BIOGENETIC AUTONOMY OF MITOCHONDRIA

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A. INTRODUCTION

The subject of mitochondrial biogenesis is rapidly becoming one of the most actively pursued in contemporary cellular and molecular biology. No fewer than two monographs,[1,2] five symposia,[3-7] and more than a dozen authoritative reviews[8-22] (cited in the list of general references) have been devoted to the subject since 1968. It would therefore be ill advised to add to this profusion just one more review dealing once again with some aspect of the general problem. Instead, what I have attempted is to survey the literature up to the end of 1972 – but including as yet unpublished material, currently in the process of publication, kindly provided by colleagues – with one end in view: to detect and describe the extent of mitochondrial autonomy and thus to discern the intrinsic limits set on the biogenesis of the organelle. The coverage is intended to be contemporary and representative rather than complete, and no doubt many errors of omission and commission have crept in during its preparation.

B. THE MITOCHONDRIAL GENOME

1. Mitochondrial DNA

a. Isolation, Structure, and Other Properties

The implication of mitochondrial DNA as a – or perhaps the principal – depository of extranuclear (extrachromosomal) genetic information in nonphotosynthetic eukaryotic cells derived from three conceptual roots: (1) the postulate dating back at least to Altman[23] that mitochondria are endosymbiotic bacterial derivatives capable of replication within the eukaryotic cell,[24,25] (2) the demonstration of extrachromosomal, cytoplasmic inheritance,[2,15-26] including a direct correlation of its manifestations with mitochondrial structure and function,[27-30] in conjunction with (3) the conclusive demonstration in the 1950's that DNA is the genetic material in prokaryotes.[31,32]

The time was therefore ripe in the early 1960's to institute a critical and systematic search for mitochondrial DNA (mtDNA) and to initiate attempts at its location and characterization at the molecular and functional level. In fact, already in

1957 Brand and I had shown[33] that purified mitochondria, isolated from chick embryos at early stages of development, contained both DNA and RNA. Similar studies with highly purified mitochondria from yeast, performed by Schatz et al.[34] in 1964, indicated that these particles contained a small but constant amount of DNA. In the previous year, M. M. K. Nass and S. Nass[35] had demonstrated by cytological techniques that fibers exhibiting properties and reactions of DNA were localized in embryonic mitochondria; similar results were obtained with mitochondria from a wide variety of metazoan animals by Nass, Nass, and Afzelius[36] and with those of plants by Kislev, Swift, and Bogorad.[37] In the same year, the mtDNA of *Tetrahymena* was shown to be capable of incorporating ^{3}H-thymidine in vivo.[38] The final proof of mtDNA as a discrete entity, different in properties from nuclear (nDNA), came with the demonstration by Luck and Reich in 1964[39] that mitochondria from *Neurospora crassa* contained a DNA of lower density than nDNA (the latter presumably present only as a contaminant). The actual separation from nDNA and isolation of such a "light" mtDNA from yeast mitochondria were reported in 1965 by Tewari, Jayaraman, and Mahler[40] and confirmed independently by other groups;[41,42] and its properties have been studied extensively ever since, both in our own laboratory[43,44] and in many others.[2,8,9,13,19,45-48]

These studies as well as those on other protists and plants have been aided considerably by the discovery that in all these species nDNA and mtDNA can be distinguished and easily separated on the basis of significant differences in their base composition. This fact can be put to good advantage for the isolation of both components even from impure mitochondria or whole cells on the basis of resulting differences in buoyant density (Table 1) (equilibrium sedimentation in CsCl or $Cs_2SO_4/HgCl_2$) or chromatographic elution patterns (on hydroxyapatite[49] or polylysine[50]). Unlike these mtDNA's, those of metazoan animals frequently exhibit an overall base composition, that is not sufficiently distinct from nDNA so as to permit identification or separation on this basis. However, they *are* distinct in nearest neighbor frequencies,[51] and what is even more important, in another and most characteristic way: They are covalently circular, and many of them in any given population are present as twisted supercoils. These properties were demonstrated first in 1966 by examination of electron micrographs of mtDNA extruded from chick,[52] mouse,[53] and rat liver[54] mitochondria. They form the basis of an extremely rapid and convenient method developed by Vinograd and his collaborators[55,56] for the isolation of such DNA's. It is based on the observation that strongly intercalating dyes, such as ethidium bromide (Etd Br), or its analogues, affect the buoyant density of circular and linear DNA's to a different extent, and so the three possible forms of the same DNA [covalently circular, relaxed circular (only one strand covalently closed), and linear] can be readily separated in CsCl/Etd Br gradients.

In consequence, the study of mtDNA and its properties has progressed rapidly and occupies a fraction of the contemporary research literature rapidly approaching that occupied by its more classical relatives, prokaryotic and nuclear DNA's. These studies have been discussed and reviewed in such great detail within the recent past[2,8,9,13,14,19,48,57-59] that little would be gained in recapitulating the same material once again. Much of the pertinent information is summarized simply in Table 1. Many of the properties and functions of mtDNA are dealt with either explicitly or implicitly in many of the discussions and interpretations in later sections of this review. In this part we restrict ourselves to three additional problems: those of its replication and repair and of the regulation of its synthesis.

b. Replication and Repair

Available experiments using the classical density shift paradigm developed by Meselson and Stahl suggest that mtDNA replicates semiconservatively in *Neurospora*,[60] animal cells,[61] *Tetrahymena*,[62] and *Saccharomyces lactis*.[63] Because of a number of experimental uncertainties (reviewed by Borst[8]), there remains some doubt concerning this conclusion in the first two instances. The experiments by Sena[63] on *S. lactis*, however, appear less susceptible to these strictures; and therefore the tentative conclusion appears justified that, at least at a gross level, the replication of mtDNA is indeed semiconservative. As concerns the steps in and a likely pathway for this process, much work remains to be done. There the most convincing experiments come from studies with animal cells.

Intermediates in replication – Two structures,

TABLE 1

Physical Characteristics of Eukaryotic DNA's[d]

	Density in CsCl (g/ml)				Contour length[c] (μm)
	nDNA	chlDNA	mtDNA	Δ[b]	mtDNA
Chlamydomonas	1.724	1.695	1.715		
Chlorella	1.716	1.692	1.712		
Euglena	1.707	1.685	1.690		
Higher plants	1.692	1.695	1.706		30
Yeast[a]	1.698		1.684		25
Neurospora crassa	1.713		1.702		20, 26
Physarum polycephalum	1.700		1.686		
Tetrahymena	1.688		1.684	6	15, 17.6
Leishmania enriettii	1.721		1.699		
Urechis caupo			1.699		5.9
Sea urchin	1.694		1.704	5	4.6–4.9
Drosophila	1.696		1.689		
Carp	1.697		1.703		5.4
Xenopus (toad)	1.700		1.702	13	5.7
Chick	1.700		1.708	42	5.1
Guinea pig	1.700		1.702		5.6
Rat liver	1.701		1.701	31	5.0–5.1
Beef liver	1.703		1.705		5.1–5.3
Man	1.700		1.706	40	4.8–5.3

	nDNA		mtDNA	
[a]Wild type *Saccharomyces cerevisiae*	Density (g/ml)	Base composition (% A + T)	Density (g/ml)	Base composition (% A + T)
Fleischmann (diploid)	1.698	66	1.684	79
DM (diploid)	1.701	62	1.685	
D310-4D (haploid)	1.698	62	1.683	83
C982-19d	1.698	60	1.683	83

[b]Density difference of separated strands of mtDNA in alkaline CsCl (mg/ml).
[c]All for circular molecules except for *Tetrahymena* and one strain of *Neurospora* (26 μ).
[d]Sources: References 2, 8, 48, and 58; the two sets of values for *Tetrahymena* and *Neurospora* are discussed in References 8 (p. 336) and 48.

Abbreviations: nDNA = nuclear DNA, mtDNA = mitochondrial DNA, chlDNA = chloroplast DNA, A = adenine, T = thymine. All buoyant densities relative to that of *E. coli* DNA take as 1.710 g/ml.

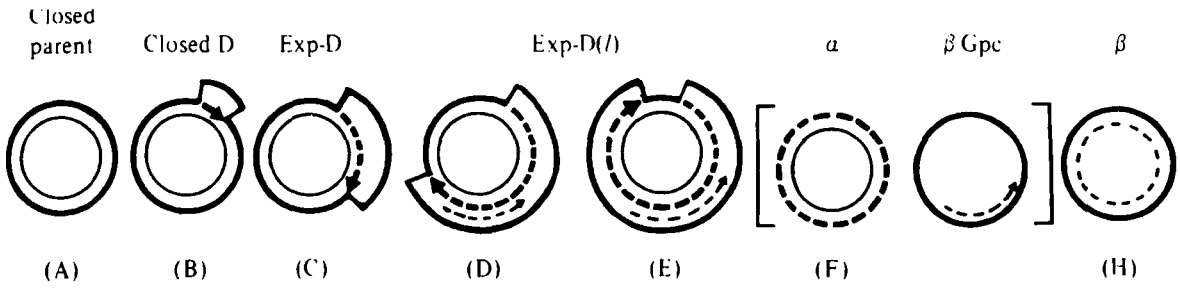

Figure 1. Replication of mitochondrial DNA – The circular replicating structures of mtDNA arranged in order of increasing degree of replication reveal certain aspects of the displacement loop model for the replication of circular mtDNA. Parental strands = solid lines; progeny strands = dashed lines; heavy strands = thick lines; light strands = thin lines. Nicks and small gaps are not indicated. Daughter molecules (F and G) are represented as having formed after separation occurred in a completely (or almost completely) expanded D-loop molecule. Exp-D(*I*) = an Exp-D molecule in which light-strand synthesis has occurred; Gpc = gapped circular molecule. (Reproduced from Robberson, D. L., Kasamatsu, H., and Vinograd, J., *Proc. Natl. Acad. Sci. U.S.*, 69, 738, 1972. With permission.)

isolated from these sources, appear to be likely candidates as possible intermediates:

1. Kirschner et al.[64] found a small number of branched circles ("theta structures") among a very large number of mtDNA molecules isolated from rat liver. These circles contain two branches of equal length, varying from 0.2 to 4.2 μm in length, and all parts of the molecule appear double stranded.

2. A different form of a branched circle, called a D (displacement) Loop DNA has been identified in large numbers, isolated, and characterized from mitochondria of mouse L cells growing in culture,[65,66] as well as in the product DNA formed by isolated mitochondria from chick liver.[67,68] Similar structures were detected also in the mtDNA isolated directly from chick liver[69] and thyroid gland[70] as well as from dividing cells of *Tetrahymena.*[71] These structures – which sometimes account for as many as 50% of the total covalent circles isolated – appear as closed, covalent duplexes with a short segment (E strand), single stranded in nature, complementary, and attached by hydrogen bonds to one of its two strands (the L strand), thus *displacing* the other (the pyrimidine-rich H strand). The E strands can be isolated by mild denaturation that leaves the duplex intact, and most of them are of the order 0.015 to 0.02 μm (3 to 5% of the total) in length. The extensive studies by Robberson, Kasamatsu, and Vinograd[66] suggest a replication scheme involving such structures as early intermediates, as shown in Figure 1.*

Repair – Mitochondrial DNA that has been irradiated by ultraviolet light, or subjected to other insults that might result in alteration in its primary or higher order structure, appears to be susceptible to various forms of repair processes to counteract the initial damage. In this regard as well, the pattern is reminiscent of that exhibited by other, more intensively studied forms of DNA. For instance, in *Saccharomyces cerevisiae* damage to the mitochondrial genome brought about by UV irradiation can be repaired by a mitochondrial system of photoreactivation[72] as well as by one (or more) other dark repair processes,[73] perhaps akin to excision and postreplication repair in bacteria.[74] These systems are specified by both nuclear and mitochondrial genes and can be shown to be effective against damage introduced not only by UV, but also by Etd Br and other mutagens.[75-77] At least one component of what might well be an analogous system in *Tetrahymena* has been identified by Westergaard and his collaborators.[78,79] They have shown that various treatments (exposure to UV or electron irradiation, to prolonged treatment with Etd Br, or to thymine starvation) induce (or result in the derepression of) the biosynthesis of a mitochondrial DNA polymerase synthesized on cell sap ribosomes. The process might thus represent a special instance of a more general phenomenon in which a shutdown of mitochondrial genes results in the eventual dilution of a repressor synthesized by the mitochondria but effective on the nuclear system of gene expression (see Section E.3.d.).

Regulation – In discussing this problem we shall

*A potentially most important finding is the recent demonstration[71a] that isolated, circular mtDNA from animal cells contains covalently inserted ribonucleotides. These may represent prime sites for the initiation of DNA chains during replication.

distinguish three separate aspects: (a) regulation of the amount of mtDNA, in particular, relative to nDNA; (b) regulation of the time of onset (and shutoff) of these two types of cellular DNA, together with (c) regulation of their rate of synthesis.

In most populations of exponentially growing cells, mtDNA appears to be present in a fixed ratio to nDNA and thus constitutes a constant fraction of total cellular DNA. These observations suggest that this stoichiometry is under nuclear control. Although experiments specifically addressing themselves to the question, say in terms of different mutant cell lines of the same organism, have yet to be performed, in *Saccharomyces cerevisiae,* at least, published data are consistent with the hypothesis.[57] Also, as shown below (Section B.3.), respiration deficient mutants, provided they retain any mtDNA, do so in normal amounts. We[81] have asked specifically what effect a doubling of the nuclear gene dosage has on the level of mtDNA by comparing isochromosomal haploids (chromosome number N, amount of nDNA equal to n) of opposite mating type with the diploid resulting from their conjugation (chromosome number 2N, amount of nDNA expected and experimentally verified equal to 2n): In all three strains growing on lactate, the ratio of mtDNA:nDNA equaled 0.15 ± 0.02. Thus, a doubling of the amount of nDNA per cell results in an identical increase in mtDNA. One can also point to possibly interesting correlations for the total amount of mtDNA per cell in widely differing species such as rat liver and yeast. For the former, Bahr[80] cites figures of 50 to 60×10^7 mitochondria/mg wet weight, with each mitochondrion containing 12×10^{-17} g mtDNA or a total complement of about 7×10^{-5} mg mtDNA/mg wet weight (7.5×10^{-15} g mtDNA per cell). For a commercial yeast strain grown similarly in the complete absence of catabolite repression (ratios mtDNA:rDNA = 0.18), the numbers determined in my laboratory[81] are $\sim 100 \times 10^7$ mitochondria/mg wet weight, and a total of 1×10^{-4} mg mtDNA/mg wet weight or $\sim 10^{-14}$ g mtDNA/cell. These values also correspond to $\sim 10^{-16}$ g mtDNA or 6×10^7 daltons per mitochondrion; since the particle weight of this DNA equals $\sim 5 \times 10^7$ daltons, each mitochondrion contains one molecule of mtDNA on the average.

The number of mitochondria per cell, of course, varies widely, both as a function of the type (species or tissue) under discussion, and also, especially in unicellular forms, as a function of the medium. For yeast cells growing rapidly in exponential phase, it does not appear to be subject to gross fluctuation in response to carbon source and state of repression[57,82,83] (for a recent contrary view for cells in chemostat cultures, see Reference 84). This consideration, together with differences in size of mtDNA between species, results in significant differences in the number of molecules of mtDNA per mitochondrion: This parameter may vary from about one (for *S. cerevisiae* in the absence of repression) to approximately ten (in vertebrate cells in culture) on to several times that[81] (in *S. cerevisiae* strongly repressed by glucose for mitochondrial number and function).

The time and rapidity of onset of mtDNA relative to that of nDNA in cells growing in synchrony appears to be subject to different controls in different cell types or, more likely perhaps, to the specific manipulations required to achieve synchrony. In the protists *Tetrahymena,*[62] *Physarum polycephalum,*[85,86] and *S. cerevisiae,*[57,87] formation of mtDNA appears to occur at about the same rate throughout the cell cycle. On the other hand, in *S.* (really the unrelated[88] *Kluyveromyces) lactis* mtDNA is synthesized discontinuously during a brief interval that precedes the one for nDNA,[89] and some preliminary evidence with *S. cerevisiae* synchronized by a different procedure has also been interpreted in these terms.[90] In synchronized animal cells, Pica-Mattoccia and Attardi,[305] as well as Bosmann,[91] have presented evidence for discontinuous synthesis of mtDNA in the S and G_2 phases by human (HeLa) and mouse (L) cells, respectively.

Although in growing cells the synthesis of mtDNA and nDNA appears to be coupled in some fashion, the two processes can be dissociated under appropriate conditions. A relevant observation by Grossman et al.[92] in yeast has turned out to be extremely useful operationally: The reinitiation of a new round of nDNA synthesis is blocked when protein synthesis is inhibited by cycloheximide (CH) (Section C.3.a.4.), but mtDNA remains unaffected. As a consequence, mtDNA increases relative to nDNA under these conditions to levels two to three times those observed in the growing cell; and if a labeled precursor is added when the previous round of replication of nDNA has already reached completion (i.e., less than one generation after CH),

almost exclusive labeling of mtDNA can be achieved. Similar effects can be obtained when the further synthesis of nDNA is interrupted by shifting appropriate temperature sensitive mutants to the nonpermissive temperature.[93] Results that are similar operationally, although their explanation and significance are much less clear, are observed when nongrowing cells of *S. cerevisiae* are shifted from an anaerobic to an aerobic atmosphere,[94,95] or are released from catabolite repression.[96] Under both these conditions, there is a substantial increase in the rate of mtDNA synthesis relative to that of nDNA without a concomitant large increase in amount (<30%) of this DNA. Similarly,[97,98] when certain tissue culture cells (kidney cells from African green monkey, and mouse line 3T3) become confluent, the rate of nDNA synthesis drops to a small fraction (2 to 5%) of the logarithmic value, while that of mtDNA continues at a much higher rate (~20%).

2. Mitochondrial Mutations

The existence of extranuclear (extrachromosomal, cytoplasmic) genes, operationally defined by non-Mendelian modes of inheritance of certain mitochondrial characteristics, is now well established. This line of investigation has been the subject of a recent monograph[2] and a comprehensive review.[15] Certain aspects of the general problem are also discussed in considerable detail in the other general references cited at the end of this article. We shall therefore restrict ourselves to a brief description of the relevant observations and their interpretations. The most important conclusion – placed on a firm experimental footing only during the most recent past, although implicit in much of the earlier thinking – is that these cytoplasmic mutations in yeast and in *Neurospora* are indeed due to alterations in mtDNA. For although this entity, first demonstrated conclusively only in 1963 and documented extensively since then (see Section B.1.a.), was shown soon thereafter to code for various mitochondrial RNA's (see Section C.1.a.), its other genetic capabilities remain to be explored. In particular, no mitochondrial gene has yet been shown to be capable of specifying an authentic polypeptide of the mitochondrion, so that it was by no means obvious that non-Mendelian cytoplasmic inheritance was *necessarily* mitochondrial with regard to localization of the determinants responsible. We first deal in some detail with what is known[1,2,11,12,22,26,29-31,99-101] concerning the nature and origin of one class of such mutants.

a. Respiration Deficiency: The Vegetative Petite (ρ^-) Mutants

1. Neutral petites – When cells of *Saccharomyces cerevisiae*, a facultatively anaerobic yeast, are plated on nutrient agar containing a readily fermentable sugar such as glucose and the resultant colonies are examined, a sizable fraction appears to be smaller (petite) than the majority (grande). Once isolated, the cells in these colonies – whether in their haploid or diploid state – pass on this particular property [colony morphology on a "nonselective" diagnostic medium such as glucose, or more strikingly glucose (0.1%) plus glycerol (3%)] to all their descendants upon vegetative growth by cell division (mitosis). Therefore, this property is controlled by a heritable alteration or mutation in the affected cells. In fact, it is but one manifestation of their phenotype, which is respiration deficient. This respiratory deficiency is due, we now know, to an alteration in the inner membrane of their mitochondria, resulting in their inability to elaborate a functional electron transport chain[29,30,102,103] (most dramatically, cytochromes *b*, c_1, and aa_3 – but not cytochrome *c*!) and its attendant system of ATP generation[83,104] (see Table 5). In consequence, these cells are unable to grow on respiratory carbon sources such as glycerol and can therefore be detected by an inability to form colonies upon replica plating to such a "selective" medium. However, they are capable of growth, albeit with a reduced yield, purely glycolytically, on glucose,* producing the small colonies described earlier.

Genetically, the mutation exhibited several unusual properties:

a. As already mentioned, it occurred spontaneously with a frequency (varying from about 0.001 to >0.1 in different strains) much higher than that characterizing common chromosomal

*Wild type cells growing aerobically on glucose do so in phases:[22,105] In the first they are supported by (aerobic) glycolysis yielding ethanol and CO_2 virtually quantitatively (i.e., the contribution due to complete oxidation of pyruvate, acetate, or acetaldehyde is insignificant); in the second the metabolism is based on the complete oxidation of the ethanol produced in the first phase.

mutations, e.g., to auxotrophy or its reverse, which are usually in the range around 10^{-6}

b. This frequency could be raised virtually to 1 (i.e., affecting every cell in a population) by treatment with appropriate mutagens at quite low concentrations. The first such agent, already used by the original French workers[26,30,106,107] in their seminal investigations in 1949, was the acridine dye euflavine (10-methyl-2,8-diamino acridine). Microdissection and clonal analysis showed it to be capable of converting the descendants of almost every bud produced in its presence into mutant cells; however, the mother cells remained unaffected.[108] Ethidium bromide (Etd Br; 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) is even more effective; in the μM range of concentration it is capable of mutagenizing without lethality virtually all cells present in either a growing or nongrowing population.[109] A large number of potential mutagens have now been examined,[110] some of which will be discussed more fully below in Section B.3.b.1. In general, agents, or treatments, most effective in producing this mutation are much less so or virtually ineffective in producing the usual chromosomal mutations and vice versa.[2] In these mutagenic events, as already mentioned, nuclear gene dosage or ploidy (haploids vs. diploids or even tetraploids[111]) does not appear to exert a significant effect.

c. Some of the original mutant strains, isolated almost 25 years ago, as well as literally thousands isolated and examined since, have been maintained in a large number of laboratories to the present day: No revertants to wild type have ever been reported in spite of a meticulous search and the obvious selective advantage this process would confer to the cells affected. Similarly, two independently isolated haploid mutants of opposite mating type – a trait specified by a nuclear determinant – can be mated to produce a zygote that upon vegetative growth (mitosis) generates diploid clones. No such zygote has ever been found to have been rendered respiration sufficient by virtue of complementation somewhere in the cell of two different hypothetical gene products with one each being provided by the two parents; nor have the two hypothetical modified genes ever been shown to be capable of producing wild type descendants by virtue of their recombination.

d. All these observations suggest, but do not prove, an extramitochondrial (extranuclear, cytoplasmic) origin of the mutation. This proof was furnished by examining the mode of inheritance of this trait and comparing it to that of characters – preferably in the same cell – of known chromosomal localization. Such traits include mating type (a vs. α), growth factor requirements (e.g., for adenine or thymine, phenotype Ade^- and Thy^-, respectively, due to modifications in a variety of *ade* and *thy* genes), or resistance to certain antibiotics.[112] As discovered more recently,[100,101,113] a variety of nuclear genes (e.g., the segregational petites of the *P/p* series) also control production of a respiration deficient phenotype.[114] The relevant genetic test is performed by examining the properties of the zygote produced by the fusion of two haploid cells, opposite in mating type, one of them mutant for the trait to be examined (Figure 2). The genetic contribution of the zygote and any of its diploid descendants can then be examined by inducing these cells to sporulate: This process (meiosis) involves first a duplication of the nuclear chromosomes and its genes, followed by their separation into four haploid ascospores enclosed in a sac (ascus). These four cells can be dissected out from the ascus, germinated, and examined for their growth requirements, etc., either immediately or upon further vegetative growth and division as haploids. Under these conditions, any single pair of nuclear alleles present in the (heterozygous) diploids should and does assort in the expected equal ratio (2:2) among the four ascospores. The type of respiratory deficiency under discussion did not exhibit this Mendelian pattern of segregation. Not only the original zygotes and all their diploid descendants, but also (with very rare exceptions, due to actual mutations in the course of the test) all four haploid ascospores and their descendants proved to be as respiration competent as the wild type (grande) parent: The petite phenotype had disappeared, and operationally the genetic determinant responsible had been eliminated. From these observations and from appropriate backcrosses of descendants with the original wild type parents, it was inferred that wild type cells contain a cytoplasmic factor (ρ^+) that is absent (ρ^-) in petites. The second part of the hypothesis has had to be modified on the basis of more recent genetic and molecular evidence.

e. The cytoplasmic nature of the mode of inheritance of the mutation was substantiated by experiments of Wright and Lederberg[115] using *S. cerevisiae* var. *ellipsoideus*. In this species the

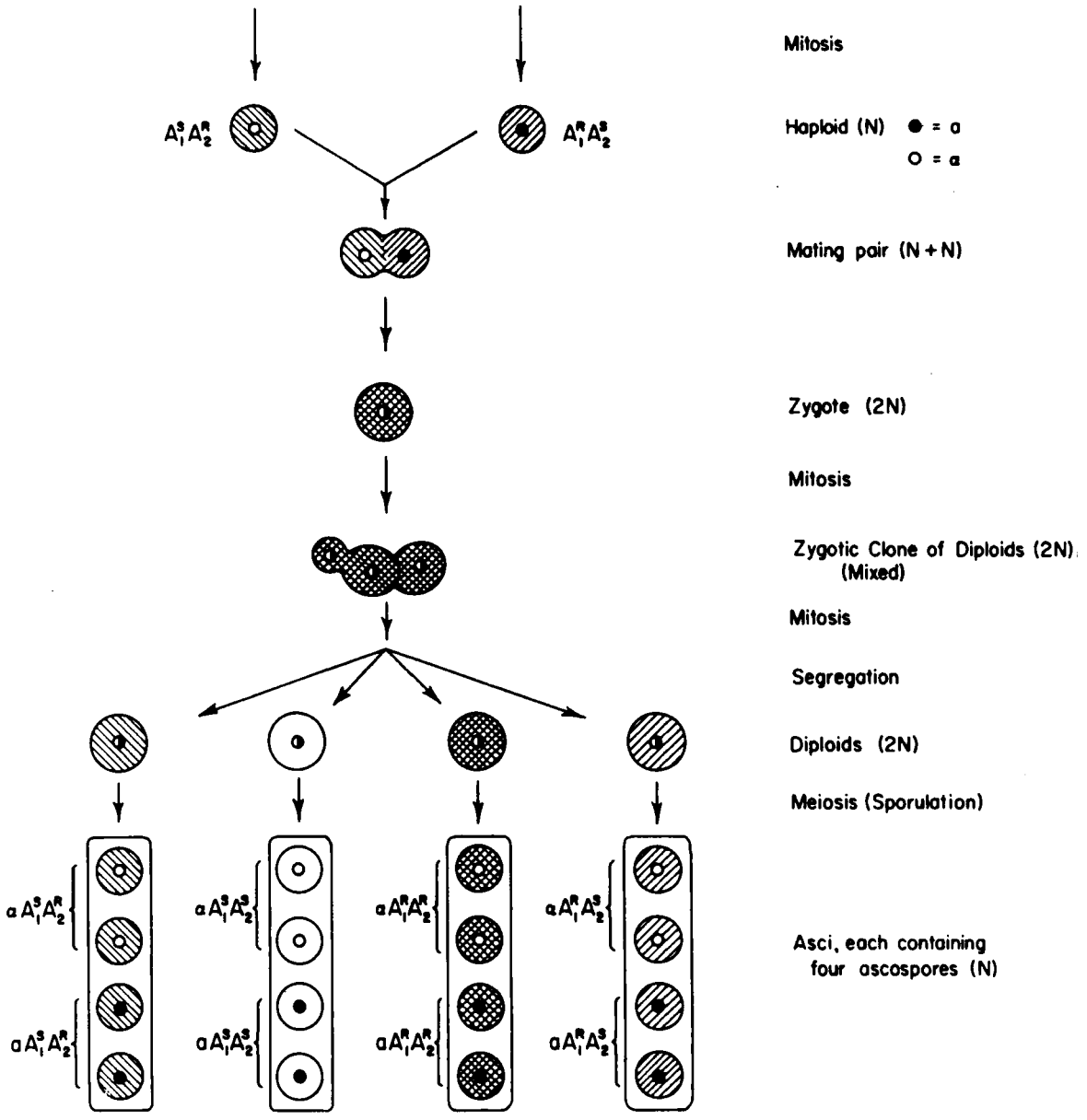

FIGURE 2. Segregation and recombination of mitochondrial markers during the life cycle of *Saccharomyces* – Two haploid strains, each capable of vegetative division (mitosis), differ in (1) an allelic pair of chromosomal markers (α and a) designating their mating type and (2) in two allelic sets of mitochondrial markers (A_1^S/A_1^R and A_2^S/A_2^R). The first part of the scheme demonstrates the formation of a zygote from the two haploids, followed by the segregation of various combinations (parental and recombinant in type) of mitochondrial markers in the course of successive mitotic divisions of the isochromosomal diploid (α/a) cell lines. The outcome of these segregational events is the formation of clones homogeneous in their mitochondrial genotype. The second part of the scheme demonstrates the complete absence of a Mendelian pattern of segregation of mitochondrial markers during meiosis, when this pattern (2:2) is readily apparent for chromosomal markers such as α and a.

The appropriate ascospores can be used to initiate all the events shown in the scheme including zygote formation. Note that some of the ascospores retain their parental mitochondrial alleles but have their nuclear alleles reversed.

mating pairs survive for an appreciable time as heterokaryons, cells with a mixed cytoplasm but with the two parental nuclei still intact. Now, occasionally, buds produced by such cells contain only one of the parental nuclei but of course share the common cytoplasm and thus generate a homozygous diploid clone: Examination of such clones originating from the fusion of wild type ($a\ arg^+\ thr^-\ \underline{\rho}^+$) cells auxotrophic for threonine, and petite cells auxotrophic for arginine ($\underline{a}\ arg^-\ thr^+\ \underline{\rho}^-$) disclosed the presence of a small number of clones that were Thr^- but respiration deficient (ρ^-), as well as an equally small number that were Arg^- but wild type (ρ^+). Thus, the trait for respiratory sufficiency had been shown to segregate independently of the nucleus and, hence, was subject to extranuclear inheritance.

f. In contrast to these cytoplasmic petite ρ^- mutants, the nuclear (segregational) petites originally discovered by Chen et al.[113] (now known to possess the genotype $p\rho^+$) exhibit, after being crossed to wild type ($P\underline{\rho}^+$), normal segregation upon meiosis (2 wild type or *P*:2 mutant or *p*). Over 20 nuclear genes, mostly unlinked, have so far been identified, all participating in mitochondrial specification in a manner so as to produce a mutant, respiration deficient phenotype qualitatively and sometimes quantitatively similar to that of ρ^- mutants.[114] When two different nuclear petites (e.g., p_x and p_y) are either crossed with one another, or any one of them is crossed with any cytoplasmic petite ($P_x\underline{\rho}^-$) to produce a zygote, the latter is capable (after some delay) of normal respiration due to complementation.[116] If such a zygote or its descendants are sporulated, the segregation pattern is 2 p_x: 2 p_y (all petites) or 2 $p_x\rho^+$ (petites):2 $P_x\rho^+$ (wild types), respectively.

2. **Suppressiveness** – The cytoplasmic petites first described exhibited complete replicative dominance of the wild type in zygotic clones and upon meiosis. Since this is not the only pattern observed with other mutant strains, this first class of petites are now known as neutral or recessive petites. More frequently, petites crossed to wild type produce zygotes that upon immediate sporulation exhibit not only the 4 ρ^+:0 ρ^- segregation described, but the reverse pattern (0 ρ^+:4 ρ^-) as well.[117] If the zygotes are not sporulated but instead allowed to undergo vegetative division on glucose plates, the resultant colonies of diploid cells are of three types: pure, or rather predominant, wild type (staining red with triphenyltetrazolium); pure mutant (white); and a mixture of both (red and white mosaics of the two types). The relative proportion of pure white (petite) colonies (or of zygotes exhibiting the 0:4 pattern on meiosis) obtained in a cross of different ρ^- petites with a standard wild type tested under carefully controlled conditions is a characteristic property of each mutant strain and can thus be used to measure its degree of dominance or suppressiveness: The latter varies continuously from zero (neutral petite) to ⩾99% (highly suppressive). The degree of suppressiveness or suppressivity is inherited coincident with the ρ^- character but is relatively easily altered, e.g., by repeated subcultures or by agents affecting cytoplasmic inheritance: It represents a second instance of extranuclear inheritance and proves that the ρ^- mutation (except for neutral petites) need not necessarily be devoid of all informational content.

Analysis at the cellular level[118,119] has shown that zygotes from ρ^- mutants of intermediate suppressiveness produce three types of cells: ρ^+ cells (which generally breed true), ρ^- cells (which always do), and cells in an intermediate, premutational state that can generate both types. Initially most, if not all, the zygotes, and probably their immediate descendants, are of the third type; and some of the cells retain this capacity for many (up to 20) generations, the exact proportion and kinetics varying with the physiological state of the cell (temperature, growth medium, etc.). Zygotic clones of such ρ^- x ρ^+ crosses thus exhibit the phenomenon of mitotic (somatic) segregation, which, as we shall see, provides us with a second general criterion for cytoplasmic inheritance, which can be used in addition to the absence of segregation during meiosis.[120,121]

Several models have been proposed to account for suppressiveness (reviewed in References 15, 122, 123): the ability of (a) either the cytoplasmic genome (mtDNA) or its product and carrier (the mitochondrion) to compete successfully in replication with its wild type counterpart in the same cell; (b) preferential transmission of either of these components to the progeny (bud); (c) the actual destruction of the wild type genome; and, contrasting with the three previous models, all of which involve some form of selection, (d) the postulate[121] that mutant and wild type genomes are capable of rapid and multiple genetic exchanges (recombination) with suppressiveness re-

lated to the probability of producing an aberrant genome as a result of these events.

b. Cytoplasmic Mutations to Drug Resistance

1. Isolation and characterization – Because of the inherent strong selection (only resistant organisms are capable of growth), mutations to drug resistance are easily isolated and have been extensively studied in bacteria. A mutant of this type resistant to streptomycin was isolated in 1954 by Sager[124] in *Chlamydomonas*, a eukaryotic protist, and shown to exhibit a non-Mendelian pattern of inheritance presumably determined by a cytoplasmic entity. By 1966 it had become apparent[125,126] that (a) mitochondria and cell sap ribosomes exhibited separate but complementary patterns of sensitivity to various antibiotics (Table 7), and that (b) only aerobic growth on ethanol and other nonfermentable carbon sources, but not on glucose, required active mitochondrial proliferation and was thus sensitive to "mitochondrial inhibitors" such as chloramphenicol (CAP) and erythromycin. These facts provided the basis for the isolation by Wilkie, Saunders, and Linnane in 1967 of mutants resistant to erythromycin during their growth on such nonfermentable carbon sources.[127-130] These studies were extended quickly in Wilkie, Linnane, and Slonimski's laboratories (for reviews see Wilkie et al.,[127] Coen et al.,[120] Linnane and Haslam,[12] Preer,[15] and Linnane et al.[22]). By now a wide variety of mutants resistant to a number of other antibiotic inhibitors of mitochondrial protein synthesis, such as CAP, spiramycin, lincomycin, mikamycin, tetracycline, etc., as well as to other drugs that interfere with mitochondrial biosynthesis by virtue of blocks on respiration or energy generation, such as oligomycin,[131-133] triethyltin, or other uncouplers,[134] have been isolated and partially characterized.[135-137]

The phenotype of many of these mutants is quite complex since they almost invariably exhibit complicated patterns of cross resistance. However, in spite of this inherent complexity, it is becoming apparent that a number of separate, perhaps eight, genes conferring drug resistance exist on the mitochondrial genome. In the next section we discuss their mode of inheritance.

2. Genetics of transmission of antibiotic resistance markers – criteria of mitochondrial inheritance (Figure 2) – The following criteria appear to be obeyed by and to define mitochondrial mutations (for detailed discussions see Coen et al.,[120] Bolotin et al.,[121] Rank and Bech-Hansen,[122a] Trembath et al.,[138] Mitchell et al.,[135] and Preer[15]):

1. Absence of meiotic segregation – Production of tetrad ratios of 4:0 and 0:4 (*antibiotic resistance* – which commonly has been abbreviated as A^R, AN^R, or ant-r* as a phenotype, and similarly in italics as an allele (gene) – to *antibiotic sensitivity*, A^S, etc.) upon sporulation (meiosis) of stable A^R or ant-r (mutant) and A^S or ant-s (wild type) diploids.

2. Mitotic segregation – The generation, in crosses between *ant-r* and *ant-s* cells of a heteroplasmic zygote, i.e., one with a mixed cytoplasm containing determinants from both parents (heteroplasmon). Not only the parental types, but various recombinant ones, become detectable if we can score for more than one marker, e.g., in the cross $A_1^R\ A_2^S \times A_1^S\ A_2^R$, we find not only these two types, but also $A_1^R A_2^R$ and $A_1^S A_2^S$, the doubly sensitive and doubly resistant ones. The various types segregate and become stably established in the course of vegetative growth. This process of reassortment of mitochondrial genomes during mitosis may not be completed until many vegetative divisions have passed.

3. Mutation to A^0 – The conversion ρ^+ to ρ^- is accompanied by either retention (i.e., of either A^R or A^S) or loss (A^0 from either) or other mitochondrial markers. Such a loss is manifested operationally by retention of the parental trait (A^S or A^R) carried by the ρ^+ parent. Since extensive vegetative growth in the presence of Etd Br leads to a complete loss of mtDNA, all other markers are necessarily lost as well. The mutagenic change $\underline{\rho}^+A^R$ (or A^S) $\rightarrow \underline{\rho}^+A^0$ is never observed. These observations permit the inference that mitochondrial resistance markers are encoded in mtDNA (or at least controlled by it in their expression).

The existence of DNA^0 (also called ρ^0) mutants can then be used to define two additional criteria for assignment of a suspected mutation to mtDNA: (a) its production by the method outlined and (b) its behavior when crossed to a mutant retaining the allele, i.e., all descendants of a cross $A^{R \text{ or } S} \times \rho^0$ will be $A^{R \text{ or } S}$.

4. Polarity – In crosses between two different ρ^+ parents carrying *ant-r* genes:

*Systematic designation.

a. Of transmission: one of the two parental genotypes is transmitted to the descendants more frequently than is the other.

b. Of recombination: provided the two parents are of opposite mating type (sign) – see below – one of the two possible recombinant genotypes will be formed preferentially: We observe that recombination is nonreciprocal, i.e., $A_1^S A_2^S / A_1^R A_2^R$ (e.g., $C^S E^S / C^R E^R$) $\neq 1$.

c. Of sign:

i. In a homosexual cross ($\omega^+ \times \omega^+$ or $\omega^- \times \omega^-$) all diploid descendants whether of parental or recombinant type are of the same sign (which is identical to that provided by the parents, i.e., ether ω^+ or ω^-).

ii. In a heterosexual cross ($\omega^+ \times \omega^-$) all recombinant descendants are ω^+ while those of parental genotype retain the sign (sex) of that particular parent. This operation serves to define the concept of mitochondrial donor and acceptor – or sign.

The following example modified from Bolotin et al.[121] shows some of the results obtained in the two types of crosses. The numbers show the percentages of various phenotypic classes (see Key) in the progeny.

C^R / E^R	$C^R_a \omega^-$		$C^R_b \omega^+$	
$E^R_a \omega^+$	8	54	44	45
	300		1.6	
	38	0.13	7	4.5
$E^R_b \omega^-$	48	42	80	3.5
	1.2		0.02	
	6	5	0.4	18

Key:

$C^R E^S$ $\quad$ $C^S E^R$

$\dfrac{C^S E^S}{C^R E^R}$

$C^S E^R$ $\quad$ $C^R E^R$

iii. Effects of UV irradiation, which converts ρ^+ to ρ^-, in heterosexual crosses prior irradiation of the ω^+ parent enhances, while that of the ω^- parent is without effect on, the transmission of parental markers.

3. Recombination and segregation – Implicit in the brief description just presented and the thinking of investigators in the field is that mitochondrial genetics shares many of the formal attributes – and perhaps also the molecular events – previously worked out for prokaryotic systems such as bacteria, their plasmids, and their viruses;[31,32,141] specifically the transfer (and attendant replication) of chromosomes and plasmids from donors to recipients, and the exchange of chromosome segments in the relatively large mating pools of vegetatively replicating phages after mixed infection. Although analogous explicit studies on mitochondrial systems are still in their infancy, several interesting questions have already emerged.

One would like to know, for instance, about the rapidity of the events that are required for the reassortment of genetic elements and culminate in the formation of recombinant genotypes, as well

as about the kinetics of transmission of these genomes together with the parental ones to stable cell lines during vegetative growth. Are they restricted to the zygote and its immediate buds, or do they continue during the mitosis of their descendants (Figure 2)? This question can be answered by a pedigree analysis of these various individual cells – and their haploid ascospores – emanating from a single cellular mating event. Experiments of this type have been performed by Coen et al.,[120] by Lukins et al.,[139] and by Wilkie and Thomas.[140] All these studies are in agreement that primary recombinational events appear restricted to the zygote and its immediate descendants, although, in occasional lines, additional reassortment and rectification may continue for a number of additional generations. Smith et al.[142] have shown also that coincident with nuclear fusion during zygote formation there is a disorganization and dedifferentiation of the mitochondrial inner membranes; these changes are reversed in older zygotes. One would also like to know whether a molecular basis, on the level of mtDNA, can be found for such reassortment. The most pertinent study is one by Shannon et al.,[143] who showed that within $\geqslant 2$ generations after a mating in mass culture between two populations varying in both the density of mtDNA and a mitochondrial marker (i.e., wild type with a suppressive ρ^- of lower density), a significant fraction of the cells in the population appeared intermediate in both these characteristics. In the interpretation of these experiments, we must not forget that all of them were performed with *S. cerevisiae*, an organism in which cellular replication is by budding and therefore inherently asymmetric. Buds initially contain only a few mitochondria (and molecules of mtDNA), which might furthermore not be representative of the mitochondrial population at large.

4. Antibiotic resistance in other organisms – Isolation of mutant cell lines, resembling the ones just described for yeast and sharing some of their characteristic attributes, have also been reported for *Paramecium*[144-146] and, more recently, for mammalian cells growing in culture.[147,148]

However, particularly in the latter case, genetic tests have not been performed and will prove to be extremely difficult to execute. Since many cells exhibiting chloramphenicol resistance (the particular trait selected for) are chromosomal even in yeast, the assignment of the mutation as mitochondrial appears premature.

3. Mutations and Mitochondrial DNA

a. Observations on Petites

1. Retention of genetic information in cytoplasmic petites – The nature of the mitochondrial mutations described so far renders impossible the usual characterization of their informational content by determining their phenotype. However, just as the degree of suppressiveness can be determined by a genetic test involving the cross of a haploid ρ^- with a suitable ρ^+ tester, so can we determine the extent to which any given allele for antibiotic resistance (or even sensitivity, with a somewhat more cumbersome testing procedure) can be contributed by a ρ^- cell, for instance by performing the cross $(a\rho^- A_1^{R(?)} A_2^{S(?)}) \times (a\rho^+ A_1^S A_2^R)$. Here A_1 and A_2 represent two different mitochrondrial loci for antibiotic resistance, with R and S indicating their resistant and sensitive alleles, respectively; and the $(a\rho^-)$ strain to be tested had been derived from one that was $A_1^R A_2^S$. We may score, for instance, for wild type diploid clones resistant to both antibiotics.* We can also ask whether any of the diploid – or haploid (through sporulation) – descendants are $\rho^+ A_1^S A_2^S$. By so doing, it can be shown that most ρ^- petites have in fact lost any given marker (they have become A^0) and – by performing the test for several markers (most conveniently with petites derived from wild types carrying a variety of different resistance alleles) – all possible markers and hence all mitochondrial information. Operationally, since the wild type descendants of the zygote are identical to the wild type parent, these petites are neutral or zero in information content. This observation would gain particular significance if it should prove possible to correlate it directly with mitochondrial DNA, for instance by isolating ρ^- mutants that lack detectable, or significant, amounts of mtDNA capable of performing a direct genetic function. As already mentioned, and discussed more fully in a later section, both types of such mutants are in fact known. In particular,

*By analoguous procedures we can also ask whether recombination can occur in petites: Here we first perform the cross $a\rho^- A_1^R A_2^S \times a\rho^- A_1^S A_2^R$, then cross the progeny into $a\rho^+ A_1^S A_2^S$ wild type and look for double resistant $(\rho^+ A_1^R A_2^R)$ *triploid* clones.

TABLE 2

The ρ^- Mutation in Yeast and mtDNA

Yeast strain	Genetic markers	Buoyant density[a] (g/ml)	Base composition (% A + T)	References
D310-4D	ρ^+	1.683	83	44
D310-4D21	ρ^-	1.673		
D310-2A-184	ρ^-	1.676	96	
DM	ρ^+	1.685	83	46
DM1	ρ^-	1.672	96	
Wild type	ρ^+	1.684		161, 169
RD-1A	ρ^-	1.671	95	
IL-8-8C	$\rho^+C^RE^R$	1.684		155, 156
IL-8-8C/R5/3	$\rho^-C^RR^R$	1.685		
IL-8-8C/E4/1	$\rho^-C^0E^R$	1.689		
IL-8-8C/C4/2	$\rho^-C^RE^0$	1.682		
IL-8-8C/	$\rho^-C^0E^0$	n.d.[b]		

[a]Relative to DNA from *Escherichia coli*, taken as 1.710.
[b]n.d. = not detectable ($\leq$ 4% of ρ^+ and other ρ^- strains).

DNA^0 (or ρ^0) mutants can be obtained relatively easily, particularly by exposing growing cells to Etd Br,[45,149-151] and these cells would be expected to lack all capacity to contribute either allele of any given mitochondrial marker in our hypothetical cross. Thus, DNA^0 mutants would be expected to be A^0 also. This expectation has been borne out by extensive studies in both Slonimski's and Linnane's laboratories;[120,121,152,153] in fact, the demonstration of the knockout of a marker coincident with the elimination of mitochondrial DNA in producing a given petite mutant provides us with a necessary (though not a sufficient) criterion for three phenomena: (1) an additional (third) criterion for cytoplasmic inheritance, (2) an indication that the latter is in fact mitochondrial in origin, and (3) that its determinants are localized in mitochondrial DNA. However, this particular proof is not complete, since, coincident with, or in consequence of, the loss of DNA, the mitochondria or the cell might undergo other changes that prevent the transmission or expression of determinants carried elsewhere in the cytoplasm. Furthermore, although DNA^0 petites are, as stated, A^0_n, the converse need not hold; other mechanisms for eliminating any given or all information in mtDNA of ρ^- cells can be envisaged and do in fact exist, e.g., in the so-called low density petites (Table 2), which contain in their DNA predominantly A and T ($\geq$ 96%). Also, it is easy to see how an absence of mtDNA might render such a "ρ^0" mutant a neutral petite as reported.[123,151,153] However, again the converse need not hold; there might exist a variety of neutral petites including some that retain a considerable portion of their wild type mtDNA sequences and meaningful information encoded in them. A low density petite (RD-1A) – which might for this very reason lack coding capabilities – has been tested by Moustacchi[154] and was, in fact, found to be neutral.

Some other ρ^- strains examined were, however, found to be genetically competent by the operational test defined above. A systematic investigation of the kinetics of loss of respiratory competence (i.e., the ρ^+ character) and two separate antibiotic resistance markers (for chloramphenicol, C^R, and erythromycin, E^R) as a function of length of exposure to low concentration of Etd Br gave the following results:[121,155] (1) Loss of antibiotic resistance (i.e., conversion to A^0) never occurred without simultaneous loss of respiratory competence ($\rho^+ \rightarrow \rho^-$), i.e., the genotype ρ^+A^0 does not exist; (2) long exposures produced predominantly clones of the type $\rho^-C^0E^0$; and (3) shorter exposures produced both this type and petites that had retained genes for antibiotic

resistance; their proportion at the maximum was $\underline{\rho}^{-}C^{0}E^{0} \cong \underline{\rho}^{-}C^{R}E^{R} >> C^{0}E^{R} \cong C^{R}E^{0}$.

These results lead to some interesting inferences concerning the mitochondrial genome and its possible modification: (1) The *A* genes are carried by the same entity that is responsible for conferring respiratory competence ($\underline{\rho}^{+}$), i.e., they are not localized on one or more additional cytoplasmic entities analogous to the plasmids that carry genes for certain antibiotic resistance traits in bacteria[141] (*R*esistance *T*ransfer *F*actors); (2) there must exist a wide variety of different ρ^{-} mutants and more than one pathway for the loss of meaningful genetic information in them; and (3) unlike the situation for marker knockout in prokaryotic (e.g., bacteriophage) systems where the probability of eliminating two determinants is low – the probability is represented by the product of the individual probabilities – the events in the mitochondrial case responsible for generating $\underline{\rho}^{-}A_n$ selectively while eliminating $A_{n'}$ from $\underline{\rho}^{+}A_nA_{n'}$ must be rare relative to ones that render large portions of the genome ineffective and produce $\underline{\rho}^{-}A_n^0A_{n'}^0$.

2. Retention of Sequences in mtDNA – The results just described raise the possibility of directly correlating such genetic findings with possible alterations in mitochondrial DNA. Such experiments have been done in a collaborative effort by Fuhuhara and Faye in Slonimski's laboratory and by Perlman in my own.[156] It was found (Table 2) that in a particular series of such mutants, $\underline{\rho}^{-}C^{R}E^{R}$, $\underline{\rho}^{-}C^{0}E^{R}$, $\underline{\rho}^{-}C^{R}E^{0}$, and $\underline{\rho}^{-}C^{0}E^{0}$, all derived from the same wild type ($\underline{\rho}^{+}C^{R}E^{R}$): The first three contained mtDNA in amounts similar to that found in the wild type, while the fourth lacked detectable amounts of this entity (< 3%). The buoyant density (and hence the G + C content) of the mtDNA's of the four strains conformed to the following order:

$$\underline{\rho}^{-}C^{0}E^{R} > \underline{\rho}^{+}C^{R}E^{R} \cong \underline{\rho}^{-}C^{R}E^{R} > \underline{\rho}^{-}C^{R}E^{0}$$

(1.689 g/ml) (1.685 g/ml) (1.684 g/ml) (1.682 g/ml)

Further studies in France disclosed that such findings did not appear restricted to this particular series of mutants but applied to others derived from the same $\underline{\rho}^{+}$ strain.[157] All $\underline{\rho}^{-}C^{R}E^{R}$ strains exhibited base compositions similar to that of the ρ^{+} parent, while the deviations for $C^{0}E^{R}$ were toward a higher and for $C^{R}E^{0}$ toward a lower G + C (higher A + T) content. The $\underline{\rho}^{-}C^{0}E^{0}$ strains either lacked all DNA or appeared to contain grossly aberrant DNA, always of much lower density (high A + T) than ρ^{+}. The latter observation confirms the existence of such strains previously discovered independently and studied in a number of laboratories.[44,46,158-161a] Since these strains appear to have lost all mitochondrial markers, these results also suggest a direct correlation between loss of coding capacity and extent of modification of mtDNA. These inferences and others, discussed below, are put in schematic form in Figure 3, which represents an elaboration and modification of a scheme first presented by Bolotin et al.[121] In their scheme, $\underline{\rho}^{-}C^{0}E^{R}$ (III) and $\underline{\rho}^{-}C^{R}E^{0}$ (IV) are formed without $\underline{\rho}^{-}C^{R}E^{R}$ (II) as an obligatory intermediate (i.e., reactions 2a, 2b, 3a, and 3b occur separately); hence, III and IV can retain a larger part of the genome and of the parental nucleotide sequences. We also show wild type sequences as being lost rather than modified in the various $\underline{\rho}^{-}$ mutants. With models such as this it is important to emphasize that what is represented in a schematic form is the eventual outcome – in terms of final, stable genotypes, or at least of genomes present in a primary mutagenized clone – and not necessarily the pathway they must traverse during mutagenesis itself, which may involve multiple steps, including ones involving possible repair and recombinational events.

Another approach is to study specifically, by means of hybridization tests, which wild type sequences, if any, are actually retained in mutant mtDNA. This can be done either by DNA-DNA[162] or by RNA-DNA hybridization[163-166] experiments. Some of the latter[165-167a] have the advantage of using actual mitochondrial gene products, namely, the various stable RNA species (Section C.1.). However, all of them are of necessity restricted to a limited portion of the genome. The tentative conclusions to be drawn from such experiments are that: (a) various mutant DNA's may retain some, but not all, of the wild type sequences; (b) the population of conserved sequences is different in different mutant lines; (c) modification of existing or generation of novel sequences of appreciable length not found in the wild type may occur but probably is not of great quantitiative significance; and (d) at least some of the sequences that are retained must be amplified relative to the wild type (i.e., present in

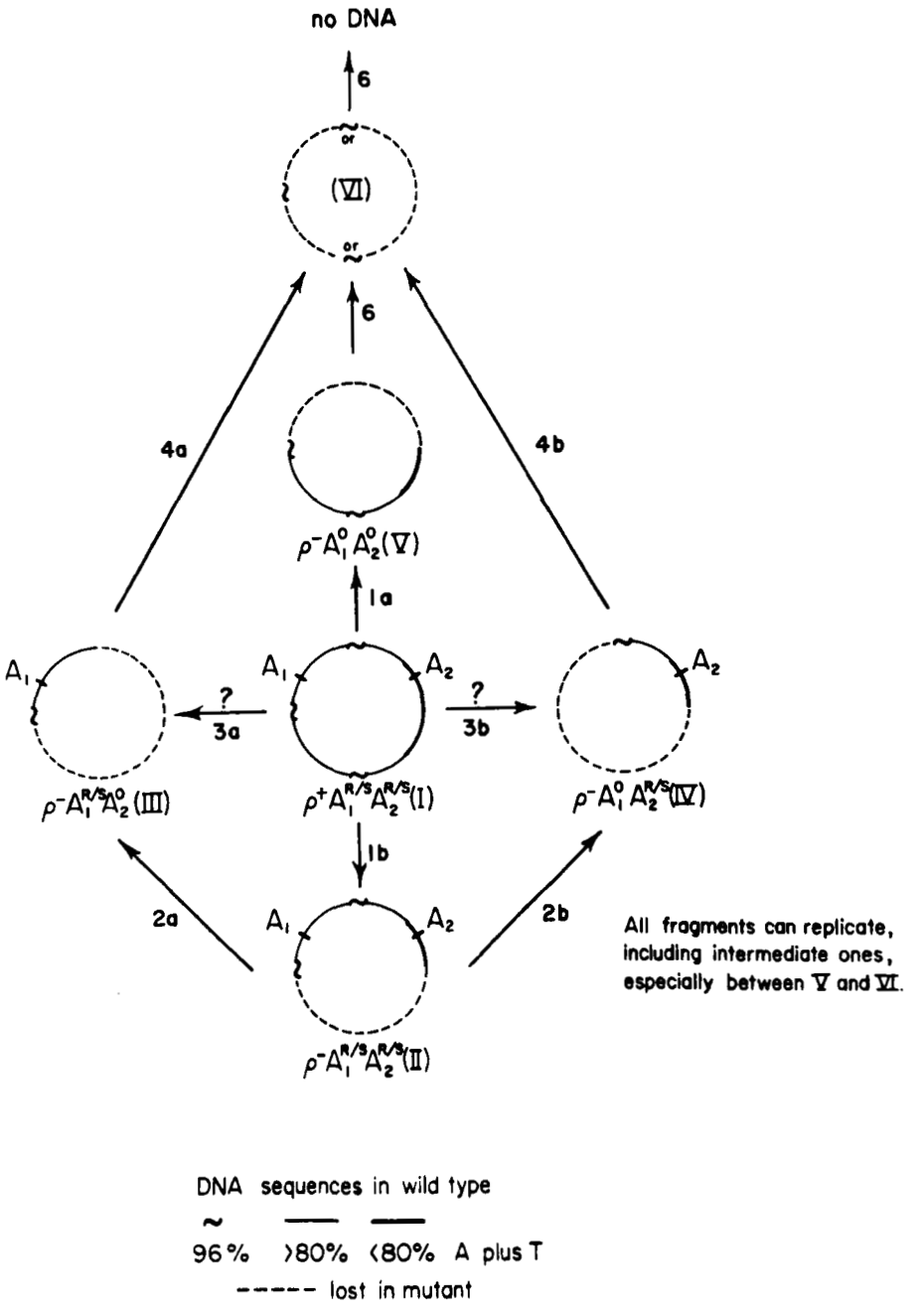

FIGURE 3. Possible molecular events during mutagenesis to ρ^- by ethidium bromide and similar mutagens – The wild type mitochondrial genome (I) is depicted for convenience as being circular and carrying two mitochondrial alleles, A_1 and A_2, as well as (noncoding, spacer) regions very high in adenine and thymine. The initial event is an incision involving both strands followed by degradative events in the order shown. Removal of the mutagen permits resumption of DNA synthesis, utilizing the population of residual sequences still present at that moment in any given cell.

multiple copies in the mutant). This last inference follows from the other three observations together with the fact that those $\underline{\rho}^-$ mutants that do contain mtDNA apparently do so in normal amounts. It is also confirmed by various experiments that show a slower rate of conversion of $\underline{\rho}^- A^R$ to $\underline{\rho}^- A^0$ by Etd Br, relative to $\underline{\rho}^+ A^R \rightarrow \underline{\rho}^- A^0$, the analogous process in the wild type.[155,156] Similar results have been interpreted by Nagley et al.[168] in terms of the presence of multiple equivalent copies of these markers. Since mutant mtDNA's have been reported to be smaller in size than wild type,[150,159,160] the possibility must be entertained that the postulated amplified sequences might not be only intra- but also intermolecular in their localization. If this supposition is correct, they may be arranged not only as tandem repeats[169] on any one molecule of mtDNA, but may also be present as separate and separable molecules, analogous to plasmids in bacteria, or to the amplified rDNA in oocytes of amphibia. Gene amplification can also be tested for – and has been so demonstrated – directly by quantitative studies of the kinetics of renaturation of mutant mtDNA.

The assumption that amplified wild type sequences are present in mutant DNA's still appears to be valid even in the most extreme cases, the low density petites, ones containing an unusually high proportion of (dA + dT) in their mtDNA (Table 2). Earlier studies in Bernardi's[46,158] and our laboratories[44] had suggested that the mtDNA of such cells consisted in large part of dA and dT tracts arranged in both alternating and nonalternating sequences. A superficially similar mutant (RD-1A) has been isolated and studied extensively in Borst's laboratory.[160-161b] Like that of others of its kind, its mtDNA shows a buoyant density in CsCl 13 mg lower than the wild type (1.671 g/ml vs. 1.684 g/ml), and its base composition is complementary and consists of 95% A + T and 5% G + C. Its strands can be separated in alkaline CsCl; the molecular weight of these separated strands is heterodisperse with a mean of $\sim 1.7 \times 10^6$ (making the extrapolated molecular weight of the duplex as isolated $\sim 3.5 \times 10^6$). Quantitative renaturation studies show the molecule to be (a) complementary to $\geqslant$0.3% of the wild type DNA, (b) not to contain any self-complementary sequences of appreciable length, and (c) to be composed of a perfectly repeating sequence of 300 or fewer nucleotides. These data, the results of pyrimidine tract analysis by van Kreijl et al.,[161a] and more recent studies by Sanders et al.[161b] suggest that this mutant DNA represents a reasonably faithful copy, containing some replication errors (~4% mismatching) of a wild type sequence, found there as a single or a very small number (<3) of copies, but repeated some 300 times in the mutant. Similar types of conserved, amplified wild type sequences, but, as anticipated, completely different in their primary structure,[167] are also present in two other low density mutants, DM1 and D310-2A-184 (Table 2). The former has been shown by Carnevali et al.[161c] to have retained in one of its sequences the wild type information for a $tRNA^{Ser}$

b. Mechanism of Mutagenesis to ρ^-

1. Types of Mutagens

A large number of different treatments and chemicals have been shown to be capable of bringing about the mutation from $\underline{\rho}^+$ to $\underline{\rho}^-$. These have been reviewed and tabulated by Nagai et al.[110] and by Sager.[2] The agents of greatest interest are ones that can induce the mutation without, at the same time, affecting either the nuclear chromosome or the viability of the treated cell. Certain polynuclear aromatic heterocyclic dyes are the most effective; among them the diaminoacridinium and phenanthridinium derivatives exemplified by euflavine (2,8-diamino-10-methylacridininium chloride) and ethidium bromide [(Etd Br), 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide], respectively, stand out. The parameters governing mutagenesis by and the structural requirements in these two series (in the concentration range ~10 μM) have been examined recently in the author's laboratory.[171] They may be summarized as follows:

a. For acridines: (1) Quaternization of the ring nitrogen (N-10) is essential; alkyl substitution of the two primary amino groups (at C-2 and C-8) is deleterious; (2) continued exposure of dividing cells is required for all diaminoacridine (alkyl proflavine) derivatives (C_1 through C_5) except for the allyl derivative; this compound is effective in the absence of growth but does require an energy supply.

b. For phenanthridines: (1) Quaternization of the phenanthridine N (N-6) by Met or Et is again essential, and alkyl substitution of the primary amino groups at C-3 and C-8 undesirable;

(2) aromatic substituents at C-6 greatly enhance activity even over bulky alkyl derivatives; (3) effective mutagens are active on either growing or nongrowing cells regardless of their current or prior carbon sources; nongrowing cells can be mutagenized rapidly in buffer even after extensive starvation.

In addition, the benzamidine derivative berenil [4,4′-(diazoamino)dibenzamidine diaceturate], a linear molecule incapable of intercalating into and hence deforming the double helix of DNA,[172] is also an effective mutagen,[173,174] reminiscent in its action to that of allyl proflavine. Therefore, intercalation is not a necessary prerequisite for chemical mutagenesis.

2. Sequence of Events

Because of its obvious advantages, especially its apparent simplicity and completeness of action, Etd Br has become the preferred mutagen in recent studies. These have included measurements of the kinetics and consequences at both the genetic and molecular levels, as well as the discovery of a variety of hitherto unexpected complexities referable to effects of the cellular and especially the mitochondrial environment on the sequence of events culminating in mutagenesis.

a. Genetic manifestations – The original findings by Slonimski, Perrodin, and Croft[109] that Etd Br appeared to be a highly effective mutagen regardless of the physiological state of the cell, active even in buffer in the absence of growth, has been confirmed and extended.[96,104,123,149,150,157,159,175,176] If we consider the retention of discrete mitochondrial genetic markers after treatment with Etd Br, three types of stable ρ^- clones can be postulated: (a) those that have lost the genetic information for all markers tested, (b) those that have retained that information, and (c) those that have retained the information selectively for one (or more) but have lost it for others. As described in Section B.3.2., this expectation has been fulfilled for a strain carrying two different antibiotic resistance markers. Kinetically, the proportion of clones of type (a) – and perhaps (c) – is increased, while that of type (b) declined as a function of increasing dose. In these studies, as well as in those of Nagley and Linnane[153] who measured suppressiveness and erythromycin resistance – and in unpublished studies by Perlman[167] on two antibiotic resistance genes in a different strain – it was also demonstrated that primary clones, i.e., colonies obtained from individual mutagenized cells, are nonidentical and heterogeneous. That is to say, these clones (after ⩾20 generations of growth) consist of a mixture of cells – all of them originating from the primary mutagenized cell or its immediate descendants – that vary in their ability to transmit a given genetic marker in a cross with a wild type tester strain. Strains exhibiting a stable genotype could only be obtained on subcloning these individual cells and their descendants.

b. Molecular effects – In the cell, Etd Br at mutagenic concentrations appears to react preferentially with mitochondrial components leading to extensive, frequently virtually quantitative, inhibition of the replication[96,149,151] and transcription[177-180] of mtDNA. However, it has little or no effect on other cellular processes, including cellular and mitochondrial proliferation, so long as an adequate energy supply is available. These effects are observed not only with yeast,[96,149,175,176,180-183] whether mutagenizable to respiratory deficiency (petite positive) or not (petite negative),[184-187] but also with a variety of other systems, including animal cells growing in culture.[179,188-190] For the purposes of this discussion, the block on DNA replication[191] is particularly pertinent since it alone already provides for the eventual generation of cells lacking in mtDNA due to the complete dilution of all such preexisting material as a function of division. However, strictly speaking, this process alone could only result in DNA^0 (ρ^0) petites. The generation of "senseful" ρ^- strains is explained by a second process, also initiated by exposure to Etd Br. This is a degradation of parental mtDNA in growing cells leading to populations of molecules decreasing in size as a function of exposure.[149,150] Again, if permitted to go to completion, the eventual outcome of growth in the presence of the mutagen is the complete disappearance of mtDNA in all mitochondria of all cells. However, upon removal of the mutagen, DNA synthesis again commences, and some of the surviving mtDNA fragments in a fraction of the cells affected (the magnitude of both these parameters being dose dependent) can serve as templates for the production of progeny mutant DNA. After a relatively short time, ⩾one cell generation, the rate of this synthesis

approaches those found in the wild type and results in wild type levels of mtDNA, corresponding to approximately 10^{-14} g (100 molecules of mol wt $\cong 5 \times 10^7$). If the degradation of mtDNA induced by Etd Br has any stochastic or random attributes, one would expect considerable heterogeneity, not only in the population, but even at the cellular level. Any one cell still containing mtDNA would be expected to possess fragments differing in length, and since they probably originate in different parts of the genome (Figure 3), they would differ in base sequence as well. This heterogeneity of DNA templates accounts for the heterogeneity of primary clones observed for retention of markers, for mtDNA, and for mtRNA as discussed earlier. Similar effects are also observed in mass cultures of mutagenized cells, and this heterogeneity is retained for many generations, with the heterogeneous mtDNA biased toward lighter molecules.[96] What is not yet clear – and remains to be established by pedigree analysis of mutated cells and their descendants, analogous to that performed on zygotic clones – is whether the eventual "rectification" process responsible for the emergence of pure cell lines takes place only in the exposed cell and its first – or first few – buds, or is a process requiring a number of cell generations for its completion.

c. **Primary vs. secondary events during mutagenesis (mutation fixation or consolidation)** – As in other mutagenic regimes,[192] the eventual expression of the initial exposure to a mutagen (here Etd Br or acridines) is under the control of and can be modified by intracellular – and intramitochondrial – events. The first indication of such potential modifications of the kinetics of mutagenesis came from studies on its temperature dependence, particularly a comparison of the effects of incubation at temperatures in the range of 35 to 40°C either during, or just subsequent to, the exposure to Etd Br.[193] Heating the mutagenized cells results, operationally, in an apparent *reversal* or *cure* of the initial mutagenic event. Mutagenesis must therefore involve at least two steps, the first susceptible to reversal at elevated temperatures. Since this first report, we, and others, have found a variety of treatments or agents that can affect mutagenesis by chemical mutagens. The treatments can be divided into three classes depending on their time of effectiveness relative to that of the mutagen: As already mentioned, we speak of cure or reversal if the second agent is found to be effective after the removal of all excess mutagen or after extensive washing by filtration or centrifugation; effects apparent on simultaneous exposure to the two agents we call *competition* or *antagonism* and, finally, we speak of *protection* if a prior treatment decreases the subsequent effectiveness of the mutagen. The current status of this type of investigation is summarized in Table 3, which also includes the results of a number of our as yet unpublished experiments. The results are quite consistent with a model that assigns a key role to the inner membrane in the mutagenic process, perhaps as an attachment site for mtDNA during its replication and repair. The membrane is implicated for the following reasons:

a. The effects on raising the temperature show a dependence[194-196] that indicates involvement of a macromolecule, but in a range that is not apposite for DNA denaturation.

b. Euflavine is antagonistic to Etd Br under conditions where it itself is completely ineffective as a mutagen.

c. Etd Br and acridines both bind avidly to lipoprotein membranes.[209-211] Similarly, chloramphenicol (CAP) may well exert its primary effect on mitochondrial membranes rather than on mitochondrial ribosomes.[22,346,349]

d. A lipophilic side chain enhances the mutagenic effectiveness of the phenanthridinium ring.[171]

e. Antimycin A, the most effective agent studied by us, appears capable of stoichiometric binding only to one site in yeast,[212] that is, to the appropriate binding site in the respiratory chain segment constituted by the cytochrome b-c_1 portion of the ubiquinone cytochrome c reductase.[213]

f. Nalidixic acid interferes with the growing point apparatus,[214] known to be localized on the membrane of bacterial cells;[215] agents known to disrupt membranes, such as dimethyl sulfoxide, are mutagenic.[216]

g. Mutagenic rates and efficiencies are exquisitely responsive to the change in metabolic state and energy supply: In part this fact might be referrable to the requirement for ATP in some of the degradative and repair processes[217-220] required for mutagenic fixation or its reversal;[175,221] and in part it might also reflect

TABLE 3

Modulation of Kinetics for $\rho^+ \rightarrow \rho^-$

Mutagen*	Treatment or state	Results** 1	2	3	Remarks	References
a, c	Heat (45°C)	+		+	Starved cells	75, 193
d	Heat (45°C)	–			Growing cells	116, 194–196
a, c	Euflavine (10 μM)	+	+	+	Starved cells	75, 171, 197
a, b	Antimycin A (<0.5 μg)	+	+	+	Starved cells, less efficient on growth	75
a	Chloramphenicol (CAP)	+		+	Strain dependent	77, 175, 197
a	Nalidixic acid		+		Extensive growth required	198, 199
a	Cycloheximide (<0.05 μg)		+		Extensive growth required	200
a	Cycloheximide (<1 μg)	+	0	0	Starved cells only	77
a	Prior exposure to C source	+			Growing vs. starved cells	77
a	Exposure to respiratory C source	+		+	Lactate > glycerol	77, 175
a	Shift to catabolite repression		+		Growing cells	201
d	Lack of intramitochondrial ATP	–				202
d	Mutant *gi* (nuclear)	–			Induction requires catabolite repression or anoxia	203
a	Mutant *ole-1* (nuclear), auxotroph for unsaturated fatty acids	–			Substitution of more "rigid" fatty acid	175
d	Mutant *1128* (nuclear), probably a membrane mutant	–			Induction by growth at low temperature (18°C) or by CAP at 28°C	204
d	CAP, erythromycin		–		Extensive growth (>15 generations) required	205, 206
a, b, c	Mutant *uvs* $\rho 5$ (nuclear), UV repair mutant (excision-deficient!)	+			Growing or starved cells	175, 207, 208
a, b, c, d	Mutant *uvs* $\rho 72$ (cytoplasmic), UV repair mutant (post-replication repair?)	–			Growing or starved cells; spontaneous on glucose	207

*Mutagens:

a	=	Etd Br
b	=	euflavine
c	=	berenil
d	=	spontaneous (i.e., no added mutagen)

**Results:

1	=	protection
2	=	competition
3	=	cure
+	=	a slower mutagenic rate
–	=	a faster mutagenic rate
0	=	no effect
Blank	=	an absence of information

different configurational states of the membrane.[213,222-224]

h. This postulate is strengthened by the behavior of certain cold sensitive mutants isolated by Weislogel and Butow:[204] These nuclear mutants are defective in what might well turn out to be a membrane protein, which in turn affects the interaction of the membrane with mtDNA. At low temperatures the membrane defect becomes amplified to a degree sufficient to render this interaction faulty and produce mutagenesis; at higher temperatures it becomes manifest only in the presence of CAP since the mutant protein results in a less efficient combining site for – and thus necessitates the continued intramitochondrial supply of – a polypeptide synthesized internally.

i. This part of the model is substantiated by two additional observations:

1. CAP or erythromycin also induces the ρ^- mutation of wild type cells in at least some strains,[205,206] but only in the presence of extensive growth on glucose; a possible interpretation is that induction requires the dilution below a critical level of some membrane component present in large excess in these strains, a component perhaps identical to the one the integration of which is modified in Butow's mutant.

2. Negrotti and Wilkie[203] have isolated a mutant (*gi*) that is readily converted to $\underline{\rho}^-$, but only under anaerobiosis or catabolite repression, conditions known to exert a substantial effect on membrane structure.

j. We have used a mutant (*ole-1*) blocked in its ability to convert saturated into unsaturated fatty acids,[225,226] essential constituents of the phospholipids of the inner membrane. When supplemented with a variety of such unsaturated acids, they can be incorporated intact into the membrane.[20,227-229] In general, mutagenesis is enhanced under conditions that lead to destabilization of the "normal" membrane structure, for instance by providing fatty acids with a higher melting point – and hence greater rigidity[228] – than the "normal" oleic acid.

Different lines of evidence suggest that one of the essential steps or sequences resulting in mutation is one concerned with mitochondrial repair processes. Two classes of mutants isolated by Moustacchi might be representative of blocks in the two types of dark repair processes for UV-induced damage in mtDNA[73,230] generally considered to be operative in prokaryotes:[192] excision repair[74,221] (which does not require extensive prior or coincident replication of DNA), and post-replication repair[217,230,231] (which does). As indicated in Table 3, *uvs ρ5*, a representative of the first class, is protected against mutagenesis by chemical mutagens; and this protection reflects itself in a considerable prolongation of the initial lag seen in all kinetic curves for this process. On the other hand, in *uvs ρ72*, a representative of the second class, this lag is virtually eliminated. Neither mutant exhibits a substantial effect on the final, extrapolated first order rate constant for the process. These observations are consistent with a model that ascribes the lag to the saturation of repair processes (rendered less efficient in *uvs ρ72*), which can affect and are themselves intimately concerned in mutagenesis. One of these processes, however, requires an initial, perhaps endonucleolytic attack, modified in *uvs ρ5*. But perhaps the strongest evidence comes from a consideration of the molecular events on the level of mtDNA known to be concomitants of mutagenesis by Etd Br and the effects thereon of some of the treatments just described.

3. Possible Mechanisms

Experiments in the author's laboratory[75,77] show that exposure of a cell population to Etd Br in the absence of growth leads to a modification in its mtDNA producing a reduction in its size, consistent with a more or less random, endonucleolytic cut of both strands (or two nicks in each strand in close juxtaposition.)* In buffer there are no further alterations over periods as long as 24 hr. However, when the mutagen is removed and cells are placed in a medium incapable of supporting growth but containing an energy source (buffer plus glucose), their mtDNA becomes rapidly degraded (<5 hr) to even smaller pieces; again, at least initially, largely by random endonucleolytic cuts, with exonucleolytic events becoming important at later times. These degradative processes are greatly accelerated in complete

*If the molecule is initially covalently circular, the event in question will require two cuts mechanistically, only the second of which is actually manifested in the experiments because of methodological limitations.

growth media.[96,149] Exposure to Etd Br under these conditions also produces a virtually complete inhibition of DNA synthesis.

As mentioned earlier, in vivo both these events – inhibition of formation of progeny strands, coincident with an Etd Br-induced degradation of parental ones – are completely specific for mtDNA and leave nDNA unaffected. The generation of mutant clones by random degradation coincident with the resumption of DNA synthesis using these collections of fragments as templates was already discussed in Section B.3.b.2. If this model is correct, we can account for many of the observations presented there and make several additional predictions:

a. Loss of mitochondrial DNA and of the information encoded therein should be a function of dose.

b. Since the postulated events are essentially random processes, so should be the position of the pieces of the genome – and to a certain extent also the distribution of the length – actually removed or retained even within a single cell of the mutagenized population. In fact, we know that primary ρ^- clones devised from a freshly mutagenized population are heterogeneous with respect to both their residual markers and their mtDNA base sequences.

c. Since the mtDNA molecules in a zygote are capable of reassortment by recombinational events even in homosexual crosses, we might postulate that nonidentical mtDNA molecules within the same cell are in or can be induced to assume a dynamic state and are hence capable of such genetic exchanges. If this is so, we should anticipate the possibility of reconstructing an intact complete genome from the random cleavage product by processes akin to the "multiplicity reactivation" and "marker rescue" known in phage genetics.[31,32,232,233]

This model might provide an explanation for the most puzzling feature of some of the phenomena described in the previous section: namely, that competition and cure do not appear to prevent the Etd Br-induced degradation of mtDNA. This finding has been documented under conditions of effective competition by antimycin,[77] nalidixic acid,[199] or cycloheximide,[200] and upon curing of the mutagenized population by the first named agent or by euflavine. It must be mentioned, however, that most of the experiments described were not performed at a sensitivity sufficient to rule out the actual retention of a small number ($\geqslant 1$) of intact parental genomes in the affected cells.

At least some of the mitochondrial enzymes postulated or implicated as participating in these various events have actually been isolated or described. Among them are the repair polymerase (DNA polymerase I),[79,191,234,235] an endonuclease capable of recognizing single-stranded or disordered regions[236] (which, however, has been shown to be of mitochondrial origin only in *Neurospora*), and an exonuclease activity activated by Etd Br or other intercalating dyes.[237]

c. Experiments on Other Organisms[2,15]

1. Respiratory deficiency in *Neurospora* – Already in 1962, Mitchell and Mitchell[238] had isolated from their stock cultures of this aerobic ascomycete a slow-growing mutant strain and found that this *poky* phenotype was maternally inherited, and hence, presumably carried by a cytoplasmic entity. The slow growth of this mutant was found to be referrable to a deficiency in respiration, specifically associated with an absence or modification of functional electron transport and of normal cytochrome *b* and cytochrome aa_3 during some part of the growth cycle.[239] In several respects then, *poky* is formally analogous, but by no means identical, to *petite*. Since then, more than ten different strains of this class of cytoplasmic mutants have been isolated in various laboratories. Many of them are in fact distinguishable from each other by details of their phenotypes and in some instances can be shown to be capable of complementation in heterokaryons: In such a common cytoplasm the separate defects are compensated by the product of the wild type gene present in the other partner. Three major groups can be distinguished.[239a] Among the now more intensively studied are the original *poky*,[239,239a] now also known as *minute-1* (*mi-1*), an example of Group I, *mi-3*, as the sole example of Group II,[239b] and *abn* (for abnormal)-*1* and *abn-2* for Group III.

2. Results of microinjection – Some of the properties of *Neurospora* render it particularly favorable for studies of cytoplasmic and mitochondrial inheritance. Among them is the discovery in Tatum's laboratory by J. F.

Wilson[240,241] that it is possible to generate artificial heterokaryons by the physical injection into a recipient cell of cytoplasm isolated from a donor. This finding was then further extended by Diacumakos et al.[242] to the injection of purified mitochondria of *abn-1* into wild type cells. As a consequence of this transfer, there occurred a gradual conversion of a fraction of the progeny cells obtained after repeated subcultures of conidia, first to a phenotype intermediate between that of wild type and *abn-1*, and later (after > 12 transfers) to that of *abn-1* itself.[242,243] Thus there is here an indication of the type of cytoplasmic replicative dominance seen in suppressive petites.

C. MITOCHONDRIAL GENE PRODUCTS

1. Mitochondrial RNA

a. Stable RNA Species (rRNA and tRNA)

1. **Nature and properties** – It is now generally agreed that mitochondria contain rRNA's and tRNA's that are different from the RNA species found in the cell sap (cytosol) of the same cell.[2,7,8,13,14,16,19,22] As concerns the mitochondrial rRNA's, these differences include base composition, nucleotide sequence, size, and a relatively low degree of secondary structure, resulting in unusual values for such parameters as sedimentation coefficients and electrophoretic mobility in acrylamide (± agarose) gels.[16,244,249,250,253] In addition, these RNA species appear to have been subjected to a variety of evolutionary changes and thus exhibit more species-specific variation and less apparent conservation (even between related species[245]) than do their bacterial[246] or even their cell sap counterparts.[247,248] To what extent this lack of conservation is in fact due to losses (or gains) in base sequences remains to be established since extensive and critical studies on the extent of interspecies homologies as a function of evolutionary divergence between say purified mt rRNA from ascomycetes and animal DNA and vice versa have not as yet been performed. As in *Escherichia coli*, the base sequence fingerprints of the small and large rRNA of mitochondria do not appear identical, and so, the latter has probably not arisen by duplication of the former. Direct DNA-DNA hybridization experiments, while showing considerable homology between the mtDNA's of various animals[16,256] (e.g., between chick and amphibians or the worm *Urechis caupo*), indicate complete absence of such homology between *Xenopus* and yeast. In more recent experiments, using RNA transcribed in vitro ("complementary RNA" or "cRNA"), rather than in vivo and hence not necessarily restricted to or even predominantly rRNA species, Dawid and Horak[258] found the following degrees of cross-hybridization:* *Xenopus laevis* – *X. laevis* (2 shipments) = 1.05 (control); *Rana pipiens* (from Vermont) – *R. pipiens* (from Wisconsin) = 0.98 (control); *X. laevis* – *X. mulleri* = 0.1; *X. laevis* – *R. pipiens* = 0.02; *X. laevis* – *Gallus domesticus* (chick) = 0.02; *X. laevis* – *Urechis caupo* = 0.003; *R. pipiens* (Vermont) – *R. pipiens* (Arizona) = 0.13. These values indicated extensive nucleotide sequence divergence among subspecies. The following observations were also reported: *Mus musculus* (mouse BALB/C) – *Cricetus cricetus* (hamster) = 0.1; *M. musculus* – *Homo sapiens* (placenta) = 0.03. Some of the properties of the more intensively studied rRNA's from ascomycetes, protozoa, and animals are summarized in Table 4.

The actual physical length of some of the rRNA species shown has also been determined directly by electron microscopy. In *Aspergillus nidulans* the two mtRNA components are 0.91 and 0.47 μm long, respectively, as compared to 1.10 and 0.52 μm for the ones of the cell sap.[259] In HeLa the length of the two mitochondrial components is 0.26 ± 0.07 and 0.26 ± 0.04 μm, respectively.[255,260] For comparison, the experimentally determined values for *E. coli* are 0.72 and 0.40 μm, respectively.[259] Molecular weights can then be calculated from these linear dimensions assuming spacings of the order of 2.45 Å between bases. It is evident that the molecular dimensions of the rRNA's of animal mitochondria are considerably smaller than those of fungi or protozoa. This observation holds for invertebrate as well as vertebrate species. For instance, the molecular weights for these two molecules in the thoracic muscle of *Locusta migratoria* have been estimated as 0.52 and 0.25×10^6 daltons by Kleinow et al.[261]

Both bacterial[246] and eukaryotic cell sap ribo-

*Obtained by averaging paired experiments of the type $mtDNA_2$ x $cRNA_1$ and $mtDNA_1$ x $cRNA_2$ (where $cRNA_1$ and $cRNA_2$ are the in vitro transcripts from DNA_1 and DNA_2, respectively).

TABLE 4

Properties of rRNA's from Eukaryotic Cells

Species	Reference	Mitochondrial: Base composition (% G + C)	Mitochondrial: Molecular weight (daltons x 10^6)	Mitochondrial: $s_{20,w}$ (S)	Cell sap: Base composition (% G + C)	Cell sap: $s_{20,w}$ (S)
Aspergillus	16, 249	30.5, 31.5	1.30, 0.70	23.5, 15.5	51	26.5, 17.0
Trichoderma viride	16, 250	31.5, 35.5		~22	50.0, 49.0	
Neurospora	16, 298	34.0, 36.5	1.28, 0.72	23 , 16	49.5 ± 0.5	26 , 17
Yeast						
(*Candida utilis*)	251	33		21 , 16	50	25 , 17
(*Saccharomyces*)	16, 48	25.0, 27.1	1.2 , 0.6	21.5[a], 14.5	47.6, 45.2	26 , 17
Tetrahymena pyriformis	16	27.9, 30.6	0.82, 0.52	21 , 14	43.2, 49.2	26 , 17
Euglena gracilis	252	29.8		21.4, 15.9	55.7	24.4, 20.1
Xenopus laevis (eggs)	253	40 , 43	0.53, 0.30	18.5, 13	63 , 53	
Man (HeLa)	254, 255	45	0.56, 0.36	16 , 12		28 , 18
E. coli[b]		54 , 54	1.04, 0.56	24.0, 16.0		

Note: If more than one value is given, the first corresponds to the rRNA of the large, the second to that of the small ribosomal subunit. Sedimentation coefficients usually determined relative to those of *E. coli* taken as 23.0 and 16.0S, respectively.

[a]Average of several different reports.
[b]And other bacteria, regardless of DNA base composition.

somes[262-264] also contain a third RNA species, integrated into the large ribosomal subunit: This molecule contains 120 bases[264,265] (40,000 mol wt) and under standard conditions exhibits a sedimentation coefficient of 5S and an electrophoretic mobility consistent with this parameter. No molecule with such a sedimentation coefficient or mobility has been found in mitochondria or their ribosomes from fungi (*Neurospora*[266] or yeast[19]) or from a wide variety of animals.[16,19,255] However, as mentioned earlier, the larger mt rRNA's, because of their structural features, frequently exhibit anomalous behavior upon sedimentation or electrophoresis. Thus, unless these measurements are performed under conditions where the molecules are completely unfolded (e.g., in the presence of formaldehyde or dimethyl sulfoxide), the presence of the mitochondrial species may well become masked under the tRNA peak. Furthermore, since mt rRNA's can be considerably smaller than their bacterial or cell sap counterparts (Table 4) without any apparent impairment of their characteristic activities, the possibility must be entertained that "5S RNA" may have undergone an analogous change. Finally, the suggestion has been advanced that the essential function in ribosome assembly usually exercised by this RNA might be performed by the large mt rRNA itself,[266] perhaps by incorporating some or all of the base sequence of 5S RNA in covalent linkage. Since bacterial and mammalian 5S RNA's do exhibit a certain amount of sequence homology,[264,265] and since the genes for 5S RNA can be isolated and purified,[262,267] it should be possible to investigate and distinguish between these various alternatives by means of appropriate hybridization experiments.

The nature and extent of methylation of the mitochondrial rRNA's have not been established with any certainty. Noll[268] claims that 5.7% of the phosphate in an alkali digest of phosphate-labeled mt rRNA from *Neurospora* can be accounted for as a 2′-O-Me dinucleotide. On the other hand, in HeLa cells Attardi et al.[254] find that in the presence of Me-labeled methionine only one Me group per 100 bases becomes labeled; this parameter for hamster kidney cells[269] is <0.5 – and may be zero.

Unique species of amino acid transfer RNA's (tRNA's) – some 25 molecules per rRNA in *Xenopus*[270] – also are found in mitochondria.[19] The basis for this claim rests on the characterization of these RNA's (and their comparison to those of the cell sap) by appropriate chromatographic and DNA-RNA hybridization techniques,

their distinct base composition,[253] and their ability to be charged selectively by mitochondrial, rather than cell sap, aminoacyl ligases (amino acid activating enzymes). In addition, Dubin and Friend[269] have recently claimed that the tRNA's – or at least the bulk of the low molecular weight ("4S") mtRNA's – a rather paucidisperse component in their preparation – of cultured hamster kidney cells might also be somewhat slower sedimenting and migrating and hence smaller (19,000 mol wt) than their cell sap or *E. coli* counterparts (mol wt. ≅ 27,500). If these findings can be substantiated for the mt tRNA's of other animal cells, the informational capabilities of their mtDNA will be higher than previously believed (see below). However, careful length and weight measurements of the mt "4S" RNA's in both *Xenopus* eggs[253,270] and HeLa cells[255,260] agree with the higher value. At least 14 discrete tRNA's (including the 2 species for methionine $tRNA_M^{Met}$ and $tRNA_F^{Met}$, see below) have been identified definitely in *Neurospora* based on their amino acid acceptance activity and ligase specificity;[272,273] the corresponding minimal number is 8 for yeast (Met, Ile, Gly, Ala, Phe, Tyr, Leu, and Val) and 5 for rat liver[274] (Leu, Tyr, Asp, Val, and Ser). In addition, the amino acylated molecules Leu-tRNA, Val-tRNA,[275] Ala-tRNA, Phe-tRNA, Ile-tRNA, and Gly-tRNA[166] from yeast, and Tyr-tRNA, Ser-tRNA, Leu-tRNA, and Phe-tRNA from rat liver[276] can be shown by hybridization tests to have been coded for only by mtDNA and not by nDNA.

One particularly important species of tRNA unique to mitochondria is the initiator tRNA (see Section C.3.a.3.) (fMet)-$tRNA_F^{Met}$. This molecule appears to be ubiquitous in the mitochondria of all species examined[13,14,277-280] and absent from their cytoplasm, as is the transformylase responsible[277] for its formation from Met-$tRNA_F^{Met}$.

2. Specification by and localization on the mitochondrial genome – Mitochondrial rRNA's appear to be exclusive gene products of mtDNA (no hybridization to nDNA); both members of the set (large and small) are present in the DNA as single copies. This now appears established[16,19] at least for *Neurospora*,[280,281] yeast,[282-284] *Xenopus*,[270] and HeLa cells.[255] For the two fungi this fraction of the DNA accounts for 2.4% of the total genome (4.8% of a single strand), while for the animal cells it corresponds to 6.5% of the total genome (13% of a single strand – 8% for the large and 5% for the small rRNA). Since the two kinds of mt rRNA saturate DNA with separate and additive plateaus, the same set of hybridization experiments has also been used to demonstrate the absence of sequence homology between them – in accord with some more direct studies discussed earlier. In the case of the animal mtDNA's which can be separated into their constituent single strands (Table 1), the rRNA genes appear to be localized exclusively on the pyrimidine-rich, heavy (H) strand.[48,255,270,283]

For tRNA's similar hybridization analyses[48] disclose the presence of ⩾20 4S (presumably tRNA) genes in yeast,[284] 15 in *Xenopus*,[270] and 12 in HeLa.[255] The values are based on hybridization plateaus of 0.9%, 3.5%, and 3.4% for these DNA's (with mol wt = 50, 11.7, and 9.6 x 10^6 daltons, respectively). Their localization has been demonstrated most convincingly by Attardi and collaborators[255,285] for HeLa, where nine of these were found on the H and three on the L strand. In fact, in a most elegant study, using the electron miscroscope for the visualization of hybrids with mtDNA, either directly with the large (rRNA) molecules, or with 4S RNA linked covalently to ferritin, Wu et al.[255] have been able to construct a physical map of the mitochondrial genome (Figure 4). As can be seen (Figure 4A), the map of the H strand (15,600 nucleotides) consists of – starting clockwise at 12 o'clock – (a) the gene for the light (12S) rRNA, 0.26±0.04 μm in length (corresponding to 1010 nucleotides); in this region there is also located a site (H2) for "4S" RNA (possibly the functional equivalent of the usual "5S" species missing in the ribosomes); (c) the gene for the heavy (16S) RNA, 0.46±0.07 μm long (1570 nucleotides); and (d) genes for seven additional "4S" RNA's, rather evenly spaced along the genome, except for two (H7 and H8), which are separated by only 120±30 nucleotides. The light (L) strand contains three sequences coding for "4S" RNA, separated from one another by 2280 and 3900 nucleotides, respectively. Their arrangement is indicated in Figure 4B. In rat liver, Nass and Buck had shown earlier that while Leu-$tRNA^{Leu}$ and Phe-$tRNA^{Phe}$ can be hybridized to the H strand, Tyr-$tRNA^{Tyr}$ and Ser-$tRNA^{Ser}$ are transcribed from the L strand.[176]

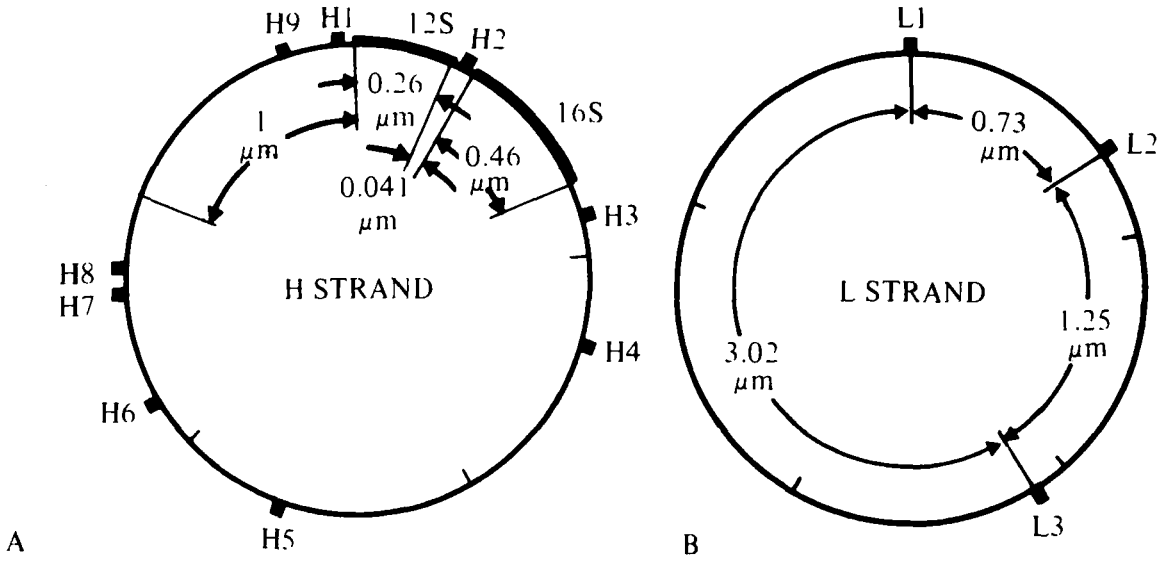

FIGURE 4. Circular map of the positions of the complementary sequences for 4S RNA's on the H and L strands of mitochondrial DNA. A. For the H strand, the positions relative to the 12S and 16S rRNA genes are shown as Hl through H9. B. For the L strand, there is no reference point on the circular molecule; only the relative positions are significant; they are shown as L1, L2, and L3. The short lines indicating the positions of the duplex regions corresponding to the 4S RNA-DNA hybrids are not drawn to scale. The total circumference of the mitochondrial DNA circle is 5.0 μm. (From Wu, M., Davidson, N., Attardi, G., and Aloni, Y., *J. Mol. Biol.*, 71, 88, 1972. With the compliments of Academic Press Inc. Limited, London, and the authors.)

3. **Precursor structures, maturation, and processing** – The rRNA's found in the ribosomes of the cell sap of eukaryotic cells are known to be specified by repetitive clusters of nuclear (specifically nucleolar) genes, which contain not only tandem sequences destined to become ultimately the large and small rRNA's ("28" plus "7S," and "18S," respectively), but also regions (spacers) between each tandem set that are never transcribed.[286-292] Furthermore, the initial transcriptional product consists of a large precursor structure (4.5 x 10^6 mol wt), larger than the sum of the final stable rRNA's combined, and differing from them in base composition and extent of methylation and other base modifications. The products arise from the precursor by a sequential series of carefully orchestrated events, including selected cleavages of the polynucleotide sequences, alterations of the constituent bases, association with various proteins,[291,298] and transport of the structures from the site of synthesis to that of utilization. Similarly, in bacteria precursor RNA's slightly larger in size than the two rRNA species (16 and 23S), and containing nucleotide sequences not conserved in the final product, have been described;[293,294] and evidence for their origin in a single transcriptional product[295-297] has been provided.

The question of the presence and nature of analogous precursor structure has been examined in *Neurospora* by Kuriyama and Luck[298] and in HeLa cells.[285,299,300] For highly purified mitochondria and their RNA, the first set of investigators identified by gel electrophoresis a minority species (32S) of mtRNA that became rapidly labeled on exposure to short pulses of radioactive uracil. The kinetics of flux of this label through this component, into two additional unstable components, and, finally, into the stable rRNA species was consistent with Figure 5, which also depicts additional physical and chemical properties of the various components disclosed by this study.

The transcriptional events in HeLa cell mitochondria have been studied in a series of elegant investigations by Aloni and Attardi. Their results may be summarized as follows:

i. Mitochondrial DNA is transcribed into two classes of products: The first consists of the various discrete, stable rRNA and tRNA species discussed earlier, the second of a heterogeneous

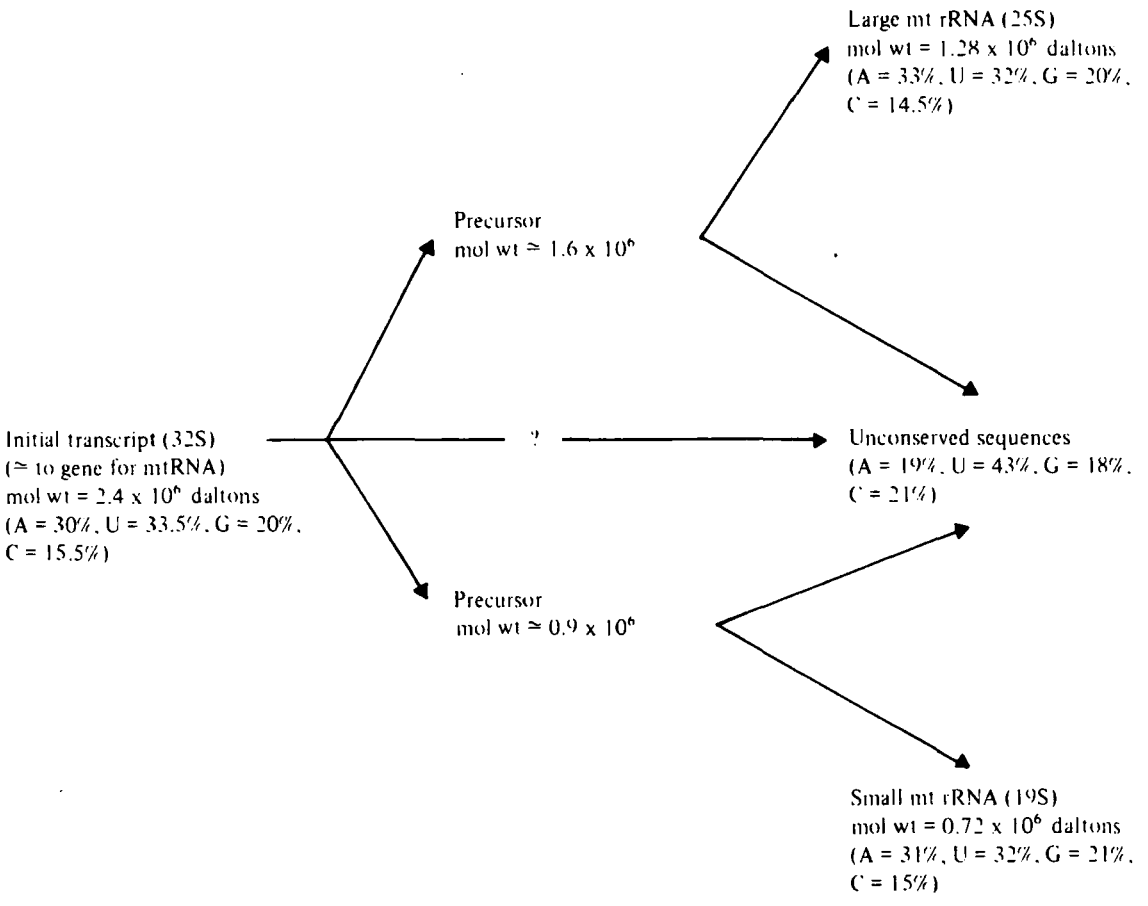

FIGURE 5. Biogenesis of mtRNA in *Neurospora crassa*. (Adapted from Kuriyama and Luck.[298])

mixture of components ranging in sedimentation coefficient from 4S to ⩾50S.

ii. Both circular and linear mtDNA molecules are transcribed, but the former appears to be subject to more intensive transcriptive activity; ⩾25 transcriptional events may be taking place simultaneously on the same template molecule.

iii. Transcription utilizes most if not all of the mtDNA molecule as a template, which is then transcribed virtually in its entirety. Furthermore, transcription is symmetric over a substantial part of, if not the entire, molecule and probably occurs simultaneously on both strands with approximately equal rates.

iv. The sequences for most of the stable rRNA species (both rRNA and most – 9 out of 12 – of the tRNA's) are encoded in and transcribed from the H strand; this transcriptive event initially produces an RNA molecule that is substantially of the same size as the entire mtDNA template.

v. The initial H strand transcript decays very rapidly ($t_{1/2} \leqslant 1$ min) in the presence of Etd Br plus actinomycin D to produce the various stable species in a coordinate fashion; among them, 4S RNA's can be shown to be derived from a precursor with a sedimentation coefficient ⩾12S.

vi. The L strand transcript is subject to even more rapid decay; some of it, however, provides the precursor of the (three) tRNA's encoded in this strand.

b. Mitochondrial Messenger RNA

1. Nature of the problem – The data presented in Section B.1.a. suggest that the informational capabilities of mtDNA are highly restricted. This is true particularly for the mitochondrial genomes of all animal cells, where the lowest value reported – for HeLa cells – does not exceed 9.6 x 10^6 daltons, equivalent to 5.00 μm or 15,600 nucleotide pairs. We also know from the studies of Attardi and collaborators sum-

marized in Sections C.1.a.2. and C.1.a.3. that about 0.86 μm or 2500 nucleotides are utilized in the specification of the two rRNA's and the spacer between them. One can also estimate that in addition a minimum of 720 nucleotides on the H strand (7 "4S" [tRNA?] species plus 1 spacer) are unavailable for productive transcription into a potential message. If we assume that base sequences complementary to stable RNA's do not and cannot function as mRNA's we not only have to add the three "4S" genes on the L strand (~250 nucleotides), but also must restrict our consideration to those sequences on either strand that do not already have a known coding function or are complementary to such a function. This consideration sets an upper limit of ~12,000 nucleotides or about 75% of the total genome on the aggregate size of all potential mt mRNA. However, the location and spacing of the various genes for 4S RNA on the H strand, as well as the demonstration of transcription of these genes, coordinate with those for the rRNA's – probably into a large transcriptional unit that might well encompass a complement to the entire H strand[285,299,300] – make it appear likely that genes coding for mRNA are even more elusive. Either they are transcribed from the H strand also and are conserved – at least occasionally – for use by integration into polysomes (see Section C.3.a.2.), while the remainder of the sequences is lost in the course of processing the large precursor structure(s) into rRNA's and tRNA's, or else they originate in the L strand and hence are complementary to these unconserved precursor sequences of the H strand. In either case mRNA sequences probably account for no more than 5000 to 6000 nucleotides and can code for a maximum of 2000 amino acids – or ten polypeptides of ~20,000 mol. wt. This is a very small – some might say insignificant – proportion not only of the total protein complement of the organelle devoted to its various metabolic functions, but even of that part of it assigned to its genetic subsistence (a minimum of 100 proteins – ribosomal proteins, amino acyl tRNA ligases, and initiation, elongation, and termination factors for translation alone – plus, conservatively, a dozen or more polypeptides concerned with replication, recombination, and repair of DNA, as well as with its transcription and the regulationo of these processes). This consideration dictates that the bulk of the genetic information for the construction and replication of the mitochondrion must reside in the nucleus in any event, and so the logical possibility can legitimately be raised that import of gene products into the mitochondria is not restricted just to proteins but might extend to mRNA's as well. In addition, Dawid[245,301] has performed very accurate hybridization experiments using the stable mtRNA's (labeled with ^{3}Me in vitro) and the mtDNA (mol wt = 11.7 x 10^6 daltons) of *Xenopus laevis* eggs. He found in addition to rRNA's and tRNA's – which account for 20% of the mtDNA sequence – only about 16% of sequences of unknown function in the steady state population of mtRNA. He has therefore explicitly suggested the possibility[245] that "coding for mitochondrial rRNA and 4S RNA is a major, and possibly the only, function of animal mRNA" and the corollary that "mitochondrial protein synthesis would then take place on messenger RNA's imported from the nucleus."

At first glance, because of the larger size of their mtDNA, the problem of mtDNA specifying the mt mRNA of protists, e.g., fungi and protozoa, appears less severe. However, the resultant greater informational content relative to animal mtDNA might be more apparent than real. In the first place, the stable RNA species (rRNA's and perhaps even tRNA's) are themselves larger, and what is more relevant, so are their precursor structures.[298] In the second, the number of tRNA's specified by mtDNA is almost certainly greater (Section C.1.a.2.). In the third, Bernardi and collaborators[302-305] have shown that as many as 20% of the sequences in yeast mtDNA have an unusually high (>90%) content in AT-rich sequences and might thus function as spacers or in some other regulatory capacity; and Sanders et al. have reached a similar conclusion from their analysis of certain mutants.[161b] Fourth, the proven capability of the mitochondria of protists, and especially of fungi[22] – in particular the ones of facultative anaerobes such as *Saccharomyces cerevisiae* – to respond rapidly to changes in environment leads to the inference that this DNA might well contain (more) regulatory sequences capable of responding to such stimuli. Finally, screening for the polypeptides – and their functional concomitants – synthesized in significant amounts by the mitochondrial translational system (see Sections C.2. and C.3.) discloses no major differences between ascomycetes and man. Thus, the question as to the origin of mt mRNA is valid

and pertinent for all species, and its answer will probably be equally applicable.

One more consideration, the question of the possible heterogeneity of mitochondrial DNA, needs to be raised and disposed of before we can address ourselves to the problem of mt mRNA itself. Given that the informational content of each individual molecule of mtDNA is small, we know that the number of such molecules per mitochondrion – and, particularly in animal cells, per cell[80,305] – may be very large indeed, varying from 100 or so in some strains of yeast to ~3000 molecules (1% of the total DNA or 3×10^{11} daltons) in some vertebrate animals. Now if the genetic continuity of the mitochondrial component is determined and controlled by its totality in the cell (the "Mitochondriome"[305a]), and if this population is itself heterogeneous, then we would have found the basis for an informational content that might be severalfold greater than that calculated from the size and properties of the individual molecule of mtDNA. This is a possibility that, in spite of the great experimental obstacle against its detection at the level say of a single heterogeneous region,[56] deserves some additional exploration. Such heterogeneity must exist at least temporarily in cells that contain a mixed mitochondrial complement, such as yeast zygotes or *Neurospora* heteroplasmons (formed by microinjection), derived from strains carrying different mt genetic markers or newly mutagenized yeast cells and primary clones derived from them (Section B.2.).

However, so far, the available evidence (summarized in Borst[19]), some based on a technique with electron microscopy that could have detected heterogeneity at a level of 100 nucleotide pairs, argues against specific large-scale heterogeneity of mtDNA at least in animals and protozoa.

2. Evidence against mitochondrial specification of its mRNA – This evidence is of two kinds: (a) an extension of the argument by Dawid[245,270] concerning the extent, nature, and function of various classes of base sequences in mtDNA of *Xenopus* oocytes, and (b) the demonstration by Swanson[306] that isolated mitochondria from this source are capable of the uptake of polyribonucleotides such as poly rU and their utilization as artificial mRNA's for their endogenous translational system. These latter experiments at best provide an indication that mitochondria can read certain highly artificial, imported messages. Furthermore, as shown by Hochberg et al.,[307] under the conditions used, mitochondrial integrity was severely compromised. We shall, therefore, not consider this evidence in detail but instead turn our attention to point (a).

The argument here is based on a comparison of the mtDNA isolated from the ovaries of animals belonging to the two species *Xenopus laevis* and *X. mulleri,* which can be crossed to yield viable (but sterile) progeny.[308] The mtDNA from both was found to be circular and indistinguishable at the level of buoyant density in CsCl or in thermal denaturation profiles, and therefore the two species were concluded to possess identical base compositions (41% G + C). The two sets of mt rRNA's in the two animals were also found to be very similar (~5% sequence divergence).

On the other hand, hybridization of the two mtDNA's either with each other or with cRNA transcribed from them in vitro showed an extensive and surprising absence of homology: Only 20% of the base sequences in the separated strands of the two DNA's reannealed to form a relatively closely matched heteroduplex (6% mismatched bases); these sequences could be identified as coding for the stable (rRNA and tRNA) RNA species since the latter occupy this fraction of the genome and since they also exhibit a capability for interspecies cross-hybridization. On the other hand, 50% of the mtDNA was capable of forming only a grossly mismatched heteroduplex (27% of bases not matched), while 30% of the sequences could not be matched up at all. Aside from the obvious implications of these findings for problems of mitochondrial evolution, mutations, and genomic stability, the inference drawn by Dawid is that

> those regions of mtDNA which are not transcribed into rRNA and 4S RNA and which evolve rapidly are spacers*. . . . An alternate hypothesis suggests that some or all of the sequences in mtDNA which do not code for rRNA or 4S RNA code for proteins whose nature has not been identified. It is possible that the sequences of such proteins are free to *evolve rapidly* (my italics) or that the changes in nucleotides are mostly changes in third letter of codons without much change in the sequence of

*Spacers as used here are nucleotide sequences not found in conserved transcripts and can be subdivided into two classes: (a) those that are never transcribed at all and (b) those that are lost during processing.

proteins . . . . By analogy with the work in T7 and T3 one would expect . . . [these] sequences to code for *non-essential* proteins (my italics) if they code for anything.

From what is known of mitochondrial translational products (Section C.2.b.), we would infer that these polypeptides are neither nonessential nor subject to rapid evolutionary change. Hence, Dawid's findings can be explained most readily either in terms of the postulated third letter change – which appears somewhat ad hoc – or in terms of import of mRNA.

3. Evidence for mitochondrial specification of its mRNA – The evidence here takes three forms: (a) the demonstration of the presence of significant amounts of minority species (non-4S, non-rRNA) of mtRNA capable of hybridization with mtDNA, (b) the isolation from mitochondria of an RNA fraction with some of the properties to be expected from a mRNA, and finally, (c) an analysis of the source of synthesis of functional (i.e., polysomal) mRNA.

The necessary condition in (a) has been met by a number of recent experiments, mostly in ascomycetes. For instance, Schäfer and Küntzel[281] demonstrated that mtRNA of high molecular weight, which can be isolated either from mitochondrial polysomes or from whole mitochondria, contains a (non-rRNA) species capable of hybridizing with approximately 10% of the mt genome of *Neurospora*; this would account for $\sim 5 \times 10^6$ daltons or $\sim 7.5 \times 10^4$ nucleotide pairs. Very similar results have been reported for *Saccharomyces cerevisiae* by Reijnders, Kleisen, Grivell, and Borst,[282] who also presented evidence that mitochondria do not contain any significant amounts ($\leqslant 0.07\%$) of stable transcripts imported from the nucleus.

As concerns (b), Perlman, Abelson, and Penman[309] have succeeded in labeling mitochondrial RNA of HeLa cells selectively in the presence of camptothecin, which suppresses the synthesis of nuclear RNA. The mtRNA – the synthesis of which was completely sensitive to Etd Br – contained, in addition to the previously identified rRNA (12S and 21S on the basis of its electrophoretic mobility, Table 4), a heterogeneous fraction (electrophoretic mobility corresponding to 15 to 30S). A considerable portion of this RNA could be shown to contain (by analogy, presumably on the 3′-OH end) a poly rA sequence 65±15 nucleotides long. This represents a unique structural feature exhibited by all eukaryotic messenger RNA's of nuclear origin[310-312] and suggests an analogous messenger function for this mitochondrial element. This inference is strengthened by the fact that polyadenylated RNA is present on mitochondrial ribonucleoprotein particles actively engaged in protein synthesis and is released from these structures by puromycin.

A related approach was used by Mahler and Dawidowicz[183] in their search for the origin of functional mt mRNA in *S. cerevisiae*. We used a temperature sensitive strain, originally isolated and characterized by Hartwell and collaborators.[313-315] Its cells are blocked in the production of all RNA (including mRNA) at the restrictive temperature of 36°C with the result that cell sap polysomes decay to monosomes and subunits with a halflife of 21 min attended by the cessation of protein synthesis on these structures. In our studies we were able to show that under conditions where nuclear RNA synthesis and polysomal functions in the cell sap were both blocked, there was no significant inhibition either of mitochondrial RNA or protein synthesis, nor any alteration of polysomal structure or function (polypeptide chain initiation or elongation). Conversely, all of these mitochondrial processes could be completely inhibited by Etd Br in the temperature sensitive strain at either the permissive temperature of 22°C – or in the parent, wild type strain at 36°C. Finally, when the temperature sensitive cells were first exposed to the elevated temperature for 60 min or longer in the presence or absence of Etd Br and then shifted down to 22°C in its presence, it was possible to demonstrate the resumption of nuclear DNA synthesis, its export to the cell sap, and the reconstruction there of functional polysomes active in protein synthesis. In contrast, no such events occurred in the mitochondria. It was therefore concluded that import of nuclear transcripts did not make a significant contribution to the population of mitochondrial messenger RNA's.

4. Critique and evaluations – The bulk of the evidence presented appears to show that mitochondrial DNA can and does code for probably most, if not all, of the polypeptide sequences translated by the mitochondrial translational system. This interpretation is strengthened by the identification of a number of mitochondrial genes that appear to specify proteins of the inner

mitochondrial membrane (Section C.2.a.1.). What remains to be accomplished, in even a single instance, is the unambiguous demonstration that one of the entities synthesized by the mitochondrial system of protein synthesis (Section C.3.) is identical to a specific polypeptide encoded in a mitochondrial gene.

c. Mitochondrial DNA-dependent RNA Polymerase (Transcriptase)

1. **Presence of unique polymerases** – Isolated, highly purified mitochondria from a variety of sources contain an enzyme capable of incorporating ribonucleotide precursors into RNA[8,13,14,19,316-318] and susceptible to significant regulation by physiological parameters.[177] In prokaryotes a great deal of useful information and much needed clarification concerning transcription and its' regulation have been derived from the observation that under appropriate conditions homologous polymerases isolated from these cells – sometimes in the presence of appropriate supplements – catalyze a reaction in vitro identical with, or analogous to, events in vivo.[319,320] The isolation, purification, and characterization of the mitochondrial enzyme(s) thus become of the greatest interest. In addition, isolation of a pure enzyme is also essential for all critical studies dealing with its possible relationships to other enzymes catalyzing the same reaction (i.e., from bacteria, from nuclei of the same or other prokaryotic cells, and mitochondrial enzymes from different species). Such studies form the basis for establishing possible evolutionary relationships and for the design of a rational strategy for selective inhibition of the various polymerases expected to be operative and active within the various organelles of a eukaryotic cell. In this context it may be useful to recall that:

a. The bacterial enzymes, most notably the one isolated from uninfected *E. coli*, are large molecules (mol wt = 5×10^5). The holoenzyme consists of four different subunits [composition: $(a_2\beta\beta')\sigma$]. It is characteristically inhibited by the antibiotic rifampicin (very closely related to rifamycin SV and sometimes also called rifampin) and a variety of other rifamycin and streptovaricin derivatives by virtue of direct binding to the β' subunit of the complex. This interaction results in a block of initiation, but not propagation, of polynucleotide chains. Antibiotics of the streptolydigin group appear to exhibit a related but not identical specificity and mode of action.

b. Nuclei of eukaryotic cells[320] (including yeast[321-324]) appear to contain a number of polymerases (frequently three or more). Among these, one (enzyme I or A) appears to be principally nucleolar in its localization and primarily concerned with the synthesis of the large precursors of rRNA. A second enzyme (II or B) is localized in the nucleoplasm in close association with the chromosomal material (chromatin) and concerned with the transcription of its DNA and, hence, is responsible for genetic expression; this enzyme, but not the nucleolar one, is inhibited by a small amount of the octapeptide α-amanitin, one of the poisonous principles of the toadstool *Amanita phalloides*. None of the three nuclear enzymes studied in a large number of organisms are inhibited by rifampicin itself, although more complex derivatives of rifamycin occasionally may function as inhibitors,[323,324] particularly at elevated concentrations.

c. In vivo, eukaryotic cells, either unicellular organisms, such as ascomycetes and other fungi, or cultured cells of metazoan animals, can be shown to be susceptible to selective inhibition of the various transcriptive systems: The (nucleolar) system leading to rRNA is inhibited by low concentrations (<1 μg/ml) of actinomycin D (not applicable to fungi);[325,326] the chromosomal (nuclear) system, by camptothecin[309] (application to fungi not yet tested); and the mitochondrial system, by low concentrations of Etd Br (≤ 1 μg/ml)[179,181,183,325,328] or by daunomycin (daunorubicin) (applicability to metazoan cell not yet tested). Although the phenomenon itself appears to be ubiquitous and to rest on a firm experimental basis, the molecular mechanism of the specificity of Etd Br for mitochondrial events remains obscure (see also Section B.3.b.). Its primary target is the DNA[105,329] and not the polymerase,[330] and a sufficient basis for a selective interaction with this template, applicable to mtDNA vs. nuclear DNA in the cells of all species examined, has yet to be revealed. The obvious explanation that nuclear, but not mtDNA, is usually associated strongly with certain proteins (histones and acidic proteins) and thus protected from the action of Etd Br[331,332] cannot be valid in this simple form; presumably, to allow RNA synthesis, the protecting proteins must themselves be removed, at

least just prior to or coincident with the attachment of the polymerase, thus rendering the DNA capable of interaction with Etd Br and resulting in an inhibition of transcription. A solution to this, as well as to many other riddles posed by mitochondrial transcription must await a study of this event in vitro with highly purified polymerases and with their homologous, intact mtDNA's as templates.

2. **Properties of the isolated enzymes** – The most highly purified and active enzymes have been isolated from the mitochondria of *Neurospora*[333] and of *Xenopus* eggs.[334] The two purified enzymes appear to be similar in activity (≤0.05 of that of the pure *E. coli* enzyme) and subunit structure (single polypeptides of 64,000 and 46,000 mol wt, respectively); both appear to aggregate readily to form oligomeric species even at low concentration in high salt; both active enzymes exhibit sedimentation coefficients of the smallest active species in high salt of ~6.3S in glycerol gradients – interpreted as dimers by Wu and Dawid;[334] for both, poly [d(A-T)] is the most effective template, while mtDNA is about half as active; and finally both are completely resistant to α-amanitin. However, significant differences also have been documented: While the *Neurospora* enzyme is sensitive to rifampicin (6 μg/ml), the animal enzyme is resistant to this inhibitor even at 100 μg/ml (it is sensitive to more complex derivatives, e.g., 3-formyl rifamycin SV *O-n*-octyloxime at concentrations ≥30 μg/ml); while the animal enzyme is active with denatured calf thymus DNA, the enzyme from *Neurospora* cannot utilize this template in either its native or denatured form.

In addition, Wu and Dawid have demonstrated that native, covalently circular DNA is the most effective mtDNA template, that the reaction is not stimulated by Mn^{2+} ions and is inhibited by high salt concentrations – and in these two respects differs from that catalyzed by either the bacterial or any of the three nuclear polymerases of *Xenopus* – and that, in any event, its chromatographic behavior on various columns is distinct from that of all the nuclear enzymes. This conclusion is in contrast to ones presented in a preliminary note by Horgen and Griffin,[335] who believe the (rifampicin sensitive) mitochondrial enzyme to be identical to one of the nuclear polymerases of the aquatic fungus *Blastocladiella emersonii*.

As concerns rifampicin sensitivity of other preparations, the situation is obscure. A relatively crude enzyme (200,000 mol wt) from mitochondria of *Saccharomyces cerevisiae* has been reported by Scragg[336] to be sensitive to rather high (≥38 μg/ml) concentrations of rifampicin, while other preparations purified from the same source, and exhibiting relatively high activity and a similar molecular weight, have been reported to be insensitive both to this inhibitor and to streptovaricin.[337] Intact, or permeabilized, mitochondria from this organism are insensitive to the inhibitor,[338] but so are ones from *Neurospora,*[339] which contains a sensitive enzyme. Permeabilized mammalian mitochondria,[340] their extracts,[341] or a partially purified enzyme[342] (from rat liver, with 65,000 mol wt) all have been reported to be inhibited by rifampicin (≥10 μg/ml). In contrast, a rather careful study of RNA synthesis on isolated rat liver mitochondria by Fukamachi, Bartoov, and Freeman[343] showed it to be inhibited 50% by 1 μg/ml of Actinomycin D, 1 μg/ml of acriflavine, 0.5 μg/ml of Etd Br, or 2 to 3 nmol/mg protein of atractyloside but to be completely resistant to rifampicin or streptovaricin up to 100 mg/ml.

3. **Biogenetic origin** – Wintersberger[338] and Tsai et al.[337] have reported on the isolation of an RNA polymerizing activity from mitochondria of cytoplasmic petite strains (ρ^-, see Section B.2.a. and B.3.a.) of *S. cerevisiae*. Since mitochondria of all such strains tested are completely incapable of supporting any intrinsic protein synthesis,[12,83,279,344,345] these experiments prove conclusively that the activity in question must have been synthesized on, and imported from, the ribosomes of the cell sap. By themselves, these experiments do not, however, permit any conclusion with regard to either a possible mitochondrial specification of the enzyme or the identity of the enzyme responsible for the activity in the wild type.

As concerns the first problem, more conclusive evidence was obtained in unpublished experiments by D. South in the author's laboratory: She demonstrated the presence of analogous activity in purified mitochondria of mutant strain 4D2l (Table 2), which contains a grossly aberrant mtDNA probably not capable of specifying senseful messages to any great extent. Thus, the relevant polymerase activity cannot have been encoded in mtDNA. A contrary opinion was expressed by Tsai et al.[337] and by Scragg[336] on

the basis of the absence of such an activity in certain ρ^- mutants not containing any mtDNA (DNA^0). However, the probable explanation of this particular phenomenon lies in the inability of these mitochondria either to retain the enzyme or, perhaps, to induce its biosynthesis. Their inner membranes are themselves aberrant and, since mtDNA is absent, might lack all reasonable points of attachment for the enzyme.[346,347] In agreement with the postulate that mtRNA polymerase is entirely of extramitochondrial origin, is the demonstration by Barath and Küntzel[349] that production of the enzyme is actually stimulated under conditions where mitochondrial transcription is inhibited by Etd Br – or mitochondrial translation by CAP.

2. Mitochondrial Polypeptides

a. Experimental Approaches

1. Genetic tests – The most direct and conclusive demonstration of mitochondrial specification for a polypeptide of the organelle would be provided by actual localization (mapping) of its structural gene in mtDNA. Eventually this will be accomplished by means of the now classical approaches of molecular genetics. This will involve the isolation of forward (nonsense and missense – the latter perhaps of the temperature sensitive variety) and backward (true and pseudorevertant) point mutants. These mutants then need to be characterized ideally at the level of both the sequence of polydeoxynucleotides in the gene and that of the amino acids in the polypeptide gene product. Less spectacular but equally useful would be the unambiguous demonstration of the absence of a well-defined polypeptide as a result of a deletion in a gene. It is fair to say that so far there is no indication that we are close to either of these goals. This lack of progress was due originally to a deficiency in appropriate mitochondrial mutants – not surprising, considering that the demonstration of the information-carrying capacity of mitochondrial DNA and even of its very existence is a relatively recent development (see Section B.1.). However, a variety of mitochondrial mutants are now available (see Section B.2.), but we know relatively little of the molecular basis of their phenotype. To recapitulate, the following classes of mutants have been described:

a. Respiration deficiency (ρ^-, petite) – This phenotype is grossly pleiotropic, with the nature of the necessary primary lesion as yet unknown. All petites so far examined,[11,12,344,345,349] even ones with mtDNA resembling the wild type in size, overall base composition, and other genetic information, are incapable of supporting intramitochondrial protein synthesis. For these two reasons, these mutants cannot be used for the identification of discrete mitochondrial genes.

b. Antibiotic resistance (ant^R, ant-r, or A^R, e.g., ERY^R, ery-r, or E^R for the phenotype "erythromycin resistance") – The discovery of mutants capable of growth on nonfermentable carbon sources in the presence of appropriate antibiotic inhibitors of mitochondrial protein synthesis (Section B.2.b.) provided the impetus for the development of the burgeoning field of mitochondrial genetics. The initial identification of a mitochondrial mutation to E^R was very quickly followed by that of a variety of mutants resistant to a whole spectrum of different antibiotics: chloramphenicol (C^R), spiramycin (S^R), paromomycin (P^R), mikamycin (M^R), etc. Many of these mutants exhibit cross-resistance to several antibiotics: For instance, among the first such mutants isolated, one was selected for its resistance to erythromycin but was quickly shown to be resistant to spiramycin, carbomycin, and lincomycin as well.[131,350,351] This phenotype (class *b-1*) is now known to be carried by an altered mt ribosome.[352] Since the mt ribosomal proteins are probably made on cell sap ribosomes, chances are that their mRNA has originated in the nucleus. Therefore, the site of the original mutation is probably on a gene for a mt rRNA (see preceding Section) rather than for a protein. Studies by Rifkin and Luck[353] indicate that the molecular basis of *poky* (*mi-1*) in *Neurospora crassa* (Section B.3.c.) might be due to a lesion in rRNA resulting in an altered, small mt ribosome.

A second class (*b-2*) of A^R mutants appears more promising. These are mutants in which cells exhibit extreme pleiotropy in their cross-resistance;[351] their isolated mitochondria (or even whole cells grown anaerobically) are, however, *sensitive* to these antibiotics, and some of them at least are also sensitive to respiratory inhibitors or uncouplers of oxidative phosphorylation (see next class). It has therefore been suggested by Linnane and collaborators[12,22,135,138] that the altered polypeptide resides in the mitochondrial (inner) membrane (see Section C.2.b.) rather than in the

ribosomes and is responsible for modifying the interaction of the former with the latter, including binding of appropriate inhibitors in vivo.

c. Resistance to respiratory inhibitors and uncouplers – The most common members of this class are mutants resistant to oligomycin, a classical inhibitor of coupled respiration and phosphorylation, a phenotype designated as O^R Recent studies[143,354,358] suggest that the component(s) responsible is a polypeptide of the inner membrane concerned with the attachment and integration of the mitochondrial ATPase (F_1) together with OSCP, a protein factor for oligomycin sensitivity, onto the appropriate membrane site. Similar considerations might also apply to mutants resistant to dicyclohexylcarbodiimide and triethyltin.

d. Repair of lesions in mtDNA induced by UV – At least one such mutant is known. It was originally isolated and characterized by Moustacchi[73] as exhibiting enhanced sensitivity to UV light, specifically in the induction in the petite (ρ^-) mutation, hence UV sensitive for ρ (*uvs* ρ). The mutant shows abnormal segregation on meiosis (Section B.2.b.2.) and is probably mitochondrial. Later collaborative studies in Moustacchi's and the author's laboratories[208] have shown that this strain appears to be deficient in a mitochondrial (post-replication) repair function and capable of interfering with the expression of a number of mutagenic treatments producing ρ^-.

All the mutants described above belonging to classes *b-2, c,* and *d* appear to be good candidates for use in the eventual identification of various mitochondrial polypeptides as true gene products of mtDNA. Of particular interest are some of the O^R mutants: Although their mitochondria contain an apparently "normal" oligomycin sensitive ATPase complex (and headpiece-stalk particles),[136] a more highly purified preparation obtainable from them consisting of membrane fragments plus soluble components (F_1 plus OSCP) retains the oligomycin resistance characteristics of the whole cell. Reconstitution experiments demonstrate that this property is present in mutant but absent in wild type membranes.[137]

e. Negative use of ρ^- mutants – The same properties that preclude the direct application of these mutants, in particular the ones lacking all, or all meaningful mtDNA, in the search for mitochondrial polypeptide gene products (see a., above) render them most useful for the converse task: the unambiguous identification of mitochondrial components as products of nuclear genes. As in the experiments to be described in the next section, these components can be recognized and identified on the basis of their size, their function – including enzymatic and optical properties – and by immunological cross-reactivity to mitochondrial components of known functions.

The conclusion based on experiments with such petite mutants is that they retain the capability of forming mitochondria that are topologically related to those found in the wild type[83,104,136,356,360] since they contain outer and inner membranes as well as the two fluid spaces. They also retain (Tables 5, 5a, and 6) the capability for synthesizing cytochrome *c*[101,103] and all the enzymes of the matrix,[104,359] the F_1 ATPase,[83a,104] and the bulk of the polypeptides usually found in the inner membrane.[2,83,101-104,359-361] They lack cytochromes *b*, c_1, and aa_3 in detectable levels, as well as components required for functional NADH:cytochrome *c* reductase, succinate:cytochrome *c* reductase, and cytochrome oxidase activity.[101-104] They are also deficient in a complete energy transducing system, which consists of the typical stalked particles normally studding the cristae and an oligomycin (rutamycin) sensitive and cold resistant, membrane bound ATPase complex.[83a,354-367] They do, however, contain many components intimately related to these activities, that is, NADH and succinate dehydrogenases[12,22,102,359] immunologically cross-reacting material to NADH:cytochrome *c* reductase[362] and cytochrome oxidase,[363,364] coenzyme Q (ubiquinone),[365] nonheme iron sulfur and copper bound to appropriate proteins,[103] and, as already mentioned, the F_1 ATPase itself.[83,104] Furthermore, the lack of cytochrome *b* may be referable at least in part to a loss of ability for its proper integration into the membrane rather than to a lack of its biosynthesis.[103]

As already mentioned, the ρ^- mutants examined, as long as they contain mtDNA, also retain the capabilities for DNA and RNA synthesis, the former in controlled and normal amounts. Some of these have also been shown[366] to retain the

TABLE 5

Distribution of Mitochondrial Activities Among the Four Compartments

Outer membrane	Intermembrane space	Inner membrane-matrix	
		Process	Marker
NADH:cytochrome b_5 reductase*	Adenylate kinase*	Citric acid cycle	Citrate synthase† (M)
Monoamine oxidase*	Nucleoside diphosphokinase		Fumarase, aconitase (M)
			Isocitrate dehydrogenase† (M)
			L-Malate dehydrogenase† (M)
			Succinate dehydrogenase (I)
Kynurenine hydroxylase			α-Keto acid dehydrogenase systems (M)
Fatty acid activating enzymes (thiokinase)		Accessory systems: Amino acid metabolism	L-glutamate dehydrogenase* (M)
			Aspartate transaminases (M)
		Fatty acid degradation	Fatty acid oxidation systems* (M)
			β-Hydroxybutyrate dehydrogenase (I)
		Heme synthesis	δ-Aminolevulinate synthetase (M)
			Ferrochelatase (I)
		Electron transport (respiratory chain)	NADH, succinate dehydrogenases and cytochrome *c* reductases (I) (inhibited by antimycin A)
			Cytochrome *c* oxidase (I)
			Fp;[a] CoQ;[b] NHI;[c] cytochrome *b*; cytochrome c_1; cytochrome *c*; cytochrome aa_3; Cu (all I)
		Energy transduction	ATPase (F_1; inhibited by oligomycin) (I)
			Oligomycin sensitivity conferring protein (I); attachment site (I)
			NADPH:NAD transhydrogenase (I)
			Transport system for cations and anions (I)
		Specific phospholipids	Cardiolipin (I)
			CTP:phosphatidic acid cytidyl transferase (M)

Abbreviations: I = inner membrane; M = matrix

*May be absent from mitochondria of *Saccharomyces cerevisiae*

†May also be present as a separate form – an iso(en)zyme – in intramitochondrial compartments

[a]Flavoprotein

[b]Coenzyme Q, ubiquinone

[c]Nonheme iron

TABLE 5a

Additional Inner Membrane-Matrix Functions

Process	Marker
Semiautonomous replication:	
DNA replication and repair	mtDNA, DNA polymerase I
RNA synthesis (transcription)	mtRNA's (see below)
	RNA polymerase
Protein synthesis	mt rRNA's and mt ribosomes
	mt tRNA's and amino acid ligases
	Transformylase and protein factors (E, F, T, and G)
	fMet

TABLE 6

The Origin of Mitochondrial Proteins in Ascomycetes

	Presence in (on)					
	ρ^- Mutants[a]			Exposure (short term) to		
	$mtDNA^+$	$mtDNA^-$	$mtDNA^0$	CAP	Etd Br	CH
Proteins of cytosol (e.g., L-glutamate dehydrogenase)	+	+	+	+	+	–
Matrix enzymes (e.g., L-malate dehydrogenase)	+	+	+	+	+	–
Outer membrane	+	+	+	+	+	–
Inner membrane	Apparently normal in amount and modified in structure			+	+	
NADH:cytochrome *c* reductase	CRM			+	±	–
Succinate, NADH dehydrogenase	+	+	+	+	+	–
Cytochrome oxidase		CRM	CRM	–	–	–
Cytochrome *b*	–	–	–	+[f]	+[f]	–
Cytochrome c_1	–	–	–	+	+	–
Cytochrome *c*	+	+	+	+	+	–
Cytochrome aa_3	–	–	–	–	–	–
F_1 ATPase	+	+		+	+	–
OSCF				+		–
mt protein synthesis	–[b]	–	–[c]	–[c,d]	–	–
RNA synthesis	+	+	–	+	–	probably +
DNA synthesis	+[e]	+	?	+[e]	–	+

Abbreviations: CAP = chloramphenicol, Etd Br = ethidium bromide, CH = cycloheximide, CRM = immunologically or functionally related proteins, + = apparently normal amounts, – = virtual or complete absence, blank = lack of information. Cytochrome oxidase refers to enzymatic activity, cytochrome aa_3 to an entity defined by its spectrum.

[a] $mtDNA^+$ designates mutants containing meaningful mtDNA, i.e., ones retaining mitochondrial markers. $mtDNA^-$ and $mtDNA^0$ designate mutants containing grossly aberrant (96% A + T) and very little ($\leq$ 4%) mtDNA. The second type is known to be and the first may be, devoid of substantial amounts of meaningful information.

[b] So far all ρ^- mutants examined have been devoid of all mitochondrial protein synthesis; however, recent studies have shown that some members of this class contain apparently normal mt r- and tRNA's. Not all of these mutants have yet been studied systematically with regard to protein synthesis.

[c] Retains ability for the synthesis of protein factors required for polypeptide chain elongation.

[d] All ribosomal proteins are synthesized.

[e] One or more components required for replication and/or repair appear to be of mitochondrial origin.

[f] Approximately 50% inhibition (cytochrome b_T).

TABLE 7

Inhibitors of Protein Synthesis

Acting on ribosomal systems of the 80S type	Acting on ribosomal systems of the 70S and 80S types	Acting on ribosomal systems of the 70S type	Acting on mitochondria
Anisomycin[a]	Actinobolin	Aminoglycosides	Aminoglycosides (?)[b]
Diphtheria toxin	Aurintricarboxylic acid	Chloramphenicol	*Chloramphenicol*
Emetine	Blasticidin S	Lincomycin	Lincomycin[c]
Endomycin	Bottromycin A_2	Macrolides	Macrolides
Glutarimide group:	Edeine	Carbomycin	Carbomycin[c]
Actiphenol	Fusidic acid	Erythromycin	Erythromycin[c]
Cycloheximide	Gougerotin	Spiramycin	Spiramycin
Streptimidone	Nucleocidin	Mikamycin	Mikamycin
Streptovitacin A	Poly-dextran-sulphate	(Vernamycin)	(Vernamycin)
Pederin	Puromycin	Siomycin	Siomycin
Phenomycin	Sparsomycin	Thiostrepton	*Thiostrepton*
Tenuazonic acid	Tetracycline group:	(Bryamycin®)	(Bryamycin®)
Tylophora alkaloids:[d]	Chlortetracycline		
Cryptopleurine	Deoxycycline		
Tylocrebrine	Oxytetracycline		
Tylophorine	Tetracycline		

[a]Most generally useful inhibitors are in italics.
[b]Incompletely studied; in yeast neomycin and paromomycin appear to be relatively effective and discriminating mitochondrial inhibitors in vitro – these are ineffective with animal mitochondria; other common members of this group (e.g., streptomycin, kanamycin) are generally ineffective.
[c]See text for restrictions.
[d]Have also been reported to be effective against yeast mitochondria.

factors required for polypeptide chain elongation.[419]

2. **Cycloheximide resistance** – One of the commonly used approaches to the identification of mitochondrial polypeptides has been the use of the inhibitor cycloheximide (CH). This glutarimide derivative selectively interferes with polypeptide synthesis on the ribosomes of the cell sap of eukaryotic cells (Table 7). It has therefore been employed in attempts at assessing the extent to which mitochondrial translation contributes to various mitochondrial structures and functions. For the former, the usual paradigm has been the study of the rate and extent of incorporation of radioactively tagged amino acids, frequently in double-label experiments. These experiments, when properly performed, have the virtue of an internal control: One class of entities continues to be made while a second is not, and they permit the identification of this special class of polypeptides against a background of all polypeptides. However, this experimental procedure suffers from two serious weaknesses, one conceptual and avoidable, the second inherent in this type of experiment in general: (1) Unless and until the entities specified by mitochondrial *genes* and those translated on mitochondrial *ribosomes* are shown to be identical (i.e., no import of nuclear mRNA; see Section C.1.b.), these experiments can tell us only about the latter and not the former. (2) There is every reason to believe a priori that in a functioning cell the two systems of gene expression must interact with one another, not just in a stoichiometric fashion by supplying products for each other's use, but also in a catalytic or regulatory manner as well; by furnishing to one another macromolecular and other entities that can act as effectors for a variety of processes, including various steps in gene expression itself. Some of these considerations will be dealt with in Section E.3.d.; however, for the present discussion it suffices to point out that the use of CH must of necessity uncouple all possible regulatory interplay and also might result in a failure to recognize components that themselves depend on a constant rate or level (pools) of other components supplied by the cell sap. In addition, CH is known to inhibit synthetic processes[368-370] other than protein synthesis in this and other compartments and to interfere with ion transport.[371] It is therefore evident that this paradigm

can at best give qualitative answers, but even the latter (e.g., the accumulation of a certain polypeptide in the mitochondria of a CH-poisoned cell) might still be difficult to interpret. Assume that formation of the final product involves processing (e.g., controlled proteolysis) by a product not available to the mitochondria of such cells: Evidently what is seen in the presence of CH, and characterized, is an intermediate, but not a product.

With these provisos we note that: (a) Accumulation of CH-insensitive products is restricted to the inner membrane of the organelle, where these products account for about 15% or less of its total protein; (b) among the polypeptides that can be dissociated and displayed by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate, it accounts for only a small fraction of all identifiable molecular weight classes ($\leqslant 10$ out of a number that may be tenfold greater) (Table 8); and (c) in general this accumulation is not accompanied by an increase in any identifiable catalytic function (Table 6) of the mitochondrion – in other words, all such functions also require a contribution by a CH sensitive function, presumably provided by one or more polypeptides formed on cell sap ribosomes.[2,12,19,22,359]

3. Sensitivity to mitochondrial inhibitors – Products tentatively identified by their insensitivity to CH as having been formed by the mitochondrial system have usually been subjected to an essential positive control experiment, their sensitivity to a typical inhibitor of mitochondrial protein synthesis such as chloramphenicol (CAP) or erythromycin (Table 7). Acriflavine and Etd Br are also frequently used in this context. In such experiments they are usually considered to act specifically as inhibitors of mitochondrial transcription, although they might possibly also interfere with translation in some more direct manner.[180]

The same compounds can also be used in the reciprocal experiment for the designation of mitochondrial entities and functions, the synthesis of which is subjected to immediate and direct inhibition in their presence. This use and the inherent pitfalls have been discussed by my collaborators and myself[187,359] and extensively reviewed by Kroon.[21,368] Some additional difficulties connected with possible effects on sizes of, and rate of transit through, pools of precursors have been pointed out by Groot et al.[361] and Schwab et al.[377]

The conclusions drawn from this approach are in agreement with ones cited earlier: Exposure to mitochondrial inhibitors does not by itself prevent cell division, nor does it interfere with the continued elaboration of the bulk of the mitochondrial components, but it does result in the gradual accumulation of increasingly faulty organelles, deficient in inner membrane structure and functions. Eventually they assume a configuration reminiscent of the petite phenotype. Obligately aerobic eukaryotic microorganisms, provided they contain inherent capabilities for an alternate, non-cytochrome-requiring pathway[239,239a] (which becomes induced or operative under these conditions), are then capable of sustained growth for many generations. Frequently normal mitochondrial structure and function can be restored reversibly upon removal of the inhibitor.[180,182] Cells of metazoan animals in culture appear to respond in an analogous manner.[188,189,389,390] Facultative anaerobes, in particular *Saccharomyces cerevisiae* and related species, are capable only of limited growth (~ 2 generations) when exposed to these inhibitors while growing on a nonfermentable (respiratory) carbon source.[181] On fermentable carbon sources such as glucose or galactose – the latter minimizing catabolite repression – growth can continue indefinitely, thus producing almost perfect petite phenocopies.[126,175,176] However, such growth is usually accompanied in due course by mutation to the p^- genotype itself; this occurs quantitatively within the first few generations in the case of Etd Br, while with euflavine (acriflavine), all buds become affected over a somewhat longer time scale. With CAP or erythromycin, continued growth for some 15 generations and very high drug concentrations are required, and the mutation is not observed in all strains.[205,206] These experiments have been interpreted in terms of the dilution by growth of an essential "replication factor" (for mtDNA), the concentration of which drops below a critical level only many generations after the cessation of its synthesis. Although this remains a tenable hypothesis, an analogous dilution of some critical inner membrane component,[204] perhaps involved in the interaction or attachment of mtDNA (Section B.3.b.), appears equally plausible.

In most cells, on short term exposure, the only

TABLE 8

Polypeptides Synthesized in Mitochondria

Organism	*Neurospora crassa*									*Saccharomyces cerevisiae*						*Saccharomyces carlsbergenesis*	Rat liver	Hamster liver		*Locusta migratoria*	
Reference	372			373		374	375–8			204	379	380, 381	381	354, 380, 382	383–4	385	385	386	386	387	387
System	c	b	a	a	a–x†	a	a	a–x††	a–y*	a	a	a	a–x†††	a–z**	a–x††††	b	b	b	a‡‡‡	a	a–x†††††
Class																					
	180	215	215‡																		
		88	(88)																		
			(78)																		
A1	58																57				
2	51										48						53				
3										44		45‡‡					45			45	
4										41					42	40					
5			(38)	38	38		~40			38											
B1						35.5	~35	32	32	34	33		33.8		34.5		35	33.5	34		
2	28		29	30	29							29		(29)			29	(31)	31.3		
3			25.5			27.7				27	(28)		25			26.5	26	28	28.2	27	(27)
4			23			(25)				24	23		23		23		23	22.5	22.5		
C1			20	20	19	(21)	~20					21		(22)		20	20			19	19
2		18	17.5			17.5		18													
3		14	15	14							15.8					15		14.5	14.5		
D1	11		(11)	10		11	~12			13	11	(12)		(12)					12		
2												7.8		7.5							

Abbreviations: Cyt = cytochrome; cyt ox = cytochrome oxidase

Systems: a = in vivo, cycloheximide-insensitive
b = in vitro, endogenous rRNA
c = in vitro, programmed with homologous mtDNA plus *E. coli* polymerase
x = isolated cyt ox
y = isolated cyt *b*
z = isolated ATPase complex

† Cyt ox bands at 38, 29, 19, 14, ⩾10, 10, <10.
†† Cyt ox bands at 36, 28, 18, 17, 13, 11, 8.
††† Cyt ox bands at 33.8 (removed by acetone), 25, 23, 13.4, 12.1, 10.5, 9.4
†††† Cyt ox bands at 42, 34.5, 23, 14, 12.5, 9.5,
††††† Cyt ox bands at 38, 24, 19, 14.5, 12.5, 10, 8.
* Cyt *b* bands at 32, 11, 10.
** ATPase bands at *58.5, 54.0, 38.5, 31.0,* 29.0, 22.0, 18.5, *12.0,* 7.5 (components of purified F_1 in

Minor bands in parentheses

‡ Five additional minor bands in this range.
‡‡ Dissociated into subunits of mol wt = 7.8 in acidic $CHCl_3$-MeOH.
‡‡‡ Emetine instead of cycloheximide.

components of the respiratory chain the elaboration of which is affected by the drugs are cytochrome oxidase, cytochrome aa_3, and a portion – but not all – of cytochrome *b* without, however, a concomitant drop in cytochrome *b* linked activities. In *Neurospora*, this phenomenon has been examined in detail by von Jagow and Klingenberg[391] and ascribed to a specific block on cytochrome b_T, the "high energy" form of cytochrome *b* required for energy transfer, without a severe effect on cytochrome b_K, which is principally concerned with electron transfer. Neither the solubilized F_1 ATPase complex nor the purified OSCP is affected by these treatments.

These studies have been extended to examine whether mitochondrial translation supplies the components required for mitochondrial nucleic acid and protein synthesis. The answer is straightforward and negative: Under steady state conditions, mitochondrial DNA and RNA continue to be synthesized at apparently normal rates and amounts,[326] as do the polypeptides required for mitochondrial protein synthesis, including all the proteins of the mitochondrial ribosomes.[392,392a]

4. Tagging by labeled formate – As mentioned in Section C.3., protein synthesis on mitochondrial ribosomes is distinguished by the presence of formylmethionyl residues in the N terminal position of nascent chains. Preliminary experiments by Polz and Kreil[393a] and by F. Feldman[393b] in the author's laboratory indicate that the mitochondria of honey bee thorax and yeast, respectively, appear to be relatively deficient in deformylase activities and therefore are capable of retaining the formyl residues even after integration of the newly synthesized polypeptides into the inner membrane. These observations render it likely that under carefully controlled conditions labeling with radioactive formate can be used as an alternate paradigm for the tagging, identification, and isolation of polypeptides of intramitochondrial origin. Since this approach does not require the use of inhibitors, it will prove useful for a study of products under physiological conditions with the retention of all regulatory interactions, and for investigating those processes themselves. The incorporation of formate into polypeptides and formylmethionyl puromycin has already been used by us to determine the upper limit of the intramitochondrial contribution to the synthesis of the proteins of the inner membrane:[279] In agreement with estimates by others,[2,12,14,19–21] it accounts for some 10% when the system is maximally active, and some 2% when it is turned down by catabolite repression.

5. Synthesis by isolated mitochondria – Although the paradigm used for the longest time, this approach has proven of the least utility. This negative assessment is conditioned by many factors, among them the inability of isolated particles to retain any synthetic capabilities dependent on coupling, a very low level of activity in general, and problems related to the purity and possible contamination by protein-synthesizing systems originating in the cell sap or from bacteria. It is also beset by a variety of other possible artifacts, including the tenacious binding of certain amino acids to membrane fragments.[307]

Earlier studies have been reviewed by Beattie;[18] in general, when interpretable, their conclusions confirm those derived from the other methods cited. One of the more recent and bold investigations is by Blossey and Küntzel[372] in which they programed mitochondrial ribosomes with a mRNA formed by the transcription of mtDNA by *E. coli* RNA polymerase. The products obtained (Table 8) bear little or no resemblance to those obtained by translating the endogenous message either in vitro, or in vivo in the presence of CH. It is not immediately obvious how to interpret these results or whether, in the absence of a large number of essential controls, they are capable of any rational interpretation at all.

b. Results

1. Functional and qualitative tests – The results of these investigations have already been anticipated and discussed in detail in previous sections. They are summarized in Tables 6 and 8.

2. Identification of specific products – In Table 8 we also report the results of several recent investigations that have attempted to identify specific polypeptide subunits synthesized by the mitochondrial system by the use of the approaches delineated in the previous section. The additional step that had to be taken was the isolation of relatively pure mitochondrial components of known function and the examination of the synthesis of their constituent polypeptides by the methods described. This approach culminated in the identification of such polypeptides in three highly purified mitochondrial enzyme complexes: cytochrome oxidase, particulate ATPase, and

cytochrome *b*.* Although these studies have thus far been crowned with success, their final evaluation must await the resolution of several apparent factual discrepancies also indicated in the table. In addition, the real function of these polypeptides remains completely unknown. All three complexes are originally tightly integrated into the inner membrane and as isolated contain several different polypeptide subunits; this composition has had to be established by the use of an exceedingly drastic technique leading to the complete denaturation and all loss of function of the protein. Those polypeptides and proteins that can be isolated in a form in which they retain testable functions – hemoprotein moieties of the cytochromes, F_1, and OSCP – all appear to have been synthesized on cell sap ribosomes. The polypeptides that *are* synthesized by the mitochondria are all highly hydrophobic, some of them to such an extent that they exhibit the solubility characteristics of proteolipids.[358,381,382,393-395] The latter, it will be recalled, were first identified and solubilized from myelin membranes by means of chloroform-methanol mixtures. The polypeptide classes (Table 8) made by the mitochondria appear to have been conserved throughout evolution, at least so far as their amounts, molecular weight distribution, and association with certain enzyme complexes are concerned. They must therefore fulfill a key function, one not necessarily related directly to the particular enzymatic activity with which they appear to be associated. As previously suggested by ourselves[359] and recently again pointed out by Borst,[19] these functions might be concerned with the interaction, integration, and regulation of the catalytic polypeptides and the inner membrane.** In the case of the ATPase, direct evidence is accumulating in support for this hypothesis.[143,354,380,382] Such a regulatory function of another component, that contained in cytochrome oxidase, also is strongly suggested by experiments of Aubert-Péré, who showed that this component can control the rate and extent of synthesis of isoenzymes of cytochrome *c*.[396]

3. **Quantitative aspects** – What fraction of the total polypeptides known, or suspected, to be synthesized by the mitochondria can actually be accounted for in terms of these identifiable components of its electron and energy transfer chain? Table 9 summarizes current estimates of the particle weights of the five relevant complexes. The data for the four respiratory complexes have been obtained from mitochondria from bovine heart muscle,[398] but it is not unreasonable to assume that their size in other mitochondria will not be grossly different: This is known to be so to a first approximation for complexes IV (cytochrome oxidase)[399,400] and V (particulate ATPase).[354] The number and kind of subunits synthesized by the mitochondria are based on the data of Table 8. We assume that the bulk of the cytochrome b_T and its mitochondrially synthesized subunit forms part of complex III and that it represents 50% of the total cytochrome *b* in this complex. The actual stoichiometry of the subunits in each complex, needed for the calculations in the last two columns of Table 9, is not known, but it is assumed to be as indicated. The results show that $\leqslant 0.41/2.2$ or some 18% of the total proteins in these five complexes is synthesized locally. Since they constitute roughly 50% of the inner membrane, local synthesis thus accounts for $\leqslant 9\%$ of its protein. This estimate is in good agreement with the results of estimates presented earlier based on: (a) fraction of incorporation of amino acids insensitive to CH but sensitive to CAP, (b) rate of formation of fMet-puromycin, and (c) fraction of total polypeptides formed by process (a).

3. The Protein Synthesizing System of Mitochondria

a. Components of the System

Implicit in the earlier discussion and spelled out in detail with regard to some specific parts and reactions is the now well authenticated fact that mitochondria of actively respiring cells contain a translational system[1,2,6-8,11-14,16,18-22] that shares the common attributes of other such systems regardless of their origins.[401-403] These attributes are inherent in, and conditioned by, the nature of the process with which they are concerned: the translation of a polynucleotide message inscribed in mRNA into a polypeptide

*Results of a recent elegant investigation by von Jagow, Weiss, and Klingenberg[239b] indicate that the mitochondrial lesion in the *mi-1 (poky)* mutants in *Neurospora*[353] results in specific deficiencies only of cytochromes aa_3 and b_T.

**This statement does not imply that they represent a distinct class of "structural proteins" accounting for a major fraction of all membrane proteins. In fact, recent studies have shown clearly that such proteins do not exist, and claims for their function and mutational alteration have been withdrawn.[397]

TABLE 9

Mitochondrial Specification of the Mitochondrial Respiratory Chain

Complex	Description	Molecular weight x 10^{-6}	% Synthesis[c] in mitochondria	Molecular weight x 10^{-6}
I	NADH-ubiquinone reductase	0.7[a]	0	
II	Succinate-ubiquinone reductase	0.2[a]	0	
III	Ubiquinone-cytochrome *c* reductase	0.23[a]	~15 (1)	0.03
IV	Cytochrome *c* oxidase	3 x 0.20[a]	~50 (3)	0.30
V	Membrane bound, oligomycin sensitive ATPase complex	0.47[b]	20 (2–3)	0.08
Totals		2.20		0.41

Sources: [a]Beef heart
[b]Yeast[354,380]
[c]See text. Number in parentheses refers to number of polypeptides (see also Table 8).

product. The translation machinery is centered on ribonucleoprotein particles (ribosomes) attached to the mRNA, uses aminoacyl tRNA's (themselves formed by the attachment of individual amino acids to the 3′ end of the RNA) as both the substrates and the translating device proper, and besides these components requires a number of enzymatic and other proteins, cosubstrates (ATP and GTP), and cofactors (Mg^+, K^+, thiol groups, etc.). The process catalyzed can be divided into three distinct phases: initiation, elongation, and termination, each utilizing a separate set of specific proteins in addition to the components intrinsic to the ribosome. However, the key reaction resulting in chain elongation by formation of the nth peptide bond – aa_n-tRNA + aa_1 – – – aa_{n-1}-$tRNA_{n-1}$ → aa_n-$tRNA_n$ + $tRNA_{n-1}$ – requires only the ribosome and is catalyzed by one of its proteins functioning as a peptide synthetase (peptidyl transferase). Mitochondrial protein synthesis has been studied and extensively reviewed recently and will therefore not be covered in detail here. However, several questions and problems, many of them conditioned by the localization of the system in close association with the inner membrane, require at least a passing discussion. This discussion will center on the properties of (a) the structures actually responsible for protein synthesis, (b) the steps and entities responsible for polypeptide chain initiation and elongation, (c) certain details concerned with its characterization by specific inhibitors, and finally, (d) the source and origin of the various required protein components.

1. The problem posed by close association of mitochondrial ribosomes with mitochondrial membranes – Mitochondrial ribosomes have now been isolated and characterized from all sources examined from protists to man (Table 10). In the cell, however, they are closely associated with and perhaps attached to the inner mitochondrial membrane, and therefore relatively drastic means are required for their release.[14,16,404,405] These observations, together with those of the behavior of certain mitochondrial mutants that exhibited antibiotic resistance in vivo, but sensitivity of their isolated mitochondria in vitro, prompted Linnane and collaborators to postulate an actual integration of the protein-synthesizing structure into the membrane.[12,135,351,406] If, as is implied in their statement, the former, in its native state, actually forms a part of the latter, then it might be expected that protein synthesis by mitochondria in vivo might exhibit features that differ qualitatively from those established for this process in bacteria, as well as in the cell sap, and even on membranes (rough endoplasmic reticulum), of eukaryotic organisms. These qualitative differences should be most striking at the level of the active ribonucleoprotein particle, leading perhaps to a complete absence of the classical poly(ribo)somal structures reported for all other systems. They might also be reflected in the interaction of the ribosome with the various soluble factors, espe-

TABLE 10

Properties of Mitochondrial and Bacterial Ribosomes*

Parameter	System		
	Bacteria	Ascomycete mitochondria	Animal mitochondria
	Sedimentation coefficient (S)		
Ribosome	70	70–74	50–60
Large subunit	50	50–58	33–45
Small subunit	30	35–40	25–35
Presence of 5S RNA in ribosome	Yes	No	No
Methylation of rRNA	Yes	?	Unsettled
Subunit exchange with bacterial ribosomes	Yes (various bacteria)	No	?
Effect of 120 m*M* NH Cl on protein synthesis	Maximal stimulation (*E. coli*)	Inhibition (90%)	?

*The data in this table are based on References 16, 19, 21, 366, and 404.

cially proteins, required for the partial reactions in protein synthesis, and in their response to characteristic inhibitors of this process.

2. **Existence of mitochondrial polysomes** – Particles exhibiting all the properties expected of polysomes have been isolated from mitochondria of *Saccharomyces cerevisiae* in our laboratory.[183, 279, 345] A typical preparation for strain A364A is shown in Figure 6. As can be seen, most of the RNA (A_{260}) is contained in structures larger than 75S (the monomeric ribosomes), with discrete peaks presumably corresponding to di- through hexamers, as well as additional larger structures. All these components carry nascent polypeptide chains since they can be labeled in vivo with short pulses of leucine or formate (as N terminal *N*-formylmethionine). In addition they exhibit the following properties: (a) This labeling pattern is blocked by prior exposure to CAP or Etd Br but not to CH; (b) exposure to ETd Br but not to CAP leads to dissociation to monomers and subunits; (c) exposure of particles carrying nascent chains to puromycin leads to dissociation with release of label as peptidyl puromycin derivatives; (d) exposure of such particles to pancreatic RNase at relatively high concentrations (> 5 μg/ml) leads to their dissociation to monomers and large subunits carrying nascent chains; and (e) they can be labeled by exposing cells to uracil under either pulse-labeling (5 min) or steady state (≥ 60 min) conditions; the tracing for radioactivity and A_{260} coincide but these profiles can be readily distinguished from those of cell sap ribosomes and polysomes by mixing mitochondrial particles labeled with one isotope (e.g., ^3H) with cell sap particles labeled with another (e.g., ^{14}C). Similar results have also been obtained with three other strains.

Analogous results have been reported by Ojala and Attardi,[408] demonstrating the presence of mitochondrial polysomes in HeLa cells. These particles account for 50% of the total ribosomes, exhibit sedimentation coefficients between 74 and 200S, centered at 120S (the monomer sediments at 64S), and correspond to a series from the dimer to the heptamer. These investigators have also provided evidence for certain unusual properties of the system that they interpret in terms of the particularly "sticky" (i.e., hydrophobic) nature of the nascent chains. These polypeptides then might interact not only with each other but also with the mitochondrial membrane, providing a second site of contact and attachment to the latter for the mitochondrial protein-synthesizing machinery. This hypothesis might provide an alternate explanation for the observations reported from Linnane's group, discussed in the previous section and more extensively by them in other publications.[22] Polysomes have also been isolated from *Euglena gracilis* by Avadhani and Buetow[409] and charac-

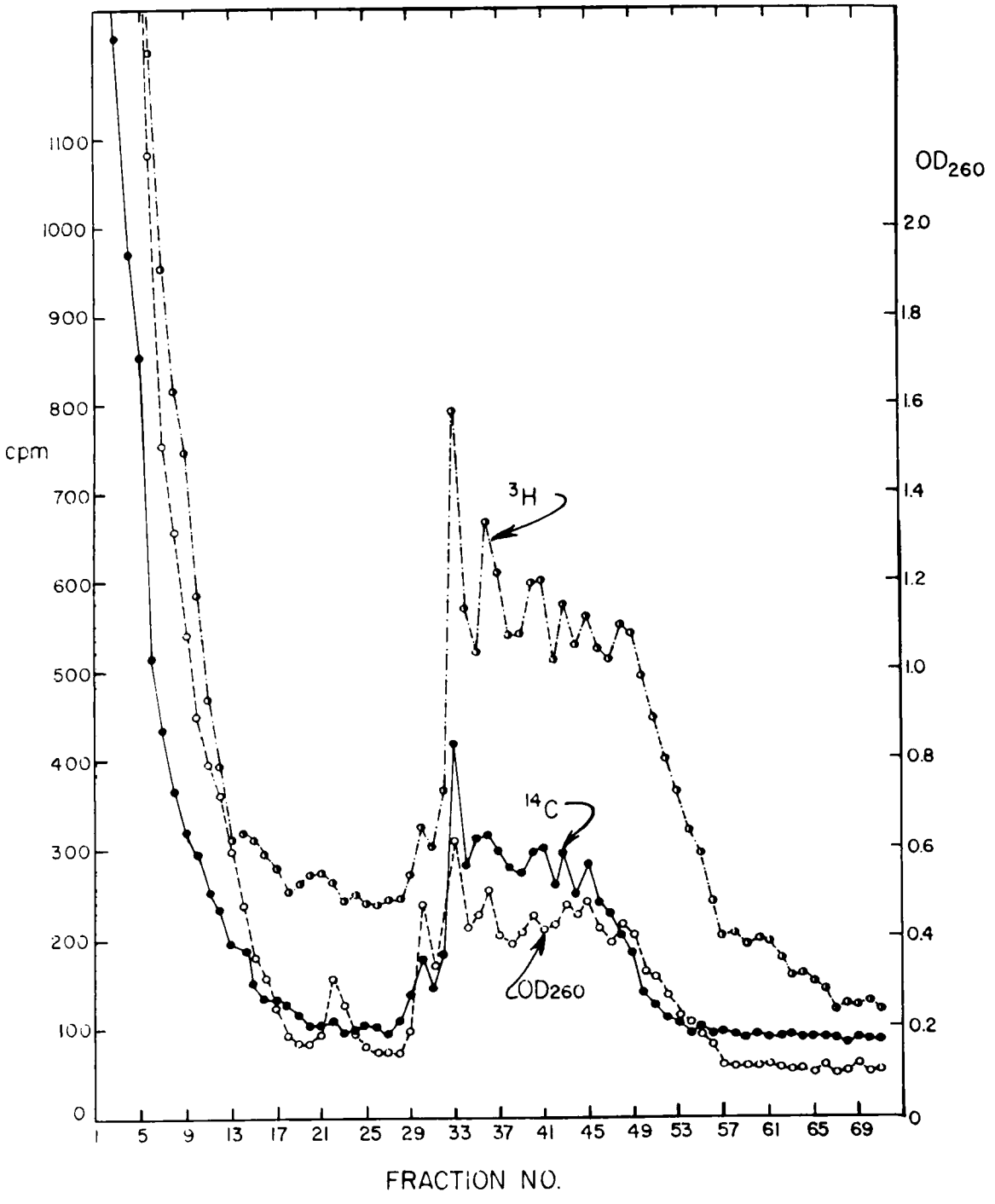

FIGURE 6. Mitochondrial polysomes in yeast – Spheroplasts obtained from exponential cultures of strain A364A growing on lactate were prepared and subjected to 5-min pulses of ^{14}C-formate and ^{3}H-leucine. Purified mitochondria were isolated from spheroplast lysates, their membranes solubilized in Triton X-100, and their contents analyzed in 5 to 30% sucrose gradients (sedimentation from left to right). Fractions were monitored for nucleic acids (OD_{260}) and for radioactivity incorporated in proteins. The incorporation of labeled precursors is prevented by prior exposure to chloramphenicol, ethidium bromide, or puromycin, but not to cycloheximide. Treatment of the isolated, labeled preparation with RNAse (10 μg/ml) leads to an accumulation of all three entities in fraction 35; and treatment with puromycin to a similar result for OD_{260}, but to a release of label to the top of the gradient. (From Dawidowicz.[345])

terized by their A_{260} profile and ability to support protein synthesis in vitro. Besides the monomer, with a sedimentation coefficient of 71S, at least six polymeric species could be distinguished in sucrose gradients.

3. Component reactions –

a. The ability of supernatant factors (usually elongation factors G and T) isolated from mitochondria of various species, the homologous cell sap, and from bacteria (*E. coli*) to support protein synthesis (usually poly Phe formation with poly rU as artificial mRNA) on ribosomes isolated from the same three sources has been investigated with ascomycetes[21,59] and *Xenopus laevis*.[410] The results are in reasonable agreement; protein factors (whether crude mixtures or purified elongation factors) of mitochondria and *E. coli* are mutually interchangeable, at least qualitatively, but cannot interact with cell sap ribosomes; conversely, elongation factors isolated from the cell sap are specific only for ribosomes from the same tissue fraction (regardless of species) and do not interact with ribosomes of mitochondria or *E. coli*. Similar results were obtained[411] also with a more complex system consisting of ribosomes from yeast mitochondria and elongation factors from this source or *E. coli*, programed with mRNA synthesized by *Neurospora* mtRNA polymerase transcribing the DNA from *E. coli* bacteriophage T3 or T7.

b. Initiation of polypeptide chains in mitochondria exhibits features reminiscent of those established for bacteria and distinct from those governing this process in the cell sap of eukaryotic organisms. These are [21,59,277,279,345,412] (i) the occurrence of a special, formylatable species of transfer RNA for Met ($tRNA^{Met}$) called $tRNA_F^{Met}$ or $tRNA^{fMet}$. This species may or may not be unique to mitochondria; (ii) the presence, exclusively in mitochondria, of a transformylase[217] capable of catalyzing the transfer of formyl residues from N^{10}-formyltetrahydrofolate to Met already linked to this tRNA, i.e., the reaction $f\text{-}FH_4 + Met\text{-}tRNA_F^{Met} = FH_4 + fMet\text{-}tRNA_F^{Met}$; and (iii) the requirement of mitochondrial ribosomes, their subunits, and initiation factors[412] for $fMet\text{-}tRNA_F^{Met}$ as the obligatory initiator of protein synthesis.

As a result of these considerations, labeled formate can be used as a specific tag for nascent polypeptide chains on mitochondrial ribosomes and polysomes and thus for these particles themselves. When supplemented with puromycin at ~ 1 m*M*, this compound can act as a quantitative acceptor for ribosome bound fMet, and thus the formation of fMet-puromycin from labeled formate added either to isolated mitochondria, or to whole cells, can be used as a rapid,convenient, and completely specific alternate assay for following the kinetics and assessing the significance of mitochondrial protein synthesis.[279,345]

4. Inhibitors and their specificity – Many recent reviews and articles have served to expand and qualify the initial postulate by Linnane and collaborators[125,413] that mitochondrial ribosomes were of the "prokaryotic, 70S type" and thus differed qualitatively in terms of possible inhibitors from those of the cell sap (from the same organism) which are of the "eukaryotic, 80S type." Information currently available[16,21,388,413,417] is summarized in Table 7. The following generalizations may be drawn:

a. The inhibitors of eukaryotic cell sap ribosomes (80S type), in particular the glutarimides, emetine and anisomycin, appear specific for this class and do not block mitochondrial or bacterial protein synthesis.

b. Similarly, the group of bacterial (70S type) inhibitors consisting of chloramphenicol, mikamycin (vernamycin), carbomycin, and spiramycin appear to be effective and specific, even in the cell, against mitochondria from all sources. Other macrolide antibiotics such as erythromycin and lincomycin, highly effective with bacteria, are potent inhibitors with certain cells and their mitochondria (e.g., yeast) but not with others (e.g., rat liver).

c. The mode of action of the inhibitors under b., although reasonably well established for bacterial systems – and centered on the large ribosomal subunit – is much more complex in mitochondria. Even where inhibition by, or binding of, these compounds can be demonstrated with isolated mitochondria in vitro, the inference that the mitochondrial ribosome or one of its proteins is the susceptible target is not always justified. Only in a few instances has a mitochondrial mutation to antibiotic resistance been shown to produce an altered ribosome, and even here the mutational change may be in the rRNA and not in a ribosomal protein (Section C.2.a.1.).

d. The inhibitor fusidic acid, which in bacteria is believed to act by fixing the usually

transient complex between 50S ribosome, elongation factor G and GDP,[418] is usually also effective with eukaryotic cell sap systems, including those of ascomycetes,[419] presumably with a similar mode of action. One would therefore expect it to be effective against mitochondria also, and that is the conclusion reached by Richter et al.[411,420] using ribosomes and elongation factors from yeast. In contrast, Grandi et al.,[421] working with an analogous system from *Neurospora*, and Dawidowicz,[345] using the formation of fMet-puromycin by yeast spheroplasts in vivo, found fusidic acid to be ineffective. Furthermore, the inhibitor has been reported to interfere also with the mitochondrial ATPase.[388] No such ambiguity exists with respect to the inhibitors thiostrepton and siomycin,[422,423] which block peptide bond formation on bacterial ribosomes by a very similar mechanism: Their specificity is restricted entirely to bacterial and mitochondrial protein synthesis.[345,419]

5. Source and origin of proteins required for protein synthesis – By applying the technique described in detail in Section C.2.a., fairly decisive answers to this question have been obtained: (a) All the proteins required for the assembly of active ribosomes in *Neurospora crassa* are synthesized on cell sap ribosomes.[59,392] Observations consistent with this postulate have also been obtained for yeast and HeLa cells.[19] The question whether all these polypeptides are encoded in nuclear genes cannot yet be answered, but the affirmative appears likely since (b) mitochondrial chain elongation factors in *Saccharomyces cerevisiae* are synthesized in this manner not only in the wild type but also in ρ^- mutants[424] including a DNA^0 mutant.[425]

D. BIOGENESIS OF MITOCHONDRIA

The problem of mitochondrial biogenesis deals with a number of questions that, although related, are in reality quite distinct and will therefore be susceptible to different conceptual and experimental approaches and solutions. These questions are (1) What is the mode of duplication of the organelle in cells that are themselves actively dividing, e.g., in a population of exponentially growing cells of unicellular eukaryotes, or of animal or plant cells in culture? (2) What, if any, is the mode of their renewal (or turnover) in cells not increasing in mass, i.e., stationary nongrowing populations of single cells, or differentiated cells of adult metazoan organisms, especially when these are themselves not subject to cellular renewal? (3) What, if any, is the mode of their differentiation, i.e., the response to the challenge presented by changes in the extra- and intracellular milieu; are they capable of responding in terms of generalized and/or specific alterations in their structure and function?

1. Duplication of Mitochondria

We start with the assumptions that (a) eukaryotic cells contain, on the average, a relatively fixed number of mitochondria per cell, or at least per unit cellular volume, and (b) such cells of appropriate organisms, when placed in appropriate media, are capable of growth and division. It follows therefore that after the completion of a division cycle, which results in a doubling in the number and mass of such cells, the total number of mitochondria must have undergone a similar doubling. This statement says nothing about the relative timing of these two replicative (or duplicating) events. This is true macroscopically, e.g., n cells (a mitochondria/cell) → n cells ($2a$ mitochondria/cell) → $2n$ cells (a mitochondria/cell) vs. n cells (a) → $2n$ cells ($a/2$) → $2n$ cells (a), or microscopically on the level of the individual cell. In the latter instance, one tries to relate mitochondrial duplication to the cell cycle[426] (i.e., whether it is continuous throughout or exhibits periodicity coincident with or separate from cellular events). At an even more fundamental level, we have also ignored the question of origin of the a mitochondria that have to be formed in order to go from a to $2a$. A priori, three alternatives can be entertained:[1,2,11,13,15,17]

a. Mitochondria are themselves capable of growth and division, and new mitochondria are therefore the direct descendants of preexisting mitochondria.

b. Mitochondria are formed *de novo* by the assembly of various bits and pieces present in the cell.

c. Mitochondria originate from other preexisting membranous organelles, which are thereby transformed into mitochondria.

Although the first hypothesis has always seemed attractive to cell biologists,[23,427,428] some

reasonably persuasive arguments could also be raised in favor of the other two alternatives. Thus, Robertson[429] and other cytologists[23,427] had long been struck by the similarity, and probable continuity, of the limiting plasma membrane (plasmalemma), internal cytomembranes, and at least the outer mitochondrial membrane: a similarity in fact that may well hint at biogenetic relationships for that particular part of the mitochondrion.[2,18,430-433] Similarly, Bell and Mühlethaler[434,435] and Wilkie[436] have suggested a precursor function for the nuclear membrane.

As concerns the third alternative, recent studies have provided conclusive evidence for the self-assembly of exceedingly complex structures such as bacteriophages. More directly, the impressive and elegant reconstruction from simple and defined components of all the enzyme complexes (Table 9) – responsible not only for the respiratory and energy transducing functions of the inner membrane but for its characteristic morphology as well – carried out mainly by Green, Racker, and their collaborators and students,[354,398,438-441] attests to the power and generality of the concept.

The strongest experimental basis for a choice between them is, however, provided by a series of elegant experiments by Luck on a choline-requiring strain of *Neurospora crassa,* using both radioautographic and density shift experiments.[442] In brief, he found that (a) mitochondria isolated from cells, prelabeled with choline and then transferred to nonradioactive medium, exhibited exponential dilution with a halflife – of phospholipids – identical to that of the cells; (b) the grain count over all the mitochondria exhibited a random distribution, with an average per mitochondrion that declined in the predicted fashion as a function of the number of cell generations in the unlabeled medium; (c) mitochondria isolated from cells grown on a medium low in choline (1 μg/ml) and then shifted to high choline (10 μg/ml) exhibited a unimodal density distribution at equilibrium that shifted gradually and continuously from that characteristic of these "low choline" particles to those that could be isolated from cells grown on high choline for several generations. Luck was careful to take cognizance of and attempt to rule out the occurrence of rapid exchange and redistribution of mitochondrial lipids – a very real possibility either as a consequence of rapid fusion and division of the particles themselves as observed by Frederic,[443] or by exchange with pools of extramitochondrial lipid (for reviews see References 444-445a). His principal conclusion that mitochondrial mass, or, more strictly speaking, mitochondrial phospholipids, increase by a continuous process of addition of new units to a preexisting framework is probably justified. Less tenable by itself is the inference that therefore mitochondria themselves arise by growth and division of the preexisting particles, explicitly stated in authoritative contemporary reviews.[11,13] However, this conclusion becomes almost inescapable in the light of the evidence cited here and elsewhere concerning the presence, continuity, and means of expression of a specifically mitochondrial genetic system in all "normal" mitochondria examined (i.e., those not exposed continuously to such agents as Etd Br) and found capable of manifesting their full potentialities (also see below). Some additional direct evidence comes from radioautographic studies on thymidine incorporation into the mitochondria of *Tetrahymena* by Parsons and Rustad[62] and of rodent liver by Bergeron and Droz.[446] Similarly, experiments by Bücher and collaborators[447] on the development of the wing depressor muscle during the imaginal molting of *Locusta migratoria* provide strong inferential support: Under conditions where the mitochondrial mass increased some 25-fold and their inner membranes some 40-fold, their total number and enzymatic composition remained essentially constant. Coincident with this increase, there was no evidence for the proliferation of any other intracellular membranous structures that might have been interpreted as incomplete mitochondria or have served as mitochondrial precursors.

In addition, there exists a large body of morphological evidence – by no means all of it equally convincing (see Baxter[17] for review) – that suggests that mitochondria are capable of enlargement, septation, and division. Finally, the structural, compositional, and functional peculiarities of the inner mitochondrial membrane – even *in extremis* in the absence of its normal respiratory and energy-transducing function[448] – that set it apart from other cytomembranes[2,449] might be taken as strong inferential proof for its continuity.

2. Mitochondrial Turnover

The most convincing results bearing on this problem come from studies of cells and tissues of metazoan animals, especially mammals. Such cells

may, in extreme cases, exhibit lifetimes comparable to that of the organism itself, i.e., be incapable of cellular turnover. Are their mitochondria capable of turnover? Studies in a number of laboratories, usually on rat tissues, have shown that the answer is in the affirmative. Turnover is readily measurable, not only with respect to components localized in the outer membrane and the matrix, but also for those, such as the cytochromes or mtDNA, that form part of or are attached to the inner membrane. Although proteins of the first class may turn over at rates as high as twice those of the second, the values for the latter are by no means inconsiderable and are more or less uniform for several components of any one tissue or cell type investigated.[13,18,20,450] For liver, the organ studied most extensively, its halflife was found to be on the order of 10 days when labeling was done with re-utilizable precursors (such as most amino acids, acetate, iron, etc.), but this value drops to about half (5 days), probably more representative of the true value, for precursors not capable of re-utilization (e.g., δ-aminolevulinic acid and guanidine-labeled arginine). The halflife for heart mitochondria is of the same order of magnitude (6 days) and that for brain only about twice that.[450,451] We conclude that mitochondria, including the framework of their inner membranes, in cells of differentiated tissues of adult animals are capable of destruction and renewal (turnover) by a synchronous process that is independent of the replicative state or capacity of the parent cell.

3. Mitochondrial Differentiation

a. General

There are a variety of physiological and genetic manipulations that produce mitochondria that have become structurally and functionally modified from the fully active, or standard, state to such an extent as to constitute one (or more) alternative forms. For the case of ascomycetes, at least some of them have been mentioned in earlier sections, and they have been tabulated and discussed in considerable detail by Linnane et al.[22] Here we shall be concerned principally with two reversible transitions in *Saccharomyces cerevisiae* that can be considered model systems for differentiation and dedifferentiation: anaerobiosis ↔ aerobiosis (respiratory adaptation) and high glucose ↔ respiratory carbon source (catabolite repression). The two phenomena and the regulatory schedules composing them, although sharing in some of their attributes, are not identical in detail: Some components of anaerobic mitochondria are still repressible by high concentrations of glucose, while others, qualitatively distinct, make their appearance even in high glucose, provided the cells have been exposed to oxygen. The results of a number of studies on these systems in several laboratories (reviewed in References 1, 2, 8, 11–13, 20, 22, 359, and 452) have produced a self-consistent body of information that may be summarized as follows:

a. All cells contain mitochondrial analogues even under conditions of maximum repression (high glucose and/or absence of oxygen);

b. these structures retain a significant fraction of the mitochondrial mass, of certain inner membrane components and functions, and of mitochondrial DNA that characterize the fully differentiated organelle;

c. these structures are themselves replicated in the course of cellular replication;

d. they can serve as direct physical precursors of their analogues in a more highly structurally and functionally differentiated state in response to the signals constituted by aerobiosis and removal of glucose (or other readily fermentable sugar, e.g., sucrose);

e. the processes in (d) do not themselves require extensive cellular proliferation or cell division;

f. this type of differentiation involves on the structural level a reorganization of the particles, especially their inner membrane, which seems to convert them from a small number of large, highly asymmetrical, convoluted structures pervading the whole intracellular space to a much larger number of smaller, more spherical particles with well-developed cristae, arranged largely along the cellular perimeter;

g. on the functional level this process of differentiation is characterized by the appearance, in a temporally defined, sequential manner, of a large number of typical mitochondrial enzymes and enzyme systems; these enzymes are localized in the matrix and the inner membrane, and their rise in activity presumably coincides with their insertion in functional form into the preexisting framework;

h. the mitochondrial system for gene expression and translation, although essential for

the full development of respiratory and energy-transducing activities associated with the inner membrane (e.g., cytochrome oxidase and oligomycin sensitive ATPase), is not responsible for providing the bulk of the required components or even for the transmission of the signals that trigger the initial response; and finally

i. the two systems for gene expression must cooperate for full effectiveness, and their coordination is also required for the correct timing and quantitation of the response; however, this coupling can sometimes be partially disrupted by artificial means (inhibitors, etc.), leading to the temporary accumulation of intermediates and precursors.

The observations and the principles underlying these studies might be applicable to a variety of other systems that produce extensive mitochondrial reorganization. These need not be restricted to protists. For instance, mitochondria of mammalian cells growing in culture respond to changes in O_2 tension[453] in a fashion reminiscent of that of yeast, and a novel population of mitochondria appears to be generated in response to thyroid hormone.[454] Similarly, changes occurring in mitochondria during the embryonic development and differentiation of chick[455] and rat liver[456] can readily be accommodated by the model presented above. Whether in fact the first example represents the development of a novel population of mitochondria is not certain, but in any event several enzymes, among them the flavine-linked α-glycerolphosphate dehydrogenase,[457,457a] not only increases in amount but might in fact represent a novel molecular species.[458] Finally, mention should be made of the important studies of Chase and Dawid[459] on mitochondriogenesis during early embryonic development of *Xenopus*, which show clearly that the earliest detectable event in this process in the biosynthesis of the two mt rRNA's, each of which exhibits an eightfold increase in its rate of synthesis at gastrulation (stage 10) and produces a doubling (to 26 and 14 ng/embryo) by stage 45 (the feeding tadpole). Synthesis of mtDNA is very slow until stage 30, increases fourfold at stage 32, and leads to a doubling in amount (to 7.5 ng/embryo) by stage 45. Finally, mitochondrial protein and cytochrome oxidase remain constant until stage 38 and then reach double their initial level by stage 45 also.

b. Specific Induction

In addition to the responses described so far, mitochondria are also capable of reacting rapidly and highly specifically to other environmental signals. For instance in yeast, D- and L-lactate dehydrogenases, both mitochondrial enzymes, the former a flavoprotein, the latter a flavoprotein linked to a specific cytochrome (cytochrome b_2), are inducible by their substrates.[460] Certain mitochondrial enzymes are also readily inducible in mammals: Alanine and ornithine amino transferases in liver respond in this manner to the administration of adrenal steroids[461] and exhibit turnover rates (~1 day) much more rapid than other mitochondrial proteins (~5 days).[4] Even more striking is the response of δ-aminolevulinate synthetase, an enzyme localized in the matrix of liver mitochondria, to certain porphyrogenic drugs such as allylisopropyl acetamide, which leads to its induction with a halflife of ~70 min.[461a] It is probably significant that none of the proteins susceptible to such rapid formation – and destruction – in mitochondria and cells in a steady state are tightly integrated into the inner membrane. However, these observations in no way aid the conceptualization, or suggest an experimentally verifiable means for a solution of a very important question, namely, that of the mechanism of transport of such proteins from cell sap ribosomes to the mitochondrion and their transfer across two sets of membrane barriers.

E. MITOCHONDRIAL AUTONOMY

We are now finally ready to attempt an answer to the problem posed in the introduction concerning the nature and the limits of mitochondrial autonomy and the biogenesis of the organelle. We shall do so by dealing with biogenetic autonomy at the following levels: (1) that of the components found in some form of stoichiometry within the mitochondria, especially with regard to mitochondrial proteins; (2) that of the information responsible for (1); (3) that of the processes which might participate in the biogenetic sequence; and (4) that of possible regulatory events that control it and relate it to other intracellular events. Finally, we shall address ourselves briefly to the question of the possible models for the evolutionary origin of mitochondria.

1. Source of Mitochondrial Components

This problem has already been covered explicitly in earlier sections. Here we wish to emphasize the severe constraints posed on strict mitochondrial autonomy both at the genetic and the translational levels. Mitochondrial DNA codes for mt rRNA, tRNA's, and a few proteins; probably none of the latter are concerned with transcription and translation and few, if any, with replication. Similarly, only a few proteins of the inner membrane are synthesized inside the particle. The corollaries of these facts are an absolute dependence of mitochondrial replication and differentiation on extramitochondrial entities and events, requiring a tightly coupled and integrated set of interactions for their successful execution. Mitochondrial replication per se, in its strictest sense, depends on a framework for which a mitochondrial contribution is not even necessary: Petites not containing any mtDNA still retain mitochondrial structures as well as the capacity for their controlled replication.

2. Autonomy from Nuclear Genes

Clearly, in such extreme cases, mitochondriogenesis requires only the genetic information encoded in nuclear genes. But then the cells, and the mitochondria in question, are highly unusual in both their history and their properties: They have been altered irreversibly and have become obligate anaerobes. Can this type of behavior be generalized to a more normal state of affairs, and in particular is there any indication of its occurring at one stage in a reversible series of events? In other words, just as a mitochondrial function can sometimes be rendered dispensable in a biochemical or physiological sense because of the existence of alternate pathways, can mitochondrial genetic information be rendered temporarily dispensable because of the existence of alternate depositories for its content?

a. The Existence of Master Copies

1. Nature of the problem – The hypothesis to be tested can be stated as follows: Can the elimination of a single or very small number of copies of the genetic information encoded in the mtDNA be rendered both necessary and sufficient for the modification (or elimination) of this information? If so, what is the localization of this copy – the "master copy"? Is it in the nucleus, in the mitochondria, or elsewhere in the cytoplasm? We have framed the question in this strong form for two reasons:

First, it provides a logical framework for the discussion of a body of recent experiments concerned mainly with two of its quite disparate aspects: (a) with the kinetics of loss of mitochondrial genetic information and nucleotide sequences, almost exclusively in yeast – it was experiments of this sort which were first interpreted explicitly[4 3 6] in terms of a nuclear master copy in 1963 by Wilkie – and (b) with a search for base sequence homologies between mtDNA on the one hand and extramitochondrial, and specifically nuclear, DNA on the other.

Second, a critical assessment of the current status of this problem – and its possible implications for such fundamental questions as cellular and mitochondrial differentiation (Section D.3.) and evolution (Section E.4.) – must be based on the internal consistency of all the reliable information available up to this point.

2. The search for base sequence homologies – In principle the specific question of the existence of nuclear master copies of mtDNA is susceptible to a straightforward answer by means of appropriate hybridization experiments: most directly by determining the degree of hybridization of mtDNA, or alternatively, of that of an authentic mitochondrial transcript, to nDNA. The difficulties that have been encountered are largely technical:

a. The experiments require pure mitochondria or some other source of uncontaminated mtDNA: this criterion, although difficult and not met in a satisfactory fashion in a number of early experiments (see Borst and Kroon[8]), no longer presents a real problem and has been satisfied in the most recent studies.

b. As can be seen from Table 11, the maximum extent of hybridization to be expected for animal DNA's is 0.1%.

c. This particular difficulty is obviated for unicellular eukaryotes such as yeast, but here it is replaced by severe handicaps in obtaining pure nuclei as a primary source of pure nDNA. Alternative means for its isolation, especially those most commonly employed, which take advantage of the differences in base composition between mtDNA and bulk nDNA, might discriminate against that very minority fraction of nDNA that carries the mitochondrial sequences – particularly if these are present on a nuclear chromosome (or chromosome

TABLE 11

Minimal Amounts of Nuclear DNA Homologous to Total mtDNA (μg)

	Chick[a]	*Xenopus*[b]	Yeast[c]
Haploid nuclear	1.3×10^{-6}	3.3×10^{-6}	1.75×10^{-8}
Mitochondrial genome	1.67×10^{-11}	1.9×10^{-11}	8.0×10^{-11}
Amount of mtDNA per 100 μg nDNA[d]	1.2×10^{-3}	5.8×10^{-4}	4.7×10^{-1}

[a]Data from Tabak, Borst, and Tabak.[462]
[b]Data from Dawid.[270]
[c]See Table 2.
[d]Assumption: One complete copy of mtDNA is present in the nuclear genome (100 μg of DNA per filter is the practical upper limit in this kind of experiment).

fragment produced during the isolation) comparable in size to mtDNA.

d. The use of mtRNA rather than DNA for hybridization experiments generates its own set of problems: Until the possibility of nuclear mRNA being translated inside mitochondria can be ruled out definitely (see Section C.1.b.), such studies must restrict themselves to RNA's that can be shown to be mitochondrial by criteria additional to their occurrence inside highly purified mitochondria free of cytoplasmic contaminants.

Three alternatives have been employed to deal with this problem: the use (i) of highly purified mtRNA, characterized specifically as mt ribosomal RNA; (ii) of prehybridization of the mtRNA with mtDNA as a preliminary step for the elimination of nuclear transcripts; (iii) of transcripts produced in vitro (so-called cRNA) by the action of a DNA-dependent polymerase (usually from *E. coli*) with mtDNA as a template. This last method, although producing "authentic" mt transcripts, does so in the absence of normal control elements; hence, it might discriminate in favor of one and against another class of products.[463,464] This might result in the preferential transcription of only a small portion of the genes or sequence ordinarily transcribed in vivo, or even, in the extreme, to the preferential transcription of sequences that are transcribed rarely, or never, inside the cell,[49,302-305] such as homopolymeric tracts, copolymeric ones such as $(dA\text{-}dT)_n$, and others that might fulfill a spacer function in vivo.[169,245] These strictures apply particularly when, as is frequently done, double-stranded DNA is used as template with a heterologous enzyme. In that case transcription is frequently symmetrical[465] and may even be from the "wrong" strand. In addition, there are pitfalls inherent in the hybridization reaction itself. Valid answers can be obtained only if it is carried out with the relevant RNA in excess, which is not always easy with a precious compound formed in vitro. Furthermore, it is essential that optimal conditions be found and maintained in both parts of the test (i.e., with the different DNA's to be compared). This is required because (i) the time dependence of both the forward and reverse reaction of this reversible system have to be taken into consideration and (ii) their rates will depend not only on the nature but also on the size of both the DNA and RNA fragments used as substrates. Finally, even if hybridization is found, it is essential to demonstrate that the homology is in fact perfect and to determine the actual length of the homologous region. At best it can then permit qualitative, but not quantitative, inferences.

3. Tests by hybridization – Data from the most reliable recent experiments all agree that the number of possible nuclear master copies, if present, must be very low, of the order of a single copy of mtDNA per nucleus,[19,48] but cannot show definitely that this low number is in fact equal to zero. The experiment in question, the techniques used, and the numbers obtained were as follows: (a) The problem was first critically examined by Fukuhara in 1970.[466] Using relatively crude mitochondria from *Saccharomyces cerevisiae* and their RNA labeled in vivo, he hybridized the latter with both n- and mtDNA purified by chromatography on hydroxyapatite. He then dissociated the hybrid formed with mtDNA to obtain a pure mt transcript and used it to challenge nuclear DNA. Hybridization was indeed found, but it was at the limit of detection

of the method (<5% of that found with mtDNA). (b) In very similar experiments, Cohen, Hollenberg, and Borst[467] also used mtRNA labeled in vivo with ^{32}P, but purified their DNA by means of equilibrium sedimentation in CsCl. They then compared the amount of hybridization of this mtRNA, purified by prior hybridization to mtDNA, against nDNA and mtDNA. They found 0.03% of the RNA bound in the former case compared to 7% in the latter, i.e., a relative efficiency of ~4%. This is also what would be predicted on the basis of single copy homology between the two DNA's $[0.07 \times (\frac{\text{mol wt (mtDNA)}}{\text{mol wt (nDNA)}} = \frac{5 \times 10^7}{1.3 \times 10^{10}})]$. No detectable hybridization was found with the mtRNA from a grossly aberrant (96% A + T) ρ^- mutant. (c) Still working with yeast, Michaelis et al.[164] used n- and mtDNA, again purified by chromatography on hydroxyapatite, but now employed these as templates for transcription by the DNA-dependent RNA polymerases from *E. coli* and yeast. They then used this relatively pure RNA synthesized in vitro for the detection of homology. Again, the extent of homology of the mt transcript with nDNA was 5 to 10% of that found with mtDNA; again, the lowest values were found when the source of the nDNA was a ρ^- mutant, this time one lacking entirely in mtDNA. (d) Storti and Sinclair[468] used both DNA-DNA hybridization and RNA transcribed in vitro, again with *S. cerevisiae*, in their attempts to answer the question. They also determined the extent of actual sequence homology by examining the properties of the hybrids. Their results suggest the existence of about two relatively faithful copies of mtDNA in the nuclear genome, integrated into and banding with the same buoyant density as bulk nDNA as long as the latter is intact, but exhibiting the expected lower density after its molecular weight is decreased by shearing. (e) Finally, Tabak, Borst, and Tabak[462] used nDNA obtained from highly purified chick erythrocyte nuclei and mtRNA synthesized in vitro with a very pure mtDNA as a template. The extent of homology found corresponded to 2±1 copies per haploid genome.

Hybridization data therefore are not sufficiently precise either to prove or rule out the existence of nuclear master copies or to tell us anything conclusive about the exact nature or function* of the postulated homology.

In any event it would be interesting to add the genetic approach to hybridization experiments and see, for instance, what happens to the kind and extent of possible nuclear homology during vegetative growth of cells extensively mutagenized by Etd Br. One would expect these to contain their full complement of master copies during the initial exposure to the mutagen, but to undergo significant alteration when different cells in the population have become stabilized as ρ^- mutants varying in the degree of aberration of their mtDNA (see below).

On the other hand, hybridization experiments can also be cited as providing evidence against the existence of master copies: Dawid and Blackler[308] found, again by means of hybridization to RNA transcribed from mtDNA by *E. coli* polymerase, anywhere from 5 to 20 equivalents of mtDNA associated with the nuclear DNA of the somatic cells of hybrid toads obtained from the crosses of *Xenopus laevis* with *X. mulleri*. These mtDNA equivalents were found to be exclusively of the maternal type. Now this is precisely the pattern exhibited by the *pure* mtDNA isolated from these animals in which this component appears to be maternally inherited. It can then be argued that, in contrast, any true *nuclear* master copy ought to exhibit biparental inheritance and that therefore *authentic* nuclear entities ought to exhibit an equal contribution from the male parent. Mixing experiments showed that the method used was sufficiently sensitive to detect a single such copy among the nuclear material: Its complete absence in the experimental set is therefore a potent argument in favor of the absence of nuclear master copies (and for mitochondrial contamination as the source of mitochondrial nucleotide sequences in this and analogous experiments). However, it must be pointed out that the tests were performed on tadpoles or mature animals and hence might have been subject to "proofreading" (see next section) during the large number of intervening mitotic divisions.

4. Genetic data – The relevant experiments

*As also pointed out by Storti and Sinclair,[468] even when there is indication of a fairly extensive homology between sequences in mt and nDNA this finding can be interpreted to indicate something other than a master copy for structural genes; it might represent regulatory sequences necessary for the control of mutual interaction between the nucleus and mitochondria, or simply a nonfunctional evolutionary relic. The recent experiments by Coon et al.[484] indicate the virtual absence of any homology in both human and rodent cells.

deal, almost exclusively, with the effect of Etd Br, and take advantage of its strong – and frequently exclusive – preference for interaction with mtDNA. They are of two kinds, the first studied exclusively with facultatively anaerobic yeasts, the other demonstrated most strikingly with aerobic yeasts.

a. Facultatively anaerobic yeasts such as *Saccharomyces cerevisiae* are also usually "petite positive,"[18,184] i.e., they are subject to respiratory deficiency and controlled by a cytoplasmic genetic determinant, a mutation brought about with high efficiency by Etd Br (Section B.3.b.2. and 3.). We have seen there that exposure to Etd Br initiates a series of (potentially reversible) steps that have to be traversed sequentially before the affected cells can be considered to be irreversibly transformed into stable mutant descendants. Various agents described there can prevent or compete against and sometimes even apparently cure the mutagenic effects produced by Etd Br. What remains to be done in all these instances, as well as in that described in the next paragraph, is to obtain a more precise estimate of the number of copies of mtDNA that actually survive the various treatments.

b. Obligately aerobic yeasts – and probably other cells, including those of vertebrates, sharing this mode of metabolism – respond to exposure to Etd Br in a qualitatively distinct manner. Not only are they "petite negative," i.e., they do not produce stable mutants deficient in their mitochondrial respiration (which might or might not be viable depending on the presence or induction of secondary respiratory pathways), but in an appropriate range of concentrations the effect of exposure to Etd Br appears reversible. That is to say that as long as Etd Br is present it blocks the continued elaboration of normal mitochondrial structural and functional parameters,[181] just as in *S. cerevisiae,* but upon its removal the synthesis of these components resumes and the cells and their mitochondria soon revert to the state existing before exposure to Etd Br.[180,182] Analysis of the mitochondrial DNA levels present after extensive exposure to these reversibly modified cells of *Saccharomyces lactis*, again by examination of total cellular DNA on CsCl gradients, shows a virtual absence of this component, which then reappears after several generations of growth in the absence of the inhibitor.[182] However, these experiments, striking as they are, could not assess accurately mtDNA at a level $\leqslant 10\%$ of that found normally, and this amount might still correspond to as many as ten copies per cell.

c. Acridines and Etd Br are also highly effective and selective against episomal genetic determinants in prokaryotes,[475] and in blocking replication and thereby eliminating the kinetoplast (a mitochondrial analogue) and its DNA[469-474] in parasitic flagellated protozoa (hemoflagelletes, trypanosomids).

d. The genetic laws, i.e., the quantitative relationships, governing the inheritance of mitochondrial genetic markers – such as the respiration deficient phenotype resistance to antibiotics and other inhibitors, as well as suppressiveness and polarity in the organisms described under a. – are unique and qualitatively different from those describing the inheritance of traits specified by nuclear genes.

e. Such mitochondrial markers can be contributed to the zygote and its offspring by mating not only two appropriate ρ^+ cells, but also a ρ^+ with a ρ^- cell containing the determinant, even though the latter is completely incapable of expressing the determinant.

Now let us examine the implication of these findings with regard to the postulated unique master copies if they are encoded in the nuclear, or some other nonmitochondrial genome. Items a. and c. above demand that – as in other cases of the inheritance of amplified (multiple copy) genes[262,289,476,477] – a mechanism must be operative at, or just subsequent to, the replication of these genes, that fulfills a *proofreading*, rectifying, or error-correcting (here really sometimes an error-introducing) function. However, in the present instance, in contrast to the "master-slave" relationship[478-481] postulated for sets of nuclear genes, this proofreading function (PRF) must be *initiated* by an alteration in the set of amplified copies (i.e., the slaves – presumably directed by Spartacus – bending the master to their will rather than the converse) since that is the site of the initial mutagenic change (item a. above). The system responsible must be able to sense and execute the whole gamut of changes (deletion and amplification of remaining sequences – Section B.3.b.) up to and including the actual elimination of mtDNA. Furthermore, item d. makes it imperative that whenever one particular

mitochondrial genotype becomes predominant, the former controls the master copy absolutely. Such processes occur during mitotic segregation subsequent to conjugation and during meiosis as well. Since the genotype can consist of any combination of a variety of possible ones – subject only to the rules of mitochondrial genetics – we must postulate that the PRF can distinguish between and be active on individual pairs of alleles (e.g., *ant*-r vs. *ant*-s). Finally, it must be equally effective on haploid or diploid cells.

Some of the facts already cited can be readily interpreted in terms of the postulated PRF: The apparent (single copy) homology to mtDNA in nuclear DNA of wild type *S. cerevisiae* is greatly reduced when the latter is converted to mutants containing either grossly modified or undetectable mtDNA. By the same token, reversibility and cure of the mutagenic effect of Etd Br might be explained in terms of an absence of the PRF in these cells, either permanently, in the case of *S. lactis*, or temporarily, at the actual moment of effective cure in *S. cerevisiae*. As a necessary corollary, since PRF is active under conditions where the mitochondrial system of gene expression is not, the specification and the expression of the entities responsible for PRF must take place outside the mitochondria. Yet for its function it must, of course, be capable of sensing changes within the mitochondria and communicate them to the nucleus.

The fact that both primary mutagenized and primary zygotic clones are genetically mixed (impure) and require many (sometimes more than 20) generations of mitotic division for the final emergence of genetically stable cell lines of established geno- and phenotype is consistent with the operation of a continuous and relatively slow process such as PRF.

Finally, the behavior of the unusual mutants studied by Mitchell et al.[135] might be taken as an additional inferential proof: These mutants, of which haploid L3000 is an example, were originally isolated as diploids (e.g., one called N1301) resistant to mikamycin. This resistance is not detectable in mitochondria isolated from this strain and is lost in vivo upon anaerobic cultivation. The diploid descendants of these mutants, as well as the resistant haploids isolated by their sporulation (to yield, e.g., L3000), also exhibited cross-resistance to the unrelated antibiotic inhibitors of protein synthesis, chloramphenicol and carbomycin, as well as to oligomycin, an inhibitor of oxidative phosphorylation.* This resistance to this battery of inhibitors, segregated 4:0 upon sporulation of N1301, was present in mixed clones upon mating of a sensitive strain with the resistant haploids (e.g., L3000), and was eliminated coincidentally with the production of ρ^- mutants by Etd Br. By these criteria (Section B.2.b.2.), the mutations in both N1301, the diploid parent, and its haploid descendant, L3000, deserved to be classified as mitochondrial. However, when the diploids resulting from the cross between L3000 and the sensitive tester were sporulated and the resultant ascospores subjected to tetrad analysis, the latter exhibited the 2:2 pattern characteristic of nuclear genes. The various findings are consistent with one of the hypotheses advanced by Mitchell et al., namely, that "all cytoplasmic genomes . . . are of a nature that they can all, on occasion, be integrated into the nuclear genome and thus all have some properties similar to the classical bacterial episome."[135] Thus, the series of events that has been postulated to have supervened in the evolution of mitochondria[366] might still be operative in the contemporary organism. PRF might then be analogous to – or require as an essential part – the proteins and structural overlaps in the DNA operative in the incision-excision mechanism of integration of bacterial plasmids (e.g., temperate bacteriophages, sex and drug resistance factors, etc.) into their chromosomal DNA.

There is an obvious alternative to the postulated PRF: The expression of the copies of mitochondrial genes residing in the nucleus is usually permanently prevented by some form of repression. This postulate, together with an occasional breakdown or elimination of this mechanism, can account for all the observations presented so far. In principle it should be possible to distinguish between the two models (PRF and repression), most conclusively in the case of ρ^0 petites, i.e., petites not containing mtDNA in significant amounts (Section B.3.a.). If there is no PRF, there should be no quantitative or qualitative

*Conversely, one of the classes of oligomycin resistant mutants isolated by Avner and Griffiths[131] shows pleiotropi resistance to a variety of antibiotic inhibitors of protein synthesis. This class also exhibits an unusual pattern o inheritance.

alteration in the extent of homology, while homology must be eliminated if there is a PRF. Similarly, in other petites the extent of nuclear homology to wild type mtDNA should bear a functional relation to the degree of aberration from the latter (and retain the homology to its own, i.e., mutant mtDNA) only if the PRF exists. So far the results obtained have been ambiguous: As mentioned, two groups claim a decrease in nuclear homology in petites, while a third has demonstrated the opposite in a ρ^0 petite.

It must be pointed out finally that alternative interpretations of the results cited above are also possible without having recourse to nuclear master copies. For instance, Mitchell et al.[135] cite the possibility that their mutation is in fact nuclear but must for its expression depend on a (membrane) component specified by mtDNA and absent in certain petites. The apparent cytoplasmic character of the mutation would then be determined in the first instance by the state of the membrane in the haploid and propagated by the transmission and segregation of this preformed resistant or sensitive mitochondrial membrane among the progeny. Although it is possible to account for mitotic segregation in a satisfactory fashion by this model – which is reminiscent of and based on the one developed for cortical inheritance by Sonneborn[482] – it is faced with more severe difficulties when it is applied to contributions – or more critically to their occasional lack – of the nonnuclear genes by various classes of ρ^- cells. The model implies that the first group retains the ability to contribute to resistance by virtue of its mitochondrial membrane structure retaining the ability to accommodate the product of the nuclear gene for resistance. Those that have lost this capability must have done so because these particulate petite mutations produce membranes that are incapable of this accommodation. The postulate of two classes of petites differing in their membrane configuration depending on the extent, presumably, of their deviations from wild type is hard to reconcile with the facts: Although petites do indeed vary greatly in the degree of abnormality and deviation from the wild type norm in their DNA and RNA's, all those examined so far do share one property, their complete inability to carry out effective mitochondrial protein synthesis and, therefore, to produce membranes containing, and structurally determined by, mitochondrial gene products. So at the least one must then add an ad hoc postulate to the hypothesis, i.e., the export of mitochondrial mRNA for translation elsewhere – and there is little, if any, evidence for such an event.

However, other hypotheses can also be entertained: All the genetic results cited might be accommodated by postulating a reduction of effective mitochondrial copies (either whole molecules or fragments capable of recombination to form such molecules) to a small number ($\geqslant 1$) under appropriate, unusual circumstances. Thus, the resistance to and recovery from exposure to Etd Br might be due to the continued survival of this limited complement. Similarly, during meiosis of the mutants discussed by Mitchell et al.,[135] we might postulate such a survival of both parental types to the same extent, in contrast to the normal situation where one predominates completely by virtue of its retention in an amplified form. It is clear that one of the more fruitful lines of future experimentation will be concerned with the survival and assortment of appropriately labeled parental mtDNA's and their component sequences during both mitotic and meiotic division cycles subsequent to the formation of a zygote.

5. The situation in higher eukaryotes – The problems presented in this section are not restricted to unicellular eukaryotes. We have already discussed the experiments by Dawid and Blackler[308] that suggest strongly that in amphibians mtDNA is inherited uniparentally, i.e., that the mtDNA in the somatic cells of the offspring is identical with that contributed to the zygotes by the maternal parent. One would like to know the sequence of events in the early embryo that culminate in these results.

Similarly, Attardi and Attardi[483] have investigated the fate of the two mtDNA's in hybrid clones of somatic cells of murine and human origin. These two mtDNA's differ in their buoyant density by 8 μg/ml and can therefore be readily distinguished and analyzed in double-label experiments with ^{3}H- and ^{14}C-thymidine as precursors. The mtDNA in the hybrids was invariably of the mouse type under conditions where the survival of a substantial number (8 to 23) of human chromosomes could be demonstrated by karyotype analysis. Therefore, as pointed out by the authors, either the presence of the murine genome is sufficient to repress completely the formation of mtDNA, or provided loss of human chromosomes is essentially random, the

survival and replication of mtDNA is a process requiring the participation of many nuclear genes distributed over a number of chromosomes.

Experiments dealing with the same problem were also performed by Coon, Horak, and Dawid[484] using the very sensitive technique of challenging cRNA, programmed by *E. coli* polymerase with pure circular mtDNA (as well as nDNA) of the two parental types, with the appropriate DNA's from the hybrid. While for the human-mouse hybrids the results obtained were in essential agreement with those of the earlier investigators, the situation was quite different for the human-rat hybrid cell lines. These fell roughly into two groups: one containing 40% nDNA and 20% mtDNA from the rat, the other with contributions of 60% and 90% from this source. In general, the proportions of nDNA were more balanced than those of mtDNA, though both showed a weakly correlated tendency to deviate from equality. This tendency towards a segregation of mtDNA was relatively slow, so that many cell lines remained hybrid for both types for 40 to 150 generations; however, it proceeded further and more rapidly than did that of the nuclear chromosomes.

Finally, there is some suggestive but by no means conclusive evidence that mitochondrial DNA might be capable of being transformed and switched to the expression of novel products by viral or oncogenic agents.[485]

3. Biosynthetic Sequences

The problem of defining biosynthetic sequences is susceptible to a logical analysis in terms of the following steps: (a) biosynthesis of components outside the mitochondria, (b) biosynthesis of components inside the mitochondria, (c) spatial and temporal integration of (a) and (b) leading to either differentiation or growth and division, and (d) regulation of (a) through (c), which fuses into the topic of the next section (E.3.d.).

a. Biosynthesis of Extramitochondrial Components

1. Proteins – From the foregoing it is clear that the bulk of the mitochondrial protein, even of its inner membrane, is synthesized on cell sap ribosomes and must then be transported from the site of its synthesis to that of its utilization and integration. The formidable topological constraints inherent in such a process have engendered much speculation as to the detailed sequence of events responsible. Kinetic studies in yeast,[486] *Neurospora*,[377] and rat tissues (liver and kidney)[18,487,488] suggest that at least some of these membrane proteins become labeled after only a very brief lag – of about the same length as the lag governing the synthesis of cell sap proteins. For these proteins the transit time must be very short. This inference, together with a wealth of morphological information[17] and certain biochemical data,[409] has prompted Kellems and Butow[490] to search for a special class of – perhaps membrane associated – cell sap (i.e., 80S) ribosomes contiguous with mitochondria and responsible for this type of synthesis. The evidence presented supports the existence of such a class of particles which, furthermore, appear capable of vectorial release of their completed polypeptide chains. This latter observation suggests secretion of such products preferentially into a compartment that is either itself mitochondrial or contiguous with the mitochondria rather than into the cell sap.[491] Such a direct transfer would obviate transit through the cell sap and a need for a selection process for isoenzymes leading to the correct apportionment of the mitochondrial and extramitochondrial forms. However, in the case of a number of mitochondrial proteins, either of the matrix, such as L-malate dehydrogenase[492] and δ-aminolevulinate synthetase[488,493] of rat liver, or associated with the inner membrane, such as cytochrome *c*[491,494,495] or the F_1 ATPase and OSCP,[354,357] there is some evidence for a form of the protein in transit between regular cell sap ribosomes and mitochondria. However, much of this evidence is either indirect or derives from experiments that, in order to demonstrate accumulation of proteins, required the use of conditions (preincubation with CAP during derepression) that might be expected to lead to a loss of structural or functional integrity of the system.[496] However, detailed kinetic studies of the appearance of the extramitochondrially synthesized polypeptides of cytochrome oxidase[377] and investigations on the transfer of ATPase subunits from the cell sap to intact mitochondria or their inner membrane in vitro[496] do suggest the likelihood of such transport processes occurring in vivo. The last named studies also point out explicitly the great difficulty of obtaining meaningful information from such in vitro studies and the variability inherent in such

parameters as mitochondrial integrity, nonspecific adsorption by various membrane fragments, etc. Of importance within this context is the fact that ferrochelatase, the enzyme responsible for the last step in the biosynthesis of the prosthethic group of *all* cytochrome hemoproteins – regardless of their actual localization within or on the inner membrane – is itself an entity tightly integrated into this membrane.[493] This fact by itself raises a separate set of problems with regard to protein transport and integration for this class of very insoluble proteins.

2. **Transport processes** – At this moment one can only speculate how membrane, and other, proteins destined for the mitochondria are transported between their site of synthesis and of utilization. They might, of course, be moved without any modification. They might undergo a modification after transport by the removal of part of their primary sequence, rendering them more hydrophobic. Finally, they might be modified by the addition – and perhaps eventual removal – of entities fulfilling specific "transporter" functions, such as other proteins, lipids, carbohydrates, or any combination thereof, with or without the formation (and breakage) of covalent bonds.

3. **The role of lipids** – Lipids, particularly phospholipids – among them most characteristically cadiolipin (diphosphatidyl glycerol)[2,20,22,228] – form an essential part of the inner membrane, as they do of all membrane systems. Both saturated and unsaturated fatty acids are required; and desaturation of the former to the latter, a reaction requiring molecular oxygen as an obligatory co-reactant, is an essential step in the biosynthetic sequence. Since petites can perform all these reactions and contain most of the normal lipids,[448] although in abnormal amounts, mitochondrial participation does not appear to be required directly. When the desaturation reaction cannot be performed, i.e., under anaerobiosis in *S. cerevisiae* or – in a variety of organisms (Table 12) – because of the absence of the requisite genetic information, unsaturated fatty acids have to be provided as essential growth factors.[225-229,497] Such "auxotrophic" cells can utilize, incorporate, and accommodate into their inner membranes a variety of fatty acids quite different sterically from the ones found under nonauxotrophic conditions, e.g., petroselinic acid, the Δ^6*cis* derivative with a melting point of 28°C, or even stearolic acid with a triple bond and a mp of 34°C instead of the "natural" oleic acid, which is Δ^9*cis*, mp <5°C. Deprival of cells of such lipid supplements or provision of nonfunctional ones, such as the Δ^9*trans*, leads to inability to form normal mitochondrial inner membranes* capable of respiration[229] and oxidative phosphorylation.[495] In extreme cases, especially under anaerobiosis, these lesions in the inner membrane may affect its ability to support normal DNA replication, transcription, and ribosome function.[22,346,497] It is therefore not surprising that other mutants blocked in a different step of lipid metabolism perhaps related to integration may even exhibit the petite phenotype.[226,227]

b. Biosynthesis of Intramitochondrial Components

Aside from the unambiguous substantiation of its occurrence, and some of the unit processes involved, little is yet known about the details concerning the formation of identifiable fractions of the inner membrane. Therefore, one will need to probe the nature and kinetics of the events starting with the release from the mitochondrial ribosome of the completed polypeptide chain and ending with its integration into a functional complex of the inner membrane containing a variety of components including polypeptides imported from the cell sap.

c. Integration and Membrane Replication

Once the questions outlined above are answered, the next major problem to be settled will be that of the steps involved in the growth and replication of the two basic membrane systems (outer and inner) confining and defining the organelle. We have seen that logically the problem can be divided into two subsets: replication of the basic framework and insertion of specialized units. Conceptually, therefore, the answers required to delineate this problem differ only in details from those inherent in the formation of all other membranes,[20,498-500] such as the plasmalemma or cytomembranes of eukaryotic or even bacterial

*Experiments of Lewin cited by Getz[20] indicate the essentiality of lipid fluidity for membrane function: Cells will grow on glucose with palmitelaidic (C_{16}, Δ^9*trans*) or linoelaidic (C_{18}, $\Delta^{9,12}$*trans*) acid at temperatures >25°C but not below 22°C; however, for derepression even the former must still be supplemented with a *cis* acid.

TABLE 12

Mutants of Yeast: Fatty Acid Growth Response

Fatty acid supplement	Anaerobic yeast	*Escherichia coli*	*ole1-1* (KD115) early	*ole1-1* (KD115) recent	*ole1-2* (KD20)	*ole-2* (KD46)	*ole1-1* (KD115) Lactic acid	*ole1-2* (KD20) Lactic acid	*Neurospora cel*	Yeast mutant *fas 1*
12:0				o		o			<+	<+
14:0			o	o		o			+	+
15:0									+	+
16:0			o	o		o			+	+
17:0									+	+
18:0			o	o		o			o	o
19:0									o	
20:0									o	
14:1 Δ^3 *cis*		+	o	+		+			o	
14:1 Δ^9 *cis*		+	+	+	+	+	+	+	o	o
16:1 Δ^9 *cis*		+	+	+	+	+	+	+	o	o
16:1 Δ^9 *trans*		+		+	+	+	+	+		
18:1 Δ^5 *cis*				o						
18:1 Δ^6 *cis*		+	o/–	+		+	+	+	o	
18:1 Δ^8 *cis*				o						
18:1 Δ^9 *cis*	+	+	+	+	+	+	+	+	o	o
18:1 Δ^9 *trans*	o	+	o	o		o			<+	o
18:1 Δ^{11} *cis*	+	+	o/–	+		+	+	+	o	
18:1 Δ^{12} *cis*				o						
18:1 Δ^{11} *trans*		+	o	o		o				
18:1 $\Delta^{9,12}$ *cis, cis*		+	+	+	+	+	+	+	o	o
18:2 $\Delta^{9,12}$ *trans, trans*			o/–	+		+				
18:3 $\Delta^{6,9,12}$ *cis, cis, cis*				+		+				
18:3 $\Delta^{9,12,15}$ *cis, cis, cis*			+	+	+	+	+	+	o	o
20:1 Δ^5 *cis*		o	o							
20:1 Δ^{11} *cis*		+	o	+		o				
22:1 Δ^{13} *cis*		o	o	o		o				
24:1 Δ^{15} *cis*		o	o/–	o	o	o	o	o		
20:2 $\Delta^{11,14}$ *cis, cis*		+		<+		o			o	

TABLE 12 (continued)

Mutants of Yeast: Fatty Acid Growth Response

Fatty acid supplement	Anaerobic yeast	*Escherichia coli*	*ole1-1* (KD115) early	*ole1-1* (KD115) recent	*ole1-2* (KD20)	*ole-2* (KD46)	*ole1-1* (KD115) Lactic acid	*ole1-2* (KD20) Lactic acid	*Neurospora cel*	Yeast mutant *fas 1*
20:3 $\Delta^{11,14,17}$ *cis, cis, cis*		+		<+		o				
20:4 $\Delta^{5,8,11,14}$ *cis, cis, cis, cis*			o	+		+				
18:1 Δ^{9} *cis*-ol				o	o					
18:1 Δ^{9} *cis*-ol-PO_4				o						
18:1 $^{-9}$	+			+		+				o
18:1 Δ^{9} *cis*, 12OH		+		+		o				
18:1 Δ^{9} *trans*, 12OH				<+		o				
18:1 Δ^{9} *cis*, 12-Acetoxy				o	o		o	o		
9,10-CH_2-18:1 Δ^{9} *cis*				o						

Symbols: + = essentially wild type growth; <+ = less than wild type growth (growth rate ≥0.5 of wild types); o = no growth; o/– = no growth and inhibitory to wild type.

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cells.* Two of the major uncertainties in all these systems are (1) whether insertion of new structural (and functional) units into the membrane can occur more or less at random anywhere along its surface, or whether they are inserted only at a small number of well-defined specialized "growing points," and (2) the size and complexity of the "biosynthetic units" capable of insertion. The two problems are of course not completely independent of one another, nor are they unrelated to a third one, namely, the detailed structure of the membrane itself.[500-504] In any event, all membrane synthesis including mitochondriogenesis cannot be adequately described without considering two vectorial components: the asymmetry of the membrane in the transverse direction (i.e., across the membrane, dividing an "outside" from and "inside") and the sequence of its construction in time: In a static sense, i.e., at any one moment, membrane surfaces appear to exhibit considerable symmetry at a particular level of resolution, i.e., to be made up of repeating units of varying complexity in the xy plane. But this observation need not argue necessarily for random insertion of such repeating subunits or their components. The membrane model currently considered most plausible by workers in the field is that of a "fluid mosaic"[503,505] or some modification thereof.[223,502] In such a model the membrane can always accommodate itself to units channeled into it at specific sites, and, given enough time, any "new" unit will equilibrate with and become equivalent to the "old" units already present at the time of such an insertion. The most reliable recent studies on bacterial systems do suggest, however, a random insertion model for membrane growth.[499,506,507] The heterogeneity of three classes of inner membrane fragments with regard to their enzyme complement and content of newly synthesized polypeptides has been taken as evidence for specialized growing regions in rat liver mitochondria by Werner and Neupert.[508] However, in view of the well-established heterogeneity of liver mitochondria[460,461,509,510] as a function of a variety of parameters such as origin, age, and method of preparation, these results are certainly open to alternate interpretations and again suggest the inadvisability of using mammalian liver for critical studies of biogenetic problems.

d. Regulation

1. Observations – Only a few instances of what must be a large body of regulatory interactions between the two intracellular systems of gene expression have come to light so far. As already mentioned, damage to mitochondrial DNA results in the enhanced synthesis of a mitochondrial enzyme (DNA polymerase I) probably involved in repair of this damage. This enzyme is extramitochondrial in its origin, and its elaboration may be under the control of a repressor specified by mitochondrial but active on nuclear DNA.[78,79] A similar interpretation has been placed by Barath and Küntzel[511] on their observations that various protein factors required for mitochondrial protein synthesis, but synthesized on cell sap ribosomes, are subject to extensive overproduction when mitochondrial macromolecular synthesis is inhibited by long-term exposure of *Neurospora* to CAP or Etd Br. In this latter case, the same phenomenon in exaggerated form also appears to be operative for the mitochondrial RNA polymerase, which then accumulates in massive amounts in the cytoplasm. These experiments form the basis of an elaborate set of postulated interlocking controls of nuclear events by mitochondrial repressors and of mitochondrial events by repressors of nuclear specification, synthesized on cell sap ribosomes.[348] Various elements of similar repressor controls had been postulated earlier by Williamson to account for the induction of the petite mutation in yeast by inhibitors of mitochondrial protein synthesis[57] and by Lloyd et al.[512] for the effects of CAP on mitochondrial proliferation in *Tetrahymena.*

An interesting example of some form of regulatory interplay is provided by the properties of *pet 494,* a nuclear mutant deficient in cytochrome oxidase activity, isolated and studied in Schatz' laboratory.[513] Analysis of inner membranes or of cross-reacting material purified by immunoprecipitation showed the absence of the polypeptide subunit of mol wt = 23,000 daltons, known from earlier studies (Table 8) and confirmed here to be a product of mitochondrial translation synthesized

*This is brought out most clearly again by a consideration of the DNA^{0} petite yeast mutants in which construction of mitochondria cannot depend on the provision of any component specified or synthesized inside the particle.

in the presence of CH. One possible interpretation is that the component lacking in this mutant is principally concerned with the proper integration of a mitochondrial translational product.

Finally in our own laboratories we are concerned with the study of two phenomena: First, during the release of yeast cells from catabolite repression (Section D.3.a.) there occurs a massive increase in the levels of a number of intra- and extramitochondrial enzymes, all concerned with aerobic respiratory metabolism. The kinetics of these biogenetic events is quite precise not only in their onset, but also in their cutoff.[359,452] The latter, unlike the former, cannot be under the control of some catabolite related to glucose levels, but is instead in all likelihood regulated by events that have their origin directly or indirectly in the mitochondria.

Secondly, we are investigating possible coupling devices between the two systems of protein synthesis by the use of *ts* mutants and specific inhibitors. For instance, initiation on cell sap ribosomes can be interrupted rapidly by shifting an appropriate mutant from the permissive to the nonpermissive temperature. Preliminary experiments by Dr. F. Feldman suggest that mitochondrial protein synthesis – as measured by the incorporation of either a labeled amino acid or of formate into membrane proteins – also comes to a halt as a result, but that this shutoff does not apply to initiation of mitochondrial polypeptides as measured by the formation of fMet-puromycin.

2. **Significance** – We also need at least to raise the question of the teleological significance of the existence and survival of the mitochondrial system of gene expression. Clearly no definite answers can be provided. The following considerations may, however, serve as a starting point for future discussions.

Evidently the dispensing of these components at the right place and at the right time is vital to the survival of the eukaryotic, respiring cell. Why should that be so? Three possible reasons come to mind.

1. Even though the polypeptides are minor constituents, they might provide essential elements for the construction of the membrane, which are required for specification of structure or proper function, or act as a regulatory device.

2. They might have to be synthesized in the immediate vicinity of the membrane since their transport from the cytoplasmic ribosomes – unlike that of other components – might be faced with insurmountable difficulties, perhaps because of their hydrophobic nature and the resultant insolubility in an aqueous environment.

3. Related to the last point is the possibility that the polypeptides might require a kind of processing which, if available elsewhere in the cell, might lead to dire consequences, perhaps because they would interact with and remove from solution other polypeptides that are not meant to be put in this position.

4. Mitochondrial Evolution

a. The Endosymbiont Model

Many investigators recently have begun to speculate about the evolutionary origin and history of this organelle. They have been struck by the many analogies between present-day bacteria and mitochondria, particularly regarding certain aspects of their protein-synthesizing systems. Most of the speculations have as their core the capture and domestication of a bacterium capable of respiration by some other primitive, anaerobic organism.[24,25,514-517] In its most explicit form,[514] this endosymbiont model postulates the following sequence of events.

1. Prokaryotic cells evolved an anaerobic metabolism in the earth's primordial reducing environment.

2. Photodissociation of water vapor in the atmosphere acted as the selective pressure that resulted in the evolution of porphyrin metabolism which ultimately led to porphyrin-containing proteins.

3. Photosynthesis in prokaryotes evolved, resulting in a gradual change in the atmosphere from a reducing to an oxidizing one.

4. Prokaryotic respiration evolved.

5. Prokaryotes, which were photosynthetic, adapted their porphyrin systems in order to respire in the dark. This step resulted in the immediate ancestors of the present-day blue-green algae.

6. An endosymbiosis involving both an aerobic and an anaerobic prokaryote was developed, which later became obligatory, presumably by a series of consolidation steps at the level of DNA. The organisms are referred to by Sagan[514] as "aerobic amitotic amoeboid organisms." She also proposes that in these cells all oxidative metabolism occurred within the symbiotic "proto-

mitochondrion," which used only its own proto-mitochondrial gene products for its construction.

7. What then followed was the evolution of these primitive amoeboid organisms into eukaryotes, a process that involved a profound modification of the symbiotic relationship between the two genomes.

b. An Alternative Model

Although suggestive and pleasing at first sight, the endosymbiont model is by no means the only possible way to account adequately for the origin of mitochondria in a prokaryotic cell. A feasible alternative has been formulated explicitly by Perlman, Raff, and myself.[104,366] The argument advanced runs somewhat as follows, with the first six points expressing factual observations on contemporary organisms and the remainder constituting the speculative part of the hypothesis.

1.Although bacteria do not contain any organelles as such, they do possess a limiting (plasma) membrane that in certain species, particularly under conditions of physiological stress, can generate elaborate and structurally highly differentiated infoldings that pervade the whole interior of the cell (cytomembranes). Such structures are particularly prevalent among marine chemi-autotrophic organisms, and can mimic either the cristate configuration characteristic of mitochondria or the thylakoid lamellae of chloroplasts.

2.The plasma membrane – and perhaps a specialized region within this structure – is responsible for electron transport and linked energy transduction (ATP formation) in both photosynthetic and nonphotosynthetic bacteria.[5,18]

3.Bacteria harbor plasmids, carriers of extrachromosomal genetic material in the form of circular DNA molecules. At least some of these plasmids are of the same size as contemporary mtDNA.

4.Plasmid DNA can either exist and replicate independently of chromosomal DNA or undergo cycles of integration into and detachment from the latter. In the course of this process, genes may become detached from the chromosome and become part of the plasmid genome, thereby becoming capable of a separate existence.

5.Replication of both chromosomal and plasmid DNA is intimately associated with and dependent on specialized structures in the plasma membrane.

6.Transcription and translation in prokaryotic cells are closely coupled events, and the enzymatic machinery required for these processes appears to be always in close association with DNA.

7.The membrane might have formed septa and isolated compartments entrapping a plasmid, its replication site, and a means for the expression of its genetic information.

8.If the plasmid happened to contain the genetic information for the construction of one or more key components of the plasma membrane associated with energy production, the sequestration described under 7. might well have conferred a distinct evolutionary advantage on the descendants of this particular cell. This advantage came about first of all because the resultant modification of the membrane led to greater efficiency in energy transduction. These events might have preceded or followed the gene sequestration.

9.The genes for ribosomal RNA (responsible for the translation of the sequestered information) might themselves become sequestered. Furthermore, the ribosomal particles so produced might themselves become integrated and secrete their product directly into the membrane.

10.Further developments conferring additional incremental advantages can be envisaged in the form of the events described under 8., these events being repeated several times within the same cell and leading to the phenomenon of amplification of sequestered genes.

In this manner are produced simultaneously both an enclosed compartment distinct from and more highly specialized and energetically efficient than the remainder of the cytomembrane system – i.e., an organelle – and a means of providing its genetic individuality and continuity.

A number of additional observations, although they do not and cannot confirm the hypothesis, are at least not inconsistent with it. Among them are the following:

1. Electron transport and phosphorylation in the membranes of present-day bacteria are qualitatively similar to the same events in contemporary mitochondria, including the requirement of the presence and structure of such key components as cytochromes, cardiolipin, and unsaturated fatty acids. There is a considerable homology between present-day bacterial and eukaryotic

cytochromes, the latter being proteins mitochondrial in their localization, but nuclear in their specification.

2. There is precedent for the advantage conferred by sequestration and amplification of ribosomal genes in a specialized organelle capable of their independent transcription – the nucleolus of eukaryotic cells.

3. The number of certain plasmids may be as high as 30 per cell; plasmids, like mitochondria of ascomycetes, are subject to environmental influences, and their number reaches a maximum as cells enter the stationary phase.

4. The origin of mtDNA as a plasmid and its inherent capabilities of exchanges with itself and with nuclear DNA might have been essential for the prolonged and continuous exchange of genetic material that must have preceded the final successful assortment of genes – in particular, genes for ribosomal RNA – into the two compartments. Even present-day bacteria possess a modest (five- to tenfold) redundancy for these sequences, and in some of them the genes are arranged in two separate clusters on the chromosome.

5. The strongest argument for the endosymbiont model is provided by the similarity in inhibition patterns and some of the other properties of the mitochondrial and bacterial translational systems; but as we have seen, closer inspection shows that mitochondrial ribosomes, even in unicellular eukaryotes, constitute a separate class, distinct from either those in prokaryotes or those in the eukaryotic cytoplasm. Even the striking analogy of inhibition patterns is by no means perfect: As more and more inhibitors of defined function become available, the analogies are beginning to break down. Furthermore, even those inhibitors that do affect both mitochondrial and bacterial protein synthesis might do so by virtue of different modes of action in the two systems. Although such agents usually interact with various ribosomal proteins in bacteria, in mitochondria they might well affect either ribosomal RNA or some component of the inner membrane that has to interact with the mitochondrial ribosomes for proper function.

6. Similarly, evolution of the ribosomal genes in prokaryotes, in the eukaryotic cell sap, and in their mitochondria appears to have proceeded independently. As measured by base composition and cross-hybridization, there appears to be a great deal more overlap of base sequences with in either of the first two groups (e.g., between yeast and animal rRNA's) then there is between either of these groups and mitochondrial RNA's – or, for that matter, between mitochondrial rRNA of yeast and of animals. Coincident with this evolution of rRNA, which is specified by mitochondrial DNA, there appears to have been a similar divergent evolution of genes for the ribosomal proteins, and these are, as we know, specified by nuclear genes.

7. Attachment of ribosomes to membranes provides the cell not only with an efficient means for the transfer of newly synthesized proteins, but also with a mechanism for the segregation of separate classes of ribosomes engaged in the synthesis of different proteins.[519]

8. Finally, one is perhaps stretching the concept of endosymbiosis to the limit – even considering the prolonged evolutionary history – in postulating a symbiont that has relegated to the nucleus the information for most of its own proteins, including all those required for the replication and expression of its own genetic messages. Mitochondrial RNA and DNA polymerases are enzyme distinct not only from their nuclear counterparts, but from those found in contemporary bacteria as well. Furthermore, the mitochondrial DNA polymerase of rat liver has been reported to be antigenically related to the extramitochondrial enzyme of a number of mammalian species but not to the enzyme from *E. coli*.[520]

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