CYTOPLASMIC GENES: OUR PRECAMBRIAN LEGACY

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I am deeply grateful to Professors GORDON KIMBER and C. SHIELDS GOWANS for inviting me to participate in this second symposium honoring DR. L. J. STADLER. Like an entire generation of genetics students I remember clearly the fantastic impact of questions raised by Stadler's classical paper "The Gene" (STADLER 1954). As an invited guest, too, I am able to utilize this opportunity to record my personal analysis of some problems of cytoplasmic heredity. I know from previous experience that these ideas might not be accepted by many standard journals and therefore I warn you not to accept uncritically anything I say here. Rather, I invite your comment.

THE FOSSIL RECORD AND CELL STRUCTURE

The earth is approximately 4500 million years old. The oldest rocks are greater than 3000 million. It has lately been realized by both sedimentary geologists (RAMSEY 1963, HOFFMAN 1968, RUTTEN 1969) and paleontologists (BARGHOORN and SCHOPF 1966, SCHOPF and BARGHOORN 1969, CLOUD 1965, 1968, LABERGE 1967) that from 3000 million years ago until virtually the present we have in many places in the world a wide distribution of unmetamorphosed sedimentary rocks. The Phanerozoic fossil record began about 620 million years ago and fossiliferous rocks deposited after that time have made immeasurable contributions to our concepts of organic evolution. I will make a plea that those of us interested in cellular evolution can no longer dismiss the utility of the Precambrian record. GLAESSNER (1968) who has spent many years studying the origin of Cambrian fossil groups has decided: "there are undisputed metazoan remains in the Vendian" ...(about 700-550 million years ago); his major conclusions of recent intensive studies of the Precambrian record of life are

> "(a) the main diversification of life occurred not earlier than in Late Proterozoic time and
> (b) only primitive plants (and bacteria) are recorded definitely from many sediments of Middle and Early Proterozoic age..."

(The Proterozoic began 2500-1000 million years ago.) (GLAESSNER 1968). Translated into modern biological terms these two paleontological conclusions are

(a) The diversification of the highest taxa (Kingdoms Animalia, Plantae and Fungi; WHITTAKER 1969) occurred in the Late Precambrian (Proterozoic). Therefore aerobic eukaryotic cells containing chromosomes with their haploid-diploid meiosis-fertilization cycles, mitochondria, chloroplasts and so forth must already have evolved.

(b) Evolution of bacteria and blue green algae occurred much earlier. The algae left "stromatolitic remains" and direct evidence for photosynthetic activity at least 2500

million years ago, perhaps as long ago as 3100 million years (BARGHOORN and SCHOPF 1966).

ORIGIN OF EUKARYOTES BY SERIAL SYMBIOSES

I have already presented an explicit and testable model for the origin of nucleated cells (SAGAN 1967). An expansion of this outline describing the evolutionary relationship between the Early Precambrian prokaryotic cells and the Late Precambrian eukaryotic cells will be published this year by Yale University Press (MARGULIS 1970b). The eukaryote cell is considered to be a product of temporally ordered, specific symbioses, outlined as equations in Table 1, shown diagramatically in Figure 1. The justification, explanations and geological implications of the model have been discussed in detail elsewhere. Based on the symbiotic theory both prokaryote and eukaryote phylogenetic trees have been drawn (MARGULIS 1968, 1969, 1970b).

Table 1 Prokaryote components of eukaryote cells (Kingdoms after WHITTAKER 1969).

I.	Kingdom MONERA (in order of evolution, from Early to Middle Precambrian)	
	(1) anaerobic heterotroph (Embden-Meyerhof fermentation=protoeukaryote)	
	(2) motile anaerobe=(spirochete-like protoflagellum)	
	(3) photoautotroph (CO_2 fixation, O_2 elimination)= photosynthetic protoplastid	
	(4) aerobic heterotroph (respiration via Krebs cycle)=protomitochondrion	
II.	Kingdom PROTISTA (in order of acquisition of organelles)	
	(1) + (4) + (2)	= protozoans, slime molds, chytrids, etc.
	(1) + (4) + (2) + (3)	= nucleated algae: brown and red seaweeds, green algae, euglenids, diatoms, etc.
III.	Kingdom FUNGI	
	(1) + (4) + (2)	= zygomycetes, ascomycetes and basidomycetes
IV.	Kingdom ANIMALIA	
	(1) + (4) + (2)	= metazoans
V.	Kingdom PLANTAE	
	(1) + (4) + (2) + (3)	= green plants: bryophytes to angiosperms

This theory was originally constructed to comprehend extrachromosomal inheritance data. Intracellular symbiosis is postulated to have been a major mechanism in the origin of eukaryotes predicting

certain discontinuities in both cell types (for example between blue green and green algae) and the fossil record. The extent to which the biological model has turned out to fit paleontological data has been gratifying.

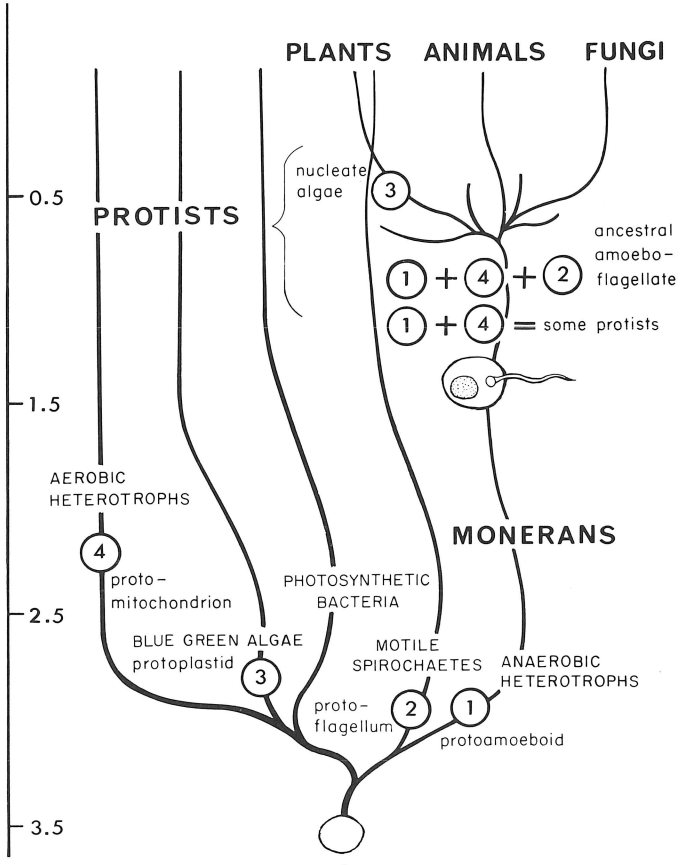

Figure 1. Evolutionary tree of organisms constructed on the basis of a symbiotic theory. Time scale indicates billions of years back from present.

It is neither possible or necessary to reiterate the symbiotic theory of the origin of higher cells from the simplest replicating entities; however, the salient points will be reviewed here in order to show its relationship to certain problems of cytoplasmic heredity. Table 2 lists in a simplified manner the minimal requirements for all chemical self-replicating systems as we know them on earth today... i.e. cells. Prokaryote cells (bacteria and blue green algae) are monogenomic and contain the elements of the cellular system (Table 2); they have been evolving by accumulation of all kinds of mutations since the Early Precambrian. The eukaryotes, however, are all considered polygenomic. A mean number of genomes of three have been postulated for the animal protists and metazoans: nucleocytoplasm, mitochondria, (9+2) homologue; and a mean number of four for the eukaryotic algae and green plants: nucleocytoplasm, mitochondria, (9+2) homologue, and photosynthetic plastids. Subsequent to the acquisition of free-living cells as endosymbionts, of course, natural

selection did not stop. Endosymbionts evolved into organelles; these symbiotic complexes evolved and diversified a great deal. "Cytoplasmic heredity" is interpreted as the observable manifestation of the multigenomes of eukaryotes. Recently many authors have explored evidence that mitochondria and chloroplasts contain elements of the cellular system (BORST, KROON and RUTTENBERG 1968, NASS 1969, COHEN 1970, WAGNER 1969, RAVEN 1970, ROODYN and WILKIE 1968). Examples of most of the items in Table 2 have been localized within mitochondria and chloroplasts from various sources. The aspects of the model that postulate independent origin of chloroplasts and mitochondria are the easiest to defend because both these organelles contain rudimentary protein synthesizing systems.

Table 2 Major minimal elements of the self-replicating cellular system

DNA (genome weight at least 2.6 x 10^8 daltons according to MOROWITZ 1967)
messenger RNA colinear with the DNA
polymerase enzymes:
DNA polymerase
DNA-dependent RNA polymerase
activating enzymes, approximately 20 different, one each for each of the 20 amino acids
transfer RNA molecules, approximately 20
incorporation of amino acids into protein. ribosomes: ribosomal RNA and protein; protein synthesis factors
lipid membrane
fermentable carbohydrate or other source of energy resulting in ATP production
particular product or activity selected by environment to perpetuate replicating system

The theory of the symbiotic origin of the mitotic eukaryotic cell, however, includes the concept of an exogenous origin for the basal body "(9+2) homologue." It is thought that the genomes of original episymbionts, selected for because they conferred motility on the ancient ancestral heterotroph, underwent profound diversification. Differentiating into basal bodies and their flagella they are considered to have finally developed into the centrioles and chromosomal centromeres of eukaryote mitosis and meiosis. That is, the entities manifesting themselves variously as (9+0) basal bodies or centrioles; axonemes; chromosomal centromeres; (9+2) cilia or longer flagella are claimed to be phenotypic expressions of ancient self-replicating spirochete-like cells. Originally motile symbionts (analogous to the similar but much later development of epibiotic cortical spirochetes in Myxotricha, GRIMSTONE and CLEVELAND 1966), by hypothesis, they differentiated to form cortical patterns of protists as well as the achromatic mitotic apparatus in nucleated cells. Superficially kinetosomes and basal bodies have been seen to divide (LWOFF 1954); however electron microscopic studies have permitted the

recognition of complex developmental cycles in which new basal bodies are formed in various specific relations to parental ones depending on the cell system involved (MIZUKAMI and GALL 1966, CROCKER and DIRKSEN 1966). There is very little direct evidence for centriolar or centromeric DNA associated with the microtubular components of these organelles (but see SMITH-SONNEBORN and PLAUT 1969, PARDUE and GALL 1970) and certainly no one has claimed basal bodies have their own independent protein synthesizing systems. Critics ask, "Upon what hard evidence can you base your claim for independent origin of the basal body-centriole system?" This confronts us squarely with the issue. Hereditary endocellular symbionts will behave at first as extrachromosomal genetic determinants. As they become progressively more integrated into the cell, however, in the limit will it be impossible to reconstruct this evolutionary history? What are possible genetic consequences of the merging of independent genomes?

EXTRACHROMOSOMAL INHERITANCE

JINKS (1964) has summarized the criteria that must be fulfilled before entities may be considered inherited extrachromosomal determinants. "They must determine some characteristic of the cell. They must duplicate and be self-duplicating in the sense that, if lost, they can not be regenerated by chromosomal material. They must be distributed to daughter cells during cell division" (JINKS 1964, p. 6). The smallest entities to fulfill these criteria are the viruses and episomes of bacteria. Viruses and episomes contain nucleic acid and protein and of course are obligate parasites on the protein synthetic system of the host cells. It is only because the reproductive rates of such duplicating particles may be different from the host bacterial genomes that their existence can at all be fathomed. The largest entities to fulfill Jinks' criteria are entire complex eukaryote cells themselves; for example, the endosymbiotic chlorellas of Paramecium bursaria (KARAKASHIAN and SIEGEL 1965). If morphology were insufficient, differential growth rates and independence of the algae from paramecium nuclear genes would allow us to identify these "plasmons." It seems that "bearers of extracellular hereditary information" must fall somewhere on the scale between naked nucleic acid and the eukaryote cell. LWOFF (1954), JINKS (1964), PREER (1969) and others have argued acceptably that basal bodies may be considered extrachromosomal genetic determinants. Of course this is necessary but not at all sufficient to prove they originated by symbiosis.

Although we are always looking at cytoplasmic genes at a single point in time, the present, they all have had a history. The most simple hypothesis of their origin is either by escape of a part of the genome from a host cell or by degeneration to organellar status from a population of once free-living cells. If the evolution of cell partners in a symbiotic complex has occurred over very long periods of time, distinguishing between these possibilities becomes extremely difficult. We claim chloroplasts and mitochondria are "plasmagenes" with an exogenous origin mainly because they are the size of and contain many of the components present in analogous free-living entities that have survived in a free-living form until the present (e.g., blue green algae and aerobic eubacteria, respectively). The origin of the (9+2) homologues is far more complex. Are they escaped fragments of chromosomal genomes or were they once a population of free-living cells? We will not be able to decide here of course, but can we determine the information we would like to have in order to eventually decide?

The meiosis-fertilization patterns of eukaryotes insures the male gamete an equal share of chromosomally determined posterity. It is the essentially negative fact, namely the lack of an equal

bi-parental contribution in a well-marked cross, that allows geneticists to identify "extrachromosomal inheritance" in the first place. Those cases where a morphologically distinct agent has been correlated with the inheritance pattern (spirochetes in sex-ratio inheritance, POULSON and SAKAGUCHI 1966; Blochmann bodies in insects, LANHAM 1968, and so forth) have been accepted as symbioses. They provide us with model systems and we must learn from them (KARAKASHIAN and SIEGEL 1965).

After the establishment of intracellular symbioses, if selection continues to act mainly on a character that is the product of two independent genetic systems and redundancies are eliminated, in what possible directions may the partners co-evolve?

Let us take for an example an isogametic eukaryotic diploid organism that undergoes gametic meoisis, such that a cross between any heterozygotes (say Aa and Aa) will give us the expected mendelian 3:1 phenotypic or 1:2:1 genotypic ratio of A-:aa or AA:Aa:aa respectively. Let us say such an organism harbors an intracellular population of about 10^3 bugs (call them β). Let us say that the product of the nuclear A gene is some nutrient, an amino acid, required in high concentrations for the continued intracellular replication of the β's. If no cytoplasmic exchange occurs at fertilization in crosses between heterozygotes in which only one partner harbors the bug, cytoplasmic inheritance will apply but of course if cytoplasmic exchange occurs β's will be inherited in all offspring of a given cross regardless of nuclear genotype. However the aa genotype will be unable to sustain the β's for more than about ten divisions.

For convenience we shall assume that in this imaginary species stable haploid cells can be propagated asexually. What sorts of mutations can occur in this system? If in a haploid line A mutates to a and the lack of the biosynthesis of the amino acid is not deleterious to the host, the host will lose the β particles in a number of generations roughly equal to N where 2^N is the number of original β's. As long as one β is present, the crossing of gene A back into this cytoplasm will permit reestablishment of the original number of particles. This sort of analysis has been made for kappa and related particles and nuclear genes like K in paramecium (see PREER 1969). It probably applies equally well to the behavior of the "ρ particle" in yeast that requires the presence of P genes for expression of the ρ+ phenotype (MOUNOLOU et al. 1968), where at least some genes of the ρ_n series are equivalent to lysine auxotrophy.

Each symbiont itself, β, of course, contains an entire genome and potentially can mutate. For example it may mutate to a form no longer requiring the product of nuclear gene A. To the geneticist this would simply mean he had lost the ability to resolve a nuclear genetic controlling element over the development of the symbiont, namely, genotype A- and aa would no longer be distinguishable. The β's might also mutate to some less effective form of beta (β^x), any selection pressure would tend to maintain the optimally adapted β's, of course, so that the probability of picking up one mutated β^x in 10^3 optimally adapted β's is vanishingly small. From a formal viewpoint the genetic element can be considered to be "a thousand ploid" instead of diploid. It is clear therefore that nuclear mutations affecting the development of the symbiont will be much easier to discover than β mutations simply as a consequence of the meiotic-fertilization mechanism that insures regular haploid-diploid alternations of the nucleus of the host. Formally this situation is quite comparable to the problem of bacterial genetics in species in which recombination systems have not been discovered. No one doubts that these bacteria have genes however, lacking alternative alleles and a recombination system it becomes impossible to observe them. These genes are not "less mutable," they simply are less available for study.

Following this example further, let us suppose that 20 independently isolated host nuclear mutations, in many different cistrons a_1-a_n can be recovered. Assume they affect different points in the biosynthetic pathway which leads to reduced intracellular concentrations of the amino acid. As long as a mutation leads to amounts of the amino acid too low to support the β's, when placed into the same diploid cell, for example, erroneously we will conclude a_3 and a_5 belong to the same cistron. If a_3 and a_5 map at very different places on nuclear chromosomes and a_3 and a_5 (Table 3) will not complement, the alternative explanation (lack of complementation does not imply different cistrons but only that the two sets of alleles affect some common need provided by the host for the symbiont) is much more likely. In discussing paralyzed flagella mutants, STARLING (1969) concludes: "...if two mutations in different cistrons, are introduced into the same diploid nucleus, complementation should occur and the progeny should be phenotypically wildtype. Conversely, if two mutations in the same cistron are introduced, the progeny should be paralyzed." He concluded that since any two of the pf18 mutants when together in diploids do not complement but give rise to paralyzed flagella that "all 7 alleles are in the same cistron." Perhaps when he finds the noncomplementing mutants map at different chromosomal loci he will consider these alternative possibilities. Comparable to the example of the β's above, different nuclear cistrons all affecting the development of normal microtubule flagellar protein, may just as easily be involved (Table 3).

Table 3 Possible explanation for noncomplementation of independent nuclear mutants (a) affecting genetically autonomous cytoplasmic particles (β)

Cytoplasmic particles carried in diploid hosts. The mutant forms (a_1, a_2, a_3, a_n) of genes (A_1, A_2, A_3, A_n) of the host organism result in lowered intracellular concentrations of a required amino acid. At least 15 units of this amino acid must be available in order for β particles to complete their life cycle normally in the host cytoplasm.

Parental genotypes and phenotypes	Genotype of offspring	Arbitrary units of amino acid*	Phenotype of offspring
$\frac{a_3}{a_3}\ \frac{+}{+}$ X $\frac{+}{+}\ \frac{+}{+}$	$\frac{a_3}{+}\ \frac{+}{+}$	15	wildtype
mutant wildtype			
$\frac{+}{+}\ \frac{a_5}{a_5}$ X $\frac{+}{+}\ \frac{+}{+}$	$\frac{+}{+}\ \frac{a_5}{+}$	15	wildtype
mutant wildtype			
$\frac{a_3}{a_3}\ \frac{+}{+}$ X $\frac{+}{+}\ \frac{a_5}{a_5}$	$\frac{+}{a_3}\ \frac{a_5}{+}$	10	mutant
mutant mutant			

*For clarity it is assumed that all + alleles contribute "five units" of the amino acid.

MUTUAL DEPENDENCY EXAMPLES FROM THE SYMBIOSIS LITERATURE

It is not possible in even several volumes to review properly the literature on intracellular symbioses. We will just mention some

known cases (discussed in HENRY 1966) to review in principle, the possible types of relationships between the partners. Many protozoans harbor prokaryote microbes, or well-adapted particles like κ,λ and u in Paramecium that presumably have ultimately originated from bacteria. Nuclear genes are known that (1) are necessary for the support of the particles (2) that destroy the particles (the "S" series) and (3) that have no effect on the particles. For the most part products of these genes are still unknown (PREER 1969). The presence of intracellular particles is often correlated with the abolishment of a nutritional requirement (e.g., lysine in Crithidia); this implies of course that the particles supply nutrients to the hosts. Very often organisms or cells harboring symbionts are able to survive under conditions that would not support the host alone, e.g., Paramecium bursaria under autotrophic conditions (KARAKASHIAN and SIEGEL 1965) and insects in the absence of nitrogenous growth requirements (LANHAM 1968). Morphological modifications may follow, e.g., the absence of excretory organs in some aphids where the Blochmann bodies are presumed to be utilizing their nitrogenous wastes (LANHAM 1968).

New metabolic products, for example the lichenic acids, seem to be products of the two partners, the algae and fungi. Replacement of the blue green alga by the green alga in the Cladonia symbiosis changes the nature of the carbohydrates stored by the lichen. Nutritive level symbioses are well known (SMITH et al. 1969) and their implications for genetics very similar to the hypothetical example, the β bugs above.

The nitrogen fixing system in legumes is another example of a process of high selective advantage (the ability to fix atmospheric N_2) under the control of two partners, the bacteroids and the leguminous plants. If DILWORTH and PARKER (1970) are correct, permanent storage of genetic material of the bacterial symbionts in the nucleus of the host plants may have evolved. We must be aware, therefore, of the possibility that exogenous symbiont DNA may ultimately be found in a nuclear location. What are the implications here for basal body origin?

Flagellar regeneration is sensitive to cycloheximide. Flagellar microtubule protein is probably entirely made on cytoplasmic ribosomes (ROSENBAUM 1970). If it were of exogenous origin the entire protein synthetic system of the "protoflagellum" may have been selected against throughout time leaving only the nucleic acid replicatory system of the original symbiont. At present the (9+2) homologue genome may code for only a few microtubule proteins and insure its own replication. We can not prove past events occurred. We may claim only that the "independent origin" concept is the optimal answer to a series of questions: why are basal bodies semi-autonomous extrachromosomal determinants? Why are they structurally related to centrioles? Why are they very constant in size and basically the same structure in all eukaryotes? Why do they have their own nucleic acids (if they have)? Why do they resemble free-living spirochetes (if they do)? Why does the centriole-flagellar system show immense variations related to mitotic division figures in protists? Why do many different chromosomal loci affecting flagella lead to the same phenotypic expression? Why do non-complementing loci affecting flagella development map at different places on the nuclear genome (if they do)? Why do certain treatments affect the numbers of centrioles per cell but none alter the size? We may never really know.

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