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

Part 2: The interaction between nuclei and mitochondrial genes during development of organelle mitochondria

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I. INTRODUCTION

To obtain clues to disclose the de-differentiation phenomena of cancer cells, developmental research on mutation in organelle mitochondria was being carried out by means of molecular biology with the focus on the morphological deterioration of mitochondria. In the first part of this paper, the author reports to have obtained an indications to conclude that the enzyme protein of mitochondrial DNA and RNA polymerase were synthesized in cytoplasma and controlled genetically by nuclear genomes (Nishihara 1970; Nishihara 2007).

Following the report by Ephrussi on a respiration deficient mutant strain, i.e., "petite mutant" in yeast in 1949 (Ephrussi 1953), reports were made on several other respiration deficient strains of different genotypes in yeast (Mortimer 1966; Yotsuyanagi 1962). Using an electron microscope, Yotsuyanagi observed these respiration deficient strains of different genotypes, and reported that

there were clear differences in the microstructure of mitochondria (McClary and Bowers 1968). The hereditary patterns of these mutant strains are complicated, and the developmental mechanisms for respective genotypes have not yet been reasonably disclosed. It is expected that, by identifying the correlation between the nuclear genomes and those of mitochondria at the formation of organelle, the developmental mechanisms of respiration deficiency should be disclosed. It could be meaningful to disclose the developmental mechanisms of mitochondrial mutation, in which its structure and functions are correlated to each other. In order to more precisely examine the correlation between nuclear and mitochondrial genomes in the development of mitochondria, the authors observed in vitro the synthesis of DNA polymerase and RNA polymerase in mitochondria of wild strain yeast and of several respiration deficient strains of yeast of different genotypes. The activities of the synthesis were determined by the measurement of ^{3}HTTD and ^{3}HUTP incorporated into macromolecular substances in the fractionation of mitochondria.

MATERIALS AND METHODS

Materials

The following strains of Saccharomyces Cerevisiae were used*

i)	Wild-type strain	106 genotype	$P\rho^+$
ii)	Cytoplasmic respiration deficient strain	106AC genotype	$P\rho^-$
iii)	Nuclear respiration deficient strain – segregational mutant	431 genotype	$p\rho^+$
iv)	Dual mutational strain – epistatic mutant 5d	5d genotype	$p_o\rho^-$

106AC in ii) means that which was obtained through acriflavin treatment of 106 and its genotype was cytoplasmic inheritance. This is called "petite mutant," as it is supposed that this treatment caused mitochondrial DNA mutation (McClary and Bowers 1968).

431 at iii) was obtained through nitrothoguanidin treatment of 106 and is a respiration deficient strain caused by nuclear genome mutation with Mendel type inheritance. It is supposed to have identical mitochondrial DNA with wild-type strain 5d at iv) which was dissociated from natural yeast, whose respiration deficiency is caused by nuclear genomes, and it is supposed that this type of mutation inevitably produces mitochondrial DNA mutation as well.

Both p and p_0 indicate that the mutation occurred in different genes on chromosomes.

Experimental methods

Each strain was incubated following the same procedures taken in the first part of this report, then the principal incubation phase took place during 15 hours for 106, and 23 hours for 106AC, 431 and 5d, which took a longer time for growth. The same measuring methods as in the first part were taken for the DNA polymerase synthesis activity and that of RNA polymerase.

RESULTS

The results of measurements are shown in Tables I and II for DNA polymerase synthetic activity and RNA polymerase synthetic activity, respectively.

The ratios of [(RNA synthesis specific activity) / (DNA synthesis specific activity)] are shown in Table III.

Table I indicates that there is no evident difference in DNA polymerase synthesis among wild-type strains, 106AC and 431. Only 5d showed higher DNA polymerase synthesis, three times as high as 106. 106AC and 5d showed lower sensitivity than 106 against ethidium bromide.

Table III indicates that all three respiration deficient strains showed lower activities than 106 in RNA polymerase synthesis. 5d showed apparent lowering against actinomycis D, while 431 showed an extreme rise in the sensitivity against actinomycis D.

Strong inhibitory effects against RNA synthesis activity was confirmed with cycloheximide and chloramphenicol in 431.

Table III shows that value of [(RNA synthesis specific activity) / (DNA synthesis specific activity)] indicates its highest figure with wild type strain, followed by 106AC, 431, and 5d, showing the lowest values.

DISCUSSIONS

There are many studies on respiration deficient strains of yeast, following the discovery of "petite mutant" by Ephrussi *et al* (1949). According to them, genes controlling the respiration function are supposed to exist both inside the nuclear chromosome as well as inside mitochondria, and it has been identified that respiration would not develop by itself when a defection or mutation in either nuclear chromosome or mitochondria occurs (Nagao and Sugimura 1966; Nagai 1968). The expression of the respiration deficient character of yeast could develop itself, when either defectiveness or deficiency exist somewhere in chromosomes concerning the energy metabolism coupled with the electron transferring system, and, therefore, the identical expression could take place among several different hereditary patterns. The electron microscopic images of mitochondria belonging to respiration deficient strains of different

genotypes reported by Yotsuyanagi (1962) evidently showed degradation in the microstructure of mitochondria in all respiration deficient strains.

5d, used in this study, which is a strain with nuclear genome mutation, is known to have mitochondrial DNA mutation as well, and is, therefore, called an 'epistatic mutant'.

In the first part of this study, the author reported that, considerably, mitochondrial DNA polymerase is controlled genetically by nuclear genomes. Therefore, it is considered that the developmental mechanism of respiration deficiency could be in the mutation of structure genes of mitochondrial DNA polymerase in chromosomes. The results of these experiments showed that a remarkable rise of DNA synthesis activity at 5a. This seems to indicate a certain anomaly in the DNA duplication mechanism. It is supposed that the incorporation value of radioactive-isotope-labeled nucleotides in TCA precipitation cannot serve to determine whether the DNA duplication status is complete or incomplete. It is impossible to distinguish any qualitative difference in incorporation activities between the cases where incomplete DNA polymerase synthesized DNA chains with a lot of defects, and where nucleotides were incorporated as a repair. The high activity of 5d could indicate that either mutations in DNA polymerase itself or anomalies in the duplication control mechanism could have caused incomplete duplication and the consequent high activity.

The developmental pattern of respiration deficiency at 431 could be interpreted as follows: although mitochondrial DNA were intact, mutation in nuclear genes could have impaired respiration ability. Therefore, in this mutation strain case, genetic information, i.e., the transcription process from mitochondrial DNA to RNA, a protein synthesis, was controlled by nuclear genomes, and somewhere in this mechanism could have mutated to disturb the respiration function. The author reported in the first part of this study that mitochondrial RNA polymerase is developed in the nucleus-cytoplasmic protein synthetic system, and its activity has a close relationship with protein synthesis inside mitochondria.

In this in vitro experiment of mitochondrial RNA synthesis, it is observed that both cytoplasmic as well as mitochondrial protein synthesis inhibitor, i.e., cycloheximide and chloramphenicol inhibited only against 431 with evident lowering, and, in particular, inhibitory action of actinomycin D inhibited remarkably against 431. From these results, it could be concluded that the metabolism of mitochondrial RNA of 431 was different from that of wild-type and other mutation strains.

From the ratios between RNA and DNA synthesis activities, apparent change was observed in the metabolism of nucleic acid of respiration deficient strains.

From the above, the genotype of nuclear respiration deficient strains could be identified as enzyme level mutation, and the developmental mechanism of mitochondrial mutation could be thought of as follows:

> Mitochondrial mutation of 431 occurred by the incorrect development of genetic information of mitochondrial DNA due to the mutation of RNA polymerase controlled by nuclear genomes.
> Mitochondrial mutation of 5d occurred due to incorrect duplication, i.e., incomplete polymerization of mitochondrial DNA, caused by DNA polymerase mutation controlled by nuclear genomes.

CONCLUSION

To disclose the developmental mechanism of mitochondrial mutants the authors carried out molecular biology experiments using wild strains yeast and several respiration deficient strains of different genotypes, in which they observed in vitro the synthesis of DNA polymerase and RNA polymerase of mitochondria. The following results were obtained through the measurement of DNA/RNA synthesis specific activities of wild-type strain and strains with mitochondrial mutation.

1. DNA synthesis of epistatic mutant 5d was extremely high.
2. Lowering of RNA synthesis was observed with 5d and segregational mutant 431.
3. Extreme inhibition by the operation of chloramphenicol and cycloheximide in RNA synthesis was observed with 431.

From the above results, the developmental mechanism of mitochondrial mutation was investigated in this report.

In an incubation medium, 0.25m moles sorbitol, 20μmoles Tris.Cl pH7.4, 1μ mole EDTA, 10μ moles $MgSO_4$, 50mμ moles dATP, dETP, dCTP, 5μC ^{3}HTTP (specific activity 12C/m mole), phosphenolypruvate 300μg, pyruvate kinase 30μg, mitochondria (protein amount) 100~200μg were contained in 250μl.
As an inhibitor of mitochondrial DNA synthesis 6μ moles ethidium bromide was contained in this medium. For the observation of the action of antibiotics, 2μg/ml of cycloheximide and 2mg/ml chloramphhemicol were added to the medium.
After 30 minutes of incubation at 37°, the reactor was stopped with 1ml 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 infrared light.

Table I: Measurement of Activities of Mitochondrial DNA Synthesis in Vitro

Strain	**106**	**106 AC**	**431**	**5 d**
In vitro system				
Complete system	1830 μμmoles	2400 μμmoles	1820 μμmoles	5690 μμmoles
cycloheximide	2610	2650	1320	4620
chloamphenicol	2260	2350	2020	5620
ethidium bromide	92	330	50	260
– d × TP	75	46	88	46
– Mg ion	50	26	85	75

Table II: Measurement of Activities of Mitochondrial RNA Synthesis in Vitro

Strain	**106**	**106 AC**	**431**	**5 d**
In Vitro system				
Complete system	1310 μμmoles	1020 μμmoles	560 μμmoles	700 μμmoles
cycloheximide	1590	750	150	620
chloamphenicol	1430	700	210	550
actiomycini D	360	262	90	700
– XTP	26	40	41	34
– Mg ion	38	25	26	28

A Beckman liquid scintillation counter (LS-133 liquid scintillation system) was used with vial 15ml scintillation liquid and ^{3}HTTP incorporation into DNA chain was measured for 5 minutes. Specific activity of reduced ^{3}HTTP was converted into μμ moles.

In an incubation medium, 0.25m moles sorbitol, 20μmoles Tris.Cl pH 7.4, 50μ moles KCl, 10μ moles $MgCl_2$, 40mμ moles CTP, GTP, 1μ mole ATP, 1μC ^{3}HUTP (specific activity 14.8C/m mole), 300μg phosphenolypruvate, 30μg pyruvate kinase, mitochondria (protein amount) 100~200μg were contained in 250μl.
For inhibition 5μg actinomycin D was added. To observe in vitro action of antibiotics 2 μg/ml of cycloheximide and 2mg/ml chloramphhemicol were added in solution.

Table III: Ratios of [(RNA synthesis specific activity) / (DNA synthesis specific activity)]

	106	**106 AC**	**431**	**5 d**
RNA synthesis activity / DNA synthesis activity	0.70	0.42	0.30	0.12

After 30 minutes of incubation at 37°, the reactor was stopped with 1ml 10% TCA, then the solution was filtered with membrane filter washing with 5ml 5% TCA followed by 5ml 10% TCA, and the filter was then dried with infrared light.
The filter was measured with a Beckman liquid scintillation counter in a 5ml vial with scintillation liquid for 5 minutes and incorporation of ^{3}HUTP into mitochondrial RNA was observed.
Specific activity of reduced ^{3}HUTP was converted into μμ mole.

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