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Host Cell Factors Involved in the Production of Slowly Sedimenting Nucleocapsids in Measles Virus-Infected Cells

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A decrease in the sedimentation rates of the measles virus nucleocapsid, and the RNA contained within, were observed during acute measles virus infection when the growth conditions of Vero cells were altered. The change in sedimentation rates of virus nucleocapsids in these experiments was apparently due to the physiological state of the cell and was independent of the history of the measles virus used for infection since: (i) the same virus stock was used to infect cells from which nucleocapsids were prepared, (ii) nucleocapsid sedimentation rates were rapid when Vero cells freshly revived from liquid nitrogen were infected, but nucleocapsid profiles showed no decrease in the amount of slowly sedimenting material using the same cells and changing the virus preparation used for infection. Frequent cell splittings and numerous medium changes were among the growth factors which appeared to correlate to slowly sedimenting particle production. Changes in the amount of self-complementarity of the measles virus RNA were also observed under these conditions.

RNA viruses which have less than a full complement of their genetic material (defective particles) have been shown to cause persistent virus infections and have been implicated in the etiology of several diseases (for a review, see 18). Measles virus is prone to producing subgenomic particles in tissue culture (7) which may be related to defective interfering particles (17) and has been implicated in, or isolated from, a number of progressive neurological disorders such as multiple sclerosis (1) and subacute sclerosing panencephalitis (14). Understandably, the question of what role measles virus might play in the establishment of latent infections and chronic diseases has received much attention (12).

Several authors have demonstrated that the host cell can have significant effects on both the amounts of defective virus progeny produced and the amount of interference with a variety of negative-stranded RNA viruses (2, 8, 11, 16). One might predict, then, that physiological alterations in a given cell line could affect the production of defective progeny and alter the status of a persistent infection. Consistent with this idea is the observation that the pretreatment of BHK cells with actinomycin D inhibited the induction of defective interfering particles of vesicular stomatitis virus (6).

Thus, the question arises as to what changes in the replication of measles virus might occur as a function of the physiology of the host cell under conditions of controlled virus infection. In the present communication we report on the effects that changing growth conditions of Vero cells can have on the production of measles virus nucleocapsids and their RNAs.

MATERIALS AND METHODS

Cells. Vero cells (7) were grown in Eagle minimal essential medium containing 10% fetal calf serum and 100 μg each of penicillin and streptomycin per ml.

Virus. Wild-strain Edmonston virus (7) was propagated in Vero cells. Plaque-purified strains were derived by plating wild-strain virus at limiting dilutions. Plaques were picked, replated, and picked twice more before pools were prepared. Working pools were prepared by infecting Vero cells with virus at a multiplicity of 0.01 to 0.05 PFU/cell. When 80% or more of the cells showed measles virus-specific cytopathic changes, cultures were quick frozen and thawed. Infected culture fluids were cleared by centrifugation at 1,500 × *g* for 10 min and stored as samples at −70°C.

Labeling infected cells and cell fractionation. Vero cell monolayers grown in roller bottles (Bellco Glass, Inc.) were infected and nucleocapsids were isolated as described previously (7). Briefly, cells were exposed to 20 μg of actinomycin D per ml 24 h after infection, and 2 h later 20 μCi of 5-[^{3}H]uridine label (Amersham Corp.) per ml was added. At the end of the labeling period, 40 h after infection, cells were harvested. A cytoplasmic extract was prepared as follows: medium was removed and the cells were scraped into 10 ml of Tris-buffered saline. The cells were then pelleted at 1,500 × *g* for 10 min, washed once in Tris-buffered saline and resuspended in 1 to 3 ml of hypotonic buffer. Cells were disrupted by 15 strokes of a Dounce homogenizer, and nuclei were pelleted at 800 × *g* for 10 min. Labeled structures in the decanted cytoplasmic extracts were separated by rate-zonal cen-

trifugation in 15 to 40% (wt/vol) sucrose gradients. Gradients were fractionated, and acid-precipitable radioactive incorporation into RNA was determined by liquid scintillation counting after collection on a 0.45-μm nitrocellulose filter (Schleicher & Schuell Co.).

The presence of nucleocapsid structures was routinely confirmed by resistance to ribonuclease A as described previously (7). Also, in random experiments, mock-labeled nucleocapsid preparations were run simultaneously with radiolabeled preparations. The pooled RNAs extracted from these unlabeled nucleocapsids hybridized extensively to complimentary DNA synthesized from highly purified measles virus 50S RNA which was reverse transcribed by the method of Taylor et al. (20). Electron microscopy of the full length (200S) and short nucleocapsid material (110S) has also been described previously (7).

Isolation of virus RNA. Designated nucleocapsid fractions were pooled, and the material was pelleted through a 10% sucrose solution (100,000 × *g*, 4 h). Pellets were suspended in 50 mM Tris buffer (pH 8.3) containing 150 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 100 μg of proteinase K (EM Labs) per ml. The samples were incubated for 30 min at 65°C, and the reactions were terminated by the addition of equal volumes of phenol and chloroform. After two phenol-chloroform and two chloroform extractions, nucleic acids were precipitated from the aqueous phase by the addition of 2.5 volumes of 95% ethanol containing 0.2 M sodium acetate (pH 5.0) at −20°C for 16 h. Samples were collected by centrifugation, suspended in 20 mM phosphate buffer (pH 7.0), and used immediately or frozen at −70°C for further use.

Sizing of Measles RNA. The RNA from measles virus nucleocapsids was sized in a sucrose gradient in one of two ways. The RNA was either layered on a 15 to 30% (wt/vol) sucrose gradient and centrifuged for 17 h at 20,000 rpm in a Beckman SW27 rotor or sized by the method of Hall and Martin (3), in which intact nucleocapsids are incubated in the presence of 1% sodium dodecyl sulfate at 65°C for 90 s, layered on a 15 to 30% (wt/vol) sucrose gradient, and centrifuged as described above.

Hybridization assays. Total complementarity was assessed in reaction volumes of 100 μl containing 0.48 M phosphate buffer and 0.5 to 5 μg of measles virus RNA per ml. Reaction samples were boiled for 5 min, quick-cooled in an ice bath, and incubated at 74°C to a C_rt of 1 to 2. Assays were terminated by cooling on ice followed by dilution with 3 volumes of distilled water. Samples were digested with RNase A (20 μg/ml; Sigma Chemical Co.) for 40 min at 37°C, and undigested RNA was collected on a 0.45-μm nitrocellulose filter after precipitation in 10% trichloroacetic acid in the presence of 5 μg of carrier RNA. Determinations for snap-back complementarity were carried out in an identical manner except that the 74°C incubations were omitted.

RESULTS

Selection of Vero cells producing low levels of slowly sedimenting particles. Initial observations made in our laboratory indicated that Vero cells which were allowed to remain confluent for 1 to 2 weeks between splittings and which were fed irregularly did not appear to produce as many slowly sedimenting particles upon infection with measles virus as did cells which were passaged on a more regular schedule. We selected for a subcell line of Vero cells which, after infection with measles virus, produced only 200S nucleocapsids. To do this, Vero cell cultures were grown in roller bottles for about 2 months. During this time, the media on these cells were changed one to two times per week, and they were not passaged until some or most of the cell sheet began to slough from the glass. When a cell culture was obtained which consistently produced a sharp 200S measles virus nucleocapsid peak, a large virus pool was made in these cells, and aliquots of the virus pool were frozen and stored at −70°C. Aliquots of the selected cell line were also frozen and stored in liquid nitrogen.

Physiological factors influencing the production of slowly sedimenting measles virus nucleocapsids. When the Vero cells were infected with identical aliquots of a measles virus pool prepared as described above, a sharp 200S nucleocapsid peak was observed initially (Fig. 1A). However, if the cells had their media changed three times and were split 1:7 each week, a more slowly sedimenting shoulder (ca. 170S) began to appear after a few cell passages (Fig. 1B). This shoulder evolved to a distinct, albeit broad, nucleocapsid peak of ca. 110S with succeeding passages (Fig. 1C) until such time that these 110 to 170S species predominated in the nucleocapsid preparation (Fig. 1D).

If a fresh aliquot of cells was taken from liquid nitrogen, the nucleocapsid peaks were once again exclusively 200S in nature at first, but began to produce increasing amounts of defective viral material after several passages. In the experiment shown in Fig. 1, the time sequence represents 3, 6, 8, and 10 cell passages in parts A, B, C, and D, respectively. In other experiments the absolute number of passages needed to produce the 110 to 170S nucleocapsid material varied somewhat, but the general pattern observed was always the same.

If the cells were grown by using either fewer feedings per week or greater time periods between cell passages (e.g., 10 versus 7 days) or both, it generally took a few more passages before slowly sedimenting nucleocapsids were initially observed, but, once they began to appear, their proliferation was rapid regardless of how the cells were grown. Once the Vero cells began to produce slowly sedimenting nucleocapsid material, infecting the cells with a different measles virus preparation had no effect on their production. Similarly, if cells which had begun

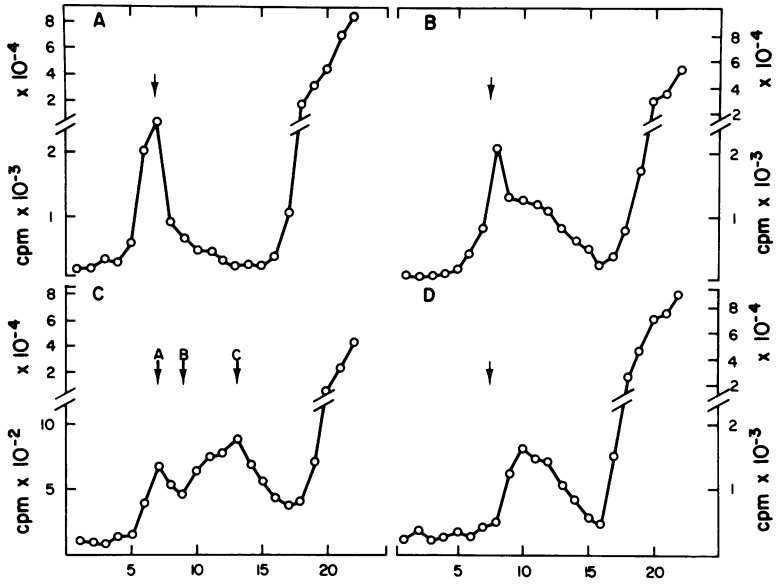

FIG. 1. *Production of defective nucleocapsid particles as a function of Vero cell passage number. Identical aliquots of a measles virus pool was used to infect Vero cells as described in the text. Ampules of Vero cells which produced no detectable defective nucleocapsid material were plated into each of two 8-ounce (ca. 236.6 ml) prescription bottles and subsequently transferred to roller bottles. These roller bottles, which were fed three times per week, were split 1:7 each week, six of the bottles being used for each nucleocapsid preparation and the other held and passaged 1:7 for subsequent preparations. Nucleocapsid profiles were determined as described in the text for Vero cells passaged 3 (A), 6 (B), 8 (C), or 10 (D) times under rapid-growth conditions. Arrows labeled A, B, and C in (C) represent fractions from which RNAs in Fig. 2 were derived. Arrows in (A), (B), and (D), and arrow A in (C) represent the position of a 200S nucleocapsid particle.*

to produce these nucleocapsids were shifted to conditions identical to those which were used to select for the Vero cells which produced 200S nucleocapsids exclusively, reversal of 110 to 170S production was not readily observed, although the rate of their formation did appear to be retarded somewhat. However, regardless of how cells were grown, if the presence of slowly sedimenting nucleocapsids was allowed to progress until it represented virtually the entire nucleocapsid population, a sudden cessation in the production of the slowly sedimenting nucleocapsids would eventually occur and the cells would cycle back to a very short-lived condition in which only 200S nucleocapsids were synthesized, but to which slowly sedimenting nucleocapsid particles began to appear within a very few (one to three) cell passages. Vero cell morphology was unchanged as determined by light microscopy for the duration of these experiments.

Characterization of Genomic RNAs. (i) Size. Under either of the techniques described in Materials and Methods, the RNA extracted from the 200S nucleocapsids sedimented through a sucrose gradient at ca. 50S; nucleocapsid material from the light side of the major peak (170S) possessed an RNA of about 18 to 28S, whereas the slowest sedimenting nucleocapsids (110S) possessed RNAs in the 10S range (Fig. 2). Figure 2 shows that the RNA derived from the 200S nucleocapsid portion of the gradient in Fig. 1C possesses a minor 10S component. This is presumably due to contamination of the 200S nucleocapsids with a more slowly sedimenting nucleocapsid species since the RNA extracted from the 200S nucleocapsid peak in Fig. 1A lacked this smaller component (date not shown). We have observed as noticed previously (7) that the correlation between the sedimentation values of the virus nucleocapsids and the RNAs extracted was very good.

(ii) Self-complimentarity of the measles RNA. A disparity occurs when different RNA purification techniques are used before the measurement of measles virus RNA self complementarity. If the RNAs are purified extensively (i.e., using proteinase K and phenol-chloroform extractions as described in Materials and Methods), significant amounts of self-complementarity are often observed (see below). However, little (<10%) self-complementarity was seen in the measles virus RNA using the sodium dodecyl sulfate-heat method of Hall and Martin (3). This may be due to the presence of some residual

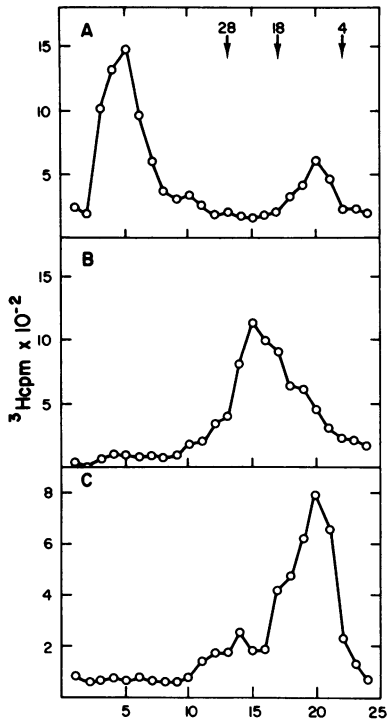

FIG. 2. *Sedimentation values of measles virus nucleocapsid RNAs. Aliquots of the nucleocapsid fractionation described in Fig. 1C were treated with 1% sodium dodecyl sulfate and heated for 90 s to release to measles RNA(s) and centrifuged as described in the text. (A), (B), and (C) represent the RNA profiles corresponding the those fractions designated with arrows in Fig. 1C. ^{3}H-labeled Vero RNA was used as 28S, 18S, and 4S size markers.*

protein remaining on the RNA which inhibits hybridization. The 260/280 spectral ratios for the RNAs derived by the sodium dodecyl sulfate-heat extraction are typically in the range of 1.6 to 1.7. The ratio obtained by the phenol extraction techniques is 1.9. All RNA extractions performed in the experiments below used this latter phenol extraction technique.

Almost all of the self-complementarity observed in the RNA extracted from measles virus nucleocapsids was of the snap-back, or hairpin, variety (data not shown). Figure 3 demonstrates that a statistically significant (r = 0.73) linear increase in the amount of snap-back complementarity was observed as a function of rapid passage (i.e., cell splittings of 1:7 and media changes three times per week) when fresh Vero cells are grown from liquid nitrogen. Table 1 shows the relationship between the nucleocapsid sedimentation rate and the degree of snap-back complementarity for a fresh Vero cell culture which had been rapid passaged for the period of several cell splittings. The appearance of snap-back complementary RNA foreshadowed the appearance of slowly sedimenting measles virus nucleocapsids. Furthermore, an appreciable amount of complementarity (>20%) was observed before the 110S nucleocapsids were produced. Interestingly, both the rapidly and more slowly sedimenting populations of nucleocapsids exhibited similar levels of snap-back RNA when extracted from a given nucleocapsid preparation.

Measles virus nucleocapsids prepared in Vero cultures that had previously produced a predominance of 110S and 170S types, but whose slowly sedimenting nucleocapsid populations had suddenly disappeared, as described above, maintained their high levels of snap-back complementarity. Incubating nucleocapsids in the presence of ribonuclease A (30 μg/ml, 37°C, 30 min) before extracting the RNA had no effect on the hybridization results, indicating that contaminating polysomal RNA was not responsible for any of the annealing results.

DISCUSSION

Measles virus, when grown in Vero cells, produces a substantial number of subgenomic particles in addition to full-sized particles as as-

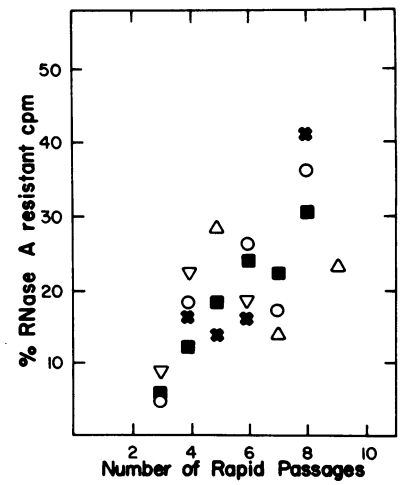

FIG. 3. *Snap-back complementarity of measles virus RNA as a function of Vero rapid cell passage. After the designated number of rapid passages, Vero cells were infected with an aliquot of measles virus, and the RNAs were purified by phenol extractions as described in the text.* ○, ▽, △, ■ *and* ×, *A series of experiments done beginning with a given ampule of cells which had been freshly revived from liquid nitrogen. For each of these respective series, the same virus pool was used, although different virus pools were used for each series. All values shown are for the 50S RNA of the 200S nucleocapsid material. The* (■) *series represents the experiment shown in Table 1. Complementarity values are averages of triplicate nuclease assays.*

TABLE 1. *Characterization of measles virus nucleocapsids as a function of Vero cell passage number*[a]

No. of rapid cell passages	Nucleocapsid class(es)	RNA size(s)	% Snap-back complementarity
3	200S	50S	6.0
4	200S	50S	12.6
5	200S (170S)	50S (18–28S)	18.3
6	200S (170S)	50S (18–28S)	24.7
7	200S 170S	50S 18–28S	22.0 23.9
8	200S 170S 110S	50S 18–28S 10S	30.6 29.2 27.8

[a] Six roller bottles of Vero cells which had been grown for the indicated number of rapid passages were infected with an aliquot of measles virus at a multiplicity of infection of 0.05. For each experiment, nucleocapsids were purified and 5% of each fraction was counted to determine the nucleocapsid profile. The remaining nucleocapsid material from each respective peak was pooled, and phenol was extracted as described in the text. An aliquot of this purified RNA was centrifuged through sucrose to determine its sedimentation value, and the self-complementarity of the RNAs was assessed using the remaining portion. The complementarity values are averages of triplicate nuclease assays. Nucleocapsid samples in parentheses represent minor slowly sedimenting shoulders on the 200S peak. Snap-back values were only determined for the RNAs in the leading portion of these nucleocapsid peaks.

sessed by the sedimentation rates of nucleocapsid structures through sucrose gradients (7; unpublished data). By growing our Vero cells under conditions which are generally considered disadvantageous for cell growth (i.e., limited feedings, irregular passage splittings, allowing cells to remain confluent until they fall off of the glass), a Vero cell culture was selected which produced only full-length 200S nucleocapsids. However, when the conditions used to grow the cells were changed to medium changes three times per week and cell splittings of 1:7, short nucleocapsid species began to appear despite the fact that the same virus pool that generated the exclusively full-length material was used for infection. Our Vero cells may have two (or more) subpopulations of cell types which effect virus replication differently, and predominance of one of these cell types may occur under different growth conditions. Since cell lines vary in their ability to support virus growth, it is not unrealistic to assume that different subpopulations of cells might also possess various abilities to properly process mature virions.

The exact physiological events which contribute to the production of these distinct subgenomic nucleocapsid species have not been determined. It is possible that any of a number of events which correlate to a slower cellular growth rate, such as DNA replication, respiration, nutritional limitations or confluency, may effect virus assembly. Other work in our laboratory has shown that certain anesthetic agents which are capable of reversibility blocking acute measles virus replication (9) will, when removed, induce the formation of slowly sedimenting nucleocapsids similar to those described above (manuscript in preparation). One of these anesthetics, halothane, has also been shown to inhibit mitochondrial respiration (13), DNA synthesis (5), and affect a number of cellular membrane changes (19). Further investigations are necessary to determine which, if any, of these parameters of cell growth are responsible for the production of slowly sedimenting nucleocapsids.

After the cells were infected with measles virus, we observed that our Vero cells, when freshly grown from liquid nitrogen, produced 200S nucleocapsid material which possesses little self-annealing RNA. However, when grown under rapid-passage conditions, they began to produce snap-back RNA after a few passages and defective particles several passages later. When Vero cells which produced a predominance of 170S and 110S nucleocapsids cycled to a physiological state which induced a rapid cessation in the production of slowly sedimenting particles, thereby producing nucleocapsid preparations which contained nothing but 200S particles, the degree of self-complimentarity remained high, and subgenomic particles appeared within a very few cell passages. This indicates that the presence of snap-back RNA in the measles population may serve in part as a trigger for short-particle production. It is possible that the production of a small amount of snap-back material is a normal event in the measles replicative cycle, and if too much of this material is produced, it may somehow lead to the formation of subgenomic progeny. Alternatively, the snap-back type of RNA may simply be characteristic of one of a number of species which expresses itself during measles replication in Vero cells. Such snap-back-type defective particles have been reported in vesicular stomatitis virus (10, 15).

The fact that slowly sedimenting nucleocapsid material can arise as a function of cell passage with a given virus pool strongly suggests that

the physiological state of the cell is involved. Other investigators have shown that the amount of defective material and interference produced during the course of a virus infection is often a function of the host cell line employed (see above). Our observations, along with these others, indicates that the production of less than full-length virus progeny likely involves a delicate interaction between the virus and the host cell.

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