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The University of Arizona, Ph.D., 1972
Biochemistry

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CHARACTERIZATION OF RAPIDLY REASSOCIATING
COMPONENTS OF THE DNA OF SOME HIGHER PLANTS

by

William Harry Thornburg

A dissertation Submitted to the Faculty of the
COMMITTEE ON AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1972

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ACKNOWLEDGMENTS

To Dr. Albert Siegel for stimulating discussion of a number of exciting areas of science and for assistance in the composition of this dissertation.

To Dr. Robert Goldberg for his continued interest in a number of biological questions, the answers to which were the objectives of both of our research efforts.

TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATIONS	vi
LIST OF TABLES	viii
ABSTRACT	ix
INTRODUCTION	1
LITERATURE REVIEW	3
Sequence Repetition in the Eukaryote Genome	3
Repetition of DNA Sequences Complementary to Cellular RNAs	4
Repetition of Other DNA Sequences	6
MATERIALS AND METHODS	10
DNA Extraction and Purification	10
DNA Renaturation and Hydroxyapatite Fractionation	12
CsCl Centrifugation	14
Preparative	14
Analytical	15
Preparation of RNA	15
Polyacrylamide Gel Electrophoresis	17
Hybridization	18
Saturation	18
Batch	19
RESULTS	20
Analysis of Pumpkin DNA	20
Preparation of HMW rRNA, 4S and 5S RNA	20
Degree of Repetition of HMW rDNA, 4S and 5S DNA	25

TABLE OF CONTENTS --Continued

	Page
Buoyant Density of Native HMW rDNA, 4S and 5S DNA	31
Characterization of Low Cot DNA	39
Buoyant Density Distribution of HMW rDNA, 4S and 5S DNA in Low Cot DNA	46
Comparison of Low Cot DNAs of Several Plant Species	50
Characterization of Low Cot Chinese Cabbage DNA	52
Characterization of Low Cot Pinto Bean DNA	56
Characterization of Low Cot Tobacco DNA	59
DISCUSSION	63
Degree of Repetition of HMW rDNA, 4S and 5S DNA	63
Linkage of Pumpkin 5S DNA	64
Function of Renatured Components of Low Cot DNA	66
LIST OF REFERENCES	69

LIST OF ILLUSTRATIONS

Figure	Page
1. Polyacrylamide gel electrophoresis of HMW rRNAs of pumpkin and tobacco	21
2. Polyacrylamide gel electrophoresis of 4S and 5S pumpkin RNA.....	24
3. Saturation hybridization of pumpkin DNA with HMW rRNA	26
4. Heterologous competition of pumpkin HMW rRNA by tobacco HMW rRNA	27
5. Saturation hybridization of pumpkin DNA with 5S RNA.....	29
6. Saturation hybridization of pumpkin DNA with 4S RNA	30
7. Hybridization of 5S RNA to buoyant density fractions of native pumpkin DNA	33
8. Hybridization of 4S RNA to buoyant density fractions of native pumpkin DNA	34
9. Hybridization of HMW rRNA and 5S RNA to buoyant density fractions of native pumpkin DNA.....	36
10. Hybridization of low molecular weight RNAs to pumpkin DNA.....	38
11. Analytical CsCl centrifugation of native, denatured and renatured pumpkin DNA	40
12. Analytical CsCl centrifugation of 1st and 2nd cycle low Cot pumpkin DNA	43
13. Hybridization of HMW rRNA to buoyant density fractions of low Cot pumpkin DNA.	47
14. Hybridization of HMW rRNA, 4S and 5S RNA to buoyant density fractions of low Cot pumpkin DNA.....	49

LIST OF ILLUSTRATIONS --Continued

Figure		Page
15.	Analytical CsCl centrifugation of native, denatured and renatured Chinese cabbage DNA	53
16.	Hybridization of HMW rRNA to Buoyant Density fractions of Chinese cabbage low Cot DNA	54
17.	Analytical CsCl centrifugation of native and renatured pinto bean DNA	57
18.	Hybridization of HMW rRNA to buoyant density fractions of low Cot pinto bean DNA	58
19.	Analytical CsCl centrifugation of native and renatured tobacco DNA	60
20.	Hybridization of HMW rRNA to buoyant density fractions of low Cot tobacco DNA	62
21.	Schematic illustration of two modes of linkage of 5S DNA	65

LIST OF TABLES

Table		Page
I	Hybridization of HMW rRNA to single stranded and low Cot DNAs obtained after several cycles of renaturation.	45
II	Correlation of the percentages of HMW rDNA and low Cot DNA of several plant species.	51

ABSTRACT

The relative number of copies of pumpkin DNA sequences which are complementary to high molecular weight ribosomal RNA (HMW rRNA), 5S and 4S RNA was found, by DNA-RNA hybridization experiments, to be approximately 1:1.2:2.5. At saturation, the levels of hybridization obtained with these RNAs were 2.4%, 0.029% and 0.044%, respectively. Based upon a haploid genomic DNA content of 4.9×10^{11} daltons; this corresponds to 3200, 3700 and 8100 copies of genes for these RNAs. These gene sequences are referred to as HMW rDNA, 5S DNA and 4S DNA.

When banded in CsCl, pumpkin DNA possesses a main band and a satellite component, the buoyant density of which are 1.695 g/cc and 1.708 g/cc, respectively. It was found that HMW rDNA and 5S DNA are contained in the satellite component, whereas 4S DNA is located in both satellite and main band DNA.

Eukaryote DNA contains many families of repetitive sequences in addition to HMW rDNA, 5S and 4S DNA. The repetitive sequences may be separated from unique sequences by renaturing the single stranded DNAs under limiting conditions such that only the most repetitive sequences reanneal. The DNA of several plant species which renatured at $Cot = 0.14$ was termed low Cot DNA and was separated from single stranded DNA by hydroxyapatite chromatography.

The proportions of the DNA of pumpkin, Chinese cabbage, pinto bean and tobacco which proved to be low Cot DNA were 23, 21, 18 and 11% respectively.

After banding in CsCl it was observed that each of the low Cot DNAs consisted of a number of renatured components which differed in buoyant density. As expected, HMW rDNA was represented in the low Cot DNAs of all species and was contained in a renatured component, the buoyant density of which is 1.706 - 1.708 g/cc (r component). Pumpkin, Chinese cabbage and tobacco also have renatured components with buoyant densities of 1.694 - 1.697 g/cc. These correspond to the main band DNAs of these species and are called m components. In addition Chinese cabbage and pinto bean DNAs have components of 1.702, and 1.699 g/cc, respectively. These correspond to the buoyant densities of portions of the dense shoulder which the native DNAs of these species display in CsCl and are called s components.

The r and m components of low Cot pumpkin DNA were shown to be distinct rapidly renatured components and constitute 19% and 4%, respectively, of pumpkin DNA. The pumpkin r component contained not only HMW rDNA, but also 5S DNA and a small amount of 4S DNA. The m component contained none of these sequences.

Under defined conditions the profiles in CsCl of the low Cot DNAs are uniquely characteristic of the species examined. These profiles, however, can be altered either by shearing the DNA or controlling the extent of renaturation. A molecular weight dependence was demonstrated by showing that whereas 7×10^6 dalton pumpkin DNA yielded only the r and m components; sheared DNA of 1.8×10^6 daltons yielded also a component intermediate in buoyant density between

r and m. Upon a sufficient lowering of the C_{ot} of renaturation it was shown that the ratio of the amounts of r to s components increases for pinto bean and decreases for Chinese cabbage DNA.

INTRODUCTION

Eukaryote DNA may be considered as consisting of a mixture of unique sequences and repetitive sequences (Britten and Kohne, 1968). Unique sequences are those which are represented once in the genome whereas the repetitive ones are those present in multiple copies of similar or identical nucleotide sequence. In plant DNAs, the sequences complementary to high molecular weight ribosomal RNA (HMW rRNA) have previously been shown to be repetitive (Matsuda and Siegel, 1967; Ingle and Sinclair, 1972). In some species, these are contained in a unique fraction of DNA, the buoyant density of which (1.702 - 1.708 g/cc) is higher than the bulk or remainder of the DNAs (1.692 - 1.697 g/cc) (Matsuda, Siegel and Lightfoot, 1970). Sequences complementary to HMW rRNA, 4S and 5S RNA (called HMW rDNA, 4S and 5S DNA) have all been found to be repetitive in some animal DNAs. Each of these sequences of Xenopus laevis DNA is contained in a fraction of DNA which is distinguishable from the others on the basis of its buoyant density (Brown and Weber, 1968a). One objective of the work reported here was to determine the degree of repetition and buoyant density of the 4S and 5S DNA as compared to HMW rDNA of a higher plant species (pumpkin).

It should be possible to separate the most repetitious sequences from the remainder on the basis of the difference of their rates of renaturation. The fractions of denatured plant DNAs which reannealed under limiting conditions ($Cot = 0.14$) are called low Cot DNAs and should contain the most repetitive sequences. The

low Cot DNAs of four plant species were found to consist of at least two renatured components which differed in buoyant density in CsCl. The low Cot DNAs were examined to determine which, if any, of the renatured components contained the repetitive HMW rDNA. Pumpkin low Cot DNA was similarly analyzed for 4S and 5S DNA.

LITERATURE REVIEW

Sequence Repetition in the Eukaryote Genome

Studies of the renaturation of DNA of various organisms have revealed a striking difference between prokaryote and eukaryote DNAs (Britten and Kohne, 1968). The genome of prokaryotes consists of an assortment of base sequences, most of which are represented once in the bacterial chromosome. One exception to this generalization has been noted; prokaryote DNAs contain several copies of sequences representing the genes for rRNAs and transfer RNA (see Bostock, 1972). A number of bacterial and viral DNAs have been investigated and these DNAs are found to renature at a rate expected on the basis of the sum of the molecular weights of unique sequences which they contain (Britten and Kohne, 1968; Wetmur and Davidson, 1968). That is, the more DNA contained within the genome of an organism, the lower is the specific rate of renaturation of its DNA.

The genome of eukaryotes contains as much as a thousand times the amount of DNA as that of prokaryotes. If this DNA consisted of unique sequences, one would expect eukaryotic DNA to renature much more slowly than viral or bacterial DNA. It has been found that only a portion of DNA of eukaryotes renatures at the expected rate. The remainder renatures more rapidly than expected, and an extreme case is represented by a 10% fraction of mouse DNA (mouse satellite) which renatures even more rapidly than bacterial or viral DNAs.

Further investigation of this phenomenon has shown that the rapidly reassociated (or renatured) DNAs consist of families of identical or related base sequences. The genome of higher organisms therefore contains, not only unique DNA, but also families of sequences which may be iterated in the genome from ten to a million times. The latter are called repetitive sequences and have been the subject of intense investigation in many laboratories. Studies have been designed to determine their pattern of organization within the genome and their biological function. Those repetitive sequences which have been studied may be arbitrarily divided into two categories according to the technique used for their detection. One category consists of the repetitive sequences which are complementary to one or more species of RNA. A second category would include those for which no complementary RNA of defined function has been isolated.

Repetition of DNA Sequences Complementary to Cellular RNAs

A number of sequences in the first category have been investigated in eukaryotes. The DNA-RNA hybridization procedure is used to determine the number of copies of DNA which are complementary to the species of RNA used in the assay. These measurements allow assignment of the repetition frequency and biological function of the sequences.

Both animal and plant DNAs have been shown to contain repetitive sequences which are complementary to rRNAs. Matsuda and Siegel (1967) showed that these sequences (rDNA) were repetitive in species of a number of genera of

higher plants. For four species, the rDNA sequences are distinguishable from other sequences because they are contained in a fraction, the buoyant density of which (1.702 - 1.708 g/cc) is greater than the remainder of the DNA. Moreover, in the DNA of pumpkin, Chinese cabbage and pinto bean, this fraction is present to such an extent that it forms a visible dense satellite band in CsCl. Sinclair and Brown (1971) have recently extended this observation to show that in several other species of plants (wheat, barley and pea) as well as many other eukaryotes, the rDNA is higher in buoyant density than the bulk of the DNAs. This reflects, at least in part, that the rRNAs of these species have a higher G+C content than that of the bulk DNA sequences. The studies of Ingle and Sinclair (1972) have confirmed that higher plant genomes contain repetitive copies of HMW rRNA. They found the haploid genomes of seven species to contain from 260 to 6,650 copies of the sequences complementary to the 25S rRNA.

Several eukaryote DNAs have also been shown to contain repetitive sequences complementary to the small ribosomal RNA (5S RNA) and to transfer RNA (4S RNA). These include Drosophila melanogaster (Tartof and Perry, 1970) human (Hela) (Hatlen and Attardi, 1971) and Xenopus laevis (Brown and Weber, 1968). These results have been summarized by Hatlen and Attardi (1971). The buoyant densities of the 4S and 5S DNA of only X. laevis have been measured. X. laevis 4S DNA is distributed with the bulk (1.698 g/cc) and to the dense side of the gradient (Brown and Weber, 1968) whereas HMW rDNA (Birnstiel et al., 1968) and 5S DNA (Brown, Wensink and Jordan, 1971) have distinctive buoyant densities of 1.724 and 1.692 g/cc, respectively.

In only one other definitive study has a purified RNA of defined function been shown to be complementary to repetitive DNA. In sea urchin DNA complements of the histone messenger RNA (9S) are repetitive and in unsheared DNA have a buoyant density (1.705 g/cc) distinct from either HMW rDNA (1.717 g/cc) or bulk DNA (1.697 g/cc) (Kedes and Birnstiel, 1971). It will be of interest to determine if other cellular RNAs, which may be required in certain organisms or tissues in large amounts, are transcribed from repetitive DNA sequences. A number of investigators have used either total or nuclear RNA preparations (Melli *et al.*, 1971) to hybridize to repetitive DNA. Since the composition and function of these RNA preparations are ill-defined these will not be discussed in detail. It should be noted, however that Britten and Davidson (1969) have postulated that at least part of the RNA confined to the nucleus has a regulatory function and should be complementary to a portion of the repetitive DNA of eukaryotes.

Repetition of Other DNA Sequences

Repetitive sequences in the second category have been detected and isolated solely on the basis of their special physical properties which differ from those of the remainder of the DNA. For example, a number of fractions of animal DNAs may be isolated because they band as satellites in CsCl (Waring and Britten, 1966) (Hennig and Walker, 1970) or complex differentially with silver ions and band at unique positions in an $\text{Ag}^+ - \text{CsSO}_4$ gradient (Corneo *et al.*, 1969). Further characterization of the isolated satellite DNAs has shown that they renature more rapidly than the remainder of the DNA and contain repetitive sequences. The

renaturation kinetics of mouse satellite would indicate that it contains a basic unit of 350 nucleotides which is repeated a million times. It is believed that these sequences are not transcribed into cellular RNA (Flamm, Walker and McCallum, 1969) but must serve some other biological function. A possible clue to this function was provided by Pardue and Gall (1970) who showed that the mouse satellite sequences were localized in the heterochromatic region of chromosomes and particularly at the region of the centromeres. The list of centromeric DNA has recently been expanded to include not only the mouse satellite sequences, but also one class of repetitive DNA from several other organisms (Yunis and Yasmineh, 1971). Because of the localization of this class of sequences, it has been speculated that their biological function is in some way involved with the maintenance of the structure or integrity of the chromosomes (Yunis and Yasmineh, 1971; Walker, 1971).

Another way in which repetitive sequences may be isolated is to control the conditions such that the renaturation of those sequences with a given frequency of repetition is favored (Britten and Kohne, 1965). The rate of renaturation of the separated strands of DNA is a function of the concentration of denatured DNA (C_0) and time (t). The extent of the reaction can thus be controlled by limiting the reaction to a given Cot . (The product of C_0 and t). One can therefore favor the renaturation of highly repetitive sequences by renaturing to a low Cot whereas sequences repeated only slightly and unique sequences require a high Cot for renaturation. In the experiments reported here, a Cot of 0.14 is assumed to favor

renaturation of sequences repeated one hundred times or more. These are referred to as highly repetitive sequences.

It is then necessary to separate the selectively renatured DNA from the single stranded DNA. Although a number of methods could be used to effect this separation, the one most often used is hydroxyapatite chromatography (Bernardi, 1965). In this method the single stranded and renatured DNAs may be separated as a result of the fact that the former elutes from a hydroxyapatite column at a lower PB concentration (0.02 - 0.1 MPB) than the latter (0.2 - 0.3 MPB).

Using the selective renaturation procedure and hydroxyapatite chromatography, it is possible to separate eukaryote DNA into fractions which differ in their degree of repetitiveness. For example, a fraction containing the mouse satellite sequences (10^6 copies) may be obtained after a very limited (low Cot) period of renaturation. The remainder of the mouse DNA may then be renatured for an additional period to give a fraction which contains sequences with a lower frequency of repetition. This is sometimes referred to as the intermediate repetitive fraction (Britten and Kohne, 1965). The repetitive DNA isolated after reannealing to a Cot at which a considerable portion (20 - 30%) of the DNA is renatured will usually consist of sequences with assorted frequencies of repetition. Depending upon the selection of Cot, it may contain sequences complementary to RNAs. For example, X.laevis DNA which is renatured to a Cot of 0.006 contains the bulk of the repetitive rDNA of this organism (Birnstiel et al., 1968). It will also include the sequences representing the animal satellite DNA type of sequences, and others which are distinguishable only on the basis of renaturation rate. A number of repetitive DNAs

have been identified in this fashion. Except for sequences complementary to known RNA species, however, there is no good evidence to suggest a function of the other types of repetitive DNA.

A considerable proportion (up to 80%) of the DNA of eukaryotes consists of repetitive sequences (Britten and Kohne, 1965). In order to explain the fundamental life processes of eukaryotes in molecular terms, it will be necessary to define the biological function of these sequences. It is likely that various portions of the repetitive DNA may serve different functions in a single organism. For example, the regulatory DNA which was mentioned briefly is believed to be part of the intermediate repetitive fraction. Possibilities for some other functions have already been discussed. These include: 1. repetitive genes, 2. DNA sequences involved in chromosome function (pairing, folding or recombination) and 3. regulatory DNA sequences. Two other proposals should be mentioned. These are: 4. that some repetitive DNA sequences represent the multiple recognition sites for the enzymes involved in the processes of replication and transcription, and 5. that in some cases, the gene sequences specifying different proteins are sufficiently similar in base sequence to renature with one another. All of these hypotheses are considered in detail in a review by Bostock (1972). It is hoped that some important functions will be revealed by future experimentation.

MATERIALS AND METHODS

DNA Extraction and Purification

Young leaves of pumpkin, Cucurbita pepo L, var, Small Sugar; Chinese cabbage, Brassica pekinensis Lowr. Rupr., var. Wong Bok; tobacco, Nicotiana tabacum L. var. Samsun; and pinto bean, Phaseolus vulgaris L. were harvested from plants which were grown in the greenhouse. Leaves were macerated in an equal weight of sucrose buffer [0.5 M sucrose, 0.01 M MgCl₂, 0.05 TRIS (tris hydroxyethylaminoethane), 0.025 M KCl, 0.01 M CaCl₂, 0.005 M mercaptoethanol, pH 8.2] by grinding with a meat grinder or by chopping. Chopping was accomplished with a modified electric slicing knife to which razor blades were fitted. The homogenate was filtered through two layers of cheesecloth and glass wool and then was centrifuged at 1000 x g for 5 minutes. The pellet was dispersed with sucrose buffer containing 3.5% Triton X-100 (Rohm and Haas) to solubilize the chloroplasts and again centrifuged at 1000 x g to pellet the nuclei. This treatment was repeated until the pellet contained little or no green pigment in order to minimize the amount of chloroplast DNA contained in these preparations. DNA was released from the nuclei by suspending the nuclear pellet in 5 - 10 ml SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) which contained 1% sodium dodecyl sulphate (SDS). The suspension was allowed to stand at room temperature for 1 - 2 hours. The volume of resuspending buffer was kept to a minimum to facilitate the eventual spooling of the DNA. An equal volume of water

saturated phenol, which had been washed with SSC, was then added and the mixture shaken for 10 minutes at 4°C. The aqueous phase was separated by centrifugation and re-extracted with phenol until there was no precipitate remaining at the interface. The aqueous phase was overlaid with two volumes of ethanol and the DNA spooled with a stirring rod.

DNA was resuspended in a minimum amount of SSC and treated with ribonuclease (50 µg/ml) for one hour at 37°C. Pronase was then added (1 mg/ml), the temperature was raised to 50°C, and the incubation continued for two hours. The incubation mixture was cooled to 4°C and extracted repeatedly with phenol as above. Finally, an equal volume of chloroform-isoamyl alcohol (24:1, V/V), which had been washed with SSC, was added and the mixture was shaken at 4°C for 15 minutes. Phases were separated by centrifugation and the DNA was spooled from the aqueous phase after addition of 2 volumes of ethanol. The RNase and pronase treatments were repeated. In some cases enzyme treatment was carried out in dialysis tubing at 50°C against frequent changes of 1 liter portions of SSC. Dialysis treatment facilitated pigment and protein removal as judged by the improved adherence of fibers to the rod, and the lack of color of the final spool. The final spool of DNA was washed with ethanol and resuspended in either 0.01 M PB (equal molar Na₂HPO₄ and NaH₂PO₄) or SSC. Spectra of the preparations at this stage were typical of nucleic acids with little contaminating protein. Concentration was estimated by assuming $A_{260}^{1 \text{ mg/ml}} = 20$.

DNAs to be used for saturation and competition experiments were further purified by banding in CsCl (Flamm, Bond and Burr, 1966). This procedure has

been found to give optimal hybridization values (Pace and Pace, 1971). The DNA solution was adjusted to a density of 1.708 g/cc by addition of CsCl and centrifuged for 62 hours at 20° C in the Spinco # 50 rotor. The entire band of purified DNA was removed with the aid of the Isco fractionator and model UA-2 ultraviolet analyzer or by dropwise collection from the bottom of the tube. The DNA-containing solution was diluted 1:5 with SSC and the DNA was pelleted by centrifugation in the Spinco #50 titanium rotor at 45,000 rpm for 18 hours at 4° C.

DNA Renaturation and Hydroxyapatite Fractionation

The method of Britten and Kohne (1965) was essentially followed. Solutions of DNA stored 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° C in sealed containers for 10 minutes in a salt water bath. Samples were then immediately incubated at 68° C for periods of time sufficient to establish the Cot value desired. In these experiments Cot is expressed as moles nucleotide-sec-liter⁻¹ and the molecular weight of nucleotides is taken as 350. The renaturation was terminated by transferring the solution to a precooled flask and the samples were kept at 0° C prior to fractionation.

Preparative or analytical water jacketed columns maintained at 64-68° C and packed with an amount of hydroxyapatite (BioRad-Biogel HT) in great excess of that required to adsorb the reassociated DNA. The capacity of these preparations of hydroxyapatite was assumed to be 80 µg/cc (Kohne, 1968). After the columns were equilibrated by washing with 0.13 M PB, the chilled solution of

DNA was loaded by one ml aliquots and allowed to enter the bed. Single stranded DNA was washed through the columns with 0.13 M PB until A_{260} of the effluent was zero. Reassociated DNA was collected by eluting with 0.30 M PB and is referred to as low Cot DNA. Two ml fractions of the 0.13 M PB and 0.30 M PB eluates were collected and the A_{260} monitored. Fractions containing the eluted DNA were pooled for subsequent analysis by CsCl centrifugation or hybridization. The percent of reassociation was calculated as the percent of the input DNA which was collected in the 0.30 M PB fraction. The method was tested by eluting a sample of partially renatured pinto bean DNA with a linear gradient of PB. Single stranded DNA was eluted at 0.03 - 0.05 M PB, while reassociated DNA was eluted at 0.22 - 0.25 M PB.

The Cot of a reference DNA was measured in order to correlate these experimental conditions to those of Britten and Kohne (1965). E. coli DNA in 0.11 M PB was sonicated (Branson sonifier-power setting of 8) to reduce the molecular weight. DNA fragments were first purified by hydroxyapatite chromatography. DNA was loaded in 0.1 M PB onto a column of hydroxyapatite at 68°C. The column was washed with 0.1 M PB until no additional U.V. absorbing material was eluted. Purified DNA was collected by washing with 0.26 M PB, diluted to a suitable concentration and denatured at 100°C for 10 minutes. At time intervals representing varying degrees of renaturation at 68°C, 30 - 50 µg aliquots of DNA were removed and the extent of renaturation was measured by estimating the amount of renatured DNA in each aliquot by HA chromatography. The Cot at which one-half of E. coli DNA had renatured was found to be equal to one.

Each of the conditions of ionic strength (0.13 M PB), temperature (68° C) and molecular weight (that resulting from sonication) used in this experiment is expected to favor an increase in renaturation rate (lower Cot) as compared to those used by Britten and Kohne. Despite these differences in experimental conditions, the value of l and 4 are in reasonable agreement.

Except for the analysis of sheared pumpkin DNA, plant DNAs were used as isolated, without intentional shearing. It is estimated that the molecular weight of these preparations was in excess of 7×10^6 .

CsCl Centrifugation

Preparative

Nine and eight tenths grams of CsCl (s) was added to 7.85 ml of native DNA in SSC or to low Cot DNAs in 0.30 M PB and the density was adjusted to 1.708 g/cc. Density was determined refractometrically at 20° C with a Zeiss refractometer (Lfft, Voet and Vinograd, 1961). Spinco # 50 (5/8 x 2 1/2 in) polyallomer tubes were coated with a solution of bovine serum albumin (100 mg/ml) or Siliclad (Clay Adams), and dried prior to use. Solutions were centrifuged in the Spinco Model L-2 ultracentrifuge at 20° C for 62 hours using the # 50 rotor. At the completion of the run, a 20 gauge needle was inserted in the bottom of the tubes and 12 drop fractions were collected. One ml of 2 x SSC was added to each fraction and the A_{260} of each fraction was recorded. DNA of the appropriate fractions was used for subsequent hybridization experiments.

The slope of the CsCl gradient, which was determined by collecting every fifth fraction on ice and immediately reading the refractive index at 20° C, was

found to be similar for all tubes. Buoyant densities were calculated with respect to a reference DNA included in each gradient.

Analytical

Three to 5 μg DNA, along with 1 μg marker DNA, were added to a CsCl solution and the final density adjusted to 1.708 g/cc in the manner described for preparative centrifugation. Samples were transferred to cells which had been packed with 12 mm, 4 $^{\circ}$ single sector Epon centerpieces and centrifuged for 18 hours at 20 $^{\circ}$ C in the Spinco Model E Ultracentrifuge. U.V. absorption photographs were traced with a Spinco Model R B analytrol equipped with a film densitometer attachment. Buoyant densities were determined by the method of Sueoka (1961), using Micrococcus luteus DNA (1.731 g/cc) as a marker. The marker DNA is not shown in the densitometer tracings.

Preparation of RNA

Early experiments, in which the position of HMW rDNA in the low Cot DNA was assayed, utilized rRNA which had been extracted from the ribosomal pellets and was a gift from A. Jaworski. An attempt to isolate low molecular weight RNAs from ribosomes proved unsuccessful, since a considerable amount of small heterogenous RNA, which presumably results from degradation of HMW rRNA, contaminates these preparations.

To overcome these deficiencies, the extraction of total RNA was undertaken. Sufficient leaves to cover the bottom of four petri dishes were washed, dried and prepared for labeling as described by Zaitlin, Spencer and Whitfield, (1968).

Each dish contained 10 ml 0.05 M MES buffer [2-(N-morpholine) ethane sulfonic acid H₂O], pH 5.8, containing 1% of a mixture of the antibiotics Rimocidin (Pfizer) and Cephaloridine (Eli Lilly). One half mC³H-5-uridine was added to each dish and incubation was continued for 32 hours under fluorescent lighting at room temperature.

Either labeled pumpkin or unlabeled tobacco leaves were washed, blotted dry and ground to a fine powder after freezing with liquid N₂ in a mortar and pestle. Two volumes per weight of tissue of each of the following were added and the slurry mixed as thawing occurred: (1) TN Mg buffer (0.1 M NaCl, 0.10 M TRIS, pH 7.6, 0.01 M MgCl₂) containing .2% SDS and 1% diethyl pyrocarbonate. (2) Redistilled, H₂O saturated phenol which had been washed with TNMg.

Phases were separated and the debris pelleted by low speed centrifugation. After a second phenol extraction, RNA was precipitated by the addition of an equal volume of isopropanol and a few drops of 3M acetate buffer. After overnight storage at -20°C the precipitate was collected by centrifugation, washed, and resuspended in STMg (0.1 M NaCl, 0.05 M TRIS, 0.01 M MgCl₂, pH 7.6). The solution was clarified by centrifugation at 5000 g for 10 minutes and treated with phenol as before. RNA was reprecipitated with 2 volumes cold 95% ethanol plus several drops of 3 M acetate buffer. The final clarified solution of RNA in STMg was centrifuged in the Model L-2 for 3×10^7 g min (45 K rpm for 4-5 hours in the 50 titanium rotor) as suggested by Payne and Dyer (1971a). This centrifugation step yielded a pellet containing predominately the HMW rRNA; whereas the supernatant was greatly enriched in the low molecular weight RNAs.

The pellet was washed with ethanol, and resuspended in a minimum volume of $2 \times \text{SSC}$. The RNA was fractionated on 2.5% gels and used for hybridization experiments where indicated.

The supernatant of the 3×10^7 g min centrifugation, which served as a convenient source of low molecular weight RNAs, was treated with RNase - free DNase ($20 \mu\text{g/ml}$) at 30°C for 30 minutes and extracted with phenol to remove the enzyme. RNA was reprecipitated with ethanol, washed and dissolved in a minimum volume of $2 \times \text{SSC}$. This RNA was fractionated by electrophoresis in 12.5% polyacrylamide gels. RNAs were further purified by removing sections containing each of the RNAs from the gel, cutting into 1.07 mm slices and shaking with several changes of 10 ml aliquots of $2 \times \text{SSC}$ containing .1% SDS in order to elute the RNA. Gel debris was pelleted by centrifugation and the solution was filtered through nitrocellulose membrane filters (Bact-t-flex-B6, Schleiker and Schuell). The RNA was precipitated, washed and dissolved in $2 \times \text{SSC}$. The final concentration of RNAs was determined from the A_{260} using a gel elution blank and assuming $A_{260}^{1 \text{ mg/ml}} = 25$.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was accomplished as described by Loening (1967). HMW rRNAs were analyzed and eluted from gels containing 2.5% acrylamide or 1.8% acrylamide - 0.5% agarose (Peacock and Dingman, 1968) and 4S and 5S RNAs were separated in 12.5% acrylamide gels. Immediately after polymerization, gels were pre-run in E buffer (0.04 M TRIS, 0.02 M Na acetate, 0.001 M EDTA, pH 7.2)

containing .2% SDS at 5 ma/gel for 2 hours. As much as .2 ml of the solutions of RNAs in 10% sucrose were applied to the gels and electrophoresed for optimal time periods. Low percentage gels were run 2 hours, removed from the apparatus and soaked in distilled H₂O for 1 hour. High percentage gels were run for 5 hours and soaked for 30 minutes. The gels were then scanned at 260 nm with a Gilford Modal 240 spectrophotometer equipped with a linear transport device. Sections containing the specific RNAs were cut from preparative gels and eluted. The distribution of radioactivity in analytical gels was monitored by freezing the gels on dry ice and cutting the frozen gels into 1.07 mm slices with a manifold of razor blades. Fractions containing the appropriate number of gel slices were then dissolved in 0.75 ml 30% H₂O₂ by incubating 12 hours at 50°C. Ten ml of a mixture of 33% Triton X-100 and 67% toluene (V/V), which contained 0.01% POPOP (1,4-bis(2-(5-phenyloxazolyl) benzene)) and 0.55% PPO (2,5-di-phenyloxazole) were added and the samples were counted in a Packard Model 3320 Tricarb scintillation spectrometer.

Hybridization

Saturation

Samples containing 1-20 pg DNA were made to a volume of 1 ml with 2 x SSC and denatured by the addition of .1 volume of 1 N NaOH. After 10-15 minutes the samples were iced and neutralized by the addition of .15 volumes of 1 M NaH₂PO₄ or KH₂PO₄. Denatured DNAs were embedded on B6 membrane filters and hybridized according to Gillespie and Spiegelman (1965). DNAs were

added by gravity flow to filters which had been presoaked in 2 x SSC and washed with 10 ml of the same buffer. Each filter was then washed with 50 ml 2 x SSC under vacuum and allowed to dry at room temperature for 1-5 hours. Filters were then dried in a vacuum oven at 80°C, cooled, and immediately immersed in glass scintillation vials which contained 1 ml of 0.1% SDS in 2 x SSC and the appropriate concentration of labeled RNA. Vials were incubated at 68°C. Hybridizations to 4S or 5S RNA were conducted for 4 hours, a period of time found by others to be sufficient for maximum hybridization (Tartof and Perry, 1970; Hatlen and Attardi, 1971). Reactions containing HMW rRNA were continued for 12 hours.

At the completion of the reactions non specific radioactivity was removed by washing the filters collectively for 15 minutes at room temperature with each of the following in succession: 300 ml 6 x SSC, 300 ml 2 x SSC (three times), 300 ml 2 x SSC containing 10 pg/ml pancreatic RNase, and 300 ml 2 x SSC. Filters were then oven dried and counted in PPO-PPOP-toluene scintillation fluid.

Each vial of the saturation experiments contained a blank filter with no DNA added.

Batch

DNA of the individual fractions of the preparative CsCl gradients was denatured and embedded as described above. Up to 25 filters were incubated in a single glass jar containing 6-10 ml of .1% SDS in 2 x SSC. The indicated amounts of RNA were added and mineral oil was overlaid to prevent evaporation. After incubation at 68°C, the filters were washed and counted. Counts were corrected for a blank filter with no DNA which was incubated in the same vial. In all cases, counts retained by the blanks were only slightly higher than background.

RESULTS

The pumpkin genome has been estimated to contain several thousand copies of HMW rDNA (Matsuda and Siegel, 1967). The proportion of pumpkin DNA which consists of these sequences is remarkably high when compared with other organisms and is greater than that reported for several species of other plant genera. It was considered that pumpkin DNA might also contain highly repetitive sequences complementary to 4S and 5S RNAs. These might be required for the production of stoichiometric amounts of HMW rRNA, 4S and 5S RNA.

Analysis of Pumpkin DNA

Experiments were initiated to determine the degree of repetition of 4S and 5S DNA, as compared to HMW rDNA. It was first necessary to isolate and purify the appropriate labeled RNAs for use in DNA-RNA saturation hybridization experiments.

Preparation of HMW rRNA, 4S and 5S RNA

It was found that the best method was to prepare a total RNA extraction from leaf tissue exposed to isotope and then separate the various RNA components from this extract by centrifugal and electrophoretic methods as described in the Materials and Methods section. The species of RNA which sedimented when the RNA extract was centrifuged for 3×10^7 g min was monitored by polyacrylamide gel electrophoresis (Fig. 1a). It can be seen that the high molecular weight RNA

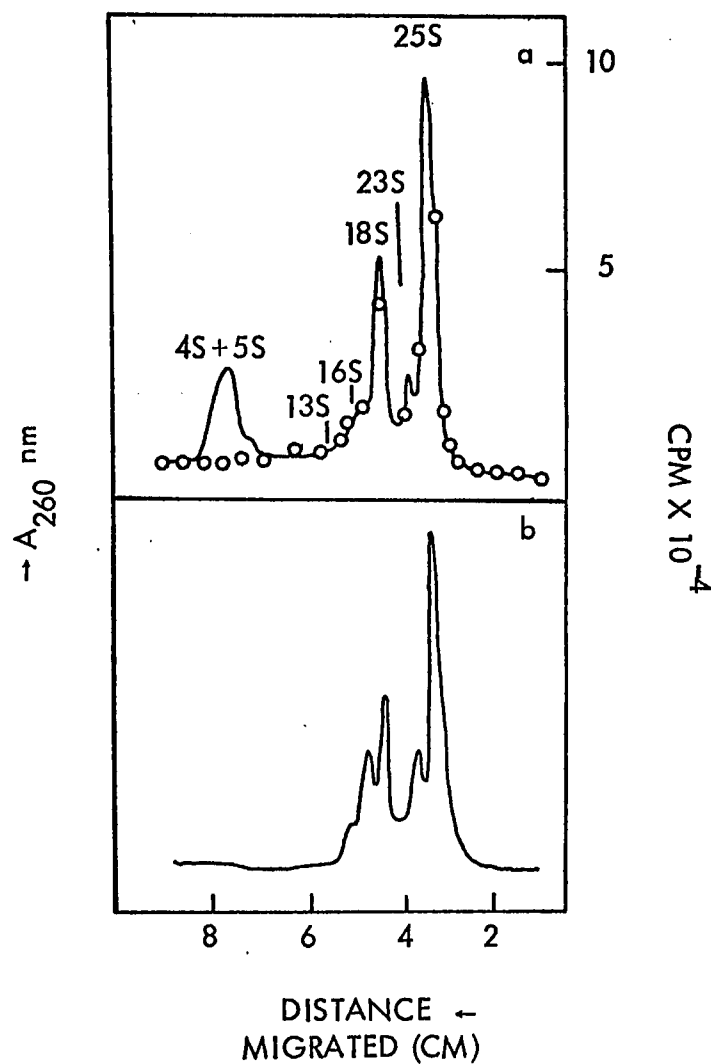


Fig. 1. Polyacrylamide gel electrophoresis of HMW rRNAs of pumpkin and tobacco.

Absorbance and radioactivity scan of (a) 1.8% acrylamide - 0.5% agarose gel containing 20 μg pumpkin RNA from the 3×10^7 g min pellet plus 20 μg added unlabeled 4S + 5S RNA marker. —, $A_{260 \text{ nm}}$; 0-0-0, cpm. (b) absorbance scan of 2.4% acrylamide gel containing 20 μg unlabeled tobacco RNA also from the 3×10^7 g min pellet.

species in this preparation are typical of plant HMW rRNA. Sixteen S and 23S RNAs are derived from 70S ribosomes, whereas 18S and 25S are from cytoplasmic ribosomes (Loening and Ingle, 1967). The 13S component is thought to be a degradation product of 23S RNA.

Analysis of the distribution of radioactivity in Fig. 1a indicates that two important criteria are satisfied by the characteristics of this RNA preparation.

(1) There is no discernable peak of RNA in the region of the gel to which 4S or 5S RNA would migrate. (2) The distribution of radioactivity is essentially coincident with the profile of absorbance at 260 nm. Contamination by bacterial RNA would be indicated by a disproportionate labeling of the 16S and 23S rRNAs, which the bacterial ribosomes have been shown to contain. Sections of 1.8% acrylamide-0.5% agarose gels containing the 13-25S RNAs were eluted with buffer and the RNA was recovered as described in Materials and Methods. This was done to insure that the preparation of HMW rRNA was not contaminated with 4S and 5S RNA or any RNAs which were excluded from the gel. RNAs prepared in this manner are collectively referred to as HMW rRNA and were used in the experiments where indicated.

It was necessary to prepare an unlabeled RNA which would serve as an effective competitor of pumpkin HMW rRNA. Fig. 1b indicates that a preparation of tobacco RNA consisted essentially of the same high molecular weight rRNA species and contained no 4S or 5S RNA. This preparation of tobacco HMW rRNA was used, without further purification on gels, as the heterologous competitor of pumpkin HMW rRNA.

Labeled pumpkin 4S and 5S RNA were also obtained from the total RNA extract for use in saturation experiments. Tritium labeled pumpkin RNA was precipitated from the supernatant of the 3×10^7 g min centrifugation, as described in Materials and Methods, and analyzed by electrophoresis on 12.5% acrylamide gels. Fig. 2a depicts the separation of two predominant low molecular weight RNAs in these preparations. A similar pattern for bean RNA has been observed by Payne and Dyer (1971) who have identified these as 5S and 4S RNA. Sections containing the 4S and 5S RNAs were removed from preparative gels similar to Fig. 2a, cut into 1.07 mm slices, and eluted with buffer as described. To establish the efficiency of this preparative technique, aliquots of the eluted sections were reapplied to 12.5% gels and the distribution of RNAs was monitored after electrophoresis. A gel containing unfractionated pumpkin 4S plus 5S was used as a marker and included in the same run (Fig. 2a).

A purified 4S RNA was collected and migrates, except for a small amount of trailing material, as a single peak (Fig. 2b). RNA eluted from the 5S section contained, in addition to a sharp 5S peak, a small component with a mobility intermediate between 4S and 5S, and considerable material at the top of the gel (Fig. 2c). Since this trailing material is present in gels of both 4S and 5S RNA at least a portion of it is believed to result from aggregation or adherence of the RNA to the walls of electrophoresis tubes.

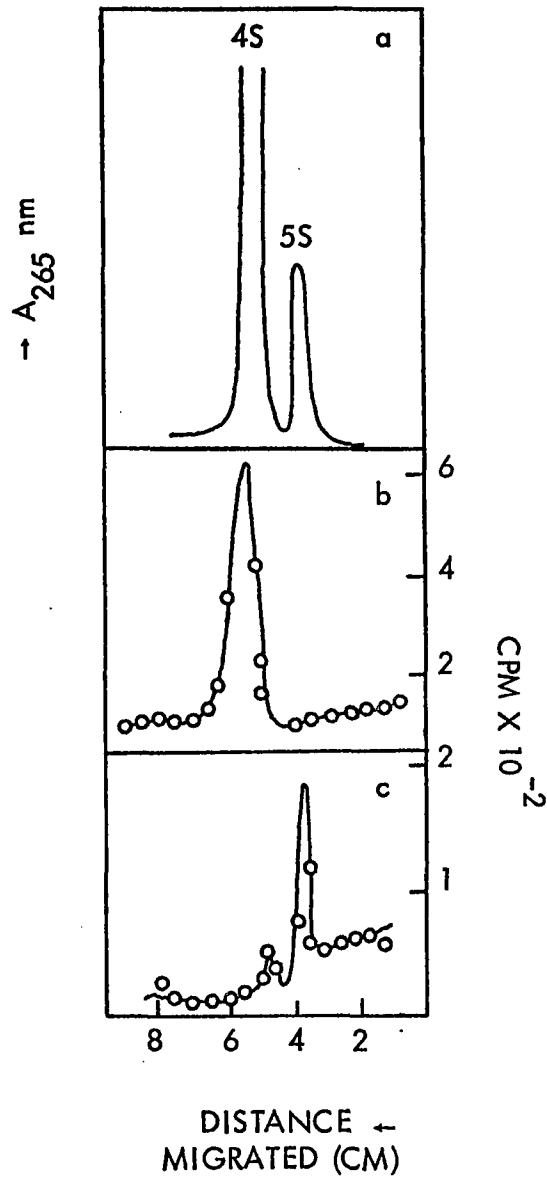


Fig. 2. Polyacrylamide gel electrophoresis of 4S and 5S pumpkin RNA

⁷ Absorbance scan of 12.5% acrylamide gel containing RNA from the 3×10^7 g min supernatant (a), and distribution of radioactivity of purified 4S (b) and 5S (c) RNAs. The three gels were run in parallel for 5 hours.

Degree of Repetition of HMW rDNA, 4S and 5S DNA

RNAs thus obtained from the total RNA extracts of pumpkin and purified by gel electrophoresis were deemed to be suitably purified for use in saturation experiments. A determination of the degree of repetition of pumpkin HMW rDNA was first undertaken. This estimate has been obtained by others and serves as a useful reference for these experiments. The saturation curve of Fig. 3 resulted from the challenge of filters containing pumpkin DNA with increasing amounts of HMW rRNA. It is estimated that, under the conditions of this experiment, 2.4% of the DNA represents HMW rDNA. This value is near that (2.2%) obtained by others (Goldberg, 1972).

It was necessary, in subsequent experiments, to employ an RNA which would serve as an effective competitor of pumpkin HMW rRNA. As Fig. 4 indicates, the preparation of tobacco leaf HMW rRNA which was monitored in Fig. 1b competes essentially in the manner expected on the basis of dilution for the HMW rDNA sites of pumpkin DNA. The capacity of rRNAs from diverse plant species to hybridize with a given DNA has been documented (Matsuda and Siegel, 1967).

A preparation of 5S RNA, obtained in an identical manner as that which was monitored in Fig. 2c was used to determine the redundancy of 5S DNA as compared to HMW rDNA. Experience of those who have conducted similar investigations with other organisms has indicated that 4S RNA and especially 5S RNA preparations, regardless of electrophoretic or chromatographic purity, contain appreciable amounts of RNA which cross reacts with the larger rRNAs. This difficulty was adequately circumvented by conducting the saturation experiments with 4S or 5S RNA in the

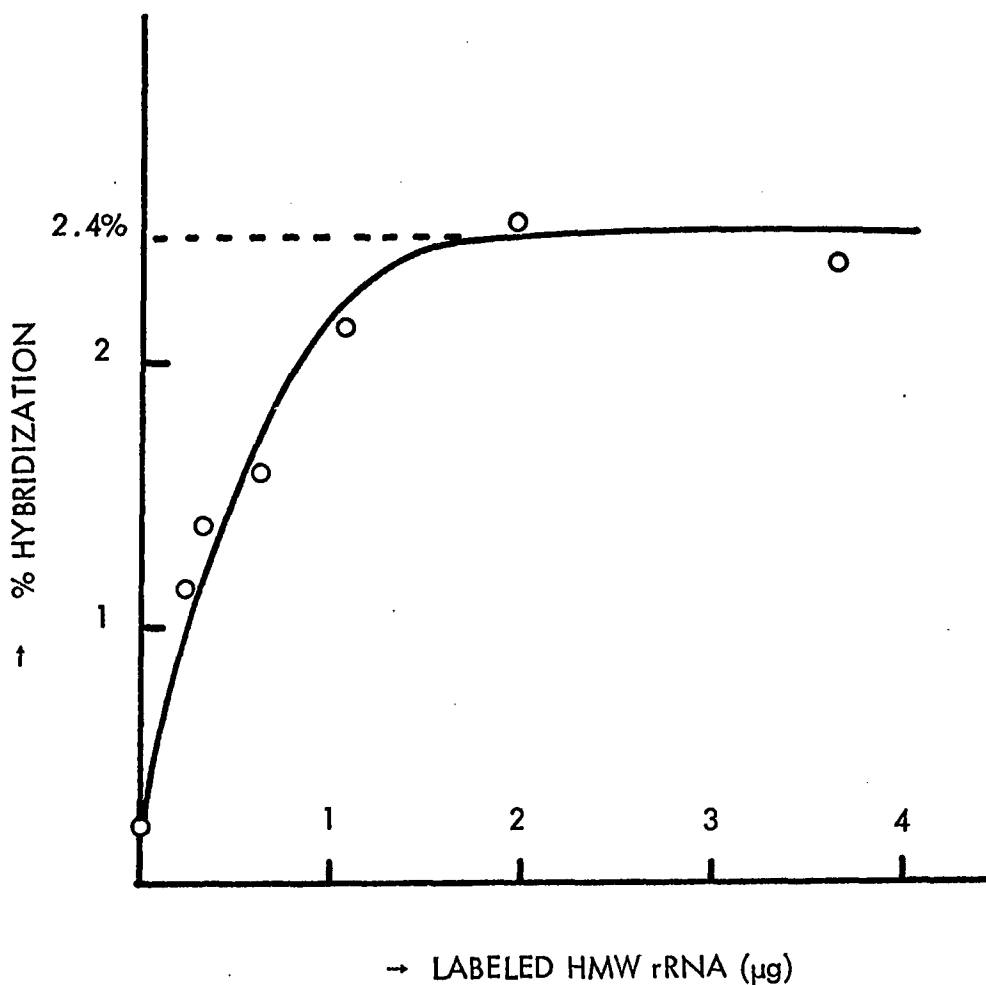


Fig. 3. Saturation hybridization of pumpkin DNA with HMW rRNA.

Increasing amounts of ^3H labeled HMW rRNA (24,478 cpm/pg) were incubated in separate vials with filters containing 1.3 pg of DNA. Conditions of this hybridization, as well as those of Fig. 4-6, are described in the Materials and Methods section. Counts retained by filters at saturation and those of blanks with no added DNA were 700 and 15 cpm, respectively. Each point represents the average of replicate determinations.

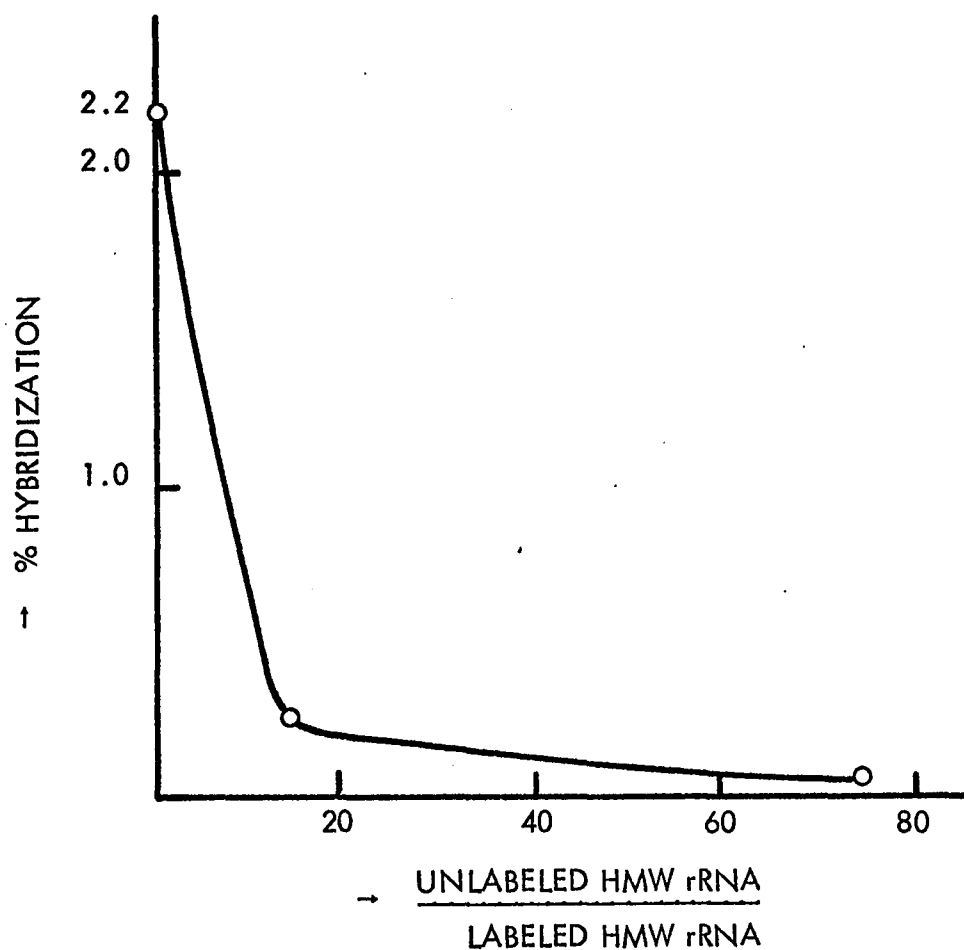


Fig. 4. Heterologous competition of pumpkin HMW rRNA by tobacco HMW rRNA

Increasing amounts of unlabeled tobacco HMW rRNA were added to vials which each contained .34 μg ^3H labeled pumpkin HMW rRNA (24,178 cpm/ μg) and a filter with 1.3 μg embedded DNA.

presence of an excess of unlabeled HMW rRNA. Increasing amounts of 5S RNA were therefore hybridized to pumpkin DNA in the presence of a one hundred fold excess of unlabeled tobacco HMW rRNA. The results are shown in Fig. 5. The rapid attainment of saturation at a RNA:DNA ratio of .03 is indicative of sequence homogeneity of 5S RNA and has been observed with other organisms (Hatlen and Attardi, 1971; Tartof and Perry, 1970). The level plateau obtained attests to the specificity of the reaction between 5S RNA and its complementary DNA sequences. From this reaction it is estimated that .029% of the DNA of these preparations consists of 5S DNA. If one assumes that the molecular weight of 5S RNA is 3.79×10^4 (Payne and Dyer, 1971b) and that the combined molecular weight of the HMW rRNAs is 3.6×10^6 (Loening, 1968), then one would expect $\frac{3.79 \times 10^4}{3.6 \times 10^6} \times 2.4$ or .026% of the DNA to be 5S DNA if there were equal numbers of copies of pumpkin 5S DNA as HMW rDNA. The value obtained (.029%) means that the number of copies of 5S DNA is at least as great as that of HMW rDNA.

It is interesting that E. coli and Drosophila DNAs contain similar numbers of copies of 5S DNA as 16 and 23S or 18 and 25S DNA. The detection of 450 copies of 18 and 25S and 27,000 copies of 5S DNA in Xenopus laevis stands in marked contrast.

The saturation of pumpkin DNA with 4S RNA occurs less rapidly than saturation with 5S RNA and requires a RNA:DNA ratio of approximately .38 (Fig. 6). This has been interpreted to suggest sequence heterogeneity of 4S RNA (Hatlen and Attardi, 1971). At saturation, .044% of pumpkin DNA is 4S DNA. If a molecular weight of 2.6×10^4 (Hatlen and Attardi, 1971) is assumed for 4S RNA, then one

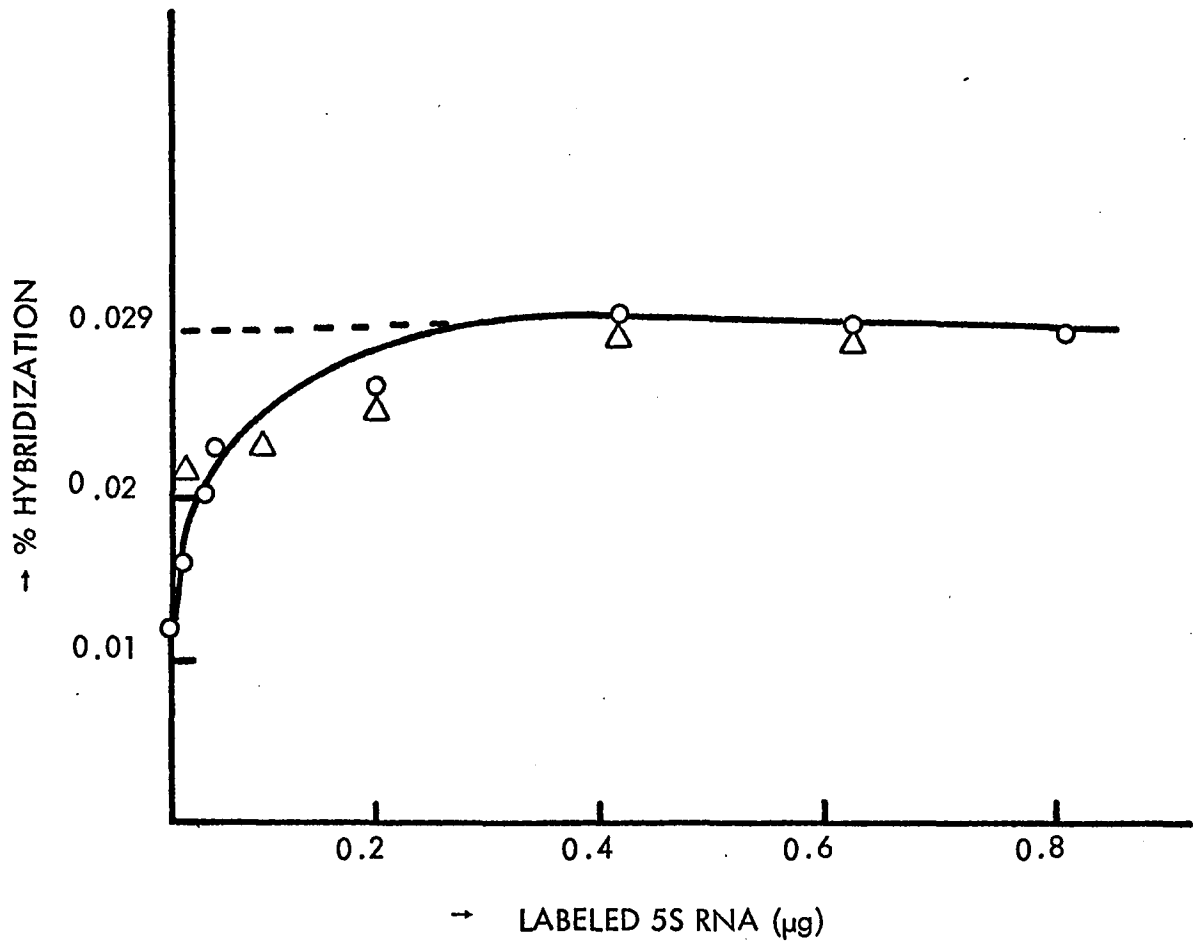


Fig. 5. Saturation hybridization of pumpkin DNA with 5S RNA.

Increasing amounts of ^3H labeled pumpkin 5S RNA (27,500 cpm/pg), together with a one hundred fold excess of unlabeled tobacco HMW rRNA were added to vials which each contained a filter with 13 pg DNA. The triangles indicate values obtained when vials of RNA were reused by incubating fresh DNA containing filters. Counts retained by filters at saturation and those of the blanks were 117 and 13 cpm, respectively.

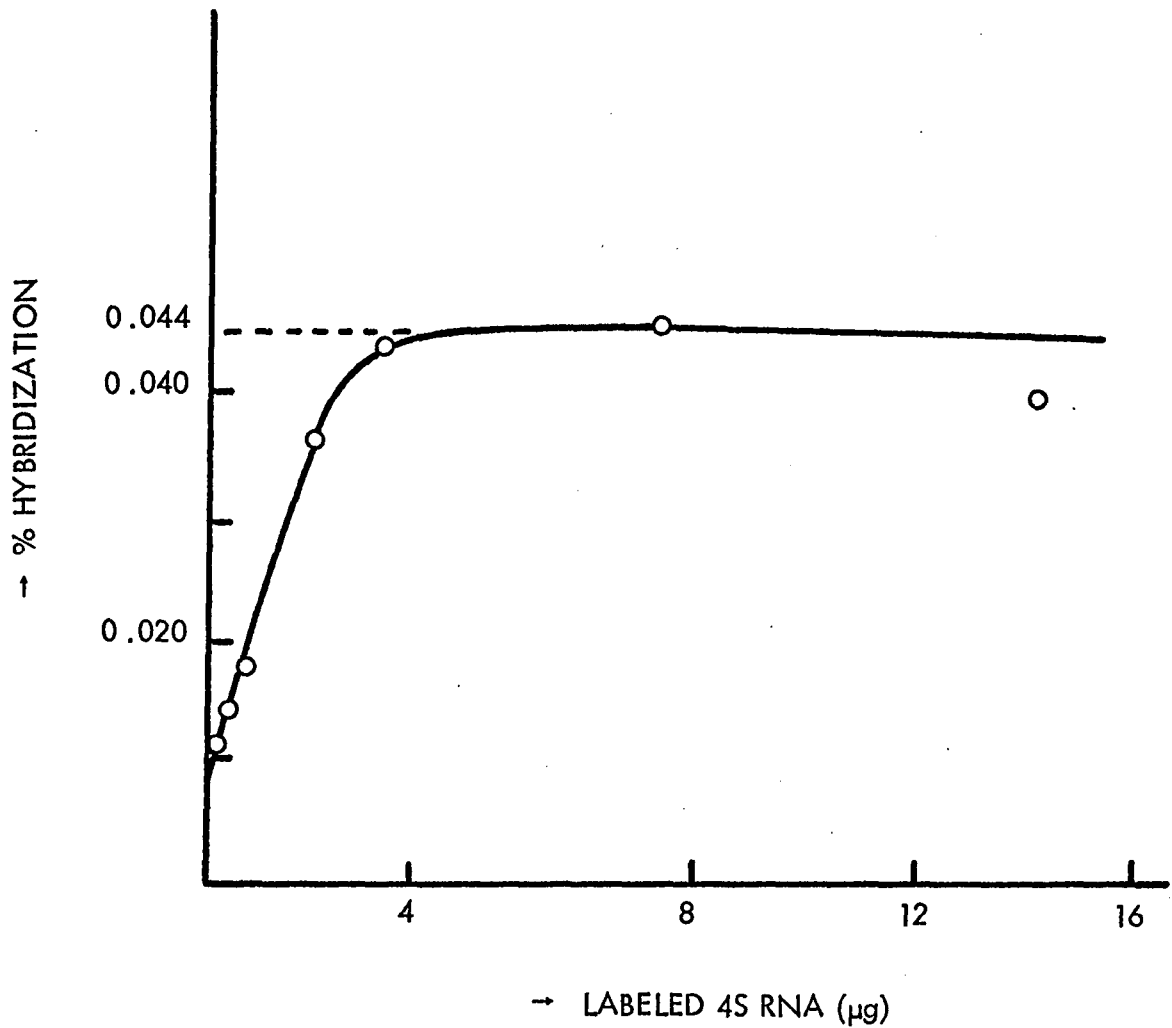


Fig. 6. Saturation hybridization of pumpkin DNA with 4S RNA.

Increasing amounts of ^3H labeled 4S RNA (27,457 cpm/ μg) together with a one hundred fold excess of unlabeled tobacco HMW rRNA were added to vials which each contained filters with 12 μg DNA. Each point represents the average of replicate determinations. Counts retained by filters at saturation and those of the blank were 144 and 14 cpm, respectively.

would expect $\frac{2.4 \times 10^4}{3.6 \times 10^6} \times 2.4\%$ or .016% for 4S DNA if the number of copies of 4S DNA and HMW rDNA were equal. The total number of copies of 4S DNA is therefore between two and three fold greater than that of HMW rDNA. This relationship is approximately obeyed in the DNAs of all organisms examined (see Hatlen and Attardi, 1971). It is important to note, however, that the degree of repetition of each of these sequences in pumpkin is remarkably high. If one assumes that there are 3,200 copies of HMW rDNA per haploid genome, then there are approximately 8,100 copies of 4S DNA in pumpkin. This may be compared to 1,150 copies in Xenopus laevis and 860 in Drosophila melanogaster.

Also, if one assumes that saturation experiments with 4S RNA measure equal numbers of sites for each of 60 transfer RNA species, it must be concluded that 135 copies of genes for each tRNA species exist.

These experiments confirm that the DNA of a higher plant contains multiple copies of 4S and 5S DNA, as well as HMW rDNA. Moreover, these sequences are repeated to such an extent that they should represent a part of the highly repetitive pumpkin DNA. It was decided to determine how these sequences were interspersed with other pumpkin DNA sequences.

Buoyant Density of Native HMW rDNA, 4S and 5S DNA

The buoyant density of the major portion (main band) of pumpkin DNA was reported to be 1.695; that of pumpkin satellite is 1.708 g/cc. HMW rDNA is contained in the satellite component (Matsuda and Siegel, 1967). The possibility was considered that 4S and 5S DNA might also be contained in fractions of pumpkin

DNA with distinct buoyant densities. To test this possibility, fractions of the preparative CsCl gradient containing native pumpkin DNA were assayed for the distribution of 4S and 5S DNA.

As shown in Fig. 7, 5S DNA is distributed on the dense side of native pumpkin DNA and is contained in the high G+C satellite fraction. In contrast, 4S DNA is distributed with the bulk of the DNA including satellite and main band (Fig. 8). Such a difference in the distribution of these sequences attests to the specificity of the assay procedure. This specificity, which is also apparent from the difference between the 4S and 5S saturation curves, suggests that these experiments measure the reaction of these RNAs with their complementary DNA sequences.

The DNA of another organism, Xenopus laevis, has been similarly analyzed for 4S and 5S DNA by Brown and Weber (1968a). Xenopus 4S DNA was found to be distributed throughout the bulk of DNA and to the dense side. This represents a similarity to the distribution of pumpkin 4S DNA. Indeed, 4S DNA of other organisms is thought to be distributed not only among sequences of varying G+C content, but is also distributed throughout the chromosomes as shown by genetic analysis in Drosophila (Ritossa et al., 1966) and an assay of 4S DNA of separated chromosomes of HeLa cells (Aloni, Hatlen and Attardi, 1971).

The way in which 5S DNA is distributed in Xenopus laevis, however, illustrates a striking dissimilarity to the present result, and may indicate a basic difference in the way in which 5S DNA is organized in the genome of animals and plants. Xenopus DNA also possesses a high G+C satellite which contains the 18S and 25S rDNAs (Birnstiel et al., 1966). Five (5) S Xenopus DNA, however, was

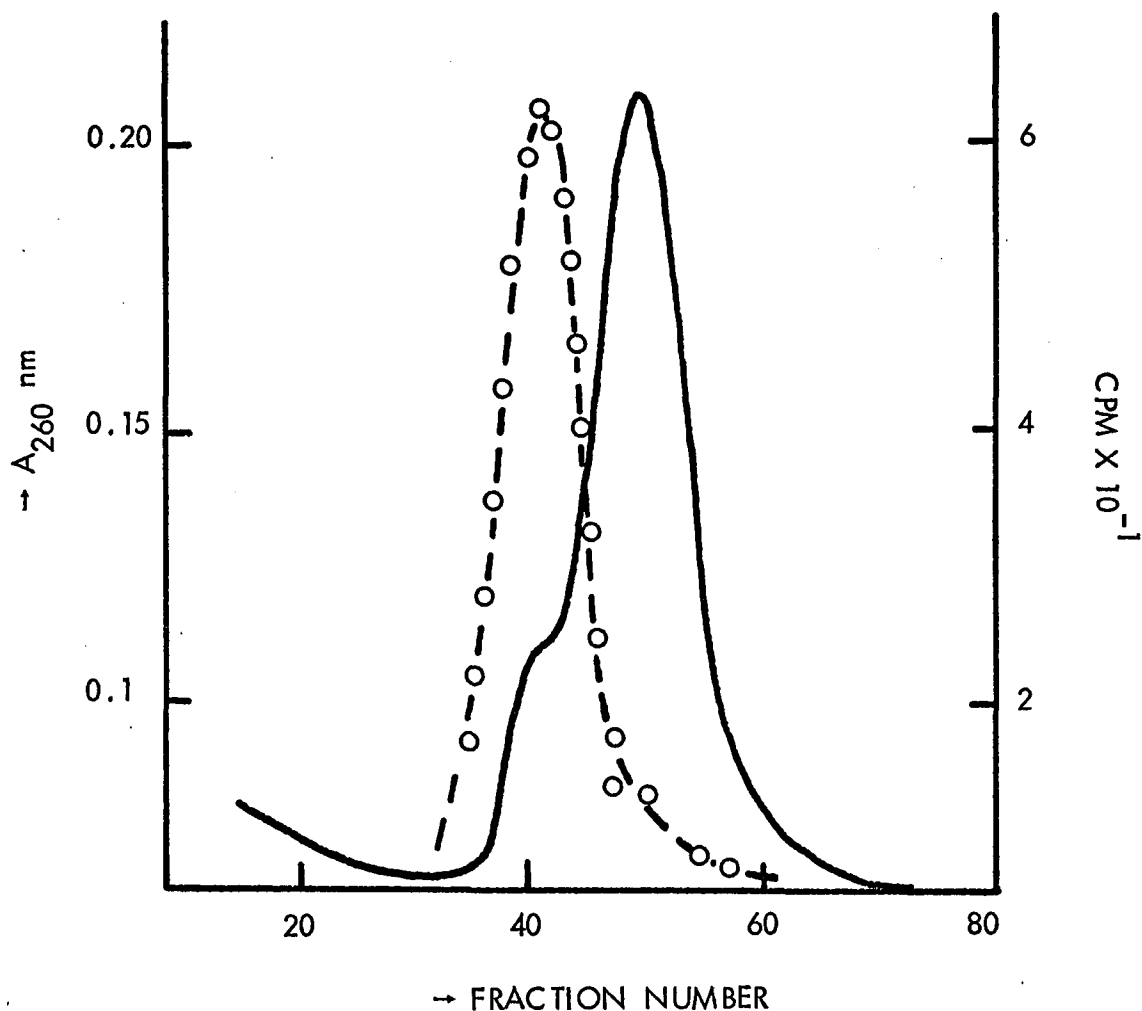


Fig. 7. Hybridization of 5S RNA to buoyant density fractions of native pumpkin DNA.

Approximately 250 μ g DNA were centrifuged to equilibrium in the preparative CsCl gradient. The absorbance at 260 nm of the collected fractions was read and the DNA of each fraction was embedded on a filter. Filters were then collectively incubated in a single jar which contained a greater than saturating amount of 3 H labeled 5S RNA (27,500 cpm/ μ g) and a one hundred fold excess of unlabeled tobacco HMW rRNA. —, A_{260} nm; 0-0-0, cpm hybridized. Details of the conditions of centrifugation and hybridization employed in this experiment and those of Fig. 8 and Fig. 9 and presented in the Materials and Methods section.

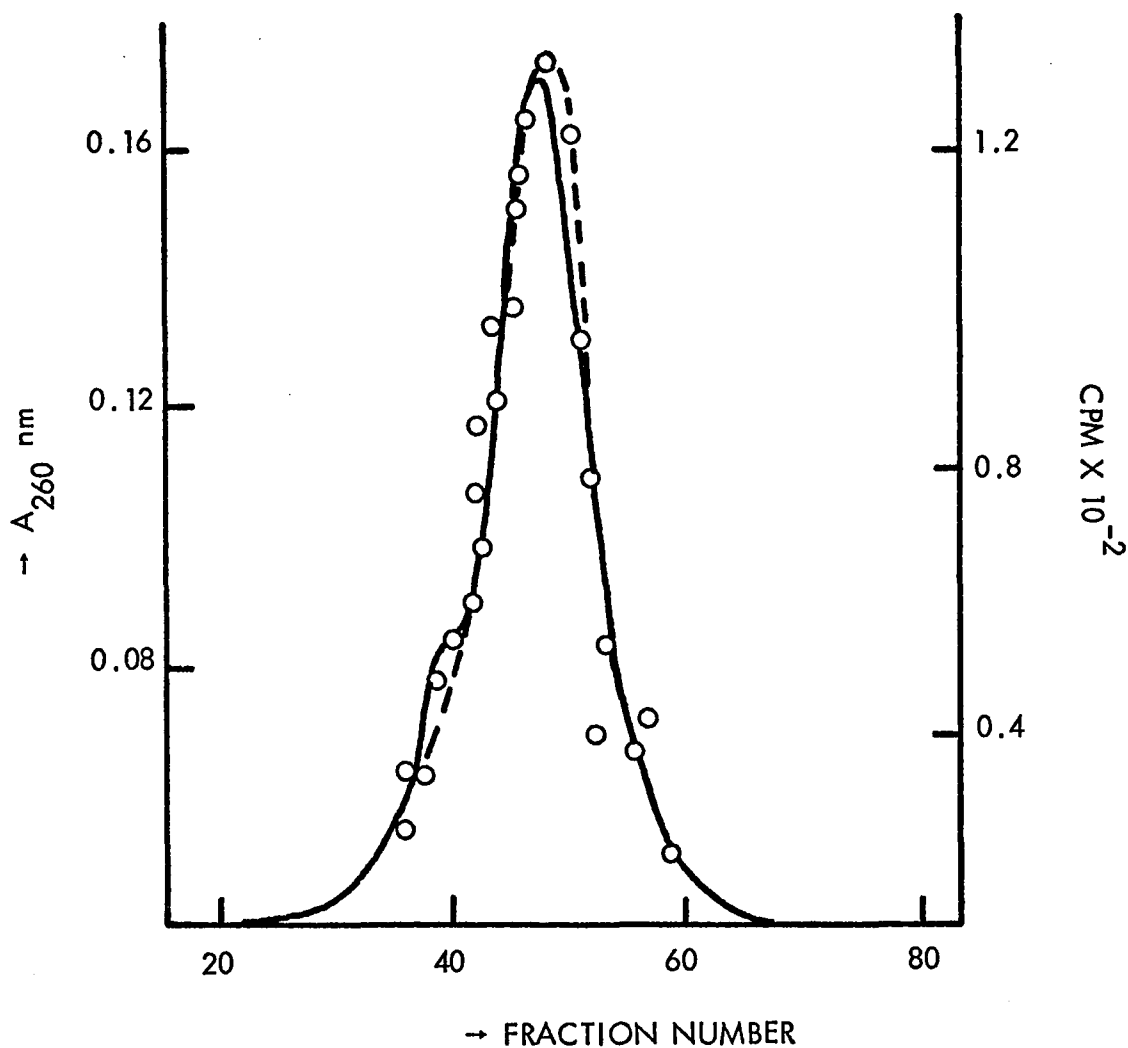


Fig. 8. Hybridization of 4S RNA to buoyant density fractions of native pumpkin DNA.

DNA of the fractions of a preparative gradient containing 250 μg native DNA was embedded on filters and batch hybridized, as described in the legend to Fig. 7, to a greater than saturating amount of ^3H labeled 4S RNA (27,457 cpm/ μg) and an excess of tobacco HMW rRNA competitor. —, $A_{260 \text{ nm}}$; 0-0-0, cpm hybridized.

found to be located in a fraction of lower buoyant density than the bulk of the DNA rather than in the satellite region. Furthermore, analysis has shown that in *Xenopus* (Brown and Weber, 1968), as well as in all other eukaryotes examined (Tartof and Perry, 1970; Aloni, Hatlen and Attardi, 1971), 5S and 18+25S rDNA are not genetically linked. The latter sequences have been shown to be contained in the nucleolar organizer region of the nuclei (Wallace and Birnstiel, 1966). Not knowing whether HMW rDNA of plants is contained in nucleoli prevents the discussion of further analogies of the nuclear localization of these sequences.

Pumpkin HMW rDNA was previously shown to band exclusively in the region of purified satellite DNA (1.708 g/cc) (Matsuda, Siegel and Lightfoot, 1970). In an attempt to ascertain whether this is also true for 5S DNA, the buoyant densities of these two sequences in a single preparative gradient were compared. Fig. 9 shows that the distribution of 5S DNA follows that of HMW rDNA and although the possibility exists that the peak fractions of these curves are displaced by .0025 g/cc in the gradient, it is highly probable that 5S DNA is also contained exclusively in the pumpkin satellite DNA. If this is so, there are at least two ways in which these sequences might be arranged in satellite DNA. (See Discussion.)

The possibility was considered, however, that the apparent coincidence of the distribution of these sequences resulted from the fact that the preparation of 5S RNA was contaminated with fragments of HMW rRNA which were not sufficiently diluted by the unlabeled competitor. Although the fact that such a level plateau at .029% was attained in the 5S saturation curve (Fig. 5) would argue against such a

Fig. 9. Hybridization of HMW rRNA and 5S RNA to buoyant density fractions of native pumpkin DNA.

Fractions of a preparative CsCl gradient containing 110 μg of native DNA enriched in satellite were collected (——, A_{260} nm) and split into two sets of filters. One set was batch hybridized as in Fig. 7 with ^3H labeled 5S RNA together with a one hundred fold excess of unlabeled HMW rRNA (0 0 0). The other set was batch hybridized with a greater than saturating amount of ^3H labeled HMW rRNA ($\Delta \Delta \Delta$).

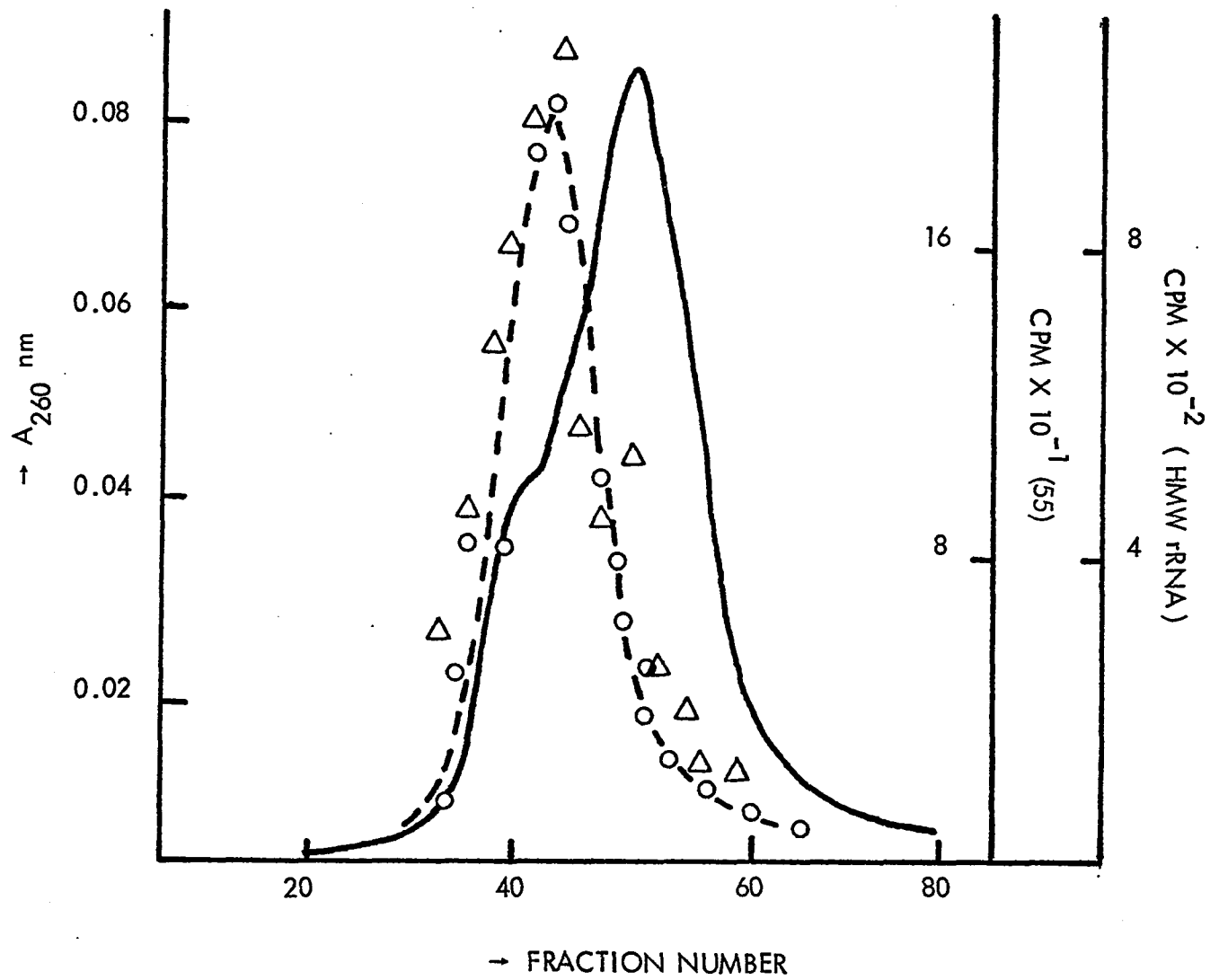


Fig. 9. Hybridization of HMW rRNA and 5S RNA to buoyant density fractions of native pumpkin DNA.

possibility, an additional experiment was designed to test the specificity of the hybridization reaction with 5S RNA.

A sample containing the low molecular weight RNAs was applied to a 12.5% acrylamide gel and the 4S and 5S RNA components were separated by electrophoresis. To insure that these components were the predominant low molecular weight species which were radiolabeled, the profile of radioactivity across the gel was monitored. The coincidence of patterns of radioactivity and $A_{265\text{nm}}$ across the gel shown in Fig. 10 demonstrates that this condition is satisfied. The RNA from selected slices of the gel was eluted and a one hundred fold excess of unlabeled HMW rRNA was added to each. Filters containing pumpkin DNA were then added to each fraction to test for hybridization across the gel. One might expect either of the following two possibilities as a result of such an experiment.

1. A constant amount of hybridization across the gel or a high baseline would indicate that heterogeneous fragments of HMW rRNA were present throughout the gel. A portion of these would have been collected with 5S and 4S RNAs and would interfere with the hybridization experiments.
2. Peaks of hybridization at the position of 5S and 4S RNAs would demonstrate the specificity of the reaction of both of these RNAs with pumpkin DNA. The result obtained is shown in Fig. 10.

Clearly, a specific reaction with both 5S and 4S RNAs is obtained and little hybridization occurs throughout the remainder of the gel. Hybridization to RNAs eluted from the top of the gel was expected, since the 3×10^7 g min supernatant contains some HMW rRNA which might barely migrate into the 12.5% gels.

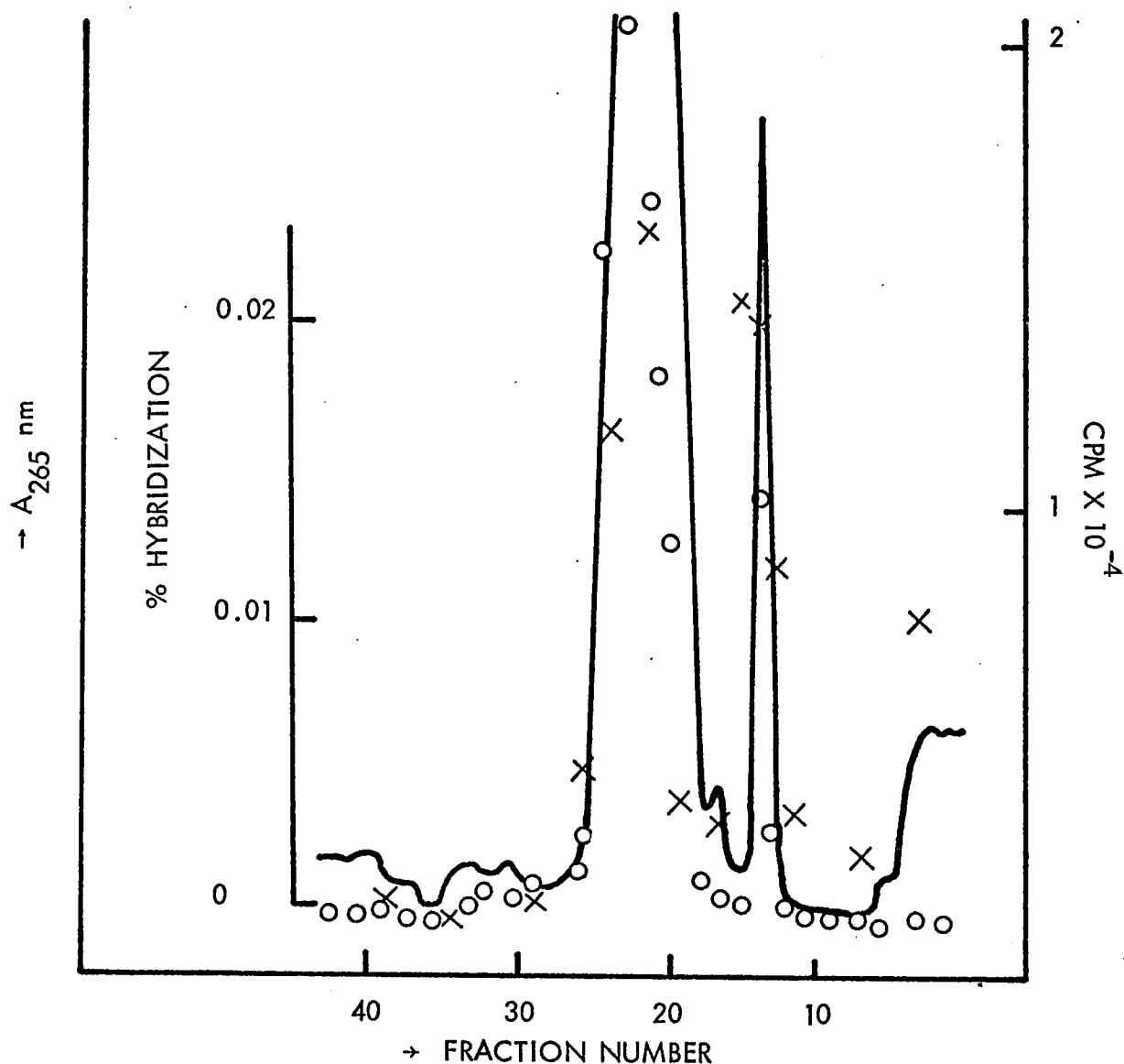


Fig. 10. Hybridization of low molecular weight RNAs to pumpkin DNA.

Approximately 80 μg of ^3H labeled RNA from the 3×10^7 g min supernatant were subjected to electrophoresis in a 12.5% acrylamide gel for 5 hours. The gel was scanned at 260 nm (—), and cut into 1.07 mm slices. RNA was eluted from each fraction (two gel slices) by adding 1 ml $2 \times \text{SSC}$ (.1% SDS) and storing in the cold for 12 hours. A .1 ml aliquot of each fraction was withdrawn and counted; 0 0 0, cpm. Another .1 ml aliquot of selected fractions was withdrawn and each was incubated, together with an excess of competitor tobacco HMW rRNA, with filters containing 22 μg of DNA at 68° for 4 hours. The percent of the DNA which hybridized with each of the fractions (x x x) is given with the assumption that the specific activities of each of the RNAs was the same (27,000 cpm/ μg).

Having demonstrated that HMW rDNA, 4S and 5S DNA each constitute families of highly repetitive pumpkin DNA sequences, it was undertaken to isolate a fraction of the pumpkin DNA which might be enriched in these sequences.

It is possible to separate the highly repetitive DNA from the remainder on the basis of the difference in renaturation rates of these fractions. The rapidly renatured DNA should be enriched in the most repetitious sequences and be amenable to further analysis. It was decided to fractionate pumpkin DNA on this basis and determine if HMW rDNA, 4S and 5S DNA were contained in the rapidly renatured fraction.

Characterization of Low Cot DNA

Pumpkin satellite contains sufficient copies of rDNA that this DNA renatures more rapidly than the bulk of the DNA (Matsuda and Siegel, 1967). Ribosomal DNA of other organisms has likewise been shown to renature rapidly (Birnstiel, Grunstein and Spiers, 1969; Patterson and Stafford, 1971; Gerbi, 1971). Experiments were undertaken to observe the characteristics of pumpkin DNA which had been denatured and then subjected to limited renaturing conditions in order to determine the rapidity with which satellite DNA would renature and whether there might be other rapidly renaturing species.

The results of such an experiment can be seen in the form of CsCl banding patterns of native, denatured and partially renatured pumpkin DNA. Fig. 11a shows the typical pattern of native pumpkin DNA with a main band of 1.695 g/cc and a satellite band at 1.708 g/cc. Fig. 11b shows the pattern of heat denatured

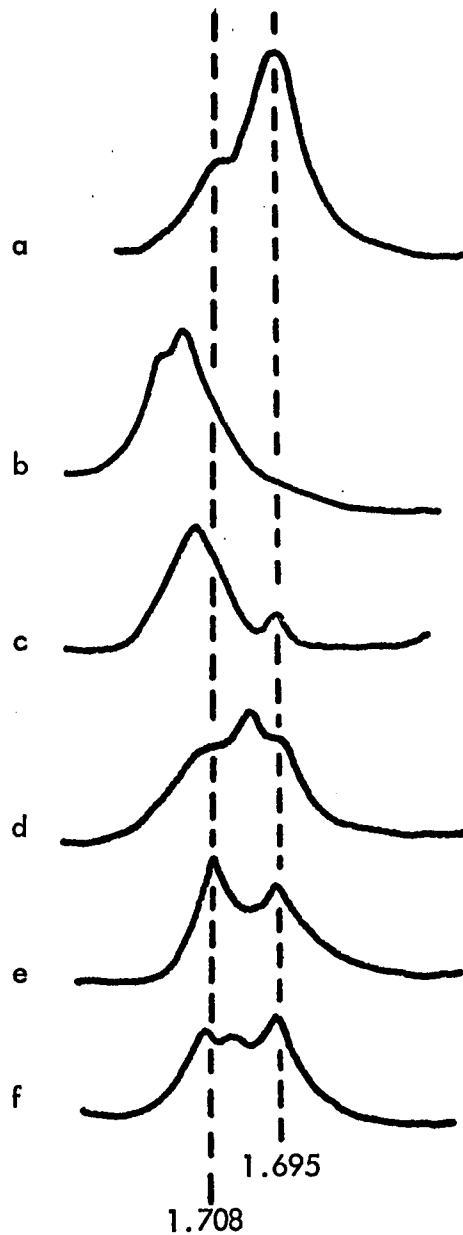


Fig. 11. Analytical CsCl centrifugation of native, denatured and renatured pumpkin DNA

Densitometer traces of U.V. photographs of 3–5 μg aliquots of the following DNAs banded in CsCl: (a) Native, (b) heat denatured for 10 min. at 100°C , (c) DNA renatured at $\text{Cot} = 0.14$ and quick cooled, (d) DNA renatured to $\text{Cot} = 1.6$ and quick cooled, (e) low Cot DNA isolated by HA chromatography, and (f) low Cot DNA resulting from sheared DNA. Details of the procedure of centrifugation employed in these and subsequent experiments are provided in the Materials and Methods section.

pumpkin DNA which has a typical buoyant density increase of 0.017 g/cc as compared with native DNA. Note that none of the DNA remains at a buoyant density of 1.695 g/cc. It can be seen in Fig. 11c that when denatured DNA is subjected to a brief period of renaturation ($Cot = 0.14$), a renatured component at 1.695 g/cc appears. If the DNA is renatured to a greater extent ($Cot = 1.6$) Fig. 11d shows that three components of renatured DNA may be detected. These have buoyant densities of 1.695, 1.700 and 1.708 g/cc.

Since it was apparent that there were rapidly renatured fractions of pumpkin DNA, it was decided to isolate those that renatured at $Cot = 0.14$ by HA chromatography and identify them by their buoyant densities. DNA collected in this fashion is referred to as low Cot DNA, and as shown in Fig. 11e consists of two components. These are designated as r (rDNA) and m (main band) components and have buoyant densities of 1.708 and 1.695, respectively. These buoyant densities suggest that r is a renatured portion of the rDNA satellite, and m represents a portion of main band DNA. The efficiency and rapidity of renaturation of these components are characteristic of fractions of eukaryotic DNA which contain highly repetitive sequences.

Different preparations of low Cot DNA showed variable amounts of a renatured component with a buoyant density intermediate between that of r and m. The appearance of this third component was shown to be dependent upon the size of the DNA being analyzed. This was demonstrated by taking two aliquots of DNA from the same stock solution. One aliquot was sheared, prior to heat denaturation, with ten passages through a 27 gauge needle. This resulted in fragments of DNA with a sedimentation coefficient in alkali of 13S (Goldberg, 1972), or a single

stranded molecular weight of 9×10^5 daltons. The second aliquot of DNA was not sheared and had a single stranded molecular weight of approximately 4×10^6 . After HA fractionation and banding in CsCl, the sheared preparation resulted in the three component product of Fig. 11f, while the unsheared product contained only r and m as shown in Fig. 11e. The origin of the molecular weight dependent component is considered in the Discussion section.

Since rDNAs, in general, renature rapidly, it was expected to obtain the renatured r component in low Cot DNA. The m component may be considered to represent a small portion of DNA which renatures at the same rate as the bulk of the main band DNA. Alternatively, it might represent a distinct minor fraction which renatures at a much greater rate than the rest of the main band.

If the second interpretation were correct, then one might not expect to find an additional quantity of m component if the DNA which was not renatured at a Cot of 0.14 was subjected to a second short period of reannealing. Fig. 12 shows the results of such a serial fractionation experiment. Fig. 12b indicates that low Cot DNA collected after the first cycle of renaturation at Cot = 0.14 consists of r and m components. The remaining single stranded DNA was heated to remove secondary structure and renatured to an additional Cot of 0.14. The renatured DNA obtained in the second cycle is shown in Fig. 12c and consists entirely of r component. Thus m is a distinct component and one cycle of incubation at Cot = 0.14 is sufficient to complete its renaturation.

To carry this investigation one step further, single stranded DNA remaining after the second cycle of reannealing was heated and renatured for an

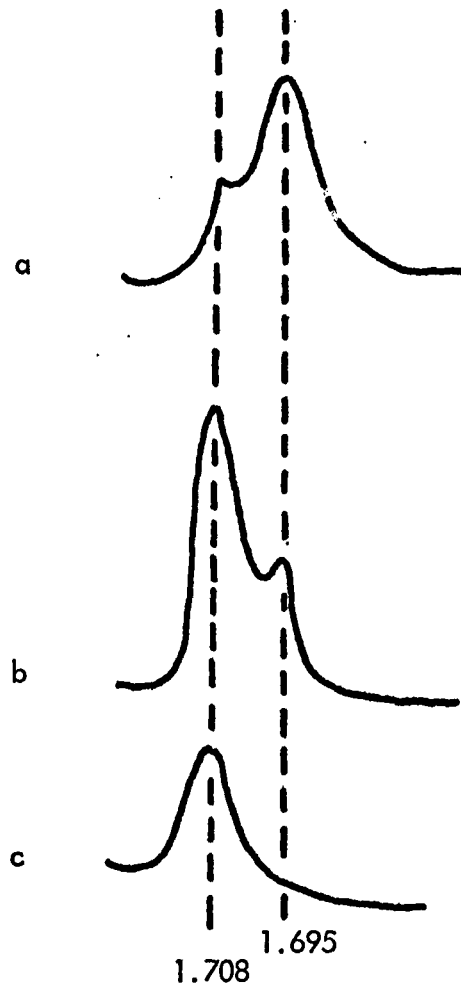


Fig. 12. Analytical CsCl centrifugation of 1st and 2nd cycle low Cot pumpkin DNA

Densitometer traces of U.V. photographs of 3-5 μg aliquots of the following DNAs banded in CsCl: (a) Native, (b) first cycle low Cot DNA, (c) second cycle low Cot DNA. The low Cot DNAs were isolated as described in the text.

additional Cot of 0.14 and fractionated as before. Little or no renatured DNA was collected by this third step. Thus, whereas one cycle of incubation is sufficient to completely renature m, two cycles were required for the complete renaturation of r component.

Table 1 shows the percentage of DNA which was obtained after each of the stages of this procedure. If r represents renatured HMW rDNA, then the low Cot DNAs of the first and second cycle fractionation should hybridize to a large extent with HMW rRNA. A small amount of DNA from the various fractionations was embedded on filters and hybridized to HMW rRNA as described in Materials and Methods. A comparison of the percentages of DNAs which hybridized indicates that low Cot DNAs of first and second cycle fractionations contain considerable HMW rDNA, but the unrenatured fraction of both cycles contained lesser amounts. This is consistent with the expectation that highly repetitive sequences (HMW rDNA) are contained in the low Cot fractions.

The reason that HMW rDNA constitutes a greater percentage of native and first cycle low Cot DNA than it does second cycle low Cot DNA has not been investigated. However two possibilities are proposed: 1. Successive heat denaturation may have led to a reduction in molecular weight and a lowering of the percent of DNA which was retained on filters. This would lower the percent hybridization value. 2. Most of the HMW rDNA was collected in the first cycle low Cot DNA, and second cycle DNA contains those non-rDNA sequences which were released from satellite DNA by fragmentation. The second possibility is considered the more likely one.

TABLE I

Hybridization of HMW rRNA to single stranded and low Cot DNAs obtained after several cycles of renaturation.

Pumpkin DNA was denatured and reannealed to 0.14 Cot. The resultant mixture was separated into fractions containing single stranded (0.13 MPB) and renatured (0.30 MPB) DNA by Hydroxyapatite chromatography. This represents one cycle of renaturation. For the 2nd cycle, DNA from the 1st cycle 0.13 MPB fraction was heated to 100°C for 10 min, renatured to 0.14 Cot and fractionated by Hydroxyapatite chromatography as before. For the 3rd cycle the procedure was repeated using the 2nd cycle 0.13 MPB fraction as the starting material. Small aliquots (1-2 µg) of each fraction were embedded on filters and hybridized with HMW rRNA as described in the Materials and Methods section.

Cycle	Fraction	Amount Collected (% of Total)	% Hybridization of each Fraction with HMW rRNA
1.	0.13 MPB	—	0.16
	0.30 MPB (low Cot DNA)	15	2.6
2.	0.13 MPB	—	0.14
	0.30 MPB (low Cot DNA)	8	1.0
3.	0.13 MPB	—	
	0.30 MPB	0	
	Native		2.5

These experiments have shown that two components, r and m, are contained within the genome of pumpkin. These are unique components which renatured more rapidly than any of the remainder of pumpkin DNA. If this were not so, renatured DNA would have been collected in the third cycle of the serial fractionation experiments. From the amounts of r and m collected in the first and second cycle of these experiments, it is estimated that these components represent 19% and 4% respectively, of pumpkin DNA. It should also be noted that a third component is obtained when the size of the DNA fragments undergoing renaturation is sufficiently reduced.

It was previously shown that while HMW rDNA and 5S DNA were confined to pumpkin satellite, 4S DNA was distributed throughout the bulk of the DNA. It was anticipated, therefore, that r component would contain HMW rDNA and 5S DNA, whereas m component might contain 4S DNA.

Buoyant Density Distribution of HMW rDNA, 4S and 5S DNA in Low Cot DNA

It was decided to investigate whether the r component of low Cot DNA contained the HMW rDNA. Low Cot DNA was prepared by HA chromatography and centrifuged to equilibrium in a preparative CsCl gradient. Fractions were collected as before and batch hybridized to HMW rRNA. The absorbance profile of the preparative gradient (Fig. 13) is similar to the analytical tracing of Fig. 11f, consisting of essentially two components. It is apparent from Fig. 13 that most of the HMW rDNA is distributed in the r component.

Fig. 13. Hybridization of HMW rRNA to buoyant density fractions of low Cot pumpkin DNA.

Approximately 40 μg of low Cot DNA were centrifuged to equilibrium in a preparation CsCl gradient. Fractions were collected and the A_{260} nm of each was measured (——). Filters embedded with the DNA of individual fractions were batch hybridized to a greater than saturating amount of pumpkin rRNA (62,000 cpm/ μg) as described in the legend to Figs. 7-9; 0 0 0, cpm hybridized. The densities of the fractions are presented for this and subsequent preparative gradients and for low Cot pumpkin DNA are shown with respect to m component (1.695 g/cc) as marker.

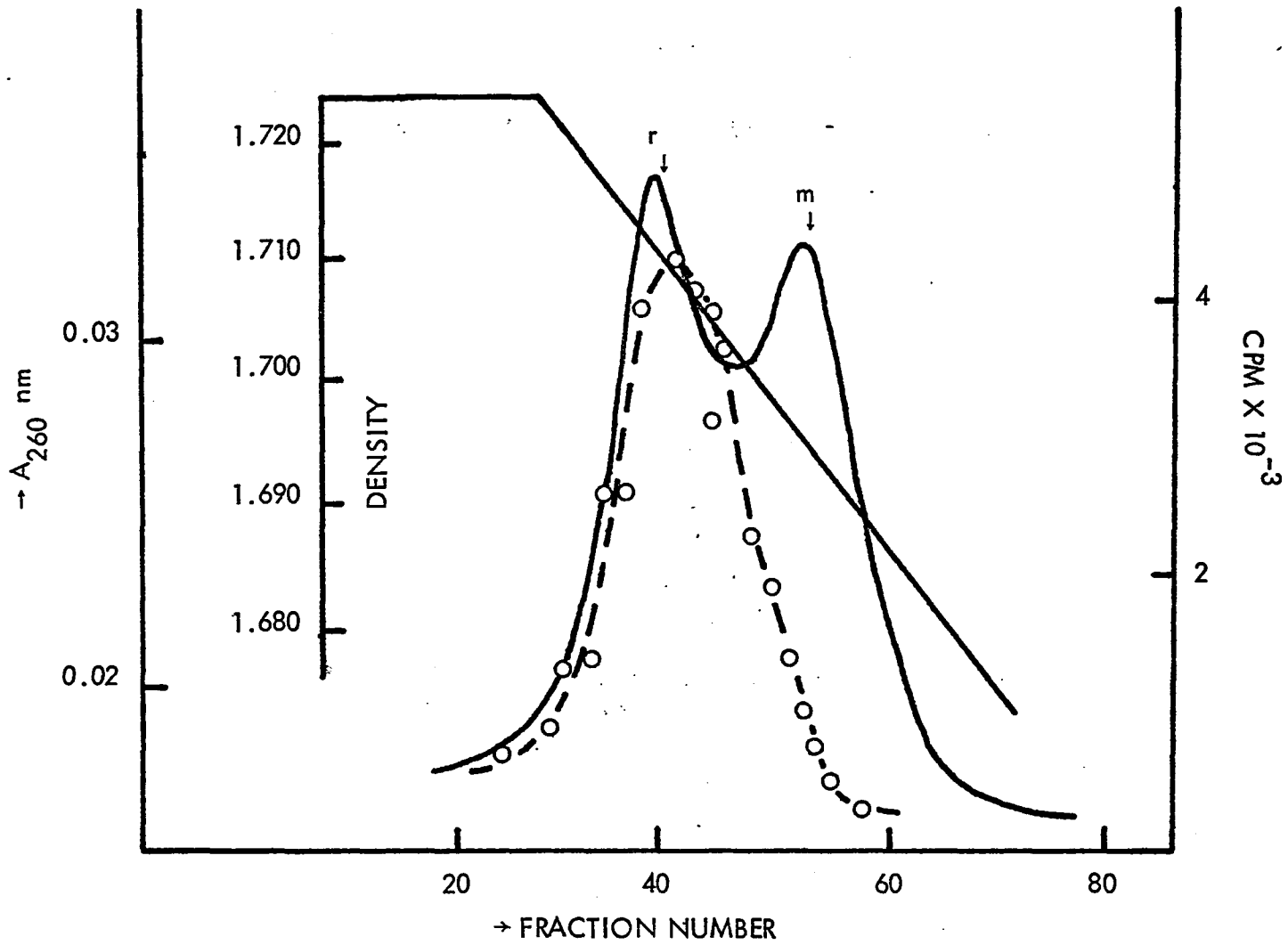


Fig. 13. Hybridization of HMW rRNA to buoyant density fractions of low Cot pumpkin DNA.

A similar experiment was performed to determine if 4S and 5S DNAs were present in low Cot DNA, and if so how these were distributed as compared with HMW rDNA. The absorbance profile of Fig. 14 shows that, in this case, a three component low Cot DNA was obtained as a result of the low molecular weight of the DNA. From Fig. 14, it is confirmed that HMW rDNA is contained in the most dense component. Hybridization to 5S RNA, in the presence of an excess of unlabeled tobacco HMW rRNA, reveals that 5S DNA is also contained in this component. Thus, 5S DNA is distributed in approximately the same buoyant density fractions as HMW rDNA in both native and low Cot DNA. Finally, hybridization to 4S RNA, in the presence of unlabeled tobacco HMW rRNA, shows that the dense component contains some 4S DNA, but m does not. This result is consistent with the fact that native satellite DNA contains a small part of the 4S DNA (Fig. 9).

These analyses have shown that pumpkin satellite DNA contains the bulk of HMW rDNA and 5S DNA, and a small portion of 4S DNA. Additional highly repetitive sequences must be contained in the pumpkin m component. The biological function of these sequences, however, has not been revealed.

Fig. 14. Hybridization of HMW rRNA, 4S and 5S RNA to buoyant density fractions of low Cot pumpkin DNA.

Approximately 80 μ g of low Cot DNA were centrifuged to equilibrium in a preparative CsCl gradient. Fractions were collected, the A_{260} was measured (—) and they were split into three sets. Each set was embedded on filters, and batch hybridized to greater than saturating amounts of either HMW rRNA (O O O), 5S RNA ($\Delta \Delta \Delta$), or 4S RNA ($\square \square \square$) as described in legends to previous figures and in the Materials and Methods section. Hybridizations with 4S and 5S RNA included an excess of unlabeled HMW rRNA and in this case were conducted for 10 hours.

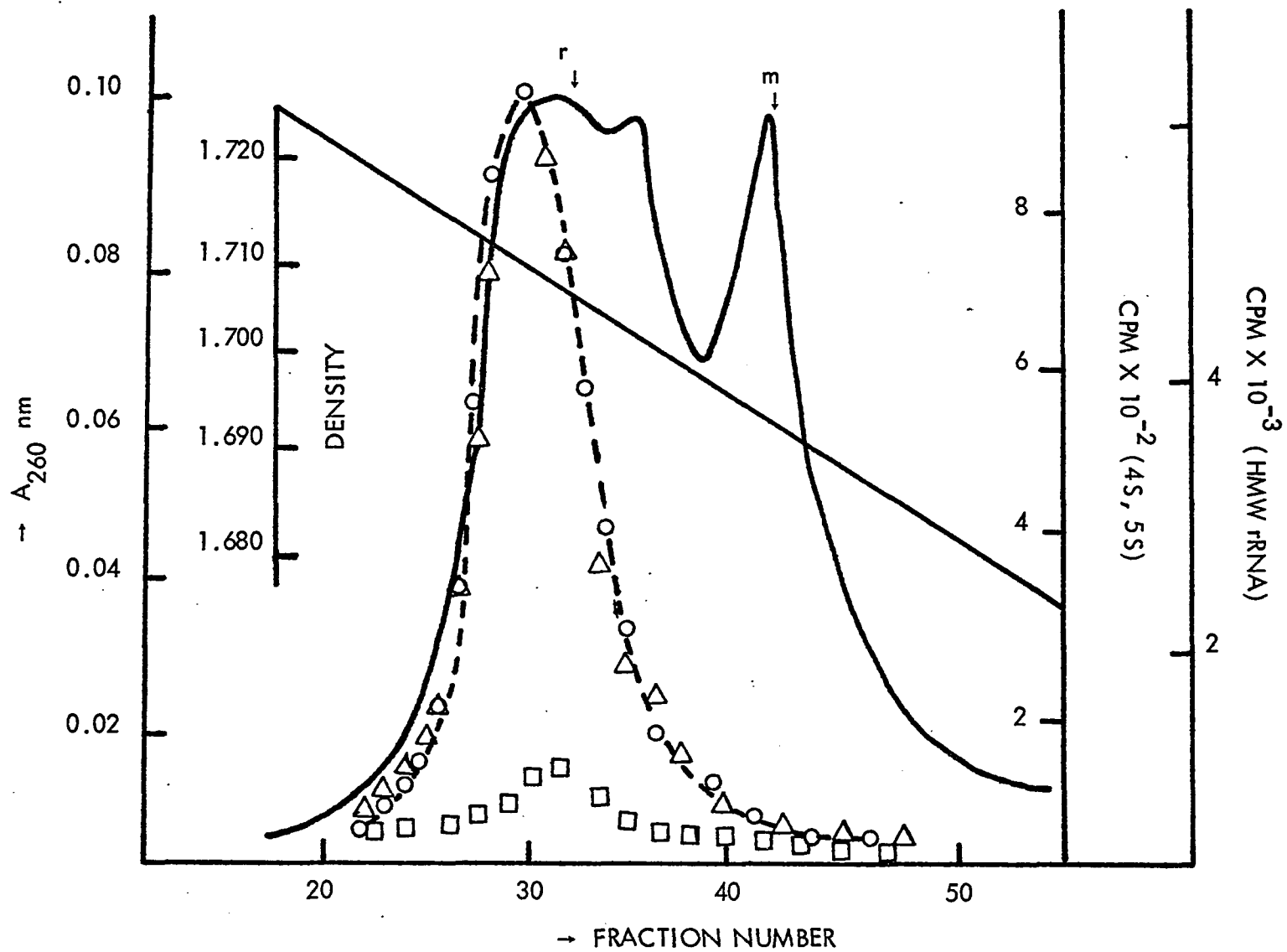


Fig. 14. Hybridization of HMW rRNA, 4S and 5S RNA

Comparison of Low Cot DNAs
of Several Plant Species

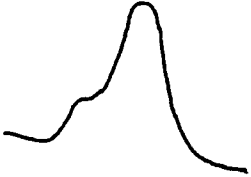



Approximately 19% of pumpkin DNA is collected, in the low Cot fraction, as renatured satellite DNA. Moreover, a substantial proportion of this renatured satellite DNA consists of HMW r DNA.

It was decided to compare the amount of low Cot DNAs of other distantly related plant species with that obtained from pumpkin to determine if these also contain renatured HMW rDNA. For these comparative studies, the DNA of Chinese cabbage, pinto bean and tobacco were chosen. This choice provides a comparison of a series of DNAs which differ in two respects: 1. These DNAs show a marked variation in their proportion of HMW rDNA as shown in Table II. 2. Tobacco DNA not only contains the lowest percent of HMW rDNA, but also lacks the prominent dense shoulder which the other species have been shown to possess. It is known that at least a part of the dense shoulder of Chinese cabbage and pinto bean contains the HMW rDNA. For comparison, densitometer traces of each of the native DNAs in CsCl are shown in Table II.

Each of the plant DNAs was heat denatured, renatured to an identical Cot (0.14) and fractionated by HA. The percent of each of the DNAs which was collected as low Cot DNA was recorded and the average values are presented in Table II. A greater proportion of the DNA of species containing a satellite component (pumpkin, Chinese cabbage and pinto bean) was collected in the low Cot fraction than was collected from tobacco, which lacks such a prominent satellite. This result was expected since, as a general rule, satellite DNAs renatured very

TABLE II

Correlation of the percentages of HMW rDNA and low Cot DNA of several plant species.

Species	Native CsCl Profile	% Hybridization to rRNA	Low Cot DNA (% of Total)	Number of Determinations
Pumpkin		2.4	23	8
Chinese Cabbage		1.8*	21	6
Pinto Bean		.47	18	1
Tobacco		.30*	11	2

* Matsuda, Siegel and Lightfoot, 1970.

rapidly due to the repetitive sequences which they contain. Also, a greater proportion of low Cot DNA was collected from those species which contained the greatest proportion of HMW rDNA (Table II).

It was decided to determine whether the low Cot DNA of each species partially consists of renatured HMW rDNA, as does pumpkin low Cot DNA, and whether these species also have renatured components analogous to the r and m components of pumpkin.

Characterization of Low Cot Chinese Cabbage DNA

Chinese cabbage DNA was analyzed in a manner similar to that of pumpkin DNA. As shown in Fig. 15c, low Cot Chinese cabbage DNA consists of prominent renatured components with buoyant densities of 1.708 and 1.702 g/cc and a minor component of 1.697 g/cc.

To determine which, if any, of these components contains the HMW rDNA, low Cot Chinese cabbage DNA was analyzed by preparative CsCl centrifugation and hybridization as was done for pumpkin DNA. As seen in Fig. 16, the 1.708 g/cc, but not the 1.702 g/cc component contains the rDNA, even though the latter has a buoyant density equal to that of the native dense shoulder of Chinese cabbage DNA. To use the same system of nomenclature as before, the 1.708 g/cc component is designated as r and the 1.697 g/cc component as m. The 1.702 g/cc component is designated as s component since this DNA may be derived from an additional renatured portion of the native dense shoulder.

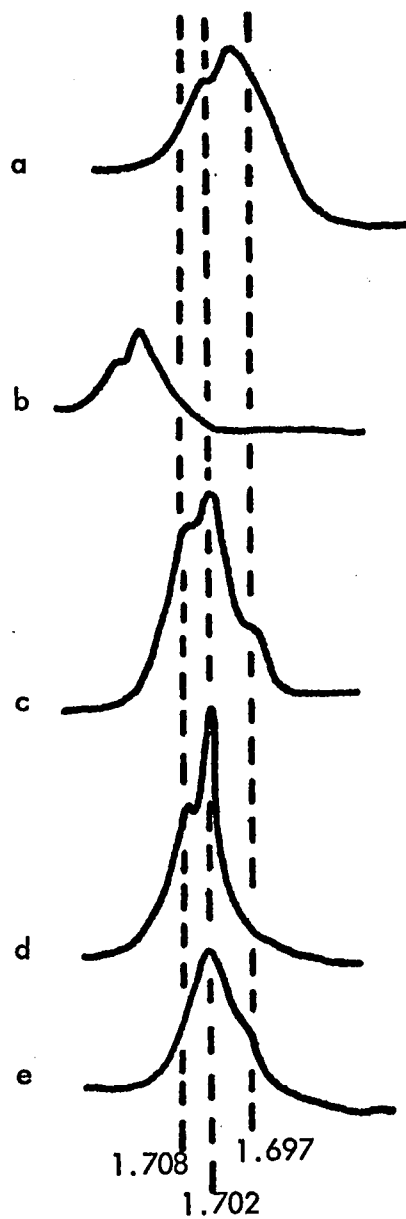


Fig. 15. Analytical CsCl centrifugation of native, denatured and renatured Chinese cabbage DNA.

Densitometer traces of U.V. photographs of 3-5 μ g aliquots of the following DNAs banded in CsCl: (a) Native, (b) heat denatured, (c) low Cot DNA isolated by HA chromatography, (d) DNA renatured to Cot = 36 and quick cooled and (e) .30 M PB fraction from HA chromatography at 4°C of quick cooled DNA.

Fig. 16. Hybridization of HMW rRNA to buoyant density fractions of Chinese cabbage low Cot DNA.

Approximately 133 μg of low Cot DNA were centrifuged to equilibrium in the preparative CsCl gradient. Fractions were collected, the A_{260} nm was measured (——) and the DNA of each fraction embedded on filters. Filters were batch hybridized to pumpkin rRNA as previously described (0 0 0, cpm hybridized). The slope of the gradient in this case is shown with respect to s component (1.702 g/cc) as marker. The rectangles indicate fractions pooled to give three separate samples each of which is greatly enriched in one of the components. These samples were used for the determination of the buoyant densities of the denatured components as described in the text.

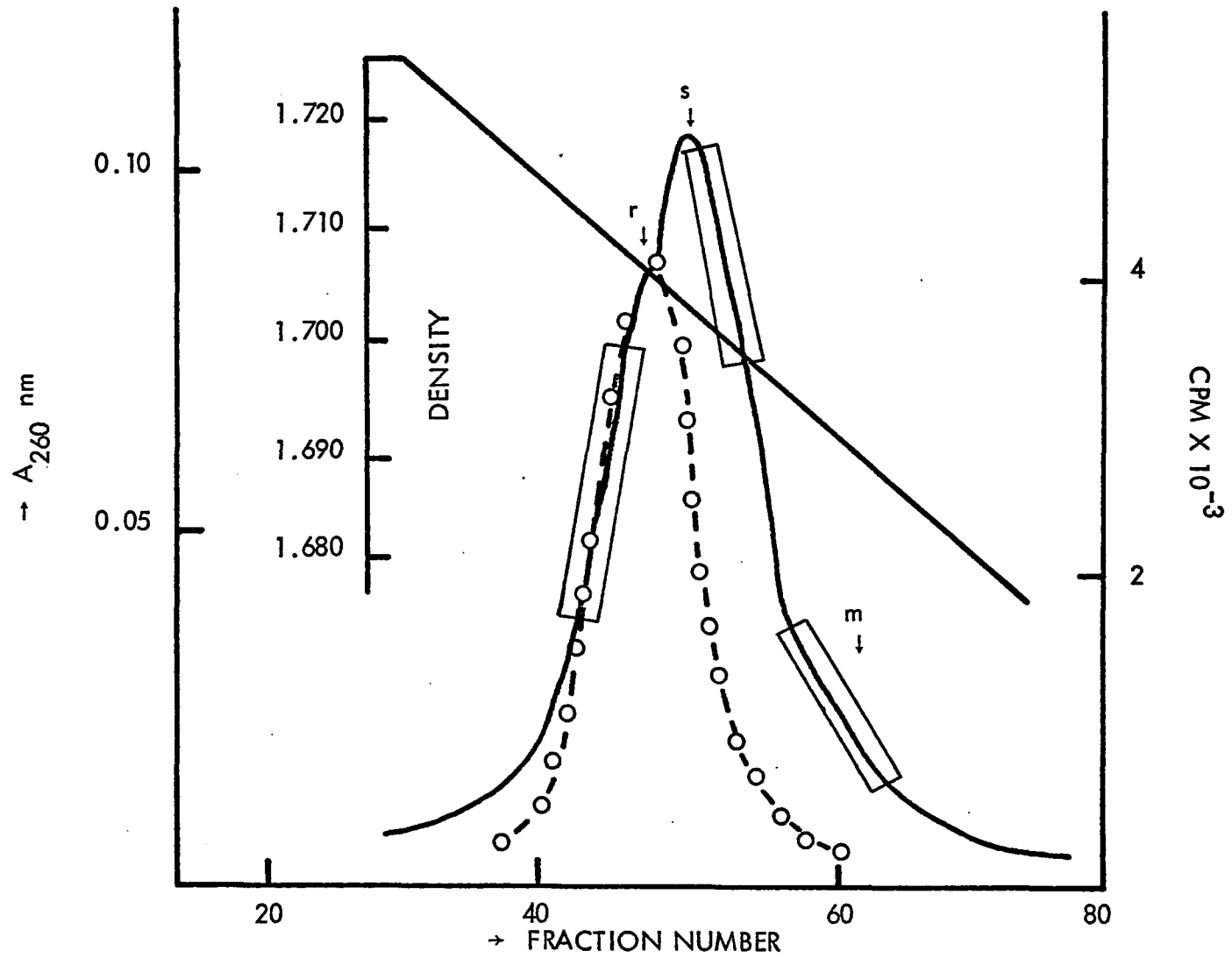


Fig. 16. Hybridization of HMW rRNA to buoyant density fractions

An alternative explanation of the origin of the s component is that this component represents a portion of native main band DNA which renatures inefficiently due to mismatching or linkage of the repetitive to unique sequences which remain single stranded under these conditions (Kram, Botchan and Hearst, 1972; Bolten et al., 1964-65). This alternative is feasible for as shown in Fig. 15 d the bulk of the DNA, even after renaturation to a Cot of 36 does not return to the position of native main band, but has a buoyant density of the s component. To distinguish between these two alternatives it would be necessary to do thermal melting experiments to determine the fidelity of base pairing in this component and to examine the effect of variation of molecular weight on the profile of the low Cot DNA of this species.

To test whether the r, s and m components actually consist of double stranded DNA, fractions included in the rectangles of Fig. 16 were pooled and the DNA contained in each pool was alkali denatured, neutralized and banded in CsCl. The buoyant densities of the denatured components were 1.718, 1.715 and 1.712 g/cc, respectively. These increases in buoyant density accompanying denaturation suggest that the renatured components consist of DNA which is at least partially double stranded.

As the Cot of renaturation is lowered, only those components containing the most repetitious sequences should be renatured. If Chinese cabbage DNA (100 $\mu\text{g/ml}$) is heat denatured, quick cooled and immediately fractionated on HA,

again the three components are obtained. Apparently the low effective Cot resulting from the time required for quick cooling or for eluting denatured DNA from the $64-68^{\circ}C$ column is sufficient to renature the three components. An attempt was made to minimize the extent of renaturation by fractionating quick cooled denatured DNA on a $4^{\circ}C$ column. Using this technique only s and m components were obtained (Fig. 15 e). Thus, as the Cot of renaturation is decreasing below 0.14, one would expect the ratio of the amounts of r to s components to decrease. These unpredictable results obtained by fractionation with the $4^{\circ}C$ column were not investigated further. However, it may be concluded from these zero renaturation experiments that the s and m components are as rapidly renatured as the r.

Characterization of Low Cot Pinto Bean DNA

Pinto bean DNA was analyzed to determine if low Cot DNA contained renatured HMW rDNA and additional components corresponding to those of pumpkin and Chinese cabbage DNAs. As shown in Fig. 17 b, two renatured components with buoyant densities of 1.706 and 1.699 g/cc are identified. The HMW rDNA component was identified, as before, by banding pinto bean DNA in the preparative centrifuge and analyzing the fractions for the distribution of HMW rDNA. It is evident from Fig. 18 that renatured HMW rDNA as well as other rapidly renatured sequences are contained in the 1.706 g/cc (r) component. It is likely that s component, by analogy to the results with Chinese cabbage DNA, corresponds to a portion of the shoulder of native pinto bean DNA which does not contain HMW rDNA.

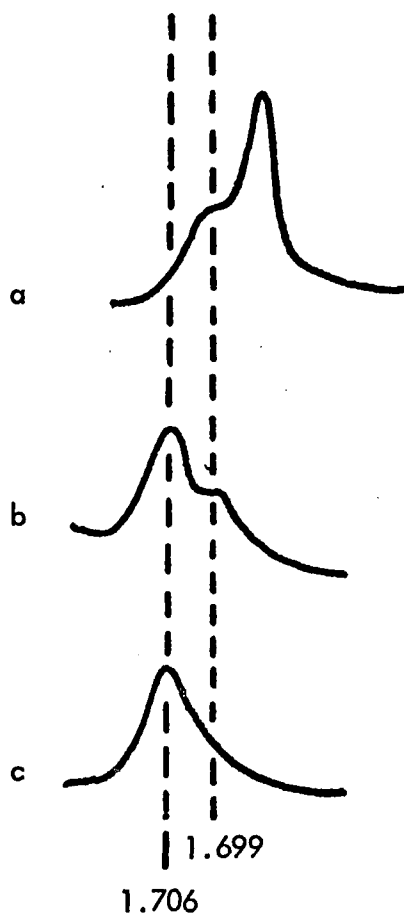


Fig. 17. Analytical CsCl centrifugation of native and renatured pinto bean DNA.

Densitometer traces of U.V. photographs of 3-5 μ g aliquots of the following DNAs banded in CsCl: (a) native, (b) low Cot, and (c) DNA which renatured at Cot = .010 and was collected by HA chromatography.

Fig. 18. Hybridization of HMW rRNA to buoyant density fractions of low Cot pinto bean DNA.

Filters embedded with the DNA of fractions of a preparative CsCl gradient of 155 μ g low Cot DNA were batch hybridized to pumpkin rRNA. —, A_{260} nm of the collected fractions; 0 0 0, cpm hybridized. The slope of this gradient is shown with respect to the r component (1.706 g/cc) which is used as a marker.

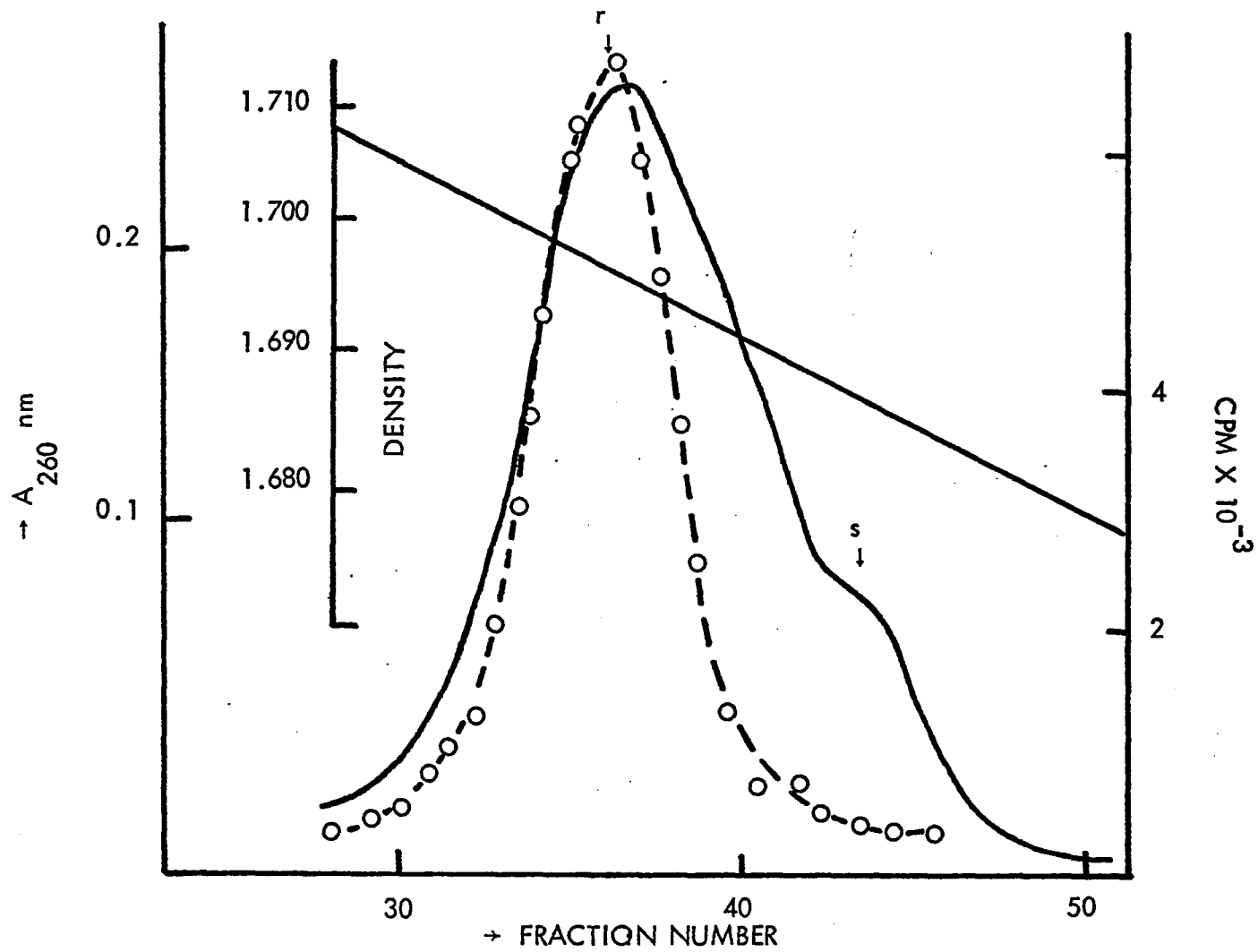


Fig. 18. Hybridization of HMW rRNA to buoyant density fractions .

An attempt was made to eliminate one or more of these components from low Cot DNA by lowering the Cot of renaturation. This should favor the renaturation of the components containing the most repetitious sequences. Fig. 17c shows that if the Cot of renaturation is lowered, the ratio of the amount of the r to s component is increased. This contrasts with the result obtained for Chinese cabbage DNA. Thus it should be possible to isolate fractions enriched in the most rapidly renatured components of a particular species by a suitable selection of Cot.

A considerable proportion of the low Cot DNAs of pumpkin, Chinese cabbage and pinto bean consists of renatured HMW rDNA. It was decided to analyze the DNA of a species which is symmetrically distributed in CsCl and also has fewer copies of HMW rDNA. Since tobacco DNA has these characteristics, low Cot tobacco DNA was prepared to see if a renatured rDNA component could be identified and whether this species also contained additional components.

Characterization of Low Cot Tobacco DNA

The profile in CsCl of low Cot tobacco DNA is shown in Fig. 19c. Two rapidly renatured tobacco components are identified. These have buoyant densities of 1.706 g/cc (r) and 1.697 g/cc (m). DNAs with these buoyant densities are also apparent in a sample of total DNA which was renatured to a higher Cot (66) and banded in CsCl (Fig. 1d). These buoyant densities suggest that r might originate from the dense side of native tobacco DNA (Fig. 19a) and that m is a part of the main band DNA. If this is so, then r component should contain HMW rDNA. The distribution of HMW rDNA in the preparative CsCl gradient of low

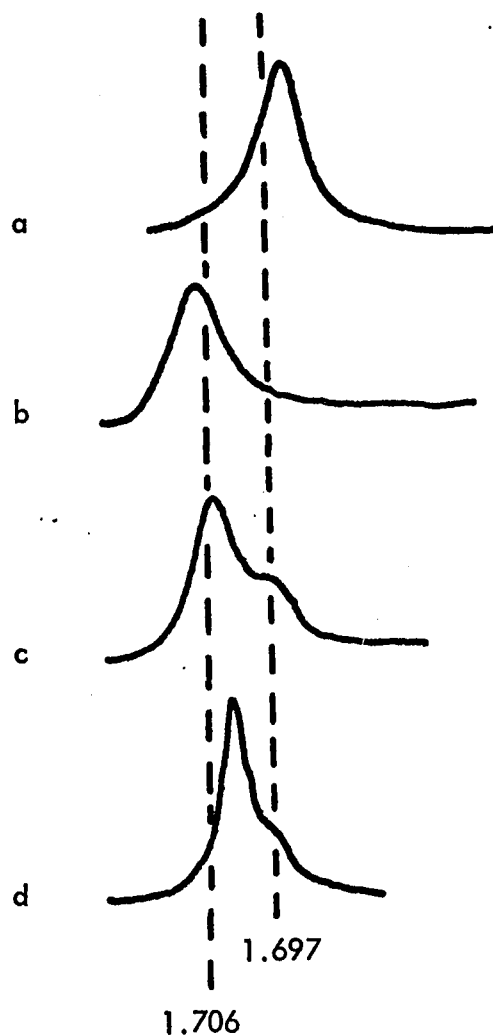


Fig. 19. Analytical CsCl centrifugation of native and renatured tobacco DNA.

Densitometer traces of U.V. photographs of 3-5 μg aliquots of the following DNAs banded in CsCl: (a) native, (b) heat denatured, (c) low Cot , and (d) DNA renatured to $Cot = 66$ and quick cooled.

Cot tobacco DNA is shown in Fig. 20. The r component does contain HMW rDNA distributed to the dense side, but must contain other renatured DNAs as well.

These analyses of low Cot DNAs have revealed that more than one rapidly renatured component is contained in the DNA of each species of plant examined. A renatured component, which contains HMW rDNA and therefore represents at least a portion of the high buoyant density fractions of native DNA, was identified in the low Cot DNAs of each species (r components). This confirms that plant HMW rDNA represents families of highly repetitive base sequences.

A considerable amount of renatured DNA, which contains highly repetitive sequences other than HMW rDNA, is represented in s and m components. The buoyant densities and abundance (relative to r) of these components is unique to the species of DNA examined and the Cot of renaturation.

The technique used here consisted of banding the rapidly renatured DNAs in CsCl. This technique permitted the detection of minor plant DNA components which were heretofore unnoticed. By this method, it should be possible to isolate such components as the pumpkin m component to determine its biological function.

Fig. 20. Hybridization of HMW rRNA to buoyant density fractions of low Cot tobacco DNA.

Filters embedded with the DNA of fractions of a preparative CsCl gradient of 75 μ g low Cot DNA were batch hybridized to pumpkin rRNA: —, A_{260} nm of the collected fractions; 0 0 0, cpm hybridized. The slope of the gradient is shown with respect to m component (1.697 g/cc).

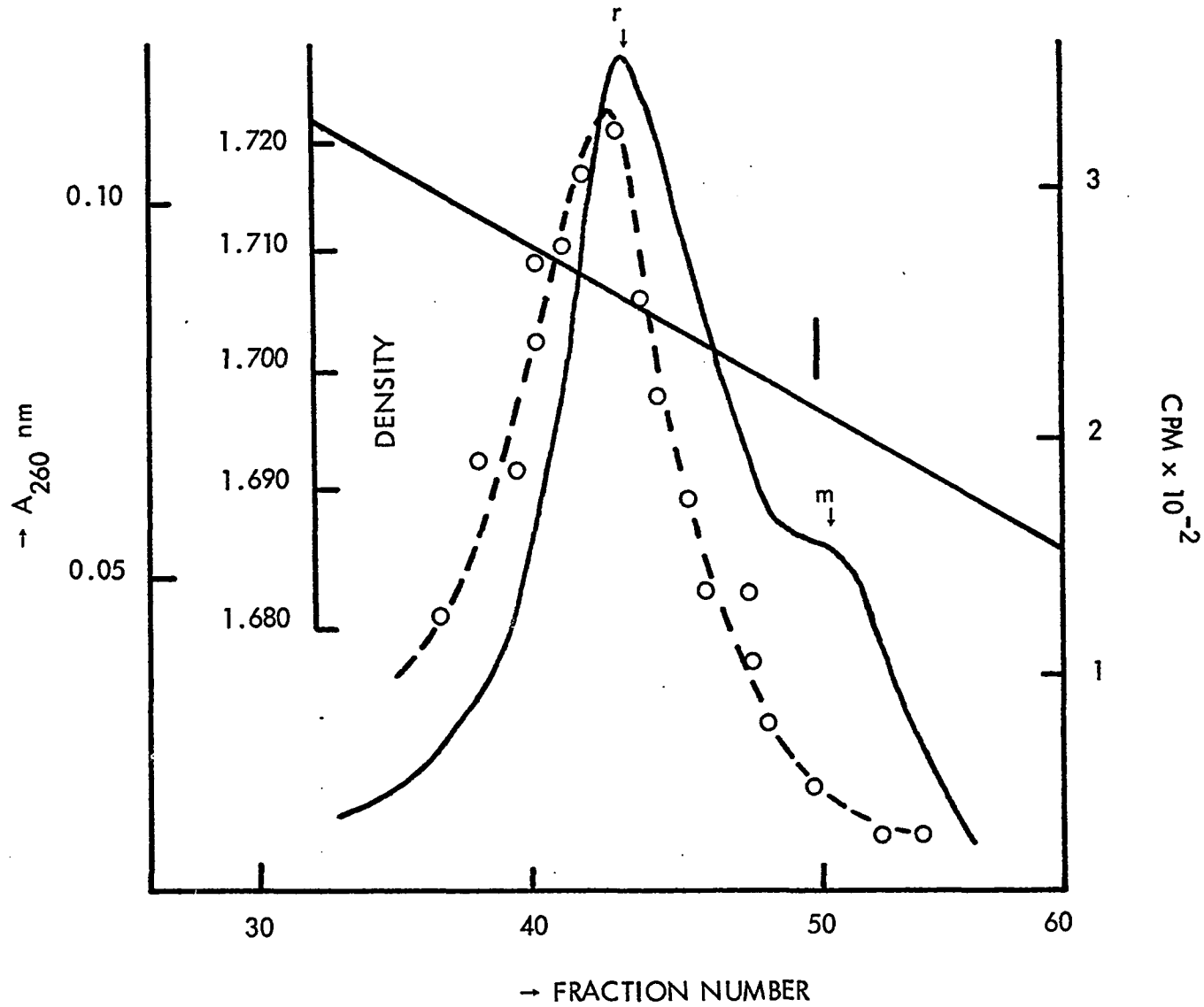


Fig. 20. Hybridization of HMW rRNA to buoyant density fractions

DISCUSSION

Degree of Repetition of HMW rDNA, 4S and 5S DNA

The accuracy of the determination of the absolute numbers of copies of HMW rDNA, 4S and 5S DNA depends upon the precise knowledge of the genomic DNA content and calibration of the conditions of the hybridization reaction. The experiments described here were designed to provide a reasonable estimate of only the ratios of the number of these sequences. Since the saturation experiments utilized a single preparation of pumpkin DNA, the differences which might be encountered due to selective loss or enrichment of these sequences from one preparation to another are minimized. The accuracy of the ratios obtained is also governed by the degree of purity of the RNAs used in the hybridization experiments and the satisfaction of several criteria which show the specificity of the reactions with each of the RNAs. These were each considered in the Results section. It would be of interest to determine if the 1:1.2:2.5 ratio is constant for a variety of plant DNAs which may differ by as much as forty fold in their content of HMW rDNA (Ingle and Sinclair, 1972).

The number of copies of 5S DNA is approximately equal to the number of units of HMW rDNA. Since pumpkin nuclear DNA contains sequences complementary to chloroplast rRNAs as well as cytoplasmic rRNAs (Matsuda *et al.*, 1970), each HMW rDNA unit is assumed to consist of a combination of 16+23S and 18+25S cistrons. Recently it has been shown that plants contain 5S RNAs in both

chloroplastic and cytoplasmic ribosomes (Payne and Dyer, 1971a). If there were one chloroplastic 5S DNA sequence associated with each 16+23S cistron and one cytoplasmic 5S DNA sequence for each 18+25S cistron, which is the arrangement which would be required if the transcription of these RNAs were controlled by their linkage in a single transcription unit, then one would find close to twice the total number of copies of 5S DNA as HMW rDNA. The fact that fewer copies than this are found suggests either that there are fewer numbers of copies of cytoplasmic or chloroplastic 5S genes than their high molecular weight counterparts, or that there are few or no sequences of chloroplastic 5S DNA in the nucleus. It might be possible to distinguish between these possibilities by hybridization experiments with the purified cytoplasmic and chloroplastic 5S RNAs.

Linkage of Pumpkin 5S DNA

In pumpkin, it was shown that 5S DNA and HMW rDNA are both contained in the dense satellite DNA. However, there are two ways in which the 5S DNA sequences may be arranged. Fig. 21 schematically depicts that (a) 5S sequences may be linked with 18 (16) S and 23 (25) S sequences or (b) may be clustered in tandem but not intermingled with the larger rDNAs. Linkage sequences marked c are non rDNA or compensation sequences, which must have a G+C content less than 5S DNA, (52% for bean; Payne and Dyer, 1971b) or HMW rDNA (52.5% for pea; Rogers, Loening and Fraser, 1970) and must be included for these sequences to band at the position of satellite DNA; which, based upon its buoyant density, would have a G+C content of only 49%. The compensation sequences would be analogous to

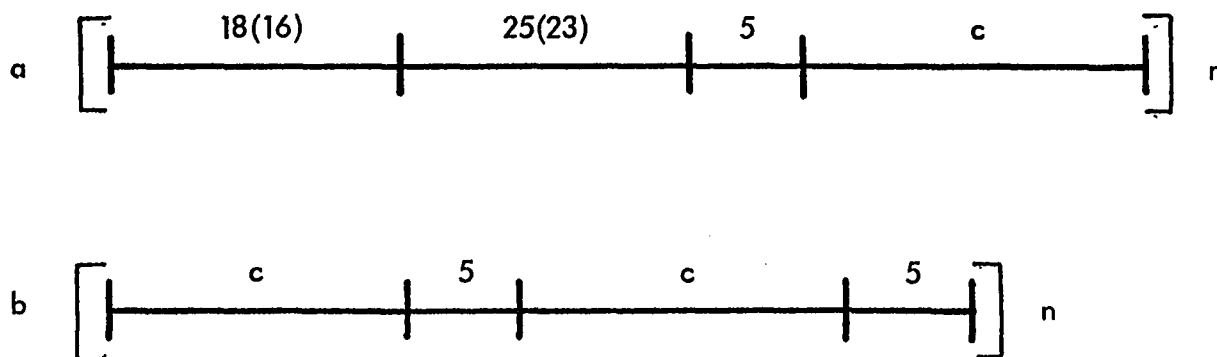


Fig. 21. Schematic illustration of two modes of linkage of 5S DNA.

(a) 5S DNA linked with HMW rDNA and spacer sequences and (b) 5S DNA alternating with spacer sequences but not linked to HMW rDNA.

the "spacer" sequences found in *Xenopus* to be linked with the high molecular weight rDNA (Brown and Weber, 1968b; Birnstiel *et al.*, 1968) and 5S DNA (Brown, Wensink and Jordan, 1971). These c sequences were detected as the rapidly renatured component resulting from low molecular weight DNA, the buoyant density of which was intermediate between that of the r and m components. (See Fig. 6f and Fig. 8).

If the first model is correct, then one is tempted to suggest a role of linkage of these sequences in the control of transcription of the rRNA. In *E. coli* (Doolittle and Pace, 1971) and *B. Subtilis* (Hecht, Bleyman and Woese, 1968) the high and low molecular weight rRNAs are believed to be transcribed as one

polycistronic precursor rRNA which is subsequently matured by selective cleavage to produce the 16, 23, and 5S RNAs. This would imply that the evolution of the control of the biosynthesis of rRNA in pumpkin followed more closely that of bacteria than animals.

If the second model is correct, then the present result would be analogous to that in *Xenopus* DNA, in which the 5S RNA cistrons are repeated in an alternating fashion with "spacer" DNA of very low G+C content. However, in pumpkin, the G+C content of the compensation sequences could be satisfied by those within the range available from the bulk of the DNA (20 - 40%).

To distinguish between these two models it would be necessary to conduct the type of analysis used for *Xenopus* (Brown and Weber, 1968b; Birnstiel et al., 1968) and *B. Subtilis* (Colli and Oishi, 1969) DNAs to determine the extent of linkage of the HMW rDNA to 5S DNA. It should also be possible to isolate the ribosomal precursor RNA (Rogers, Loening and Fraser, 1970) to determine by DNA-RNA hybridization experiments, whether 5S RNA sequences are included in this transcript.

Function of Renatured Components of Low Cot DNA

It was expected that the dense rDNA containing fractions of each species would renature under the conditions employed. The rDNA containing renatured components (r) therefore form a part of the rapidly renatured DNA of these species. To this component, we may assign, at least in part, the gene function for the production of the HMW rRNAs. In addition, the r component of pumpkin also

contains the genes for 5S RNA and perhaps a few for 4S RNA. Also included in this component are the presumed compensation (c) sequences which can be resolved by shearing the DNA prior to renaturation.

However, no functional assignment can be made for the s or m components. A number of rapidly renatured DNAs have been observed from a wide variety of other organisms (Bond et al., 1967; Maio, 1971; Corneo, Ginelli and Polli, 1970 and Votavona, Sponar and Sormova, 1970). Similar to the results obtained with plant DNAs, these may be derived from satellite fractions or portions of main band DNA. It is interesting to note that while the buoyant density of r components is nearly constant among the species of plants examined, which represent separate phylogenetic orders, the buoyant densities and abundance (relative to r) of the remaining renatured components is uniquely characteristic of the species. This lack of relatedness might have been expected from the observation of Hennig and Walker (1970) that the rapidly renatured satellite DNAs of closely related species of two families of rodents differed in buoyant density and abundance.

Specific rapidly renatured fractions of animal DNAs have been shown to be localized in the heterochromatin portion of chromosomes and especially concentrated at the region of centromeres (see Yunis and Yasmineh, 1971). Moreover, centromeric DNAs of mouse (Pardue and Gall, 1970) and Drosophila melanogaster (Botcham et al., 1971) represent the most repetitious DNA of each of these organisms. It is interesting to compare the properties of rapidly renatured plant DNAs with the centromeric DNAs of animals. Presumably, centromeric DNAs should serve some

function which is essential to the integrity of chromosomes; therefore analogous highly repetitive DNAs should also be contained in plants. The most likely candidate for this role is the pumpkin m component. Its renaturation characteristics and homogeneity in CsCl resemble centromeric DNA of other organisms. However, if centromeric DNA is the most repetitious DNA of an organism then it must be contained in the r component of pinto bean since r renatures more rapidly than s component of this species. It is also possible that centromeric DNAs, if contained at all in the low Cot DNAs of these plants, might be masked by the more prominent s and m components.

It will be necessary to transcribe the renatured plant components into RNA and determine if these represent centromeric DNA by the "in situ" hybridization experiments (Gall and Pardue, 1969). Also, as techniques become available for the isolation of other RNAs of defined function, such as messenger or regulatory RNA, it would be worthwhile to see if the renatured DNA components contain multiple genes for these sequences.

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