THE LOCALIZATION OF [3H]THYMIDINE INCORPORATION IN THE DNA OF REPLICATING SPINACH CHLOROPLASTS BY ELECTRON-MICROSCOPE AUTORADIOGRAPHY

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SUMMARY

Electron-microscope autoradiography has been used to obtain information on the localization of DNA labelled with [3H]thymidine in chloroplasts known to be replicating and concomitantly synthesizing and segregating DNA, in cultured leaf disks. The studies were made using both Microdol-X developer and a 'compact' developer which gave a smaller grain size. About 80 % of the grains were associated with the granal membranes and with presumptive DNA regions (3-nm fibril material in clear areas). Few grains occurred in association with the chloroplast envelope. We suggest that the DNA of chloroplasts is associated with the grana DNA regions and extends into the stroma. Some light-microscope autoradiographs of whole chloroplasts show spiral or helical-like labelling patterns. We interpret these patterns as demonstration of the possibility that DNA occurs along the length of a continuous lamellar membrane system. Chloroplast fractionation experiments provided data consistent with the electron-microscope autoradiographic studies as most of the label was associated with chlorophyll-containing lamellae.

We consider an association of chloroplast DNA molecules along the length of a continuous lamellar system would ensure an orderly segregation of DNA to daughter chloroplasts, during the binary fission of spinach chloroplasts by constriction division.

INTRODUCTION

It is now well established that in the higher plant chloroplast there are many copies (20-30) of the chloroplast genome (Wells & Birnstiel, 1969; Kirk, 1972). The replicating chloroplasts of light-grown spinach leaf disks actively synthesize DNA and during the division cycle segregate it in similar amounts to the daughter chloroplasts Rose, Cran & Possingham, 1974, 1975; Possingham & Rose, 1976. It is therefore possible that the chloroplast DNA (cDNA) of higher plants is located in the chloroplast in such a way as to permit such a segregation.

The organization of DNA in the chloroplasts of a higher plant has been studied by light-microscope autoradiography and electron microscopy of serial sections (Herrmann, 1970; Herrmann & Kowallik, 1970; Kowallik & Herrmann, 1972). In Beta vulgaris chloroplasts they found several distinctly separate DNA nucleoids, separated from each other by lamellate thylakoid stacks. As the number of nucleoids was much less than the number of chloroplast genomes, Kowallik & Herrmann (1972)
concluded that each nucleoid contained 4–8 cDNA molecules. Earlier electron-microscope studies of Swiss chard (Kislev, Swift & Bogorad, 1965) and *Avena sativa* (Gunning, 1965) revealed a number of DNA areas in a single chloroplast section. There appears to be no evidence to-date for a regular or definite pattern of DNA throughout higher plant chloroplasts as occurs in certain algae which have a continuous ring-shaped nucleoid lying inside the peripheral thylakoids which loop around the rim of the chloroplast (Bisalputra & Bisalputra, 1969; Gibbs, Cheng & Slankis, 1974). In these algae it appears that there are associations between the cDNA and thylakoids which facilitate this arrangement. Close associations between thylakoid membranes and DNA also occur in dinoflagellates (Kowallik & Haberkorn, 1971; Bibby & Dodge, 1974). In higher plant chloroplasts observations of cDNA membrane associations have been made in spinach (Woodcock & Fernández-Morán, 1968) and in *Pelargonium* (Knoth, Herrmann, Böttger & Börner, 1974), though the regularity of this relationship and which chloroplast membranes are involved has not been clearly defined.

To obtain more information on the location of cDNA in relation to the segregation process in replicating spinach chloroplasts, we have studied the incorporation of [3H]thymidine ([3H]-TdR) into chloroplasts using light- and electron-microscope autoradiography and chloroplast isolation and fractionation techniques.

**Materials and Methods**

*Culture of leaf disks*

The growth of the spinach plants (*Spinacia oleracea*), and subsequent leaf disk culture on sterile nutrient agar has been previously described (Possingham & Smith, 1972). Growth in the light refers to a 14 h-day, at 20000 lux, with day and night temperatures of 25 and 22 °C, respectively. Growth in continuous darkness was at 25 °C.

*Autoradiography*

The disks were incubated in 50 μCi [6-3H]thymidine (27 Ci/mM, Radiochemical Centre, Amersham, U.K.), added in 1 ml under sterile conditions to the 20 ml of nutrient agar medium in 9-cm Petri dishes. The light-microscope autoradiographic techniques have been described previously, as has the specificity of incorporation of [3H]-TdR into DNA (Rose et al. 1975).

For electron-microscope autoradiography, tissue from the disks was fixed in 6:25 % glutaraldehyde, postfixed in osmium and embedded in Araldite (Cran & Possingham, 1972 a). Sections were mounted on carbon-coated copper grids, and stained in uranyl acetate in 50 % ethanol, followed by lead citrate. The stained sections were then coated with a thin layer of carbon, and fixed to coverslips with double-sided tape. The grids were coated with an approximate monolayer of diluted Ilford L-4 emulsion, by passing the coverslip through a film formed at the top of a 20-ml specimen tube (a modification of the Caro & van Tubergen (1962) loop method). The coverslips were attached to glass slides by double-sided tape, and the grids exposed at 5 °C for 6–15 weeks in light-tight boxes containing silica gel. Development was carried out with Microdol-X for 3 min or with the 'compact' development mixture of Lettré & Pawelec (1966), for 1 min; in both cases at approximately 20 °C. The 'compact' developer mixture (containing Phenidone as the developing agent) gave backgrounds too high for routine use with chloroplasts, which in general have a low level of labelling.

Grain locations over chloroplasts were determined using Microdol-X development. Photogra phs of 131 chloroplasts from random locations in background-free areas were obtained by 2 different operators. We used the central region of the filamentous grain as the estimated
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Position of the original latent image (Caro & van Tubergen, 1962). We also scored photographs as to whether the grains were touching different membranes, as done by Comings & Okada (1973), in their nuclear DNA replication studies.

[3H]thymidine incorporation into isolated chloroplast fractions

Five-day-old light-grown disks (30 per plate) were incubated 24 h in 3H-TdR as in the autoradiography studies, and usually 180 with a total fresh weight of about 4 g used for chloroplast isolation.

Chloroplasts were obtained by a razor chopping method designed to give a high proportion of intact chloroplasts free of contaminating nuclei (Spencer & Wildman, 1964). Disks were chopped in a medium containing 0.25 M sucrose, 25 % Ficoll, 5 % dextran, 25 mM Tris-HCl pH 7.8, 1 µg/ml Cleland’s reagent, 2 mM CaCl2, 1 mM magnesium acetate, 0.1 % bovine serum albumin and 0.5 mg/ml cold thymidine (Honda, Hongladarom & Laties, 1966). The resulting brei was filtered twice through fine mesh (100-µm) steel gauze, and 3-ml aliquots were layered on the top of discontinuous sucrose gradients. The gradient consisted of 6 ml of 60 % sucrose, 2 ml of 45 % sucrose and 6 ml of 20 % sucrose in modified Honda medium. After centrifugation in a swinging bucket rotor for 20 min at 10,000 rev/min (Beckman model L2-65B, SW 27.1 rotor), the chloroplast bands at the bottom and top of the 45 % sucrose interfaces were collected. A double banding pattern was characteristic of chloroplasts isolated from disks, as many contain large amounts of starch.

The isolated chloroplasts were suspended in the modified Honda medium, pelleted at 10,000 rev/min and resuspended using a glass rod, in 17 ml of bursting medium (10 mM bicine-NaOH buffer, pH 7.6, 4 mM MgCl2), similar to that described by Douce, Holtz & Benson (1973). The chloroplasts were left in this medium in ice for 30 min to ensure detachment of most chloroplast envelopes. While causing loss of the envelope, the osmolarity of the medium is designed to cause minimal disruption of the lamellar system (Douce et al. 1973). Following the treatment in bursting medium, the suspension was centrifuged in a SW 27.1 rotor for 10 min at 3500 g to obtain a lamellae-rich fraction, and for 40 min at 38,000 g to obtain an envelope-rich fraction (Joy & Ellis, 1975).

The 3 fractions (lamellae, envelope and supernatant) were collected on Whatman GF/A glass-fibre filters, and washed with 25 ml of bursting medium. The chlorophyll was extracted with 80 % acetone and estimated spectrophotometrically (Arnon, 1949). The filters were then washed with 5 % TCA (containing cold thymidine) ether-ethanol and finally ether before air drying and counting in a toluene-based scintillation fluid (Rose et al. 1975).

RESULTS

Electron-microscope autoradiographs of 3H-TdR incorporation into chloroplasts are shown in Figs. 2-7. These photographs show the type of incorporation patterns we obtained, using disks of different ages, different physiological conditions and different isotope incorporation times; variables which did not in any consistent way alter grain localization patterns. We found that starch grains serve as a useful control against random silver grain formation as they are seldom labelled. It is also apparent that the grains do not occur at the periphery of the organelle in association with the chloroplast envelope.

By quantifying the autoradiographic data (see Materials and methods), we obtain the silver grain distribution pattern shown in Fig. 1. Most of the grains occur over the clear areas containing fibrils (about 3 nm) (e.g. Figs. 2-4) or grana lamellae (Figs. 2-7). If we score the autoradiographs in another way (Comings & Okada, 1973), 5 % of the grains touch the chloroplast envelope, while 72 % touch the lamellar system of grana and stroma membranes.
Electron-microscope autoradiographs of constricting chloroplasts are shown in Figs. 8 and 9. Silver grains occur on either side of the constriction, as do presumptive DNA areas (Fig. 9 in particular).

![Histogram showing the location of silver grains in relation to the different chloroplast components. 332 grains scored.](image)

Resolution with Microdol-X development is to a degree hampered by the size of the silver grains, and a 'compact' developer was used in an attempt to overcome this problem. Although we commonly had high backgrounds with this developer, the results were essentially similar to those obtained with Microdol-X. This is illustrated in Fig. 10 which shows a labelled chloroplast, while in Fig. 11 a labelled nucleus is shown, indicating that the round silver grains formed with compact developer are not simply osmiophilic lipid bodies, which are commonly present in chloroplasts.

We could not tell from the electron-microscope autoradiography data whether there is a regular or repeating arrangement of the cDNA along chloroplast lamellar membranes. There was no clear evidence that DNA is associated with particular grana of the lamellar membrane regions as grains frequently occurred throughout the length of a given section. However, we found spiral or helical-like labelling patterns (Fig. 12) in some light-microscope autoradiographs. We observed these patterns over individual chloroplasts on a number of occasions, but to-date have not been able to relate them to particular treatments.

As there are always some uncertainties with resolution when electron-microscope autoradiography is used (Rogers, 1971) we isolated chloroplasts and separated them into lamellae-rich and envelope-rich fractions, as an alternative means of localizing $^3$H-TdR incorporation. We found that the label in these experiments is mainly
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associated with the chloroplast lamellae fraction, which is high in chlorophyll (Table 1). A much smaller proportion of the label is associated with the chloroplast envelope and small membrane fragment fraction. Some of this latter incorporation is probably due to contamination with chlorophyll-containing lamellae fragments. A proportion of the labelled DNA is liberated into the supernatant but this proportion is variable, probably because of variations in the severity of osmotic disruption.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incorporation into TCA-insoluble material, cpm (% of total)</th>
<th>Chlorophyll, µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>3500-g pellet, chloroplast lamellae fraction high in chlorophyll</td>
<td>15657 (61 %)</td>
<td>8.34</td>
</tr>
<tr>
<td>38000-g pellet, chloroplast envelope and small membrane fragments</td>
<td>2460 (9 %)</td>
<td>0.86</td>
</tr>
<tr>
<td>Supernatant, soluble components</td>
<td>7736 (30 %)</td>
<td>—</td>
</tr>
</tbody>
</table>

DISCUSSION

Our electron-microscope autoradiography data indicate that in spinach chloroplasts there is an association of cDNA with the chloroplast lamellae system, and in particular with grana lamellae. As well, the fibril-containing areas of low electron density were heavily labelled. These latter areas are usually recognized on morphological grounds as DNA-containing regions following the work of Ris & Plaut (1962) with Chlamydomonas. More recently, Gibbs et al. (1974) have shown by electron-microscope autoradiography that all plastid DNA is localized in the nucleoid of Ochromonas danica. We suggest that our labelling patterns could result from the association of cDNA with lamellar membranes and with its extension from these into the stroma, where it is present in the fibril regions. Our autoradiographic data are consistent with observations on the location of the DNA regions based on morphological criteria (Figs. 2–4, 8–10). It is also consistent with the conclusion of Woodcock & Fernández-Morán (1968) obtained by spreading osmotically disrupted chloroplasts in a protein monolayer.

It could possibly be argued that grain locations over lamellae are fortuitous because of the relatively large amount of these membranes in chloroplasts. However, in the spinach disks used in our experiments granal stacking is not extensive (which in part may be related to the high light intensity at which the disks are grown) and although the variation is large, on average about 20% of the area of a chloroplast section is occupied by lamellar membranes, whereas almost 50% of the grains occur here. Histogram analysis shows that there is not a uniform distribution of grains throughout the chloroplast, as the DNA regions recognized on morphological grounds contain a large proportion of the grains, whereas starch regions which occupy a large area are rarely labelled.

We believe that the light-microscope autoradiographs showing helical-like labelling patterns are of considerable significance. One possible interpretation is that this pattern
represents incorporation along the lamellar system, and evidence is available which favours a continuous helical arrangement of the chloroplast lamellar system in higher plants (Spencer & Wildman, 1962; Paolillo, 1970; Wildman, Jope & Atchison, 1974; S. G. Wildman, personal communication, 1974). Such labelling patterns might arise when there is synchrony of synthesis of cDNA molecules and of new regions of the chloroplast lamellae.

The data we have obtained from the fractionation of isolated chloroplasts are consistent with the electron-microscope autoradiography. Clearly, little DNA is associated with the chloroplast envelope and spinach chloroplasts thus do not appear to segregate their DNA in the same way as proposed for bacteria, where the DNA is attached to the limiting membrane (Ryter, 1968).

Our fractionation data are in agreement with the report of Spencer & Whitfeld (1967) that primer DNA and DNA polymerase are tightly associated with a particulate fraction free of external chloroplast membranes. Our finding that some of the cDNA can be released free of membranes by osmotic shock is supported by the work of Wong & Wildman (1972) who used osmotic shock as the basis of a method for the isolation of cDNA.

The data we have obtained suggest a segregation mechanism based on the association of the cDNA with the lamellar system. The cDNA molecules would be located at intervals along the lamellar system, probably being associated with the thylakoids of the grana. It is possible, from the light-microscope autoradiographs, that the cDNA associations could occur at regular intervals. If the lamellar system is indeed a continuum, its breakage at a single, central point would ensure that similar amounts of DNA would distribute to each daughter chloroplast during binary fission by constriction division (Cran & Possingham, 1972a, b). Such a system would account for the results of our light-microscope autoradiographic studies (Rose et al. 1974; Possingham & Rose, 1976) where on average DNA doubles during the chloroplast division cycle and distributes fairly equally to daughter chloroplasts. In principle, the type of segregation mechanism would be similar to that in the brown alga, Spacelaria (Bisalputra & Bisalputra, 1970). Here the peripherally located ring-like genophore becomes elongated with the lengthening of the chloroplast and segregates into 2 daughter loops, each being transmitted to the daughter half of the chloroplast. We consider that in spinach the difference lies in the organization of the cDNA which forms associations along the length of the lamellar system.

We do not consider the evidence obtained by Kowallik & Herrmann (1972) by serial section conflicts with our observations. The discrete DNA regions they have observed may well all be associated with grana (see their fig. 8). The arrangement of cDNA found in serial section might depend on whether there is a periodicity along the membrane and this could vary from plant to plant. Essentially what is important is the exact distribution of DNA molecules in relation to the lamellar system of chloroplasts.

Although in spinach chloroplasts the cDNA is predominantly associated with lamellar membranes rather than the outer membrane, the situation may well be different in other plastid types. Etioplasts have been shown to segregate their DNA to
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daughter plastids and observations have been made of DNA-membrane associations (Sprey, 1968; Sprey & Gietz, 1973). At least some of the DNA appears to be associated with the etioplast envelope (Herrmann & Kowallik, 1970). In a mutant of Pelargonium zonale known to have few lamellar structures, associations between cDNA and the plastid envelope have also been reported (Knoth et al. 1974). It is at least possible that changes which occur in plastid membrane systems during their differentiation from proplastids or etioplasts to chloroplasts (such as the invagination of the outer membrane) could lead to changes in the location of cDNA.

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REFERENCES


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Fig. 4. Disks grown in darkness for 5 days, transferred to the light for 1 day, and then incubated in \(^{3}H\text{-TdR}\) for 24 h in the light. Autoradiograph exposed 10 weeks. \(\times 25000\).

Fig. 5. As for Fig. 4. \(\times 18000\).

Fig. 6. Disks excised and incubated in \(^{3}H\text{-TdR}\) for 72 h in the light. Autoradiograph exposed 12 weeks. \(\times 29000\).

Fig. 7. As for Fig. 6. \(\times 17000\).
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Fig. 8. Disks excised and incubated in $^3$H-TdR for 24 h in the light. Autoradiograph exposed 15 weeks. $\times 14\,000$.

Fig. 9. Disks grown in darkness for 5 days, transferred to the light for 1 day, and then incubated in $^3$H-TdR for 24 h in the light. There is a single silver grain on either side of constriction. Note fibril-containing clear areas. Autoradiograph exposed 10 weeks. $\times 25\,000$. 
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Fig. 10. Electron-microscope autoradiograph of chloroplast. Compact development. Disks excised and incubated in $^3$H-TdR for 24 h in the light. The grey halo around most of the black grains distinguishes them from lipid droplets. Autoradiograph exposed 12 weeks. $\times 33000$.

Fig. 11. Electron-microscope autoradiograph of a nucleus. Compact development. Disks excised and incubated in $^3$H-TdR for 24 h in the light. Autoradiograph exposed 12 weeks. $\times 11000$.

Fig. 12. Light-microscope autoradiograph of a cell, showing grain patterns which are located over chloroplasts. Disks were grown in the dark for 5 days, before being incubated in $^3$H-TdR for 24 h in the light. Autoradiograph exposed 13 days. $\times 950$. 
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