DNA SYNTHESIS IN ISOLATED CHLOROPLASTS

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There is now ample evidence that chloroplasts from both higher and lower plants contain DNA (Granick and Gibor, 1967). This finding suggests the possibility that chloroplasts are to some extent capable of directing the synthesis of their own ribonucleic acids and, in turn, their own proteins. A capacity for both protein and RNA synthesis (Spencer and Wildman, 1964; Spencer, 1965; Boardman et al., 1965; Kirk, 1964; Semal et al., 1964), and in particular, for messenger RNA synthesis (Spencer and Whitfeld, 1967), has already been demonstrated in isolated chloroplasts. Evidence is now presented that isolated spinach chloroplasts incorporate ^{3}H-thymidine triphosphate into an acid-insoluble, alkali-stable product, indistinguishable from DNA. Incorporation requires dGTP, dCTP, and dATP, and is inhibited by DNase and actinomycin D, but not by RNase. The radioactive product bands with chloroplast DNA in CsCl and, like chloroplast DNA, it can be denatured and renatured.

Spinach plants (Spinacia oleracea) were grown in nutrient solutions, and leaf homogenates were prepared as described elsewhere (Spencer and Whitfeld, 1967) using a sucrose solution containing Tris, KCl, $MgCl_2$ and mercaptoethanol. Leaf homogenates were filtered through Miracloth (Chicopee Mills Inc., New York) to yield a cell-free extract from which was prepared the washed 1000 g pellet. Chloroplasts and nuclei were isolated from this fraction, the former by density gradient centrifugation, and the latter by treatment with Triton X-100 (Rohm and Haas) as described elsewhere (Spencer and Whitfeld, 1967). In the present experiments the youngest leaves (up to 2" long) were harvested from plants 2 to 3

weeks after transplanting seedlings into nutrient solutions. DNA synthesis was assayed by the incorporation of ^{3}H-thymidine triphosphate (TTP) into trichloracetic acid-insoluble material using the following reaction mixture: 0.2 ml of a solution containing 0.01 M Tris pH 7.4, 0.002 M.$MgCl_2$, and 0.004 M mercaptoethanol, 10 µl of a solution containing 30 µg each of dGTP, dATP and dCTP, 5 µl of ^{3}H-TTP (Schwarz Bioresearch Inc., 1 millicurie/ml, 4.8 curies/mmole) and 0.2 ml of chloroplasts (equivalent to 50 to 150 µg of chlorophyll) or 0.2 ml of nuclei (equivalent to 10 to 15 µg of DNA) suspended in the above tris-$MgCl_2$-mercaptoethanol solution. Incubation was at 25° for 10 to 15 minutes. The reaction was stopped by the addition of 0.5 ml of 0.1 M sodium pyrophosphate and 1 ml of cold 10% trichloracetic acid (TCA). The TCA-insoluble fraction was sedimented, and radioactivity measured in a TriCarb Liquid Scintillation Spectrometer as described elsewhere (Spencer and Whitfeld, 1967). The DNA content of various leaf fractions was determined by the method of Burton (1956) after decolorization with ethanol, acetone and ether.

When the washed 1000 g pellet fraction, which contains both chloroplasts and nuclei, was incubated under the above assay conditions significant amounts of ^{3}H-TTP were incorporated into an acid-insoluble product (Table 1). Fractionation

Table 1

Distribution of ^{3}H-thymidine triphosphate incorporating activity in cell-free fractions from spinach leaves

Leaf Fraction	^{3}H-TTP Incorporation (cpm/µg DNA)
Washed 1000 g pellet (chloroplasts + nuclei)	305
Chloroplasts	645
Nuclei	49

of the 1000 g pellet into the two types of organelle showed that, on a DNA basis, the isolated chloroplasts were more than 13 times as active as the isolated nuclei, and twice as active as the 1000 g pellet fraction from which they were derived. The lower specific activity of the nuclear fraction was not due to the fact that nuclei had been treated with Triton X-100. Separate experiments showed that this treatment had no effect on the ^{3}H-TTP incorporating activity of nuclei. We have found considerable difficulty in removing all nuclei from our chloroplast preparations, especially where very young leaves are used. The ratio of nuclear DNA to chloroplast DNA in our purified chloroplasts from this kind of leaf material has varied between 0.5 : 1 to 2 : 1, as compared to a ratio of approximately 10 : 1 in the original 1000 g pellet fractions. In the experiment in Table 1 this ratio was 1.9 : 1. The measured specific activity of the total chloroplast fraction is therefore a low estimate of the true specific activity of the chloroplast polymerase system. When corrected for the contribution of the residual nuclear DNA, the specific activity of chloroplasts in the experiment was calculated to be 1775 c.p.m. µg chloroplast DNA.

The characteristics of the ^{3}H-TTP incorporation reaction carried out by isolate chloroplasts provide strong evidence that the acid-insoluble, radioactive reaction product is in fact DNA. The incorporation of ^{3}H-TTP is almost completely dependent on the presence of the other three deoxyribonucleoside triphosphates, it is strongly inhibited by both DNase and actinomycin D, and is unaffected by pancreatic RNase (Table 2). A post-incubation treatment of the reaction mixture with DNase (10 µg/m for 20 minutes at 25°) rendered 92% of the incorporated radioactivity acid-soluble, whereas after alkali treatment (0.3 M NaOH for 1 hour at 37°) 87% of the incorporate counts remained acid-precipitable.

Equilibrium density gradient centrifugation in CsCl confirmed that the labelled product of the chloroplast reaction was DNA. For this purpose, 80 µg of unlabelled carrier chloroplast DNA was added to an incubated reaction mixture (7-fold the normal assay) and total DNA was extracted, using a modified Marmur procedure (Marmur, 1961). Sodium dodecyl sulfate treatment was carried out at room temperatur

Table 2

Characteristics of ^{3}H-thymidine triphosphate incorporation by isolated spinach chloroplasts

	Assay Ingredients	^{3}H-TTP Incorporation (cpm/assay)
Exp. 1	Complete reaction mixture*	3607
	Omit dGTP, dCTP	75
	" dATP, dCTP	43
	" dATP, dGTP	59
	" dGTP, dATP, dCTP	41
Exp. 2	Complete reaction mixture*	2830
	+ DNase, 0.76 µg/ml	1995
	+ DNase, 7.6 µg/ml	271
	+ actinomycin D 0.76 µg/ml	2227
	+ actinomycin D 7.6 µg/ml	886
	+ RNase 0.76 µg/ml	2881
	+ RNase 7.6 µg/ml	2677

* Components of complete reaction mixture are detailed in text.

phenol rather than chloroform was used as the denaturing agent, and the isopropyl alcohol precipitations were omitted. The carrier chloroplast DNA had been prepared, by the same procedure, from the Triton-soluble fraction of a 1000 g pellet. When the purified reaction product was subjected to density gradient centrifugation in CsCl, the radioactivity peak coincided with the absorbance peak of the chloroplast DNA. The double-stranded nature of the DNA product was revealed by an increase in buoyant density which resulted from its alkali denaturation. A difference of 9 fractions was found between the reference DNA and the denatured product DNA, as against a difference of 14 fractions between the reference DNA and the undenatured product DNA. A characteristic of chloroplast DNA is its ability to completely renature following denaturation (Tewari and Wildman, 1966). When the denatured, radioactive chloroplast product was subjected to renaturing conditions its buoyant density decreased to that of the native product and coincided

with renatured carrier chloroplast DNA absorbance peak.

DNA-synthesising activity in isolated chloroplasts implies the presence of both a native DNA primer and associated DNA polymerase. In the course of chloroplast isolation the plastids undergo several washings in low osmotic medium (Tris-$MgCl_2$-mercaptoethanol) which cause swelling and loss of the external chloroplast membranes with concomitant loss of internal soluble components. It follows then that both the primer DNA and the DNA polymerase must be tightly associated with the particulate, non-mobile phase of the chloroplasts.

With the present finding of DNA synthesis in isolated chloroplasts, the list of requirements for the potential autonomy of the chloroplast is complete. It now remains to discover what is the specific nature of the information contained in chloroplast DNA, to what extent it is replicated independently of the nucleus, and what is the proportion of chloroplast components whose synthesis is directed by chloroplast DNA. Recent evidence from hybridization experiments indicates only partial homology of chloroplast DNA with nuclear DNA in Euglena (Richards, 1967). This strongly supports the notion that a significant proportion of the chloroplast DNA is replicated within the chloroplast, independently of the nucleus.

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