Short Communication

DNA REPLICATION AND SYNTHESIS OF DNA-BINDING PROTEINS IN THE CHLOROPLASTS OF A CALLUS CULTURE

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Abstract

Continuous labelling of callus with ^{3}H-thymidine results in intermittent peaks of ^{3}H-DNA per chloroplast, showing synchrony of division. The increase in ^{3}H-DNA could be due to several replication rounds, and the drop to successive plastid divisions without intervening DNA synthesis. The level of DNA-binding proteins in the chloroplast parallels the peaks of plastidal DNA synthesis; such proteins could hence comprise factors necessary for plastid multiplication in tissue cultures.

Introduction

Soon after plastid DNA was discovered, Green and Gordon [1] observed that its replication was independent from that of nuclear DNA. Among the specific external influences, light seemed to promote only the actual division of plastids in leaf discs [2] while high sucrose or mannitol was inhibitory [3]. The action of phytohormones was noted, especially in tissue cultures [4,5]. The influence of internal but unidentified factors was seen in the correlation of nuclear DNA quantity with the number of chloroplasts but not with plastid DNA quantity [6] and synthesis [7]. Evidence for cytoplasmic influence on plastid DNA synthesis and division was obtained by the use of specific inhibitors [8,9].

We have followed plastid DNA synthesis in callus cultures. Indeed, an efficient reproduction of plastomes should be ensured during propagation by *in vitro* methods, to avoid a defective photosynthetic apparatus as in the frequent albinism reported in androgenetic cereals. Following Mitchison's [10] suggestion that the appearance of particular proteins during the G phase of a cell cycle could represent specific signals for division, we based our approach on the DNA-binding capacity of many protein factors involved in DNA replication [11]. These proteins could play an important role in the compacting organization of proplastid nucleoids [12,13] and plastid nucleoids [14] (chloroplast DNA protein complex). These could be isolated from plastids as an empirical category using appropriate preparations of DNA-cellulose.

Keywords: Callus culture; Chloroplast division; Chloroplast DNA synthesis; DNA-binding proteins, *Psophocarpus tetragonolobus*

Materials and Methods

Callus tissue from a stem explant of the winged bean (*Psophocarpus tetragonolobus* L) with about 8 to 12 very large chloroplasts of equal size in each cell [15] was maintained on agar medium [16] containing indole-acetic acid (0.2 mg/l) and N_6 benzyl-aminopurine (2 mg/l) at 24°C under an intensity of 1600 lx and a photoperiod of L/D: 16/8. The precursors ^{3}H(methyl)-thymidine (sp. act. 46 Ci/m Mol) and a mixture of ^{14}C-amino acids (mean sp. act. 80 mCi/mMol) were added to the medium during experiments to label DNA and proteins, respectively.

Samples of approximately 15 g of callus were removed on appropriate days and chloroplasts prepared from them by homogenization in buffer (tris-HCl 5mM pH 8.0, sorbitol 30mM, EDTA 3mM, β-mercaptoethanol lmM, bovine serum albumin 0.1%) straining through four layers of cheesecloth, and three cycles of low and high speed

centrifugation (Sorvall, rotor SS 34: 1000 rpm/45 s and 3500 rpm/90 s) and further purification in a two-phase partition system containing 5.8% polyethylene glycol 4000, 5.8% dextran T 500, 30mM sorbitol, 5mM KCl and 5mM $(K)PO_4$ buffer at pH 7.4 [17]. Intact chloroplasts from the interphase were diluted in excess homogenization buffer and repelleted. Aliquots of this preparation were taken for counting the number of chloroplasts.

Both DNA and DNA-binding proteins were extracted by a technique enabling their separation from the same sample of chloroplasts. Chloroplasts were lysed by dialysis against low osmolal buffer (tris-HCl 20mM pH 7.4, NaCl 50mM, EDTA lmM, β-mercaptoethanol lmM) for 1 h, the dialysate brought to 1.7M NaCl and 15% polyethylene glycol 6000, and centrifuged (8000 rpm/20 min) to separate the proteins in the supernatant from the DNA in the pellet.

DNA in the pellet was resuspended in dialysis buffer, deproteinized by repeated extraction against buffer-saturated phenol and finally against chloroform, brought to 0.3M KOH and incubated at 37°C for 18 h and precipitated in cold 10% TCA on to glass fibre discs (Whatman GF/C) whose ^3H radioactivity was counted in a scintillation mixture (Amersham, PCS).

The DNA-binding proteins in the supernatant which could be solubilized by 1.7M NaCl, were dialysed extensively against lysis buffer and isolated by passage over a column (3 ml bed volume) of DNA-cellulose [18] prepared from double-stranded calf thymus DNA (1 mg DNA fixed per g moist cellulose). After washing, the column was eluted in a minimal volume of 2.5M NaCl and the total eluate counted for ^{14}C in a scintillation mixture.

Results

Callus was cultured on medium containing ^3H-thymidine and ^{14}C-amino acids. Samples of callus were removed at regular intervals during the culture period, which for this tissue is normally about 30 days before transfer, and their chloroplasts were isolated. ^3H and ^{14}C radioactivities were measured respectively in plastid DNA and in the DNA-binding protein fraction extracted from the same preparation of chloroplasts, and expressed in terms of the number of chloroplasts isolated from each sample. A suitable sequence of sampling was determined from preliminary experiments over various periods (6 to 28 days of culture) and different intervals (daily to every 5 days) and followed in the experiment illustrated in Figure 1.

Discussion

Plastids may be distributed inhomogenously in a callus. This may be disregarded by considering the whole plastid population as one entity, but nevertheless undergoing certain physiological constraints inherent to development *in vitro*.

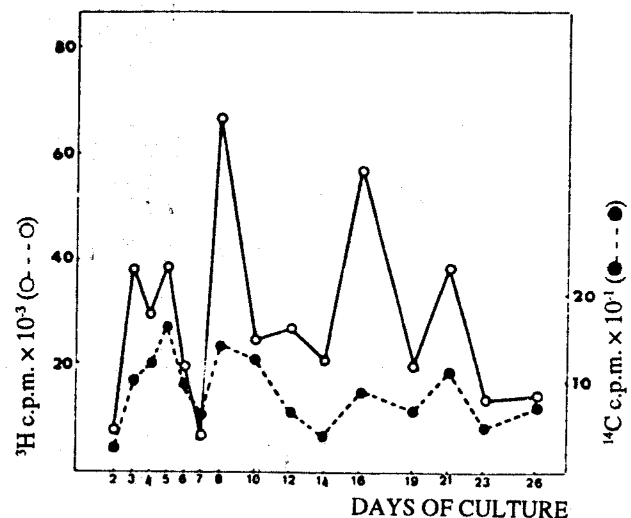

Figure 1. Synthesis of DNA (O- - - O) and DNA-binding proteins (●- - - ●) in chloroplasts of winged-bean callus tissue. Radioactivity is expressed in terms of the number of chloroplasts isolated, which ranged from 3 to 12×10^6 for each sampling. Values indicated are per 10^6 chloroplasts. ^3H counts correspond to plastid DNA and ^{14}C to binding-proteins.

DNA synthesis in a synchronous system is recorded as intermittent peaks after pulse-chase labelling with precursor and as a stepwise profile after continuous labelling [10]. The intermittent peaks we have obtained after continuous labelling can however be interpreted when the radioactivity is expressed per chloroplast; they should represent an increase in the genome copies of a single organelle. The latter then undergoes one or more successive divisions resulting in daughter plastids with fewer copies and a drop in the radioactivity to a basal value. Thus, in the higher peaks of Figure 1, corresponding to an approximately eight-fold increase in radioactivity, three successive divisions would suffice to come down to the basal copy number per plastid.

Chloroplast genomes have already been reported to be highly polyploid, meaning that they often consist of several copies of the circular DNA molecule [19,20,21]. However, during normal development of buds, estimation of level of DNA in chloroplasts gives constant values [22], that is, plastid division ensues closely upon DNA synthesis in an asynchronous system.

DNA synthesis and organelle division obviously alternate in the system we have studied, but there is no direct coupling in the sense that each replicative doubling is not necessarily followed by immediate division as in most other systems. Indeed, plastid division has been shown to

be quite indifferent to the accomplishment of a synthetic phase since illuminated leaf-disc etioplasts, whose DNA synthesis was inhibited by FdUR, still underwent two divisions [5]. Plastids could apparently divide more than twice without intervening DNA synthesis if they already possessed a multi-copy genome. Active plastid DNA synthesis in cultured tobacco cells has been found to occur only during the first day of culture, increasing the copy number of plastid DNA per cell, which then decreased gradually with successive plastid and cell divisions [23]. Inversely, a partial loss of DNA provoked by nalidixic acid does not affect subsequent plastid division [24]. An increase of ploidy in chloroplasts [25] is also attributed to a lack of coupling between DNA synthesis and division, while light, which prevents polyploidy, does so by promoting plastid division [22]. Nevertheless, the plastid DNA content is related to the plastid differentiation state and is not affected by light [26].

Synchrony of plastid DNA synthesis in winged-bean callus tissue is clear from our results. Its interval is seen to gradually increase with the age of the culture. However, synchrony of plastid division has not been reported in previous studies of excised leaf discs [2,3,27]. Our present observations should nevertheless be viewed in the context of callus development *in vitro*. In fact, the first three nuclear divisions and their concomitant DNA synthesis are partially synchronous when Jerusalem artichoke explants are cultured *in vitro* [28]. Since the developmental context of winged-bean callus cultures is more likely to resemble Jerusalem artichoke explants rather than leaves or leaf discs, we presume that plastids of green callus tissue also respond to the trauma of excision and the stimulus of transfer to fresh medium by exhibiting initial synchrony.

A majority of proteins functioning in chloroplasts are imported although a chloroplast-specific γ-polymerase [29] and a topoisomerase [30] have been isolated. Several DNA-binding proteins of *Nicotiana tabacum* plastids have also been isolated and classified into two types according to their solubilization in 2M NaCl [14]. Our DNA-binding proteins fraction, as isolated by solubilization in 1.7M NaCl, could be regarded as a "loosely bound"-type of *Nicotiana tabacum* plastid DNA-binding proteins. The labelling profile of the DNA-binding proteins of chloroplasts, as determined by us, also parallels that of plastid DNA synthesis and division to a great extent. However, the resolution of the curve of protein-labelling is not fine enough for us to choose with any certitude between its correlation with the increase in copies of the plastid genome or the actual division of plastids. Perhaps the use of more specific DNA's on the cellulose support would yield finer and more significant correlations. The present results point to the interest of looking into the pool of chloroplast proteins possessing DNA affinity for factors influencing plastid multiplication in tissue cultures.

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