Characterization of Deoxyribonucleic Acid Species from Castor Bean Endosperm

INABILITY TO DETECT A UNIQUE DEOXYRIBONUCLEIC ACID SPECIES ASSOCIATED WITH GLYOXYSONES

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ABSTRACT

Three DNA buoyant density species (nuclear, 1.692 g cm⁻³; mitochondrial, 1.705 g cm⁻³; and plastid, 1.713 g cm⁻³) can be detected in extracts from castor bean endosperm. No other buoyant density species can be identified. DNA extracts from sucrose density gradient purified glyoxysomes exhibit varying amounts of each of the three identified DNAs but no other distinguishable DNA species. RNA synthesized in vitro by Escherichia coli RNA polymerase using purified castor bean nuclear DNA as a template, hybridizes equally well with its template and with the 1.692 g cm⁻³ species from glyoxysome fractions. These results are discussed in terms of their relevance to microbody biogenesis.

It is well established that, in addition to the DNA species resident in the nucleus of higher plants, there are DNA components which are characteristic of their mitochondria and chloroplasts. Furthermore there is considerable evidence that these DNAs serve some role in the biogenesis of these organelles.

Glyoxysomes represent a third organelle found in some cells of higher plants and are members of a class of organelles generally termed microbodies. Microbodies are widely found in both plant and animal cells, and it is therefore of interest to know if they, too, have an associated DNA species involved in their biogenesis.

Ching (7) has claimed that glyoxysomes from gametophytes of Pinus sp. are capable of in vitro protein synthesis and that they contain a DNA component of approximately the same buoyant density as the nuclear DNA (personal communication). Recently, Clarke-Walker (9) has isolated a circular DNA from yeast mitochondrial preparations with properties distinct from those normally described for mitochondrial DNA. He suggested that this DNA species, with a buoyant density identical to that of yeast nuclear DNA, might be associated with peroxisomes, the microbodies described in yeast (25). No evidence was presented to confirm this postulation, however, as neither enzymatic markers nor cytologic characteristics of the particles used for isolation of this DNA were studied. Identification of these particles as peroxisomes, based solely upon sedimentation characteristics, has been shown to be unsatisfactory (16). However, since these reports suggest that microbodies (glyoxysomes or peroxisomes) may contain information contributing to their organization and biogenesis, we have investigated further the question of DNA associated with glyoxysomes.

The studies reported here describe the fractionation of subcellular organelles from castor bean endosperm tissue in which a clearly identifiable glyoxysomal preparation can be characterized (3). DNA species associated with the nucleus, mitochondrion and plastid from the castor bean endosperm are clearly evident. Data are presented, however, that there is no unique DNA species associated with and characteristic of castor bean glyoxysomes.

MATERIALS AND METHODS

Castor bean mitochondria, plastids, and glyoxysomes were purified in large quantities by procedures to be detailed elsewhere (D. Branton, P. DeSilva and R. W. Breidenbach, in preparation) based on a modification of the procedure of Breidenbach et al. (4). Endosperm tissue, weighing 1 to 2 kg, from 5-day-old castor beans was homogenized gently in a tissue slicing homogenizer designed and built by Branton and DeSilva and kindly loaned to us. Differential centrifugation of the homogenate was carried out as soon after homogenization as possible by simultaneous use of four refrigerated RC2B Sorvall centrifuges with GSA rotors. The low speed pellet obtained after centrifugation at 2500g for 10 min, containing cell walls, debris, nuclei, and plastids, was directly extracted to obtain DNA by the method of Bernardi et al. (2). The nuclear DNA in this fraction was separated from organelle DNA by hydroxyapatite column chromatography, repetitively chromatographing six times with linear sodium phosphate gradients (2). The high speed pellet (high speed pellet; 10,000g for 15 min) containing mostly mitochondria, glyoxysomes, some plastids and minor contaminants, was fractionated on a complex sucrose gradient in a Beckman Ti15 zonal rotor. The gradient consisted of the following sequence of sucrose solutions (0.01 M in Tricine, pH 7.5, 1 mM in EDTA) pumped into the rotor in order: 500 ml of linear gradient from 30 to 38%. 100 ml 38%, 250 ml 43%, 250 ml 48%, 250 ml 55%, and
the volume of 60% necessary to completely fill the rotor. The high speed pellet suspension in 30% sucrose was then loaded, and the rotor was accelerated and run at 35,000 rpm for 4 hr. Approximately 8 hr were required to obtain the organelle fractions once homogenization of the endosperm material was initiated. The purified organelle fractions were then extracted to obtain DNA by one of two methods. The first was the aforementioned method of Bernardi et al. (2) through the step prior to column chromatography. A second and generally more suitable method involved direct lysis of the organelles by addition of CsCl to a suspension of the organelles.

The lysates were spun at 20,000 rpm in a Sorvall SS-34 rotor for 10 min, and the protein layer was discarded. The liquid was adjusted to 1.7 g cm\(^{-3}\) and centrifuged for 48 hr in either a Spinco Ti 65 fixed angle rotor operated at 55,000 rpm (Fig. 2a) or in a Spinco SW 56 rotor operated at 40,000 rpm (preparation 2 in Table 1 and Fig. 2b). The bottoms of the centrifuge tubes were punctured and 3-5 drops (Fig. 2a) or 1 drop (Fig. 2b) fractions were collected. The A\(_{260}\) of the 5-drop fractions was measured directly, and the A\(_{260}\) of the 1-drop fractions was measured after addition of 0.2 ml of water to each fraction. Concentrations of protein and DNA in the various organelle fractions were measured by the methods of Lowry et al. (18) and Burton (6), respectively, before the DNA was extracted.

**Enzymic Analysis of Specific Enzyme Markers in Organelle Fractions.** Identification of specific organelles isolated by sucrose gradient centrifugation was made by assay of the following marker enzymes: glyoxylose, isocitrate lyase (11); plastochnid, aldolase assayed by the enzymic method described by Taylor (27) using fructose diphosphate in 0.5 M Tricine buffer, pH 7.6, and glyceraldehyde-3-phosphate dehydrogenase obtained from Calbiochem lot 1003070; mitochondria, succinic acid oxidase assayed polarographically by the procedure of Raison et al. (22).

**Analytical CsCl Density Gradient Centrifugation.** The methods described by Schildkraut et al. (23) and Szybalski (26) were followed for CsCl density gradient centrifugation. A saturated stock solution of CsCl was made with 10 mM tris, pH 8.5. Stock solutions of CsCl, DNA sample, *Micrococcus lysodeikticus* DNA, and H\(_2\)O were mixed to achieve a final density of 1.70 g cm\(^{-3}\) in all runs. Two Beckman Model E ultracentrifuges were used, one equipped with the Beckman monochromatic light source and scanner and the other with photographic absorption optical system. For the photographs, Kodak commercial film was exposed for 4 min at 265 nm and developed for 4 min in D-19 developer at 22 C. With this latter system we could detect as little as 0.1 \(\mu\)g of DNA in a 1-ml sample using 12-mm path length cells. The cells were loaded with 1 to 40 \(\mu\)g of DNA.

Cesium chloride, code 62, was obtained from the Rare Earth Division of American Potash and Chemical Corp., West Chicago, Ill.

*Micrococcus lysodeikticus* DNA was obtained from Miles Laboratories, Kankakee, Ill. This was dissolved in SSC\(^2\) to make a stock solution of 100 \(\mu\)g/ml which was used without further treatment.

**Transcription of DNA to Prepare RNA and Isolation of RNA Products.** Transcription was carried out as described by Cohen et al. (10) except that the reaction mixture of Tsai et al. (28) was used. The reaction mixtures were incubated for 120 min at 37 C with *Escherichia coli* DNA-dependent RNA polymerase. *E. coli* RNA polymerase was prepared by the method of Burgess (5).

**Preparation of DNA-containing Filters and Hybridization Studies.** Hybridization of RNA to denatured DNA was carried out using the filter technique of Gillespie and Spiegelman (15) as modified by Church and McCarthy (8) and Michaelis et al. (19). Membrane filters (50 or 25 mm in diameter) were loaded with sufficient heat-denatured DNA, that 6-mm filters, containing 5 \(\mu\)g of DNA each, could be punched from the large filter. The retention of DNA on each filter was monitored by the method of Burton (6) after each hybridization study. In a typical hybridization experiment, a small vial containing: 0.20 ml of formamide, 0.10 ml of 20 \(\times\) SSC, 0.09 ml of H\(_2\)O, 0.010 ml of \(^{14}\)H-RNA (20,000 cpm) and up to six 6-mm filters. The mixture was covered with 0.2 ml of paraffin oil and was incubated 24 hr at 37 C. After the hybridization, filters were washed twice in 5 ml of 5 \(\times\) SSC which was 50% formamide (at 37 C) and twice in 5 ml of 5 \(\times\) SSC (at 37 C). After drying in vacuum, each filter was counted in 10 ml of toluene scintillation fluid. Filters were then washed twice with toluene and dried in vacuum. Each filter was treated with 4 ml of 2 \(\times\) SSC and 0.020 ml of pancreatic RNAse solution (4 mg/ml) for 1 hr at room temperature, washed twice with 5 ml of 5 \(\times\) SSC, dried in vacuum and counted.

**RESULTS**

DNA species of three different buoyant densities are distinguishable in whole cell castor bean preparations. Identification of the major buoyant density species of DNA characteristic of the nuclei, mitochondria, and plastids was based upon the enrichment of a particular DNA species concurrent.

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\(^2\) SSC is 0.15 M NaCl plus 0.015 M Na Citrate buffer; 20 \(\times\) SSC or 5 \(\times\) SSC refer to the citrate-saline buffer at 20 or 5 times the above concentrations.

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**Fig. 1.** Typical distribution of protein and marker enzymes obtained by sucrose gradient buoyant density centrifugation. A suspension of high speed pellet centrifuged for 4 hr at 35,000 rpm in a Beckman Ti15 rotor.
Table 1. DNA Associated with Organelle Fractions Separated on Sucrose Gradients in the TiI5 Zonal Rotor

<table>
<thead>
<tr>
<th>Organelle Fraction</th>
<th>DNA Density²</th>
<th>µg/mg protein and/or % total DNA³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.692</td>
<td>1.705</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.25</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>31%</td>
<td>69%</td>
</tr>
<tr>
<td>Proplastid</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Nuclei</td>
<td>&gt;95%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Glyoxysome</td>
<td>Preparation 1</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>34%</td>
<td>1.5%</td>
</tr>
<tr>
<td></td>
<td>0.65</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>49%</td>
<td>26%</td>
</tr>
</tbody>
</table>

¹ Determined by equilibrium centrifugation in the analytical ultracentrifuge.
² Amounts of each buoyant density species as well as total DNA with fractions were determined by area measurements on the analytical ultracentrifuge using the marker DNA as an internal standard (except as noted in footnote 3). Total DNA was confirmed in some instances by the Burton method (6).
³ Amounts of DNA determined directly by the A₆₅₀.

with the purification of a particular organelle. Purified samples of the organelles studied were obtained from preparations separated in the zonal rotor. A typical separation as indicated by the distribution of protein and marker enzymes is shown in Figure 1.

The density of the DNAs found in the extracts from nuclear mitochondrial, proplastid, and glyoxysomal fractions obtained in this fashion are summarized in Table I. The preparation of DNA from an enriched nuclear fraction showed 95% of the DNA isolated to have the buoyant density of 1.692 g cm⁻³. Both nuclear and mitochondrial DNA are normally found in the mitochondrial fractions with their proportions varying somewhat from preparation to preparation. However, in each case the 1.705 g cm⁻³ species predominated. The proplastid fraction contained a single species of buoyant density 1.713 g cm⁻³. The glyoxysomal fractions contained varying amounts of all three buoyant density species but no DNA with buoyant density distinguishably different from the nuclear, mitochondrial, or proplastid DNA densities. Figure 2 shows the A₆₅₀ profile from two different preparative CsCl gradients of glyoxysome associated DNA illustrating the varying proportions of the different buoyant density species found in different glyoxysome preparations.

As a further test of the identities of the DNA found in the glyoxysomal fractions, labeled RNA was synthesized in vitro using RNA polymerase and highly purified nuclear DNA as a template. The labeled RNA was tested for complementarity to castor bean nuclear DNA and to the 1.692 g cm⁻³ species purified from glyoxysomal fractions. Yeast nuclear DNA was used as a control in these experiments to define a background level of binding to noncomplimentary DNA. Table II shows that

Table II. Hybridization of RNA Homologous to Castor Bean Nuclear DNA with DNA of the Same Density from Castor Bean Glyoxysomal Fractions

<table>
<thead>
<tr>
<th>DNA Type on Filter</th>
<th>cpm/Filter</th>
<th>DNA Specific Radioactivity³</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Yeast nuclear</td>
<td>40</td>
<td>0⁵</td>
</tr>
<tr>
<td>Castor bean nuclear</td>
<td>5197</td>
<td>1040³</td>
</tr>
<tr>
<td>Castor bean nuclear</td>
<td>3654</td>
<td></td>
</tr>
<tr>
<td>Glyoxysome (ρ = 1.692)</td>
<td>3568</td>
<td>1018⁴</td>
</tr>
<tr>
<td>Glyoxysome (ρ = 1.692)</td>
<td>4784</td>
<td></td>
</tr>
</tbody>
</table>

¹ Replicate filters were pooled for Burton DNA assays and the values divided into the sum of the cpm/filter of each type.
² 10 µg of yeast nuclear DNA.
³ 8.5 µg DNA/2 filters.
⁴ 8.2 µg DNA/2 filters.
the amount of RNA hybridized per microgram of the 1.692 g cm⁻³ species from the glyoxysome fractions was identical to the amount of RNA hybridized with the nuclear DNA that served as the template for its synthesis.

**DISCUSSION**

Our results clearly demonstrate that only three species of DNA with unique buoyant densities could be detected in castor bean endosperm. In each case one of these buoyant density species can be shown to be concentrated in fractions enriched with one of the three organelles well known to contain unique DNA species, i.e., nuclei, mitochondria, and proplastids. The values obtained for DNA densities agree well with those of Suyama and Bonner (24) who showed nuclear densities in the range of 1.688 to 1.691 g cm⁻³ for Phaseolus aureus, Brassica rapa, Ipomoea batatas, and Allium cepa. These same species also showed a buoyant density of 1.706 g cm⁻³ for mitochondrial DNA. On the other hand, the DNA found in fractions enriched with glyoxysomes does not contain any components with unique buoyant densities. Variable amounts of density species, indistinguishable from those identified with other organelles, are the only species observed in the glyoxysomal preparation, and no single buoyant density species predominates in all preparations. In contrast, our preparations of purified mitochondria and proplastids always contain a predominance of their characteristic buoyant density species with lesser and variable amounts of contaminating species. It should be noted that the DNA component suggested by other workers to be characteristic of glyoxysomes (peroxisomes) (T. M. Ching, personal communication; Clarke-Walker, [9]) was indistinguishable in density from the nuclear DNA. Furthermore, hybridization studies show that this component, when isolated from castor bean glyoxysomes, contains all of the base sequences transcribed in vitro from purified castor bean nuclear DNA since equal amounts of labeled RNA hybridized with both. From this evidence we are led to conclude that there is no DNA species uniquely associated with glyoxysomes and that all of the DNA found in glyoxysomes can most simply be ascribed to varying amounts of contamination of this fraction by other organelles or their DNAs. Nuclei of euakaryotic organisms typically contain 200 to 300 μg of DNA per mg of protein (12) so that very small numbers of nuclei contaminating the glyoxysomes are all that are necessary to give the amounts of DNA reported. In higher plants, proplastids are a serious contaminant of most preparations of glyoxysomes. Since they also contain relatively high amounts of DNA (2.5–5 μg/mg of protein) (17), they too make a significant contribution to the amount of DNA found in glyoxysome fractions.

One aspect of the report by Clarke-Walker (9) merits further consideration. The DNA species described was obtained as a closed-circular species from yeast preparations homogenized and centrifuged in the presence of ethidium bromide. This species had a buoyant density after removal of the dye that was characteristic of yeast nuclear DNA rather than mitochondrial DNA and could also be found in respiratory-deficient mutants which lacked mitochondrial DNA (neutral pettl). The author found only one particulate band sedimenting in a sucrose gradient and suggested that this material and the DNA contained in it could be from the peroxisomes described by Avers and Federman (1). However, such considerations must also include the possibility of contamination by other intact or fragmented organelles. Structures with characteristic mitochondrial enzyme markers can be readily isolated from neutral pettles (21) and would contribute to the particulate preparation. Moreover, we have shown that even purified preparations of glyoxysomes from castor bean are badly contaminated with nuclear, mitochondrial, and proplastid DNA. Thus, while the observation of a circular DNA with particulate association in yeast is interesting, the suggestion of a specific peroxisomal DNA based on the data presented seems unwarranted at this time.

Two other pieces of information must be discussed with respect to glyoxysome biogenesis. First, Ching (7) reported rapid incorporation of labeled amino acids into the glyoxysome fractions from Pinus sp. gametophytes. Second, both Ching (7) and Gerhardt and Bevers (14) found significant levels of RNA associated with glyoxysome preparations. These data could be explained by a model in which newly synthesized enzymatic proteins are synthesized on restricted regions of the endoplasmic reticulum which then invaginate to enclose them. In such a model, it is possible that RNA, and some capacity for continued protein synthesis, is retained. Electron micrographs frequently show association of microbodies with the rough endoplasmic reticulum further supporting such a model (13, 20, 29).

In any case, it appears that the reports of unique glyoxysomal DNA with a buoyant density identical to that of nuclear DNA are not substantiated. We have not, however, ruled out the possible existence of a unique DNA species with a buoyant density identical to either the mitochondrial or proplastid buoyant densities. Since the glyoxysome preparations do not exhibit an enrichment of either of these species, this possibility appears unlikely.

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**LITERATURE CITED**