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Reversible biophysical changes of DNAs from *in vitro* cultured non-tumour cells

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We have previously shown [1, 2] that DNA from plant crown-gall tumour tissues is highly relaxed and thereby very susceptible to various DNA-destabilizing agents which, in contrast, do not affect DNA from healthy tissues. *In vitro* DNA strand separation (chain unpairing), the rate of *in vitro* DNA synthesis and *in vivo* increase in tumour cell multiplication are closely correlated [3–5].

Here we report that plant non-tumour cell DNA may temporarily undergo conformational changes when these cells are cultured *in vitro* in the presence of opines (octopine, nopaline, lysopine which accumulate in crown-gall tissues [6]) or dl-ethionine (a known carcinogen in mammalian tissues [7]). The induced DNA double-strand destabilisation and consequent increase in template activity are shown to be reversible under our experimental conditions.

Materials and methods: Deoxyribonucleoside-5'-triphosphates dXTP [^{12}C] and dTTP [^{3}H]-lithium salt (sp.act. 25 Ci/mol) were obtained from Amersham, UK. Octopine (oct), nopaline (nop) and α-naphthalene acetic acid (NAA) came from Sigma Co., USA. Lysopine (lys) was synthesized by the organic chemistry laboratory, Institut Pasteur, Paris. Kinetin (kin) was from Fluka A.G., Switzerland, dl-ethionine from Hoechst, Germany, para-aminobenzoic acid from Eastman Kodak Co., USA, myoinositol from Prolabo and biotin and thiamine from Serlabo, France.

The plant tissue used was a cambium line isolated from birch trunk (*Betula verrucosa* Ehrh.). Hand-cut sections of fresh stock (Fontainebleau forest) were aseptically isolated according to the procedure of Gautheret and Morel [8]. Explants were cultured in tubes containing 25 ml of basic Nitsch medium [9] supplemented with 5×10^{-4} M myoinositol, 3×10^{-6} M thiamine, 4×10^{-7} M biotin, 7×10^{-6} M para-aminobenzoic acid, 5×10^{-6} M NAA and 20 g/l glucose and solidified with 0.7% Difco agar. Nutrient medium pH was adjusted to 5.8 before autoclaving (15 lb pressure for 20 min). The explants kept at 25 ± 2°C under continuous fluorescent light (6.7 W/m^2) were transferred onto fresh medium every 45 ± 5 days and tested for the absence of bacterial contamination. After 6–7 successive transfers (stabilized strain), calluses were transferred onto medium either not containing (–) (control) or containing (+) (step 1) octopine (3.2×10^{-5} M final concentration), nopaline (2.6×10^{-5} M final concentration) or dl-ethionine (6.1×10^{-6} M final concentration) (24 calluses for each condition). All substances were dissolved in distilled water, sterilized by Millipore filter and added to the sterile medium. NAA was omitted in order to avoid interference between auxin and tested substances; birch explants are by nature able to grow for a certain length of time on culture medium which does not contain auxin. Six or eight samples of tissue were removed after 35 days and immediately used for DNA extraction (control and step 1). The remaining explants (from step 1 set) were then transferred onto octopine (or nopaline, or dl-ethionine) (–) medium (step 2) for one 21-day period, at the end of which they were used for DNA extraction. Each culture was duplicated and DNAs of the same type were pooled.

DNAs were isolated and purified in the presence of a standard saline citrate solution (2 × SSC) as previously described [2]. Only those purified DNA preparations (absorbance 260/280 nm = 2.0) having a hyperchromic effect (40–50% in the presence of 0.2 M KOH at 260 nm) were used. Conditions for measurement for *in vitro* DNA chain relaxation (hyperchromicity) and contraction (hypochromicity) were as follows [2]. UV absorbance at 260 nm of treated and control tissue DNA (10 μg in 1 ml of Tris-HCl buffer 10^{-2} M pH 7.65) was measured before and after addition of increasing concentrations of compounds (opines, plant hormones, carcinogen). The blank cuvette contained the equivalent amount of the same compounds. The results were expressed as UV absorbance percent increase. The incubation conditions with all compounds required for *in vitro* DNA synthesis have been described previously [2, 10]. The synthesized product is measured as [^{3}H]-TNP (cpm × 10^{-3}) in acid-precipitable material using treated and control tissue DNA as template. Analyses previously carried out have shown that the *in vitro* effect of tested substances on DNA chain separation depends on the duration of plant culture on (+)

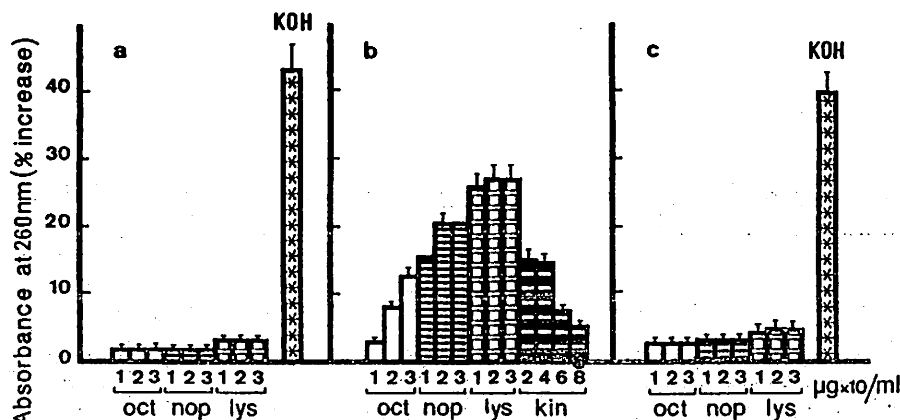

Figure 1: Birch trunk cambium non-tumour cell DNA in vitro *chain opening and closing. Effects of octopine, nopaline, lysopine and kinetin on DNAs isolated from tissues cultured on octopine (–) medium (a: control), on octopine (+) medium (b: step 1), on octopine (–) medium (c: step 2). UV absorbance (expressed in % increase) of DNAs was measured in the absence or presence of different concentrations of the compounds successively added to the blank and the DNA solution (see [5] (bars indicate ± SEM; n = 3).*

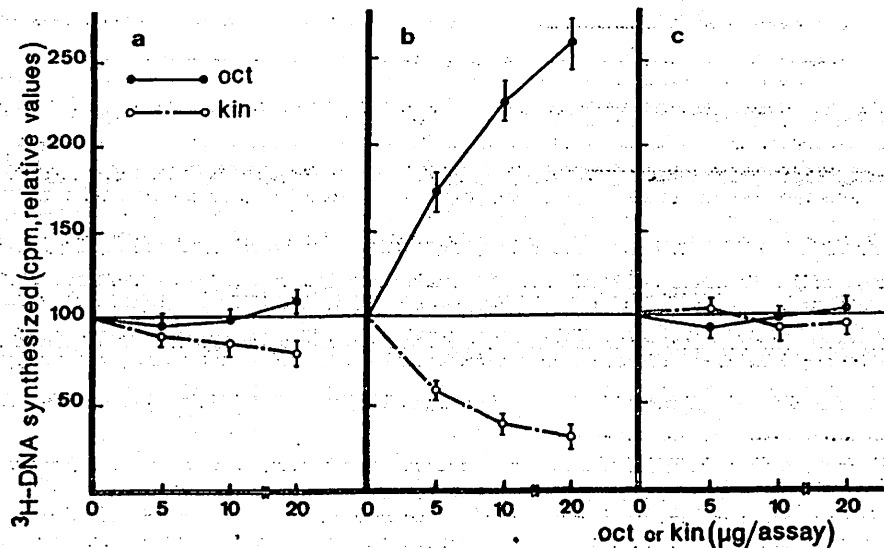

Figure 2: Effects of octopine (●——●) and kinetine (○– · –○) on in vitro *DNA synthesis. DNA originated from calluses cultured on octopine (–) medium (a: control), calluses cultured on octopine (+) medium (b: step 1), calluses recultured on octopine (–) medium (c: step 2). Each type of DNA was used as a template (see [3]) in the absence or presence of compounds as indicated. Results are expressed relative to the values observed in the absence of octopine or kinetin in the incubation mixture (bars indicate ± SEM; n = 3).*

medium: it is about twice as large for 35 days of culture as for 15 days. The working concentrations were shown to be optimal for observation of DNA relaxation in the concerned tissues. The *in vitro* experiments were repeated three times and statistical significance of differences between control and experimental values was assessed by Student's *t* test.

Results and discussion: DNA isolated from plant non-tumour cells cultured on standard medium (without NAA) never exhibited increase in *in vitro* UV absorbance in the presence of octopine, nopaline or lysopine (Figure 1a). In the presence of 0.2 M KOH used as a reference, this same DNA undergoes complete strand separation. In contrast, DNA extracted from cells cultured on octopine (+) medium underwent, *in vitro*, a progressive increase in UV absorbance in the presence of octopine (Figure 1b) and this effect was potentiated by nopaline or lysopine. L-arginine (octopine precursor) or L-lysine (nopaline precursor) had no effect on chromicity. Kinetin induced hypochromicity of this DNA (Figure 1b) as observed with plant tumour DNA [2, 11]. The antagonism between both plant hormones kinetin and indole-3-acetic acid (IAA) on *in vitro* crown-gall DNA structure and activity [2] was verified *in vivo*: crown-gall pea cells treated with kinetin were not susceptible to further growth stimulation by IAA (unpublished results).

Since octopine when present in the culture medium induced DNA relaxation, better template activity of DNA isolated from calluses cultured on octopine (+) medium was to be expected. The amout of radioactive acid-precipitable product synthesized on this calluses DNA was indeed always higher (Figure 2b) than the amount synthesized on DNA isolated from control tissues (Figure 2a). That DNA from octopine-targeted non-tumour tissue is destabilized was further demonstrated by antagonistic effects induced *in vitro* by octopine or kinetin: hyperchromicity and increase of template activity in the presence of octopine; hypochromicity and decrease of template activity in the presence of kinetin. These effects were the same as those observed when tumour DNA is assayed *in vitro* [2, 11].

However, the most striking observation of the present study was that DNA from calluses which had been transferred from an octopine (+) to an octopine (–) medium was no longer sensitive to octopine, nopaline or lysopine as evid-

Table 1: In vitro *DNA strand separation after successive incubations with nopaline plus octopine or with dl-ethionine. DNAs derived from trunk cambium non-tumour cells cultured on medium containing (+) or not containing (–) nopaline or dl-ethionine (results are expressed in % increase relative to UV absorbance of DNA (10–15 µg) measured in the absence of compound; means ± SEM; n = 3)*

Amount of compound added to incubation mixture (µg/assay)		Culture medium		
		Nopaline (–) (control)	*Nopaline (+) (step 1)*	*Nopaline (–) (step 2)*
No compound		0	0	0
+ Nopaline	10	2.2 ± 0.4	8.0 ± 0.5**	1.7 ± 0.7
	20	4.0 ± 0.5	18.0 ± 0.5**	2.7 ± 0.1
	30	7.8 ± 0.5	25.0 ± 0.5**	3.9 ± 1.0
+ Octopine	10	8.0 ± 0.5	30.0 ± 0.9**	5.2 ± 1.1
	20	7.8 ± 0.1	32.7 ± 1.0**	7.6 ± 1.3
	30	7.5 ± 0.6	35.5 ± 0.6**	8.4 ± 1.4
		dl-Ethionine (–) (control)	*dl-Ethionine (+) (step 1)*	*dl-Ethionine (–) (step 2)*
No compound		0	0	0
+ dl-Ethionine	10	3.8 ± 0.1	4.3 ± 0.3	1.9 ± 0.4
	20	4.1 ± 0.2	6.7 ± 0.5*	1.8 ± 0.6
	30	4.3 ± 0.3	14.8 ± 0.4**	1.9 ± 0.6

*In comparison with control values, p <: *0.01; **0.001.*

Table 2: Effects of nopaline and dl-ethionine on in vitro *synthesis of DNAs derived from trunk cambium non-tumour cells cultured on medium containing (+) or not containing (−) nopaline or dl-ethionine. Each type of DNA (0.5–1.0 μg) was used as template in the absence or presence of increasing concentrations of nopaline or dl-ethionine (results are expressed in cpm: means ± SEM; n = 3).*

Amount of compound added to incubation mixture (μg/assay)		Culture medium		
		Nopaline (−) (control)	*Nopaline (+) (step 1)*	*Nopaline (−) (step 2)*
No compound		1082 ± 24	1134 ± 32	883 ± 55
+ Nopaline	5	1024 ± 37	1580 ± 89**††	919 ± 10
	10	1022 ± 33	1234 ± 46**	939 ± 10
	20	924 ± 22	1045 ± 26*	889 ± 50
		dl-Ethionine (−) (control)	*dl-Ethionine (+) (step 1)*	*dl-Ethionine (−) (step 2)*
No compound		795 ± 34	1042 ± 23**	936 ± 54
+ dl-Ethionine	5	818 ± 52	1544 ± 112**††	934 ± 33
	10	812 ± 19	1489 ± 112**†	797 ± 30
	20	732 ± 26	1094 ± 84*	841 ± 18

In comparison with no compound values, p <: †*0.02;* ††*0.01. In comparison with control values,* p <: **0.02;* ***0.01.*

enced by their non-response *in vitro*. When DNA came from calluses grown first on octopine (+) medium for 35 days, then subcultured on octopine (−) medium for 21 days (these being the optimal duration for maximal expected effects), we observed, respectively, (Figure 1c and Figure 2c) a similar amount of DNA chain opening and DNA synthesis to that obtained with control DNA from untreated calluses (Figure 1a and Figure 2a). The enhancement of DNA strand separation and DNA template activity produced in these non-tumour cells by octopine temporarily present in their culture medium was thus a reversible process.

Similar results were obtained when octopine was replaced in the culture medium by nopaline or by the carcinogen dl-ethionine (Tables 1 and 2).

In previous experiments [1, 2, 5], it was shown that octopine increases *in vitro* over-relaxation of crown-gall DNA and *in vivo* crown-gall cell multiplication. In the present work, the effect of octopine, nopaline and dl-ethionine added in the culture medium on the *in vitro* behaviour of DNA from plant non-tumour cells is significant although *in vivo* growth on agar medium is not significantly changed. In this latter respect, cultured tissue of a woody species may respond differently from tissue of a herbaceous species.

The finding that *in vitro* DNA from birch cambium grown on octopine (or nopaline or dl-ethionine) (+) or (−) medium could undergo strand-relaxation or strand-contraction indicates that the structure of this DNA is apparently close to that of a "pre-cancerous" DNA [1]. It is possible that these substances, if permanently present in the culture medium of plant non-tumour tissue, may contribute to irreversible changes in these cells. Our results on DNA chain opening and closing corroborate recent findings that auxin temporarily alters gene expression which results in transitory synthesis of specific proteins in excised plant seedlings [12].

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