STUDIES WITH CHLOROPLAST AND MITOCHONDRIAL DNA

I. Evidence of Sequence Homology between Chloroplast and Nuclear DNA (Broad Bean) and between Mitochondrial and Nuclear DNA (Rat Liver)

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ABSTRACT A mixture of broad bean chloroplast and nuclear DNA or rat liver mitochondrial and nuclear DNA was taken through a heating and annealing cycle, and examined by CsCl density gradient centrifugation. The formation of intermediates between the two DNAs during joint annealing was used as a method of detecting sequence homology in different DNA samples. Homology was found between chloroplast and nuclear DNA from broad bean and between mitochondrial and nuclear DNA from rat liver. Since this method produces only qualitative data no implication for possible nucleocytoplasmic relationship can be assessed.

INTRODUCTION

DNA has been shown to be present in cellular organelles other than nuclei by a number of workers. Chiba (1) first found DNA in chloroplasts of a variety of plant species. Nass and Nass (2) demonstrated that DNA also existed in mitochondria. At the time there was some doubt about the validity of these reports since it was argued that the observed results could be explained by the presence of a small amount of contaminating DNA from the nucleus. However, with the discovery of the different renaturation behavior of mitochondrial and nuclear DNA by Borst and Ruttenberg (3) and chloroplast and plant nuclear DNA by Tewari and Wildman (4), it appears reasonably well established that these two organelles, mitochondria (or chloroplasts) and nuclei, contain different types of DNA (5–7).

DNA in cellular organelles differs from that found in the nucleus in a number of ways. Pertinent to the work reported here is the difference in renaturation behavior. Thus, under identical conditions nuclear DNA from broad bean does not

renature, while chloroplast DNA renatures readily. A similar situation has been found for rat liver nuclear DNA which does not renature and mitochondrial DNA which does. This difference in renaturation behavior has been used in the present study as a means of determining the presence of each in a mixture when studied by the CsCl density gradient technique.

An analysis of the buoyant densities of organelle and nuclear DNAs has revealed a close similarity. Thus, chloroplast DNA of many plants, such as broad bean (8), tobacco and spinach (9), snapdragon (10), and sweet pea and lettuce (11), were reported to have buoyant densities in CsCl very similar to the corresponding nuclear DNA. Mitochondrial DNA from rat liver, beef heart, beef liver, and mouse liver (12) from various guinea pig tissues (5) and from sheep heart (13) also appeared to have very similar buoyant densities to their corresponding nuclear DNAs. Such similarity in buoyant density implies a similarity in base composition; however, a similarity in base composition does not imply a similarity in base sequence.

Sequence homology between different DNAs has been studied by Schildkraut et al. (14, 15) by studying the formation of hybrid DNA molecules during annealing. The hybrid was detected by its position in the analytical ultracentrifuge after banding in CsCl density gradient. It was later shown by Britten and Waring (16) that the presence of hybrid DNA molecules in a gradient is a valid test for sequence homology between two DNAs.

Recently Dawid and Wolstenholme (17) used renaturation and hybridization techniques to study sequence relationships between mitochondrial DNAs of different species and between mitochondrial and nuclear DNAs of the same species. We have followed essentially this technique in our studies. We have found that rat liver mitochondrial DNA anneals with rat liver nuclear DNA and that broad bean chloroplast DNA anneals with its own nuclear DNA. On the other hand chloroplast DNA does not form hybrids when annealed with rat liver mitochondrial or nuclear DNA implying that hybridization is more than simply a loose association resulting from a chance occurrence.

MATERIALS AND METHODS

Preparation of Chloroplasts, Mitochondria, and Nuclei

Chloroplasts from young broad bean leaves were prepared as described by Kung and Williams (8) applying their criteria of purity for the preparations. Mitochondria from rat liver were prepared according to the procedure of Borst et al. (18). The purity of the preparation was monitored by electron microscopy. The mitochondria were incubated with 100 μg/ml DNase I for 30 min at 25°C before the extraction of DNA.

Nuclei of broad bean and rat liver were prepared by the methods of Bonner (19) and Sadowski and Steiner (20) respectively. Extraction of nuclear DNA was carried out by the procedure of Marmur (21). DNA from broad bean chloroplasts and rat liver mitochondria were prepared by the same procedure except that the ethanol precipitation steps were omitted (18, 22). After several deproteinization steps with chloroform–isoamyl alcohol (24:1) the

yellow-brown aqueous layer was dialyzed for 48 hr against 0.15 M sodium chloride + 0.015 M trisodium citrate (SSC) until the yellow color was dialyzed out. The sac contents were incubated for 60 min with 50 μg/ml RNase at 37°C and deproteinized further with chloroform–isoamyl alcohol.

Thermal Denaturation, Renaturation, and Hybridization

DNAs at a concentration of 20 μg/ml in SSC were denatured by heating for 10 min at 100°C and then cooled rapidly in ice water (23). Renaturation was carried out in double-strength SSC for 2 hr at 60°C and cooled slowly to room temperature according to the method of Marmur and Doty (24). In the hybridization experiments, the incubation time used was 16 hr.

CsCl Density Gradient Centrifugation

This was carried out according to Meselson et al. (25) in the Spinco Model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 44,770 rpm and 25°C for 20–24 hr. Densities were calculated from the equation of Schildkraut et al. (26) using *Micrococcus radiodurans* as marker (ρ = 1.728 g/cm^3) as determined by Schien (27).

RESULTS

Chloroplast and Nuclear DNA from the Broad Bean

In Figs. 1 *a–e* are represented the Analytrol tracings obtained from the ultraviolet (UV) photographs of the density gradient centrifugations.

Chloroplast and nuclear DNA from the broad bean have identical buoyant densities in CsCl, banding at 1.696 g/cm^3 (8, 28). The banding pattern of native and denatured chloroplast and nuclear DNA is shown in Figs. 1 *a* and *c* respectively. The sharper peak in Fig. 1 *a* as compared with Fig. 1 *c* is consistent with the view that chloroplast DNA is much more homogeneous than the corresponding nuclear DNA.

The buoyant densities of the denatured chloroplast and nuclear DNA are also the same, banding at 1.712 g/cm^3 (Figs. 1 *a* and *c*). Those of the corresponding renatured material are very different. Since chloroplast DNA renatures almost completely, the buoyant density is near the native value of 1.696 g/cm^3 (Fig. 1 *b*). Nuclear DNA does not renature under the same conditions, therefore the buoyant density of this material remains near 1.712 g/cm^3 (Fig. 1 *d*). Although nuclear DNA did not renature, the sharpness of the band is interpreted to represent the formation of high molecular weight networks.

When chloroplast and nuclear DNA were annealed together a band of intermediate density was observed at 1.705 g/cm^3 (Fig. 1 *e*). A band representing renatured chloroplast DNA was observed at 1.699 g/cm^3. The band at 1.705 g/cm^3 was intermediate between the renatured chloroplast (1.699 g/cm^3) and the denatured nuclear (1.712 g/cm^3) bands and therefore represents the hybrid formed by annealing these two types of DNAs. The formation of the hybrid suggests that chloroplast and nuclear DNA from the broad bean have nucleotide sequences in common.

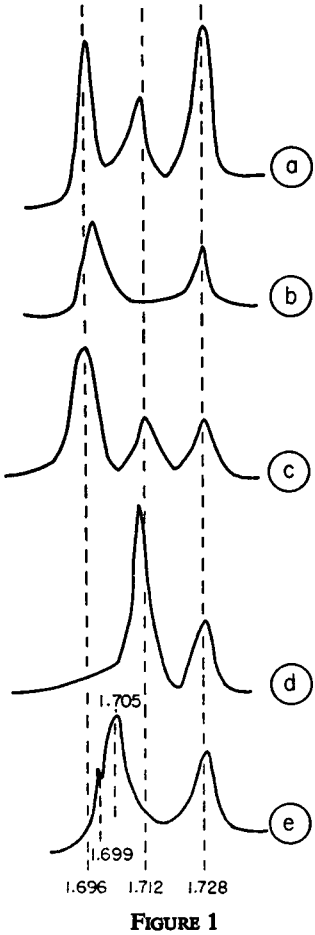

FIGURE 1

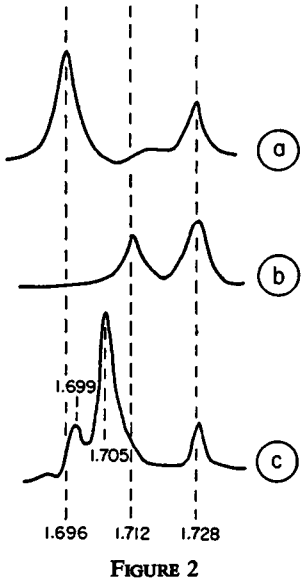

FIGURE 2

FIGURE 1 Hybridization experiments of chloroplast and nuclear DNA of broad bean. Densitometer tracings of UV absorption photographs of DNA banded in CsCl density gradients. (*a*) Native and denatured chloroplast DNA; (*b*) renatured chloroplast DNA; (*c*) native and denatured nuclear DNA; (*d*) "renatured" nuclear DNA; (*e*) hybrid formed between chloroplast and nuclear DNAs. The chloroplast and nuclear DNAs were prepared separately and then mixed at equivalent amounts just before the hybridization experiment. The marker band to the right is native DNA of *M. radiodurans*, the density of which is 1.728 g/cm^3.

FIGURE 2 Hybridization experiments of chloroplast and nuclear DNA of broad bean. Densitometer tracings of UV absorption photographs of DNA prepared from a mixture of chloroplasts and nuclei. (*a*) Native; (*b*) denatured; (*c*) annealed.

Further evidence indicating the formation of a hybrid between the chloroplast and nuclear DNA is presented in Fig. 2. When DNA was prepared from the 1000 *g* fraction which was a mixture of chloroplasts and nuclei (8) it exhibited a single symmetrical peak at the native state (Fig. 2 *a*) and an increase in density of 0.016 g/cm^3 when thermally denatured (Fig. 2 *b*). In the annealing experiment a hybrid

with an intermediate buoyant density of 1.705 g/cm³ was observed (Fig. 2 *c*). As suggested by the density value, the hybrid was formed between the renatured chloroplast and denatured nuclear DNA. The remaining portion of the renatured chloroplast DNA (1.699 g/cm³) appeared to be constant in all experiments. It is possible that this small portion of renatured chloroplast DNA may represent a fraction in which renaturation took place between two equally sized complementary strands. No unpaired single strands therefore are available for any further base pairing with the denatured and single-stranded nuclear DNA.

Mitochondrial and Nuclear DNA from Rat Liver

The buoyant densities for nuclear and mitochondrial DNA were found to be identical at 1.704 g/cm³ in this laboratory.[1] The Analytrol tracings are shown in Fig. 3. A much sharper peak was observed for mitochondrial DNA (Fig. 3 *a*) than for nuclear DNA (Fig. 3 *c*), indicating a higher degree of homogeneity for the former species.

Denaturation, renaturation, and hybridization studies involving nuclear and mitochondrial DNA from rat liver are also shown in Fig. 3. In Fig. 3 *a*, the banding positions of native and denatured mitochondrial DNA are shown. It can be seen that the density of the denatured mitochondrial DNA was shifted to 1.721 g/cm³. Denatured nuclear DNA was also banded with a density of 1.721 g/cm³ as shown in Fig. 3 *c*. This is because the densities of both the native and denatured mitochondrial and nuclear DNA from rat liver are identical. The difference in renaturation behavior of the two DNAs can be seen from the tracings in Figs. 3 *b* and *d*. Mitochondrial DNA renatured almost completely (Fig. 3 *b*) while nuclear DNA failed to renature under the same conditions (Fig. 3 *d*). The increase in sharpness of the peaks seen in Figs. 3 *b* and *d* are a reflection of the formation of high molecular weight networks.

When nuclear and mitochondrial DNA from rat liver were isolated separately but annealed together the formation of a hybrid was readily detected. In Fig. 3 *e* the hybrid is shown to band with a density of 1.715 g/cm³, intermediate between that of renatured mitochondrial DNA (1.705 g/cm³) and that of denatured nuclear DNA (1.721 g/cm³). When DNA was isolated from a homogenate of rat liver so that mitochondrial and nuclear DNA were isolated together, a similar hybrid was observed banding at 1.715 g/cm³ after denaturation and annealing. As before, a band at 1.705 g/cm³ representing renatured mitochondrial DNA and one at 1.721 g/cm³ representing denatured nuclear DNA was observed.

Broad Bean and Rat Liver DNA

We found that broad bean chloroplast (1.696 g/cm³) and rat liver mitochondrial DNA (1.704 g/cm³) renatured almost completely and they were observed to form

[1] Kung, S. D., and M. A. Moscarello. Manuscript submitted for publication.

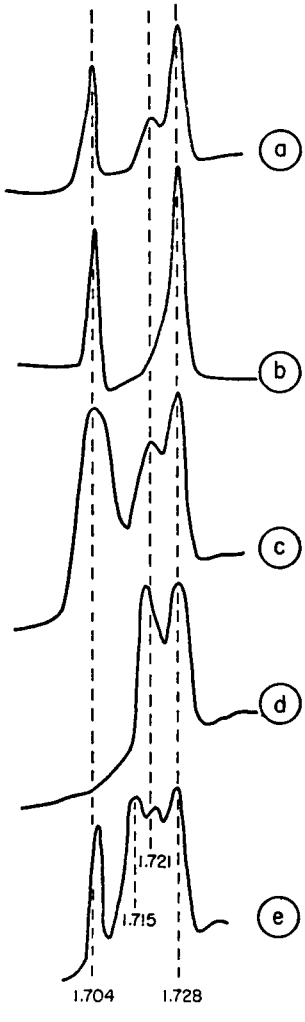

FIGURE 3

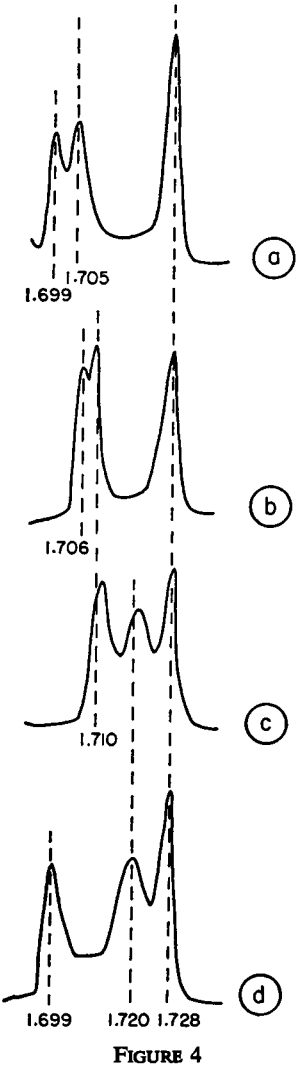

FIGURE 4

FIGURE 3 Hybridization experiments of mitochondrial and nuclear DNA of rat liver. Densitometer tracings of UV absorption photographs of DNA banded in CsCl density gradients. (*a*) Native and denatured mitochondrial DNA; (*b*) renatured mitochondrial DNA; (*c*) native and denatured nuclear DNA; (*d*) "renatured" nuclear DNA; (*e*) hybridized mitochondrial and nuclear DNA. The mitochondrial and nuclear DNAs were prepared separately and then mixed in equivalent amounts just before the hybridization experiment.

FIGURE 4 The absence of sequence homology between broad bean and rat liver DNAs. Densitometer tracings showing: (*a*) chloroplast (1.699 g/cm^3) and mitochondrial (1.705 g/cm^3) DNA annealed together; (*b*) broad bean nuclear (1.710 g/cm^3) and rat liver mitochondrial DNA (1.706 g/cm^3); (*c*) broad bean and rat liver nuclear DNA (1.720 g/cm^3); (*d*) broad bean chloroplast (1.699 g/cm^3) and rat liver nuclear DNA (1.720 g/cm^3).

hybrids with their corresponding nuclear DNAs. That these hybrids were not simply due to the entanglement of DNA strands can be seen by the experiment represented in Fig. 4. When chloroplast and mitochondrial DNAs were denatured and renatured together, the tracing in Fig. 4 *a* was obtained. It is clear that no hybrid was formed. Only two bands were observed, banding at the respective densities of 1.699 and 1.705 g/cm^3 for renatured chloroplast and mitochondrial DNA. This suggested that the formation of hybrid was species specific. Such a suggestion was further supported by the evidence obtained from the following experiments illustrated in Figs. 4 *b–d*. No hybrid was observed between the rat liver mitochondrial and broad bean nuclear DNA (Fig. 4 *b*) or between nuclear DNAs from both rat liver and broad bean (Fig. 4 *c*). In addition, when chloroplast DNA from broad bean and nuclear DNA from rat liver were annealed together, they formed separate bands at their respective renatured and denatured densities of 1.699 and 1.720 g/cm^3 (Fig. 4 *d*). The absence of hybrid indicated a lack of sequence homology between DNAs prepared from broad bean and rat liver. A more detailed study of the species specificity and other properties of the formation of hybrid DNA molecules will be reported in the near future.

DISCUSSION

Since Schildkraut et al. (14) reported the use of CsCl density gradient techniques for the study of DNA hybrids as an indication of DNA homology, the method has been widely used (15, 17). Dawid and Wolstenholme (17) showed that nonspecific aggregation of DNA molecules did not occur during annealing in their experiments. The strong species specificity of the hybrid formed indicates that they are held together by the association of complementary sequences. It can therefore be used as a valid test for sequence homology. The data presented in this communication and a subsequent one support this conclusion.

A number of studies have been presented on chloroplast and nuclear DNA from broad bean (8, 28) and mitochondrial and nuclear DNA from rat liver (footnote 1). Some of the data are collected in Table I. The buoyant densities of native, denatured, and renatured DNAs as well as other parameters such as G + C content, Tm, hyperchromicity, and molecular weight are recorded. In both broad bean and rat liver, the buoyant density of the organelle (chloroplast or mitochondrial) DNA was the same as the corresponding nuclear DNA. The density of the denatured organelle DNA was the same as that for the nuclear DNA. Whereas the organelle DNA renatured readily, the nuclear DNA did not.

When hybrids were formed in our experiments, these were the result of the association between chloroplast DNA and broad bean nuclear DNA or mitochondrial DNA and rat liver nuclear DNA. When chloroplast and nuclear DNAs were annealed separately and then mixed, no hybrids were observed. This observation implies that the formation of hybrids is relatively specific. Further support for the

TABLE I

SOME PHYSICOCHEMICAL PROPERTIES OF CHLOROPLAST AND NUCLEAR DNA FROM BROAD BEAN AND MITOCHONDRIAL AND NUCLEAR DNA FROM RAT LIVER

Organism	DNA	Buoyant density			Hybrid	G + C content	Tm	Hyperchromicity	$s_{20,w}$	Mol wt $\times 10^{-6}$	References
		Native	Denatured	Renatured							
		g/cm³	*g/cm³*	*g/cm³*							
Broad bean	Chloroplast	1.696 ±0.001	1.712 ±0.001	1.699 ±0.001	1.705	37.0	86.5	34	16.0	5.6	28
Broad bean	Nuclear	1.696 ±0.001	1.712 ±0.001	1.710 ±0.001		37.0	86.3	36	25.0	12.2	
Rat (liver)	Mitochondrial	1.704 ±0.001	1.721 ±0.001	1.705 ±0.001	1.715	44.0		42.0			This study
Rat (liver)	Nuclear	1.704 ±0.001	1.721 ±0.001	1.719 ±0.001		44.0	87.0	36.5	32.0	22.6	

specificity of hybridization was obtained by annealing chloroplast and mitochondrial DNA. In this case, too, hybrids were not observed. Using the different techniques of DNA-agar and DNA-filter, DuBuy and Riley (29) reported the formation of a hybrid between nuclear and mitochondrial DNA of mouse liver. A hybrid between chloroplast DNA and nuclear DNA from *Euglena gracilis* was observed by Richards using the filter technique (30). The experiments reported in this communication corroborate and extend these findings. Although the density gradient technique used by us is not quantitative, it does allow one to visualize the hybrid and compute its buoyant density.

According to our data and that of others, three types of hybrids have been detected by CsCl density gradient centrifugation:

(*a*) Hybrids with densities intermediate between the densities of the two renatured strands. When DNA isolated from one of chloroplasts, mitochondria, bacteria, viruses, or satellite DNA, all of which are known to renature readily, is annealed with another DNA which also renatures readily, the density of any hybrid formed will be intermediate between those of the two renatured strands. This is supported by the data of Dawid and Wolstenholme (17) who observed hybrid formation between mitochondrial DNA from frog annealed with mitochondrial DNA from chick. Since the density of the hybrid was intermediate between those of the two renatured DNAs this suggested that renaturation occurred first and hybrid formation second. This is supported by the finding of Britten and Kohne (31) that concatenation occurred at the later stages of reassociation. The data of Schildkraut et al. (14) support the view that renaturation occurs first. They ob-

served that renaturation can be achieved within 30 min while hybridization took several hours.

(*b*) Hybrids with densities intermediate between the renatured and denatured DNAs have been observed in our laboratory. When chloroplast and nuclear DNA isolated from the broad bean were annealed, the hybrid formed had a density intermediate between that of renatured chloroplast (which is known to renature readily) and denatured nuclear DNA (which does not show any appreciable renaturation). Likewise, when rat liver mitochondrial and nuclear DNA were annealed, the resulting hybrid had a density intermediate between that of renatured mitochondrial DNA and denatured nuclear DNA.

(*c*) Hybrids with densities between those of two denatured components. Nuclear DNA is not known to renature extensively. It is interesting therefore, that Dawid and Wolstenholme (17) reported that a hybrid was formed between frog nuclear DNA and mouse nuclear DNA. A homology of 2–4% was apparently sufficient to result in hybrid formation. This finding is difficult to explain. One would not expect that high molecular weight DNA from two very different sources would hybridize since neither of the two alone renatures extensively. It would be of interest to know more about this hybrid.

The possibility that hybrid formation can occur in vivo has been reported by Straun et al. (32). When foreign, double-stranded DNA from bacteria (*Agrobacterium tumefaciens*, $\rho = 1.722$ g/cm^3) was injected into growing tomato plants (buoyant density of tomato DNA = 1.692 g/cm^3) a hybrid DNA was isolated with a density intermediate between that of the two native DNAs. The density was found to have a value of 1.707 g/cm^3. A further report by Ledoux and Huart (33) showed that DNA from *Micrococcus lysodeikticus* formed a hybrid with barley DNA after the bacterial DNA had been injected into the germinating seed. As suggested by Straun et al. (32) such hybrid DNA molecules may play a role as templates for replication, which could in turn result in cellular abnormalities.

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