Comparative Studies on Ribonucleic Acid Dependent RNA Polymerases in Cucumber Mosaic Virus Infected Cucumber and Tobacco and Uninfected Tobacco Plants†

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ABSTRACT: RNA-dependent RNA polymerases have been isolated in almost pure form from cucumber mosaic virus (CMV) infected cucumber cotyledons and from tobacco leaves and were compared with the less pure enzyme from uninfected tobacco. The purified polymerase from cucumber shows on sodium dodecyl sulfate gels two peptide chains of about 100 and 112 kdaltons. The enzyme from tobacco shows a close doublet of about 125 kdaltons, which is also present in the less pure preparation from healthy tobacco. While both the cucumber and tobacco enzymes can use many polynucleotides and RNAs as templates, considerable quantitative differences exist, poly(C) being by far the most effective template for the cucumber enzyme but of low activity with the tobacco enzyme and poly(UG) being highly active with the latter but not the former. Poly(A) and poly(G) are inactive. Different viral RNAs, including CMV RNA, show smaller differences. The sedimentation rates of the enzyme from both sources are the same as that of γ-globulin. A uridine 5′-triphosphate (UTP) terminal transferase also present in both plants sediments much more slowly and can be completely removed from the RNA polymerases. However, slight nucleolytic activity remains associated with the purified polymerases and appears to be proportional to the polymerase activity. The conclusion derived from these data is that the RNA-dependent RNA polymerases of different plants differ and are not detectably affected by virus infection in qualitative terms while being produced in greatly increased amounts upon some virus infections. Similar conclusions were previously reached with less purified enzyme preparations from tobacco as compared to cowpea, infected with different viruses, if any.

RNA-dependent RNA polymerase activity has been found in Chinese cabbage, tobacco, cowpea, cauliflower, cabbage, tomato, barley, and possibly at a very low level in cucumber. Infection by various plant RNA viruses, e.g., turnip yellow mosaic (TYMV), tobacco mosaic (TMV), tobacco necrosis (TNV), alfalfa mosaic (AMV), cowpea mosaic (CpMV), and bromegrass mosaic (BMV), has been found to increase the amounts of these enzymes in their respective hosts by 2–6-fold. Cucumber mosaic virus (CMV) has been reported to elicit particularly high enzyme activity in cucumber cotyledons (Gilliland & Symons, 1968; May et al., 1969, 1970). Since we and others [reviewed in Fraenkel-Conrat (1979)] have produced much support for the hypothesis that these enzymes are completely host specific rather than virus coded, it appeared important to compare the CMV-stimulated cucumber enzyme with the enzyme produced in tobacco upon CMV infection. CMV was found to greatly increase the RNA-dependent RNA polymerase activity not only in cucumber but also in tobacco, and it was possible, by a modified purification procedure, to obtain near-pure preparations of both CMV-stimulated cucumber and tobacco enzyme. When these were compared in regard to molecular weights on denaturing gels and other properties, they were clearly different, while the CMV-stimulated tobacco enzyme was clearly similar to the healthy tobacco enzyme. We thus have further evidence for the concept that plants produce RNA-dependent RNA polymerases, the amounts, but not the nature, of which are increased to a very variable extent by virus infection.

Materials and Methods

Materials. [8-^{3}H]ATP,[1] [8-^{3}H]GTP, [5-^{3}H]CTP, and [5-^{3}H]UTP were purchased from Schwarz BioResearch; unlabeled nucleoside triphosphates and actinomycin D were obtained from Sigma Chemical Co. Poly(A), poly(C), and poly(CU) (1:1) were obtained from Miles Laboratories, and poly(G), poly(UG) (1:1), and poly(UG) (5:1) were purchased from P-L Biochemicals. Phosphocellulose P-11 was purchased from Whatman Biochemicals; Blue-Sepharose CL-6B was from Pharmacia Fine Chemicals. Tobacco mosaic virus (TMV) RNA and turnip yellow mosaic virus (TYMV) RNA were prepared by standard methods from the viruses isolated from their host plants.

Plant and Virus Strain. Cucumber (*Cucumis sativas* L.) and tobacco (*Nicotiana tabacum* L. cv. Samsum) plants were grown in a greenhouse. The yellow strain of CMV (CMV-Y) (Tomaru & Hidaka, 1960) was inoculated onto cucumber cotyledons and onto tobacco plants, which were 10–15 cm high, by rubbing with the aid of Carborundum (600 mesh), and the plants were kept in the greenhouse (20–30 °C). The inoculated leaves were harvested 7 days after inoculation and stored frozen at −70 °C before use. CMV was purified according to the method of Takanami (1981), and the RNA was prepared by the $NaDodSO_4$–phenol method. The RNA was dissolved in distilled water and stored frozen at −70 °C.

Assay of RNA Polymerase Activity. Polymerase assays were done essentially according to the method of Ikegami & Fraenkel-Conrat (1979a), except that the specific activity of the ^{3}H-labeled nucleoside triphosphates in the reaction mixture (0.1 mL) was reduced by adding 12 nmol of the corresponding unlabeled triphosphates instead of 0.4 nmol, 70 nmol of the other triphosphates being used. A unit of RNA polymerase is defined as that amount which incorporated 1 nmol of UMP/h at 30 °C into high molecular weight RNA adsorbed onto a DEAE-cellulose paper disk (Whatman DE-81) when

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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using CMV RNA as template at a concentration of 100 μg/mL. Specific activity is defined as units of enzyme activity per milligram of protein. Protein concentration was estimated by the method of Bradford (1976) with bovine serum albumin as the standard.

Results

Purification of RNA-Dependent RNA Polymerase from Cucumber and Tobacco. Initial Extract. All preparations were performed at 0–4 °C. Healthy or CMV-infected tobacco leaves or cucumber cotyledons (100–200 g) were homogenized in a Waring blender for 2 min after adding per gram of plant material 1 mL of extraction buffer (100 mM Tris-HCl, pH 8.0, 200 mM NH_4Cl, 180 mM 2-mercaptoethanol, 4 mM EDTA, and 10% glycerol) mixed with an equal volume of saturated ammonium sulfate. This primary extraction of the tissue with about one-third saturated ammonium sulfate instead of half-saturated ammonium sulfate (Ikegami & Fraenkel-Conrat, 1979a) seems advantageous in removing uridyl terminal transferase-like activity and a large amount of other contaminating proteins.

The homogenate was squeezed through several layers of cheesecloth and centrifuged at 10000*g* for 10 min. The pellet was resuspended in the original volume of extraction buffer 50% saturated with ammonium sulfate and again centrifuged. The solids were then suspended in 0.15 mL (per gram of plant material) of 2-fold-diluted extraction buffer, the suspension was centrifuged at 10000*g* for 10 min, and this low-salt extraction was repeated with 0.1 mL. The two extracts were combined and used for the following steps.

Step 1: *Poly(ethylene glycol) Precipitation.* Low-salt poly(ethylene glycol) (PEG) precipitation and high-salt solubilization of RNA polymerase was originally proposed for the purification of *Escherichia coli* RNA polymerase (Gross et al., 1976) and adopted for CMV RNA replicase isolation by Kumarasamy & Symons (1979). In these procedures a final concentration of 11% PEG is used. However, we found that the higher concentration of PEG (22%) was necessary for complete precipitation of the enzyme from both tobacco and cucumber plants, which was also used by Gill et al. (1981) for the solubilized particulate CMV replicase. Thus an equal volume of 44% (w/w) PEG 6000 in 30 mM 2-mercaptoethanol–1 M NH_4Cl was added to the initial extract and stirred for 30 min at 0 °C. The precipitate was collected by centrifugation at 12000*g* for 15 min and extracted with about 0.1 mL (per gram of leaf material) of 20 mM Tris-HCl, pH 8.0, 5% PEG 6000, 2 M NH_4Cl, 1 mM EDTA, and 30 mM 2-mercaptoethanol. A clear supernatant was obtained after centrifugation at 10000*g* for 10 min and stored frozen at −70 °C. Its enzymatic activity was quite stable under these conditions. It was completely dependent on added RNA template.

Step 2: *Blue-Sepharose CL-6B Column Chromatography.* Frozen step 1 enzyme was thawed and any precipitate (which was found not to contain RNA polymerase activity) was removed by centrifugation at 10000*g* for 10 min. The supernatant was dialyzed against two changes of 1 L of 20 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 10% glycerol, 25 mM NH_4Cl, 1 mM EDTA, and 0.1 mM dithiothreitol (DTT) at 0 °C for 4 h. The dialyzate was applied onto a 5-mL column of Blue-Sepharose previously equilibrated with the same buffer. After the column was washed with 12 mL of the equilibrating buffer, the enzyme was eluted with a linear 0.025–1 M NH_4Cl gradient in the same buffer (total 70 mL) containing 30% glycerol but lacking the $MgCl_2$; 2.5-mL fractions were collected at a flow rate of about 9 mL/h. Active fractions were pooled to give the step 2 enzyme.

Blue-Sepharose has affinity for a wide variety of proteins. Thompson et al. (1975) reported that it was able to bind *E. coli* DNA polymerase. Kumarasamy & Symons (1979) successfully used it for purification of CMV RNA replicase. Our attempt to purify RNA polymerase using a linear-gradient elution with NH_4Cl on Blue-Sepharose resulted in considerable purification by removing many other proteins and gave at least a 10-fold purification and up to 80% recovery of the enzyme activity. Under our experimental conditions, the activity of the enzymes from the three different sources was bound completely to the column and upon gradient elution with NH_4Cl eluted at 0.37 M salt concentration.

Step 3: *Stepwise Chromatography on Phosphocellulose Column.* Step 2 enzyme was dialyzed against 1 L of 20 mM Tris-HCl, pH 8.0, 30% glycerol, 0.1 M NH_4Cl, 1 mM EDTA, and 0.1 mM DTT at 0 °C overnight. The dialyzate was applied onto a small column of phosphocellulose (0.8 × 5.5 cm, Whatman P-11) previously equilibrated with the dialyzing buffer. The column was then washed successively with 3 mL of buffer, 3 mL of buffer containing 0.2 M NH_4Cl, 3 mL of buffer containing 0.4 M NH_4Cl, and 6 mL of buffer containing 0.6 M NH_4Cl. RNA polymerase from cucumber plants bound completely to the column and eluted at 0.4 M salt concentration. The enzymes from healthy and CMV-infected tobacco plants, however, failed to bind to the phosphocellulose column. Therefore, in case of the tobacco enzymes, the RNA polymerase in the first fraction after this chromatography was concentrated by using a small column (0.6 × 5 cm) of Blue-Sepharose with elution by 0.6 M NH_4Cl.

Step 4: *Glycerol-Gradient Centrifugation.* An equal volume of saturated ammonium sulfate was added to the step 3 enzyme and the mixture held overnight at 0 °C. The resultant precipitate was collected by centrifugation at 10000*g* for 10 min and dissolved in a small amount of 20 mM Tris-HCl, pH 8.0, 250 mM KCl, 25 mM NH_4Cl, 1 mM EDTA, 0.1 mM DTT, and 5% glycerol. The concentrated enzyme was subjected to glycerol-gradient centrifugation using 7.5–20% glycerol in the same buffer at 36 000 rpm (SW 41) for 24 h at 4 °C. Twenty to twenty-two fractions of 0.55 mL were collected from the bottom of the gradient and assayed for RNA polymerase. A small aliquot of each fraction was set aside for protein analyses by $NaDodSO_4$–polyacrylamide gel electrophoresis. Glycerol was added to the combined peak fraction to give a final concentration of about 30%.

Upon glycerol-gradient centrifugation, all of the three enzymes behaved similarly, their activity appearing in a peak very near to the position where γ-globulin sedimented. The enzyme in healthy and CMV-infected tobacco plants always showed exactly the same sedimentation rate. Up to 10-fold purification was achieved at this step, but recovery of the activity was usually only about 50%, probably due to the low concentration of protein at this stage. This step is especially useful for removing contaminating leaf nucleases and uridyl terminal transferase if they are present.

Table I shows a summary of the results for purification of RNA-dependent RNA polymerase by this procedure from three different sources, i.e., CMV-infected cucumber and CMV-infected and healthy tobacco plants. Upon infection with CMV, the RNA-dependent RNA polymerase activity in extracts from tobacco plants was increased by more than 20-fold. This enhancement was considerably higher than that found with tobacco plants infected with tobacco necrosis (Fraenkel-Conrat, 1976), tobacco mosaic (Romaine & Zaitlin, 1978), or alfalfa mosaic virus (LeRoy et al., 1977). We also obtained very high enzyme activity in extracts from cucumber

Table I: Summary of Purification of RNA-Dependent RNA Polymerases from Three Different Sources[a]

	CMV-infected cucumber			CMV-infected tobacco			healthy tobacco		
	total act.[b] (units)	total protein[c] (mg)	sp act.[d]	total act. (units)	total protein (mg)	sp act.	total act. (units)	total protein (mg)	sp act.
initial extract, ammonium sulfate precipitation	2200	73	30	443	125	3.5	21	92	0.2
step 1, PEG 6000 precipitation	3000	50	60	237	10	23.7	13	46	0.3
step 2, Blue-Sepharose chromatography	2440	3.6	680	112	0.34	331	6.6	1.6	4.1
step 3a, phosphocellulose chromatography	1550	0.42	3680						
step 3b, Blue-Sepharose chromatography				69	0.16	421	2.4	0.35	6.9
step 4, glycerol-gradient centrifugation	694	0.07	≃9900	38	0.01	≃3800	1.2	0.01	100

[a] Calculations are made by assuming that enzyme preparations were started from 100 g of leaves. [b] The total activity is given in nanomoles of UMP incorporated per hour at 30 °C with CMV RNA as the template. [c] The amount of protein was estimated according to Bradford (1976). [d] The specific activity is expressed in nanomoles of UMP incorporated per milligram of protein. Because of the very low protein concentrations of the step 4 enzymes, their specific activities are only approximate or minimal figures.

Table II: Requirements for RNA Polymerase Action[a]

	[^{3}H]UMP incorporation for enzyme from					
	CMV-infected cucumber		CMV-infected tobacco		healthy tobacco	
enzyme from	cpm	% cpm	cpm	% cpm	cpm	% cpm
complete mixture[b]	12650	(100)	3405	(100)	1930	(100)
−CMV RNA	63	0	35	1	49	3
+10 mM sodium phosphate (pH 7)	13684	108	3228	95	1694	88
+10 mM sodium pyrophosphate (pH 7)	52	0	33	1	29	2
−ATP	269	2	481	14	228	12
−CTP	212	2	87	3	350	18
−GTP	250	2	265	8	477	25
−ATP, CTP, and GTP	38	0	15	0	182	9

[a] Assays were carried out with step 3a or step 3b enzymes after dilution to obtain appropriate counts. [b] The complete mixture is a 0.1-mL sample containing 50 mM Tris-HCl, pH 8.0, 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 7.5 mM dithiothreitol, 7 μg of actinomycin D, 5 μCi of [^{3}H]UTP, 70 nmol each of ATP, CTP, and GTP, 12 nmol of UTP, 10 μg of CMV RNA, and 30 μL of enzyme. Of this mixture 30 μL was transferred, before and after 1 h of incubation at 30 °C, respectively, to DEAE-cellulose paper disks. The counts were corrected by subtracting the counts of the unincubated aliquots (50–70 cpm).

Table III: Relative Template Activity of RNAs and Synthetic Polyribonucleotides[a]

		[^{3}H]NTP incorporation for enzyme from					
		CMV-infected cucumber		CMV-infected tobacco		healthy tobacco	
	[^{3}H]NTP	cpm	%	cpm	%	cpm	%
no template	UTP	23	0	0	0	9	0
CMV RNA	UTP	10698	(100)	2273	(100)	2099	(100)
TYMV RNA	UTP	10720	100	2717	120	2074	99
TMV RNA	UTP	13498	126	1959	86	1639	78
Qβ RNA	UTP	3425	32	1449	64	1019	49
poly(A)	UTP	47	0	42	2	68	3
poly(C)	GTP	76369	714	1224	54	386	18
poly(G)	CTP	71	1	12	1	17	1
poly(U)	ATP	2504	23	2298	101	2015	96
poly(CU) (1:1)	ATP	47360	443	7441	327	7298	348
poly(UG) (1:1)	ATP	1833	17	2749	121	2570	122
poly(UG) (5:1)	ATP	9193	86	7794	343	8346	398

[a] Assays were carried out as described in Table II. [^{3}H]UTP was replaced by [^{3}H]ATP, [^{3}H]CTP, or [^{3}H]GTP according to the kind of synthetic polyribonucleotides used as template, always being accompanied with the presence of 12 nmol of the corresponding unlabeled triphosphate and 70 nmol of the other three unlabeled triphosphates.

plants infected with CMV, in confirmation of the results of Kumarasamy & Symons (1979), though using a different extraction procedure. The final purification, compared to the initial extract, ranges from 300- to 1000-fold.

Comparison of Properties of RNA-Dependent RNA Polymerases Obtained from Three Different Sources. The enzyme preparations obtained from CMV-infected cucumber and tobacco, as well as from uninfected tobacco, show the typical characteristics required for RNA-dependent RNA polymerase (Table II). The activity is dependent on template RNA and on the presence of all four triphosphates and is inhibited by adding pyrophosphate but not by orthophosphate.

Table III summarizes the activity of the enzymes with a variety of templates, including viral RNAs and synthetic polyribonucleotides. None of the three enzymes shows template specificity when tested with three plant viral RNAs; Qβ RNA

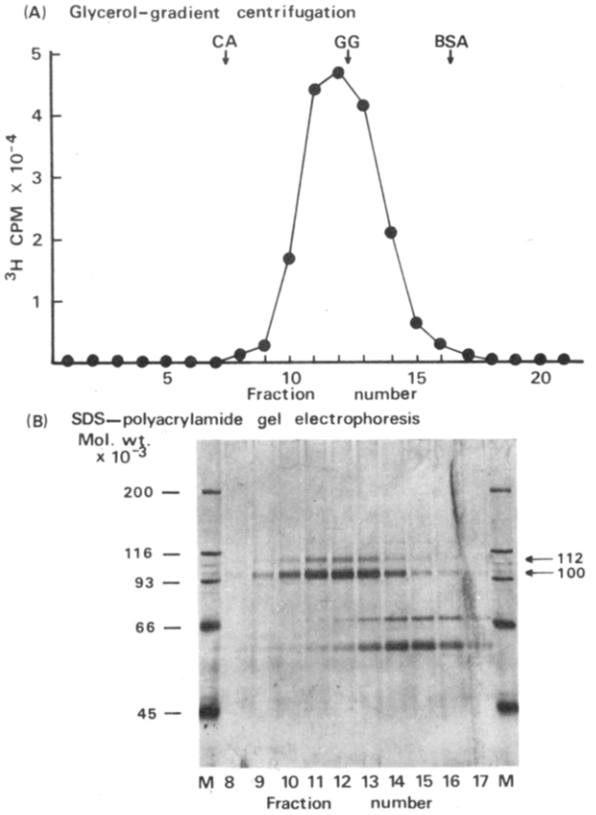

FIGURE 1: Glycerol-gradient centrifugation and protein analysis of enzyme preparation obtained from CMV-infected cucumber plants. (A) Step 3a enzyme was centrifuged through a gradient of 7.5–20% glycerol in 20 mM Tris-HCl, pH 8.0, 250 mM KCl, 25 mM NH_4Cl, 1 mM EDTA, and 0.1 mM DTT with a Beckman SW 41 rotor at 36 500 rpm for 24 h at 4 °C. Twenty-drop fractions (0.55 mL) were collected from the bottom of the tube, and the RNA polymerase activity of each fraction was assayed as described in Table II. CA, GG, and BSA with arrows show the positions of the molecular weight markers catalase (200K), γ-globulin (150K), and bovine serum albumin (66K), respectively, which were centrifuged through a sister tube of the gradient. Sedimentation was from right to left. (B) The proteins in a small aliquot of each fraction obtained by glycerol-gradient centrifugation were analyzed by $NaDodSO_4$–polyacrylamide (7.5%) slab gel electrophoresis. Each fraction number corresponds to that of the gradient. Lanes M contained a mixture of the following molecular weight markers (0.1 μg of each protein): myosin (200K), β-galactosidase (116K), phosphorylase B (93K), bovine serum albumin (66K), and ovalbumin (45K). Lane 12 contained enzyme proteins equivalent to approximately 7 units of activity.

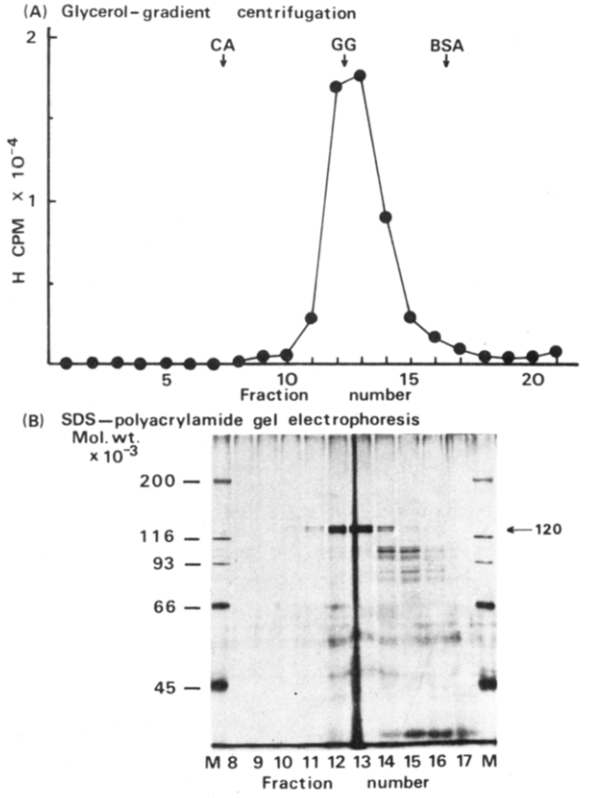

FIGURE 2: Glycerol-gradient centrifugation and protein analysis of enzyme preparation obtained from CMV-infected tobacco plants. (A) Step 3b enzyme was subjected to glycerol-gradient centrifugation as described in Figure 1. (B) The proteins were analyzed as described in Figure 1. Lane 12 contained enzyme proteins equivalent to approximately 0.5 unit of activity.

also serves as a template though its efficiency is lower than those of the plant viral RNAs. As for the synthetic polyribonucleotides, poly(C) is a very active template for the enzyme from infected cucumber plants as reported by May & Symons (1971) but not for the enzymes in healthy or infected tobacco plants. The random copolymers poly(CU) (1:1) and poly(UG) (5:1) both serve as a very effective template for the enzymes in tobacco, as reported by Ikegami & Fraenkel-Conrat (1979a), whereas the cucumber enzyme greatly prefers poly(CU) to poly(UG) as its template. No significant differences are found in template preferences between the enzymes in healthy and CMV-infected tobacco plants.

The sedimentation rate of the three enzyme activities is very close to that of γ-globulin. The slightly faster sedimentation observed with the cucumber as compared to the tobacco enzyme in Figures 1–3 is not regarded as significant, since in many other experiments no difference was observed (see Figure 4).

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis of Proteins. Discontinuous $NaDodSO_4$ gel electrophoresis on 7.5% acrylamide gels was done according to Laemmli (1970). The proteins in each fraction after glycerol-gradient centrifugation were precipitated by adding cold 50% trichloroacetic acid containing 0.2% sodium deoxycholate to give a final concentration of 10–15%. The mixtures were held on ice for 30 min and centrifuged at 10000*g* for 10 min with polyethylene microcentrifuge tubes. The precipitate was washed 3 times with acetone at 0 °C and resuspended in 20–25 mL of Laemmli's sample buffer. Protein bands were revealed on the gels by the ultrasensitive silver staining method of Merril et al. (1981), by which less than 10 ng of a single protein was detectable.

The electrophoretic patterns of the enzyme extracted from CMV-infected cucumber plants show a main band of about 100 kdaltons and a lesser one at 112 kdaltons, with a very faint one at about 50 kdaltons (Figure 1). The presence of the two larger proteins in similar proportion, together with many other strong bands, including one at about 50 kdaltons, was shown by Kumarasamy & Symons (1979), and the large ones were regarded as probable enzyme components. The presence of our two large proteins clearly coincides with the peak of enzyme activity on the glycerol gradient (Figure 1). In contrast, the enzyme activity of a gradiented CMV-stimulated tobacco preparation correlates with a strong double band of protein of about 125 kdaltons, and only very faint bands of smaller proteins are seen in these fractions at the peak tubes of enzyme activity. Coelectrophoresis of the tobacco and cucumber enzymes (not shown) makes the difference in band locations more evident. The corresponding preparation from healthy tobacco contains very many more proteins at the present stage of purification, but the doublet is clearly visible in the enzymatically active fractions of the gradient (Figure 3).

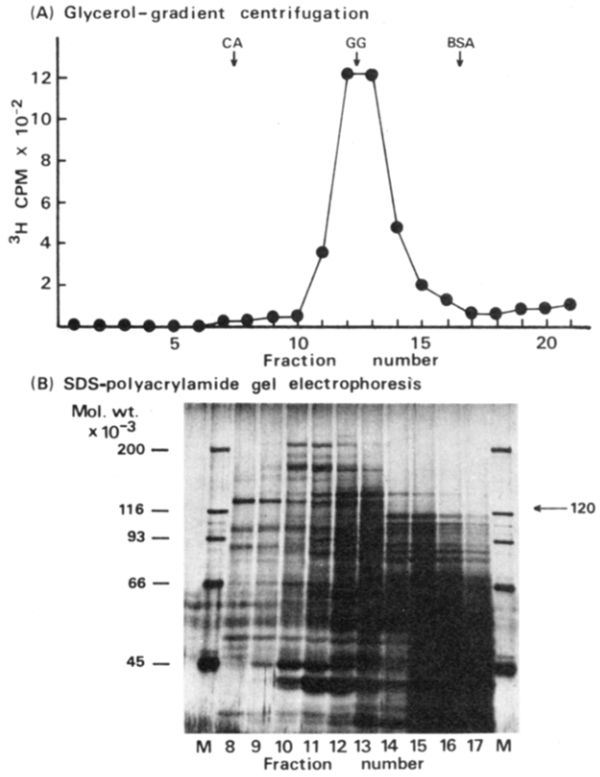

FIGURE 3: Glycerol-gradient centrifugation and protein analysis of enzyme preparation obtained from healthy tobacco plants. (A) Step 3b enzyme was subjected to glycerol-gradient centrifugation as described in Figure 1. (B) The proteins were analyzed as described in Figure 1. Lane 12 contained enzyme proteins equivalent to approximately 0.2 unit of activity.

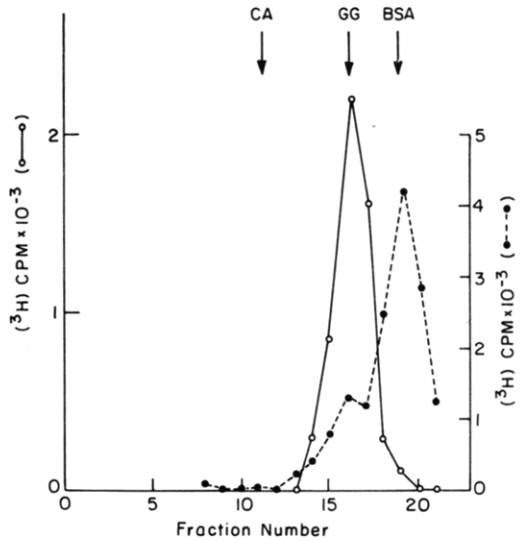

FIGURE 4: Glycerol-gradient centrifugation of [^{3}H]UMP incorporating activity obtained from CMV-infected and healthy cucumber plants. The methods were essentially as described in Figure 1 except that centrifugation was only for 18 h. (○) The enzyme was obtained from infected cucumber plants; (●) the enzyme was obtained from healthy plants. The reaction mixture (0.1 mL) used for assay of the enzyme obtained from healthy plants contained only 0.8 nmol of unlabeled UTP instead of 12 nmol; thus it was of higher specific activity. The amount of activity in the 150-kdalton peak of healthy plants was insufficient for enzymatic characterization. Further attempts to isolate an RNA-dependent RNA polymerase from cucumber cotyledons are in progress.

Endonuclease Activity of Plant RNA Polymerase Preparations. Since the products obtained with RNA-dependent RNA polymerase, though largely double stranded, are always found to be smaller than those with the template, the presence

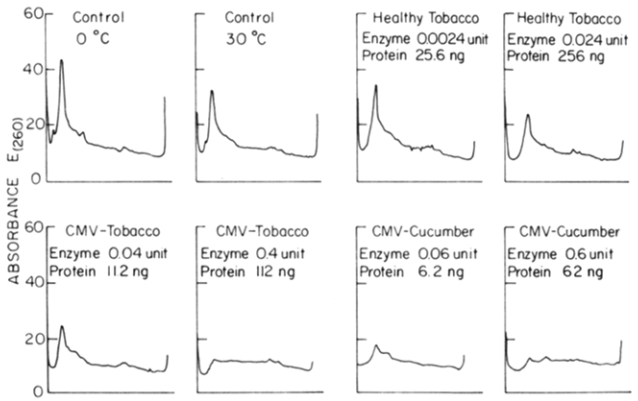

FIGURE 5: Demonstration of endonucleolytic action of purified RNA-dependent RNA polymerase. Polyacrylamide gel (2.4%, 5 mA/gel, 3.25-h) scans of TYMV RNA are shown after exposing it to the assay buffer alone for 90 min at 0 °C (control) or at 30 °C with no enzyme or with varying amounts of various enzymes, as indicated. The abscissa is in arbitrary units; the ordinate corresponds to the length of the gel.

Table IV: Differentiation between RNA Polymerase and Uridyl Transferase[a]

enzyme	substrate	template-primer	incorporation (cpm) U	C	A	G
RNA polymerase	all four NTPs	TYMV RNA oligo(U)	794 126	480	1118	1256
	only one NTP	TYMV RNA oligo(U)	206 153	86	116	153
uridyl transferase	all four NTPs	TYMV RNA oligo(U)	1079 4388	107	403	120
	only one NTP	TYMV RNA oligo(U)	3146 5709	310	276	200

[a] The enzyme preparations were from tobacco, isolated by glycerol-gradient centrifugation, yielding peaks of about 150 and 50 kdaltons, respectively. When all four NTPs were used, only one at a time was ^{3}H labeled. For conditions of incorporation see Tables II and III.

of nucleases has generally been surmised. To test for the action of such enzymes, we have incubated viral RNA templates, such as TYMV RNA, with enzyme preparations in the absence of triphosphates and then subjected the mixture to polyacrylamide gel electrophoresis. The gels were scanned with UV light to determine to what extent the 2×10^6 dalton viral RNA had become degraded. As shown on Figure 5, the presently available partially purified cucumber and tobacco enzyme preparations caused increasing degradation of the template in the order of their specific RNA polymerase activities—the cucumber more than the CMV-infected tobacco enzyme and the healthy tobacco enzyme less, even though considerable amounts of contaminating proteins are present in the latter (see Figure 3). These data suggest that endonucleolytic degradation is caused by the RNA-dependent RNA polymerase itself and not by contaminating RNase, although alternate explanations for these preliminary observations are by no means ruled out.

Tests for Terminal Uridyl Transferase. The presence of enzymes in plants that polymerize UTP has been reported some time ago for tobacco (Brishammar & Juntti, 1975) and more recently for tomato (Boege & Sanger, 1980). These enzymes mimic RNA polymerases in that they utilize the triphosphate, thus being inhibited by pyrophosphate, and they also require a primer, a function that typical viral RNAs with free 3′-hydroxyl groups can serve. If the radioactive triphosphate used is UTP, such enzymes can thus simulate RNA-dependent RNA polymerases. However, the retention

of UMP incorporation activity even in the absence of the other triphosphates and particularly the high primer activity of poly(U) and oligo(U) clearly identify this enzyme as a terminal transferase (see Table IV).

In our previous methods of tobacco RNA polymerase purification we got rid of this enzyme during the first steps, poly(ethylene glycol)–dextran two-phase separation and DEAE-cellulose chromatography. When we started to develop a new purification method with particular reference to avoiding the two-phase method that we suspected of causing partial denaturation and labilization of the enzyme, we obtained polymerase preparations that retained some uridyl transferase activity. These enzymes could then be separated as clearly distinct peaks upon glycerol-gradient centrifugation, since their apparent molecular weights differ by 3-fold (150 vs. 50 kdaltons) (see Figure 4). An experiment illustrating the different nature of these two enzymes is shown in Table IV. The present enzyme purification method removes the terminal transferases, as stated, at early steps. Apparently tobacco contains small amounts of transferases also for the other nucleotides (see Table IV).

Discussion

The purified preparations of cucumber and tobacco RNA-dependent RNA polymerase appear, on grounds of protein composition, to approach homogeneity. They are also clearly different. The cucumber enzyme consists almost only of a 100-kdalton and less of a 112-kdalton protein, the same that were regarded by Kumarasamy & Symons (1979) as characteristic for the enzyme among at least eight other partly strong protein bands. The tobacco enzyme consists almost only of a doublet of about 125 kdaltons. The main question concerning the physical nature of these enzymes is raised by their sedimentation rate of glycerol gradients being the same as that of γ-globulin, which suggests an M_r of about 150K. No evidence for the presence of stoichiometric amounts of a smaller subunit to account for this difference can be detected on the electrophoretic patterns, although the presence of much smaller subunits that might have run off the gels cannot be excluded. The RNA replicase of cauliflower has been reported to show a single-chain protein of 140 kdaltons on nondenaturing and denaturing gels (Astier-Manifacier & Cornuet, 1978). We are inclined to believe that this may be the case also for the cucumber and tobacco enzymes and that the discrepancy of the native and denatured molecular weight, with γ-globulin and β-galactosidase as respective markers, is due to conformational differences or some technical problem. As often stated, molecular weights of macromolecules can only be established by sequencing the molecule.

The dual bands seen with these enzymes, seemingly equal and very close together in the tobacco enzyme, also pose problems. Their complex with a molecular weight of about 250K would be in greater discord with the sedimentation rate suggesting 150K than is the concept that they are separate entities. It seems possible that these are two forms derived from the same precursor, processed to slightly different lengths. Again, only sequencing of the proteins, or their gene(s), can solve this problem.

The specific activities of the enzymes from virus-infected plants are in the same range as those of Qβ replicase. The only enzyme obtained in pure form from uninfected plants (cauliflower) is reported to be 1 order of magnitude less active (Astier-Manifacier & Cornuet, 1978). The fact that all of these enzymes are very active with homopolynucleotides and/or random heteropolynucleotides [poly(C), poly(UC), poly(UG)] shows that in vitro no specific initiation is required or used. Primers, such as are found essential with polio RNA replicase [oligo(U) with poly(A) template] (Flanegan & Baltimore, 1979), were found to be ineffectual with the plant enzymes (Ikegami & Fraenkel-Conrat, 1979a). However, it must be recognized that the mode of action of these enzymes in the cell, which probably is associated with membranous structures, is probably very different from the in vitro use of the isolated enzyme.

The enzymes have nucleolytic activity even at the highest level of purification, in that some degradation of large templates is observed when these are incubated with the enzymes in the absence of triphosphates. This activity actually appears to be proportional to the polymerase activity rather than to the amount of plant protein. It thus seems possible, but as yet not proven, that the nucleolytic activity of the enzymes is an inherent property rather than being due to trace contamination with nuclease(s). The finding of nucleolytic activity accounts for the largely double-stranded products of enzyme action being always heterogeneous and no larger than the template, although the newly synthesized strands are largely between 5×10^5 and 10^6 daltons when purified enzyme is used (data not shown).

In all plants except cucumbers, RNA-dependent RNA polymerases of indistinguishable properties have been found in non-virus-infected healthy tissue. The evidence for the presence of such an enzyme in cucumber is as yet inconclusive. We are in the process of attempting its isolation from healthy cucumber cotyledons (see Figures 3 and 4). Its activity at all stages of purification is only 0.1–0.3% of that in CMV-infected cotyledons and has not yet been clearly shown to represent a true RNA-dependent RNA polymerase. We do not yet know the biological role of such enzymes in plant cell biology. It has been suggested that they may serve to make small double-stranded RNAs, possibly with regulatory functions (Ikegami & Fraenkel-Conrat, 1979b); in that case the slight nucleolytic activity might not be harmful, but it must surely serve a specific purpose. If these enzymes upon virus infection are taken over for viral RNA replication, this also appears to occur on membranes, and the template is presumably replicated without or before becoming degraded. The question of whether these enzymes serve as viral RNA replicases is still controversial. We have in no instance found any indication of the existence of other RNA replicating activities in infected plants than the plant RNA-dependent RNA polymerases, and we have found no evidence for the latter to be altered or added to upon viral infection.

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Replicative Conformation of Parental Nucleosomes: Salt Sensitivity of Deoxyribonucleic Acid–Histone Interaction and Alteration of Histone H1 Binding†

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ABSTRACT: The transiently altered DNA–histone interaction of parental chromatin during replication was studied by micrococcal nuclease digestion. A large amount of nuclease-resistant pulse-labeled DNA and a small fraction of nonreplicating DNA are released from chromatin fragments by treatment with 0.5 M NaCl and appear as protein-free DNA. As shown by reconstitution experiments, the salt sensitivity of digested nascent chromatin is most probably a consequence of the shorter DNA fragment size (55 ± 15 base pairs) in these complexes. This new DNA is associated with parental chromatin fragments which are structurally changed in such a way that parts of nucleosomal DNA were more susceptible to nuclease attack. The core histones of these particles are probably not distinct from those of salt-stable nucleosomes. However, histone H1 and probably high-mobility group proteins appear to be more weakly bound during replication as shown by electrophoresis under nondenaturating conditions. The results agree with the assumption that the transient alteration of nucleosomal conformation describes a state in which DNA could be replicated without leaving the associated core histone complexes. A possible attachment of pulse-labeled chromatin with nuclear matrix is discussed.

Newly replicated chromatin is transiently changed in chromatin structure [for a review, see DePhamphilis & Wassarman (1980)]. The higher nuclease susceptibility (Seale, 1975, 1976, 1978; Hildebrand & Walters, 1976; Weintraub, 1976; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Seidman et al., 1979; Klempnauer et al., 1980) and the altered nuclease-resistant cleavage products of newly replicated chromatin (Hildebrand & Walters, 1976; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Schlaeger & Knippers, 1979; Klempnauer et al., 1980) are two important features of the structural differences from mature chromatin and have been observed in several systems (DePhamphilis & Wassarman, 1980).

A portion of the nascent chromatin is cleaved by micrococcal nuclease to DNA fragments which are shorter in size than those produced under the same conditions from nonreplicating chromatin, indicating that the pulse-labeled DNA is differently organized (Seale, 1978; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Klempnauer et al., 1980). The amount of nascent chromatin which differs in the DNA–protein interaction could be measured by the release of protein-free short DNA fragments (4–5 S) in 0.5 M salt as previously described (Schlaeger & Knippers, 1979).

From the salt-sensitive behavior of nascent chromatin fragments, it is suggested that almost all parental chromatin regions associated with new DNA are subjected to a conformational change during replication (E.-J. Schlaeger and R. L. Seale, unpublished experiments).

In an effort to understand the altered DNA–protein interaction of newly replicated chromatin, the structural properties of pulse-labeled DNA have been studied in more detail by using micrococcal nuclease as a probe of chromatin structure. In the first section of this work, pulse-labeled chromatin was nuclease digested in intact nuclei and analyzed, whereas in the second part, the structural properties of nascent DNA on isolated nucleosomes were investigated.

From the data obtained, it is suggested that during DNA replication the parental nuleosome structures are subjected to a conformational change in such a way that parts of nucleosomal DNA become more susceptible to nuclease cleavage cuts. This altered DNA–histone interaction was also observed for a minor class of nonreplicating chromatin subunits. The unusual DNA fragments are released from salt-sensitive nucleosomes as a consequence of the shorter size. Evidence will be presented showing that the histone H1 is transiently more

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