The strain used for this study was an uracil auxotroph, uracil permease-defective mutant MD.171-1C. The presence of both uracil and permease mutations ensures a derepression of the ATCase biosynthesis which is ~10-fold that of the FL-100 wild type. It was verified by the in vitro test using ^{14}C-aspartate (18) that the spheroplasts fixed as described under Materials and Methods retain the ATCase activity which proceeds with its initial velocity for at least 1 h. Upon incubation of these fixed spheroplasts in the presence of substrates and lead acetate, the picture observed under the electron microscope was as shown in Fig. 1a. An abundant precipitate of lead phosphate was visible, localized essentially inside the nuclei. Very often the precipitate appeared concentrated at one pole of the nucleus, which could correspond to the nucleolus (Fig. 1b). The specificity of the reaction observed was controlled using two mutant strains devoid of ATCase activity: FL-100 ura2-19 and FL-100 ura2-20. Both gave pictures such as that in Fig. 1c, where the nuclei did not contain any visible precipitate. This specificity was further demonstrated by the absence of any intracellular precipitate in spheroplasts incubated in a mixture from which the substrate aspartate was omitted (Fig. 1d). The slight contaminating precipitate visible inside the vacuoles and on the periplasmic membrane is most probably due to residual phosphatase activities using carbamyl phosphate as substrate. This supposition was supported by the observation that the precipitates that were relatively important in cells fixed by the glutaraldehyde alone were strongly reduced or completely absent when paraformaldehyde, known as a powerful inhibitor of phosphatase activities (16), was added to the fixative mixture. However, a nonenzymatic origin of the extracellular precipitate, as postulated by Bernhardt and Davis (13), cannot be excluded.

Nuclear Localization of ATCase in a Strain Lacking the Proteinase B Activity

The process leading to an intranuclear accumulation of a protein supposed to be synthesized on cytoplasmic ribosomes is at this time completely unknown and the possibility of its investigation very limited.

By analogy with other membrane transport processes, the involvement of a proteolytic step in the nuclear compartmentation of ATCase was considered. We have examined here the possibility of participation of the proteinase B, which was suggested by recent work from this laboratory (18), showing that in S. cerevisiae the heavy bifunctional protein bearing both the uracil path-specific CPSase and ATCase activities can be cleaved, by the action of proteinase B, into two independent molecular species each catalyzing one of the two reactions. In strain HP.232-2B, lacking the proteinase B activity, only the heavy bifunctional form of ATCase is detectable. It was interesting to check whether this proteinase B–defective strain, unable to cleave the heavy form of ATCase, can accumulate this protein inside the nucleus. Fig. 2 shows that this is actually the case. It can therefore be concluded that the pyrimidine path-specific CPSase accompanies the ATCase in the nucleus and that the proteinase B activity is not necessary for the nuclear accumulation of this bifunctional protein.
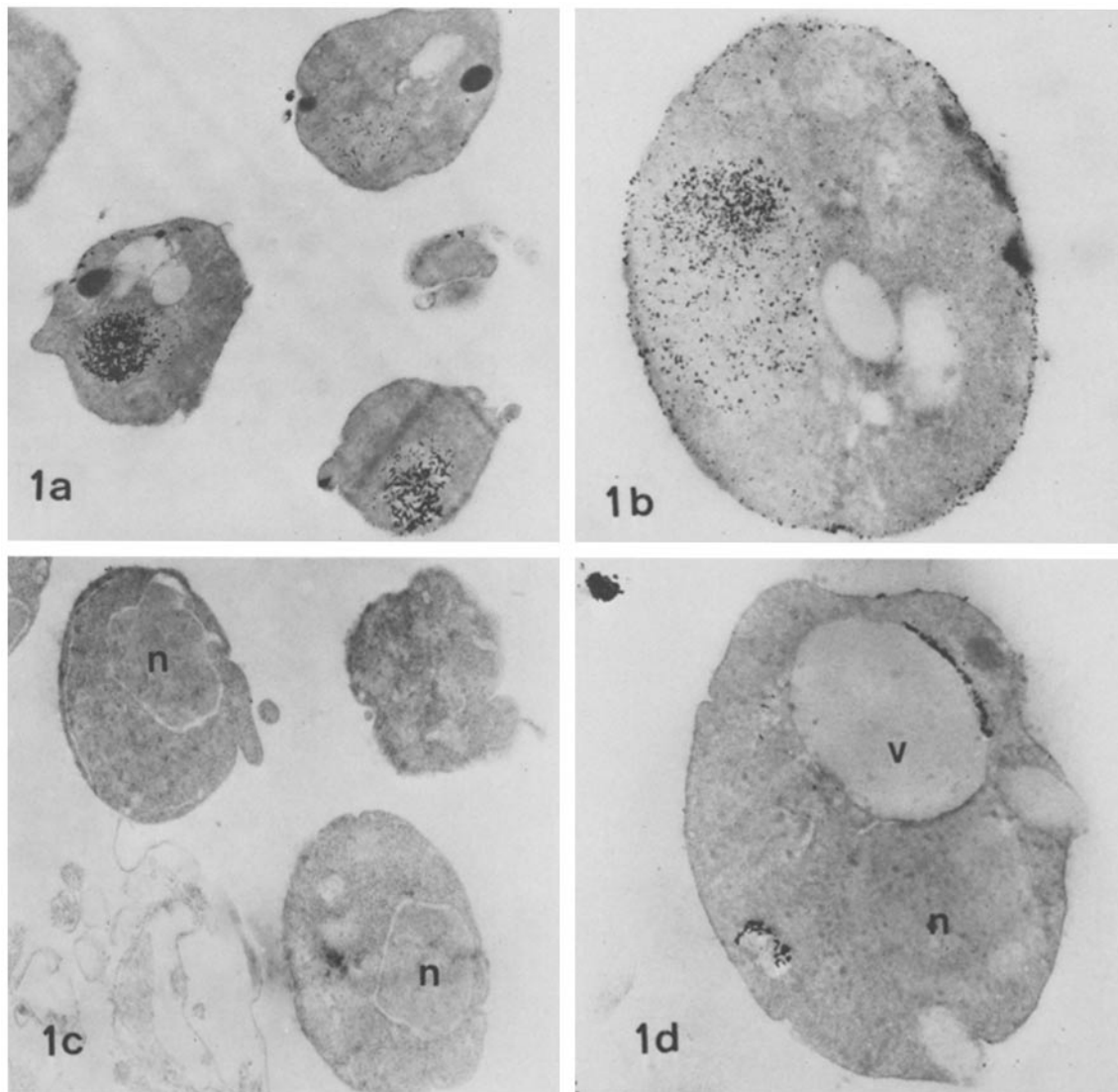
FIGURE 1 Localization of the lead phosphate precipitate appearing in the fixed protoplasts during the incubation for ATCase activity in the presence of lead acetate. (a and b) Strain MD.171-1C; (c) strain FL-100 ura2-19, devoid of ATCase activity; (d) strain MD.171-1C incubated in the absence of the substrate L-aspartate. n, Nucleus; v, vacuole. (a and c), × 8,000; (b and d) × 14,000.

Cytoplasmic Localization of ATCase in a ura2 Strain Transformed by pFL1 Plasmids

The transformed strain used here contains multiple extra-chromosomal copies of the pFL1 plasmid bearing the URA2 gene. It produces large amounts of ATCase, the specific activity of which is 5–10 times that of the MD.171-1C strain. It was interesting to verify whether this cell is still able to concentrate such an overproduced amount of ATCase inside the nucleus. As shown in Fig. 3a and b, this is not the case, most of the very dense lead phosphate precipitate observed being located in the cytoplasm. The intranuclear concentration of the precipitate does not exceed the cytoplasmic concentration in this type of cell (Fig. 3b). Here, again, the reaction is dependent upon the presence of aspartate (Fig. 3c).

The possible interpretations of this finding are considered in the Discussion. Its immediate interest is in providing a control for the cytochemical technique used here, suspected to be able to produce artifactual results. The possibility of artifacts, reviewed by A. Worbrodt (19), could arise from the displacement of an originally cytoplasmic reaction product that would mi-

grate and adsorb onto the nuclear structures during the dehydration step. The cytoplasmic localization of the precipitate observed in the transformed strain invalidates this possibility.

Subcellular Fractionation and Isolation of Nuclei

Obviously, the best support for the nuclear localization of ATCase in *S. cerevisiae* would be the recovery of its cellular activity in isolated nuclei. Despite considerable effort, we have been unable to isolate such nuclei. All attempts made to adapt to our purposes the conditions of preparation of yeast nuclei, i.e., the formation and lysis of protoplasts as well as the techniques of purification of isolated nuclei (20), did not increase the recovery of nuclear ATCase activity. Fig. 4 reports the profile of ATCase in the Percoll gradient, which allows a very good separation of organelles, chromatin liberated from lysed nuclei, and unbroken cells or protoplasts. The profile of ATCase activity parallels that of alcohol dehydrogenase (ADH), used as cytoplasmic marker. 93% of the recovered ATCase activity and 87% of ADH activity remain on the top of the Percoll gradient, i.e., they are not bound to the nuclei
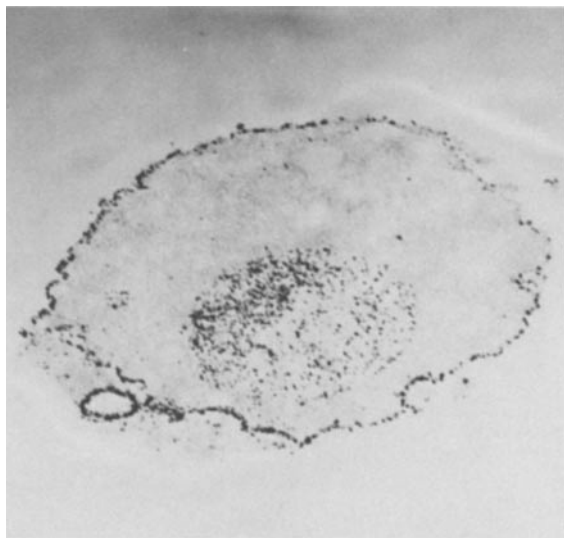
FIGURE 2   Localization of the lead phosphate precipitate in the fixed protoplasts of strain HP.232-2B devoid of proteinase B activity. The incubation was performed in the complete medium containing the ATCase substrates and lead acetate. × 14,000.

that sediment in the gradient between fractions 10 and 13. The peak of the DNA-dependent RNA polymerase activity, chosen as nuclear marker, coincides with the microscopically observed band of nuclei and represents 44% of the recovered activity. Even for this enzyme, 35% of the recovered activity remains on the top of the gradient.

DISCUSSION

The fact that we find the ATCase of *S. cerevisiae* to be located in the nucleus supports the observation of Bernhardt and Davis concerning *N. crassa* (13). The transformed strain used here represents an important control eliminating the possibility of a technical artifact, thus providing supplementary evidence for the authenticity of this cellular distribution.

The absence of a significant peak of ATCase activity in the fractions of the Percoll gradient that contain the isolated nuclei is not necessarily in contradiction with the in vivo cytochemical finding. This result could simply reflect a nucleoplasmic local-ization of this protein involving only weak interactions with the nuclear structures. The release of enzymes from the nucleo-plasm during the isolation of nuclei in isoosmotic aqueous media was frequently observed. For instance, the DNA polym-erase from several types of mammalian cells was completely washed out from nuclei isolated in aqueous media, and the demonstration of its nuclear localization required the use of nonaqueous conditions (22). Similarly, the DNA-dependent RNA polymerase III as well as the poly(A) polymerase from rat liver nuclei were virtually absent from "isotonic nuclei" but
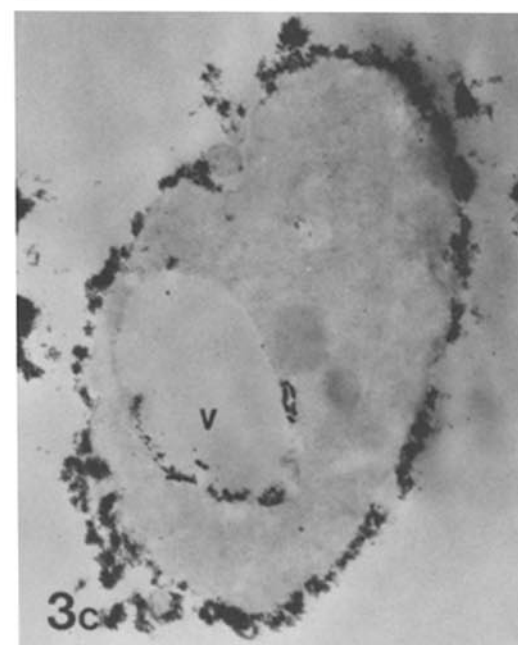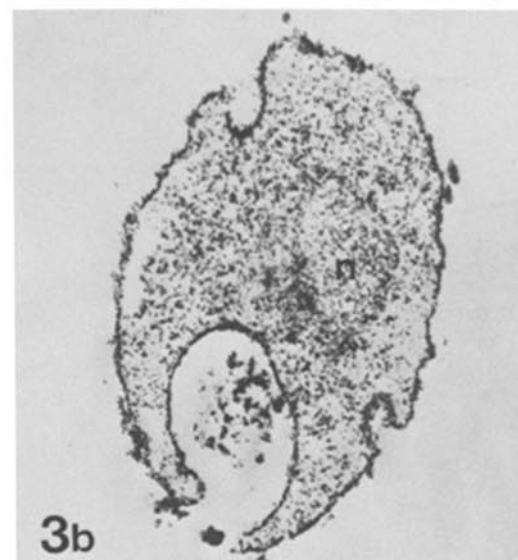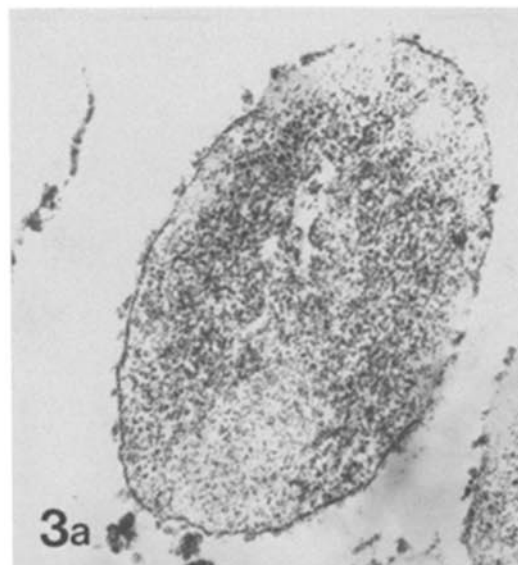

FIGURE 3   Localization of the lead phosphate precipitate in the fixed protoplasts of strain FL-100 cpa1, cpa2, ura2 triple nonsense transformed by the plasmid pFL1 bearing sal × h01. (a and b) Protoplasts incubated in the complete medium containing the ATCase substrates and lead acetate, (c) same conditions minus aspartate. n, Nucleus; v, vacuole. × 14,000.
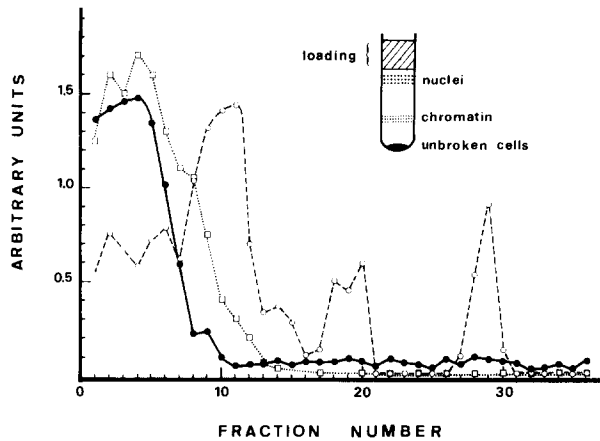
FIGURE 4 Distribution of ATCase activity after cell fractionation. Lysate from strain MD.171-1C was layered on a preformed 0-30% Percoll gradient and centrifuged as described in Materials and Methods. The resulting gradient is diagramed in the *inset*. (●) ATCase activity (1 U = 100 nmol of carbamyl aspartate formed/h). (○) The nuclear marker DNA-dependent RNA polymerase activity determined as described by Wintersberger (21) (1 U = 1 nmol of UTP incorporated/h). (□) The cytoplasmic marker alcohol dehydrogenase (1 U = 10 nmol NADH₂ formed/h).

present in nuclei isolated in hyperosmotic sucrose media (23, 24).

The significance of the ATCase compartmentation in *S. cerevisiae* cannot be accounted for in terms of channeling of the two arginine and uracil path–specific carbamoylphosphate pools as was suggested for *N. crassa* (13). Whereas in *N. crassa* the specificity of the two carbamoylphosphate pools has been well-demonstrated (25), this is not the case in *S. cerevisiae*, where the respective loss of the two CPSase activities does not lead to auxotrophy for arginine or for pyrimidine (26). The arginine path–specific CPSase of *N. crassa* was found, both by the cytochemical technique and by differential centrifugation, to be localized in mitochondria, whereas in *S. cerevisiae* this enzyme was found, by differential centrifugation studies, in the soluble, cytoplasmic fraction (5).

Thus, the actual physiological significance of the observed nuclear compartmentation of ATCase in *S. cerevisiae* is unclear. As a nuclear localization of UTP kinase has been already reported in mammalian cells and as the enzyme complex concerned here is regulated by feedback inhibition by UTP, one would be tempted to speculate that the reason for its nuclear compartmentation would be the existence of a nuclear UTP pool, allowing a more efficient regulation of these enzymatic activities. In fact, a small nuclear UTP pool, independent of the cytoplasmic one, has been already suggested by pulse-chase experiments in Novikoff rat hepatoma cells (27).

Several hypotheses can be considered in explanation of the cellular distribution of ATCase in the ura2 strain transformed by pFL1 plasmids. The limiting factor in the nuclear compartmentation of ATCase might be either the intranuclear solubility of the protein or the activity of a putative transport system

in the nuclear membrane. Another possibility is that the protein encoded by the plasmid might differ in some aspects from that coded for by the chromosomal gene and would not be recognized by the transport system. Such a protein could not be accumulated inside the nucleus over the concentration gradient but could still penetrate through the pore system of the nuclear membrane by free diffusion.

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