Special short dual-action RNA fragments can both induce and inhibit crown-gall tumors

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Abstract

When ribosomal RNA which contains an excess of purine nucleotides is subjected to mild degradation by a specific ribonuclease, we can isolate specific RNA-fragments which, *in vitro*, can be transcribed into complementary DNA by an RNA dependent DNA polymerase partially purified from *Escherichia coli* extracts. These RNA-fragments can either stimulate or inhibit development of crown-gall tumors induced by inoculation of *Agrobacterium tumefaciens* (oncogenic strain B_6) into decapitated germinating pea seedlings (*Pisum sativum* L. cv. Annonay). Stimulation or inhibition of tumor development depends on the time at which the RNA-fragments have been introduced into the bacteria-infected wound. Interaction between RNA-fragments and auxin was also studied. Results obtained both *in vitro* and *in vivo* will be described.

Introduction

In 1971, we described a procedure for the detection and isolation of a special DNA-associated RNA and of another RNA which is bound to a reverse transcriptase-like enzyme and which we have found in small amounts in *Escherichia coli* (Beljanski *et al.*, 1971a) and *Agrobacterium tumefaciens* (Beljanski *et al.*, 1972; Aaron da Cunha *et al.*, 1976). The size of both these RNAs is about 6 S, they both contain an excess of purine over pyrimidine nucleotides and, *in vitro*, they can both be transcribed into a complementary DNA by the reverse transcriptase-like enzyme found in bacterial and plant extracts (Beljanski, 1972; Beljanski *et al.*, 1974c). For DNA synthesis, the bacterial enzyme requires Mg^{++} and the plant enzyme requires Mn^{++} (Beljanski *et al.*, 1974c). Both short-chain RNAs can initiate transplantable tumors when they are inoculated into young *Datura stramonium* inverted stem sections (or whole plants) axenically cultured on solid synthetic medium containing auxin and kinetin (Beljanski *et al.*, 1974a; Aaron da Cunha *et al.*, 1975). The overgrowth tissues obtained under the influence of these special short-chain RNAs can be shown to be characteristic crown-gall tissue, according to the following criteria defined by phytopathologists:

1) the overgrowth tissue in a host is not selflimiting;
2) the overgrowth tissue can be indefinitely maintained by grafting onto healthy plants of the same species.
3) the overgrowth tissue grows on a minimal culture medium that does not support the growth of normal cells of the type from which the tumor cells were derived.

In more recent studies, we showed that some particular RNA fragments, smaller in size than the 6 S "transforming RNA" (Beljanski *et al.*, 1976), and originating from species totally unrelated to *Agrobacterium tumefaciens*, can also act as tumor inducing agents in *Datura stramonium* axenically cultured on medium containing auxin and

chloroform and the aqueous phase was adsorbed on Sephadex G-25 fine column (3 x 60 cm) previously equilibrated with Tris-HCl buffer 0.01 M pH 7.5. This same buffer was used for elution of RNA fragments from the column as described for RNA fragments obtained through the action of pancreatic RNase (Beljanski *et al.*, 1975). U_2 RNA fragments eluted from the column form a rather large peak, detected by U.V. absorbance at 260 nm. The U.V. absorbing material was arbitrarly divided into 4 fractions; each fraction was freeze-dried then dissolved in a small volume of distilled water, treated again with chloroform and dialysed against distilled water at 4 °C under axenic conditions. The amount of RNA fragments was determined by absorbance at 260 nm.

Characterisation of U_2 RNA fragments

Polyacrylamide gel (3.7 %) electrophoresis was performed under conditions described in the legend to figure 1. 4 S RNA was used as a marker. Nucleotide analysis of r-RNA and U_2 RNA fragments was performed in the following way: RNA (100-150 μg) was hydrolysed in 0.5 ml of N HCl at 100° for 1 hr. Purine bases and pyrimidine nucleotides were separated by thin layer chromatography according to Björk and Svensson's method (1967). U.V. absorbing spots were located and eluted with 0.1 N HCl and the amount of each base was determined using the following extinction coefficients: A = 13.8; G = 11.8; C = 13.6; U = 9.89 (Hori, 1967).

Diphenylamine reaction was negative with 2 mg of r-DNA or U_2 RNA-fragments while it was positive with 3 μg of thymus DNA. On this basis, U_2 RNA fragments are free of DNA.

Cs_2SO_4 density gradients

Samples of RNA, of ^{3}H-DNA-RNA hybrid and also of ^{3}H-DNA separated from an RNA-^{3}H-DNA hybrid synthesized *in vitro* with the help of RNA-directed DNA polymerase, underwent Cs_2SO_4 density gradient centrifugation at 20 °C for 64 hr at 30,000 rpm in a SW 39 Spinco rotor. Fractions were collected and ^{3}H-product precipitated with TCA (5 % final concentration) filtered on a glass filter and dried. Radioactivity of samples was measured in a Packard spectrometer.

Enzyme preparation used for transcription of U_2 RNA fragments

E. coli RNA free RNA directed DNA polymerase (Fraction II from DEAE cellulose column) precipitated with $(NH_4)_2SO_4$ and dialysed as described (Beljanski *et al.*, 1974b) was used for transcription of RNA fragments. When needed DNA dependent DNA polymerase I was partially purified from *E. coli* extracts (Beljanski *et al.*, 1974b).

Incubation conditions for transcription of U_2 RNA fragments

The 0.15 ml incubation medium contained: Tris-HCl 25 μM (pH 7.65); $MgCl_2$ 2 μM; each XTP 5 nMoles (d-TTP ^{3}H, 50,000 cpm); RNA fragment U_2 4 μg; enzyme (fraction II from DEAE cellulose column) 60 μg. After incubation at 36° for 20 mn, the radioactive product was precipitated with TCA (5 % final concentration), washed with TCA on a glass filter and dried. Radioactivity of samples was measured with a Packard spectrometer.

Induction of crown-gall tumors on a germinating pea seedlings

Germinating pea seedlings, experimentally infected with *Agrobacterium tumefaciens* (strain B_6) were used because they are cheap and easy to handle. Pea seeds (*Pisum sativum* cv. Annonay) were disinfected and allowed to germinate according to techniques previously described (Le Goff *et al.*, 1976). When the epicotyls reached a height of 5 mm, seedlings were decapitated, and bacterial suspension immediately applied to the wound. *A. tumefaciens* cells, strain B_6 were harvested during exponential growth and used for a single inoculation (5 x 10^7 cells/wound).

U_2 RNA fragments were dissolved in sterile distilled water and filtered on a millipore filter before use. They were applied to the wound at various times and either

Results

I. Characterization of RNA fragments

U_2 RNA fragments are single stranded short chain RNAs as judged by absorbance at 260 nm at neutral and alkaline pH (absence of hyperchromicity). Polyacrylamide gel electrophoresis of several U_2 RNA fragment preparations and densitometer tracings show that these fragments migrate slightly more rapidly than 4 S RNA, suggesting that their size is smaller than that of 4 S RNA (Fig. 2). There is apparently no detectable difference between RNA fragments from four arbitrary fractions, each separately analysed by this technique. Base ratio analysis of *E. coli* r-RNA used for degradation by ribonuclease U_2 and of RNA fragments eluted from Sephadex G-25 column shows an excess of purine over pyrimidine nucleotide content (Table 1).

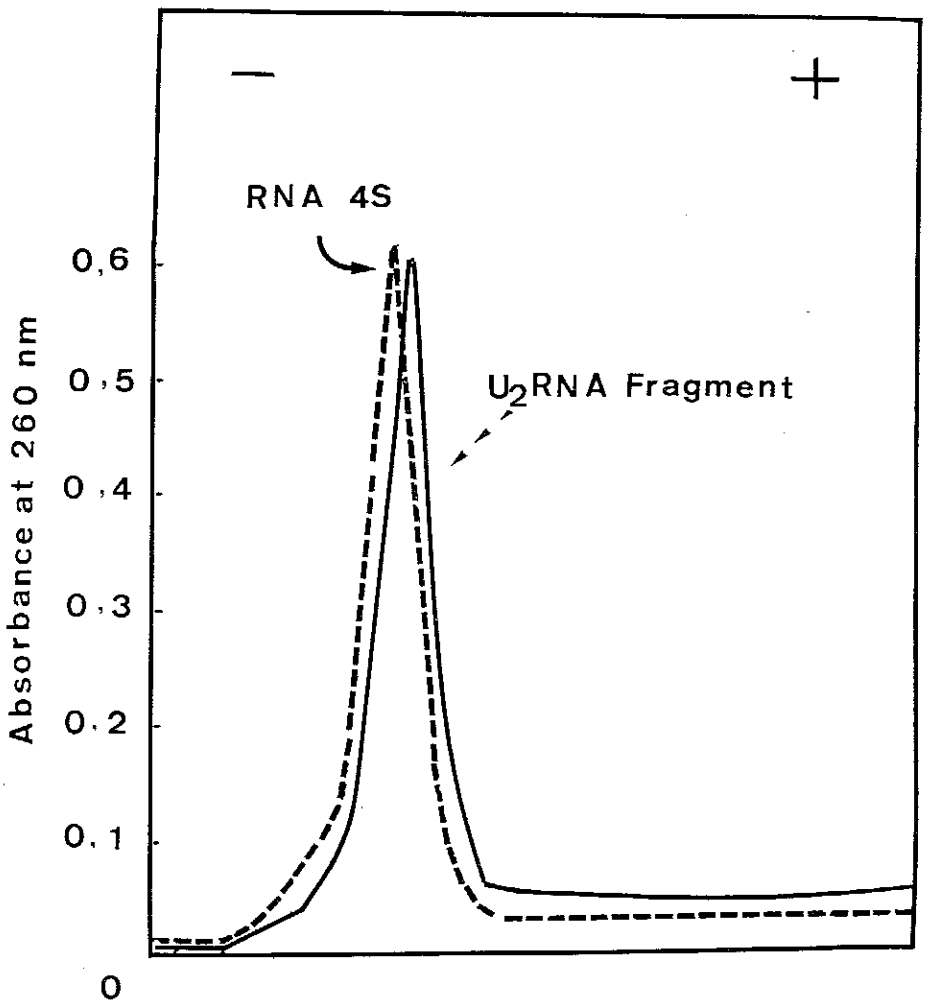

FIG. 2. – *Electrophoretic mobility of U_2 RNA fragments obtained from ribosomal RNA of* E. coli *M 500 Sho-R.*

E. coli *4 S RNA (4 µg) and U_2 RNA fragments (5 µg) were submitted to polyacrylamide gel (3.7 %) electrophoresis for 90 mn and densitometer tracings determined as described (Beljanski* et al., *1972, 1974c). Each sample was separately run under the same conditions and the densitometer tracings were superposed on the same figure.*

periodate (Beljanski *et al.,* 1975) removes its template activity; this suggests that a 3′ OH terminal group is required for transcription of RNA into DNA. It should be underlined that, under conditions used here, r-RNAs and 4 S RNA are not transcribed into DNA. The same is true for RNA fragments obtained through RNA degradation with either pancreatic RNase A or RNase T_1. The fact that U_2 RNA fragments can act as a template for DNA synthesis suggests that the 3′ terminal nucleotide (A or G) has an important part to play in relation with reverse transcriptase-like enzyme activity and with U_2 RNA fragment transcription. It should be recalled that messenger RNAs from various origins (Furudni *et al.,* 1975; Ross, 1975) contain methylated guanine in the 5′ position and that they can be transcribed *in vitro* into a complementary DNA (Ross *et al.,* 1972; Kacian *et al.,* 1972).

The amount of ^3H DNA synthesis is proportional to the amount of U_2 RNA fragments used as a template (figure 3) and increases as a function of time (figure 4). After electrophoretic migration on polyacrylamide gel, isolated U_2 RNA fragments fully retain their template activity for DNA synthesis as shown on figure 3.

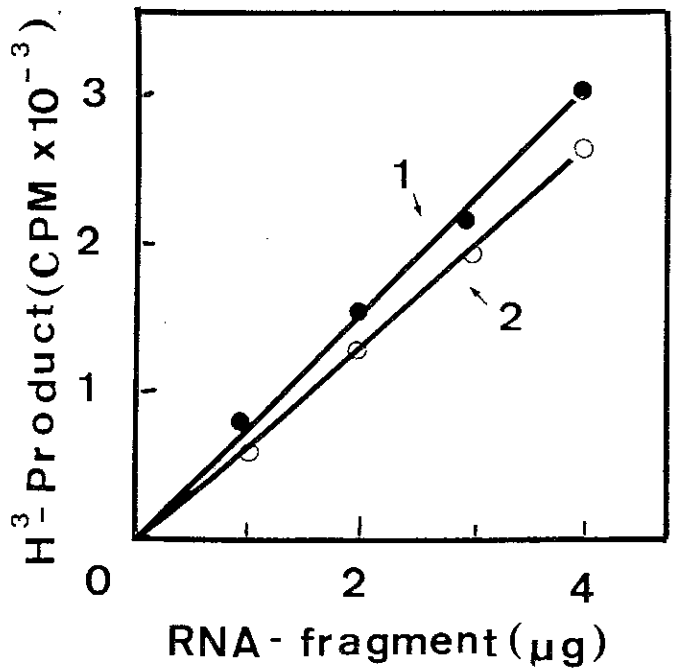

FIG. 3. – *Template activity of U_2 RNA fragments used at different concentrations. Different concentrations of U_2 RNA fragments were used for in vitro DNA synthesis. Incubation conditions are the same as described in material and methods. U_2 RNA fragments (2) were first run on polyacrylamide gel electrophoresis, eluted with 3 M NaCl, dialysed and then assayed for template activity. U_2 RNA fragments (1) did not undergone gel electrophoresis.*

synthetic and of the enzymatically formed ^{3}H-DNA-U_2 RNA fragment hybrids were identical. From the above experiments we may conclude that ^{3}H-DNA synthesized on the template U_2 RNA fragment is complementary to that RNA fragment. This conclusion is also strengthened by the fact that the base compositions of the ^{3}H-DNA product and of the RNA fragment are complementary (Table 1).

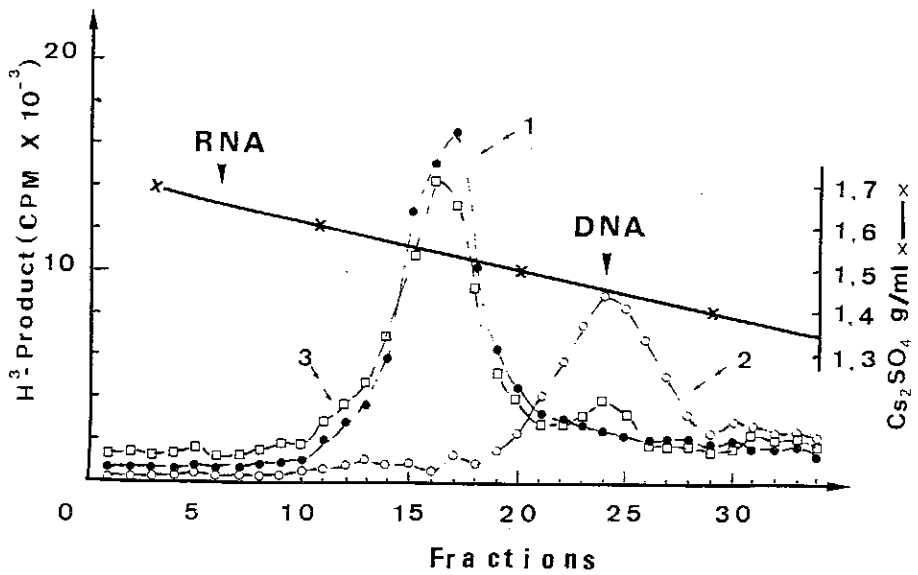

FIG. 5. – *Centrifugation of the ^{3}H DNA-U_2 RNA fragment hybrid and ^{3}H DNA in Cs_2SO_4 density gradient.*

^{3}H DNA-U_2 RNA fragment hybrid was synthesized as described in material and methods. After elimination of the enzyme by treatment with chloroform, upper aqueous phase containing ^{3}H DNA-U_2 RNA hybrid was divided into two fractions. One fraction was treated with 0.3 N KOH at 80° for 20 mn, the other one was not. Treated and untreated ^{3}H-labeled material were mixed with Cs_2SO_4 (1.8 gr, pH 7.3, final volume 3 ml) and separately centrifuged at 30,000 rpm at 20 °C for 64 hr in a Spinco SX 39 rotor. Fractions were collected and precipitated with TCA (5 % final concentration), filtered on a glass filter and dried; radioactivity was measured in a Packard spectrometer.
1 (●——●), ^{3}H product (untreated enzymatic hybrid);
2 (○——○), ^{3}H product treated with alkali;
3 (□——□), ^{3}H DNA-U_U RNA fragment synthetic hybrid.

U_2 RNA fragments act as primers for in vitro replication of DNA from various sources

In addition to their ability to act as an RNA template for DNA synthesis when RNA dependent DNA polymerase is present in the incubation medium, U_2 RNA fragments can also be utilized *in vitro* by DNA dependent DNA polymerase I (from *E. coli*) for the replication of several special DNAs. When partially purified *E. coli* DNA polymerase I is incubated with all components necessary for DNA synthesis, no significant amount of DNA is synthesized (Table 3). But addition of U_2 RNA fragments to the incubation medium strongly stimulates synthesis of acid-precipitable ^{3}H-DNA, which can be characterized as DNA according to all conventional criteria.

disappears when they are given 24 hr after bacterial inoculation. It is generally admitted that a 24 hr lapse is needed to transform a healthy plant cell into a tumorous one (Kurkdjian *et al.*, 1975). However, it is striking to observe that when these same U_2 RNA fragments are inoculated on the 4th or 8th day following infection with *A. tumefaciens,* they strongly inhibit tumor development and intense necrosis follows. A simple wound affecting cells on the surface of a tumor always leads to slight necrosis; but it is far less important than that observed after inoculation of U_2 RNA fragments. This is patent at the end of the 12 days routine experimental period, but it is remarkably evident when tests are continued up to 18 days after bacterial infection. Necrosis induced by U_2 RNA fragments is by then almost complete in 100 % of individual tumors, while only about 17 % of untreated tumors are necrotic. It should be kept in mind that it is difficult, when inoculating RNA fragments into a tumor, to achieve an even distribution in the tissues. It is also important to note that U_2 RNA fragments injected into wounded pea seedlings which are not infected with *A. tumefaciens* show neither oncogenic nor toxic effects: no tumor appears and plant growth is normal.

Table 4. Effects of U_2 RNA fragments and IAA incubated separately or together during induction or development of crown gall tumors

Treatment with	Time between moment of infection with *A. tumefaciens* (time 0) and treatment			
	time 0	24 hr	4 days	8 days
Distilled water	100* ± 6	100 ± 8	100 ± 10	100 ± 11
IAA, 1 μg/wound	157 ± 13	138 ± 12	95 ± 9	83 ± 13
U_2-RNA fragments, 80 μg/wound	162 ± 9	110 ± 9	61 ± 6	61 ± 7
IAA, 1 μg + U_2 RNA fragments, 80 μg/wound	131 ± 11	168 ± 13	87 ± 11	104 ± 18

Experimental conditions (see material and methods).
(*) Weight of tumors (relative values).

It must be pointed out that during the first 24 hr of tumor induction the activity of U_2 RNA fragments is concentration-dependant. Tumor weight increase is observable with 20 μg of RNA fragments per wound, it is higher with 80 μg and lower with 160 μg (Table 5). For a given concentration of U_2 RNA fragments, their inhibitory action on tumor development is the same whether tumors are treated on the 4th or 8th day following bacterial infection.

Table 5. Effect of different concentrations of U_2 RNA fragments during appearance and development of crown gall tumors induced with *A. tumefaciens* B_6

Treatment with:		Time between moment of infection with *A. tumefaciens* (time 0) and treatment		
		Time 0	4 days	8 days
Distilled water		100* ± 10	100 ± 14	100 ± 16
U_2 RNA fragments	20 μg/wound	134 ± 18	81 ± 15	80 ± 17
	80 μg/wound	173 ± 15	64 ± 15	67 ± 16
	160 μg/wound	125 ± 8	44 ± 18	62 ± 11

Experimental conditions (see material and methods).
(*) Weight of tumors.

and, on the contrary, ribonuclease U_2 separates A and G nucleotides. With these two enzymes, we prepared, from the same r-RNA, fragments which show distinct biological activities. RNA fragments obtained with pancreatic RNase act only as primers for DNA replication. U_2 RNA fragments act both as primers and as templates for DNA synthesis when *E. coli* reverse transcriptase-like enzyme is present and the incubation medium contains the four deoxyribonucleoside-5'-triphosphates. Physical and chemical analysis showed the synthesized product to be DNA. This template activity shown by U_2 RNA fragments suggests that a 3' terminal nucleotide (G or A) may be required for binding these fragments to RNA dependent DNA polymerase (*E. coli* reverse transcriptase). We do not know yet whether the active U_2 RNA fragments have a G or an A terminal nucleotide. But it is interesting to recall that messenger RNAs which can be transcribed into complementary DNA have at their 5' end a G terminal nucleotide. Therefore, we plan to separate the 3' G and 3' A terminated U_2 RNA fragments in order to find which of them functions as a template for DNA synthesis.

The dual properties of U_2 RNA fragments suggested to us that, being primers and templates, they could play a part in the induction and development of tumor initiated by *Agrobacterium tumefaciens* B_6. This approach seemed justified by the facts that auxin (plant hormone), which plays an important part in plant tumor development, binds *in vitro* to 4 S RNA but not to ribosomal RNA and that the size of U_2 RNA fragments is close to the size of 4 S RNA, though they are different in nature (4 S contains some unusual bases). We followed *in vivo* the activity of U_2 RNA fragments when exogenous auxin was added at various stages of tumor induction and development in germinating pea seedlings. Interaction between auxin and RNA fragments was to be expected, at least during certain stages of tumor genesis. When U_2 RNA fragments and IAA (indolylacetic acid) were separately inoculated at time 0 (start of *A. tumefaciens* infection) into a pea seedling wound, each of these substances stimulated tumor tissues formation. U_2 RNA fragments seem to act as primers for DNA replication and differ from the naturally occuring primer of the plant host. The stimulation due to either U_2 RNA fragments or IAA lasts only 24 hours after bacterial infection; this seems to be the time needed for *A. tumefaciens* to transform a normal plant cell into a tumor cell. During this time, there is no synergy between IAA and U_2 RNA fragments. When the latter fragments are inoculated 24 hours after bacterial infection, they have no more action on tumor development, while IAA still stimulates it. During the induction period, U_2 RNA fragments showed a stimulating activity only if they were intact or very slightly degraded. Intact r-RNA from which they were obtained is completely devoided of activity.

When U_2 RNA fragments are added to the wound on day 4 or day 8 after *A. tumefaciens* infection, they strongly inhibit tumor development and an intense necrosis is observed. IAA supresses the inhibiting action of U_2 RNA fragment. These findings suggest that IAA and U_2 RNA fragments associate in some way during certain stages of tumor development. In seated tumors, U_2 RNA fragments may be degraded; auxin does not bind any more those fragments. Results given here strongly suggest that binding of IAA to U_2 RNA fragments is possible only when these fragments are intact or slightly degraded. An optimal or suboptimal auxin concentration could thus be obtained. The consequences of this association or of its absence can be seen from the experiments described in this paper.

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