DNA methylation in dedifferentiating plant pith tissue

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Summary. A dedifferentiating system in plant tissue (*Nicotiana glauca* pith tissue grown 'in vitro') is described, where DNA amplification was demonstrated both cytologically and biochemically. In this system a DNA satellite is synthesized and reaches its maximum around 48 h of culture. This satellite is highly methylated and there are 2 methylation peaks around 24 and 72 h of culture. The results are discussed in relation to recent evidence of the involvement of methylation in differentiation processes.

5-Methylcytosine (m^5Cyt) has been detected in animal[1] and plant DNA[2-4], the highest content being localized at the level of satellite DNAs[5,6] and highly repeated sequences[7,8], in comparison with main band DNA and unique sequences. The functional significance of DNA methylation in eukaryotic cells is still unclear. As stressed by several authors[9-11], the distribution of m^5Cyt could be involved in the fine regulation of gene expression and differentiation. Looking at this interesting hypothesis, we have studied the incorporation of the labeled methyl group in a dedifferentiating plant system which has been under investigation for several years in our laboratory. *Nicotiana glauca* pith tissue grown 'in vitro' and allowed to dedifferentiate under controlled conditions seems to us to be a particularly suitable test system for a preliminary approach to methylation studies during phase change from a differentiated stage to a dedifferentiated one.

In the 1st 72 h of culture of the primary explant, the pith tissue shows an extra DNA synthesis partially localized around nucleoli, followed by DNA extrusion, nuclear fragmentation and formation of meristematic centers[12]. Moreover, microdensitometric and autoradiographic analysis of cell proliferation confirmed the occurrence of an early DNA amplification process during the 1st days of culture[13]. This period is characterized by a transient DNA synthesis of a heavy satellite DNA ($\rho = 1.722$ g/ml)[14], whose function seems to be strictly correlated with dedifferentiation processes, as evidenced by BrdU incorporation experiments[15]. In relation to the above-mentioned results, we report here a preliminary study on DNA methylation patterns during the 1st dedifferentiating phases.

Materials and methods. *Nicotiana glauca* pith tissue was aseptically grown on agar medium[16] supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 0.4 mg/l). The tissue was collected at different culture times (0, 24, 48, 72, 96, 120, 196 h) and transferred to the same liquid medium containing [methyl-^{3}H] methionine (30 µCi/ml, sp. act. 15 Ci/mmole, from the Radiochemical Centre, Amersham): for each culture time at least 5 tissue samples (1 g each) were transferred into 15 ml of the liquid medium. DNA extractions were carried out as previously described[14]. DNA solutions were ultracentrifuged on CsCl preparative gradients in the Beckman rotor 30 at 25,000 rpm for 4 days at 20 °C; afterwards, fractionation was carried out under continuous recording at 260 nm. With the extraction technique used, the average DNA recovery is of the order of about 100 µg per g of tissue fresh weight. Each fraction was analyzed for OD and refractive index; radioactivity was checked in a liquid scintillation spectrometer.

In order to avoid protein contamination, separately pooled fractions containing satellite and main peak DNAs respectively were pelleted at 35,000 rpm in a Beckman rotor 40 for 20 h after correction of the refractive index of the CsCl solution to a value of 1.381. After solubilization in $0.1 \times$ SSC, the fractions were used for spectrophotometric and radioactivity measurements. Analytical ultracentrifugation was performed in the Spinco Model E ultracentrifuge at 44,000 rpm for 20 h at 25 °C using as a reference marker the DNA from *Bacillus subtilis* phage $_e$ ($\rho = 1.742$ g/ml).

Results and discussion. An example of the densitometric profiles after CsCl preparative and analytical ultracentrifugation is shown in figure 1. As previously reported[14], a heavy satellite ($\rho = 1.722$ g/ml) appears in the early h of culture, besides a main band DNA ($\rho = 1.695$ g/ml) and a constantly-present additional satellite ($\rho = 1.706$ g/ml). The pattern of ^{3}H-m^5Cyt labeling at 48 h of culture is shown after CsCl preparative gradient (fig. 2). The amount of the 1.722 g/ml satellite DNA reaches a maximum around 48 h (about 15% of total DNA) as evidenced in figure 3.

The specific activity of satellite DNA from tissue cultures of *Nicotiana glauca* labeled with [methyl-^{3}H] methionine for 2 h is reported in figure 3; 2 methylation peaks are present around 24 and 72 h. Higher methylation of heavy satellite DNA compared with that of main peak DNA, comprising 1.706 g/ml satellite, has been observed; as shown in the table, the sp. act. ratio between satellite and main peak band DNA ranges from 1.72 to 2.48. Tentative-

Incorporation ratio of ^{3}H-methyl group into DNA

Hours of culture	0	24	48	72	96
Specific activity ratio between satellite and main band DNA ± SE	2.48 ±0.46	1.80 ±0.08	2.14 ±0.04	1.89 ±0.06	1.72 ±0.02
Number of analyzed fractions	5	5	6	5	5

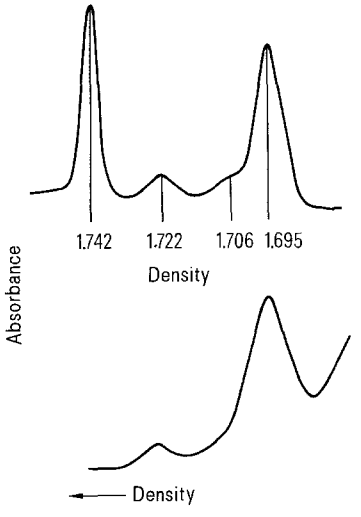

Figure 1. Densitometric profiles after preparative (lower tracing) and analytical (upper tracing) CsCl ultracentrifugations.

ly, a rough estimate of ^{3}H-methyl group incorporation in the 1.706 satellite DNA as compared to main peak DNA was carried out and we obtained a mean value around 1.5. The specific activity ratio between satellite and main peak DNA suggests preferential satellite methylation. It is worth noting that the G-C content ratio between satellite and main peak DNA is 1.74, so the high labeling ratio could be explained by the high G-C content of the heavy satellite. ^{3}H-thymidine incorporation experiments on the same material and culture conditions showed generalized labeling of the same extent both in satellite and main peak DNA[17]. Taking into account these results, satellite methylation could play an important role in the processes of DNA differential synthesis observed at the cytological level[12,13].

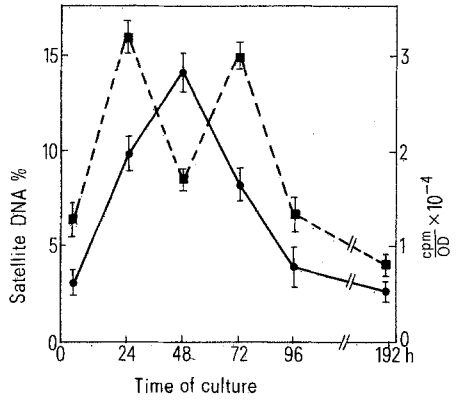

Figure 2. Absorbance (solid line) and radioactivity (dashed line) profiles after CsCl preparative ultracentrifugation of DNA from 48 h pith culture.

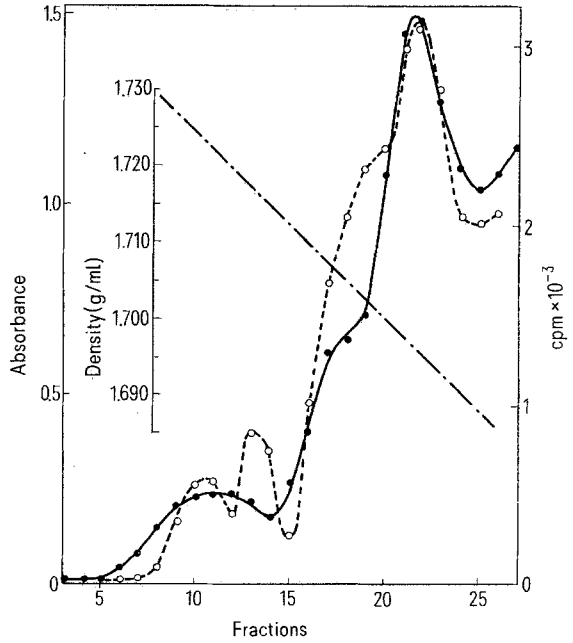

Figure 3. Percent of satellite DNA (solid line) in comparison with total DNA at different culture times. The dashed line represents the sp. act. of ^{3}H-m^{5}Cyt labeled satellite. Vertical bars represent the SE: the number of analyzed fractions at each point is: 8 (0 h); 8 (24 h); 7 (48 h); 6 (72 h); 4 (96 h); 5 (192 h).

In this context the lowering of ^{3}H-methyl group incorporation after 48 h of culture (about one half in comparison with 24 h) seems to parallel the massive RNA synthesis (particularly rRNA) observed at the same time of culture[18]. These results seem to be in agreement with the specific undermethylation observed on DNA from wilt-infected cotton plants as compared to DNA from noninfected plants[3].

The relationships between specific DNA amplification and methylation on one side and dedifferentiation on the other introduce some interesting problems, since the phenomenon of nuclear fragmentation followed by mitosis seems to be a general process in the induction of 'in vitro' cell proliferation[19].

It is worth noting that the amplification process of the heavy satellite DNA and consequently the high extent of satellite DNA methylation may be due to the presence of 2,4-D in the culture medium as found by Nagl and Rucker[20] in Cymbidium; on the other hand it is not possible in our system to analyze a control without phytohormones or phytohormone-like substances, since the pith tissue does not proliferate in a hormone-free culture medium. The only indirect evidence that 2,4-D can affect the methylation pattern could be the fact that habituated callus (i.e. normal hormone dependent callus that acquires at very low frequency the ability to proliferate in a hormone-free medium) presents a lower level of DNA methylation in comparison to normal callus, as evidenced by Msp I and Hpa II restriction enzymes analysis (unpublished results). Experiments are now in progress to check whether different phytohormone concentrations or other phytohormones can affect the pattern of DNA methylation.

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