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Mitochondrially-imported cytoplasmic tRNA^{Lys}(CUU) of *Saccharomyces cerevisiae*: *in vivo* and *in vitro* targetting systems

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ABSTRACT

The cytoplasmic tRNA^{Lys}(CUU) (tRNA$_1^{Lys}$) is the single yeast tRNA species to be traffiked from the cytoplasm into the mitochondrial compartment of the cell. To study mechanisms of this targetting we worked out two test systems. The *in vivo* system based on the electroporation of intact yeast cells was used to introduce labelled tRNAs into the cytoplasm. All tRNA species tested were effectively introduced into the cytoplasm, but only the cytoplasmic tRNA$_1^{Lys}$ was found in the mitochondrial compartment within 1–2 hours after the electroporation procedure. The *in vitro* system permits specific transfer of the tRNA$_1^{Lys}$ into isolated mitochondria. Contrary to the known systems for protein transport into isolated mitochondria, mitochondrial import of tRNA$_1^{Lys}$ *in vitro* requires the presence of soluble cellular proteins in the reaction mixture. The translocation proved to be ATP-dependent and to require the presence of an ATP-generation system in the reaction. Preincubation of the tRNA with the total cellular extract of the cell markedly increases the rate of the translocation. Two protein fractions are necessary to direct the import *in vitro*. The first one has high heparin-binding affinity, while the other protein fraction is not retained by heparin-Sepharose.

INTRODUCTION

One of the characteristic features of the eukaryotic cell is the presence of distinct genomes in the nucleus and organelles. These genomes are in continuous interaction. A great deal of data exists on protein transfer from the cytoplasm into chloroplast and mitochondrial compartments of the cell (see (1–2) for review). Several receptors and signal excising enzymes were characterized biochemically or on the level of gene and mRNA sequences (3–8). The process of small RNA molecules traffiking into the mitochondrial compartment of the cell is, on the contrary, not understood at all. This transfer, however, seems to be of high interest as a model of intercompartmental exchange with RNA molecules and also as a basis for novel vector systems development. Up to now, a nuclear-encoded RNA (MRP RNA) which takes part in mitochondrial RNA processing in mammals (9–10) and numerous cytoplasmic tRNAs in plants (11), protozoans (12–16) and yeast (17,18) were found to be present in mitochondria. All the previous data on the intercompartmental targetting of small RNAs were based on the results of hybridization and sequence analyses. In the case of the yeast *Saccharomyces cerevisiae* subcellular and mitochondrial fractionations were done to prove the presence of a cytoplasmic tRNA in the inner compartment of mitochondria (18). To study the mechanisms of RNA mitochondrial import, *in vivo* and *in vitro* test systems have to be developed. Here we present the attempt to work out such models using the yeast *S. cerevisiae*. This choice is explained by the simplicity of manipulating yeast cells and by the fact that the single cytoplasmic tRNA$_1^{Lys}$ was previously reported to be present in mitochondria (17–18). This very tRNA represents one of the puzzles of yeast molecular biology, since it is of 'cytoplasmic type' (the same as its counterpart found in the cytoplasm), while the 'mitochondrial type' tRNA^{Lys} is encoded by the mitochondrial genome. The hypothesis suggesting that it takes part in mitochondrial RNA splicing (19) remains without direct demonstration. So, this tRNA seems to be of a great interest not only as a model of intercompartmental targetting, but also from the point of view of yeast biology. On the basis of the electroporation procedure and of the system of protein transfer into isolated mitochondria we have elaborated two test systems of specific mitochondrial targetting of the exogenous cytoplasmic tRNA$_1^{Lys}$. These systems can be further used to study the mechanisms of the traffiking pathway and to clarify possible role(s) of the imported cytoplasmic tRNA in mitochondria.

MATERIALS AND METHODS

Yeast strains, cultivation and isolation of mitochondria

Most of the work was done on the wild type yeast *Saccharomyces cerevisiae* strain S288C and the *rho*° strain JC8/55 (20). Yeast cells were grown on the standard rich medium YPG (1% yeast extract, 2% bacto-peptone, 3% glycerol; except for the *rho*°-cells, for which 2% glucose was used instead of glycerol). The wild type cells were grown to the density of 0.8–1.0 A550, pelleted, washed several times with water and disrupted by glass beads in 0.44M sorbitol, 10 mM Tris-HCl (pH8.0), 1 mM EDTA (STE-buffer). After removing the cellular debris by low speed centrifugation (2000×g for 5 min.) the mitochondria were purified by two subsequent sucrose gradient (0.6–2.1M) centrifugations, diluted to 300 mM of sucrose with TE-buffer (10mM Tris-HCl, pH8.0, 1mM EDTA) and repelleted. The

isolated organelles were then treated with RNase (18) and repurified. For the *in vitro* targetting experiments the RNase treatment was done only after the reaction of transfer.

Isolation and characterization of tRNAs

Cytoplasmic tRNA was extracted by water-saturated phenol from the *rho*°-cells suspended in water. Mitochondrial tRNAs were extracted by the same procedure from purified organelles suspended in STE buffer. High molecular weight RNAs were removed by two steps of LiCl (5M) precipitation (−20°C, 30 min.) and the tRNAs were finally purified by DEAE-cellulose chromatography. The resulting tRNA was fractionated by the preparative 2D gel electrophoresis (21) with the following modifications: the 1st dimention was 7M urea, 1×TBE-buffer (22), 15% polyacrylamide (acrylamide/methylen-bis-acrylamide=40/1.5); the 2nd—4M urea, 0.5×TBE-buffer, 20% polyacrylamide.

The lysine tRNAs were specifically labelled in the total cytoplasmic and mitochondrial tRNA (23). This method includes aminoacylation with lysine, followed by periodate oxydation, alkaline deacylation and 3′-labelling with T4 RNA ligase and pCp. Only the lysine tRNAs become thus ^{32}P-labelled. The labelled tRNAs were then used as standards for preparative electrophoresis of the unlabelled cytoplasmic tRNA enriched with the lysine tRNAs by the chromatography on monoQ. The bands corresponding to the unlabelled $tRNA_1^{Lys}$ (coinciding in the labelled mitochondrial and cytoplasmic preparations) were cut out the gel and eluted (the difference of the pCp-labelled and unlabelled tRNA species by one nucleotide was taken into account). Individual tRNAs obtained were labelled with T4 polynucleotide kinase (22), purified by preparative electrophoresis and used for targetting experiments. Their identity was verified the by partial sequencing using the Pharmacia RNA-sequencing kit (ribonuclease digestion method). The crude preparation of cellular and mitochondrial amino acyl tRNA-synthetases and aminoacylation were done as described in (24−25).

Electroporation of intact yeast cells

The wild type cells were grown overnight in 100 ml of YPG medium to a density of 0.8−1.2 A550, pelleted by centrifugation, washed three times with deionized water and suspended in 1 ml of deionized water (pretreated with diethylpyrocarbonate (Serva) and sterilized). 10^6 Cerenkov cpm of labelled individual tRNAs (0.2−0.5 μg) or 10^7 Cerenkov cpm per 10−20 μg of total bacterial or yeast tRNA and 0.8 ml of 60% PEG4000 (Merk) were added and gently mixed. The suspension was incubated at room temperature for 15 min. and subjected to 5−10 subsequent electric 10 msec pulses of 2.5 kV/sm in a self-made apparatus with 20/20/3 mm chambers. The electroporated yeast cells were removed from the chambers, washed twice with sterile water and suspended in 100 ml of fresh YPG medium. After incubation at 30°C with slow shaking for appropriate time the cells were pelleted, washed with water and treated with pancreatic RNase (250 μg/ml, room temperature, 5 min). The cells were then washed twice with water, suspended in STE, broken and fractionated as described above.

Extracted total cellular and mitochondrial tRNA were fractionated by denaturing polyacrylamide gel electrophoresis, corresponding to the 1st-dimension run as described in 'Materials and methods', the gel was dryed and exposed to the HS11 (Assofoto) X-ray film for appropriate time at −70°C with intensifying screens.

Transport of tRNAs into isolated mitochondria

Mitochondria freshly isolated from 1 l. of culture (0.8−1.0 A550) were suspended in 3 ml of 0.44M sorbitol, 20mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mM DTT, 2 mM PMSF, 1 mM ATP, 1 mM phosphoenol pyruvate and 4 units of pyruvate kinase (Serva). After 5 min. of preincubation 300 μl aliquots of the suspension were mixed with labelled tRNAs ($0.1-0.2\times10^6$ cpm, 20−100 ng in a volume of 10 μl of water) and 50 μl of the appropriate protein fraction (1 mg/ml in 20 mM HEPES (pH 7.4), 2 mM PMSF, 1 mM DTT, 50% glycerol) were added. After incubation for appropriate time at 30°C the pancreatic RNase (Serva) and phosphodiesterase (Pharmacia) were added to 200 and 100 mg/ml, respectively. The RNase treatment was carried out at 4°C for 10 min and mitochondria were layered onto the 0.4−0.6−2.0M sucrose step gradient (made in 10 mM Tris-HCl (pH 8.0), 2mM EDTA) and centrifuged at 40000×g for 30 min. The band of mitochondria was recovered and the organelles were resuspended in STE-buffer and pelleted. tRNA was extracted and electrophoresed as described above.

The initial protein preparation represents the extract of wild-type cells: an overnight culture was centrifuged, washed twice with ice cold water, suspended in 20 mM potassium phosphate (pH 6.8), 100 mM KCl,1 mM DTT, 2 mM PMSF, 10% glycerol and broken with glass beads. The cellular debris was removed by the low speed centrifugation (2000×g). The supernatant was centrifuged at 100000×g for 30 min and subjected to gel-filtration through Sephadex G25 (coarse) (Pharmacia) equilibrated with 20 mM HEPES (pH 7.4), 2mM PMSF, 1mM DTT and 50% glycerol. The fractions containing 1 mg/ml of protein or more were pooled and stored at −20°C. For fractionation the S-100 supernatant was dialyzed against 20mM HEPES, 2mM PMSF, 1mM DTT, 10mM NaCl, 10% glycerol and applied to a heparin-Sepharose column (LKB) equilibrated with the same buffer. Proteins were eluted stepwise by 100, 200 and 500mM NaCl in the same buffer. The pooled fractions were dialyzed against 20mM HEPES, 2mM PMSF, 1mM DTT, 10mM KCl and 50% glycerol and stored at −20°C.

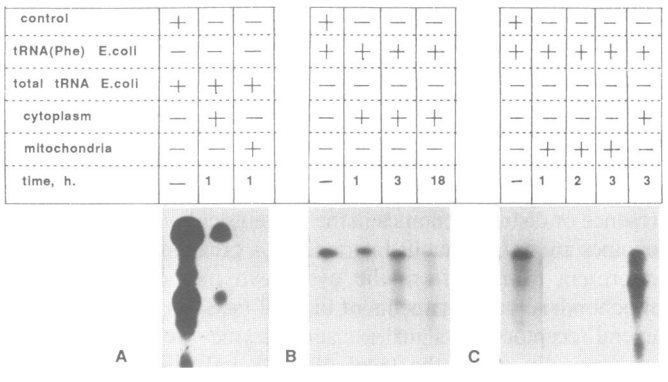

Figure 1. Results of the introduction of various tRNAs into intact *S.cerevisiae* cells. The (+)s correspond to the presence of the component in the electroporation mixture or to the cellular compartment from which tRNA was isolated ('mitochondria' or 'cytoplasm'). The 'control' corresponds to an aliquot of the labelled RNA used for electroporation. '**A**'—the cells were electroporated in the presence of labelled total *E.coli* tRNA (commercial, Sigma), '**B**' and '**C**'—of labelled individual $tRNA^{Phe}$ of *E.coli* . The indicated time corresponds to the time of incubation of electroporated cells in rich medium after the electric shock.

RESULTS

$tRNA_1^{Lys}$ mitochondrial targetting *in vivo*

We applied the technique commonly used for electroporation of intact yeast cells to introduce labelled tRNAs into the cytoplasm. Under our conditions of electroporation the% of survival is between 40 and 60. We therefore expected that the intracellular processes, including RNA targetting, would proceed normally in electroporated cells. Fig.1(A) shows results of introducing total *E.coli* tRNA in the *S.cerevisiae* wild-type cells. *E.coli* tRNA extracted from the cells does not seem to be degraded when compared with the control. No labelled tRNA was found in the mitochondrial compartment of the cells several hours after the electroporation procedure. Fig.1(B) demonstrates that foreign tRNA (individual $tRNA^{Phe}$ of *E.coli*) injected into the cytoplasm remains undegraded for at least 1 hour after the procedure. 3 h. after electroporation the major band still corresponds to the undegraded tRNA. After 18 hours most of the introduced tRNA is degraded, while a minor band corresponds to the undegraded tRNA. Therefore, we expected that the injected tRNA species would have enough time to be targetted into the mitochondria. The procedure used for tRNA labelling does not lead to the loss of biological activity of tRNAs in aminoacylation and protein synthesis (data not shown), so we also expected the features of tRNA crucial for targetting to be retained by labelled molecules. *E.coli* tRNAs (either from total preparation or individual ones) do not penetrate into the mitochondrial compartment of the cells within 3 h (we have not tried longer times of incubation of the electroporated cells in the rich medium for reason of RNA degradation rate (Fig.1(C)). Labelled total tRNAs of *S.cerevisiae* were also easily introduced into the cytoplasm by electroporation. We have not succeeded in detecting the label in the mitochondrial compartment in this case either (not shown). This can be due to the low specific activity of labelled individual tRNA species in total preparation when compared with labelled individual tRNA used in further studies or to insufficient amount of the $tRNA_1^{Lys}$ to be able to detect the import. The main serie of tests was done with individual *S.cerevisiae* lysine tRNAs: two cytoplasmic (anticodons CUU and U*UU) and one mitochondrial (Fig.2(A)). All the three species are effectively injected into the cytoplasm and show only slight signs of degradation within the 1st hour of incubation after the electroporation procedure. Only the labelled $tRNA_1^{Lys}$ (anticodon CUU) was found to be present in mitochondria. As this is the cytoplasmic tRNA that was previously found to be present in mitochondria (17−18), we conclude that the process of targetting takes place in our model system without loss of specificity. In fact, we tested at least 16 other different individual tRNAs of *S.cerevisiae* in the described system: 2 serine, 2 valine, 2 leucine, one tyrosine, one methionine and one tryptophane tRNAs from the cytoplasm and methionine and tyrosine tRNAs from mitochondria were identified by partial sequencing, the other 5 corresponding to the individual electrophoretic bands.

The transported tRNA remains stable in mitochondria for at least one hour (Fig.2(B)). After 3 hours it is degraded up to 70−80%, in average. 18 hours after the electroporation procedure there is no detectable amount of undegraded labelled cytoplasmic $tRNA_1^{Lys}$ in the mitochondrial compartment. The rate of degradation of the introduced tRNA is thus slightly higher in mitochondria than in the cytoplasm. The described system, while highly specific, was not shown to be very efficient. When using up to 5×10^6 cpm of labelled tRNA per standard reaction we obtained, in average, $0.5-1.0\times10^3$ cpm of label in the cytoplasm and only 120−250 cpm in mitochondria. Such yield corresponds to the very low yield of mitochondrial import of the labelled $tRNA_1^{Lys}$ present in the cytoplasm. This can be due to the interference of the injected species with the resident ones, to the low efficiency of the traffiking in electroporated cells and/or to the low efficiency of the targetting process *in vivo*, in general. This method is thus hardly useful for identification of steps and factors taking part in targetting.

$tRNA_1^{Lys}$ mitochondrial targetting *in vitro*

We tested if the $tRNA_1^{Lys}$ can be specifically incorporated into isolated mitochondria as many mitochondrially-imported proteins do. Fig.3 shows results of incubation of isolated mitochondria

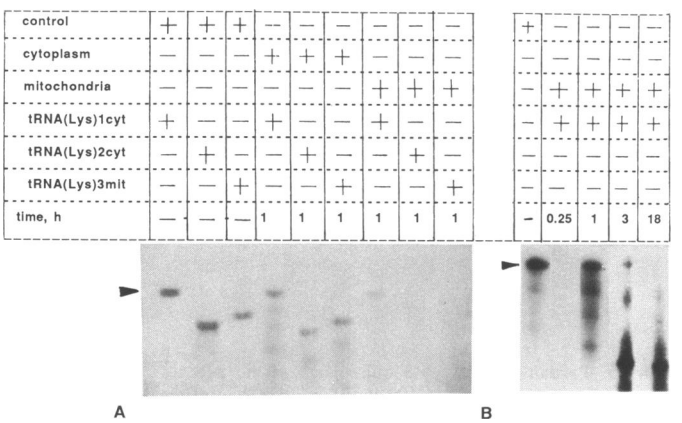

Figure 2. Results of the introduction of labelled individual yeast tRNAs into intact cells by electroporation. '**A**'—testing various tRNAs (tRNA(Lys)1cyt and tRNA(Lys)2cyt correspond to the two cytoplasmic isoacceptors, with CUU and U*UU anticodons, respectively and tRNA(Lys)3mit—to a single mitochondrial species). '**B**'—electroporation of the cells by the $tRNA_1^{Lys}$ with different time of incubation in rich medium after the electric shock. The arrows show the band corresponding to the cytoplasmic $tRNA_1^{Lys}$.

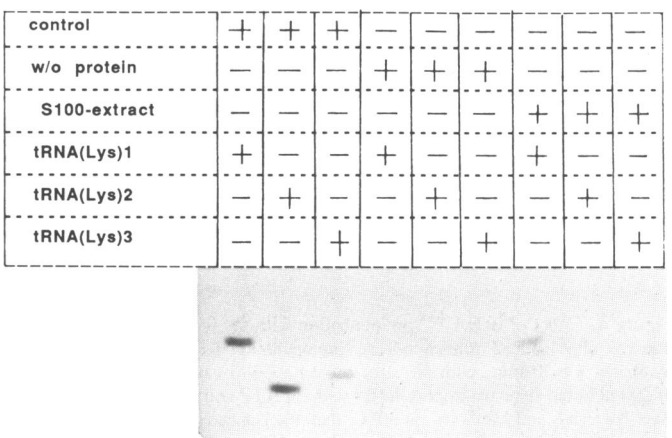

Figure 3. Results of the individual yeast tRNAs transfer into isolated mitochondria. tRNA numbers correspond to those of Fig.2. 'w/o protein'—the conditions of protein transfer into isolated mitochondria (26) were used; 'S-100 extract'—the same conditions completed by addition of 50 μl of S-100 supernatant (50 μg of protein) per standard transfer reaction.

with labelled individual tRNAs in conditions directing mitochondrial import of proteins (26). The conditions similar to those used for proteins did not lead to the introduction of labelled tRNAs into isolated mitochondria at least within 2 hours (longer incubations were not used for the reason of tRNA degradation). We tested various fractions of total cellular extract and found that the addition of the S-100 supernatant to the reaction mixture makes the cytoplasmic the $tRNA_1^{Lys}$ unaccessible to RNase within 1 hour of incubation. Since the incubation of this tRNA with the extract but without mitochondria did not lead to such a result, this would mean that the $tRNA_1^{Lys}$ was introduced into the mitochondria (Fig.3). The other two lysine tRNAs (the cytoplasmic lysine tRNA with the U*UU anticodon and the mitochondrial one) were always degraded by RNase and thus did not penetrate into the mitochondria. Subsequent fractionation of mitochondria revealed that the labelled tRNA was associated with mitosol and/or with the inner mitochondrial membrane (not shown). This result proves that the isolated organelles supplemented with soluble cellular proteins can direct the targetting with the same specificity as *in vivo* (18). The efficiency of the described *in vitro* system for specific $tRNA_1^{Lys}$ targetting is significantly higher than that of the electroporation system. Using 0.5×10^6 cpm of the labelled tRNA per standard reaction we obtained up to $0.5-1.0\times10^3$ cpm of the label in the mitochondria. The markedly higher yield can be explained by higher concentrations of the labelled tRNAs and mitochondria and/or by the absence of competing host tRNAs.

The transport of the cytoplasmic $tRNA_1^{Lys}$ takes place only in the presence of ATP, which proves the energy-dependance of the import. The ATP-generating system also should be present in the import reaction, which indicates the requirement for an 'energized' state of mitochondrial membranes for translocation (see two last lanes of Fig.5).

To obtain detectable amounts of the labelled $tRNA_1^{Lys}$ in mitochondria we had to use at least 30 min-incubations (Fig.4). Incubation of $tRNA_1^{Lys}$ with the extract prior to the addition of isolated mitochondria permitted to decrease the time needed for the appearance of detectable amounts of the labelled tRNA in the mitochondria to 10 min. (Fig.4). Preincubation of the tRNA with the mitochondria in the absence of the transport-directing extract with subsequent addition of the latter did not lead to the decrease of the time needed for import (not shown). Therefore, it seems plausible that the tRNA interaction with soluble protein(s) is at least one of the rate-limiting steps of the targetting. Such a result strongly suggests the existence of an RNA-binding carrier. Preliminary attempts to fractionate the S-100 extract on heparin-Sepharose show that the fractions with high affinity for heparin (eluted from immobilized heparin by 100, 200 and 500 mM of salt) cannot direct the targetting by themselves, as well as the fraction that is not retained on heparin at 10mM of salt (Fig.5). When the protein fraction eluted from Heparin-Sepharose with 500mM of salt was added to the total extract the efficiency of the transport markedly encreased (Fig.5). The increase in intensity of the band corresponding to the imported tRNA upon addition of the Heparin-500 protein fraction was estimated as 90−120%, in average. The addition of more than 5 μg of the Heparin-500 protein fraction to the S-100 extract did not lead to a further increase of import efficiency. A similar result can be obtained when complementing the flow-through protein fraction with that eluted at 500mM. Taking into account that heparin-binding properties usually correlate with the RNA-binding ones, the obtained results can be explained by the requirement of at least two factors—one with and the other

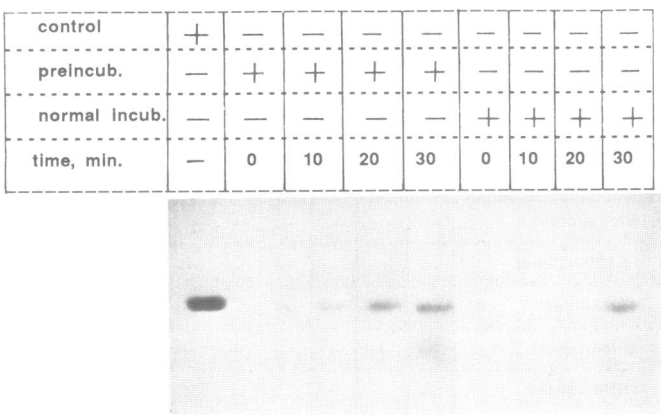

Figure 4. Effect of $tRNA_1^{Lys}$ preincubation with the S-100 extract prior to the transfer into isolated mitochondria. 'preincubation (+)'—the $tRNA_1^{Lys}$ was incubated for 30 min. with 50 μl of protein (S-100 extract) in a final volume of 200 μl in the standard import buffer without ATP and ATP-generation system (see 'Materials and Methods') at 30°C, then the isolated mitochondria suspended in the same buffer containing ATP and an ATP-generation system were added in a volume of 100 μl. After the indicated time RNase was added and tRNA from mitochondria was isolated as described in 'Materials and methods'. 'normal incubation (+)'—the import reaction was carried out in the presence of tRNA, S-100 extract and mitochondria as described in 'Materials and Methods', but the incubation time was as indicated in the line 'time'. After the time indicated RNase was added and tRNA was isolated. '0' min. time corresponds to the time needed to mix the ingredients of the reaction.

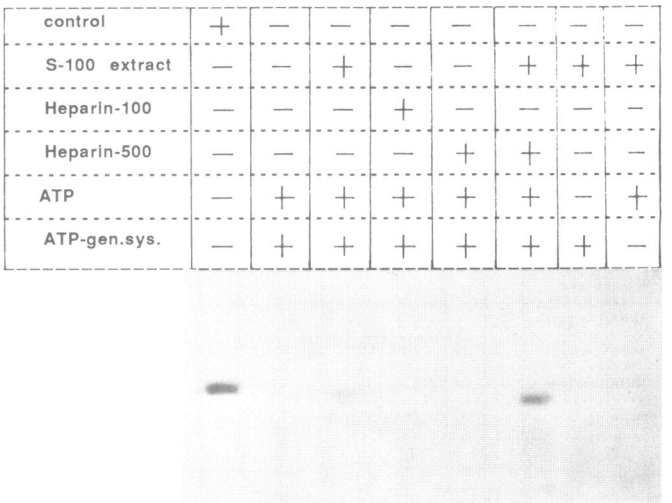

Figure 5. Protein and energy requirements of the $tRNA_1^{Lys}$ transport into isolated mitochondria. Heparin-100 and Heparin-500 correspond to the fractions of S-100 extract bound to heparin-Sepharose at 10 mM of salt and eluted by the salt concentration of 100 and 500mM, respectively. The fractions, after being prepared as described in 'Materials and methods', were added to the standard transfer reaction in the amount of 50 μg (in 50 μl). The incubation was carried out for 1 h. S-100(+)Heparin-500(+) line corresponds to the simultaneous addition of 50 μl of S-100 extract and 5 μl of the Heparin-500 fraction. The amounts of mitochondria and labelled tRNA were the same in all the cases, as well as the volumes of the aliquots taken for the electrophoresis. ATP(−) and ATP gen.sys.(−) lines correspond to the standard transfer mixture devoid of ATP or ATP-generating system, respectively.

without RNA-binding activity—for the targetting process. Nevertheless, to demonstrtate it clearly additional experiments are needed.

DISCUSSION

We have set up two test systems allowing specific transfer of the yeast cytoplasmic $tRNA_1^{Lys}$ into mitochondria. The specificity of the transport correlates with the previous finding of this very cytoplasmic tRNA inside the mitochondrial compartment of the yeast cell and the absence of the corresponding gene in the mitochondrial genome (17–18). To our knowledge, our systems give the first direct proofs for the existence of a specific pathway for mitochondrial targetting of tRNAs, since almost all the previous data concerning the mitochondrial RNA import were based on results of hybridization and of sequencing tRNAs isolated from the cytoplasm and from the mitochondria. The described systems give an exciting possibility to study the mechanisms of this targetting process.

We found the targetting process to be ATP-dependent and requiring the presence of an ATP-generating system. The same requirements were shown for protein mitochondrial import, since the need for ATP and for the energized state of the mitochondrial membranes are the characteristic features of the protein precursor translocation mechanism (26–28). The time needed for the mitochondrial import of the tRNA also correlates well with the time needed for the protein mitochondrial import (26). This correlation can be explained in terms of the hypothesis suggesting that the mitochondrial protein import machinery is involved at least in some steps of the tRNA mitochondrial import.

Contrary to the mitochondrial import of proteins, the $tRNA_1^{Lys}$ import was found to be mediated by soluble protein factors at least one of which seems to have RNA binding activity (at least to have strong polyanions–binding properties). The interaction of the tRNA with the soluble proteins seems to be the rate-limiting step of the targetting, while the translocation itself proceeds relatively quicker. We do not have now a clear idea of what these factors can be. The mitochondrial lysyl-tRNA synthetase seems to be ruled out as it cannot aminoacylate the cytoplasmic imported tRNA (18) and, therefore, cannot serve as a carrier. Its cytoplasmic counterpart can be one of the factors (at least as a carrier) but it is probably unable to direct the transfer (data not shown).

To speculate on the possible pathways of mitochondrial $tRNA_1^{Lys}$ import one can suggest that the main peculiarities of this process are restricted to the 'targetting' process (meaning the process of transport of the tRNA to the mitochondria). On the other hand, the translocation of the tRNA through the mitochondrial membranes seems to be related to the protein mitochondrial import mechanisms. In fact, results obtained on mitochondrial import of covalent DNA-protein conjugates (27) suggest that the pathway of mitochondrial protein import can, in principle, serve to introduce into the mitochondrial compartment of the cell other molecules than an unfolded polypeptide chain. If so, these mechanisms can also serve for tRNA translocation through mitochondrial membranes.

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