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Efficient reverse transcription of cowpea mosaic virus RNAs

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ABSTRACT

Conditions are described which give an efficient synthesis of DNA copies of cowpea mosaic virus (CPMV) RNAs, using avian myeloblastosis reverse transcriptase and oligo(dT) primers. Maximum incorporation of dAMP into cDNA is attained with 0.4 to 0.8 mM of each deoxynucleoside triphosphate, 12 mM Mg^{++} and 60 mM K^{+} ion concentrations. High enzyme concentrations (up to 100 units/ml) were used. Under these conditions over 1000 pmoles of dAMP were incorporated per reaction. The cDNA:RNA molar ratio approached 0.3 when 1 pmole CPMV RNA was used as template.

The products were heterogeneous but large. Bottom component RNA (about 6000 nucleotides long) was copied into cDNA molecules ranging from about 1000 to 4000 nucleotides, and middle component RNA (about 4000 nucleotides long) was copied into cDNA mostly between 500 and 2000 nucleotides long, on average about 1500, which can be cleaved by restriction endonuclease Hae III into two fragments of 880 and 540 nucleotides.

INTRODUCTION

Avian myeloblastosis virus RNA-dependent DNA polymerase (reverse transcriptase) can be used with an oligo dT primer to synthesize DNA complementary to an RNA which has a 3' terminal poly(A) sequence. RNAs with which this has been investigated include eukaryote mRNA such as that of globin genes (1, 2), RNAs of tumour viruses (3, 4), and poliovirus RNA (5, 6).

Like poliovirus, the two RNAs of cowpea mosaic virus (CPMV) are possibly translated into large precursor polypeptides (7), have no 5' terminal cap structure (8) and have 3' terminal poly(A) sequences (9). These characteristics are almost unique among plant viruses. We are investigating the structure and function of these nucleic acids, for which *in vitro* synthesis of cDNA would be an invaluable asset. DNA copies of the viral genome can be used as hybridization probes

in the search for possible sub-genomic messengers or relationships with other viruses; DNA rapid sequencing methods (10, 11, 12) can be applied thus providing alternative approaches to RNA sequence studies. Restriction enzyme mapping and construction of bacterial plasmids carrying a cDNA insert are also feasible.

Reverse transcription of CPMV RNA has been reported (13), and some of the cDNA approached the full-length size of the RNAs, at least of the M-RNA. However, the two RNAs were not studied individually. We have tested each of the two CPMV RNAs separately, have increased the cDNA synthesis about 120 fold, and describe conditions suitable for the synthesis of cDNA more than 1000 bases long, including molecules of up to 4000 bases.

MATERIALS AND METHODS

Purification of virus and RNA

Cowpea mosaic virus (CPMV) yellow (Nigerian) strain, was grown in Vigna unguiculata (L) variety "Blackeye Early Ramshorn" and purified as described previously (8, 14). The middle and bottom (M and B) virion components were separated by cesium chloride density gradients (1.31 to 1.48 g/ml) in 50 mM Tris-HCl, pH 8.0 buffer. The isolated components were dialysed to remove CsCl and pelleted through 40% sucrose, in the same buffer. The resuspended pellets were dialysed against 50 mM Tris-HCl or Tris-borate buffer pH 8.8 or 9.0. The virions were disrupted by adding an equal volume of 4% sarkosyl NL 97, 2% sodium tri-isopropylnaphthalene sulphonate, 0.2 M NaCl, 0.02 M EDTA, 0.02 M Tris, and heating at 50° for 5 min. The RNA was purified by three phenol (saturated at pH 8.8 or 9.0) extractions, the first of which was with 1:1 chloroform-phenol. The RNA was precipitated with two volumes of ethanol at -20°, and washed three times with 95% ethanol at 0°. The RNA precipitate was dried and then dissolved in water.

If the initial virion separation in CsCl was particularly good, further purification of the RNA was not essential. However, usually a sucrose gradient purification was also done (8) to check the purity of RNAs and to remove any breakdown products.

Avian myeloblastosis reverse transcriptase

The enzyme was gratefully received as a gift from Dr. J.W. Beard (Life Sciences Inc., St. Petersburg, Fl., U.S.A.) via Dr. J. Gruber (National Cancer Institute, Bethesda, MD., U.S.A.). Aliquots were diluted to 1000 to 4000 U/ml in 25 to 50% glycerol, 50 to 100 mM potassium phosphate buffer pH 7.2, 0.5 to 1 mM DTT and 0.05 to 1% Triton X-100, and stored in small volumes at -80°.

cDNA synthesis

Initially, conditions were based on those described previously for CPMV RNA (13) and poliovirus RNA (5,6), and consisted of: 50 mM Tris-HCl pH 8.4 (20°), 50 mM KCl, 5 to 8 mM $MgCl_2$, 0.4 to 4 mM dithiothreitol (DTT), 0.2 mM dTTP, dCTP, dGTP, dATP, oligo$(dT)_{12-18}$ 5 μg/ml, RNA 50 to 80 μg/ml, and reverse transcriptase, 10 units/ml. The final volume was 100 μl and incubation was at 37° for 1 hr.

Throughout the course of experiments of the type described in the text (see Figs. 2-6), modifications were made to the system. Our improved conditions finally consisted of: 50 mM Tris-HCl pH 8.4, 60 mM KCl, 12 mM $MgCl_2$, 1 mM DTT, 0.4 to 0.8 mM dNTPs, oligo$(dT)_{10}$ 2 to 5 μg/ml or oligo$(dT)_8$dA 4 to 10 μg/ml; RNA at 60 to 120 μg/ml (40 to 60 pmoles/ml), 100 units reverse transcriptase/ml, actinomycin D at 100 μg/ml and bovine serum albumin (BSA) 50 to 100 μg/ml. The final volume was 50 μl; incubation was for 40 min.

The enzyme was diluted from its stock solution (see above) by about 1/50, thus this contributed 0.5 to 1% glycerol, 1 to 2 mM potassium phosphate buffer, 0.01 to 0.02 mM DTT and 0.001 to 0.002% Triton X-100, to each reaction mixture.

Tritiated dATP or dTTP (usually about 5 μCi) contributed about 0.004 mM of that dNTP in addition to the concentration of unlabelled dNTP quoted above (α-^{3}H-dATP, α-^{3}H-dTTP 20 to 25 Ci/mmole; Radiochemical Centre, Amersham). α-^{32}P-dATP was 175 to 350 Ci/mmole. These isotopes were supplied in 50% ethanol. This was removed by evaporation in a nitrogen gas stream, or by drying aliquots in the reaction tubes before each incubation.

For the analysis of incorporation of ^{3}H dNTPs, 25 μl aliquots of reactions were spotted onto Whatman 3MM filters, which were washed three times in cold (5-10°) 10% trichloroacetic acid (approximately 50 ml solution per filter per wash). The filters

were held in a perforated holder in a magnetically stirred percolator. The first two washes were for 30 min each, the third for 10 min. This was repeated with 95% ethanol.

The filters were dried and incubated at 60° for 1 hr in Soluene 350 (Packard) then scintillation counted in Instafluor (Packard) in a Packard Tri-Carb 3375. Counting efficiency for tritium was about 33%.

Analysis of cDNA products

Reactions were stopped by the addition of 1/10 volume 100 mM EDTA. About 50 μg ribonuclease A (previously heated to 80° to destroy deoxyribonuclease activity) was added and incubated at 37° for 10 min. This was to digest RNA not hybridized to DNA.

Protein was then extracted with phenol (pH 8). After an ether extraction, the aqueous phase was made to about 0.2 M ammonium acetate (or sodium acetate if alkaline gels later to be used), and 10 μg wheat germ tRNA or yeast RNA added before precipitating with ethanol (see above). The nucleic acid pellets were redissolved in water.

Aliquots of 20 μl or less were mixed with 5 μl of a 20% ficoll solution, and loaded into 5 x 2 mm slots (3 mm deep) in 120 x 120 x 4 mm horizontal agarose (0.7 or 1%) gels. Electrophoresis was for 1 to 2 hr at 30 V (60 mA) using a buffer of 40 mM Tris-acetate, pH 7.7 and 1 mM EDTA (15) and containing 0.5 μg ethidium bromide per ml (16). Non-radioactive marker DNAs were visualized by ethidium bromide fluorescence under ultra violet illumination. Gels were then soaked for at least 2 hrs in 7% acetic acid, and for 1 hr in water, to remove ^{32}P-labelled mononucleotides not incorporated into DNA. Gels were then dried *in vacuo* and autoradiographed using Kodak Safety IRD or Sakura A 7C film.

As the electrophoretic mobility in 1% agarose of both the double-stranded and single-stranded DNA markers used were almost identical, the size of single-stranded cDNA products (and DNA-RNA hybrids) was calculated from the mobility of double-stranded DNA markers.

Treatment of single-stranded cDNA with restriction endonuclease Hae III (BioLabs, New England) was performed in 6 mM Tris-HCl pH 7.4, 6 mM NaCl, 6 mM $MgCl_2$, and 6 mM β-mercaptoethanol, using 4 units of enzyme per μg DNA, at 37° for 1 hr.

Preparation of labelled marker DNAs

Bacteriophage λ DNA Hind III restriction nuclease fragments (Boehringer) were labelled as follows.

To 10 µl DNA (2 pmoles) was added 20 µl 0.5 M Tris-HCl pH 8.0, 5 µl (0.5 units) E. coli alkaline phosphatase (P&L Biochemicals Inc., Milwaukee, Wisc., U.S.A.) and 65 µl water. After 30 min at 37°, the reaction was stopped by adding NTA (nitrilo-triacetic acid) to a concentration of 8 mM. Following a further 10 min incubation at 37°, the mixture was heated to 100° for 3 min. Tris-HCl (100 mM pH 8.0) containing 1 mM EDTA was then added and the protein extracted (three times) with phenol saturated with the same buffer. The aqueous layer was extracted with ether and the DNA precipitated with ethanol (at -20° in the presence of 0.25 M ammonium acetate).

Labelled α-^{32}P-dATP was dried in a small plastic reaction tube (about 4 pmoles if 2 pmoles DNA 5' ends. Specific activity 250 Ci/mmole). The dried 5' dephosphorylated DNA was redissolved in 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ with 6 mM β-mercaptoethanol, and added to the α-^{32}P-dATP along with 2.8 units of T4 polynucleotide phosphokinase (P&L Biochemicals). The mixture was incubated at 37° for 30 min, then passed through a Sephadex G-25 column. The large molecular weight material just after the void volume was then precipitated and washed with ethanol, dried, and dissolved in water.

RESULTS

The activity of the enzyme using poly (A) as a template with oligo(dT)$_{10}$ primer

Under the conditions used for these initial experiments (see Materials and Methods), very efficient synthesis of poly(dT) occurred, over 1000 pmoles dTMP being incorporated in a 50 µl reaction (Table 1). This activity was stimulated slightly by DTT and BSA.

CPMV RNA as a template under conditions suitable for reverse transcription of poly (A)

Under similar conditions, CPMV RNA was also a good template but 2 to 3 times less active than poly (A), as measured by dTMP incorporation. The incorporation of dAMP into CPMV cDNA was 4 to 5 times less than the dTMP incorporation. However, the results in Table 1

TABLE 1. Activity of the reverse transcriptase with a poly (A) template[1].

	Reaction mixture	cpm/25 µl	pmoles dTMP	pmoles dAMP
Exp. I	complete	698,215	1,536	-
	without BSA	598,802	1,317	-
	without DTT	385,799	849	-
	without enzyme	741	2	-
Exp. II	complete	315,778	1,926	-
	complete	6,167	-	37
	CPMV RNAs	114,044	695	-
	CPMV RNAs	23,763	-	145

[1]The reaction mixture contained poly (rA), and where indicated CPMV RNAs, at a concentration of 100 µg/ml. The 50 µl reaction also contained 5 mM $MgCl_2$, 50 mM KCl, 0.25 µg oligo$(dT)_{12-18}$, and 1 unit of reverse transcriptase (1 unit is defined as that amount of enzyme giving an incorporation of one nanomole dTMP into acid precipitated material, using poly (rA) as template, in 10 min at 37°). All four dNTPs, 0.2 mM. In Exp. II, about half as much ^3H dNTP was used as in Exp. I. Complete reactions contained 1 mM DTT and 50 µg/ml BSA. Final volumes 50 µl. 25 µl aliquots were counted, but incorporation in pmoles is given as pmoles per 50 µl (total) reaction.

also show that a reasonable amount (over 100 pmoles) of dAMP was incorporated. These data are consistant with the possibility that some of the CPMV RNA sequence is copied, but that much of it consists of short copies from the 3' end poly (A) region, with little synthesis of extensive sequences of the CPMV RNA. (The average A:U ratio of CPMV RNA is 28:31). The size of these products was analysed on sucrose gradients and agarose gels (not shown). Much of the cDNA consisted of short transcripts (probably about 50 to 500 nucleotides long).

We wished to improve the conditions such that CPMV RNA-directed dAMP incorporation approached that of dTMP incorporation, and longer transcripts were made.

The effect of bovine serum albumin and dithiothreitol on dAMP incorporation into CPMV cDNA

Fig. 1 shows the effect of BSA and DTT on the activity of reverse

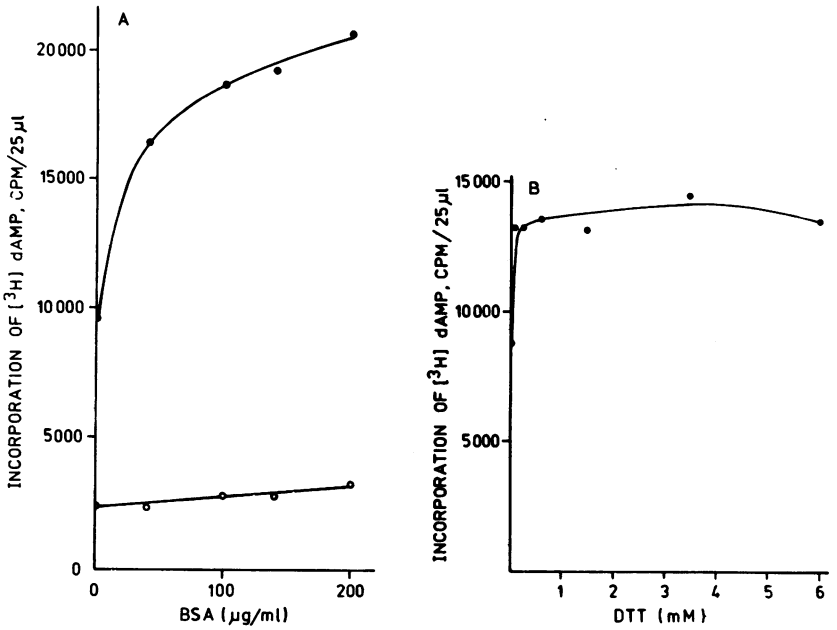

Fig. 1. The effect of bovine serum albumin and dithiothreitol. The reaction mixtures were as for Table 1. A. Bovine serum albumin concentration curve. • CPMV RNAs M + B, 100 µg/ml. o No RNA. B. Dithiothreitol concentration curve. These reactions contained BSA at 50 µg/ml.

transcriptase with CPMV RNAs. A concentration of 1 mM DTT was chosen for further experiments, and BSA was included at 100 µg/ml although in later experiments with much higher enzyme concentrations, its effect was less marked. These reactions contained actinomycin D (100 µg/ml), as did all further incubations.

The optimum concentration of deoxynucleoside triphosphates

The concentration of the mixture of all four deoxynucleoside triphosphates (dNTPs) was varied. The optimum concentration for ^{3}H dAMP incorporation was 0.8 mM of each dNTP (Fig. 2A), but even at 0.4 mM, 80% of the maximum was attained. Most further experiments were performed with 0.4 mM dNTPs, or higher (up to 1 mM).

Variation of the oligo(dT) primer concentrations, and comparison with other oligo(dN) primers

It would be expected that if indeed the reverse transcription com-

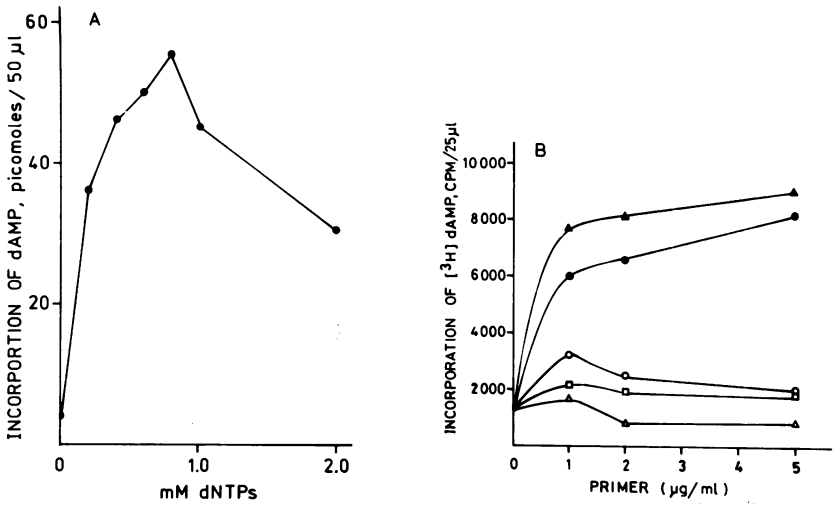

Fig. 2. The optimum deoxynucleoside triphosphate concentration, and the effect of concentration of several primers. The reaction mixtures were as for Table 1, with CPMV RNAs M + B at 100 µg/ml, and BSA 100 µg/ml. A. Using 5 µCi of ^{3}H dAMP (20 Ci/mmole), the concentration of all four unlabelled dNTPs was varied. Oligo(dT)$_{12-18}$ was used as primer. B. At 0.2 mM dNTPs, the concentration and type of primer was varied. ▲ oligo(dT)$_{10}$, ● oligo(dT)$_{12-18}$, o oligo(dC)$_{12-18}$, □ oligo(dG)$_{12-18}$, △ oligo(dA)$_{12-18}$.

mences on the poly (A) region of CPMV RNA, then oligo(dT) should prime far better than oligo(dC), (dG) or (dA), which would not prime unless there are complementary oligo sequences in the RNAs. Fig. 2B shows that oligo(dT) is an efficient primer, oligo(dT)$_{10}$ being marginally better than oligo(dT)$_{12-18}$. The activity increases little with concentrations above 1 µg primer/ml, with an RNA (M + B) concentration of 50 µg/ml. This is equivalent to about 30 pmoles oligo(dT) and 1 to 2 pmoles RNA. Oligo(dT) at 1 µg/ml in fact gave 75% the activity due to 5 µg/ml, was used for most further experiments. Oligo(dA) did not act as primer. Oligo(dG) and oligo(dC) primed a very low level of incorporation. It cannot be ruled out that an oligo (or even poly) C or G tract or at least a C or G rich region, occurs not far from the 5' end of the RNA, resulting in small 5' end cDNA copies of RNA. This has not yet been further investigated.

Dependency of the reaction upon enzyme concentration, primer and CPMV RNAs

Using a 1 μg/ml concentration of oligo(dT)$_{12-18}$ as primer, a dependency enzyme and its concentration was shown (Table 2). The activity was similar for both B-RNA and M-RNA, but negligible in their absence. Although without RNA or enzyme, about 900 cpm were detected on filters (see Table 2), this level of activity was regularly found with zero-time controls in other experiments, and we believe it to be 'background' radioactivity (such as entrapped, unincorporated dAMP). Unless the washing procedure described in Materials and Methods was rigorously followed, this background was higher. All further results are corrected for zero time controls.

The optimum potassium and magnesium ion concentrations

The synthesis of CPMV reverse transcripts was not completely dependent on K^+ ions but was stimulated, with a maximum effect at about 60

TABLE 2. Dependency of CPMV cDNA synthesis on enzyme concentration, primer and CPMV RNAs.

Template[1]	Reaction mixture[2]	Incorporation of dAMP cpm/25 μl
B-RNA	complete; enzyme 48 U/ml	25,981
	8 U/ml	11,086
M-RNA	complete; enzyme 48 U/ml	24,874
	8 U/ml	7,113
Both RNAs	complete; enzyme 48 U/ml	16,718
	24 U/ml	10,876
	16 U/ml	8,517
	8 U/ml	4,812
	without primer	1,789
None	complete	961
	without enzyme	891

[1] RNAs were at a concentration of 50 μg/ml, with oligo(dT)$_{12-18}$ at 1 μg/ml.

[2] The reaction mixture also contained 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 1 mM DTT, 100 μg/ml BSA, and 100 μg/ml actinomycin D. Reactions without primer or template contained reverse transcriptase at 48 U/ml.

mM KCl (Fig. 3A), which was used in further experiments. The maximum effect of increasing the Mg^{++} concentration was reached by 4 mM (Fig. 3B), with either $MgCl_2$ or $Mg(CH_3COO)_2$. However, it is known that Mg^{++} and NTPs can complex causing interdependent concentration effects. Having established the NTP concentration response (Fig. 2A) and that of Mg^{++} ions (Fig. 3B), it was necessary to re-evaluate one with respect to the other. An example of such an experiment is given in Fig. 4. It was confirmed that at 0.2 mM dNTP concentration, the Mg^{++} ion optimum was about 4 mM, but at higher dNTP concentrations (0.4 mM to 0.6 mM) the Mg^{++} ions optimum was about 12 mM. This concentration was used for further experiments.

The rate of cDNA synthesis, and the effect of high enzyme concentrations

The time course of the reaction is shown in Fig. 5A. Most of the cDNA synthesis occured in the first 30 min, in the presence of actinomycin D.

We had shown (Table 2) that the cDNA synthesis increased with increasing enzyme concentrations, at least up to 48 units/ml. We inves-

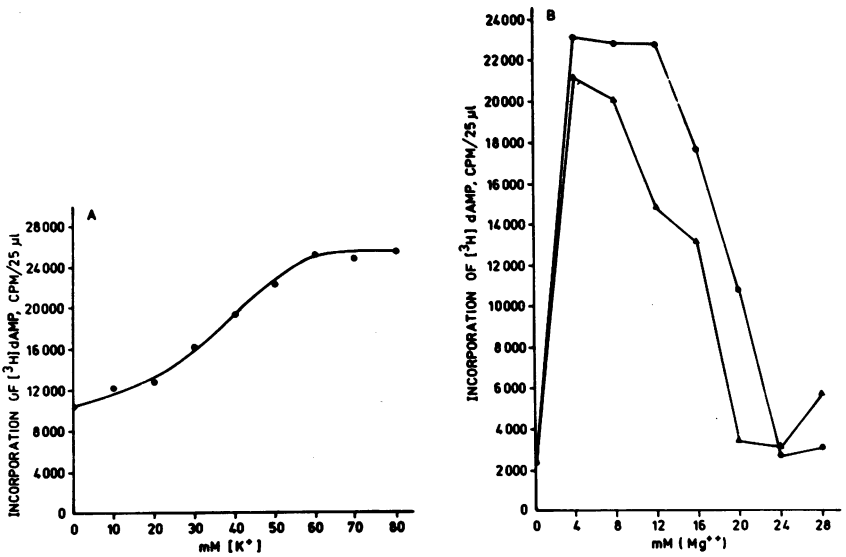

Fig. 3. The optimum potassium and magnesium ion concentrations. The reaction mixtures were as for Table 1, but using 0.4 mM dNTPs, and enzyme concentration of 20 units/ml, oligo$(dT)_{12-18}$ 2 µg/ml and BSA 100 µg/ml. A. Potassium (KCl) concentration curve. B. Magnesium concentration curves. ● $MgCl_2$ ▲ $Mg(CH_3COO)_2$.

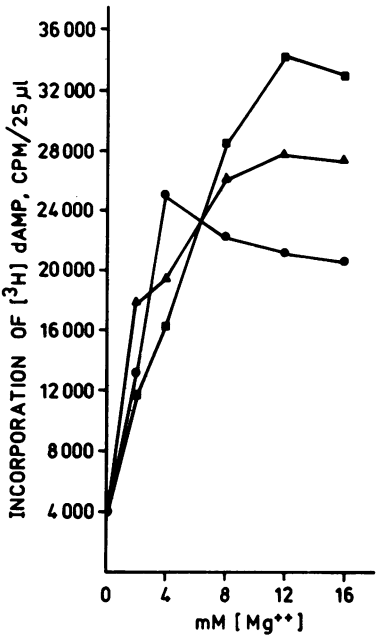

Fig. 4. The magnesium ion optimum concentration at several deoxynucleoside triphosphate concentrations. The incubation reactions were as for Fig. 3, but with 60 mM KCl, an enzyme concentration of 50 units/ml, and CPMV RNAs M + B at 60 µg/ml. Tritiated dATP was kept at a constant specific activity of 0.5 Ci/mmole. ● 0.2 mM dNTPs, ▲ 0.4 mM dNTPs, ■ 0.6 mM dNTPs.

tigated the effect of raising the enzyme concentration even higher, in 30 min reactions. This is shown in Fig. 5B. The activity increased almost linearly up to 5 units per 50 µl reaction (100 units/ml concentration). Probably the activity could be further increased with even more enzyme, but since 100 units/ml gives an excellent level of cDNA synthesis, we chose this concentration for further investigations although we are aware that it is not maximal.

The effect of CPMV RNA concentration, and the cDNA:RNA ratio

Most experiments so far described were with 50 to 60 µg/ml concentration of CPMV RNA. The effect of CPMV RNA concentration was investigated at optimal ionic conditions and high dNTP and high enzyme concentrations. The concentration giving maximum cDNA synthesis from the mixture of both RNAs was 160-360 µg/ml (Fig. 6A). For B-RNA it was about 40 µg/ml, and for M-RNA 100 µg/ml. This is equivalent to

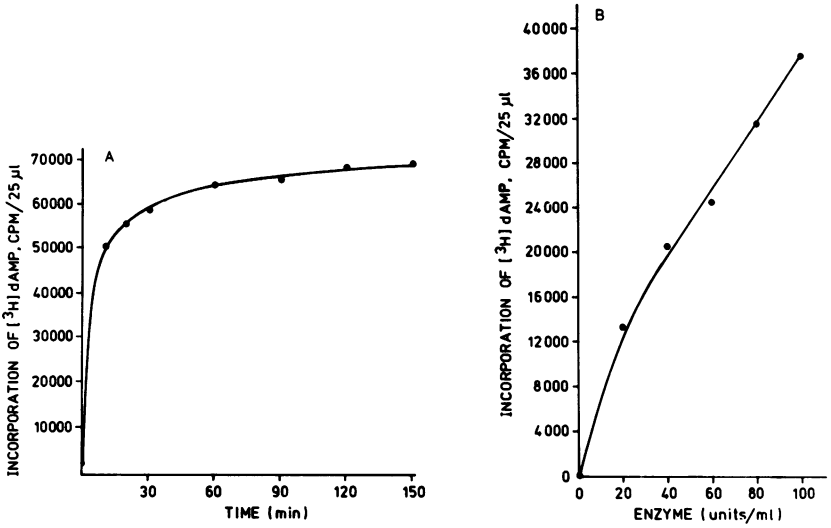

Fig. 5. The reaction kinetics and enzyme concentration curves. A. A 250 µl reaction was used, and 25 µl aliquots were taken at the times indicated. The reaction contained 12 mM $MgCl_2$, 60 mM KCl, enzyme at 80 units/ml, oligo$(dT)_{10}$ 2 µg/ml, CPMV RNAs M + B 100 µg/ml, 0.8 mM dNTPs and actinomycin D at 100 µg/ml. B. The reaction (50 µl) was as in A, except that 60 µg CPMV RNA/ml was used with oligo$(dT)_{12-18}$ as primer and enzyme as indicated.

about 1.0 and 3.6 pmoles per 50 µl reaction for B and M RNAs respectively. However, the template copying was more efficient at low concentrations (Fig. 6B). The molar ratio cDNA:RNA approached a value of 0.3 with 40 µg/ml (1 pmole/50 µl) of B-RNA, and 20 µg/ml (1.5 pmole/50 µl) of M-RNA.

Efficiency of dAMP and dTMP incorporation into CPMV cDNA

Using CPMV RNA concentrations of 60 to 150 µg/ml, we regularly obtained an incorporation of over 1000 pmoles dAMP per 50 µl reaction, similar to the incorporation of dTMP. An example is shown in Table 3. The T:A in this cDNA is almost 1:1 ratio, as is the A:U ratio of the RNA. This is in contrast to the results of Table 1, when the T:A was about 4:1. This was an indication that the improved conditions perhaps allow more extensive synthesis of cDNA molecules.

Reverse transcription of CPMV RNAs with a specific primer

Our initial primer concentration studies (Fig. 2B) indicated that

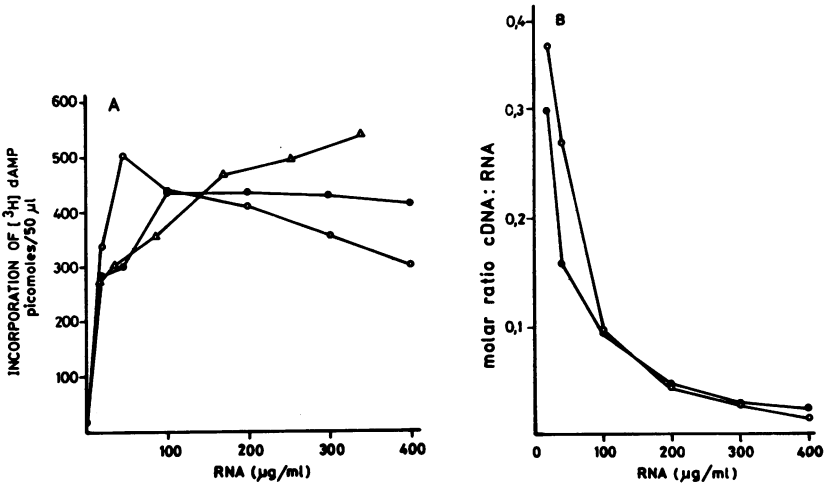

Fig. 6. The effect of CPMV RNA concentration and the cDNA:RNA ratio. A. The reaction mixtures were as for Fig. 5; using oligo$(dT)_{10}$ as primer. CPMV RNAs were varied as indicated. o B-RNA, • M-RNA, △ M + B RNAs. B. The reaction was the same as A. The amount of cDNA synthesized was calculated from the molar amount of dAMP incorporated and the $_{d}A\%$ (base ratio) assuming CPMV RNAs to have 31% U, and that they are copied accurately their full-length. The cDNA (synthesized) to RNA (input) ratio was then calculated.

1 µg/ml was sufficient for an efficient priming, but that higher levels would increase the activity. We re-evaluated this effect with

TABLE 3. Efficiency of CPMV RNA reverse transcription

Reaction mixture[1)]	pmoles incorporated dAMP	dTMP
without enzyme	4	-
without template	52	75
both CPMV RNAs, 60 µg/ml	966	-
both CPMV RNAs, 60 µg/ml	-	1320
both CPMV RNAs, 100 µg/ml	995	-
B-RNA, 150 µg/ml	1170	-
M-RNA, 115 µg/ml	1330	-

[1)]The reaction mixtures contained 1 mM DTT, 100 µg BSA/ml, 60 mM KCl, 12 mM $MgCl_2$, 0.4 mM dNTPs, 100 U reverse transcriptase/ml, 2 µg oligo$(dT)_{10}$/ml and 60 µg CPMV RNAs/ml in a final volume of 50 µl.

the optimal reaction conditons, using high concentrations of B and M-RNAs separately which gave a high yield of cDNA. We also used oligo $(dT)_8dA$ as primer, since for both RNAs, the two nucleotides adjacent to the poly A sequence are U (Stanley et al., unpublished results). A result is shown in Fig. 7. An efficient synthesis is attained with 4 μg/ml (120 pmoles/ml), but increases at least up to 20 μg/ml. In this experiment, the RNA concentrations were 100 μg/ml, equivalent to about 73 pmoles/ml and 100 pmoles/ml B-RNA. Thus, the efficient synthesis requires an oligo$(dT)_8dA$:CPMV RNA molar ratio of about 120 or more. (Oligo$(dT)_{10}$ or oligo$(dT)_{12-18}$ were required at about 15 to 30 molar ratio to template, see above). Oligo$(dT)_7dAdA$ was also an efficient primer, and this was used for some product analysis experiments.

Analysis of the products of CPMV RNA reverse transcription

DNA was purified from the reaction mixture by phenol extraction, followed by ethanol precipitation (see Materials and Methods). Aliquots were analysed on agarose gels. Fig. 8 shows the cDNA products of CPMV B and M-RNAs primed with oligo$(dT)_{10}$, using a concentration of 0.4 mM of all four NTPs. The products were heterogenous but large.

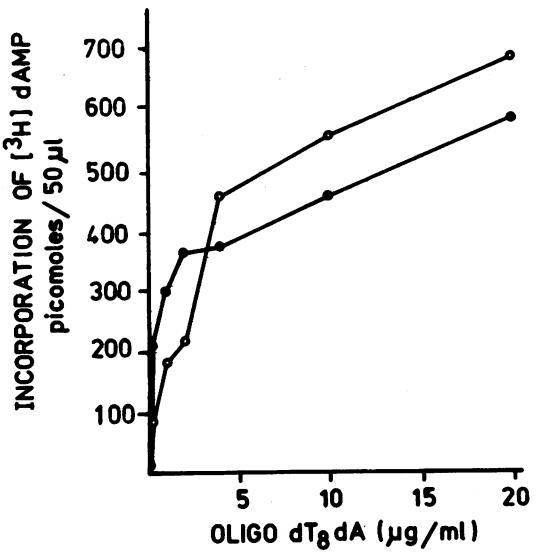

Fig. 7. Reverse transcription of CPMV RNAs primed with oligo$(dT)_8dA$: the primer concentration curves. The reaction mixtures were as for Fig. 5. • M-RNA (100 μg/ml). o B-RNA (100 μg/ml).

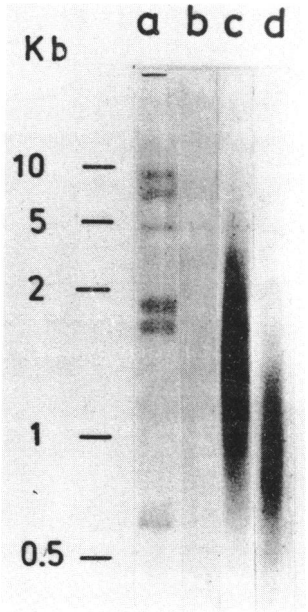

Fig. 8. Analysis of CPMV M and B-RNA reverse transcripts, primed with oligo$(dT)_{10}$, by electrophoresis on a 0.7% agarose gel. The reaction conditions were as described for Table 3, using 60 μg/ml concentrations of each RNA. They were prepared for analysis, electrophoresed with ^{32}P-labelled λ DNA markers and autoradiographed as described in Materials and Methods. a. λ DNA Hind III cleavage products. b. Control reaction without CPMV RNA template. c. Products of B-RNA. d. Products of M-RNA. Kb refers to the number of base pairs (= number of single-strand bases) in kilobases.

Those of B-RNA range from about 700 bases to 5000. Those of M-RNA from 500 to about 1500 bases. The control incubation with no RNA template yielded no detectable products in this size range. The products primed with the specific primer oligo$(dT)_8$dAdA in the presence of 0.8 mM NTPs were also analysed, using a 1% agarose gel. The size range was estimated with respect to both λ DNA and ØX 174 DNA restriction endonuclease fragments (Fig. 9). The M-RNA products included molecules from about 400 to 2000 bases long, and the B-RNA products ranged from about 500 to 2000 bases, with some material over 4000 bases long. Several such experiments were done, and it is concluded that most of the cDNA has a length between 500 and 2500 bases. Fig. 9 also shows that cDNA transcribed from CPMV M-RNA is cut into (at least) two discrete fragments, by restriction endonuclease Hae III, which cleaves

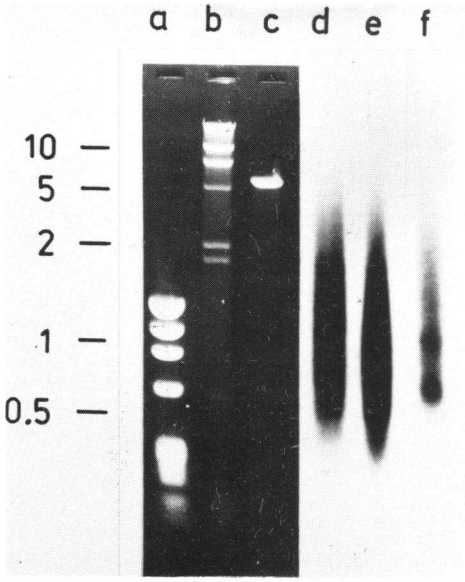

Fig. 9. Analysis of CPMV M and B-RNA reverse transcripts, primed with oligo(dT)$_7$dAdA, by electrophoresis on a 1% agarose gel. The reaction conditons were as described for Fig. 5, but using RNA concentrations of 100 μg/ml and 10 μg/ml concentration of oligo(dT)$_7$dAdA (P and L Biochemicals, Milwaukee, U.S.A.) as primer, and an enzyme concentration of 100 units/ml. The figure shows half of the gel with marker DNAs visualized by ethidium bromide fluorescence (slots a-c), aligned with the autoradiogram of the other half of the same gel, containing the cDNA products (slots d-f).
a. ØX 174 DNA cleaved with Hae III. b. λ DNA cleaved with Hind III. c. ØX 174 DNA cleaved with XhoI. d. cDNA transcripts from CPMV B-RNA. e. cDNA transcripts from CPMV M-RNA. f. cDNA transcripts of CPMV M-RNA, as in e, after incubation with Hae III restriction endonuclease.
Kb refers to number of base paires (= number of single-strand bases) in kilobases. In this experiment, the cDNA products were heated to 100° for 3 min and rapidly cooled before ribonuclease A treatment (see Materials and Methods) to minimize DNA:RNA hybrids. The products probably consist of single-stranded cDNA.

not only duplex, but also single-stranded DNA (17, 18). The fragments are 880 and 540 bases long, confirming that cDNA molecules longer than 1400 bases can be transcribed from the M-RNA. The B-RNA transcripts do not seem to be cleaved by Hae III.

DISCUSSION

While large RNAs of tumour viruses may be copied by tumour virus reverse transcriptase (19, 20) and small heterologous RNAs such as

globin messengers (1, 2) may also be efficiently copied, it might be expected that large heterologous RNAs, such as those of CPMV, might not be copied so efficiently into cDNA. The middle component (M)-RNA has a molecular weight (21) of 1.37×10^6 (about 4000 nucleotides) and that of bottom component (B)-RNA is 2.02×10^6 (about 6000 nucleotides). We were encouraged to investigate CPMV RNA reverse transcription since recently, it has been reported that avian myeloblastosis virus reverse transcriptase can synthesize full-length copies of poliovirus RNA (5). CPMV RNAs may have some similarity, or at least analogy to poliovirus RNAs (see Introduction). Furthermore, reverse transcription of CPMV RNA had previously been reported (13) with promising results; the cDNA consisted mostly of molecules about 600 to 1800 nucleotides long, and there was a suggestion of a small amount of material possibly representing full-length transcripts (13). Using conditions similar to those reported for poliovirus (5, 6) or CPMV RNA (13), we found that most of the cDNA was small and had a T:A ratio of 4:1, suggesting that a lot of the chains consisted of copies of 3' end poly (A) sequences, which probably did not extend much further.

In the case of poliovirus RNA, Kacian and Myers (5) reported that the maximum rate of synthesis and maximum product size are obtained under the same conditons. We developed conditions which gave a very efficient incorporation of dAMP into large products. Some of the requirements for this high yield synthesis of long cDNA transcripts comply with requirements reported for other systems, such as relatively high dNTP concentrations (1, 19), and high enzyme concentrations (2). We have worked with 0.4 to 0.6 mM dNTPs, and primer at 1 μg/ml. These are slightly sub-optimal concentrations (see Figs. 2A and 2B) and clearly our highest enzyme concentration of 100 units/ml is not saturating (Fig. 5B), thus certainly the activity and possibly the size of the products could be increased further. In contrast to the results with poliovirus RNA, we found that pyrophosphate (at for example 4 mM, which inhibited synthesis by 50%), did not increase the amount of large cDNA synthesized (results not shown).

Using 1 pmole B-RNA, or 2 pmoles M-RNA about 1000 pmoles of dAMP per assay were incorporated. Both CPMV RNAs contain about 30% U, thus 1000 pmoles dA in cDNA is equivalent to about 3000 pmoles of all four nucleotides. If this were full-length cDNA, this would be equivalent to about 0.5 pmole B-cDNA and 1 pmole M-cDNA. Thus we

have a synthesis theoretically sufficient to give full-length copies of 50% of the RNA molecules present, or if all RNA molecules are used as templates, then the products would be about 50% the length of the RNA molecules. In fact we find a mixture; on average much of the cDNA synthesized is about half the size of the templates (Fig. 8 and 9), but we also find a small amount of longer transcripts.

In this report, we were mainly concerned with single-stranded cDNA synthesis and all reactions therefore contained actinomycin D at 100 μg/ml. In its absence, twice as much cDNA is synthesized (results not shown), and this additional synthesis occurs mainly after the first 30 min, when most of the cDNA synthesis reported here occurs. We believe that in the absence of actinomycin, double-stranded DNA may be synthesized. We are currently investigating this phenomenon.

The preliminary restriction enzyme analysis of CPMV cDNA indicates that reverse transcription of CPMV RNA with a specific primer (oligo $(dT)_7dAdA$) and subsequent physical mapping of fragments may provide an approach to the determination of nucleotide sequences of defined regions within the genome. Primed synthesis with these specific primers also provides a means of sequence determination of the 3' end region of the RNAs beyond the poly (A) sequences.

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