RNA-DNA HYBRIDIZATION STUDIES WITH THE CROWN GALL BACTERIA AND THE TOBACCO TUMOR TISSUE*

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Received December 20, 1968

Summary. The DNA from Agrobacterium tumefaciens strains 4-32, B_6 (tumorogenic) and 11BNV$_6$ (nontumorogenic) showed respectively 0.44, 0.38 and 0.24 percent hybridization with RNA fraction "C" (14-18S RNA) from the tobacco tumor tissue but no significant hybridization with any RNA fraction from the normal tissue. It is suggested that crown gall tumors may contain "infective agent" from the bacteria. The "infective agent" in different tumorogenic strains of crown gall bacteria may be related and the nontumorogenic strain may carry an "infective agent" with some defect.

Introduction. Transformation of a normal plant cell into a tumor cell by Agrobacterium tumefaciens results in the permanent activation of several biosynthetic systems so that a tumor cell is able to synthesize auxin, cytokinin, and other substances (Braun, 1962) in abundance which must be supplied to the normal cells for growth in vitro. Since these modifications could occur by a persistant de-repression of a segment of normal cell genome or by the incorporation of a self-replicating "infective genome" into host genome the work in this laboratory was designed to distinguish between these

* Supported by United States Atomic Energy Commission Contract #AT(30-1)37221. G. E. Milo was supported by a post-doctoral fellowship (CA-5016-11) from the United States Public Health Service.

two alternatives. In the present work RNA-DNA hybridization technique has been used to examine homology between the tumor tissue and the crown gall bacteria.

Materials and Methods. Three week old cultures of the normal tissue and of the bacteria-free crown gall tumor tissue of tobacco (Nicotiana tabacum L. var. Wisconsin 38) maintained on complete and minus indoleacetic acid and kinetin media respectively (Srivastava, 1968) were used in the present work. Except for the use of greater amount of ^{32}P (500 μc) and an incubation period of 4 hours the procedures for the labelling, extraction and sucrose density gradient fractionation of nucleic acids were as described by Srivastava (1968). Fractions 1-10, 11-20, 21-32, and 42-52 (from the bottom of the tube) were pooled to give RNA fractions A, B, C, and D, respectively. Fractions 33-41 containing DNA were discarded. The pooled RNA fractions were extensively dialysed against 2 x SSC (SSC, standard saline citrate = 0.15 M NaCl, 0.015 Na citrate, pH 7.0) and after determining the $O.D._{260m\mu}$ and the trichloroacetic acid precipitable radioactivity on aliquots of RNA fractions they were used for RNA-DNA hybridization.

Tumorogenic strains 4-32 and B_6 and non tumorogenic strain $11BNV_6$ of A. tumefaciens were grown for 48 hours in nutrient broth plus 0.5% glucose. Bacteria were recovered by centrifugation and used for the isolation of DNA by the procedure of Marmur (1961). The isolated DNA was purified by chromatography on methylated albumin coated Hyflosupercel column as described by Srivastava (1967).

Highly polymerized calf thymus DNA used in some experiments was obtained from California Biochemicals Corp.

The denaturation of DNA, its immobilization on nitrocellulose filters, the formation of RNA-DNA hybrids and the post annealing ribonuclease treatment were carried out as described by Gillespie and Spiegelman (1965). Fifty micrograms of denatured DNA per filter was used in all experiments. and usually 3-4 DNA filters and one blank were incubated (18 hrs. at 67° C.)

in each scintillation vial containing 16-20 μg of radioactive RNA in 2 ml of 2 x SSC. Retention of DNA on nitrocellulose filters during immobilization was 90% and no measureable loss of DNA during annealing was noted.

All radioactivity measurements were done in a Nuclear Chicago 720 Counter using the toluene based scintillation fluid (Srivastava, 1967).

Results and Discussion. A typical set of data on RNA-DNA hybridization presented in Table I show no significant hybridization between any RNA fraction from the normal tissue and the DNA from bacteria or the calf thymus. On the other hand significant hybridization between DNA from tumorogenic strains 4-32 (tumor inducing strain for our tobacco tumors) and B_6 and RNA fraction "C" from tumor tissue was noted. Furthermore, the DNA from strain 4-32 also showed some hybridization with RNA fraction

Table I

Hybridization of DNA* from tumorogenic and nontumorogenic strains of crown gall bacteria and from calf thymus with ^{32}P labelled RNA fractions for normal and tumor tissue cultures of tobacco.

DNA source	RNA fractions	Normal tissue				Tumor tissue			
		cpm/μgRNA	RNA input (μg)	cpm bound	percent hybridization	cpm/μgRNA	RNA input (μg)	cpm bound	percent hybridization
A. tumefaciens strain 4-32	A	860	16	7	0.010	2194	16	105	0.096
	B	364	20	9	0.041	1465	20	93	0.132
	C	440	20	7	0.034	1328	20	300	0.441
	D	2805	20	7	0.005	3336	20	67	0.039
A. tumefaciens strain B_6	A	740	16	6	0.016	1965	16	4	0.004
	B	247	20	2	0.016	1147	20	3	0.005
	C	400	20	3	0.015	1000	20	189	0.380
	D	1327	20	3	0.004	4876	20	10	0.004
A. tumefaciens strain $115Nv_6$	A	860	16	6	0.014	2194	16	6	0.005
	B	364	20	2	0.015	1465	20	5	0.006
	C	440	20	2	0.009	1328	20	160	0.240
	D	2805	20	2	0.007	3336	20	10	0.006
Calf thymus	A	503	16	6	0.020	1751	16	14	0.014
	B	245	20	2	0.011	1061	20	11	0.016
	C	342	20	11	0.060	1092	20	29	0.047
	D	1313	20	10	0.013	3214	20	68	0.042

* Fifty micrograms DNA per filter was used.

"B" from the tumor tissue. These results not only support the suggestion of Schilperoort et al., (1967) that the tumorous state may be associated with the presence of the genetic material of the inducing bacteria but they also show that "infective agent" in different tumorogenic strains of crown gall bacteria may be related. It should however, be noted in Table 1 that in addition to significant hybridization between tumor RNA fraction "C" and the DNA from the tumorogenic bacteria considerable, though somewhat less, hybridization between tumor RNA fraction "C" and DNA from nonpathogenic strain $11BNV_6$ was also observed. This may suggest that nonpathogenic strain may carry an "infective agent" with some defect. In this respect it is noteworthy that tumor formation in Pinto bean leaves was stimulated if $11BNV_6$ cells were added to the inoculum containing B_6 cells (Lippincott, 1968).

Although virus like particles have not been detected in crown gall tumor cells (Lipetz, 1967; Gee et al., 1967) the increase of bacterial virulence on ultraviolet irradiation (Heberlein and Lippincott, 1967a) or on mitomycin C treatment (Heberlein and Lippincott, 1967b) support the concept of a virus being the actual tumorogenic agent in crown gall bacteria. In spite of these considerations the possibility of the transfer of the part of crown gall bacterial genome itself to the plant cell as the cause of cellular transformation should not be excluded as Ledoux and Huart (1968) have reported the integration and replication of DNA of M. lysodeikticus in DNA of germinating barley.

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