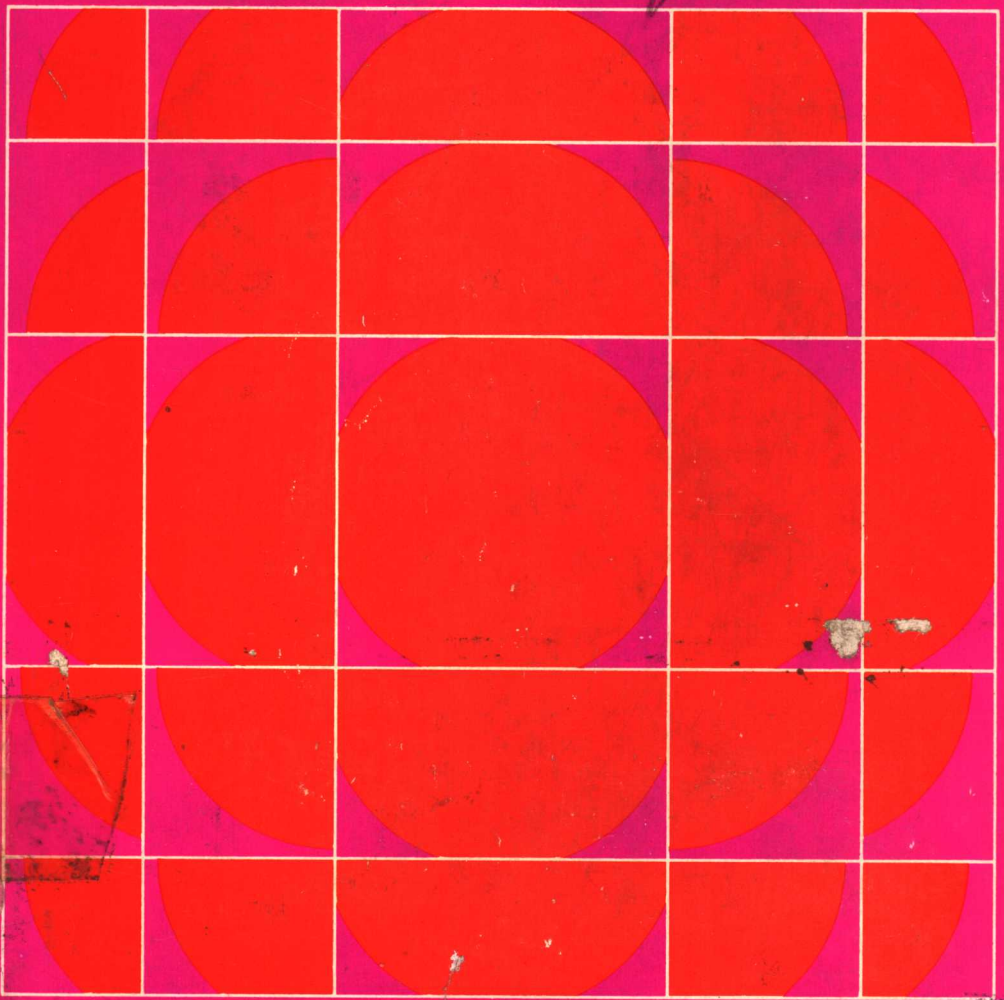
Techniques of Biochemical and Biophysical Morphology

VOLUME TWO

Edited by David Glick and Robert M. Rosenbaum



TECHNIQUES OF
BIOCHEMICAL AND
BIOPHYSICAL MORPHOLOGY

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PREFACE

A pressing need has evolved for review volumes published at regular intervals, usually annually, to aid scientists in keeping up-to-date with developments in instrumentation, methodology, and techniques of biochemical and biophysical morphology. This need has been particularly accentuated by the remarkable expansion of the field in recent years and the growing importance of its contributions to many areas of biological and medical science. Therefore, we have initiated this series of review volumes to serve as an international authoritative source in the field. *Techniques of Biochemical and Biophysical Morphology* is designed to cover important new developments systematically and in a self-modernizing manner.

Each volume will be made up of chapters contributed by recognized authorities having intimate knowledge of, and experience with, the subjects they review. The general plan of the chapters will follow explanation of underlying principles and theory, a critical evaluation of past work, and a presentation of details of the instrumentation, methods, or techniques recommended by the author in a fashion to furnish the qualified scientist with practical laboratory information. To reach the greatest number of interested readers all chapters will appear in English.

The Editors and members of the Advisory Board wish to make this series as useful as possible, and therefore welcome any and all suggestions.

DAVID GLICK
ROBERT M. ROSENBAUM

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TECHNIQUES OF BIOCHEMICAL
AND BIOPHYSICAL MORPHOLOGY VOLUME 2

A Survey of Methods and a Critique of the Study of the Biogenesis of Mitochondria in Yeast

ANTHONY W. LINNANE, G. M. KELLERMAN, AND H. B. LUKINS, *Department of Biochemistry, Monash University, Clayton, Victoria, Australia*

I. PERSPECTIVE SURVEY OF MITOCHONDRIOGENESIS IN YEAST

1. Introduction

A. STATEMENT OF THE PROBLEM

The problem posed in elucidating the mechanism of biosynthesis of the mitochondrion represents a very complex one, involving the incorporation of many components manufactured in different parts of the cell into a multicompartment membrane-limited structure. The organelle has a limited degree of autonomy, in that is possesses its own DNA with genetic functions separate from those of the nuclear DNA, and a protein synthetic system different in many properties from the cytoplasmic ribosomal system. However, the great majority of the mitochondrial protein is manufactured on the cytoplasmic ribosomes, and it is then incorporated into the organelle along with the mito-

chondrially synthesized protein, cytoplasmically synthesized lipid, and cofactors.

B. NATURE OF THE ORGANELLE

From the ultrastructural point of view, the mitochondrion has an outer membrane, an intermembrane space, an inner membrane system with complex folds called cristae, and finally an innermost or matrix space. The membranes have selective permeability properties so that they, and the spaces they delimit, contain specific ions, cofactors, and enzymes. Recent investigations on both mammalian and yeast mitochondria have shown that the inner membrane has extremely plastic properties; its general morphology varies widely and can undergo pronounced conformational changes depending on its metabolic state (1–5). Although much work has been performed on fractionating mitochondria and assaying the enzymatic composition of the fractions, we do not yet have a detailed description of the location of the various components even of the mammalian organelle, on which most of the studies have concentrated. However, there would seem to be general agreement on the localization of some enzymes (for a review, see ref. 6). Thus the outer membrane in the mammalian mitochondrion has been shown to contain monoamine oxidase and rotenone insensitive NADH-cytochrome *c* reductase; work on the outer membrane has been hampered by the difficulty of ensuring that there is no contamination by other cell membranes such as endoplasmic reticulum or plasma membrane fragments. In the inner membrane are localized the enzymes and carriers of the electron-transport chain, including the flavo proteins such as NADH dehydrogenase and succinate dehydrogenase, the cytochromes b, c_1, c, a, and a_3 with their associated nonheme iron and copper proteins, the oxidative phosphorylation system including the "knobs and stalks" and the "translocases" such as the atractyloside sensitive site. The inner membrane has readily recognizable asymmetry; at the morphological level knob and stalk projections are observed on the inside surface, and at the functional level the electron-transport system has been shown, at least in the mammal, to be accessible to succinate and NADH only from the inner (matrix) side, whereas cytochrome *c* appears to have preferential access to the outer face of the inner membrane.

There has been considerable interest in the "structural proteins," which were originally described as apparently a single major constituent of the mitochondrial membrane, devoid of any enzymatic role (7). Their status is now uncertain, as more recently it has been suggested that they are a series of constituents of similar composition, which may have different specific roles in the electron-transport process (8). Further

work has also shown that a significant proportion of the protein originally isolated in the structural protein fraction actually represents a denatured form of the mitochondrial ATPase (9,10). Recently it has been suggested that "miniproteins" of molecular weight of the order of 5000 daltons or less make up a significant proportion of the structural protein of mitochondrial and other cell membranes, in which they may form a series of aggregates (11,12). The position of these miniproteins is still uncertain and their artifactual nature has not yet been excluded with certainty. It would, however, seem reasonable at present to suggest that there are several different insoluble proteins of fairly similar amino acid composition with some role in the organization of the membrane-bound enzyme complexes; there is as yet no unanimity on the exact number or detailed functions. Of special relevance to the present discussion are the suggestions from a variety of experiments that the products of the mitochondrial protein synthesizing system may indeed be proteins of this "structural" type (13–19), which become located in the inner membrane; consistent with this there is good circumstantial evidence that the many components of the mitochondrial protein-synthesizing system and the mitochondrial DNA are themselves closely associated with the inner membrane (15,18). Much current research effort is directed toward the elucidation of the exact localization of these components and the nature and extent of their interaction with other membrane components.

The real problem of mitochondrial biogenesis is that the component proteins are largely coded by nuclear DNA and synthesized on the cytoplasmic ribosomes, yet they become located not only in the readily accessible outer membrane but also in the inner membrane and matrix space of the organelle. The two genetic and protein synthesizing systems—mitochondrial and extramitochondrial—are closely coordinated and controlled during this biogenesis, so that a balanced output of the many components is achieved during all phases of cell growth and differentiation. The study of such complex interacting systems is difficult, and so the most suitable experimental situations have been exploited as much as possible.

C. ADVANTAGES OF *Saccharomyces cerevisiae* AS AN EXPERIMENTAL ORGANISM

The facultative anaerobic yeast *Saccharomyces cerevisiae* offers perhaps the most suitable single system for a multidirectional attack on the problem of mitochondrial biogenesis. One of its major advantages is that it can survive, grow, and reproduce in the complete absence of mitochondrial oxidative functions by utilizing fermentation processes

as a source of energy, whereas under normal aerobic conditions the organism can grow on a variety of nonfermentable substrates, utilizing its mitochondrial oxidative phosphorylation pathways as a source of energy. Cells cultured under any of a variety of conditions which lead to the elaboration of incompletely formed mitochondria, enable the effects of appropriate factors to be studied *in vivo* at the intact cell level, as well as with isolated mitochondria *in vitro*. The ability of the mitochondria to generate energy can be abolished by a variety of treatments, such as anaerobiosis, the presence of certain drugs and antibiotics that inhibit the elaboration of protein or nucleic acids by the organelle, and by genetic mutations, some nuclear and some mitochondrial in location, which lead to the loss of essential components of the mitochondrion itself. As an additional variable parameter, the organism is subject to catabolite repression, so that even under conditions of aerobic growth there is an incomplete maturation of mitochondria in the presence of readily fermentable substrate in the culture medium. Another major advantage is in the genetic field, where investigations are facilitated by the fact that both haploid and diploid forms of the organism can grow vegetatively so that it is relatively easy to study both nuclear and cytoplasmic genetic phenomena. The organism has a conveniently short generation time, of the order of a couple of hours, and mostly it grows as discrete cells rather than large mycelial aggregates so that studies involving plating are simple. It thus becomes possible to exploit many of the advantages of bacterial genetic technology in the study of cellular organization and control in a eucaryote, and the knowledge gained in this way represents an important step towards an understanding of the mammalian system.

D. SCOPE OF PRESENT REVIEW

In the present review we have concentrated our discussion on the techniques currently employed in our laboratory, especially with reference to those aspects of the problem for which yeast is particularly suitable. Thus we have devoted space to the details of genetic manipulations in yeast and we have collected information on cultural conditions and the control of these factors. We have not written in extensive detail on procedures widely used in other fields, such as nucleic acid handling, but we have concentrated on those modifications of existing technology of special importance in yeast mitochondrial investigations. Again, we have concentrated on description of methods tested in our laboratory and have merely quoted other methods with which we have no personal expertise.

A theoretical discussion of the general problem of mitochondrial biogenesis is included but has been held to the minimum sufficient to give perspective and coherence to the technical descriptions; several detailed theoretical reviews of the field have been published recently (20–27), and proceedings of three meetings devoted to the discussion of these problems have also appeared (28–30).

2. Mitochondrial DNA

A. SIZE AND PHYSICAL NATURE

There is as yet no final definition of the information content of mitochondrial DNA in any organism; there is at least very strong evidence for the existence of 5-μ closed circles as the basic component in mammalian mitochondria, but the evidence from yeast mitochondria is less definite. The largest circles reported from this organism are of the order of 28 μ, corresponding to 54×10^6 daltons or 27×10^6 daltons in the sense strand (21,31,32). However, these large circles are accompanied by considerable amounts of DNA in linear and also some smaller circles in all preparations; it has not yet been established whether these are all true components of the organelle DNA or whether they reflect fragmentation during the isolation procedure. Even if it is accepted that the basic units are of the order of 5 μ in the mammal and 28 μ in yeast, the problem remains as to whether these are single species, or whether there are multiple types of DNA in any given mitochondrion. In yeast, calculations based on organelle number and total DNA content or on total extruded length of DNA from one organelle (33) suggest that there may well be at least two pieces of DNA per mitochondrion. On the other hand, estimation of information content based on renaturation kinetics of heat-denatured DNA has been interpreted to indicate that the total information content of the mitochondrial DNA is of the order of 5 μ in the mammal and about 28 μ in yeast, corresponding to the presence of the one unique species of DNA in each mitochondrion (34). As the precision of the method is limited, the question is still open, and any calculations of possible genetic roles of this DNA can thus refer only to a minimum position.

B. COMPOSITION OF MITOCHONDRIAL DNA

Wild-type (ρ^+) yeast mitochondrial DNA has a buoyant density of 1.683 g/ml and is readily separated from the major nuclear band at 1.700 g/ml on a CsCl gradient. Although this buoyant density, corresponds to a guanine + cytosine (G + C) content of 23% on the usual

formula (35), there is direct chemical analytical evidence that the actual G + C content is lower, estimates of 17% (36) and 21% (37) having been published. There is evidence, then, not only of an extremely low G + C content, but also of some difference in structure, at present uncharacterized, leading to unusual relationships between buoyant density and base composition (36). Mitochondrial DNA usually comprises of the order of 15% of the whole cell DNA. The DNA of crude isolated mitochondrial fractions is usually contaminated with about 50% of nuclear DNA (38).

C. MITOCHONDRIAL DNA IN PETITE (ρ^-) CYTOPLASMIC MUTANTS

The mitochondrial DNA in a variety of petite (ρ^-) cytoplasmic mutants has been found to have a buoyant density equal to or less than that of the ρ^+ cell by a number of laboratories (39–42); there was a single report some time ago of one petite mutant whose DNA had a higher buoyant density than normal (39). There have been reports of G + C contents from 4 to 15% in petite *m*DNA samples. The size of these DNA molecules has been reported to include 0.5- to 2.5-μ circles (31–33) and linear pieces up to 10 μ or even larger, although all pieces seem to be significantly smaller than the normal, now believed to be 28-μ circles (32). It is of particular interest that we have found in several petites containing *m*DNA, that the absolute content of this component in the cell is about the same as in the wild type, or only a little less (38). The combination of a normal amount per cell and a smaller size of the individual pieces implies that there are many more pieces of *m*DNA per petite cell than in the normal cell. Whether these extra pieces of *m*DNA represent random scission of the normal molecule, or selective replication of a limited region, is not established, but we have some suggestive evidence that the latter possibility may obtain (42*a*).

D. CYTOPLASMIC PETITE MUTANTS (ρ^0) THAT LACK *m*DNA

Of special interest in the study of the petite mutation has been the isolation, from cultures treated with high levels of mutagen, of several such mutants that lack any demonstrable mitochondrial DNA (38,43). This conclusion is based on the absence of any peaks from a CsCl density gradient of whole-cell DNA other than the nuclear DNA peaks; the lack of any peaks, other than nuclear DNA, in a gradient run with DNA extracted from isolated mitochondria; the complete destruction of all mitochondrion-associated DNA by DNAase, which presumbably has access only to the nuclear DNA contaminating the mitochondria.

Even the cycloheximide method for the preferential radioactive labeling of mitochondrial DNA (44) has not enabled the demonstration of this component in these mutants. In all these cases, gradients were extended to include a region of density well below any feasible alteration in G + C percentage, even including the region of the dAT polymer. There is the theoretical possibility that extremely small pieces of DNA material (of the order of 2×10^5 daltons or less) could be present but not seen because of their inability to form a recognizable band due to their high diffusibility; however, there was no excessive A_{260} in the region in the form of a raised base line, which this hypothesis would require. Even if such small pieces of material were present, they may well be too small to have any informational significance.

This ρ^0 mutant offers the possibility of definitive identification of which of the mitochondrial proteins are nuclear coded, since any component found in such a cell must have its information coded in the nucleus. Futhermore, as the mitochondrial ribosomal RNA is coded by *m*DNA (*vide infra*), all proteins present in the ρ^0 mutant must have been synthesized on the cytoplasmic ribosomes.

E. NATURE OF GENE PRODUCTS OF *m*DNA

The mitochondrial DNA has been shown to hybridize with mitochondrial RNA, both ribosomal and transfer (45–48). Assuming that the ribosomal RNA contains between 1 and 1.5×10^6 daltons (see page 43) and that notionally 20 *t*RNA molecules contain 0.5×10^6 daltons, a minimum of 1.5 to 2×10^6 daltons remain in the mammalian organelle and 23×10^6 daltons in yeast. These could potentially code for other products such as proteins, or be taken up in repetitive cistrons for RNA fractions. Evidence on the number of cistrons in *m*DNA for ribosomal RNA is not conclusive, as calculations based on published hybridization percentages in yeast give apparent values of 0.5 to 2 cistrons/genome (45,48), but it appears likely to be one copy per genome. In one sense, the very low G+C percentage of the yeast *m*DNA (36) does limit the amount of usable information it contains. It has been calculated (49) that as a functional messenger RNA should have at least 40% G+C even when using the codons with the lowest G+C for each amino acid, less than one-half of the total yeast *m*DNA (G+C less than 20% could be transcribed into messenger RNA. Even this amount requires long stretches of almost pure A+T content to lie between the functional G+C rich regions, so that there may well be even less than one-half of the total genome capable of coding for protein molecules. The difference in the sizes of yeast and mammalian *m*DNA [whose G+C content (21) is sufficiently high for it to be considered as all of

potential coding function] is thus probably much greater than the difference in their actual coding potential. Whatever the ultimate answer may be, there is strong circumstantial evidence from studies on antibiotic resistant mutants in yeast that at least some of the mitochondrial ribosomal proteins may be coded by the *m*DNA (50) and also that some membrane proteins may also be mitochondrially coded (51). By analogy with some mutations in *Escherichia coli* (52) where erythromycin resistance results from changes in the degree of methylation of ribosomal RNA, we suggest that similar changes may also be responsible for certain antibiotic resistance mutations in yeast mitochondria. Although in yeast the available information appears on the surface to be more than enough for a complete set of ribosomal proteins, in the mammal the information left after deducting that transcribed into ribosomal and transfer RNA corresponds to only about 3000 to 3500 amino acids *in toto*. It is conceivable that the "miniribosome" recently described in mammalian mitochondria (53,54) actually does have a small number of protein components, representing the minimum number for a functional ribosome, and corresponding to this small amount of information; alternatively, it may well be that only some of the ribosomal proteins are coded by the *m*DNA, and the others by the nucleus. In either case, there appear to be major evolutionary differences between yeast and mammal in the role of the *m*DNA.

The other approach to identification of the gene products of the *m*DNA has been the study of mutants with alterations in this component; these include the petite mutation originally described by Ephrussi (55) and the more recently discovered cytoplasmic mutants for antibiotic resistance (50,51,56–58). The interplay of these mutations has enabled us to recognize that there is a whole range of petites (59,60), the characteristic single feature of which appears to be lack of the mitochondrial protein-synthesizing system including the mitochondrial ribosomal RNA (13,45). The information sites for antibiotic resistance may or may not be involved in the petite mutational event, as in some strains they can be recovered after appropriate genetic crossing whereas in others they are lost (59,60). As already discussed, there is evidence that the various petite mutants possess different lengths of *m*DNA (32,33), different in base composition and buoyant density (39–42), and that the extreme position occurs with the ρ^0 cell that possesses no demonstrable *m*DNA (38,43). To date, investigation of the ρ^0 cell has shown that it contains the mitochondrial RNA polymerase (61), which appears to establish that this enzyme is neither coded nor manufactured in the organelle. Experiments on enzyme identification in the "petite" will now have to be reinterpreted in terms of the actual genetic lesion

in the petite mutant used; in many cases only the ρ^0 mutant will be able to supply a definitive answer, as already discussed.

3. The Mitochondrial Protein-Synthetic System

A. NATURE OF THE MITOCHONDRIAL RIBOSOME

The RNA of the yeast mitochondrial ribosome has been found to sediment at rates corresponding to approximately 21 and 15*S*, with a G+C content of about 26%; these figures are quite distinct from those of the yeast cytoplasmic ribosomes, whose components sediment under the same condition at 25 and 17*S* and whose G+C content is 47% (48,62, 63). In size, the mitochondrial ribosomal RNA would seem to resemble the bacterial species, whose sedimentation rates under these conditions are 23 and 16*S*. There have been many attempts to isolate yeast mitochondrial ribosomes in an active form free from other constituents (48,64–70); these have had limited success and the apparent size of the ribosome has varied from 70 to 80*S*. A recent report (71) describes successful isolation of the active ribosome and finds it to be 74*S* in type. During subsequent evolution the mitochondrial ribosome and its RNA appear to have decreased in size, as reports on the mammalian system suggest a 55 to 60*S* ribosome in which the smaller species of RNA sediments at only 12*S* while the larger species of RNA has been described variously as 21*S* (54) or 16*S* (53). In *Neurospora crassa* some *t*RNA molecules have been found to be specific for the mitochondrion and several of the aminoacyl *t*RNA synthetases are also specific for the organelle (72–74). To date, a complete count is not available for either of these components, but since at least some of the *t*RNAs have been shown to hybridize with *m*DNA (45), one may anticipate that ultimately the organelle will be shown to possess a complete set of specific *t*RNA species with at least one for each amino acid. The initiation mechanism in mitochondria has been shown to involve the formyl methionyl *t*RNA mechanism (75), and this specific *t*RNA has been shown in yeast to hybridize with the mitochondrial DNA (76). One difference between bacterial and mitochondrial ribosomes is seen in the reports that no 5*S* RNA species could be isolated from *N. crassa* mitochondria (77) or yeast mitochondrial ribosomes (71).

Further resemblances between the yeast mitochondrial protein synthetic system and the *E. coli* system are seen when the elongation factors (G,T_s,T_u) are examined; a fraction isolated from mitochondria that contains all three factors stimulates protein synthesis by isolated *E. coli* ribosomes, whereas the similar fraction isolated from the cytoplasm has little stimulatory effect at comparable protein concentration; only at