SYMPOSIUM NO. 8: NON-CHROMOSOMAL INHERITANCE

GENETIC CONTROL OF MITOCHONDRIA IN PARAMECIUM

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ABSTRACT

Mitochondrial mutations for resistance to various antibiotics (erythromycin, chloramphenicol, spiramycin, mikamycin) have been obtained in *Paramecium aurelia* and their properties are reviewed. Using these mitochondrial markers, the interactions between nucleus and mitochondria have been studied in two ways: by microinjection of mitochondria from one stock or species into other stocks and species of *P. aurelia* and by a genetic study of a nuclear mutation affecting mitochondrial multiplication. Both types of experiments show: (1) that there may exist incompatibility between a given type of mitochondria and the cell into which they are introduced and (2) that through multiplication in the host cell, mitochondrial properties can be modified. The possible basis for incompatibility and host-induced modifications is discussed.

PARAMECIUM AURELIA is a unique organism from a genetical point of view, because nearly all the phenomena that have been studied with it for almost 30 years involve some kind of cytoplasmic determination. Beginning with the kappa particles and the killer trait, various characters—mating types, serotypes and the pattern of organization of its surface structures—have been shown to be, at least partly, cytoplasmically inherited. (See for review PREER 1968). Last in this series of “non-Mendelian” phenomena come certain mitochondrial characteristics. Needless to say however we do not wish to imply that orthodox nuclear heredity is less important in *Paramecium* than in any other organism.

With respect to mitochondrial genetics, *Paramecium* offers various advantages. (1) the cell is rather big (100 μ long), and microinjection techniques have been developed (KOISUMI and PREER 1966; KNOWLES 1973). (2) The process of conjugation, which may or may not be accompanied by varying amounts of cytoplasmic exchange, provides a range of situations for following the transmission of mitochondrial markers. (3) Finally, the name *Paramecium aurelia* actually covers a number of genetically isolated “species” (formerly called *syngens*), which provide, by the use of the microinjection technique, a unique opportunity to study the degree of autonomy of mitochondria in relation to the nuclear genomes.

This paper briefly reviews the main properties of the mitochondrial mutations so far studied in *Paramecium* (species 1 and 4) and presents some recent data on nucleo-mitochondrial interactions.

RESULTS

Mitochondrial Mutations in Paramecium

Mutants have been obtained by screening cells resistant to various antibiotics known for their interaction with the 50S moiety of bacterial ribosomes: erythromycin (ERY), chloramphenicol (CAM), spiramycin (SPI), mikamycin (MIK). These mutants grow in the presence of certain concentrations (100–250 γ /ml) of the antibiotics which completely inhibit the multiplication of wild type cells. Doubly-resistant mutants to chloramphenicol and erythromycin ($C^R E^R$) or mikamycin and erythromycin ($M^R E^R$) have also been obtained by two step selection procedures.

1) *Genetic basis of the mutations*

Genetic analysis has shown that all these mutations are cytoplasmically inherited (BEALE 1969; ADOUTTE and BEISSON 1970) and microinjection experiments demonstrated that they were mitochondrial. This demonstration was made by the following procedure.

A partially purified preparation of mitochondria extracted from a resistant strain, E^R for instance, is injected into a sensitive cell (E^S). The recipient cell is placed in medium containing erythromycin. The injected cell at first behaves as a sensitive one and does not divide, then after a lag of 2–3 or many more days, depending mainly on the number of injected mitochondria, it resumes growth and becomes resistant. This process of “transformation” has been extensively studied cytologically and genetically both on microinjected cells (KNOWLES 1972) and on E^S cells which have received E^R mitochondria through the more “natural” channel of a cytoplasmic bridge at conjugation (PERASSO and ADOUTTE, 1974). Both cytological and genetical data show that, under the pressure of the antibiotic, there is a selective multiplication of the few initial E^R mitochondria along with an elimination of those containing the E^S genome. After a few fissions, the ex- E^S cell has become purely erythromycin-resistant.

Doubly-resistant mitochondria have also been transferred into sensitive cells: for instance $M^R E^R$ mitochondria have been microinjected into sensitive cells in species 1, and $C^R E^R$ mitochondrial has been introduced through cytoplasmic exchange into sensitive cells in species 4. The recipient sensitive cell always acquires the doubly-resistant phenotype ($M^R E^R$) or ($C^R E^R$), regardless of the selective agent applied to the recipient: ERY or MIK in the first case, ERY or CAM in the second case (BEALE 1973).

On the whole, it is most likely that this process does not involve a genetic transformation of the sensitive mitochondria but rather their progressive replacement by the resistant ones.

2) *Phenotype of the resistant mutants*

Mutants resistant to a given antibiotic may vary in their level of resistance: while highly resistant mutants grow as well in the presence of the antibiotic as in its absence, less resistant mutants may have a reduced growth rate in the presence of the antibiotic and even show slight alterations in the structure of their mitochondria (ADOUTTE *et al.* 1972).

Besides the resistance itself, the mutations may have pleiotropic effects bearing mainly on cross resistance to other antibiotics (ADOUTTE, 1974), thermosensitivity and growth rate. When observed, this thermosensitivity is also mitochondrially inherited and undissociable from the resistance determinism (ADOUTTE and BEISSON 1970). In species 4, various E^R mutants were found to be thermosensitive while all C^R mutants appear to be thermoresistant; but the reverse has been found for some E^R and C^R mutants in species 1. Finally the effect of some mutations on growth rate is worth mentioning. It has been shown (ADOUTTE and BEISSON 1972) that several mutations towards resistance confer on the mitochondria a reduced rate of replication. Within the same cell, mitochondria of different genotype may multiply at different rates. Moreover, cells pure for such resistant mutations may have a reduced rate of replication in the absence of the antibiotic.

3) *Molecular basis of the resistant mutations*

Some at least of these mutations seem to affect mitoribosomal properties. Mitoribosomes of some E^R strains fail to bind radioactive ERY *in vitro* while mitoribosomes of E^S cells do (TAIT 1972). For one E^R strain of species 1, a change of one band on polyacrylamide gels electrophoresis of mitoribosomal proteins has been obtained as compared with the wild type (BEALE, KNOWLES and TAIT 1972). This fact may be interpreted as an indication that one or more mitoribosomal proteins are altered in resistant cells, but other interpretations are possible e.g., there might be a secondary effect of an altered mitoribosomal RNA. This idea, which was first developed by BORST (1972), and seems more and more in favor, is also consistent with the pleiotropic effects of many E^R , C^R and M^R mutations obtained in Paramecium, as was also observed in yeast (GRIVELL *et al.* 1973).

A different class of mutation may be exemplified by the mikamycin-resistant strains. Preliminary experiments indicate that mitoribosomes from some mikamycin-resistant mutants, which are also weak E^R , do bind radioactive ERY (BEALE and SPURLOCK, *in press*). This result suggests that the M^R mutations could affect the membranes of the mitochondria rather than the mitoribosomes themselves. Again, however, this interpretation should not be regarded as conclusively established, since changes not affecting the antibiotic-binding properties of mitoribosomes could result in changes in resistance.

In summary, the various classes of mitochondrial mutations obtained in Paramecium look very similar to those described in yeast. With these mutations, we concentrated on using the various experimental possibilities offered by Paramecium to investigate the interactions between nuclear and mitochondrial genomes.

Interactions between Nucleus and Mitochondria

This important aspect of mitochondriology has been attacked by two techniques: (1) by microinjection of mitochondria into cells of different genotype and into different "species" (KNOWLES 1973) and (2) by a genetic study of a peculiar nuclear mutation affecting mitochondrial multiplication (SAINARD 1973, SAINARD *et al.* 1974).

1) *Transfer of mitochondria between stocks and species by microinjection*

The general methodology is as follows. Mitochondria carrying a resistance marker are isolated from species A and injected into sensitive cells of species B. After injection, the recipient cell is placed in the antibiotic (for example ERY); at least two questions can be raised *a priori*: Will the mitochondria of A origin develop in B cells? If they do, what will be the properties of A mitochondria after multiplication in B cells?

FIGURE 1.—Microinjection of resistant mitochondria from species 1 into species 5. The dotted cells represent resistant cells and the white ones sensitive recipient cells; the black "dumbbells" symbolize the purified mitochondria extracted from a resistant culture and injected into a sensitive cell. Solid arrow symbolizes a high efficiency of transformation of the injected cell and dashed arrow symbolizes a low efficiency of transformation.

Extensive experiments have been carried out in nearly all directions using species 1, 5 and 7, as well as between two stocks of species 1.

Three types of situation have been found in connection with the first question raised: (1) the injected mitochondria fail to transform the recipient cell; (2) the injected mitochondria transform the recipient cells as well as they transform cells of their own species: this is termed *high efficiency* of transfer ($> 50\%$ of injected cells); (3) the injected mitochondria show a *low efficiency* of transfer: in all experiments, less than 10% of recipient cells are transformed, and interestingly, the lag before the injected cells resume growth is much increased (19–29 days instead of 5–6). It is interesting to note also that reciprocal transfers do not always give similar results: the transfer $A \rightarrow B$ can have a low efficiency while the transfer $B \rightarrow A$ has a high efficiency.

With regard to the properties of mitochondria of one species multiplying in cells of another species, two types of situations have been found. The first situation is illustrated in Figure 1. When erythromycin-resistant mitochondria from species 1 are microinjected into sensitive species 5 cells, erythromycin resistance is transmitted to species 5 with low efficiency, while injection of similar mitochondria into species 1 gives a high efficiency of transfer. In contrast, after growth in species 5, species 1 mitochondria are found to give a high efficiency of transfer into both species 1 and 5. Further, species 1 mitochondria, after growth in species 5 and retransfer back to species 1, again show low efficiency transfer into species 5 and high efficiency transfer into species 1.

Therefore, the high or low efficiency of transfer is not an intrinsic property of E^R mitochondria but is determined by the nucleo-cytoplasmic environment in which the mitochondria multiply.

FIGURE 2.—Microinjection of mitochondria from species 1 or 5 into species 7 and of mitochondria from species 1 (after a passage in species 5) into species 7. Symbols as in Figure 1.

The second situation is illustrated in Figure 2. The transfer of species 5 mitochondria ($5E^R m_5$) into cells of species 7 shows a high efficiency, while the transfer of species 1 mitochondria ($1E^R m_1$) into cells of species 7 has a low efficiency. The transfer of mitochondria from species 1, after a period in cells of species 5 ($5E^R m_1$), into species 7, also has a low efficiency. In this case, it appears that the ability for $E^R m_1$ mitochondria to grow in species 7 is a mitochondrially inherited property and is not modified in the nucleo-mitochondrial hybrid.

These facts raise a number of questions which are not easy to answer yet. The most intriguing phenomenon is the basis for low efficiency transfers and the "host induced" modifications of mitochondrial phenotypes.

Very similar phenomena which will now be described have been observed in a completely different type of situation; both sets of facts will be discussed together at the end.

2) Interactions between a nuclear gene and mitochondrial development

In species 4, a slow growing mutant, cl_1 , has been isolated. Homozygotes cl_1/cl_1 are thermosensitive and have about 1/10th of the wild-type amount of spectroscopically detectable cytochrome oxidase. This complex phenotype is controlled by a single nuclear recessive gene. But as illustrated in Figure 3, crosses $cl_1/cl_1 \times$

FIGURE 3.—Crosses $cl_1/cl_1 \times cl_1^+/cl_1^+$. 1: conjugation without cytoplasmic exchange. After autogamy, the heterozygous F_1 clones (●) yield homozygous F_2 cells cl_1/cl_1 (○) and cl_1^+/cl_1^+ (●) in a 1:1 ratio. The generation time of the parental strains and of the F_1 and F_2 clones is indicated on both sides of the diagram.

$+/+$ also reveal a cytoplasmic component. In F_2 , cl_1/cl_1 or $+/+$ homozygotes can be obtained either in combination with cytoplasm derived from the cl_1 parent or with cytoplasm derived from the wild-type parent. While cl_1 homozygotes in cl_1 cytoplasm are identical to the cl_1 parent, cl_1 homozygotes in wild type cytoplasm are different: they have a much more reduced growth rate (1–2 fissions a day *vs.* 3 fissions a day for the cl_1 parent) and their mitochondria are spectacularly altered, with very few or no cristae. However, after 15–25 cell generations, these sick F_2 clones resume a 3-fission-a-day growth rate and their mitochondria revert to normal structure.

More can be learned if both strains carry different mitochondrial markers, for instance if mitochondria in cl_1 strains are E^S ($M^{cl}E^S$) and mitochondria in wild-type strain are E^R (M^+E^R), or *vice versa*, and if cytoplasmic exchanges occur at conjugation (Figure 4).

a) If the cl_1 exconjugant which contains a majority of mitochondria of type $m^{cl}E^S$ and a minority of M^+E^R is placed in erythromycin (Figure 4, IA), one observes a low efficiency of transformation, with the same two features as in the

FIGURE 4.—Crosses $cl_1/cl_1 \times cl_1+/cl_1+$. 2: conjugation with cytoplasmic exchange. I: the parents cl_1/cl_1 (○) and cl_1+/cl_1+ (●) are respectively E^S (white cytoplasm) and E_1^R (dotted cytoplasm). E_1^R is an erythromycin-resistant mutation, selected in the wild type strain. II: The parents cl_1/cl_1 (○) and dcl_1+/cl_1+ (●) are respectively E_{201}^R (starred cytoplasm) and E^S (white cytoplasm). E_{201}^R is an erythromycin-resistant mutation selected in the cl_1 strain. Symbols for high and low efficiency of transformation as in Figures 1 and 2.

inter-species microinjection experiments: long lag and fewer transformations than normal in presence of the cl_1^+ gene. In the reciprocal situation (wild-type exconjugant receiving $M^{cl}E^R$ mitochondria) there is a high efficiency of transformation (Figure 4, IIB).

b) If the wild-type exconjugant which contains a majority of M^+E^R and a minority of $M^{cl}E^S$ mitochondria is grown in non-selective medium and if an F_2 is obtained (Figure 4, IB), the two classes of homozygotes cl_1/cl_1 and cl_1^+/cl_1^+ segregate. When these F_2 homozygotes are tested for resistance to ERY, it appears that all cl_1^+/cl_1^+ segregants are pure E^R while all the cl_1/cl_1 segregants are pure E^S . All these F_2 clones derive from the same heterozygous F_1 clone: the only difference between them is due to the nuclear reorganization (autogamy) which made them become cl_1 or cl_1^+ homozygotes. It can therefore be concluded: (1) that both types of mitochondria can be maintained in a cl_1/cl_1^+ heterozygote (Figure 4, IB or IIA) but (2) that once a cell becomes homozygous, each allele rapidly selects the type of mitochondria with which it was previously associated in the parental strain. A comparable phenomenon has been described for chloroplasts in *Pelargonium* (TILNEY-BASSETT 1973).

There is no doubt that the cl_1 mutant differs from the wild type both by a nuclear gene and by a mitochondrial difference. But the nature of this mitochondrial difference is not at all clear. It does not seem to be strictly dependent on mitochondrial genetic factors: in cl_1/cl_1 homozygotes, the wild-type mitochondrial condition is slowly converted, through 15–25 cell generations, into an apparently compatible condition.

As a conclusion to this report, we will now try to analyze the basis for the modifications of mitochondrial properties observed in both the interstock-interspecies microinjection experiments and in those with the cl_1 mutant.

CONCLUSION

The striking facts emerging from the two groups of data just described can be summarized in the following way.

A sort of *incompatibility* can be observed between a given type of mitochondria and the cell into which they are introduced, reminiscent of phenomena observed for chloroplasts in *Oenothera* (SCHÖTZ 1970). The manifestations of this incompatibility are twofold: (1) a difficulty for the "foreign" mitochondria to multiply in the host cell. Even under the selective pressure of the antibiotic, they often cannot multiply or if they eventually multiply, a long lag is required before they can do so. In the case of wild-type mitochondria confronted with cl_1 genome, this incompatibility is expressed by an abnormal mitochondrial structure. (2) By multiplying in a foreign cytoplasm, mitochondria (although they retain their original marker genes) can be *modified*. In the microinjection experiments, mitochondria from one species multiplying in cells of another species may have their compatibility with the cytoplasm of another species modified. In the case of the cl_1 mutant, the wild-type mitochondria eventually lose their incompatibility with the cl_1 genotype.

To explain the molecular basis of this incompatibility and of these "host induced" modifications of mitochondrial properties, two different types of hypotheses can be proposed. Either these phenomena involve differences and modification in mitochondrial nucleic acids or, alternatively, in the organization of mitochondrial membranes.

In the first type of hypothesis, at least two kinds of processes can account for the data: incompatibility results from a lack of mutual fitness between products of mitochondrial and of nuclear origin and the modification of the mitochondrial properties depends on mutation and selection of a more fitting mitochondrial genotype. Another alternative is a process of host induced modification of the mitochondrial genome similar to that described for phage (ARBER 1969). Genetic experiments using more mitochondrial mutants and biochemical study of mitochondrial nucleic acids might provide relevant information.

In the absence of such data, it is also interesting to consider the other type of hypothesis, based only on differences in the pattern of organization of molecules or molecular complexes in the mitochondrial membrane. As shown in Figure 5, the genetic differences in the mitochondria or in the nucleus, are expressed by a different pattern of organization. It is reasonable, as discussed by HERSHEY (1970), to admit that membranes have a material continuity which results in some type of membrane inheritance. This implies that membranes grow through

FIGURE 5.—Pattern differences in membrane structure. It is not specified whether the dots and circles are molecules or molecular aggregates or complexes (like cytochrome oxidase, for instance). This scheme does not necessarily require that an ordered arrangement extends all over the membrane: the influence of the preexisting organization need not extend over more than a limited group of molecules or molecular complexes. It may be enough to assume that around a spot showing the A type of organization, the available interacting sites show a reduced or zero affinity for accepting the B type building blocks.

localized insertion of new molecules which find their place according to their physiochemical properties within the established framework. Therefore, whenever type A building blocks only are available for membranes of type B, or *vice versa*, these building blocks cannot easily get inserted into the preexisting framework and the conversion from one type of organization to the other is a long and difficult process.

Decisive experiments in favor of this type of hypothesis are not yet easy to obtain. However, the hypothesis deserves consideration and it is specially appealing to us because it is in *Paramecium* that the only experimental evidence for the inheritance of surface patterns, unrelated to any nucleic acid modification, has been given (SONNEBORN 1963; BEISSON and SONNEBORN 1965).

It is not unlikely that mitochondrial heredity as well as cell heredity in general is based both on nucleic acid specificity and on membrane continuity.

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