

# Biosynthetic Pathway of Mitochondrial ATPase Subunit 9 in *Neurospora crassa*

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**ABSTRACT** Subunit 9 of mitochondrial ATPase (Su9) is synthesized in reticulocyte lysates programmed with *Neurospora* poly A-RNA, and in a *Neurospora* cell free system as a precursor with a higher apparent molecular weight than the mature protein ( $M_r$  16,400 vs. 10,500). The RNA which directs the synthesis of Su9 precursor is associated with free polysomes. The precursor occurs as a high molecular weight aggregate in the postribosomal supernatant of reticulocyte lysates. Transfer in vitro of the precursor into isolated mitochondria is demonstrated. This process includes the correct proteolytic cleavage of the precursor to the mature form. After transfer, the protein acquires the following properties of the assembled subunit: it is resistant to added protease, it is soluble in chloroform/methanol, and it can be immunoprecipitated with antibodies to  $F_1$ -ATPase. The precursor to Su9 is also detected in intact cells after pulse labeling. Processing in vivo takes place posttranslationally. It is inhibited by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazide (CCCP).

A hypothetical mechanism is discussed for the intracellular transfer of Su9. It entails synthesis on free polysomes, release of the precursor into the cytosol, recognition by a receptor on the mitochondrial surface, and transfer into the inner mitochondrial membrane, which is accompanied by proteolytic cleavage and which depends on an electrical potential across the inner mitochondrial membrane.

Subunit 9 of the oligomycin-sensitive mitochondrial ATPase (Su9), also known as "proteolipid" or "dicyclohexylcarbodiimide (DCCD)-binding protein," is part of the membrane piece  $F_0$  of the enzyme complex (1, 2). It is a highly hydrophobic polypeptide and contains 80–90 amino acids, depending on the species (3). A prominent characteristic of the protein is its solubility in chloroform/methanol mixtures, a property which it shares with only a few other mitochondrial proteins (4, 5). Su9 is thought to be a constituent of the channel through which protons are guided across the mitochondrial membrane to drive the synthesis of ATP (6–8). The protein is also known for its property of binding DCCD (4, 9–11), whereby the ATPase activity of the complex is lost (12, 13). The amino acid sequence of Su9 from a number of organisms has been determined (3, 14).

The biogenesis of this subunit has been studied in yeast (2, 5), *Neurospora* (1), and in higher eukaryotes (15, 16). In yeast this polypeptide is coded for by a mitochondrial gene, which has been isolated and sequenced, and is translated on mitochondrial ribosomes (17, 18). In *Neurospora* this polypeptide is

coded for by a nuclear gene and translated on cytoplasmic ribosomes (1, 19). With higher organisms there are conflicting reports, but the sequence data of the mitochondrial DNA from HeLa and mouse cells clearly support the nuclear localization of this gene (20, 21). This situation raises the intriguing question, how proteins which are homologous in structure and identical in function can be transferred into the inner mitochondrial membrane from the matrix side in a yeast cell and from the cytoplasmic side in a *Neurospora* cell. Protein and DNA sequencing have demonstrated that in yeast the subunit is made without an additional amino- or carboxyterminal sequence (17, 18); even the initiator formylmethionine is retained in the mature protein (3, 22). Translation of *Neurospora* messenger RNA in a wheat germ extract and in a reticulocyte lysate has yielded a product with a higher apparent molecular weight suggesting that a larger precursor form is the primary product (19, 23).

Here we report experiments designed to elucidate the transport pathway of the protein from the ribosome to its functional site in the inner membrane. They show that in heterologous

and homologous translation systems the protein in *Neurospora* ( $M_r$  10,500) is made as a larger precursor with an  $M_r$  of 16,400. The precursor in the postribosomal supernatant of the reticulocyte lysate is probably present as an aggregate. This precursor is posttranslationally imported into mitochondria in vitro. During this transport, it is correctly processed to the size of the mature protein. After transfer in vitro the protein acquires the following properties of the mature assembled Su9: it is resistant to added protease, it is soluble in chloroform/methanol in contrast to the precursor form, and it can be precipitated with antibodies to  $F_1$ -ATPase. It is shown that the precursor can also be detected in vivo in pulse-labeled cells. The processing in vivo occurs posttranslationally and is energy dependent.

## MATERIALS AND METHODS

Growth of *Neurospora*, radioactive labeling, subfractionation of cells, incorporation of [ $^3$ H]leucine in a cell free system, and protein synthesis in reticulocyte lysates were performed as described earlier (24-26). Isolation of mitochondria, microsomes, and free polysomes for subsequent extraction of RNA was carried out according to Scheele et al. (27). RNA from these fractions was obtained as described in references 28-30.

**Sucrose Density Gradient Centrifugation:** Linear sucrose gradients were prepared by mixing 5.5 ml of 5% sucrose with 5.5 ml of 35% sucrose (wt/wt). The solvent was 0.3 M KCl, 10 mM Tris-HCl, pH 7.5. Samples to be centrifuged were made 0.3 M in KCl. Centrifugation was carried out in a Beckman ultracentrifuge (rotor SW41 Ti; Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 207,000 g for 20 h at 1°C. Gradient fractions (1.2 ml) were obtained by puncturing tubes at the bottom. Density of fractions was determined in a Zeiss refractometer. Fractions were subjected to immunoprecipitation.

**Immunoprecipitation:** Su9 and  $F_1$ -ATPase were isolated according to the procedures described by Sebald and co-workers (4, 31). Antibodies against Su9 were prepared as described in reference 32. Immunoprecipitation was carried out under the following conditions: mitochondria (0.25-0.5 mg of protein) were lysed in 50  $\mu$ l of 2% SDS, 60 mM Tris-HCl, pH 6.8, 5% (vol/vol) 2-mercaptoethanol by boiling for 3 min. Then 1 ml of Triton buffer (1% Triton X-100, 0.3 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5) was added. The mixture was adjusted to 1 mM PMSF and freed from insoluble material by centrifugation for 15 min at 27,000 g. 0.5 ml of antiserum was added to the supernatant and the mixture was kept at 4°C for 16 h. The precipitate was collected and washed three times with Triton buffer and once with 10 mM Tris-HCl, pH 7.5.

Immunoprecipitations from reticulocytes lysate supernatants and from fractions of sucrose density gradients were performed according to Zimmermann and Neupert (26), except that KCl was replaced by NaCl.

The immunoprecipitates were dissolved either in 80  $\mu$ l of 2% SDS, 60 mM Tris-HCl, pH 6.8, 5% (vol/vol) 2-mercaptoethanol or in 40  $\mu$ l of 2% SDS, 100 mM Tris-HCl, pH 8.0, 5% (vol/vol) 2-mercaptoethanol depending on the gel system used (see below). The samples were shaken for 1 h at 4°C and for 2 min at 95°C.

Preparation of antibodies against  $F_1$ -ATPase and immunoprecipitation was carried out as previously described (4).

**Transfer In Vivo of Su9:** *Neurospora* cells were grown at 25°C for 14 h as described above. 90 ml of the culture (containing ~150 mg of cells) were then cooled to 8°C and 5  $\mu$ Ci/ml [ $^3$ H]leucine (40-60 Ci/mmol; New England Nuclear Chemicals, Boston, MA) was added. After a pulse period of 3 min, the assay was divided into three equal portions. The first portion was immediately filtered at 0°C. The second sample was incubated for another 5 min at 25°C in the presence of 0.05 mg/ml unlabeled leucine and 0.1 mg/ml cycloheximide. The third sample received 8 nmol/ml carbonylcyanide *m*-chlorophenylhydrazide (CCCP) and the same amounts of unlabeled leucine and cycloheximide as sample two. Then portions two and three were filtered at 0°C. The hyphae were immediately homogenized in 1 ml boiling 3% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM *o*-phenanthroline, pH 7.5, using an Ultra-Turrax (Janke und Kunkel UG, IKA-Werk, Staufen, W. Germany). After boiling for 5 min, the samples were cooled to room temperature, diluted with 30 vol of Triton buffer, and centrifuged at 27,000 g for 15 min. The supernatant was incubated with antiserum overnight at 4°C. Then the appropriate amount of swollen Protein A-agarose (Sigma Chemical Co., St. Louis, MO) was added. The mixture was shaken for 1 h at room temperature. The immune complex was collected by centrifugation and washed three times with Triton buffer and once with 10 mM Tris-HCl, pH 7.5.

**Analytical Procedures:** Electrophoresis in 15% polyacrylamide horizontal and vertical slab gels was performed according to Korb and Neupert (24)

and Laemmli (33), respectively. Unless otherwise indicated, gels were autoradiographed (23). Fluorography was carried out according to Chamberlain et al. (34). Microscale radio-sequencing was carried out as described by Tarr (35). Protein was determined according to Lowry et al. (36).

## RESULTS

### *Su9 Is Synthesized as a Larger Precursor in Cell Free Systems*

A reticulocyte lysate programmed with *Neurospora* RNA in the presence of [ $^{35}$ S]-methionine synthesizes a product which can be immunoprecipitated with antibodies against isolated Su9. Fig. 1 shows the gel electrophoretic and autoradiographic analysis of this product in comparison with the mature Su9. The mature form, both the isolated protein as well as the protein immunoprecipitated from [ $^{35}$ S]sulfate labeled mitochondria, displays a single band with an  $M_r$  of 10,500. The precursor form migrates with an  $M_r$  of 16,400. This confirms the earlier observation by Michel et al. (19) that in a wheat germ cell free system Su9 is synthesized as a larger precursor.

Synthesis of Su9 was also investigated in a cell free homol-

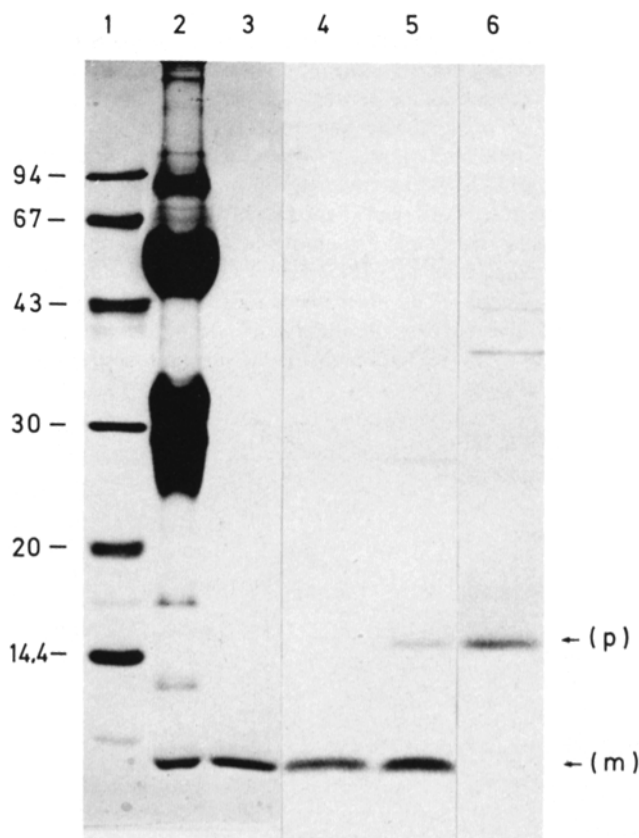


FIGURE 1 Synthesis in vitro of Su9. (Lane 1) Molecular weight marker mix. Marker proteins were: phosphorylase *b* (94,000 daltons), albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), trypsin inhibitor (20,000 daltons), and  $\alpha$ -lactalbumin (14,400 daltons). (Lane 2) Immunoprecipitate of Su9 from mitochondria, Coomassie Blue stain. (Lane 3) Isolated protein with Coomassie Blue stain. (Lane 4) Autoradiography of an immunoprecipitate of mature Su9 from [ $^{35}$ S]sulfate labeled mitochondria. (Lane 5) Autoradiography of an immunoprecipitate of Su9 transferred and processed in vitro (for details, see legend to Fig. 6). (Lane 6) Autoradiography of an immunoprecipitate of the precursor to Su9 synthesized in a reticulocyte lysate. (p) Precursor. (m) Mature protein.

ogous system from *Neurospora*. A postmitochondrial supernatant of *Neurospora*-cells was incubated for 10 min with [<sup>3</sup>H]leucine. It has been shown previously that completion of polypeptide chains occurs within the first 10 min of incubation (25). Then the ribosomes were spun down and the precursor of Su9 was immunoprecipitated from the supernatant. As shown in Fig. 2, the immunoprecipitated band displays the same  $M_r$  as the precursor synthesized in the reticulocyte lysate system.

### Precursor to Su9 Is Detected after Pulse Labeling In Vivo

A *Neurospora* culture was adjusted to 8°C and labeled leucine was added. After 3 min, the culture was divided into three portions. One portion was immediately harvested. To the second and third portion, excess unlabeled leucine and cycloheximide were added and incubation was continued for another 5 min at 25°C before harvesting. The third portion received in addition the uncoupler CCCP. All portions were harvested and treated with boiling SDS-containing buffer. After adding Triton buffer to the extracts, immunoprecipitation was performed with antibodies against Su9. As shown in Fig. 3, lane 1, the main part of radioactivity was found in the mature protein but the precursor was also labeled. After the chase, the labeled precursor disappears and the radioactivity in the mature protein increases (Fig. 3, lane 2). CCCP blocks the conversion of precursor to mature protein (Fig. 3, lane 3), apparently by a breakdown of the mitochondrial membrane potential (32). The radioactivity in the precursor is higher in the CCCP-treated cells than in the pulse-labeled cells, presumably because transfer does not immediately stop when the cells are harvested. CCCP and the chase are effective within 5 s (unpublished data). The decrease of radioactivity in the precursor and the increase in the mature form do not agree quantitatively. The reason for this is the incomplete precipita-

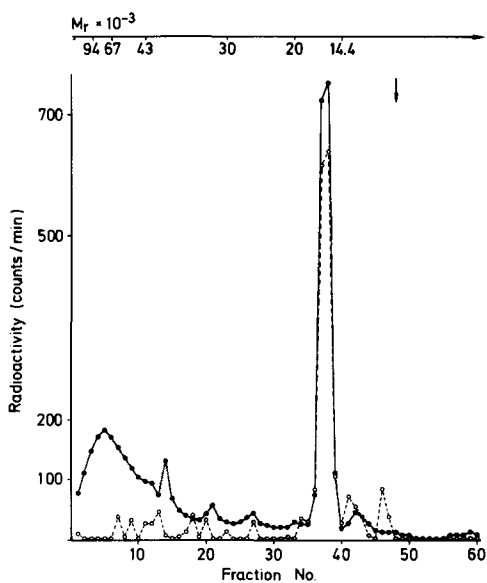


FIGURE 2 Synthesis of the precursor to Su9 in a homologous cell free system. Synthesis in vitro was carried out in a homologous system in the presence of [<sup>3</sup>H]leucine, and in a reticulocyte lysate in the presence of [<sup>35</sup>S]methionine. Immunoprecipitates were mixed and subjected to electrophoresis. Gels were sliced and radioactivities were determined. Arrow indicates position of mature Su9. (○) <sup>3</sup>H Radioactivity. (●) <sup>35</sup>S Radioactivity.

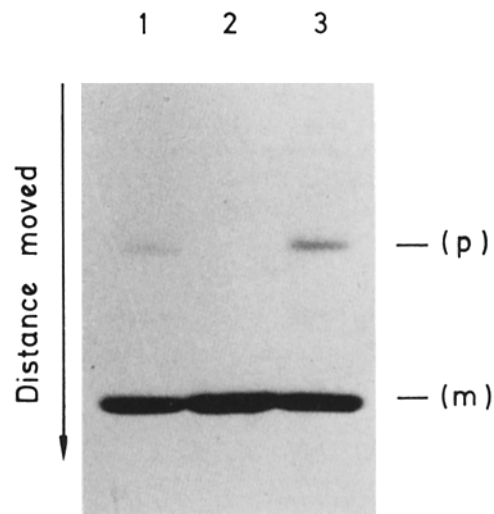


FIGURE 3 Synthesis of Su9 precursor and its processing in vivo. *Neurospora* cells were pulsed at 8°C with [<sup>3</sup>H]leucine for 3 min. Then the cell suspension was divided into three aliquots and treated as described below. Su9 was immunoprecipitated after boiling and disrupting the cells in SDS containing buffer. The precipitates were subjected to SDS PAGE and fluorographed (34). (Lane 1) Pulse for 3 min. (Lane 2) Pulse for 3 min, then addition of cycloheximide and unlabeled leucine and further incubation at 25°C for 5 min. (Lane 3) Pulse for 3 min, then addition of cycloheximide, unlabeled leucine and CCCP and further incubation at 25°C for 5 min. (p) Precursor. (m) Mature Su9.

tion of the precursor form by the antibody raised against the mature Su9. The quantity of precursor present after a 3-min pulse was estimated by comparing the amount of label in the mature form after chase to the amount of mature Su9 after chase in the presence of CCCP. This was done by cutting out bands and counting. The label in the mature Su9 band was greater by a factor of 1.8 following the chase in the absence of CCCP than it was in the presence of CCCP (data not shown). This means that after the 3-min pulse ~60% of the labeled chains were already processed and therefore already imported into the mitochondria, since processing occurs within the mitochondria (see below). These data clearly show that the precursor is not an in vitro artifact, and that it is posttranslationally processed and its pool size is extremely small.

### Precursor of Su9 Is Synthesized by Free Polysomes

Nucleic acids were isolated from the following subcellular fractions: free polysomes, microsomes, and mitochondria. The yield was 9.6, 0.9, and 0.8  $A_{260 \text{ nm}}$  U/g of cells, respectively. Reticulocyte lysates were incubated with equal amounts of the respective nucleic acids (27  $A_{260 \text{ nm}}$  U/ml translation mixture). RNAs from free and bound polysomes were almost equally effective in stimulating protein synthesis and two different sets of proteins were obtained (37). Precursor to Su9 was immunoprecipitated from the postribosomal supernatant of the translation assays and analysed by gel electrophoresis and autoradiography. The precursor protein was mainly synthesized under direction of the message from free polysomes (Fig. 4). Considering the relatively small amounts of membrane bound ribosomes in *Neurospora* we conclude that the translation of Su9 occurs by free ribosomes in the cytosol.

### Precursor to Su9 Forms Aggregates

Precursor to Su9 was synthesized in a reticulocyte lysate and the postribosomal supernatant was prepared. The supernatant was layered on a sucrose gradient and centrifuged for 20 h.

The fractions of the gradient were subjected to immunoprecipitation. Fig. 5 shows the distribution of the precursor protein over the gradient. The precursor migrates in a molecular weight range between about 80,000 and 240,000 (5 to 15 times the  $M_r$  of the precursor in SDS-containing gels).

A similar situation has been found with the precursor of the mitochondrial ADP/ATP carrier (26). In both the cases, however, demonstration that these higher  $M_r$  forms are homooligomers is lacking due to the difficulties in analysing the extremely small amounts of protein synthesized in the cell-free systems.

### Precursor Is Transferred into Mitochondria and Processed In Vitro

The postribosomal supernatant of a reticulocyte lysate containing Su9 precursor synthesized in vitro was incubated with

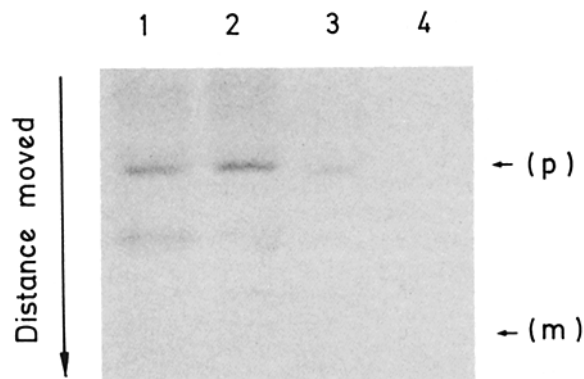


FIGURE 4 Synthesis of Su9 by free ribosomes. Reticulocyte lysates were programmed with nucleic acids from (lane 1) total polysomes, (lane 2) free polysomes, (lane 3) membrane bound polysomes, and (lane 4) mitochondria-associated polysomes. Su9 precursor was immunoprecipitated from each of the four lysates. (p) Precursor to subunit 9. (m) Mature Su9.

mitochondria from *Neurospora* spheroplasts. After increasing time periods of incubation, mitochondria and supernatant were separated again by centrifugation and Su9 was immunoprecipitated from both fractions. As shown in Fig. 6, the precursor disappeared from the supernatant and a labeled protein appeared in the mitochondria which had the molecular weight of mature Su9 (see also Fig. 1). After 60 min of incubation, >90% of the precursor had disappeared from the supernatant and the radioactivity was recovered in the mature protein in the mitochondria.

Transfer of Su9 was also observed in a homologous system. A cell free homogenate containing the majority of the mitochondria was prepared from cells grown on [ $^{35}$ S] sulfate to label the assembled protein and incubated with [ $^3$ H]leucine. After various time periods mitochondria were isolated from the homogenate and Su9 was immunoprecipitated. The immunoprecipitates were subjected to gel electrophoresis and the gels

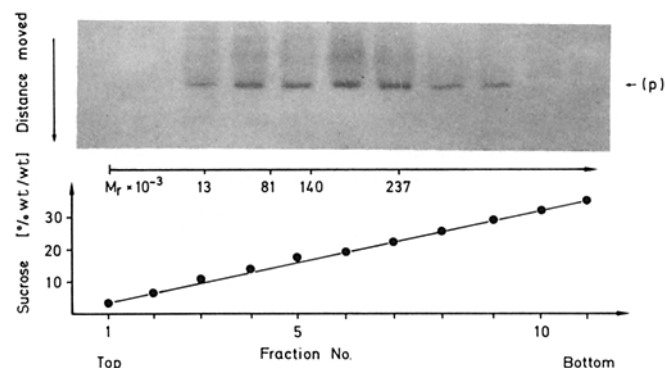


FIGURE 5 Density gradient centrifugation of the precursor to Su9. A reticulocyte lysate supernatant containing radio-labeled *Neurospora* proteins was centrifuged on a sucrose density gradient. 11 fractions (1.2 ml each) were obtained and the sucrose concentration was determined in a Zeiss refractometer. Immunoprecipitation of Su9 was carried out with each fraction. Molecular weight markers were: cytochrome *c* (13,000 daltons), creatine kinase (81,000 daltons), lactate dehydrogenase (140,000 daltons), pyruvate kinase (237,000 daltons). Marker proteins were centrifuged in parallel tubes. The positions of the respective proteins were determined by measuring the absorbancy at 280 nm  $A_{280\text{nm}}$ . The proteins were recovered in a volume of 2.4 ml (two fractions). (p) Precursor to Su9.

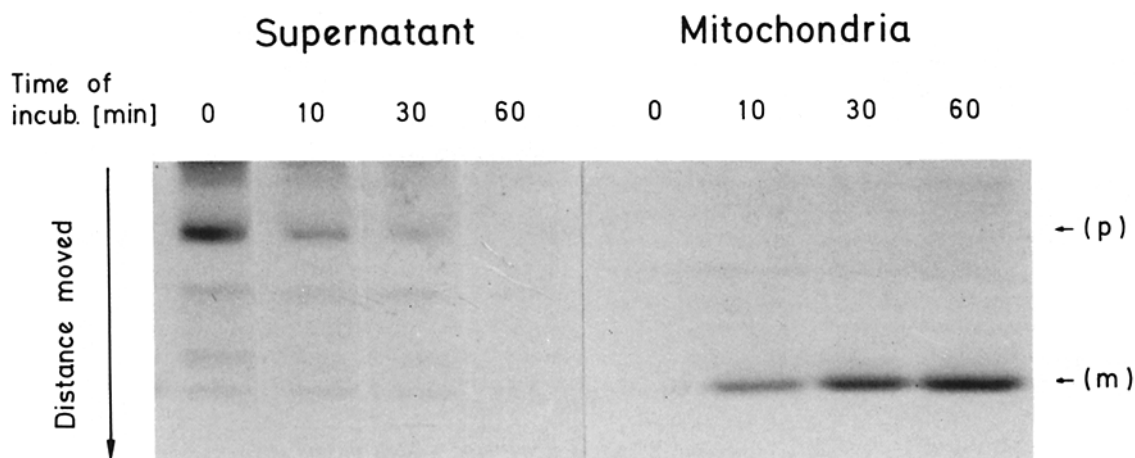


FIGURE 6 Kinetics of transfer in vitro of Su9 into isolated mitochondria. A postribosomal supernatant was prepared from a reticulocyte lysate containing [ $^{35}$ S]methionine-labeled *Neurospora* precursor proteins. Sucrose and methionine were added to final concentrations of 0.2 M and 25  $\mu$ M, respectively. Mitochondria isolated from spheroplasts were resuspended in this mixture at a concentration of about 1 mg/ml protein. After incubation for 0, 10, 30, and 60 min at 25°C, mitochondria and supernatant were again separated by centrifugation. Immunoprecipitation was carried out with the supernatant and with the mitochondrial pellet. (p) Precursor. (m) Mature protein.

were sliced (Fig. 7). During prolonged incubation the  $^3\text{H}:^{35}\text{S}$  ratio increased in the 10,500 dalton peak of the mature protein indicating that [ $^3\text{H}$ ]leucine-labeled precursor protein was processed and transferred into mitochondria.

### Su9 Transferred In Vitro Displays Properties of the Mature Protein

Treatment of mitochondria after transfer in vitro with Proteinase K (50  $\mu\text{g}/\text{ml}$ ) shows that the protein has been transferred into a location where it is largely if not totally protected from protease. The mature assembled Su9 was resistant to protease in a similar fashion. The precursor in the supernatant, however, was degraded under these conditions. Depending on the amount of protease added, the precursor was digested to fragments which were not picked up by the antibody (see also reference 23), or, at lower concentrations, to a fragment migrating slightly faster than the mature protein (Fig. 8). This 9,900-dalton fragment was much better recognized by the antibody than the precursor.

Su9 can be extracted from mitochondrial membranes by chloroform/methanol mixtures (4) (see also Fig. 9). We checked whether this property of the mature protein was shared by the precursor in the supernatant and the protein transferred in vitro. Fig. 9 demonstrates that after extraction of the postribosomal supernatant the precursor was not recovered in the chloroform/methanol phase but remained in the residual protein fraction. In contrast, Su9 transferred and processed in vitro could be extracted by chloroform/methanol.

### Precursor to Su9 Is Correctly Processed In Vitro

The apparent molecular weight of the protein transferred in vitro is indistinguishable from that of the protein labeled in vivo (see Fig. 1). The sequence of the mature protein starts with tyrosine and has a second tyrosine in position 56 (3). To establish that the proteolytic cleavage of the precursor actually occurs at the correct site, protein synthesis in the reticulocyte lysate was carried out in the presence of [ $^3\text{H}$ ]tyrosine. After

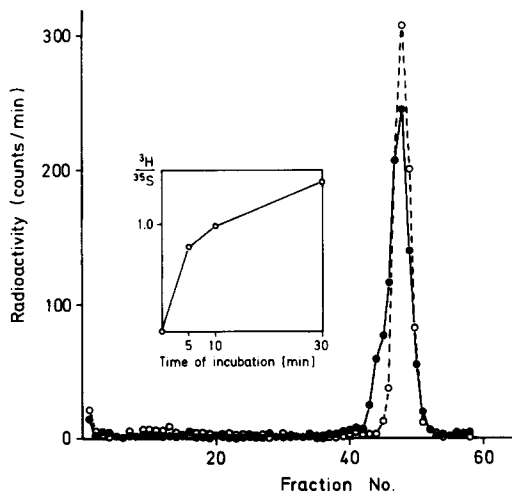


FIGURE 7 Transfer of Su9 in a homologous *Neurospora* cell free system. A cell free homogenate of  $^{35}\text{S}$ -labeled *Neurospora* cells was incubated with [ $^3\text{H}$ ]leucine. After 0, 5, 10, and 30 min of incubation at 25°C, mitochondria were isolated by centrifugation and Su9 was immunoprecipitated. The immunoprecipitates were analyzed as described in the legend to Fig. 2. (○)  $^3\text{H}$ -Subunit 9 transferred after 30 min. (●)  $^{35}\text{S}$ -preassembled mature protein. The insert shows the increase of the  $^3\text{H}:^{35}\text{S}$  ratio in the peak of the mature Su9.

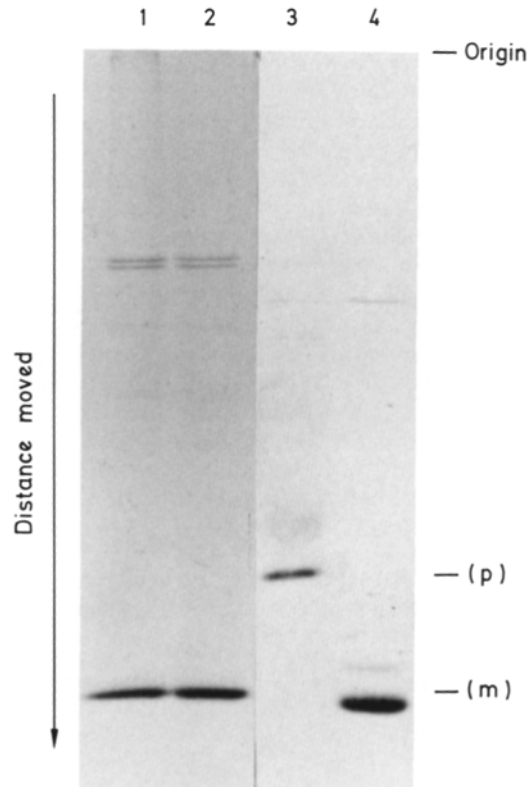


FIGURE 8 Sensitivity to protease of Su9 precursor and Su9 transferred into mitochondria in vitro. Transfer in vitro was carried out for 60 min as described in the legend to Fig. 6. To one half of the mixture, Proteinase K was added (50  $\mu\text{g}/\text{ml}$ ), and both halves were kept at 4°C for 1 h. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. Mitochondria were isolated and Su9 was immunoprecipitated. In a parallel experiment, a reticulocyte lysate supernatant containing radio-labeled Su9 precursor was treated with Proteinase K in the same way. (Lane 1) Su9 transferred in vitro, control. (Lane 2) Su9 transferred in vitro, Proteinase K treatment. (Lane 3) Precursor, control. (Lane 4) Precursor, Proteinase K treatment. (p) Precursor. (m) Mature protein.

transfer of the precursor into isolated mitochondria, the protein was immunoprecipitated and subsequently analysed by manual Edman degradation. Table I gives the radioactivities recovered in the first three Edman steps. About 30% of the total activity employed in this experiment is recovered in the first cycle. The same result was obtained when mature Su9 which was labeled in vivo with [ $^3\text{H}$ ]tyrosine was subjected to the same procedure. When the precursor was analyzed, no significant amounts of radioactivity were found in the first three Edman steps. As a control [ $^{35}\text{S}$ ]methionine labeled precursor and mature protein were also sequenced. No significant amounts of methionine were released during the first three steps. Apparently, the initiating methionine is absent from the precursor. The mature protein contains four methionines in positions 9, 18, 73, and 77 (3). We conclude from these data that processing in vitro occurs at the right position.

### Antibodies against $F_1$ -ATPase Precipitate a Large Portion of Su9 Transferred In Vitro

When mitochondria from cells grown on [ $^{35}\text{S}$ ]sulfate were dissolved in a Triton X-100 containing medium with low ionic strength, antibodies from rabbits immunized with  $F_1$ -ATPase co-precipitated labeled Su9 together with the large subunits of  $F_1$  and with a number of unidentified peptides (Fig. 10). It has

been shown that under these conditions the  $F_1F_0$  complex is partially preserved. About 50% of total Su9 was found in the immunoprecipitate (4). When mitochondria were first dissolved in hot SDS in order to dissociate the ATPase complex and then treated with Triton X-100, the antibody did not precipitate any Su9.

When transfer in vitro of labeled precursor to Su9 had been carried out, the antibody against  $F_1$  precipitated a significant part of labeled mature Su9. In light of the above mentioned partial dissociation of the complex after lysis of mitochondria

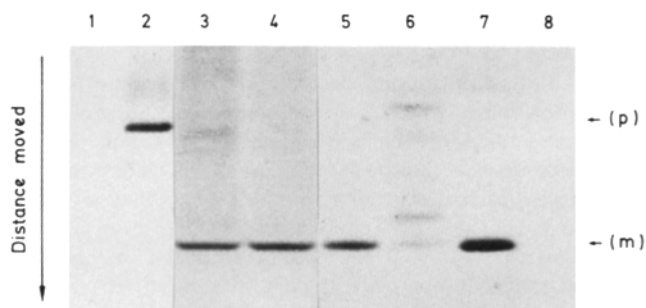


FIGURE 9 Differential solubility of precursor and mature Su9 in chloroform/methanol. (Lanes 1 and 2) A reticulocyte lysate supernatant (600  $\mu$ l) containing [ $^{35}$ S]methionine labeled precursor was precipitated with acetone, and 0.5 mg of acetone precipitated unlabelled mitochondrial protein was added. The dried precipitate was extracted five times with chloroform/methanol (2:1, vol/vol). The extract was evaporated to dryness and resolubilized in 2.6 ml 2% SDS, 60 mM Tris-HCl, pH 6.8. The dried precipitate was dissolved in the same buffer. After dilution with Triton buffer (see Materials and Methods) immunoprecipitation of Su9 from both fractions was carried out. (Lanes 3 and 4) The same experiment described for lanes 1 and 2 was carried out, except that the mitochondrial protein was labeled (in vivo labeling with [ $^{35}$ S]sulfate) and not the reticulocyte lysate. This experiment served as a control, to make sure that in the presence of the large amounts of reticulocyte lysate protein, Su9 can be extracted with chloroform/methanol. (Lanes 5 and 6) Transfer in vitro was carried out for 60 min as described in the legend to Fig. 6. Mitochondria were extracted with chloroform/methanol. The extract and the residue were treated as described for lanes 1 and 2. (Lanes 7 and 8) Mitochondria labelled with [ $^{35}$ S]sulfate in vivo were treated as described for lanes 5 and 6. (Lanes 1, 3, 5, and 7) Immunoprecipitation from chloroform/methanol extracts. (Lanes 2, 4, 6, and 8) Immunoprecipitation from residual protein fractions. (p) Precursor. (m) Mature protein.

with detergent, it can be concluded that a considerable part of total Su9 transferred in vitro becomes associated with the  $F_1$ -piece. The remainder of the protein could be precipitated with antibodies to Su9 from the supernatant of the anti- $F_1$  precipitation sample (Fig. 10). To verify the identity of Su9 transferred in vitro and recovered in the immunoprecipitate obtained with antibodies against  $F_1$ -ATPase, the immunoprecipitate was re-dissolved in SDS and immunoprecipitation with antibodies against Su9 was carried out. Although the recovery was quite low due to the high tendency of Su9 to attach to centrifuge tubes, etc., a band co-migrating with mature Su9 was seen (Fig. 10).

In a further experiment, transfer in vitro of  $^{35}$ S-labeled Su9 was performed with mitochondria from cells grown in the presence of [ $^3$ H]leucine. After transfer, immunoprecipitation with antibodies against  $F_1$  and against Su9 was carried out in parallel. The immunoprecipitates were electrophoresed and the gels were sliced. The  $^{35}$ S: $^3$ H ratios of the Su9 peaks were determined. The ratios were 0.29 for the anti- $F_1$  precipitate and 0.61 for the anti-Su9 precipitate. This again indicates that a significant part of total Su9 transferred in vitro can be immunoprecipitated with antibodies to  $F_1$ -ATPase.

These data suggest that after transfer and processing the mature Su9 becomes associated with the ATPase complex. It is not known whether this occurs by exchange of subunits before or after lysing the mitochondria. In any case, the data show that Su9 which is transferred and processed in vitro acquires properties which allow it to interact with other subunits of the ATPase complex.

## DISCUSSION

Su9 of the oligomycin sensitive ATPase in *Neurospora* is one of the mitochondrial proteins whose extramitochondrial precursors carry an additional sequence (38, 39). In the case of Su9, the additional sequence is relatively large and may comprise more than one third of the precursor. Antibodies prepared against the mature Su9 interact only weakly with the precursor but strongly with the processed form, indicating that there is a major difference between the conformations of the mature protein and the precursor form. The sequencing data of mature and precursor form suggest that the additional sequence is located at the amino terminus. The precursor form occurs in the supernatant of the reticulocyte lysate as an aggregate with a broad range in  $M_r$ . This aggregate apparently does not

TABLE I  
Edman Degradation of Different Forms of Radio-labeled Su 9

Label	Precursor				Protein transferred in vitro				Mature protein			
	$^3$ H]Tyrosine		$^{35}$ S]Methionine		$^3$ H]Tyrosine		$^3$ H]Tyrosine		$^{35}$ S]Methionine			
	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%		
Radioactivity employed	2,799	100	4,617	100	1,250	100	1,640	100	2,060	100		
Radioactivity released												
First cycle	137	4.9	63	1.4	373	29.8	560	34.1	96	4.7		
Second cycle	32	1.1	29	0.6	68	5.4	124	7.6	25	1.2		
Third cycle	25	0.9	33	0.7	40	3.2	41	2.5	20	1.0		

Precursor proteins were synthesized in reticulocyte lysates in the presence of 0.45 mCi/ml [ $^{35}$ S]methionine (1,000 Ci/mmol) or 0.08 mCi/ml [ $^3$ H]tyrosine (76 Ci/mmol, NEN Chemical, Boston, MA). The postribosomal supernatants were subjected to immunoprecipitation. Mature proteins were obtained by labeling intact cells with [ $^3$ H]tyrosine (100  $\mu$ Ci/100 ml of culture) or [ $^{35}$ S]sulfate\* (100  $\mu$ Ci/100 ml of culture) for 15 min or 16 h, respectively. Mitochondria were isolated and Su9 was immunoprecipitated. In vitro transferred Su9 was obtained by carrying out a transfer experiment for 60 min as described in the legend to Fig. 6, except that the precursor proteins were labeled with [ $^3$ H]tyrosine (see above). Su9 was immunoprecipitated after reisolating the mitochondria. The immunoprecipitates were dissolved and subjected to gel electrophoresis. The gels were dried and fluorographed (34). The radioactive bands were cut out, washed in water, and extracted with 80% (vol/vol) formic acid. After removal of the formic acid, the proteins were subjected to manual Edman degradation.

\* Incubation of cells with [ $^{35}$ S]sulfate leads only to a labeling of methionine in Su9 since cysteine is not present in the mature protein (3).

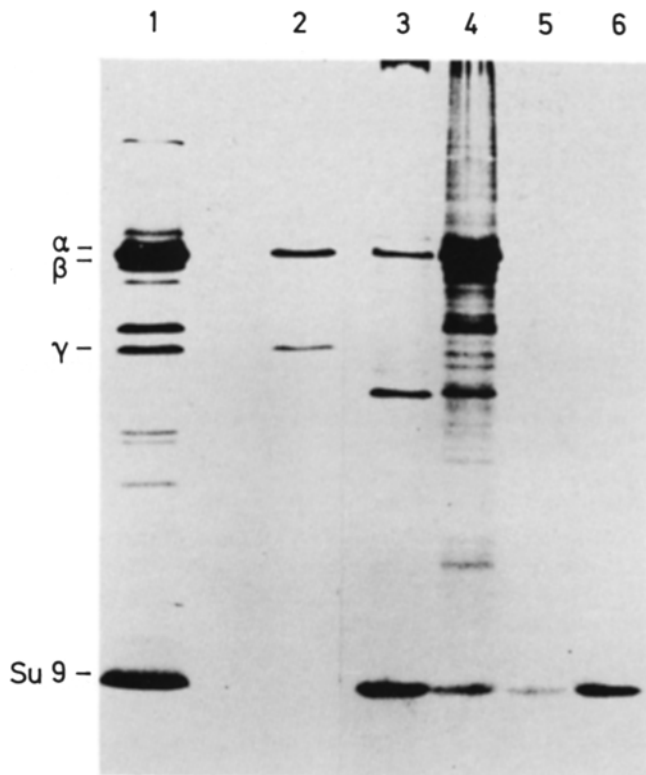


FIGURE 10 Immunoprecipitation from mitochondria of Su9 transferred in vitro with antibodies to  $F_1$ -ATPase. (Lane 1) Immunoprecipitation of the  $F_1F_0$  complex with antibodies to  $F_1$ -ATPase from [ $^{35}$ S]sulfate-labeled mitochondria. (Lane 2) [ $^{35}$ S]sulfate-labeled mitochondria were boiled for 2 min in 50  $\mu$ l of 2% SDS, 5% 2-mercaptoethanol, 60 mM Tris-HCl, pH 6.8. 1 ml of Triton buffer was added and immunoprecipitation with antibodies to  $F_1$ -ATPase was carried out. (Lanes 3-6) Transfer in vitro of Su9 into isolated mitochondria was carried out for 1 h as described in the legend to Fig. 6. Immunoprecipitations were performed with reisolated mitochondria. (Lane 3) Immunoprecipitation with antibodies to Su9. (Lane 4) Immunoprecipitation of  $F_1F_0$  complex with antibodies to  $F_1$ -ATPase. (Lane 5) The same experiment described in lane 4 was carried out. The immunoprecipitate was dissolved in 50  $\mu$ l of 2% SDS and diluted with Triton buffer. Then immunoprecipitation with antibodies to Su9 was carried out. (Lane 6) The  $F_1F_0$  complex was immunoprecipitated as in lane 4. The supernatant was saved and immunoprecipitation was performed with antibodies against Su9. ( $\alpha$ )  $\alpha$ -Subunit of  $F_1$ -ATPase. ( $\beta$ )  $\beta$ -Subunit of  $F_1$ -ATPase. ( $\gamma$ )  $\gamma$ -Subunit of  $F_1$ -ATPase. (Su9) Su9.

represent denatured material, because it can be transferred into mitochondria and processed to the mature size. Aggregates of extramitochondrial precursor forms have been described for other mitochondrial proteins such as ADP/ATP carrier (26) and cytochrome *c* oxidase subunit V (38). The physiological significance of this aggregation remains unclear.

On the basis of experiments presented here and in other publications (23, 32), it is possible to propose a mechanism for the transfer of ATPase Su9 into the mitochondria. First, Su9 is synthesized as a precursor with an additional sequence at the amino terminus by free cytoplasmic ribosomes and is released into the cytosolic fraction, where it enters an extramitochondrial precursor pool. From this pool it is transferred into the mitochondria. The intracellular transfer of this protein therefore is a posttranslational event as already shown before for a number of other proteins (39, 41).

The second step in the transfer process is the recognition of

the precursor by the mitochondrion, i.e., a specific binding to a receptor on the outer mitochondrial membrane. This step can be resolved when the potential across the inner mitochondrial membrane is dissipated. This was achieved by including in the in vitro transfer system protonophores, or valinomycin plus  $K^+$ , or a combination of the inhibitors antimycin A and oligomycin. Under these conditions, binding of the precursors to the surface of the mitochondria still takes place (26, 32, 41). The bound precursor is very sensitive to added proteinase, in contrast to the transferred and processed Su9 (23). Specific binding to receptorlike sites has already been demonstrated for apocytochrome *c*, the extramitochondrial precursor to holo-cytochrome *c* (42).

The third step is the translocation of the precursor into the inner membrane, which is accompanied by the proteolytic removal of the additional sequence. Differentiation of these two processes has not been possible as yet. They may be intrinsically linked. Furthermore, processing may occur in more than one step, as demonstrated for other mitochondrial precursors (43, 44). Su9 is correctly processed in vitro and thereby acquires properties of the mature subunit. For example it is soluble in chloroform/methanol, in contrast to the precursor form.

After transfer in vitro Su9 interacts with other components of the  $F_1F_0$  complex, i.e., partial assembly appears to occur. Assembly in vitro of proteins imported into chloroplasts has been reported. Chua and co-workers (45, 46) have demonstrated that in isolated chloroplasts the small subunit of ribulose-1,5-bisphosphate carboxylase becomes associated with the large subunit after transfer and processing in vitro (45). Furthermore, the polypeptides 15 and 16 of the light harvesting complex in chloroplasts were shown to acquire the ability to bind chlorophyll after transfer in vitro (46). With mitochondria it was observed that ADP/ATP carrier transferred in vitro acquires the ability to bind carboxyatractylate and to pass through hydroxyapatite columns (M. Schleyer and W. Neupert, unpublished observations). Whether Su9 becomes part of a fully assembled and functional ATPase remains unclear in light of the considerable complexity of this multisubunit enzyme.

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