NUCLEAR DNA AND CYTOPLASMIC DNA FROM TISSUES OF HIGHER PLANTS

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ABSTRACT

Young wheat roots were labeled with ^{32}P-inorganic phosphate. Following the labeling period, roots were homogenized in a sucrose medium and fractionated into nuclei, cytoplasmic particles (including proplastids and mitochondria), and a soluble fraction containing most of the microsomes. DNA prepared from the particles had a higher buoyant density than that from the nuclei and showed a marked loss in total label if the roots were exposed to non-radioactive medium for 48 hours prior to fractionation of the cells.

INTRODUCTION

Various reports are current in the literature concerning the existence of a DNA fraction in cells which is distinct from that commonly associated with chromosomes. DNA fibrils have been detected in chloroplasts and mitochondria by electron microscopy (10, 12). Under certain deficient nutritional conditions, cytoplasmic DNA has been observed in protozoa (14). Of all the presumed non-chromosomal forms of DNA, that of the chloroplasts has been most extensively studied. Chloroplast DNA and nuclear DNA have been shown to be separable by equilibrium density centrifugations using CsCl solutions as solvent (1, 6). And, direct determinations of DNA content have been performed in the chloroplasts of *Acetabularia* (2). The presence of DNA in one or more types of cytoplasmic structure seems to be reasonably well established.

Although one may extrapolate from the demonstrated genetic role of chromosomal DNA and interpret the presence of DNA in cytoplasmic structures as providing a physical basis for their genetic continuity, such a sweeping interpretation should remain open to question. A few reports point to the presence in plant tissue of a form of DNA with physiological properties different from those characterizing chromosomal DNA. In growing roots of wheat and corn, this type of DNA has been isolated as a product of relatively low molecular weight manifesting a high degree of turnover (13). The evidence against conservation of such DNA negates the conclusion that the function of all forms of naturally occurring DNA function is identical with that of DNA constituting the genetic moiety of the chromosome. The observation in *Chlorella* that a fraction of DNA rich in GC (guanine-cytosine) labels rapidly under illumination is at least consistent with the observations made on roots (4). This report is addressed to the question of whether the metabolically labile DNA found in roots is cytoplasmic in origin.

MATERIALS AND METHODS

PREPARATION OF DNA: For preparation of DNA from unfractionated tissues, fresh roots were cut into small lengths (about 1 cm) and dropped into 95 per cent ethanol previously cooled to at least -10°C. A blending device was used for partially disintegrating the tissue, but no attempt was made

to fragment the cells. The alcoholic suspension was centrifuged and the residue washed several times with more ethanol. Roots thus prepared were either used immediately or stored under ethanol at −20°C.

A solution of 5 per cent (w/v) sodium lauryl sulfate containing 0.5 M sodium citrate and 0.05 M Tris buffer was adjusted to a pH of 7.4 and heated to approximately 70°C. The tissue, which was freed of most of the alcohol by filtration, was plunged into five times its volume of detergent solution with rapid mixing. The temperature of the solution fell quickly and was subsequently maintained at 60°C. After standing for 10 minutes, the suspension was filtered through nylon fabric (35-micron mesh). The filtrate was retained for use as extractant. The residue was transferred to a preheated mortar and ground with the aid of sand. Grinding was continued until few intact cells could be seen under the microscope. Hot extractant was added to the thick slurry and the suspension centrifuged at room temperature. The residue was re-extracted at 60°C for 10 minutes, and the extracts combined. Two volumes of 95 per cent ethanol were added to the extracts and the precipitate collected by centrifugation. Although fibers may be spooled at this stage, the practice was not followed because the recovery of DNA in such fibers is variable. Except for embryonic tissues, the comparatively large amounts of materials extracted by the detergent solution interfere with the spooling of all the DNA present in the extract.

The precipitate was suspended in a minimal volume of 0.15 M NaCl containing 0.015 M sodium citrate, 0.05 M Tris buffer, 10 per cent (v/v) ethanol and 0.25 mg/ml of Pronase (11). A stock solution of the protease, Pronase, was prepared as previously described (3) and the suspension containing the DNA was incubated in the presence of the enzyme for 2 to 4 hours at 55–60°C. After incubation, the solution was made 2.5 M with respect to NaCl, and deproteinized with chloroform: isoamyl alcohol (24:1 by volume). Two volumes of ethanol were layered over the aqueous solution and the DNA fibers were spooled on a glass rod. The fibers were redissolved and treated with RNase according to the procedure of Marmur (8). Subsequent steps were those in Marmur's procedure except for the use of ethanol in the final fiber collection. However, isopropanol was found to be equally effective in this last step.

Additional purification was achieved by dissolving the DNA in 0.15 M NaCl–0.015 M sodium citrate to a concentration of 0.2 to 0.5 mg/ml and passing the solution through a column of G-50 Sephadex. The saline-citrate solution was used to wash the column (1 to 2.5 × 30 cm). DNA appeared in the effluent immediately after the void volume and was collected in 2 to 3 times the original volume of solution. Colored material, which is generally present in whole root preparation, elutes after the DNA. The eluted DNA was precipitated with alcohol and collected as fibers. If the concentration in the eluate was too low for fiber collection, the solution was placed in a dialyzing bag which was then covered with dry Sephadex to remove water.

The yield of such preparations was 80 ± 8 per cent of the original DNA content. The latter was determined on a sample of the alcohol-washed residue prior to extraction with sodium lauryl sulfate solution. The residue was fractionated by the Schmidt-Thannhauser procedure and the DNA determined by the diphenylamine reaction (13). Since, frequently, interfering materials are present in crude extracts of roots which yield spurious diphenylamine values, the RNA-free residue was also treated with DNase and the DNA was determined by the absorbance at 260 mμ and the diphenylamine reaction. In the case of "Pawnee" variety, unlike other varieties of wheat tested, the two methods yielded the same values.

CELL FRACTIONATION: Wheat seeds (var. Pawnee) were germinated on screens and the labeling experiments were conducted as previously described (13). At the conclusion of the labeling period, roots were chopped with scissors and ground with sand in a mortar. The suspension medium was 0.8 M sucrose containing 0.001 M of each of the chlorides of magnesium, calcium, and manganese. After completion of grinding, the suspension was filtered through cheesecloth and then through a 35-μ nylon mesh. The filtered suspension was layered in 40-ml centrifuge tubes which could be accommodated in the swinging bucket rotor (H-4) of a Servall centrifuge. The tubes were prepared in the following way: 10-ml portions of 2.0, 1.5, and 1.0 M sucrose with cation supplement were successively layered in the tube. The 1.0 M layer was then overlaid with the tissue suspension and the tubes centrifuged for 60 minutes at 8,000 RPM. At the conclusion of the run, three zones were found in the centrifuge tubes—an upper turbid layer which under the microscope contains very few, if any, visible particles, a concentration of particles at the interface between the 1.0 and 1.5 M layers, a similar concentration between the 1.5 and 2.0 M layers, and a sediment. The latter contains most of the intact nuclei. The upper turbid layer was removed by a syringe and the remaining zones less the nuclear sediment were pooled. The suspension of particles was diluted by dropwise addition of 0.8 M sucrose to a concentration of approximately 1.2 M. The 1.2 M suspension was layered over 2.0 M sucrose, and the tubes centrifuged as above. The interfacial zone between the 1.2 M and 2.0 M layers contains the cytoplasmic particles and no intact nuclei. None of the particles stain

with aceto-orcein but, as will be seen, such observation does not necessarily mean the absence of very small nuclear fragments. The particles thus obtained were examined with the electron microscope. The nuclear fraction was purified by flotation in supersaturated sucrose solution as described earlier (5).

Standard analytical techniques for determining RNA and DNA were identical with those previously used (13).

RESULTS

When DNA prepared from whole roots was fractionated on a methylated albumin kieselguhr column (Fig. 1), two characteristics were noted which were not previously observed in preparations made with unheated solutions of sodium lauryl sulfate and without Pronase treatment (13). No DNA eluted at 0.4 to 0.45 M NaCl, the concentration range at which most of the "non-fibrous" metabolically labile DNA was previously found. On the other hand, the elution profile showed two peaks, one at approximately 0.5 M NaCl, the other at 0.6 M. Since the profile was reproducible with several DNA preparations, it was regarded as a characteristic of wheat root DNA. Whether the previously reported non-fibrous DNA represented a partial breakdown product of the material found in the first peak has not been satisfactorily established. The recovery of total DNA by the present method was about 80 per cent of the amount originally present. Some of the non-fibrous material might have therefore been lost by this method. However, chromatographic analysis of DNA prepared by the old procedure did not show the first peak but, instead, showed a gradual rise in optical density of the eluate beginning at about 0.4 M NaCl. Since, DNA with higher GC content would be expected to elute earlier (for the same molecular weight), it appeared probable that at least part of the high GC non-fibrous DNA represented a partial breakdown product of the material under the first peak. If so, the DNA prepared by the "improved" procedure should contain a metabolically labile fraction. We were interested in determining the cellular site of this fraction as well as ascertaining whether such a fraction was actually present.

DNA prepared by the same method from isolated nuclei did not show two peaks when analyzed chromatographically. The result suggested that

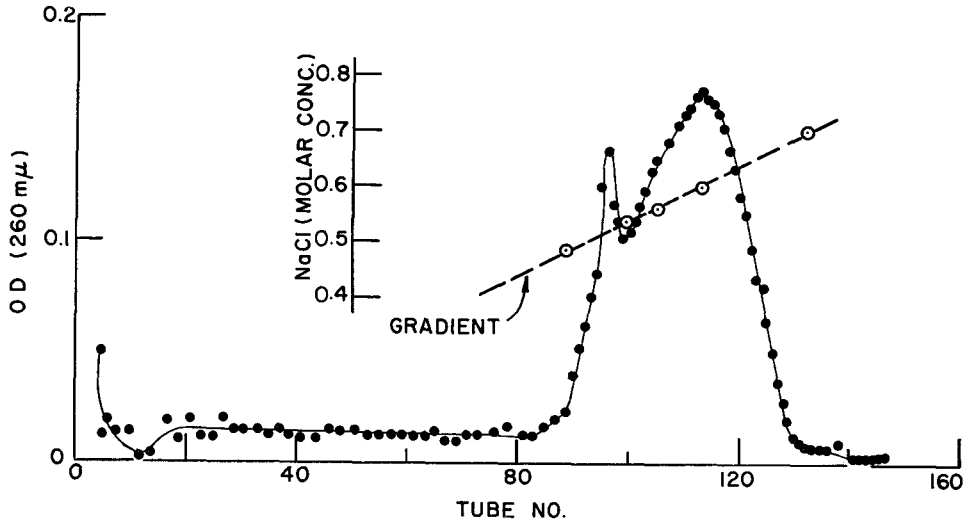

FIGURE 1 Elution profile of DNA prepared from 62-hour-old wheat seedlings. The DNA was dissolved in approximately 30 ml of buffered 0.1 M NaCl and adsorbed on a 10-ml methylated albumin column prepared according to Sueoka and Cheng (15). After washing with an equal volume of buffered saline, the material was eluted by a salt gradient generated from two cylinders containing 250 ml of buffered 0.1 M NaCl and 1.2 M NaCl, respectively. Two peaks are evident, the first at 0.52 M NaCl, the second at 0.6 M. DNA prepared from isolated nuclei does not show this first peak. Other physical properties of DNA prepared by the procedure used here have been described elsewhere (3).

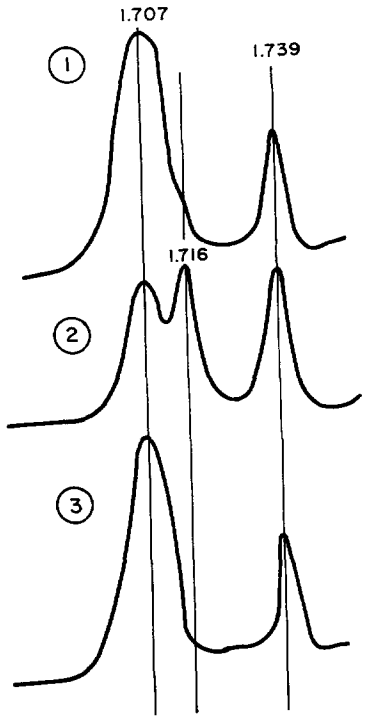

FIGURE 2 Densitometer tracings of DNA prepared from different fractions of root cells and centrifuged in CsCl according to the procedure of Meselson, Stahl, and Vinograd (9). *1*, Whole tissue; 2, Partially purified fraction of cytoplasmic particles; *3*, Nuclei. The numbers indicate mean density values in gm/cc., 1.707, 1.716, and 1.739. The reference band (1.739) is DNA prepared from *Pseudomonas aeruginosa* which had been grown on minimal medium with N^{15}-NH_6Cl as sole nitrogen source. The cytoplasmic fraction analyzed was the same as that used in the preparative procedure (Fig. 3).

the peak which was absent from the nuclear preparation was cytoplasmic in origin. We, therefore, analyzed the DNA prepared from whole tissue, isolated nuclei, and cytoplasmic particles by centrifugation in CsCl (Fig. 2). The results obtained were similar to those reported by others (1, 6), namely, that the minor component was much enriched in concentration in the cytoplasmic particulate fraction. Both peaks were abolished by treatment with DNase. Examination of the cytoplasmic fraction by electron microscopy revealed the following components: mitochondria, proplastids, and membranous material. Most of the membranous material from the tissue was, however, present in the "upper turbid layer" (See Methods). And, since no DNA was detected in this layer we conclude that the DNA present in the particulate fraction was then present in the mitochondria and/or proplastids, or in some other group of particles which sediment with the cytoplasmic organelles. Exposure of the particles to "osmotic shock" by rapid dilution of the sucrose medium resulted in a release of the DNA into the supernatant fluid. It would, therefore, appear that the particles in question are bounded by membranes. These studies permit us to state that a DNA fraction of higher buoyant density than the major component is found in the particulate cytoplasmic fraction of wheat roots, and is not found to a detectable degree in purified nuclei, microsomes, or the soluble fraction of the cells. On the other hand, these studies do not permit us to specify the particular cytoplasmic structure in which the DNA of higher density is found.

The principal question to which this report is addressed is whether the behavior of this cytoplasmic DNA is similar to or different from that of nuclear DNA. To answer this question, two groups of 4- to 5-day-old wheat seedlings were labeled with ^{32}P-inorganic phosphate for 4 hours. At the end of this period, the roots of one group were removed and fractionated as described under Methods. A second group was freed of external radioactive phosphate by washing the roots with 0.01 M phosphate and then by allowing the seedlings to grow for an additional 48 hours in nonradioactive inorganic phosphate. At the end of this period the roots were treated like those of the first group.

The results of this experiment are shown graphically in Fig. 3. After centrifuging the DNA preparations from the cytoplasmic fractions in a solution of CsCl, two peaks of DNA were obtained by stepwise collection of the centrifuged samples. The presence of two peaks has already been noted in one of the preparations analyzed by equilibrium density centrifugation (Fig. 2, *2*). Two types of tests were made to determine whether the fraction of higher buoyant density was a product of denaturation. On treatment with heat or alkali, the optical density of the DNA from the nuclear fraction increased by 37 per cent; that of the mixed DNA in the cytoplasmic fraction increased by 39 per cent. No changes in optical density were noted on heating to 70°C. Virtually all of the DNA (97 to 99 per cent) from

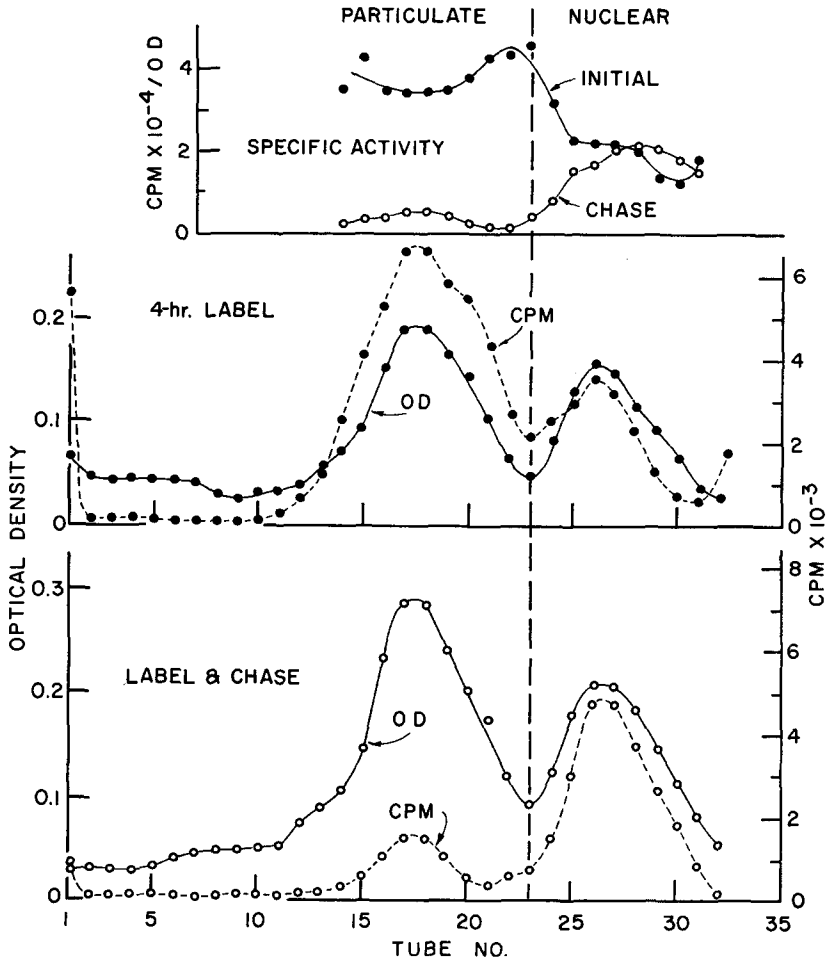

FIGURE 3 Changes in ^{32}P labeling pattern of DNA derived from cytoplasmic particles and nuclei. Four-day-old wheat seedlings were exposed to 5 μc/ml of carrier-free ^{32}P in standard Hoagland's solution less phosphate component. At the end of 4 hours, one group was removed for analysis and the second group was washed twice with Hoagland's solution containing 100 X normal phosphate concentration. This second group was removed for analysis after growing for an additional 48 hours in standard Hoagland's solution. Root particles were isolated as described under Methods and the DNA prepared from these was centrifuged in a solution of CsCl (density = 1.715 gm/cc) at 30,000 RPM for 10 hours followed by 20,000 RPM for 80 hours. A SW 39 Spinco rotor was used, the temperature during the run being 22°C. Fractions were removed by piercing the bottom of the tube and collecting the drops. Sufficient nuclear DNA contaminates the preparation (see text) to permit a comparison of the two types using the particulate fraction alone. Each collecting tube contained 0.1 ml; the total volume in each of the tubes centrifuged was 5.0 ml with a layer of about 0.5 ml of paraffin oil at the top. Only the first 32 collecting tubes are shown in the figure, as the remaining 18 tubes (toward the lighter end of the gradient) had negligible radioactivity and no appreciable absorbance at 260 mμ.

either sample passed through a membrane filter (Bac-T-Flex membrane filter B-6, size 27 mm, produced by Carl Schleicher & Schuel Co., New York City) as determined by the optical density of the filtrate and the residual ^{32}P counts in the filter disc. On heating replicates of the samples at 100°C for 20 minutes followed by quick cooling, 94 per cent of the DNA from the nuclear fraction and 90

per cent of that from the cytoplasmic fraction were retained by the filter. We, therefore, concluded that the fraction of higher buoyant density which constituted about half of the DNA in the cytoplasmic preparation consisted mainly of double-stranded DNA. It is this fraction which analytical studies have shown to be absent from isolated nuclei (Fig. 2).

Each of the preparations shown in Fig. 3 appears to contain a mixture of cytoplasmic DNA and nuclear DNA. The behavior of what we presume to be the nuclear component is identical with that observed on comparing nuclear fractions from labeled seedlings with nuclear fractions from labeled and "chased" seedlings. We have, therefore, omitted the set of curves for DNA from purified nuclei. The nuclear component in the cytoplasmic fraction may be taken to reflect accurately the behavior of nuclear DNA. The total DNA increase in wheat roots over a period of 48 hours is about 5 per cent. Since the extent of initial labeling would vary within that range from one batch of plants to another, the absence of any marked change in specific activity of the nuclear DNA is entirely consistent with the assumption of DNA conservation. This, however, is not the case with the DNA of higher buoyant density found in the cytoplasmic fraction. One may observe that the total counts in the DNA fraction have decreased markedly, following the chase. This decrease cannot be explained by a gross difference in yield, since the recoveries as shown by the optical density plots are similar for both batches. The simplest conclusion one may draw is that label has been lost and that consequently a marked reduction has occurred in specific activity following the chase.

DISCUSSION

The apparent absence of DNA conservation in the particulate components of root cytoplasm is puzzling because of the formidable evidence for genetic continuity in chloroplasts and mitochondria (6, 7) and because of the evidence from electron microscope studies that both these organelles contain DNA. A number of possibilities exist to explain this seeming contradiction. The labile DNA may not be identical with the DNA identified in chloroplasts or mitochondria. Or, one may suppose that, in the maturing cells of roots, the DNA in these organelles is undergoing both replication and degradation. Alternatively, one may take the unorthodox position that the original template for this DNA is found in the chromosomes, but that copies are made in the cytoplasmic particles. These experiments do not discriminate between these possibilities, but they do point to the existence of a form of DNA which occurs in cytoplasmic particles and which has distinctive physiological characteristics.

This work was supported by a grant from the National Science Foundation (GB 1381).

Received for publication, September 16, 1964.

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