REVERSE TRANSCRIPTION OF A PLANT VIRAL RNA

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1. Introduction

It is now well established (for reviews see [1,2]) that poly A-containing RNAs such as eucaryotic messenger RNAs (mRNA) or RNAs from oncorna viruses may be efficiently used as templates for DNA synthesis catalyzed by an RNA-directed DNA polymerase (reverse transcriptase) in the presence of an oligothymidylic acid (oligo dT) primer. The recent observation that the RNA genome of the plant virus Cowpea Mosaic Virus (CPMV) contains a poly A sequence of 150 to 250 nucleotides at its 3′ terminus [3] prompted us to verify whether this RNA might similarly function as template for complementary DNA synthesis.

The classification of the various plant RNA viruses is based primarily on the shape of these viruses as observed by electron microscopy, on their single or plurimolecular genome composition, and in some cases on their antigenic properties. Very little is known about the structure of their genomes. Thus, their DNA transcripts used as probes could represent very useful tools for the comparative study of these genomes and for the search of possible structural similarities between a part of the permissive host cell genome and that of the virus.

The aim of this work has been to synthesize and partially characterize the DNA transcripts obtained using CPMV RNA as template, oligo dT as primer, and the reverse transcriptase purified from Avian Myeloblastosis Virus as enzyme source.

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2. Materials and methods

2.1. *Materials*

CPMV was a kind gift of Dr A. Van Kammen. The extraction procedure of the RNA from the virion is based on the one described by El Manna and Bruening [3]. To the virus suspension (8 mg/ml), one tenth of the volume of a macaloid suspension (10 mg/ml) was added, followed by addition of a 5% solution of sodium dodecyl sulphate (SDS) to a final concentration of 1%. After intensive mixing, the suspension was incubated for 5 min at 37°C to achieve complete lysis of the virion. The solution was brought to 0.1 M with 1.5 M Tris–HCl (pH 9.0) followed by addition of an equal volume of water-saturated phenol. After 40 min of shaking at 4°C, the phases were separated and the phenol phase re-extracted with half its volume of 0.1 M Tris–HCl (pH 9.0). The combined aqueous phases were treated again with phenol and the complete procedure repeated. The RNA was precipitated at −30°C from the combined aqueous phases by a K-acetate (pH 5)-alcohol treatment. The yield was 2.6 A_{260} units per mg of virus. Analysis by formamide electrophoresis in 4% acrylamide of the material thus obtained revealed two bands whose relative mobilities (26S and 34S) compared to 16S rRNA were in accordance with published data [3,4]; they correspond to the two species of high molecular weight RNA that make up the split genome of CPMV.

Ribosomal 16S and 5S RNAs were a gift of Dr D. Hayes, deoxytriphosphates and tRNA came from Boehringer Mannheim GmbH, and (dT) $_{12-18}$ from Pabst Laboratories. Turnip Yellow Mosaic Virus (TYMV) and Tobacco Mosaic Virus (TMV) RNAs were prepared in the laboratory as described by

Gierer and Schramm [5], and Alfalfa Mosaic Virus (AMV) RNA was provided by Dr L. Bosch. Rabbit globin mRNA was purchased from Searle Laboratories and duck globin mRNA was a gift of Dr K. Scherrer. [^{3}H]DNA from λ phage ($23 \cdot 10^3$ cpm/μg) was a gift of Dr E. Cassuto; denaturation of the DNA was achieved at low (~ 0.01 μ) ionic strength during 15 min in a boiling water bath followed by immediate cooling in a dry ice-acetone mixture. S_1 nuclease isolated from *Aspergillus oryzae* was a gift of Dr E. Cassuto; one unit of S_1 nuclease activity converted 10 nmol of single-stranded DNA to acid soluble form in 10 min at 37°C under standard conditions. RNase A was purchased from Worthington and [^{3}H]dATP from Radiochemical Center, Amersham.

2.2. *Methods*

2.2.1. Preparation of RNA-dependent DNA polymerase

The enzyme was isolated from Avian Myeloblastosis Virus kindly sent to the laboratory by Dr J. Beard. The enzyme was purified by stepwise elution from the DE-52 column as described [6] followed by salt gradient chromatography on a P-11 column as indicated by Rougeon et al. [7]. After fractionation, a second short P-11 column was used to concentrate the enzyme. In part of the experiments, an enzyme isolated in the laboratory by Dr F. Rougeon according to the published procedure [7] was used. One enzyme activity unit was defined as the amount of enzyme sufficient for the incorporation of 1 nmol of TMP in 30 min with poly rA-oligo dT under standard conditions [7].

2.2.2. DNA synthesis

The mixture (0.1 ml) for DNA synthesis contained 50 mM Tris–HCl (pH 7.9), 50 mM KCl, 10 mM $MgCl_2$, 20 μg of serum albumin, 0.4 mM TTP, 0.2 mM each of dGTP and dCTP, 0.05–0.1 mM [^{3}H]dATP (spec. act. 100–1000 cpm/pmole), 4 mM dithiothreitol, 0.4–0.8 activity unit of RNA-dependent DNA polymerase, and various amounts of RNA, oligo dT, actinomycin D and RNase A as indicated. Incubation was performed at 37°C for 90 min. For analytical purposes the reaction was stopped by addition of carrier RNA and 10% cold trichloroacetic acid (TCA), and the radioactivity retained on nitrocellulose filters determined in the presence of Bray's solution.

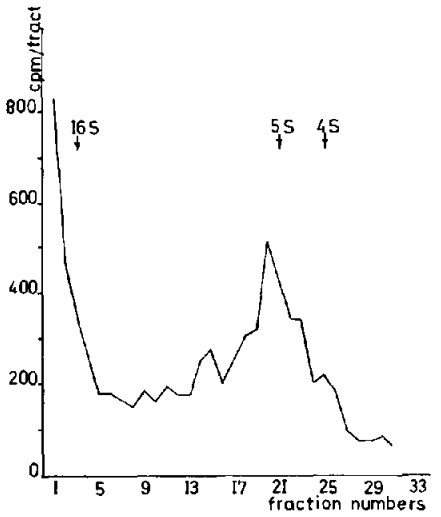

Fig.1. Sucrose gradient centrifugation analysis of c[^{3}H]DNA. *E. coli* 16S, 5S and 4S RNAs were used as markers. Experimental conditions are as described in Materials and methods.

When the material was to be further analyzed, the reaction carried out in the presence of actinomycin D (80 μg/ml) was stopped by adding aqueous phenol or SDS and the DNA was recovered after ethanol precipitation in the presence of carrier RNA. It was then treated in a boiling water bath for 15 min in 0.3 M KOH to remove template RNA.

2.2.3. Analysis of DNA

For sucrose gradient analysis, samples of c [^{3}H] DNA (50 000 cpm in 0.05 ml) were layered over 5 ml sucrose gradients (10 to 35% sucrose in 50 mM Tris–HCl (pH 7.7), 0.1 M NaCl) and run in an SW-50 rotor (Spinco) at 39 000 rev/min for 15 to 18 h. Four-drop fractions were collected, carrier RNA added followed by precipitation with 10% cold TCA. The radioactive material retained on nitrocellulose filters was measured in the presence of Bray's solution. *E. coli* 16S, 5S and 4S RNAs were included in a parallel sucrose gradient as markers, and their position determined at A_{260}.

Slab gels (4% acrylamide) were performed in formamide essentially as described by Pinder et al. [8]. The heavy c [^{3}H]DNA fraction from a sucrose gradient similar to the one presented in fig.1 (about 50 000 cpm) was spun down to remove water. It was dissolved in 0.05 ml formamide, heated for 5 min to 90°C, rapidly cooled to 0°C and applied onto the gel.

After electrophoresis at 15 mA and 450 V for 20 h at room temperature, the gels were stained with methylene blue to reveal the unlabelled RNA markers, and cut into 0.5 cm-long slices. Each slice was put into a glass vial, Soluene (Packard) was added and the samples counted in a toluene-Triton X-100 scintillation mixture.

DNA-RNA hybridization experiments (volume: 0.05 ml) were performed in 0.3 M NaCl, 30 mM Na-acetate (pH 7.0), 0.1% SDS, 40% formamide and a 100–500 excess (4–20 μg) RNA. After incubating for 9 min at 60°C the temperature was decreased to 25°C over a period of 16 h. The reaction was stopped by adding two volumes of ethanol, the precipitate collected and dissolved in 10 mM NaCl, 0.3 mM $ZnSO_4$, 50 mM Na-acetate (pH 4.8) and 10 enzyme activity units of S nuclease, and the mixtures (0.08 ml) incubated for 90 min at 37°C. After addition of carrier RNA the cold 10% TCA precipitable material was collected on nitrocellulose filters and counted in the presence of Bray's solution.

3. Results and discussion

It is evident from the data summarized in table 1 that CPMV RNA is an efficient template for DNA synthesis catalyzed by the RNA-directed DNA polymerase. In the absence of RNA, or after treatment of CPMV RNA with DNase-free RNase, no DNA synthesis was observed. The system completely depends on the presence of Mg^{2+} ions. Only a very low level of incorporation was noticeable in the absence of the oligo dT primer, or when one of the four deoxyribonucleoside triphosphates was omitted.

The behaviour of the system investigated here is thus practically the same as that of the eucaryotic mRNA-dependent DNA synthesis catalyzed by the same enzyme (for reviews see [1,2]). However, the fact that only a very low level of DNA synthesis is observed in the absence of added primer contrary to what is observed with oncogenic RNA viruses, suggests that as in the case of RNA phages [9], CPMV RNA does not contain an endogenous primer which could be utilized by the reverse transcriptase.

In table 2, various RNAs were compared for their ability to serve as template for DNA synthesis using oligo dT as primer. The complete lack of template activity observed with other plant viral RNAs, such as TYMV and AMV RNAs is most probably due to the absence in their primary structures of sufficiently long poly A-containing tracks which could have served for the binding of the oligo dT primer.

Table 1
Requirements for DNA synthesis in the presence of CPMV RNA and reverse transcriptase

Additions	[^{3}H]dAMP (pmoles) incorporated
Complete mixture	50.0
– oligo dT	2.5
– $MgCl_2$	0.0
– CPMV RNA	0.1
– dCTP	3.3
– dGTP	2.0
– dTTP	2.7
+ actinomycin D 20 μg/ml	38.5
actinomycin D 50 μg/ml	30.0
+ RNase A 1 μg/ml	0.2

The conditions of these analytical assays are described in Materials and methods. The complete incubation mixture contained CPMV RNA: 30 μg/ml, and oligo dT: 3 μg/ml.

It is surprising that in spite of the difference in size between CPMV RNA and globin mRNA (26S and 34S versus 9-10S, respectively), their template efficiencies are practically the same under identical experimental conditions.

By sucrose gradient centrifugation, two fractions of c [^{3}H]DNA were recovered (fig.1). The lighter fraction with a sedimentation coefficient of 5.5–6S

Table 2
Comparison of various RNAs as templates for reverse transcription

RNA added	[^{3}H]dAMP (pmoles/μg RNA) incorporated
CPMV	10.0
globin messenger (duck)	12.0
globin messenger (rabbit)	8.3
TYMV	0.1
AMV	0.1

The analytical assays were carried out as indicated in Materials and methods. The concentration of the RNAs was 20 μg/ml and of oligo dT, 0.6 μg/ml.

is typical of many cDNAs prepared with other templates [1,2]. The heavier fraction is recovered partly in the bottom of the tube, suggesting that its sedimentation coefficient is higher than 16S. The possibility that this fraction is an aggregate could however not be excluded in spite of the fact that prior to the run the incubation mixture was deproteinized then boiled for 15 min in 0.3 M KOH to hydrolyze the RNA. To verify this point, the heavier fraction was further analyzed by gel electrophoresis either in formamide or in urea at 60°C.

Even in formamide, some non-covalent interactions between nucleic acids can still subsist [10]. To disrupt such linkages, the sample of c [^{3}H]DNA corresponding to the heavier fraction was dissolved in formamide and heated to 90°C prior to the electrophoresis. The profile of the DNA thus obtained (fig.2) shows that all the labelled DNA is heavier than 5S. There appears a broad band of material of sedimentation coefficient between 9S and 16S which consists of several discrete bands. The mol. wt. of this cDNA is roughly 0.2×10^6 to 0.6×10^6, or 600 to 1800 nucleotides. Its genetic information is sufficient to code for one to two proteins of average size. Its apparent lower sedimentation constant observed in the formamide gel may result from the elimination

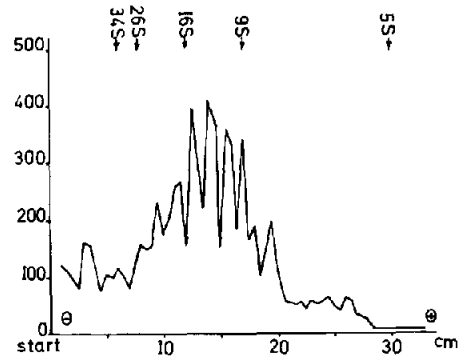

Fig.2. Slab gel electrophoresis of c[^{3}H]DNA in the presence of formamide. Experimental conditions were as described in Materials and methods. Markers: *E. coli* 16S and 5S rRNAs, duck globin 9S mRNA, and CPMV 34S and 26S RNAs.

of non-covalent interactions by the formamide treatment. The relatively small amount of material migrating between 16S and 26S probably reflects the existence of very long cDNA chains approaching in length a considerable portion of the CPMV genome. The nature of the minute amount of cDNA with a sedimentation constant above 34S is unknown.

The analysis in 8 M urea at 60°C of the heavier fraction of c [^{3}H]DNA revealed that most of the

Table 3
DNA-RNA hybridization

Exp.	c[^{3}H]DNA input (cpm)	RNA added	[^{3}H]dAMP incorporated into DNA resistant to S_1 nuclease (cpm)	(% of input)
1	1350	CPMV	1192	88
		E. coli 16S	162	12
		E. coli 4S	215	16
		TMV	209	16
		AMV	588	44
		none	192	15
2	39 950	CPMV	39 650	99
		E. coli 5S	7900	20
		AMV	20 540	51
		none	5880	15

Experimental conditions were as described in Materials and methods. To verify the activity of S_1 nuclease, phage λ [^{3}H]DNA (1500 cpm) was incubated with S_1 nuclease and the cold TCA precipitable material analyzed in the same conditions as all other RNA samples: native and denatured (see Materials and methods) λ [^{3}H]DNA yielded 1390 and 43 cpm respectively.

radioactive material lay between 10S and 18S (not shown here) in good agreement with the formamide system.

Table 3 shows the results obtained when CPMV c [^{3}H]DNA was hybridized to various RNAs. About 90% of the DNA synthesized becomes resistant to S_1 nuclease digestion after annealing with an excess of CPMV RNA. This observation together with the results presented in table 1 exclude the possibility of a terminal addition reaction to CPMV RNA, or of non-complementary synthesis. This is also supported by the absence of hybridization between different RNAs and CPMV cDNA, as compared with the level of self-annealing.

In hybridization experiments, two additional observations have been made. First, a certain amount of c[^{3}H]DNA is resistant to S_1 nuclease digestion after the heating and cooling step in the absence of added RNA (self-annealing). This could not be explained on the basis of limiting amount of S_1 nuclease added, since in the same experiment λ phage [^{3}H]DNA denatured by prior heating was fully hydrolyzed. Presumably, the existence of a nuclease-resistant fraction reflects the presence of certain elements of secondary structure in the cDNA as already observed with cDNA synthesized with globin mRNA as template [11]. Secondly, the level of hybridization achieved with AMV RNA considerably exceeds the value of self-annealing of the cDNA as well as the hybridization value obtained with other control RNAs tested so far. This observation suggests the presence of some common nucleotide sequences in AMV and CPMV RNAs.

The data presented in this paper prove that a plant viral RNA can serve as template for DNA synthesis catalyzed by an RNA-directed DNA polymerase. It is likely that other poly A-containing plant viral RNAs, for instance the RNA of Bean Pod Mottle Virus [4], another representative of the Comovirus group, can also function as template for DNA synthesis in vitro.

Since plant viral RNAs can be obtained in relatively large quantities, they offer many more possibilities than mRNAs of animal origin for large scale cDNA preparation and their use for various analytical or experimental purposes. Moreover, the apparently long cDNA transcripts obtained probably correspond to more than one gene. Insertion of such fragments into the genome of plant DNA viruses, for instance, might present some interest in plant genetic engineering.

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