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GENETIC TRANSFORMATION OF AGROBACTERIUM TUMEFACIENS B_6 BY RNA AND NATURE OF THE TUMOR-INDUCING PRINCIPLE

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It has been shown that free RNA or RNA associated with DNA can mediate genetic information in different organisms leading to heritable changes of recipient cells (1)(2)(3)(4)(5)(§)(7). Our investigations have established that genetic information can be transferred to certain bacterial species by a specific transforming RNA found as a product excreted into the culture medium by showdomycin-resistant E.coli(2)(3). The most attractive results are illustrated by A.tumefaciens B_6, transformed by E.coli transforming RNA. These transformants have acquired new biochemical properties and have partially or completely lost their tumor inducing capacity in plants (4). Further, evidence was presented that information carried by transforming RNA could be stored as DNA in transformant A.tumefaciens B_6-Tr-1 (8). However these data did not indicate if new DNA was integrated either into bacterial genome or into plasmid of the same cells .

Data presented here show that wild type E.coli contains a DNA-bound RNA which also possesses transforming potential toward A.tumefaciens B_6. We also report that two RNA fractions isolated from oncogenic and non-oncogenic strains of A.tumefaciens are capable of initiating the formation of transplantable tumors in Datura stramonium.

"Episomal RNA " acting as transforming RNA. We have described in 1971 a procedure which permits detection of an RNA associated with bacterial DNA and termed "episomal RNA" (9). This RNA * resembles transforming RNA excreted by showdomycin-resistant bacteria by its size, base ratio (table 1) and transforming potential (2)(4); it can

+ "Episomal RNA" is defined as a DNA-bound RNA which once free from DNA is capable of inducing bacterial transformation and can be transcribed in vitro into a complementary DNA by a reverse transcriptase-like enzyme.

also be transcribed in vitro into DNA by bacterial reverse transcriptase (table 1) (10).

Table 1 : Base ratio of E.coli "episomal RNA " and of ^{3}H-DNA synthesized on E.coli " episomal RNA" .

Base	E.coli episomal RNA	^{3}H-DNA synthesized on RNA	E.coli DNA	A.tumefaciens DNA
	Moles per 100 moles of nucleotides			
A	29.7	16.7	24.5	21.8
G	35.2	17.0	24.5	30.1
C	17.6	36.2	25.0	28.2
U(T)	17.5	30.1	26.0	19.9
	$\frac{G+A}{C+U}$ = 1.87	$\frac{C+T}{G+A}$ = 1.98	$\frac{C+T}{G+A}$ = 1.04	$\frac{C+T}{G+A}$ =0.93
	G+C/A+U(T)= 1.11	1.13	0.96	1.39

" Episomal RNA" (100-200 µg) was analysed as described in the legend to table 4. ^{3}H-DNA synthesized in vitro from 4 labelled d-XTP on "episomal RNA " was analysed as described (8).

After incubation of A.tumefaciens strain B_6 with "episomal RNA" bacteria were plated on solid medium and clones analysed (4). All clones are keto-lactose positive and react with anti-B_6 serum (Table 2) . About half of clones have practically lost the tumor-inducing capacity and have acquired new biochemical properties. Densitometer tracings of polyacrylamide gel (fig.1) show that the amount of 23 S RNA of transformed cells is much higher than that of 23 S RNA of the wild type. Quantitative and qualitative differences exist between 70 S ribosomal proteins of transformants and those of wild type B_6 (fig.2). It is remarkable that transformed cells have practically lost their tumor-inducing capacity detected utilizing pea seedlings in germination (Table 3).With time these transformants became completely non-oncogenic. The above data suggest that "episomal RNA" have similar genetic potential as that of excreted transforming RNA (2).

Table 2 : Specific tests for A.tumefaciens (32)

Strains of A.tumefaciens	3-keto-lactose formation	Serological test
Wild type B_6	++++	++++
Transformant B_6-Tr-Ep.	++++	++++
a year after : Transformant B_6-Tr-Ep.A	++++	++++

Table 3 : Oncogenic capacity of A.tumefaciens B_6 and transformants B_6-Tr-Ep.

Strains of A.tumefaciens	N° of bacteria per wound host	Confidence limit of the average of adjusted tumor weights (cg)(33)	N° of plants
B_6 wild type	1.2×10^6	$15.3 < 20.68 < 26.33$	42
B_6-Tr-Ep.	1.2×10^6	$3.48 < 5.96 < 8.44$	56
a year after			
B_6-Tr-Ep-A	1.2×10^6	no tumors	45

Tumor inducing capacity was tested on pea seedlings in germination(33).

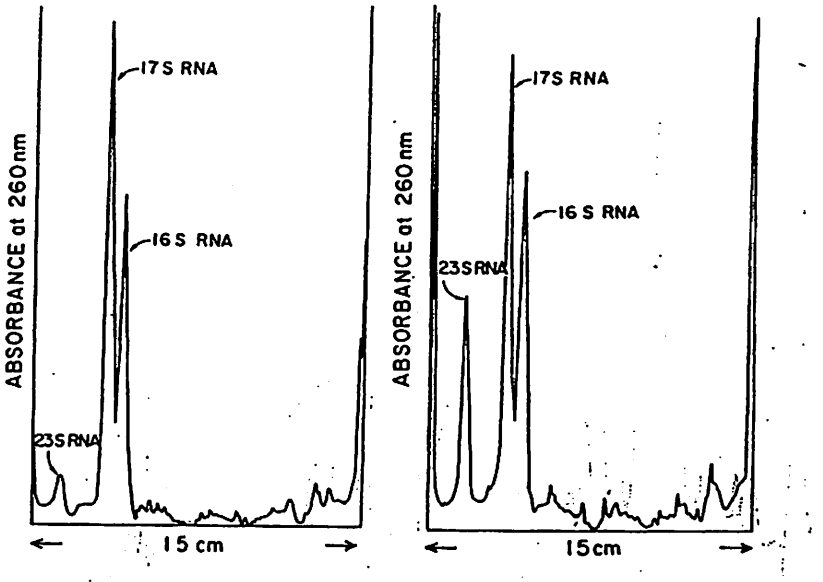

Fig.1 Densitometer tracingsof ribosomal RNAs of A.tumefaciens wild type B_6 and transformed A.tumefaciens B_6-Tr-Ep. Ribosomal RNAs isolated from washed ribosomes were separated by electrophoresis as previously described (4).Left : wild type B_6 ; right : transformed B_6-Tr-Ep .

Presence of the tumor inducing RNA in oncogenic and non-oncogenic strains of Agrobacterium tumefaciens . It is known since 1907 that oncogenic strains of A.tumefaciens possess a capacity to initiate the formation of transplantable tumors when inoculated into plant tissues (11). Once obtained , tumor cells can proliferate autonomously in the absence of the inciting bacteria. Attempts were made to show that the tumor inducing principle (TIP) (12) in A.tumefaciens is DNA (13)(14)(15)(16)(17)(18)(19)(20), although no conclusive evidence was presented. Data suggesting that an RNA rather than DNA may be an important part of the TIP in A.tumefaciens have been presented by Braun and Wood (21). These data received support from the findings that total RNA from A.tumefaciens can induce overgrowth in tomato plants although no transplantation of tissue was done (22).

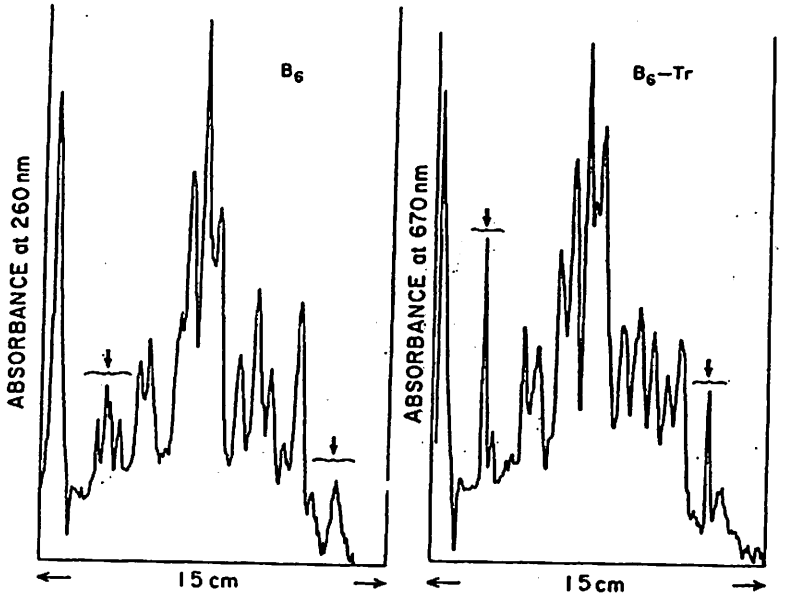

Fig.2 : Densitometer tracings of 70 S ribosomal proteins of A.tumefaciens B_6 and transformant B_6-Tr-Ep. Ribosomal proteins were isolated and separated by gel electrophoresis (4). Left : wild type B6 ; right : transformant B6-Tr-Ep

In view of our studies on transformation of bacteria by RNA it was conceivable that A.tumefaciens contains a tumor-inducing RNA bound either to DNA or to a reverse transcriptase like enzyme. In fact, two RNA fractions have been isolated and purified from oncogenic strain B_6, from non-oncogenic transformant B_6-Tr-1 (4) and from a mutant II BN V_6 (generously supplied by Dr.A.C.Braun). Both RNA fractions initiate the appearance of transplantable tumors when inoculated under axenic conditions into tissues of young D.stramonium cultured on solid synthetic medium (23).These RNAs were termed tumor inducing RNA (TI-RNA).One RNA fraction was isolated from a RNA-bound RNA directed DNA polymerase preparation present in small amounts in oncogenic and non-oncogenic strains tested as described for E.coli (24)(25).The active fractions synthesize a DNA-like, acid precipitable material when incubated in the presence of Mg^{++} ions and four deoxyribonucleoside-5'-triphosphates. No exogenous RNA or DNA are required. Fractions from the peak were mixed and treated with phenol in order to separate an RNA fraction from proteins to which it is bound.Obtained in very small amount (25) the RNA fraction is free from DNA judging by the fact that the diphenylamine reaction was negative with 1 mg of this RNA although it was positive with 1 µg of thymus DNA.

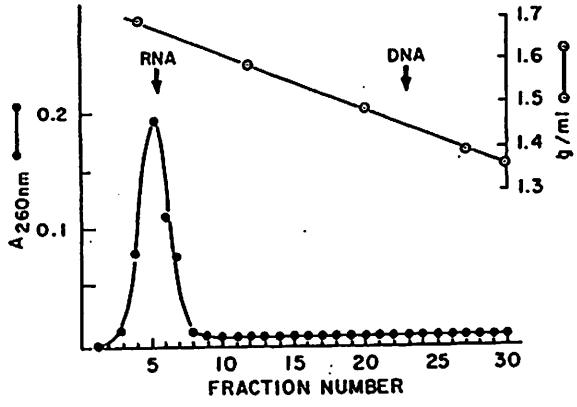

Fig.3: Cs_2SO_4 density gradient of TI-RNA isolated from reverse transcriptase of A.tumefaciens B_6-Tr-1. 30 µg of TI-RNA were mixed with Cs_2SO_4 and centrifuged at 30.000 rpm(20°) for 64h (8).Fractions were collected and the absorption was determined at 260 nm.

A second RNA fraction has been found to be linked to A.tumefaciens DNA ("episomal RNA") ; this was also isolated and analysed (25). TI-RNA were characterized in several respects : Cs_2SO_4 density gradient (fig.3) showed that TI-RNA sedimented in the density region of RNA (1.642) and that no U.V. absorbing material was found either at the density of RNA-DNA hybrid or at the density of DNA (1.420). TI-RNAs are rich in purine nucleotides (table 4) and are single stranded as judged by the absence of hyperchromicity in the presence of RNase A at low (0.001 x SSC) ionic strength (SSC : 0;15 M NaCl + 0.015 M sodium citrate-Na_3). On sucrose gradients , TI-RNA sediments between 5 - 6 S (25) value which was also obtained by gel electrophoresis (fig.4). In its size, TI-RNA resembles "viroid RNA " capable of inducing the potato spindle tuber disease discovered by Diener (26).

Table 4 Base ratio analysis of TI-RNA isolated from RNA-bound reverse transcriptase of A.tumefaciens and that of TI-RNA transcript.

moles per 100 moles of nucleotides

Bases	Ribosomal RNA (23 S + 16 S) of B_6 strain	TI-RNA (B_6 strain)	TI-RNA (B_6-Tr-1 strain		DNA synthesized upon TI-RNA B_6-Tr-1
A	25.2	26.0	29.8		21.0
G	29.8	34.6	31.8		20.2
C	23.5	20.4	21.2		28.2
U (T)	21.5	18.9	17.2		30.6
G+A/C+U	1.22	1.43	1.62	$\frac{C+T}{G+A}$ ..	1.46
G+C/A+U	1.16	1.20	0.97		

Nucleotides from TI-RNA and ribosomal RNA (100-200 µg) were analysed by thin layer chromatography (34). ^{3}H-DNA synthesized from 4 ^{3}H d-XTP on TI-RNA was analysed as described (8).

Transcription of TI-RNA into a complementary DNA by bacterial and "plant reverse transcriptase" like enzyme. Table 5 shows that TI-RNA can be used in vitro as a template by bacterial reverse transcriptase for synthesis of DNA . The reaction requires the presence of 4 d-XTP and Mg^{++} ions. DNA polymerizing activity seems

also to be present in the extract from normal tobacco plant cells (in vitro cultured tobacco cells) fractionated on a DEAE-cellulose column. For this latter enzyme Mg^{++} cannot replace Mn^{++}(table 5). The amount of ^3H-product is greatly decreased in the presence of RNase and the ^3H-product is not detected in the presence of DNase. On neutral Cs_2SO_4 gradient, the ^3H-product separated from the enzyme sedimented at the densities of both DNA-RNA hybrids and free DNA (Fig.5).After treatment with 0.3 N KOH (heating at 80° for 20 mn) all of the ^3H-product sedimented at the density of DNA (fig.4). On sucrose gradients the ^3H-product pretreated with alkali sedimented at 5 - 6 S ,a value which corresponds to that obtained for TI-RNA itself (25).

Table 5 Transcription of TI-RNA into a DNA like material by reverse transcriptase

	CPM of ^3H-d-TTP incorporated in 20 mn	
	E.coli enzyme	Plant enzyme
Complete	3730	398
" - Mg^{++}	220	-
" - Mn^{++}	-	67
" - d-GTP,d-CTP,d-ATP	195	72
" + RNase A and T_1 (20 µg each)	240	81
" + DNase (5 µg)	232	57

Incubation conditions (see ref.8); E.coli reverse transcriptase, 60 µg (fraction II)(24) or Tobacco "reverse transcriptase," 200 µg (fraction II from DEAE-cellulose column.

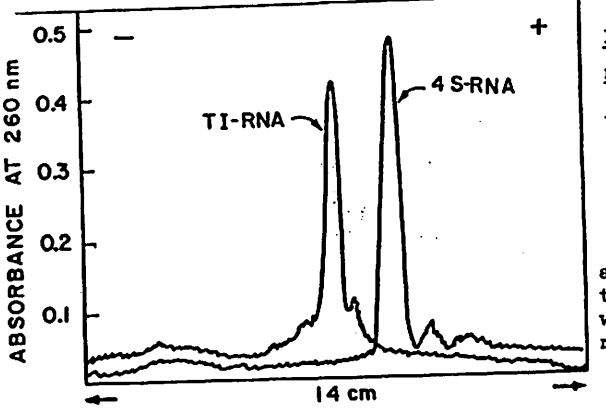

Fig.4:Densitometer tracings of TI-RNA(B_6-Tr-1) 15 µg of RNA separated on polyacrylamide gel (7.5 % w/v)for 2h at 5 mA per tube at 4°(4). 4 S RNAmet.was used as marker.Before electrophoresis TI-RNA was heated for 10 mn.at 100°.

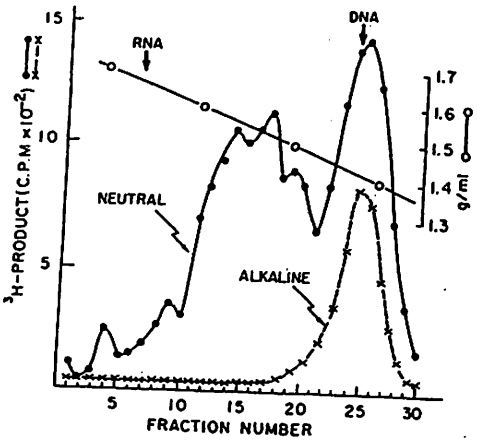

Fig.5 Cs_2SO_4 gradient analysis of the ^{3}H-product. The product synthesized on B_6Tr-1 TI-RNA by E.coli reverse transcriptase (see table 5) was separated from the enzyme by chloroform. After dialysis against 0.2 M KCl it was analysed as previously described (8).TCA precipitable material was counted.

Induction of tumors in Datura stramonium with TI-RNA.TI-RNA removed from RNA-bound reverse transcriptase and "episomal RNA" both originating from A.tumefaciens B_6 strain were injected (4-5 ug per wound) into stems of D.stramonium grown under axenic conditions. Tumors induced with TI-RNA from oncogenic and non-oncogenic strains (table 6) generally appeared latter in all plants than those with A.tumefaciens B_6 cells. They did not appear when TI-RNA was pretreated with alkali or with rather high amount of RNase A. Ribosomal RNA from E.coli is inactive and total RNA from strain B_6 induces a callus. DNA synthesized in vitro on TI-RNA seems to be non-oncogenic (table 6).Overgrowth tissues are considered as tumorous only if they can be indefinitely maintained by grafting on young D.stramonium (25).Tumors induced with TI-RNA were grafted successively at least three times with always positive results.On certain stems, tumors appeared after grafting at a distance from the point of graft, indicating that a transmissible tumor inducing agent is present in Crown Gall tumor tissue as already shown by others (27)(28).Tumors induced with TI-RNA can be poorly cultured in vitro on a medium which does not allow a healthy plant tissue to grow .

Table 6 :Tumor induction in young D.stramonium with TI-RNA isolated from A.tumefaciens strains

Source of RNA	Tumors
B_6 wild type (TI-RNA from rev.trans.*)	+++
B_6-Tr-1 " " " "	+++
B_6-Tr-1 " " " " treated with RNases A+T_1	0
B_6-Tr-1 " " " " + E.coli rev.trans.	+++
II BN V_6 " " " ".....	+++
B_6 wild type (Ep.RNA)	+++
B_6-Tr-1 " "	+++
B_6-Tr-1 " " treated with 0.3 M KOH	0
II BN V_6 (Ep.RNA)	+++
Total RNA from B_6 wild type	callus
Ribosomal RNA from E.coli K 12	0
DNA synthesized in vitro on TI-RNA from rev.trans.(B_6Tr-I)	0

* Rev.trans.= reverse transcriptase.For details see ref.25

Discussion and Conclusions

Evidence has been presented that E.coli wild type "episomal RNA" originally bound to DNA but separated from DNA during purification is capable of transforming A.tumefaciens oncogenic strain B_6 into transformants which have acquired new biochemical properties. After transformation they have lost 70 % of tumor inducing capacity.With time they become completely non-oncogenic as it was observed with partial transformant B_6Tr-4 and B_6 Tr-4 A (7). The fact that E.coli "episomal RNA " acts as transforming agent is not surprising since this RNA can be transcribed (in vitro) into a complementary DNA. In vivo newly made DNA may be integrated into DNA of recipient cells, as demonstrated for transformants B_6-Tr-1 (4).

We have established that two RNA fractions isolated from A.tumefaciens strains and characterized by different methods, are effective in initiating the formation of transplantable tumors in Datura plants.

What is the basic mechanism by which TI-RNA from A.tumefaciens provokes the tumorogenic state in normal plant cells ? Once inside the recipient cell TI-RNA could be transcribed into DNA by a reverse transcriptase like enzyme and then either associated with cell DNA or integrated into the cell genome or some genetic extrachromosomal element and replicate, thus leading to cell transformation.

It is also conceivable that TI-RNA inside the plant cells can act as a primer for replication of host cell DNA either by competing with the host RNA primers required for DNA replication or by initiating the new sites.During this process primer RNA could be bound to DNA by a covalent linkage thus interfeering in the replication process (20). The observation that cells derived from Crown Gall teratoma (30)may revert to normal state and that there appears to be a transmissible agent in Crown Gall tumorous tissue, suggested that the tumor inducing principle introduced into transformed plant cells could be maintained as a autonomous epigenic element (31). The fact that TI-RNA was found to be present in oncogenic and non-oncogenic strains of A.tumefaciens suggests : 1) that it is not released in biologically active form from the non-oncogenic strains and(2) that the oncogenic strains have some special mechanisms for introducing the TI-RNA into host cells that is not possessed by the avirulent strains .

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