The Nucleoside Triphosphate–Ribonucleic Acid Nucleotidyltransferase (EC 2.7.7.6) of *Agrobacterium tumefaciens* (Smith and Townsend) Conn

PURIFICATION AND PROPERTIES OF THE ENZYME FROM THE TUMORIGENIC STRAIN B6806

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The RNA nucleotidyltransferase (RNA polymerase) of the plant-tumorigenic bacterium *Agrobacterium tumefaciens* was purified. The method involves the disruption of the bacterial cells with glass beads in a Waring Blendor, treatment with DEAE-cellulose, fractionation with $(\text{NH}_4)_2\text{SO}_4$, protamine sulphate precipitation, DEAE-cellulose column chromatography and either glycerol-gradient centrifugation or phosphocellulose chromatography. The subunit structure of the highly purified enzyme is similar to, although not identical with, the RNA nucleotidyltransferase of *Escherichia coli*. It can be described as β' , β , χ_1 and α (mol.wts. 160000, 150000, 98000 and $41000 \pm 10\%$ respectively). χ_1 is the temporary designation for a protein subunit, which might have the same functions as the σ subunit in *E. coli*. The enzyme of *A. tumefaciens* is rifampicin-sensitive, has a temperature optimum *in vitro* of $41 \pm 1^\circ\text{C}$ and a pH optimum of 8.2 ± 0.1 . Mg^{2+} and Mn^{2+} are activators. The enzyme transcribes with different efficiencies artificial, viral, bacterial, plant and animal templates.

Agrobacterium tumefaciens (Smith & Townsend) Conn is a Gram-negative bacterium, classified by Bergey (1957) in the family of Rhizobiaceae of the order of Eubacteriales. It induces tumours on plants called crown galls (Smith & Townsend, 1907).

According to current experimental interpretations, *A. tumefaciens* releases DNA (Jajko & Hegeman, 1971) which is then taken up by plant cells (Stroun *et al.*, 1967; Stroun & Anker, 1971); subsequently the bacterial DNA and perhaps a prophage become integrated into the plant genome (Schilperoort *et al.*, 1967; Milo & Srivastava, 1969; Quétier *et al.*, 1969; Srivastava & Chadha, 1970; Stroun & Anker, 1971; Schilperoort *et al.*, 1973; Petit *et al.*, 1970) and are transcribed into RNA (Stroun & Anker, 1971). On the basis of experiments with the drug rifampicin, an inhibitor of bacterial RNA nucleotidyltransferases (RNA polymerases) (Mauro *et al.*, 1969), Stroun (1971) postulated that transcription of the bacterial DNA in plant cells takes place by means of a bacterial RNA nucleotidyltransferase. It was therefore decided to investigate whether the RNA nucleotidyltransferase of the tumourigenic strain B6806 of *A. tumefaciens* has properties that distin-

guish it from the RNA nucleotidyltransferase of *Escherichia coli*.

Experimental

Materials

Chemicals. Calf thymus DNA (grade A) was from Calbiochem, La Jolla, Calif., U.S.A. Barley nuclear and chloroplast DNA were gifts from Professor R. Criddle, Department of Biochemistry, University of California, Davis. *E. coli* DNA was a gift from Professor S. Linn, Department of Biochemistry, University of California, Berkeley, bacteriophage T2 DNA was a gift from Professor M. Chamberlin, Department of Biochemistry, University of California, Berkeley, 16S rRNA was a gift from Professor R. Traut, Department of Biological Chemistry, University of California, Davis. Poly(dA-dT) was synthesized as described by Schachman *et al.* (1960). Poly(U) was from Miles Laboratories, Elkhart, Ind., U.S.A. Labelled nucleotides were from the New England Nuclear Corp., El Cerrito, Calif., U.S.A. Unlabelled nucleotides were from Sigma Chemical Corp., St. Louis, Mo., U.S.A. DNA nucleotidyltransferase (EC 2.7.7.7) was a gift from Professor M. Chamberlin. RNA nucleotidyltransferase from *E. coli* was a gift from Dr. W. Mangel, Department

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of Molecular Biology, University of California, Berkeley. Deoxyribonuclease (EC 3.1.4.5) was from Calbiochem. Ribonuclease (EC 3.1.4.22) from bovine pancreas was from Calbiochem and was heated to 80°C for 10 min before use. Lysozyme (grade 1, EC 3.2.1.17) and Pronase were from Sigma. Crystallized bovine serum albumin was from Pentex, Kankakee, Ill., U.S.A. Cytochrome *c* (type VI) was from Sigma. Ovalbumin was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Protamine sulphate was from Eli Lilly and Co., Indianapolis, Ind., U.S.A., and was dissolved in water at 37°C before use. $(\text{NH}_4)_2\text{SO}_4$ (enzyme-grade) was from Schwarz/Mann Laboratories, Van Nuys, Calif., U.S.A. Phosphocellulose (from H. Reeve Angel and Co., Clifton, N.J., U.S.A.) was washed and then equilibrated with buffer E. DEAE-cellulose (type 40) (from Schleicher and Schuell, Keene, N.H., U.S.A.) was washed and then equilibrated with buffer B. Hydroxyapatite was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Chromatographic-grade phenol (from the Fisher Company, Burlingame, Calif., U.S.A.) was neutralized and saturated by shaking it just before use with 0.3 vol. of 0.1 M-potassium phosphate buffer, pH 7.0, containing 0.1 M-NaCl and 0.001 M-EDTA. Acrylamide reagents were from Bio-Rad Laboratories. Sodium dodecyl sulphate was from BDH Chemicals Ltd., Poole, Dorset, U.K.

Biomaterials. *A. tumefaciens* B6806 was a gift from Professor Lippincott, Northwestern University, Evanston, Ill., U.S.A. The bacteriophage PS-8 was a gift from Professor R. E. Beardsley, Manhattan College, New York, U.S.A.

Other materials. Whatman GF/C glass-fibre filters (2.4 cm diameter) were used. Super-Brite glass beads, with a medium diameter of 200 μm , were from Lapine, Berkeley, Calif., U.S.A., and prepared before use as described by Burgess (1969).

Media and buffers. Medium H contains (per litre): nutrient broth, 8 g; NaCl, 5 g; sucrose, 5 g; casamino acids, 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; K_2HPO_4 , 2 g; silicone antifoam emulsion, 0.1 ml. Buffer A is 50 mM-Tris-HCl (pH 8.0)–10 mM- MgCl_2 –0.1 mM-dithiothreitol–0.1 mM-EDTA–5% glycerol. Buffer B is the same as buffer A, but contains 20 mM-Tris-HCl, pH 8.0. Buffer C is the same as buffer B, but contains 50% glycerol. Buffer D is 50 mM-Tris-HCl (pH 8.0)–0.1 M-NaCl–1 mM-EDTA. Buffer E is 50 mM-Tris-HCl (pH 8.0)–0.1 mM-dithiothreitol–50 mM-KCl–0.1 mM-EDTA–5% glycerol.

Methods

Growth of bacteria. *A. tumefaciens* B6806 was grown at 30°C under vigorous aeration in flasks containing 15 litres of medium H. The cells were harvested in late exponential-growth phase with a Sharples flow centrifuge and immediately stored at –20°C.

Identification of *A. tumefaciens*. Before and after growth in large batches the bacteria were identified by: (a) the colony morphology; (b) their characteristic pink color; (c) the ketolactose test (Bernaeerts & DeLey, 1963); and (d) the infectivity test. Infectivity tests were done by putting 20 μl of bacterial suspension on freshly cut carrot (*Daucus carota*) slices or 20 cm high sunflowers (*Helianthus annuus*), previously wounded with a needle of 1 mm diameter. An automatic Eppendorf pipette was used. The carrot slices were kept in sterile water and Petri dishes at room temperature. Commercial sunflowers were grown under greenhouse conditions. Tumour formation took place 2–3 weeks after inoculation of the bacteria.

Isolation of DNA from bacteria. The cells were washed twice in buffer D and repeatedly frozen and thawed. Finally they were suspended in buffer D (50 ml of buffer/6 g of cells) and 5 mg of lysozyme/50 ml of suspension was added. After addition of 7.5 ml of 25% (w/v) sodium dodecyl sulphate/50 ml of suspension, the solution was heated at 70–75°C for 10–20 min, cooled slowly and then placed in the cold room for 20 min. Then an equal volume of phenol was added and the mixture was shaken for 20 min at 4°C. After centrifugation (Sorvall centrifuge, 4°C, SS 34-rotor, 17000 g, 10 min), the upper aqueous phase was collected and re-extracted with an equal volume of phenol, followed by centrifugation as before. The DNA was precipitated from the aqueous phase by adding 1.5 vol. of ice-cold propan-2-ol and was then collected on a glass stirring rod. The DNA was dissolved in SSC medium* and then dialysed against 500 vol. of the same solution with five subsequent changes. Ribonuclease (50 $\mu\text{g}/\text{ml}$) was added and the solution was incubated for 1 h at 35°C. After addition of Pronase (100 $\mu\text{g}/\text{ml}$) and incubation for 3 h at 35°C further deproteinization was done by shaking the enzyme-treated DNA solution with an equal volume of chloroform–3-methylbutan-1-ol (24:1, v/v) and centrifuging as above. This was repeated until no more white precipitate was visible in the interphase. The DNA was precipitated with 1.5 vol. of ice-cold propan-2-ol and dissolved in SSC medium followed by dialysis against buffer D.

Isolation of DNA from bacteriophage PS-8. Bacteriophage PS-8 was purified as described by Knopf (1974). The DNA was extracted with phenol by the method of Kaiser & Hogness (1960). Further purification of the DNA was by hydroxyapatite chromatography as described by Bernardi *et al.* (1970).

RNA nucleotidyltransferase assay. Assay solution 1 contained 0.2 M-Tris-HCl, pH 8.0, 0.1 M- MgCl_2 , 0.05 M-2-mercaptoethanol and 5 mg of bovine serum albumin/ml. Assay solution 2 contained 0.05 M-Tris-HCl, pH 8.0, 5 mM-2-mercaptoethanol, 0.52 mg

* Abbreviation: SSC medium, 15 mM-NaCl–1.5 mM-sodium citrate.

of bovine serum albumin/ml and 0.5mM-EDTA. Assay tubes (standard assay) contained 0.02ml of assay solution 1, 0.04ml of assay solution 2, 40nmol each of CTP, GTP, UTP and ATP (one of the nucleotides being labelled with either ^{32}P or ^3H), 40nmol of a template and enzyme solution in a final volume of 0.1ml. Synthesis was initiated by adding the enzyme solution and was terminated after 10min at 37°C by adding 3ml of ice-cold 3.5% (v/v) HClO_4 containing 0.1M- $\text{Na}_4\text{P}_2\text{O}_7$. The tubes were immediately chilled on ice. After 20min the precipitates were collected on Whatman GF/C filters (previously soaked in 0.1M- $\text{Na}_4\text{P}_2\text{O}_7$) and were washed five times with 3ml portions of ice-cold 2M-HCl containing 0.1M- $\text{Na}_4\text{P}_2\text{O}_7$ and once with a 3ml portion of ice-cold ethanol. The filters were dried for 20min under a Fisher infrared radiator. The radioactivity on the filters was determined by using a toluene-based solvent [4g of 2,5-diphenyloxazole (PPO) and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) per litre of toluene] in a Nuclear-Chicago liquid-scintillation counter.

Protein determination. The amount of protein was determined by the methods of Lowry *et al.* (1951) and Warburg & Christian (1941).

Purification of the enzyme. The purification procedure was adapted from the one of Berg *et al.* (1971). All steps described were carried out at 4°C. All centrifugations were made in a Sorvall centrifuge with an SS34 rotor, for 1h at 27000g unless stated otherwise.

(1) Extraction of the enzyme from bacterial cells. *A. tumefaciens* B6806 (60g of frozen cells) were mixed with 100ml of buffer A and 480g of glass beads in a Waring Blender. The mixture was blended at high speed (three-quarters of maximum speed), the speed being controlled by a rheostat. By periodically cooling the blender in a solid CO_2 -methanol bath, or by using a blender cooled by a refrigeration jacket, the temperature was kept below 6°C. After 10min of blending, 50ml of buffer A was added and the blending was continued for a further 5min. The beads were allowed to settle and the supernatant was decanted. The beads were rinsed with 120ml of buffer A and the supernatant and rinse fractions were combined and centrifuged. The dark-yellow supernatant was collected (fraction 1, 240ml).

(2) DEAE-cellulose treatment. Fraction 1 was made 0.4M with respect to KCl. DEAE-cellulose (4g/100ml of solution) was added and stirred for 45min (the amount of DEAE-cellulose in the slurry was determined by drying a known volume in a dialysis bag of known dry weight and then weighing). The slurry was centrifuged and the supernatant was passed through two columns of glass-wool previously washed with buffer A. The removal of the nucleic acids and proteins was checked by measurement of the E_{260} and E_{230} respectively (fraction 2, 235ml).

(3) First $(\text{NH}_4)_2\text{SO}_4$ fractionation. To fraction 2 was slowly added 36g of $(\text{NH}_4)_2\text{SO}_4$ /100ml of enzyme solution and simultaneously 0.37ml of 1M-NaOH/100ml of enzyme solution. After being stirred for 45min the solution was centrifuged. The pellet was resuspended in 120ml of buffer A containing 0.5M- $(\text{NH}_4)_2\text{SO}_4$, stirred for 30min and then centrifuged. The supernatant was collected and the $(\text{NH}_4)_2\text{SO}_4$ concentration raised to 2.5M. After centrifugation the precipitate was collected [the $(\text{NH}_4)_2\text{SO}_4$ supernatant having been drained as thoroughly as possible] and resuspended in buffer B. If necessary it was diluted with buffer B so that the conductivity equalled that of a 40mM- $(\text{NH}_4)_2\text{SO}_4$ solution (fraction 3, 120ml).

(4) Protamine sulphate precipitation. A 1% (w/v) protamine sulphate solution was slowly added to fraction 3 to a ratio of 40 μl /ml. The solution was stirred at 4°C for 20min, then a 1ml portion was centrifuged and the supernatant was assayed for activity. Protamine sulphate additions and trial centrifugations with small samples were repeated until 90% of the activity disappeared from the supernatant. A total of 7.2ml of protamine sulphate/120ml of fraction 3 was added before the whole precipitate was collected by centrifugation. The precipitate was rinsed by suspending it in 36ml of buffer A containing 0.1M-magnesium acetate. After being stirred for 20min the precipitate was collected by centrifugation and then extracted for 60min with 50ml of buffer A containing 0.35M- $(\text{NH}_4)_2\text{SO}_4$. After centrifugation the supernatant was collected (fraction 4, 50ml).

(5) Second $(\text{NH}_4)_2\text{SO}_4$ fractionation. To fraction 4 was added 10ml of saturated (at 25°C) and neutralized (with NaOH) $(\text{NH}_4)_2\text{SO}_4$ /7.9ml of enzyme solution. After being stirred for 15min the precipitate was collected by centrifugation and then successively extracted with 2.0, 1.8, 1.6, 1.5 and 1.3M- $(\text{NH}_4)_2\text{SO}_4$ in buffer A. Each extraction was continued for 15min and the precipitates were collected by centrifugation for 20min. The first two extractions were carried out with 23ml samples and the last three with 15ml samples. Fractions with a high specific activity of RNA nucleotidyltransferase (usually the 1.6 and 1.8M fractions) were precipitated by adjusting them to 2.5M- $(\text{NH}_4)_2\text{SO}_4$, and finally pooled. The precipitate was collected by centrifugation and dissolved in buffer B (fraction 5, 30ml).

(6) DEAE-cellulose chromatography. A column (1cm $\times$ 13cm) of DEAE-cellulose was prepared and equilibrated with 400ml of buffer B. The flow rate was adjusted to 1ml/min. Then 37ml of enzyme solution (fraction 5) was layered on the column at a protein concentration of 1mg/ml. Before elution the column was washed with 2 column volumes of buffer B. Elution was done with a linear gradient (0.04–0.36M-KCl in buffer B, 150ml total volume). Frac-

tions of volume 2ml were collected and immediately assayed for RNA nucleotidyltransferase activity and protein content. Fractions of high specific activity (Fig. 1, fractions 19–24) were precipitated with 0.45g of $(\text{NH}_4)_2\text{SO}_4$ and 0.004ml of 1M-NaOH per ml of solution. The precipitate was collected by centrifugation and dissolved in buffer C at a final concentration of 5mg/ml (fraction 6, 3ml). Further purification was done by either of the following methods.

(i) Glycerol-gradient centrifugation. This was done with a 15–35% (v/v) glycerol–buffer B gradient. A gradient which does not contain KCl is designated a low-salt gradient. A gradient which in addition contains 0.4M-KCl is designated a high-salt gradient. Fraction 6 (100–500 μg of enzyme) was precipitated with 1.5vol. of satd. $(\text{NH}_4)_2\text{SO}_4$ solution, centrifuged and then redissolved in buffer B. Then the enzyme solution was carefully layered on top of the gradient and centrifuged in a Beckman ultracentrifuge with a SW 41 rotor, at 4°C, for 24h at 32000rev./min. Fractions of volume 0.5ml were collected with an Isco density-gradient fractionator (fraction 7.1).

(ii) Phosphocellulose chromatography. A small column (6cm $\times$ 1.5cm) was prepared. In order to recover a possible σ factor, a small column (5cm $\times$ 0.8cm) of DEAE-cellulose was directly connected to the outflow of the phosphocellulose column. Both columns were equilibrated with 20 column volumes of buffer E. After precipitation of the enzyme with $(\text{NH}_4)_2\text{SO}_4$, centrifugation and careful removal of residual $(\text{NH}_4)_2\text{SO}_4$ from the tubes, the enzyme was suspended in buffer E and applied to the column at a concentration of 1.7mg/ml (volume 1ml), the flow rate being adjusted to 20ml/h. Before elution of the protein the columns were equilibrated with buffer E for 1h. The elution was done with a step gradient of 0.2, 0.3, 0.4, 0.5 and 0.6M-KCl. Fractions with a high activity (Fig. 2, fractions 8–10) were concentrated by an $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by resuspension in buffer B (fraction 7.2).

Storage of the enzyme. The enzyme was stored in buffer C at -20°C , where it was stable over several months.

Polyacrylamide-gel electrophoresis and determination of the molecular weights of the subunits. Samples were prepared in 0.06ml containing 0.033M-sodium phosphate buffer, pH7.1, 0.125% (w/v) sodium dodecyl sulphate, 0.016M-2-mercaptoethanol and 0.004% (w/v) Bromophenol Blue. Samples were incubated for 30min at 37°C or for 15min at 67°C before electrophoresis. Electrophoresis in 7.5% (w/v) or 5% (w/v) polyacrylamide gels (8cm $\times$ 0.5cm tubes) was done at room temperature as described by Shapiro *et al.* (1967). The current was 5mA/tube for the first 15min and 10mA/tube thereafter for 4–6h. The gels were fixed, stained and destained as described by Burgess (1969). The molecular weights of the subunits were determined as described by Shapiro *et al.* (1967).

Tests for enzymic impurities. All tests were made at the same time under the same conditions. Enzyme fraction 7.1 was used. The assay for the DNA-dependent DNA nucleotidyltransferase was done as described by Lehman *et al.* (1958), and RNA-dependent DNA nucleotidyltransferase was assayed by the method of Goodman & Spiegelman (1971). Polyadenylate nucleotidyltransferase (EC 2.7.7.19) was assayed as described by Ohasa & Tsugita (1972) and polynucleotide phosphorylase (EC 2.7.7.8) was assayed by the method of Reiner (1969).

Results and Discussion

Purification procedure

The results of an extraction of the RNA nucleotidyltransferase are shown in Table 1. Three steps are critical in the purification of the enzyme. (1) Optimal extraction from the bacterial cells is the basis for high yield. Good breakage of the cells can be achieved only if the glass bead–cell mixture has a thick consistency. (2) The use of DEAE-cellulose for the

Table 1. Purification of the RNA nucleotidyltransferase from *A. tumefaciens* (60g of cells)

In the present system the unit is defined as the amount of enzyme that catalyses the incorporation of 1nmol of nucleotide monophosphate/h at 37°C into an acid-precipitable product with bacteriophage T2 DNA as template.

Fraction	Volume (ml)	$10^{-3} \times$ Total units	Recovery	E_{260}	E_{280}	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg of protein)
(1) Extract	240	140	100	1.15	0.73	14.8	3552	40
(2) DEAE-cellulose treatment	235	119	85	0.69	0.45	14.0	3290	35
(3) First $(\text{NH}_4)_2\text{SO}_4$ fraction	120	80	57			7.8	936	85
(4) Protamine eluate*	50	76	54			4.5	225	340
(5) Second $(\text{NH}_4)_2\text{SO}_4$ fraction	30	54	39			2.5	75	720
(6) DEAE-cellulose fractionation	3	28	20			10	30	900

* The enzyme was assayed in 0.35M- $(\text{NH}_4)_2\text{SO}_4$.

Fig. 1. DEAE-cellulose column profile

A column (1 cm $\times$ 13 cm) of DEAE-cellulose was prepared and equilibrated with 300 ml of buffer B. The enzyme solution (37 ml) was layered on top of the column at a concentration of 1 mg/ml. After equilibration with buffer B, elution was done with a linear gradient (0.04–0.36 M-KCl in buffer B). Fractions (2 ml) were collected and immediately assayed for RNA nucleotidyltransferase activity and protein content. $\circ$, Enzyme activity; $\bullet$, E_{280} ; —, [KCl].

Fig. 2. Glycerol-gradient profiles

(a) High-salt glycerol gradient. The gradient was 15–35% (v/v) glycerol in buffer B and contained 0.4 M-KCl. Centrifugations, from the left to the right, were done in a Beckman ultracentrifuge with an SW 41 rotor, for 24 h, 32000 rev./min at 4°C. Fractions (0.8 ml) were collected and immediately assayed for activity. $\circ$, Poly(dA-dT) was used as a template; Δ , bacteriophage T2 DNA was used as a template; $\bullet$, E_{280} . (b) Low-salt glycerol gradient. The conditions are the same as (a) except that this gradient did not contain KCl. $\circ$, Poly(dA-dT) was used as a template; $\bullet$, bacteriophage T2 DNA was used as a template; Δ , E_{280} .

removal of nucleic acids, which would interfere in the protamine sulphate precipitation, results in the improvement of the assay conditions for the enzyme. Complete removal of the DEAE-cellulose is, however, necessary for a successful protamine sulphate precipitation. (3) It is also necessary that the conductivity of the protein solution before precipitation with protamine sulphate is exactly adjusted. Although it is possible to elute the enzyme from the protamine sulphate precipitate with 0.1 M-(NH₄)₂SO₄ for *E. coli* (Berg *et al.*, 1971) it was necessary to elute the *A. tumefaciens* enzyme with 0.35 M-(NH₄)₂SO₄. The DEAE-cellulose column profile is shown in Fig. 1. The enzyme was eluted at a KCl concentration of 0.14–0.20 M in two peaks. The reasons for these two peaks are not clear at present. Since RNA nucleotidyltransferase is inactivated in dilute solution, the precipitation of the enzyme after purification by DEAE-cellulose chromatography has to be done immediately. At this stage the enzyme was judged to be about 50% pure (Plate 1a). Further purification by either glycerol-gradient centrifugation or phosphocellulose chromatography resulted in an enzyme which was judged to be almost 100% pure (Plates 1b and 2b). Glycerol-gradient centrifugation under low-salt conditions, yielded an enzyme with a specific activity of 1040 (units/mg of protein), whereas phosphocellulose chromatography yielded an enzyme with a specific activity of 1150 (units/mg of protein). Glycerol-gradient centrifugation is efficient because the recovery is almost 100%. The yield is smaller with phosphocellulose chromatography, but this procedure results in a slightly more purified enzyme. The profile of the enzyme-glycerol gradient is shown in Fig. 2(a) (high-salt conditions) and Fig. 2(b) (low-salt conditions). As expected (Berg & Chamberlin, 1970) the enzyme sediments as a relatively broad peak under low-salt conditions, whereas it clearly sediments as a narrower peak under high-salt conditions. The elution profile from the phosphocellulose column is shown in Fig. 3. The activity was eluted in a large peak at approx.

Fig. 3. Phosphocellulose column profile

A small column (6cm×1.5cm) was prepared and equilibrated with 20 column volumes of buffer E. The enzyme (1 ml), resuspended in buffer E, was applied to the column at a concentration of 1.7 mg/ml. Elution of the enzyme was done with a step gradient of 0.2, 0.3, 0.4, 0.5 and 0.6M-KCl in buffer E (3 ml samples). Fractions (1 ml) were collected and immediately assayed for RNA nucleotidyltransferase activity. ●, Enzyme activity; —, [KCl].

Table 2. Template and nucleotide dependence of the RNA nucleotidyltransferase from *A. tumefaciens*

The standard reaction mixture with 40nmol of bacteriophage T2 DNA as template was used. Incubation was for 20min at 37°C except where stated in the Table. The reaction mixture contained 0.5 unit of enzyme (fraction 6).

Additions to or omissions from the standard reaction mixture	AMP incorporated (pmol)
None	156 and 166
+ Pancreatic RNAase* (5μg in the reaction mixture) added at the beginning of the reaction	<6
+ Pancreatic RNAase (5μg in the reaction mixture) added at the end of the reaction and incubated for a further 20min at 37°C	<6
+ DNAase (5μg in the reaction mixture) added 20min before the start of the reaction with RNA nucleotidyltransferase	<6
- CTP	26
- GTP	12
- UTP	6
- CTP and UTP	<6
- GTP and UTP	<6
- GTP and CTP	<6

* Abbreviations: RNAase, ribonuclease; DNAase, deoxyribonuclease.

Table 3. Transcription of different templates with the RNA nucleotidyltransferase from *A. tumefaciens*

Standard assay conditions were used in this experiment, but different templates were substituted in excess for bacteriophage T2 DNA

Templates	AMP incorporated (nmol/30min per E ₂₆₀ unit of template)
Barley nuclear DNA	122
Barley chloroplast DNA	127
Calf thymus DNA	4
<i>E. coli</i> DNA	73
<i>A. tumefaciens</i> DNA	20
Bacteriophage T2 DNA	39
Bacteriophage T4 DNA	10
Bacteriophage PS-8 DNA	205
Poly(dA-dT)	820
16S rRNA	<0.07
Poly(U)	<0.01
None	<0.01

Table 4. Effect of rifampicin on the RNA nucleotidyltransferase-catalysed reaction

The standard reaction mixture with 40nmol of bacteriophage T2 DNA as template and 1.4 units of enzyme (fraction 6)±addition of rifampicin was used. Rifampicin was added to the reaction mixture before the enzyme. Incubation was for 15min at 37°C.

Amounts of rifampicin in the reaction mixture (μg/unit of enzyme)	AMP incorporated (nmol)	Inhibition (%)
0	348	0
0.014	237	32
0.14	82	76
1.4	38	89
14	0.2	99

0.4M-KCl followed by a small peak at 0.5–0.6M-KCl. The gel-electrophoresis pattern of fraction 9 is shown in Plate 1(b). Both the enzyme purified on a glycerol gradient (Plate 2b) and that on a phosphocellulose column consisted, after this step, of four [counting β' and β as two subunits (Plate 3)] protein subunits.

Biochemical evidence for the isolation of the RNA nucleotidyltransferase of *A. tumefaciens*

The results from the deoxyribonuclease digest (Table 2) and the other transcription experiments (Table 3) show that DNA is required as a template. Polyribonucleotides cannot serve as templates. The fact that rRNA directed slight incorporation of AMP could be due to slight contamination with DNA. The reaction is ribonucleotide-dependent and the reaction product can be degraded by pancreatic ribonuclease (Table 2), which shows that the reaction

EXPLANATION OF PLATE I

Polyacrylamide-gel-electrophoresis profiles of the RNA nucleotidyltransferase from A. tumefaciens at different purification stages

(a) Enzyme purified on a DEAE-cellulose column (20 μ g). (b) Enzyme purified on a phosphocellulose column (20 μ g). Electrophoresis was done from the top to the bottom as described in the text.

EXPLANATION OF PLATE 2

Comparison of the RNA nucleotidyltransferase from E.coli with the RNA nucleotidyltransferase from A. tumefaciens

(a) RNA nucleotidyltransferase from *E. coli* (40 μ g of enzyme); (b) RNA nucleotidyltransferase from *A. tumefaciens* (20 μ g of enzyme; fraction 7.1). Gel electrophoresis from the top to the bottom was conducted as described in the text.

EXPLANATION OF PLATE 3

*Resolution of the high-molecular-weight protein from *A. tumefaciens* RNA nucleotidyltransferase in two distinct subunits (β' and β)*

A sample (10 μ g) of enzyme (fraction 7.1) was put on the gel. Electrophoresis from the top to the bottom was conducted as described in the text.

Fig. 4. Determination of the molecular weights of the subunits of the RNA nucleotidyltransferase from *A. tumefaciens*

Electrophoresis on 5% sodium dodecyl sulphate-polyacrylamide gels was done as described in the text. The following proteins served as molecular-weight markers: (1) horse heart cytochrome *c*, mol. wt. 13400 (Atlas & Farber, 1956); (2) *E. coli* RNA nucleotidyltransferase, α -subunit, mol.wt. 41000 (Berg & Chamberlin, 1970); (3) chicken ovalbumin, mol.wt. 45000 (Warner *et al.*, 1954); (4) *E. coli* RNA nucleotidyltransferase, σ -subunit, mol.wt. 86000 (Berg & Chamberlin, 1970); (5) and (6) *E. coli* RNA nucleotidyltransferase, β and β' subunits, mol.wt. 150000 and 160000 (Burgess, 1969; Berg & Chamberlin, 1970). ●, Mol.wt. markers; ○, subunits of *A. tumefaciens* RNA nucleotidyltransferase. The migration is given relative to the marker dye.

product is RNA. The enzyme is sensitive to rifampicin (Table 4); 1.4 μg of rifampicin/unit of enzyme was sufficient to inhibit 89% of the enzyme-catalysed reaction.

Purity of the enzyme

The homogeneity of the enzyme was investigated by polyacrylamide-gel electrophoresis, which is a sensitive method for the detection of contaminating components (Richardson, 1969). The enzyme purified by phosphocellulose chromatography especially (Plate 1*b*), and also the one purified on a glycerol gradient (Plate 2*b*), gave a very distinct banding pattern after gel electrophoresis showing three or four (distinguishing β' and β) bands. The peak activities from a low- and high-salt glycerol gradient (Figs. 2*a* and 2*b*) were found in the same fractions, regardless of whether poly(dA-dT) or bacteriophage T2 DNA was used as a template.

Tests for enzymic contaminations

Ten poly(dA-dT) enzyme units (fraction 7.1) incorporated less than 20 pmol of AMP/h in a polyadenylate nucleotidyltransferase assay and less than 100 pmol of AMP/h in a synthetic polynucleotide

phosphorylase assay. The same amount of RNA nucleotidyltransferase catalysed the incorporation of 2 pmol of dAMP/h in a DNA-dependent assay for DNA nucleotidyltransferase whereas 10 μg of DNA nucleotidyltransferase catalysed the incorporation of 306 pmol of dAMP in the same time. No significant incorporation of dAMP was observed in the RNA-dependent DNA nucleotidyltransferase assay.

Subunit composition of the enzyme and the molecular weights of the subunits

Plate 2*b*) shows the subunit structure of the enzyme from *A. tumefaciens* purified on a glycerol gradient, compared with the one from *E. coli*. RNA nucleotidyltransferase of *A. tumefaciens* consists of at least four subunits. The high-molecular-weight subunits have, within experimental error, the same migration characteristics in a 5% sodium dodecyl sulphate-polyacrylamide gel as the β' , β and α subunits of the RNA nucleotidyltransferase from *E. coli* (Plate 2 and Fig. 4). These subunits of the enzyme from *A. tumefaciens* have therefore been designated β' , β and α and their molecular weights were estimated to be 160000, 150000 and 41000 ($\pm 10\%$) respectively (Plate 2 and Fig. 4). Both the glycerol-gradient-purified enzyme and the phosphocellulose-purified enzyme from *A. tumefaciens* contain another subunit, which has been designated χ_1 , with mol.wt. $98000 \pm 10\%$ (Plates 1 and 2*b* and Fig. 4). The fact that this subunit co-sediments with the other subunits and occurs in every enzyme preparation in stoichiometric amounts is strong evidence for χ_1 being part of the RNA nucleotidyl-

Fig. 5. Determination of the optimal temperature

Standard assay conditions, but with 2.2 nmol of poly(dA-dT) as template and 1.3 units of enzyme (fraction 6), were used in this experiment. Incubation was for 30 min at the corresponding temperature.

Fig. 6. Determination of the pH optimum

The pH assay solution was 0.1M-Tris-HCl at the appropriate pH (measured with a Radiometer/Copenhagen 26 pH meter) with 0.5 mM-2-mercaptoethanol. The reaction mixture (final volume 0.1 ml) contained 0.05 ml of pH assay solution, 30 nmol of $MgCl_2$, 40 nmol each of CTP, UTP, ATP and [^{32}P]GTP, 40 nmol of bacteriophage T2 DNA and 2.3 units of enzyme (fraction 7.1).

Fig. 7. Determination of the optimal bivalent metal ion concentration

The assay mixture (0.1 ml final volume) contained 0.05 ml of modified assay solution 2 (modified assay solution 2 does not contain EDTA), 0.01 ml of $MgCl_2$ or $MnCl_2$ solution of the appropriate concentration, 40 nmol each of CTP, GTP, UTP and ATP (one of the nucleotides being radioactive), 40 nmol of bacteriophage T2 DNA and 1.8 units of enzyme (fraction 6). To exhaust Mg^{2+} ions present in the storage buffer, the enzyme was diluted in 0.01M-Tris-HCl, pH 8.0, before use. ○, $MgCl_2$; ●, $MnCl_2$.

transferase molecule. Comparison of the enzyme from *A. tumefaciens* with the one from *E. coli* and other bacterial RNA nucleotidyltransferases which have a similar subunit structure (Losick *et al.*, 1970; Johnson *et al.*, 1971; Hermoso *et al.*, 1972) suggested

Fig. 8. Effect of the nucleotide concentration on the RNA nucleotidyltransferase-catalysed reaction

(a) Standard assay conditions were used in this experiment, except that poly(dA-dT) (4.4 nmol) was the template and ATP (40 nmol) and UTP (in various amounts) were the only nucleotides. Seven poly(dA-dT) units of RNA nucleotidyltransferase (fraction 7.1) were used in this experiment. Incubation was for 10 min. (b) Standard assay conditions with various amounts of nucleotides (GTP, UTP, CTP), 40 nmol of bacteriophage T2 DNA and 1.2 units of RNA nucleotidyltransferase (fraction 7.1) were used in this experiment. Incubation was for 20 min.

that χ_1 corresponds to σ . For *E. coli* and other bacterial RNA nucleotidyltransferases, this subunit can be separated out from the core enzyme by phosphocellulose chromatography (Burgess *et al.*, 1969). For *A. tumefaciens*, however, χ_1 binds to phospho-

Fig. 9. Effect of the DNA concentration on the RNA nucleotidyltransferase-catalysed reaction

Standard assay conditions, but with various amounts of bacteriophage T2 DNA and 6.3 units of enzyme (fraction 7.1), were used in the experiment. Incubation was for 10 min.

cellulose. This fact alone does not exclude χ_1 from having σ functions since at least one other prokaryotic σ factor behaves in the same way (Herzfeld & Zillig, 1971). Both the phosphocellulose-purified enzyme and the glycerol-gradient-purified enzyme transcribe bacteriophage T2 DNA. This supports the suggestion that χ_1 might have σ functions, but ultimately σ has to be defined by a functional assay. Therefore more experimental evidence is needed to sustain the suggestion that χ_1 is σ . In all preparations of *E. coli*, except those fractionated on hydroxyapatite and possibly on phosphocellulose by means of a shallow KCl gradient, a small protein, designated ω (mol.wt. 9000–12000), could be detected (Burgess, 1969; Berg & Chamberlin, 1970). No similar subunit was detectable in highly purified preparations of *A. tumefaciens* RNA nucleotidyltransferase.

Other characteristics of the enzyme

The conditions for the following experiments are described in the corresponding Tables and Figures. The temperature optimum for the standard assay was found to be $41 \pm 1^\circ\text{C}$ with a relatively broad peak from 37° to 41°C (Fig. 5). Temperatures below or above this range did inhibit the reaction significantly. The pH optimum for the standard assay was 8.2 ± 0.1 . On variation of the pH, only a slight effect on the incorporation of AMP was observed in the range pH 7.8–8.2 (Fig. 6). Mg^{2+} as well as Mn^{2+} can serve as activators. The optimal bivalent metal ion concentration for the standard assay was found

to be 8 mM for MgCl_2 and 4 mM for MnCl_2 . From 20 mM upwards for MgCl_2 and 4 mM upwards for MnCl_2 the reaction is strongly inhibited (Fig. 7). The incorporation of AMP as a function of the nucleotide concentration is shown in Fig. 8. By using poly(dA-dT) as template and variable amounts of UTP (Fig. 8a) the theoretical $V_{\max}$, as derived from the Lineweaver-Burk (1934) plot is 1.35 nmol/10 min. The apparent K_m was $8 \times 10^{-5}\text{M}$. Fig. 8(b) shows that the nucleotides were used in saturating amounts in the standard assay (0.4 mM of each). Fig. 9 shows the incorporation of AMP as a function of the bacteriophage T2 DNA concentration. With 6.3 units of enzyme $V_{\max}/2$ is reached with about 6 nmol of DNA/0.1 ml of reaction mixture. $V_{\max}$ was reached with about 40 nmol (amount used in the standard assay) of deoxynucleotides/0.1 ml of reaction mixture. The theoretical $V_{\max}$ from the Lineweaver-Burk (1934) plot is 1.15 nmol/10 min; this value is near to the observed values and indicates that an excess of DNA is not inhibitory. The results of the transcription of different templates by the RNA nucleotidyltransferase of *A. tumefaciens* are summarized in Table 3. Poly(dA-dT) and PS-8 bacteriophage DNA are the most efficient templates. Plant DNA, purified by hydroxyapatite chromatography, was a better template than commercial animal DNA. Whether this difference or the one observed in the transcription between plant nuclear and chloroplast DNA reflects a difference in the template specificity or is due to other differences remains to be established.

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