Purification and characterization of chromatin-bound DNA-dependent RNA polymerase I from parsley (*Petroselinum crispum*)

INFLUENCE OF NUCLEOSIDE TRIPHOSPHATES

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The isolation and purification of DNA-dependent RNA polymerase I (EC 2.7.7.6) from parsley (*Petroselinum crispum*) callus cells grown in suspension culture is described. The enzyme was solubilized from isolated chromatin. Purification was achieved by using DEAE- and phospho-cellulose in batches, followed by column chromatography on DEAE- and phospho-cellulose (two columns) and density-gradient centrifugation. The highly purified enzyme was stable over several months. The properties of purified parsley RNA polymerase I were investigated. Optimum concentration for Mn^{2+} was 1 mM, and for Mg^{2+} 4–6 mM, Mn^{2+} was slightly more stimulatory than Mg^{2+} . The enzyme was most active at low ionic strengths [10–20 mM-(NH_4)₂SO₄]. The influence of various phosphates was tested: pyrophosphate inhibited RNA polymerase at low concentrations, whereas orthophosphate had no effect on the enzyme activity. ADP was slightly inhibitory, and AMP had no effect on the enzyme reaction. Nucleoside triphosphates and bivalent cations in equimolar concentrations in the range 4–11 mM did not influence the RNA synthesis *in vitro*. Free nucleoside triphosphates in excess of this 1:1 ratio inhibited the enzyme activity, unlike free bivalent cations, which stimulated RNA polymerase I.

Eukaryotic cells contain multiple forms of DNA-dependent RNA polymerase (EC 2.7.7.6). Three forms (I, II, III) were described in many animal cells and yeast. In higher-plant cells, in several cases only a partial purification of the enzyme has been achieved (for review see Duda, 1976). Freely suspended callus cells of parsley (*Petroselinum crispum*), grown heterotrophically in fully synthetic liquid medium, had been used as an ideal source for the isolation of RNA polymerase II (Link & Richter, 1975).

In the present work we describe the isolation of RNA polymerase I from actively dividing parsley cells. The optimum metal ion concentrations, the behaviour with different ionic strengths and the subunit structure of the highly purified RNA polymerase I were determined.

In isolated nuclei from parsley a strong dependence of the transcription on ATP and Mg^{2+} was observed (Grossmann *et al.*, 1979). In the present paper the optimum conditions for RNA synthesis *in vitro* as regards nucleoside triphosphates and bivalent cations were investigated.

Materials and methods

Buffers

The extraction medium (buffer A) contained 0.05 M-Tris/HCl (pH 8.0), 0.25 M-sucrose, 0.001 M-MgCl₂ and 0.7% 2-mercaptoethanol. Buffer B contained 0.05 M-Tris/HCl (pH 8.0), 0.001 M-EDTA, 0.001 M-dithiothreitol and 30% (v/v) glycerol.

Cell culture

Freely suspended callus cells from *Petroselinum crispum* were propagated in Erlenmeyer flasks on a rotary shaker or in a fermenter (Microferm, New Brunswick) as described by Seitz & Richter (1970).

Preparation of ion exchangers

DEAE-cellulose was prepared for chromatography as described by Whatman. For column chromatography, buffer B containing 0.05 M-(NH_4)₂SO₄ was used for equilibration. For the batch treatment DEAE-cellulose was equilibrated also with buffer B containing 0.25 M-(NH_4)₂SO₄. Phospho-cellulose was treated with 0.01 M-KOH for 10 min

and washed three times with distilled water; afterwards 0.01 M-HCl was added. After 10 min the ion exchanger was washed three times with water and adjusted to pH 7.4 with concentrated KOH. Then the phosphocellulose was incubated overnight with buffer B containing 0.5 M-(NH₄)₂SO₄ and equilibrated for 6–8 h with buffer B containing 0.1 M-(NH₄)₂SO₄.

Refractive index and protein determination

(NH₄)₂SO₄ concentrations were measured with a Zeiss refractometer. Protein determinations were carried out by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

RNA polymerase I purification

(1) *Chromatin preparation.* Frozen parsley cells (2 kg) were suspended in 3 litres of buffer A and homogenized with an Ultra-Turrax apparatus (Janke and Kunkel, Staufen i.Br., West Germany). The homogenate was filtered through four layers of surgical gauze. After addition of Triton X-100 (40 ml/litre of homogenate), the filtrate was stirred, filtered and centrifuged for 30 min at 13 000 g. The resulting crude chromatin was gathered from the pellet, suspended in buffer A and centrifuged once more for 30 min at 10 000 g. The pellet was re-suspended in buffer A with an Ultra-Turrax homogenizer and centrifuged again for 30 min at 13 000 g. The resulting purified chromatin was suspended in 30 ml of buffer B, then glycerol was added to a final concentration of 50% (v/v). The chromatin could be stored at –60°C.

(2) *Batch technique.* After thawing of the chromatin, increasing amounts of (NH₄)₂SO₄ were added (final concentration 0.5 M) to the solution and stirred for 2 h. The insoluble material was removed by centrifugation at 180 000 g for 45 min in a Beckman 60 Ti rotor. The supernatant was diluted with the same volume of buffer B (without glycerol) and treated batchwise with DEAE-cellulose [10 g equilibrated with buffer B containing 0.25 M-(NH₄)₂SO₄]. The solution was stirred for 15 min, and then centrifuged for 10 min at 1800 g. The supernatant was diluted again with the same volume of buffer B and treated as a batch with phosphocellulose [to 500 ml of solution, 40 ml of the ion exchanger, equilibrated with buffer B containing 0.1 M-(NH₄)₂SO₄, was added]. The batch sediment (after centrifugation at 1800 g for 10 min) was suspended in 15 ml of buffer B containing 0.1 M-(NH₄)₂SO₄ and used for the subsequent column chromatography.

(3) *First phosphocellulose chromatography.* The suspension was loaded on a column (1.5 cm × 30 cm) of phosphocellulose equilibrated with buffer B containing 0.1 M-(NH₄)₂SO₄. The column was washed with 100 ml of the same buffer. The enzymes were eluted with 200 ml of 0.3 M-(NH₄)₂SO₄ dis-

solved in buffer B; 2.5 ml fractions were collected. The fractions were assayed for polymerase activity and protein content. The fractions of the first peak (10–17), which contained the polymerase activity, were combined and diluted with buffer B to a final concentration of 0.05 M-(NH₄)₂SO₄.

(4) *DEAE-cellulose chromatography.* The active fractions from step (3) were loaded on a column (1.5 cm × 30 cm) of DEAE-cellulose equilibrated with buffer B containing 0.05 M-(NH₄)₂SO₄. The column was washed with 100 ml of the same buffer. The enzyme was eluted with a linear gradient (100 ml) of 0.05–0.5 M-(NH₄)₂SO₄ dissolved in buffer B. The fractions (1.7 ml) containing α-amanitin-resistant RNA polymerase activity were combined (9–17) and adjusted to 0.1–0.12 M-(NH₄)₂SO₄ in buffer B.

(5) *Second phosphocellulose chromatography.* The active fractions eluted from DEAE-cellulose were layered on a column (1.5 cm × 20 cm) of phosphocellulose equilibrated with buffer B containing 0.1 M-(NH₄)₂SO₄. The column was washed with 100 ml of the same buffer. The enzyme was eluted with buffer B containing 0.3 M-(NH₄)₂SO₄; 1.7 ml fractions were collected and assayed for RNA polymerase activity. Fractions 10–16 were combined, adjusted with glycerol to a final concentration of 50% and stored at –60°C.

(6) *Sucrose-density-gradient centrifugation.* A sample (1.5 ml) of the active fractions from step (5) was layered on a linear sucrose gradient (5–20%) and centrifuged as described by Guilfoyle *et al.* (1976).

Polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide gels were prepared as described by Laemmli (1970). The slab gel included a stacking gel of 2.4% acrylamide. The running gel was a mixed one, in which the upper half contained 5% and the lower half 10% acrylamide. Purified RNA polymerase I from sucrose-gradient fractions was subjected to electrophoresis under denaturing conditions as described by Schwartz & Roeder (1974).

RNA polymerase I assay

The procedure described by Guilfoyle *et al.* (1976), with several modifications, was used. The standard assay mixture contained, unless indicated otherwise, in a final volume of 500 μl, 0.05 M-Tris/HCl (pH 8.0), 25% (v/v) glycerol, 0.001 M-MnCl₂, 0.006 M-MgCl₂, 0.02 M-(NH₄)₂SO₄, 0.02 M-dithiothreitol, heat-denatured salmon sperm DNA (100 μg/ml), 0.4 mM-ATP, -CTP and -GTP, 0.04 mM-[5'-³H]UTP (2 μCi) and 20 μl of the enzyme (9.4 μg of protein). The mixture was incubated at 29°C for 30 min and subsequently treated as described by Guilfoyle *et al.* (1976). One unit of

enzyme activity is the amount of protein that catalyses the incorporation of 1 nmol of [^3H]UMP into RNA at 29°C in 30 min.

Results

Purification procedure for RNA polymerase I

Link & Richter (1975) demonstrated that parsley cells are an ideal source for the isolation of RNA polymerase II. During their purification procedure RNA polymerase I was unstable. In the present work we were able to purify RNA polymerase I by using the methods described by Guilfoyle *et al.* (1976).

Chromatin isolated by this method contained most of the nucleolar RNA polymerase I, whereas RNA polymerase II was soluble and could be detected in the supernatant of the chromatin pellet (Lin *et al.*, 1974). The RNA polymerase I could also be solubilized by treating the chromatin with 0.5 M-(NH₄)₂SO₄. Further treatments with DEAE-cellulose and phosphocellulose in batches removed most of the proteins, and a high purification of RNA polymerase I was achieved. This could be seen when parsley chromatin-bound RNA polymerase was fractionated on a phosphocellulose column. The enzyme activity was eluted at 0.12 M-(NH₄)₂SO₄ together with the protein peak (Fig. 1a). After this chromatographic step the enzyme was purified 74-fold; the yield was 94% (Table 1). These values were calculated with reference to chromatin; with reference to the crude extract, the values would be much higher. From the subsequent DEAE-cellulose column the enzyme was eluted at (NH₄)₂SO₄ concentrations of 0.1–0.15 M (Fig. 1b). The enzyme was insensitive towards α -amanitin, even at high concentrations (100 $\mu\text{g}/\text{ml}$). This insensitivity towards the inhibitor, and the elution patterns from the phosphocellulose and DEAE-cellulose columns,

characterized the enzyme activity as RNA polymerase I. After the next purification step on a second phosphocellulose column, the enzyme was purified 93-fold, with a recovery of 42% (Fig. 1c; Table 1).

Sucrose-density-gradient ultracentrifugation of a sample of the preparation resulted in substantial purification, 134-fold. The specific activity after this step was 80 units/mg of protein, as shown in Table 1.

Polyacrylamide-gel electrophoresis

In order to determine the subunit structure of purified RNA polymerase I, denaturing polyacrylamide gels were used. The enzyme was separated on 'mixed' gels consisting of 5% (upper half) and 10% (lower half) acrylamide. Six bands have been designated as components of the enzyme, and showed mol.wts. of 185 000, 130 000, 70 000, 47 000, 37 000 and 30 000. The polypeptide of high mol.wt. (185 000) gave a very weak band, indicating an instability of this component during gel electrophoresis (results not shown).

Properties of RNA polymerase I from parsley

The kinetics of purified RNA polymerase I from parsley was linear up to 30 min (Fig. 2a). Also a linear relationship between polymerase concentration in the assay (protein content) and enzyme activity was detected. RNA polymerase I from parsley utilized denatured and undenatured salmon sperm DNA as template. There existed a slight preference for native DNA (results not shown).

With denatured salmon sperm DNA as template, the enzyme activity was slightly higher with Mn²⁺ than with Mg²⁺ at the optimum concentration of these bivalent cations (Fig. 2b). The optimum concentration for Mn²⁺ was 1 mM, and for Mg²⁺ 4–6 mM. At optimum concentrations for Mn²⁺ and Mg²⁺ with denatured DNA as template, the enzyme was more active at low salt concentration. Under

Table 1. *Purification of parsley RNA polymerase I*

The Table summarizes the purification procedure for RNA polymerase I from 2 kg of frozen parsley cells. One unit of enzyme activity is 1 nmol of [^3H]UMP incorporated into RNA in 30 min at 29°C. Specific activity is expressed as units/mg of protein. Heat-denatured (100°C) salmon sperm DNA was used as template. For standard assay conditions see the Materials and methods section.

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Recovery (%)
Chromatin	134	1900	1135	0.6	1	100
(NH ₄) ₂ SO ₄ precipitation						
DEAE-cellulose (batch)						
Phosphocellulose (batch)						
First phosphocellulose column	20	24	1060	44	74	94
DEAE-cellulose column	15	17	910	53	89	80
Second phosphocellulose column	12	8.6	480	56	93	42
Sucrose gradient (sample)				80	134	28

Fig. 1. Purification of RNA polymerase I from parsley

(a) First phosphocellulose chromatography. The column was eluted with 200 ml of 0.3 M- $(\text{NH}_4)_2\text{SO}_4$ dissolved in buffer B; the fraction size was 2.5 ml. $\bullet$, RNA polymerase activity; $\cdots$, protein content (determined by the method of Lowry *et al.* (1951); —, $(\text{NH}_4)_2\text{SO}_4$ concentration. (b) DEAE-cellulose chromatography. Active fractions from the first phosphocellulose chromatography were loaded on to the DEAE-cellulose and eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ from 0.05 to 0.5 M in buffer B. The fraction size was 1.7 ml. $\bullet$, RNA polymerase activity; $\circ$, activity in the presence of α -amanitin (100 $\mu\text{g}/\text{ml}$); —, $(\text{NH}_4)_2\text{SO}_4$ concentration. (c) Second phosphocellulose chromatography. Active fractions from DEAE-cellulose chromatography were layered on phosphocellulose and eluted with 0.3 M- $(\text{NH}_4)_2\text{SO}_4$ in buffer B. The fraction size was 1.7 ml. $\bullet$, RNA polymerase I activity; $\cdots$, transmission at 280 nm (protein); —, $(\text{NH}_4)_2\text{SO}_4$ concentration.

these conditions the optimum was 10–20 mM- $(\text{NH}_4)_2\text{SO}_4$ (Fig. 2d). Pyrophosphate was a very potent inhibitor of RNA polymerase I from parsley; 50% inhibition was reached at 1 mM-pyrophosphate, whereas sodium orthophosphate was only slightly inhibitory at concentrations exceeding 8 mM

(Fig. 2c) and showed even a weak stimulation of polymerase activity at 2 mM.

Influence of nucleoside triphosphates and bivalent cations on RNA polymerase I activity

In a series of experiments, the influence of

Fig. 2. Characterization of RNA polymerase I from parsley

Purified RNA polymerase I after the second phosphocellulose chromatography was used. (a) Kinetics of $[^3\text{H}]\text{UTP}$ incorporation into trichloroacetic acid-insoluble material. (b) Effect of bivalent cations: $\bullet$, MnCl_2 ; $\circ$, MgCl_2 . (c) Effect of various phosphates: $\bullet$, sodium orthophosphate; $\circ$, sodium pyrophosphate. (d) Effect of $(\text{NH}_4)_2\text{SO}_4$. Optimum bivalent-cation concentrations were applied (1 mM- MnCl_2 and 6 mM- MgCl_2).

Fig. 3. Influence of nucleoside triphosphates and bivalent cations on purified RNA polymerase I activity from parsley

In these experiments, 0.4 mM-GTP, 0.4 mM-CTP, 0.04 mM-UTP and 1 mM- MnCl_2 were kept constant, whereas ATP and MgCl_2 were varied. (a) and (b) Increasing nucleoside triphosphate concentrations at different bivalent-cation concentrations in the ranges (a) 4-6 mM [$\bullet$, 4 mM-bivalent cations (3 mM- MgCl_2); Δ , 5 mM-bivalent cations (4 mM- MgCl_2); $\blacksquare$, 6 mM-bivalent cations (5 mM- MgCl_2)] and (b) 7-10 mM [$\blacktriangle$, 7 mM-bivalent cations (6 mM- MgCl_2); $\bullet$, 9 mM-bivalent cations (8 mM- MgCl_2); $\circ$, 10 mM-bivalent cations (9 mM- MgCl_2)]. (c)-(e), Increasing bivalent-cation concentrations at different nucleoside triphosphate concentrations in the ranges (c) 2-3 mM [$\circ$, 2 mM-nucleoside triphosphates (1.16 mM-ATP); $\bullet$, 3 mM-nucleoside triphosphates (2.16 mM-ATP)], (d) 4-9 mM [$\square$, 4 mM-nucleoside triphosphates (3.16 mM-ATP); $\bullet$, 5 mM-nucleoside triphosphates (4.16 mM-ATP); $\circ$, 7 mM-nucleoside triphosphates (6.16 mM-ATP); $\blacktriangle$, 9 mM-nucleoside triphosphates (8.16 mM-ATP)] and (e) 11-15 mM [$\bullet$, 11 mM-nucleoside triphosphates (10.16 mM-ATP); $\circ$, 13 mM-nucleoside triphosphates (12.16 mM-ATP); $\blacktriangle$, 15 mM-nucleoside triphosphates (14.16 mM-ATP)].

nucleoside triphosphates and bivalent cations on RNA synthesis *in vitro* by RNA polymerase I from parsley was investigated. The ATP concentration was varied in the presence of 0.84 mM other

Fig. 4. Influence of nucleoside triphosphates and bivalent cations maintaining a 1:1 ratio on the activity of RNA polymerase I from parsley

In these experiments 0.4 mM-CTP, 0.4 mM-GTP, 0.04 mM-UTP and 1 mM-MnCl₂ were kept constant, whereas ATP and MgCl₂ were varied. Each of the two groups of substances (nucleoside triphosphates and cations respectively) were present at the concentrations indicated in the abscissa. ●, Nucleoside triphosphates (NTP) and bivalent cations in a 1:1 ratio; □, 1 mM-bivalent cations in excess of this ratio (cation_{exc.}); ■, 3 mM-cation_{exc.}; □, 5 mM-cation_{exc.}; △, 2 mM-nucleoside triphosphates in excess of the 1:1 ratio (NTP_{exc.}); ▲, 5 mM-NTP_{exc.}.

nucleoside triphosphates (0.4 mM-GTP, 0.4 mM-CTP and 0.04 mM-UTP) at different bivalent-cation concentrations (1 mM-MnCl₂ and different MgCl₂ concentrations). As shown in Figs. 3(a) and 3(b), the activity of RNA polymerase I decreased if the nucleoside triphosphate concentration exceeded the 1:1 molar ratio of cations to triphosphates. Maximum activity was obtained at 4–9 mM-nucleoside triphosphates if bivalent-cation concentration was 10 mM. In a second experiment the bivalent-cation concentration was varied (MnCl₂ was kept constant at 1 mM, MgCl₂ was varied in the range 4–11 mM) at different nucleoside triphosphate concentrations (0.4 mM-GTP, 0.4 mM-CTP and 0.04 mM-UTP, whereas ATP was varied). The results are shown in Figs. 3(c) 3(d) and 3(e). Optimum RNA polymerase activity was reached again at 10 mM-bivalent cations and 4 mM-nucleoside triphosphates. At lower nucleoside triphosphate concentrations the dependence of the enzyme activity on bivalent cations was not so prominent (Fig. 3c). At higher nucleoside triphosphate concentrations, exceeding 10 mM (11, 13 and 15 mM), the influence of bivalent cations was weak (Fig. 3e). It should be emphasized that under these circumstances the total enzyme activity was inhibited; the values measured under such conditions were

only half those determined at lower nucleoside triphosphate concentrations. On the other hand, a strong dependence of RNA polymerase I activity on bivalent cations was observed if nucleoside triphosphates were present in the range 4–9 mM (Fig. 3d).

In a further experiment the nucleoside triphosphates (0.4 mM-GTP, 0.4 mM-CTP and 0.04 mM-UTP were constant, ATP was varied) and bivalent cations (1 mM-MnCl₂ was constant, MgCl₂ was varied) were increased simultaneously, maintaining a cation: triphosphate ratio of 1:1. In some tests the nucleoside triphosphates or bivalent cations were present in excess of this ratio. Fig. 4 demonstrates that RNA polymerase activity was not affected by increasing concentrations of both components present in a 1:1 ratio in the range 4–11 mM; increasing nucleoside triphosphate concentrations present in excess inhibited the RNA polymerase, whereas increasing amounts of bivalent cations applied in excess of this 1:1 ratio had a stimulatory effect on the enzyme reaction.

Discussion

In contrast with the methods applied by Link & Richter (1975) for the isolation of RNA polymerases, during our purification procedure RNA polymerase I was stable and could be stored over several months without loss of activity. A first very effective purification was achieved by two treatments with DEAE-cellulose and phosphocellulose applied in batches. These procedures were followed by ion-exchange chromatography on phosphocellulose, DEAE-cellulose and a second phosphocellulose column.

The purified RNA polymerase I from parsley had a putative subunit structure similar to that of the enzymes isolated from soya bean (Lin *et al.*, 1974) and cauliflower (Guilfoyle, 1976), and also showed similar characteristics regarding metal ions, ionic strength and template preference.

As previously described, isolated nuclei from parsley contained a very high pyrophosphatase activity (Grossmann *et al.*, 1979) which is able to remove very rapidly pyrophosphate originating from nucleoside triphosphates during RNA synthesis; the resulting orthophosphate should not inhibit RNA polymerase activity. The inhibitory effect of sodium pyrophosphate on RNA synthesis *in vitro* is in agreement with the assumption made by Peller (1975). Orthophosphate had only slight effects on the activity of RNA polymerase from parsley. AMP, one of the compounds produced during RNA synthesis, also had no effect on the RNA polymerase I from our cells; ADP was slightly inhibitory at concentrations above 2 mM.

In a series of experiments, the influence of

nucleoside triphosphates and bivalent cations on RNA synthesis *in vitro* was tested. RNA polymerase I from parsley was not affected in the range 4–11 mM if nucleoside triphosphates and bivalent cations were present in a 1:1 ratio. Nucleotides added in excess of this inhibited the enzyme activity, whereas bivalent cations in excess had a stimulatory effect on the RNA polymerase reaction.

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