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Persistent Infection of Cells in Culture by Measles Virus

III. Comparison of Virus-Specific RNA Synthesized in Primary and Persistent Infection in HeLa Cells

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The pattern of actinomycin D-resistant RNA synthesis was examined during primary infection of HeLa cells by virulent Edmonston measles virus and in two HeLa clones persistently infected by the same strain of virus. One of these clones, K11, produces infectious virus of low virulence for HeLa cells, and the other, K11A-HG-1, has thus far failed to yield infectious virus. The patterns of virus-specific RNA synthesized in these three types of infection are qualitatively similar to each other and to the patterns of virus-specific RNA synthesis in other paramyxovirus infections. There were, however, quantitative differences. In addition, virions of the virulent Edmonston strain of measles virus were found to contain high-molecular-weight RNA with a sedimentation constant identical to that of Newcastle disease virus.

Measles virus infection in a variety of cell cultures is characterized by a latent period of 10 to 18 h followed by a period of virus production which lasts for 24 to 48 h under optimal conditions (17). Only a few infectious units per cell may be recovered, even though there are extensive cytopathic effects (16, 18, 21, 25).

In infected HeLa cultures, a few cells survive and multiply to give rise to new cell populations with persistent infection (20). Studies with clones of HeLa cells persistently infected by the Edmonston strain of measles virus established that the development of persistent infection was associated with the selection of a variant virus with low virulence for HeLa cells (21). When one of these clones was grown in the presence of measles antibody, a persistently infected subclone was obtained which produced no detectable infectious virus (22). The capacity of measles virus to induce persistent infection in HeLa cells and the virus-host cell events which occur in such infections may reflect the host-measles virus relationships leading to the development of a subacute sclerosing panencephalitis (1, 12, 13).

We are now trying to determine the factors which control persistent infection in HeLa cells by Edmonston measles virus employing the K11 clone as the prototype of persistently infected HeLa cells which produce complete virus (21) and the K11A-HG-1 clone as the prototype of persistently infected HeLa cells which produce little, if any, complete virus (22).

As a first step, we analyzed the patterns of virus-specific RNA synthesized in these two persistently infected clones and compared them to those synthesized in HeLa cells during primary virulent infection by parent Edmonston virus. Because the RNA synthesis of this putative paramyxovirus has not been studied previously, a necessary prelude to this study was a comparison of the virus-specific RNA produced during primary infection with those produced during infection by a model (26) paramyxovirus-Newcastle disease virus (NDV). Virion RNA of Edmonston measles virus and NDV were also compared.

The results indicate that the patterns of virus-specific RNA synthesized in HeLa cells during virulent infection by Edmonston virus and in both types of persistent infection are qualitatively similar to each other and to the pattern of virus-specific RNA synthesized in NDV infection. However, quantitative differences have been observed. In addition, we have found that, as reported by Schluederberg (23), measles virions like those of other paramyxoviruses possess high-molecular-weight RNA (2, 5, 9, 11), and that the sedimentation constant of this RNA is identical to that of NDV.

MATERIALS AND METHODS

Cells. HeLa cells and the persistently infected HeLa clones K11 and K11A-HG-1 were routinely cultured as described previously (21) in Eagle basal medium supplemented with 10% heated fetal calf serum (Gibco), 100 units of penicillin per milliliter, 100 μg of streptomycin per milliliter, and 2 μg of amphotericin B per milliliter (FCE medium). The $NaHCO_3$ concentration in Eagle medium was reduced to 0.1%. The Vero cell line, from kidneys of African green monkeys (24), was supplied by M. D. Daniels of the Harvard University Primate Center. Cultures of these cells were routinely prepared in the same manner as for HeLa cells in FCE medium with the fetal calf serum at a final concentration of 5%. Cell suspensions for culture were prepared with 0.1% trypsin and 0.005 M versene in saline A. Saline A (19) was modified by replacement of the $NaHCO_3$ with 0.02 M Tris to give a final pH of 7.7 to 7.8. After 2 to 3 days of Vero cell growth, the $NaHCO_3$ concentration in the medium was increased to 0.2%. Secondary chicken embryo cultures were prepared as previously described (4, 7).

Viruses. A stock of parent Edmonston measles virus previously passaged in HeLa cells (21) was prepared by infecting roller bottle cultures containing approximately 10^8 Vero cells. After the development of marked cytopathic effects, virus was harvested by freezing and thawing the cells twice in 10 ml of culture fluid, followed by homogenization of the cell suspension for 3 min by means of a motor-driven Teflon tissue grinder (Tri-R Industries) immersed in an ice bath. The homogenate was centrifuged at 1,500 × *g* for 10 min at ambient temperature, and the supernatant containing 5×10^8 PFU/ml in Vero cells was stored at −50 C.

NDV (Australia-Victoria, 1932) was grown in embryonated hen eggs and concentrated as described previously (4, 7).

Measles-infected HeLa cultures. Cultures of HeLa cells with primary infection by parent Edmonston virus and of persistently infected HeLa K11 and K11A-HG-1 clones were prepared in 100- by 15-mm Pyrex dishes with 2×10^7 cells in 10 ml of FCE medium for RNA studies, production of cell-associated virus, and cell counts. For determination of viral intracellular immunofluorescence, cultures were prepared in 60- by 15-mm pyrex dishes containing cover slips with 10^6 cells in 5 ml of FCE medium. Primary infection of HeLa cells was achieved by adding virus at an input multiplicity of 10 to trypsinized log-phase cells suspended in FCE medium in siliconized screw-capped vials. The volume of the virus-cell mixture was 5 to 7 ml with a final cell concentration of 2×10^7/ml. The mixture was then stirred for 2 h at 36.5 C by means of a magnetic Teflon-coated bar, and cultures were then prepared after sedimentation of the cells and suspension in fresh FCE medium. Cultures of the K11 clone between its 234th and 242nd serial passages and of the K11A-HG-1 clone between its 32nd and 40th serial passages were prepared directly from trypsinized stock cultures in log phase. All cultures were incubated in an atmosphere of 5% CO_2 in air.

Measles virus infection of the HeLa cells in each experiment was monitored (i)by measurement of viral intracellular immunofluorescence; (ii)by recovery of cell-associated virus as described previously (21); and (iii)by the development of cytopathic effects in the primary infected cultures. Cell-associated virus was obtained by homogenizing cells scraped from two dishes in saline A, pH 7.2 to 7.4. Fetal calf serum was then added to the homogenates giving a final concentration of 10%, and the homogenates were then stored at −50 C prior to plaque assay. Plaque assays were performed in Vero cells (18, 24) rather than in primary human amnion, since the latter are too resistant to overt infection by variant virus from the K11 clone (21).

Labeling of intracellular RNA. HeLa cell cultures 20 h after primary infection and 20- to 22-h old cultures of uninfected HeLa cells or of the persistently infected HeLa K11 and K11A-HG-1 clones were treated for 2 h with actinomycin D (courtesy of Merck, Sharp and Dohme, Rahway, N.J.) at a concentration of 30 μg/ml of medium. The cells were then labeled for 2 h with ^{3}H-uridine at a concentration of 50 μg/ml (New England Nuclear Corp., 20 Ci/mM) in FCE medium.

Labeling of the NDV intracellular RNA was done in secondary cultures of chicken embryo cells infected with virus at a multiplicity input of 5. Cells were treated with ^{14}C-uridine (New England Nuclear Corp., 50 μCi/mM) between 6 and 8 h after infection and 40 min after the addition of actinomycin D (10 μg/ml).

Labeling of virion RNA. Measles viron RNA was labeled by preparing 100- by 15-mm glass petri dish cultures of an infected HeLa cell suspension in medium containing 25 μCi of ^{3}H-uridine per milliliter. Culture fluids were collected from 10 cultures 24 and 48 h after infection. The culture fluids were pooled, and the supernatant virus was purified by differential centrifugation as described for NDV (6, 10). NDV was labeled with ^{32}P (New England Nuclear Corp.) in embryonated hen's eggs and purified in the same manner (6, 10).

Isolation and fractionation of RNA. Labeled cells and viruses were solubilized in pH 8.5 buffer containing 1% sodium dodecyl sulfate (SDS, Eastman) and 1% 2-mercaptoethanol (Eastman), then extracted in cold phenol, and alcohol precipitated as previously described (6, 10). Extracted RNA was fractionated by velocity sedimentation in SDS containing sucrose gradients (8). For each fraction absorbancy was monitored at 260 nm, and trichloroacetic acid-precipitable radioactivity was determined as previously described (8). RNase resistance (50 μg of pancreatic RNase per milliliter, Sigma Chemical Co.; and 25 μg of T1RNase per milliliter, Calbiochem) was also determined as previously described (8).

RESULTS

Comparison of virion RNA of measles virus and NDV. The use of the techniques employed by Duesberg and Robinson (10) for the purification of NDV revealed that HeLa cell-grown

measles virus has a buoyant density of 1.22 g/cm³ in linear 15 to 65% sucrose gradients containing D_2O. This value is in good agreement with that of 1.225 g/cm³ obtained by Schlueder-berg (23) for trichloroacetic acid precipitability of ³H-uridine-labeled Vero cell-grown measles virus. Figure 1 shows the distribution of tritium counts and densities obtained after similar purification from fluids of ³H-uridine-labeled cultures of either infected or uninfected HeLa cells. Whereas the gradient from the uninfected cultures (panel B) contains little trichloroacetic acid-precipitable tritium, that from the infected cultures (panel A) contains considerable amounts which band very broadly in the density range of 1.21 g/cm³.

Pools of fractions 7 to 9 (average density, 1.22 g/cm³) and fractions 10 to 12 (average density, 1.19 g/cm³) from gradient of infected cells (Fig. 1A) and fractions 8 to 13 from the uninfected cell gradient (Fig. 1B) were designated A, B, and C, respectively. They were solubilized in SDS and phenol extracted in the presence of similarly solubilized ³²P-labeled NDV added for comparison. After alcohol precipitation, the precipitates were resolubilized and fractionated by velocity sedimentation. In Fig. 2, panels A and B, respectively, show the distribution of trichloroacetic acid-precipitable counts for RNA isolated from the 1.22 and 1.19 g/cm³ regions of the density gradient from the infected cell cultures (Fig. 1A). Both panels show trichloroacetic acid-precipitable tritium which cosediments exactly with the 50*S* ³²P-labeled NDV-RNA. In addition, each panel contains much larger amounts of slower sedimenting trichloroacetic acid-precipitable tritium. Panel C, representing the trichloroacetic acid-precipitable tritium from the uninfected cells, contains no counts in the 50*S* region and small amounts in the more slowly sedimenting regions. Therefore, as in the case of other paramyxoviruses, measles virus contains 50*S* RNA. The fact that the trichloroacetic acid-precipitable tritium

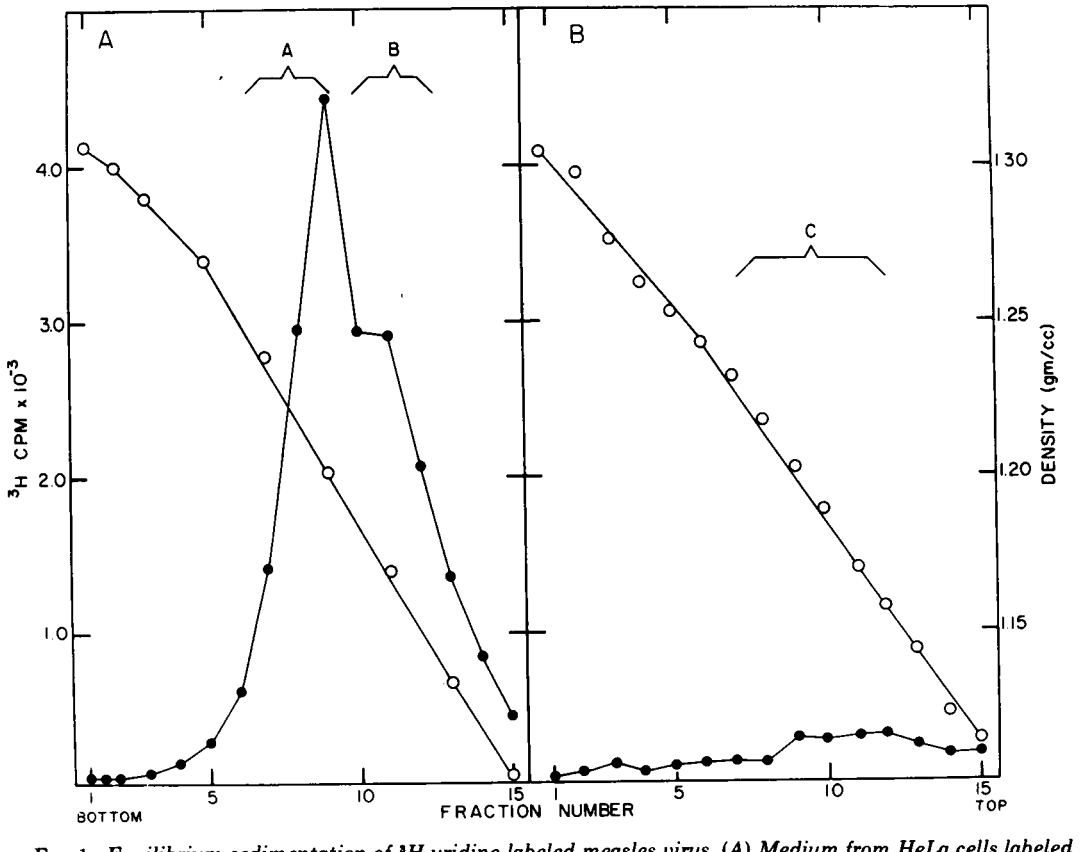

FIG. 1. *Equilibrium sedimentation of ³H-uridine-labeled measles virus. (A) Medium from HeLa cells labeled with ³H-uridine during primary infection by measles virus, as described in Materials and Methods, was subjected to low-speed centrifugation (10,000 × g for 10 min), layered over 15 to 65% sucrose gradients containing D_2O, and centrifuged at 4 C for 4 h at 180,000 × g in a Spinco SW41 rotor. (B) Medium from uninfected HeLa cell cultures treated in a similar manner.*

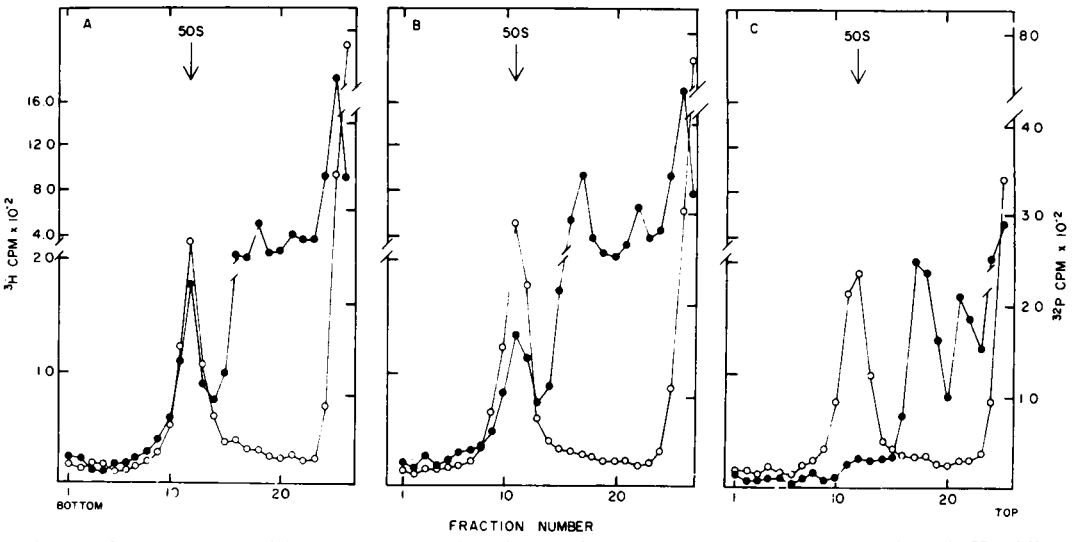

FIG. 2. *Comparison of virion RNA of measles virus and NDV. (A) Velocity sedimentation of ^{3}H-uridine-labeled measles virus RNA extracted from the fractions designated "A" in Fig. 1A (average density of 1.22 g/cm^3) (●) and NDV-RNA labeled with ^{32}P (○) centrifuged at 22 C for 3.5 h at 180,000 × g in a Spinco SW41 rotor. (B) Same as (A) but RNA extracted from the fractions designated "B" in Fig. 1A (average density of 1.19 g/cm^3). (C) Same as (A) but RNA extracted from the fractions designated "C" in Fig. 1B (uninfected cultures).*

extracted from the uninfected culture fluids shows a sedimentation profile similar to that of the more slowly sedimenting material from the infected cultures suggests that the latter material may be of host cell origin and released in larger quantities as a result of the cytopathic effects of measles virus.

Comparison of virus-specific RNA produced in primary infection by measles virus and NDV. Preliminary experiments revealed that as in other paramyxovirus infections measles virus-specific RNA synthesis can only be detected in the infected cell if the background of the host cell RNA synthesis is suppressed by treatment with actinomycin D. In the measles virus-HeLa system, a 2-h pretreatment with 30 μg of actinomycin D per milliliter was found to suppress the host cell RNA synthesis by 99.8%.

Figure 3 shows the distribution of trichloroacetic acid-precipitable tritium from actinomycin D-treated uninfected or measles virus-infected HeLa cells cosedimented with ^{14}C-labeled virus-specific RNA from NDV-infected chicken embryo cells. It can be seen that the measles virus-infected cells contain considerable amounts of trichloroacetic acid-precipitable tritium species not found in similarly treated uninfected HeLa cells, and that these species cosediment with NDV-specific RNA from NDV-infected cells. Thus, as previously shown for NDV infection (5), the measles primary infected cells contain little trichloroacetic acid-precipitable tritium in the 50*S* region and greater amounts in more slowly sedimenting regions with a major peak at 18*S*.

Figure 4 shows that the measles virus-specific RNA is mostly RNase sensitive and that, as in NDV infections (5, 6), the extent of RNase resistance increases as the sedimentation constant increases. There appears to be a peak of RNase resistance between the 28 and 50*S* regions.

Comparison of primary virulent and persistent virus infection in HeLa cells (cytopathic effects, viral intracellular immunofluorescence, and recovery of cell-associated virus). These findings are shown in Table 1 for cultures treated with actinomycin D and ^{3}H-uridine and are essentially the same as in untreated cultures. The percentage of cells of both the K11 and K11A-HG-1 clones with viral intracellular antigen is nearly the same as the percentage of cells in the primary virulent virus-infected cultures. However, the yields of infectious virus from disrupted K11 cells are less than 5% of that recovered from disrupted primary infected cells 24-h postinfection and less than 1% of that recovered 48 h after infection. No infectious virus was recovered from a total of 2×10^7 equivalents of K11A-HG-1 cells.

Comparison of virus-specific RNA produced in primary and persistent measles virus infection. Figure 5 contains velocity sedimentation profiles of actinomycin D-resistant RNA synthesized in primary and persistent measles-infected HeLa cells. Figure 5A shows that both the virus-yielding K11 cells and

the nonyielding K11A-HG-1 cells contain 50*S*, 30 to 35*S*, and 18*S* species characteristic of primary Edmonston virus infection. Figure 5B shows a similar experiment in which cells undergoing virulent measles virus infection were also included. The total amount of ^{3}H-uridine incorporated into the uninfected cells, persistently infected cells, and those undergoing primary infection represents less than 1%, 1 to 2%, and 5% of the total amount of ^{3}H-uridine incorporated into these cultures when not treated with actinomycin D (data not shown). Figure 5B shows that, although the patterns of incorporation of ^{3}H-uridine are qualitatively similar in all three types of infection and nearly equivalent amounts of incorporation have taken place in all three, there is considerably more ^{3}H-uridine incorporated into the low-molecular-weight RNA species in primary infection.

Figure 5C compares the incorporation of ^{3}H-

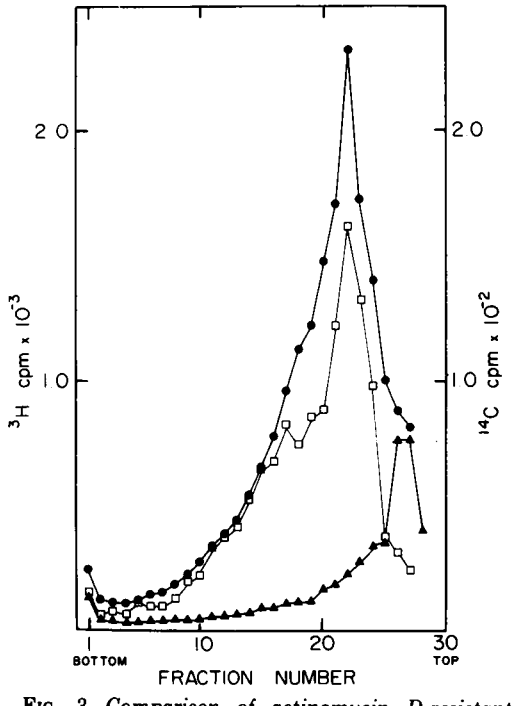

FIG. 3. *Comparison of actinomycin D-resistant virus-specific RNA synthesized in HeLa cells infected with virulent Edmonston strain measles virus and the RNA synthesized in NDV-infected chicken embryo cells. ^{3}H-uridine-labeled RNA from measles virus-infected (●) cells cosedimented (at 42,200 × g for 15 h at 20 C) with ^{14}C-labeled NDV-specific RNA (□). ^{3}H-uridine-labeled RNA from similarly treated uninfected cells (▲) run in a parallel gradient. Conditions of infection and labeling for this and subsequent figures are described in Materials and Methods.*

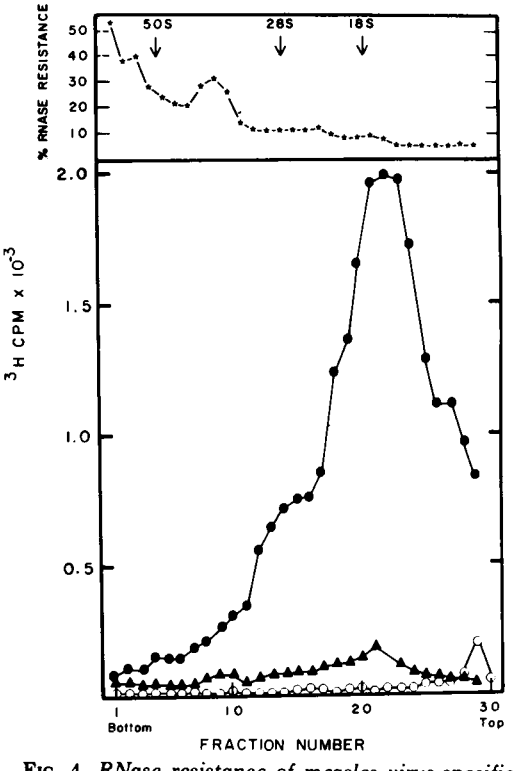

FIG. 4. *RNase resistance of measles virus-specific RNA synthesized in HeLA cells. After sedimentation as in Fig. 3, fractions were divided equally. One sample was treated for 45 min at 37 C with 50 μg of pancreatic RNase per milliliter and 25 U of T1 RNase per milliliter. The other sample was incubated under similar conditions but without RNase. Trichloroacetic-precipitable radioactivity of all fractions was measured. Total trichoroacetic acid-precipitable counts (●), RNase-resistant counts (▲), uninfected cell RNA (O) run in a separate gradient.*

TABLE 1. *Cytopathic effects, viral intracellular immunofluorescence, and infectious virus in HeLa cells with primary virulent and persistent infection by Edmonston measles virus*

HeLa cultures	Passage level	Hours post-infection	CPE	Viral intracellular antigen (%)	PFU/cell equivalent
Primary infected[a]	–	24	± or +	98 to 100	1-3
Persistently infected	–	48	++ or +++	100	5-20
K11 clone[a]	234 to 242	–	– or ±	87 to 98	$1-3 \times 10^{-2}$
K11A-HG-1 clone[a]	32 to 40	–	–	83 to 94	$<2 \times 10^{-7}$

[a] Representative of from 5 to 8 experiments.

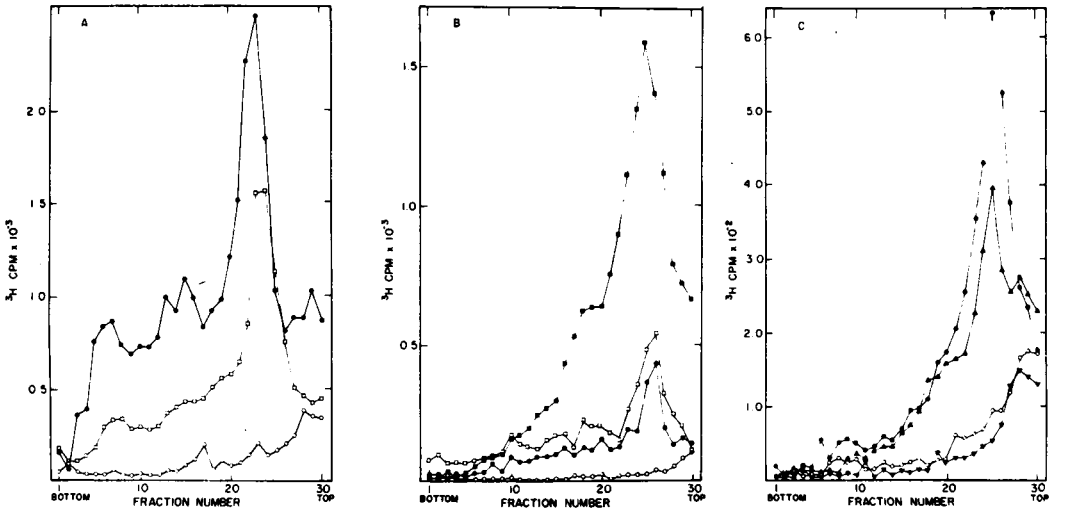

FIG. 5. *Actinomycin D-resistant RNA extracted from HeLa cells with persistent measles virus infection. (A) Velocity sedimentation (in parallel gradients) of ³H- uridine-labeled RNA from actinomycin D-treated K11 (●) and K11A-HG-1 (□) clones and uninfected HeLa cells (○). (B) Comparison of actinomycin D-resistant RNA synthesized in primary virulent (■) and persistent infection (as in "A"). (C) Comparison of actinomycin D-resistant RNA extracted from K11 cells (●) and clones K11-VP1, virus producing, (▲), and K11-VF1, virus free, (▼), as well as uninfected HeLA cells (●).*

uridine into the RNA of a virus-producing subclone, K11-VP1, and a virus-free subclone, K11-VF1, to the ³H-uridine incorporation of the parent K11 clone and uninfected HeLa cells. The K11-VP1 subclone was established from a single hemadsorption-positive K11 cell (Yamamoto and Rustigian, unpublished data). Ninety-six percent of the cells in this subclone showed viral intracellular immunofluorescence. The K11-VF1 subclone was obtained from an hemadsorption-negative colony of K11-VP1. None of the cells in K11-VF1 showed viral intracellular immunofluorescence. K11-VP1 incorporates ³H-uridine into RNA with patterns similar to that of parent K11 cells (Fig. 5C), whereas K11-VF1 shows patterns which are undistinguishable from those of uninfected HeLa cells.

DISCUSSION

In measles virus infection, as in NDV (5), Sendai (2), and mumps (11) infection, the majority of the RNA synthesized in actinomycin D-treated infected cells sediments more slowly than the 50*S* RNA of the virus. The major peak of this RNA is at 18*S*, and, in addition, the usual 28 to 35*S* RNA is found. As in the case of other paramyxoviruses, the majority of these other RNA species appear to be single stranded, as indicated by their lack of resistance to RNase. Limited amounts of RNase resistance are found in the higher-molecular-weight species, with a major peak between 35 and 40*S*. However, because the amount of this RNase-resistant material is quite small, it has not been possible to determine whether they are involved as intermediates in the synthesis of single-stranded species as suggested for the base-paired RNA species extracted from NDV-infected cells (6). We are presently attempting to obtain enough 50*S* virion RNA for use in annealing studies with the intracellular virus-specific RNA and with the slowly sedimenting species found in virion preparations. However, by analogy with other paramyxovirus infections, it appears likely that the majority of the intracellular measles virus-specific species are complementary in base sequence to the 50*S* RNA of the virion.

A comparison of virus-specific RNA synthesis in primary virulent and persistent measles virus infection of HeLa cells shows that, although both types of infection produce qualitatively similar virus-specific RNA, the primary Edmonston virus infection yields proportionally three times more of the low-molecular-weight RNA species. This observation is similar to those of Blair and Robinson (2) and Bratt (3) that, for other paramyxovirus infections, the ratio of low- to high-molecular-weight species is lower in avirulent or mildly virulent infection than in virulent infection.

The finding of qualitatively similar patterns of intracellular virus-specific RNA provides no insight into the differences in virus production and other biological properties found in HeLa

cells, primary virulent measles infection, and those with the two types of persistent infection. This may, in part, be due to the limitations of the velocity sedimentation procedure to resolve individual species within the 18*S* peak which, for NDV and Sendai virus infection, appears to contain virus-specific mRNA as indicated by intimate association with polyribosomes (2, 5). Separation of this 18*S* RNA into distinct species using electrophoresis in SDS-containing polyacrylamide gels as has been done for the 18*S* RNA of NDV (Collins and Bratt, in preparation) may allow for a more precise definition of qualitative and quantitative differences in the virus-specific RNA produced in primary and persistent infections which may in turn be correlated with differences in virus-specific proteins. In this connection, studies on the synthesis of intracellular virus-specific proteins in these different types of infection are currently underway.

The inclusion of measles virus in the paramyxovirus group has previously been based primarily on morphological criteria. Additional biochemical data have been provided here. It has been shown that measles virus contains a high-molecular-weight RNA species which, by cosedimenting with 50*S* NDV-RNA, appears to be similar in size to the RNA of other paramyxoviruses (2, 9, 10, 11, 14). It is not yet clear, however, whether the measles virus RNA is limited to this species, because, as in the case of mumps virus (11) or of Sendai virus preparations containing incomplete virus (15), we and Schluederberg (23) have found that lower-molecular-weight species of RNA are also present in virion preparations. Although it is clear that in the case of Sendai virus these smaller species are virus specific, in the case of measles virus it remains to be determined whether these species are of viral or host origin.

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