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RNA FROM CALLUS CULTURES AND LEAVES OF *NICOTIANA TABACUM*

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Abstract—MAK column chromatography has been used to analyse RNA from normal and crown gall callus cultures and leaves of *Nicotiana tabacum*. To determine the elution behaviour of well-defined DNA-like RNAs with different GC content, complementary RNAs (*c*-RNA) synthesized on *Agrobacterium tumefaciens* DNA and crown gall DNA were used. The elution profile of the RNA from all three tissues followed a similar pattern. By salt gradient elution the RNA in the *t*RNA region showed a remarkably high CMP content which was significantly higher for the normal tissues than for crown gall tissue. RNA from the callus cultures contained more DNA-like RNA (D-RNA) with a higher turnover rate than RNA from leaves. Because of its relatively low poly A content, measured as RNase A + T_1 resistance, as well as its high turnover rate, the salt-eluted D-RNA is thought to be heterogeneous nuclear RNA (Hn-RNA) and not *m*RNA. RNA molecules that might represent the *m*RNA population, having intramolecular poly A tracts, were subsequently eluted by a salt gradient, a low salt buffer and with the chaotropic agent guanidine thiocyanate, which removed tenaciously bound (TB-RNA) in two fractions, α and β. Crown gall RNA showed both a different labelling behaviour and a higher poly A content in the α and β fractions compared to the normal tissues. *c*-RNAs may be eluted at different salt concentrations because of their different GC content. They give rise to a considerable fraction of TB-RNA which in the presence of tobacco leaf RNA was split into fractions similar to α and β. No fraction was found amongst these RNAs which did have intramolecular poly A tracts.

INTRODUCTION

The crown gall tumor is induced by virulent *Agrobacterium tumefaciens* cells inoculated into plants. The transformation results in a permanent activation of several biosynthetic processes [1] and the synthesis of new enzyme [2]. Tumors also contain either octopine and lysopine or nopaline, which are found neither in normal and habituated callus cultures nor in healthy plants [3, 4]. The plant species used does not determine which of these compounds is present, but this is completely dependent upon the type of bacterial strain used for transformation. However, recent results indicate that these guanidine derivatives are not specific for crown gall cells [5, 6]. Nucleic acid hybridization experiments suggest that genes of the inciting bacterium are present in the genome of the tumorous cells [7–11]. *A. tumefaciens*-specific RNA [11, 12] and proteins [13–15] have also been detected. A large plasmid, present in virulent *A. tumefaciens* cells, may be transferred to the plant cells during crown gall induction [10, 11, 16].

The mechanism of tumor formation is still unclear. Possibly the introduction and subsequent expression of exogenous genes may lead to permanent epigenetic changes and tumorous growth. This problem might be clarified by studying both the DNA-like RNA (D-RNA) population from normal and transformed cells, and the RNA encoded by the bacterial genes in the tumorous cells. Interference in the regulation of gene expression will induce changes in the composition of

the D-RNA, and possibly also in its turnover rate.

Hybridization experiments between D-RNA and plant DNA might reveal changes in the RNA population. However, the feasibility of this technique is complicated by the fact that large amounts of a well-defined type of DNA, selected for unique sequences must be used. Moreover, one has to devise a technique for the large scale isolation of D-RNA, starting from total RNA. We prefer to use total RNA because of our interest in both polysomal *m*RNAs and their precursors present in nuclei. The isolation of subcellular fractions only might result in uncontrolled losses.

MAK column chromatography may be used for separating D-RNA from total RNA [17–22]. If substantial changes in the D-RNA have taken place in transformed cells, there might have been a change in base composition which could be detected by analysing the base composition of the MAK column fractions.

As nothing is known about the D-RNA population in crown gall tissue, we started our studies by analysing total RNA with sucrose gradient centrifugation and MAK column chromatography. Distinct labelling periods with ^{32}Pi and uridine-[^{3}H] as well as chase experiments were used to study labelling kinetics and turnover rates. MAK column fractions were analysed for specific radioactivity (sp act) and base composition. RNase resistance was also tested to see whether poly A rich sequences are present in D-RNA from plants, as recently described for animal cells and viruses [23–26].

Because of a large difference in GC content, the RNA transcribed from the *A. tumefaciens* genes in transformed cells might elute from the MAK column at another salt concentration than the plant D-RNA. This possibility was studied by fractionating crown gall *c*-RNA-[^{3}H] and *A. tumefaciens c*-RNA-[^{3}H]; the latter in the presence and absence of tobacco leaf RNA.

RESULTS

Labelling kinetics and sedimentation characteristics

For a reliable base composition analysis of rapidly labelled RNA-[^{32}P] in those MAK column fractions also containing *r*RNA, a low sp. act. of the *r*RNA and a sufficiently high sp. act. of

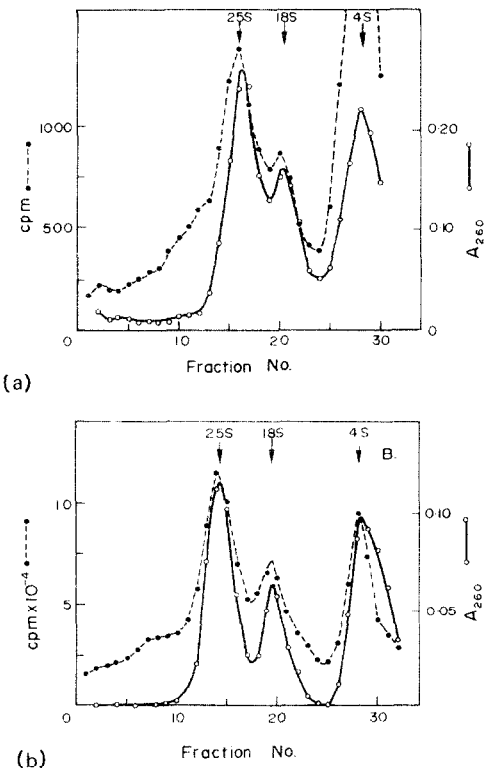

Fig. 1. Sedimentation profile of total crown gall RNA performed in 5–20% sucrose for 5 hr at 41000 rpm at 4°. (a) Labelled for 3 hr with ^{32}Pi. (b) Labelled for 7 hr with uridine-[^{3}H].

the rapidly labelled RNA are both essential, especially since the uptake of ^{32}Pi from the medium is slow for plant tissue [18]. After a starvation time of 3 hr, a labelling period of at least 3 hr was needed, using 1 mCi ^{32}Pi to reach usable sp. act. values, ranging from 100 to 300 cpm/μg purified RNA. The lower values were always found for tobacco leaves.

Sucrose density gradient sedimentation of the RNA-[^{32}P] preparations labelled for 3 hr, as shown for crown gall in Fig. 1a, revealed fast-sedimenting rapidly-labelled RNA (>25S). Although *r*RNA (25 and 18S) is also labelled, the sp. act. of rapidly labelled RNA is much higher for both callus cultures. In the case of tobacco leaves this difference was less pronounced, as also appeared from MAK column fractionation (see below) of the same RNA batches.

In all cases a more efficient labelling of RNA was obtained by using uridine-[^{3}H]. Figure 1b shows the sucrose gradient profiles of crown gall

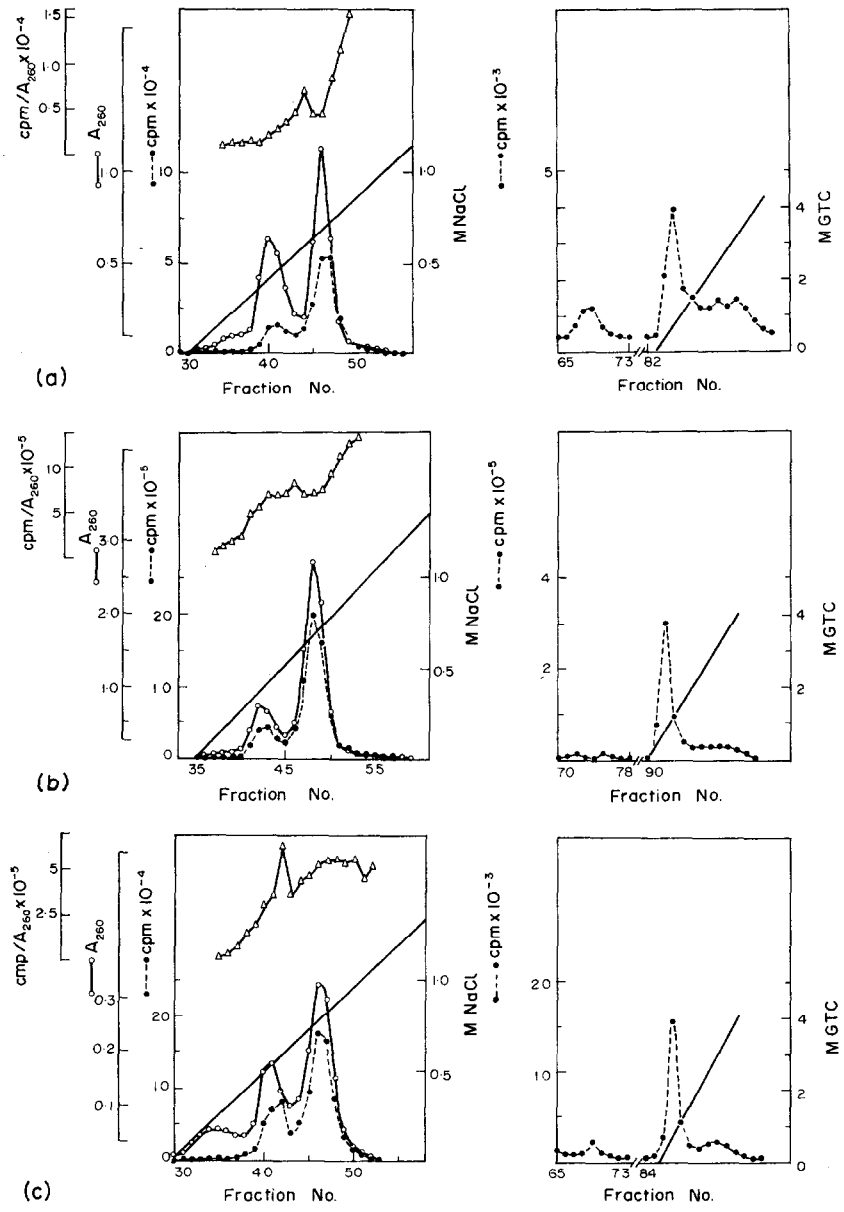

Fig. 2. Elution profile of MAK fractionated total crown gall RNA, labelled for different periods with uridine-[^{3}H]. The sp. act. profile of the RNA, eluted by the NaCl gradient is shown at the top. M GTC = molar guanidine thiocyanate. (a) Labelled for 2 hr. (b) Labelled for 24 hr. (c) Labelled for 2 hr, followed by a chase of 24 hr in fresh medium.

RNA-[^{3}H] labelled for 7 hr with 1 mCi uridine-[^{3}H] (sp. act. 26000 cpm/μg). After 1·5 hr the sp. act. was only 1450 cpm/μg, but at 2 hr the *r*RNA is hardly labelled. Uridine-[^{3}H] was preferred, therefore, over ^{32}Pi to detect deviations in sp. act. in MAK column fractions of RNA either labelled for short times or obtained after a chase incubation in fresh medium. An additional advantage of the use of uridine-[^{3}H] over ^{32}Pi, is that inositol hexaphosphate, which often contaminates RNA preparations, is not labelled. This compound hinders the interpretation of MAK column elution profiles [27], and might also be the origin of the label found at the 4S position in the sedimentation

Table 1. Base composition of different RNA MAK column fractions of crown gall callus tissue, normal callus tissue and tobacco leaf after 3 hr labelling with ^{32}Pi

Group	Base composition (% ^{32}P) Crown gall CMP	AMP	GMP	UMP	AU/GC	Normal tissue CMP	AMP	GMP	UMP	AU/GC	Tobacco leaf CMP	AMP	GMP	UMP	AU/GC
A	31·6	21·3	27·0	20·1	0·70	35·9	20·2	25·3	18·6	0·63	43·6	18·4	22·1	15·9	0·53
B	22·8	27·1	26·0	24·1	1·05	26·8	25·9	24·8	22·5	0·95	36·3	21·6	24·4	17·7	0·65
C	20·5	29·3	27·5	22·7	1·08	22·2	30·1	27·4	20·3	1·02	21·6	25·9	30·5	22·0	0·93
D	18·9	31·1	25·6	24·4	1·25	16·4	32·0	27·5	24·1	1·28	20·8	26·5	30·1	22·6	0·97
Tris-wash						15·9	41·6	20·9	21·6	1·79					
α	17·1	35·4	24·4	23·1	1·42	17·3	29·7	28·4	24·6	1·19	19·8	28·1	27·0	25·1	1·03
β	18·6	39·6	21·1	21·7	1·58						18·2	30·4	25·9	25·5	1·26

These results are average of the values of the individual fractions, grouped together as indicated in Fig. 3 to compare different MAK columns.

Table 2. Base composition of the different RNA MAK column fractions of tobacco leaf RNA, labelled for 50 hr with ^{32}Pi

RNA fraction	Base composition (% ^{32}P) CMP	AMP	GMP	UMP	AU/GC
A and B	26·2	19·9	30·9	23·0	0·75
C and D	20·5	24·9	31·5	23·1	0·92
α	21·5	26·0	28·0	24·5	1·02
Total RNA	22·6	23·4	31·4	22·6	0·85
Ribosomal RNA*	25·3	23·8	30·5	20·4	0·79
Crown gall DNA					1·50

* See Ref. 28.

profile of RNA-[^{32}P] (Fig. 1a) even though the RNA applied to the gradient was extensively dialysed.

MAK column chromatography

Typical MAK column elution profiles of crown gall RNA labelled for distinct periods of time with uridine-[^{3}H] are shown in Fig. 2. A chase experiment was included by taking half of the batch of tissue labelled for 2 hr for a chase of 24 hr in fresh medium. The RNA bound to the column was eluted subsequently with a 0–2 M NaCl gradient, a wash with Tris–HCl buffer (pH 7·4) and a 0–5 M guanidine thiocyanate gradient.

The sodium chloride gradient. In order to distinguish different types of RNA in the three tissues investigated, we looked for changes in A 260 nm profile, sp. act. and base composition with different labels and labelling times. For all three tissues the A 260 profile shows two peaks, the first being smaller than the second. Both *t*RNA and 5S RNA are expected in the first peak [18, 27]. The RNAs in this peak have a relatively high CMP content (Table 1) after short labelling times with ^{32}Pi, which is not found after a labelling period of 50 hr (Table 2). This is especially so for crown gall tissue RNA (Table 1). The second peak shows a drop in sp. act. and AU–GC ratio, which is very pronounced in the case of crown gall and normal tissue RNA (Fig. 3). This feature is found for short labelling times (Figs. 2a and 3a) and much less, either for longer labelling times or after a chase of 24 hr (Fig. 2b and c). The second peak represents the bulk of the ribosomal RNA, not separated into lr (light ribosomal) and hr (heavy ribosomal) fractions [19–22, 27].

The gradual increase in AMP content of the RNA eluted with increasing salt concentration is a typical feature of the separation on MAK columns. RNA that is eluted first is characterized by a high CMP content, while more AMP-rich RNA is eluted at higher ionic strength, or is tenaciously bound (TB-RNA).

Between the two peaks and again after the second peak a rise in sp. act. and AU–GC ratio can be seen, especially after short labelling times: this is not found to the same extent for tobacco leaf RNA (Fig. 3). Although for crown gall RNA dur-

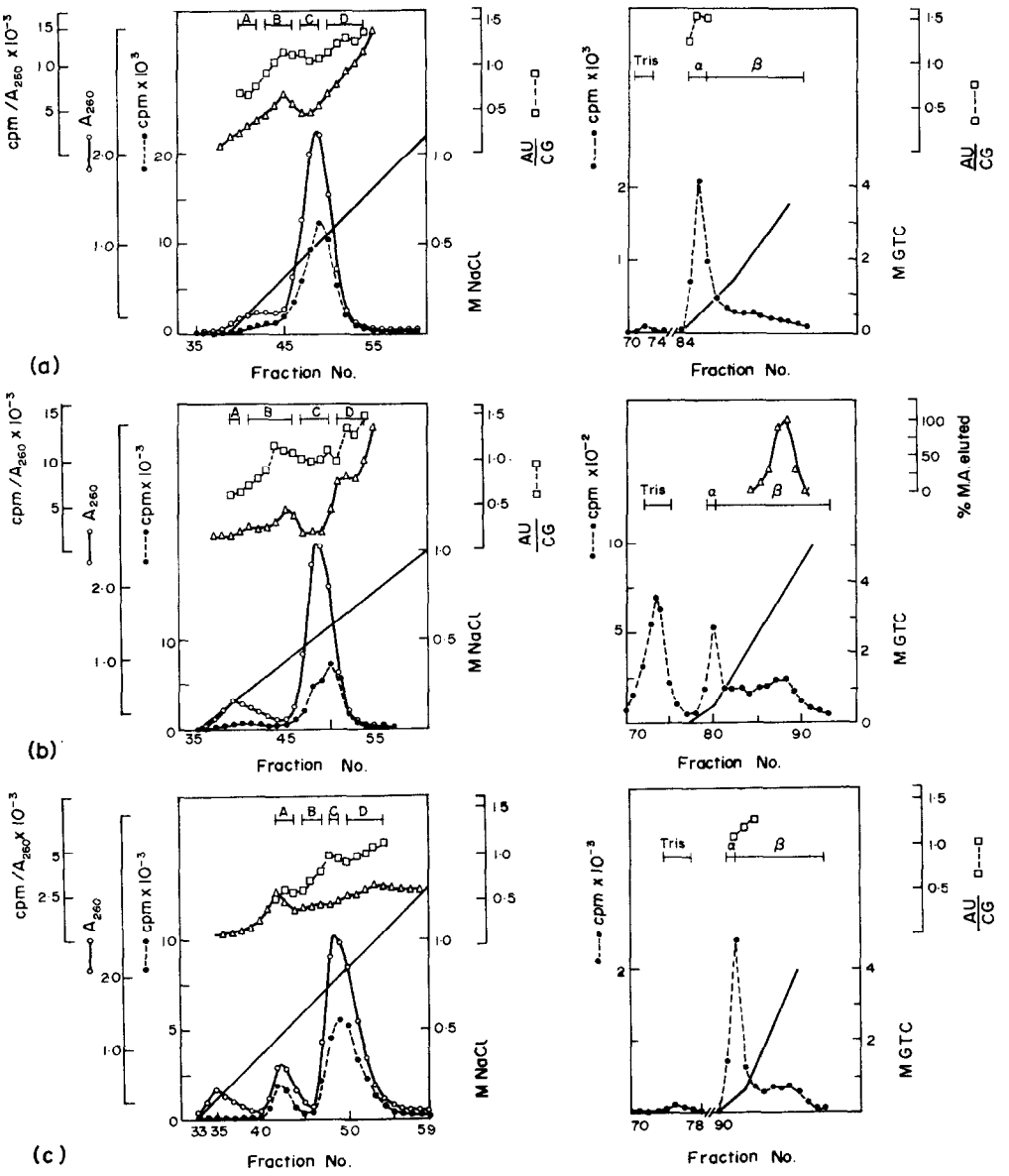

Fig. 3. Elution profile of MAK fractionated total RNA of different tissues, labelled for 3 hr with ^{32}Pi. Besides the sp. act. profile, also the AU–GC profile and the different classes of RNA are shown. In the case of normal tissue RNA the portion of methyl albumin (MA) eluted in the TB-fractions, is shown, expressed in the percentage of the fraction in which the most methylalbumin was lost. (a) Crown gall. (b) Normal tissue. (c) Tobacco leaf.

ing the 24 hr chase, incorporation of uridine-[^{3}H] into low MW RNAs as well as into *r*RNA has continued as if the tissue was labelled for 24 hr, no rise in sp. act. and AU–GC ratio was observed, contrary to the 24 hr labelling experiment (Fig. 2b and c). This clearly demonstrates that a special class of rapidly labelled RNA with a relatively short half life and therefore sensitive to chase treatment, starts to elute before the *r*RNA. On account of the large amount of *r*RNA with a low sp. act. and a high GC content, the overall sp. act. and AU–GC ratio drops down for a few fractions, causing the observed minimum. The turnover rate and the ratio of D-RNA : RNA may therefore be far higher in the callus cultures than in the tobacco leaves. The sp. act. of the *r*RNA from tobacco leaf and normal

tissue is nearly the same, indicating that the availability of precursor for RNA synthesis cannot be very different in both systems.

Tris wash. No RNA is eluted from the column by the higher salt concentrations. Unexpectedly we found that a variable small amount of labelled RNA was freed from the column by a wash with a low ionic strength buffer containing 0·05 M Tris–HCl (pH 6·7) only. This treatment precedes the elution of TB-RNA with guanidine thiocyanate.

The amount of RNA recovered in this way was always too small to measure its A at 260 nm and could be detected only by its radioactivity, so its sp. act. is likely to be high. This RNA fraction was detected even in RNA-[^{32}P] preparations which have a low sp. act. compared to uridine-[^{3}H] labelled RNA. In the case of RNA-[^{32}P] from normal callus tissue, the amount of Tris-eluted RNA was suitable for base composition analysis (Fig. 3b, Table 1). It has a DNA-like base composition with an extremely high AMP content and a relatively low GMP content compared with the salt-eluted D-RNA.

Guanidine thiocyanate gradient. Part of the RNA is still bound to the column after salt gradient elution and Tris wash. This TB-RNA is freed by a guanidine thiocyanate gradient, as shown in Fig. 2 and 3. For both RNA-[^{32}P] and RNA-[^{3}H] from the various tissues studied the radioactivity profile consists of two peaks, called α and β. The RNA in these fractions was measured by its radioactivity alone, because guanidine thiocyanate and the methylalbumin in these fractions interfere with the A 260 nm measurement. Purification of TB-RNA resulted in a variable loss, making a comparative analysis of the RNA from different tissues unreliable. Therefore we measured the percentage of radioactivity in both fractions and the base composition (Tables 1 and 3).

The α fractions contained more radioactivity than the β fractions for crown gall RNA, especially after short labelling periods with uridine-[^{3}H] (1·5 and 2 hr) or ^{32}Pi (2 and 3 hr) but also after prolonged labelling periods with uridine-[^{3}H] (24 hr and chase experiment). The reverse phenomenon was observed for crown gall RNA labelled with adenosine-[^{3}H] for a short time (1·5 hr) or with ^{32}Pi for a longer period (50 hr).

From the base composition the α fractions of crown gall TB-RNA have both a significantly higher UMP content and a lower AMP content than the β fractions. This explains the higher amount of radioactivity in the α fractions when labelled with uridine-[^{3}H] and in the β fractions when labelled with adenosine-[^{3}H]. The data obtained by labelling with ^{32}Pi indicate that especially the α fractions of crown gall RNA contain some kind of RNA that is labelled more rapidly than the RNA in the β fractions. Tobacco leaf RNA and normal tissue RNA, however, have β fractions with a higher amount of radioactivity than the α fractions, irrespective of precursor and labelling period used. Base analysis showed both a higher UMP and a higher AMP content in the β fractions, compared to the α fractions. The base composition of the TB-RNA clearly identifies it as D-RNA (Table 1). Crown gall TB-RNA is distinguished by a remarkably high AMP content, not found to the same extent for RNA from both normal tissues. The fact that the percentage of radioactivity of the TB-RNA decreases with duration of labelling time demonstrates that the

Table 3. Percentage of RNA, eluted by the guanidine thiocyanate gradient and labelled with different precursors

Precursor	Labelling time (hr)	% Total RNA Crown gall α	Crown gall β	Normal tissue α	Normal tissue β	Tobacco leaf α	Tobacco leaf β
Uridine-[^{3}H]	1·5	7·3	4·4				
	2	5·6	3·9				
	7	2·8	2·1	3·4	5·1	4·3	5·3
	24	2·8	1·9				
	2 and 24 chase	2·8	2				
Adenosine-[^{3}H]	1·5	6·5	8·4				
^{32}Pi	2	6·8	4·6				
	3	5·6	4·4	2·4	9·1	5·4	10·7
	16			1·8	3·4		
	50	1·6	3·3			2·3	4·2

RNA is rapidly labelled. The turnover rate of the TB-RNA, however, must be far less than that of the D-RNA eluted by the salt gradient. In contrast to the latter, no difference was found in the percentage of radioactivity in total TB-RNA whether the crown gall tissue is labelled continuously for 24 hr or for 2 hr with a chase in fresh medium for 24 hr.

Contrary to the results of Johnson [20] with yeast cells, we did not find that TB-RNA mainly consists of *r*RNA. The base composition of the α fractions of tobacco leaf RNA-[^{32}P] labelled for 50 hr is still not similar to that of *r*RNA (Table 2). We also analysed from this long term labelled RNA the α fractions as well as the A + B fractions in Cs_2SO_4 density gradients [29], using formaldehyde to prevent precipitation of RNA. The A + B fractions mainly contain *t*RNA, 5S RNA and *r*RNA, which all sediment to the bottom under the conditions employed. No such "bottom component" could be detected in the α fractions, indicating that contamination by *r*RNA, if present, must be very small.

RNase resistance

The resistance to RNase A + T_1 digestion has been used by several investigators [23–26] as an indication for the presence of polyadenylate (poly A) tracts in RNA. MAK column fractions of crown gall RNA labelled for 1·5 hr with adenosine-[^{3}H] and uridine-[^{3}H] respectively, examined by this procedure (Table 4) showed that RNase-resistant sequences labelled by adenosine and to a much lesser extent by uridine were indeed present. The percentage of RNase resistance follows closely the increase in AMP content of the RNA eluted successively by the various treatments (Table 1).

It should be noted that the RNase resistance is not due to the presence of DNA since insoluble radioactivity was never detected on the electropherograms of alkaline hydrolysates. RNase resistance due to double-strand structures in RNA was prevented by using heat-treated RNA.

Salt-eluted D-RNA has lower RNase resistance than both "Tris-RNA" and TB-RNA, which resemble each other very strongly, indicating that they might represent the same kind of RNA. The chase experiment with uridine-[^{3}H] labelled RNA (Fig. 2c) seems to support this idea, since "Tris-RNA" is still labelled while most of the label has vanished from salt eluted D-RNA.

MAK column chromatography of c-RNAs

To study the fractionation behaviour of D-RNAs with a different GC content, not containing poly A tracts, cytosine-[^{3}H] labelled RNAs were synthesized *in vitro* on *A. tumefaciens* DNA and on crown gall DNA using *E. coli* DNA-dependent RNA polymerase. Both *c*-RNAs have a high sp. act., show the same sedimentation value of 8–10S in neutral gradients, and have GC contents of 60 and 40% respectively.

The top fraction of crown gall *c*-RNA-[^{3}H] elutes at 0·72 M NaCl, while a large amount of *c*-RNA-[^{3}H] is eluted at higher salt concentrations thus behaving like salt eluted D-RNA from this tissue, the main fraction of which is eluted just behind the *r*RNA peak. No *c*-RNA was freed from the column with the Tris wash, and about 11% was tenaciously bound.

A. tumefaciens *c*-RNA also did not contain any so-called "Tris-RNA". The top fraction eluted with 0·51 M NaCl, showing that if *A. tumefaciens* RNA without intramolecular Poly A tracts were present

Table 4. The influence of RNase treatment on adenosine and uridine labelled crown gall tissue RNA

	TCA precipitable counts			
	Uridine-[^{3}H] labelled		Adenosine-[^{3}H] labelled	
Group	After RNase	% RNase resistant	After RNase	% RNase resistant
A	60	11		
B	65	4·5	292	14·5
C	120	1·2	1242	15·3
D	125	3	1005	21·4
Tris-wash	14	6	60	36
α	81	9·8	303	38·8

RNA, prepared after 1·5 hr labelling, was treated with RNase A + T_1 after fractionation on MAK columns. The results are the average of the values of the individual fractions, grouped together to compare different MAK columns.

in the crown gall RNA, it would elute *before* *r*RNA. To see whether this would occur in the presence of large amounts of *r*RNA, a mixture of 1 μg *A. tumefaciens* *c*-RNA and 1·2 mg tobacco leaf RNA was fractionated. However, although the *A. tumefaciens* *c*-RNA eluted at a somewhat higher salt concentration (0·55 M NaCl) than previously, it still came slightly before *r*RNA. Approximately 30% of *A. tumefaciens* *c*-RNA was tenaciously bound. In the presence of total tobacco leaf RNA, its elution profile is changed in a way that is very like the α and β fractions of the plant TB-RNA, showing that tobacco leaf RNA brings about a tighter binding of the *c*-RNA to the column.

DISCUSSION

There is a contradiction in the nature of the TB-RNA and its relation to the salt-eluted D-RNA. For mammalian [19] and yeast [20] cells, a precursor-product relationship exists for this D-RNA towards TB-RNA; TB-RNA was found in the cytoplasm, while salt-eluted D-RNA was not. Other work with mammalian cells [21] and those from soybean and carrot [31], suggested that TB-RNA is not in the cytoplasm, and that it is the precursor of salt-eluted D-RNA.

In trying to solve this contradiction, we looked for the presence of poly A sequences. For animal cells a relationship between heterogeneous nuclear RNA (Hn-RNA) and *m*RNA is supported by the finding of poly A sequences covalently linked to both classes, relatively more poly A being found in *m*RNA [23–25]. Recent studies with plant RNA have also shown the presence of intramolecular poly A tracts [11, 32, 33]. Therefore, the high AMP content and the remarkably high percentage of RNase resistant sequences that are labelled with adenosine-[^{3}H] and only to a slight extent with uridine-[^{3}H], found in crown gall TB-RNA, suggest that *m*RNA is present in these fractions.

Our results show that TB-RNA, like salt-eluted D-RNA, is rapidly labelled but has a longer half-life. The latter property need not be in disagreement with a *m*RNA-like function since recent studies [34] on cytoplasmic *m*RNA in Hela cells have demonstrated that the stability of the bulk of the free polysomal *m*RNA population is much greater than previously reported, with an estimated average half-life of *ca* 3 days.

A small portion of the TB-RNA with extended intramolecular poly A tracts presumably interacts with *r*RNA rather than with MAK and therefore elutes with high salt. This RNA fraction is first bound to the MAK section of the column when the RNA is applied at low ionic strength. Subsequently it is bound to the cellulose bottom layer at the high ionic strength that has freed the RNA from the MAK section. This cellulose-bound RNA is then eluted by the low ionic strength Tris-wash. The binding capacity of this RNA to cellulose at high ionic strength and the elution at low ionic strength most likely is the result of the intramolecular poly A tracts [35]. According to Sullivan [36] poly A tracts of considerable length (at least more than 50 nucleotides) must be present in RNA, if it is to bind effectively to cellulose. The influence of *r*RNA on the elution profile of a different RNA is shown in the experiments where *A. tumefaciens* *c*-RNA was mixed with a large amount of tobacco leaf RNA.

From the percentage of RNase resistance, the high turnover rate and the base composition we conclude that D-RNA starts to elute before the bulk of *r*RNA in the salt-eluted fractions. They may do so because of differences in GC content (compare *A. tumefaciens* *c*-RNA eluting before crown gall *c*-RNA) and by having heterogeneous MWs. Since these D-RNAs are not trapped by the cellulose bottom layer it is supposed that any poly A tracts present in this type of RNA must be relatively small (less than 50 nucleotides). We presume, therefore, that the bulk of Hn-RNA molecules are released by the salt gradient.

Interesting differences are found between the TB-RNA fractions of crown gall RNA and RNA from normal cells. Crown gall TB-RNA has a remarkable high AMP content, associated with the presence of poly A sequences. This is also expressed in a higher percentage of radioactivity in crown gall TB-RNA when it is labelled with adenosine-[^{3}H] instead of uridine-[^{3}H] or ^{32}Pi. Moreover, crown gall TB-RNA showed different labelling kinetics of RNA in α- and β-fractions compared with the normal tissue types. These data indicate that the population of TB-RNA in transformed cells is different from normal.

A further difference between RNA from crown gall cells and normal cells was found for the *t*RNA population. It has been reported that plant *t*RNA

has an abnormally high CMP content when relatively short periods of labelling were used [23]. This result was interpreted in terms of a rapid exchange of cytidylic and adenylic acids in the 3'-end (CCA-end) of the *t*RNA molecules. The same phenomenon was observed in our experiments for tobacco leaf and normal callus *t*RNA. However, crown gall tissue *t*RNA had a significantly lower CMP content suggesting that nucleotide exchange reactions are far less active in the transformed cells. This phenomenon might be related to the role that *t*RNA and *t*RNA modifications may play in differentiation and neoplasia [37].

EXPERIMENTAL

Incorporation of radioactive precursors in plant tissue RNA. Crown gall callus tissue, induced by *A. tumefaciens* strain A_6 and normal callus tissue of *Nicotiana tabacum* L. cv. White Burley, were grown *in vitro* [38] and checked for sterility as previously described [10]. Tobacco plants were grown in a greenhouse.

Labelling was performed in 40 ml sterile liquid medium, with the same composition as that used in agar medium for *in vitro* growth. 50 g of *in vitro* cultured tissue or 5 g of 2-month-old tobacco leaves were used. The liquid medium of normal callus tissue and tobacco leaves was supplemented with 2 mg/l IAA and 0·2 mg/l kinetin. The medium for tobacco leaf segments was supplemented with a mixture of 100 U/ml penicillin, 100 U/ml streptomycin, 0·005% pimafucin and 20 μg/ml rifampicin, to suppress microbial growth.

After a pre-incubation period in medium without radioactive precursors, (3 hr for ^{32}Pi and 1 hr for uridine-[^{3}H] and adenosine-[^{3}H]) labelling was performed with 25 μCi/ml carrier free ^{32}Pi (Philips Duphar, Petten, Holland) or 25 μCi/ml adenosine-[^{3}H] or uridine-[^{3}H] (Radiochemical Centre, Amersham; sp. act. varying from 17·25 Ci/mmol to 30 Ci/mmol). After incubation, the radioactive medium was removed and checked for sterility [10]. The tissue was washed 2× with fresh medium and frozen in liquid N_2.

RNA isolation. RNA was extracted from the plant tissues by a modified phenol–detergent method [39, 40]. The final pellet was dissolved in 0·1 M Tris–HCl pH 7·4; 0·1 SSC (1 SSC = 0·15 M NaCl; 0·015 M Na citrate). The amount of RNA recovered for all tissues was 70–75% of the total percentage of RNA in these tissues, determined by the method of Munro and Fleck [41].

MAK columns (12 × 2·5 cm) were prepared [42] with a 1 cm bottom layer of cellulose on which a layer of kieselguhr was placed to adsorb residual proteins eluted from the column. Unless stated otherwise, 2 mg RNA was applied to the column at 0·05 Tris–HCl, pH 6·7 (20 μg/ml). Non-bound products were removed by a wash with 100 ml buffer and the RNA eluted at room temp with a 150 ml linear gradient of buffered 0–2 M NaCl, and 3 ml fractions were collected. The columns were then washed with 50 ml buffer and TB-RNA eluted with a 60 ml linear gradient of buffered 0–5 M guanidine thiocyanate, at 35° [30].

Base ratios. MAK fractionated RNA-[^{32}P] was mixed with 0·5 mg purified carrier *E. coli* rRNA and precipitated with 3 vol cold (−20°) EtOH [43]. The ppt was collected by centrifugation, washed with 75% EtOH, dissolved in 8 μl 0·9 M KOH and hydrolysed in a 5 μl micropipet for 16–24 hr at 37° in a desiccator above 0·5 M KOH. The vol remained constant during incubation. The digest was applied directly to a sheet of Whatman no 1 paper and the mononucleotides separated by electrophoresis at 2100 V for 2·5 hr in a buffer containing 50 ml HOAc (99%), 5 ml pyridine and 0·01 M EDTA, pH 3·50–3·65. The electropherogram was dried at 140° for 5 min, freed from pyridine by washing with 96% EtOH and re-dried. The nucleotide spots were localized under UV light. The complete electropherogram was cut into pieces of 0·5 × 0·5 cm^2 and placed in liquid scintillation vials with 10 ml of toluene containing 0·4% PPO.

RNase resistance of fractionated RNA was determined by taking 3 samples of 0·25 ml of each fraction. The samples were brought to a final conc. of 0·1 M NaCl buffered with 0·05 M Tris–HCl, pH 6·7. To avoid RNase resistance due to secondary structures in RNA, the solns were boiled and quickly cooled, so that double stranded RNA was melted out. One sample was supplemented with 500 μg BSA and RNA precipitated by the addition of an equal vol of 10% TCA (control). The other 2 samples were incubated with 1 μg RNase A + 0·3 U RNase T_1 per μg RNA for 30 min at 37° and precipitated with TCA. Ppts were collected on Whatman GF/C glass-filters, washed with cold 5% TCA, dried and counted for radioactivity in toluene–0·4% PPO.

Complementary RNA (c-*RNA*) was synthesized *in vitro* with *E. coli* DNA-dependent RNA polymerase and native DNA as previously described [10]. The sp. act. was about 6 × 10^5 cpm/μg.

Cs_2SO_4 *formaldehyde gradients* were prepared [29], the refractive index adjusted to 1·5860, and the gradient performed in a 50 Ti rotor in 48 hr at 4° at 40000 rpm.

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