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Visualization of a Spinach Plastid Transcriptionally Active DNA-Protein Complex in a Highly Condensed Structure

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ABSTRACT

A transcriptionally active DNA-protein complex isolated from spinach *Spinacia oleracea* plastids is visualized by electron microscopy in different conditions. This structure, after glutaraldehyde fixation, is highly condensed. DNA is supertwisted with proteins bound to it producing a beaded substructure. When glutaraldehyde fixation is omitted this structure is less condensed and DNA fibrils come out from a proteinous central body. The DNA-protein complex can be separated into two populations by CsCl centrifugation: one with a buoyant density of 1.570 grams per cubic centimeter and the other of 1.610 grams per cubic centimeter. By visualization of these two populations, it is concluded that proteins are either firmly bound to DNA in the central body, or more loosely bound to the DNA fibrils. These latter proteins could play a role in enzymic functions and/or in the supercoiling of DNA.

The DNA from the DNA-protein complex possesses all fragments that belong to pure circular chloroplast DNA hydrolyzed by two restriction enzymes: Bam HI and Eco RI. Some molecules observed in a supercondensed form with a beaded substructure probably contain entire chloroplast DNA molecules.

A hydrolysis test with micrococcal nuclease gives no indication of the presence of 'nucleosome-like' structures. Thirty-six polypeptides with molecular weights ranging from 12,000 to 180,000 are present in the complex, and seven of them are highly soluble in 0.4 N H_2SO_4; their molecular weights range from 14,000 to 46,000 as shown by two-dimensional gel electrophoresis.

No linolenic acid can be detected in the preparation, indicating the absence of chloroplast membranes.

The spinach plastid DNA (ctDNA[1]) is known to be a circular molecule of 95×10^6 daltons whose restriction map is established and on which several genes coding for rRNA, tRNA, ribulose-1,5-bisphosphate carboxylase and a thylakoid polypeptide of 32,000 daltons (photogene) are located (7, 12, 20).

The structure of a deproteinized spinach plastid chromosome has been visualized by Yoshida *et al.* (22) and by Herrmann *et al.* (11). It appears in a folded form organized around a central body composed of proteins not extracted by usual procedures. The structure of a transcriptionally active DNA-protein complex from plastids has not yet been observed. In *Escherichia coli*, Stonington and Pettijohn (19) have isolated and characterized such a structure which appears to be folded and organized in domains of supercoiling. This chromosome has been visualized by Delius and Worcel (4). More recently, Griffith (9) has shown that prokaryotic DNA can be visualized in a regularly condensed chromatin-like fiber on the basis of a 130 Å repeated beaded substructure. A transcriptionally active DNA-protein complex from plastids can be obtained as described by Hallick *et al.* (10) with *Euglena* chloroplasts and by Briat *et al.* (2) with spinach plastids. Such structures contain ctDNA, RNA polymerase, and other proteins (3) and preferentially transcribe rDNA (2, 3, 18).

This paper reports the visualization, using the method of Dubochet *et al.* (8), of the transcriptionally active DNA-protein complex from spinach plastids and the characterization of the different molecular components of such structures. The results show that the plastid chromosome is highly condensed and that a beaded substructure is observed.

MATERIALS AND METHODS

Purification of the Transcriptionally Active ctDNA Protein Complex. Chloroplasts were isolated from 1 to 2 kg of spinach leaves from a local market using a method described previously (3). The ctDNA-protein complex was purified according to Briat *et al.* (2).

Electron Microscopy. Pellets of ctDNA-protein complex were solubilized with 0.5 SSC (0.15 M NaCl/0.015 M trisodium citrate). Unfixed samples were diluted with 0.5 SSC to a final concentration of 0.5 to 1 μg ctDNA/ml. Other samples were fixed with 0.1% glutaraldhedyde for 1 h at room temperature and then diluted to 0.5 to 1 μg ctDNA/ml with sterile H_2O. Copper grids, covered with a carbon film, were charged with vapors of amylamine following the method of Dubochet *et al.* (8). Samples were spread on these grids, stained with 2% uranyl acetate, shadowed with platinium at an angle of 7°, and observed in a Siemens 101 electron microscope. Enlargement was calibrated with a carbon replica of 2,160 lines per mm.

CsCl Centrifugation. For CsCl centrifugation, the complex pellets were solubilized with 10 mM triethanolamine, pH 7.8, 1 mM DTT, 1 mM EDTA, 0.1 M $(NH_4)_2SO_4$, and fixed by incubation for 1 h at room temperature in presence of formaldehyde added to a final concentration of 0.35%.

ctDNA-protein complexes were banded in CsCl density gradients as follows: 300 μl of each sample were mixed in SW 50 tubes with a CsCl solution containing the medium described above adjusted to 0.3% formaldehyde. The final density was adjusted to 1.630 g/cm^3. After centrifugation at 34,000 rpm for 45 h at 20°C in a Beckman SW 50 rotor, gradients were collected and analyzed at 260 nm with an ISCO UA 5. The density of each fraction was determined by measuring its refractive index.

[1] Abbreviations: ctDNA, chloroplast DNA; SSC, sodium saline citrate; DEGS, diethylglycol succinate.

Protein Solubilization and Analysis. All proteins from the complex were solubilized and analyzed by a modification of a procedure described previously (3). Pellets of complex from 1 kg of leaves were solubilized in 100 μl of 0.125 M Tris-HCl, pH 6.8, 2% SDS, 1 mM EDTA, 10% 2-mercaptoethanol, and 20% glycerol and heated 2 min at 100°C. Samples were deposited on a 8 to 20% polyacrylamide-SDS gradient in the system of Laemmli (14). Electrophoresis was performed at 80 v for 16 h at room temperature.

Basic proteins from the complex were solubilized twice with 0.4 N H_2SO_4 for 45 min at 0°C. After centrifugation at 30,000*g* for 25 min, 5 volumes of cold ethanol were added to the supernatants. After 48 h at −20°C, the slurry was centrifuged and pellets were solubilized with 46 mM glycine, 6 M urea, 1% 2-mercaptoethanol, pH 4. The first dimension of electrophoresis was performed for 4 h at 100 v on 4% acrylamide gel in 6 M urea, 0.44 M acetic acid, pH 4.5. The electrophoresis buffer was 93 mM glycine, pH 4, and 0.02% 2-aminoethanethiol. The second dimension of electrophoresis was performed in 8 to 20% polyacrylamide-SDS gradient in the system of Laemmli (14) for 16 h at 40 mamp. Gels were stained with 0.1% Coomassie blue in 10% acetic acid-50% methanol and destained with 10% acetic acid-50% methanol.

ctDNA Content Analysis. The complex was digested for 6 h in a 30-μl final volume with 60 units, either of Bam HI or Eco RI (Miles) representing 10 times the number of units necessary to digest the same amount of purified DNA, and analyzed by 0.7% agarose gel electrophoresis in the following buffer: 0.02 M sodium acetate, 0.05 M Tris, 0.002 M EDTA, 0.018 M NaCl. Gel was run for 12 h at 80 v at room temperature. The DNA fragments were visualized with ethidium bromide under ultraviolet light and photographed.

Prior to digestion of the complex by micrococcal nuclease (Worthington), the pellets of ctDNA-protein complex were solubilized in a medium allowing transcription activity (2). Two μg of the complex per assay, determined by *A* at 260 nm, were incubated for 2 min at 37°C with 2 mM $CaCl_2$. Then, the micrococcal enzyme was added at five different concentrations ranging from 0.05 to 2 units of enzyme per unit of *A* at 260 nm of complex. Several concentrations of the enzyme were used for screening the conditions necessary to obtain typical patterns as it is usual for chromatin studies (15). After 10, 30, and 60 s of incubation, the digestion was stopped with 50 mM EDTA. The DNA from these different samples was extracted by incubation one night at 37°C in 0.2% SDS, 100 μg/ml proteinase K, and 10% glycerol. Then, 0.1 M KCl was added, and samples, after cooling, were centrifuged at 10,000*g* for 2 min. The supernatants were loaded on a 2% agarose slab gel (150 × 150 × 4 mm) in 40 mM Tris, pH 7.8, 20 mM NaCl, and 2 mM EDTA. Electrophoresis was done at 100 v for 8 h at room temperature. The DNA and the digestion products were visualized by ethidium bromide fluorescence and photographed.

Fatty Acids Determination. The DNA-protein complex (150 μg ctDNA, 400 μg proteins) was transferred into a vial containing 4 ml of the transesterification mixture (CH_3OH/H_2SO_4/benzene; 10:5:5, v/v) and 20 μg behenic acid as an internal standard. The vials were bubbled with N_2, securely tightened with a Teflon-lined cap, and placed in an oven at 68°C for 2 h. The separation of fatty acid methyl esters by GC (Hewlett Packard 5750, 10% DEGS on Varaport 30 column; temperature, 175°C) was done according to Douce and Joyard (5).

RESULTS

Visualization of the Spinach Plastid Transcriptionally Active DNA-Protein Complex. Electron microscopy visualization of the ctDNA-protein complex which is spread on grids charged by amylamine vapors according to Dubochet *et al.* (8) leads to a series of figures showing various transitions between isolated linear DNA fragments and highly condensed DNA protein structures (Figs. 1 and 2).

If the visualization occurs after the structure has been fixed by 0.1% glutaraldehyde (Fig. 1), proteins and DNA appear in a highly condensed form having a beaded substructure (Fig. 1, A and B). It seems that proteins are attached on the surface of a supercoiled condensed DNA structure. These structures can be partially decondensed as shown in Figure 1B, possibly because of nicks in the DNA. Figure 1C shows a less condensed complex largely organized around a central body. Proteins are seen as beads along the DNA. In some parts, proteins seem to be more or less regularly distributed. In other parts, these proteins are irregularly disposed. DNA fibrils often show twisted branches. It should be noted that condensed and decondensed parts are continuous. In the absence of glutaraldehyde fixation, the DNA-protein complex appears to be composed of a central body from which double strands of DNA, with some proteins fixed on them, are derived (Fig. 2, A and B). As can be seen on these two micrographs, the ratio of DNA to each central body is variable because stretches of DNA have been broken. These figures are very similar to those of Yoshida *et al.* (22) and of Herrmann *et al.* (11). After DNase or proteinase K treatments of the complex, linear double-stranded DNA and proteinous central bodies can be easily observed (Fig. 2, C and D).

Isopycnic Centrifugation Analysis of the ctDNA-Protein Complex. The formaldehyde-fixed ctDNA-protein complex analyzed in CsCl was composed, in three different experiments, of two defined classes of macromolecules (Fig. 3A): the first with a buoyant density of 1.570 g/cm^3 and the second of 1.610 g/cm^3. The complexes contained in each of the two classes were diluted with H_2O to a final concentration of 1 μg ctDNA/ml and visualized as described in "Materials and Methods."

Macromolecular components with a buoyant density of 1.570 g/cm^3 are constituted of protein associated to ctDNA. A dilution necessary for electron microscopy preparation, reverses the formaldehyde fixation and the complexes appear partially dissociated (Fig. 3B). The complexes isolated with a buoyant density of 1.610 g/cm^3 contain only strongly associated proteins (Fig. 3C); the weakly bound proteins have been probably lost during the preparation before ultracentrifugation in CsCl gradients. In both cases, the central body is still firmly linked to the ctDNA (Fig. 3, B and C).

Analysis of the Complex DNA. Extensive digestions of the complex by restriction nucleases Eco RI and Bam HI give the same digestion pattern that is obtained with purified spinach ctDNA (Fig. 4A) (21, 23).

We look for the presence of a repeated substructure in the ctDNA-protein complex by using the same method as for chromatin (16) in which typical patterns are observed after digestion by micrococcal endonuclease. No repetitive 'nucleosome-like' structure can be observed in our experiments (Fig. 4B) using five different concentrations of enzyme (see "Materials and Methods"). The results obtained with only one concentration (0.05 unit) after three different times for digestion (10, 30, and 60 s) are shown in Figure 4B. All other concentrations of enzyme we have tested gave the same results.

Characterization of the Polypeptides of the ctDNA-Protein Complex. After SDS-polyacrylamide gel electrophoresis, 36 polypeptides with mol wt ranging from 12,000 to 180,000 are always present (Fig. 5A), as previously observed (3).

After solubilization of the proteins of the complex with 0.4 N H_2SO_4 and two-dimensional gel electrophoresis, seven major polypeptides of mol wt ranging from 14,000 to 46,000 are seen (Fig. 5B). If the first dimension is performed at pH 8.3 instead of 4.5, only four polypeptides (14, 16, 20, and 21 × 10^3 daltons) are present (not shown).

Lipids Analysis. The fatty acids of leaf chloroplast membranes

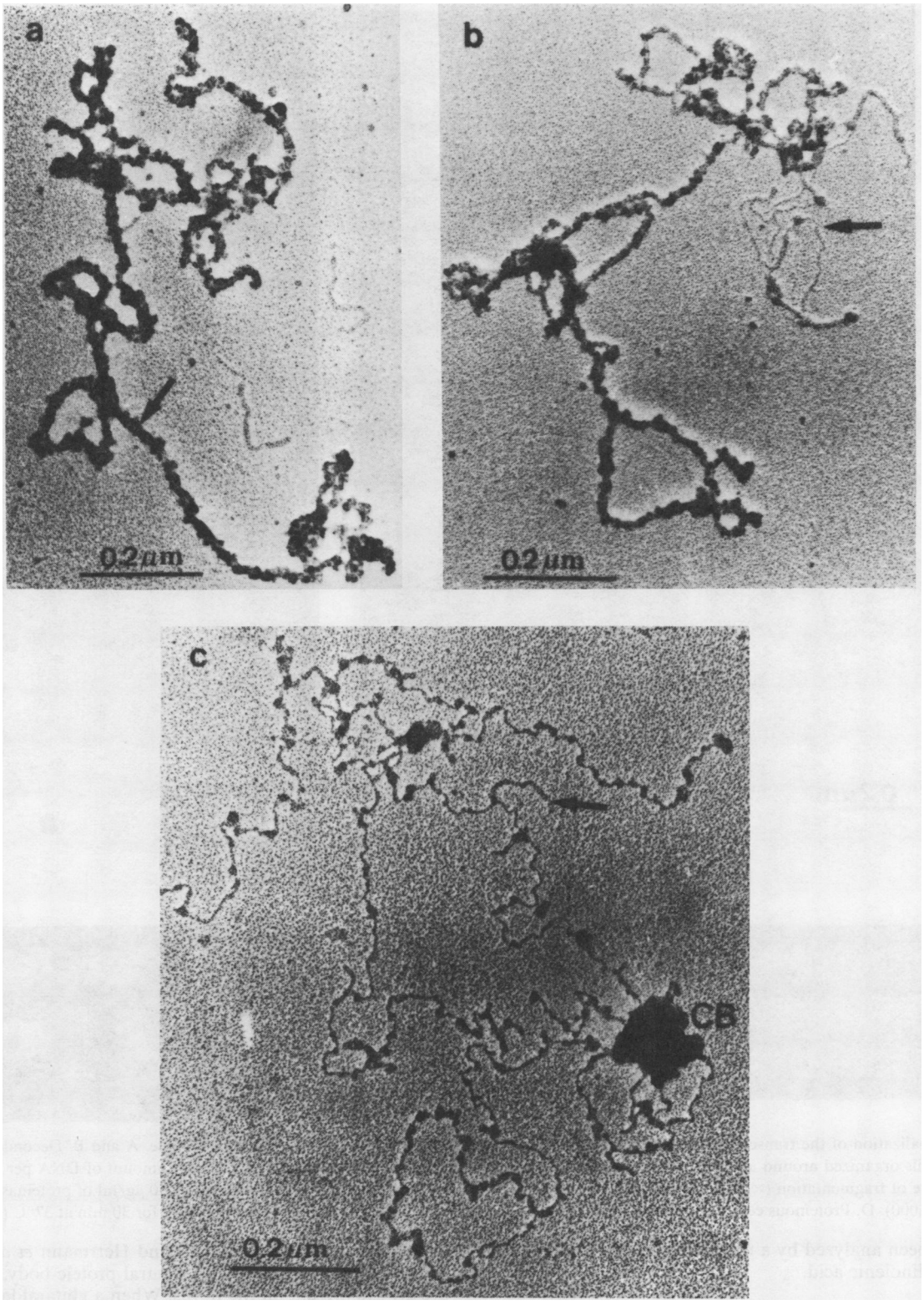

FIG. 1. Visualization of the glutaraldehyde fixed transcriptionally active DNA-protein complex from spinach plastids. A, Transcriptionally active DNA-protein complex in a very high condensed form; arrow indicates beaded substructure. B, Condensed transcriptionally active DNA-protein complex with a part of decondensed DNA as shown by the arrow. C, Less condensed structure organized around a central proteinous body (CB); proteins are visible on the DNA fibrils; arrow indicates twisted branches. Magnification of these micrographs is × 100,000.

are unusual in their high degree of unsaturation. Trienoic acids, particularly the α-linolenic acid (all cis-9,12,15-octadecatrienoic acid), predominate. The α-linolenic acid can account for over 90% of the total chloroplast membranes (6). α-Linolenic acid is not detectable in our isolated ctDNA-protein complex; therefore, we conclude that membranes are absent from our preparation. Chloroplast membranes contain 0.25 mg of linolenic acid/mg of protein (R. Douce, personal communication). Our sample (400 μg of

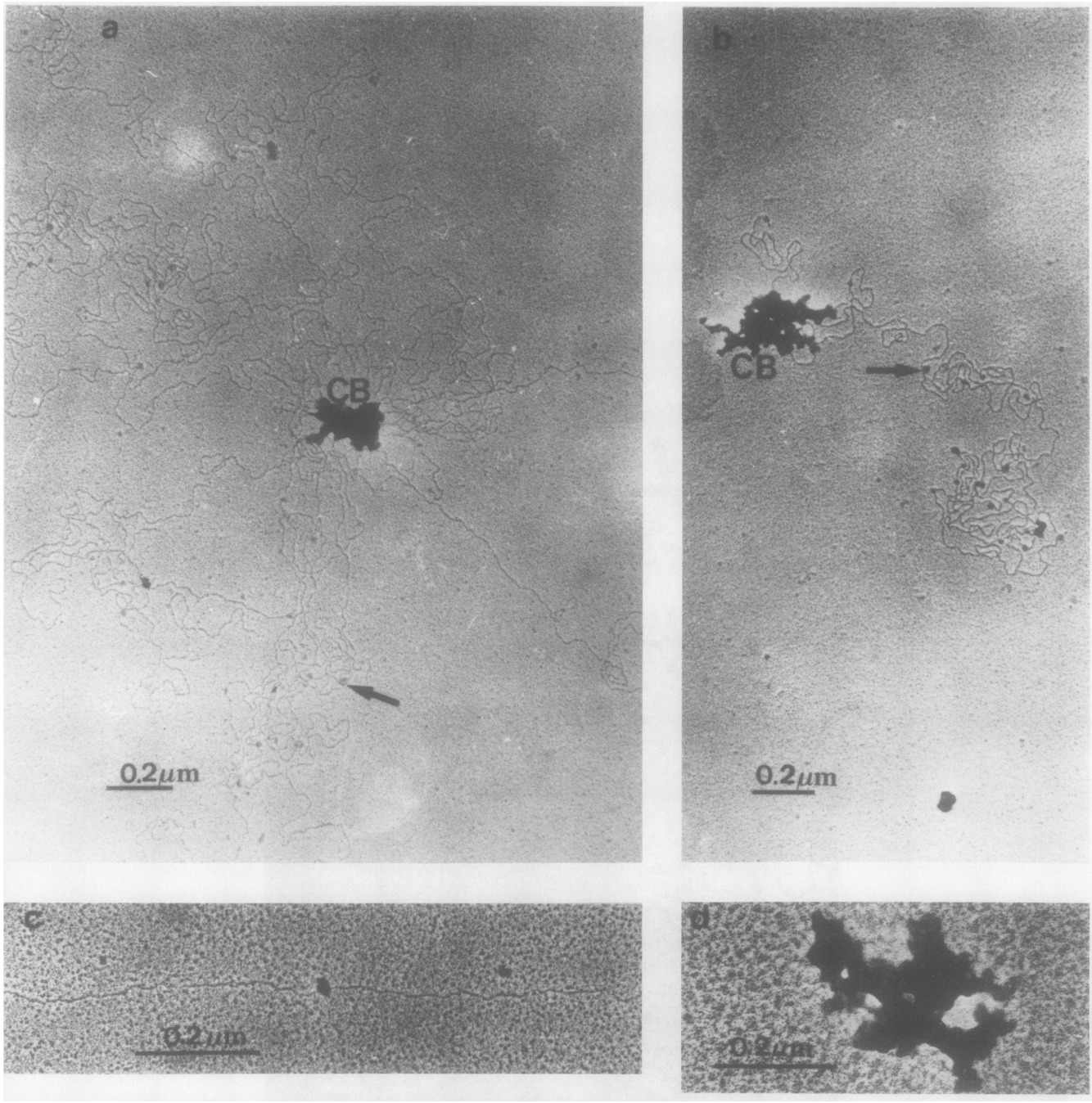

FIG. 2. Visualization of the transcriptionally active DNA-protein complex which is not fixed by glutaraldehyde. A and B, Decondensed structure with DNA fibrils organized around a central body (CB); arrows indicate proteins bound to the DNA filaments; amount of DNA per central body is variable because of fragmentation (× 50,000). C, Linear double-stranded DNA after digestion of the complex by 50 μg/ml of proteinase K for 30 min at 37°C (× 100,000). D, Proteinous central body visualized after digestion of the complex by 50 μg/ml of DNase I for 30 min at 37°C (× 100,000).

protein) has been analyzed by a method whose limit of detection is <0.1 μg of linolenic acid.

DISCUSSION

We have described previously a transcriptionally active DNA-protein complex from pure and intact spinach chloroplasts. Transcriptional properties of this complex have been studied (1–3, and we now report the visualization of this structure spread on grids charged previously with amylamine vapors according to Dubochet *et al.* (8).

When the DNA-protein complex is not fixed with glutaraldehyde (Fig. 2, A and B), the structure observed resembles the one reported by Yoshida *et al.* (22) and Herrmann *et al.* (11). DNA fibrils are organized around a central proteic body, and proteins are always present on the fibrils. When a glutaraldehyde fixation is performed, two important features are noted: (a) DNA fibrils are supertwisted (Fig. 1A and 2); (b) many proteins are present on the DNA and these give a rough aspect to the structure. This structure can appear in a very highly condensed form (Fig. 1A) with a beaded substructure upon supertwisted DNA. This condensed structure is very similar to the *Escherichia coli* chromosome as visualized by Griffith (9). When the DNA is decondensed, proteins are dispersed along the extended fibrils (Fig. 1C). The role of these proteins is unknown; perhaps they participate in the supercoiling of the ctDNA. The supercoiled DNA covered with

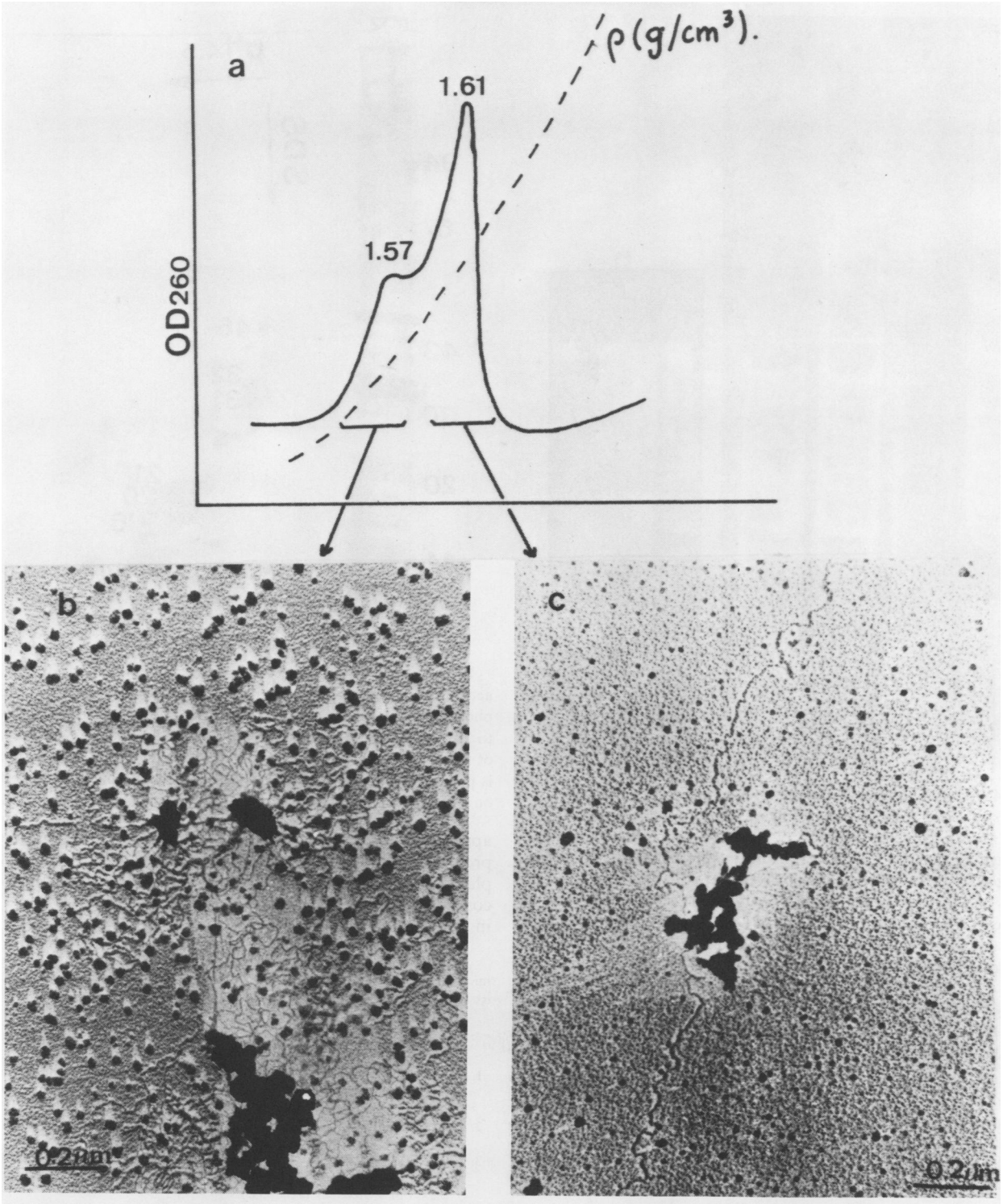

FIG. 3. Visualization of formaldehyde-fixed DNA-protein complex after isopycnic centrifugation in CsCl. A, Evidence of two populations of molecules after centrifugation, at equilibrium, in CsCl for 45 h at 34,000 rpm at 20°C in a SW 50 Beckman rotor. B, Visualization of the molecules which sediment, at equilibrium in CsCl, at a buoyant density of 1.570 g/cm^{-3} (× 60,000). C, Visualization of the molecules which sediment, at equilibrium in CsCl, at a buoyant density of 1.610 g/cm^{-3} (× 60,000).

proteins (Fig. 1A) could be an intact plastidial chromosome, but we do not have definitive evidence to prove this point.

Chromatin contamination can be excluded in our preparations for the following reasons. Each sample visualized possessed a transcriptional activity insensitive to 100 μg/ml of α-amanitin which is a specific inhibitor of eucaryotic RNA-polymerases II and III; micrococcal nuclease experiments (Fig. 4) showed the absence of repetitive DNA fragments in the complex. Furthermore, to prevent nuclear chromatin binding to chloroplasts, all the purification steps were carried out in the presence of 4 mM EDTA (R. G. Herrmann, personal communication).

Figure 3B indicates clearly that two types of DNA-protein interactions occur in this structure. First, there is a class of firmly bound proteins that are always present in the complex. These

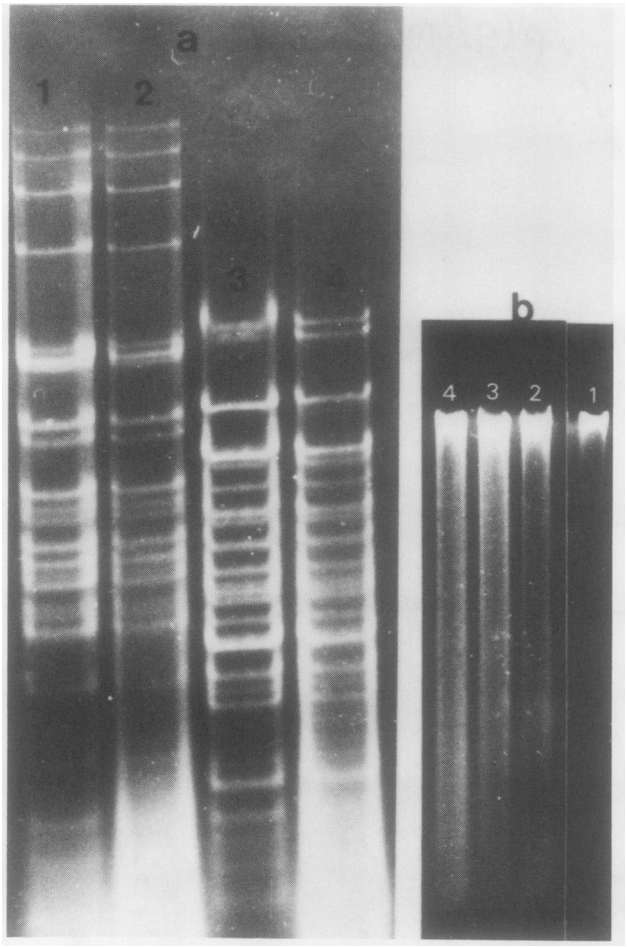

FIG. 4. Electrophoretograms of the complex after digestion by different nucleases. A, Electrophoresis on 0.7% agarose gels of deproteinized spinach ctDNA and of complex digested by Bam HI and Eco RI: complex hydrolyzed by Bam HI (1), ctDNA hydrolyzed by Bam HI (2), complex hydrolyzed by Eco RI (3), ctDNA hydrolyzed by Eco RI (4). B, Electrophoresis on 2% agarose gel of ctDNA from the complex digested by micrococcal nuclease: control (1), digestion for 10 s at 37°C of the complex by 0.05 unit of micrococcal nuclease (2), digestion for 30 s at 37°C (3), digestion for 60 s at 37°C (4).

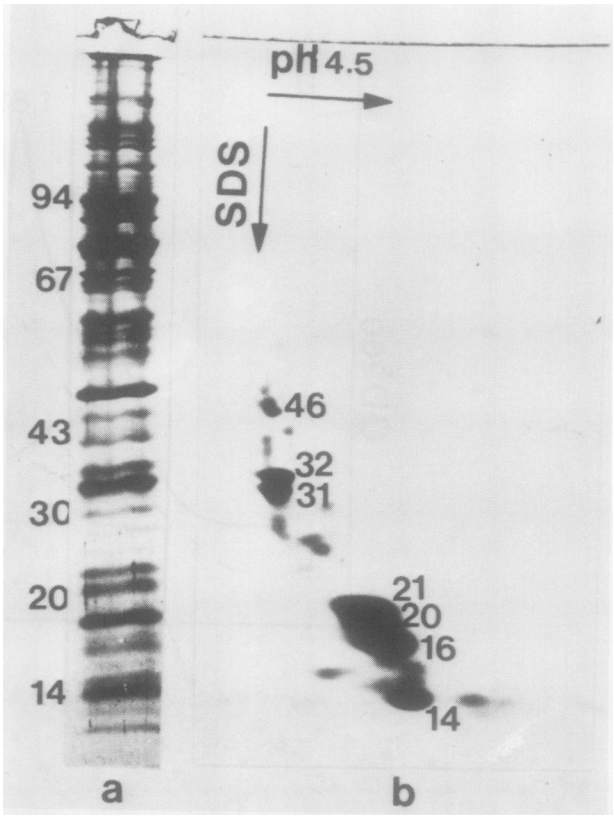

FIG. 5. Characterization of the polypeptides of the transcriptionally active complex by polyacrylamide gel electrophoresis. A, SDS-gel electrophoresis of the polypeptides of the transcriptionally active complex in a 8 to 20% polyacrylamide gradient. B, Two-dimensional gel electrophoresis of the complex polypeptides soluble in 0.4 N H_2SO_4. The first dimension is performed at pH 4.5 without SDS. The second dimension is performed on a 8 to 20% SDS polyacrylamide gel. Mol wt are multipled by 10^{-3}.

proteins belong to the 'central body' and probably correspond to central bodies observed previously after a drastic deproteinization of ctDNA (22). The central body can be involved in the membrane attachment of the nucleoid but cannot represent a chloroplast membrane fragment since no α-linolenic acid has been detected in our preparations. Second, there is a class of proteins which is bound to the DNA with weaker interactions and detached from the DNA after dilution of the formaldehyde (Fig. 3B) or during the preparation (Fig. 3C). These proteins are involved in enzymic functions such as transcription since RNA-polymerase is present in this complex (2, 3). They could also play a role in replication and condensation of the DNA.

The condensation of the plastome could be related with the presence of basic proteins in the complex (Fig. 5B). We have not found ribosomes, rRNA, or RNAs in our preparations using electrophoresis under denaturing (methyl mercury) or nondenaturing conditions (not shown). The presence of prokaryotic histone-like proteins is now well documented for *E. coli* since Rouvière-Yaniv and Gros (17) have isolated the basic Hu protein, and, more recently, Hübscher *et al.* (13) have characterized a histone H2A-like protein in this bacteria. DNA protein structures appear to be organized in a condensed form in chromatin in prokaryotic chromosomes, and, as we show, in plastome of higher plants. Basic proteins could play an essential function in the condensed form of plastid DNA as they do in *E. coli* (13, 17) and in nuclear chromatin.

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