Experimental Cell Biology
Editors: J.R. Rüttner, Zürich; G.V. Sherbet, Newcastle upon Tyne;
H. Ramseier, Zürich; L. Helson, New York, N.Y.; A. Wolsky, New York, N.Y.

Reprint
Publisher: S. Karger AG, Basel
Printed in Switzerland

Expl Cell Biol. *53:* 335–350 (1985)

0304–3568/85/0536–0335$2.75/0

The in vitro Effects of Opines and Other Compounds on DNAs Originating from Bacteria, and from Healthy and Tumorous Plant Tissues

Liliane Le Goff, Mirko Beljanski

Laboratoire de Pharmacodynamie, Faculté des Sciences biologiques et pharmaceutiques, Châtenay-Malabry, France

Key Words. Opines · DNA synthesis · DNA conformation · Auxin · Tumorous state · Gene expression · *Agrobacterium tumefaciens* · Crown gall

Abstract. Purified total DNAs were isolated from oncogenic or nononcogenic *Agrobacterium tumefaciens* cells as well as from normal and crown gall tissues. Opines (octopine, nopaline, lysopine), plant hormone (auxin IAA) and some carcinogenic compounds were used in order to correlate their effects on in vitro strand separation and synthesis of DNAs with in vivo tumorous cell multiplication. Octopine (or nopaline) induced chain opening of DNAs originating from octopine (or nopaline)-metabolizing bacteria and from same bacteria strain-induced tumorous cells. This phenomenon was measured by the increase in DNA hyperchromicity which is concentration dependent. The tested compounds stimulated the in vitro synthesis of the same DNAs. Under the same conditions, in vitro strand separation and synthesis of healthy plant DNA was not (or only slightly) enhanced, except in the case of particular hormone-connected healthy cell DNA. IAA and carcinogens stimulated in vitro synthesis and induced in vitro strand separation (dose-dependent effect) of DNAs isolated from crown gall cells and inducing bacteria. Compared to healthy cell DNAs, these DNAs were thus susceptible to structurally very diversified molecules and in this way behave as do mammalian tissue DNAs. The opine and IAA actions observed here were specific for plant tissue DNA; cancerous human or animal tissue DNAs were insensitive. By their presence in the crown gall cells, opines possibly maintain destabilized areas (required for rapid growth and division) on tumor cell DNA. The cooperative actions of IAA and opines as well as small RNA and RNA fragments on gene activation, might explain the autonomy of plant tumor cells.

Introduction

Unusual amino acids called opines are synthesized in crown gall tumors induced by the bacteria *Agrobacterium tumefaciens* [37, 42]. Lysopine, octopine and nopaline were identified first [10, 19, 35] but in the meantime many other opines have been discovered. Detailed reviews were recently published on this subject [14, 16, 17, 23, 38]. A given *A. tumefaciens* strain has the property of inducing tumors which specifically synthe-

size the type of opine(s) which may be metabolized by this bacterial strain [32, 41]. A great deal of research has been carried out to try and to correlate crown gall induction in plants and the capacity of these tumorous tissues to synthesize specific opines [18, 46]. Whereas these compounds are characteristic of most crown gall tumors, their biochemical role in such tissues is unknown. It was of great interest to ascertain that octopine may stimulate the multiplication of crown gall tissue while the amino acid from which it derives was inactive [15, 31, 47].

We have previously shown the close correlation that exists between DNA chain opening, enhancement of DNA in vitro synthesis and acceleration of in vivo cell multiplication [7, 27, 28]. That correlation was most obvious in the case of cancer mammalian cells which are known to synthesize special proteins, mainly embryonic proteins [22]. Thus we were particularly interested in looking for a possible relation between DNA secondary structure relaxation, enhancement of tumor cell DNA in vitro synthesis and tumor cell multiplication in vivo. In fact, we present here the very close and selective effect of each metabolite-dependent bacterial strain on the DNA isolated from the corresponding induced tumor. In addition, the same interdependence may also be evidenced between the metabolized tumor opine and the effect on the DNA of the *A. tumefaciens* strain used for its induction.

Material and Methods

Chemicals

Pancreatic RNase A (4 × crystallized) was purchased from Worthington Co, Freehold, N.J., USA. Deoxyribonucleoside-5'-triphosphates (d-XTP) ^{12}C and ^{3}H-lithium salt (specific activity 25 Ci/mol) were obtained from Amersham, UK. *L*-Arginine and *L*-lysine were products of Hoffmann-La Roche, Basel, Switzerland. Octopine and mitomycin C were purchased from Sigma Co, St Louis, Mo., USA. Nopaline was kindly supplied by Dr. *L. Firmin*, John Innes Institute, Colney Lane, Norwich, UK. Lysopine was synthesized by the organic chemistry laboratory at the Institut Pasteur. 9,10-Dimethyl-1,2-benzanthracene (DMBA) was a product of Nutritional Biochemicals Co, Cleveland, Ohio, USA. Daunorubicin was supplied by Rhône-Poulenc, Ivry, France. Cyclophosphamide (Endoxan) was obtained from Laboratoire Lucien, Colombes, France. Indolacetic acid was from Prolabo, France. Sodium lauryl sulfate was from Serlabo, France and 8-hydroxychinolin from Merck, FRG. Phenol used was from Baker Chemicals, Deventer, The Netherlands.

Bacterial Strains and Culture Media

The sources of the *A. tumefaciens* used are listed in table I. Bacterial cultures were obtained by suspending a single colony in 5 ml of sterilized NB medium (Nutrient broth, Difco, 8 g/l; NaCl 5 g/l) and cultured by shaking for 24 h at 28 °C. This was followed by subculture in the same fresh medium overnight. Bacteria were harvested by centrifugation during the logarithmic phase and the pellet washed with a physiological serum solution. The stock of harvested bacteria was immediately used or frozen at −20 °C.

Healthy and Tumorous Plant Material

Two-day-old etiolated decapitated epicotyls of *Pisum sativum* L. (cv. Annonay) were experimentally infected with *A. tumefaciens* tumorigenic strains B6 and C58 as already described [24] and crown gall tissues used to prepare DNA. Light-grown pea shoots (2 weeks old) were used to extract healthy DNA. Callus tissues from *Datura stramonium* (var. Tatula) inverted stem sections cultured in vitro and apical segments of Datura stems from greenhouse plants were also used for the isolation of healthy tissue DNA. All these tissues were either immediately used or frozen at −20 °C.

Isolation and Purification of Bacterial and Plant DNAs

Bacterial cells were homogenized in sterile distilled water and lysed in the presence of sodium lauryl sulfate (2% final concentration). A phenol solution containing 0.2% of 8-hydroxychinolin was added (v/v) and the mixture shaken for 10 min at 4 °C. After centrifugation at 5,000 *g* for 10 min the upper phase was saved. The interphase was retreated in the same way and centri-

Table I. *A. tumefaciens* strains used

Strains	Sources	Characteristics
Octopine-type		
B6	1	[41, 43]
B2	1	[41, 43]
A6	1	[41, 43]
Nopaline-type		
T37	1	[41, 43]
IIBV7	1	[41, 43]
42 IV	1	[41]
A. delft	2	[41]
C58	3	[13, 43]
A 114	3	[13, 43]
A 115	3	[13, 43]
Octopine/nopaline-type		
A 66	1	[41]
A. rubi	3	[41]
AT 181	4	[32, 43]
EU 6	4	[32, 43]
nul (octopine/nopaline)-type		
AT1	4	[32]
AT4	4	[32, 43]

1 = *P. Manigault*, CNRS, Gif-sur-Yvette, France; 2 = *A. Faivre-Amiot*, INRA, Versailles, France; 3 = *E.W. Nester*, University of Washington, Seattle, USA; 4 = *J.A. Lippincott*, Northwestern University, Evanston, Ill., USA.

fuged. The upper phases were mixed and then treated twice with chloroform-isoamylic alcohol (24:1, v/v). DNA was precipitated with 2 vol of 96% alcohol and dissolved in a 0.2 saline standard solution (SSC). RNA-contaminating DNA preparations were eliminated by incubation with 20 µg/ml of RNase A (previously heated for 10 min at 95 °C to remove DNase activity) for 30 min at 36 °C. RNase was then removed by phenol and three chloroform treatments. DNA was precipitated with 2 vol of alcohol, dissolved in a 2SSC solution and dialyzed against the same solution for 24 h at 4 °C. Purified DNA was stored at −20 °C. Crown gall and healthy tissues from pea and Datura were crushed with a masticator in a minimum volume of Lerman buffer pH 8.0 [30] supplemented with 8-hydroxychinolin and lauryl sulfate (concentrations mentioned above) and incubated for a few minutes at 37 °C. The plant fragments were then cautiously broken down in a Potter and the supernatant was treated in the same way as the bacterial cells.

The amount of purified DNAs dissolved in the 2 SSC solution was determined by absorbance at 260 nm (260/280 = 2.0). The integrity of DNAs was verified before use [7].

Hyperchromicity of DNAs

The UV absorbance of DNAs (10 µg in 1 ml of Tris-HCl buffer 10^{-3} *M* pH 7.65) at 260 nm was measured at 24 °C before and after the addition of different concentrations of a given compound as described for mammalian DNA [7]. Several compounds may be successively introduced with the tested DNA preparation. Controls (without DNA) contained the equivalent amounts of the same compound. Results are expressed as UV absorbance increase (%). The hyperchromic effect of the DNAs after incubation with KOH 0.2 *N* varies from 42 to 56% at 260 nm (maximum DNA chain opening).

In vitro DNA Synthesis

The incubation medium [3] contained per 0.15 ml: Tris-HCl buffer (pH 7.65) 25 µmol; $MgCl_2$ 2 µmol; 4 d-XTP 5 nmol each (plus TTP, 50,000 cpm); DNA 0.5 µg; enzyme DNA-dependent DNA polymerase I, whose preparation from *Escherichia coli* has been described [5] 60 µg. After incubation (10 min at 26 °C) TCA (5%) precipitable material was filtered on a Whatman GF/C glass filter, washed with a TCA solution and 96% alcohol, then dried. Radioactivity was measured in a Packard liquid scintillation counter.

Results

Effects of Opines, Plant Hormone IAA and Carcinogenic Compounds on the DNAs of Different A. tumefaciens *Strains*

On DNA Strand Separation

Each bacterial DNA used separately was tested for DNA strand separation measured by the UV absorbance increase in the absence or presence of either octopine, nopaline, lyso-

pine, *L*-arginine or *L*-lysine. IAA and certain anticancer drugs or various compounds known to be carcinogenic were also used.

Octopine Tumor-Inducing Strains That Metabolize Octopine. As illustrated in figure 1a DNA originating from *A. tumefaciens* B6 incubated with octopine undergoes DNA strand separation resulting in a UV absorbance increase with octopine concentrations. Nopaline or lysopine cannot replace the octopine. Also DNA from *A. tumefaciens* B2 responds to octopine which in vitro separates the chains of this DNA, and the increase in UV absorbance is about 20–22% in the presence of 20 µg of octopine (fig. 1b). In this last case, lysopine produced the same effect but the nopaline did not. Incubated with KOH 0.1 *N*, these DNAs undergo complete DNA strand separation. This amounts to a 45–50% increase in UV absorbance. There is no increase in UV absorbance when the DNAs are incubated with *L*-arginine or *L*-lysine.

Nopaline Tumor-Inducing Strains That Metabolize Nopaline. A. tumefaciens T37 possesses DNA whose strands may be separated only by nopaline (fig. 2a) and the maximum DNA chain opening (20%) is observed in the presence of 20 µg of nopaline. Identical results are obtained with DNA from IIBV7 bacteria (fig. 2b). Two other DNAs isolated from *Agrobacterium delft* and 42 IV bacteria which selectively metabolize nopaline were tested in the presence of these opines. Only the nopaline induces strand separation of *A. delft* DNA (fig. 2c) while nopaline and octopine are effective for 42 IV DNA strand separation (fig. 2d).

The above DNAs respond to IAA at small concentrations (2.5–5 µg): added before the tested opines (fig. 1b, 2b), it leads to an UV absorbance increase; added after opines (fig. 2a, c, d), it leads to an additional UV

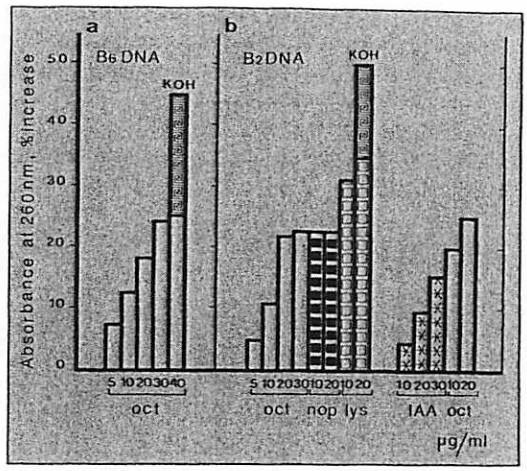
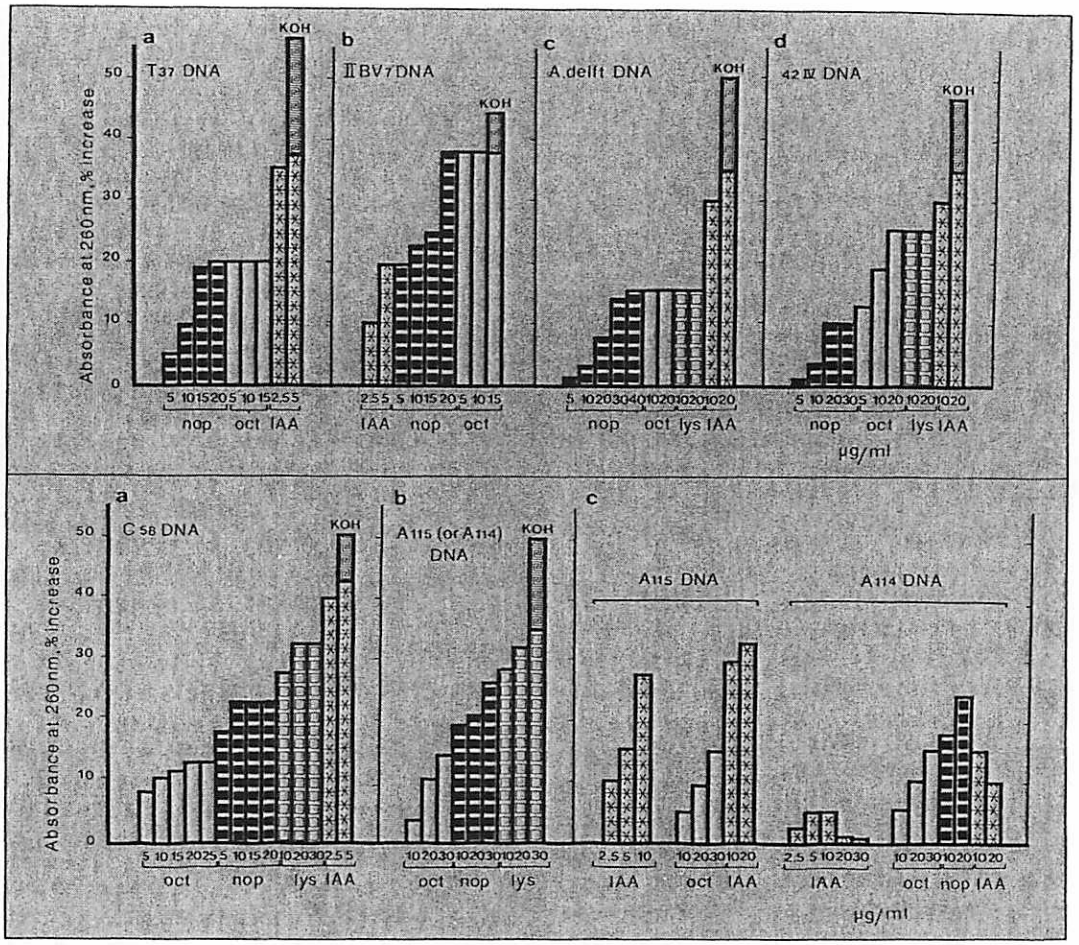


Fig. 1-7. Effects of opines, IAA and carcinogenic compounds on strand separation of *A. tumefaciens* (different strains) DNAs. UV absorbance of DNAs was measured (260 nm) in the absence or presence of each compound added as the indicated concentrations. oct = Octopine; nop = nopaline; lys = lysopine; dauno = daunorubicin; cyclo = cyclophosphamide; mito = mitomycin.

absorbance increase that reached about 40%, i.e. close to the maximum opening.

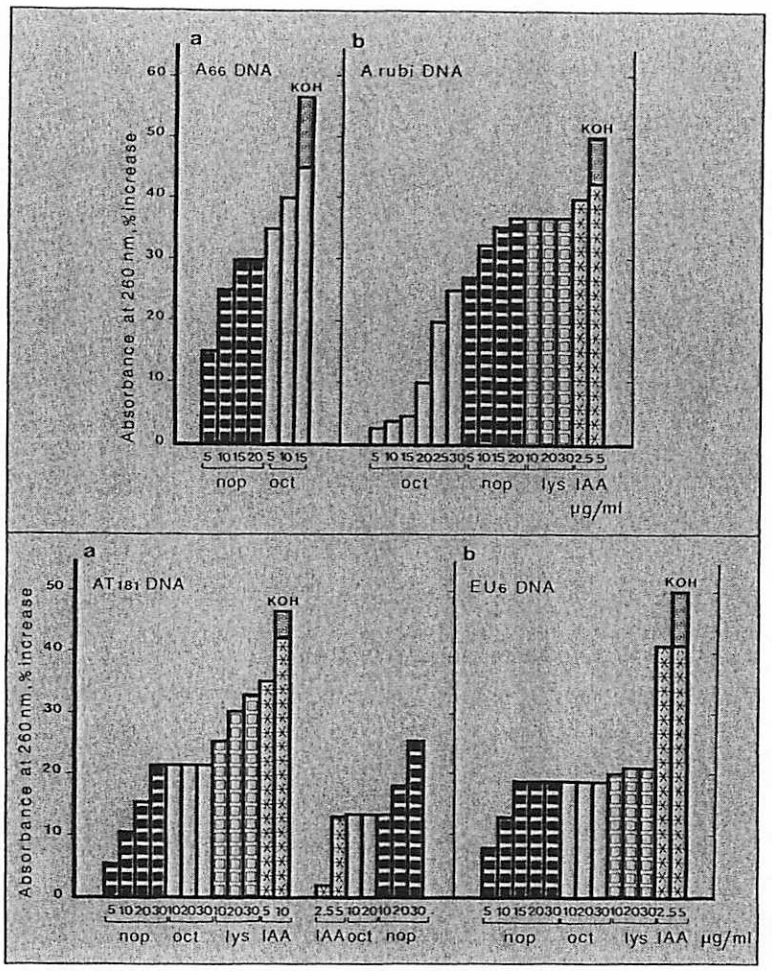
The DNA from C58, another nopaline-catabolizing bacteria, failed to respond to selective opening by nopaline alone: octopine, nopaline, lysopine or IAA used separately one after the other, are capable of producing an additive effect on the strand separation of DNA (fig. 3a). Strand separation of the DNAs isolated from A 114 and A 115 (plasmid-less derivatives from an C58 thermoculture) is strongly induced with either octopine, nopaline or lysopine (fig. 3b). However, these DNAs behave differently in the presence of IAA: DNA from A 115 bacteria undergoes substantial chain separation (25–30%) in the presence of IAA, while A 114 DNA responds poorly to IAA (fig. 3c). It is interesting to note that these two nononcogenic strains lead to the appearance of tumors when introduced



(For legend, see p. 338.)

close to an auxinic callus developed in vitro on an inverted fragment of plant stem (data not shown here).

Octopine and Nopaline (or Octopine-Nopaline-Free) Tumor-Inducing Strains That Metabolize Octopine or Nopaline. Both opines, octopine and nopaline, are present in the tumors induced by the octopine-catabolizing attenuated strain A 66 (whose IAA restores its absolute oncogenic potential). The DNA originating from these bacteria responds to the effect of each: thus A 66 DNA undergoes considerable chain opening (30%) in the presence of nopaline (or octopine) and this opening continued when increasing concentrations of octopine (or nopaline) were added thereafter (fig. 4a). This subsequent increase in UV absorbance is about 45% while in this case KOH induces UV absorbance of up to 56%. Identical results were obtained with DNA from the nopaline-catabolizing oncogenic strain *Agrobacterium rubi* (fig. 4b);



4

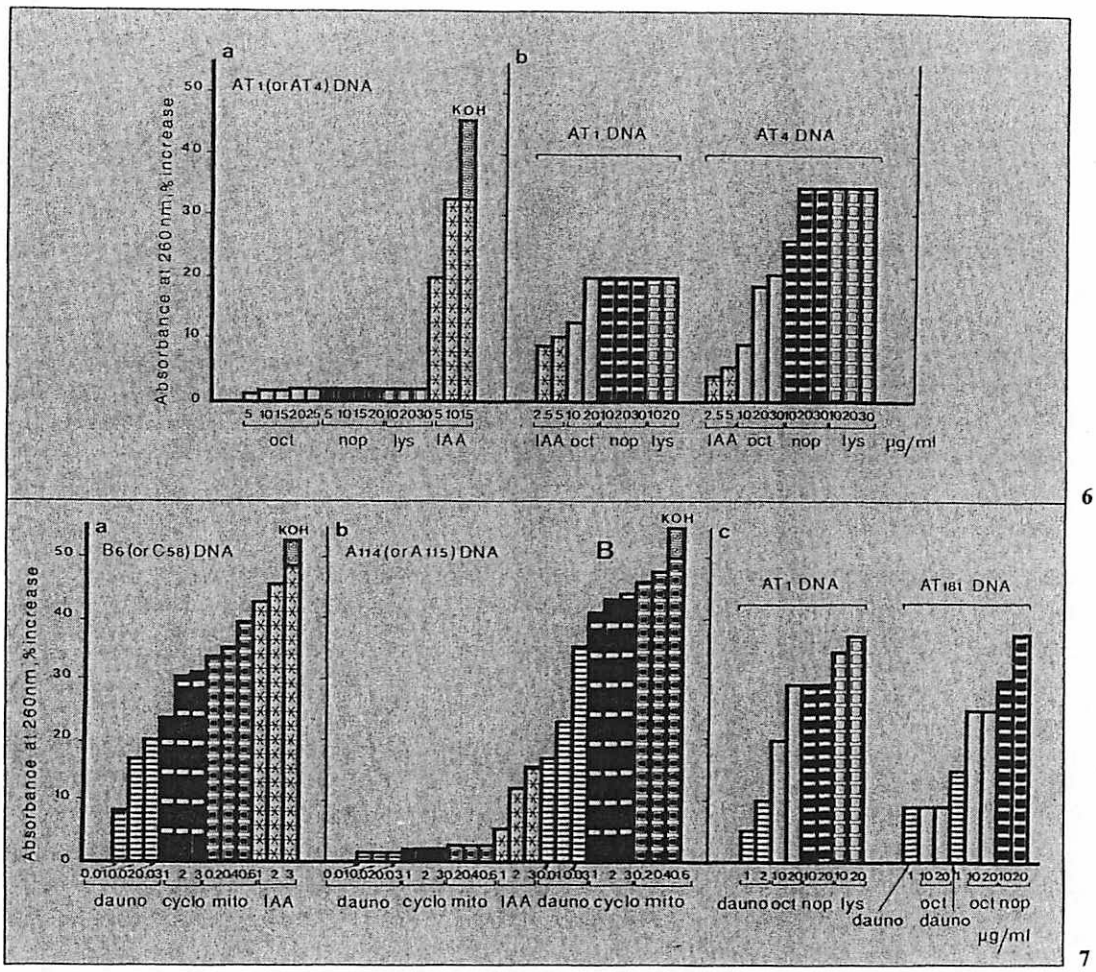
5

(For legend, see p. 338.)

for this last DNA, 30 µg of octopine are necessary to obtain a chain opening of 25% while 15 µg of nopaline added first induce the same degree of opening. Lysopine is without effect but IAA is effective on DNA strand separation.

AT 181 and EU 6 bacteria utilize nopaline and produce tumors containing neither octopine nor nopaline. DNA isolated from AT 181 undergoes a strand separation in the presence of nopaline and lysopine but not of octopine (fig. 5a). In spite of a preincubation in the presence of IAA, this DNA remains insensitive to the action of octopine. EU 6 DNA responds only to nopaline (fig. 5b). Octopine and lysopine are without action on the strand separation of this DNA, even after an IAA preincubation.

Octopine-Nopaline-Free Tumor-Inducing Strains That Do Not Metabolize Any of These Opines. DNAs isolated from AT1 and AT4 bacteria which are incapable of metabolizing



(For legend, see p. 338.)

either octopine or nopaline and whose induced tumors are free of these opines, do not undergo chain separation when incubated with octopine, nopaline or lysopine (fig. 6a). But when these DNAs are preincubated with IAA (2.5–5 µg) one observes (fig. 6b) an UV absorbance increase of about 10% in the presence of the opines: thus, AT1 DNA responds to octopine but not to nopaline or lysopine; AT4 DNA responds to octopine and nopaline but not to lysopine.

Under identical conditions, we have investigated the effect of daunorubicin, cyclophosphamide (Endoxan) and mitomycin C which are anticancer agents or carcinogens according to the concentration used [7, 9, 26, 45] on DNA strand separation. When successively added to the incubation mixture, these substances induce a strong enhancement in UV absorbance (about 40%) of B6 (octopine strain) and C58 (nopaline strain) DNAs (fig. 7a), in the presence of alkali, this UV

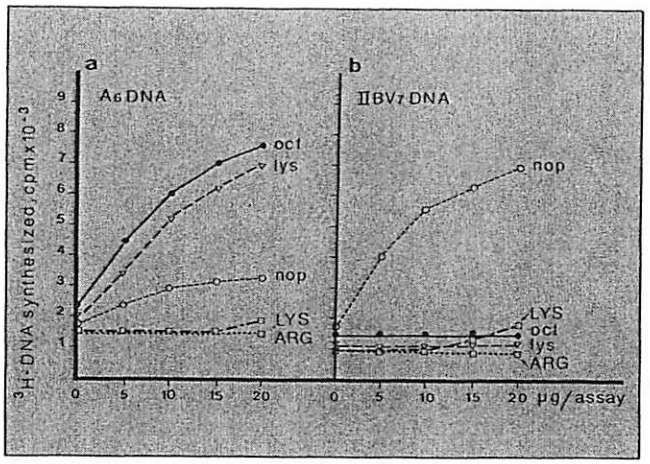


Fig. 8. Effects of opines on *A. tumefaciens* in vitro DNA synthesis. ARG = *L*-Arginine; LYS = *L*-lysine. Other abbreviations as in figures 1–7.

absorbance increase is 50–52%. As shown above, IAA behaves as a carcinogen and exhibits an important effect on the strand separation of these DNAs (fig. 7a). In contrast, DNAs from attenuated A 114 and A 115 bacteria respond to the hyperchromic action of the substances mentioned above but *only* after a preincubation in the presence of IAA: 2–3 µg of IAA induce an increase of about 15% in the UV absorbance; subsequently carcinogens added in small concentrations (0.06–1 µg) highly enhance (45–50%) the hyperchromicity of these DNAs (fig. 7b).

We have already mentioned a sensibilization of AT1 DNA after the plant hormone action: only when preincubated in the presence of IAA this DNA was susceptible to octopine (fig. 6). We observed a similar pre-effect in the case of carcinogens: when AT1 DNA is first incubated with daunorubicin, it responds to octopine and lysopine but not to nopaline (fig. 7c). AT 181 DNA remains insensitive to the octopine action even after an IAA preincubation (fig. 5a) while it responds to it after the addition of daunorubicin to the incubation mixture (fig. 7c).

On DNA in vitro Synthesis

It is known that DNA replication requires DNA chain opening [36]. We have used DNAs from bacteria that selectively metabolize octopine or nopaline in order to determine if they would be better templates for DNA in vitro synthesis in the presence of respective opines.

The results are illustrated in figure 8. A6 (octopine-metabolizing strain) DNA, incubated at 26 °C with all necessary components and DNA-dependent DNA polymerase is a much better template in the presence of octopine than in its absence (fig. 8a). Nopaline has practically no effect on the in vitro synthesis of this DNA (fig. 8a). In contrast, IIBV7 (nopaline-metabolizing strain) DNA is an excellent template for DNA synthesis in the presence of nopaline (fig. 8b), while in its absence or in the presence of octopine, DNA synthesis is rather poor. It should be stressed that lysopine, which separates the strands of DNA from A6 bacteria, stimulates in vitro the synthesis of the same DNA (fig. 8a). IIBV7 DNA is insensitive to lysopine that does not undergo DNA chain separation nor an increase

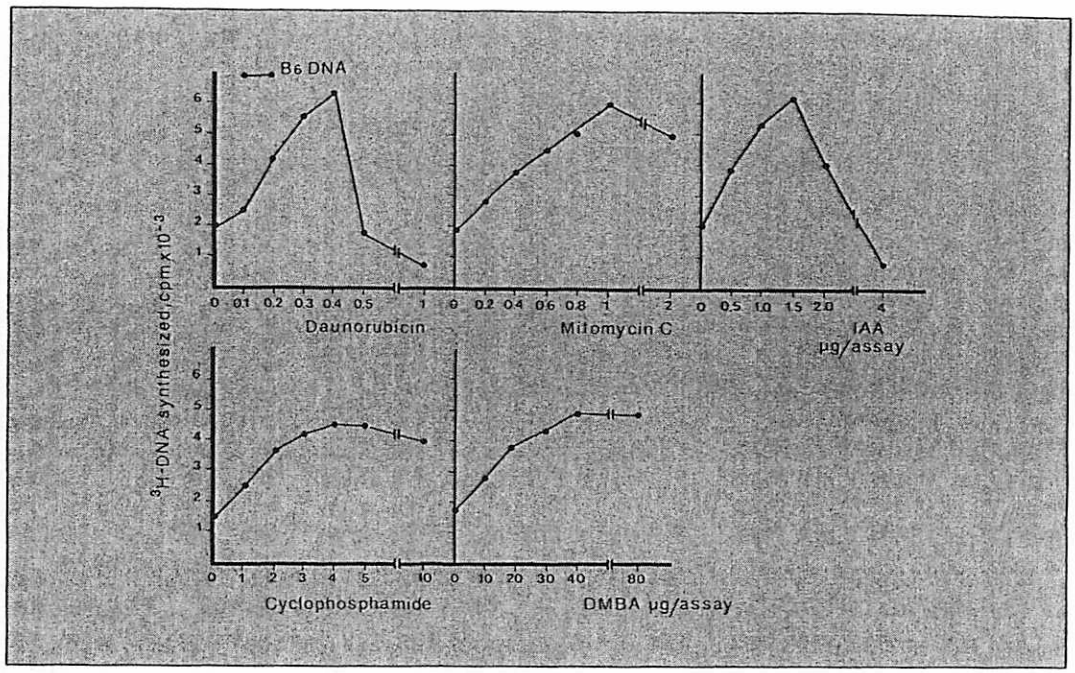


Fig. 9. Effects of different carcinogenic compounds on *A. tumefaciens* in vitro DNA synthesis.

in DNA in vitro synthesis (fig. 8b). *L*-Arginine or *L*-lysine has no effect on the synthesis of the DNAs from these strains. These data demonstrate a close correlation between DNA in vitro strand separation induced by opines and DNA-increased synthesis in vitro in the presence of these compounds.

Similar results were obtained with *A. delft* and 42 IV (nopaline-metabolizing strains). Their DNAs, incubated at 26 °C with all compounds required for in vitro DNA synthesis, are better templates in the presence of nopaline than in its absence, but at 36 °C nopaline is without effect. In any case, there is always a much better stimulation (approximately 3 times) of *A. tumefaciens* DNA in vitro synthesis in the presence of an opine (depending on the strain used) at 26 °C, compared to results observed at 37 °C (no stimulating effect). It should be remembered that bacterial growth takes place at 26–28 °C but is stopped at 37 °C. An identical temperature effect is observed for cancer induction.

We investigated the in vitro synthesis of DNA from *Agrobacterium* B6 cells in the presence of the carcinogenic agents cyclophosphamide, daunorubicin, DMBA, mitomycin C and IAA (fig. 9). At low concentrations, each of the compounds substantially stimulates the synthesis of the DNA. At high concentrations, each induced a strong inhibiting action. Used under identical conditions, DNA originating from *E. coli* and *A. tumefaciens*-transformed B6 Trl [4] did not respond to any opine or carcinogenic compound either for in vitro DNA strand separation or DNA synthesis.

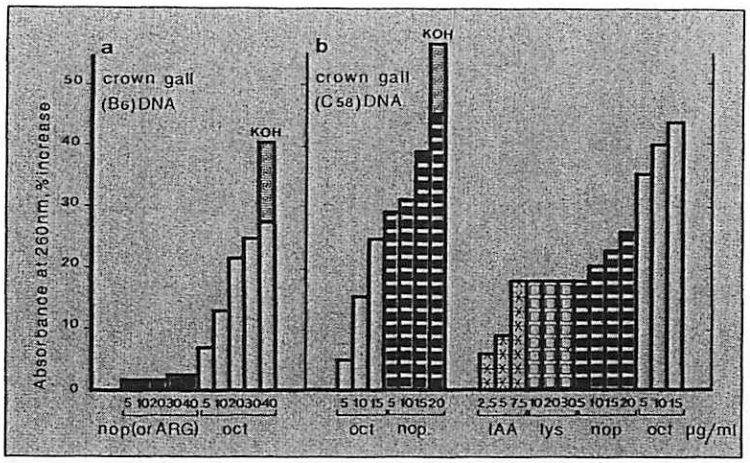


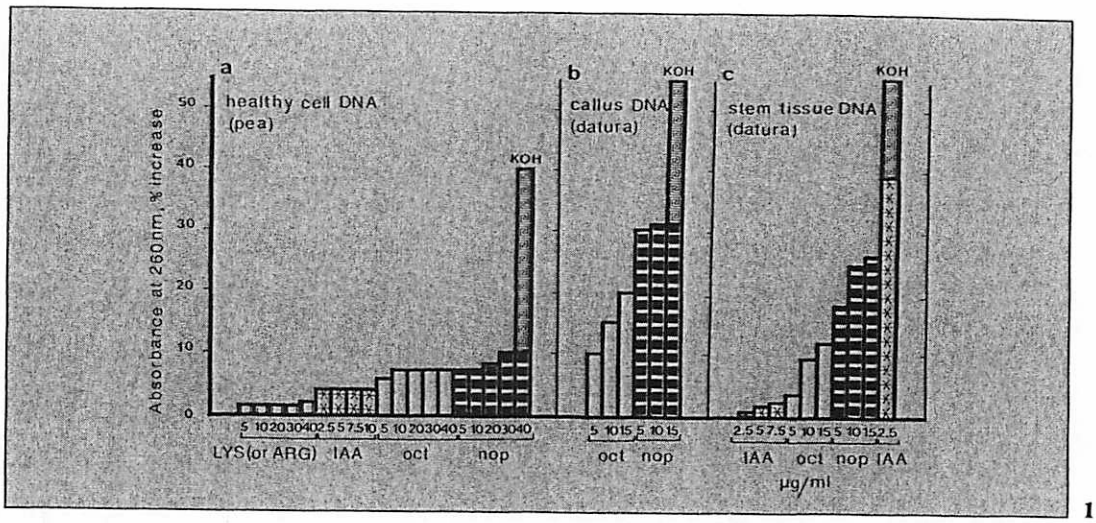
Fig. 10. Effects of opines and IAA on crown gall DNA strand separation. **a** B6-induced tumor DNA. **b** C58-induced tumor DNA. Abbreviations as in previous figures.

Effects of Opines, Hormone IAA and Carcinogenic Compounds on Healthy and Crown Gall Plant Tissue DNAs

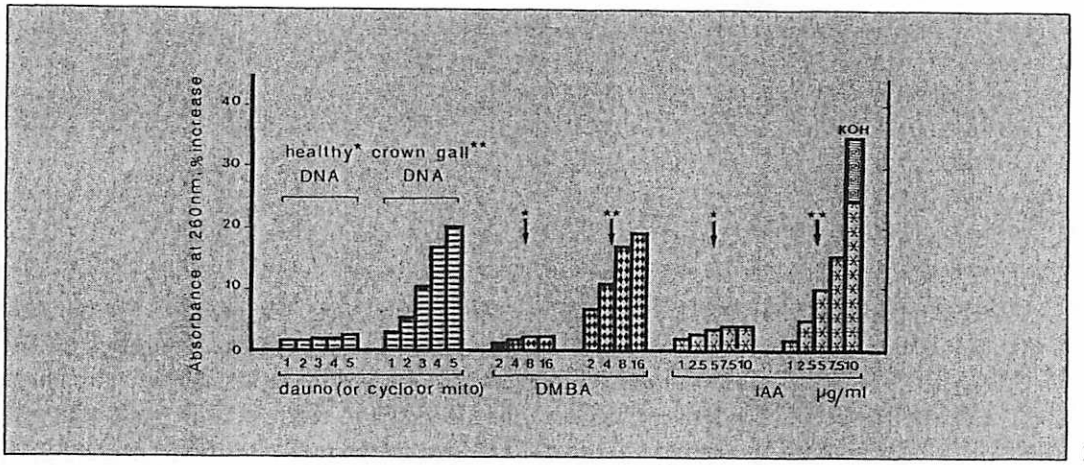
On DNA Strand Separation

For DNA chain opening we used DNAs from crown gall tissues induced by B6 of C 58 bacteria and developed on germinating pea seedlings. Figure 10a indicates that only octopine induces in vitro strand separation of DNA isolated from B6-tumor tissues to a high degree (26% UV absorbance increase). Nopaline and *L*-arginine are without effect. Tested under the same conditions, DNA from C58-induced crown gall tissues undergoes a UV absorbance increase in the presence of both octopine and nopaline (fig. 10b). After the IAA is added, this DNA undergoes strand separation (18% UV absorbance increase in the presence of 7.5 µg IAA per assay) (fig. 10b). Lysopine is without effect. The opines which specifically induce strand separation of the DNA from these tumor tissues are the same as those effective for DNA strand separation of the inducing bacteria. This demonstrates a close correlation between a given opine and the chain opening of DNAs from the inducing *A. tumefaciens* strain and induced crown gall tumors.

It was often discussed [12, 18, 46] if the presence is true of very small and hardly detectable amounts of opines in healthy plant tissues. It was therefore important to determine to what extent DNA from healthy plant tissue will respond (or not) to the action of opines. Thus, strand separation of DNA from growing pea stems was studied in the presence of either octopine, nopaline, *L*-arginine or *L*-lysine. Results illustrated by figure 11a show that this DNA responds slightly (7–9%) to either octopine or nopaline but not at all to arginine or lysine (for this DNA the UV absorbance increase is 40% with KOH). IAA also induces a slight but detectable chain opening of this DNA. On the other hand, DNA originating from callus tissues developed on axenic inverted stems of *D. stramonium* strongly responds to octopine and nopaline (fig. 11b) (15 µg of octopine leads to a 20% UV absorbance increase that reached 30% when we subsequently added 15 µg of nopaline; the maximum DNA chain opening



11



12

Fig. 11. Effects of opines and IAA on healthy DNA strand separation. **a** Pea stem DNA. **b** Datura auxinic callus DNA. **c** Datura top-stem DNA. Abbreviations as in previous figures.

Fig. 12. Crown gall and normal DNA strand separation in the presence of some carcinogenic compounds. Abbreviations as in previous figures.

is 55% with KOH). So, the apical stem segments of Datura contain DNA that in vitro undergoes UV absorbance increase (30%) in the presence of octopine or nopaline (fig. 11c). After preincubation with opines, this latter DNA becomes two or three times more sensitive to a later IAA action for chain opening: the UV absorbance increase reaches approximately 45% (up to 56% in the presence of KOH) (fig. 11c).

We studied the hyperchromic effect of several carcinogenic compounds on healthy and cancer pea DNAs. Figure 12 shows that in the presence of either daunorubicin, cyclo-

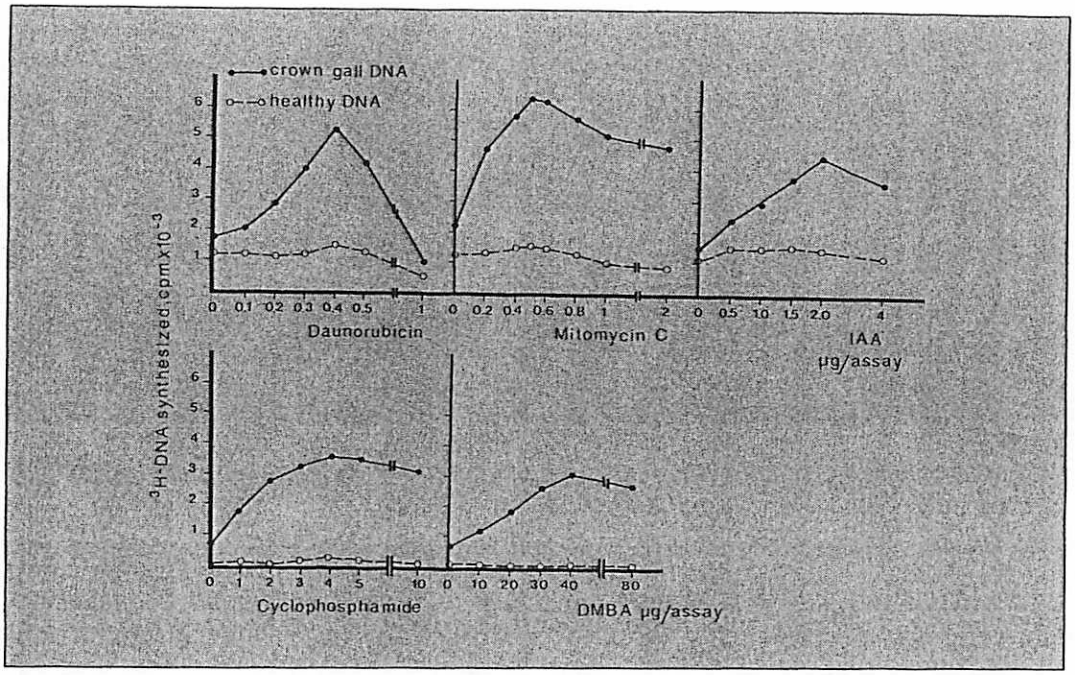


Fig. 13. Crown gall and healthy in vitro DNA synthesis in the presence of various compounds.

phosphamide, mitomycin C or IAA, UV absorbance increases considerably with crown gall DNA but not at all or only slightly with healthy DNA. There are optimal concentrations for the extent of DNA strand separation which vary according to the different substances used. It is important to note that the hormone IAA exhibits a small but detectable hyperchromic effect on healthy plant DNA.

On DNA in vitro Synthesis

Fast development of crown gall tumors implies an accelerated replication of DNA from these cells compared to that of healthy cells, cell division being interdependent on DNA replication. Using template DNA from healthy or tumorous cells of pea seedlings and DNA-dependent DNA polymerase, we compared the template activites of both during DNA in vitro synthesis. Tumorous DNA exhibits a higher template activity compared to that of DNA from healthy plant cells (see origin of curves in fig. 13). This indicates that crown gall DNA contains a larger number of single-stranded DNA regions required for enzyme activity. The in vitro crown gall DNA synthesis is highly enhanced by cyclophosphamide, daunorubicin, mitomycin C and IAA used at small concentrations. Used at the same concentrations these substances have only a slight effect on healthy DNA synthesis. High concentrations always inhibit the synthesis of the DNAs of both tissues.

We have shown above (fig. 10) that octopine and nopaline enhanced the chain opening of crown gall DNAs (B6 and C58 DNA,

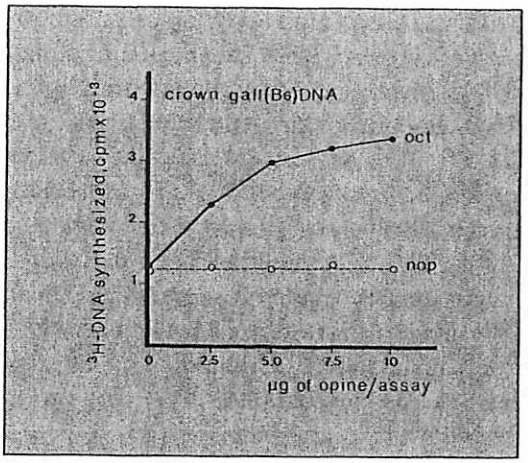


Fig. 14. In vitro synthesis of *A. tumefaciens* B6-induced tumor DNA in the presence of opines. Abbreviations as in previous figures.

respectively). This correlation was corroborated by the observation of a selective stimulating effect of opines on crown gall DNA synthesis in vitro (fig. 14). Octopine (or nopaline) are effective on the DNA of tumorous cells induced by the strain of *A. tumefaciens* which metabolizes octopine (or nopaline). Both opines have only a very slight stimulating effect on the in vitro synthesis of DNA from healthy plant tissues. Octopine, nopaline and lysopine have no effect on DNA from healthy or cancerous mammalian cells.

Discussion and Conclusion

An attempt was made to characterize the nature of the mechanism by which opines may be involved in the maintenance of the crown gall plant cells. We focused our attention on plant and *A. tumefaciens* DNAs mainly on the in vitro interaction of these DNAs and opines. Why this approach? Firstly, in contrast to the normal plant cells, tumor plant cells synthesize a large amount of opines [18, 46]. Secondly, DNAs from plant tumor tissues are 'destabilized' and react easily with various naturally occurring or chemically synthesized molecules [27–29]. Thirdly, the weight of crown gall tissues increases in the presence of octopine [31, 47] or when induction takes place with the *A. tumefaciens* B6 oncogenic strain grown in the presence of octopine [15]. *L*-Arginine, a precursor of octopine, was without effect in this process. All these observations suggest that opines may somehow play a role in the maintenance of crown gall tumor tissues. What facts support this hypothesis? The transition from a normal cell to tumor cell involves reorientation resulting in increased synthesis of nucleic acids [7, 34] and of some specialized mitotic [22] and enzymatic proteins [2, 27, 48, 49] in order to assure continued cell growth and division of tumor cells. Opines which have been considered as accidental metabolic substances whose amount largely increases in tumor cells [12, 44] were not taken into account as important agents for the maintenance of tumoral state. The fact that opines stimulate the development of crown gall cells and also but slightly that of normal cells suggested that they may induce changes in the physicochemical structure of DNAs, without modifying their primary structure. This implies that the mechanism involving opines in the activation of the opine-synthesizing system (DNA) in normal cells is similar to that involved in crown gall cells.

Moreover, we have seen that some healthy tissues (Datura callus tissues) had 'destabilized' (relaxed) DNA (in comparison with the stable DNA of other tissues (pea stems)) that strongly respond in vitro to the action of opines. This correlates with the

finding that callus tissues possess some of the histological characteristics of tumorous tissues, and once grafted to healthy plants of the same origin induce the appearance of tumors [*Aaron-Da Cunha,* pers. commun.]. On the basis of all our results it is possible to contend that the synthesis of very small amounts of opines by healthy plant cells is feasible. This may be connected with the physiological state of the tissues which are dependent upon the effects of endogenous plant hormones.

We have shown here an interaction between plant and *Agrobacterium* DNAs and opines. The DNA of a healthy plant cell, insensitive to octopine, becomes very reactive when normal cells of the plant are cultivated in a medium containing octopine [unpubl. results]. This healthy DNA becomes 'destabilized' (measured in vitro using the UV absorbance technique). Once octopine is removed from the culture medium, the DNA of these normal cells again becomes stabilized and no longer responds to octopine [unpubl. results]. Thus, gene activation by an opine in crown gall cells and in normal cells results in DNA relaxation. This may also be induced under the influence of IAA and small RNA (RNA fragments) [6, 25]. Opines do not react in vitro with DNAs from *E. coli,* from *A. tumefaciens* strain made nononcogenic by *E. coli* RNA [4] and from healthy and cancerous human and animal tissue DNAs. It may be concluded from this that plant and *A. tumefaciens,* oncogenic or not, possess DNAs which have specific and maybe common binding sites for opines.

It is well established that gene activation requires DNA chain opening in local areas, thus allowing RNA polymerase to transcribe a given portion of DNA into mRNA and offering more binding sites on chains for DNA replication [20, 39, 40]. Both of these processes are needed for cell growth and division. Opines, RNA fragments or small RNAs, carcinogens, antimitotics, antibiotics, IAA and opines interfere in chain opening [7, 27–29]. They possess a weak additive effect on DNA strand separation from normal cells, while this effect is aggressive with cancer cells DNA. IAA, first initiating DNA separation, allows opines further to destabilize DNA. This might explain why some *A. tumefaciens* strains may induce tumors only in the presence of this hormone. It is important also to recall that tumorigenesis requires IAA for tumor induction with small RNAs [6, 25] and that the presence of an IAA gene is required for bacteria oncogenicity expression [1, 33]. The question thus arises of the existence of plant oncogenes – as in the eukaryotic cells of vertebrates [11, 21] – and of the function of their products. Is there one or several common denominator(s) for the activation of oncogenes in both the mammalian and plant kingdoms? Opines, the products of crown gall cells, induce the opening of plant tumor cell DNA which correlates with gene activation and may allow a shift in the preferred site for initiation of transcription, resulting in the production of a longer RNA transcript. This problem was recently discussed by one of us in connection with the role of trigger molecules in normal and malignant gene expression [8]. All these results go to make up a general model for understanding the basic underlaying mechanism of the maintenance of the tumorous state.

Acknowledgements

We thank *A. Faivre-Amiot, J.A. Lippincott, P. Manigault* and *E.W. Nester* for generously providing strains of *A. tumefaciens.* Datura tissues were kindly supplied by *M.I. Aaron-Da Cunha.*

References

1 Akiyoshi, D.E.; Morris, R.O.; Hinz, R.; Mischke, B.S.; Kosuge, T.; Garfinkel, D.J.; Gordon, M.P.; Nester, E.W.: Cytokinin/auxin balance in crown-gall tumors is regulated by specific loci in the T-DNA. Proc. natn. Acad. Sci. USA *80:* 407–411 (1983).

2 Antoniw, J.F.; Ooms, G.; White, R.F.; Wullems, G.F.; Vloten-Doting, L. v.: Pathogenesis-related proteins in plants and tissues of *Nicotiana tabacum* transformed by *Agrobacterium tumefaciens*. Plant Mol. Biol. *2:* 317–320 (1983).

3 Beljanski, M.: Oncotest: a DNA assay system for the screening of carcinogenic substances. IRCS Med. Sci. *7:* 476 (1979).

4 Beljanski, M.; Beljanski, M.; Manigault, P.; Bourgarel, P.: Transformation of *Agrobacterium tumefaciens* into a non-oncogenic species by an *Escherichia coli* RNA. Proc. natn. Acad. Sci. USA *69:* 191–195 (1972).

5 Beljanski, M.; Beljanski, M.: RNA-bound reverse transcriptase in *Escherichia coli* and in vitro synthesis of a complementary DNA. Biochem. Genet. *12:* 163–180 (1974).

6 Beljanski, M.; Aaron-Da Cunha, M.I.: Particular small size RNA and RNA-fragments from different origins as tumor inducing agents in *Datura stramonium*. Mol. Biol. Rep. *2:* 497–506 (1976).

7 Beljanski, M.; Bourgarel, P.; Beljanski, M.: Correlation between in vitro DNA synthesis, DNA strand separation and in vivo multiplication of cancer cells. Expl Cell Biol. *49:* 220–231 (1981).

8 Beljanski, M.: The regulation of DNA replication and transcription. The role of trigger molecules in normal and malignant gene expression; in Wolsky, Expl Biol. Med., vol. 8 (Karger, Basel 1983).

9 Bertazzoni, C.; Chieli, T.; Solcia, E.: Different incidence of breast carcinomas or fibroadenomas in daunomycin or adriamycin treated rats. Experientia *27:* 1209–1210 (1971).

10 Biemann, K.; Lioret, C.; Asselineau, J.; Lederer, E.; Polonsky, J.: Sur la structure chimique de la lysopine, nouvel acide aminé isolé des tissus de crown-gall. Bull. Soc. Chim. biol. *42:* 979–991 (1960).

11 Bishop, M.: Les oncogènes. Pour la Science *55:* 28–41 (1982).

12 Braun, A.C.: The relevance of plant tumor systems to an understanding of the basic cellular mechanisms underlying tumorigenesis. Prog. exp. Tumor Res. *15:* 165–187 (1972).

13 Currier, T.C.; Nester, E.W.: Evidence for diverse types of large plasmids in tumor-inducing strains of *Agrobacterium*. J. Bact. *126:* 157–165 (1976).

14 Chilton, W.C.; Tempé, J.; Matze, M.; Chilton, M.D.: Succinamopine: a new crown-gall opine. J. Bact. *157:* 357–362 (1984).

15 Da-Costa, C.: Contribution à l'étude de l'action des deux opines, octopine et nopaline, sur le métabolisme des tissus végétaux sains et tumoraux; DEA Paris (1982).

16 Ellis, J.G.; Murphy, P.J.: Four new opines from crown-gall tumors. Their detection and properties. Molec. Gen. Genet. *181:* 36–43 (1981).

17 Firmin, J.L.; Fenwick, G.R.: Agropine – a major new plasmid-determined metabolite in crown-gall tumors. Nature, Lond. *276:* 842–844 (1978).

18 Goldmann, A.: Les métabolites anormaux des tissus de crown-gall «marqueurs» de la transformation tumorale; in La culture des tissus et des cellules des végétaux, pp. 202–209 (Masson, Paris 1977).

19 Goldmann, A.; Thomas, D.W.; Morel, G.: Sur la structure de la nopaline, métabolite anormal de certaines tumeurs de crown-gall. C.r. hebd. Acad. Sci., Paris, sér. D *268:* 852–854 (1969).

20 Hagen, G.; Kleinschmidt, A.; Guilfoyle, T.: Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta *162:* 147–153 (1984).

21 Hunter, T.: Les protéines des oncogènes. Pour la Science *84:* 90–101 (1984).

22 Ibsen, K.H.; Fishman, W.H.: Developmental gene expression in cancer. Biochim. biophys. Acta *560:* 243–280 (1979).

23 Knopf, U.C.: Crown-gall and *Agrobacterium tumefaciens*: survey of a plant cell transformation system of interest to medicine and agriculture; in Roodyn, Subcellular biochemistry, vol. 6, pp. 143–173 (Plenum Press, New York 1979).

24 Kurkdjian, A.; Manigault, P.; Beardsley, R.: The pea seedling as a model of normal and abnormal morphogenesis. Am. Biol. Teach. *36:* 13–19 (1974).

25 Le Goff, L.; Aaron-Da Cunha, M.I.; Beljanski, M.: Un ARN extrait d'*Agrobacterium tumefaciens*, souches oncogènes et non oncogènes, élément indispensable à l'induction des tumeurs chez *Datura stramonium*. Can. J. Microbiol. *22:* 694–701 (1976).

26 Le Goff, L.; Beljanski, M.: Cancer/anti-cancer dual action drugs in crown-gall tumors. IRCS Med. Sci. *7:* 475 (1979).

27 Le Goff, L.; Beljanski, M.: Crown-gall tumor stimulation or inhibition: correlation with DNA strand separation. Proc. 5th Int. Conf. Plant Path. Bacteria, Cali. (Columbia) 1981, pp. 295–307.
28 Le Goff, L.; Beljanski, M.: Agonist and/or antagonist effects of plant hormones and an anticancer alkaloid on plant DNA structure and activity. IRCS Med. Sci. *10:* 689–690 (1982).
29 Le Goff, L.; Roussaux, J.; Aaron-Da Cunha, M.I.; Beljanski, M.: Growth inhibition of crown-gall tissues in relation with the structure and activity of DNA. Physiol. Plant. *64:* 177–184 (1985).
30 Lerman, L.S.; Tolmach, L.J.: Genetic transformation. I. Cellular incorporation of DNA accompanying transformation in *Pneumococcus.* Biochim. biophys. Acta *26:* 68–82 (1957).
31 Lippincott, J.A.; Lippincott, B.B.; Chi-cheng Chang: Promotion of crown-gall tumor growth by lysopine, octopine, nopaline and carnosine. Plant. physiol. *49:* 131–137 (1972).
32 Lippincott, J.A.; Beiderbeck, R.; Lippincott, B.B.: Utilization of octopine and nopaline by *Agrobacterium.* J. Bact. *116:* 378–383 (1973).
33 Liu, S.T.; Perry, K.L.; Schardl, C.L.; Kado, C.I.: *Agrobacterium* Ti plasmid indoleacetic acid gene is required for crown-gall oncogenesis. Proc. natn. Acad. Sci. USA *79:* 2812–2816 (1982).
34 Loeb, L.; Gelboin, H.V.: Stimulation of amino acid incorporation by nuclear ribonucleic acid from normal and methylcholanthrene-treated rats. Nature, Lond. *199:* 809–810 (1963).
35 Ménagé, A.; Morel, G.: Sur la présence d'octopine dans les tissus de crown-gall. C.r. hebd. Acad. Sci., Paris, sér. D *259:* 4795–4796 (1964).
36 Meselson, M.; Stahl, F.V.: The replication of DNA. Cold Spring Harb. Symp. quant. Biol. *23:* 9–12 (1958).
37 Morel, G.; Duranton, H.: Le métabolisme de l'arginine par les tissus végétaux. Bull. Soc. Chim. biol. *40:* 2155–2167 (1958).
38 Nester, E.W.; Gordon, M.P.; Amasino, R.M.; Yanofsky, F.: Crown gall: a molecular and physiological analysis. Ann. Rev. Plant Physiol. *35:* 387–413 (1984).
39 Neubort, S.; Liebeskind, D.; Mendez, F.; Elequin, F.; Hsu, K.C.; Bases, R.: Morphological transformation, DNA strand separation and antinucleoside immunoreactivity following exposure of cells to intercalating drugs. Molec. Pharmacol. *21:* 739–743 (1982).
40 Patel, G.L.; Thompson, P.E.: Immunoreactive helix destabilizing protein localized in transcriptionally active regions of Drosophila polytene chromosomes. Proc. natn. Acad. Sci. USA *77:* 6749–6753 (1980).
41 Petit, A.; Delhaye, S.; Tempé, J.; Morel, G.: Recherches sur les guanidines des tissus de crown-gall. Mise en évidence d'une relation biochimique spécifique entre les souches d'*Agrobacterium tumefaciens* et les tumeurs qu'elles induisent. Physiol. vég. *8:* 205–213 (1970).
42 Smith, E.F.; Townsend, C.O.: A plant tumor of bacterial origin. Science *25:* 671–673 (1907).
43 Sciaky, D.; Montoya, A.L.; Chilton, M.D.: Fingerprints of *Agrobacterium* Ti plasmids. Plasmid *1:* 238–253 (1978).
44 Schilperoort, R.A.; Kester, H.C.M.; Klapwijk, P.M.; Rörsch, A.; Schell, J.: Plant tumors induced by *A. tumefaciens.* A genetic approach. Semaine d'étude: Agriculture et hygiène des plantes, Gembloux 1975.
45 Schmähl, D.; Habs, M.: Carcinogenic action of low-dose cyclophosphamide given orally to Sprague-Dawley rats in a life time experiment. Int. J. Cancer *23:* 706–712 (1979).
46 Scott, I.M.; Firmin, J.L.; Butcher, D.N.; Searle, L.M.; Sogeke, A.K.; Eagles, J.; March, J.F.; Self, R.; Fenwick, G.R.: Analysis of a range of crown-gall and normal plant tissues for Ti-plasmid-determined compounds. Molec. Gen. Genet. *176:* 57–65 (1979).
47 Swain, L.W.: Crown-gall enhancement by octopine. Bull. Torrey bot. Club *99:* 31–33 (1972).
48 Weickmann, J.L.; Olson, E.M., Glitz, D.G.: Immunological assay of pancreatic ribonuclease in serum as an indicator of pancreatic cancer. Cancer Res. *44:* 1682–1687 (1984).
49 Wilson, C.M.: Plant nucleases. A. Rev. Pl. Physiol. *26;* 187–208 (1975).

Received: January 23, 1985
Accepted: April 3, 1985

Dr. Liliane Le Goff,
Laboratoire de Pharmacodynamie,
Faculté des Sciences biologiques et pharmaceutiques,
Rue J.B.-Clément,
F-92290 Châtenay-Malabry (France)