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Characterization of Messenger RNAs of Measles Virus

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SUMMARY

Ten different RNA species associated with measles virus were detected in virus-infected cells. The largest RNA (no. 1) was considered to be genomic RNA and the other nine RNAs were found to have a methylated cap as well as a poly(A) tail. Northern blot hybridization indicated that RNAs no. 2 and 7 to 10 correspond to mRNAs encoding structural proteins, and that RNAs no. 3 to 6 are intermediate-sized (IS) RNAs. The possibility that the ISRNs represent defective interfering RNA or a precursor form of the mRNA seems to be unlikely since the amounts of IS RNAs did not increase after undiluted passages or decrease in a pulse-chase experiment. These results indicate that IS RNAs are readthrough transcripts of neighbouring cistrons.

The molecular nature of measles virus (MV)-associated RNAs has not been well investigated. Baccko *et al.* (1983) estimated that the size of the genomic RNA of MV is 4.5×10^6 daltons by agarose gel electrophoresis of denatured nucleocapsid RNA. Hall *et al.* (1978) first showed six major MV-associated RNAs in infected cells by polyacrylamide gel electrophoresis and speculated that they represent mRNAs encoding structural proteins. Subsequently, we and another group noted that at least ten different virus-associated RNA species are produced in MV-infected cells, by analysis of viral RNA synthesis in actinomycin D-treated cells (Udem & Cook, 1984; Yoshikawa & Yamanouchi, 1984).

MV cDNA which corresponds to parts of the genome or mRNAs of the virus has recently become available for the molecular analysis of MV and subacute sclerosing panencephalitis (SSPE) virus (Baccko *et al.*, 1984; Bellini *et al.*, 1984; Billeter *et al.*, 1984; Carter *et al.*, 1983; Gorecki & Rozenblatt, 1980; Rozenblatt *et al.*, 1982, 1985), and evidence has been obtained that in infected cells not only genomic RNA and mRNA are produced but also low levels of intermediate-sized (IS) RNAs. To explain the nature of the IS RNAs, Carter *et al.* (1983) proposed the possibilities of defective interfering (DI) RNA, precursor forms of mRNA, RNAs produced by misreading by the polymerase, or polycistronic mRNA. To examine these hypotheses, further characterization of MV-associated RNAs has been attempted in this study.

The Edmonston strain of MV was propagated in Vero cells, and a stock with a titre of 1×10^6 p.f.u./ml was used. IMR-32 human neuroblastoma cells were infected with the virus except in the undiluted passage experiments in which Vero cells were used. Details of the preparation of the stock virus and maintenance of cells have been described previously (Yoshikawa *et al.*, 1983).

Radioisotope labelling of viral RNA was performed as described previously (Hirayama *et al.*, 1985; Yoshikawa & Yamanouchi, 1984). The amount of isotope incorporation into RNA was measured from exposed X-ray films using a densitometer (Asuka Manufacturing Co., Tokyo, Japan). To analyse RNA synthesis, virus-infected cells were labelled for 2 h with ³²P; after starvation and the cells were sampled until 5 h at 30 min intervals. In pulse-chase experiments, the cells were labelled for 2 h and chased for 3 h at 30 min intervals.

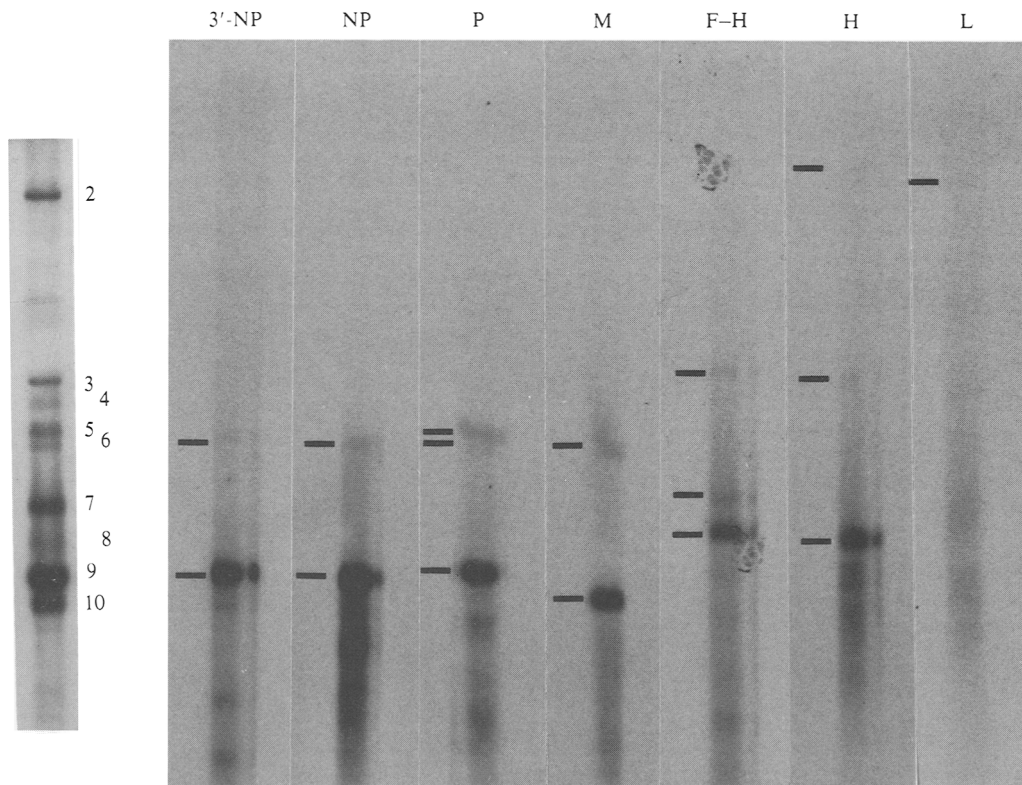


Fig. 1. Northern blot analysis of viral mRNAs. Poly(A)⁺ RNAs were denatured, electrophoresed and blotted onto nitrocellulose membranes. The membrane was cut into strips and incubated with different cDNA probes. Bars indicate positive signals.

Northern blot analysis was performed as described by Rozenblatt *et al.* (1985). Messenger RNAs extracted from virus-infected cells were denatured, electrophoresed and blotted onto nitrocellulose membranes. RNAs on the membrane were hybridized to labelled cDNA probes (10^7 to 2×10^7 c.p.m./ μ g), kindly supplied by Drs S. Rozenblatt and M. Billeter.

Cap structure was examined as described by Mizumoto & Lipmann (1979). Briefly, viral mRNAs labelled with $^{32}\text{P}_i$ were digested with RNase, and the fraction containing the cap structure was obtained by DEAE-Sephadex A25 column chromatography. The lyophilized sample was further treated with nuclease P1 and electrophoresed on DEAE-cellulose paper at pH 3.45, with a constant voltage (500 V) for 3 h together with authentic cap markers.

Poly(A)-containing [poly(A)⁺] viral RNAs were subjected to Northern blot analysis using cDNAs corresponding to genomic 3'-NP, L, F, H, NP, P and M regions (Fig. 1). Strong hybridization was observed at positions corresponding to the molecular sizes of RNAs no. 2 and 7 to 10. The result suggests that they are mRNAs encoding structural proteins as speculated by Udem & Cook (1984). [Note that NP and P mRNAs ran at the same position (no. 9).] The sum of the mol. wt. of these six mRNAs after subtraction of the mol. wt. of six poly(A) tails is 5.56×10^6 . This value is about the same as the mol. wt. of genomic RNA. The remaining four subgenomic RNAs (no. 3 to 6), which correspond to the IS RNAs reported by Carter *et al.* (1983) and Bacsko *et al.* (1984), were recognized as weak bands.

The possibility that the IS RNA represents a precursor form of mRNA was checked by pulse and pulse-chase labelling investigation of viral RNA synthesis. The amount of labelled IS RNAs was increased in parallel with those of other viral RNAs between 2 and 5 h in the pulse experiment (Fig. 2a). Moreover, the amount of pulse-labelled IS RNAs was not changed during

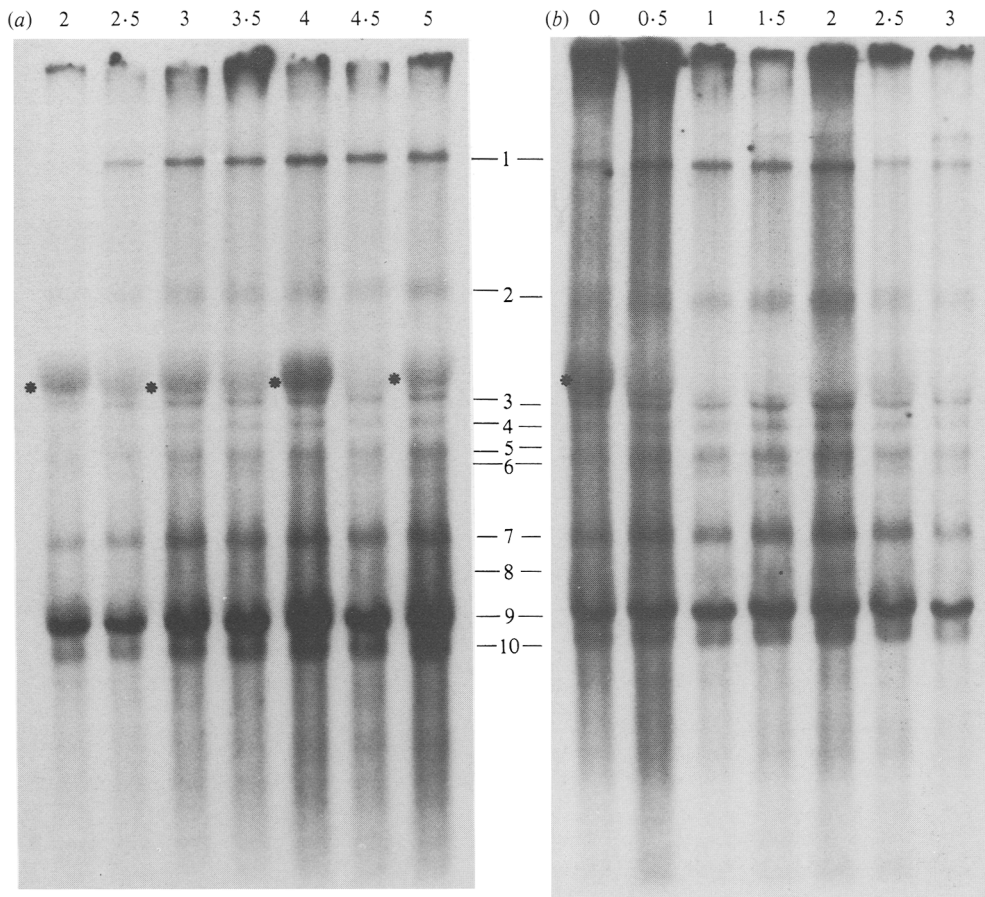


Fig. 2. Analysis of processing of virus-associated RNAs. (a) Pulse labelling of virus RNA during synthesis. MV-infected cells were starved and treated with 15 $\mu\text{g/ml}$ actinomycin D for 2 h. The cells were labelled with $^{32}\text{P}_i$ for the times shown (h) in the presence of actinomycin D and sampled at 30 min intervals. (b) Pulse-chase labelling of virus-associated RNAs. The cells labelled as above were chased for up to 3 h at 30 min intervals. Asterisks indicate traces of 28S ribosomal RNA.

the 3 h chase period (Fig. 2b). These results suggest that no IS RNA is a precursor of an mRNA, nor is it processed into smaller RNAs.

To investigate whether IS RNA represents DI RNA, undiluted MV was serially passaged for 10 generations. The virus yield was markedly decreased at early passages, increased again during several subsequent passages and then showed a cyclical fluctuating pattern (Fig. 3a). The viral RNA patterns were examined at each passage level and were found to be basically similar to that of the original virus (Fig. 3b). Since the stock virus obtained at passage levels that showed a marked decrease in virus titre might have contained DI RNA, correlation with the synthesis of IS RNA was examined, but no increase of IS RNA was observed in these isolates. Co-infection of cells with the original stock virus and the low titre stock virus did not cause an increase in IS RNA (data not shown).

To examine methylation of the cap and the cap structure of mRNA, viral RNA was labelled with [$\text{Me-}^3\text{H}$]methionine or $^{32}\text{P}_i$. The amount of methyl methionine-labelled RNA was markedly different from that of P_i -labelled RNA (Fig. 4a, b). Provided only the cap portion is methylated, the amount of P_i incorporation into a molecule of mRNA should correlate with its mol. wt. and the amount of incorporation of methyl residues should correlate with the number of

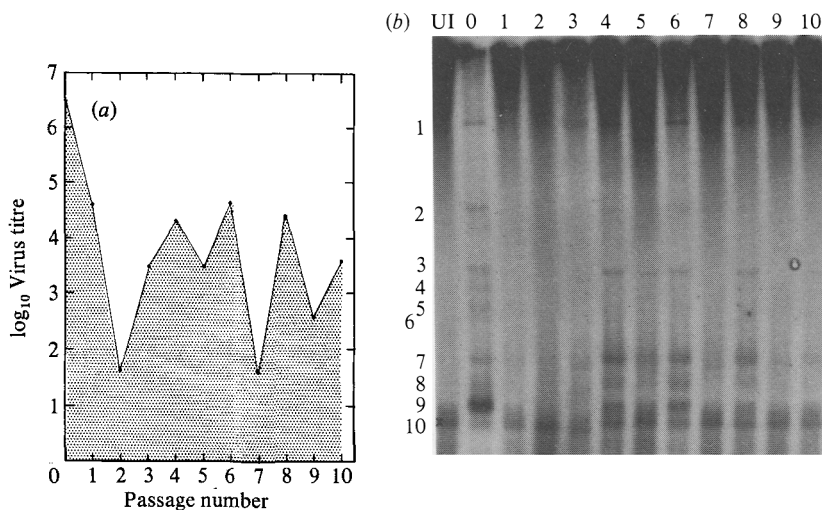


Fig. 3. Undiluted passage of MV. (a) Virus yield during undiluted passage. Vero cells were infected with undiluted MV serially for 10 passages and virus titre was assayed at each stage. (b) MV-associated RNAs. Virus RNAs were labelled from 25 to 30 h post-infection. UI, Uninfected control. 0 to 10 (top) indicate passage number. \times , Cellular RNAs which remained after treatment with 20 $\mu\text{g}/\text{ml}$ actinomycin D.

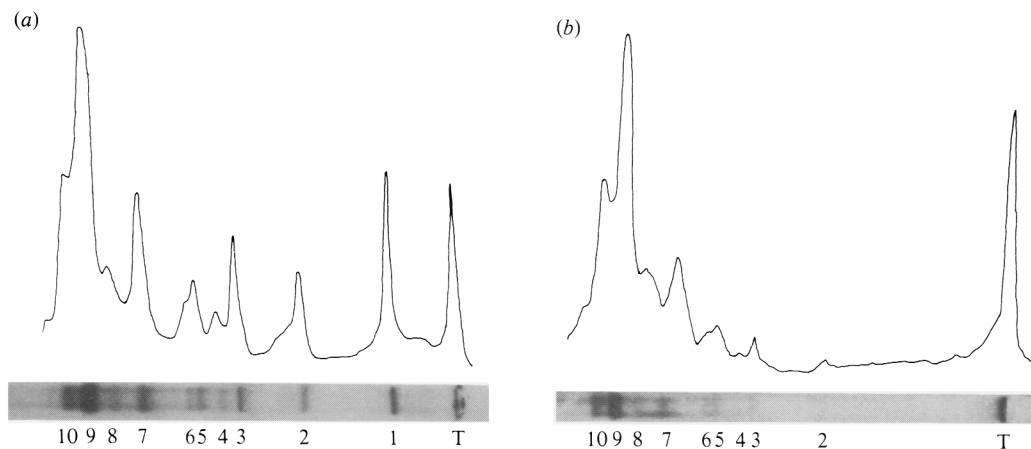


Fig. 4. Amount of radioisotope-labelled MV-associated RNAs. (a) Pattern of RNA labelled with $^{32}\text{P}_i$ and its amount measured by densitometry. T, Top of the gel. (b) MV-associated poly(A)⁺ RNA labelled with [$\text{Me-}^3\text{H}$]methionine and its amount.

mRNA copies. As shown in Table 1, the molar ratio of each mRNA calculated from P_i incorporation, which varied among mRNAs (abundant in RNA no. 7 to 10 and poor in no. 3 to 6), was well correlated with the incorporation of methyl residues. When the relative molar ratios and the relative amount of incorporation of methyl residues were plotted for each RNA, the correlation index was about 1.0. These results indicate that RNAs no. 2 to 10 have a methylated cap and poly(A) tail. The mean of the relative molar ratio of the four IS RNAs was 4.55 and that of the monocistronic mRNAs (RNA no. 7 to 10) was 36.28. Thus, the number of copies of these monocistronic mRNAs is nearly ten times greater than that of IS RNAs. This explains why the IS RNAs were easily detected by labelling the virus-infected cells with $^{32}\text{P}_i$ or [^3H]uridine but rarely by [^3H]methyl residue incorporation or Northern blot hybridization.

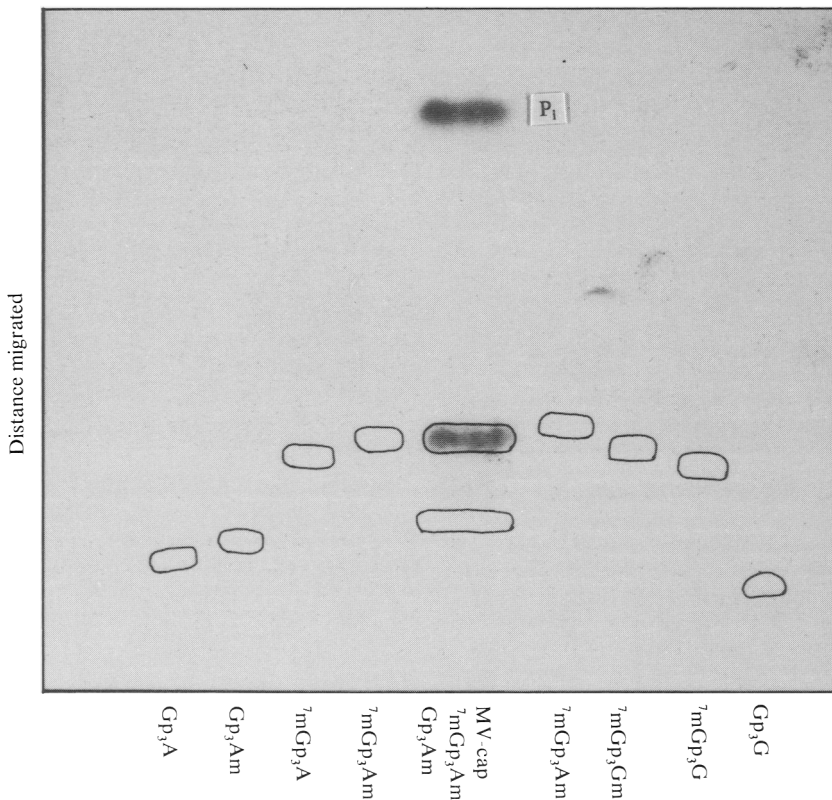


Fig. 5. Analysis of the cap structure of MV mRNAs. The virus mRNA labelled with $^{32}\text{P}_i$ was purified and treated with RNase mixture. The cap structure was recovered by DEAE-Sephadex chromatography. P_i indicates free phosphoric acid released by nuclease P1.

Table 1. Quantification of MV-associated RNAs

No.	^{32}P -labelled RNA				^3H -methyl-labelled mRNA	
	^{32}P amount	Mol. wt. $\times 10^{-6}$	Molar ratio*	Relative ratio†	^3H amount	Relative amount†
1	185.0	4.95	3.74	3.6	—	—
2	81.0	2.65	3.05	2.6	4.0	0.8
3	108.0	1.62	6.67	6.4	11.0	2.2
4	40.0	1.39	2.88	2.8	2.5	0.5
5	76.0	1.21	6.28	6.0	32.0	6.4
6	36.5	1.15	3.17	3.0	20.0	4.0
7	235.0	0.81	29.00	27.7	176.0	35.1
8	126.0	0.66	19.09	18.2	131.5	26.2
9-1,2	586.0	0.56	104.64	100	501.0	100
10	193.0	0.52	37.12	35.5	169.0	33.7

* Amount of ^{32}P divided by mol. wt.

† Expressed as a percentage taking the value for RNA no. 9 (9-1,2) as 100%.

The results of DEAE-cellulose paper electrophoresis clearly showed that all the viral mRNAs had only one type of cap structure, m^7GpppAm (Fig. 5). This suggests that all MV mRNAs start from the same nucleotide (adenine). Methylated cap structures and a lack of methylation of internal ribose residues in a paramyxovirus mRNA has previously been found in Newcastle disease virus (Colonno & Stone, 1975, 1976).

As to the nature of the IS RNA, the results of the pulse and pulse-chase labelling experiments suggest that IS RNA is not a precursor form of mRNA. Moreover, during undiluted passages of the virus in which efficient production of DI RNA was expected, the amount of IS RNA was not increased. Therefore, it is unlikely that IS RNA represents DI RNA.

The IS RNAs appear to be dicistronic mRNAs, for the following reasons. (i) Each IS RNA had a molecular size comparable to the sum of a combination of two monocistronic mRNAs. (ii) IS RNAs were labelled with [$Me\text{-}^3H$]methionine and also bound to an oligo(dT)-cellulose column, suggesting that they have the complete structure of mRNA with both a methylated cap and a poly(A) tail. (iii) The cDNAs to different cistrons hybridized to the same size of IS RNA in Northern blot hybridization. Similar results have previously been reported (Baczko *et al.*, 1984). (iv) Recently, we have obtained a cDNA clone to the mRNA of SSPE virus-infected cells and found that it consists of 626 bases of the P gene, the intercistronic trinucleotide, 1461 bases of the M gene and 75 bases of the poly(A) sequence (unpublished results).

The function of the IS RNAs of MV is unknown. To determine whether they function as mRNA, experiments on *in vitro* translation of purified IS RNAs are now in progress. Baczko *et al.* (1984) reported that an IS RNA consisting of the P + M genes is increased in the brain of a patient with SSPE. The significance of this for the pathogenesis of SSPE also remains to be clarified.

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