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A centrifugation method for separation of plant viral genomic and subgenomic RNAs

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Using alfalfa mosaic virus (AMV) as a model, a simple method for separating plant viral genomic RNAs from their subgenomic counterparts was established. The method relies on sucrose gradient fractionation under carefully selected conditions of centrifugation and fraction collection. The RNA components are recovered in nearly quantitative yield and have full biological activity as measured by infectivity of the reconstituted RNAs in suitable protoplasts and plant hosts. The individual RNAs, on the other hand, show no such infectivity, indicating that the separation is indeed complete.

Sucrose gradient centrifugation; AMV; Multipartite genome; Coat protein mRNA

1. INTRODUCTION

Most plant viruses possess a single-stranded RNA genome (+ sense) which may be either mono- or multipartite. Often, individual RNAs of these viruses contain more than one open reading frame (ORF), but only the 5′-proximal ORF can be translated. In these cases, a subgenomic RNA is produced in vivo. Not only are these subgenomic RNAs translatable, but some of them, especially those encoding viral coat proteins, are among the most efficiently translated plant viral RNAs [1].

Unfortunately, given their distribution and biological function, these subgenomic RNAs can often be a hindrance in structure-function studies of viral RNAs. Clean separation of the subgenomic and genomic RNAs is often required, and several methods have been used, with varying degree of success, to effect such separations.

We, therefore, have studied the use of sucrose density gradient centrifugation as a separation method for plant viral RNAs. Alfalfa mosaic virus (AMV), a member of the Tricornaviridae family, was used as a model for these studies. AMV encapsidates three genomic RNAs, designated 1, 2 and 3 in decreasing order of size, and a subgenomic RNA, designated RNA 4, which encodes the viral coat protein and is derived from the 3′-end of RNA 3. Since the molecular mass of the AMV subgenomic RNA 4 is 0.28×10^6 Da while that of the smallest genomic RNA (RNA 3) is 0.68×10^6 Da, it was decided that a sucrose density gradient centrifugation method would be appropriate. Accordingly, a simple, reproducible centrifugation method, in which yield, purity, integrity and infectivity of the AMV RNAs are maintained, has been developed.

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2. MATERIALS AND METHODS

[^{3}H]ATP (1.85 TBq/mmol) was purchased from NEN, poly(A) polymerase from BRL, RNASIN inhibitor from Promega and diethylpyrocarbonate from Sigma.

2.1. *Virus purification*

Alfalfa mosaic virus (strain 425) was purified from infected *Nicotiana tabacum* L. var. Samsun NN by a modification of the method of Van Vloten-Doting et al. [2].

2.2. *RNA extraction*

Sterile, disposable plastic ware was used where possible.

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Glassware and utensils were baked overnight. All solutions were either treated with 0.1% diethylpyrocarbonate (DEPC) or were made with water that had been DEPC-treated. Viral RNA was extracted from the purified virions using a phenol-chloroform-SDS procedure as described [3]. RNA was precipitated with ethanol 3 times, and stored in water at −70°C.

2.3. *Sucrose gradient centrifugation*

The gradient apparatus (Auto Densi-Flow IIC, Buchler Instruments) and all associated tubing were pre-rinsed with 30% H_2O_2 followed by 2 rinses with water. The gradient solutions were 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.1% SDS containing either 5% or 23% sucrose and were held on ice at all times. Linear gradients were prepared using 5.8 ml of each solution. The tubes were filled from the bottom at a rate of approx. 1 ml/min and gradients were kept vibration-free at 4°C.

Total viral RNA was dissolved in the gradient buffer without sucrose, at a concentration of 0.5 mg/ml. Each gradient was loaded with 200 μg of the RNA. Centrifugation was carried out in a Beckman SW41 rotor at 40000 rpm for 6.5 h at 4°C.

2.4. *Fraction collection*

The centrifuge tubes were closed from the top with an Econo column cap fitted with a 3-way stopcock (Bio-Rad). The outside of the tube was thoroughly washed with 30% H_2O_2, rinsed with water, and gently pierced with a 26 gauge needle. Fractions of approx. 300 μl (usually about 7 drops per tube) were collected over a 15 min period by regulating the speed with the stopcock. Approx. 50 fractions were collected per gradient tube. Fractions were stored at −70°C until analyzed.

The separation was monitored by electrophoresis of 10 μl aliquots of selected fractions on 4% polyacrylamide gels containing 7 M urea [4]. The RNAs were stained with ethidium bromide. Appropriate fractions were adjusted to 0.2 M Na acetate, pH 4.5, and the RNA precipitated once with 2.5 vols ethanol and stored in H_2O at −70°C.

2.5. *Polyadenylation of RNA*

RNA was polyadenylated using a modification of the method of Barker et al. [5]. The final reaction volume (100 μl) contained the following components, which were added in order: 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 250 mM NaCl, 0.5 mg/ml BSA, 0.2 mM [^{3}H]ATP (0.5 Ci/mmol), 50 units RNASIN RNase inhibitor, 2–5 μg AMV RNA, 2.5 mM $MnCl_2$ and 1–3 units poly(A) polymerase. Incubation at 37°C for 30 min usually resulted in 80–180 residues added to the 3′-termini of the RNAs.

2.6. *Local lesion assays*

AMV RNA fractions were tested for their ability to infect a host capable of supporting local lesions rather than systemic infection. The local lesion assay was performed as described [6], using half-leaves of 8-day-old *Phaseolus vulgaris* var. Berna. The number of lesions per half-leaf were counted after 7 days.

2.7. *In vitro infection of tobacco protoplasts*

Protoplasts were prepared from *N. tobacum* var. Carlson as described [7]. Infection was according to Loesch-Fries and Hall [8], except that 2 μg of RNA was added to the protoplast pellet before polyethyleneglycol 4000 (Polyscience) and the protoplasts were cultured without antibiotic supplementation in K3 medium [9] with mannitol instead of sucrose. After three days, protoplasts were attached to a microscope slide using poly-D-lysine ([10]; Levy, A. and Vicentini, A.M., unpublished). The presence of AMV coat protein was detected using anti-AMV coat protein antibodies which had been labelled with fluorescein isothiocyanate (FITC) [6]. Infected protoplasts were counted under UV light.

3. RESULTS

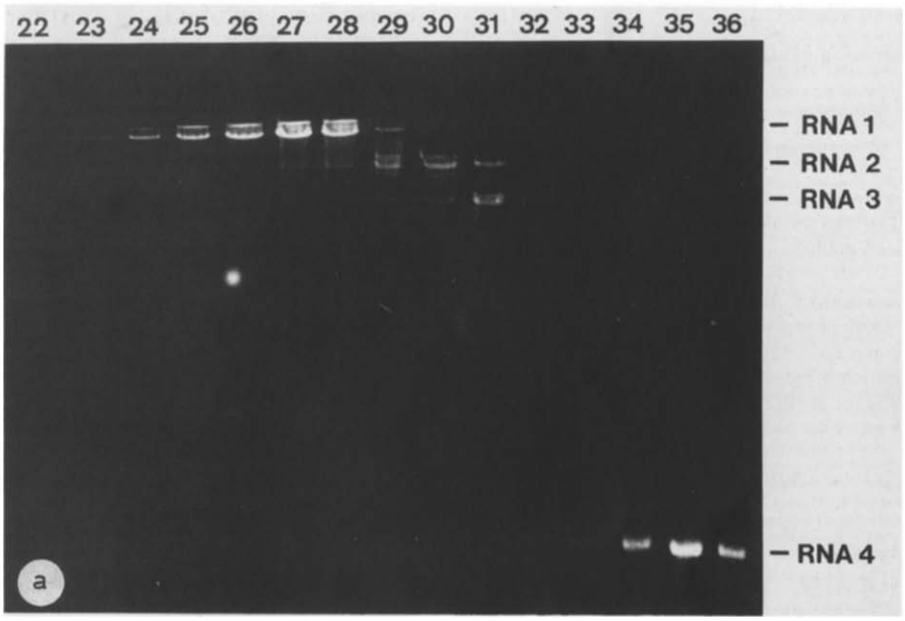
3.1. *RNA purification*

Several types of sucrose gradients, both linear and non-linear, and several centrifugation procedures were studied (not shown). The 5–23% linear gradient was found to be the most effective for separating the subgenomic from genomic RNAs of AMV. After centrifugation, problems were encountered in fraction collecting when an automated device was used to collect from the top of the gradient since early RNA fractions seriously contaminated later ones. This problem was eliminated by manually collecting fractions from the bottom of the gradient.

Polyacrylamide gel electrophoretic analysis of the gradient fractions revealed that under the above conditions the subgenomic RNA 4 was well separated from genomic RNAs 1, 2 and 3 (fig.1a). The gradient fractions containing RNAs 1, 2 and 3 were pooled (pools A + B) as were those containing RNA 4 (pool D), and were re-analyzed by polyacrylamide gel electrophoresis (fig.1b). No cross contamination could be seen between the genomic and subgenomic RNAs. Moreover, when pools A + B + D (RNAs 1,2,3 + RNA 4) were reconstituted, the resultant mixture (fig.1b) could not be distinguished from unfractionated total AMV RNAs, indicating that no RNA degradation had occurred during purification. We estimated the yield of intact RNA to be at least 80%.

3.2. *Polyadenylation of the RNA*

Previous results have indicated that polyadenylation of poly(A)$^-$ RNAs via the enzyme poly(A) polymerase, is very sensitive to contaminants in the reaction. Therefore, it was decided that this assay would be a good qualitative test of the 'cleanliness' of the separated AMV RNAs. RNA, from gradient fractions, was subjected to enzymatic polyadenylation and the results, summarized in table 1, show that the RNAs were all efficiently polyadenylated before and after gradient purification.



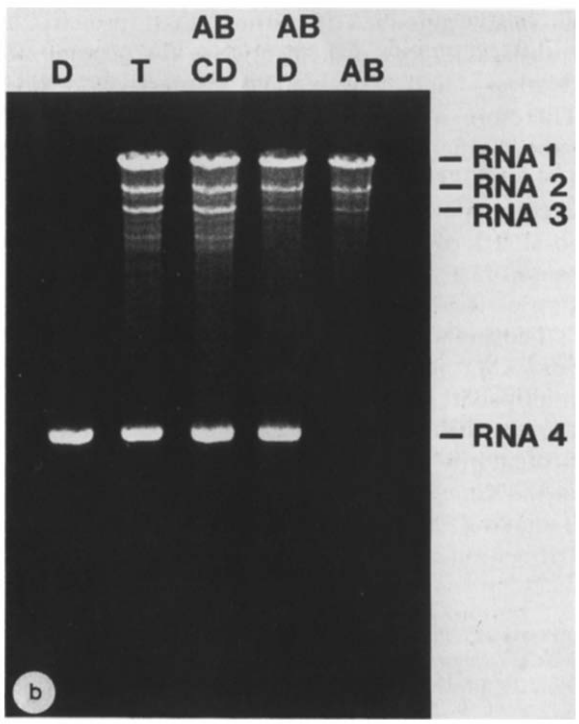


Fig.1. Gel electrophoretic analysis of AMV RNAs separated by sucrose gradient centrifugation. (a) Total AMV RNA was separated as described. Aliquots (10 μl) from selected gradient fractions were subjected to electrophoresis on gels of 4% polyacrylamide, 7 M urea. Gradient fraction numbers were indicated along the top of the gel. AMV RNA components are indicated to the right of the gel. The following fractions were pooled: pool A, fractions 22–28 inclusive; pool B, fractions 29–30; pool C, fractions 31–33; pool D, fractions 34–36. (b) The pooled fractions were subjected to electrophoresis under the same conditions as a. The pools or combined pools are indicated above the gel. T represents total AMV RNA before separation. AMV RNA components are indicated to the right of the gel.

3.3. *Biological activity of the RNA*

3.3.1. Local lesion assays

In order to test the infectivity of the separated vs intact and reconstituted RNAs, the local lesion assay was performed using half leaves of a local lesion host, *Phaseolus vulgaris*. As shown in table 2, the reconstituted fractions were able to sustain at least 80% of the control level infectivity, whereas

Table 1

Polyadenylation of separated AMV RNAs

	cpm	A residues/pmol RNA[a]
No RNA	1466	0
Total AMV RNAs	64337	215
AMV RNAs 1,2,3	45408	198
AMV RNA 4	70103	125

[a] The number of A residues/pmol of RNA was calculated after a 30 min incubation, using the known molecular mass of RNA 4, and the average known molecular masses of RNAs 1,2,3. The values reported are relative to the 'No RNA' control, which was set at '0'

Table 2

Infectivity of separated AMV RNAs

Source of RNA	Number of local lesions[a]	Percentage of infected protoplasts[b]
Total AMV RNA	630	37.3 (106/284)
Mock infected	0	0 (0/1000)
AMV RNAs 1,2,3	0	0 (0/651)
AMV RNA 4	0	0 (0/200)
Reconstituted AMV RNAs 1,2,3 + 4	492	37.7 (20/53)

[a] 10 μg RNA was used per half leaf, and the number of lesions per half leaf is shown

[b] 2 μg RNA was used, and the percentage of treated protoplasts which become infected is shown. The actual numbers of protoplasts counted and infected are given in parentheses

the separated RNAs were able to produce no infection at all, indicating their total functional separation.

3.3.2. Protoplast infection

Nicotiana tabacum protoplasts were also infected with intact, separated and reconstituted AMV RNAs. The results shown in table 2 again confirm that the separation of the RNAs was complete. Separated RNAs did not produce any infection at all, whereas after reconstitution, approx. 100% of control level infectivity was regained.

4. DISCUSSION

Among the methods most commonly employed for RNA separation are polyacrylamide gel electrophoresis followed by electro-elution [11] or $CaCl_2$ precipitation [12], or alternatively, selective precipitation with $MgSO_4$ [2]. None of these existing procedures have been found to be completely satisfactory, however. In some cases, the RNAs were not well separated from one another, and in other cases the yield of undegraded RNA was unacceptable or its biological activity was too low. Sucrose density gradient centrifugation conditions allowing separation of plant viral genomic RNAs from the subgenomic RNA were therefore established. Since the method described is simple and efficient, and gives nearly quantitative yields of pure, infectious RNAs, many structure-function studies that depend upon complete separation of these components may now be more easily undertaken.

AMV was chosen as a model system due to the fact that separation of genomic and subgenomic RNAs could be tested under conditions of maximal stringency. AMV has an absolute requirement for a small amount of either coat protein or translatable coat protein mRNA for genome activation and consequent infectivity [13]. Therefore, even the slightest amount of subgenomic RNA4 contamination in the genomic RNA 1,2,3 fractions would lead to the latter RNAs becoming infectious. The ability to demonstrate a total lack of infectivity after RNA fractionation is therefore a stringent criterion for complete separation of these RNA components.

The method developed should be useful not only for AMV but for other + strand mono- or multipartite plant viruses as well. In most of them, the size difference between the subgenomic coat protein mRNA and the smallest genomic RNAs is in the same range as seen with AMV RNAs 4 and 3 making them suitable candidates for this centrifugation method.

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