*FUNCTION OF CHLOROPLAST DNA, I. HYBRIDIZATION STUDIES INVOLVING NUCLEAR AND CHLOROPLAST DNA WITH RNA FROM CYTOPLASMIC (80S) AND CHLOROPLAST (70S) RIBOSOMES**

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Tobacco chloroplasts have been shown to have (1) a specific DNA (ρ = 1.703 compared to 1.698 for nuclear DNA, as well as differences in renaturation behavior and base composition);[1] (2) characteristic ribosomes (sedimentation constant of 70*S* in contrast to 80*S* for cytoplasmic ribosomes, and also requiring 10 mM Mg^{++} for stability compared to 1 mM for cytoplasmic ribosomes[2, 3]); (3) independent DNA → RNA polymerase, localized within the organelle, and differing in many properties from the corresponding nuclear enzyme;[4, 5] and (4) DNA polymerase capable of making chloroplastlike DNA *in vitro*,[6] such properties being consistent with the concept that chloroplasts are autonomous in nature. To assess the functional role of chloroplast DNA, experiments have been carried out to measure the amount of hybridization which occurs between chloroplast and nuclear DNA's versus RNA's obtained from highly purified 70*S* and 80*S* ribosomes from tobacco leaves. The results show that chloroplast DNA contains the complements of RNA from 70*S* ribosomes but does not contain segments coding for RNA from 80*S* cytoplasmic ribosomes. In contrast, nuclear DNA seems to have many cistrons for chloroplast ribosomal RNA, in addition to having specific regions for cytoplasmic ribosomal RNA.

Materials and Methods.—Isolation of DNA: Purification of nuclei, chloroplasts, and DNA was the same as described before[1, 6], except that after treating the DNA with 50 μg RNase (Sigma) and 20 units/ml RNase T_1 (Cal Biochem), the mixture was incubated with 50 μg/ml of Pronase (Cal Biochem) followed by deproteinization with phenol, alcohol precipitation, and extensive dialysis against SSC. DNA samples showing A_{260} = 0.20–0.22 for 10 μg/ml (assayed by Burton's method[7]) were used for hybridization studies.

Labeling ribosomal RNA: Using the technique of Hirai and Wildman,[8] 50 μc of H^3-uracil in 0.25 ml of water was supplied to a 2- to 3-cm long, single leaf per small tobacco plant on each of 3 successive days. To allow for the disappearance of any rapidly labeled RNA, the leaves were not harvested until 2 days after the isotope treatment. In some cases, a chase of 50 μg of nonradioactive uracil was given to each leaf 6 hr before harvest.

Isolation of ribosomes: 80S: Each gram of leaves was homogenized by mortar and pestle in the presence of 1 ml of 10 mM Tris-HCl (pH 8.0) and 10 mM Mg^{++}. After centrifugation for 20 min at 17,000 × *g*, supernatants equivalent to 0.5 gm of leaves were layered on 5–20%, linear sucrose gradients containing 10 mM Mg^{++}, centrifuged for 2 hr at 24,000 rpm in the Spinco SW25.1 rotor, and fractions collected by pumping out the gradient and monitoring the OD_{260} according to Chen and Wildman.[9]

70S: Chloroplasts isolated in Honda medium from 5 gm of leaves, as described by Chen and Wildman,[9] were resuspended in 1 ml of 10 mM Tris, 10 mM Mg^{++}, and 0.4% DOC and ground in a glass homogenizer. After centrifugation at 17,000 × *g* for 45 min, the supernatant was dialyzed 4 hr against 1000 vol of 10 mM Tris and 10 mM Mg^{++}, and then the ribosomes were resolved on a gradient and collected as described for 80*S* ribosomes. Both radioactive and nonradioactive 70*S* and 80*S* ribosomes were obtained as described.

Isolation of ribosomal RNA: The combined fractions from sucrose gradients were diluted with an equal volume of 10 mM Tris and 10 mM Mg^{++} in the case of 70*S* (2 mM Mg^{++} for 80*S*), and the ribosomes pelleted by centrifuging 3 hr at 140 kg. Ribosome pellets corresponding to 10 gm of leaves (70*S*) or 1 gm of leaves (80*S*) were suspended in 1 ml SSC to which was added 0.1 ml of 10% SDS and 0.1 ml bentonite (10 mg/ml). The mixture was shaken with 1.5 ml of water-saturated phenol. After 3 phenol extractions, the RNA in the aqueous phase was precipitated with 2 vol of alcohol and kept in the freezer overnight. The small amount of precipitate was collected by centrifugation, dissolved in 1.0 ml SSC, and dialyzed against 1000 vol of SSC which was changed several times. The RNA solution contained the same amount of radioactivity regardless of whether it was dried and measured directly or precipitated and washed with cold acid.

Hybridization: The technique used was essentially that of Gillespie and Spiegelman[10] in a final volume of 2.0 ml 6 SSC. Preliminary experiments using H^3-thymidine DNA indicated that up to 250 μg of DNA could be retained by the filter. After hybridization, the filters were treated with 5 ml of 20 μg/ml RNase at 30° for 6 hr, and then each side of the filters was washed with 100 ml of 2 SSC.

Experimental Results.—Isolation of pure ribosomes and ribosomal RNA's: The OD_{260} and radioactivity profiles shown in Figure 1 are typical of those used for the isolation of 70*S* and 80*S* ribosomes. In Figure 1*a*, a large peak of 80*S* ribosomes is resolved from a small peak of 70*S* ribosomes. Only those ribosomes in the leading edge of the 80*S* peak (fractions 15-20) were collected and used for RNA extraction. In a typical experiment, 360μg of RNA containing 1350 cpm/μg was isolated from the ribosomes collected from four such gradients. Seventy per cent of the OD_{260} in the ribosomes was recovered as RNA. A profile typical of those used for isolation of 70*S* chloroplast ribosomes is presented in Figure 1*b*. Only the ribosomes in the trailing edge of the major, 70*S* monosome peak (fractions 10–14) were collected to avoid contamination by either the small amount of ribosomes on the leading edge of the 70*S* monosome peak or the heavier polysomes. In a typical experiment, 80 μg of RNA containing 1420 cpm/μg was isolated from the 70*S* ribosomes collected from two such gradients, the recovery being 65 per cent on an OD basis.

When H^3-RNA from 70*S* ribosomes was mixed with nonradioactive RNA from 70*S* ribosomes, or H^3-RNA from 80*S* ribosomes with unlabeled RNA from 80*S* ribosomes, and then both mixtures centrifuged in 5–20 per cent sucrose contain-

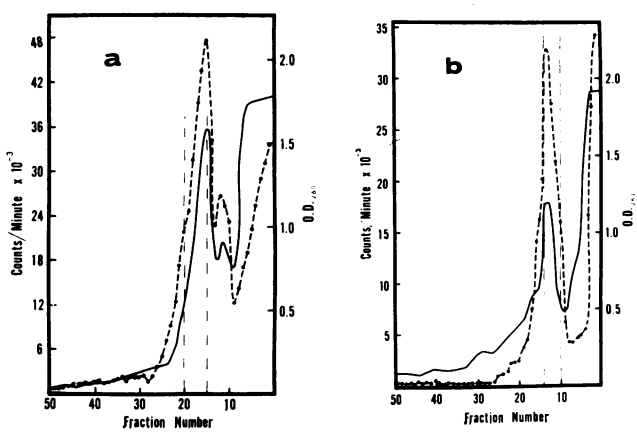

FIG. 1.—Method used for isolation of pure 70*S* and 80*S* ribosomes. *Continuous line* = OD_{260}; *dashed line*, cpm. Space between parallel broken lines represents fractions used for isolation of ribosomes. (*a*) Total leaf ribosomes; (*b*) ribosomes from isolated chloroplasts, both after sucrose density gradient centrifugation.

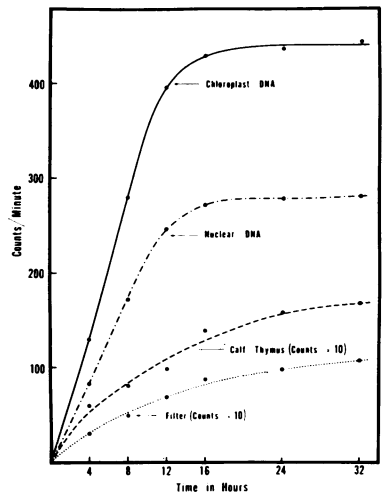

FIG. 2.—Time required for maximum hybridization. Radioactivity retained by filters as a function of time of annealing. One hundred μg. of nuclear, chloroplast, or calf thymus DNA contained on filters annealed with 5 μg of RNA's. Specific activity of RNA's from 80*S* and 70*S* ribosomes was 1120 and 900 cpm/μg, respectively. Chloroplast DNA vs. H^3-RNA from 70*S* ribosomes; nuclear DNA vs. H^3-RNA from 80*S* ribosomes; calf thymus DNA vs. H^3-RNA from 80*S* ribosomes (comparable result obtained with chloroplast ribosomal RNA). The ordinate scale for calf thymus and filter counts per minute is one tenth that for nuclear and chloroplast DNA's.

ing 0.05 *M* NaCl at 24,000 rpm for 16 hours, the radioactivity peaks matched the OD_{260} peaks, thus showing the radioactive RNA's to be free of heterogeneous pulse-labeled RNA.

Time for maximum hybridization: As shown by the data in Figure 2, maximum binding of H^3-RNA from 70*S* ribosomes to chloroplast DNA, or H^3-RNA from 80*S* ribosomes to nuclear DNA, is achieved in 16 hours and does not increase as the time of annealing is extended. In each case, 100 μg of DNA was present on the filters. When a similar amount of calf thymus DNA on the filters was used, binding was negligible, amounting to less than 4 per cent of that which bound to the tobacco DNA's. With no DNA on the filters, less than 3 per cent of the H^3-RNA's that bound to the leaf DNA's adsorbed nonspecifically to the filters. Thus, the binding of the two ribosomal RNA's to tobacco leaf DNA's appears to be specific. In all subsequent experiments, 16 hours was used for annealing.

Saturation of DNA's with ribosomal RNA's: One hundred μg of either chloroplast or nuclear DNA were adsorbed to filters and hybridization was carried out with increasing amounts of RNA from 80*S* and 70*S* ribosomes. The data in Figure 3 show an almost linear increase in the amount of H^3-RNA's bound to DNA's until saturations are reached at 3–4 μg of RNA. Apparently, all of the binding sites on the DNA's are saturated when the DNA/RNA ratio is about 20. The fact that clear saturation levels are reached is a further indication that the ribosomal RNA's are not contaminated with messenger RNA.

Proportionality of ribosomal RNA binding as a function of DNA concentration: The data in Figure 4 were obtained by varying the amount of chloroplast or nuclear DNA's on the filters in the presence of H^3-RNA's from 70*S* or 80*S* ribosomes in a ratio of 20 DNA to 1 RNA. In the case of nuclear DNA versus RNA from 80*S* ribosomes, binding is proportional up to a concentration of 60 μg of DNA and then departs from proportionality as the DNA is further increased. In the case of chloroplast DNA versus RNA from 70*S* ribosomes, proportionality occurs up to the limit that chloroplast DNA was adsorbed on the filters. All further experiments were performed in the region of proportionality.

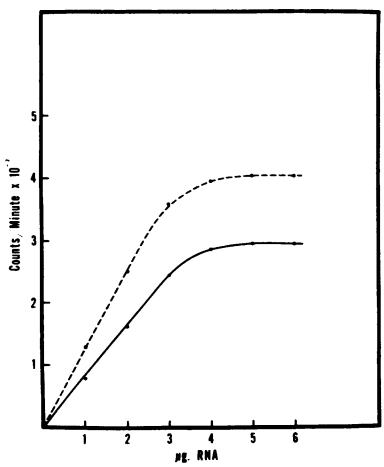

FIG. 3.—Amount of RNA's required for saturation of DNA's. One hundred μg of nuclear DNA vs. cytoplasmic ribosomal RNA (· —— ·), or chloroplast DNA vs. chloroplast ribosomal RNA (· - - - ·). Specific activity of RNA's are as in Fig. 2.

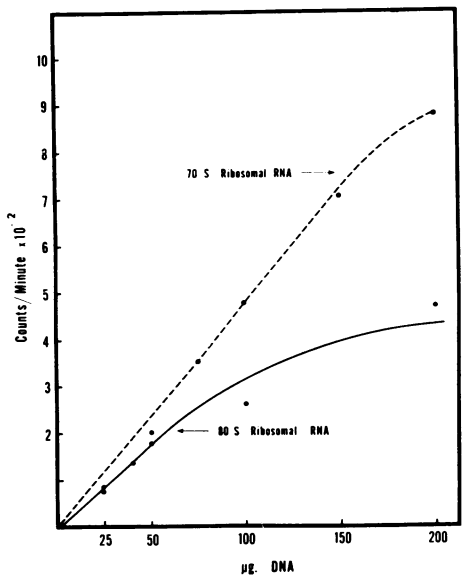

FIG. 4.—Proportionality of ribosomal RNA binding as a function of DNA concentration. Solid line, nuclear DNA; dashed line, chloroplast DNA. Specific activities of RNA's from 80*S* and 70*S* ribosomes were 1220 and 900 cpm/μg, respectively. Ratio of DNA/RNA = 20/1.

Extent of hybridization with ribosomal RNA's and DNA's: Hybridization levels for nuclear DNA versus RNA from 80*S* ribosomes and chloroplast DNA versus RNA from 70*S* ribosomes are tabulated in Table 1. The data presented have been obtained from three independent preparations of 80*S* and 70*S* ribosomes and utilize three different concentrations of DNA. The RNA from 80*S* ribosomes hybridized with nuclear DNA ranged from 0.21 to 0.29 per cent. These values are close to the 0.3 per cent hybridization between ribosomal RNA cistrons and DNA of several bacteria,[11, 12] a higher plant,[13] an insect,[14] and HeLa cells.[15] Matsuda and Siegel[16] report values ranging from 0.06 to 0.80 per cent for four higher plants, one being tobacco where the range was from 0.07 to 0.13 per cent.

The per cent chloroplast DNA hybridized with RNA from 70*S* ribosomes ranges from 0.45 to 0.65 and is therefore much higher than in the case for RNA from 80*S* ribosomes versus nuclear DNA. Scott and Smillie[17] report a value of 1 per cent for *Euglena* chloroplast DNA versus chloroplast ribosomal RNA. However, the chloroplast ribosomes do not appear to have been purified to the extent as were those used in the present experiments.

Cross-hybridization experiments: As shown by the data in Table 2, significant hybridization did not occur between chloroplast DNA and RNA from 80*S* ribosomes. Even with a concentration of 100 μg of chloroplast DNA, binding occurred to a level of 0.02 per cent which was only 7 cpm above that observed with calf thymus DNA. Thus, chloroplast DNA does not seem to have any comple-

TABLE 1. *Extent of hybridization between nuclear and chloroplast DNA's and RNA's from 70S and 80S ribosomes.*

Preparation	Nuclear DNA on filters (μg)	Cytoplasmic ribosomal RNA bound (cpm)	Per cent hybridization
1	25	81	0.27
	50	180	0.28
	100	260	0.21
2	25	64	0.24
	50	144	0.26
	100	307	0.27
3	25	71	0.21
	50	154	0.23
	100	294	0.22
Preparation	**Chloroplast DNA on filters (μg)**	**Chloroplast ribosomal RNA bound (cpm)**	**Per cent hybridization**
1	25	90	0.40
	50	204	0.45
	100	481	0.54
2	25	174	0.59
	50	383	0.65
	100	660	0.56
3	25	181	0.51
	50	340	0.48
	100	738	0.52

Specific activities (cpm/μg) for preparations 1, 2, and 3: RNA from 80S ribosomes, 1220, 1120 and 1350, respectively; RNA from 70S ribosomes, 900, 1180, and 1420, respectively.

TABLE 2. *Cross hybridization between nuclear and chloroplast DNA's and RNA's from 70S and 80S ribosomes.*

	DNA on Filters		RNA from			
Preparation	Nuclear	Chloroplast	80S	70S	Cpm bound	Per cent hybridization
1	+	−	−	+	53	0.11
	−	+	+	−	14	0.02
2	+	−	−	+	52	0.09
	−	+	+	−	13	0.02
3	+	−	−	+	68	0.09
	−	+	+	−	14	0.02

Specific activities of ribosomal RNA's are the same as in Table 1 and are used in the same order. (Fifty μg of nuclear or chloroplast DNA's on the filters.)

mentary base sequences corresponding to the RNA of 80S cytoplasmic ribosomes. On the other hand, nuclear DNA hybridized to a significant extent (0.09–0.11%) with the RNA of 70S chloroplast ribosomes. To ensure that this result could not be attributed to a slight contamination of the nuclear DNA by chloroplast DNA, nuclear DNA was further purified by banding in CsCl and still yielded the same level of hybridization with 70S ribosomal RNA.

Competition studies between nuclear DNA, chloroplast DNA, and RNA from 80S and 70S ribosomes: Since nuclear DNA appeared to have nucleotide sequences complementary to ribosomal RNA's from both 70S and 80S ribosomes, competitive inhibition experiments were conducted to ascertain whether the base sequences for both kinds of RNA's were overlapping, or occupied different regions on the DNA. That the latter is the situation is borne out by the data in Figure

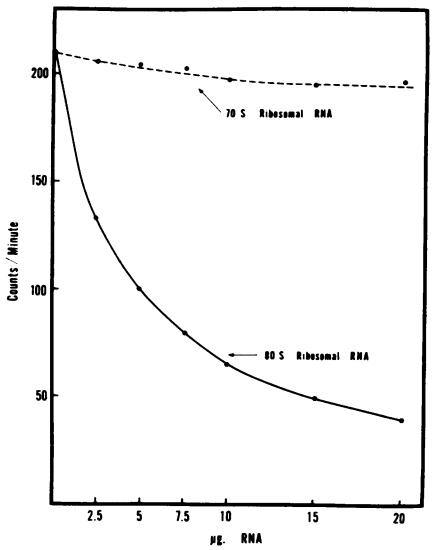

FIG. 5.—Competition for cytoplasmic ribosomal RNA hybridization sites on nuclear DNA. Fifty μg nuclear DNA and 5 μg of H^3-cytoplasmic ribosomal RNA (1220 cpm/μg) in the presence of increasing amounts of nonradioactive cytoplasmic ribosomal RNA (● —— ●) or chloroplast ribosomal RNA (● - - - ●).

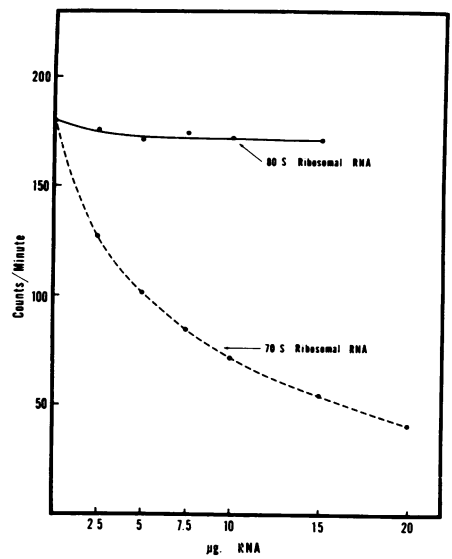

FIG. 6.—Competition for chloroplast ribosomal RNA hybridization sites on chloroplast DNA. Twenty μg chloroplast DNA and 5 μg of H^3-chloroplast ribosomal RNA (1420 cpm/μg) in the presence of increasing amounts of nonradioactive chloroplast ribosomal RNA (● - - - ●) or cytoplasmic ribosomal RNA (● —— ●).

5, where a constant amount of nuclear DNA on the filters was annealed with H^3-RNA from 80*S* ribosomes in the presence of varying amounts of nonradioactive RNA from 80*S* and 70*S* ribosomes. Only 80*S* ribosomal RNA competed with the H^3-RNA from 80*S* ribosomes, the data corresponding to a theoretical curve for competitive inhibition. Chloroplast ribosomal RNA does not appear to be a competitive inhibitor for the sites on nuclear DNA complementary to cytoplasmic ribosomal RNA. The data in Figure 6 show the results when a constant amount of chloroplast DNA was incubated with H^3-RNA from 70*S* ribosomes together with nonradioactive RNA from 80*S* or 70*S* ribosomes. In accordance with the results obtained by cross-hybridization, cytoplasmic ribosomal RNA shows no evidence of competing for the sites on chloroplast DNA which are complementary to chloroplast ribosomal RNA.

Discussion.—Scott and Smillie[17] have reported that chloroplast DNA from *Euglena* contained cistrons for chloroplast ribosomal RNA. Thus, the experiments reported here for tobacco chloroplasts are consistent with the *Euglena* findings. Of more interest, however, is the finding that nuclear DNA also contains cistrons complementary to the ribosomal RNA from chloroplasts, the extent of hybridization being 30 per cent of that found for nuclear DNA versus cytoplasmic ribosomal RNA. In fact, the cross-hybridization of nuclear DNA versus chloroplast ribosomal RNA amounts to 20 per cent of the hybridization

between chloroplast DNA versus chloroplast ribosomal RNA. A somewhat comparable situation appears to exist in yeast where mitochondrial DNA hybridized with RNA from a particulate fraction equivalent to mitochondria, but not with cytoplasmic RNA, and yeast nuclear DNA hybridized with both species of RNA.[20] Since chloroplast DNA constitutes only 10 per cent of the total DNA in a tobacco leaf, the total information in the nuclear DNA capable of coding for chloroplast ribosomal RNA is about three times greater than that contained in the chloroplast DNA. There are about 300–500 chloroplasts for each nucleus in tobacco leaf cells. Therefore, the amount of coding information for chloroplast ribosomal RNA in a nucleus compared to a single chloroplast becomes about 1000 times greater.

The hybridization between nuclear DNA and 80*S* cytoplasmic ribosomal RNA ranges around 0.3 per cent. Assuming 10×10^{-12} gm of DNA to be present in diploid tobacco nuclei,[19] calculation reveals there to be about 2000 cistrons complementary to the RNA from 80*S* ribosomes. This value indicates a very large repetition of ribosomal RNA coding units which could be similar or identical in sequence, depending on whether all the ribosomal RNA is similar or identical. Such a large number of complementary sites for ribosomal RNA appears to be characteristic of higher organisms. For example, the value reported for *Drosophila melanogaster* is from 100 to 500 and 200 to 400 for HeLa cells. In contrast to the large number of repetitive coding units for cytoplasmic ribosomal RNA contained in nuclear DNA, the number of repetitive coding units for chloroplast ribosomal RNA in chloroplast DNA is very small. The hybridization experiments show a maximum of 0.65 per cent of chloroplast DNA has nucleotide sequences complementary to chloroplast ribosomal RNA. Taking the amount of DNA per chloroplast to be 4.7×10^{-15} gm, and assuming that RNA is complementary to only one of the two DNA strands in any given region, 8.4×10^6 daltons of DNA is complementary to chloroplast ribosomal RNA, which is equivalent to about four sites for each of the two ribosomal RNA components. This number is the lowest reported for any DNA coding for ribosomal RNA, being about one-tenth of that obtained for *E. coli* and far lower than for higher organisms. If the DNA in tobacco chloroplasts were localized at two ends of the organelle in a manner comparable to that recently reported for an algal chloroplast,[18] the number might be reduced to two sites. In any event, there do not seem to be many repeating units in the chloroplast DNA capable of coding for chloroplast ribosomal RNA. Evidently, most of the chloroplast DNA (*ca.* 3×10^9 daltons) could be available for other purposes such as mRNA formation. Since chloroplast DNA does not bind with cytoplasmic RNA to a significant extent, it may also be inferred that the sequences of nucleotides in the RNA of 70*S* ribosomes are vastly different from those in the RNA of 80*S* ribosomes.

The question remains as to whether the codons in nuclear DNA complementary to the RNA in 70*S* ribosomes are functional in the formation of chloroplast ribosomes, or represent a nonfunctional relic of past evolution. That there are common nucleotide sequences between chloroplast DNA and nuclear DNA has been reported by Richards for *Euglena*[21] and also observed by us in hybridization experiments involving nuclear DNA and *in vitro* DNA synthesized by tobacco

chloroplasts.[6] If the information required for a photosynthetic apparatus was originally contained in a primeval nuclear DNA, and subsequent evolution resulted in the origin of chloroplast DNA from nuclear DNA when the photosynthetic apparatus became enclosed within an organelle, it would be no surprise to find the two DNA's sharing complementary sequences of nucleotides. However, the nucleus does have a role in the development of chloroplasts, as attested by the many nuclear mutants which affect the biosynthesis of chlorophyll as well as the structure and enzymatic components of chloroplasts.[22] Thus, it is conceivable that the nucleus may exert control over chloroplasts at the chloroplast ribosomal level. In view of the very small number of sites for formation of chloroplast ribosomal RNA on chloroplast DNA, compared to the large number on nuclear DNA, we are attracted to the idea that chloroplast DNA may code for a small number of highly specific ribosomes, whereas most of the other ribosomes in the chloroplast are derived from coding information contained in nuclei.

Summary.—Hybridization experiments were performed with chloroplast and nuclear DNA's and H^3-RNA's isolated from highly purified 70*S* chloroplast and 80*S* cytoplasmic ribosomes from tobacco leaves. Chloroplast DNA hybridized with chloroplast ribosomal RNA (0.5%) but not with cytoplasmic ribosomal RNA. Nuclear DNA hybridized with cytoplasmic ribosomal RNA (0.25%) and also with chloroplast ribosomal RNA (0.1%). The coding information contained in nuclei for chloroplast ribosomal RNA is about three times greater than that contained in chloroplast DNA on a per cell basis. The chloroplast ribosomal RNA cistrons contained in nuclear DNA occupy sites separated from the many repetitive cistrons for cytoplasmic ribosomal RNA.

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