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# Regulation of Cytoplasmic and Mitochondrial Leucyl-Transfer Ribonucleic Acid Synthetases in *Neurospora crassa*

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The production of cytoplasmic leucyl-transfer ribonucleic acid synthetase activity was found to be reciprocally proportional to that of the corresponding mitochondrial enzyme during logarithmic growth of strains of *Neurospora crassa* with normal mitochondria. In the presence of *cni-3* mutant mitochondria, production of mitochondrial enzyme activity was greatly increased whereas cytoplasmic enzyme production remained constant. The effect of *cni-3* on the yield of the two enzyme activities indicated that the regulatory mechanism involved is a complicated one that cannot be accounted for by the relatively simple transcription competition model proposed previously.

The structure of most mitochondrial proteins is specified by nuclear genes and translated on cytoplasmic ribosomes. In many instances these proteins duplicate functions of enzymes that are structurally different and localize elsewhere in the cell. Following Barath and Küntzel's (1, 2) suggestion that mitochondria produce a regulatory element which governs negatively the transcription rate in the nucleus of mitochondrial structural genes, we showed that the level of the mitochondrial leucyl-tRNA synthetase (L-leucine:tRNA$^{Leu}$ ligase [AMP-forming], EC 6.1.1.4) activity of *Neurospora crassa* was markedly elevated during chloramphenicol inhibition of mitochondrial protein synthesis. The increase in mitochondrial enzyme, however, was accompanied by a decrease in cytoplasmic enzyme activity (3). Subsequently, we found that the two structural genes are closely linked and demonstrated that the enzymes are structurally as well as immunologically different (4). To account for these observations as well as the pleiotropic phenotype of a *leu-5* mutant, 45208t, which produces only a trace amount of the mitochondrial enzyme while producing a cytoplasmic enzyme with a lower than normal affinity for leucine, we proposed (4) that (i) the two genes are physically adjacent, (ii) each gene has its own promoter, (iii) there is a binding site for the regulatory element of mitochondrial origin which affects the transcription rate from the "mitochondrial" promoter and, to accommodate the phenotype of the *leu-5* mutant and its revertants, (iv) the direction of transcription is from the cytoplasmic to mitochondrial region and (v) the function of the mitochondrial gene promoter depends upon some structural feature near the 5′ end of the "cytoplasmic" gene. Accordingly, reciprocal control would result from competition between transcription initiation of the mitochondrial message and completion of transcription of the message of the cytoplasmic gene. We show here that, in conformance to the transcription competition model, the activities of the two enzymes vary reciprocally during growth in the absence of inhibitors in strains containing normal mitochondria. However, contrary to expectation, over the range of considerably higher levels of production of mitochondrial enzyme activity characteristic of strains containing *cni-3* mutant mitochondria, the level of cytoplasmic enzyme activity remains nearly constant.

The *cni-3* mutation used in these studies was isolated by Rosenberg et al. (8) as one that leads to the partial constitutive production of the mitochondrial cyanide-insensitive respiratory system. The mutation was shown to be maternally inherited and to be recessive in heteromitochondrial strains, as would be expected if some repressor-like element were involved in regulating the nuclear structural genes for the cyanide-insensitive respiratory system (5). On the assumption that the repressor affected by *cni-3* was the same as that proposed by Barath and Küntzel (1, 2), we measured mitochondrial and cytoplasmic leucyl-tRNA synthetase production by *cni-3* and found that the level of mitochondrial enzyme activity was high, whereas the specific activity of the cytoplasmic enzyme was significantly less than usually found in extracts of our standard *cni-3*$^{+}$ strains (4).

The behavior of the *cni-3* mutant offered the opportunity to test our transcription competition model which required, in its simplest form, a decrease in cytoplasmic enzyme production proportional to an increase in the production of the mitochondrial enzyme over a wide range of enzyme production levels. To collect enough

reliable data, we developed a simple rapid procedure for separating the two activities which allowed direct quantification. The chromatographic procedure used and the characteristic distribution of the two enzyme activities in extracts of *cni-3*$^+$ and *cni-3* are illustrated in Fig. 1. The patterns presented are those obtained from extracts of *inl cni-3*$^+$ strains, chosen for comparison from among those used in the determinations plotted in Fig. 2 because they contained nearly the same amount of cytoplasmic leucyl-tRNA synthetase activity. The chromatographic patterns illustrate clearly that the specific activity of the mitochondrial leucyl-tRNA synthetase is much higher in *cni-3* than *cni-3*$^+$.

Figure 2A is a plot of the specific activities of the two enzymes found in extracts obtained from a variety of different *cni-3*$^+$ strains during logarithmic growth. These strains vary in growth rate, but no systematic variation in the production of the two enzymes has been observed to be dependent upon growth rate or individual supplements, e.g., inositol or leucine. The data clearly demonstrate the reciprocal relationship between the specific activities of the two enzymes during unperturbed growth that we observed previously to occur during chloramphenicol inhibition. The passive nature of the determinations eliminates one of the serious concerns that we had about our previous data, namely that chloramphenicol, in addition to inhibiting

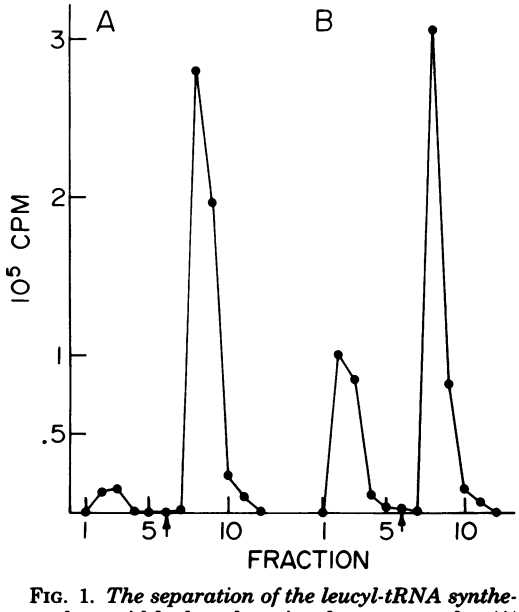


FIG. 1. *The separation of the leucyl-tRNA synthetases by rapid hydroxylapatite chromatography. (A) Chromatographic pattern of inl::cni-3*$^+$*; (B) inl::cni-3. Short columns (0.9 × 12.5 cm) of hydroxylapatite equilibrated with 0.05 M $KPO_4$ (pH 7.7) were loaded with 10 mg of protein which was desalted by passage through Sephadex G25 equilibrated against the same buffer. The mitochondrial enzyme was eluted quantitatively in the first six 5-ml fractions whereupon 0.15 M $KPO_4$ buffer was applied to the column (↑) for elution of the cytoplasmic enzyme. Strains, growth, preparation of extracts, and assay procedure were as described in Fig. 2.*

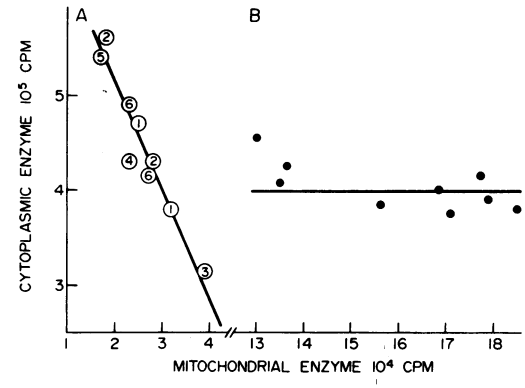


FIG. 2. *Cytoplasmic leucyl-tRNA synthetase activity as a function of mitochondrial leucyl-tRNA synthetase activity in extracts of cni-3*$^+$ *and a cni-3 mutant strain. Each point represents the two activities found in extracts chromatographed as in Fig. 1 and therefore represents the specific activity found in the 10 mg of protein applied to the column. The data in A were obtained with several different cni-3*$^+$ *strains (noted by number) chosen because of slightly different growth rates. In the case of the leucine auxotrophs, different amounts of leucine were supplied during growth. The leucine auxotrophs used were as follows: 1, R59 (leu-4); 2, R156 (leu-3). The fluoroleucine-resistant mutants (leucine permeability defective) used were as follows: 3, flr 16; 4, flr 101; 5, flr 185; and 6, inl (89601, inositol requiring). The cni-3 strain used in B was cni-3-2-28A, an inl::cni-3 derivative of the original mutant crossed into our standard genetic background by two successive crosses in which cni-3 served as the maternal parent. The growth regimens employed, which included varying concentrations of leucine and harvesting at different times during logarithmic growth, were without any systematic effect on enzyme production levels. The preparation of the 0 to 75% ammonium sulfate precipitates and assay procedure were essentially as previously described (6, 9) except that the 0.05 M $KPO_4$ fractions were diluted with water by a factor of two and the 0.15 M $KPO_4$ fractions by a factor of five to eliminate the differential inhibition of the two activities by $KPO_4$ that has been noted previously (9). The rates of reaction with diluted fractions under the conditions employed was found to be linear for more than 10 min. For convenience the reaction was allowed to proceed for 6 min. Each fraction was assayed individually, and the summed activity was confirmed by measurement in duplicate of the pooled activity from each of the peaks.*

mitochondrial protein synthesis, might set in motion some degradative reactions which could affect differentially the endogenous levels of the two enzymes. We reported that autolysis was quite severe under combined cyclohexamide and chloramphenicol inhibition (3), and even though no overt autolysis was observed during inhibition by either of the antibiotics alone, considerable endogenous degradation could have gone unnoticed.

Although the activity of the two enzymes appears to be reciprocally proportional over a twofold range in *cni-3*$^+$ strains, the situation is quite different in the *cni-3* mutant strain. The data of Fig. 2B show that the specific activity of the cytoplasmic enzyme produced by *cni-3* remains reasonably constant at a level close to 0.75 of the maximum produced by *cni-3*$^+$, whereas the activity of the mitochondrial enzyme varies between three- and fivefold higher than the maximum observed in *cni-3*$^+$ strains. These observations conflict directly with the expectation from the transcription competition model. Furthermore, extrapolation of the data obtained with *cni-3*$^+$ would lead to an expectation of little, if any, cytoplasmic enzyme in extracts of *cni-3* at high levels of mitochondrial enzyme production.

Clearly the regulatory interactions involved are more complicated than previously supposed, for it appears, overtly at least, that the *cni-3* mutation not only increases the yield of mitochondrial enzyme activity but also uncouples its production from that of the cytoplasmic enzyme. To check this, we replaced, by appropriate crosses, the normal mitochondria of the *leu-5* mutant 45208t with *cni-3* mitochondria to determine whether *cni-3* mitochondria could reverse the mitochondrial enzyme deficiency. As stated above, the *leu-5* mutation appears to be in the coding sequence for the cytoplasmic enzyme (4). The *leu-5*::*cni-3* strains were found to produce no more than the trace amounts of the mitochondrial enzyme characteristic of *leu-5* mutants with normal mitochondria. It seems unlikely, then, that *cni-3* uncouples transcription of the two genes.

The proximity of the two genes as well as the enzymatic phenotype of *leu-5* point to interdependent transcription of the two coding sequences, and the reciprocal relationship between the specific activities of the two enzymes in *cni-3*$^+$ strains suggests this as well. The behavior of *cni-3*, however, is inconsistent with simple transcription regulation models and raises the question of whether, in addition to transcriptional regulation, some post-transcriptional regulatory steps may be involved at the level of either message processing, translation efficiency, or post-translational modification. Indeed, the mitochondrial enzyme deficiency of *leu-5* might result from a sequence change in the primary transcript which prevents proper post-transcriptional processing, perhaps, for example, the excision of an intervening sequence (7). There is, as yet, no indication of the involvement of post-translational modification steps in the production of the leucyl-tRNA synthetases. The synthesis of both enzymes is completely inhibited by cycloheximide, and no increase in mitochondrial enzyme activity has been observed in the presence of chloramphenicol unless cytoplasmic protein synthesis is allowed to proceed (3). We have reported, however, that the production of mitochondrial phenylalanyl-tRNA synthetase activity involves some post-translational modification step which proceeds in the absence of cytoplasmic and mitochondrial protein synthesis (3). The levels of mitochondrial phenylalanyl-tRNA synthetase activity have also been found to be higher in strains containing *cni-3* mitochondria rather than *cni-3*$^+$ (data not presented), suggesting that the mutation in the mitochondrial genome, like chloramphenicol, might affect several different processes in the eventual production of mitochondrial proteins from information in the nucleus. The analysis of the specific regulatory interactions involved in the production of the two leucyl-tRNA synthetases now awaits isolation and characterization of the primary transcript(s) and messages derived therefrom.

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