Characterization of the Rapidly Reassociating Deoxyribonucleic Acid of *Cucurbita pepo* L. and the Sequences Complementary to Ribosomal and Transfer Ribonucleic Acids†

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ABSTRACT: The numbers of DNA sequences in the pumpkin genome which are complementary to high molecular weight ribosomal RNA, 5S RNA, and 4S RNA have been found to be present in the ratio 1:1.2:2.5. Based upon a haploid genomic DNA content of 5.1×10^{11} daltons, there are 3400 copies of the combined 16S, 18S, 23S, and 25S ribosomal RNA cistrons, 3900 copies of 5S, and 8600 copies of 4S RNA cistrons per genome. Both high molecular weight (16, 18, 23, and 25 S) and low molecular weight (5 S) ribosomal RNAs are contained in a dense satellite component which has a buoyant density in CsCl of 1.708 g/cm^3. Complements of the transfer RNA are distributed throughout both the bulk or main band DNA (1.695 g/cm^3) and the satellite DNA. At least two classes of highly repetitive sequences, r and m, can be identified when pumpkin DNA is denatured and then reassociated at low Cot. These have buoyant densities of 1.708 and 1.695 g/cm^3 and correspond, respectively, to reassociated satellite DNA and a portion of main band DNA. The complements for high molecular weight ribosomal, 5S, and some 4S RNA are located in r but not m. The r and m components comprise 19 and 4%, respectively, of the total DNA.

The numbers of DNA sequences complementary to HMW rRNA[1] and 5S and 4S RNA have been reported for several organisms (see Hatlen and Attardi, 1971). *Xenopus laevis* DNA, which serves as a useful model for comparison, contains, respectively, 450, 27,000, and 1000 copies of the genes for 18 + 25S rRNA and 5S and 4S RNA per haploid genome (Birnstiel *et al.*, 1966; Brown and Weber, 1968). These sequences differ not only in number but also in buoyant density in CsCl. Thus, while 4S DNA species have a broad range of buoyant densities similar to and greater than bulk DNA (1.698 g/cm^3), HMW rDNA and 5S DNA have distinctive buoyant densities of 1.724 and 1.692, respectively. The former is at the buoyant density of the rDNA satellite, while the latter has a lower buoyant density than that of the bulk of the DNA. These differences have permitted the isolation of *Xenopus* HMW rDNA (Birnstiel *et al.*, 1968) and 5S DNA (Brown *et al.*, 1971).

It was previously reported that the DNA of *Cucurbita pepo* L. contains several thousand copies of HMW rDNA (Matsuda and Siegel, 1967). These sequences are confined to the pumpkin satellite DNA (Matsuda *et al.*, 1970), the buoyant density of which (1.708 g/cm^3) is greater than that of the main band (1.695 g/cm^3).

We report here the results of experiments designed to measure the number of copies and buoyant densities of HMW rDNA and 5S and 4S DNA.

Investigation of reassociation kinetics (Britten and Kohne, 1968) has shown that the most rapidly reassociated fraction of the DNA of a higher organism contains the most repetitious nucleotide sequences. Rapidly reassociated pumpkin DNA was isolated to determine if HMW rDNA and 5S and 4S DNA were contained in this fraction. We show that rapidly reassociated DNA is multicomponent and that HMW rDNA and 5S and some 4S DNA are present in one of these components.

Materials and Methods

Preparation of DNA. Young leaves of pumpkin, *Cucurbita pepo* L. var. small sugar, were macerated with an equal weight of sucrose buffer (0.5 M sucrose–0.01 M $CaCl_2$–0.05 M Tris–0.025 M KCl–0.005 M mercaptoethanol, pH 8.2) by either grinding in a meat grinder or chopping with an electric slicing knife to which razor blades had been fitted. The macerate was filtered through two layers of cheesecloth and glass wool and the filtrate was centrifuged at 1000*g* for 5 min. The pellet, containing nuclei and chloroplasts, was dispersed with sucrose buffer containing 3.5% Triton X-100 (Rohm and Haas) to solubilize the chloroplasts and the nuclei were pelleted by centrifugation. This treatment was repeated until the pellet contained little or no green pigment.

DNA was isolated by suspending the nuclei in 5–10 ml of SSC (0.15 M NaCl–0.015 M sodium citrate, pH 7.0) containing 1% sodium dodecyl sulfate. The suspension was allowed to stand 1–2 hr at room temperature and was then repeatedly extracted with redistilled phenol until no protein remained at the aqueous–phenol interface.

The aqueous phase was overlaid with 2 vol of ethanol and the DNA, after spooling with a stirring rod, was resuspended

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[1] Abbreviations used are: rRNA, ribosomal RNA; HMW rRNA, a high molecular weight preparation of RNA consisting of 16S, 18S, 23S, and 25S plant rRNAs; HMW rDNA, sequences complementary to HMW rRNA; 5S DNA, sequences complementary to 5S RNA; 4S DNA, sequences complementary to 4S RNA; SSC, standard saline–citrate; PB, equimolar Na_2HPO_4 and NaH_2PO_4; TNMg, 0.1 M NaCl–0.1 M Tris (pH 7.6)–0.01 M $MgCl_2$; STMg, 0.1 M NaCl–0.05 M Tris–0.01 M $MgCl_2$; E buffer, 0.04 M Tris–0.02 M NaOAc–0.001 M EDTA.

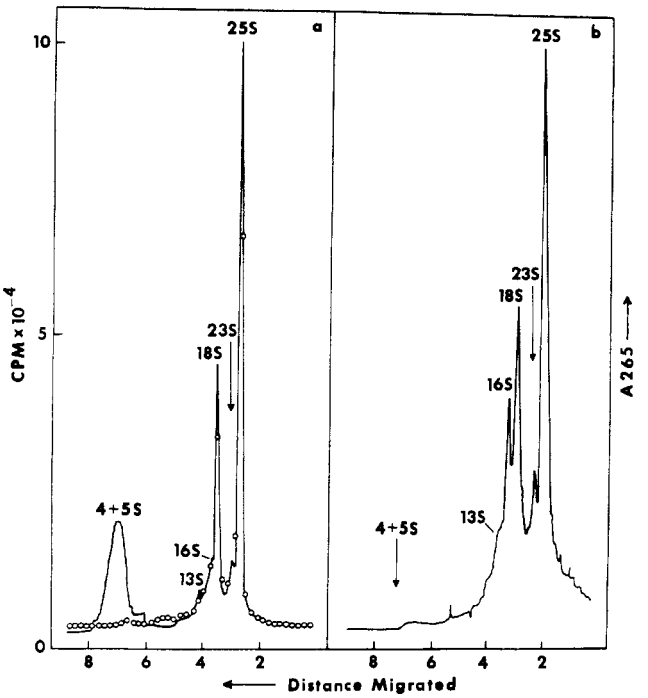

FIGURE 1: Electrophoresis of HMW rRNA. Absorbance and radioactivity scan of (a) 1.8% acrylamide-0.5% agarose gel containing 20 μg of pumpkin HMW rRNA from the $3 \times 10^7 \times g \times$ min pellet plus unlabeled 4S + 5S RNA marker: (—) A_{260nm}; (O) counts per minute; and (b) absorbance scan of 2.4% acrylamide gel containing 20 μg of unlabeled tobacco HMW rRNA. Both gels were run for 2 hr.

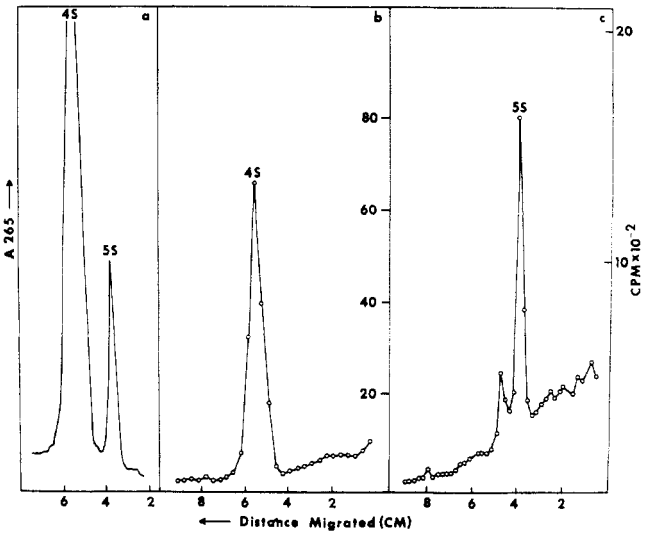

FIGURE 2: Electrophoresis of 4S and 5S RNAs. Absorbance scan of 12.5% acrylamide gel containing RNA from the $3 \times 10^7 \times g \times$ min supernatant (a) and radioactivity distribution of purified 4S (b) 5S (c) RNAs. The three gels were run in parallel for 5 hr.

in a minimum amount of SSC and treated with pancreatic ribonuclease (50 μg/ml) for 1 hr at 35°. Pronase was then added (1 mg/ml), the temperature raised to 50°, and the incubation continued for 2 hr. The incubation mixture was then cooled to 4° and extracted in succession with phenol and chloroform–isoamyl alcohol (24:1, v/v), and the DNA was spooled after the addition of ethanol. The enzyme treatment was repeated and in some cases was carried out in dialysis tubing at 50° against frequent changes of SSC. The final spool of DNA was washed with ethanol and resuspended in either 0.01 M PB (equimolar Na_2HPO_4 and NaH_2PO_4) or SSC.

DNA to be used for hybridization experiments was further purified by preparative CsCl centrifugation (Flamm *et al.*, 1966). This procedure has been shown to enhance purity and give optimal hybridization values (Pace and Pace, 1971).

RNA Preparations. A sufficient number of young leaves to cover the bottom of four 15-cm petri dishes were washed and prepared for labeling as described by Zaitlin *et al.* (1968). Each dish contained 10 ml of 0.05 M 2-(*N*-morpholino)ethanesulfonic acid–H_2O, pH 5.8, containing 1% of a mixture of the antibiotics Rimocidin (Pfizer) and Cephaloridine (Eli Lilly). 5-[^{3}H]Uridine (0.5 mCi) was added to each dish and incubation was continued for 32 hr under fluorescent lighting at room temperature. All glassware and solutions were sterilized prior to use.

Either labeled (pumpkin) or unlabeled (tobacco) leaves were washed and ground to a fine powder after freezing with liquid N_2 in a mortar and pestle. Two volumes per weight of tissue of each of the following were added and the slurry was mixed as thawing occurred: (1) TNMg buffer (0.1 M NaCl–0.1 M Tris (pH 7.6)–0.01 M $MgCl_2$), containing 0.2% sodium dodecyl sulfate and 1% diethyl pyrocarbonate; (2) redistilled phenol saturated with TNMg.

The aqueous phase was collected after centrifugation and subjected to a second phenol extraction. RNA was precipitated by the addition of an equal volume of isopropyl alcohol and a few drops of 3 M acetate buffer, pH 5. After overnight storage at −20°, the precipitate was collected by centrifugation, washed with ethanol, resuspended in STMg buffer (0.1 M NaCl–0.05 M Tris–0.01 M $MgCl_2$, pH 7.6), and reextracted with phenol. RNA was then precipitated with 2 vol of cold 95% ethanol, resuspended in STMg, and clarified. This solution was centrifuged in the Spinco Model L-2 for $3 \times 10^7 \times g \times$ min (45,000 rpm for 4–5 hr in the 50 Ti rotor) to sediment the HMW rRNA as suggested by Payne and Dyer (1971a). The pellet contains the 16S, 18S, 23S, and 25S rRNAs, whereas the supernatant contains mostly the low molecular weight RNAs.

The pellet was washed with ethanol and resuspended in a minimum volume of 2 × SSC. The supernatant solution was treated with DNase (RNase free, Worthington, 20 μg/ml) at 30° for 30 min and extracted with phenol to remove the enzyme. The RNA was then precipitated with ethanol, washed, and dissolved in a minimum volume of 2 × SSC.

The $3 \times 10^7 \times g \times$ min pellet material was analyzed by polyacrylamide gel electrophoresis (Figure 1) and was found to be minimally contaminated by 4S or 5S RNA. It contains the 16- and 23S rRNAs from chloroplast ribosomes and the 18- and 25S rRNAs from cytoplasmic ribosomes (Loening and Ingle, 1967). The 13S component is a known product of the degradation of 23S RNA. Analysis of the distribution of radioactivity in the gel shows that the rRNAs are approximately uniformly labeled. Contamination by bacteria would be indicated by a disproportionate labeling of 16S and 23S rRNA. Although this RNA preparation is apparently free of 4S and 5S RNA, additional precaution was taken by eluting RNA from the block of gel containing only the 13–25S rRNAs for use as HMW rRNA in hybridization experiments.

It was necessary to employ an unlabeled competitor of HMW rRNA in hybridization experiments. Unlabeled tobacco leaf HMW rRNA was isolated and, as shown in Figure 1b, consists of the same RNA species as the labeled pumpkin HMW rRNA.

Polyacrylamide gel electrophoresis of the RNA obtained

from the $3 \times 10^7 \times g \times$ min supernatant revealed the presence of two predominant low molecular weight RNAs (Figure 2a). A similar pattern was observed by Payne and Dyer (1971a), who have shown these to be 4S and 5S RNAs. Sections of the gel containing the two species were removed and the RNAs were eluted with several changes of 2 × SSC containing 0.1% sodium dodecyl sulfate. Gel debris was pelleted by centrifugation and the solutions were filtered through nitrocellulose membrane filters (S&S, B-6). The RNA was concentrated by ethanol precipitation and its concentration determined by assuming $A_{260\ nm}^{1\ mg/ml} = 25$. To test the efficiency of this technique, aliquots of the eluted RNAs were applied to gels and rerun. As seen in Figure 2b, 4S RNA migrates as a single peak except for a small amount of labeled material toward the top of the gel. 5S RNA also contains some trailing label and a minor component which migrates to a position intermediate between that of 4 and 5 S (Figure 2c).

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed essentially in accordance with Loening (1967). HMW rRNAs were monitored on gels containing either 2.5% acrylamide or 1.8% acrylamide–0.5% agarose (Peacock and Dingman, 1968). Low molecular weight RNAs were separated on 12.5% polyacrylamide gels. Immediately after polymerization, gels were prerun in E buffer (0.04 M Tris–0.02 M sodium acetate–0.001 M EDTA, pH 7.2) containing 0.2% sodium dodecyl sulfate at 5 mA/gel for 2 hr. Up to 0.2 ml of RNA solution containing 10% sucrose was applied to the gels and run for optimal time periods. After electrophoresis, the gels were scanned at 260 or 265 nm in a Gilford Model 240 spectrophotometer equipped with a linear transport device. Distribution of radioactivity in the gels was monitored by freezing the gels on Dry Ice and cutting them into 1.07-mm slices with a manifold of razor blades. Fractions containing an appropriate number of gel slices were dissolved in 0.75 ml of 30% H_2O_2 by incubating for 12 hr at 50°. Ten milliliters of a mixture of 33% Triton X-100 and 67% toluene (v/v) containing 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene and 0.55% 2,5-diphenyloxazole was added and the samples were counted in a Packard Model 3320 scintillation spectrometer.

DNA–RNA Hybridization. SATURATION EXPERIMENTS. Samples containing 1–20 μg of DNA were made to a volume of 1 ml with 2 × SSC and denatured by the addition of 0.1 vol of 1 N NaOH. After 10–15 min the samples were chilled and neutralized by the addition of 0.15 vol of 1 M NaH_2PO_4. Denatured DNAs were then embedded on 24-mm B-6 nitrocellulose membrane filters (Schleicher and Schuell) and hybridized with ^{3}H-labeled RNA according to Gillespie and Spiegelman (1965) in 2 × SSC–0.1% sodium dodecyl sulfate at 68°. Hybridizations to 4S and 5S RNA were conducted for 4 hr, since others (Tartof and Perry, 1970; Hatlen and Attardi, 1971) have found this time to be sufficient for maximum hybridization. Reactions containing HMW rRNA were continued for 12 hr.

At the completion of the incubation, filters were treated with pancreatic RNase (10 μg/ml) at room temperature, washed exhaustively with 2 × SSC, oven dried, and counted in 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene scintillation fluid. Each vial of the saturation experiments contained a blank filter with no DNA added.

BATCH HYBRIDIZATION. This procedure has been described (Brown and Weber, 1968). DNA in individual fractions of a preparative CsCl gradient was denatured and embedded on filter membranes. As many as 25 filters were incubated in a single jar with sufficient 2 × SSC containing 0.1% sodium dodecyl sulfate to cover the filters. The indicated amounts of RNA were added and mineral oil was overlaid to prevent evaporation. After incubation, filters were washed and counted as above and the counts corrected by subtracting those retained by a filter which contained no DNA.

DNA Reassociation and Hydroxylapatite Fractionation. Isolation of the rapidly reassociated DNA was accomplished according to Britten and Kohne (1965). Solutions of DNA in 0.01 M PB were adjusted to 0.13 M PB and diluted to a concentration of 10–50 μg/ml. DNA was denatured by heating to 100° in sealed containers for 10 min in a salt–water bath. Samples were then immediately incubated at 68° for a period of time sufficient to establish a Cot value of 0.14, where Cot is expressed as moles of nucleotide × second per liter.

Reassociation was terminated by transferring samples to a pre-cooled flask, and the DNA was loaded onto a bed of hydroxylapatite (Bio-Rad Bio-Gel HT) maintained at 64–68° in a water-jacketed column. The single-stranded DNA was washed through the column with 0.13 M PB. Reassociated DNA was collected by elution with 0.30 M PB and is referred to as rapidly reassociated DNA. To standardize the conditions, we have measured the Cot at which sonicated *Escherichia coli* DNA was half-renatured, and find this value to be equal to one. This value is in reasonable agreement with the value of 4 obtained by Britten and Kohne (1965).

CsCl Centrifugation. For preparative gradients, 9.8 g of CsCl was added to 7.85 ml of SSC or PB which contained either native or rapidly reassociated DNA and the density was adjusted to 1.708 g/cm^3 with the aid of a refractometer (Ifft *et al.*, 1961). Spinco polyallomer tubes ($^5/_8$ × 2.5 in.) were coated with a solution of bovine serum albumin (100 mg/ml) or Siliclad (Clay-Adams) and dried prior to use. Solutions were centrifuged at 35,000 rpm for 62 hr in the No. 50 rotor at 20°. At the completion of the run, a 20 gauge needle was inserted in the bottom of the tube and 12-drop fractions were collected. One milliliter of 2 × SSC was added to each fraction and the absorbance at 260 nm measured. The slope of the gradient was determined at the end of the run by refractometry and buoyant densities were calculated by using a DNA of known buoyant density as a marker.

For analytical gradients, 3–5 μg of DNA along with 1 μg of marker DNA were added to a solution of CsCl and the density was adjusted to 1.708 g/cm^3. Samples were centrifuged for 18 hr at 20° in the Model E centrifuge and buoyant densities were calculated as described by Sueoka (1961) using *Micrococcus luteus* DNA as a marker ($\rho = 1.731$), which is not shown in the microdensitometer tracings.

Results

Saturation Hybridization Experiments. The proportion of DNA which is complementary to HMW rRNA was estimated from the data in Figure 3a to be 2.4%. This value is similar to that (2.2%) obtained by others in this laboratory (Goldberg, 1972).

Similar determinations for the proportion of DNA complementary to 5S and 4S RNAs yielded values of 0.029 and 0.044% (Figures 3b and c), respectively. To ensure that contaminant fragments of HMW rRNA were not affecting the results (Tartof and Perry, 1970; Hatlen and Attardi, 1971), an excess of unlabeled HMW rRNA was added to the hybridization reaction vessels. Although this precaution was taken routinely, in the instance where it was tested, the presence of competitor HMW rRNA had only a small effect.

It can be seen in Figure 3b that there is rapid attainment of a saturation with 5S RNA at a RNA/DNA ratio of 0.03,

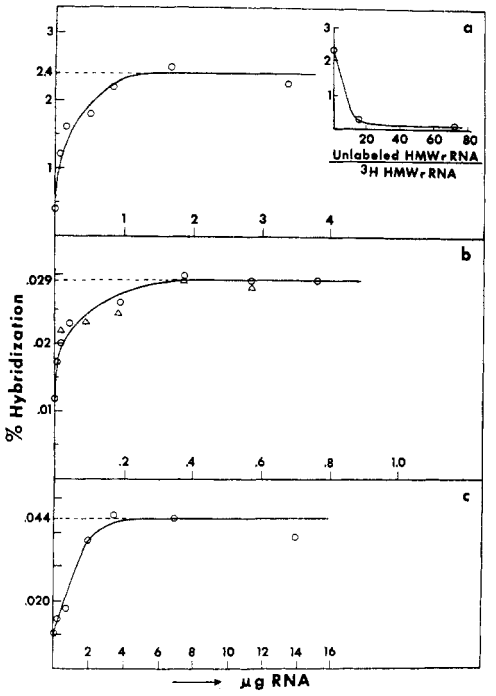

FIGURE 3: Saturation of pumpkin DNA with ^{3}H-labeled HMW rRNA and 5S and 4S RNA. Increasing amounts of RNAs were hybridized in separate vials with filters containing the indicated amounts of DNA: (a) HMW rRNA (24,178 cpm/μg) with 1.3 μg of DNA; (b) 5S RNA (27,500 cpm/μg) with 13 μg of DNA; (c) 4S RNA (27,457 cpm/μg) with 12 μg of DNA. Inset to a, HMW rRNA (unlabeled) plus 0.34 μg of tritiated HMW rRNA with 1.3 μg of DNA. In a and c each point represents the average of replicate determinations. In b the triangles indicate values obtained when vials of RNA were reused by incubating fresh filters. Each vial in b and c contained at least a 100-fold excess of unlabeled HMW rRNA competitor. The number of counts per minute retained by filters at saturation and those of the blank were, respectively: (a) 700 and 15 cpm; (b) 117 and 13 cpm; (c) 155 and 14 cpm.

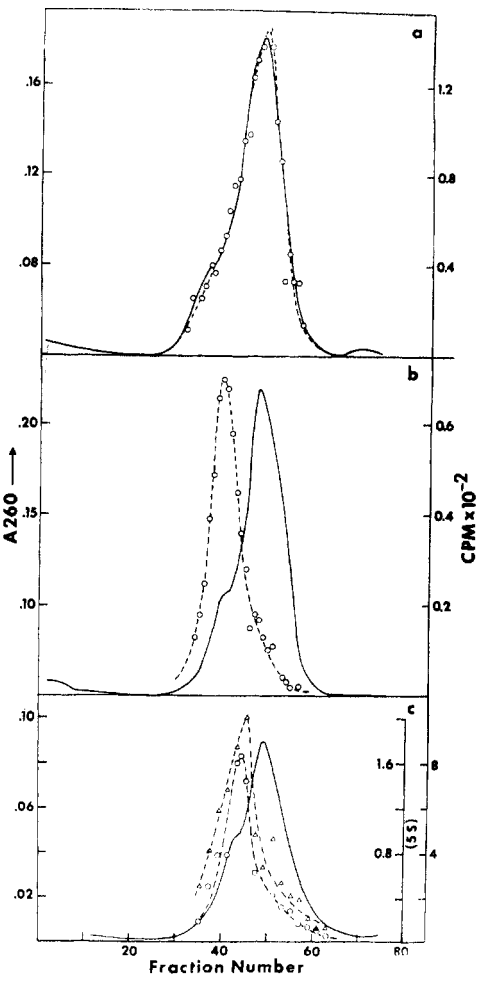

FIGURE 4: Buoyant density distribution of HMW rDNA and 5S and 4S DNA. Approximately 250 μg of native pumpkin DNA was centrifuged in each of two tubes. Fractions were collected and "batch" hybridized to (a) tritiated 4S RNA and (b) tritiated 5S RNA: (—) A_{260nm}; (○) counts per minute hybridized. In c, fractions of a gradient which contained 110 μg of a preparation of DNA enriched in satellite were split into two sets and hybridized to tritiated HMW rRNA (△) or tritiated 5S RNA (○). This enriched preparation was obtained by heating native DNA at 86° for 10 min, quick cooling, and recovering the undenatured DNA from hydroxylapatite after the denatured DNA had been washed through the column. The details of the centrifugation and hybridization are given under Methods and Materials. The amount of RNA used for each hybridization was estimated to be in excess of the saturation level. Labeled 4S and 5S RNAs were mixed with a 100-fold excess of unlabeled HMW rRNA. Blank values were in each case less than 15 cpm.

whereas the comparable value for 4S RNA is 0.38. These values are typical for saturation with these RNAs (Hatlen and Attardi, 1971; Tartof and Perry, 1970) and suggest a much greater sequence heterogeneity of 4S RNA than 5S RNA. The ratio of 1.0 obtained for HMW rRNA verifies that this preparation consists of RNA sequences with a large kinetic complexity (Birnstiel *et al.*, 1972).

These data are summarized in Table I and are converted to show the number of copies of the sequences. For these calculations we use 10.2×10^{11} daltons as the estimate of cellular DNA content (Keener, S., personal communication) and we assume that the mature leaf cells, from which the DNA was extracted, were arrested in the G1 stage of the cell life cycle. The cells would thus contain genomic complements of DNA, each comprising 5.1×10^{11} daltons.

TABLE I: Number of HMW rDNA and 5S and 4S DNA Sequences of Pumpkin.

	HMW rRNA	5S RNA	4S RNA
% DNA hybridized	2.4	0.029	0.044
Assumed mol wt of RNA	3.6×10^6 [a]	37,900 [b]	2.6×10^4 [c]
Approximate no. of genes per haploid genome	3400	3900	8600
Rel no. of genes	1	1.2	2.5

[a] Loening, 1968. [b] Payne and Dyer, 1971a. [c] Hatlen and Attardi, 1971.

Buoyant Density of Native HMW rDNA and 5S and 4S DNA. To determine the buoyant density of each of the sequences, fractions of a preparative CsCl gradient containing native DNA were "batch" hybridized to RNAs. As seen in Figure 4a, 4S DNA is distributed throughout the main band and satellite regions of the gradient. In contrast, 5S DNA (Figure 4b) is confined to the satellite region. Figure 4c shows that both MHW rDNA and 5S DNA have buoyant densities

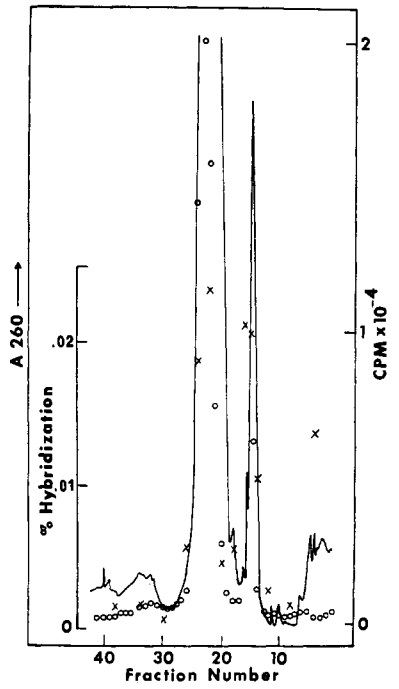

FIGURE 5: Distribution of hybridization to low molecular weight RNAs. Electrophoretic pattern of 80 μg of tritiated RNA from the $3 \times 10^7 \times g$/min supernatant in a 12.5% gel after 5 hr. RNA was eluted from each fraction (two 1.07-mm gel slices) by adding 1 ml of 2 × SSC containing 0.1% sodium dodecyl sulfate and storing in the cold for 12 hr. A 0.1-ml aliquot of each fraction was withdrawn and counted (O). Another 0.1-ml aliquot of selected fractions was withdrawn and each was incubated, together with an excess of competitor HMW rRNA, with filters containing 22 μg of DNA at 68° for 4 hr. The per cent of the DNA which hybridized with each of the fractions (×) is given with the assumption that the specific activities of each of the RNAs was the same (27,000 cpm/μg).

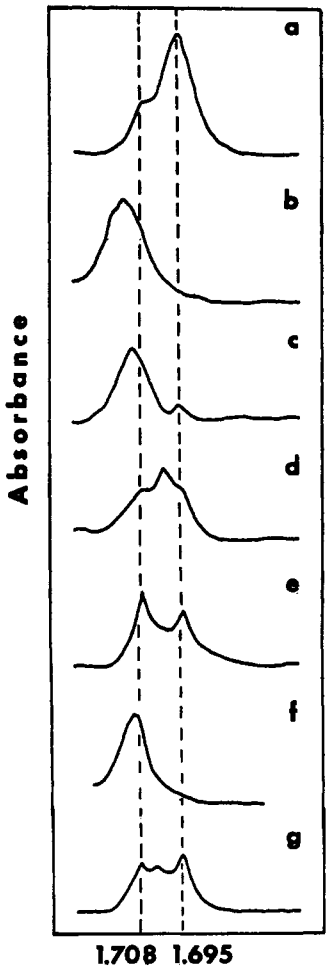

FIGURE 6: Isopycnic analyses of pumpkin nuclear DNA subjected to different treatments: (a) native; (b) heat denatured for 10 min at 100° in 0.13 M PB and quick cooled; (c) heat denatured and annealed to a Cot of 0.14 in 0.13 M PB; (d) heat denatured and annealed to a Cot of 1.6 in 0.30 M PB; (e, f) DNA that had been annealed to a Cot of 0.14 (as in c, above) was fractionated by hydroxylapatite chromatography into duplex and single-stranded DNA. The reassociated duplex DNA is seen in e. The single-stranded DNA was again annealed to a Cot of 0.14 and additional reassociated duplex DNA recovered by hydroxylapatite chromatography is seen in f; (g) reassociated duplex DNA as in e, above, but prepared from sheared DNA.

which are indistinguishable from each other and from the satellite component.

In order to eliminate the possibility that fragments of tritiated HMW rRNA which were insufficiently diluted by competitor HMW rRNA might be responsible for the 5S hybridization observed, selected slices cut from a gel containing the low molecular weight RNAs were eluted and hybridized with DNA. In this experiment one would obtain either a high background of hybridization with fragments of contaminating HMW rRNA or specific peaks of hybridization in the regions of the reactive RNA species. It is shown in Figure 5 that specific hybridization occurs with both 5S and 4S RNAs, but little if any occurs at other regions of the gel. Some hybridization occurs at the top of the gel since, prior to fractionation on gels, these RNA preparations contain some HMW rRNA.

Characterization of Rapidly Reassociated DNA. The dense satellite component contains, among other things, highly repetitive sequences complementary to HMW rRNA, 5S rRNA, and some of the 4S RNA and has been shown to reassociate rapidly (Matsuda and Siegel, 1967). The following experiments were performed to determine whether the dense satellite is unique in its reassociation behavior or whether there might also be DNA of other buoyant densities containing repetitive nucleotide sequences. Figures 6a–c are analytical isopycnic profiles, respectively, of native DNA, denatured DNA, and DNA which has been annealed to a Cot of 0.14. It can be seen that with limited annealing to Cot 0.14 a band, probably consisting of reassociated material, appears at the native main band buoyant density of 1.695 and that reassociated dense satellite, if present, is obscured by the mass of denatured DNA. DNA annealed to a somewhat greater Cot (Figure 6d) presents a rather complex pattern with much of the DNA shifting to lower buoyant density.

DNA annealed to Cot 0.14 was fractionated on hydroxylapatite to determine whether the 1.695-g/cm^3 band seen in Figure 6c does represent reassociated DNA and whether the native dense satellite had also reassociated under these conditions. The isopycnic banding pattern of the recovered duplex DNA is shown in Figure 6e. It consists of two bands, one with the density of native satellite component and the other of native main band density, called r and m, respectively. It should be noted that the profile seen in Figure 6e was obtained with DNA of single-stranded mol wt of approximately 3.5×10^6 or greater. DNA intentionally sheared to a single-stranded mol wt of 9×10^5 (Goldberg, 1972), prior to denaturation, resulted in the profile of Figure 6g, in which there is an apparent splitting of the r component into two bands. We conclude that there is, indeed, another buoyant density

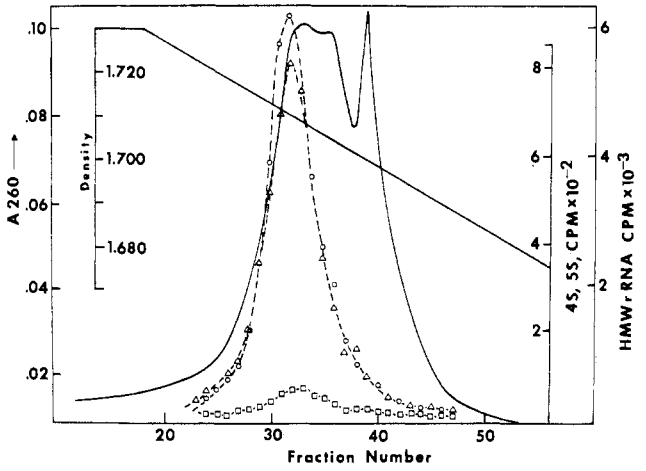

FIGURE 7: Buoyant density distribution of HMW rDNA and 5S and 4S DNA in rapidly renatured DNA. Approximately 80 μg of rapidly reassociated DNA, collected by hydroxylapatite chromatography after annealing to Cot = 0.14, was centrifuged in the no. 50 rotor for 62 hr. Fractions were collected and split into three sets. Each set was "batch" hybridized to either HMW rRNA (○), 5S RNA (△), or 4S RNA (□) in jars which contained 10 ml of 2 × SSC and 0.1% sodium dodecyl sulfate, and a greater than saturating amount of the RNA. Hybridization with 4S and 5S RNA included an excess of unlabeled HMW rRNA.

species present in addition to the dense satellite, which reassociates rapidly.

The single-stranded DNA recovered from the hydroxylapatite column was heated to 100° to remove secondary structure and was annealed for an additional Cot of 0.14. The DNA so treated was fractionated on hydroxylapatite to determine whether additional duplex DNA is formed by this second annealing cycle. Some was formed and, as seen in Figure 6f, it has a buoyant density very close to the r component. No additional reassociated DNA is formed upon a third cycle of annealing to a Cot of 0.14. We conclude that all of the m component is reassociated at a Cot of 0.14, whereas it probably takes a somewhat greater Cot to complete the reassociation of the r component. Measurement of r and m components yields estimates of 19 and 4%, respectively, of the total DNA.

Buoyant Density Distribution of Reassociated HMW rDNA and 5S and 4S DNA. Since r and m components are distinct in their ability to reassociate more rapidly than the remainder of pumpkin DNA, these components should contain the most repetitious nucleotide sequences. The results of an experiment which determine the density distribution of HMW rDNA and 5S and 4S DNA between the r and m components of reassociated DNA (purified by hydroxylapatite chromatography after annealing to Cot = 0.14) are shown in Figure 7. By comparison with the data presented in Figure 4, many of the cistrons for HMW RNA and 5S RNA appear to have reassociated, whereas only a few of those for 4S RNA have done so. Each of the sequences is contained in the most dense fraction of DNA but none is contained in the m component. In this case the absorbance profile of renatured DNA was similar to that shown in Figure 6g because this preparation of DNA had a low molecular weight. These results substantiate the finding that the HMW rDNA and 5S DNA, as well as a small portion of the 4S DNA, have the same buoyant density as satellite DNA.

Discussion

Number of HMW rDNA Sequences. The results reported here agree in some respects with those reported for other eukaryotes and they are unique in other respects. It has been reported previously (Matsuda and Siegel, 1967) that a large proportion of the *C. pepo* genome is complementary to HMW rRNA. We have confirmed this phenomenon and have established this value to be 2.4%. The HMW rRNA used in our analysis contained material from both chloroplast and cytoplasmic ribosomes and if we assume that there are an equal number of cistrons for the RNAs from the two sources (Matsuda *et al.*, 1970) then the calculation can be made that there are 3400 cistrons for each class of rRNA. This value is higher than that reported for animal species but falls within the range of values determined for several plant species (Ingle and Sinclair, 1972).

Number and Distribution of 4S DNA Sequences. The ratio of 4S DNA to HMW rDNA or 5S DNA is approximately 2.5. This compares to ratios in the range of 2–7 (see Hatlen and Attardi, 1971) for other eukaryotes. As expected, 4S DNA is interspersed with other sequences with buoyant densities of the bulk pumpkin DNA. As a comparison, a substantial portion of the 4S DNA of *Xenopus* was also found to be distributed with the bulk DNA. Indeed, in two cases, 4S DNA has been found to be distributed throughout the genome as demonstrated by an analysis of the size classes of chromosomes of Hela cells (Aloni *et al.*, 1971) and genetic analysis in *Drosophila* (Ritossa *et al.*, 1966). It is important to note that the number of copies of each of the sequences measured is remarkably high in pumpkin DNA. For example, if one assumes that equal numbers of copies of an estimated 60 species of transfer RNA were measured in the hybridization with 4S RNA, then there are approximately 145 copies for each species. It is of interest to note that almost none of the 4S RNA species reassociated under the limited annealing conditions employed here (Cot = 0.14). There is a suggestion that perhaps only that small portion of the sequences with densities great enough to be scattered among highly repetitive sequences in the dense satellite component reassociated. The data indicate that there is little cross-reassociation between the coding sequences for the different tRNAs under the restrictive annealing conditions employed.

Number and Distribution of 5S DNA Sequences. The number of copies of 5S DNA is only slightly greater than the number of units of HMW rDNA. Since nuclear DNA of plants contains sequences complementary to chloroplast rRNAs (Tewari and Wildman, 1968) as well as cytoplasmic rRNAs, each unit is assumed to consist of one 16 + 23S and one 18 + 25S cistron. Also, 5S RNA has recently been shown to be located in both chloroplast and cytoplasmic ribosomes (Payne and Dyer, 1971b). We assume that the 5S RNA preparation used in these experiments contained 5S RNAs from both sources but which were not resolved by our conditions of electrophoresis. If this is true, then the total number of copies of 5S RNA is less than the number needed to assign one 5S sequence to each 16 + 23S and 18 + 25S cistron, an arrangement which would be required if the synthesis of 5S and HMW rRNA was coordinately controlled by their inclusion in a single transcription unit. Indeed, in *Xenopus* (Brown and Weber, 1968; Brown *et al.*, 1971) and all other eukaryotic organisms examined, 5S RNA cistrons are clustered and are not linked with those for the HMW rRNAs (Aloni *et al.*, 1971; Tartof and Perry, 1970), whereas, in bacteria, cistrons for the 16S, 23S, and 5S rRNAs are tightly linked in that order (Doolittle and Pace, 1971; Colli *et al.*, 1971). It is possible that both situations exist in plants, the former for cistrons for the 5S RNA contained in cytoplasmic ribosomes and the latter for 5S RNA contained in the 70S ribosomes.

The buoyant density of pumpkin DNA containing the 5S RNA cistrons is the same as that of the dense rDNA satellite, and contrasts sharply with *Xenopus* where 5S DNA has a lower buoyant density than bulk DNA (Brown *et al.*, 1971). The low buoyant density of this DNA in *Xenopus* has been shown to result from high A-T spacer DNA which separates the 5S RNA cistrons from each other. Such spacer DNA, if it exists in pumpkin, must have quite different properties from that of *Xenopus*.

Functional Significance of the r and m Components. It was expected that satellite DNA would reassociate under the conditions employed. Satellite sequences (r component) therefore form a part of the rapidly reassociating DNA of this species. This component contains, among other things, the gene functions for the production of HMW rRNA, 5S RNA, and perhaps some 4S RNA but no functional assignment can as yet be made for the rest of the r component or for the m component.

A number of rapidly reassociated DNAs have been observed from a wide variety of organisms (Bond *et al.*, 1967; Maio, 1971; Corneo *et al.*, 1970; Votavona *et al.*, 1970). These may be derived from satellite fractions or portions of main band DNAs. It is believed that these DNAs are not transcribed in the cell but in some way are involved in the maintenance of structure of chromosomes (Yunis and Yasmineh, 1971). We have shown here, in agreement with others (Wells and Birnstiel, 1969; Bendich and McCarthy, 1970), that such a rapidly reassociating component may also exist in the DNA of a higher plant. However, we do not exclude the possibility that structural or controller gene functions may be found for the unassigned part of the r component and for the m component.

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