*HYBRIDIZATION OF PLANT RIBOSOMAL RNA TO DNA: THE ISOLATION OF A DNA COMPONENT RICH IN RIBOSOMAL RNA CISTRONS**

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Several studies of artificial hybridization between ribosomal RNA and homologous and heterologous DNA's from animal and bacterial sources have been reported. These studies indicate that there is a multiplicity of cistrons coding for ribosomal RNA,[1-8] that these cistrons are located at the nucleolar organizer region of higher organisms,[2, 8] and that nucleotide sequences of ribosomal RNA tend to be conserved during evolution.[5, 7] We report here artificial hybridization studies between ribosomal RNA and nuclear DNA isolated from several higher plants. The data permit us to conclude that: (1) the nuclear DNA's from different plant species vary over a tenfold range in their content of ribosomal RNA cistrons; (2) ribosomal RNA's from different plant species appear to be remarkably similar to each other in hybridization behavior with several plant DNA's; and (3) those plant DNA's which show high hybridization values with ribosomal RNA contain a minor or satellite component. The satellite component of pumpkin DNA has been isolated and has been shown to be greatly enriched in ribosomal RNA coding material.

Materials and Methods.—Extraction of DNA: DNA was obtained from wheat germ, *Triticum vulgare*, L.; leaves of tobacco, *Nicotiana tabacum*, L. var. *Samsun;* pinto beans, *Phaseolus vulgaris*, L.; pumpkin, *Cucurbita pepo*, L. var. *Small Sugar;* and Chinese cabbage, *Brassica pekinensis* (Lour. Rupr.) var. *WongBok*. Wheat germ, or deribbed well-expanded leaves, was ground with a half-weight of sucrose-tris buffer (0.5 *M* sucrose, 0.01 *M* $MgCl_2$, 0.05 *M* Tris, 0.025 *M* KCl, 0.005 *M* mercaptoethanol, pH 8.2)[9] using sand and an ice-cold mortar and pestle. The homogenate was filtered through two layers of cheesecloth and glass wool and centrifuged at 1000 × *g* in an SS-34 head (Sorvall RC-2) for 5 min at 1°C. The pellet, containing nuclei and chloroplasts, was resuspended, washed, and centrifuged as above for 3 successive times with one half of the grinding volume of sucrose-tris buffer containing 3.5% Triton X-100 (Rohm and Haas) to remove the chloroplasts.[10] The nuclear pellets from 100 gm of tobacco or wheat tissue were then resuspended in 40 ml saline-EDTA (0.15 *M* NaCl, 0.1 *M* ethylenediaminetetraacetate (EDTA), pH 8.0) and lysed by adjusting to 2% sodium dodecylsulfate (SDS) for 10 min at 25°C. The DNA was then extracted and purified using Marmur's procedure.[11] All other nuclei were initially defatted as outlined by Hotta and Bassel[12] for bull sperm nuclei. The defatted pellet from 100 gm tissue was then resuspended in 20 ml BPES[13] buffer (0.006 *M* Na_2HPO_4, 0.002 *M* NaH_2PO_4, 0.001 *M* disodium EDTA, 0.179 *M* NaCl, pH 9.0) that contained 0.3 *M* 4-aminosalicylic acid and 1% SDS. An equal volume of H_2O-saturated phenol containing 0.1% 8-hydroxyquinoline was added and the total sample was mixed by gentle inversion for 20 min before centrifuging to separate the layers. The phenol treatment was repeated twice before the DNA was spooled after adding 2 vol of 95% ethanol. The DNA was then purified by Marmur's technique except that BPES buffer was used instead of SSC (0.15 *M* NaCl, 0.015 *M* trisodium citrate, pH 7.0). The chloroform and phenol were both washed with pH 9.0 BPES buffer before use.

Extraction of RNA: Labeled RNA was obtained from leaves of tobacco and Chinese cabbage. Plants were grown in sand with complete nutrient medium[14] until 5–7 weeks of age. The complete medium was then removed and replaced with −P medium for 2–3 days prior to labeling each plant with 1 mc carrier-free $H_3P^{32}O_4$ for 3–5 days in the greenhouse. Alternatively, detached leaves were allowed to absorb label (0.3–0.5 mc P^{32}/leaf) through the petiole for 4 days under 400 ft-c continuous fluorescent lighting. The initial grinding and centrifugation procedure after

harvest was identical to that used for DNA extraction. The 1000 × *g* supernatant was centrifuged at 20,000 × *g* for 30 min and the resulting supernatant fraction was centrifuged at 105,000 × *g* for 3 hr. The ribosomal pellet was then extracted for RNA using phenol,[15] and the RNA fraction was purified further by precipitating overnight in 1.5 *M* NaCl in M/60 Sorenson's phosphate buffer, pH 7.0. The RNA fractions were then monitored on sucrose density gradients to ensure that the radioactivity profile was identical to the 28*S* and 18*S* patterns shown for total ribosomal RNA.

Hybridization of RNA to DNA: Hybridizations were performed according to Gillespie and Spiegelman.[16] The DNA samples (50 μg of total nuclear, 10 μg of satellite DNA) were denatured by heating in 0.1 × SSC for 10 min at 90°C, then quick cooling in ice water before embedding on membranes. Hybridizations were conducted using 10 μg labeled RNA in 5 ml 2 × SSC for 8–10 hr. This amount of RNA was found to be sufficient for saturation for each nuclear DNA fraction. The radioactivity was assayed by gluing membranes onto planchets and counting in a Nuclear-Chicago thin-window gas-flow counter. The radioactivities of samples incubated at 0°C were subtracted from those obtained at hybridization temperature (68°) before calculating for per cent hybridization.

Determination of buoyant density and T_m of DNA: The technique described by Meselson, Stahl, and Vinograd[17] was followed for the determination of buoyant densities in CsCl. Densities were calculated by using the position of standard *Micrococcus lysodeikticus* DNA (ρ = 1.731 gm/cc) as a reference.[18]

Denaturation of DNA as a function of temperature was determined in SSC according to the procedure of Marmur and Doty.[19] The behavior of denatured DNA samples was also studied by following the absorbancy upon slow cooling after melting.

Results.—Artificial hybridization of tobacco ribosomal RNA to nuclear DNA's of several plant species: Hybridization experiments between tobacco leaf ribosomal RNA and tobacco nuclear DNA revealed that about 0.1 per cent of the DNA was complementary to the RNA. Several experiments were then conducted to determine whether and to what extent other plant DNA's might prove to have regions complementary to tobacco ribosomal RNA. The results of these experiments are shown in Table 1. Two features of these results are of interest. (1) All of the DNA's tested prove to have regions which are complementary to tobacco ribosomal RNA. This is so even though the DNA's were extracted from plants which belong to different taxonomic families. (2) The proportion of the different DNA's which are complementary to tobacco ribosomal RNA varies over a tenfold range. Surprisingly, heterologous hybridization with pinto bean, pumpkin, and Chinese cabbage DNA reveals a much larger extent of hybridization than does the homologous hybridization with tobacco DNA. The value for pinto bean is about three times that for tobacco, whereas Chinese cabbage and pumpkin DNA's apparently have

TABLE 1

HYBRIDIZATION OF TOBACCO RIBOSOMAL RNA TO DNA FROM DIFFERENT PLANT SPECIES

DNA source	Net cpm*	Specific hybridization (cpm)†	RNA hybrid (μg) / 100 μg DNA
Expt. 1			
Tobacco	89	76	0.07
Pumpkin	897	879	0.80
Wheat	87	68	0.06
Pinto bean	309	290	0.26
Expt. 2			
Tobacco	349	325	0.076
Chinese cabbage	3270	3238	0.76

* Average of duplicate determinations. Ten μg labeled tobacco leaf rRNA (2200 cpm/μg, expt. 1; 8500 cpm/μg, expt. 2) were incubated with membranes containing 50 μg of DNA for 8 hr at 68°C.

† Values obtained in hybridization condition (68°C) were corrected for nonspecific hybridization by subtracting cpm of identical samples incubated in duplicate at 0°C.

TABLE 2

HYBRIDIZATION OF SEVERAL DNA'S TO RRNA FROM TOBACCO AND CHINESE CABBAGE

DNA source	rRNA source	Hydridization (% of DNA)
Tobacco	Tobacco	0.13
Tobacco	Chinese cabbage	0.10
Chinese cabbage	Tobacco	0.82
Chinese cabbage	Chinese cabbage	0.93
Pinto bean	Tobacco	0.28
Pinto bean	Chinese cabbage	0.28

Average of duplicate hybridization reactions. Corrected for nonspecific binding as indicated in Table 1.

ten times the percentage of complementary region for tobacco ribosomal RNA than does tobacco DNA. Wheat, a monocot, proves to have DNA which hybridizes to about the same extent as does tobacco DNA.

Artificial hybridization of several plant DNA's with tobacco and Chinese cabbage ribosomal RNA: The data presented in Table 1 suggest that the ribosomal RNA's from phylogenetically diverse plant species may be remarkably similar to one another. In addition, it appears that the nuclear DNA's of different species may contain different proportions of ribosomal RNA complementary material. To further test this hypothesis, experiments were performed to determine the extent of hybridization of tobacco, pinto bean, and Chinese cabbage DNA's with ribosomal RNA from both tobacco and Chinese cabbage. The results are presented in Table 2 where it can be seen that the homologous and heterologous hybridization values are remarkably similar for both tobacco and Chinese cabbage DNA's. Only heterologous hybridizations were performed with pinto bean DNA but it can be seen that this DNA has the same proportion of complementary region (0.28%) for both tobacco and Chinese cabbage ribosomal RNA. The value 0.28 per cent is the same as that found by Chipchase and Birnstiel[20] for the homologous hybridization of pea (another legume) DNA to pea ribosomal RNA. Since hybridization reactions have been shown to be highly specific,[21] one may infer from the data presented in Tables 1 and 2 that the base sequences of ribosomal RNA obtained from different plant species may be identical over long stretches of the polynucleotide chain and that different plant DNA's contain different proportions of ribosomal RNA complementary regions.

The presence of a satellite component in DNA's with high hybridization values for ribosomal RNA: The DNA's of the several plant species whose hybridization values for ribosomal RNA have been herein reported were banded by isopycnic centrifugation in CsCl gradients. This was done with the idea that perhaps DNA's with high hybridization values would contain a satellite component which might prove to be responsible for the excessive proportions of ribosomal RNA cistron content. The results are shown in Figure 1 where it can be seen that tobacco DNA, a low hybridizer, bands as a single component. This is also true of wheat DNA, another low hybridizer. The DNA's from pumpkin and Chinese cabbage which contain a large percentage of complementarity to ribosomal RNA, however, do contain satellite components as does pinto bean DNA, which exhibits an intermediate value in the hybridization experiments. Although low hybridizing DNA's prove not to contain a detectable satellite component and the intermediate and high hybridizers do clearly contain such a component, the amount of the more dense minor DNA com-

ponent does not seem to be quantitatively related to the extent of hybridization. It is possible, therefore, that the observed correlation between hybridization value and presence or absence of a satellite component might be fortuitous. Thus, it was decided to isolate and characterize the satellite component from one of the DNA's in order to test whether it might be rich in ribosomal RNA cistrons.

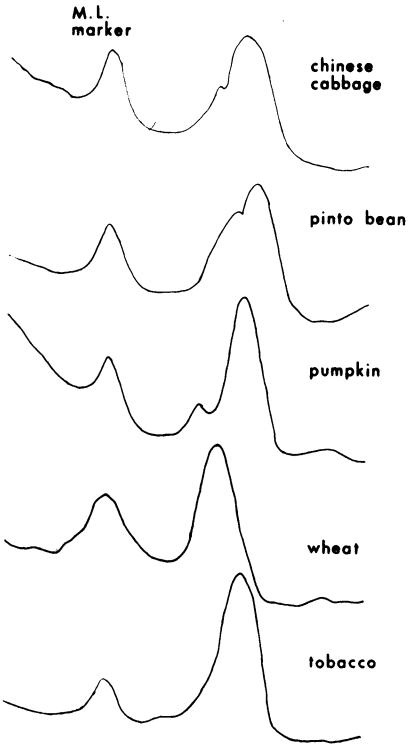

FIG. 1.—Microdensitometer tracings of UV-absorption photographs of nuclear DNA's from several plant species in CsCl density gradient. The photographs were taken after 18 hr centrifugation at 44,770 rpm at 20°C in a Spinco model E analytical ultracentrifuge. The marker DNA is from *Micrococcus lysodeikticus* (ρ = 1.731).

The isolation and partial charatcerization of pumpkin satellite and main band DNA's: A decision was made to isolate the pumpkin satellite component because, of the satellites observed (Fig. 1), it is most different in density from its main component. Separation of the pumpkin satellite component from the main band DNA was effected by using a slight modification of the angle-head preparative isopycnic centrifugation procedure of Flamm, Bond, and Burr.[22] Figure 2 shows a typical optical density profile obtained after centrifuging in CsCl, density 1.7, containing 300 μg pumpkin nuclear DNA in the small no. 50 Spinco angle-head rotor at 35,000 rpm for 70 hours. The satellite component was found to comprise approximately 6 per cent of the total DNA. The tubes containing satellite component were pooled as were the tubes containing the main band component. The two components were reconcentrated by ethanol precipitation and aliquots were examined for efficiency of component separation by banding in the analytical centrifuge. The densitometric tracings are shown in Figure 3 where it can be seen that the procedure used yields a remarkable separation after a single cycle.

Figure 4 presents the results of a melting and annealing study of the pumpkin

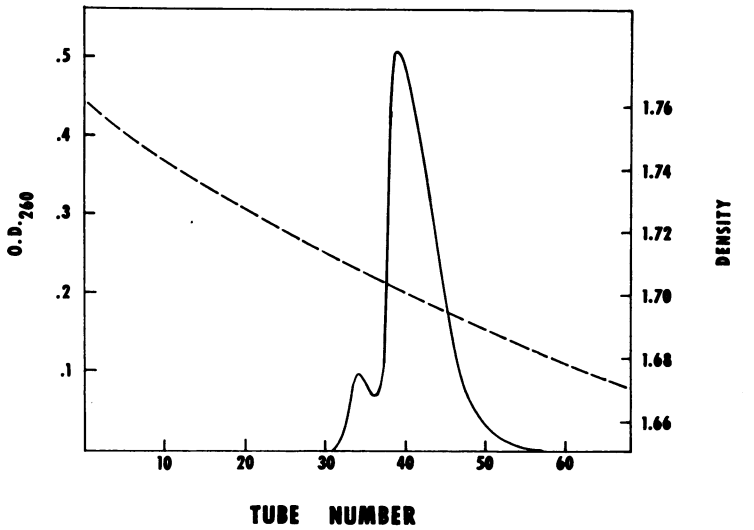

FIG. 2.— Profile of pumpkin DNA after angle head (Spinco no. 50) centrifugation in CsCl at 35,000 rpm for 70 hr at 20°. At the conclusion of centrifugation, a 20-G needle was inserted into the bottom of the tube and 12-drop fractions were collected. The optical density at 260 mμ of each fraction was determined after addition of 1 ml 0.1 SSC. The density of every seventh fraction was determined by refractometry immediately as it was collected. ——, OD_{260}; ——, density.

satellite and main band components. Like the satellite component found in mouse liver nuclear DNA, the pumpkin satellite DNA proves to renature readily on slow cooling in contrast to the main band DNA. The guanine + cytosine (GC) contents of the satellite component estimated on the basis of buoyant density (1.707) and melting temperature (T_m) (92°) are, respectively, 48 per cent and 55.7 per cent. This sort of discrepancy has been noted for mouse liver satellite component and has been attributed to a possible content of 5-methyl cytosine.[23] The estimated GC content of the satellite component is considerably greater than that estimated for the main band component (35.7% and 40.7% on the basis of buoyant density (1.695) and T_m (86°), respectively) and more closely resembles that reported for plant ribosomal RNA.[24, 25]

The pumpkin satellite DNA component proves to be exceedingly rich in regions complementary to ribosomal RNA as compared with total pumpkin nuclear DNA, and the main band is correspondingly poor in such regions (Table 3). The data indicate that approximately 4 per cent of the satellite component is composed of cistrons for ribosomal RNA, whereas only 0.2 per cent of the main band component will hybridize with tobacco ribosomal RNA. This phenomenon appears to be somewhat similar to that reported by Wallace and Birnstiel,[8] who observed that only the more dense fractions of *Xenopus* DNA would hybridize with ribosomal RNA, although there was an insufficient amount of this DNA component to appear as a satellite in banding experiments. It is possible to isolate reasonable quantities of this satellite DNA component from pumpkin DNA for further characterization.

Discussion.—The data presented in Figures 1 and 2 indicate that the ribosomal RNA's from diverse plant species are remarkably similar to one another in artificial hybridization behavior. We conclude, therefore, that these RNA's are probably

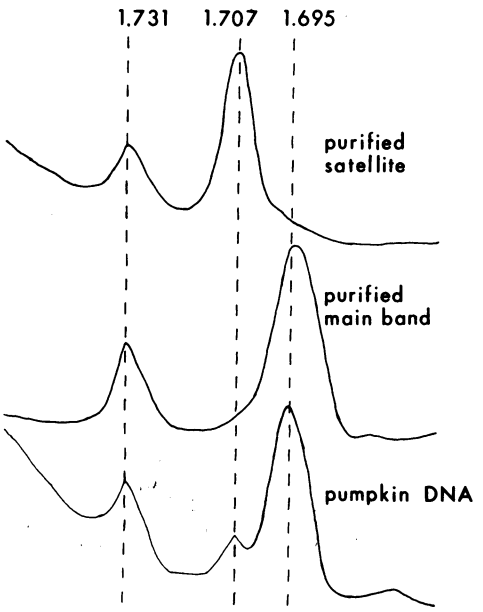

FIG. 3.—Microdensitometer tracings of UV-absorption photographs of unfractionated, satellite, and main band pumpkin DNA in CsCl density gradient. Conditions of density gradient centrifugation were the same as in Fig. 1.

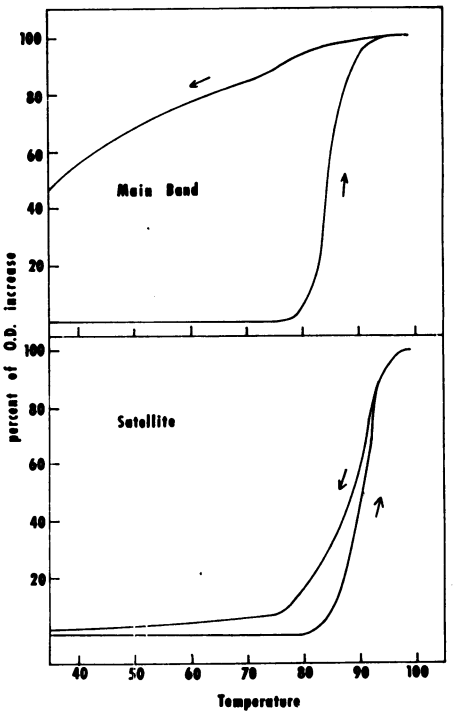

FIG. 4.—Melting and annealing of main band and satellite components of pumpkin DNA in SSC. DNA solutions in stoppered cuvettes were slowly heated and slowly cooled in the Gilford 2000 spectrophotometer chamber equipped with thermospacers. Hypochromicity of the satellite and main band components were, respectively, 25% and 39%. Arrows pointing up and down indicate the curves obtained during heating and cooling, respectively.

identical over long stretches of the polynucleotide strands. A similar, but not identical, situation has been found to prevail in a microbial system.[5, 7] Considerable heterologous hybridization has been found to occur among species of *Bacillus* with regard to ribosomal and transfer RNA. However, the extent of intergeneric hybridization with ribosomal RNA found in microorganisms is considerably less than we have found for phylogenetically well-separated plant species. Some heterologous ribosomal RNA hybridization has been observed to occur between animal

TABLE 3

HYBRIDIZATION OF TOBACCO RIBOSOMAL RNA TO DIFFERENT DNA FRACTIONS FROM PUMPKIN

DNA source	Hybridization (% of DNA) Expt. 1	Expt. 2
Tobacco	0.086	0.082
Whole pumpkin	0.44	0.34
Main band, pumpkin	0.21	0.23
Satellite band, pumpkin	3.1	4.0

Each experiment represents values using independent sources of RNA and DNA. Membranes contained 10 μg satellite DNA and 50 μg of all other DNA's. The hybridization procedures and subsequent treatments are outlined in Table 1 and *Materials and Methods*.

species[3] but, again, not to the extent observed in the present instance. It would appear, on the basis of the limited data available, that there may be a greater evolutionary conservation of ribosomal RNA in plants than there is in other groups of organisms.

The fraction of plant nuclear DNA that is composed of coding sequences for ribosomal RNA varies over a tenfold range among the different species examined. We calculate that tobacco nuclei contain about 1500 copies of the cistrons for ribosomal RNA on the basis of an estimate of 6×10^{12} daltons of DNA per nucleus.[26] If Chinese cabbage and pumpkin nuclei contain about the same amount of DNA, they would contain about ten times this number of ribosomal RNA cistrons. We are at a loss to explain such probable high numbers in reasonable physiological terms. It is interesting to note, however, that minor DNA components enhanced in GC content are present in those DNA's which have high hybridization values. These minor, or satellite, components probably contain the bulk of ribosomal RNA complementary material. In the case of pumpkin DNA the satellite component has been shown to contain somewhat over half the nuclear content of cistrons for ribosomal RNA.

The satellite components may comprise a portion of chromosomal DNA, probably from the nucleolar organizer[2, 6] region of the chromosome. An alternate possibility, however, is that they are composed largely of extrachromosomal DNA which has arisen as a result of asynchronous replication of a portion of the chromosomal DNA and which is possibly sequestered in the nucleolus. The latter hypothesis may not be too unlikely in view of the observation that DNA is present in amphibian oöcyte nucleoli that are well separated from chromosomes.[27] The variable hybridization values that we have found for whole pumpkin DNA (compare Tables 1 and 3) may be a reflection of different amounts of asynchronous DNA replication.

The satellite component of pumpkin DNA renatures readily in SSC in contrast to other higher organism DNA's but in a manner similar to that of the mouse liver nuclear satellite. Waring and Britten[28] have interpreted this behavior to signify the presence of repeating short nucleotide sequences. Such an interpretation is compatible with an hypothesis that the pumpkin nuclear satellite is composed in part of repeating cistrons for ribosomal RNA. We have observed by means of CsCl banding experiments that the Chinese cabbage nuclear satellite also renatures readily even in the presence of the main band. The main band does not renature to an appreciable extent but forms large networks as described by Waring and Britten [28] for mouse liver DNA.

Total unfractionated leaf ribosomal RNA has been used in the experiments reported herein. Leaves, however, contain two main classes of ribosomes. Seventy *S* ribosomes are found in chloroplasts and 80*S* ribosomes in the cytoplasm.[29] We are unable to report at this time the relative amount of nuclear DNA which is complementary to each of the species of ribosomal RNA. However, preliminary data obtained with the use of root ribosomal RNA, which contains the 80*S* ribosome,[30, 31] suggest that low hybridizing DNA's, such as tobacco, have approximately equal amounts of material complementary to the two types of ribosomes. The high hybridizing DNA's, such as Chinese cabbage and pumpkin, appear to contain primarily regions coding for ribosomal RNA contained in 80*S* ribosomes.

Summary.—Homologous and heterologous hybridization studies between plant nuclear DNA's and ribosomal RNA's reveal that nuclear DNA's from different plant species vary over a tenfold range in their content of ribosomal RNA cistrons and that the ribosomal RNA's from different plant species are remarkably similar to each other. Those plant DNA's which have high hybridization values with ribosomal RNA contain a satellite component. The pumpkin nuclear satellite has been isolated and has been shown to be greatly enriched in cistrons for ribosomal RNA.

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