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In Vitro Replication of Cowpea Mosaic Virus RNA

III. Template Recognition by Cowpea Mosaic Virus RNA Replicase

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Cowpea mosaic virus (CPMV) RNA replicase has been purified about 200-fold from CPMV-infected *Vigna unguiculata* leaves. Optimal reaction conditions for replicase activity have been established that allow RNA synthesis to proceed for at least 15 h. Using a polymerase assay under conditions optimal for CPMV RNA-directed RNA synthesis, all natural RNA species tested appeared to be able to direct the incorporation of labeled ribonucleotides, whereas synthetic homoribopolymers were either inactive or only slightly active. Using a nitrocellulose membrane filter assay to measure complex formation between the replicase preparation and various RNA species, all natural RNA species tested, except that of the comovirus radish mosaic virus, appeared to be unable to compete with ^{32}P-labeled CPMV RNA in binding to replicase. We propose that CPMV replicase is actually template specific but does not display this property in a polymerase assay, since labile complexes between heterologous templates and replicase become stabilized by the formation of phosphodiester bonds. From homoribopolymer competition binding experiments we conclude that the polyadenylic acid on the CPMV genome might be a part of the replicase binding site.

Cowpea mosaic virus (CPMV), the type member of the comoviruses, contains a single-stranded RNA genome of the plus type, consisting of two molecules with molecular weights of 1.37×10^6 and 2.02×10^6, respectively (28), which are encapsidated in two physically separable nucleoprotein particles (5, 35). Some features of CPMV are distinguishable from several other single-stranded RNA plant viruses and reminiscent of the animal picornaviruses. First, the CPMV genome contains a polyadenylic acid [poly(A)] tract at its 3'-ends (5, 9). Second, the 5'-end of the CPMV genome is conspicuous by the absence of either a "cap" structure [m^7G(5)ppp(5')Np] or a free tri-, di-, or monophosphate (19). From recent studies it is known that there is actually a protein covalently linked to the 5'-terminus (J. Stanley, P. Rottier, J. W. Davies, P. Zabel, and A. van Kammen, submitted for publication), as has been demonstrated for the virion RNAs of poliovirus (10, 20, 26, 27), foot-and-mouth disease virus (31), and encephalomyocarditis virus (12, 17, 26). Thus, questions arise about the possible roles these characteristic 3'- and 5'-termini are playing in the replication of the CPMV genome. Since knowledge about eucaryote virus RNA replication is still in its infancy and a detailed understanding of the mechanism of RNA replication will depend upon the availability of an in vitro replicase system, we have focused our attention on the purification and characterization of CPMV replicase.

We have shown previously that *Vigna unguiculata* plants infected with CPMV contain a membrane-bound, RNA-dependent RNA polymerase (replicase) complex that is synthesizing virus-specific RNAs in vitro and is most probably involved in CPMV replication in vivo (39). This RNA polymerase activity seems to derive, at least in part, from the virus genome as judged from the following observations. (i) The time course of appearance of the replicase is related to the growth of the virus (39). (ii) No significant replicase activity has been detected in the corresponding fraction from uninfected plants (39). (iii) Plants infected with a temperature-sensitive mutant of CPMV do not contain this RNA polymerase activity at the nonpermissive temperature (30°C), whereas the enzyme activity developing in leaves at the permissive temperature (22°C) disappears upon a shift to the nonpermissive temperature, not as a result of the temperature sensitivity of the enzyme itself but most likely by a defect in the synthesis of the replicase (7).

In a previous report we have demonstrated that the membrane-bound replicase can be solubilized from the cytoplasmic membranes and obtained in a highly stable and template-dependent form provided no detergents are used (38).

The major goal of the experiments presented

in this paper was to describe some of the basic properties of CPMV replicase. We have examined first the assay conditions favorable for replicase activity. We then tested a variety of RNA species for their capacity to direct the incorporation of labeled ribonucleotides. Finally, we studied the affinity of the replicase for various RNA species, using a nitrocellulose filter-binding assay.

MATERIALS AND METHODS

Materials. Cowpea seeds [*V. unguiculata* (L.) Walp. var. "Blackeye Early Ramshorn"] were obtained from W. Atlee Burpee Co., Philadelphia, Pa. Ribonucleoside triphosphates, rifampin, cordycepin (3'-deoxyadenosine), phosphocreatine, creatine phosphokinase (140 U/mg of protein), RNase A (from bovine pancreas, 5× crystallized, type A), RNase T1 (from *Aspergillus oryzae* grade II, 480,000 U/ml of protein), and dithioerythritol (DTE) were purchased from Sigma Chemical Co.; DEAE-Bio-Gel A (control no. 13758) was from Bio-Rad; poly(A) ($s_{20,w}$, 9.5), polyuridylic acid [poly(U); $s_{20,w}$, 8], polyguanylic acid [poly(G); $s_{20,w}$, 10 to 11], and polycytidylic acid [poly(C); $s_{20,w}$, 6.2] were from Boehringer Mannheim GmbH; phenylmethylsulfonylfluoride (PMSF) was from Merck and Co.; aurintricarboxylic acid-ammonium salt was from Serva; Soluene-350 sample solubilizer and Instafluor were from Packard Instrument Co, Inc.; and [5-^{3}H]UTP (11 to 24 Ci/mmol), [5-^{3}H]-CTP (17 Ci/mmol), [8-^{3}H]GTP (15 Ci/mmol), and [2-^{3}H]ATP (20 Ci/mmol) were purchased from The Radiochemical Centre. Actinomycin D was kindly donated by Merck Sharp and Dohme. α-Amanitin was a generous gift from H. Stunnenberg (Department of Genetics, Wageningen, The Netherlands).

Buffers. Buffer $TK_{10}EDP$ contained 0.05 M Tris-hydrochloride (pH 7.4), 0.01 M KCl, 0.001 M EDTA, 0.01 M DTE, and 0.5 mM PMSF. Buffer $TG_{25}K_{50}EDP$ contained 0.05 M Tris-hydrochloride (pH 8.2), 25% (vol/vol) glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE, and 0.5 mM PMSF. Buffer $TG_{50}K_{50}EDP$ was identical to $TG_{25}K_{50}EDP$ except 50% (vol/vol) glycerol instead of 25% was used. Buffer $TG_5K_{250}EDP$ contained 0.05 M Tris-hydrochloride (pH 8.2), 5% (vol/vol) glycerol, 0.250 M KCl, 0.001 M EDTA, 0.01 M DTE, and 0.5 mM PMSF. PMSF and solid DTE were added and dissolved just before use of the buffer. The former was added from a 0.2 M stock solution in 96% ethanol at −20°C.

RNAs. CPMV and CPMV RNA were isolated as described by Klootwijk et al. (19). Radish mosaic virus (RaMV) RNA was isolated from RaMV by the same method used to isolate CPMV RNA. RaMV was kindly donated by R. Hull (Norwich, England). Semliki Forest virus RNA was a gift from M. Pranger (University of Utrecht, Utrecht, The Netherlands); J. Klootwijk (Free University of Amsterdam, Amsterdam, The Netherlands) donated 17S rRNA from yeast, and L. van Vloten-Doting (University of Leiden, Leiden, The Netherlands) donated RNAs from brome mosaic virus, tobacco streak virus, satellite tobacco necrosis virus, tobacco mosaic virus (TMV), cowpea chlorotic mottle virus, cucumber mosaic virus, alfalfa mosaic virus, and turnip yellow mosaic virus (TYMV).

Virus and plants. *V. unguiculata* plants were cultivated in pots in a growth chamber under controlled conditions of light, temperature, and humidity as described by Hibi et al. (15). Eight days after sowing, the primary leaves were inoculated with crude sap from plants infected with a yellow strain isolate of CPMV (5, 33, 35) and further cultivated at 30°C with 75% relative humidity and continuous light. The primary leaves were harvested on day 4 after inoculation and used directly for the isolation of the replicase.

Replicase purification procedure. All steps were carried out at 0 to 4°C unless specified otherwise.

(i) Isolation of membrane-bound replicase. The isolation of the membrane-bound replicase was performed as described previously (39) except that PMSF, a protease inhibitor, was included in the homogenization buffer and DTE was used instead of β-mercaptoethanol. Briefly, portions (12 g) of freshly harvested leaves from which the midribs were removed were homogenized with 35 ml of $TK_{10}EDP$ in a prechilled mortar. The homogenate was squeezed through two layers of Miracloth (fraction 1) and centrifuged at 1,000 × *g* for 15 min in a Sorvall SS34 rotor. The green-colored supernatant was carefully pipetted off, adjusted to 20% (vol/vol) glycerol (fraction 2), and centrifuged at 31,000 × *g* for 30 min. The 31,000 × *g* pellet was resuspended with the aid of a Thomas homogenizer in $TG_{25}K_{50}EDP$ (1 ml for each gram of leaf tissue used; fraction 3) and used for the solubilization procedure. This crude replicase suspension could be stored at −70°C for several months without loss of enzyme activity, providing a convenient stage to interrupt the purification if desired. (After storage at −70°C, the enzyme was slowly thawed at 0°C, whereupon the solubilization procedure was started.)

(ii) Solubilization of membrane-bound replicase. Solubilization of the replicase providing fraction 4 was carried out by washing the membranes with an Mg^{2+}-deficient buffer as described previously (38).

(iii) DEAE-Bio-Gel A column chromatography. Thirty milliliters of a packed DEAE-Bio-Gel A slurry was washed with $TG_{25}K_{50}EDP$, poured into a Pharmacia K16/20 column equipped with a flow adaptor, and equilibrated with $TG_{25}K_{50}EDP$. The solubilized enzyme (fraction 4) was applied to the column (1.6 by 15 cm) at a flow rate of about 20 to 25 ml/h. Up to 475 ml of fraction 4 containing 0.6 to 0.9 mg of protein per ml could be applied to this column without loss of enzyme activity in the flowthrough. During the flow of the sample through the column, the top layer turned brown at about 0.5 cm. After application of the sample, the column was washed with $TG_{25}K_{50}EDP$ to remove unbound material. Then the bound material was eluted with a 0.05 to 0.4 M KCl gradient, generated by an LKB Ultrograd gradient mixer. In comparison with the linear 0.05 to 0.4 M KCl gradient previously used (38), the nonlinear gradient as shown in Fig. 1 was found to improve the resolution of the DEAE column considerably, thus providing a replicase peak which was sharp, symmetrical, and completely separated from the nucleic acids eluting at the end of the gradient. When 200 to 400 g of leaf tissue had been

used for the preparation of replicase, a 160 to 200-ml gradient was applied, and 3- to 3.25-ml fractions were collected at a flow rate of 16 to 20 ml/h and assayed for polymerase activity, using 25-μl samples. The eluate of the column was continuously monitored at 280 nm by means of a Uvicord II (LKB). The fractions containing the bulk of the polymerase activity were pooled (fraction 5) and dialyzed overnight against 1 liter of $TG_{25}K_{50}EDP$ saturated with $(NH_4)_2SO_4$. After collection of the precipitate by centrifugation at 31,000 × *g* for 20 min, the precipitate was either prepared for storage in liquid nitrogen as described previously (38) or prepared for glycerol gradient centrifugation. In the latter case, the pellet was dissolved in 1 to 2 ml of $TG_5K_{250}EDP$ and dialyzed for 4 h against 500 ml of $TG_5K_{250}EDP$.

(iv) Glycerol gradient centrifugation. The dialyzed enzyme solution (0.5 to 0.75 ml) was layered on a 11.4-ml, linear 15 to 30% (vol/vol) glycerol gradient in $TK_{250}EDP$ and centirfuged in the polyallomer tube of a Beckman SW41 rotor at 40,000 rpm for 17 h at 1°C. Up to 7.5 mg of protein may be layered on each gradient in an SW41 rotor. Fractions were collected through a hole punctured in the bottom of the tube, monitored continuously at 280 nm by means of a Uvicord II, and assayed for polymerase activity, using 10-μl samples. Peak fractions, which were completely colorless, were pooled and dialyzed overnight against two changes of 300 ml each of $TG_{50}K_{50}EDP$. The dialyzed enzyme solution (fraction 6) was divided into samples (100 to 150 μl) and stored in liquid nitrogen.

Replicase assay. The standard assay mixture contained, in a final volume of 0.24 ml: 0.05 M Tris-hydrochloride (pH 8.2); 5 to 10% glycerol; 0.008 M magnesium acetate; 0.005 to 0.02 M KCl; 0.06 M $(NH_4)_2SO_4$; 0.001 M EDTA; 5 μg of actinomycin D; 0.8 to 2 mM DTE; 0.25 μmol each of ATP, GTP, and CTP; 0.01 μmol of UTP; 4 μCi of [^{3}H]UTP (specific activity, 11 to 24 Ci/mmol; 1 mCi/ml); CPMV RNA; and enzyme as indicated in the figure legends and tables. In some experiments (see figure legends and tables) the final volume of the reaction mixture was 0.12 ml, with half of the amounts of ribonulceoside triphosphates and actinomycin D specified above. Unless specified otherwise, CPMV RNA is a mixture of B-RNA and M-RNA.

The reaction mixtures were incubated at 30°C for 60 min and assayed for trichloroacetic acid-precipitable counts by spotting samples on Whatman 3MM filter disks, which were immediately immersed and collected in ice-cold 5% trichloroacetic acid containing 2% $Na_4P_2O_7$ and washed batchwise as described previously (38). Each filter was treated with 0.75 ml of Soluene-350 for 1 h at 50°C to solubilize the precipitates and was subsequently counted with 7 ml of Instafluor. Under these conditions the counting efficiency for tritium was 40% in a Packard Tri-Carb scintillation counter. The values given are all corrected for zero-time values.

Protein determination. Protein was measured by the method of Lowry et al. (22), using bovine serum albumin as a standard. Because several buffer constituents strongly interfere with this assay, protein was first precipitated with ice-cold 10% trichloroacetic acid and then washed with ice-cold acetone. The precipitates were collected by centrifugation, dissolved in 1 N NaOH, and then used in the protein assay.

Replicase-RNA binding assay. Binding of replicase to ^{32}P-labeled CPMV RNA was assayed on the basis of ability of nitrocellulose filters to retain RNA-protein complexes. Incubation mixtures were prepared on ice and contained, in a total volume of 0.24 ml, all the ingredients required for the polymerase assay except the four ribonucleoside triphosphates and actinomycin D. [^{32}P]RNA and replicase were used at concentrations specified in the figure legends. The reaction was started by the addition of replicase and incubated at 30°C. After incubation for 15 min, two separate 100-μl portions were taken from each incubation mixture and filtered slowly through membrane filters (Millipore HAMK 02412), which had been presoaked for at least 30 min in washing buffer containing 0.05 M Tris-hydrochloride (pH 8.2), 25% (vol/vol) glycerol, 0.05 M KCl, and 0.001 M EDTA. After the sample had passed through, the filters were washed with 2× 3.5 ml of ice-cold washing buffer, dried, and counted with Instafluor. In more recent experiments, incubation mixtures of 0.06 ml were used and filtered in whole through one filter.

In the absence of replicase 2 to 3% of the amount of radioactivity applied to the filter was retained, whereas replicase at saturating concentrations was able to retain about 50 to 55% of the RNA. In preliminary experiments we have found that a low background retention (2 to 3%) only occurred when Mg^{2+} ions were omitted from the washing buffer. The reason for this is unknown, but we think that the unusual nature of the 5'-end of CPMV RNA (19) might be responsible for the sticking of the RNA to the filters in the presence of Mg^{2+} ions.

In competition binding experiments replicase was held fixed so as to be at one-half of the saturation plateau, and various amounts of unlabeled competitor RNA were mixed with the ^{32}P-labeled CPMV RNA before replicase was added. All data have been corrected for background retention.

RESULTS

Purification of replicase. We have improved and extended the purification procedure described previously (38) by eluting the DEAE–Bio-Gel column with a nonlinear KCl gradient (Fig. 1) and subsequent glycerol gradient centrifugation, respectively (Fig. 2). A final purification of approximately 150- to 200-fold was achieved (Table 1). Protein purity was assessed by polyacrylamide gel electrophoresis under denaturing conditions (data not shown). From this, we conclude that the replicase has not yet been purified to homogeneity and requires additional purification steps to allow its identification.

Assay conditions favorable for replicase activity. (i) Characteristics of replicase reaction. Table 2 shows the general characteristics of CPMV replicase. The synthesis of RNA

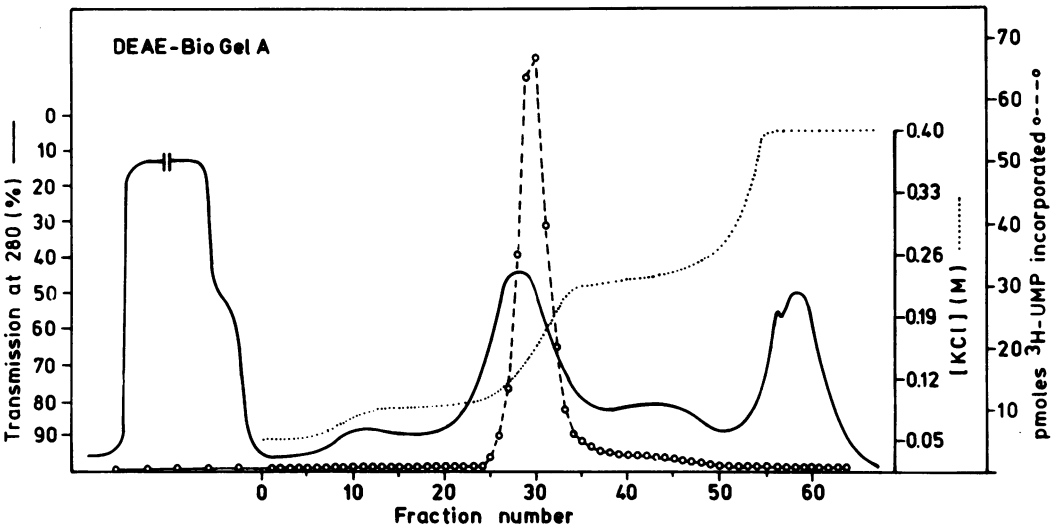

FIG. 1. *DEAE-Bio-Gel A column chromatography of solubilized RNA replicase. Fraction 4 (350 ml, 210 mg of protein) was passed through a DEAE-Bio-Gel A column (1.6 by 15 cm) followed by $TG_{25}K_{50}EDP$ to wash out unbound material. The column was eluted with 200 ml of a nonlinear 0.05 to 0.4 M KCl gradient in the same buffer. Fractions of 3.0 ml were collected and assayed for replicase activity on 25-μl samples for 60 min under standard conditions. Enzyme activity* (○) *is expressed as picomoles of [³H]UMP incorporated per 25 μl of column fraction per 60 min.*

showed an absolute requirement for RNA and a divalent cation. Enzyme activity was not inhibited by actinomycin D, cordycepin, α-amanitin, rifampin, DNase, and orthophosphate. In contrast, RNA synthesis was completely suppressed by pyrophosphate, RNases, and aurintricarboxylic acid. Thus, these results are consistent with the properties of an RNA-dependent RNA polymerase.

(ii) Effect of Mg^{2+} and Mn^{2+} ions. Using $MgCl_2$ as the source of Mg^{2+} ions, optimal replicase activity was obtained at 12 mM, whereas higher concentrations diminished the RNA synthesis (Fig. 3). When Mg^{2+} ions were added as acetate salt, RNA synthesis proceeded optimally at 8 mM and displayed a higher rate than the $MgCl_2$-catalyzed incorporation at all concentrations tested, suggesting an inhibitory action of chloride ions (Fig. 3). The replicase preferred Mg^{2+} to Mn^{2+} ions for optimal activity (Table 2, Fig. 3). Replacement of Mg^{2+} ions by Mn^{2+} ions caused a 2.5-fold decrease in RNA synthesis at the optimum $MnCl_2$ concentration (3 mM). Addition of Mn^{2+} ions to an Mg^{2+}-activated reaction reduced the [³H]UMP incorporation by about 75% (Table 2).

(iii) Effect of K^+ and $(NH_4)^+$ ions. Monovalent cations were either stimulatory or inhibitory depending on the type of salt (Fig. 4). Low concentrations of $(NH_4)_2SO_4$ slightly stimulated the enzyme activity. Optimal activity was achieved with 60 mM, but above this concentration the enzyme activity strongly decreased. RNA synthesis was not affected by potassium acetate concentrations up to 100 mM. Higher concentrations, however, caused inhibition (Fig. 4). Potassium ions added as the chloride salt strongly decreased the enzyme activity (Fig. 4). Replicase activity was optimal at the lowest KCl concentration, again suggesting the inhibitory effect of Cl^- ions on RNA synthesis. It should be emphasized that it is the RNA-synthesizing reaction which is sensitive to Cl^- ions rather than the replicase per se, since the replicase could be centrifuged and stored at high salt (250 mM KCl) and remained active when assayed at low salt concentrations.

(iv) pH. The replicase exhibited a rather broad pH optimum, ranging from pH 7.6 to 9.1. Maximal activity occurred at pH 8.2.

(v) Temperature. To measure the effect of the temperature on the replicase activity, the time course of RNA synthesis was determined at different temperatures ranging from 18 to 42°C. At 18 to 30°C RNA synthesis proceeded for at least 15 h, demonstrating the pronounced stability of the enzyme, whereas at 38 and 42°C RNA synthesis stopped after 3 h. At 30°C the enzyme was still able to perform RNA synthesis after 17 h. The rate of RNA synthesis was linear for at least 2 h at all temperatures tested and maximal at 30 to 34°C.

(vi) RNA synthesis as a function of enzyme concentration. The rate of RNA synthe-

sis increased linearly with the amount of enzyme added from 0.5 to 120 μg of protein per 0.12 ml, indicating that the enzyme remains stable upon dilution.

(vii) RNA synthesis as a function of template concentration. Replicase activity increased linearly with template concentration up to about 0.4 μg (0.24 pmol) of CPMV RNA per 15 μg of protein. Template saturation occurred at about 5 to 10 μg of RNA per 15 μg of protein. From the linear double-reciprocal plot it was calculated that about 0.4 μg of CPMV RNA (0.24 pmol) is required to obtain half V_{max}.

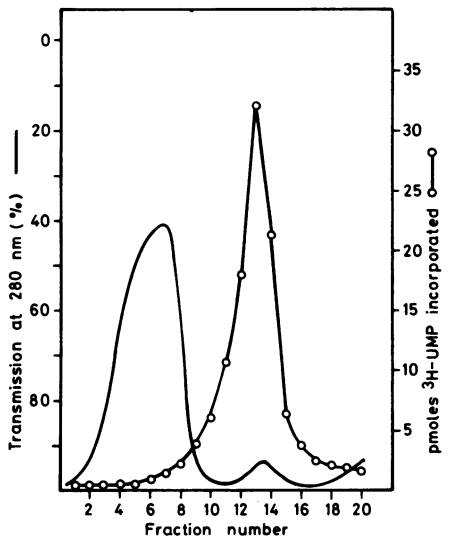

FIG. 2. *Glycerol gradient centrifugation of DEAE-purified replicase. DEAE-purified replicase (fraction 5; 0.75 ml, 7.5 mg of protein) was layered on 11.4 ml of a 15 to 30% glycerol gradient in $TK_{250}EDP$. The gradient was centrifuged at 40,000 rpm in a polyallomer tube of an SW41 rotor for 17 h at 1°C. Fractions of approximately 0.6 ml were collected through a hole in the bottom of the tube and assayed for replicase activity on 10-μl samples for 60 min under standard conditions. Enzyme activity* (○) *is expressed as picomoles of [³H]UMP incorporated per 10 μl of gradient fraction per 60 min. The bottom of the tube is at the left. When a DEAE-purified protein preparation from mock-infected leaves corresponding to the DEAE-purified replicase was analyzed by glycerol gradient centrifugation, a similar optical density profile was obtained, but no polymerase activity was detected throughout the gradient.*

TABLE 2. *Characteristics of CPMV replicase reaction*

Reaction conditions	% of control
Complete[a]	100[b]
$-Mg^{2+}$	1.8
$-Mg^{2+}$ + Mn^{2+} (3 mM)	42.9
$+Mg^{2+}$ (8 mM) + Mn^{2+} (3 mM)	24.0
−RNA	1.2
$+PP_i$ (4 mM)	0
$+P_i$ (4 mM)	92.4
+Phosphocreatine (10 mM) + phosphokinase (10 μg/ml)	85.8
+RNase A (10 μg/ml) + RNase T_1 (10 U/ml)	1.5
+EGTA (0.1–5 mM)[c]	100
−Actinomycin D	118.8
−Actinomycin D + cordycepin (50 μg/ml)	116
−Actinomycin D + α-amanitin (25 μg/ml)	123
−Actinomycin D + rifampin (50 μg/ml)	110.6
−Actinomycin D + DNase (30 μg/ml)	109
−Actinomycin D + ATA (10 μM)[d]	4.2

[a] The complete reaction mixture (0.12 ml) containing 1.5 μg of protein and 10 μg of CPMV RNA was assayed for 60 min at 30°C, as described in the text.
[b] 100% = 7.6 pmol of [³H]UMP incorporated.
[c] EGTA, Ethyleneglycol-2-(2-aminoethyl)-tetraacetic acid.
[d] ATA, Ammonium aurintricarboxylate.

(viii) Ribonucleoside triphosphate requirement. Analysis of the replicase activity by monitoring the incorporation of [³H]CTP, [³H]GTP, or [³H]ATP in the absence of one or all three other unlabeled ribonucleoside triphosphates revealed that the replicase required all four ribonucleoside triphosphates (Table 3). In-

TABLE 1. *Summary of purification of CPMV replicase*[a]

Fraction no.	Fraction description	Vol (ml)	Total protein (mg)	Total activity (U)[b]	Sp act (U/mg)	Purification (fold)	Yield (%)
1	Homogenate	265	1,458	25,896	17.8	1	100
2	1,000 × *g* supernatant	324	1,134	22,585	19.9	1.1	87
3	Membrane-bound replicase	84	160	14,051	87.8	4.9	54
4	Solubilized replicase	94	89	12,369	139	7.8	48
5	DEAE–Bio-Gel column pooled peak	22	13	6,897	530.5	29.8	27
6	Glycerol gradient pooled peak after dialysis	3.6	1.7	5,313	3125	175.6	21

[a] From 84 g of *Vigna* leaves.
[b] One unit of RNA replicase activity is defined as the amount of enzyme catalyzing the incorporation of 1 pmol of labeled UMP into acid-precipitable form in 30 min at 30°C. Fractions were assayed immediately after collection.

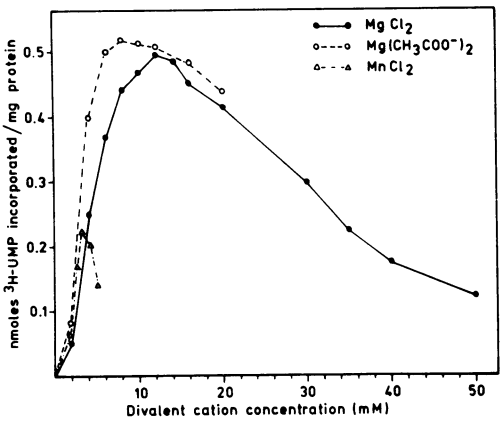

FIG. 3. *Effect of Mg^{2+} and Mn^{2+} ions on CPMV replicase activity. Reaction mixtures (0.24 ml) containing 42 μg of protein, 10 μg of CPMV RNA, and various amounts of Mg^{2+} or Mn^{2+} were assayed for 60 min at 22°C as described in the text.*

corporation with only [^{3}H]CTP, [^{3}H]GTP, or [^{3}H]ATP present in the reaction mixture was only 0.5 to 3% that of the complete reaction mixture. However, when we measured the incorporation of [^{3}H]UTP in the absence of ATP, GTP, and CTP, we occasionally found a considerable residual activity (up to 20%; Table 3), depending on the batch of CPMV RNA used as template.

Because of the poly(A) tracts present at the 3' end of the CPMV RNA genome, it was conceivable that initiation of complementary strand synthesis preferentially occurs at the poly(A) tail of the template (+) strand and results in the synthesis of (−) strand, which will be relatively enriched in poly(U) when nucleotide sequences adjacent to the poly(A) segment are only partially transcribed. This implies that a TMV RNA-directed incorporation of [^{3}H]UTP might respond differently to the omission of the three other unlabeled ribonucleotides because of the poly(A)-deficient nature of TMV RNA. Table 3 shows that indeed much less activity was left (7.5%) when only [^{3}H]UTP was present with TMV RNA instead of CPMV RNA as template. It was found previously (39) that [^{3}H]UTP incorporation activity of the membrane-bound CPMV RNA replicase, which mainly elongates (+) strands complementary to endogenous (−) strands, and thus does not involve poly(A)-di-

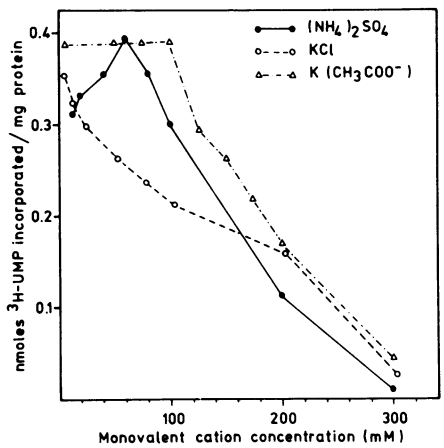

FIG. 4. *Effect of K^+ and NH_4^+ ions on CPMV replicase activity. Reaction mixtures (0.24 ml) containing 42 μg of protein, 10 μg of CPMV RNA, 12 mM $MgCl_2$, 13 mM $(NH_4)_2SO_4$, and various amounts of KCl (3 to 303 mM) were assayed for 60 min at 22°C as described in the text. The reaction containing various amounts of $(NH_4)_2SO_4$ (13 to 300 mM) was carried out in the presence of 42 μg of protein, 10 μg of CPMV RNA, 12 mM $MgCl_2$, and 13 mM KCl. The reaction mixtures (0.21 ml) containing various amounts of potassium (0 to 300 mM) were assayed in the presence of 24 μg of CPMV RNA, 8 mM magnesium acetate, 2.5 mM KCl, and 60 mM $(NH_4)_2SO_4$.*

TABLE 3. *Ribonucleoside triphosphates requirement*

Reaction conditions	Replicase activity (%) with labeled substrate:					
	[^{3}H]CTP	[^{3}H]GTP	[^{3}H]ATP	[^{3}H]UTP	[^{3}H]UTP[a]	[^{3}H]UTP[b]
Complete[c]	100	100	100	100	100	100
−CTP		14.1	4.6	19.9	18.4	24
−GTP	5.2		16.6	27.1	24.4	20
−ATP	1.8	4.6		21.8	23.7	22
−UTP	2.9	3.5	13.2			
−(GTP, ATP, UTP)	1.5					
−(CTP, ATP, UTP)		0.5				
−(CTP, GTP, UTP)			2.6			
−(CTP, ATP, GTP)				20	7.5	6

[a] 10 μg of TMV RNA was used as template instead of CPMV RNA.
[b] Data for the membrane-bound replicase were taken from Zabel et al. (39).
[c] Complete reaction mixtures (0.12 ml) containing 20 μg of protein, 10 μg of CPMV RNA, 0.005 μmol of labeled ribonucleoside triphosphate, and 0.125 μmol each of the other three unlabeled ribonucleotides were assayed at 30°C for 60 min as described in the text.

rected poly(U) synthesis, is only 6% in the absence of all three unlabeled ribonucleoside triphosphates (Table 3).

These results and the ones presented below suggest that the poly(A) tract is involved in the initiation step of CPMV replication.

Template activity of various RNA species. (i) Template activity of poly(A), poly(U), poly(C), and poly(G). In a first attempt to study the possible template specificity of CPMV replicase, several synthetic, viral, and nonviral RNAs were assayed in time course experiments for their capacity to direct the synthesis of RNA. Since both CPMV RNAs contain a poly(A) tract at their 3'-ends (5, 9) we first investigated the template activity of poly(A) at two different concentrations (20 and 100 μg/0.24 ml). At the highest concentration the incorporation of [^{3}H]UMP was about 30% that with CPMV RNA as template (Fig. 5A), whereas at the lower concentration (20 μg/0.24 ml) the poly(A)-directed incorporation of [^{3}H]UMP amounted to about 17% that of CPMV. These results demonstrate that CPMV RNA replicase can utilize poly(A) as a template although the efficiency is very low, since on a molar basis very high amounts of this artificial template are required.

The template activity of poly(U) as measured by the incorporation of [^{3}H]AMP was low (10 to 15%) and only manifest at high concentrations (Fig. 5A). Addition of poly(G) at both high (100 μg/0.24 ml) and low (20 μg/0.24 ml) concentrations did not stimulate the incorporation of [^{3}H]CMP (2%) (Fig. 5B). The template activity of poly(C) as measured by the incorporation of [^{3}H]GMP was negligible (7%) (Fig. 5B).

(ii) Template activity of natural RNAs. After having established that synthetic polynucleotides were either inactive or very inefficient templates, in comparison with CPMV RNA, we then examined the template activity of a variety of nonviral and viral RNAs all added in equimolar amounts. The kinetics of RNA synthesis are shown in Fig. 6. All the RNAs tested, except tRNA from yeast (not shown), were able to

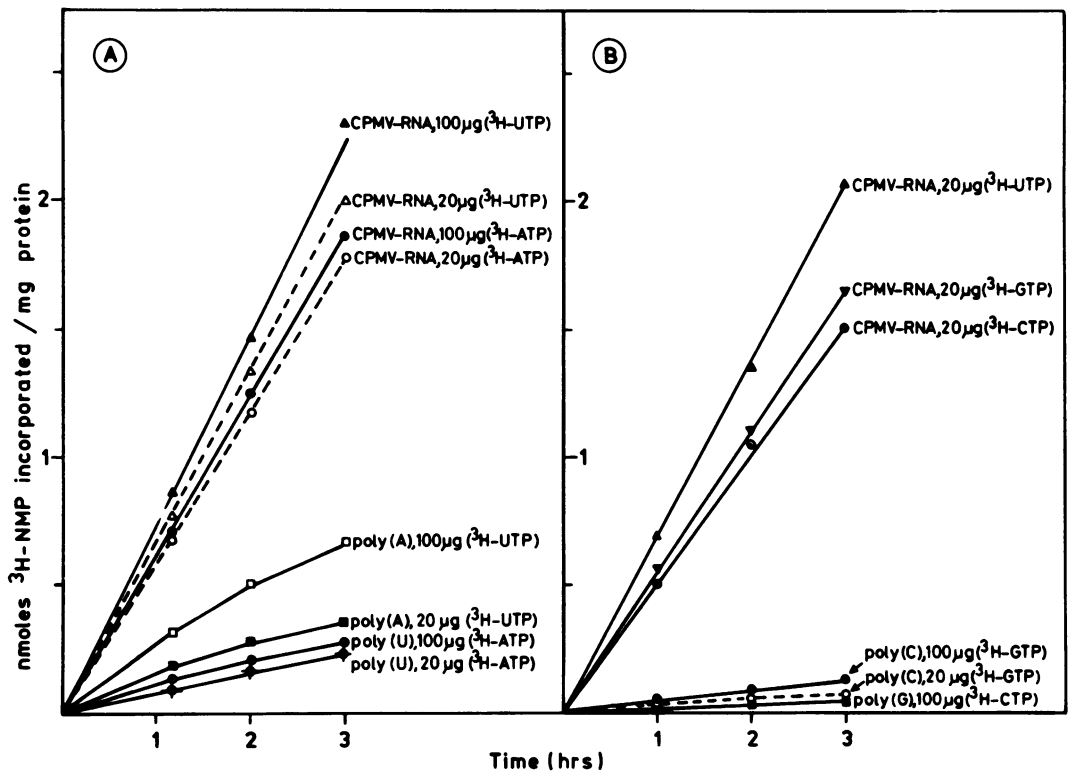

FIG. 5. *Time course of RNA synthesis directed by poly(A), poly(U), poly(G), and poly(C). Reaction mixtures (0.24 ml) containing 48 μg of protein, 0.01 μmol of labeled ribonucleoside triphosphate, and 0.25 μmol each of the other three unlabeled triphosphates were assayed at 30°C as described in the text in the presence of various polynucleotide templates as indicated. Duplicate samples (30 μl) were taken at intervals for determination of acid-insoluble radioactivity.*

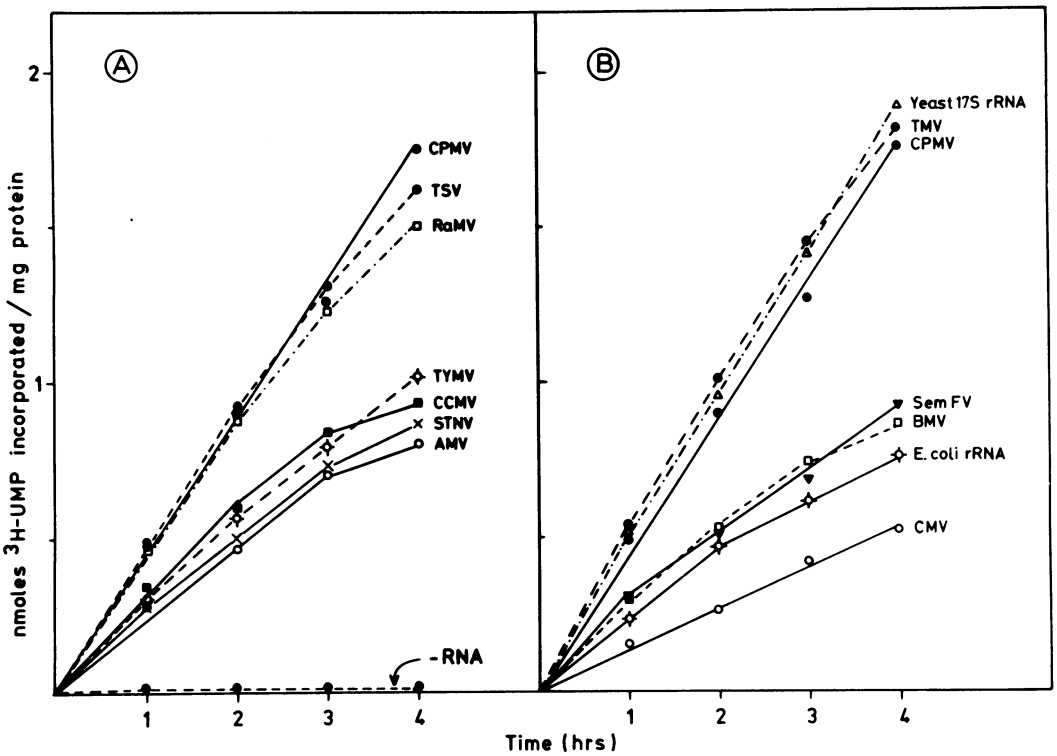

FIG. 6. *Time course of RNA synthesis directed by various template RNAs. Reaction mixtures (0.24 ml) containing 22 μg of protein were assayed at 30°C in the absence (●) (A) or presence of 6 pmol of the indicated template RNAs as described in the text. At the specified times, duplicate samples (35 μl) were removed and assayed for acid-insoluble radioactivity. Abbreviations: TSV, tobacco streak virus; STNV, satellite tobacco necrosis virus; AMV, alfalfa mosaic virus; SemFV, Semliki Forest virus; BMV, brome mosaic virus; CMV, cucumber mosaic virus; RaMV, radish mosaic virus; CCMV, cowpea chlorotic mottle virus.*

stimulate CPMV replicase. The RNA synthesis directed by the RNAs from the plant viruses TMV, tobacco streak virus, and RaMV and 17S rRNA from yeast was comparable (80 to 110%) to the reaction catalyzed by CPMV RNA. The rRNA from *Escherichia coli* and the RNAs from TYMV, cowpea chlorotic mosaic virus, brome mosaic virus, satellite tobacco necrosis virus, alfalfa mosaic virus, and Semliki Forest virus were about 45 to 60% as effective, whereas cucumber mosaic virus RNA showed the lowest efficacy (20 to 25%). The same results were obtained with two different batches of replicase. With most templates tested, the RNA synthesis continued at a linear rate for 4 h.

Replicase-RNA binding. As far as the incorporation of [^{3}H]UMP is concerned, CPMV replicase apparently does not display template specificity. However, the possibility exists that under more selective and stringent conditions, the replicase might exhibit a more fastidious behavior in the selection of templates, or in other words, the polymerase assay might not meet the requirements to measure template specificity properly. Therefore, we studied the affinity of the replicase preparation for various RNA species, using a nitrocellulose membrane filtration assay, based on the ability of nitrocellulose filters to retain nucleic acid-protein complexes but not free nucleic acids (2, 11, 16, 29, 37). This provides a simple and sensitive assay to measure the binding of replicase to RNA species.

(i) Characteristics of the binding reaction. When increasing amounts of replicase were added, in the absence of ribonucleotides, to a constant amount of [^{32}P]CPMV RNA, increasing amounts of [^{32}P]RNA were retained on the filter until a saturation plateau was reached (Fig. 7). At this plateau region 50 to 55% of the RNA was bound. A double-reciprocal plot of the binding curve data yielded a straight line (Fig. 7, inset), suggesting that all binding sites are similar and independent. When [^{32}P]CPMV RNA was passed through filters in the absence of replicase, about 97 to 98% of the radioactivity was found in the filtrate.

Since the DEAE-purified replicase prepara-

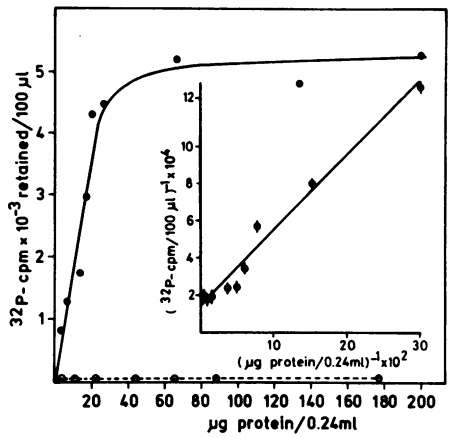

FIG. 7. *Binding of CPMV replicase and the corresponding DEAE-purified protein fraction from uninfected Vigna leaves to [^{32}P]CPMV RNA. Binding assays (0.24 ml) containing 1 µg of [^{32}P]CPMV RNA (24,000 cpm/µg) and increasing amounts of either CPMV replicase (●) or "healthy" protein (⊙) were prepared and incubated as described in the text. After incubation at 30°C for 15 min, two 100-µl samples were taken, filtered through membrane filters (Millipore Corp.), washed, and counted. A blank value of 227 cpm obtained from a binding assay run in the absence of protein has been subtracted. The insert shows the double-reciprocal plot of the data.*

tion used for this experiment is not homogenous, it was conceivable that the binding results from nonspecific interaction of contaminating proteins. Therefore, we examined the RNA binding capacity of a corresponding DEAE-purified protein preparation from mock-inoculated leaves. This protein preparation completely failed to bind [^{32}P]CPMV RNA (Fig. 7). These data clearly demonstrate that the retention of the CPMV RNA is specific for the replicase preparation and most likely involves the binding of the polymerase to the binding site(s) on the RNA. This conclusion is supported by the finding that complex formation was inhibited about 50 and 80% by high salt (0.2 M KCl) and aurintricarboxylic acid (10 µM), respectively, both agents which were also inhibitory in the polymerase reaction. Aurintricarboxylic acid, a triphenylmethane dye, has been shown to inhibit Qβ replicase (3) as well as other RNA and DNA polymerases (3, 13) by binding to the template binding sites on the polymerases, thereby preventing template binding and initiation.

(ii) Competition binding assay. On the basis of the binding assay, it became possible to reinvestigate the possible template specificity of CPMV replicase by performing competition binding assays. Thus, increasing amounts of unlabeled heterologous RNA species were mixed with a constant amount of [^{32}P]CPMV RNA and then presented to the replicase preparation. By determination of the amount of [^{32}P]CPMV RNA retained on the filter, the ability of heterologous RNAs to compete with CPMV RNA for binding to the replicase was measured. A control experiment, showing homologous RNA to be an efficient competitor, demonstrated the validity of this approach (Fig. 8A).

The RNAs from brome mosaic virus, tobacco streak virus, TYMV, and Semliki Forest virus, added up to a 50-fold molar excess of [^{32}P]CPMV RNA, completely failed to compete with CPMV RNA in binding to the replicase (Fig. 8B). On the other hand, the RNA of RaMV, which is also a member of the comovirus group (5), was capable of competing with CPMV RNA quite efficiently (Fig. 8B). These data clearly show that CPMV replicase, if challenged with mixtures of different RNA species, recognizes and selects CPMV RNA and the closely related RaMV RNA.

To determine whether the poly(A) tract of the CPMV genome was involved in the binding of the replicase, a series of competition binding reactions was carried out, in which synthetic poly(A) and poly(U) were used as competitors. About 1 µg of poly(A) added to 1 µg of [^{32}P]-CPMV RNA was able to reduce the labeled complex with 50%, whereas only 20% of the labeled complex was formed in the presence of 20 µg of poly(A) (Fig. 9A). Complex formation was also inhibited by poly(U) (Fig. 9A), probably, in part, in consequence of hybrid formation with the poly(A) on the viral genome. From the double-reciprocal plots (Fig. 9B) it was calculated that 0.64 µg of poly(A) or 3.75 µg of poly(U) per 0.24 ml, respectively, was required to obtain half of the maximal competition, using 1 µg of CPMV RNA.

In contrast to the strong decrease in complex formation caused by either poly(A) or poly(U), poly(C) displayed a much lower competition ability (Fig. 9A). At about a 300-fold molar excess of poly(C), 70 to 75% of the replicase-CPMV RNA complex remained.

DISCUSSION

Although we have not succeeded so far in purifying CPMV replicase to homogeneity, the template dependence in addition to the great stability of the replicase preparations prompted us to characterize the replicase reaction with special emphasis on the template recognition. This was of interest since animal and plant virus RNA replicases isolated so far lack stringent template specificity, as judged by the ability of a wide variety of viral and nonviral RNA species to direct the incorporation of labeled ribonucleotides (4, 6, 14, 21, 23, 25, 34, 36, 40). Thus, the

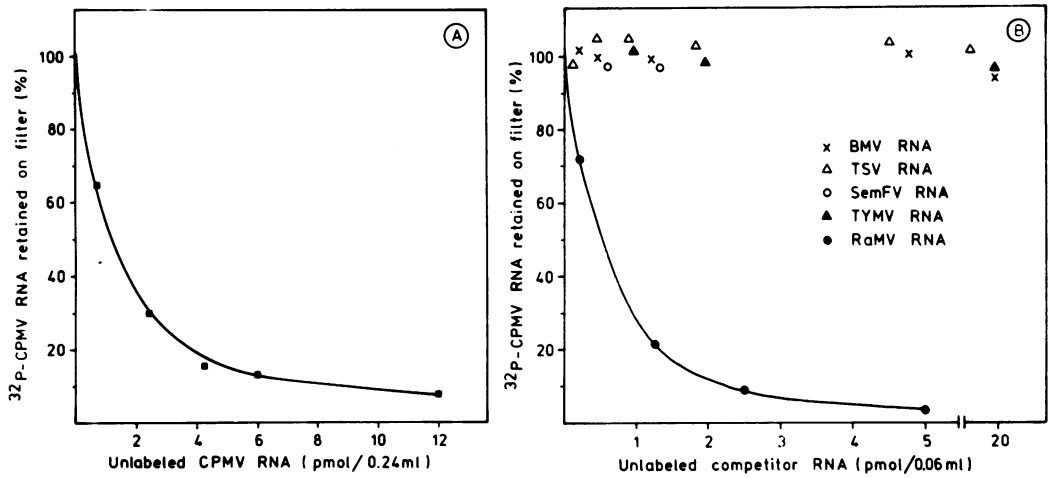

FIG. 8. *Competition between [^{32}P]CPMV RNA and either CPMV RNA, brome mosaic virus (BMV) RNA, tobacco streak virus (TSV) RNA, Semliki Forest virus (SemFV) RNA, TYMV RNA, and RaMV RNA for binding to CPMV replicase. (A) Binding assays (0.24 ml) containing 0.6 pmol (1 μg) of [^{32}P]CPMV RNA (21,400 cpm/μg), varying amounts of unlabeled CPMV RNA as specified, and 16.5 μg of protein were prepared and incubated as described in the text. [^{32}P]CPMV RNA was mixed with the unlabeled CPMV RNA before the addition of replicase. After incubation at 30°C for 15 min, duplicate 100-μl samples were taken, filtered through membrane filters (Millipore Corp.) washed, and counted. (B) Binding assays (0.06 ml) containing 0.29 pmol of [^{32}P]CPMV RNA (43,000 cpm/μg), varying amounts of unlabeled RNA species as specified, and 5 μg of protein were prepared and incubated as described in the text. [^{32}P]CPMV RNA was mixed with the unlabeled species before the addition of replicase. After incubation at 30°C for 15 min, the reaction mixtures were filtered through Millipore filters, washed, and counted.*

reliability of the replicase preparations for the analysis of virus RNA replication in vitro has been questioned. To become acquainted with the basic properties of CPMV replicase, we have initially studied CPMV RNA-directed [^{3}H]UMP incorporation under various incubation conditions, thus providing a well-defined basis on which the studies on the template specificity could be performed. As far as the incorporation of ribonucleotides is concerned, our results on the template activity of various RNA species do not differ significantly from those obtained with several other replicase preparations. However, using the nitrocellulose membrane filter assay, our results on the recognition of template RNAs by partially purified CPMV replicase show the contrary. Viral RNAs, which, to different extents, all stimulated the incorporation of [^{3}H]UMP, appeared to be unable to compete with CPMV RNA in the binding reaction, except for the RNA from RaMV, another member of the comovirus group. This indicates that the CPMV replicase preparation harbors a protein binding preferentially to CPMV RNA. Whether this protein is identical to the replicase itself is not yet proven, and its identification has to await further purification. In our opinion, however, the following observations strongly favor the replicase as being the protein involved in the specific binding of CPMV RNA. (i) The polymerase reaction as well as the binding reaction were both inhibited by high ionic strength (0.2 M KCl) and aurintricarboxylic acid (10 μM), a triphenylmethane dye known to inhibit template binding and initiation of nucleic acid synthesis (3, 13). (ii) No CPMV coat proteins could be detected in the replicase preparations by polyacrylamide gel electrophoresis under denaturing conditions. (iii) No binding of CPMV RNA occurred in the presence of corresponding protein preparation from uninfected leaves.

Even if we assume that it is not the replicase itself that is responsible for the RNA binding, the presence per se in the replicase preparation of a CPMV RNA specific binding protein distinct from coat protein strongly supports the existence of a virus-coded protein, somehow involved in virus replication. In considering that (i) the CPMV replicase preparation derives from a cellular membrane fraction, previously shown to be involved in virus-specific RNA synthesis in vitro (39) as well as in vivo (1, 8), and (ii) plants infected with a temperature-sensitive mutant of CPMV do not contain replicase activity at the nonpermissive temperature (7), our results presented in this report do not favor the idea of virus RNA replication mediated only by a host RNA-dependent RNA polymerase, as suggested by Ikegami and Fraenkel-Conrat (18). At this stage, however, we cannot completely

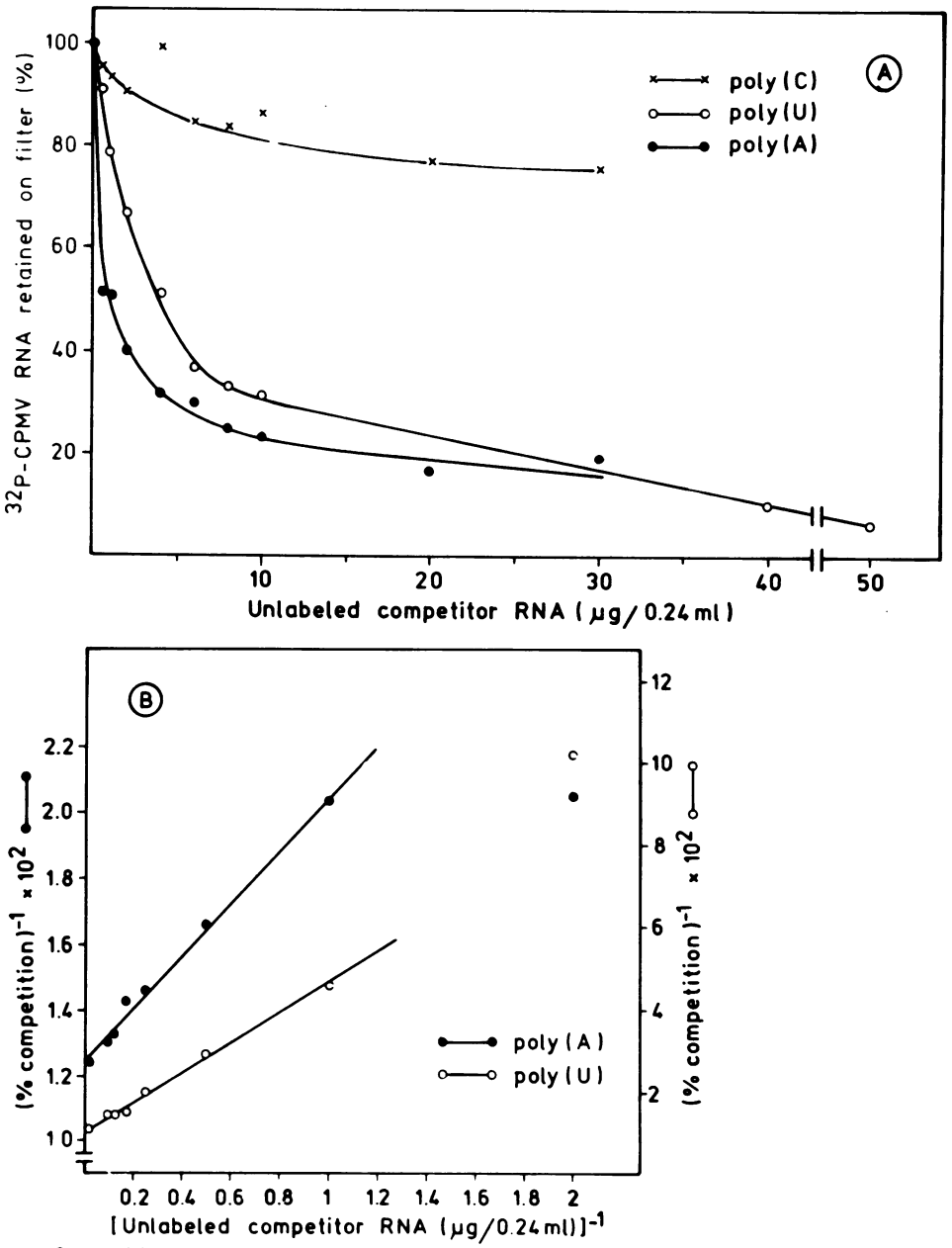

FIG. 9. *Competition between CPMV RNA and poly(A), poly(U), or poly(C) for binding to CPMV replicase. (A) Binding assays (0.24 ml) containing 1 μg of [^{32}P]CPMV RNA (18,000 cpm/μg), varying amounts of unlabeled polynucleotide, as specified, and 16.5 μg of protein were prepared and incubated as described in the text. [^{32}P]CPMV RNA was mixed with the unlabeled polynucleotides before the addition of replicase. After incubation at 30°C for 15 min, duplicate 100-μl samples were taken, filtered through membrane filters (Millipore Corp.), washed, and counted. (B) Double-reciprocal plots of the data.*

rule out the possibility that the replicase is composed of a core polymerase encoded by the host and a virus-coded subunit indispensable for specific template recognition (30).

It is not known why heterologous templates which are unable to compete in the binding reaction stimulate the incorporation of [^{3}H]-UMP. In this respect, it is of interest to mention that the formation of a single phosphodiester bond has been shown to stabilize the complex between DNA and the RNA polymerase from *E. coli* (32). Therefore, it is quite conceivable

that in the replicase assay, the replicase forms very labile complexes with the heterologous templates, complexes which are not viable but which become stabilized by allowing the replicase to incorporate ribonucleotides, thereby artificially increasing the affinity of the replicase for these templates. It can be proposed that RNA chain initiation occurs nonspecifically and on numerous sites along heterologous templates but specifically and on one site of CPMV RNA. We are currently investigating the initiation specificity of CPMV replicase. Using the same nitrocellulose filter binding technique, Mouchès et al. (24) have also described preferential recognition of homologous and related template RNA by TYMV replicase, although in that case the unrelated TMV and brome mosaic virus RNA appeared to be able to compete for binding with TYMV for about 40 and 50%, respectively, at a fivefold weight excess. In view of the competition binding data obtained with synthetic poly(A) and poly(U), it should be kept in mind that the poly(A) tail on the viral RNA comprises only about 2% of the complete genome. Taking into account the amount of homopolymers required to compete with CPMV RNA, it will be evident that the affinity of the replicase for CPMV RNA far exceeds that for synthetic poly(A), indicating that the poly(A) tract on the CPMV RNA is not the only part of the genome involved in replicase binding. The cooperation of other sites on the CPMV genome appears to be required in binding the replicase properly and specifically. From the efficient competition ability of RaMV RNA, it may be concluded tentatively that these two comovirus RNA genomes have some structural features in common, specifying the binding site(s) for the replicase.

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