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Isolation of the Tumor-Inducing RNA from Oncogenic and Nononcogenic *Agrobacterium tumefaciens*

(crown gall disease/bacterial RNA/transplantable tumors)

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ABSTRACT Two RNA fractions have been isolated and purified from both oncogenic and nononcogenic strains of *Agrobacterium tumefaciens*. Both RNAs are capable of inducing the formation of transplantable tumors when introduced at wound sites in stems of *Datura stramonium* plants. One of these RNA fractions was found to be bound to an RNA-directed DNA polymerase, while the other was associated with the bacterial DNA. Physical evidence suggests that both are single stranded and small in size; linear sucrose gradients show that their size corresponds to a value of 5–6 S. A concentration of 4–5 μg of the RNAs dissolved in 0.01 ml of water is effective in initiating the formation of transplantable tumors in *Datura* plants.

Oncogenic strains of *Agrobacterium tumefaciens* possess a capacity to regularly initiate the formation of transplantable tumors when inoculated into tissues of many different plant species (1). The bacteria are concerned only with the initiation of the tumors, and once the cellular transformation has been accomplished the tumor cells proliferate autonomously in the absence of the inciting bacteria. These findings suggest that a factor of considerable biological interest passes from the bacteria to the host cells and brings about a heritable change in the affected cells. This hypothetical factor is known as the tumor-inducing principle (2). A number of reports have been published showing that DNA preparations isolated from oncogenic strains of *A. tumefaciens* may induce hyperplasia in plants, although the initiation of tumors of a transplantable type was not demonstrated in any case (3–11). Data suggesting that RNA rather than DNA may play a role in the transformation process were presented by Braun and Wood (12). This suggestion appeared to receive support from the findings of Swain and Rier (13), who reported that total RNA isolated from an oncogenic strain of *A. tumefaciens* induced the formation of overgrowths in tomato plants. No attempt was made in that study, however, to learn whether cells of the new growths were transplantable.

Evidence is presented in the present study to show that two RNA fractions, one isolated from RNA-bound RNA-directed DNA polymerase and the other associated with the DNA of *A. tumefaciens*, have a capacity to initiate the formation of transplantable tumors when inoculated into stems of *Datura stramonium* plants. These RNAs have similar physical and chemical properties and are termed tumor-inducing RNA (TI-RNA). The RNAs are found in both oncogenic and nononcogenic strains of *A. tumefaciens*. The possible mechanism by which the TI-RNAs operate is discussed.

Abbreviations: TI-RNA, tumor-inducing RNA; dNTP, deoxyribonucleoside-5′-triphosphates; episomal RNA, DNA-bound RNA possessing tumor-inducing potential; 2× SSC, 0.3 M NaCl–0.03 M sodium citrate.

MATERIALS AND METHODS

Chemicals Used. Deoxyribonucleoside-5′-triphosphates (dNTP), ^{12}C and ^{3}H-lithium salt (specific activity 17.5 Ci/mole), were obtained from Schwarz/Mann, Orangeburg, N.Y.; DEAE–cellulose DE-52 and glass filter GF/C from Whatman; deoxyribonuclease and ribonuclease A from Worthington Biochemical Corp., Freehold, N.J.; Pronase from Calbiochem, Los Angeles, Calif.; Bacto-tryptone and yeast extract from Difco Labs., Detroit, Mich.; kinetin from Nutritional Biochemical Corp., Cleveland, O.; and indoleacetic acid from Touzart et Matignon, France.

A. tumefaciens Strains. Oncogenic strain B_6 and nononcogenic strains B_6-Tr-1 obtained by transformation of strain B_6 with RNA (14) and IIBNV6 (generously supplied by Dr. Armin C. Braun) were grown aerobically at 30° in Bacto-tryptone–yeast extract–NaCl (10:5:5 g/liter, pH 7.3). After the strains were plated on solid synthetic medium (5), their purity was tested by the 3-keto-lactose reaction (15), by a serological test with B_6 antiserum, and by a capacity for tumor induction.

Escherichia coli Strain K-12 Hfr, grown as described (16), was also used for isolation of certain species of RNA as well as for isolation of RNA-free RNA-directed DNA polymerase.

Isolation of Tumor-Inducing RNA from A. tumefaciens. For isolation of the tumor-inducing principle, overnight cultures were concentrated by centrifugation, and the bacterial pellet (80-100 g) was used to prepare the tumor-inducing RNA either linked to RNA-directed DNA polymerase or associated with DNA.

Isolation of tumor-inducing RNA bound to RNA-directed DNA polymerase. Extracts of *A. tumefaciens* cells were prepared and fractionated on a DEAE–cellulose column as described for *E. coli* (17). This procedure allowed us to isolate from *A. tumefaciens* the complex of RNA-directed DNA polymerase bound to tumor-inducing RNA (see Fig. 1). RNA was separated from the enzyme by the phenol method, precipitated by alcohol, and dialyzed against distilled water containing 0.2 M KCl (yield in RNA, about 100 μg). The amount of RNA was determined by UV absorption at 260 nm and by orcinol reaction. Diphenylamine reaction was negative with 1 mg of TI-RNA although it was positive with 1 μg of thymus DNA.

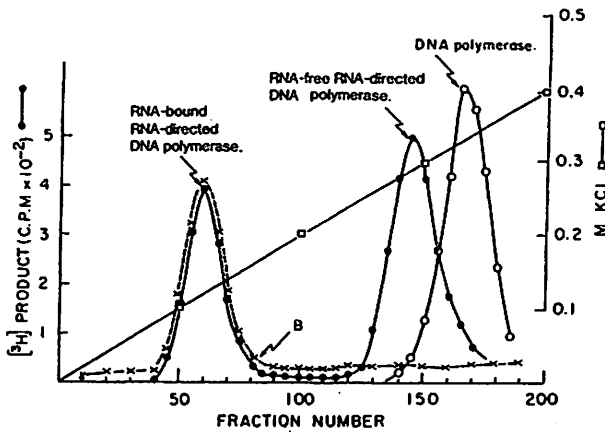

Fig. 1. Isolation of RNA-bound RNA-directed DNA polymerase) from *A. tumefaciens* on a DEAE-cellulose column. Dialyzed proteins (3 g) corresponding to the second $(NH_4)_2SO_4$ fraction from crude bacterial extracts were fractionated on a DEAE-cellulose column (14). The activity of RNA-bound RNA-directed DNA polymerase was determined (in the absence of exogenous template RNA) by using all four dNTPs ([³H]dTTP) and Mg^{++} ions. Fractions of RNA-bound RNA-directed DNA polymerase were mixed, and the RNA was isolated by phenol (see *Methods*). (*B*) X---X, without nucleic acid; ●——●, with nucleic acid. Values for cpm have been multiplied by 10^{-2}.

Isolation of tumor-inducing RNA associated with DNA. The general principle of the procedure for isolation and purification of DNA containing RNA (DNA-bound RNA, also called "episomal RNA") described for *E. coli* was used (18). *A. tumefaciens* (80 g), suspended in 150 ml of sterile distilled water†, was lysed with aqueous sodium lauryl sulfate (0.2% final concentration) added slowly with stirring. The mixture of lysed bacteria was diluted with an equal volume of distilled water, stirred (10 min at 4°), and centrifuged at 10,000 × *g* for 10 min. The supernatant containing DNA was removed and mixed with two volumes of cold 96% ethyl alcohol. The lower phase was reextracted four times with sterile distilled water. The upper phase containing DNA was removed and mixed with two volumes of cold 96% ethyl alcohol. DNA was collected on a glass rod and dissolved in a solution of 2× SSC (0.3 M NaCl-0.03 M sodium citrate), then treated twice with an equal volume of phenol and twice with an equal volume of chloroform. After precipitation with alcohol, DNA was dissolved in 2× SSC and dialyzed for 20 hr at 4° against 2 liters of 2× SSC.

Such a DNA preparation contains a large amount of RNA, most of which is removed by successive treatments with RNase A (20 μg/ml of enzyme heated at 100° for 10 min) and RNase T1 (5 μg/ml), for 30 min at 37°. These treatments were carried out in 2× SSC, which prevents the complete removal by RNase of RNA associated with DNA. After extensive purification with phenol and chloroform (absence of interphase) and dialysis against 2× SSC for 20 hr at 4°, the amount of residual RNA did not exceed 10% (orcinol reaction) of the total nucleic acid content of the DNA preparation. Such a DNA preparation, dialyzed at 4° for a few hours against distilled water, was sonicated (Bronson sonicator)

† In Tris buffer or in solution containing 1 M NaCl or KCl, there is a rapid degradation of *A. tumefaciens* DNA.

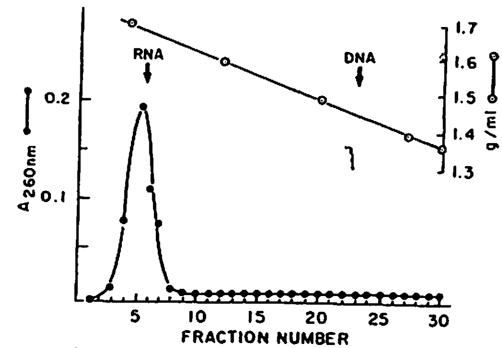

FIG. 2. Cs_2SO_4 neutral density gradient of TI-RNA isolated from RNA-bound RNA-directed DNA polymerase of *A. tumefaciens* B_6-Tr-1. 30 μg of TI-RNA isolated from RNA-bound RNA-directed DNA polymerase of *A. tumefaciens* B_6-Tr-1 was mixed with Cs_2SO_4 and centrifuged at 30,000 rpm (20°) for 64 hr (30). Fractions were collected and the absorbance was determined at 260 nm.

for 10 min and treated with 20 μg of DNase/ml in the presence of Mg^{++} (10 mM) at 36° for 30 min. DNase was removed with chloroform (2-3 treatments; treatments with DNase were repeated if necessary). The supernatant containing RNA was precipitated with 2 volumes of cold alcohol and left for several hours at −20°. The precipitate was concentrated by centrifugation and dialyzed for 24 hr at 4° against sterile distilled water (yield in RNA, about 1 mg). The RNA preparation in Cs_2SO_4 density gradient sedimented at the density of RNA.

Before being used for tumor induction, RNA preparations were treated with chloroform for 30 min and dialyzed overnight at 4° against distilled water under sterile conditions. No *A. tumefaciens* cells were found in these preparations.

Cs_2SO_4 Density Gradient. Samples of RNA were subjected to Cs_2SO_4 (1.8 g/3 ml) density gradient centrifugation at 20° for 64 hr at 30,000 rpm in an SW39 Spinco rotor. Fractions were collected and analyzed by UV absorption at 260 nm.

Sucrose Gradient Analysis. RNA size was determined by centrifugation on 5-20% linear sucrose gradient. Sucrose was dissolved in a solution of 10 mM Tris·HCl (pH 7.65) containing 0.1 M NaCl and 10 mM $MgCl_2$. When DNA was

TABLE 1. *Base ratio analysis of TI-RNA isolated from RNA-bound RNA-directed DNA polymerase of A. tumefaciens B_6 and B_6-Tr-1*

	Moles per 100 moles of nucleotides		
Nucleotides	Ribosomal RNA (23 S + 16 S) of B_6 strain	TI-RNA (B_6 strain)	TI-RNA (B_6-Tr-1 strain)
A	25.2	26.0	29.8
G	29.8	34.6	31.8
C	23.5	20.4	21.2
U	21.5	18.9	17.2
(G+A)/(C+U)	1.22	1.43	1.62
(G+C)/(A+U)	1.16	1.20	0.97

Base ratio of TI-RNA was determined as described in *Methods*, and that of ribosomal RNA of *A. tumefaciens* B_6 as described (14).

analyzed, $MgCl_2$ was replaced by 1 mM EDTA. Centrifugation was carried out in a SW39 Spinco rotor for 16 hr at 25,000 rpm. Collected fractions were analyzed by UV absorption at 260 nm.

Nucleotide Analysis. RNA (100-200 μg) dissolved in 1 N HCl was hydrolyzed for 60 min at 100° in a boiling water bath. After drying, the products were separated by thin-layer chromatography in two dimensions according to the methods of Björk and Svenson (19). UV-absorbing spots were eluted in 0.1 HCl and analyzed by A_{280}/A_{260} ratio.

Plants Used for Tumor Induction. In order to determine the homogeneity and tumor-inducing potential of different strains of *A. tumefaciens* (wild-type B_6, transformant B_6-Tr-1, and mutant IIBNV6), clones were tested on *Pisum sativum* (variety Annoney) and on *D. stramonium*. B_6 strain was oncogenic while transformant B_6-Tr-1 and mutant IIBNV6 were nononcogenic for tumor induction. The procedure for inoculation of bacterial cells into pea seedlings as well as quantitative estimation of tumors have been described (20). Pea seedlings were kept at 24° in the dark.

When RNA preparations were tested for tumor induction, young plants of *D. stramonium* were used because they respond actively to tumor induction. In addition, these plants require rather small amounts of the RNA fractions for the induction of tumors. Plants were kept constantly illuminated in a room at 24°, under axenic conditions.

Growth of D. stramonium in Tubes. After seed coats were removed, seeds were carefully made aseptic with Ca-hypochlorite (30 g/liter) for 3 min at room temperature and allowed to germinate in tubes containing solid medium (MS) without carbon source (21). After 8 days at 24° in the dark, the length of the stem was approximately 5-6 cm. Several transfers of the young plants to fresh MS medium containing kinetin, indoleacetic acid (each at 0.3 μM), and sucrose (30 g/liter), allowed us to obtain (with illumination) plants having a stem diameter large enough (2 mm) for inoculation of samples and grafting of tumors (sterility was checked in all tubes). Callus never appeared when distilled water was injected instead of RNA preparations or *A. tumefaciens* B_6.

Inverted Stem Sections for Tumor Induction. Two-centimeter sections of *D. stramonium* stems (basal extremity turned up) were introduced into tubes containing medium (MS) as described by De Ropp (22). When axenic callus appeared after a few days on both extremities of the stem sections, the RNA (4-5 μg, in water solution) was introduced into a stem that had been wounded by making a lateral cut. As controls, several inverted stems were inoculated with distilled water. Proliferation of cells occurred at the site of inoculation. Callus was small when distilled water was inoculated on inverted stem sections.

RESULTS

General Characteristics of Tumor-Inducing RNA Isolated from A. tumefaciens. Tumor-inducing RNA (TI-RNA) isolated from RNA-bound RNA-directed DNA polymerase (material in peak I from DEAE-cellulose column, see Fig. 1) and episomal RNA separated from DNA of *A. tumefaciens* were characterized in several respects. First, Cs_2SO_4 density gradient analysis showed (Fig. 2) that TI-RNA sedimented in the density region of RNA (1.642 g/cm^3) and that no UV-absorbing material was found either at the density of RNA·DNA hybrid or at the density of DNA itself (1.420 g/cm^3). The same results were obtained with both types of TI-RNAs. Second, base ratio analysis of TI-RNA showed that purine nucleotides were present in excess over pyrimidines (Table 1) when compared to ribosomal RNA of *A. tumefaciens* B_6. Third, TI-RNA is single-stranded, as judged by the absence of hyperchromicity in the presence of RNase A at low (0.001 × SSC) ionic strength.

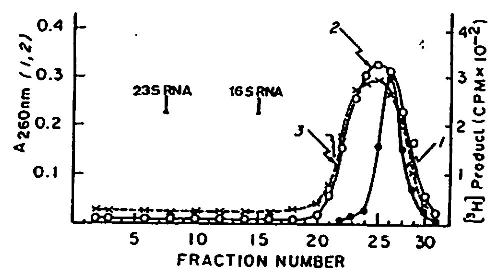

FIG. 3. Sucrose gradient analysis of TI-RNA isolated from RNA-bound RNA-directed DNA polymerase from *A. tumefaciens* B_6-Tr-1. 100 μg of TI-RNA were layered on a 5-ml, 5-20% sucrose gradient (see *Methods*) and centrifuged at 25,000 rpm in a Spinco SW39 rotor at 4° for 16 hr. $tRNA^{Met}$ was used as marker. Fractions were collected, and the UV-absorbing material was detected at 260 nm. (*1*) ●——●, $tRNA^{Met}$; (*2*) ○——○, TI-RNA. (*3*) X----X, the position of [^{3}H]DNA transcript was determined by trichloroacetic acid-precipitable radioactivity.

TI-RNA isolated from RNA-bound RNA-directed DNA polymerase has a tendency to aggregate. It was therefore heated for 10 min at 100° before being analyzed by polyacrylamide gel electrophoresis.

The size of TI-RNA determined on linear sucrose gradients corresponds to a value of 5-6 S (Fig. 3). In this respect TI-RNA resembles "viroid RNA," which is capable of inducing the potato spindle tuber disease (23).

Tumors on Stems of D. stramonium Inoculated with TI-RNA. TI-RNA removed from RNA-bound RNA-directed DNA polymerase and episomal RNA, both free of DNA and originating from *A. tumefaciens* B_6 strain, were injected once into *D. stramonium* stems as described in *Methods*. In certain experiments tumors appeared in all plants that were inoculated with TI-RNA; in others, three or six of ten plants produced tumors that generally appeared later than those induced with intact *A. tumefaciens* B_6.

Our main results are presented in Fig. 4 and Tables 2 and 3. Those presented in Table 3 were obtained by inoculating the episomal RNA as such or in association with *E. coli* RNA-directed DNA polymerase into *Datura* stems. Tumors appeared on plants inoculated with episomal RNA originating either from oncogenic strain B_6 or from nononcogenic strain B_6-Tr-1 and IIBNV6. Inoculation of *E. coli* RNA-directed DNA polymerase at the same time as episomal RNA did not seem to influence tumor induction to a significant degree. Tumors did not appear when TI-RNA was pretreated with KOH or high amounts of RNase. It should be recalled that TI-RNA is highly resistant to RNase A because of the high A-G nucleotide content. Ribosomal RNA from *E. coli* did not induce tumors. Total RNA from strain B_6 has been found to induce callus-like overgrowths on tomato plants (13).

Results presented in Table 2 were obtained with RNA preparations isolated from RNA-bound RNA-directed DNA

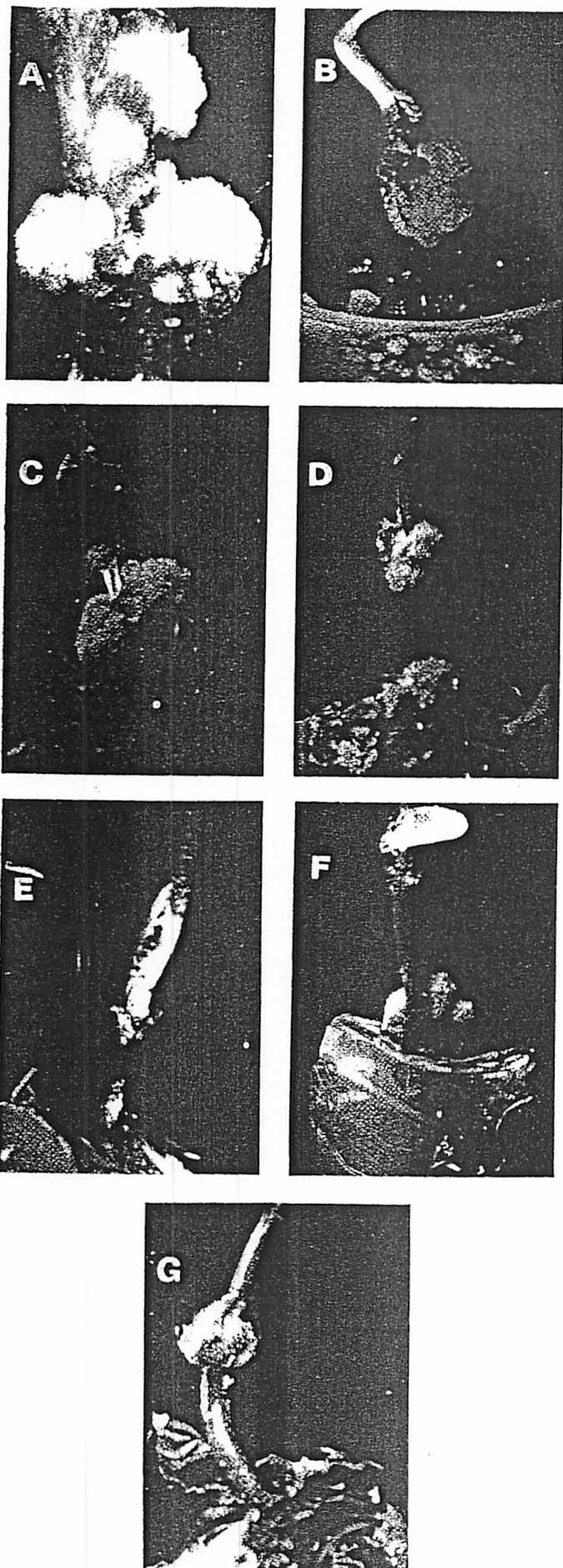

TABLE 2. *Tumor induction with RNA episome isolated from strains of A. tumefaciens*

Source of RNA	Tumors
B_6 wild type	+++
B_6-Tr-1 transformant	+++
B_6-Tr-1 transformant, treated with 0.3 M KOH	0
II BNV6	+++
E. coli (Hfr K 12) ribosomal RNA	0
Total RNA from B_6	callus

TI-RNA (4–5 μg) in 0.01 ml of water isolated from DNA (episomal RNA) was used (see *Methods*).

polymerase (see Fig. 1). Tumors appeared whether the TI-RNA originated from oncogenic strain B_6 or nononcogenic strains B_6-Tr-1 and IIBNV6. These results, as well as those obtained with episomal RNA, show that tumor-inducing RNA is present in all strains used and that both RNA fractions have similar properties.

Transplantability of Tumor Tissue. Tumorous tissues that appeared after 2-3 weeks were grafted into healthy *D. stramonium* stems in the absence of TI-RNA. We consider our results as positive when the implanted cells develop into a tumorous growth and can be maintained by grafting on young *D. stramonium* plants cultured on solid medium in tubes (Fig. 4). Tumors that were induced with episomal RNA or RNA from RNA-directed DNA polymerase were grafted successively at least three times. On certain stems tumors appeared at a distance from the point of the graft. Callus obtained after inoculation with distilled water could not be maintained by grafting.

DISCUSSION AND CONCLUSIONS

Evidence presented here demonstrates that a specific RNA found in both oncogenic and nononcogenic strains of *A. tumefaciens* initiates the formation of transplantable tumors when inoculated into stems of *D. stramonium* plants. This tumor-inducing RNA is rich in A-G nucleotides and is found associated not only with the bacterial DNA but is also bound to a RNA-directed DNA polymerase found in those organisms. This RNA has been characterized with the use of Cs_2SO_4 density gradient centrifugation, base ratio analyses, and sedimentation properties on sucrose gradients. The TI-RNA appears to be single-stranded, and the size of the RNA as determined on linear sucrose gradients corresponds to a value of 5-6 S. In this respect it resembles the "viroid RNA," which is the causal agent of the spindle tuber disease of potato (23).

FIG. 4. Demonstration of the oncogenic capacity of the tumor-inducing RNA isolated from *A. tumefaciens*. (*A*) Tumors obtained by inoculation of episomal RNA (strain B_6) into a fragment of inverted stem of *D. stramonium*. Development of secondary tumors after grafting of the primary tumorous tissue that was obtained with the following TI-RNA: (*B*) episomal RNA (from oncogenic strain B_6); (*D*) RNA isolated from RNA-bound RNA-directed DNA polymerase (nononcogenic strain IIBNV6); (*F*) RNA isolated from RNA-bound RNA-directed DNA polymerase (nononcogenic strain B_6-Tr-1); (*G*) episomal RNA (from nononcogenic strain B_6-Tr-1); (*C*) oncogenic bacteria B_6 used instead of TI-RNA; (*E*) absence of tumorous tissue after graft of an axenic callus tissue.

TABLE 3. *Tumor induction with RNA isolated from RNA-bound RNA-directed DNA polymerase of A. tumefaciens.*

Source of RNA	Tumors
B_6 wild type	+++
B_6 wild type + *E. coli* RNA-directed DNA polymerase	+++
B_6-Tr-1 transformant	+++
B_6-Tr-1 transformant treated with RNases A and T1	0
B_6-Tr-1 transformant + *E. coli* RNA-directed DNA polymerase	+++
II BNV6 mutant	+++
DNA transcribed upon TI-RNA from B_6-Tr-1	0

TI-RNA (4-5 μg) in 0.01 of ml water isolated from RNA-bound RNA-directed DNA polymerase was used (see *Methods*). 50 μg of TI-RNA were incubated with RNases A (25 μg) and T1 (10 μg) for 3 hr at 36°. RNases were removed by chloroform. The aqueous phase, after it was dialyzed, was used for inoculation.

It is generally agreed that to be considered truly tumorous experimentally produced overgrowths in plants should possess the following properties: (*1*) development of tumorous tissue in a host should be nonself-limiting (24); (*2*) cells of the new growths should be transplantable (24); and (*3*) such cells should grow indefinitely on a minimal culture medium that does not support the growth of normal cells of the type from which the tumor cells were derived (25). Tumor tissue transformed with TI-RNA preparations satisfies two of these criteria (*1* and *2*). Difficulty was experienced in maintaining cells from these tumors *in vitro*. The same difficulty was experienced in culturing cells from tumors initiated with the intact oncogenic B_6 strain of *A. tumefaciens*. This may mean that tissue from *Datura* plants contains some as yet uncharacterized nutritional requirement that was not satisfied by the culture medium used in these studies.

The fact that TI-RNA is found to be present in nononcogenic as well as in oncogenic strains of *A. tumefaciens* suggests: (*1*) that it is not released in biologically active form from the nononcogenic strains of the bacteria; or (*2*) that it is released from those strains but is inactivated before accomplishing a transformation; or (*3*) that the oncogenic strains have some special mechanism for introducing the TI-RNA into host cells that is not possessed by the avirulent strains. Other possibilities, of course, exist.

The tumor-inducing RNA obtained from *A. tumefaciens* could be involved in inducing the tumorous state either by being transcribed into DNA by a RNA-directed DNA polymerase-like enzyme where it would then be physically closely associated with or integrated into and replicate with the genome of the host cell, or it could be present in the form of a more or less autonomous entity. The finding that there appears to be a readily transmissible tumor-inducing agent present in crown gall tumor tissue (26, 27) and that crown gall tumor cells may, under certain special conditions, revert to normal cell types (28, 29) appears to favor the second alternative. The results reported here, nevertheless, suggest that a specific RNA may play an important role in the tumor cell transformation in the crown gall disease.

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