Light-driven DNA biosynthesis in isolated pea chloroplasts

W. Ronald Mills and Brian J. Baumgartner

Program in Biological Science, University of Houston-Clear Lake, Houston, TX 77058, USA

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Isolated chloroplasts from *Pisum sativum* leaves catalyze [^{3}H]thymidine incorporation into acid-insoluble material using light as the sole energy source. Neither ribonucleotides, deoxyribonucleotides nor deoxyribonucleosides (other than [^{3}H]thymidine) are required. However, it is necessary that the plastids be intact and photosynthetically competent, as little synthesis occurs in lysed preparations or in the presence of photophosphorylation inhibitors. Thymidine incorporation is markedly reduced by the DNA synthesis inhibitors rifampicin, nalidixic acid, ethidium bromide and *N*-ethylmaleimide. Plant age is an important factor, since rates of synthesis are 3–10-times higher in plastids isolated from young plants (6–8 days old) than in chloroplasts from older ones (9–14 days old). The maximum rates using plastids from young leaves of 60–70 pmd/mg chlorophyll/h are 30–60-times greater than those previously reported [1].

Pisum sativum *Pea chloroplast* *Light-driven DNA synthesis* *Chloroplast replication*

1. INTRODUCTION

Although the synthesis of DNA in isolated chloroplasts was first noted in the 1960's [2–6], there has been little subsequent work [1,7,8]. Most studies have required ATP (sometimes plus an ATP-generating system), all the deoxyribonucleotides, $MgCl_2$ and other factors [2–8]. Under these conditions, it is quite difficult to establish unequivocally that DNA synthesis is occurring in the chloroplasts per se and not in nuclei or mitochondria which often contaminate plastid preparations [9]. Fortunately, a less ambiguous system is available; in this case, a nucleoside (for example [^{3}H]thymidine) can be used as the radioactive precursor and light can be employed as the sole energy source to drive DNA synthesis. A similar approach has been used successfully to study various aspects of both protein synthesis [10,11] and RNA synthesis [12] in isolated chloroplasts. A brief report of light-driven DNA synthesis in isolated chloroplasts appeared in 1974 [1]; to our knowledge, additional studies have not been published. We report the light-stimulated synthesis of DNA in isolated pea chloroplasts at rates markedly higher than those previously noted.

2. MATERIALS AND METHODS

Plants (*Pisum sativum* L. var. Little Marvel) were grown as in [13]; leaves from 6–14 day old plants were harvested 30–60 min into the light cycle. Leaf material was homogenized as in [14]. Following an initial centrifugation at 2000 × *g* for 1 min, chloroplasts were prepared as in [13]. Samples were incubated in light with [*methyl*-^{3}H]thymidine according to [11] except the following incubation medium was used: 300 mM sorbitol, 50 mM EPPS buffer (pH 8.2) and 30 mM KCl. Reactions were stopped by addition of an equal volume of 10% (w/v) trichloroacetic acid containing 2% (w/v) sodium pyrophosphate. Samples were extracted using a modification of the procedure in [8] and radioactivity in DNA was determined by scintillation counting. Chlorophyll was determined as in [14], while chloroplast intactness was estimated to be 70–90% using phase contrast microscopy [15] or the ferricyanide method [16]. Biochemicals were obtained from Sigma, and [*methyl*-^{3}H]thymidine (80 Ci/mmol) was supplied by ICN Chemical and Radioisotope Division.

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3. RESULTS AND DISCUSSION

Chloroplasts isolated from 6–14 day old pea shoots were able to incorporate [^{3}H]thymidine into acid-insoluble material using light as the sole energy source, as little synthesis occurred in the dark (fig.1, table 1); stimulation in light ranged from 18–80-fold. Incorporation rates were 5–60 pmol/mg Chl/h; rates began to approach zero after 10–25 min, depending on the preparation (fig.1). Thus, the initial rate (over the linear region) could be 150 pmol/mg Chl/h or more. The highest rates were observed in plastids isolated from young (6–8 day old) shoots with peak activity at day 8 (fig.2); synthetic capacity in chloroplasts from older plants (9–14 days old) was much lower. These data are consistent with those in [17] showing that the level of plastid DNA in intact pea leaves increased until day 8 or 9 and either stabilized or declined thereafter. This may also explain why our maximum rates were 30–60-times those in [1] obtained on light-stimulated DNA synthesis in 10–30 day old spinach plants. Since our data suggest that high rates of DNA synthesis only occur in chloroplasts isolated from young pea shoots, plastids from older spinach leaves may not catalyze incorporation at high rates.

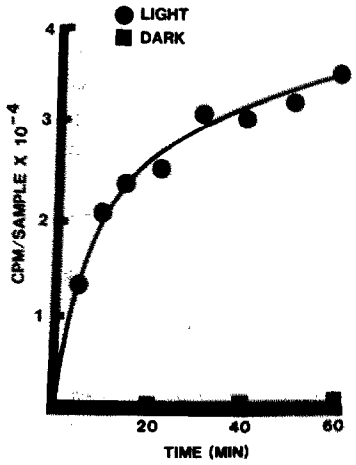

Fig.1. Time course for light-driven incorporation of [^{3}H]thymidine into DNA in isolated pea chloroplasts. A final volume of 2 ml contained 940 μg Chl and 45 μCi [^{3}H]thymidine. At the times indicated, 200 μl aliquots were removed, added to 10% trichloroacetic acid and processed for scintillation counting. Dark tubes were wrapped in aluminum foil.

Table 1

Characteristics of light-driven DNA biosynthesis in isolated pea chloroplasts

Treatment	Thymidine incorporation (% of light control)[a]
Dark	2
Osmotically lysed	4
Triton X-100 (1.0%)	3
DNase (10 μg/ml)	71
RNase (10 μg/ml)	100
DCMU (5 mg/ml)	3
NH_4Cl (10 mM)	4
ATP (10 mM)	109
ATP (10 mM) + $MgCl_2$ (10 mM)	253
Deoxyadenosine (1 mM) + deoxyguanosine (1 mM) + deoxycytosine (1 mM)	33

[a] Control tubes containing 96 μg Chl (0.2 ml final volume) were incubated in light (45 min) in the following standard incubation medium: 300 mM sorbitol, 50 mM EPPS (pH 8.2), 30 mM KCl and 3.3 μCi [^{3}H]thymidine. Mean cpm light control was 52640

Several lines of evidence suggest that synthesis was occurring mainly in intact chloroplasts (fig.1, table 1). First, since broken chloroplasts do not synthesize ATP in the absence of exogenous substrates [18], the use of light as the only energy source essentially ensures that incorporation is occurring in intact plastids. Moreover, ATP alone did not reproducibly stimulate synthesis (table 1), although ATP plus $MgCl_2$ did enhance incorporation. This is not surprising since it was recently shown [19] that MgATP complex stimulates CO_2 assimilation by isolated pea chloroplasts, probably because MgATP more readily penetrates the plastid membrane than ATP alone. Second, broken plastids showed little capacity for DNA synthesis as both osmotically lysed and Triton X-100-treated chloroplasts were inactive. Triton X-100 sensitivity is particularly interesting since it solubilizes chloroplasts but does not disrupt nuclei or bacteria [20]. Since Triton X-100 treatment almost totally blocked synthesis (table 1), nuclear or bacterial contamination was insignificant.

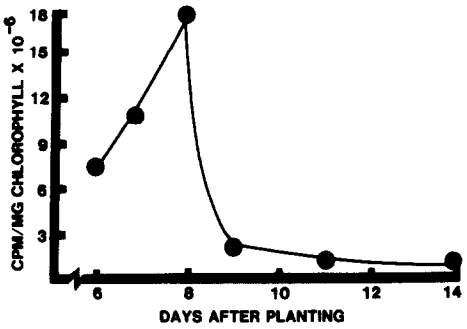

Fig.2. Effect of plant age on light-driven DNA biosynthesis in isolated pea chloroplasts. Plastids were isolated from pea plants of various ages and incubated. Chlorophyll was determined for each plastic preparation and radioactivity incorporated into DNA was expressed on a chlorophyll basis.

Third, when DNase was included in the incubation medium, incorporation was only slightly reduced (table 1). This is the expected result if synthesis occurs in chloroplasts with intact outer membranes, which would prevent exogenous enzymes from penetrating the organelle. By contrast, when chloroplasts were osmotically disrupted after incubation and then treated with DNase, acid-precipitable radioactivity was reduced to near zero (data not shown), indicating that the DNase was active and that most of the radioactivity was indeed in DNA. RNase had little or no effect when included in the incubation medium. Finally, DNA synthesis did not require the addition of exogenous deoxyribonucleotides or deoxyribonucleosides, other than [^{3}H]thymidine. This contrasts with most other studies (e.g., [7,8]) where chloroplastic DNA synthesis was strongly dependent on exogenous deoxynucleotides. However, this result was not unexpected, since intact chloroplasts should retain their stromal components, including deoxynucleotides and deoxynucleosides. A mixture of deoxynucleosides (deoxyadenosine, deoxycytosine and deoxyguanosine at 1 mM each) inhibited thymidine incorporation rather than stimulating it (table 1); the basis for this inhibition is not clear. One possible explanation is competition for uptake between [^{3}H]thymidine and the other deoxyribonucleosides in the mixture.

If light is indeed the sole energy source, incorporation should be quite sensitive to photophosphorylation inhibitors. This was the case, as both the uncoupler NH_4Cl and the electron flow inhibitor DCMU nearly totally blocked activity (table 1).

Table 2

Effect of DNA and protein synthesis inhibitors on light-driven DNA biosynthesis in isolated pea chloroplasts

Treatment	Thymidine incorporation (% of light control)[a]
Dark	2
Ethidium bromide (0.5 mM)	1
Nalidixic acid (1000 μg/ml)	76
N-Ethylmaleimide (8 mM)	2
Rifampicin (100 μg/ml)	38
Cycloheximide (50 μg/ml)	107
D-*threo*-chloramphenicol (50 μg/ml)	135

[a] Control tubes containing 86 μg Chl (0.2 ml final volume) were incubated in light (45 min) in the following standard medium: 300 mM sorbitol, 50 mM EPPS (pH 8.2), 30 mM KCl and 3.3 μCi [^{3}H]thymidine. Mean cpm in light control was 9363

Light-driven thymidine incorporation was reduced by several DNA synthesis inhibitors previously shown to lower ATP-driven synthesis in isolated chloroplasts. The intercalator ethidium bromide and the sulfhydryl group inhibitor *N*-ethylmaleimide were most effective at the concentrations tested, inhibiting incorporation by 99 and 98%, respectively (table 2). These substances were previously shown to inhibit NTP-stimulated DNA synthesis in isolated plastids [7,8]. Nalidixic acid and rifampicin which inhibited DNA synthesis in isolated *Chlamydomonas reinhardii* chloroplasts [7], also reduced incorporation in pea chloroplasts (table 2), although these inhibitors were less effective than ethidium bromide and *N*-ethylmaleimide at the concentrations studied. By contrast, corn plastids were reported to be relatively insensitive to both nalidixic acid and rifampicin [8]. The protein synthesis inhibitors chloramphenicol and cycloheximide did not inhibit thymidine incorporation (table 2).

We have presented data consistent with the idea that chloroplasts isolated from young pea shoots catalyze light-driven thymidine incorporation into

DNA at high rates. Neither exogenous deoxynucleotides nor other energy sources were required, indicating that synthesis occurred in unbroken plastids. Since intact chloroplasts retain their outer membranes as well as their stromal enzymes, cofactors and substrates, it has been suggested [21] that the environment in these plastids is more likely to allow correct initiation, elongation and termination (and thus production of identifiable DNA molecules in vitro) than broken plastids. To date, isolated chloroplasts have not been used widely to investigate plastid DNA synthesis [9], probably because of low activity. Since we have found that intact plastids from young shoots have much greater synthetic capacity, we now believe that they should be useful in studying both the mechanism of DNA synthesis and the regulation of this process in chloroplasts.

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