

WHAT THE PAPERS SAY

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ROOTS

The Discovery of Chloroplast DNA

John T. O. Kirk

'Roots' presents articles on landmark discoveries that laid the basis for contemporary molecular and cellular biology. In the October issue of *BioEssays* (vol. 3, no. 4, p. 180), Ruth Sager described the early genetic work on chloroplast DNA in *Chlamydomonas*. Here, J. T. O. Kirk describes the early parallel work on the discovery of higher plant chloroplast DNA with biochemical methods.

Summary

In the space of three years – from 1962 to 1964 – the proposition that chloroplasts contain their own DNA made the transition from being a controversial hypothesis to an accepted dogma. The crucial evidence came from biochemical analyses of the organelles themselves and from cytological studies. These discoveries revolutionized our views on the distribution of genetic information within the cell, and gave rise to the vigorous new field of chloroplast molecular biology. It is nevertheless ironic to recall that of the biochemical papers published on this topic in 1962–64, the one which was probably the most influential in creating the new paradigm was in fact quite wrong, and attributed to the chloroplast, DNA species which did not in reality belong to this organelle at all.

Chloroplast DNA was not discovered all of a sudden. There is no one experiment that we can point to – such

as the 'Pajama' experiment in the case of gene repression, described by Arthur Pardee in an earlier paper in this series – which revealed its presence. Rather there was first an accumulation of suggestive, but inconclusive, evidence over quite a long period, which was then followed by a crystallization in the form of a few decisive biochemical and ultrastructural studies leading, after only minor initial resistance, to the universal acceptance that chloroplasts do indeed contain their own genetic material in the form of DNA.

The presence of DNA in chloroplasts and other plastids had long been suspected. The discovery of maternal, and other non-Mendelian patterns of inheritance of some chloroplast defects in higher plant species by Correns, Baur and others in the early years of the century led to the supposition that these organelles contained within themselves at least some of the genetic information which determined their development. When, in the late 40s and 50s, it became clear that, except in certain RNA viruses, genetic information is stored in the form of DNA, people naturally began to wonder whether chloroplasts also contained DNA. Many cytological and chemical studies were carried out but gave conflicting and inconclusive results (I give an account of this early period elsewhere).¹ As so often happens, the matter had to await development of new techniques – in this case of organelle

purification, DNA isolation and analysis, and electron microscope methodology – before it could be resolved. Its resolution – so far as the biochemical evidence was concerned – was, however, by no means as clear-cut as at first seemed to be the case. Indeed the biochemical world generally, although accepting unquestioningly that chloroplast DNA existed, laboured for some years under a serious misapprehension as to which of the various DNA species that had been described in the literature actually belonged to the chloroplast. It is to this part of the history of chloroplast DNA that I propose mainly to direct my attention in this essay.

To the historian of science, the most piquant aspect of the discovery of chloroplast DNA is that the paper which was most influential in persuading the scientific community that chloroplasts really do contain their own DNA was, in its scientific content, if not its broad message, substantially incorrect, while another paper on the same topic from a different laboratory at about the same time, which got the science right, was for several years largely ignored. The crucial period within which the proposition that there is DNA in chloroplasts made the transition from being a popular hypothesis to an accepted dogma was 1962–4. In 1962, Ris and Plaut² at the University of Wisconsin published light and electron microscope pictures of *Chlamydomonas*

chloroplasts which provided much more persuasive evidence for the presence of DNA than any of the previous cytological studies. At the same time as this cytological work was going on, attempts were being made in various laboratories to isolate chloroplasts free of nuclear material, extract DNA from them, and determine whether it differed in its properties from nuclear DNA (as evidence that it did not originate simply in nuclear contamination). In 1963 two papers appeared independently reporting that DNA could be extracted from isolated higher plant chloroplasts which differed in its base composition from the nuclear DNA. One was by Chun, Vaughan and Rich³ of M.I.T., using spinach and beet leaves as starting material and CsCl buoyant density centrifugation as the technique for characterizing the DNA. The other⁴ was by myself, at that time in Oxford, using leaves of the broad bean and a new chemical method for base composition determination of greatly improved accuracy. It is to these two papers and a fundamental discrepancy between them, the significance of which was not at the time appreciated, that we shall in a moment return.

In addition to their higher plant studies Chun *et al.* reported in their paper that the total DNA from the alga *Chlamydomonas reinhardtii* exhibited, on buoyant density centrifugation in CsCl, a minor DNA band of density 1.695 g cm^{-3} , in addition to the major, presumed nuclear, DNA band of density 1.723 g cm^{-3} . They had no evidence as to the cellular location of this minor species, but Sager and Ishida⁵ at Columbia were subsequently able to show that it was greatly enriched in isolated chloroplast fractions, indicating that it did in fact originate in the chloroplasts.

Also in 1963, Leff, Mandel, Epstein and Schiff, at Brandeis, reported⁶ that in another alga, *Euglena gracilis*, the whole cell DNA in CsCl gradients possessed, in addition to the main band of density 1.708 g cm^{-3} , a minor satellite of density 1.688 g cm^{-3} . This minor species was absent from a strain of *Euglena* which had lost the ability to form chloroplasts, which led Leff *et al.* to suggest that the minor DNA might be 'associated with the ability to form chloroplasts'. In the following year it was demonstrated independently by Ray and Hanawalt⁷ at Stanford, Brawerman and Eisenstadt⁸ at Yale, and Edelman, Cowan, Epstein and Schiff⁹ at Brandeis, that this minor DNA formed a much higher proportion of the DNA extracted from isolated

chloroplasts than it did of total cell DNA, indicating that it was in fact located in the chloroplasts.

Thus by the end of 1964 the presence in both higher plant and algal chloroplasts of a unique DNA, distinct from nuclear DNA, was well established and indeed was never again questioned. The good news that chloroplasts really did contain DNA spread rapidly not only through the primary scientific literature, but also into the textbooks, and the most compelling piece of evidence quoted in support of this (see for example, Wilkie's 1964 book on *The Cytoplasm in Heredity*)¹⁰ was usually the paper of Chun *et al.* on spinach and beet chloroplasts. Of that set of papers referred to above, published in the 1962-4 period, this was undoubtedly the most influential, and was commonly quoted as the cornerstone of the chloroplast DNA edifice not only throughout the remainder of the sixties, but even into the seventies. Things were, however, not all as they seemed, and to understand why this is so we must go back to the two original higher-plant papers referred to above.

In my paper I had reported that the DNA isolated from purified broad bean chloroplasts had an adenine/guanine ratio of 1.67, corresponding to 37.4% GC (guanine-cytosine), and the DNA from nuclei had an adenine/guanine ratio of 1.54, corresponding to 39.4% GC. The difference although small, was undoubtedly real, being statistically significant at the 0.1% level. Chun *et al.*, in their studies on spinach and beet leaves, had found that the DNA from chloroplast preparations consisted in CsCl gradients predominantly of a component of density 1.695 g cm^{-3} but also contained 5-15% of a DNA with density 1.705 g cm^{-3} and 5-40% of a DNA with density 1.719 g cm^{-3} . Since the nuclear DNA from these plants also had a density of 1.695 g cm^{-3} , Chun *et al.* concluded that the 1.695 component in chloroplast DNA preparations was really contaminating nuclear DNA, and that the 1.705 and 1.719 components must together constitute the true chloroplast DNA. Since these buoyant densities correspond to GC contents of 46 and 60%, respectively, this meant that Chun *et al.* were attributing a GC content to chloroplast DNA in these species, which was not only much higher than that of the corresponding nuclear DNA (~36% GC), but also much higher than the value (37% GC) I had measured for broad bean chloroplast DNA.

At the time this difference did not seem to be important: after all, we knew

that the nuclear DNA base composition could vary from one plant species to another, so why should not the chloroplast DNA base composition vary similarly? However, in the next few years several other papers appeared, all reporting - from various higher plant species - chloroplast DNAs of relatively high buoyant density (and therefore high GC content), substantially higher in every case than that of the corresponding nuclear DNA. The broad bean result was looking increasingly out of line, and the view was expressed to me personally, although not in the literature, that my data must be wrong.

Things were, however, about to change, and from 1967 onwards, studies using more rigorous methods were reported which completely altered the picture. The two studies which were mainly responsible for clarifying the issue were those of Wells and Birnstiel in Edinburgh,^{11,12} and Whitfeld and Spencer in Canberra.¹³ They showed that DNA extracted from properly purified chloroplasts isolated from healthy leaves of a number of higher plant species, including spinach, yielded in every case only one DNA band, density about 1.697 g cm^{-3} , on CsCl centrifugation: the 1.705 and 1.719 forms described by Chun *et al.* were absent. The 1.697 g cm^{-3} DNA lacked the base 5-methylcytosine which is present in nuclear DNA but which had already been shown to be absent from chloroplast DNA in *Euglena*.^{7,8} The buoyant density of 1.697 g cm^{-3} observed for chloroplast DNA from broad bean and other species by Wells and Birnstiel corresponds to a GC content of 37.8%, which is in excellent agreement with the value of 37.4% GC obtained by chemical analysis and reported in my original paper. Subsequent studies in many laboratories have amply confirmed that higher plant chloroplast DNAs are distinct from the high-GC-content DNA species described by Chun *et al.* and others in the 1963-7 period. I have given a detailed account of the way opinion in this field developed and changed from 1963 onwards elsewhere.¹⁴

On the basis of those few studies available by the end of the sixties which I regard as valid, I proposed the generalization^{14,15} that throughout the higher plants the base composition bears no particular relation to that of the nuclear DNA in the same plant but has a rather constant value in the region of $37.5 \pm 1\%$ GC, and a buoyant density in CsCl of about $1.697 \pm 0.001 \text{ g cm}^{-3}$. The nuclear DNA seems to vary much

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more widely and can have a GC content well above, or below, that of the chloroplast DNA.

What went wrong in 1963–7? What were those high-density DNA species which people were detecting in DNA extracted from higher plant chloroplasts, and wrongly identifying as chloroplast DNA? With hindsight we can now say that the 1.705 g cm⁻³ band was probably contaminating mitochondrial DNA, which in higher plants has a buoyant density of about this value. The 1.719 g cm⁻³ species (observed by Chun *et al.* but not by others) may have been due to bacterial contamination, resulting from the use of shop-bought rather than freshly grown leaves. It seems very likely that the main component in the chloroplast DNA preparations (e.g. the 1.695 g cm⁻³ band observed by Chun *et al.*), which people assumed was entirely due to contaminating nuclear DNA, was in fact a mixture of nuclear DNA with the true chloroplast DNA. It was bad luck that in the species chosen – spinach and beet, very commonly used of course for photosynthesis studies – the nuclear and the chloroplast DNAs have about the same buoyant density in CsCl.

After the initial chemical and buoyant density studies pointing to the presence of a unique DNA in chloroplasts, a wealth of corroborative evidence soon appeared confirming its existence and delineating its special characteristics. Chloroplasts were shown to be able to carry out the DNA-dependent synthesis of both RNA^{16,17} and DNA,^{18,19} and the actual intact chloroplast chromosome was isolated and shown by electron microscopy to be circular.²⁰ The molecular genetics of the chloroplast has now advanced to the point at which individual genes are being isolated and cloned and their nucleotide sequence determined.

In retrospect it is not surprising that the discovery of DNA in chloroplasts was accepted so readily. The evidence which appeared in 1962–4 was compelling, the ground had been prepared by the geneticists, it was an idea whose time had come. To anyone interested in the reality of how science progresses, as opposed to the tidied-up version normally presented to posterity, there is nevertheless a pleasing irony in the fact that the most clear-cut piece of evidence for

DNA in chloroplasts, the scientific paper most influential in launching the now vigorous field of chloroplast molecular biology, later turned out to be quite wrong.

Although there was no rearguard action in the literature by the nuclear lobby (by which in this context I mean that school of thought which maintained that DNA was confined to the nucleus), there was certainly some resistance, and properly so, at the personal level to what was after all a revolutionary change in the concepts of plant cell biology. So far as my own contribution was concerned, I first presented it to my scientific peers at a Biochemical Society meeting in Leiden, Holland, early in 1963. An eminent British nucleic acid chemist who was present then stood up and assured the audience that they could disregard these findings since he did not believe that my analytical method would work. Later in the same year I gave a seminar on my findings in one of the best-known centres for chloroplast biochemistry in Germany. At the end the professor, eminent in this field since the thirties, announced that in his laboratory too they had searched for chloroplast DNA, and on the basis of their measurements they were confident that 'there was not even one molecule per chloroplast!'. In fairness I should add that in that laboratory they then went back to the drawing board, tried new techniques, and shortly thereafter succeeded in finding DNA in chloroplasts after all.

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