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Disclosure of Major Causes of Mitochondrial Mutation by means of Molecular Biology

Part 3: Mitochondrial Genes

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I. INTRODUCTION

In the first part of this study, the author carried out experiments using yeasts to disclose the developmental mechanism of mitochondrial mutation, i.e., respiration deficient petite mutant, at the molecular level.

Following these experiments the author concluded that respiration deficient petite mutant can be developed by inhibitor of protein synthesis of not mitochondrial, but nucleic cytoplasmic protein synthesis, especially mitochondrial DNA as well as RNA polymerase, which is controlled by nucleic DNA.

For the second part, the authors observed more precisely the interaction between nuclear genes and mitochondrial genes during development of mitochondrial mutations. They fractionated mitochondria from several respiration deficient mutant strains, whose degradations were caused by anomalies of nuclear genes as well as mitochondrial genes. Following that, they observed in vitro mitochondrial DNA and RNA synthesis to interpret the developmental

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mechanism in respective petite mutants in comparison with those of the wild type.
As a way to identify the function of mitochondrial genes in the development of mitochondrial organelle, the authors carried out experiments using inhibitors cycloheximide against protein synthesis in cytoplasm, chloramphenicol against mitochondrial protein and ethidium bromide, which conjugates with mitochondrial DNA at low concentrations and inhibits the development of hereditary information (Meyer & Simpson 1969; Lepecq & Paoletti 1969; Li & Crothers 1969), and observed how these antibiotics affected the respiration ability of yeast. To quantify the above-mentioned results, ATPase activities of fractionated mitochondria as its coupling Factor F_1, which plays a very important role in oxidative phosphorylation, of wild type yeast, which were cultured in a medium containing inhibitors of protein synthesis, were measured.
From these experiments, genetic information contained in mitochondrial DNA were investigated.

II. MATERIALS AND METHODS

1. Materials

Uniform haploid Saccharomyces cerevisiae wild-type strain 106 was used for the experiment. The culture medium and incubation condition were reported in the first part (Nishihara 1970a). Secondary potassium phosphate, ammonium molybdate, isobutyl alcohol, ethanol and sulfuric acid were Wako first class. Primary tin chloride and magnesium sulfate were Kosou Chemical special grade. Tris-aminometan was Sigma chemical company reagent grade. PEP, pyruvate kinase and ATP were from Boehringer. Sodium silicate was Wako special grade. Sodium tungstate was Kosou chemical special grade. Ethidium bromide was from Calbiochem B grade. Cycloheximide was from Kaken pharmaceutical, chloramphenicol was from Sankyo,, and Streptovaricin was from Toyo Jozo.

- Preparation of molybdenum acid reagent: 50g of ammonium molybdate was added to 10N 400ml of sulfuric acid, then water was added in order to make the solution volume 1 l.
- Preparation of silica tungstate solution: 1.9g of sodium silicate and 26.5g of sodium tungstate were added to 170ml of water. 5ml of concentrated sulfuric acid was added little by little to this solution. It was then heated to reflux with a reflux cooling tube inserted for 5 hours. After it cooled, water was added to make the solution volume 333ml.
- Isobutyl alcohol benzen solution: isobutyl alcohol was mixed with the same amount of benzen.

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- Ethanol sulfuric acid solution: 20ml of sulfuric acid was added to ethanol to make the solution amount 1 l.
- For quantification of protein, the Lowry method was taken with bovine albumin used as a standard.
- For quantification of phosphorus, the Martin-Doty method was taken with secondary potassium phosphate used as a standard.

2. Experimental method

i) The influence of antibiotics on respiration

Each glucose agar culture medium contains 0.2μg/ml of cycloheximide, 2mg/ml of chloramphenicol, 0.2mg/ml streptovaricin and 1μg of ethidium bromide, respectively. Wild-type strain 106 which had been pre-incubated in a glucose liquid culture medium was spread on a respective glucose agar culture media to grow for 2 to 3 days at 30° C. TTC agar was stratified upon the grown-up colonies, which was kept for 10 minutes at 30° C and respiration ability was then observed. While antibiotics caused a slight number of 'petite mutant' on the agar culture media, the observation of respiration ability was done with colonies without mutation.

ii) The measurement of activities of mitochondrial ATPase

Wild-type yeast strain 106 was incubated in the same procedures taken in the first part of this report. As it was reported that the respiration ability of yeast under incubation increased in the middle of the log phase, and that it became constant at the end of the log phase, therefore, the above antibiotics were added to the culture media at the end of log-phase, at 16^{th} hour after the start of incubation, i.e., 2μg/ml of cyclohexmide, 2mg/ml chloramphenicol, and 1μλ/ml of ethidium bromide, respectively. Then, they were further incubated for 5 more hours.
For the fractionation of Mitochondria the same procedures were taken as with the first part of this report.

iii) The measurement of mitochondrial ATPase

In 1ml of incubation media, 50μ moles of Tris-Cl pH 7.4, 2μ moles of ATP, 1μ moles of $MgSO_4$, 5μ moles of PEP, 7.5μg of pyruvate kinase, 20~40μg (protein amount) of mitochondria are supposed to be contained.
This culture media was incubated for 10 minutes at 30° C, then cooled with ice, and immediately 0.5ml of silicotungstic acid as a protein remover agent was added to stop the reaction.

In addition, 0.5ml of ammonium molybdate, 0.5ml of water and 2.5ml of iso-butanol-benzen were added and stirred well.
Thus, inorganic phosphorus, which had been produced by ATPase function, was extracted by an organic solvent. Then 0.5ml of the above iso butanol-benzen was removed, using mikroliterpipette of Eppendorf, mixed well with 2ml of alcohol solution sulfuric, and, finally, 10m M $SnCl_2$ was added in order to develop color and the absorbance at 750nm was measured.
As ADP, which was generated during the decomposition process, caused product inhibition to ATPase, PEP and Pkinase were added in the reaction in order to regenerate ADP into ATP during the measurement of ATPase activities.
ATPase activities which were equivalent to the Pi generation of 1μ mole of Pi / minute were regarded as 1 unit.

III. THE RESULTS OF THIS STUDY

The results of experiment (i) are shown in Table 1.
Inhibition against respiration ability was observed in the system where ethidium bromide, streptovaricin and chloramphenicol were present. No inhibitory effect to respiration ability was observed in the cycloheximide system.
The results of experiment (ii) are shown in Table 2.
Significant decrease in the activities of ATPase compared to the control was observed in the system where chloramphenicol and ethidium bromide were present. In the cycleheximide system, no decrease in ATPase activity was observed compared to the control.

IV. DISCUSSIONS

Since Simpton reported that mitochondria of the higher order organisms had the ability to synthesize protein (Simpson 1955), numerous studies with regard to protein synthesis of mitochondria have been carried out. (Wintersberger 1965; Linnane *et al*., 1968; Woodward & Munkres 1967) It is known that the protein synthetic mechanism of mammalian mitochondria is different from cytoplasmic type 80s type ribosome system, (Linnane *et al*., 1968; Linnane 1967) similar to those of yeast, fungi and mitochondria and chloroplast of plants, but are bacteria type 70s system. In addition, it was reported to be highly possible that mitochondria had its own DNA-like mesosome of bacteria (Yotsuyanagi 1966; Nass & Nass 1963; Nass 1969), had its own enzymes for syntheses of DNA, RNA and protein, (Linnane 1967; Yotsuyanagi 1966; Nass & Nass 1963; Nass 1969; Windersberger 1966; Rifkin *et al.,* 1967; Luck *et al.,* 1964; Wintersberger & Tuppy 1965) and are extra-nuclear self-multiplication system synthesizing protein using its own genetic information. It is possible

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Table 1. The effects of antibiotics against respiration ability

Antibiotics	Concentration	TTC Color
control		+
cyclohexmide	0.2 μg/ml	+
chloramphenicol	2 mg/ml	–
ethidium bromide	1 μg/ml	–
streptovaricin	0.2mg/ml	±

Table 2. Measurement value of mitochondrial ATPase activities

Antibiotics	Concentration	Activities of ATPase unit/mg	Activity compared to the control (%)
control	0.2 μg/ml	1.15 0.95	100
cyclohexmide	2 mg/ml	1.08 0.97	100
chloramphenicol	1 μg/ml	0.55 0.50	50
ethidium bromide	0.2mg/ml	0.19 0.24	20

to regard mitochondria as a bacteria like organism, i.e., Procaryota which coexists inside the cells of 80s type ribosome protein synthesis organisms, i.e., Eukaryota.

However, the author inferred, in the first and second part of this study, that the genetic information of mitochondria did not have all the necessary structures for its own self-multiplication, but that enzymes in charge of multiplication of mitochondrial DNA and synthesis of mitochondrial RNA, namely, mitochondrial DNA polymerase and RNA polymerase, were generated by the nucleus-cytoplasmic protein synthetic system and were under the control of nuclear genes (Nishihara 1970a; Nishihara 1970b). It has not yet been disclosed which types of protein are synthesized by the protein synthetic system of mitochondria, which are independent from the cytoplasmic protein synthetic system. In general, it was thought that the structural protein of mitochondria were produced (Woodward & Munkers 1966). But the definition of structural protein is not clear and its substance cannot be imagined (Blair *et al*., 1968). It was reported recently that inactivated enzyme as an artifact was included in the fraction of this protein (Schatz 1969).

While mitochondria of higher order organisms have multiple functions, their essential function is oxidative phosphorylation. This phosphorylation is coupled with respiration phenomenon, in which hydrogen ions based in a cellular substratum were transferred to enzymes via the electron transporting system. Most energy needed for the life process is produced by the respiration function of mitochondria.

The mechanism of oxidative phosphorylation has yet to be fully disclosed. There are studies trying to disclose this mechanism by extracting a series of proteins involved in the process as coupling factors and by reconstituting them from each other (Schatz *et al*., 1967; Kagawa & Racker 1966b). Row forming 90 Å diameter particles observed on the inner membrane of mitochondria were identified to be the coupling factor F_1 , which plays the most essential role in synthesizing ATP coupled with electron transporting (Kagawa & Racker 1966a). The coupling factor F_1 was extracted as soluble enzyme and was disclosed to be the identical enzyme with ATPase of mitochondria (Kagawa & Racker 1966). Mitochondria ATPase, when conjugated to the inner membrane of mitochondria, are distinctly inhibited by oligomycin, and, reportedly, with these characteristics, it can be distinguished from other ATPase (Kagawa & Racker 1966). With regard to the biological distribution of this ATPase (coupling factor F_1), it is found, in mitochondria of animals, plants and fungi as well as chloroplast of plants as eukaryota, and, in plasmic membrane of bacteria and its continuation as prokaryota.(Altmann 1890)) Therefore, an entire protoplast is a bacteria which could be regarded as a single mitochondria (Altmann 1890). Phylogenetically it is said that the energy acquiring mechanism of living organisms started when archaeorganism appeared on the earth, as an anaerobic heterotrophic organism, and it developed itself into a heterotrophic carbonic acid assimilation, a chemical reduction, a photosynthetic organism, and finally an aerobic heterotrophic organism. It is also said that the oxidation reduction mechanism of photosynthetic bacterium was used as an aerobic respiration mechanism of aerobic organisms without any modification (Gaffron 1962). Mitochondria can also be regarded as an intracellular parasite, as Altmann investigated (Altmann 1890), which parasitized in anaerobic organisms since the dawn of time, having lost most biological functions, and mainly became responsible for respiration ability. This interpretation corresponds well with the unique functions of this organelle and the biological distribution of coupling factor F_1 (ATPase) which performs those unique functions. From the above discussion, it is not unreasonable to conclude that coupling factor F_1, which is a characteristic enzyme for functions of mitochondria and the independent protein synthesis mechanism of mitochondria have a very close relationship to each other.

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On the other hand, it is reported that with the differentiation of the internal microstructure of mitochondria, the respiration ability of yeast increased (Yotsuyanagi 1962) the activities of ATPase were intensified, and that, when respiration ability was inhibited by removing enzymes, mitochondria and the activities of ATPase disappeared (Solmo 1968). It is also known that the inhibition of protein synthetic mechanism of mitochondria caused disability in respiration. It could, therefore, be concluded that respiration ability, protein synthesis of mitochondria and ATPase activities have close relationships to each other.

The experiments in this study confirmed that there was no respiration disturbance where cycloheximide inhibited the protein synthesis in cytoplasm to a certain extent, and that there was an evident decrease in respiration ability where antibiotics inhibited mitochondrial protein synthesis, such as chloramphenicol and streptovaricin. It could, therefore, be inferred that the mitochondrial protein synthetic mechanism contributed to the energy transformation mechanism of respiration either as an electron transport system or in coupling factor system.

In experiment (ii), in order to observe the effects of the above antibiotics against F_1 mitochondrial ATPase, the most important enzyme among coupling factors, during incubation, a preliminary experiment had been conducted to confirm that the 90% of yeast mitochondrial ATPase was inhibited by oligomycin, therefore ATPase activities measured in this study derived from F_1.

In experimental results, there was a distinct decrease of ATPase activities in the system where ethidium bromide (Meyer & Simpson 1969; Lepecq & Paoletti 1969; Li & Crothers 1969), which conjugated to mitochondrial DNA, and was expected to disturb its genetic information, was administered in culture . As ethidium bromide was administered at the end of a log phase, an increase of occurrence of petite mutant was not observed.

No decrease of mitochondrial ATPase activity was confirmed in the system where the cytoplasmic protein synthetic system was inhibited by cycloheximide.

An evident decrease in ATPase activities was seen in the system where chloramphenicol inhibited the mitochondrial protein synthetic system. Schatz reported similar results, however, he concluded that it was not the inhibition of ATPase synthesis, but that oligomycyin sensitivity conferring protein, which connected ATPase to the mitochondria's inner membrane, and were

produced in mitochondria, could be inhibited by chloramphenicol. If we accept this conclusion, it is expected that ATPase activities should be lowered with antibiotics against the cytoplasmic protein synthesis system, but no such results were obtained in this experiment. On the other hand, Tzagoloff carried out the similar experiments using chloramphenicol and cyclohexiemide with glucose derepression system, and reported recently that ATPase activity was also lowered with cycloheximide administration, and that this enzyme could probably be synthesized in cytoplasma (Tzagoloff 1969). Therefore, it can possibly be interpreted that ATPase is synthesized in cytoplasma, and that the protein synthetic mechanism had some relationship with its absorption into mitochondria. However, in the first part of the study, it was investigated that essential proteins to produce mitochondria, such as mitochondrial RNA synthesizing enzymes, were synthesized in cytoplasma. Therefore, we could interpret this as follows: in a derepression system, where undifferentiated mitochondria gradually differentiated and became multi-functional, cycloheximide controlled the cytoplasmic protein synthesizing mechanism and affected the synthesis of the enzyme protein essential for the production of mitochondria. As a result, the genetic informational function of mitochondria was controlled and the protein synthesizing mechanism of mitochondria did not work, which caused an eventual lowering of ATPase activity. In this experiment, antibiotics were administered at the maturity period of mitochondrial production. This appears to have avoided the timing when the cytoplasmic protein synthetic mechanism is involved in mitochondrial production in the derepression system.

In this study, the lowering of ATPase activities was confirmed with the inhibition of both the protein synthesis mechanism of mitochondria and the functions of mitochondrial DNA, while the lowering of ATPase activities was not observed in the system where cytoplasmic protein synthesis was inhibited. It may be concluded that the coupling factor F_1 (ATPase) in charge of oxidative phosphorylation was under the control of mitochondrial genes, and that its genetic information was functioning in the protein synthesis mechanism of mitochondria. This needs to be verified with more direct experiments. From the above study, it was investigated that mitochondrial genes could control the protein of coupling factor systems.

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V. CONCLUSION

1. The activities of yeast mitochondrial ATPase with the administration of cycloheximide, cytoplasmic protein synthesis inhibitor did not show any difference from that of the control.

2. The activities of yeast mitochondrial ATPase with the administration of chloramphenicol, a mitochondrial protein synthesis inhibitor, showed evident lowering.

3. The activities of yeast mitochondrial ATPase with the administration of ethidium bromide, which is supposed to conjugate to mitochondrial DNA at low concentration and to control its genetic information, showed evident lowering.

With the above results, an interpretation would be allowed that coupling factor F_1 (ATPase) is controlled by mitochondrial genes, and that its genetic information is translated by the protein synthesis system of mitochondria. By disclosing the functions of mitochondrial genetic information, it is expected to obtain clues to differentiate whether morphology transformation of mitochondria in cancer cells is derived by the mutation of nuclear genes or that of mitochondrial genes.

With the continuation of this study, it is expected to obtain clues to disclose dedifferentiation phenomena of cancer cell organelle.

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