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PARTICULAR SMALL SIZE RNA AND RNA FRAGMENTS FROM DIFFERENT ORIGINS AS TUMOR INDUCING AGENTS IN *DATURA STRAMONIUM*

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Abstract. Particular RNA fragments obtained by action of pancreatic ribonuclease on purified RNAs originating from species totally unrelated to *Agrobacterium tumefaciens* (*Escherichia coli*, rabbit, monkey) are capable of inducing the formation of transplantable tumorous tissue when introduced at wounded sites in inverted stems of *Datura stramonium* maintained under axenic conditions on a medium containing auxin and kinetin. Reovirus RNA and a small size RNA (5–6 S) isolated from RNA bound RNA directed DNA polymerase from *Escherichia coli* also induced the appearance of tumorous tissues which grow on solid synthetic medium in the absence of auxin and kinetin.

I. INTRODUCTION

In our previous studies, evidence was presented that a small size RNA (tumor inducing RNA, TI-RNA) isolated and purified from *Agrobacterium tumefaciens*, oncogenic and non-oncogenic strains, regularly induced the appearance of tumorous proliferation in *Datura stramonium* grown under axenic conditions on a solid synthetic medium containing auxin and kinetin [1]. Tumorous tissue induced with TI-RNA was indistinguishable from that induced with oncogenic *A. tumefaciens* B_6 cells: (1) tumorous tissue in a host was not self-limiting (2) cells of the new overgrowth were indefinitely transplantable, (3) they have been transferred serially on a minimal culture medium not supplemented with auxin and kinetin [2, 3]. Our observation that an RNA fraction is indeed an essential component in tumorogenesis of plants is supported by Braun and Wood's report [4] that RNase does not allow tumorous tissue to appear while DNase is without effect. This observation was confirmed in peas by Kurkdjian (personal communication). It was also shown by Swain and Rier [5] that total RNA isolated from an oncogenic strain of *A. tumefaciens* induced the formation of overgrowths in tomato plants. In that study no transplantation of overgrowth tissue was performed. It should be mentioned that recent studies failed to demonstrate that purified DNA from oncogenic strains of *A. tumefaciens* is tumorogenic in plants [6, 7, 8].

Here we demonstrate that a special type of RNA and RNA fragments isolated from sources other than *A. tumefaciens* regularly induce the appearance of tumorous proliferation which has been characterized according to established criteria.

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II. MATERIALS AND METHODS

1. *Bacterial strains and enzymes*

Agrobacterium tumefaciens strains were grown aerobically as previously described [1]. *E. coli* strain K-12 Hfr wild type, grown as previously described [9] was used for isolation of RNA bound to RNA directed DNA polymerase. *E. coli* M 500 Sho-R [11] was used for isolation of ribosomal RNAs (both 23 S and 16 S) which contain an excess of purine nucleotides over pyrimidines.

2. *Isolation of Tumorogenic RNA from E. coli wild type*

RNA which is bound to RNA directed DNA polymerase was isolated from *E. coli* and characterized as described in detail elsewhere [9].

Reoviral RNA, (single stranded) composed of essentially three fractions: large 2.2×10^6, medium 1.3×10^6, small 0.8×10^6 (plus RNA fragment $\neq 2 \times 10^4$) [10] was kindly supplied by Dr. Joklik.

DNA dependent DNA polymerase I: enzyme was partially purified from extracts of *E. coli* M 500 Sho-R as described elsewhere [9].

3. *Isolation of ribosomal RNA from E. coli M 500 Sho-R ribosomes*

Ribosomal RNAs (23 S and 16 S) were isolated from free ribosomes by phenol and chloroform method [11]. The presence of both 16 S and 23 S RNA was checked by polyacrylamide gel electrophoresis [11].

4. *Isolation of total RNA from rabbit and monkey liver*

Liver from rabbit ('Fauve de Bourgogne') and monkey (Papio-papio) was homogenized in Tris-HCl buffer 10^{-2} M containing 10^{-2} M $MgCl_2$ and 0.1 M KCl (pH 7.5) for 5 min at 4°C (in turmix). Lauryl sulfate (0.2% final conc.) was added and RNA isolated and purified as described for the isolation of bacterial RNA [11]. The amount of RNA was determined by U.V. absorbance at 260 and 280 nm. 260/280 ratio must be equal or superior to 2. No DNA was found in these preparations.

5. *Preparation of RNA fragments from RNAs*

RNA fragments were obtained by mild degradation of ribosomal RNA (*E. coli* M 500 Sho-R) and from total RNA using pancreatic RNase (RNase solution was previously heated at 100°C for 10 min). 20 mg of RNA dissolved in 4 ml distilled water were incubated with 300 μg of crystalline RNase for 30 min at 36°C. RNase was removed by several chloroform treatments followed by centrifugation at 2000*g* for 5 min. The upper aqueous phase containing RNA fragments was passed through Sephadex G-25 fine column (70 X 3 cm) (Figure 1) equilibrated with Tris-HCl buffer 10^{-2} M (pH 7.5). RNA fragments were eluted with the same buffer solution. 3–5 ml fractions were collected. The U.V. absorbing material of each peak was detected by its absorption and designated as P_1, P_2, P_3, P_4 and P_5 according to the order of elution from the column. Fractions from each peak were mixed and freeze-dried. Dried material was dissolved in a small volume of distilled water, sterilized by treating with chloroform and extensively dialysed against 1 l of sterile distilled water with two changes under axenic conditions. RNA fragments were

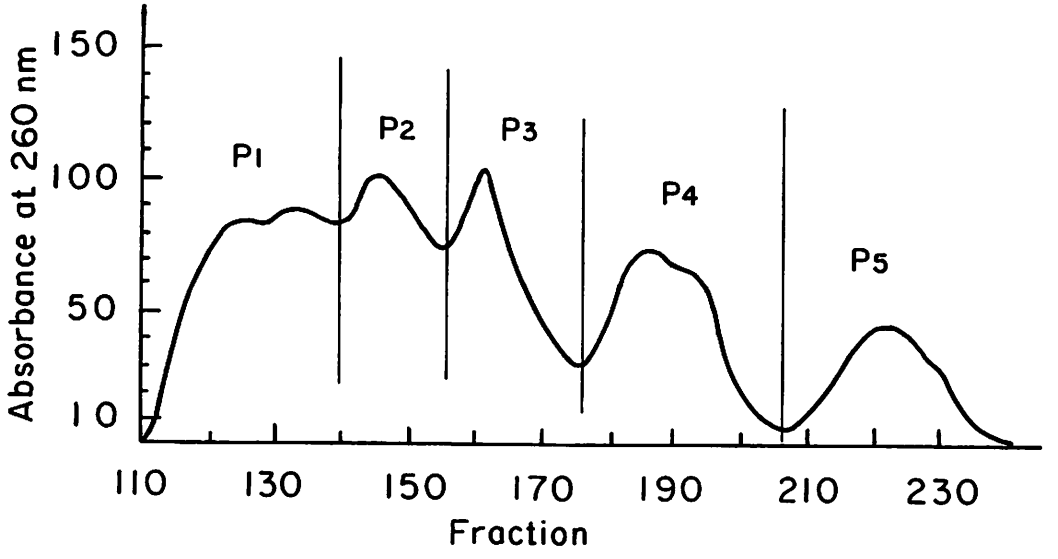

Fig. 1. Separation of RNA fragments obtained by 'mild' degradation with RNase A. 20 mg of r-RNA (*E. coli* M 500 Sho-R) dissolved in 4 ml of distilled water were incubated with 300 μg of RNase A for 30 min at 36° C. RNase was eliminated by several treatments of the incubation mixture with chloroform alternated with centrifugations. The upper aqueous phase containing the products of degradation was immediately separated on Sephadex G-25 fine column using Tris solution (10^{-2} M) as eluent.

characterized by chemical and physical means as previously described [12]. Diphenylamine reaction was negative with 2 mg of RNA fragments although it was positive with 2 μg of purified thymus DNA.

6. *Isolation of DNA from Datura stramonium*

Whole Datura plants or auxinic callus tissue from inverted stems of Datura plants, grown under axenic conditions were placed in a mortar containing 2 SSC [1] solution plus lauryl sulfate (0.2% final concentration) and gently broken up by mechanical means for 10 min at 4 °C. DNA was extracted and purified as previously described for the isolation of bacterial DNA [1].

7. *Plants used for Tumor induction*

Inverted stem sections of *Datura stramonium* plants used for Tumor Induction were obtained by De Ropp's method [13] and maintained on solid synthetic medium containing auxin and kinetin [14]. Sterility of RNA and RNA fragments was checked by using solid agar medium. No bacterial cells were found in these preparations.

8. *Tumorous tissue culture*

Autonomy for auxins and cytokinins is a characteristic of cultured Crown Gall cells. Tissue isolated from overgrowths induced either by RNA or by RNA fragments from different origins were transferred to two media for plant cell growth: one contained auxin and kinetin, the other did not [14]. Only those overgrowth tissues which could grow in the absence of auxin and kinetin were considered as tumorous.

III. RESULTS

9. *Tumor Inducing RNA*

(TI-RNA) isolated and purified from *E. coli* RNA-bound RNA directed DNA polymerase [9] has several characteristics which are close or identical to those previously found for TI-RNA isolated from Agrobacterium tumefaciens B_6 [1]. First, polyacrylamide gel electrophoresis (Figure 2) shows that *E. coli* TI-RNA is small in size (around 5–6 S). Second, it shows an excess of purine nucleotides content (Table I). Third, it can be transcribed *in vitro* into DNA like material by an *E. coli* RNA directed DNA polymerase [9]. Last, TI-RNA from *E. coli* (as TI-RNA from *A. tumefaciens* strains) induces transplantable tumors in *Datura stramonium* grown under axenic conditions on a medium containing auxin and kinetin (Table III). Once degraded TI-RNA is no longer active as a tumor inducing agent (Table III).

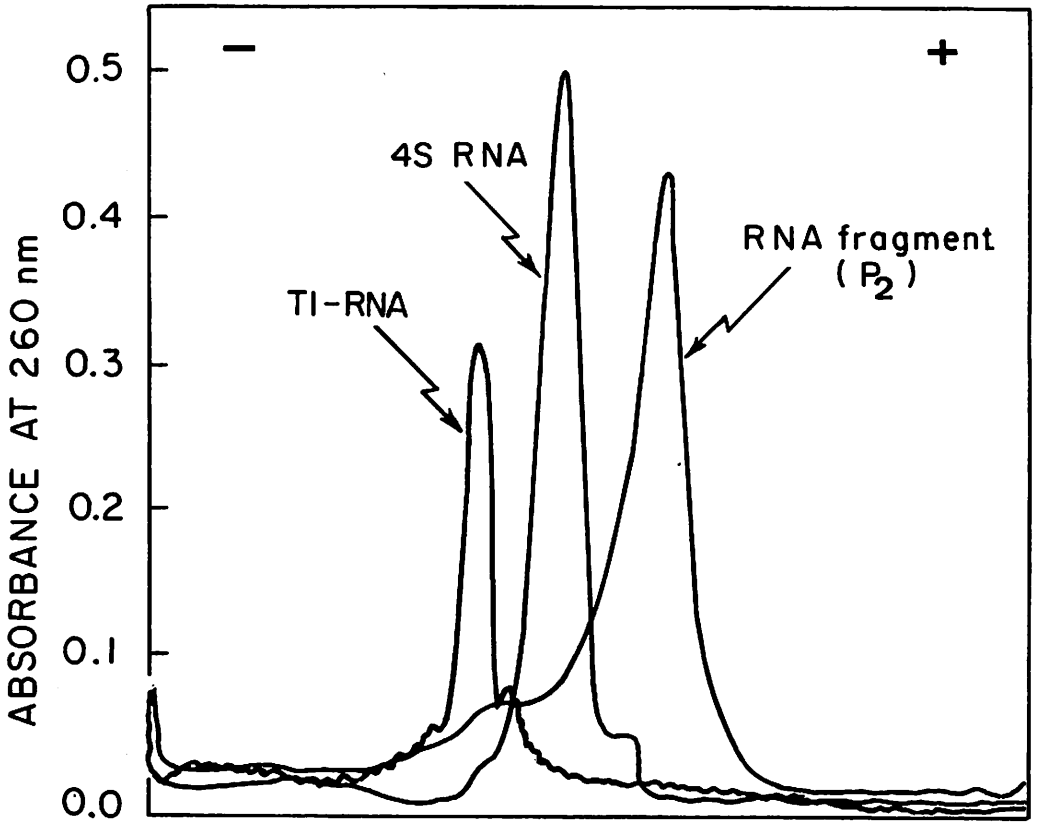

Fig. 2. Electrophoretic mobility of RNA fragment P_2 from ribosomal RNA of *E. coli* M 500 Sho-R. *E. coli* 4 S RNA (t-RNA met, 4 μg) and RNA fragment P_2 (6 μg) were separated on 4.5% polyacrylamide gel and densitometer tracings determined as described [11]. *E. coli* TI-RNA (4 μg) was separately run under the same condition and the densitometer tracings were superposed in the same figure.

TABLE I: Base ratio analysis of RNA molecules used for tumor Induction in Datura Stramonium

Bases	moles per 100 moles of nucleotides							
	E. coli M 500 Sho-R		RNA fragment P_2 *E. coli* M 500 Sho-R	RNA fragment P_1		Reoviral RNA[b]		RNA bound to reverse transcriptase of *E. coli*
	23 S RNA	16 S RNA		rabbit[a]	monkey	A-rich	total	
A	31.0	32.0	36.1	24.8	20.1	88	27.5	31.2
G	34.3	35.0	43.2	45.2	46.2	0	21.9	35.1
C	17.7	16.7	10.3	13.7	15.2	15	21.9	16.6
U	16.1	16.3	10.4	16.3	18.5	10.5	28.7	17.0
G+A/C+U	1.96	2.01	3.32	2.30	2.10	7.30	0.99	1.99

[a] total RNA from rabbit liver: A = 24.3 G = 27.6 C = 23.5 U = 24.6
[b] Bellamy and Joklik, 1967 [10]

RNA molecules (150–200 μg) were hydrolysed with 1 N HCl at 100°C for 1 hr. Bases and nucleotides were separated by thin layer chromatography [15].

10. *RNA-fragments as tumor inducing agent*

RNA fragments originating from species totally unrelated to *A. tumefaciens* can act as a tumor inducing agent in *Datura stramonium* under particular conditions. Obtained from ribosomal RNA of *E. coli* M 500 Sho-R, from rabbit and monkey total liver RNA, RNA fragments used in the present studies contain purine nucleotides in excess over pyrimidines (Table I). This is not surprising since all these RNA fragments have been obtained under the same experimental conditions using pancreatic RNase which cleaves RNA chains on C and U nucleotides. RNA fragments contain 25–50 nucleotides as suggested by electrophoretic mobility of RNA fragment P_2 obtained from *E. coli* Sho-R. They are single stranded. From the biological point of view, RNA fragments can be, *in vitro*, easily distinguished from TI-RNA by the fact that while TI-RNA from *E. coli* or reoviral RNA is inactive (Table II) RNA fragments act as primers for replication of DNA from Datura plants. In fact, Datura plant DNA, incubated in the presence of DNA dependent DNA polymerase I (*E. coli*) and necessary components is poorly replicated (Table II). Addition of RNA fragments from different sources highly stimulates the DNA synthesis which is not detectable in the presence of DNase. When pretreated with RNase or periodate (the latter destroys the 3′ OH group) they have no effect anymore. Intact ribosomal RNA which has no effect on DNA replication (Table II) and is not transcribed into DNA did not induce tumors [1] while RNA fragments from different sources act as tumor inducing agents (Table II). It should be underlined that RNA fragment P_2 from *E. coli* M 500 Sho-R maintains its tumor inducing capacity after electrophoretic separation on polyacrylamide gel and proper isolation (Table III).

11. *Tumors on stem of D. stramonium inoculated with TI-RNA from E. coli, reoviral RNA or with RNA fragments from various sources*

TABLE II: Effect of RNA fragments on *in vitro* replication of DNA from Datura stramonium

Origin of DNA	cpm of H^3 TTP incorporated in 10 min at 36°				
	without RNA fragments	with RNA fragments			
		P_1	P_2	P_3	P_4 (from *E. coli*)
Datura plant	565	11.024	14.783	10.136	13.102
Datura plant + DNase (5 μg)	78	372	465	–	–
Datura plant + RNase T_1 (20 μg)	503	1.730	1.690	–	–
Tobacco culture	637	2.970	5.630	5.990	6.990
Datura inverted stems	576	9.632	16.343	8.076	9.016 (from *E. coli*)
Datura inverted stems	576	617 with P_1 pretreated with periodate			
Datura inverted stems	532	4.676	7.130	6.996	1.320 (rabbit liver)
Datura inverted stems	560	3.076	4.896	4.326	1.032 (monkey liver)
Datura inverted stems	560	+ TI-RNA (2 μg): 676			
Datura inverted stems	560	+ r-RNA (2 μg): 596			
Datura inverted stems	560	+ Reoviral RNA (2 μg): 716			

Incubation mixture (0.2 ml final volume): Tris-HCl buffer 10^{-2} M, 25 μM; $MgCl_2$, 2 μM; each deoxyribonucleoside-5'-triphosphate 5 nMoles (one labelled with $^3H \neq 50.000$ cpm); DNA from *Datura* plants, 0.25–0.5 μg; RNA fragments, 2 μg. DNA dependent DNA polymerase I partially purified from *E. coli*, 80 μg; incubation 10 min at 36°C. Reaction was stopped with TCA. Precipitable product was washed on glass filter, dried, and radioactivity determined with a Packard spectrometer.

RNA and RNA fragments were inoculated into wounded inverted stems of Datura plants as described in the legend to Table III. Extensive cellular proliferation appeared two or three weeks after inoculation. The overgrowth tissues induced by RNA molecules tested are not self-limiting in growth as they are in the case of auxinic callus induced with distilled water (Table III and Figure 3). Each overgrowth tissue has been grafted successfully one or two times onto healthy plants of the same species (Table III). Crown Gall tissue free of *A. tumefaciens* was used for comparison (Table III).

12. *In vitro culture of tumorous tissue*

An established criterion for proving that tissues are tumorous is the demonstration that they will grow on a medium that does not contain plant hormones required for the growth of normal plant cells. In all cases reported here tumorous tissues were cultured and transferred several times without adding any growth factor to the medium (Table III).

TABLE III: Tumor Induction in *Datura stramonium* with TI-RNA and RNA-fragments from various origins

Sources of RNA molecules	Development of overgrowth Primary tissue	Development of secondary tumors after grafting of the primary tumorous tissue	Growth of tumorous tissue. Number of transfers on culture medium without addition of hormones
TI-RNA from transformant B_6 Tr-1 (non oncogenic)	$4^+/4$	$3^+/7$	12 (from grafted tissue)
TI-RNA from mutant 5 gly of *A. tumefaciens* (non oncogenic)	$4^+/4$	$3^+/8$	6
TI-RNA from *E. coli* K 12 wild type	$4^+/4$	$2^+/6$	6 (from grafted tissue)
Reoviral RNA (total)	$4^+/4$	$6^+/10$	6 (from grafted tissue)
RNA fragment P_2 from *E. coli* M 500 Sho-R[a]	$4^+/4$	$4^+/7$	6
RNA fragment P_2 from *E. coli* M 500 Sho-R	$4^+/4$	$2^+/5$	7
RNA fragment P_1 from monkey liver	$4^+/4$	$2^+/7$	5
RNA fragment P_1 from rabbit liver	$4^+/4$	$3^+/16$	5
Crown Gall cells free of *A. tumefaciens*	$4^+/4$	$6^+/9$	7
Distilled water (inoculated without RNA)	small callus	no tumor 0/5	no growth
TI-RNA (B_6 Tr-1) pretreated with KOH	small callus	no tumor 0/5	no growth

RNA molecules (5–15 μg) in 0.05 ml of sterile distilled water were inoculated at wound site of inverted stems of *D. stramonium* (5 stem sections for each type of RNA molecules). As a control, we inoculated, in place of RNA molecules, either distilled water or RNA pretreated with 0.3 N KOH for 16 hr at 24°C and dialysed against distilled water.

[a] RNA fragment P_2 from *E. coli* M 500 (5 μg) was separated by electrophoresis on polyacrylamide gel (4.5%) eluted with 1 M NaCl solution, dialysed against distilled water, concentrated and inoculated. Minimal culture medium of Murashige and Skoog [14] was modified: glycine and casein hydrolysate were omitted; medium containing 3×10^{-8} M kinetin and 10^{-7} M indol acetic acid. 300 μg of TI-RNA (B_6-Tr-1) were incubated in 0.05 ml of 0.3 N KOH for 16 hr at 37°C. Solution was neutralized with 0.3 N HCl, diluted 10 fold and used for inoculation (10 μg).

Fig. 3. Demonstration of the oncogenic capacity of the tumor-inducing RNA fragments on Datura stramonium. (1) Development of secondary tumors after grafting of the primary overgrowth tissue that was obtained with RNA fragment P_2 from ribosomal RNA of *E. coli* M 500 Sho-R. (2) Development of secondary tumors after grafting of the primary overgrowth tissue that was obtained with RNA fragment P_1 from total RNA of monkey. (3) Development of secondary tumors after grafting of the primary overgrowth tissue that was obtained with Crown Gall cells (free *A. tumefaciens*). (4) Absence of tumorous tissue after graft of an auxinic callus tissue.

IV. DISCUSSION

Data presented here demonstrate that both purified special small size RNA and RNA fragments isolated from different origins (*E. coli*, *A. tumefaciens* non oncogenic strains, rabbit and monkey liver, reovirus RNA) induced, in a reproducible manner, tumorous transformation in tissues of *Datura stramonium* inverted stem sections grown under axenic conditions on a solid medium containing auxin and kinetin. No tumors were obtained when RNA molecules were replaced by distilled water. Inoculation of active RNA molecules on Datura plants grown under ordinary conditions in a greenhouse did not result in non-self limiting tumors, which suggests that plant hormones (auxinic callus) are required for tumor induction.

Each tumorous transformation induced either by RNA or RNA fragments was apparently undistinguishable from that induced with *A. tumefaciens*, oncogenic strain B_6: in both cases the overgrowth was not self-limiting, the transformed tissue could be maintained by grafting onto healthy plants of the same species and the induced tumorous tissue could be cultured indefinitely *in vitro* in the absence of the causal agent, of auxin and of kinetin. In all experiments reported here sterile conditions were rigorously maintained.

The fact that RNA fragments introduced into recipient cells lead to a different phenotype of the cell suggested that they might interfere in some way in the replication of cellular DNA, initiating for example new replication sites on DNA molecules (Table II) or interfering at the transcriptional level. In contrast to RNA fragments, TI-RNA from *E. coli* does not act *in vitro* as primer for DNA replication. However, it can be transcribed *in vitro* into DNA by an appropriate enzyme, as previously observed for TI-RNA from *A. tumefaciens* [1]. The same could happen *in vivo*. Another possibility is that TI-RNA might be degraded by plant enzyme into smaller fragments which in turn could act as RNA primers for DNA replication. In this respect we do not know which of the normal constituents of reoviral RNA, the large, medium or small size RNA fragment is active as the tumor inducing agent in *Datura* plant.

The most striking fact reported here is that certain RNA fragments originating from species totally unrelated to *A. tumefaciens* can act as tumor inducing agents in *D. stramonium* under particular conditions only. This would indicate that they possibly share some common base sequences allowing them to interact with plant DNA as demonstrated by their priming effect on *in vitro* replication of Datura DNA.

On the basis of our results, one is inclined to consider the active RNA fragments (and possibly some particular small size RNA) as a class of fundamental regulatory molecules which, depending on their nature, their affinity for a given DNA and the prevailing physiological conditions, may force a cell, through their action on DNA, to lose or to recover the ability to follow the normal regulation process.

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