RNA-DEPENDENT RNA POLYMERASE FROM HEALTHY TOMATO LEAF TISSUE

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1. Introduction

In searching for the replicase of the genome of RNA plant viruses, an RNA-dependent RNA polymerase activity was found to be present at low levels in uninfected, healthy plants [1–4]. This enzyme has been partially purified and characterized from healthy tobacco leaves [5–8] but its physiological role is still unclear.

Since we are interested in the replication of viroids, the smallest agents of disease presently known, we attempted to purify an RNA-dependent RNA polymerase from healthy tomato plants which are experimental hosts for several viroids. Because viroids do not contain enough genetic information to code for their own replicase [9] viroid replication must proceed via the RNA synthesizing machinery pre-existing in the host cell. Therefore the RNA-dependent RNA polymerase of healthy tomato plants is one of the enzymes possibly involved in viroid replication in this host.

Partial purification of the tomato RNA-dependent RNA polymerase was achieved by a modification of the procedure originally developed in [8] for the corresponding tobacco enzyme. Our modification consists mainly of the addition of Mg^{2+} before the DEAE-chromatography step. This improves the solubility of the tomato enzyme markedly and changes its chromatographic behaviour in such a way that it now passes through the DEAE-column and can be concentrated to high yields and purity by subsequent heparin–Sepharose chromatography. Moreover a contaminating uridilyl transferase activity and the bulk of the protein are retained on DEAE–Sephadex and thus eliminated from the RNA-dependent RNA polymerase preparation.

2. Materials and methods

Dextran T500 and DEAE–Sephadex-A25 were from Pharmacia, polyethylene glycol-6000 (PEG) from Serva and phosphocellulose p11 from Whatman. Tobacco mosaic virus (TMV)-RNA was prepared by phenol extraction from TMV propagated in *Nicotiana tabacum*. Ribulose 1,5 bisphosphate carboxylase was isolated as in [10]. The templates used were purchased from Boehringer, P-L Biochemicals and Collaborative Research. Heparin– Sepharose was prepared according to [11]. ^{3}H-Labeled ribonucleoside 5′-triphosphates were purchased from the Radiochemical Center, Amersham. Templates were oxidised with sodium metaperiodate according to [12].

2.1. *Purification of the RNA-dependent RNA polymerase*

For the purification of the RNA-dependent RNA polymerase the procedure in [8] was adopted with the following modifications: Batches of 100 g healthy tomato leaves (cultivar 'Rentita' grown for ~6 weeks in a greenhouse) were not ground in buffer but frozen in liquid nitrogen and homogenized in the frozen state in a 1 l Waring blendor. One volume of extraction buffer (50 mM Tris/HCl (pH 8.1), 100 mM NH_4Cl, 90 mM mercaptoethanol, 2 mM EDTA, 5% glycerol) was then stirred into the frozen leaf powder, which was allowed to warm up to 0°C and squeezed through linen. After the PEG–dextran phase partitioning the PEG high salt supernatant (470 ml) was brought to 20 mM $MgCl_2$ and dialyzed against two changes of 5 l Mg-buffer (20 mM Tris/HCl (pH 7.5), 20 mM mercaptoethanol, 10% glycerol, 20 mM $MgCl_2$). The resultant enzyme solution (800 ml) was applied to a DEAE–Sephadex-A25 column (5 cm × 7.5 cm) equilibrated with Mg-buffer. The eluate from the DEAE-column which contains the RNA-dependent RNA

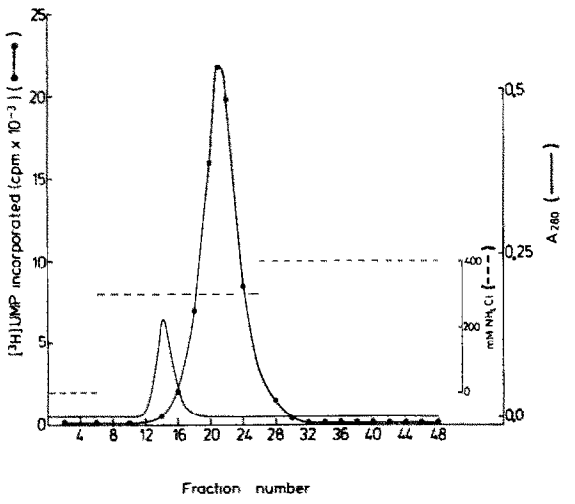

Fig.1. Heparin–Sepharose chromatography of RNA-dependent RNA polymerase from healthy tomato leaf tissue. The enzyme was bound to and eluted from the column as in section 2. (——) Absorbance at 280 nm; (- - -) NH_4Cl concentration; (●——●) [³H]UMP incorporation. In the polymerase assay the unlabeled UPT was omitted.

polymerase was allowed to directly enter and bind to a heparin–Sepharose column (2.5 cm X 2.5 cm) which had been coupled to the DEAE-column outlet. The heparin–Sepharose column was washed with 200 ml Mg-buffer and the enzyme was then eluted with the same buffer containing 300 mM NH_4Cl. The main peak fractions (fractions 20–22 of fig.1 = pool 1) and the adjacent fractions (16–19 and 23–28 = pool 2) were isolated separately and stored frozen in liquid nitrogen until use.

2.2. *Enzyme assay*

Reaction mixture of 50 μl contained: 50 mM Tris/HCl (pH 8) at 22°C, 0.3 mM ATP, CTP, GTP, 0.15 mM UTP and 5 μCi [³H]UTP (40 mCi/μmol), 0.14 mg/ml actinomycin D, 0.16 mg/ml TMV-RNA, 5 mM DTT, 10 mM $MgCl_2$ and 10 μl enzyme solution. After 30 min incubation at 37°C samples of 45 μl were withdrawn and transferred onto GF/A filters on dry ice. Then the filters were soaked for 5 min in 10% trichloroacetic acid and afterwards washed by two changes in 5% trichloroacetic acid and acetone, followed by a final wash in acetone and dried under a heat lamp. Alterations of the composition of the reaction mixture are marked in the legends to the figures.

2.3. *Other methods*

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 7.5% gel as in [13]. Prior to electrophoresis the samples of pooled fractions were concentrated 100-fold. Protein was estimated according to [14].

3. Results and discussion

3.1. *Purification of the tomato RNA-dependent RNA polymerase*

When the purification procedure for the isolation of the RNA-dependent RNA polymerase from healthy tobacco leaves in [8] was applied as closely as possible to leaf tissue from healthy tomato we obtained very low amounts of enzyme. When the DEAE–Sephadex and phosphocellulose-concentrated enzyme was dialyzed the preparations became turbid and upon centrifugation for 5 min at 10 000 X *g* this low enzyme activity was found in the pellet. The main protein component present in the pellet, however, is the enzyme ribulose 1,5-bisphosphate carboxylase which follows from SDS–PAGE as shown in fig.2 (C,D) and also from immunological assay. This enzyme is known to constitute up to 50% of the protein extractable from green plant tissue. Most probably the RNA-dependent RNA polymerase is co-precipitating with the carboxylase during purification in the absence of Mg^{2+}. It is possible that the reported existence of a 'bound' or 'particulate' form of the polymerase is actually a problem of the reduced solubility of the enzyme rather than its postulated association to membranes which is still a matter of controversy [4].

In a systematic search for the reasons of the loss of polymerase activity we found that it can be simply avoided by the presence of 20 mM $MgCl_2$ in the corresponding solutions. Mg^{2+} obviously solubilizes the RNA-dependent RNA polymerase and thus prevent its precipitation. In addition a change in the chromatographic behaviour of the enzyme during DEAE–Sephadex chromatography was observed. In the presence of 20 mM $MgCl_2$ the RNA-dependent RNA polymerase does not bind any longer to DEAE–Sephadex, whereas the bulk of the protein in the solution is retained on the DEAE-column.

Under these conditions a contaminating uridilyl transferase activity could be eliminated from the RNA-dependent RNA polymerase preparation. This poly(U– polymerase is bound to DEAE–Sephadex

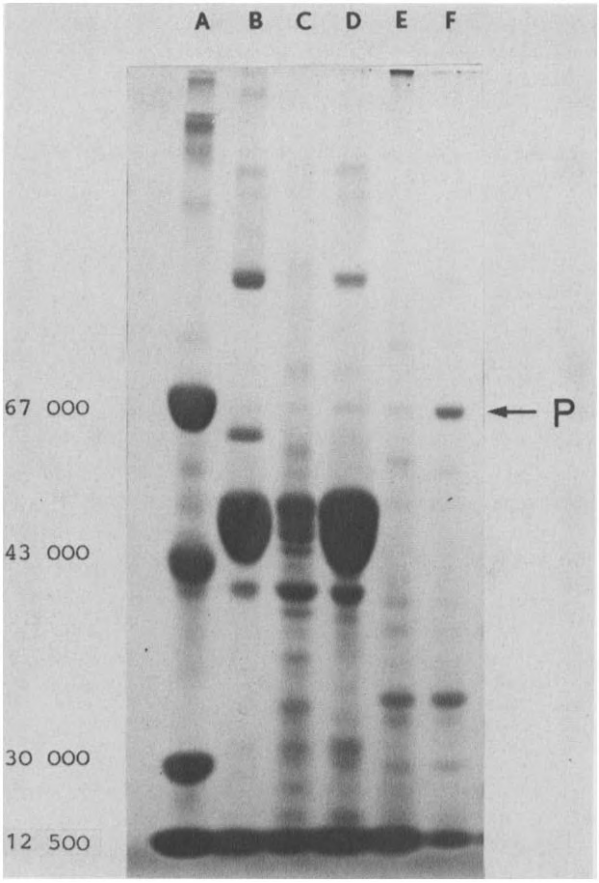

Fig.2. SDS–PAGE of RNA-dependent RNA polymerase from tomato leaf tissue on a 7.5% gel at various stages of purification. (A) Marker proteins: 12 500, cytochrome *c*; 30 000, carbonic anhydrase; 43 000, ovalbumin; 67 000, bovine serum albumin. (B) Large subunit of ribulose bisphosphate carboxylase from tobacco leaves (55 000). (C,D) Preparations obtained after polymerase precipitation as a consequence of dialysis against Mg^{2+}-deficient buffer, as described in the text, and subsequent centrifugation for 5 min at 10 000 × *g*. (C) Supernatant. This solution, which shows no polymerase activity, was concentrated 100-fold before SDS–PAGE. (D) Polymerase containing pellet after resolubilization with Mg^{2+} buffer. (E,F): Heparin–Sepharose purified polymerase after 100-fold concentration. (F) Main peak fractions of polymerase activity corresponding to fractions 20–22 of fig.1. (E) Adjacent fractions 16–19 and 23–28 of fig.1. (P) Presumptive subunit of polymerase.

and can be eluted from the column by one step of 200 mM NH_4Cl in Mg-buffer. Its characterization is under way [15].

Since the RNA-dependent RNA polymerase appears in the flow through it was convenient to couple the DEAE-column directly to the heparin–Sepharose column from which the enzyme can be eluted after the bulk of the protein by one step of 300 mM NH_4Cl (fig.1). The main peak fractions 20–22 (pool 1) contained the RNA-dependent RNA polymerase of highest activity (table 1) and purity as shown in the SDS–PAGE analysis (fig.2(F)). The enzyme in these fractions is purified ~120-fold and its specific activity is 5.9 nmol . 30 min^{-1} . mg^{-1} with TMV-RNA as template. Thus it is ~10-times higher in specific activity than the enzyme from healthy tobacco. Furthermore, the overall yield of the partially purified tomato enzyme appears to be ~12-times higher than the yield of the tobacco enzyme which is evidently due to the improved solubility in the presence of $MgCl_2$. On the other hand, if compared to RNA-dependent RNA polymerases from other sources such as the Qβ replicase for example, the specific activity of the RNA-dependent RNA polymerase from healthy tomato is rather low. This could either be based on a lower turnover number of the enzyme or/and the presence of residual non-enzyme protein(s) in the preparation.

From the ~64 000 M_r reported for a subunit from the RNA-dependent RNA polymerase from tobacco [8] and from the protein pattern in fig.3(F), one must assume that the upper protein band in the region of the bovine serum albumin marker (67 000) is the presumptive subunit of the corresponding enzyme from healthy tomato. The nature of the additional proteins with lower M_r remains to be elucidated.

3.2. *Catalytic properties of the RNA-dependent RNA polymerase from tomato*

From the results given in table 2 it is evident that the heparin–Sepharose purified enzyme is dependent on RNA templates and gives the highest incorporation of [^{3}H]UMP into trichloroacetic acid-precipitable material with all four ribonucleoside triphosphates (RNTP). The residual incorporation of ~20% [^{3}H]-UMP in the absence of one of the four RNTPs can be explained by the premature termination of transcription of the TMV-RNA template. Low levels of incorporation of radioactivity are also observed if [^{3}H]-GTP or [^{3}H]ATP are present in the assay while the other RNTPs are omitted. This incorporation (25–36%) is due to terminal addition of GMP or AMP by corresponding transferases, respectively, because it needs the presence of an RNA with an intact 3′-OH terminus. If the 3′-OH terminus is destroyed by treatment with sodium metaperiodate which results in a

Table 1
Activity of the RNA-dependent RNA polymerase from healthy tomato at different purification steps

Step	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg protein)	Purification[b]	Yield (%)
Ammoniumsulfate precipitate 0–50%	600	28.6	0.048	1	100
PEG high salt supernatant	–[c]	34.9	–	–	122
DEAE–Sephadex	–[c]	34.7	–	–	121
Heparin–Sepharose					
pool 1 + 2	3.8	14.6	3.8	80	51
pool 2	0.43	2.8	5.9	123	

[a] Specific activity units are expressed as nmol UMP incorporated in 30 min at 37°C with TMV-RNA as template

[b] Since no reliable data on the replicase activity of the crude tissue extract could be obtained all activities are compared to the 50% $(NH_4)_2SO_4$ precipitate

[c] The protein estimation of this step is impaired by the presence of PEG [17,18]

Table 2
Requirements of purified RNA-dependent RNA polymerase from healthy tomato leaf tissue

Ribonucleoside triphosphates				Templates	Heparin–Sepharose purified polymerase	
ATP	CTP	GTP	UTP		(cpm)	(cpm %)
+	+	+	^{3}H	TMV-RNA	2030	100
–	–	–	^{3}H	TMV-RNA	61	3
–	^{3}H	–	–	TMV-RNA	0	0
–	–	^{3}H	–	TMV-RNA	730	36
^{3}H	–	–	–	TMV-RNA	507	25
–	+	+	^{3}H	TMV-RNA	467	23
+	–	+	^{3}H	TMV-RNA	426	21
+	+	–	^{3}H	TMV-RNA	447	22
+	+	+	^{3}H	–	0	0
+	+	+	^{3}H	TMV-RNA oxidised	2400	100
–	–	^{3}H	–	TMV-RNA oxidised	295	12
^{3}H	–	–	–	TMV-RNA oxidised	49	2
^{3}H	+	+	+	poly(rU)	7064	294
^{3}H	+	+	+	poly(rU) oxidised	2362	96

Heparin–Sepharose purified polymerase was used in the assay, which was carried out as in section 2, except that 20 μCi respective radioactive precursor was added. Each test was started by the addition of 10 μg of the corresponding template. –, RNTP omitted; +, unlabeled RNTP present at 0.15 mM; ^{3}H, tritium-labeled RNTP present at 0.15 mM final conc.

Table 3
Relative template activity of ribo- and desoxyribopolynucleotides with tomato RNA-dependent RNA polymerase

Ribonucleoside triphosphates				Templates	Heparin–Sepharose purified polymerase	
ATP	CTP	GTP	UTP		(cpm)	(cpm %)
+	+	+	^{3}H	TMV-RNA	2030	100
+	+	+	^{3}H	poly(rA)	284	14
+	^{3}H	+	+	poly(rG)	183	9
+	+	^{3}H	+	poly(rC)	264	13
+	^{3}H	+	+	oligo(rC) · poly(rG)	61	3
+	+	^{3}H	+	oligo(rI) · poly(rC)	20	1
+	+	+	^{3}H	oligo(rU) · poly(rA)	61	3
+	+	+	^{3}H	poly(rA) · poly(rU)	0	0
+	^{3}H	+	+	poly r(U,G) 10:1	832	41
+	^{3}H	+	+	poly r(U,G) 1:1	1360	67
+	+	+	^{3}H	poly r(A,U,G)	3573	176
+	+	+	^{3}H	poly r(A-U)	832	41
+	+	+	^{3}H	poly r(A-C)	1258	62
+	+	+	^{3}H	calf thymus-DNA denat.	41	2
+	^{3}H	+	+	poly(dG) · poly(dC)	0	0
+	+	+	^{3}H	poly(dA) · poly(dT)	0	0
+	+	+	^{3}H	poly(dA)	0	0
+	^{3}H	+	+	oligo(rC) · poly(dG)	122	6
+	+	+	^{3}H	oligo(rU) · poly(dA)	20	1

For assay conditions see legend to table 2

terminal dialdehyde, the incorporation of [^{3}H]GMP or [^{3}H]AMP is inhibited. Interestingly the terminal addition of AMP becomes more prominent if the TMV-RNA is replaced by poly(rU). This can be explained by the recent finding that poly(A) polymerases can catalyze the synthesis as well as the hydrolysis of poly(rA) but not the hydrolysis of poly(rA) · poly(rU) [16].

From the comparison of the [^{3}H]UMP incorporation in the presence of all four RNTPs with untreated and sodium metaperiodate treated TMV-RNA as template it follows, on the other hand, that no terminal addition of UMP takes place. This means that there is no poly(U) polymerase activity present so that the [^{3}H]UMP incorporation is only based on the action of the RNA-dependent RNA polymerase.

In addition of TMV-RNA a series of synthetic ribo- and desoxyribopolynucleotides were tested for their suitability as templates with the purified RNA-dependent RNA polymerase. Table 3 shows that from the RNA templates the homopolynucleotides and their complexes with the corresponding complementary oligonucleotides proved to be rather poor templates. The double-stranded complex poly(rA) · poly(rU) was not accepted at all. It is evident that the random and alternating copolymers serve as more efficient templates than the homopolymers. Interestingly, the poly[r(A,U,G)] is the most efficient template for the tomato RNA-dependent RNA polymerase. As expected no DNA templates are transcribed by the tomato enzyme. Inhibition studies with TMV-RNA as template finally showed, that the tomato polymerase is almost completely inhibited in the presence of 8 mM pyrophosphate. Actinomycin D (140 μg/ml) reduced the [^{3}H]UMP incorporation to ~70% and rifampicin (100 μg/ml) and α-amanitine (200 μg/ml) showed no inhibitory effect.

Our data show that the RNA-dependent RNA polymerase from healthy tomato leaf tissue exhibits characteristics which are very similar to those of the corresponding enzyme from healthy tobacco leaves [8]. Moreover they demonstrate that rigorous tests for contaminating nucleotidyl transferase activities are necessary before reliable conclusions about the RNA-dependent RNA polymerase activity can be drawn.

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