INDUCTION OF CROWN-GALL : PARTIAL HOMOLOGY BETWEEN TUMOR-CELL DNA, BACTERIAL DNA AND THE G+C-RICH DNA OF STRESSED NORMAL CELLS.

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INTRODUCTION

Crown-gall is a unique case of a true tumor induced by bacteria : tumorous transformation arises when a virulent strain of Agrobacterium tumefaciens (*) is introduced into a susceptible plant previously conditioned by wounding. The wounded cells generally die and are not the prospective tumor cells; but their wound juice is claimed to act upon the neighbouring cells , making them conditioned and thus able to be transformed.

Braun (1947) on the basis of thermal studies proposed the hypothesis that a Tumor Inducing Principle (*) is provided by the bacteria. Attempts to isolate cell-free extracts containing A.t.DNA and endowed with tumor-inducing ability led initially to failures or to controversy. Kovoor recently succeeded in inducing auxin-prototrophic (1967) and transplantable (1968) overgrowths by introducing large amounts of A.t.DNA into normal plant tissue cultures.

The dependence of tumor size on certain characteristics (duration and temperature) of the conditioning and inception periods led Braun and Stonier (1958) to set up several proposals,one of which suggested a possible complex between the TIP and the "active sites" elaborated by the conditioned host cells. Kupila and Stern (1961) detected by global estimation a 40 % transient increase of the DNA content ; it occured between 24 and 48 hours after the plants were wounded and was independant of the later infection. Lipetz (1967) observed by autoradiography two waves of DNA synthesis also preceding the first wound division. We recently reported the

*Abreviations: A.t.,Agrobacterium tumefaciens; TIP, Tumor Inducing Principle; SSC, Standard Saline Citrate; Nh DNA, Nuclear heavy satellite DNA; c-RNA, complementary RNA.

relative and transient increase of the nuclear G+C-rich satellite DNA (Nh DNA, see Quétier et al., 1968) in 48 hours old wounded tissue of numerous dicotyledonous (Guillé et al, 1968). It seems likely that this specific synthesis may be the main component of the mentioned DNA synthesis. Furthermore, Bopp (1964) was able to prevent the tumor induction by inhibiting specifically the amitotic DNA synthesis occuring between stress and healing, thus indicating that this unusual DNA synthesis is an obligatory and early step in the transformation process.

On the other hand the idea of an unequivocal role played by the bacterial DNA is strongly supported by Schilperoort and al (1967) who report that while hybridization does not occur significantly between normal tobacco leaf DNA and the RNA copied on A.t.DNA by E.Coli RNA-polymerase , it does occur between tumorous tobacco tissue culture DNA and A.t. c-RNA. Although E.Coli RNA-polymerase may copy some cistrons more specifically, their results provide evidence for common sequences between bacterial DNA and the DNA of transformed cells. These sequences do not seem to be detectable in normal plant DNA.

On the basis of all these results it seemed of interest :

- to obtain direct evidence for partial homology between A.t.DNA and the transformed cell DNA;
- to determine if homologous base sequences exist between A.t. and Nh DNA's, i.e. is there some analogy with the transformation by mammalian viruses or with the lysogeny process ?

MATERIALS and METHODS

DNA isolation procedure : plant DNA was extracted and purified as previously described (Quétier and Guillé, 1968) from normal and crown-gall tissue cultures of Scorzonera hispanica and Nicotiana tabacum (strains kindly given by Dr Morel) and from leaves of green-house grown N.tabacum. Agrobacterium tumefaciens, strain B6 (kindly provided by Dr Manigault) was grown on synthetic media in which $^{32}PO_4$ is added at a concentration up to 10 mC/5mg P/500ml. DNA was extracted and purified as in the case of plant DNA with the exception of an additional treatment (lysozyme and Na-dodecylsulfate) to remove teichoic acids.

Further purification was achieved by preparative Cs Cl equilibrium density gradient ultracentrifugation according to Bond, Flamm and Burr (1966): Possible minute contamination by protein and RNA is ruled out. Samples were controlled in an analytical ultracentrifuge

DNA-DNA hybridizations : The procedure given by Denhardt (1966) was found to be quite effective. Single stranded DNA was fixed on HA millipore filters according to Gillespie and Spiegelman (1965). Filters were put in small capped tubes containing 1 ml of " preincubation medium " and incubated 6 hours at 67°C. Sonicated radioactive single stranded A.t.DNA was added and allowed to hybridize during 14 hours. Filters were washed on each side with 50 ml of SSC (*), dried and counted on a low back-ground (1 to 2 cpm) Tracerlab counter.

RESULTS :

I. Homology between tumorous and A.t. DNA's

Results shown in Fig. 1 confirm the conclusions of Schilperoort et al (1967). There are common sequences between tumor cell DNA and bacterial DNA (tissue cultures are bacteria-free). TIP would thus appear to be a fraction of bacterial DNA integrated into the genome of the plant cell. This integration of genetic material might account for the acquisition by tumorous cells of such properties as the accelerated synthesis of growth factors and the appearance of new nitrogen-compounds.

An estimation of the amount of bacterial DNA integrated into the host genome may be attempted in the following way. The saturation value is about 0,2 % (*DNA x 100/DNA) : if the DNA content of a diploïd is assumed to be $6. 10^{-12}$ g , the calculated value of bacterial DNA integrated amounts to $\simeq 1. 10^{-14}$ g , a value of the same order of magnitude as an entire bacterial genome. This number (the equivalent of one bacterial DNA content per transformed cell DNA) is to be considered as a very coarse estimation however because of the little-known nature of nitrocellulose hybrids.

The possible integration of an entire bacterial genome into the DNA of a higher organism seems surprising : the following must be kept in mind however : 1°) there is a similar ratio of DNA content between virus and bacteria as between bacteria and higher organism (500 to 1000). 2°) virus DNA may be integrated in the genome of both bacteria (lysogeny, see Cowie and Szafranski , 1967) and cells of higher organisms (transformation, see Fujinaga and Green, 1968) ; there is thus no a priori impossibility for the entire integration of bacterial DNA into the genome of a higher organism.

II. Homology between A.t. DNA and Nh DNA of stressed normal cells.

Results shown in table I illustrate the second point set out in our interpretation.They indicate a partial homology between A.t. DNA and the

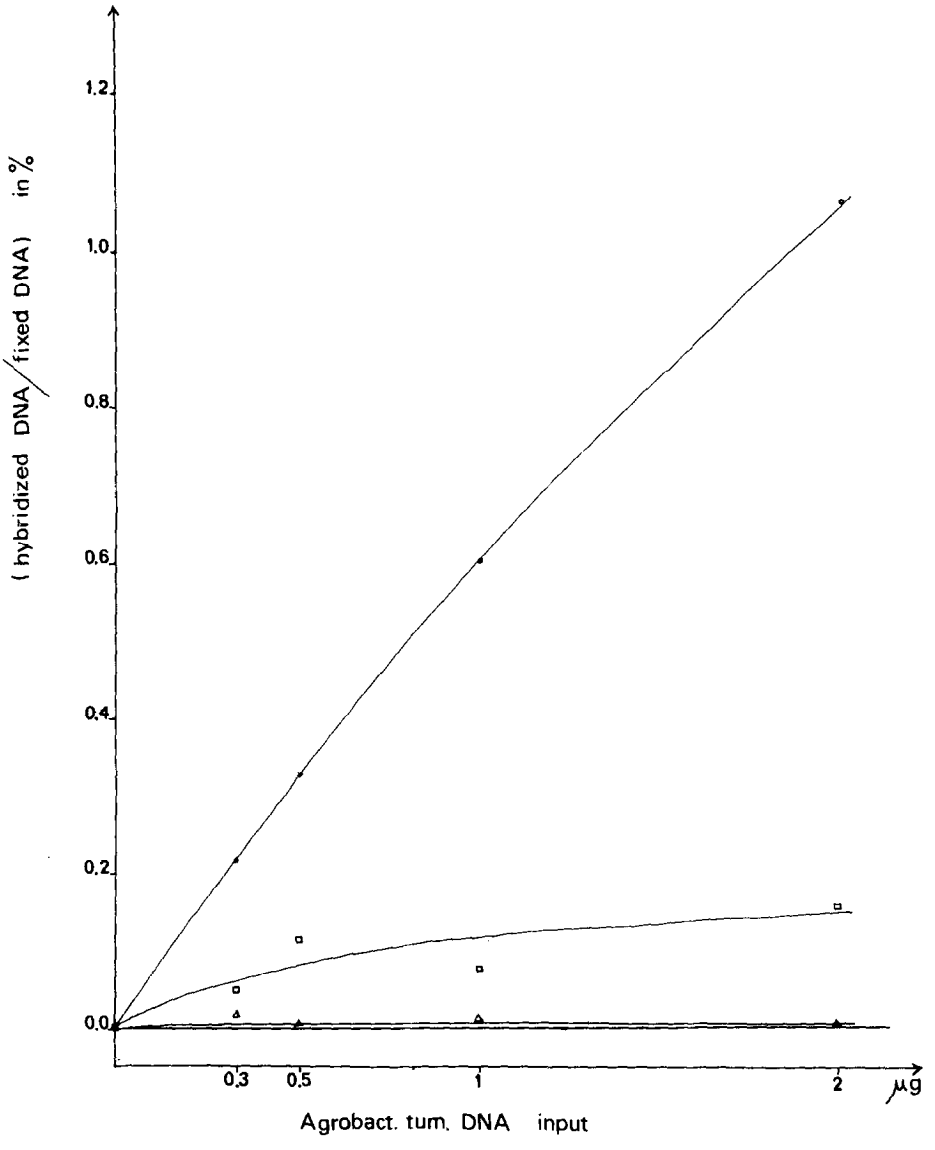

Fig.1: Hybridizations between bacterial DNA and tumorous or normal cell DNA: Each membrane filter carrying 50 µg of unlabelled DNA of specified origin was allowed to hybridize with increasing amounts of ^{32}P labelled bacterial DNA:

o——o, fixed unlabelled A.t.DNA x ^{32}P A.t. DNA, no saturation visible in this range.

△——△, fixed unlabelled nuclear(main band)DNA of normal tobacco cell x ^{32}P A.t.DNA; no homology detectable(background).

□——□, fixed unlabelled nuclear DNA of tumorous tobacco cell x ^{32}P A.t.DNA. This hybridization (□——□) was repeated three times with tobacco and twice with Scorzonera, using ^{32}P A.t.DNA of various specific activities(up to 63,000 cpm/ug).

Table I: Hybridization between bacterial DNA and Nh satellite DNA of stressed normal cells: Each membrane filter carried 50 μg of normal cell DNA or Nh satellite DNA isolated from 48-hour-old wounded Glycine max. Hybridization seems to occur in this range without a plateau; but previous assays gave a hybridization ratio between Nh DNA and ribosomal RNA indicating that Nh DNA possess a high level of redondancy (Quétier et al, 1968 and unpublished results).

^{32}P LABELLED BACTERIAL DNA INPUT (in μg)	DNA FIXED ON FILTER (50 μg)			
	N DNA of normal cell		Nh satellite DNA (stressed normal cell)	
	Hybridized DNA in μg	% of input hybridized	Hybridized DNA in μg	% of input hybridized
0.5	background	not detectable	0.006	1.2
1	" "	" " "	0.017	1.7
1.5	" "	" " "	0.027	1.8

Nh satellite DNA appearing in stressed normal cells: this supports the hypothesis of a complex between bacterial DNA and the "active sites" appearing in conditioned cells, if they are indeed Nh DNA. Time-course similarity between the variation in the number of "active sites" per cell (Braun and Stonier, 1958), the rate of conditioning and the amitotic DNA synthesis agrees with this assumption. This homology between A.t. DNA and Nh DNA calls for two remarks: 1°) if the same locus is involved in the two hybridizations that we have observed, it is to be expected that Nh DNA would compete with the annealing between bacterial DNA and tumor cell DNA. Preliminary evidence bears out this expectation; 2°) while there is homology between tumor cell DNA and bacterial DNA, homology also exists between bacterial DNA and conditioned host cell DNA; this raises the question of the origin of the new sequences occuring in tumor cell DNA: are they of bacterial origin or are they provided (at least partly) by the host itself? The fact that Nh DNA is not detectable by analytical ultracentrifugation in both unstressed normal and tumorous plant DNA does not rule out the possibility that even when its rare sequences are amplified in tumorous plant DNA, they may go undetected.

These two sets of results afford the following comments:
-Hybridization with bacterial DNA and/or host Nh DNA seems to be a more suitable test for transformation than those currently used and may be an approach to a genetical definition of the disease(s): rescue of hybridi-

zed strands from the filter should yield further information.

-Such hybridizations should lead to a better knowledge of the characteristics that inter-relate the different plant neoplastic diseases which include the classical crown-gall tissue and its slowly growing strains, the teratoma induced by attenuated bacteria, the tissue transformed by A.t. DNA and tissue anergized to auxin in-vitro.

-This model of transformation has to be confirmed by the isolation of the natural complex between Nh DNA and bacterial DNA that should occur in the induction phase. Numerous studies published by Ledoux and Huart (1967) are in agreement with this view. A more complete description of the scheme proposed for general tumor inception will be pubished elsewhere.

SUMMARY

The mechanism of Crown-gall tumor inception has been studied by DNA-DNA hybridizations : 1°) there are common sequences between bacterial DNA and the transformed(bacteria free)cell DNA, while no detectable homology occurs with normal cell DNA; 2°) homology is shown to exist also between bacterial DNA and the Nuclear heavy satellite DNA appearing in normal stressed cells; this raises the question of the origin of the integrated new sequences. These results confirm on molecular grounds the hypothesis of a Tumor Inducing Principle and of active sites provided by the wounded host.Formation of a duplex between bacterial DNA and the Nh satellite DNA synthetized during wounding is proposed to be an obligatory step before the true integration of genetic material, provided by the bacteria and/or the host itself.

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