Plant Physiol. (1980) 65, 234–237
0032-0889/80/65/0234/04/$00.50/0

Sequence Homology between Chloroplast DNAs from Several Higher Plants

Received for publication May 29, 1979 and in revised form August 23, 1979

DAVID BISARO AND ALBERT SIEGEL
Department of Biology, Wayne State University, Detroit, Michigan 48202

ABSTRACT

An estimate has been made of the amount of sequence homology present in the chloroplast DNA (ctDNA) of several higher plants by the technique of DNA-DNA hybridization. Approximately 85% of tomato, 60% of spinach, 45% of kale, and 15% of barley ctDNA sequences were found to hybridize with tobacco ctDNA under conditions in which maximum hybridization in homologous reactions reached 85%. All heteroduplexes contained significant amounts of sequence mismatch as indicated by a 3 to 9 C decrease in melting temperature as compared to homoduplex.

The data suggest that considerable sequence homology exists between the ctDNAs of these plants and that some sequences are held in common among all of the species tested.

The DNA found in the chloroplasts of higher plants exists as a covalently closed circle with a mol wt, depending on the species, of 85–95 × 10^6 daltons (12). Several chloroplast-specific components are now known to be coded for by higher plant ctDNA[1]. These include the chloroplast ribosomal RNAs (2, 20, 21), a nearly complete set of transfer RNAs (9), the large subunit of the enzyme ribulose-bisP carboxylase (6, 7) and possibly several other as yet unidentified soluble and membrane bound proteins (4).

Investigations comparing chloroplast ribosomal RNAs (21) and the large subunit of ribulose-bisP carboxylase (13, 14) from different plant species suggest that the primary structure of these molecules is highly conserved. Although this result would indicate that a certain amount of sequence homology exists between different ctDNAs, the coding sequences required to specify these molecules probably represent only a small fraction of the chloroplast genome (11). In contrast, distinct fragmentation patterns result when ctDNA from different species are digested with restriction endonucleases (1, 22). These studies clearly show that ctDNAs differ in base sequence, but provide little quantitative information about the extent of the difference.

Here, we present an estimate of the amount of sequence homology present in the ctDNA of several higher plants. Homology measurements were obtained by the technique of DNA-DNA hybridization in solution followed by digestion of unreacted DNA with single-strand-specific nuclease S1. The data are discussed with respect to the evolution of the chloroplast genome and the phylogenetic relationships of the plants in which they are found. Our results are also compared with those from recent similar experiments by Walbot (23) and Lamppa and Bendich (15).

[1] Abbreviations: ctDNA: chloroplast DNA; PB: sodium phosphate buffer; HAP: hydroxylapatite; Pipes buffer: piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

MATERIALS AND METHODS

Preparation of Chloroplast DNA. Chloroplasts were prepared from tobacco (*Nicotiana tabacum* L., cv. Xanthi), tomato (*Lycopersicon esculentum* Mill.), spinach (*Spinacia oleracea* L.), kale (*Brassica oleracea* var. acephala D.C.), and barley (*Hordeum vulgare* L.) plants. Young leaves were chopped in Honda medium (10) with an electric knife fitted with razor blades and the resulting brei was sedimented through discontinuous gradients of 60, 45, and 20% sucrose (19). Chloroplasts were collected from the 20–45% discontinuity. ctDNA was prepared by a modification of the urea phosphate DNA extraction procedure (5). Chloroplasts from 50 g of leaf tissue were lysed in a solution containing 8 M urea, 0.24 M PB, 1 mM EDTA, and 1% SDS. The lysate was extracted three times with phenol saturated with 50 mM Tris. The aqueous fraction was loaded on a column containing 2 g of HAP and washed successively with 80–100 ml of 8 M urea—0.24 M PB, 40 ml of 10 mM PB, and 10 ml of 0.4 M PB to elute DNA. DNA-containing fractions were pooled and centrifuged for 14 h at 45,000 rpm in a Beckman SW 50 rotor. The resulting pellets were resuspended in 1 ml of 5 mM PB and stored at 4 C over a drop of chloroform. Purity of ctDNA preparations was monitored by cesium chloride density gradient analysis in a Beckman model E analytical ultracentrifuge. Native, denatured and reassociated (Cot 0.32) samples were run simultaneously. Failure of reassociated samples to renature and thus recover to within 4 mg/ml of native buoyant density was taken as evidence of contamination by nuclear DNA (12, 19).

Labeling of ctDNA. ctDNA was labeled *in vitro* by a modification of the nick translation procedure described by Macgregor and Mizuno (16). ^{3}H- or ^{14}C-labeled TTP was incorporated into DNA in the presence of equimolar amounts of unlabeled nucleotides by the combined action of DNase I and DNA polymerase I. Labeled DNA was separated from unincorporated nucleotides by HAP chromatography; free nucleotides were eluted with 0.12 M phosphate buffer at 50 C, and DNA at 98 C. DNA-containing fractions were pooled, denatured at 100 C for 3 min, and again applied to a HAP column at 50 C. DNA eluted at this temperature was used in hybridization experiments. Foldback DNA (10–20% of input DNA) was removed by this procedure. The specific activity of ^{3}H- and ^{14}C-labeled DNA prepared by this method was 2–5 × 10^6 and 6–10 × 10^3 cpm/μg, respectively. Average single-strand fragment size obtained was 200–400 and 450–650 nucleotides for ^{3}H- and ^{14}C-labeled DNA as determined by sedimentation through alkaline sucrose gradients (18).

Hybridization Reactions. Hybridization reactions were carried out at 60 C in 10 mM Pipes buffer, 0.375 M NaCl, 0.1 mM EDTA (pH 6.7) (17). Cot values were corrected to 0.18 M Na^+ (5). Small amounts of heterologous [^{3}H]ctDNA were mixed with at least a 750-fold excess of tobacco [^{14}C]ctDNA, and 25 μl samples incubated for appropriate time intervals in siliconized capillary tubes. The reactions were terminated by chilling the capillaries on ice.

The extent of hybridization was determined by digestion of reaction samples with single-strand-specific nuclease S1 according to the procedure of Zimmerman and Goldberg (24). Following digestion, samples were spotted on Whatman 3MM filter paper discs, washed at 4 C with 5% trichloroacetic acid (three times), ethanol-ether (1:1) and ether, then dried and counted.

Thermal Denaturation Analysis. Melting profiles of homo- and heteroduplexes were obtained by reacting [^{3}H]ctDNA in the presence of excess unlabeled tobacco ctDNA in 0.12 M phosphate buffer. Reacted samples were placed in a large water bath and the temperature slowly increased from 50–99 C with a Haake E-52 temperature programmer assisted by an electric heating coil. Samples were removed at appropriate temperatures and chilled on ice. The amount of DNA remaining in duplex form was determined by S1 nuclease digestion as described previously.

RESULTS

Hybridization of [^{3}H]ctDNA with Excess Tobacco [^{14}C]ctDNA. [^{3}H]ctDNAs prepared from tobacco, tomato, spinach, kale, and barley plants were reacted in the presence of a 750-fold excess of tobacco [^{14}C]ctDNA as described under "Materials and Methods." Tobacco driver DNA, as followed by ^{14}C label, reassociated as a single component with typical second order kinetics at a rate expected for a DNA with a complexity of about 95 × 10^6 daltons (Fig. 1). At completion, duplex formation reached 85%. An equal amount of duplex formation was observed when tobacco or tomato tracer DNA was hybridized in the presence of tobacco driver DNA (as followed by ^{3}H label). On the basis of these experiments, ctDNAs from tobacco and tomato plants are enough alike to cross-hybridize completely under the conditions used (Fig. 1). Approximately 60% of spinach, 45% of kale, and 15% of barley ctDNA sequences were capable of hybridizing with sequences present in tobacco ctDNA. In control incubations in which no tobacco driver DNA was present less than 6% self-hybridization of ^{3}H-tracer DNAs could be detected (data not shown). The hybridization data presented in Figure 1 primarily represent heteroduplex formation, except in the case of the homologous tobacco-tobacco reaction.

Completion of Reactions. The hybridization experiments described here result in the formation of heteroduplexes which contain significant amounts of sequence mismatch (discussed further below). As sequence mismatch may retard the rate of heteroduplex formation, it is conceivable that the driver tobacco ctDNA sequences could react to completion before reaction with complementary (but mismatched) tracer sequences is completed (3). This would result in an underestimation of the homology actually shared by the ctDNA molecules. To determine whether heterologous reactions were in fact proceeding to completion, kale [^{3}H]ctDNA which had been previously reacted in the presence of excess unlabeled tobacco ctDNA to Cot 22 was challenged by a second addition of either tobacco or kale ctDNA and further reacted to Cot 10. After further reaction with tobacco ctDNA, little additional hybridization was observed (47% as compared to 42% after initial reaction) illustrating that almost all of the tracer DNA sequences that could have reacted with heterologous DNA did so during the first reaction. That the unhybridized tracer DNA was still reactable was shown by the second reaction with homologous driver DNA. In this experiment, 85% of the [^{3}H]DNA was in hybrid form after further reaction to Cot 10. We conclude that differential rates of hybridization do not seriously affect the data or the conclusions derived from them.

Hybridization of [^{3}H]ctDNAs With Mixed Driver DNAs. To determine if there are highly conserved nucleotide sequences held in common by all ctDNAs tested, hybridization experiments were performed in which various unlabeled ctDNAs were present in excess, either singly or in combination. The presence of driver

Table I. *Heteroduplex Formation in the Presence of One or Two Driving DNAs*

[^{3}H]ctDNA was reacted in the presence of excess unlabeled tobacco, kale, or tobacco and kale ctDNA. The concentration of driver DNA in tobacco + kale reactions was twice that of single driver reactions. Data are expressed as per cent [^{3}H]DNA double-stranded.

[^{3}H]ctDNA Tracer	ctDNA in Excess		
	Tobacco	Kale	Tobacco + Kale
Tomato	85	47	80
Spinach	60	52	64
Barley	15	10	16

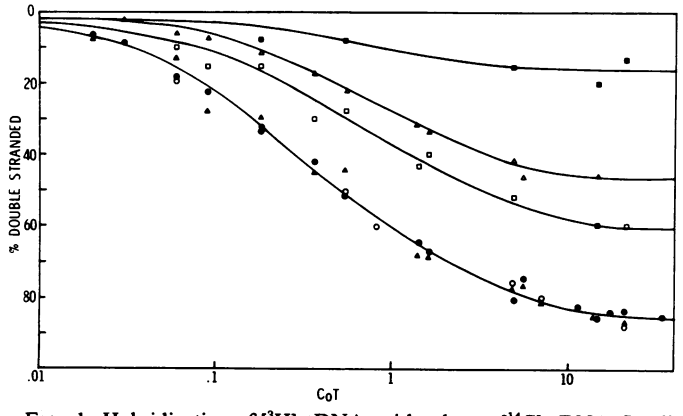

FIG. 1. Hybridization of [^{3}H]ctDNAs with tobacco [^{14}C]ctDNA. Small amounts of [^{3}H]ctDNA from tobacco (▲), tomato (○), spinach (□), kale (△), or barley (■) were reacted in the presence of a 750-fold excess of tobacco [^{14}C]ctDNA (●), and the amount of DNA in double-stranded form determined by digestion of reaction samples with S1 nuclease. Data were corrected for reactability of tracer [^{3}H] DNAs.

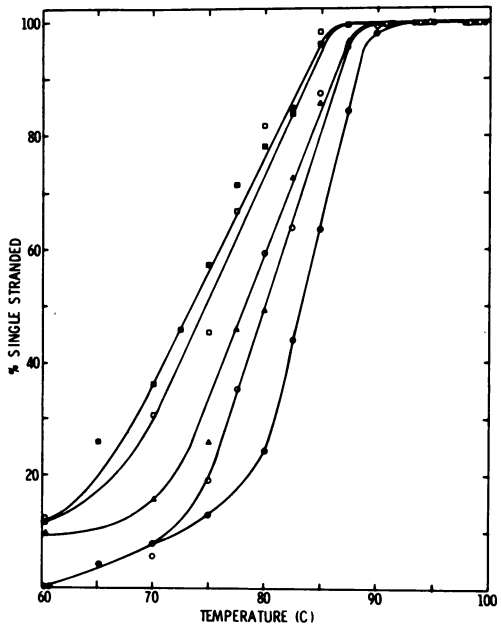

FIG. 2. Thermal denaturation of heteroduplexes. [^{3}H]ctDNAs were reacted to completion in the presence of a 750-fold excess of unlabeled tobacco ctDNA in 0.12 M phosphate buffer. Reacted samples were heated to appropriate temperatures and digested with S1 nuclease to determine the amount of DNA in single-stranded form. Melting profiles of tomato (○; Tm, 80 C), spinach (▲; Tm, 79 C), kale (□; Tm, 75 C), and barley (■; Tm, 74 C) heteroduplexes are shown. Tobacco homoduplex (●; Tm, 83 C) is included for comparison.

DNAs in combination did not result in additive increases in the extent of hybridization observed (Table I). On this basis, it appears that there are sequences within these ctDNAs which are preferentially conserved.

Thermal Denaturation Analysis. From the data presented above we see that some of the nucleotide sequences present in these ctDNAs have diverged to the extent that they will no longer hybridize under the experimental conditions used. To determine whether and to what extent divergence has occurred within those sequences which do hybridize, thermal denaturation profiles of the heteroduplexes and tobacco homoduplex were constructed as described under "Materials and Methods." Tobacco homoduplex showed a relatively sharp helix coil transition upon melting with a Tm of 83 C (Fig. 2). Heteroduplex transitions were not as sharp; depression of the Tm was somewhat greater at lower temperatures than at higher ones (Fig. 2). Observed Tm depressions were 3 C for tomato, 4 C for spinach, 8 C for kale, and 9 C for barley-tobacco heteroduplexes. Taking a 1 C Tm depression to represent 1% sequence mismatch (5), about 3–9% mismatch is present in these heteroduplexes.

DISCUSSION

In this study, the amount of sequence homology present in the chloroplast genomes of several higher plants was estimated by the technique of DNA-DNA hybridization. About 85% of tomato, 60% of spinach, 45% of kale, and 15% of barley ctDNA sequences were found to be enough alike to hybridize with tobacco ctDNA. Under our experimental conditions, maximum hybridization values reached 85% in homologous reactions. All heteroduplexes contained significant amounts of sequence mismatch, as indicated by a 3–9 C depression in Tm relative to homoduplex.

These results indicate that ctDNAs from higher plants contain substantial amounts of sequence homology. At least 15% of the chloroplast DNA of a monocot (barley) is capable of hybridizing with ctDNA from a dicot (tobacco). Even more striking is the extensive homology observed between ctDNAs from closely related species. The chloroplast genomes of tobacco and tomato (both members of the family Solanaceae) are apparently completely homologous as judged by the ability of tomato ctDNA to anneal with tobacco ctDNA to the same extent observed in homologous reactions. However, a 3 C difference in Tm was detected between tobacco homoduplex and tomato-tobacco heteroduplex. ctDNA from spinach and kale also share considerable homology with tobacco ctDNA, although, as might be expected, not nearly as much as that observed between DNA from tobacco and tomato chloroplasts. The species examined here are quite widely dispersed through the plant kingdom. According to the classification scheme of Cronquist (8), tobacco and tomato are in the subclass Asteridae, spinach in the Caryophyllidae, and kale in the Dilleniidae of the class Magnoliatae. Barley is even more distantly related to these, belonging to the subclass Commelinidae of the class Liliatae. The examination of species belonging to subclasses not represented here or elsewhere should provide a more complete assessment of the extent of divergence of ctDNA sequences through evolution.

Results similar to those reported here have been obtained by Walbot (23). She has shown that the ctDNAs from two species of grass share about 95% homology, while about 45% of the nucleotide sequences present in cabbage ctDNA are capable of hybridizing with grass ctDNA. The amount of homology (45%) reported between cabbage (a dicot) and grass (a monocot) ctDNA is somewhat greater than that observed between the monocot and dicot tested here (15% between barley and tobacco). At least some of this difference may be to the different experimental conditions used. Lamppa and Bendich (15) have also recently reported on similar experiments. In addition to comparing ctDNAs of different species by S1 digestion after hybridization, they also estimated sequence diversity by HAP chromatography. The results they obtained with HAP chromatography are reminiscent of those reported here, although different in detail. For instance, they observed only 75% homology between two species of the same family (compared with complete homology observed by us) and 34–57% homology between monocots and a dicot (compared with 15% observed by us). The species and subclasses chosen were different than those we examined and it seems likely that the extent of sequence homology observed in any experiment may depend on the particular organisms investigated. However, their results obtained by S1 digestion of heteroduplexes are quite different from ours. They observed about 30% homology between pea ctDNA and all other ctDNAs from species outside of the pea family (Leguminoseae), including both monocot and dicot classes. The disparate results may have been obtained as a consequence of different experimental procedures, *i.e.* stringency of S1 conditions, size of DNA fragments, etc.

Some of the sequences present in these ctDNAs have diverged to the extent that they will not hybridize under the experimental conditions employed in these experiments. Perhaps a greater or lesser extent of hybridization would be observed were the hybridization criterion made more relaxed or more stringent. The sequences which do hybridize have also diverged from each other (as indicated by the Tm depression of heteroduplexes) but obviously not to the extent that they will no longer hybridize. One might be drawn to the conclusion, therefore, that nucleotide change is a random process during the course of evolution. However, this does not appear to be so because evidence is presented for a family of ctDNA sequences which are more resistant to change than the rest. The data in Table I show that tomato, spinach, and barley ctDNA sequences which hybridize with kale ctDNA are part of a family of sequences which also hybridize with tobacco ctDNA. To take the most extreme example, barley (a monocot) shares a set of recognizable nucleotide sequences with tobacco and kale ctDNA (both dicots). There seems to be little doubt that the dicots tested are considerably more closely related to each other than any of them are to barley and, further, that it is likely that barley lies on a different evolutionary branch than the four dicots. We conclude that there exists a region of ctDNA comprising at least 10–20% of the total which is more resistant to change than the rest.

Acknowledgments—We wish to acknowledge K. Kolacz and W. E. Timberlake for their welcome advice and helpful discussions.

LITERATURE CITED

1. Atchison BA, PR Whitfield, W Bottomley 1976 Comparison of chloroplast DNAs by specific fragmentation with Eco RI endonuclease. Mol Gen Genet 148: 263–269
2. Bedbrook JR, R Kolodner, L Bogorad 1977 *Zea mays* chloroplast ribosomal RNA genes are part of a 22,000 base pair inverted repeat. Cell 11: 739–749
3. Bonner TI, DJ Brenner, BR Neufeld, RJ Britten 1973 Reduction of the rate of DNA reassociation by sequence divergence. J Mol Biol 81: 123–135
4. Bottomley W, D Spencer, PR Whitfield 1974 Protein synthesis in isolated spinach chloroplasts: comparison of light driven and ATP-driven synthesis. Arch Biochem Biophys 164: 106–117
5. Britten RJ, DE Graham, BR Neufeld 1974 Analysis of repeating DNA sequences by reassociation. Methods Enzymol 29: 363–418
6. Chan PH, SG Wildman 1972 Chloroplast DNA codes for the primary structure of the large subunit of fraction I protein. Biochim Biophys Acta 277: 677–680
7. Coen DM, JR Bedbrook, L Bogorad, A Rich 1977 Maize chloroplast DNA fragment encoding the large subunit of ribulosebiphosphate carboxylase. Proc Nat Acad Sci USA 74: 5487–5491
8. Cronquist A 1968 The Evolution and Classification of Flowering Plants. Houghton Mifflin, Boston
9. Haff LA, L Bogorad 1976 Hybridization of maize chloroplast DNA with transfer ribonucleic acids. Biochemistry 15: 4105–4109
10. Honda SI, T Hongladarom, GG Laties 1966 A new isolation medium for plant organelles. J Exp Bot 17: 460–472
11. Kirk JTO 1971 Chloroplast structure and biogenesis. Annu Rev Biochem 40: 161–196
12. Kolodner R, KK Tewari 1975 The molecular size and conformation of the

chloroplast DNA from higher plants. Biochim Biophys Acta 402: 372–390
13. KUNG SD, CL LEE, DD WOOD, MA MOSCARELLO 1977 Evolutionary conservation of chloroplast genes coding for the large subunits of fraction I protein. Plant Physiol 60: 89–94
14. KWOK SY, SG WILDMAN 1974 Evolutionary divergence in the two kinds of subunits of ribulose diphosphate carboxylase isolated from different species of *Nicotiana*. J Mol Evol 3: 103–108
15. LAMPPA GK, AJ BENDICH 1979 Chloroplast DNA sequence homologies among vascular plants. Plant Physiol 63: 660–668
16. MACGREGOR HC, S MIZUNO 1976 *In situ* hybridization of "nick translated" ^{3}H-ribosomal DNA to chromosomes from salamanders. Chromosoma 54: 15–25
17. ROZEK CE, WC ORR, WE TIMBERLAKE 1978 Diversity and abundance of polyadenylated RNA from *Achlya ambisexualis*. Biochemistry 17: 716–722
18. STUDIER FW 1965 Sedimentation studies of the size and shape of DNA. J Mol Biol 11: 373–390
19. TEWARI KK, SG WILDMAN 1966 Chloroplast DNA from tobacco leaves. Science 153: 1269–1271
20. TEWARI KK, SG WILDMAN 1968 Function of chloroplast DNA. I. Hybridization studies involving nuclear and chloroplast DNA with RNA from cytoplasmic (80S) and chloroplast (70S) ribosomes. Proc Nat Acad Sci USA 59: 569–576
21. Thomas JR, KK Tewari 1974 Conservation of 70S ribosomal RNA genes in the chloroplast DNAs of higher plants. Proc Nat Acad Sci USA 71: 3147–3151
22. VEDEL F, F QUETIER, M BAYEN 1976 Specific cleavage of chloroplast DNA from higher plants by EcoRI restriction endonuclease. Nature 263: 440–442
23. WALBOT V 1977 The dimorphic chloroplasts of the C_4 plant *Panicum maximum* contain identical genomes. Cell 11: 729–737
24. ZIMMERMAN JL, RB GOLDBERG 1977 DNA sequence organization in the genome of *Nicotiana tabacum*. Chromosoma 59: 227–252