Purification of Mengovirus and Identification of an A-rich Segment in its Ribonucleic Acid

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Poly (A) segments form a part of mammalian mRNA (Darnell, *et al.* 1971; Edmonds, Vaughan & Nakazato, 1971; Mendecki, Lee & Brawerman, 1972), vaccinia virus mRNA (Kates, 1970) and the RNA of a number of RNA viruses (Armstrong *et al.* 1972; Gillespie, Marshall & Gallo, 1972; Johnston & Bose, 1972; Lai & Duesberg, 1972). The potential significance of the poly (A) segment in mRNA function is unknown, but it is of interest that the genomes of RNA tumour viruses contain a much greater proportion of poly (A) than the RNAs of other viruses and that poly (A) segments have not been detected in virus RNAs that do not function directly as mRNA, such as the RNAs of influenza virus, Newcastle disease virus and vesicular stomatitis virus (Gillespie *et al.* 1972; Lai & Duesberg, 1972). It has been reported that the poly (A) segment of poliovirus RNA represents 0.75 % (Armstrong *et al.* 1972) or 0.25 % (Gillespie *et al.* 1972) of the total nucleotide content of the virus genome. Our data show that mengovirus RNA contains an A-rich segment of approximately 16 nucleotides, which represents only about 0.19 % of the total genome.

Mengovirus and mengovirus RNA were prepared as follows. Cultured Novikoff rat hepatoma cells (subline N1S1-67) were infected with 10 to 30 p.f.u./cell of mengovirus as described previously (Plagemann, 1968), and suspended to 4×10^6 cells/ml in basal medium 42 (BM 42) (Plagemann & Swim, 1966). The suspensions were supplemented with 2 μ g of actinomycin D/ml at 30 min after infection and with 0.5 μ M-[3 H-8]-adenosine (2 mCi/ μ -mol) or with 0.5 μ M-[3 H-5]-uridine (2 mCi/ μ -mol; Schwarz/Mann) at 1 h after infection. At 7 h after infection the suspensions were frozen and thawed and then clarified of cell debris by centrifuging at 1000 g for 10 min at 4 °C. Mengovirus was further purified from the supernatant fluid by a procedure similar to that recently described for EMC virus (Kerr & Martin, 1972). One litre of clarified cell lysate was supplemented with 6 % (w/w) of polyethylene glycol (6000, Baker Chemical Co.) and 0.01 % (v/v) of mercapto-ethanol and stirred with a magnetic stirrer at 0 °C for 1 h. The precipitate was collected by centrifuging at 5000 g for 20 min and suspended in 10 ml of a solution composed to 100 mM-tris-HCl (pH 8.2) and 20 mM-EDTA (B 13). The suspension was treated with a 20-Kc MSE sonic oscillator (1 cm probe) at 0 °C for 30 s and 5 ml samples were layered over 33 ml linear 0.5 to 1.6 M gradients of sucrose in B 13. The gradients were centrifuged in a SW 27 rotor in a L-2 Beckman ultracentrifuge at 24000 rev/min at 4 °C for 6 h. One ml fractions were collected from the gradients by means of an ISCO gradient fractionator (Model 183) which was attached to a continuously recording spectrophotometer and a fraction collector. Samples of the fractions were analysed for HA activity (Plagemann & Swim, 1966) and radioactivity in acid-insoluble material (Plagemann, 1968). The fractions which contained the virus were pooled from one or more gradients, diluted eightfold with B 13 and concentrated by ultrafiltration on a Diaflo membrane (XM 300, Amicon Corp., Lexington, Mass.) to approximately 10 ml. The virus suspension was again centrifuged through a sucrose density gradient and subsequently concentrated by ultrafiltration as previously described. The results from a typical purification of [3 H]-uridine-labelled virus are summarized in Table 1. About 50 % of the original virus was recovered in the final preparation. The amount of protein in 0.5 ml

Table 1. *Purification of mengovirus*

Purification step*	Total				HA units/ μg protein	Virus recovery %
	Volume (ml)	HA (units)	[^3H] (ct/min)	Protein (μg)		
1. Clarified cell lysate	1000	13×10^5	18×10^6	245 000	5.2	100
2. Polyethylene glycol precipitate	10	10×10^5	12×10^6	9 100	113	77
3. First sedimentation and ultrafiltration	10	8.2×10^5	11×10^6	500	1640	63
4. Second sedimentation and ultrafiltration	10	6.4×10^5	9×10^6	< 200	< 3200	49

* The virus was purified as described in the text.

Fig. 1. Sucrose density gradient sedimentation of purified mengovirus (A) and mengovirus RNA (B). (A) [^3H]-uridine-labelled mengovirus was purified as described in the text. A second sucrose density gradient was monitored for E_{260} (○—○) and 0.1 ml samples of each fraction from the gradient were analysed for HA activity (▲—▲) and radioactivity in acid-insoluble material (●—●). (B) A sample of RNA extracted from [^3H]-uridine-labelled virus was mixed with a sample of purified [^{14}C]-labelled 29 S rRNA and centrifuged through a linear 0.15 to 0.9 M gradient of sucrose in B 6 (Plagemann, 1970) in a SW 27 rotor, at 24 000 rev/min, 20 °C for 12 h. Fractions from the gradient were analysed for [^{14}C] (○—○) and [^3H] (●—●) in acid-insoluble material.

of the virus suspension (3×10^4 HA units) was below that detectable by the method of Lowry *et al.* (1951). The specific activity was therefore at least 3200 HA units/ μg protein. The radioactivity, extinction and HA profiles from the second sucrose density gradient sedimentation (Fig. 1A) also indicate that the virus was free of contaminating material.

The virus RNA was isolated from purified virus by extraction with sodium dodecylsulphate and phenol as described previously (Plagemann & Swim, 1968). As indicated in Fig. 1B, the RNA sedimented as a homogeneous fraction. A sedimentation coefficient of about 34 S was estimated for the virus RNA by the method of Martin & Ames (1961) using cosedimented [^{14}C]-labelled purified 29 S rRNA from N1S1-67 cells (Plagemann, 1970) as a

Fig. 2. Sucrose density gradient sedimentation of $[^3\text{H}]$ -adenosine (A) and $[^3\text{H}]$ -uridine-labelled (B) mengovirus RNA without and with prior treatment with RNase. Samples of 3 ml of virus RNA in $\times 2$ SSC were supplemented with $40 \mu\text{g}$ RNase A and $20 \mu\text{g}$ RNase T1 per ml and incubated at 27°C for 60 min. These samples and samples of untreated RNA in $\times 2$ SSC were then analysed by sucrose density gradient centrifugation as described in the legend to Fig. 1B, except that the gradients were centrifuged for 11 rather than 12 h.

marker. Based on the Spirin equation (Spirin, 1963), the mol. wt. of mengovirus RNA was estimated as 2.8×10^6 . Samples of virus RNA extracted from $[^3\text{H}]$ -adenosine or $[^3\text{H}]$ -uridine-labelled virus were suspended in a solution composed of 0.3 M-NaCl and $0.03 \text{ M-sodium citrate}$ ($\times 2$ SSC) and incubated with $40 \mu\text{g}$ of RNase A (Sigma Chemical Co.) and $20 \mu\text{g}$ of RNase T1 (Worthington Biochemical Corp.) per ml at 27°C for 60 min. As indicated by the data in Fig. 2B, uridine-labelled virus RNA was completely degraded to acid-soluble material, whereas approximately 0.75 % of the adenosine-labelled virus RNA was not solubilized and was recovered in a low-molecular weight fraction (Fig. 2A). Similar results were obtained when the virus RNAs were suspended in $\times 0.1$ SSC and incubated with $2 \mu\text{g}$ each of RNase A and T1 at 27°C for 30 min (not shown). Samples of RNA were also hydrolysed in NaOH and the nucleotides were separated by high-voltage electrophoresis (Plagemann, 1972). The results showed that 100 % of the adenosine label in RNA was associated with AMP, and that 86 % of the uridine label was associated with UMP and 14 % with CMP.

Based on a mol. wt. of 2.8×10^6 for mengovirus RNA, an average mol. wt. of the monophosphonucleotides of 350 and an overall base composition of the RNA of approximately 25 % of each of the four bases (unpublished data), we estimated that mengovirus RNA is composed of approximately 8000 monophosphonucleotides, of which 2000 would be AMP.

Fig. 3. Sedimentation analysis of 4 S tRNA and of poly (A) released from mengovirus RNA by RNase treatment. A sample of [^3H]-adenosine-labelled mengovirus RNA (72 000 ct/min) was incubated in $\times 2$ SSC containing $40\text{ }\mu\text{g}$ of each RNase A and T1 per ml at 27°C for 60 min and then analysed by sucrose density gradient centrifugation as described in the legend to Fig. 1B, except that the gradient was centrifuged for 58 rather than 12 h (●—●). A sample of [^3H]-labelled 4 S tRNA in $\times 2$ SSC was co-sedimented in an accompanying gradient (○—○). The graph is a composite of the radioactivity profiles of the two gradients.

Since 0.75 % of the AMP in the virus RNA is resistant to degradation by RNase A and T1 we estimate that mengovirus RNA contains an A-rich segment containing approximately 15 AMPs. This calculation is based on the assumption that every virus RNA molecule contains a single A-rich segment of equal length. This conclusion is supported by a more direct analysis of the mol. wt. of the A-rich segment released from virus RNA by the RNase treatment. The A-rich segment sedimented only about half as rapidly in a sucrose density gradient as 4 S tRNA (Fig. 3). According to the method of Martin & Ames (1961), we estimated a sedimentation coefficient of 1.9 S for this material which is equivalent to a mol. wt. of approximately 6000 as estimated by the Spirin formula and a content of approximately 17 nucleotides. Thus both methods of calculating the size of the A-rich segment of mengovirus RNA yielded similar results. The results therefore are consistent with the conclusion that each mengovirus RNA molecule contains a single segment composed of 15 to 17 AMPs. The data reported by Gillespie *et al.* (1972) suggest that poliovirus RNA contains a poly (A) segment of similar length whereas Armstrong *et al.* (1972) estimated from their data that the poly (A) segment of poliovirus RNA consists of 50 to 70 AMPs. Present work is directed towards determining the location of the poly (A) segment in mengovirus virus RNA and in mengovirus double-stranded RNA.

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