Purification and Molecular Weight Determination of Measles Virus Genomic RNA

By K. BACZKO, M. BILLETER1 AND V. TER MEULEN*

Institute of Virology and Immunobiology, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany and 1Institute of Molecular Biology 1, University of Zürich, CH-8093 Zürich, Switzerland

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SUMMARY

A purification procedure for genomic measles virus RNA, free of contaminating smaller RNA and of DNA, is described. Viral nucleocapsids were prepared from MA160 cells infected in spinner cultures with measles virus (Edmonston strain). Nucleic acid was extracted, treated with DNase and RNA sedimenting at about 50S in sucrose gradients was isolated. This method yielded 0.5 to 1.5 μg of genomic RNA per litre of culture. A molecular weight of 4.5 × 10^6 was determined by gel electrophoresis under fully denaturing conditions.

Measles virus, a paramyxovirus (Kingsbury et al., 1978) is a human pathogen causing acute and subacute infections. The association of this agent with a variety of human diseases has raised great interest amongst immunologists and virologists and many studies have been performed to unravel the pathogenic mechanisms which can lead to these different disease processes (ter Meulen & Carter, 1982). However, virological approaches have been hampered by the fact that measles virus grows poorly in tissue culture and the low yield of virus has so far prevented the application of modern molecular virological techniques to the search for measles virus genetic information present in diseased organs. No reliable method has been available for the isolation of sufficient quantities of pure, stable genomic RNA to permit cloning of viral nucleic acids in appropriate vectors. The lack of purified measles virus RNA has also prevented the exact determination of molecular weight: this has been estimated as 6.2 × 10^6 from the sedimentation of RNA in sucrose gradients (Schluederberg, 1971), or length measurements of nucleocapsids (Nakai et al., 1969), but no determination has been performed under fully denaturing conditions.

In this report, we describe a method for the preparation of genomic RNA free of contaminating smaller RNAs and DNA, and present a molecular weight determination based on mobility during electrophoresis in fully denaturing methylmercury-agarose gels (Bailey & Davidson, 1976). Virus RNA was purified from intracellular viral nucleocapsids prepared by a modification of the method of Compans & Choppin (1967). This approach was used since only a small percentage of genomic RNA is packaged and released from infected cells as mature virions.

All buffers and solutions were treated with 0.05% diethyl pyrocarbonate, autoclaved and stored frozen before use. Reaction vials were siliconized Corex glass or Eppendorf plastic tubes. The whole purification procedure was performed under conditions that rigorously excluded contamination by RNase. The Edmonston strain of measles was a gift from W. Bellini (Bellini et al., 1979). It was plaque-purified three times and propagated twice in Vero cells at a low multiplicity of infection (0.1), generating a stock containing few defective particles. Growth of virus was as described in detail elsewhere (Baczko & Lazzarini, 1979). MA160 cells (Microbiological Associates) were suspended in spinner culture and adjusted to 2 × 10^5 to 3 × 10^5 cells/ml. After 2 days, cells were centrifuged and resuspended in 0.2 vol. for infection at a multiplicity of 0.1. After 1 h of constant stirring at 37 °C, medium was added to produce a cell density of 2 × 10^5 to 3 × 10^5 per ml.

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Two days later, cells were collected by centrifugation, and resuspended in the original volume. At 3 days post-infection [3H]uridine (27 Ci/mmol, Amersham Buchler) was added to a concentration of 0.5 μCi/ml. Cells were harvested 4 to 5 days post-infection, when the cytopathic effect was complete, and all subsequent steps leading to the extraction of nucleic acids were carried out as quickly as possible in a cold room (4°C). Cells were pelleted at 400 g for 10 min, washed with ice-cold phosphate-buffered saline and sedimented again at 700 g for 5 min. Pellets were resuspended in ice-cold 10 mM-HEPES pH 7-0, 5 mM-MgCl₂ at a concentration of 3-2 × 10⁷ cells/ml and the cells were allowed to swell for 10 min on ice. Nonidet P40 was added to a final concentration of 1%. After 5 min on ice cells were carefully disrupted (to avoid destruction of nuclei) by two slow passages in a Dounce homogenizer. Cellular debris and nuclei were removed by centrifugation for 5 min at 700 g. The supernatant was adjusted to 7.5 mM-EDTA and layered onto a step gradient consisting of 40, 30 and 25% (w/v) caesium chloride (Serva; ultrapure) in SEH (100 mM-NaCl, 10 mM-HEPES pH 7-0, 1 mM-EDTA). Centrifugation was performed at 36000 rev/min in a SW41 rotor at 4°C. The visible nucleocapsid band in the 30% caesium chloride region was carefully removed by aspiration in a siliconized Pasteur pipette, diluted at least threefold with SEH and pelleted onto a 65% (w/v) sucrose/SEH cushion for 20 min at 36000 rev/min in a SW41 rotor at 4°C. The firm pellet was removed in a shortened siliconized Pasteur pipette and resuspended in at least five times its volume of SEH containing 2% SDS, at 37°C. Pronase (Calbiochem; B grade, 20 mg/ml, self-digested for 2 h at 37°C in 0.15 M-EDTA) was added to a final concentration of 1 mg/ml and incubated for 10 min at 37°C with repeated shaking, until most of the visible pellet was solubilized. Nucleic acids were extracted four times with SEH-saturated phenol and the phenol phases re-extracted with the original volume of SEH, 2% SDS. The aqueous phases were pooled in a siliconized Corex tube and nucleic acids were precipitated overnight with ethanol at −20°C before collection by centrifugation. Pellets were washed once with 90% ethanol, dried under reduced pressure and resuspended in 100 mM-NaCl, 10 mM-HEPES pH 7-0, 10 mM-MgCl₂ in the proportion of 100 μl per litre of original spinner culture.

The nature of the nucleic acids present at this stage was determined by DNase or RNase digestion followed by electrophoresis under denaturing conditions. For each test, 1 to 2 μg of nucleic acids from the nucleocapsid preparation was mixed with 0.1 μg of a marker DNA as an internal control for successful nuclease function. One sample was kept on ice, one was digested with 1000 U/ml DNase I [Worthington; treated with Macaloid to remove any trace of RNase (Schaffner, 1980)] and one was treated with pancreatic RNase A (Worthington; heated 10 min at 90°C to inactivate DNase) at a concentration of 1 μg/ml. After incubation for 5 min at 37°C, samples were adjusted to 20 mM-methylmercury hydroxide, heated for 5 min at 50°C, cooled, adjusted to 10% glycerol, and 0.025% bromophenol blue and subjected to electrophoresis through a vertical 1% agarose gel (10 × 10 × 0.2 cm) in denaturing buffer (0.05 M-boric acid, 0.005 M-sodium borate, 0.01 M-sodium sulphate, 0.001 M-sodium EDTA, 6 mM-methylmercury hydroxide). After a run of 1 to 2 h at 30 mA, 70 V, gels were stained with ethidium bromide (1 μg/ml) in 0.2 M-ammonium acetate, 18 mM-2-mercaptoethanol, and photographed. As seen in Fig. 1 (a), the nucleocapsid preparation contained considerable amounts of DNA and smaller than genome-size RNAs. The larger band was not abolished entirely by either RNase or DNase and therefore contained both RNA and DNA which co-migrate in the gel.

The amount and size of contaminating nucleic acids were found to vary in different preparations. Apparently they are only loosely associated with the nucleocapsid protein. Treatment of nucleocapsid preparations with DNase or RNase degraded the DNA and the low molecular weight RNA species respectively, whereas the genomic RNA was only slightly affected by RNase (results not shown). Repeated banding in caesium chloride reduced the contaminating nucleic acids; however, the yield of genomic RNA was also decreased. Therefore, we routinely used only one density gradient step. The contaminating RNAs were mostly of ribosomal origin; the prominent bands co-migrated precisely with the 28S and 18S bands obtained in cytoplasmic extracts from uninfected cells. The nature of the DNA remains unclear and was not investigated further; on some occasions a DNA band was observed migrating even above the genomic measles virus RNA. We assume that the contaminating DNA consists of relatively high mole-
Fig. 1. Characterization of nucleic acid preparations isolated from measles virus nucleocapsids using electrophoresis in 1% agarose under fully denaturing conditions. (a) Lane 1, nucleic acids extracted from nucleocapsids; lane 2, marker DNA (pBR322 linearized; 4362 base pairs); lane 3, mixture of material in lanes 1 and 2; lane 4, mixture of material in lanes 1 and 2, digested with DNase I; lane 5, mixture of material in lanes 1 and 2, digested with RNase A. (b) Lane 1, RNA from nucleocapsid preparation (after DNase I treatment); lane 2, 50S RNA isolated from RNA shown in lane 1 by centrifugation in a sucrose gradient.

Circular weight fragments of different sizes which run artefactually as one band (Lehrach et al., 1977). This DNA invariably contaminated the viral RNA after sucrose gradient sedimentation (see below) unless it had been previously removed by DNase I digestion.

For the further purification of measles virus genomic RNA in routine preparations, the nucleic acids extracted from nucleocapsid preparations derived from caesium chloride, without RNase or DNase treatment, were resuspended in 100 μl of 100 mM-NaCl, 10 mM-HEPES pH 7.0 and 2% SDS per litre of original spinner culture. Any contaminating heavy metals were removed using a Chelex 100 (Bio-Rad) column (0.3 x 0.3 cm) (omission of this step led to degradation of the RNA, even in the presence of ethanol at −20 °C). Nucleic acids from the flow-through were phenol-extracted three times, ethanol-precipitated, collected by centrifugation, washed with 90% ethanol and dried under reduced pressure. The pellet was resuspended in 25 μl per litre of original spinner culture of 100 mM-NaCl, 10 mM-HEPES pH 7.0, 10 mM-MgCl2, and DNase I was added to a final concentration of 1000 U/ml.

After incubation for 5 min at 37 °C, the reaction mixture was adjusted to 1% SDS and 15 mM-EDTA, Pronase was added to 1 mg/ml and the solution incubated for 10 min at 37 °C. RNA was extracted three times with phenol, ethanol-precipitated, collected by centrifugation, washed with 90% ethanol and dried under reduced pressure. The pellet was resuspended in 100 μl of 10 mM-HEPES pH 7.0, 1 mM-EDTA per litre of original culture and heated for 1.5 min in a boiling water-bath. [Denaturation of RNA was necessary, since genomic RNA was partly double-stranded at this stage (30% RNase-resistant) and, consequently, would have been lost as material sedimenting more slowly than 50S during the following sucrose gradient.]

Centrifugation was carried out in polyallomer centrifugation tubes containing 10 to 30% (w/v) sucrose in 100 mM-NaCl, 10 mM-HEPES pH 7.0, formed as a step gradient (five steps) in a Spinco SW55 rotor at 50000 rev/min and 15 °C for 100 min. Fractions were collected by a sterile syphon system and aliquots were scintillation-counted. Fractions containing genomic RNA sedimenting around 50S were pooled, passaged through a Chelex 100 column (0.3 x 0.5 cm),
adjusted to 1 mM-EDTA and ethanol-precipitated. As seen in Fig. 1(b) it is possible by this method to isolate intact genomic RNA. This RNA remains intact after incubation for 1 h at 41 °C in 10 mM-HEPES pH 7-0, 1 mM-EDTA or for several weeks as an ethanol precipitate at −20 °C; it is fully sensitive to RNase digestion (results not shown). In addition to genomic minus strands, it contained considerable amounts of full-length plus strands and was 50% RNase-resistant after self-annealing. The yield of genomic RNA varies from infection to infection, but was of the order 0·5 to 1·5 µg per litre of spinner culture. This is low compared to yields of other negative-strand viruses, e.g. vesicular stomatitis virus (VSV), but it is enough for cloning experiments, which are in progress (K. Baczko, M. Billeter & V. ter Meulen, unpublished results).

To determine the molecular weight of measles virus genomic RNA, nucleocapsid RNA (after treatment with DNase I) was mixed with VSV genomic RNA, denatured and electrophoresed into a 1% agarose gel containing methylmercury hydroxide (Bailey & Davidson, 1976) for 3 h at 30 mA, 70 V (Fig. 2a). Migration distances were plotted against the logarithm of the molecular weights of VSV (Repik & Bishop, 1971) and 28S and 18S ribosomal reference RNAs (Stewart & Letham, 1973). The graph (Fig. 2b) shows a clear linear relationship in three experiments between the known molecular weights of the marker RNAs and their distance of migration. Therefore, we feel confident in our estimation of the molecular weight of measles virus genomic RNA as 4·5 × 10⁶. The relationship between log of molecular weight and distance migrated under the conditions used is known for RNA molecules up to 6·67 × 10⁶ (Wege et al., 1981). The value of 4·5 × 10⁶ for measles virus genomic RNA was also obtained (results not shown) using coronavirus RNAs (a gift from H. Wege) as size markers. The molecular weight found here differs significantly from the published value of 6·2 × 10⁶ (Schluederberg, 1971). However, that figure was derived from sedimentation in zonal sucrose gradients, which may be subject to some inaccuracy, due to the non-linearity of the gradient and the particular conformation of the RNA. Measles virus RNA has a molecular weight intermediate between that of other negative-strand viruses, e.g. VSV 3·8 × 10⁶ (Repik & Bishop, 1971) and respiratory syncytial (RS) virus 5 × 10⁶ (Stewart & Letham, 1973) which is in agreement with the estimated number of virus-coded proteins: five for VSV (Wagner et al., 1972), six for measles (Graves et al., 1978; Stephenson & ter Meulen, 1979) and seven for RS virus (Huang & Wertz, 1982).
Short communication

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