STUDIES ON RNA SYNTHESIS IN CHLOROPLAST PREPARATIONS

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It has been found (Kirk, 1964) that highly purified preparations of broad bean chloroplasts incorporate radioactivity from ^{14}C-ATP into a substance having the properties of RNA. The reaction is similar in certain respects (requirement for all four nucleoside triphosphates, sensitivity to deoxyribonuclease and actinomycin D) to the RNA polymerase enzymes that have been found in other organisms (Weiss, 1960; Hurwitz et al., 1960; Stevens, 1960; Huang et al., 1960; Chamberlin and Berg, 1962). It was suggested that the activity is due to a chloroplast RNA polymerase, working with chloroplast DNA as a template. Evidence has now been obtained by other workers (Dr. S.G. Wildman, personal communication) for RNA synthesis in tobacco chloroplast preparations.

In this paper, further studies on the enzyme are described, with particular reference to apparent differences between the reactions catalyzed by the chloroplast and nuclear fractions.

METHODS

Chloroplast and nuclear fractions were isolated from broad bean leaves by high speed density gradient centrifug-

ation, DNA was estimated and RNA polymerase activity was measured as already described (Kirk, 1963; 1964).

In the experiments on the ratio of incorporation of adenine and guanine, the chloroplast and nuclear fractions were incubated for 10 min at 30° in a medium containing 50 μmoles Tris (pH 8.0), 8 μmoles $MgSO_4$, 3 μmoles cysteine.HCl, 40 mμmoles CTP, 40 mμmoles UTP, 8.7 mμmoles ATP and 8.7 mμmoles GTP in a total volume of 0.8 ml. In some tubes the ATP, and in others the GTP, was labelled with ^{14}C in the purine ring atom 8 (specific activity 2.3 μC/μmole, Schwarz BioResearch). Incorporation of radioactivity into material insoluble in 0.2 N $HClO_4$ was measured as already described (Kirk, 1964). The ratio of radioactivity incorporated from ^{14}C-ATP to that incorporated from ^{14}C-GTP was assumed to be a measure of the ratio of incorporation of adenine and guanine into the RNA. In other experiments chloroplast and nuclear fractions were incubated as above but with ^{14}C-ATP and ^{14}C-GTP present in the same tube. RNA was then isolated from the incubation mixtures by lauryl sulphate treatment and deproteinization (Marmur, 1961), followed by addition of 1 mg carrier yeast RNA, and precipitation by addition of ethanol (final concentration, 67% v/v) plus $HClO_4$ (final concentration, 0.1 N). The RNA was washed successively with 0.2 N $HClO_4$, 67% ethanol, 80% ethanol and absolute ethanol, and then dried. The RNA was hydrolysed with N HCl (Smith and Markham, 1950), and the purine bases were separated by paper chromatography (Wyatt, 1951), and the radioactivity estimated.

RESULTS

Time course. Under the standard assay conditions (Kirk, 1964), incorporation of radioactivity from ^{14}C-ATP by the

chloroplast fraction proceeds rapidly for about 15 min, and then levels off and stops between 20 and 30 min.

Effect of added DNA. Addition of 50 or 750 μg of broad bean DNA (prepared as described by Marmur, 1961) to the assay system had no stimulatory effect on the incorporation obtained with the chloroplast fraction. Addition of 750 μg DNA abolished the inhibition (46%) of incorporation produced by 3 μg actinomycin D.

Effect of divalent cations.

Table 1. Standard assay conditions except for variation in divalent cation concentration. Expt.i: In each tube, 11.1 mg dry weight chloroplasts containing 38 μg DNA. Expt.ii: 13.8 mg dry weight chloroplasts containing 62 μg DNA. Nuclear fraction containing 83 μg DNA.

		μμmoles ^{14}C-adenine incorporated/10 min	
Expt.	Additions (final conc.)	Chloroplasts	Nuclear fraction
i.	-	12	
	0.002 M $MgCl_2$	125	
	0.004 M $MgCl_2$	170	
	0.01 M $MgCl_2$	231	
	0.002 M $MnCl_2$	110	
	0.01 M $MnCl_2$	39	
ii.	0.01 M $MgSO_4$	316	76
	0.01M $MgSO_4$ + 0.002M $MnCl_2$	181	67

Bacterial RNA polymerase requires a divalent cation for activity: at a concentration of 2 mM, $MnCl_2$ gives about twice the activity obtained with $MgCl_2$ (Chamberlin and Berg, 1962).

The chloroplast activity also requires a divalent cation: however, 2mM $MgCl_2$ and 2 mM $MnCl_2$ gave approximately the same reaction rate (Table 1.i). In the presence of 10 mM $MgSO_4$, 2mM $MnCl_2$ inhibited the chloroplast activity by 40-49%; the nuclear reaction, on the other hand, was inhibited by only 12-20% (e.g. Table 1.ii).

Incorporation ratio of adenine to guanine. If the RNA synthesis in the chloroplast preparations is due to a chloroplast RNA polymerase using chloroplast DNA as a template, then by analogy with other RNA polymerases (Furth et al., 1961; Weiss and Nakamoto, 1961; Stevens, 1961; Chamberlin and Berg, 1962), it might be expected that the RNA formed would have a base ratio more similar to that of the chloroplast DNA than to that of the nuclear DNA. To test this hypothesis, chloroplast and nuclear fractions were incubated in the presence of the 4 ribonucleoside triphosphates with either the ATP or the GTP labelled. The molar ratio of ^{14}C-adenine to ^{14}C-guanine incorporated was 1.99 (mean of 5 determinations) for the chloroplasts, and 1.57 (mean of 5 determinations) for the nuclear fraction. Application of Student's t test (s=0.126, t=5.26) indicated that this difference was significant at the 0.1% level. To check this result incubations were carried out with both the ATP and the GTP labelled, the RNA was isolated and the radioactivity in the adenine and guanine was measured: this method gave an adenine to guanine incorporation ratio of 1.95 (2 analyses) for the chloroplasts, and 1.57 (2 analyses) for the nuclear fraction.

It seemed possible that these results might be due to the presence of a substantial pool of GTP in the chloroplast fraction, or ATP in the nuclear fraction, thus causing

dilution of the appropriate labelled nucleoside triphosphate. To investigate this hypothesis, chloroplast and nuclear preparations were resuspended in hypotonic medium (10 mM Tris, pH 7.4, 1.88 mM cysteine.HCl) for 15 min to liberate any residual pool materials, and were centrifuged down (20,000 g, 23 min). RNA polymerase was then assayed with either the ATP or the GTP labelled, as described. The molar ratio of ^{14}C-adenine to ^{14}C-guanine incorporated was 2.10 (4 determinations) for the chloroplasts and 1.54 (4 determinations) for the nuclear fraction, i.e. the incorporation ratio was not significantly affected by this treatment.

The above results suggest that the RNA formed by the chloroplast preparations has a higher ratio of adenine to guanine than the RNA formed by the nuclear preparations. This is in accordance with the known difference in base ratio between the chloroplast and nuclear DNAs (adenine/guanine is 1.67 for chloroplast DNA and 1.54 for nuclear DNA - Kirk,1963).

DISCUSSION

The fact that neither the chloroplast nor the nuclear activity is stimulated by addition of DNA is not surprising since both fractions already have appreciable amounts of DNA. The ineffectiveness of actinomycin in the presence of large amounts of exogenous DNA is presumably due to the drug combining with this DNA (J.M. Kirk, 1960) with a consequent lowering of the effective actinomycin concentration.

The different inhibitions of the chloroplast and nuclear reactions produced by $MnCl_2$ (in the presence of $MgSO_4$), and the difference in the incorporation ratio of adenine and guanine, support the suggestion previously made (Kirk, 1964) that the activity in the chloroplast preparations is not due

to contaminating nuclear material. However, further investigation will be required to establish this with certainty.

In the case of the nuclear preparations there is good agreement between the ratio of adenine to guanine incorporated (mean of all determinations, 1.56) and the adenine to guanine ratio of the DNA (1.54). This agreement may be somewhat fortuitous since the base ratio of the RNA formed might depend upon just which genes in the nuclear fraction are not repressed. In the chloroplast reaction the ratio of adenine to guanine incorporated (mean of all determinations, 2.02) is appreciably higher than the adenine to guanine ratio of the DNA in such preparations (1.67): possibly not all of the DNA in these preparations is active in RNA synthesis.

It is hoped to study in more detail the RNAs formed by the chloroplast and nuclear fractions with a view to determining whether they are, as these findings suggest, different.

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