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

Part 1.

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INTRODUCTION

Characteristic morphological changes at the cellular level in intractable immune diseases, including cancers, the deterioration and deformities of organelle mitochondria, are well known (Brachet 1961; Mercer 1961; Morris 1963). A decrease in the numbers of mitochondria and the simplified structure of cristae are known in cancer cells (Aisenberg 1961; Reid 1962; Bernhard 1958; Schulz 1957; Weissenfeels 1957). The author sought to disclose the mechanisms of the causes of mitochondrial mutation at the molecular level by using yeasts, i.e., Saccharomyces Cerevisiae. The essential function of mitochondria is oxidative phosphorylation of energy metabolism, which is coupled with cellular respiration, in the inner membranes of mitochondria (White & Handler 1964). Mitochondria have their own DNA, and own RNA, which are independent from cytoplasm nuclear, and have DNA polymerase, and DNA-dependent RNA polymerase, with a protein synthetic system resembling that of bacteria and independent from that of the cytoplasm system (Nass & Nass 1963; Schatz *et al*, 1964; Luck & Reich 1964; Kalf 1964; Wintersberger 1966; Wintersberger & Tuppy 1965; Luck 1967; Barnett *et al*, 1967; Schweizer *et al*, 1969) (Rogers *et*

* This research is the author's PhD doctoral thesis in 1971 at the University of Tokyo, Faculty of medicine.

al, 1967; Wintersberger 1965; Simpson 1955; Linnane *et al*, 1968). Therefore, it is said that mitochondria are characteristic independent organelle with a self-reproductive system without regulation of nucleic DNA (Fletcher *et al*, 1961). However, it is also said that mitochondria are not independent from nuclei, but have a close correlation with them (Ephrussi 1953; Brachet 1967). It is not disclosed (Halder & Freeman 1966; Kalf 1964) what kind of genetic information mitochondrial genomes have (Schatz *et al*, 1964; Woodward & Munkres 1967). To disclose the causes of morphological change of mitochondria, i.e., undifferentiation of mitochondria in diseased cells, which are intracellularly infected cells observed in immune diseases including cancer cells, it is necessary to know the genetic information of the mitochondrial genome. In addition, it is necessary for mitochondrial development to know the correlation between nuclear and mitochondrial genomes.

Ephrussi reported a respiration defect mutant strain. i.e., petite mutant. in yeast in 1949 (Ephrussi *et al*, 1949). This petite mutant has the genotype of cytoplasmic inheritance and marked deformities are observed in the microstructure of the mitochondria. (Yotsuyangi 1962; McClary & Bowens 1968) (Monoulou *et al*, 1966). The sedimentation constant of DNA extracted from mitochondria of petite mutant is different from that of wild strains. The author studied the mechanism of development of mitochondrial mutation using yeast, i,e., Saccharomyces Cerevisae.

Using protein synthesis inhibiters, the author investigated how the respiration deficient strain of yeast develops. Following that, to support the phenomena observed in these experiments, mitochondrial fraction was obtained: mitochondria were separated from investigated yeasts. Using the mitochondrial fraction in vitro, synthesis of DNA and RNA in mitochondria were observed to determine the amount of ^{3}HTTP and ^{3}HUTP incorporation.

MATERIALS AND METHODS

1. Materials

Uniform haploid Saccharomyces Cerevisiae wild-type strain 106 and adenine-required strain 414d were used. ATP, CTP, GTP, α-ATP, and GTP were used with Boehringer & Soehne Gmbtt Mannheims Co. and HTTP ^{3} (relative activity 12^{c}/m mole) and ^{3}HUTP (relative activity 14.8^{c}/m mole) were used with Schwarz Bioresearch Co. Ethidium bromide was used with Calbiochem, Actinomycin D with Merk Sharp & Dohme, Cycloheximide with

Kakenkagaku, Chloramphenicol with Sankyo Co., and Streptovalicine was used with Toyojozo Co.

- Preparing snail enzyme.
  To separate mitochondria from yeast cells it is necessary to digest the yeast cellulose cell walls with snail enzyme. The preparation of snail enzyme for experimental use was as follows: Extirpation of digestive tracts from 100 snails were carried out. These were then soaked in 0.05M Tris-Cl buffer and centrifuged. Following that, the supernatant was separated and added to the buffer to make final the 100ml volume. Then ammonium sulfate was added to the solution to make 80% saturation and left at 4° C overnight. Following that, the solution was centrifuged and sedimentation was recovered, and added the buffer 30ml reserved at –20° C in a refrigirator.
- Preparing a scintillation liquid: In 1,000ml toluene, 4.2g diphenyloxazole and 0.52g phenyloxazolyl benzene were added.
- Preparing a glucose liquid culture medium: 0.1g (NH4)2SO4, 0.2g KH2PO4, 0.35g of pepsin, 0.35 g of yeast extract and 10g glucose were dissolved in 1,000ml of distilled water.
- Preparing a glycerin liquid culture medium: 10g of glycerin instead of glucose was added to the above-mentioned glucose liquid culture medium.
- Preparation of a glucose agar culture medium: 1.5g of agar was added to 100ml of the above-mentioned glucose to a liquid culture medium.
- Preparation of a glycerin agar culture medium: 1.5g of agar was added to the above-mentioned 100ml of glycerin liquid culture medium.
- Preparation of a TTP agar culture medium for pile-up method, 500mg of TTC, 5g of glucose, and 10g agar were added to 1,000ml of distilled water.

For quantitative analysis of protein, the Lowry method was used to standardize bovine albumin (Lowry *et al*, 1951).

2. Experimental methods

1) Experiment for the development of a respiration-deficient strain

For certainty, the same experiment was carried out with two different strains of yeast. Each Saccharomyces Cerevisiae wild-type strain 106 and adenine-requiring strain 414d was suspended in 3ml distilled water and 0.2ml of the suspension were incubated in the following four culture mediums:

i) 5ml of glucose liquid culture medium containing 0.1μg/ml, 0.15μg/ml, and 0.2 μg/ml cycloheximide.
ii) 5ml of glucose liquid culture medium containing 2mg/ml chloramphenicol.
iii) 5ml of glucose liquid culture medium containing 200mμg/ml of streptovalicine.
iv) 5ml of glucose liquid culture medium without antibiotics as a control.

After 24 hours at 30° C, cycloheximide-containing, and streptovalicine-containing and the control culture medium were diluted into 5×10^5 times, after that they were spread on an agar glucose culture medium to grow 50~200 colonies. These agar media were cultured 3 to 5 days at 30° C; thereafter, the number of respiration-deficient strain colonies were counted.

Adenine-requiring strain 414d are red in colony color in normal respiration and white in the respiration deficient strain. In this experiment, the respiration ability of the 414d strain was detected in this way. For certification, the incubation medium was changed in a glycerin culture medium, whether growing or not. Following that, respiration ability was detected. It is well known that a respiration deficient strain cannot grow in a glycelin medium. It is well known that a respiration normal strain can reduce TTC and change its color to red in culture. However, a respiration deficient strain cannot reduce TTC, therefore no change of color occurs (Meyer & Simpson 1968) In this way, the development of a respiration deficient strain from the 106 strain was observed.

The culture medium was poured on 3~5mm at 50° C, on colonies growing a glucose agar culture medium, with melting TTC agar and after 20~40 minutes incubation at 30° C, a color change was observed. For certainty, sterilized colored and non-colored colonies with the same count of agar culture were incubated in a glucose culture medium for 24 hours.

The yeast cells were then washed with sterilized water and were then incubated in a glycerin culture medium. Following that, the development of a respiration deficient strain was observed.

2) Measurement of activities of mitochondrial DNA synthesis and mitochondrial RNA synthesis

- Fractionation of mitochondria: Separation of mitochondria from whole cell homogenate

Wild-type yeast Saccharomyces Cerevisiae 106 strain was cultured in 10ml glucose liquid medium at 30° C for 24 hr. This 10ml medium was then cultured in a 1000ml glucose medium at 30° C with aeration. The growth curve of the

106 strain indicates a log phase of 13 to 15 hrs after incubation to reach a stationary phase. At a later log phase 15 hrs after incubation, yeast cells of the 106 strain were obtained. These cells were called **experiment 2)–A** To observe the direct reaction of used reagents of inhibitors upon DNA synthesis as well as RNA synthesis of mitochondria in vitro, yeast cells of late log phase, namely 15 hrs after incubation, were used as **experiment (2)–A b)**

Further, obtained experiment 2) –A cells were cultured for 8 hrs at 30° C in a culture medium with i) cycloheximide 2μg/ml, ii) chloramphenicol 2mg/ml, and iii) no antibiotics for control. These cells were called **experiment (2)–B**. To observe reactions of cycloheximide and chloramphenicol upon in vitro DNA synthesis as well as RNA synthesis of mitochondria during development of petite mutant yeast cells of **experiment (2)–B** were used to separate mitochondrial fraction. Following that, cells were recovered by centrifugation.

Mitochondrial fractionation of **experiment 2)–A b)** as well as **experiment 2)–B** group cells were carried out to obtain sphenoplasts, which were then homogenized by Ohnish-Hagiwara (Ohnishi *et al*, 1966) methods on which the cellulose cell-wall of yeast was digested by snail enzyme.

The 10g of yeast cells obtained from a 1,000ml medium were washed twice in 4° C water, then washed with SET (1.3M sorbilol, 0.1 m MEDTA, 10 m M Tris-Cl, ph 5.7). After that, they were suspended in 20ml SET, then incubated at 30° C for 30 minutes with 0.3 ml βmercaptoethanol, to which was added 70mg snail enzyme and incubated 1.5 hours at 30° C. After centrifugation, yeast cells were washed twice with SET at 4° C, then suspended in 20ml SET. This suspension was poured into a tephrone-paste 27mm-diameter Pievce-type homogenizer in an ice box, then homogenized 10 times with a slow up and down rotation. The homogenate was centrifuged at 5,500rpm (rotor No. 4) for 5 minutes at 0° C, and the supernatant was again centrifuged with 11,000rpm (rotor No. 4) for 9 minutes. The obtained sedimentation was mitochondrial fraction.

This mitochondrial fraction was again suspended in 20 ml SET. This suspension was centrifuged 2100×g for 5 minutes. Again the supernatant was centrifuged at 8500×g for 9 minutes and the obtained sedimentation was used as mitochondrial fraction for measurement of activities of mitochondrial DNA synthesis.

(1) Measurement of activities of mitochondrial DNA synthesis

For measurement of activities of mitochondrial DNA synthesis of **experiment (2)–A b)** as well as **experiment (2)–B** group cells was carried out. For this purpose, Wintersberger's (Wintersberger 1966; Wintersberger & Tuppy 1965; Clark-Walker & Linnane 1966; Rifkin *et al*, 1967) method was used, referring

to Simpson's [35] and Rabinowitz [35] methods. In an incubation medium, 0.25 ml moles sorbilol, 20μmoles Tris-Cl butter ph 7.4,1μmole EDTA, 10μmoles MgSO4, 50mμmoles dATP, dGTP, dCTP,5μc^{3}HTTP (relative activities 12 c/m mole), 300μg phosphoenol pyruvate and 100~250μg protein amount of mitochondria were contained in 250μℓ.

As inhibitor of mitochondrial DNA synthesis 6μ moles ethidium bromide was contained in medium. Observation of the action of antibiotics and reaction on mitochondrial DNA synthesis was carried out with 2μg/ml cycloheximide and 2mg/ml cholramphenicol in medium. After 30 minutes of incubation at 37° C, the reactor was stopped with 1ml 0° C 10% TCA, then the solution was filtered with membrane filter washing with 5ml 5% TCA then 5ml 10% TCA, and the filter was then dried with ultrared light.
A Beckman liquid scintillation counter (LS-133) was used with vial 15ml scintillation liquid and ^{3} HTTP incorporation into DNA chain was measured for 5 minutes.

(2) Measurement of activities of mitochondrial RNA synthesis

For measurement of activities of mitochondrial RNA synthesis of **experiment 2)–A b)** as well as **experiment 2) –B** groups cells was carried out.
For this purpose Winterberger's method was used (Wintersberger & Tuppy 1965).

In an incubation medium, 0.25m moles sorbilol, 20 μmoles Tris-Cl buffer ph7.4, 5.0μmoles KCl, 10μmoles Mg Cl_2 40μ moles CTP,GTP, 1 μmoles ATP, 1μ C ^{3}HUTP (relative activity 14.8c/m moles) 300μg phosphoenol pyruvate, 30μg pyruvate kinase and 100~200μg protein-amount mitochondria were contained in 250mℓ solution. For inhibition 5μg actinomycinD was added. To observe in vitro action of antibiotics 2μg/ml cycloheximide, 2mg/ml chloramphenicol were added in solution. After 30 minutes at 37° C the incubation reaction was stopped by adding 10% TCA 0° C. The solution was filtered with a membrane filter, washed with 5ml 5% TCA and 5ml 10% TCA The membrane filter was then dried with infrared light. The filter was measured with a Beckmann liquid scintillation counter in 5ml vial with scintillation liquid for 5 minutes and incorporation of ^{3}HUTP into mitochondrial RNA was observed.

RESULTS

1) Results of experiment 1) on development of respiration deficient strain is shown in ***Table I***.

In eukaryota cells there are two systems of protein synthesis. One is the nucleus-cytoplasmic protein synthesis system of eukaryote-type and the other

Table I. Experiment (1) Experiment for the development of a respiration deficient strain. Mutation Rate of Respiratory Deficient Strains (Petite Mutant).

Strain	Reagents	Concentration	Total Colony numbers	Resp. deficit. Colony numbers	Petite mutant rate (%)	Mutation rate average (%)
414d	–	–	103	1	0.97	1.2
			240	3	1.2	
			198	3	1.5	
	Chloramphenicol	2mg/ml	182	2	1.1	1.1
			220	2	0.9	
			137	2	1.4	
	Cyclohexmide	0.15µg/ml	43	5	11.6	11.5
			52	7	13.4	
			60	6	10.0	
			115	13	11.3	
		0.2µg/ml	213	25	11.7	11.3
			52	7	13.4	
			135	12	8.8	
106	–	–	132	2	1.5	2.1
			115	3	2.6	
			173	4	2.3	
	Chloramphenicol	2mg/ml	136	3	2.2	2.4
			192	5	2.6	
			191	6	3.1	
			350	7	2.0	
	Cyclohexmide	0.15µg/ml	149	15	10.0	10.0
		0.2µg/ml	107	15	14.0	13.5
			115	15	13.0	
	Streptovalicine	0.2 mg/ml	136	3	2.2	2.2
			107	3	2.8	
			177	3	1.7	

is the mitochondrial protein synthesis system resembling that of bacteria, i.e., prokaryota-type.

For inhibition of protein synthesis of the former type cycloheximide can be used and for the latter chloramphenical can be used.

The mutation rate of control of strain 414d as well as 414d cultured in a chloramphenicol containing medium, the was 1.1~1.2% , or almost the same as the spontaneous mutation rate.

The cycloheximid culture medium of strain 414d the mutation rate was 11.4%, namely about 10 times higher than the spontaneous mutation rate.

On strain 106, the mutation rate of the control and the chloramphenical culture medium were 10.0% and 13.5%, respectively, namely 5 times higher than the spontaneous mutation rate.

And that of streptovalicine was 2.2%, namely almost the same as the spontaneous mutation rate.

Table II. Experiment 2).
(1) Measurement of Activities of mitochondrial DNA synthesis of experiment 2) A b)

① Direct reaction of ethidium bromide, cycloheximide, and chlorampenicol to mitochondrial DNA synthesis in vitro.

In vitro system, reagent	Incorporation into mitochondrial DNA
Complete system (without) control	1830 μμmoles
Ethidium bromide	55 μμmoles
Cycloheximide	2150 μμmoles
Chloram phenicol	1930 μμmoles
-dXTP	76 μμmoles
-Mg^{++}	52 μμmoles

(2) Measurement of Activities of mitochondrial DNA synthesis in vitro of experiment 2)-B

In vitro system	Incorporation into mitochondrial DNA	ethd	-dXTP	-Mg^{++}
15hrs + 8hrs No reagents, Control	2600 μμmoles	46	21	25
15hrs + 8hrs Cycloheximide	1100 μμmoles	59	46	55
15hrs + 8hrs Chloramphenicol	9200 μμmoles	84	53	38

Refer to experimental methods. Specific activities of incorporated ^{3}HTTP activities into mitochondrial DNA were read asμμ mole.
Counted ^{3}HTTP in the system without XTP as well as without Mg ion is considered to be absorbed, therefore regarded to zero.

On both strains 414d and 106 in the cycloheximid group, a higher development of respiration deficient mutation was observed.

2) Results of experiment 2)

(1) Measurement of activity of mitochondrial DNA synthesis in vitro of **experiment (2)–A b)** and **experiment 2)–B** group cells is shown in ***Table II***.

From the result of **experiment 2)–A b)**, without dXTP and Mg ion, the measured count of ^{3}HTTP was considered not to be incorporated into DNA, but to be absorbed; therefore, the count was considered to be zero.

Ethidium bromide combined with mitochondrial DNA makes inert DNA activity; therefore, the culture medium containing it, ^{3}HTTP incorporation

Table III.
(2) Measurement of Activities of mitochondrial RNA synthesis of experiment 2) -A b)

① Direct reaction of ethidium bromide, cycloheximide, and chlorampenicol to mitochondrial RNA synthesis in vitro.

In vitro system, reagent	Incorporation into mitochondrial RNA
Complete system (without) control	1280 μμmoles
Ethidium bromide	83 μμmoles
Cycloheximide	1630 μμmoles
Chloram phenicol	1520 μμmoles
-dXTP	26 μμmoles
-Mg^{++}	38 μμmoles

② Measurement of Activities of mitochondrial RNA synthesis in vitro of experiment 2)-B

In vitro system	Incorporation into mitochondrial RNA	ethd	-dXTP	-Mg^{++}
15hrs + 8hrs No reagents, Control	111μμmoles		26	40
15hrs + 8hrs Cycloheximide	23μμmoles	25	38	24
15hrs + 8hrs Chloramphenicol	388μμmoles	28	18	21

Refer to experimental methods,
Specific activities of incorporated ^{3}HTTP activities into mitochondrial DNA were read asμμ mole.
Counted ^{3}HTTP in the system without XTP as well as without Mg ion is considered to be absorbed, therefore regarded to zero.

observed 55 was to be zero. No marked direct action of cycloheximide nor as choramphenicol upon mitochondrial DNA synthesis in vitro, were observed. From the result of **experiment 2)–B**, comparing the specific activity of DNA synthesis between **experiment 2)–A b) control** and **experiment 2)–B control**, a slight enhancement of the latter was observed.

Comparing specific activity of DNA synthesis between **experiment 2)–A b) control** and cycloheximide containing medium group, that of the latter was observed to be half of that of the former.

Comparing the specific activity of control and that of the chloramphenicol containing medium group, that of the latter was observed to be 3.5 times higher than the former.

Results of experiment 2)

(2) Measurement of activity of mitochondrial RNA synthesis of **experiment 2)–A b)** and **experiment 2)–B** group in vitro was exhibited in ***Table III***.

A marked difference between **experiment 2)–A b) control** and **experiment 2)–B control** namely 1/10 decrease of the former comparing the latter was observed.

No activity of mitochondrial RNA synthesis in medium containing cycloheximide was observed.

Comparing activity of **experiment 2)–B control** and that of a medium containing chloramphenicol, the latter was 3 to 4 times higher than that of the former.

DISCUSSION

The protein synthetic system in mitochondria is quite similar to that of bacteria. Mitochondria have ribosome of 70S type (Clark-Walker & Linnane 1966; Rifkin *et al*, 1967). Antibiotics such as chloramphenicol, which inhibit the bacteria type 70S ribosome protein synthetic system, specifically inhibit mitochondrial protein synthesis (Meyer & Simpson 1969; Mager 1960). The protein synthetic system in yeast resembles that of mammalian cells, 80S type ribosome (Rogers *et al*, 1967), and is inhibited by 80S type ribosome protein inhibitor such as cycloheximide (Meyer & Simpson 1969). Petite mutant, namely the respiration deficient mutant having cytoplasmic genotype, exhibits marked changes in the microstructure in mitochondria, (Yotsuyangi 1962) (McClary & Bowens 1968) and has changed mitochondrial DNA (Monoulou *et al*, 1966). Therefore, it is considered that in the development of the organelle mitochondria, mitochondrial DNA has a specific important role (Woodward & Munkres 1967).

The developmental mechanisms of petite mutant by acriflavin as well as ethidium bromide treatment, is thought that by intercalation of these reagents DNA polymerase of mitochondria is inhibited (Meyer & Simpson 1969; Lepecq & Paoletti 1967). It is not certified whether the enzymes of mitochondrial DNA synthesis, i. e., mitochondrial DNA polymerase is synthesized in mitochondria or in cytoplasm. Hypothesizing it is synthesized in mitochondria, petite mutant can be induced at a high rate by the inhibitor of mitochondrial protein synthesis, i.e,. chloramphenicol. Hypothesizing it in cytoplasm, petite mutant can be induced in high rate by the inhibitor of cytoplasmic protein synthesis, i.e., cycloheximide. Also, it is well known that the spontaneous mutation rate of petite mutant is 1% therefore 10^5 times higher than the spontaneous mutation rate of nucleus genome. From the experiment of the development of respiration

deficient petit mutant, it was known that a high rate of development of mutant from wild type strain cultured in a cycloheximide-containing medium, which inhibits the 80S type ribosome protein synthetic system, namely the cytoplasmic system of yeast. Inducement of petite mutant by cycloheximide had been reported by Lomander in 1963, using Saccharomyces pastorianus (Lomander & Gundersen 1963). However, he had no idea nor comment concerning the mechanisms in the development of the mutant (Lomander & Gundersen 1963). No increase of the mutation rate from wild type strain cultured in a chloramphenicol containing medium was observed in this experiment.

This is closely coincides with experimental results carried out by Linnane (1968). From **experiment 2) A b)A control** of in vitro, it was known that cyclohexmide had no direct action to mitochondrial DNA polymerase.

Therefore, the developmental mechanism of petite mutant by cycloheximide is considered quite different from that of ethidium bromide and acriflavin.

Streptovalicine, (Mizuno *et al*, 1968) i.e., inhibitor of DNA-dependent RNA polymerase of Gram-positive bacteria, showed not only depression of respiration in yeast in the preliminary experiment, but also in vitro inhibition in DNA dependent RNA polymerase activity. If mitochondrial genome have genetic information concerning mitochondrial DNA polymerase, it is possible to develop petite mutant by means of streptovalicin. Following this experiment, streptovalicin induce no petite mutant. Attardi reported using HeLa cells that information of mitochondrial DNA are translated in cytoplasma by mitochondrial mRNA (Attardi & Attardi 1968). From my experiment, mitochondrial DNA polymerase are synthesized in a different way from Attardi's result. Following these facts, it is considered that mitochondrial DNA polymerase is synthesized in cytoplasma and is controlled genetically by nuclear genomes.

In 1957, Kornberg disclosed DNA polymerase, and synthesized DNA chain in the cell-free system (Lehman *et al*, 1957). This enzyme needs primer DNA, and four kinds of nucleotides for the DNA chain. (A+T)/(G+C) ratio of synthesized DNA chain is equal to primer and the enzyme needs Mg ion for synthesizing (Lehman *et al*, 1957). The author certified in preliminary experiments using whole yeast homogenate, primer DNA dependency, four kinds of nucleotides dependency, and Mg ion dependency. DNA synthesis in isolated mitochondria can be carried out without a primer DNA chain. DNA-dependent RNA polymerase, which was reported by Weiss (Weiss & Gladstone 1959), are known as the above-mentioned dependency just the same as DNA polymerase. After

the author certified these dependencies using whole homogenate of yeast in the preliminary experiment, activities of mitochondrial RNA synthesis were measured. From the result, activities of mitochondrial DNA synthesis in culture containing cycloheximide was 42% lower compared to **experiment 2)-B control.** This result supports **experiment 1)** Namely, inducement of petite mutant by cycloheximide in culture.

Activities of mitochondrial DNA synthesis in culture containing chloramphenicol was 3.5 times higher compared to **experiment 2)-B control**. From these two inhibitors of protein synthesis, i.e., groups of cyclocheximide and chloramphenicol, development of petite mutant is considered to be dependent upon the activities of mitochondrial DNA polymerase. It is not correct that activities of DNA, synthesis observed by incorporation of ^{3}HTTP into high molecular substance, indicate normal polymerization of DNA, Cycloheximide is a quite different substance from ethidium bromide which intercalate into the mitochondrial DNA chain and inhibit respiration. The increased mutation rate of petite mutant by cycloheximide is considered to be a disturbance of cytoplasmic protein synthesis of nuclear DNA inducing deteriorated mitochondrial DNA polymerase, consequently uncompleted DNA polymerization of mitochondria occurs, after which petite mutation development occurs. Marked increase of activities of mitochondrial DNA synthesis in **experiment 2)-B** in yeast cultured with cholramphenicol in medium which disturb mitochondrial protein synthesis, is important, However, to explain this mechanism is very difficult. In a complete independent self–reproductive organism like bacteria, duplication of DNA is disturbed by inhibition of protein synthesis at some division cycle stage (Ward & Glaser 1969). As Altman thought, mitochondria can be seen as an independent parasitic self-reproductive organism in eukaryote cells (Altman 1890). From this experiment, it is considered that mitochondria have a different self-reproductive system from that of the completely independent system of bacteria, because mitochondrial DNA polymerase are synthesized in the cytoplasmic protein system out of mitochondria, which has main role of duplication of mitochondrial gene substance, i.e,. DNA, Hypothesizing that sequential enzymes of coupling factor and the electron transmitting system are synthesized in the mitochondrial system, if these enzymes have aging and deteriorate or inhibited synthesizing, it can be thought synthesis of mitochondrial DNA polymerase promote, consequently mitochondrial DNA duplication promote and mitochondrial reproduction promote.

Yotsuyanagi (1962) reported that mitochondrial number in yeasts increase in the late log phase, at the same time start development of inner structure in

mitochondria and complete in stationary phase observing transmitting electron microscopic pictures. In this experiment, activities of mitochondrial RNA synthesis were lowered in the stationary phase comparing high level of late log phase. Mitochondrial RNA synthesis is thought to be lower in completion of mitochondrial development. In yeast cultured in cycloheximide-containing medium no activities of mitochondrial RNA synthesis were observed, therefore, mitochondrial RNA polymerase is considered to be synthesized in the cytoplasmic protein system just like mitochondrial DNA polymerase. In yeast cultured in chloramphenicol containing medium three times the increased activities of RNA polymerase was observed.

Therefore, at the mitochondrial reproduction mitochondrial RNA polymerase have a quite different role from common prokaryote, i.e., bacteria, complete independent system resembling to mitochondrial DNA polymerase.

From this experiment the author proposes that the development of petite mutant in the experimental system have a close correlation of inhibition of special protein synthesis in cytoplasm, namely mitochondrial DNA polymerase and RNA polymerase, which are controlled by nuclear DNA and synthesized in cytoplasm not in mitochondria. The author considers that instead of a cytoplasmic protein inhibitor of cycloheximide, the intracellular infection by non-pathogenic common enteromicrobes can induce mitochondrial mutation, which disturb cytoplasmic protein synthesis resulting in incomplete mitochondrial DNA polymerase.

SUMMERY AND CONCLUSION

To disclose the mechanism of mitochondrial mutation, deformities and deterioration in diseased cells including cancers, the author carried out following experiments using yeast, i.e., Saccharomyces Cerevisiae.

Experiment for development of respiration deficient strain using inhibitor of protein synthesis in culture. 2) Measurement of activities of mitochondrial DNA and RNA synthesis in vitro during development of respiration deficient strain using inhibitor of protein synthesis in culture.

The following results were obtained:

1. Using cycloheximide, respiration deficient strain, i.e., petite mutant, can be obtained at a high rate.
2. Using chloramphenicol, no marked development of respiration deficient strain is obtained.

3. Using streptovalicin, no marked development of respiration deficient strain is obtained.
4. In yeast cultured in cycloheximide containing medium decreased activities of mitochondrial DNA polymerase, and no activities of mitochondrial RNA polymerase are observed.
5. In yeast cultured in chloramphenicol containg medium marked increased activities of mitochondrial DNA as well as mitochondrial RNA are observed.

From these experiments the author concluded that petite mutant, i.e., respiration deficient 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.

Consequently the author proposes that the major cause of intractable immune diseases, including cancer, is result of mitochondrial deterioration due to entangled complicated intracellular contamination of low virulent pathogenic as well as nonpathogenic common enteromicrobes such as viruses, mycoplasma, and bacteria.

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