ABSENCE OF DETECTABLE MITOCHONDRIAL RECOMBINATION IN PARAMECIUM

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ABSTRACT

An extensive search for recombination between mitochondrial markers was carried out in Paramecium tetraurelia. Thirty-two combinations, altogether involving 24 different markers, were studied. The markers belonged to the three main categories of mitochondrial mutations presently available in this organism. (a) Spontaneous or UV-induced antibiotic resistance mutations, most probably affecting mitochondrial ribosomes, (b) nitrosoquaguanidine-induced antibiotic resistance markers displaying thermosensitivity or slow growth, enabling easy selection of possible wild-type recombinants, and (c) mitochondrial partial suppressors of a nuclear gene, probably corresponding to molecular alterations distinct from the preceding two categories. In addition, different genetic configurations were analyzed (i.e., mutant × mutant, double-mutant × wild-type, etc.).—None of the combinations yielded any evidence for the occurrence of recombined genomes despite the fact that: (1) all of them were studied on a large scale involving the screening of at least several thousand mitochondrial genomes (often several millions), (2) in many of them the detection level was sufficiently high to enable the isolation of spontaneous mutants in control cells, and (3) in several of them, reconstitution experiments carried out in parallel show that the conditions were fully adequate to detect recombinant genotypes. The results are in marked contrast with those obtained on the few other organisms in which mitochondrial recombination has been studied, particularly Saccharomyces cerevisiae, in which mitochondrial recombination is intense. —The most likely basis for the various manifestations of mitochondrial genetic autonomy in Paramecium, described in this as well as in previous publications, is that the chondriome of this organism is made up of thousands of structurally discrete, noninteracting units.

THE isolation of mitochondrial antibiotic-resistant mutations in yeast (WILKIE, SAUNDERS and LINNANE 1967; THOMAS and WILKIE 1968a; LINNANE et al. 1968) was rapidly followed by the discovery of recombination between these mitochondrial markers (THOMAS and WILKIE 1968b; COEN et al. 1970) and has since led to extensive experimental and theoretical analysis of this process (reviewed by GILLHAM 1978; DUJON and SLONIMSKI 1976; BIRKY 1978). This analysis has revealed a number of interesting features of the mitochondrial recombination process (DUJON, SLONIMSKI and WEILL 1974; HOWELL et al.

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and, in conjunction with biochemical methods, has provided a powerful tool for analyzing the informational content of mitochondrial DNA (examples can be found in several recent symposia: Bücher et al. 1976; Saccone and Kroon 1976; Bandlow et al. 1977; Bacilla, Horecker and Stopani 1978). The occurrence of mitochondrial recombination has since been documented in other fungi, using genetic methods (Aspergillus nidulans: Rowlands and Turner 1974; Podospora anserina: Belcour and Begel 1977; Schizosaccharomyces pombe: Seitz-Mayr, Wolf and Kaudewitz, 1978; Kluyveromyces lactis: Brunner, de Cobos and Griffiths 1977) and in one type of mammalian somatic-cell hybrids, using biochemical techniques (Horak, Coon and Dawid 1974).

Mitochondrial antibiotic-resistant mutations have been available for several years in Paramecium, both in species 1 (Beale 1969; Beale, Knowles and Tait 1972) and 4 (Adoutte and Beisson 1970, 1972). These mutants were initially isolated with the aim of extending the study of mitochondrial recombination to a quite different organism that, unlike yeast, was an obligate aerobe.

No recombination was found between these mutations in early studies (Adoutte and Beisson 1972; Beale 1973) except for preliminary results involving one particular \(C^r \times E^r\) combination (Adoutte 1974). However, for two reasons, the combinations of markers available at that time were not fully satisfactory. First, in most combinations, only the double-mutant genotype was readily detectable (i.e., \(C^rE^r\) in a \(C^rE^r \times C^sE^s\) combination). Identification of presumptive wild-type \(C^sE^s\) recombinants could be achieved only after they had segregated vegetatively, and this was known to be a very lengthy process in Paramecium for these markers. The fact that recombination was not detected in these experiments might therefore have been due to low viability or even lethality of the double-mutant recombinants. (It was known that double mutants isolated by mutation often displayed physiological perturbations.) Second, all the available mutations had been screened for resistance to mitoribosomal drugs, and it had been shown for several of them that they affected the mitoribosomes (Tait 1972; Spurlock, Tait and Beale 1975). Because of this it was possible that all the mutants might be closely linked.

Since then, a number of new mitochondrial markers have been isolated in species 4 of Paramecium and some of them, at least, most probably correspond to nonmitoribosomal functions (Sainsard-Chanet 1978; Knowles and Adoutte, unpublished). Several of the new markers display a strong selective disadvantage with respect to wild-type and several are highly thermosensitive, enabling the rapid selection of possible wild-type recombinants in mutant \(\times\) mutant combinations. Finally the availability of the technique of mitochondrial microinjection (Knowles 1974) has provided a convenient way of constructing a large number of "mixed" mitochondrial clones.

This paper summarizes the results of the analysis of 32 pair-wise combinations of mitochondrial markers in several different genetic configurations (mutant \(\times\) mutant, double mutant \(\times\) wild type, etc.). The combination that had earlier

* The nomenclature of mitochondrial mutations used in the present paper is described in the Materials and Methods.
apparently yielded recombinants was also investigated. No recombination was detected in any of these combinations despite the fact that control experiments performed in parallel by microinjection show that the conditions were more than adequate to detect recombination events between the markers under study. Furthermore, in several experiments, the screening was sufficiently powerful to detect spontaneous reverse mutations.

These results, taken together with other genetic data pointing to the considerable genetic autonomy of mitochondria in Paramecium, indicate that genetic recombination between mitochondria probably does not occur in that organism. The possible structural basis of this autonomy, as well as the evolutionary significance of these findings, is discussed.

MATERIALS AND METHODS

The strains used in the present study, their origin and their phenotype are listed in Table 1. The following conventions have been adopted for strain nomenclature.

1. Mitochondrial antibiotic-resistant mutants are designated by a capital letter referring to the drug to which they are the most resistant (E: erythromycin; C: chloramphenicol; S: spiramycin) followed by a capital R superscript and a number subscript (e.g., ER_R). Since there are presently no genetic means of distinguishing different genes in the Paramecium mitochondrial genome, the numbers simply designate independent mutant isolates. When mitochondrial mutants were isolated after a nitrosoguanidine mutagenesis (see below), their strain number is preceded by “NG” and followed by a number referring to a particular mutagenesis experiment (e.g., ER_NO5-91).

2. Since most of the crosses (or microinjections) were carried out between isonuclear strains, the nuclear genotype is usually not given, and the strains are designated by the name of the mitochondrial marker they harbor. When necessary the diploid nuclear genotype is given in brackets.

3. Most of the work reported involves combinations of markers carried out within stock d4-2 of Paramecium tetraurelia (Sonnewborn 1975). However, some combinations involve stocks 29 and 32 of the same species. Only in these cases have the number of the stocks been indicated (e.g., 29 ER_R).

4. Markers of the cl system have been defined elsewhere (Sainsard-Chanet 1976, 1978) and are discussed in the text. They are designated M cl, M mu and M sup and can be briefly defined as mitochondrial partial suppressors of the nuclear mutation cl.

Several of these strains have been previously described (Adoutte and Beisson 1970; 1972; Adoutte 1974; Adoutte and Doussiére 1978; Sainsard 1975; 1976, 1978) while others correspond to newly isolated markers. Much effort has been put into isolating mutants resistant to drugs acting on elements other than the mitochondrial ribosomes (i.e., ethidium bromide, oligomycin, etc.). This approach has been unsuccessful in Paramecium (Sainsard-Chanet and Adoutte, unpublished; discussed in Adoutte 1977). Other methods were therefore sought to obtain new types of mitochondrial markers. In particular, it was found that N-methyl-N’nitro-N-nitrosoguanidine (NG) was an extremely powerful mitochondrial mutagen in Paramecium, yielding up to 100% ER or 100% CR mutants from cells grown for 24 or 48 hr in 1 to 2 μg/ml of NG (Knowles and Adoutte 1977). Among 100 newly isolated ER and 100 CR mutants, a few displayed new phenotypical characteristics with respect to the previously isolated mitochondrial mutants; some are highly thermosensitive, others are clearly slow growing. Those used in the present study have been included in Table 1.

Growth conditions: All the techniques for handling the cells, carrying out crosses, thermosensitivity and antibiotic resistance test, etc. have been previously described (Sonnewborn 1970; Adoutte and Beisson 1970; Perasso and Adoutte 1974).
<table>
<thead>
<tr>
<th>Name of Strain</th>
<th>Type</th>
<th>Selective Agent</th>
<th>400</th>
<th>600</th>
<th>800</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₄, TR₄</td>
<td></td>
<td>ERY</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>36°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>U.V.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>Spont.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>U.V.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>Spont.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>CAP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>CAP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>then ERY</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>ERY</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>NG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>NG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E₄</td>
<td></td>
<td>high thermosensitive</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Phenotypes of the mitochondrial mutants**

<table>
<thead>
<tr>
<th>CLASS</th>
<th>Name of Strain</th>
<th>Origin</th>
<th>27°C Growth rate at 35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>E₄, TR₄</td>
<td>UV</td>
<td>+/—</td>
</tr>
<tr>
<td>CLASS</td>
<td></td>
<td>36°C</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>U.V.</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spont.</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U.V.</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spont.</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAP</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAP</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>then ERY</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERY</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERY</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NG</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NG</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>highly thermosensitive</td>
<td>—</td>
</tr>
</tbody>
</table>
The mitochondrial markers used in the present study have been grouped into three main classes. Class I corresponds to spontaneous or UV-induced antibiotic-resistant mutants; Class II to nitrosoguanidine-induced antibiotic-resistant mutants displaying additional unusual phenotypes (see text); and Class III corresponds to a set of suppressors of the nuclear cl, mutation. Except when indicated (i.e. 29 or 32), all markers were isolated in stock d4-2 of *Paramecium tetraurelia* (see Materials and Methods). The following symbols have been used:

--- spont = spontaneous mutant
--- UV or NG = ultraviolet or nitrosoguanidine induced mutant
--- ERY = erythromycin
--- SPI = spiramycin
--- CAP = chloramphenical
--- MIK = mibamycin
--- OLI = oligomycin

The numbers placed below the various inhibitors represent concentrations in μg per ml

--- +++ = four to five fissions/day (identical to wild type grown in normal medium)
--- + + = three fissions per day
--- + = one to two fissions per day
--- ε = one fission per day or less
--- ++/---, +/---, +/----, +/------ indicate that the cells undergo, respectively, 10, 7 to 8, 5 to 6 or 3 to 4 residual fissions at 36° then die.

<table>
<thead>
<tr>
<th>Name of Strain</th>
<th>Type</th>
<th>Selective Agent</th>
<th>Origin</th>
<th>ERY</th>
<th>SPI</th>
<th>CAP</th>
<th>MIK</th>
<th>OLI</th>
<th>Growth rate at 27°</th>
<th>Growth rate at 36°</th>
</tr>
</thead>
<tbody>
<tr>
<td>M*</td>
<td>&quot;gene cl;&quot;</td>
<td>spont</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>M^{cl}</td>
<td>&quot;gene cl;&quot;</td>
<td>spont</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++/ε</td>
</tr>
<tr>
<td>M^{su}</td>
<td>&quot;gene cl;&quot;</td>
<td>spont</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++/ε</td>
</tr>
<tr>
<td>M^{cl,201}</td>
<td></td>
<td>ERY</td>
<td>spont</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>+ ++</td>
<td>++/ε</td>
</tr>
<tr>
<td>M^{su,ER}</td>
<td></td>
<td>ERY</td>
<td>spont</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
<td>++/ε</td>
</tr>
</tbody>
</table>

**TABLE 1—Continued**
Microinjection: Microinjection was carried out as described by Knowles (1974) except that in most instances purification of the mitochondria to be injected was not carried out. A fraction of cytoplasm was taken from a donor cell and injected immediately into a recipient cell. The number of mitochondria thus transferred can be roughly estimated to be of the order of 100. The frequency of successful mitochondrial transfers using this method is extremely high; usually 90 to 100% of the cells survive the injection.

RESULTS

General methodology

The search for mitochondrial recombination obviously requires the prior generation of cells containing at least two types of mitochondrial genomes differing at two loci. Such cells were obtained either by crossing strains having different mitochondrial markers and selecting pairs that underwent cytoplasmic exchange through cytoplasmic bridges at the end of conjugation, or by introducing "foreign" mitochondria into recipient cells by microinjection. In both cases, the input of the two markers is very biased, the marker present in the recipient cell being predominant. Most generally, however, the two markers display different replication rates during cell growth in normal medium (see Adoutte and Beisson 1972) and, if the introduced marker replicates faster during vegetative multiplication of cells containing a "mixture," one obtains a progressive enrichment for that marker. At certain stages, depending on the particular combination studied, roughly equal amounts of the two types of markers will be present. This process usually expands over many cell generations (from 10 to over 100). By adequately selecting the direction in which the initial mixing is carried out, starting from a small number of unsymmetrically mixed cells, one can obtain a large number of "well mixed" ones. Furthermore, this enables the two types of markers to co-exist within the same cytoplasm for extended periods, thereby favoring the possible occurrence of genetic exchange.

Once cells containing roughly equal proportions of the two mitochondrial types were obtained, different protocols for identifying recombinants were used depending on the availability of a positive, or a semi-positive screening system. For instance, starting with cells containing \( C^r + E^r \) mitochondria, \textit{a priori} there exists a good selective system for finding \( C^rE^r \) recombinants. The cells were placed directly in medium containing both antibiotics. To improve the possibility of detecting rare recombinants, the cells were often placed in subinhibitory concentrations of both antibiotics, thereby enabling slow cell growth and expression of presumptive recombinants. In addition, large numbers of cells were tested. Alternatively, one can start with \( C^rE^r + C^sE^s \) mitochondrial mixtures and search for \( C^sE^s \) or \( C^rE^s \) recombinants. In that case, only a half-selective screen was available, consisting of growing cells in one of the two antibiotics, then determining whether the second marker had remained associated with the selected one. In that case, one has to try to exert a strong selective pressure in favor of the \( C^rE^s \) recombinant, for instance, with respect to the \( C^rE^r \) parent, by carrying out successive cell transfers in medium containing only chloramphenicol. One can also search for \( C^sE^s \) recombinants in a \( C^r + E^r \) combination.
In this case, there usually is no positive screening and the mixed cells must be grown in nonselective medium for extended periods to allow the vegetative segregation of \( C^r E^r \) recombinants. This is possible only if both the \( C^r \) and \( E^r \) parents show a selective disadvantage with respect to wild type. Each of these systems had advantages and drawbacks, which will be discussed later.

Most of the combinations analyzed involved markers isolated from the same original wild-type stock (different stocks of Paramecium are descendants of cells isolated at different geographical locations; see Sonneborn 1975). Since differences in mitochondrial recombination properties were found to occur between different isolates of \textit{Saccharomyces cerevisiae} (Coen et al. 1970), we extended the search for recombination in Paramecium to mutations isolated in different stocks. Finally, we also took advantage of the interactions occurring between the \textit{cl}, nuclear gene and its mitochondrial suppressors (Sainsard 1975; Sainsard-Chanet 1976, 1978) to search for mitochondrial recombination.

In these experiments it was of critical importance to be able to distinguish recombination from back mutation by using combinations of markers and number of cells that made the results significant. These aspects will be discussed in detail below.

All the combinations analyzed are summarized in Table 2 with their respective advantages and disadvantages.

\textit{Detailed analysis of a few combinations}

Since a detailed description of the 32 combinations analyzed would require too much space, especially in view of the fact that none of them provided any evidence for recombination, we describe here eight types of crosses and provide details for the most significant ones only.

\textit{Search for \( C^r E^r \) recombinants in \( C^{r_2} \times E^{r_1}, C^{r_3} \times E^{r_1} \) and \( C^{r_4} \times E^{r_1} \) crosses:}

These three crosses were analyzed according to the protocol described in Figure 1, with only minor variations. As can be seen on that figure, we searched for \( C^r E^r \) recombinants at three stages: (1) At the first post-conjugal fission or, after a few post-conjugal fissions, by placing one (or a few) cells derived from conjugants that had undergone cytoplasmic exchange in doubly selective medium in order to detect early recombinational events. (2) After the ex-\( E^r \) or ex-\( C^r \) conjugant had started to grow in chloramphenicol- or erythromycin-containing medium, respectively, with the idea that during multiplication of \( C^r \) mitochondria in an \( E^r \) host (and \textit{vice versa}), recombinational events may occur. (3) After over 20 generations of growth of the ex-\( E^r \) conjugant in normal medium and its enrichment in \( C^r \) mitochondria, which enabled testing a large number of cells containing roughly equal amounts of the two types of mitochondria.

It should be mentioned that a test of one Paramecium cell in doubly selective medium corresponds to the observation of a population of several thousands of mitochondria. The results of the three crosses are given in Table 3. It can be seen that except for two \( C^r E^r \) clones isolated at one stage from the \( C^{r_2} \times E^{r_2} \) cross, the results were negative (two \( C^r E^r \) clones out of 835 cells tested). Verification before the test (or during the test, on sister cells) that the tested cells indeed
### TABLE 2
Crosses in which mitochondrial recombination was searched for

<table>
<thead>
<tr>
<th>Type of combination</th>
<th>Phenotype selected for</th>
<th>Crosses</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Intrastock without cl&lt;sub&gt;1&lt;/sub&gt;</td>
<td>A&lt;sub&gt;R1&lt;/sub&gt;A&lt;sub&gt;R2&lt;/sub&gt;</td>
<td>CR&lt;sub&gt;3&lt;/sub&gt; × ER&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Positive screen</td>
<td>a&lt;sub&gt;1&lt;/sub&gt; — The mutational sites may be very closely linked, therefore recombination frequency may not be sufficiently different from mutation rate (A&lt;sup&gt;R1&lt;/sup&gt;A&lt;sup&gt;S2&lt;/sup&gt; → A&lt;sup&gt;R1&lt;/sup&gt;A&lt;sup&gt;R2&lt;/sup&gt; or A&lt;sup&gt;S1&lt;/sup&gt;A&lt;sup&gt;R2&lt;/sup&gt; → A&lt;sup&gt;R1&lt;/sup&gt;A&lt;sup&gt;R2&lt;/sup&gt;).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CR&lt;sub&gt;3&lt;/sub&gt; × ER&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CR&lt;sub&gt;3&lt;/sub&gt; × ER&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A&lt;sub&gt;R1&lt;/sub&gt;A&lt;sub&gt;S2&lt;/sub&gt; × A&lt;sub&gt;S1&lt;/sub&gt;A&lt;sub&gt;R2&lt;/sub&gt;</td>
<td>CR&lt;sub&gt;3&lt;/sub&gt; × ER&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Recombinants correspond to a wild-type genotype (no a&lt;sub&gt;2&lt;/sub&gt; disadvantage)</td>
<td>b&lt;sub&gt;1&lt;/sub&gt; — No positive screen. Have to rely on vegetative segregation of recombinant genotype. Should therefore combine two counterselected markers.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CR&lt;sub&gt;3&lt;/sub&gt; × ER&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A&lt;sub&gt;S1&lt;/sub&gt;A&lt;sub&gt;R2&lt;/sub&gt; and/or rapid growth</td>
<td>A&lt;sub&gt;S1&lt;/sub&gt;A&lt;sub&gt;R2&lt;/sub&gt;</td>
<td>CR&lt;sub&gt;RNG-12&lt;/sub&gt; × ER&lt;sub&gt;NGS-6&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CR&lt;sub&gt;RNG-21&lt;/sub&gt; × ER&lt;sub&gt;NGS-6&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CR&lt;sub&gt;RNG-12&lt;/sub&gt; × ER&lt;sub&gt;NGS-10&lt;/sub&gt;</td>
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<td></td>
<td>CR&lt;sub&gt;RNG-21&lt;/sub&gt; × ER&lt;sub&gt;NGS-10&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A&lt;sub&gt;R1&lt;/sub&gt;-tsA&lt;sub&gt;S2&lt;/sub&gt; × A&lt;sub&gt;S1&lt;/sub&gt;A&lt;sub&gt;R2&lt;/sub&gt;-ts</td>
<td>thermoresistant → A&lt;sub&gt;S1&lt;/sub&gt;A&lt;sub&gt;S2&lt;/sub&gt; if A&lt;sub&gt;R&lt;/sub&gt; and ts correspond to the same mutation)</td>
<td>ER&lt;sub&gt;RNG-21&lt;/sub&gt; × ER&lt;sub&gt;NGS-6&lt;/sub&gt;</td>
<td>Positive screen</td>
<td>c — As a&lt;sub&gt;1&lt;/sub&gt;.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER&lt;sub&gt;RNG-29&lt;/sub&gt; × ER&lt;sub&gt;NGS-6&lt;/sub&gt;</td>
<td>Recombinant genotypes are presumably wild type, therefore functional</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER&lt;sub&gt;RNG-21&lt;/sub&gt; × ER&lt;sub&gt;NGS-10&lt;/sub&gt;</td>
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<td></td>
<td></td>
<td>ER&lt;sub&gt;RNG-29&lt;/sub&gt; × ER&lt;sub&gt;NGS-10&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER&lt;sub&gt;RNG-21&lt;/sub&gt; × ER&lt;sub&gt;NGS-13&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER&lt;sub&gt;RNG-29&lt;/sub&gt; × ER&lt;sub&gt;NGS-13&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of combination</td>
<td>Phenotype selected for</td>
<td>Crosses</td>
<td>Advantages</td>
<td>Disadvantages</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------------------</td>
<td>------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>(\text{A}^{R}_1\text{A}^{R}_2 \times \text{A}^{S}_1\text{A}^{S}_2)</td>
<td>(\text{A}^{R}_1\text{A}^{S}_2) or (\text{A}^{S}_1\text{A}^{R}_2)</td>
<td>(\text{C}^{R}_1\text{E}^{R}_2 \times \text{C}^{S}\text{E}^{S}(\times 4))</td>
<td>(\text{A}^{R}_1\text{A}^{S}_2) recombinants are advantaged with (\text{A}^{R}_1\text{A}^{R}_2) in the presence of antibiotic (\text{A}_1)</td>
<td>(d_1) — The system is only half selective: the test must be carried out in two steps.</td>
</tr>
<tr>
<td>(\text{A}^{R}_1\text{su} \times \text{A}^{S}_1\text{-su})</td>
<td>(\text{A}^{R}_1\text{-su})</td>
<td>(\text{E}^{S}_1 \times \text{E}^{S}(\times 3))</td>
<td>Positive screen: one looks for reappearance of high erythromycin resistance</td>
<td>(d_2) — As (a_1), (c) — As (a_1),</td>
</tr>
<tr>
<td>(\text{A}^{R}_1\text{ts-su} \times \text{A}^{S}_1\text{-su})</td>
<td>(\text{A}^{R}_1\text{ts-su})</td>
<td>(\text{E}^{R}_1\text{TR}_1 \times \text{E}^{S})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{A}^{R}_1\text{ts} \times \text{M}^*)</td>
<td>thermostable</td>
<td>(\text{E}^{R}_{\text{NG5-10}} \times \text{M}^*)</td>
<td>Positive screen; Recombinant genotypes are wild type</td>
<td>(f_1) — Semi-selective system: one looks for non-thermo-resistant (\text{E}^{R}_1), (f_2) — As (a_1),</td>
</tr>
<tr>
<td>II. Interstocks</td>
<td>(\text{A}^{R}_1\text{A}^{R}_2) and (\text{A}^{S}_1\text{A}^{S}_2)</td>
<td>(29\text{ER}_6 \times \text{d}4\text{-2CR}_4(\times 4))</td>
<td>Recombination stimulated in interstock combinations?</td>
<td>Those listed in intrastock crosses plus possible negative interactions with nuclear genes.</td>
</tr>
</tbody>
</table>
TABLE 2—Continued

<table>
<thead>
<tr>
<th>Type of combination</th>
<th>Phenotype selected for</th>
<th>Crosses</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>III. Intrastocks with cl1</td>
<td>(cl1/cl1) M&lt;sup&gt;cl&lt;/sup&gt;E&lt;sup&gt;S&lt;/sup&gt;</td>
<td>(cl1/cl1) M&lt;sup&gt;cl&lt;/sup&gt;E&lt;sup&gt;R&lt;/sup&gt;201 × (cl&lt;sup&gt;+&lt;/sup&gt;/cl&lt;sup&gt;+&lt;/sup&gt;) M&lt;sup&gt;cl&lt;/sup&gt;E&lt;sup&gt;S&lt;/sup&gt;</td>
<td>1 — M&lt;sup&gt;cl&lt;/sup&gt; and E&lt;sup&gt;R&lt;/sup&gt; probably located in different genes</td>
<td>Non-selective or semi-selective system (see text).</td>
</tr>
<tr>
<td>M&lt;sup&gt;clA&lt;sub&gt;1&lt;/sub&gt;&lt;/sup&gt; × M&lt;sup&gt;+&lt;/sup&gt;A&lt;sup&gt;S&lt;/sup&gt;</td>
<td>M&lt;sup&gt;clA&lt;sub&gt;1&lt;/sub&gt;&lt;/sup&gt;</td>
<td>(cl1/cl1) M&lt;sup&gt;cl&lt;/sup&gt;E&lt;sup&gt;R&lt;/sup&gt; × (cl1/cl1) M&lt;sup&gt;+&lt;/sup&gt;E&lt;sup&gt;S+&lt;/sup&gt;</td>
<td>2 — M&lt;sup&gt;cl&lt;/sup&gt; has a selective advantage over M&lt;sup&gt;clE&lt;sup&gt;R&lt;/sup&gt;&lt;/sup&gt; and should therefore segregate with relative ease.</td>
<td></td>
</tr>
</tbody>
</table>

The following symbols have been used: A<sup>R</sup><sub>1</sub> and A<sup>R</sup><sub>2</sub> stand for antibiotic resistance markers displaying distinct phenotypes (usually resistance to two different antibiotics). A<sup>R</sup><sub>1</sub>-su is the presumptive genotype of partial revertants of some A<sup>R</sup> mutants, A<sup>R</sup><sub>1</sub> and su being located at two distinct mutational sites (see text). A<sup>R</sup><sub>1</sub>-ts represents the genotype of thermosensitive antibiotic-resistant mutants, no assumption being made as to whether A<sup>R</sup> and ts are at two distinct sites or whether the A<sup>R</sup> and ts phenotypes are due to a single molecular lesion. These symbols are all hypothetical, but they correspond to the simplest hypothesis for each individual strain, facilitate the description and do not introduce major bias in the discussion. The † sign indicates that the combination has been achieved by micro-injection. When a bracketed number is given after a cross, it represents the number of independent repeats of the cross that were carried out.
Figure 1.—This figure illustrates the way cells were handled and the stages at which they were tested in a typical CR x ER cross. Ex-conjugants from pairs that were observed to form a cytoplasmic bridge were separated and usually allowed to undergo one fission in normal medium (N). One of the two sister cells was kept in normal medium, yielding a clone from which subsequent subclones were isolated. The other was placed in medium containing chloramphenicol and erythromycin (CAP + ERY) to select for CRER recombinants. From the clone grown in normal medium, cells were transferred into medium containing either chloramphenicol (CAP), erythromycin (ERY) or both antibiotics, as well as into normal medium. Tests in single antibiotics allowed the identification of the parental origin of the clone, as well as the verification that cytoplasmic exchange had indeed occurred (for instance, cells derived from the CR parent multiplied immediately in chloramphenicol containing medium and, if mixed, multiplied but only after a lag of one to a few days in erythromycin-containing medium; see ADOUTTE and BEISSON, 1970). Tests in the double-selective medium were again aimed at isolating CRER recombinants. Regular successive transfers of the ex-ER conjugant in normal medium yielded large clones of mixed cells and also possibly allowed for the vegetative segregation of presumptive CSES recombinants (see Text). “Transformed clones” refer to ER cells that acquired chloramphenicol resistance after growth in CAP-medium and vice-versa. Such cells were also further tested in CAP + ERY (not shown on the figure). Additional transfers and tests were often carried out on cells displaying some residual growth in CAP + ERY medium (not shown).
TABLE 3

<table>
<thead>
<tr>
<th>Stage of the test</th>
<th>Cross</th>
<th>( C^R x E^R_1 )</th>
<th>( C^R x E^R_2 )</th>
<th>( C^R x E^R_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/24</td>
<td>0/140</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0/22</td>
<td>0/160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0/45</td>
<td>2/48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0/24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 div.</td>
<td>0/15</td>
<td></td>
<td>0/240*</td>
<td></td>
</tr>
<tr>
<td>60 --</td>
<td>0/15</td>
<td></td>
<td>0/120</td>
<td></td>
</tr>
<tr>
<td>70 --</td>
<td>0/15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80 --</td>
<td>0/15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0/175</td>
<td>0/140</td>
<td>2/568</td>
<td></td>
</tr>
</tbody>
</table>

The figures indicate the number of \( C^R E^R \) cells obtained over the number of mixed cells that were tested. The "stages of the test" refers to those indicated in Figure 1. The * indicates that the 240 tested cells had been UV irradiated (with the idea of stimulating recombination).

The two types of mitochondria was always obtained. For example, such was the case of the 140 cells belonging to 20 different pairs tested at level (1) of the \( C^R_1 x E^R_1 \) cross, the 45 cells tested at level (4) of the \( C^R_2 x E^R_3 \) cross, etc. In a few cases, it was ascertained that the mixed cells, even after being blocked in doubly selective medium, were still mixed when tested in singly selective medium. Furthermore, about 50% of the cells reported in Table 3 were first placed in "weakly" doubly selective medium, enabling residual growth.

The two \( C^R E^R \) strains obtained from the \( C^R_1 x E^R_1 \) cross appeared after prolonged growth of mixed cells at 18° in very weakly selective medium (a step not carried out in the two other crosses). These conditions are known to favor the accumulation of \( E^R \) mutations (Adoutte 1977). One spontaneous \( C^R E^R \) mutant appeared in the \( C^R_1 \) parental controls grown under the same conditions. No other \( C^R E^R \) strains were isolated at later stages from the same cross. In addition, these two \( C^R E^R \) strains differ from each other in their levels of resistance to erythromycin and chloramphenicol (as is frequently the case between spontaneous mutants, but is not expected if the strains result from recombination). It is most likely therefore that these two strains arose by mutations and not recombination.

Search for \( C^R E^R \) recombinants in \( C^R_1 \) \& \( E^R \) combinations: These combinations, which were generated by microinjection, were the most favorable ones to detect wild-type recombinants, since the three markers used were either very strongly \( (E^R_{NG7-9}) \) or strongly \( (C^R_{NG7-12}, C^R_{NG7-21}) \) counter-selected with respect to wild type. They were studied on a very large scale, i.e., several million mixed cells were kept in constant growth for over 100 generations by regular transfers of aliquots of approximately 1000 cells. As a control, specially constructed cells were grown under identical conditions; they consisted of \( C^R + E^R \) mixed cells into which a small number (1–10) of wild-type mitochondria were introduced by microinjection to ascertain that the experimental conditions were favorable for the expression of this class of mitochondria.
<table>
<thead>
<tr>
<th>Combinations</th>
<th>1</th>
<th>25</th>
<th>60</th>
<th>89</th>
<th>120</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C^R_{NG7-12} \rightarrow E^R_{NG5-6}$</td>
<td>$\text{t}$</td>
<td>+</td>
<td>+</td>
<td>t</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$C^R_{NG7-21} \rightarrow E^R_{NG5-6}$</td>
<td>t</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$E^R_{NG5-6}$</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C^R_{NG7-12}$</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C^R_{NG7-21}$</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C^8E^8 \rightarrow (C^R_{NG7-12} + E^R_{NG5-6})$</td>
<td>+</td>
<td>+</td>
<td>t</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$C^8E^8 \rightarrow (C^R_{NG7-21} + E^R_{NG5-6})$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The table indicates the evolution of mixed ($C^R + E^R$) or ($C^R + E^R + C^8E^8$) cells obtained by microinjection. The $\rightarrow$ sign indicates the direction of microinjection. Clones of about 1,000 mixed cells (or parental controls) were transferred to flasks containing 1 l of medium. In such flasks the clones can undergo about ten fissions, then about 1,000 cells were transferred again to 1 l of fresh medium, etc. At various stages, samples of cells were tested for their resistance toward chloramphenicol (CAP) and erythromycin (ERY). The $+$ sign indicates that the cells started growing immediately in the antibiotic containing medium (i.e., they are fully resistant). The $t$, $T$ and $t$ signs indicate that the cells started multiplying in the corresponding medium after a lag ($t$: one to two days lag; $T$: three to five days lag; $t$: over five days lag), indicating that the cells have less and less amounts of the selected marker. The $-$ sign indicates that the cells were completely blocked.
The results are summarized in Table 4. It can be seen that the mixed \( C^R + E^R \) state extended over a large number of generations, the proportion of \( C^R \) mitochondria progressively increasing with respect to the \( E^R \) ones. After 60–80 cell generations, the cells have lost all \( E^R \) mitochondria but remain thereafter \( C^R \).

The opportunity for the occurrence of \( C^S E^S \) recombinants and for their accumulation therefore extended over a long period. In contrast, in the control experiment, when mixed cells were injected with small number of wild-type mitochondria, it was observed that all the cell population evolved toward a \( C^S E^S \) genotype fairly rapidly.

**Search for thermoresistant recombinants in \( E^R_{N5-21} \times E^R_{N5-1} \), \( E^R_{N5-29} \times E^R_{N5-1} \), \( E^R_{N5-3} \times E^R_{N5-21} \times E^R_{N5-29} \), \( E^R_{N5-29} \times E^R_{N5-1} \), \( E^R_{N5-21} \times E^R_{N5-13} \), \( E^R_{N5-29} \times E^R_{N5-13} \) combinations:** Crosses were carried out, using microinjection, between the most highly thermosensitive \( E^R \) mutants isolated after NG mutagenesis (see MATERIAL AND METHODS), and thermoresistant recombinants were screened for. In most combinations, there were only minor differences in the phenotypes of the two confronted markers, and it was not always possible to ascertain that the injected cells were mixed. That they were mixed is highly probable, however, since the method is extremely reliable. In the same series of injections, when the markers could be distinguished, the cells always proved to be mixed.

For the six combinations studied, a total of 41 cells were microinjected, giving rise to 41 clones. These clones were transferred into tubes, fed with 8 ml of medium and placed at the nonpermissive temperature (36°C). In that way, several thousand cells derived from the initial microinjected mixed cell were tested, increasing the probability of detecting recombinants (each tube contained 2000–4000 cells). In 39 of these 41 tubes, the cells declined and eventually died. In the remaining two tubes, there were a few surviving cells that died in a second test. In parallel with this experiment was a set of four tubes of thermosensitive \( M^+ \) cells injected with the thermoresistant \( C^R_{N5-13} \) marker; all gave rise to pure \( C^R \) and thermoresistant clones. The experimental design therefore permitted the expression of thermoresistant genotypes.

**Search for \( E^R \) recombinants in the \( E^S \) \( \times \) \( E^S \) combination:** Strain \( E^S \) corresponds to a spontaneous partial reversion of \( E^R \). It has kept a very low level of erythromycin resistance, is still thermosensitive as \( E^R \), was and displays a generation time slightly shorter than that of \( E^R \). All these new properties are mitochondrialy inherited. It is clear that strain \( E^S \) does not correspond to a true reversion, and it may be hypothesized that it harbors a second-site mutation that has improved growth rate (and hence was selected for in the stock tube), while diminishing erythromycin resistance.

The simplest hypothesis is to consider that its genotype is \( E^R \) \( su \), without making any assumption as to the molecular nature or location of the \( su \) mutation, although it is possible that \( su \) is located within the same gene as \( E^R \). This strain was crossed to a wild-type strain with the aim of recovering \( E^R \) \( su^+ \) recombinants that would re-express erythromycin resistance at a high level. Here, the screening was easy. After conjugation, pairs that had undergone cytoplasmic exchange were retained, the ex-conjugants were separated and grown for a few
fissions in normal medium, and then samples of cells were tested individually in low concentrations of erythromycin. A total of 63 cells belonging to five different pairs were tested. Whenever the slightest sign of erythromycin resistance was displayed, several transfers and further tests were carried out. Such was the case for 31 lines. Of these, eight yielded stable \( E^a \) strains. However, a careful phenotypic characterization showed that none of them displayed the original \( E^a \) phenotype. They were either more strongly or more weakly erythromycin resistant. Furthermore, \( E^a \) strains also appeared at high frequency in the control cells: six out of ten lines that were followed; their phenotypes were similar to those obtained from the crossed cells. It is clear that in attempting to obtain re-expression of the \( E^a \) marker, we used conditions that favored the selection of spontaneous \( E^a \) mutations. This cross was repeated and the tests were carried out in higher concentrations of erythromycin, preventing residual growth. None of the 18 pairs analyzed yielded \( E^a \) lines. (One was, however, obtained from an \( E^a \), control!) In addition, several dozen cells derived from \( E^a \), conjugants that had received wild-type mitochondria were placed in erythromycin after they had undergone 10, 20 and 30 fissions in normal medium, thereby enabling the formation of well-mixed cells. None of these mass tests yielded any \( E^a \) clones.

If our hypothesis concerning the \( E^a \), strain is correct, i.e., that it corresponds to a \( E^a \), \( su \) mutant, it can be concluded that the frequency of recombination between \( E^a \), and \( su \) is not higher than the mutation rate of an \( E^a \) phenotype.

Search for \( C^aE^a \) or \( C^aE^a \) recombinants in the \( C^aE^a \times C^aE^a \) and \( C^aE^a \times C^aE^a \) combinations: Strain \( C^aE^a \) was obtained by isolating an \( E^a \) mutation in the \( C^a \) strain. Strain \( C^aE^a \) has a reduced growth rate, is highly thermosensitive and its mitochondria are very quickly eliminated from mixed mitochondrial populations in contrast to those of the \( C^a \) strain, which are extremely stable (Aboutte and Beisson 1972). \( C^aE^a \times C^aE^a \) is a partial revertant of this strain, which has retained chloramphenicol and erythromycin resistance, but grows faster and is less thermosensitive. The aim of these crosses was to isolate \( C^aE^a \) recombinants on the basis of two expectations: (1) Such recombinants should show a clear advantage with respect to \( C^aE^a \) or \( C^aE^a \) in normal medium and even more so in the presence of chloramphenicol, since the original \( C^aE^a \) mutation displays a slightly higher resistance to the drug than to the double mutants. (2) The \( C^aE^a \) recombinants should be relatively stable with respect to wild type.

Mixed \( C^aE^a + C^aE^a \) cells were therefore first grown in normal medium and then transferred into medium containing chloramphenicol. Two situations occurred: (1) The cells became chloramphenicol resistant. In that case, after a few additional transfers in chloramphenicol-containing medium, they were tested in erythromycin-containing medium to determine whether the \( E^a \) marker had remained associated to the \( C^a \) one. (2) The \( C^a \) marker was lost very quickly from the mixed cells. This implied that no \( C^aE^a \) recombinants were formed since these should be stable. A second approach consisted in growing ex-\( C^aE^a \) conjugants at 36°, thereby favoring the multiplication of \( C^aE^a \) mitochondria and then searching for \( C^aE^a \) thermoresistant recombinants that might have been formed during this process.
Both approaches were first used in four independent $C^{R_e}E^{R_e} \times C^{S}E^{S}$ crosses. A total of 87 mixed cells were grown in chloramphenicol. All remained $E^{R_e}$. In addition, 100 individual cells and over 6000 cells were tested in mass; All of the ex-$C^{R_e}E^{R_e}$ conjugants that had become thermoresistant at 36°, proved to be pure $C^{S}E^{S}$.

The first approach was also used extensively on microinjected cells: five $C^{S}E^{S}$ cells were injected with $C^{R_e}E^{R_e}$-b mitochondria. After 3-4 fissions in normal medium, they were cloned in erythromycin or chloramphenicol. A total of 66 cells acquired erythromycin resistance and 72 acquired chloramphenicol resistance. The 66 and 72 clones were grown in erythromycin and chloramphenicol, respectively, for 40 generations (with transfers every 10 fissions). Then the $E^{R_e}$ cells were tested in chloramphenicol, and vice versa. The 138 clones proved to be $C^{R_e}E^{R_e}$, although the conditions for expressing $C^{S}E^{S}$ or $C^{R_e}E^{S}$ recombinant genotypes were quite favorable.

Search for $C^{S}E^{R_e}$ and $C^{S}E^{S}$ recombinants in the $29$ $E^{R_e} \times d_{4-2}$ $C^{R_e}$ interstock combination: The idea of carrying out crosses between markers isolated in different geographical stocks of Paramecium came from observations made on yeast mitochondrial crosses. It was demonstrated that a specific mitochondrial genetic factor called $\omega$, which exists in nature in two forms, $\omega^+$ and $\omega^-$, strongly influences the frequency of recombinants in the genetic region that is adjacent to it (the region carrying the $C^{R_e}$ and $E^{R_e}$ alleles) depending on whether the cross is of the $\omega^+ \times \omega^+$ ($\omega^- \times \omega^-$) or $\omega^+ \times \omega^-$ type (Boilotin et al. 1971). If such a situation existed in Paramecium, recombination might be stimulated between mitochondrial genomes of different origin.

Eleven combinations involving markers isolated in stocks $d_{4-2}$, 29 and 32 were analyzed (see Table 2), six being carried out on a large scale similar to the type (2) crosses described above. No clear case of recombination was observed in these experiments.

However, one combination, $29$ $E^{R_e} \times d_{4-2} C^{R_e}$, yielded some rare double-sensitive cells. The results were summarized in a previous publication (Adoutte 1974) at a time when the data suggested that recombination had occurred. Since this was the only case yielding presumptive recombinants, the analysis was pursued and extended. Additional studies have now cast doubt on the previous interpretations. The results will now be discussed in some detail to draw a definitive conclusion about this work.

Four $29$ $E^{R_e} \times d_{4-2} C^{R_e}$ crosses were carried out. Two were extensively analyzed and will be summarized. The $E^{R_e}$ marker confers a high level of erythromycin resistance and no cross resistance to chloramphenicol. The $C^{R_e}$ marker confers a high resistance to chloramphenicol and a slight cross-resistance to erythromycin. Both are only slightly disadvantaged with respect to wild-type.

The protocol used was essentially that described in Figure 1. A few differences should be noted, however: (1) A very high mortality usually occurred after autogamy of the $F_1$ ex-conjugants. It is due to the fact that these are hybrids between two very distinct stocks with different chromosomal numbers (Dippell 1954). The $F_2$ clones that were analyzed therefore derived from a very limited
number of F₁ cells that survived autogamy and in which important nuclear rearrangement probably occurred. (2) In the two crosses, emphasis was placed on the search for $C^sE^s$ recombinants. This implied numerous successive transfers of the initially mixed cells because of the great stability of the two parental markers. (3) In both crosses, after having carried out these successive transfers, i.e., after a few months, we reisolated surviving cells from the initial clones (that had not undergone transfers) to repeat the analysis.

The results were as follows: From the first cross, only one clone derived from an $E^r$, ex-conjugant grown in normal medium yielded well-mixed $C^r + E^r$ cells after autogamy (25 fissions after conjugation). Fifteen subclones were isolated at the 60th fission. These subclones progressively lost the $E^r$ determinants in favor of the $C^r$ ones and later appeared to be purely $C^r$ (80th fission). Then, at about the 100th fission, 14 of these clones were noted as displaying lowered chloramphenicol resistance (that is, they required a lag in chloramphenicol-containing medium before starting to grow and displaying full resistance, as is typical of mixed $C^r + C^s$ cells). At the 140th generation, the 14 clones had become completely chloramphenicol and erythromycin sensitive, the 15th remaining $C^r$. The $C^sE^s$ character behaved exactly like wild-type mitochondria in all respects.

In view of this result, 36 subclones were reisolated from the initial post-autogamous clone. These cells had undergone a very prolonged stationary phase during which they had become enriched with $C^r$ mitochondria. The 36 clones were grown for 215 generations in normal medium, but none yielded any $C^sE^s$ cells.

It should be mentioned that extensive search for $C^rE^r$ recombinants was carried out at various stages of the evolution of the mixed cells. Negative results were obtained in the sense that, although one $C^rE^r$ strain was obtained from mixed cells, a similar clone was observed in parental controls, showing that the level of detection of double-resistant clones was at the level of spontaneous mutation.

In summary, this first cross yielded many double-sensitive lines, but they were all derived from the same initial clone, and it was not possible to obtain them again when reisolating cells from the same clone after prolonged starvation.

The central feature of the second cross is that the same initially mixed clones, kept in tubes, were studied three times successively, over a period of several months (see Figure 2).

(1) Ex-conjugant clones (7 to 8 fissions after conjugation) belonging to 40 pairs that had undergone cytoplasmic exchange were first transferred to tubes (=80 tubes). After about 10 additional fissions, a sample of cells from each tube was tested and 22 pairs in which at least one of the two ex-conjugant clones was mixed were identified. Six progeny cells from each member of such pairs (i.e., from 44 ex-conjugants) were isolated in normal medium (=264 cells) and gave rise to clones from which successive transfers of a single cell were carried out every 10 fissions, first for 50 fissions at 36° then for 200 fissions at 27°. A total
of 198 clones were maintained until the latest test (about 270 fissions after conjugation). None of them was double sensitive.

However, as soon as antibiotic resistance tests were carried out on post-autogamous cells (that is after about 30 post-conjugal fissions), phenotypes of chloramphenicol and erythromycin resistance different from those of the two parents were observed. This phenomenon was also observed in the three other ER CR, d4-2 CR, crosses. The new phenotypes appear immediately after autogamy, then remain stable. Most probably they correspond to the segregation of nuclear genes that are different between the two stocks that modify the expression of mitochondrial markers. These nuclear genes also appear capable of inducing different types of evolution of mixed mitochondrial populations. In usual crosses between isonuclear strains, the occurrence of autogamy does not introduce any modification of the regular pattern of evolution of the mixed F₁ cells, while in this inter-stock cross, the mixed CR + ER F₁ cells evolve quite differently after autogamy. Some very quickly become pure CR, others ER, while others remain mixed CR + ER over numerous fissions, the CR marker slowly becoming more prevalent with respect to ER. This may again be due to the formation of particular combinations of nuclear genes after autogamy exerting a selective effect on one or the other mitochondrial genotype. Nuclear modifiers of the segregation and expression of mitochondrial genes have been described in crosses of nonisonuclear strains of yeast (AVNER et al. 1973; WOLF, DJON and SLONIMSKI 1972; CALLEN 1974).

(2) After seven months of stationary phase interrupted by short periods of growth, cells of 26 ex-conjugants (a subset of the initial 44) were reisolated from the tubes for a second analysis. For six of these ex-conjugants, successive transfers of 15 cells per ex-conjugant in normal medium were carried out during about 260 generations. In one of these six ex-conjugants, three clones out of the 15 that
were followed gave rise to doubly sensitive cells at about 120th generation. (One of the three was pure $E^R$ when reisolated from the tube, the other two were still mixed $C^R + E^R$). All of the other clones ($75 + 12$), as well as the 15 clones of each of the two parental controls, had kept their initial phenotype.

(3) A third analysis was carried out to try to reisolate double-sensitive clones. Three different mixed $E^R$ ex-conjugants were chosen (including the one that had yielded double-sensitives at the second analysis). Nine cells from each were reisolated from the tubes, 11 months after the initial cross, yielding a total of 27 clones. These clones were maintained by regular transfer of single cells for about 150 generations in normal medium and did not yield any $C^S E^S$ lines.

In summary, the second cross yielded a very small number of $C^S E^S$ lines, all belonging to the same original ex-conjugant clone, after several months of nearly complete stationary phase of the mixed cells.

Do the double-sensitive clones isolated in the two crosses correspond to recombinants or to revertants? The only argument in favor of the recombinational origin of the $C^S E^S$ clones rests in the fact that the two parental strains, $d_{4-2} C^R,4$ and 29 $E^R,4$, have never yielded any revertants, either when grown as controls in the experiments just described or in the stock tubes in which they have been kept for over six years in the laboratory. If one considers this argument as decisive (as we did in our preliminary report of this cross), one can try to account for the low frequency of $C^S E^S$ clones as follows: (1) the $C^R,4$ and $E^R,4$ markers are both only slightly counter-selected with respect to wild type. If recombination frequency is low, rare $C^S E^S$ genomes may be lost because of the drift introduced by the transfer of only one cell every 10 fissions. (2) It was mentioned above that nuclear genes appear to have been segregating in this cross and that they may modify the evolution of a mixed mitochondrial population. It is possible that only a few nuclear genotypes allow the formation or the expression of $C^S E^S$ recombinants, thereby limiting the number of "significant" clones that were analyzed.

Several lines of information suggest however, that the $C^S E^S$ clones correspond to back mutations. The number of clones that evolved towards double sensitivity in the second cross remained very low with respect to the total number of clones followed in these three analyses of this cross, 3 out of 300, although these 300 clones were known to have gone through a mixed $C^R + E^S$ state in $F_1$.

In the second cross, the double-sensitive lines appeared only at the second analysis, that is after the cells had undergone a prolonged stationary phase in tubes, which is known to be favorable for the appearance of back mutations.

Although no reversions of the $E^R,4$ or $C^R,4$ markers were observed in their original nuclear background, several other $E^R$ markers have been observed to revert throughout the years, and the $C^R,4$ marker has reverted in some $C^R E^R$ double-mutant strains.

The mitochondrial DNA of two double-sensitive clones obtained in the first and second cross, as well as that of the 29 $E^R,4$ and $d_{4-2} C^R,4$ parental strains, was analyzed by EcoRI restriction-endonuclease digestion. Strains 29 $E^R,4$ and $d_{4-2} C^R,4$ displayed quite distinct patterns, and one of the double-sensitive strains yielded
a pattern identical to that of \( C^R_4 \), the other yielding a pattern identical to that of \( E^R \). No evidence for the formation of a "hybrid" pattern was obtained from the double-sensitive strains (R. Makki and D. Cummings, personal communication). This does not completely rule out the possibility that these strains arose by recombination, since it could be either a recombinational event through a conversion-like process or through the integration of a small region of one genome into the other. However, it is simpler to assume that the two double-sensitive lines originated from a reversion of \( E^R \) and \( C^R_5 \), respectively.

Finally, in order to increase the possibility of detecting \( C^8 \) recombinants, markers \( 29 \ E^R_6 \) and \( 29 \ E^R_7 \) were microinjected in strains \( d_{4-4} C^R_{SG7-12} \) and \( d_{4-4} C^R_{SG7-21} \) (which correspond to two \( C^R \) markers that are counter-selected with respect to wild type). These combinations were studied both by transfers of individual cells and in "mass" (as detailed in the crosses described in Figure 2). They did not yield any double sensitives.

In summary, although the recombinational origin of the double-sensitive lines cannot be totally excluded, they are more simply accounted for by back mutations of one or the other parental marker. To account for the fact that no such reversions were recorded in the uncrossed parental lines, it can be hypothesized that they were favored and selected for in some particular nuclear genetic backgrounds issued from the interstock cross.

**Search for recombination between the M\( ^e \) and E\( ^R \) markers in the M\( ^e \)E\( ^8 \) × M\( ^+ \) E\( ^R \) and M\( ^e \) F\( _{201} \) × M\( ^+ \) E\( ^8 \) combinations:** These crosses have been reported by Sainsard-Chanet (1976) in a quite different context. They are summarized here because they involve the M\( ^e \) mitochondrial marker, which displays distinct properties from the antibiotic resistance markers and most likely has a different molecular basis (Sainsard-Chanet 1978).

The aim of the experiments was to try to detect recombination between the M\( ^e \) and E\( ^R \) markers. However, since M\( ^e \) does not display a clear cellular phenotype when placed in a wild-type nuclear background, the cl\( _1 \) nuclear gene, which discriminates very efficiently between M\( ^e \) and M\( ^+ \) mitochondria, was introduced in the cross. Starting from (cl\( _1 \)/cl\( _1 \))M\( ^e \)E\( ^8 \) × (cl\( _1^+ \)/cl\( _1^+ \))M\( ^+ \) E\( ^R \), and cl\( _1 \)/cl\( _1 \)M\( ^e \)E\( ^8 \) × cl\( _1^+ \)/cl\( _1^+ \)M\( ^+ \) E\( ^R \) crosses, cl\( _1 \)/cl\( _1^+ \) exconjugants containing mixed cytoplasm were retained. The M\( ^e \) marker was therefore associated with an E\( ^R \) allele in one of the combinations and with an E\( ^R \) one in the other. At auto-gamy these exconjugants yielded (cl\( _1 \)/cl\( _1 \)) and (cl\( _1^+ \)/cl\( _1^+ \)) homozygotes that were distinguished by their growth rate and thermosensitivity. These homozygotes were then tested in erythromycin to identify their mitochondrial genotype.

From F\( _1 \) cells containing a mixture of M\( ^e \)E\( ^8 \) and M\( ^+ \) E\( ^R \) mitochondria, 84 of 95 cl\( _1 \)/cl\( _1 \) F\( _2 \) clones became pure for M\( ^e \)E\( ^8 \) mitochondria, and the 11 others for M\( ^+ \)E\( ^R_1 \) mitochondria. Of 110 cl\( _1^+ \)/cl\( _1^+ \) F\( _2 \) clones, 80 became pure for E\( ^R \), mitochondria, and 30 others became erythromycin sensitive between the 15th and the 110th generation. In the second cross, from F\( _1 \) cells containing a mixture of M\( ^e \)E\( ^R_201 \) and M\( ^+ \) E\( ^8 \) mitochondria, all 92 cl\( _1^+ \)/cl\( _1^+ \) F\( _2 \) clones became pure for E\( ^8 \) mitochondria, and of 112 cl\( _1 \)/cl\( _1 \) F\( _2 \) clones, 106 became pure for M\( ^e \) E\( ^R_201 \).
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mitochondria, four for $M^+E^8$ mitochondria, and two acquired a $M^{cl}E^8$ phenotype between the 70th and the 90th generation.

These results show that in a large number of $F_2$ clones, after going through a mixed stage, the $M^{cl}$ marker was still found associated with the $E^8$ or $E^8$ allele it was originally linked to. The only cases that would result from a recombination between these two markers were the 30 $cl_1^{+}/cl_1^{+}$ clones derived from the first cross, which ended by selecting sensitive mitochondria although the $E^8$ marker was initially associated with the $M^{cl}$ and not with the $M^+$ allele, and the two $cl_1/cl_1$ clones derived from the second cross, which also ended by selecting sensitive mitochondria although the $E^8$ marker was initially associated with the $M^+$ and not the $M^{cl}$ allele. However, other hypotheses can account for these exceptions.

A correlation has been established between mitochondrial and cellular growth rates and, knowing the growth rate of any mitochondrially mutated strain, the behavior of these mitochondria in a mixed mitochondrial population can be predicted (Adoutte and Doussiere 1978). The growth rates of cells pure for $M^+E^8$, or $M^{cl}E^8$ mitochondria is nearly the same (Sainsard-Chanet 1978). The evolution towards erythromycin sensitivity of the 30 $cl_1^{+}/cl_1^{+}$ clones all derived from the same ex-conjugant could result not only from the selection of recombinant $M^+E^8$ mitochondria, but also from the selection of the original $M^{cl}E^8$ mitochondria. These might not have been eliminated in this ex-conjugant because they initially represented the major mitochondrial type and were eventually selected because their replication rate is similar to that of the few $M^+E^8$ mitochondria.

For the two $cl_1/cl_1$ clones that became erythromycin sensitive instead of becoming pure for $M^{cl}E^8_{201}$ mitochondria, it can be assumed that they have not selected recombinant $M^{cl}E^8$ mitochondria but “modified” mitochondria derived from the initial $M^+E^8$ mitochondria, which were not all eliminated during the first stages when they were associated with the $cl_1/cl_1$ nucleus. It is now known that mitochondria are modified regularly between about 2 and 15 fissions from an incompatible towards a compatible state in a $cl_1/cl_1$ nuclear context and that these modified mitochondria have approximately the same replication rate as $M^{cl}E^8_{201}$ mitochondria; they are, therefore, not eliminated when mixed with these last ones (Sainsard-Chanet and Knowles 1979).

In summary, the cases that could be considered to represent a recombinational event can also be satisfactorily interpreted in other ways and cannot be used as proof for recombination. The fact that, in a very large number of $F_2$ clones in which the cells had been mixed, the $M^{cl}$ marker was still found associated with the $E^8$ or $E^8$ allele to which it was originally linked indicates that recombination between these two markers is a very rare event, if it occurs at all.

In addition, it can be pointed out that the “recognition” and very active selection by the $cl_1$ gene in $F_2$ of mitochondria that are genotypically $M^{cl}$, implies that these mitochondria have remained phenotypically $M^{cl}$ in spite of the 20 cell generations they have undergone mixed with $M^+$ ones. This result is in good agreement with the idea of functional autonomy of mitochondria in
Paramecium derived from quite distinct types of experiments (Adoutte and Doussiere 1978; see also discussion).

**Search for M^aE^r recombinants in the M^aE^r × M^cE^s combinations:** E^r mutations were isolated in strains already harboring the M^a mitochondrial mutation, which is a mitochondrial suppressor of the cI marker gene (Sainsard 1975). One of the mitochondrial types obtained, designated M^aE^rO was injected into (cI/cI) M^cE^s cells. It was known that the cI gene actively selects mitochondria harboring the M^a mutation (Sainsard 1975). It was, therefore, expected that, if M^aE^r recombinants were formed during the selection phase, they would be at an advantage with respect to M^aE^r (in normal medium) and would eventually segregate vegetatively. Nineteen cI/cI M^cE^s cells were injected with M^aE^rO mitochondria, and their progeny were cloned two fissions after the injection. From each injected cell, three cells were isolated in normal medium, yielding clones that were repeatedly transferred in that medium, and one cell was placed in erythromycin to be sure that the injection had been successful. Such was the case for 15 of the 19 cases. The 45 corresponding clones were followed by regular transfer of a single cell every 10 fissions for 120 generations. All the clones became E^r and acquired a rapid growth-rate at about the 15th fission after injection, which corresponds to the replacement of the M^cE^s mitochondria by M^aE^r ones. None of the 45 clones had evolved towards sensitivity after 120 fissions, although this should have provided ample time for any M^aE^s mitochondria to take over from M^aE^r ones.

**DISCUSSION**

This paper reports on the search for mitochondrial recombination in 32 different crosses altogether involving 24 distinct mitochondrial markers. The results were negative in that not a single case of appearance of recombinant genotypes in a frequency that could be clearly distinguished from spontaneous mutations was observed. The only cases that might have resulted from recombination between the M^c and E^r markers can also be interpreted by another mechanism. We will, therefore, first discuss to what extent this negative result is significant, then draw a parallel between these and other results bearing on the question of mitochondrial autonomy in Paramecium. The situation will be compared to that of other organisms in which mitochondrial genetics has been studied, and hypotheses accounting for the results in Paramecium will be discussed. Finally, the possible evolutionary significance of the results will be outlined.

Are the results significant? In view of the negative results obtained, two questions can be raised concerning their significance: Were the markers used appropriate? Were the screening procedures used sufficiently powerful?

Because of the absence of detectable recombination and/or complementation in all the crosses analyzed, the classical genetic tools for discriminating between our various markers are not available. We therefore have to rely on the phenotypic characteristics of the mutations and their physiological consequences to
try to obtain some insight into the possible number of distinct genes that have been studied.

The initial mitochondrial mutants isolated were spontaneous or UV-induced mutants resistant to antibiotics affecting the mitochondrial ribosomes and known to affect the large subunit of bacterial ribosomes. These are mainly erythromycin-, chloramphenicol- and spiramycin-resistant mutants. Over 100 such mutants have been isolated throughout the years, and the ones used in the present study are representatives of the best-defined phenotypic classes, in particular those that show little or no cross resistance to the other antibiotics. Analogous mutants, isolated in *Paramecium primaurelia*, were shown to have altered mitochondrial ribosomes (TAIT 1972; SPURLOCK, TAIT and BEALE 1975) and the same is true for mutants $E^r$, and $E^{r, 162}$ of *Paramecium tetrarurelia*, used in the present study (CURGY and TAIT, personal communication). In addition, many of the antibiotic-resistant mutants used in this study were shown to display a typical pattern of physiological alterations (decreased amount of cytochrome oxidase, increased rates of cyanide-insensitive respiration during the exponential phase of growth, and decreased growth rate) that can all be attributed to decreased efficiency of mitochondrial protein synthesis, in agreement with the idea that all of these mutations affect the mitochondrial ribosomes (ADOUTTE and DOUSSIERE 1978). Only the severity of the alterations varies between these mutants. Very similar antibiotic-resistant mutants were extensively analyzed in yeast, and one $E^r$ class at least was shown to be located in the mitochondrial DNA segment coding for 28S mitoribosomal RNA. The other loci of resistance to antibiotics acting on the large ribosomal subunit are also located either in the same ribosomal DNA segment or very close to it (FAYE, KUJAWA and FUKUHARA 1974; NAGLEY et al. 1974; HEYTING and SANDERS 1976; JACQ et al. 1977). If the situation is the same in Paramecium, we would be dealing with a set of closely linked mutations.

In addition to these spontaneous or UV-induced antibiotic-resistant mutants, a set of nitrosoguanidine-induced mutants were also used. These were particularly useful because they were either clearly thermosensitive or strongly counter-selected in mixed mitochondrial populations. Their phenotypic properties may all be due to the mutation conferring antibiotic resistance or, more probably, considering the efficiency of action of nitrosoguanidine, they may be double or multiple mutants. In that case, the additional mutation(s) conferring thermostability or slow growth could either be close to that conferring antibiotic resistance or distant from it. However, knowing that NG induces closely-linked multiple mutations in bacteria (GUEROLA, INGRAHAM and CERDA-OLMEDO 1971), they also may all be located in a limited portion of the mitochondrial genome.

In summary, the most conservative estimate is that all the antibiotic-resistant mutations, whether spontaneous, UV or NG induced, as well as their partial suppressors, lie in a limited region of the genome, possibly all being located in the gene for the large mitochondrial ribosomal RNA.

On the other hand, three mitochondrial mutations, $M^{cl^1}, M^{au}$ and $M^*$, were isolated on a completely different basis: they are partial suppressors of a nuclear gene mutation, $cl^1$, which interacts very strongly with wild-type mitochondria
leading to their complete disorganization (Sainsard, Claissé and Balme-Frezol 1974). The three suppressors relieve this incompatibility to different extents (Sainsard 1975; Sainsard-Chanet 1978). The important point is that these suppressors, when placed in a wild-type nuclear background, display physiological properties quite distinct from those of the antibiotic-resistant mutations: whole cells carrying the \( M^{cl} \) mutation, in particular, have normal amounts of cytochrome oxidase in the exponential phase of growth, but become deficient in stationary phase, which is just the opposite of \( A^R \) mutants (Sainsard-Chanet 1978). Because of the great regularity of the physiological alterations observed in the ribosomal mutants, it is possible that these mitochondrial suppressors do not correspond to ribosomal alterations, but rather to a quite distinct molecular class.

On the whole, then, we can consider that the mutations used in the present study belong to at least two distinct mitochondrial genes. There is no way of knowing, however, whether these genes are closely linked or distant. Therefore, it becomes critical to ascertain whether the screening methods used were sufficiently powerful to detect rare recombinants.

In several of the combinations analyzed, positive screening was available to detect possible recombinant genomes. Such was the case, in particular, in the \( C^R \times E^R \) combinations in which \( C^aE^r \) recombinants were sought and in the \( A^R \, t^s \times A^r \, t^s \) combinations in which thermoresistant recombinants were sought. It should be stressed that testing a single mixed \( C^n + E^n \) cell in double-selective medium screens a population of several thousand \( C^R + E^R \) mitochondria, and several hundred such cells were tested. In several of the \( C^n \times E^n \) crosses, the tests were sufficiently extensive to permit the detection of spontaneous \( C^R E^R \) mutants within the parental control cells. This shows that the screening procedures used were large enough to detect very rare genotypes. We estimate that \( C^n E^n \) genotypes occurring with a frequency as low as \( 10^{-3} \), and possibly \( 10^{-4} \), per mitochondria could have been detected. (This should be compared with the frequency of recombination of several percent occurring between \( C^R \) and \( E^R \) markers of yeast.) This estimate is determined on the basis that there were about 10,000 mitochondria in a Paramecium cell, each probably containing several mitochondrial genomes (Suyama and Preer 1965). By testing a single cell, one screens of the order \( 5 \times 10^4 \) genomes. If the frequency of \( C^R E^R \) recombinants were \( 10^{-4} \), each cell would contain about five \( C^R E^R \) genomes. Allowing for the fact that this would be a dynamic situation (i.e., \( C^R E^R \) recombinants might be recombining again to yield the parental genotypes and might also be counter-selected) and for the fact that more than one \( C^R E^R \) genome might be necessary for the cell to start growing in double-selective medium, this leads to the very rough estimates presented.

However, one serious objection can be made to these particular \( C^R \times E^R \) combinations. It relates to the fact that selection for only the double-mutant genotype was possible. Such recombinants may have been defective physiologically, or even lethal, since it is known that \( C^R E^R \) strains isolated by mutation often show defects (Adoutte and Douzière 1978).
This objection does not apply to the case of the $ts \times ts$ crosses in which thermoresistant, that is wild-type, recombinants were sought. Six combinations of that type involving several tens of thousands of cells were screened, none of which showed evidence of recombination. In addition, the introduction of a small number of wild-type mitochondria in such $ts$ cells enables them to acquire rapidly a thermoresistant phenotype when grown at the nonpermissive temperature. In order to account for the negative results in this series of experiments, one must assume that the recombination frequency is below $10^{-4}$. Now, several independently isolated $ts$ mutations were crossed, and there were slight differences in the thermosensitivity of several of the mutants. It is, therefore, difficult to assume that the combinations involved only homoalleles so that the absence of thermoresistant recombinants is highly significant.

Objections concerning the possible lethality of recombinants or the identity of the mutated sites do not hold for a set of crosses in the double-mutant $\times$ wild-type configuration in which single-mutant recombinants were sought. Such was the case in the $CRE \times$ wild-type, $E^s \times$ wild-type and $M^{su}E^r \times M^+E^s$ combinations. In these three cases, the double-mutant strain was obtained, starting from the single-mutant one. It is reasonable to assume that one is dealing with two distinct mutational sites. These sites may be closely linked in the case of $CRE$ and $E^s$, (that is, $E^r_{su}$), but they need not be so in the case of $M^{su}E^r$. In the three cases, it was possible to provide single-mutant recombinants with a clear selective advantage. Although quantitative estimates are difficult to make in these systems, it can be assumed by analogy with other systems that even a small number ($\leq 10$) of $CRE$ or $M^{su}E^r$ mitochondria per cell would have been detected in view of the strong selective advantage these single mutants have over the double-mutant parent. In the case of $E^s \times$ wild-type, it was shown that recombination, if it existed, had to occur at a much lower frequency than that of mutations towards erythromycin resistance.

Finally, in a few cases no real positive screening was available. Such is the case of the $CRE \times E^r$ combinations in which double sensitives were sought. In several of these combinations, the two markers used were clearly counter-selected with respect to wild type, the experiments were carried out on very large numbers of cells and special attention was taken to limit drift. In addition, in reconstitution experiments carried out in parallel, it was shown that the injection of a small number of wild-type mitochondria into these mixed cells led fairly rapidly to the evolution of the whole cell population towards a double-sensitive stage. In spite of the absence of a positive screen, these experiments also appear to be quite significant.

In conclusion, the markers and the screening procedures used enable us to conclude that the nondetection of recombinants is significant at a level of $10^{-3}$ (in some cases $10^{-4}$) recombinant mitochondria per cell per generation. It must be admitted that, if recombination does exist, it has yielded in most of the studied crosses extremely low numbers of recombinant mitochondria, indistinguishable from mutated ones.
Although less extensively studied, no recombination was found in *Paramecium primaurelia* either (Beale, Knowles and Tait 1972; Beale 1973).

**Independent evidence for the absence of mitochondrial recombination:** Beale and Knowles (1976) have shown that the erythromycin resistance character can be transferred by microinjection from some species of the *Paramecium aurelia* complex into some others. Make and Cummings (1977) have recently analyzed the mitochondrial DNA restriction enzyme pattern of several of these species, as well as that of "hybrids," containing $E^r$ markers derived from another species. They made the important observation that, in all the "hybrids" they analyzed, the mitochondrial DNA pattern was entirely that of the donor species. This result indicates that whenever transfer of resistance is successful between two species, the whole mitochondrial DNA of the donor species replaces that of the recipient one. Now, if recombination is possible, this is a system in which one would expect the recipient cell to keep most of its original mitochondrial genome, presumably best fitted to its nuclear genome, and acquire only the $E^r$-confering region from the donor mitochondrial DNA. The results provide evidence for the lack of genetic recombination. In fact, this may be the reason underlying the failure to transfer the resistance markers between many of the species of the *P. aurelia* complex. Since this would imply the multiplication of the complete donor genome in the recipient cell, too much nucleo-mitochondrial incompatibility may arise if the species are too distantly related.

**Other evidence of mitochondrial genetic autonomy in Paramecium:** Several previously obtained lines of evidence point to a large extent of functional autonomy of mitochondria in Paramecium. They can be summarized as follows: (1) By introducing a very small number of erythromycin-resistant mitochondria into a sensitive cell, then placing this cell in erythromycin-containing medium, one obtains an intensive selective multiplication of the resistant mitochondria in the virtual absence of cell growth. At some stages, sensitive and resistant mitochondria are seen side-by-side in the transforming cell (Knowles 1972; Perasso and Adoutte 1974). When the cell resumes growth in the antibiotic-containing medium, genetic tests fail to reveal any residual sensitive genomes, indicating that the presence of a large number of resistant mitochondria does not provide any "protection" for the sensitive ones in the presence of antibiotic (Perasso and Adoutte 1974), as would be expected if there were a lack of genetical and functional interactions between the two types of mitochondria.

(2) By constructing cells containing two genetically distinct types of mitochondria, then growing the cells in normal medium, one always observes the progressive loss of one of the two types. The kinetics of loss, however, strictly depend on the particular markers used, some being lost very rapidly, others very slowly (Adoutte and Beisson 1972; Adoutte and Doussière 1978; this paper). Cases of strong counter-selection of mutated mitochondria with respect to wild-type ones point to a lack of "complementation" between the two types. The data become more striking when replication rates of mutated mitochondria in mixed cytoplasm are compared to those occurring in pure mutant cytoplasm.
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They are found to be very similar, if not identical, indicating a complete absence of interaction in the mixed cytoplasm (Adoutte and Doussiére 1978).

(3) The very active and rapid selection of Mez mitochondria under the action of the nuclear cl gene provides additional evidence for the maintenance of autonomous genetic and physiological properties by a given class of mitochondria in a mixed cytoplasm (Sainsard-Chanet 1976).

The situation in other organisms: The situation in Paramecium is in marked contrast to that of a set of organisms in which mitochondrial genetics has been studied, particularly yeast. In Saccharomyces cerevisiae, both genetical (Dujon, Slonimski and Weill 1974; Linnane, Howell and Lukins 1974; Perlman et al. 1976) and biochemical (Shannon et al. 1972; Michaelis, Petrochilo and Slonimski 1973; Williamson and Fennel 1974; Sena et al. 1975; Prunell et al. 1977) evidence demonstrate the occurrence of extensive recombination between mitochondrial genomes. Recombination frequencies are quite high even within restricted portions of the mitochondrial genome (Kotylak and Slonimski 1976; Jacq et al. 1977). The genetic data fit quite satisfactorily a model postulating that mitochondrial genomes constitute a panmictic pool of molecules undergoing several rounds of recombination (Dujon, Slonimski and Weill 1974), although a number of factors tend to restrict the complete randomness of mating of genomes (Birky 1978).

Mitochondrial recombination has also been recently described in the fission yeast, Schizosaccharomyces pombe (Wolf et al. 1978). Two filamentous fungi, Aspergillus nidulans (Rowlands and Turner 1974; 1975) and Podospora anserina (Belcour and Begel 1977) can yield very high frequencies of mitochondrial recombinants in some crosses.

Finally, a striking result was described in a special type of mammalian somatic-cell hybrids. Horak, Coon and Dawid (1974) have shown that in mice × rat cell hybrids retaining both parental sets of chromosomes, a very high frequency of clones display “hybrid” mitochondrial DNA molecules containing covalently linked DNA sequences from both species. The contrast between these results and those obtained in the interspecies transfers of mitochondria in Paramecium described above is worth stressing. More recently, Wallace et al. (1976) have shown reassociation of a human mitochondrial Crc marker with mouse mitochondrial DNA in human × mouse somatic hybrids. The Crc sequence, however, does not appear to be co-linear with the mouse mitochondrial DNA (Molloy, Francisco and Eisenstadt 1978).

In summary, both in a number of fungi and also probably in mammalian cells in culture mitochondrial recombination occurs readily.

A hypothesis to account for the absence of genetic exchanges between Paramecium mitochondria: Three types of hypotheses can account for the failure to detect mitochondrial recombinants in Paramecium: (1) Mitochondrial recombination does exist in Paramecium, but all the markers used are so closely linked as to render recombination frequency indistinguishable from mutation frequency. (2) Mitochondrial recombination exists, but recombined genomes (even wild-type ones) are systematically defective. (3) Mitochondrial recombination
does not exist or is extremely rare for one of the two following reasons: (a) "Hybrid" mitochondria containing the two types of DNA are formed, but no recombination occurs because either of a lack of recombination enzymes or the structure of the mitochondrial DNA molecules prevents it. (b) No hybrid mitochondria are formed, that is each mitochondrion keeps its structural autonomy and does not exchange DNA with the surrounding ones.

Hypotheses 1, 2 and 3a cannot be formally excluded, although hypothesis 1 appears unlikely in view of the large number of combinations studied, and hypothesis 2 has no clear precedent except for the systematic appearance of a small number of defective recombinant genomes in particular mitochondrial crosses of Podospora (Belcour and Begel 1978). Hypothesis 3a may be related to the peculiar structure of Paramecium mitochondrial DNA, which is a linear molecule (Goddard and Cummings 1975, 1977), analogous to Tetrahymena's mitochondrial DNA (Arnberg et al. 1974; Upholt and Borst 1974) and in contrast to that of all other species studied so far that have circular molecules (Borst 1972).

In fact, in favor of hypothesis 3b, a body of evidence has been accumulated. Paramecium mitochondria display considerable "structural individuality" at all stages of their life cycle; when observed by phase contrast microscopy in living cells, they appear as more or less elongated dumbbells, quite distinct from each other, as already noted by Fauré-Fremiet (1910) in his extensive monograph. They are strikingly illustrated in a recent paper (Perasso and Beisson 1978) and also visible in an available motion picture (Adoutte and Knowles 1977). While it is difficult completely to exclude the occurrence of transitory fusions of a small number of units, it can definitely be stated that networks do not exist under the varied experimental conditions used. At the electron microscopic level, in several hundred random sections obtained in J. André's laboratory through the years, as well as in all available pictures in the literature, no Y- or T-shaped mitochondria that may suggest branching or fusion were observed. Furthermore, the fact that Perasso and Beisson (1978) were able to establish that the mean mitochondrial length undergoes a clear shift during the cell cycle (it doubles during the first quarter of the cell cycle, then progressively decreases until the next cellular division) provides an indirect argument in favor of mitochondrial individuality: if constant fusion, reassociation and breakdown were occurring, no clear pattern would have emerged from their measurements. Finally, serial-section reconstruction of the Paramecium subcortical layer (C. Omoto, personal communication) shows an abundance of distinctly separated mitochondria, with general morphology in excellent agreement with that derived from phase contrast observations.

This set of observations is in marked contrast to those made in several organisms displaying "giant chondriomes." In some organisms such as Euglena, under particular growth conditions, and Scuticociliates, grown in similar conditions as Paramecium, they are so obvious as to be evident in transverse sections observed by transmission electron microscopy without restoring to serial sectioning (Calvayrac et al. 1974; Calvayrac, Butow and Lefort- Tran 1972; de Puytorac
et al. 1974; Didier and Detcheva 1974; Rodrigues de Santa Rosa and de Puytorac 1976; Kaneshiro and Holtz 1976). In several other species, extensive mitochondrial networks were revealed by high-voltage electron microscopy or by serial-section reconstruction (Keddie and Barajas, 1969; Osafune 1973; Atkinson, John and Gunning 1974; Bromberg 1974; Rosen et al. 1974; Burton and Moore 1974; Grobe and Arnold 1975; Paulin 1975; Rancourt, McKee and Pollack 1975; Davison and Garland 1975; Gaffal and Kreutzner 1977; Gaffal and Schneider 1978).

The case of yeast and cultured animal cells are particularly interesting since they display mitochondrial recombination. In yeast, the detailed studies of Stevens (1977), in parallel with those of Hoffman and Avers (1973) and Grimes, Mahler and Perlman (1974), show that the number of mitochondria varies from less than 10 (exponential growth phase) to 30–50 (stationary phase), but, more importantly, that in all cases one of the mitochondria is highly branched and much bigger than all the others. This organism is, therefore, characterized by a giant chondriome undergoing cycles of fusion and fragmentation, providing an appealing basis for the genetic data. The dynamics of such a chondriome had already been analyzed in detail by Frederic (1958) on cultured chicken fibroblasts observed by phase-contrast microscopy. The author described constant changes in shape and size of mitochondria: fragmentation, sealing, swelling, branching, etc. These observations were reproduced and accompanied with striking images by Barasa et al. (1973). More recently, the observation of cultured human cells by high-voltage electron microscopy has provided clear confirmation of the heterogeneity of shape of mitochondria, as well as of the existence of “fused” groups (Kilarski and Koprowski 1976).

In summary, then, we suggest that the failure to detect mitochondrial recombination in Paramecium, as well as their other manifestations of functional autonomy, is caused by their particular organization as discrete, independent elements.

Biological implications of the features of the mitochondrial genetic system of Paramecium: The results described in the present paper, associated to those previously obtained, all point to an extensive genetic autonomy of mitochondria in Paramecium. Both the genetical and the cytological data lead us to picture the Paramecium chondriome as made up of thousands of distinct entities having little or no interaction with each other. On the other hand, a given subset of genetically distinct mitochondria is quite capable of responding to a selective pressure by strong differential multiplication. It appears that, by the simple interplay of mutation and selection, the mitochondrial genome of this organism possesses a considerable potential for adaptation. Under such conditions, recombination may lose its adaptative importance.

Change in the mitochondrial population may occur in response either to an extracellular selective pressure or to nuclear modifications. This may lead to co-adapted nucleo-mitochondrial systems. Such systems may then constitute barriers to further exchange of nuclear (and mitochondrial) markers because of the nucleo-cytoplasmic incompatibility such exchanges would generate. This
type of phenomenon has been described in the case of chloroplasts, and the possible role of cytoplasmic genomes in speciation has already been stressed (Stubbe 1959; 1964; Grün 1976).

The ecological genetics of Paramecium is poorly known, and the extent of genetic exchanges in nature is unknown for nuclear, as well as for cytoplasmic, markers. It is possible that, even if conjugation is a relatively frequent event, it is seldom accompanied by cytoplasmic exchange. Paramecium would then resemble the majority of living organisms displaying sexuality, in which the paternal contribution to the zygote cytoplasm is small or nonexistent (see Sager 1973 and Birky 1975 for a discussion of this question). This property, associated with the selective processes described above, may rapidly lead to the formation of a large number of distinct, genetically isolated mitochondrial "clones." This may be one of the bases for the heterogeneity of mitochondrial genomes observed within the same species or between closely related ones (Dawid 1972; Jakovcic, Casey and Rabinowitz 1975; Potter et al. 1975; Cummings, Goddard and Maki 1976; Sanders et al. 1976; Goldbach et al. 1977; Upholt and Dawid 1977; Fonty et al. 1978), and the nucleo-cytoplasmic incompatibility phenomena detected by microinjection of mitochondria between different species of Paramecium (Beale and Knowles 1976).

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LITERATURE CITED


Atkinson, A. W., Jr., P. C. L. John and B. E. S. Gunning, 1974 The growth and division of the single mitochondrion and other organelles during the cell cycle of Chlorella, studied by quantitative stereology and three dimensional reconstruction. Protoplasma 81: 77–109.


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Nagley, P., P. L. Molloy, H. B. Lukins and W. A. Linnane, 1974  

Osapune, T., 1973  

Paulin, J. J., 1975  
The chondriome of selected trypanosomatids. A three-dimensional study based on serial thick sections and high-voltage electron microscopy. J. Cell Biol. 66: 404–413.

Perasso, R. and A. Adjouette, 1974  


Pérès, R., C. Birky, D. Demko and R. Straussberg, 1976  

Potter, S., J. Newbold, C. Hutchinson and M. Edge, 1975  

The mitochondrial genome of wild-type yeast cells V. Genome evolution. J. Mol. Biol. 110: 17–52.

Rancourt, R. W., A. P. McKee and W. Pollack, 1975  

Rodrigues, de Santa Rosa, M. and P. de Puytorac, 1976  

Rosen, D., H. Edelman, E. Galun and D. Oanon, 1974  

Rowlands, R. T. and G. Turner, 1974  

Saccone, C. and A. Kroon, 1976  

Sager, R., 1973  

Sainsard, A., 1975  

Sainsard, A., M. Claisse and M. Balmefrehol, 1974  

Sainsard-Chanet, A., 1976  


Sanders, J. C., Hetting, A. DiFranco, P. Borst and P. P. Slonimski, 1976  


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