WestVirginiaUniversity
THE RESEARCH REPOSITORY @ WVU

Faculty Scholarship

1990

Import of the iron-sulfur protein of the cytochrome b. c1 complex into yeast mitochondria.

W Fu

S Japa

D S Beattie

Follow this and additional works at: https://researchrepository.wvu.edu/faculty_publications

Digital Commons Citation

Fu, W; Japa, S; and Beattie, D S, "Import of the iron-sulfur protein of the cytochrome b. c1 complex into yeast mitochondria." (1990). *Faculty Scholarship*. 192.
https://researchrepository.wvu.edu/faculty_publications/192

This Article is brought to you for free and open access by The Research Repository @ WVU. It has been accepted for inclusion in Faculty Scholarship by an authorized administrator of The Research Repository @ WVU. For more information, please contact ian.harmon@mail.wvu.edu.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1990 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 265, No. 27, Issue of September 25, pp. 16541-16547, 1990
Printed in U.S.A.

Import of the Iron-Sulfur Protein of the Cytochrome $b \cdot c_1$ Complex into Yeast Mitochondria*

(Received for publication, February 9, 1990)

William Fu, Shanker Japa, and Diana S. Beattie‡

From the Department of Biochemistry, West Virginia University School of Medicine, Morgantown, West Virginia 26506

The yeast gene for the Rieske iron-sulfur protein of the cytochrome $b \cdot c_1$ complex was subcloned into the expression vector, pSP64, then transcribed and translated *in vitro* in a reticulocyte lysate in the presence of [^{35}S]methionine. Import studies *in vitro* of the newly synthesized precursor form of the iron-sulfur protein into isolated yeast mitochondria revealed that the precursor form of the iron-sulfur protein is processed into the mature form via an intermediate form. After the import reaction at 18 or 27 °C, treatment of mitochondria with exogenous protease indicated that both intermediate and mature forms had been internalized into mitochondria where they were resistant to digestion by external protease. Import and processing of the iron-sulfur protein into mitochondria also occurred at temperatures ranging from 2 to 27 °C in a temperature-dependent manner. Processing of the precursor form to the intermediate form appeared to be less sensitive to temperature than the processing of the intermediate form to the mature form. Moreover, at temperatures of 12 °C or lower, the mature form produced was completely digested by exogenous protease suggesting that it was assembled incorrectly in the membrane and not assembled into the $b \cdot c_1$ complex. The successive disappearance of first the mature form and then the intermediate form of the iron-sulfur protein by increasing concentrations of the metal chelators, EDTA and *o*-phenanthroline, suggested that two different proteases requiring divalent metal ions are involved in the two-step processing of the presequence of the iron-sulfur protein. Furthermore, mitoplasts containing only the matrix/inner membrane fraction were able to import and process the precursor form of the iron-sulfur protein indicating that both proteolytic processing events occur in the matrix/inner membrane fraction.

The majority of mitochondrial proteins are encoded by nuclear genes and synthesized on free cytoplasmic ribosomes, despite the fact that mitochondria contain their own unique DNA. Mechanisms of import and processing of nuclear-coded mitochondrial proteins have been extensively investigated since the discovery in 1979 (1) that subunits of the F_1-ATPase are synthesized in the cytosol as larger precursor proteins containing amino-terminal extensions (presequences) prior to import into mitochondria. Subsequent studies have provided evidence that the import and processing of precursor proteins into mitochondria occur through several distinct steps (2–6). After completion of translation, precursor forms of mitochondrial proteins containing targeting signals (amphiphilic structures composed of hydrophobic regions linked by basic residues) are directed to the mitochondrial outer membrane (6–9). A second step in the import pathway requires the binding of these precursor proteins to proteinaceous receptors exposed at the cytoplasmic face of the outer membrane (10–12). The precursor proteins are then unfolded by a mechanism involving ATP into a translocation-competent conformation (13, 14). The eventual translocation of precursor proteins into the mitochondrial matrix via contact sites between the outer and inner membranes is dependent on an electrochemical potential ($\Delta\psi$) across the inner membrane (15). During or after translocation, precursor proteins are processed to their mature forms by either one or two separate cleavages by matrix-localized protease(s) (16–18). Finally, the processed mature proteins are assembled into functional complexes in their proper submitochondrial compartment.

The Rieske iron-sulfur protein, one of the catalytic subunits of the cytochrome $b \cdot c_1$ complex, is localized on the outer surface of the inner mitochondrial membrane (19, 20). This protein contains two Fe-S clusters and is required for electron transfer from ubiquinol to cytochrome c_1 in the $b \cdot c_1$ complex (21). Our laboratory made the initial observation in intact yeast cells that the precursor form of the iron-sulfur protein is processed *in vivo* into the mature form via an intermediate form (22). This proteolytic two-step cleavage of the precursor iron-sulfur protein was subsequently observed during experiments both *in vitro* and *in vivo* in *N. crassa* (23). Moreover, the intermediate form of the iron-sulfur protein was shown to be localized in the matrix fraction as it fractionated with the matrix marker fumarase upon treatment of mitochondria with digitonin (23).

Recently, the nuclear gene encoding the Rieske iron-sulfur protein has been isolated and sequenced from both *N. crassa* (24) and *S. cerevisiae* (25). In the current study, we have subcloned the iron-sulfur protein gene into a pSP64 expression vector to study the import and processing of the precursor form of the iron-sulfur protein *in vitro* into isolated yeast mitochondria. The results obtained confirm the two-step cleavage of the precursor iron-sulfur protein in yeast mitochondria and suggest different temperature sensitivities for each step. Moreover, treatment of mitochondria with exogenous protease after import has indicated that both intermediate and mature forms of the iron-sulfur protein are localized in a protease-resistant compartment. The precursor form of the iron-sulfur protein can also be imported and processed to both intermediate and mature forms in mitoplasts (mitochondria from which the outer membrane has been removed) indicating that both processing steps occur in the inner membrane/matrix fraction.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed. Tel.: 304-293-7522.

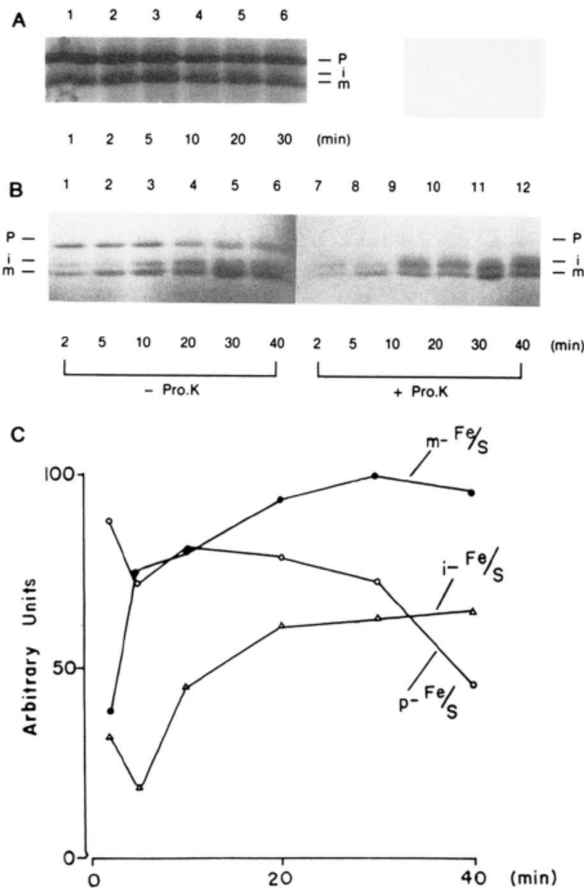

FIG. 4. **Time course of the Rieske iron-sulfur protein import into mitochondria.** Translation mixture containing ^{35}S-labeled iron-sulfur protein precursor was centrifuged at 105,000 x *g* for 45 min and incubated with isolated yeast mitochondria at 27 °C (*A*) or 18 °C (*B*) for the times as indicated. Mitochondria were added at a final concentration of 1.0 mg/ml for each import reaction. *A*, at each time point, 300 μl of import mixture was withdrawn and analyzed as described under "Experimental Procedures." *B*, one-half of each import mixture (400 μl) was removed (*lanes 1–6*) as described in *A* while the remaining half was treated with proteinase K (*lanes 7–12*). The positions of precursor (*p*), intermediate (*i*), and mature (*m*) forms of the iron-sulfur protein are indicated. *C*, the bands of the three forms of the iron-sulfur protein from the autoradiograph of the gel shown in *B*, *lanes 1–6*, were quantified by laser densitometry. The mature form observed at 30 min was arbitrarily set at 100.

EXPERIMENTAL PROCEDURES AND RESULTS[1]

Import of the Iron-Sulfur Protein Precursor—The time course of import of the precursor form of the iron-sulfur protein into mitochondria was studied by incubating yeast mitochondria with translation mixtures containing radiolabeled precursor. At the times indicated, aliquots of the import mixture were withdrawn and analyzed by SDS-PAGE[2] with or without proteinase K treatment. Fig. 4*A* shows that the import and processing of the iron-sulfur protein into mitochondria occurred rapidly at 27 °C. Maximum amounts of the labeled mature form were observed after a short incubation, times of 1 or 2 min, while only traces of labeled intermediate form were observed at all times studied. When the import incubation was performed at 18 °C, the presence of the intermediate form became apparent. Labeling of both the intermediate and mature forms increased with time in a parallel fashion with the mature form more heavily labeled at each time point. The amount of the precursor form remained essentially constant for 20–30 min reflecting continued binding of the precursor form to the mitochondria during this time as processing occurred (Fig. 4*B*, *lanes 1–6*). Consequently, the total amount of radiolabeled iron-sulfur protein, the sum of the precursor, intermediate, and mature forms, increased more than 50% from the 2- to the 20-min time point at which time a decrease in the amount of precursor was observed. At all times of import at 18 °C, the precursor form was digested by exogenous proteinase K; however, both the intermediate and mature forms were resistant to digestion by exogenous proteinase K indicating that they are present in a protease-resistant compartment of the mitochondria (Fig. 4*B*, *lanes 7–12*). A slight loss in the amount of the mature form after proteinase K digestion suggests possible damage to the outer membrane of the mitochondria allowing some digestion of the mature form which is exposed to the outer surface of the inner membrane.

The profound effect of temperature on the import and processing of the iron-sulfur protein into yeast mitochondria prompted us to examine the effect of even lower temperatures on the import reaction. Clearly, import and processing of the iron-sulfur protein into yeast mitochondria were temperature-dependent (Fig. 5, compare *lanes 1, 4,* and *7*). Both the intermediate and mature forms of the iron-sulfur protein were resistant to proteinase K treatment after import at 27 °C (*lane 8*), suggesting that both forms have been internalized at this temperature. Surprisingly, mature form as well as the precursor form were accessible to exogenous protease when the import reaction was performed at 2 or 12 °C (*lanes 2* and *5*); however, the intermediate form remained resistant to protease treatment after the import at these temperatures. The intermediate form produced at the lower temperatures of incubation appears more diffuse after proteinase K digestion, but does not represent digested precursor form as it is clearly localized in the matrix as discussed under "Submitochondrial

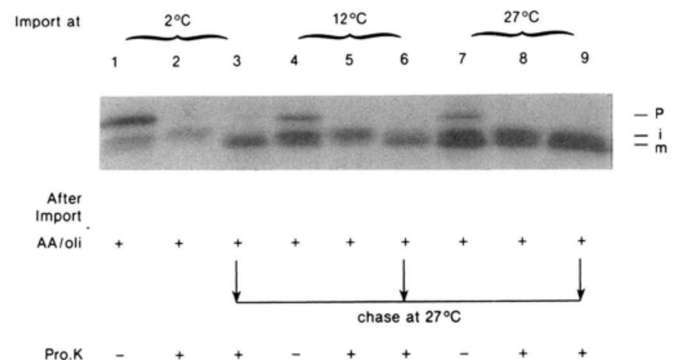

FIG. 5. **Effect of temperature on the sensitivity of the various forms of the iron-sulfur protein to proteinase K digestion.** Mitochondria were incubated with the high speed supernatant of the translation mixture for 30 min at 2, 12, or 27 °C. The samples were placed on ice, antimycin A and oligomycin were added, and the samples were divided into three groups. For the first group, mitochondria were immediately analyzed by SDS-PAGE (*lanes 1, 4,* and *7*). The second group was treated with proteinase K, prior to SDS-PAGE (*lanes 2, 5,* and *8*). The third group was further incubated for 20 min at 27 °C, placed on ice, and treated with proteinase K. Mitochondria were repelleted by centrifugation and analyzed by SDS-PAGE (*lanes 3, 6,* and *9*). *Abbreviations* are as in Fig. 4.

[1] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; kbp, kilobase pair(s).

Localization of the Different Forms of the Iron-Sulfur Protein." Moreover, the amount of intermediate remains the same before and after proteinase K digestion which would be unlikely if the intermediate form represented digested precursor form. After the initial import of the precursor form at 2 or 12 °C, the incubation mixture was further incubated for 20 min at 27 °C in the presence of antimycin A and oligomycin. The chase at 27 °C resulted in the processing of the precursor and intermediate forms to the mature form which was then resistant to added proteinase K (*lanes 3* and *6*). These data suggest that the processing of the intermediate to the mature form is more sensitive to temperature than the processing and import of the precursor form into a protease-resistant intermediate form.

The sensitivity of the intermediate and mature forms of the iron-sulfur protein to exogenous proteinase K was further explored by studying the time course of import at 12 °C. The results obtained confirm that only the intermediate form remained resistant to exogenous proteinase K after import at 12 °C (Fig. 6, *A* and *B*), while at all times of incubation the mature form was sensitive to digestion. As mentioned above, the intermediate form labeled under these conditions appeared very diffuse after proteinase K digestion for reasons which remain obscure. Perhaps the protease has attached a portion of the intermediate form such that it now runs more slowly on the gels. However, the absolute amounts of the intermediate form before and after proteinase K digestion are almost identical at all time points examined indicating that no loss of intermediate form resulted from treatment with the exogenous protease. The appearance of both intermediate and mature forms increased with time during the incubation reaching a maximum after 40 min. The amount of the precursor form remained constant throughout the incubation reflecting the continued binding of precursor form to the mitochondria during the incubation at 12 °C. These results suggest that formation of the mature form occurs normally at 12 °C; however, the processed mature form produced at 12 °C is assembled incorrectly in the mitochondrial membrane where it remains accessible to proteinase K.

Effects of Inhibitors on Import of the Iron-Sulfur Protein—The effects of inhibitors which block formation of a membrane potential plus chelators shown previously to inhibit the matrix-localized mitochondrial processing proteases were studied during import of the iron-sulfur protein precursor into mitochondria at 18 °C. In the presence of antimycin A and oligomycin, only the precursor form was observed in the reisolated mitochondria. In addition, the precursor was completely digested by proteinase K treatment (Fig. 7*A*, *lanes 5* and *10*) indicating that the import and processing of the iron-sulfur protein requires the presence of a membrane potential. Addition of 10 mM EDTA, a nonpenetrating metal chelator, or 0.5 mM *o*-phenanthroline, a membrane-permeable metal chelator, to the import mixture separately had little effect on processing of the precursor form (*lanes 2, 3* and *7, 8*); however, addition of both EDTA and *o*-phenanthroline caused a considerable inhibition of the processing of the intermediate to the mature form (*lanes 4* and *9*). Some inhibition of processing the precursor to the intermediate form was also observed, but the precursor form remained protease-sensitive (*lane 9*).

Increasing concentrations of both EDTA and *o*-phenanthroline added to the import reaction at 18 °C resulted in the successive disappearance of first the mature and then the intermediate form of the iron-sulfur protein (Fig. 7*B*). The addition of 2.5 mM EDTA and 0.5 mM *o*-phenanthroline (*lane 1*) inhibited partially the conversion of intermediate to mature form. Increasing the concentration of EDTA to 10 mM and *o*-phenanthroline to 2 mM completely inhibited the conversion of intermediate to mature form and partially inhibited the conversion of precursor to intermediate form (*lane 3*). Furthermore, when the concentration of *o*-phenanthroline was increased to 4 mM, processing of the precursor to intermediate

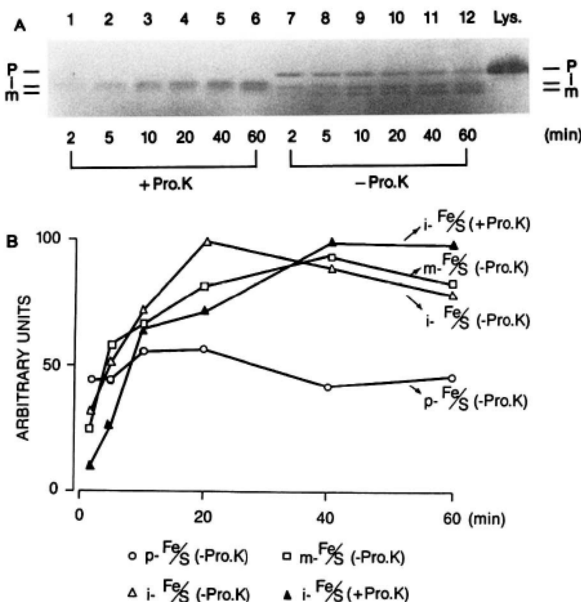

FIG. 6. **Time course of import of the iron-sulfur protein import into mitochondria at 12 °C.** *A*, experimental conditions were identical with those of Fig. 4*B*, except that the incubation temperature was at 12 °C for the times indicated. *B*, the bands of the three forms of the iron-sulfur protein from the autoradiograph of the gel shown in *A* were quantified by laser densitometry and are presented in arbitrary units. The amount of intermediate form after a 20-min incubation was normalized to 100 units. *Abbreviations* are as in Fig. 4.

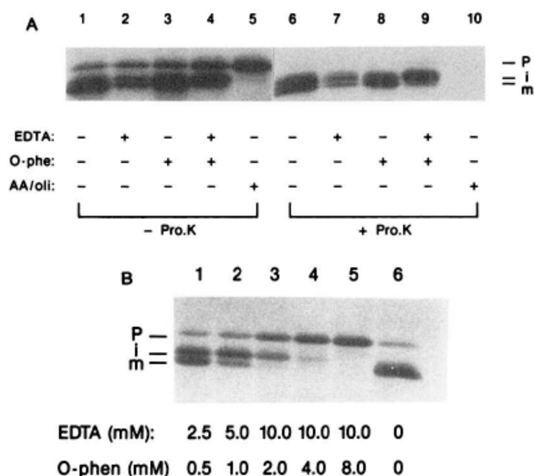

FIG. 7. *A*, effect of metal chelators on the import of the iron-sulfur protein into mitochondria. Mitochondria were preincubated with an appropriate amount of import buffer at a final concentration of 1.0 mg/ml at 18 °C for 5 min. Where indicated, EDTA, *o*-phenanthroline (*o-phe*), or antimycin A and oligomycin (*AA/oli*) were added. The import reactions were carried out at 18 °C for 30 min. At that time, one-half of each import mixture was analyzed by SDS-PAGE (*lanes 1–5*), while the second half was treated with proteinase K and then analyzed by SDS-PAGE (*lanes 6–10*). *B*, mitochondria at a final concentration of 1.0 mg/ml were preincubated in an import buffer in the presence of EDTA and *o*-phenanthroline at 18 °C for 5 min. After addition of reticulocyte lysate containing ^{35}S-labeled precursor iron-sulfur protein, the import reactions were further incubated at 18 °C for 20 min. Mitochondria were reisolated from each import mixture and analyzed by SDS-PAGE. *Abbreviations* are as in Fig. 4.

form was almost completely blocked. As the processing protease converting the precursor to the intermediate form was inhibited, the precursor form became resistant to exogenous protease suggesting that it had been translocated into the mitochondria (data not shown).

Submitochondrial Localization of the Different Forms of the Iron-Sulfur Protein—To localize both the intermediate and mature forms of the iron-sulfur protein within the mitochondria, mitochondria were subfractionated by digitonin treatment after the import reaction. Enzymes present in the intermembrane and the matrix spaces are released sequentially by successive increases in digitonin concentration. Fumarase was used as an enzymatic marker for the mitochondrial matrix space. As shown in Fig. 8, *A* and *B*, treatment of mitochondria with concentrations of digitonin ranging from 0.075–0.1% resulted in the concomitant release of both the mature and intermediate forms of the iron-sulfur protein from mitochondria. At a digitonin concentration of 0.15%, more of the mature form was removed from the membrane than intermediate form; however, both the mature and intermediate forms were totally released by treatment of mitochondria with 0.2% digitonin. By contrast, the precursor form of the iron-sulfur protein was not removed from the membrane fraction with increasing concentrations of digitonin. The release of fumarase, the matrix marker, from mitochondria into the supernatant with increasing digitonin concentration paralleled the loss of the intermediate and mature forms of the iron-sulfur protein (Fig. 8*B*). After treatment with 0.075% digitonin, about 85% of the mature and intermediate forms were retained in the mitochondria while about 20% of the fumarase activity was released. Almost 85% of fumarase activity was released after treatment with 0.15% digitonin, which released 75% of the intermediate form and all of the mature form. These results suggest that the intermediate form of the iron-sulfur protein is present in the matrix space. The mature form, however, is more susceptible to solubilization by higher concentrations of digitonin suggesting that it is not in the matrix but loosely associated with the inner membrane. Interestingly, an identical release of the intermediate and mature forms with increasing concentrations of digitonin was observed after import at 12 and 27 °C, as well as at 18 °C (Fig. 8).

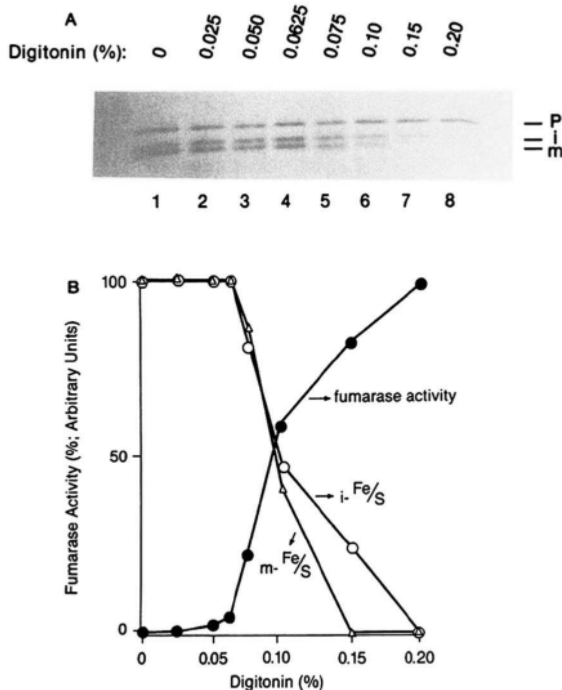

FIG. 8. **Release of both imported intermediate and mature forms of the iron-sulfur protein from mitochondria by treatment with digitonin.** Mitochondria were incubated with the newly synthesized iron-sulfur protein at 18 °C for 20 min. The reaction mixture was divided into 8 equal portions and mitochondria were reisolated by centrifugation. The reisolated mitochondria were washed twice with a buffer containing 0.6 M sorbitol and 20 mM Hepes/KOH (pH 7.4) and then resuspended at 0.2 ml of above buffer in the presence of the indicated concentrations of digitonin as indicated under "Experimental Procedures." Each fraction was separated into pellet and supernatant by centrifugation. The pellets were analyzed by SDS-PAGE while the supernatants were assayed for fumarase activity. An autoradiograph of the dried gel is shown in *A*. The bands of both intermediate and mature forms of the iron-sulfur protein quantified by laser densitometry and the fumarase activities are shown in *B*. *Abbreviations* are as in Fig. 4.

Import of the Iron-Sulfur Protein into Mitoplasts—Import and processing of the precursor form of the iron-sulfur protein into isolated mitochondria had been successfully demonstrated at temperatures ranging from 4 to 27 °C. We next attempted to demonstrate whether the precursor form of the iron-sulfur protein could be imported into mitoplasts which lack the mitochondrial outer membrane. The time course of the import of the precursor form of the iron-sulfur protein into mitoplasts at 18 °C indicated that mitoplasts can import the precursor form as efficiently as mitochondria (Fig. 9). Both the intermediate and the mature forms were observed in the absence of proteinase K during the different time intervals suggesting that both proteases involved in the conversion of the precursor form to the intermediate form and the intermediate form to the mature form are present in the mitochondrial matrix-inner membrane fraction. The intermediate form was resistant to digestion by exogenous proteinase K, while both precursor and mature forms were sensitive to digestion suggesting that conversion of the intermediate form into the mature form occurred in the matrix/inner membrane fraction. Moreover, the mature form is assembled on the outer surface of the inner mitochondrial membrane where it was accessible to exogenous proteinase K.

Next, the inhibitors of the proteases involved in the two-step cleavage of the iron-sulfur protein were studied during the import reaction in mitoplasts (Fig. 10). Raising the concentrations of EDTA and *o*-phenanthroline resulted in the successive inhibition of the two proteases as previously observed in mitochondria (Fig. 7*B*). After addition of exogenous proteinase K (*lanes 6–9*), the intermediate form remained resistant to proteinase K. As the processing enzyme converting precursor to intermediate form became inhibited, the precursor form also became resistant to proteinase K. The import of the precursor iron-sulfur protein into mitoplasts was investigated at 4, 12, and 18 °C (Fig. 11, *lanes 1, 2,* and *3,* respectively). Only the precursor form of the iron-sulfur protein was observed at 4 and 12 °C in contrast to the results obtained using mitochondria (Fig. 5). Significant import and processing to both intermediate and mature forms were ob-

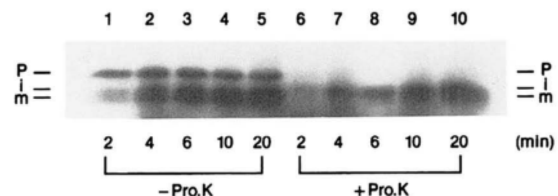

FIG. 9. **Time course of import of the iron-sulfur protein into mitoplasts.** Translation mixture containing ^{35}S-labeled precursor iron-sulfur protein was incubated with mitoplasts prepared as described under "Experimental Procedures" at 18 °C for the indicated times. *Abbreviations* are as in Fig. 4.

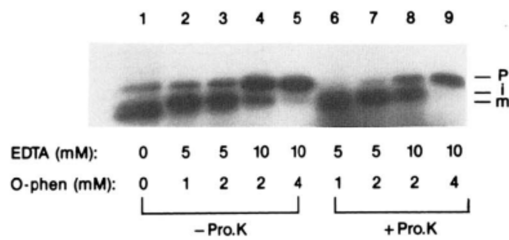

FIG. 10. **Effect of EDTA and *o*-phenanthroline on the proteolytic two-step processing of the iron-sulfur protein in mitoplasts.** This experiment was identical with that of Fig. 7*B*, except that mitoplasts were used in the incubation. *Abbreviations* are as in Fig. 4.

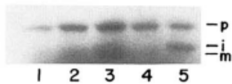

FIG. 11. **Effect of different temperatures on the import of the iron-sulfur protein into mitoplasts.** The import reactions were performed at various temperatures: 4 °C (*lane 1*), 12 °C (*lane 2*), and 18 °C (*lanes 3–5*). Antimycin A and oligomycin were added to the import mixture in *lane 4*, while 10 mM EDTA and 2.0 mM orthophenanthroline were added to *lane 5*. After the import incubation, the reisolated mitoplasts were analyzed by SDS-PAGE. *Abbreviations* are as in Fig. 4.

served at 18 °C. In addition, when the membrane potential was blocked by the addition of antimycin A and oligomycin (*lane 4*), the import of the precursor form into mitoplasts was inhibited suggesting that a membrane potential is necessary for the import of the precursor form into mitoplasts as well as into mitochondria.

DISCUSSION

Both intermediate and mature forms of the iron-sulfur protein were observed during import studies *in vitro* of the newly synthesized precursor form of this protein into isolated yeast mitochondria. These results confirm previous results obtained in yeast *in vivo* (22) and *N. crassa* both *in vivo* and *in vitro* (23) indicating that the precursor form of the iron-sulfur protein is processed to the mature form via an intermediate form. This apparent two-step proteolytic processing has also been reported for other mitochondrial proteins encoded by nuclear genes and synthesized in the cytoplasm such as cytochrome c_1 (33, 34), cytochrome b_2 (34, 35), and subunit 9 of ATPase (36). Treatment of mitochondria after the import reaction with exogenous protease or digitonin extraction suggested that both intermediate and mature forms have been internalized into mitochondria at 18 or 27 °C where they are insensitive to protease digestion and released by digitonin treatment concomitantly with the matrix marker fumarase. Moreover, the precursor form of the iron-sulfur protein was imported and processed to both intermediate and mature forms in mitoplasts indicating that both proteolytic processing events occur in the matrix/inner membrane fraction. Treatment of mitoplasts with exogenous protease after import reaction resulted in the disappearance of both precursor and mature forms of the iron-sulfur protein suggesting their localization on the outer surface of the inner membrane; however, the intermediate form remained resistant to added protease suggesting its localization in the matrix fraction. Mitoplasts containing only the matrix/inner membrane fractions thus appear competent to perform both processing steps after import of the iron-sulfur protein including the translocation of the mature form to the outer surface of the inner membrane where it became sensitive to exogenous protease.

Previous reports had indicated that the processing protease(s) responsible for the cleavage of the presequences of mitochondrial precursor proteins were localized in the matrix of yeast (37) and rat liver mitochondria (38). This matrix protease is inhibited by metal chelators such as EDTA and *o*-phenanthroline and is dependent on divalent metal ions such as Co^{2+} and Mn^{2+} suggesting that the enzyme is a metalloendoprotease (37). In the present study, addition of both 10 mM EDTA and 0.5 mM *o*-phenanthroline resulted in considerable inhibition of processing of the intermediate to the mature form and only slight inhibition of processing of the precursor to the intermediate form (Fig. 7*A*). Increasing the concentration of *o*-phenanthroline to 8 mM completely blocked the processing of the precursor form to the mature form via the intermediate form (Fig. 7*B*). The successive disappearance of first the mature form and then the intermediate form of the iron-sulfur protein by increasing the concentrations of the metal chelators suggests that two proteases, both requiring divalent metal ions, are involved in the two-step processing of the presequence of the iron-sulfur protein. Furthermore, the processing of the precursor to both the intermediate and mature forms by mitoplasts indicated that both proteases appear to localize in the matrix/inner membrane fraction. Interestingly, in both mitochondria and mitoplasts, complete blockage of processing of the precursor to the intermediate form resulted in the translocation of the precursor form into a compartment where it was resistant to digestion by exogenous protease.

Import and processing of the iron-sulfur protein into mitochondria occurred at temperatures ranging from 2 to 27 °C in a temperature-dependent manner. Processing of the precursor form to the intermediate form occurred readily at all temperatures and was apparently less sensitive to temperature than the processing of the intermediate form to the mature form. For example, the intermediate form was barely detectable during import reactions at 27 °C suggesting that once the precursor form is cleaved in the matrix, the intermediate form is almost immediately processed to the mature form and assembled with other subunits of the $b \cdot c_1$ complex in the inner membrane. At an import temperature of 18 °C, processing of the intermediate to the mature form occurred more slowly as indicated by the presence of the intermediate form at all times during the incubation. The amount of intermediate form was considerably lower than that of the mature form at all times investigated, again suggesting a rapid conversion of intermediate to mature form which is slowed sufficiently by the 18 °C temperature to permit observation of the intermediate form. Further lowering of the temperature of the incubation to 12 °C resulted in the presence of approximately equal amounts of the intermediate and mature forms which increased in parallel during a 40-min incubation (Fig. 6). After import at 12 °C, the intermediate form remained insensitive to digestion by external proteinase K and was released by digitonin concomitantly with the matrix marker fumarase (data not shown) suggesting that processing of the precursor to the intermediate form results in the translocation of the protein into the mitochondrial matrix. At import temperatures of 12 °C or lower, the mature form produced was completely digested by exogenous protease. We suggest that the mature form produced during processing of the intermediate form at import temperatures at or below the transition temperature of the inner membrane is incorrectly translocated back across the membranes perhaps via the contact sites, where it becomes accessible to proteinase K. Previous studies in *N. crassa* had indicated that at 2 °C to 8 °C no formation of the mature form of the iron-sulfur protein occurred (23).

In that system, all species of the iron-sulfur protein were largely sensitive to added proteinase K at incubation temperatures below 12 °C (23).

The results of this study suggest that the precursor form of the iron-sulfur protein in yeast first associates tightly with the mitochondrial membrane and is translocated into the matrix in a process dependent on an electrical potential across the inner membrane. In the matrix space, the precursor form is first processed into the intermediate form by a matrix protease. The subsequent processing of the intermediate form to the mature form is catalyzed by a second protease located in the matrix/inner membrane fraction and appears to be coupled to the translocation of the mature form back across the inner membrane where it is assembled into the $b \cdot c_1$ complex. The exact intramitochondrial localization of the second protease and the mechanism of assembly of the mature form of the iron-sulfur protein with other subunits of the $b \cdot c_1$ complex is currently under investigation in our laboratory.

Acknowledgment—We wish to thank Dr. Bernard Trumpower of Dartmouth College of Medicine, Hanover, NH for the generous gift of the RIP1 gene.

REFERENCES

1. Maccecchini, M.-L., Rudin, Y., Blobel, G., and Schatz, G. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 343–347
2. Hay, R., Bohni, P., and Gasser, S. (1984) *Biochim. Biophys. Acta* **779,** 65–87
3. Douglas, M. G., McCammon, M. T., and Vassarotti, A. (1986) *Microbiol. Rev.* **50,** 166–178
4. Pfanner, N., Hartl, F.-U., and Neupert, W. (1988) *Eur. J. Biochem.* **175,** 205–212
5. Hartl, F.-U., Pfanner, N., Nicholson, D. W., and Neupert, W. (1989) *Biochim. Biophys. Acta* **988,** 1–45
6. Roise, D., and Schatz, G. (1988) *J. Biol. Chem.* **263,** 4509–4511
7. Von Heijne, G. (1986) *EMBO J.* **5,** 1335–1342
8. Schatz, G. (1987) *Eur. J. Biochem.* **165,** 1–6
9. Roise, D., Theiler, F., Horvath, S. J., Tomich, J. M., Richards, J. H., Allison, D. S., and Schatz, G. (1988) *EMBO J.* **7,** 649–653
10. Zwizinski, C., Schleyer, M., and Neupert, W. (1984) *J. Biol. Chem.* **259,** 7850–7856
11. Gillespie, L. L. (1987) *J. Biol. Chem.* **262,** 7939–7942
12. Ohba, M., and Schatz, G. (1987) *EMBO J.* **6,** 2109–2115
13. Eilers, M., and Schatz, G. (1986) *Nature* **322,** 228–232
14. Eilers, M., Hwang, S., and Schatz, G. (1988) *EMBO J.* **7,** 1139–1145
15. Gasser, S. M., Daum, G., and Schatz, G. (1982) *J. Biol. Chem.* **247,** 13034–13041
16. Yang, M., Jensen, R. E., Yaffe, M. P., Oppliger, W., and Schatz, G. (1988) *EMBO J.* **7,** 3857–3862
17. Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U., and Neupert, W. (1988) *Cell* **53,** 795–806
18. Ou, W. J., Ito, A., Okazaki, H., and Omura, T. (1989) *EMBO J.* **8,** 2605–2612
19. Sidhu, A., Clejan, L., and Beattie, D. S. (1983) *J. Biol. Chem.* **258,** 12308–12314
20. Bell, R. L., Sweetland, J., Ludwig, B., and Capaldi, R. A. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 741–745
21. Trumpower, B. L., and Edwards, C. A. (1979) *J. Biol. Chem.* **254,** 8697–8706
22. Sidhu, A., and Beattie, D. S. (1983) *J. Biol. Chem.* **258,** 10649–10656
23. Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H., and Neupert, W. (1986) *Cell* **47,** 939–951
24. Harnisch, U., Weiss, H., and Sebald, W. (1985) *Eur. J. Biochem.* **149,** 95–99
25. Beckmann, J. D., Ljungdahl, P. O., Lopez, J. L., and Trumpower, B. L. (1987) *J. Biol. Chem.* **262,** 8901–8909
26. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) *Biochemistry* **16,** 4743–4751
28. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13
29. Gasser, S. M. (1983) *Methods Enzymol.* **97,** 329–336
30. Racker, E. (1950) *Biochim. Biophys. Acta* **4,** 211–214
31. Côté, C., Solioz, M., and Schatz, G. (1979) *J. Biol. Chem.* **254,** 1437–1439
32. Japa, S., Zhu, Q., and Beattie, D. S. (1987) *J. Biol. Chem.* **262,** 5441–5444
33. Ohashi, A., Gibson, J., Gregor, I., and Schatz, G. (1982) *J. Biol. Chem.* **257,** 13042–13047
34. Hartl, F.-U., Ostermann, J., Guiard, B., and Neupert, W. (1987) *Cell* **51,** 1027–1037
35. Daum, G., Gasser, S. M., and Schatz, G. (1982) *J. Biol. Chem.* **257,** 13075–13080
36. Schmidt, B., Wachter, E., Sebald, W., and Neupert, W. (1984) *Eur. J. Biochem.* **144,** 581–588
37. McAda, P. C., and Douglas, M. G. (1982) *J. Biol. Chem.* **257,** 3177–3182
38. Miura, S., Mori, M., Amaya, Y., and Tatibana, M. (1982) *Eur. J. Biochem.* **122,** 641–647

SUPPLEMENTAL MATERIAL TO - "IMPORT OF THE IRON-SULFUR PROTEIN OF THE CYTOCHROME BC, COMPLEX INTO YEAST MITOCHONDRIA"

William Fu, Shanker Japa and Diana S. Beattie

EXPERIMENTAL PROCEDURES

Construction of pSP64-RIP

The recombinant plasmid, pSP64-RIP, containing the gene for Rieske iron-sulfur protein was constructed as follows. The gene for the iron-sulfur protein of yeast (RIP1) present in the plasmid YEp352dR-RIP was a generous gift of Dr. Bernard L. Trumpower. The plasmid, YEp352dR-RIP (7.2 kbp), was digested with the restriction enzymes, HindIII and SacI, into 2 fragments corresponding to 5.2 and 2 kbp; the latter contained the RIP1 gene. The 2 kbp HindIII-SacI fragment was directionally subcloned into the plasmid pSP64 previously digested with HindIII and SacI. The resulting recombinant, pSP64-RIP, contained the entire RIP sequence as well as the ampicillin resistant gene and the SP6 promoter of pSP64 (Fig.1).

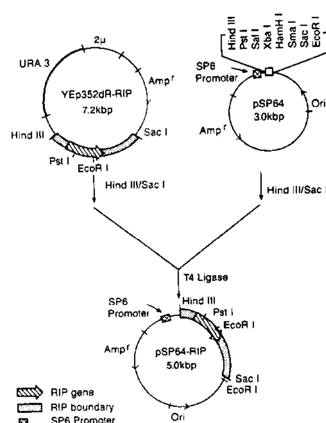

Fig. 1. Construction of pSP64-RIP. YEp352dR-RIP containing the gene coding for the Rieske iron-sulfur protein, was digested with HindIII and SacI restriction enzymes. The HindIII-SacI fragment was directionally subcloned into pSP64 at the HindIII and SacI sites in the presence of T4 DNA ligase. The resulting plasmid was designated pSP64-RIP including a SP6 promoter. Amp^r = ampicillin resistent gene, Ori= Origin.

In vitro Transcription and Translation

Plasmid pSP64-RIP was digested with SacI and the linearized gene extracted with phenol/chloroform and precipitated with ethanol. The linearized plasmids containing 3'-protruding overhangs were converted to blunt ends using T4 DNA polymerase (2.5 units/ug) in 5XSP64 transcription buffer containing 5mM DTT and all 4 dNTPs (0.1 mM each) for 10 min at 37°C. The reaction was terminated by incubating for 5 min at 70°C. The blunt-ended DNA was transcribed in a medium containing 1mM each of ATP, CTP, and UTP, 0.25 mM GTP, 0.5mM ^{m7}GpppG, 100 units RNase inhibitor and 60 units SP6 polymerase in a final volume of 100 ul. The transcription mixture was incubated for 1h at 37°C, then an additional 30 units of RNase inhibitor and 15 units of SP6 polymerase were added, and the incubation was continued for another hour. The reaction was stopped by the addition of 10 units of DNase I. The reaction products were extracted with phenol-/chloroform and precipitated with ethanol. Generally, 30-40 ug of capped mRNAs were obtained from 1 ug of linearized DNA. The capped mRNAs were suspended at a concentration of 0.5 ug/ml in autoclaved water containing ribonuclease inhibitor and stored at -70°C.

Translation in vitro was performed in a nuclease-treated rabbit reticulocyte lysate. RNA (1.0 ug) was added to a mixture containing 35 ul of reticulocyte lysate, 7 ul H_2O, 1 ul of a 1mM amino acid mixture minus methionine, and 5 ul of [^{35}S]-methionine (10 mCi/ml) and incubated for 1h at 30°C. The reaction mixture was centrifuged at 105,000xg for 45 min at 4°C to remove ribosomes.

Northern Blot Hybridization

A probe was synthesized by nick-translation of the PstI-EcoRI fragment of the RIP gene from plasmid with alpha-[^{32}P] CTP using standard procedure (26). The mRNA was separated on 1% agarose gel electrophoresis containing 2.2M formaldehyde as described by Lehrach et al. (27). The resulting denatured mRNA was transferred to Bio-Trace RP Nylon 66 membrane by capillary flow. Following the transfer, the membrane was rinsed in 2x SSC (0.3M NaCl, 0.037M sodium citrate, pH 7.0) and allowed to air-dry. The mRNA was cross-linked to the membrane by UV irradiation. Hybridization was performed at 68°C under conditions described by Feinberg et al. (28). Following hybridization, the membrane was washed twice in 0.5x SSC containing 0.1% SDS for 30 min at room temperature and twice in the same buffer for 30 min at 55°C. The membrane was dried at room temperature and then autoradiographed.

Growth of Yeast and Isolation of Mitochondria

The method used to isolate mitochondria is a slight modification of that described by Gasser (29). The wild-type strain (777-3A) of S. cerevisiae was grown aerobically at 30°C in 1L of semisynthetic medium containing 1g of glucose, 22ml of 90% lactate, 3g of yeast extract (Difco), 0.4g of $CaCl_2$ H_2O, 0.5g of NaCl, 0.7g of $MgSO_4$ $7H_2O$, 1g of KH_2PO_4, 1.2g of $(NH_4)_2$ SO_4, 5mg of $FeCl_3$, and 80 mg of adenine adjusted to pH 5.0 with NaOH.

Cells were grown to the early logarithmic phase (OD_{650} =0.9-1.2), harvested by centrifugation at 3500xg for 5min at room temperature and washed once with distilled water. Cells were preincubated by soaking in a buffer containing 0.1M Tris-HCl (pH 9.4) and 10mM DTT at a concentration of 1g wet weight cells per 3 ml. After preincubation for 15 min at 30°C, the cells were reisolated by centrifugation at 3500xg for 5 min and washed once with 1.2M sorbitol and 20mM KH_2PO_4 (pH7.4). After suspension of cells at a concentration of 0.15g wet weight per ml in the above buffer, Zymolyase 20,000 was added at a concentration of 1 mg per gram cells and the mixture incubated for 1h at 30°C with gentle shaking. The spheroplasts, thus obtained, were isolated by centrifugation at 1500xg for 5min at room temperature and washed twice with the same buffer. The spheroplasts were resuspended in a chilled breaking buffer containing 0.6M mannitol, 20mM Hepes/KOH (pH7.4), 0.1% bovine serum albumin (BSA), and 1mM PMSF at a concentration of 0.3 g per ml. The suspension was homogenized on ice by 10-15 strokes in a tight-fitting Dounce glass homogenizer. The homogenate was diluted with an equal volume of breaking buffer and centrifuged at 1000xg for 10 min at 4°C. The supernatant from the low-speed centrifugation was centrifuged at 10,000xg for 5 min at 4°C and the pellet containing mitochondria was washed once with 0.6M sorbitol and 20mM Hepes/KOH (pH7.4). The final mitochondrial pellet was suspended in the above washing buffer at a concentration of 10 mg protein per ml. The approximate protein content was measured by diluting 10 ul of the mitochondria suspension with 1ml of 0.6% SDS. A 10mg per ml concentration of the original suspension gives an absorbance of 0.2 at 280nm. Only mitochondria with a respiratory control ratio with succinate as substrate of 2.0-2.75 were used for import studies.

Preparation of Mitoplasts

Mitochondria, prepared as described above, were suspended in 0.1M sorbitol and 20mM Hepes/KOH (pH 7.4), incubated at 0°C for 30 min, and centrifuged at 10,000xg rpm for 5 min. The pellet, containing mitoplasts, was resuspended in 0.6M sorbitol and 20mM Hepes/KOH (pH 7.4) at a concentration of 10mg protein per ml.

In vitro Import of the Iron-sulfur Protein into Mitochondria

Isolated mitochondria (200 ug of protein) were preincubated in a 200 ul reaction mixture containing 0.6M sorbitol, 20mM Hepes/KOH buffer (pH 7.4), 40mM KCl, 1mM DTT, 10mM $MgCl_2$, 10mM succinate, 10mM malate, and 2mM ATP for 5 min at various temperatures. Where indicated, EDTA and o-phenanthroline were added during the preincubation to inhibit the processing proteases present in the mitochondrial matrix; 4uM antimycin A and 10uM oligomycin were added to destroy the membrane potential. After the preincubation, a known amount of centrifuged rabbit reticulocyte lysate (3-5% v/v) was added and the incubation continued for 30 min. Mitochondria were reisolated from the import mixture by centrifugation at 10,800 xg for 5 min in a refrigerated microcentrifuge, washed once with 200 ul of 0.6M sorbitol, 20mM Hepes/KOH buffer (pH 7.4), and resuspended in the same buffer. Where indicated, proteinase K (30 ug/ml final concentration) was added to the mitochondria resuspended after the initial centrifugation and the samples were incubated for 30 min at 0°C. Proteinase K activity was stopped by addition of the combination of PMSF and TLCK (1mM each final concentration). The mitochondria were reisolated by centrifugation at 10,800 xg for 5 min and were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Mitochondria were fractionated with digitonin after incubation with rabbit reticulocyte lysate containing ^{35}S-labeled iron-sulfur precursor under various conditions. Mitochondria were reisolated by centrifugation, as described above, washed once with 0.6M sorbitol and 20mM Hepes/KOH (pH 7.4), suspended in the different concentrations of digitonin (derived from a 10% digitonin stock solution), placed on ice for 3 min and then centrifuged for 5 min in a refrigerated microcentrifuge. The pellets were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography while the supernatants were assayed for fumarase activity (30).

Miscellaneous Methods

Plasmid construction, agarose gel electrophoresis were performed by standard techniques as described by Maniatis et al. (26). Autoradiographs were quantitated by measuring relative optical densities using a BioImage laser scanning densitometer.

Materials

L-[^{35}S]Methionine (1200-1400 Ci/mmol) was obtained from Amersham Corp. Nuclease-treated rabbit reticulocyte lysate and amino acid mixture minus methionine were purchased from Promega. HEPES, EDTA, antimycin A, oligomycin, PMSF, TLCK, digitonin and Proteinase K were from Sigma. o-phenanthroline was from Fisher. Zymolyase 20,000 was obtained from ICN Biomedicals, Costamesa, CA.

RESULTS

Transcription and translation of pSP64-RIP

The identity of the recombinant plasmid pSP64-RIP was confirmed by restriction analysis using endonucleases SacI, HindIII and PstI in various combinations. Figure 2 indicates that a 5 kb linearized gene was obtained by the digestion of pSP64-RIP with SacI (lane 2). Digestion of the plasmid with both SacI and HindIII resulted in the formation of a 2 and a 3kb fragment (lane 3). In addition, fragments of 4.48 and 0.52 kb (lane 4) and 3.52 and 1.48 kb (lane 5) were obtained by the digestion of pSP64-RIP with HindIII/PstI and SacI/PstI, respectively. These results indicate that the RIP gene had been successfully subcloned into the expression vector pSP64.

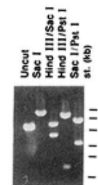

Fig. 2. Restriction analysis of the plasmid. The plasmid, pSP64-RIP was digested with HindIII, SacI, and PstI in various combinations. The fragments produced were separated on a 0.8% agarose gel.

The linearized pSP64-RIP produced by the digestion of SacI was transcribed in vitro in the presence of ^{m7}GpppG and SP6 polymerase. The capped mRNA, thus produced, was verified by both electrophoresis and Northern blot hybridization. A single band corresponding to approximately 1.2 kb in size (Fig. 3A, lane 4) which hybridized to the nick-translated PstI-EcoRI fragment of the RIP gene was observed (Fig. 3A, lanes 1,2,and 3). This mRNA preparation was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]-methionine. Analysis of the translation products by SDS-PAGE followed by autoradiography revealed a single band corresponding to 27.2 kDa (Fig. 3B, lane 2). A band with the same migration was also obtained after immunoprecipitation of the translation mixture with specific antibodies against the iron-sulfur protein (lane 1). The observed molecular weight of the newly-synthesized radiolabeled polypeptide is similar to that reported previously for the precursor form of the iron sulfur protein in yeast (22,31).

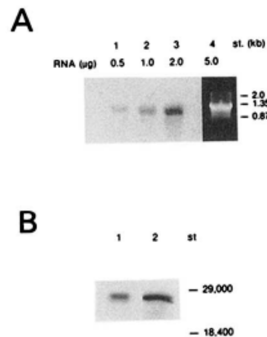

Fig. 3. In vitro transcription and translation of the pSP64-RIP gene. A. The SacI-digested linearized pSP64-RIP was transcribed in vitro as described in "Methods". Various amounts of the capped mRNA were directly loaded and separated on a 1% agarose gel (lane 4) or transferred onto nylon membrane (lanes 1-3). The Northern blots were hybridized with a nick translated DNA probe containing the PstI-EcoRI portion of the coding region of the RIP gene (lanes 1,2,3) as described in "Methods". B. Two ul of the translation mixture after centrifugation at 105,000 xg for 45 min was immunoprecipitated using a specific antiserum against the Rieske iron-sulfur protein (lane 1) as described by Japa et al. (32). One ul of the translation products synthesized in a nuclease treated reticulocyte lysate in the presence of [^{35}S]-methionine (lane 2) was loaded on a 12.5% SDS-PAGE which was dried and subjected to autoradiography. The positions of the prestained marker proteins are indicated on the right side as follows: Mr=29,000, carbonic anhydrase; Mr=18,400, lactoglobulin.