Ribonucleic Acid Species of Intracellular Nucleocapsids and Released Virions of Vesicular Stomatitis Virus

MICHAEL P. KILEY¹ AND ROBERT R. WAGNER

Department of Microbiology, The University of Virginia School of Medicine, Charlottesville, Virginia 22901

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Infection of chicken embryo cells with vesicular stomatitis (VS) virus resulted in variable production of three classes of intracellular viral ribonucleocapsids with sedimentation coefficients of approximately 140S, 110S, and 80S, as well as three corresponding classes of released virions designated B, LT, and T. Intracellular nucleocapsids of each class contained three proteins of which the major N protein was firmly bound, and the minor L and NS1 proteins were readily dissociated with 0.5 M NaCl. The ribonucleic acid (RNA) species extracted from B, LT, and T virions, and from corresponding intracellular nucleocapsids, contained RNA species with approximate molecular weights of 3.2×10^6 , 2.0×10^6 , and 10^6 , respectively, as determined by polyacrylamide gel electrophoresis. These values are roughly equivalent to sedimentation coefficients of 42S, 28S, and 23S for each of the virion and nucleocapsid RNA species. Cells infected at high multiplicity with undiluted passage VS virus gave rise primarily to virions and nucleocapsids containing 23S RNA, whereas cells productively infected with purified B virions produced predominantly B and LT virions and nucleocapsids. At late stages in the productive cycle of infection, more virions containing 42S RNA were produced, but the intracellular pool of nucleocapsids containing 28S and 23S RNA remained relatively constant. Additional studies by more refined techniques are required to test the hypothesis that nucleocapsids containing 28S and 23S RNA are precursors of the 42S RNA in infectious VS-B virions and that production of defective T and LT virions results from failure of ligation of the RNA precursors.

Cells infected with vesicular stomatitis (VS) virus produce infectious, bullet-shaped (B) virions, and defective, truncated (T) virions (6). Also described more recently (16) is another defective virion, called long T (LT), which is produced by L cells infected with a variant strain of Indiana VS virus. All three VS virion types contain a single molecule of single-stranded ribonucleic acid (RNA), the molecular weights of which have been estimated to be ($\pm 10\%$) in the range of 3.6×10^6 for B RNA, 2×10^6 for LT RNA, and 10^6 for T RNA (1, 8, 16). The molecular weights of the RNA species in B and T virions are consistent with the lengths of the nucleocapsids enclosed in the respective virions (14). Ribonucleocapsid structures can also be isolated from cytoplasm of infected cells and have sedimentation coefficients of approximately 140S, 110S, and 80S (9, 16, 20, 24), which are quite

similar to those of the nucleocapsids extracted from B, LT, and T virions (9, 16). These values, as well as those for the sedimentation coefficients of the RNA species extracted from nucleocapsids and virions, can be considered only approximate and, in fact, may vary considerably among different types, strains, and mutants of VS virus (18, 19), as well as by the degree of secondary structure (13). Therefore, we shall refer to these virion RNA species by their estimated sedimentation coefficients of 42S for B RNA, 28S for LT RNA, and 23S for T RNA (8, 16, 20).

All three major types of VS virions can be produced simultaneously by infected cells, but the proportionate yields of each virion type are dependent on a variety of factors, including multiplicity of infection (4, 6), proportion of defective particles in the inoculum (4, 5), strain of virus or mutant type (16, 18), and cell type (4). Many laboratories are studying the mechanisms by which the parental 42S RNA is transcribed to

¹ Present address: Department of Epidemiology, University of Michigan, Ann Arbor, Mich.

make messenger RNA species (2, 5, 7, 9, 12, 13, 16, 19, 20, 24), as well as exploring the events which control replication of the various classes of progeny RNA. Experiments reported here represent an attempt to relate the synthesis of intracellular nucleocapsids to the RNA packaged in virions which are released as the three principal types of VS viral particles.

MATERIALS AND METHODS

Virus, cells, and media. Virus, cells, and media materials have been described in previous communications (21–23). The Indiana serotype of VS virus was used throughout and was grown in primary cultures of chicken embryo (CE) fibroblasts prepared from 9-day-old minced, trypsinized embryos. Highly infectious preparations of VS virions were prepared by low multiplicity infection (~ 0.001); virions harvested at 17 hr after incubation at 37 C were concentrated by centrifugation at $90,000 \times g$ and were purified by rate zonal centrifugation for 90 min at $31,000 \times g$ in a 0 to 40% sucrose gradient. Virions collected from the lower light-scattering band are designated infectious B virions (6) with titers of 2×10^{10} to 7×10^{10} plaque-forming units (PFU) per ml. Defective T virions (6) were prepared by harvesting virus released from CE cells infected with the third undiluted passage of VS virus and then were separated from infectious particles by rate zonal centrifugation in 0 to 40% sucrose. CE cells were grown in lactalbumin hydrolysate supplemented with 5% fetal calf serum in petri plates (100 mm in diameter). Minimal essential medium with 5% fetal calf serum was used for incubation of infected cells and for labeling with RNA and protein precursors. Media were purchased from Grand Island Biological Company, Grand Island, N.Y.

Radioisotopes. ^3H -uridine (58 Ci/mmole), ^3H -leucine (58 Ci/mmole), ^3H -tyrosine (50 Ci/mmole), and ^{14}C -amino acids (54 mCi/matom) were obtained from Schwarz BioResearch, Orangeburg, N.Y. ^3H -adenosine (10.5 Ci/mmole) and ^{14}C -uridine (507 mCi/mmole) were obtained from Amersham/Searle, Arlington Heights, Ill.

Cell fractionation. Cytoplasmic extracts were prepared essentially by the method of Penman et al. (15). CE cells were harvested at intervals after infection by washing twice with 5 ml of ice-cold phosphate-buffered saline (PBS) solution and then by scraping the cells into 5 ml of cold Earle's balanced salt solution (BSS). Cells were pelleted by centrifugation at $1,000 \times g$ for 10 min, resuspended in 10 ml of cold BSS, and pelleted. This cell pellet was resuspended in 2 to 4 ml (depending on number of cells) of reticulocyte standard buffer (RSB) and allowed to swell with intermittent mixing for 15 to 20 min. Cells were then disrupted by 15 to 20 strokes of the tight-fitting pestle of a 7-ml Dounce homogenizer (Kontes Glass Co., Vineland, N.J.). Nuclei were pelleted at $800 \times g$ for 10 min, and the cytoplasmic extract was decanted.

Labeled viral nucleocapsids in the cytoplasm of infected cells were isolated either by rate zonal or isopycnic centrifugation. For rate zonal centrifugation, the cytoplasmic extract was layered onto 31 ml of 15 to 30% sucrose (w/v) in cellulose nitrate tubes; with

some preparations, as indicated in the text, the extracts were made 0.5% with respect to the detergents deoxycholate and BRIJ-58, and the sucrose was dissolved in RSB with or without ethylenediaminetetraacetic acid (EDTA). Centrifugation in the 15 to 30% sucrose gradients was for 3 hr at $95,000 \times g$ and at 4 C in the SW27 rotor. Gradient fractions of 1.3 ml each were collected from the bottom of the tube at a constant flow rate and monitored by absorption at 260 nm. Viral specific nucleocapsids were also isolated by equilibrium centrifugation in CsCl (starting specific gravity 1.3 g/ml) for 48 hr at $160,000 \times g$ in the SW65 rotor. Fractions of about 0.3 ml were collected from the bottom of the tube, and density was determined by weighing in 25- μ liter or 50- μ liter pipettes.

Isolation of released virions. VS virions released into the medium of infected cultures were partially purified by clarifying centrifugation at $1,000 \times g$ for 10 min followed by pelleting of the virions at $80,000 \times g$ for 90 min. The virus pellet was resuspended in 1 ml of high salt buffer (HSB: 0.01 M Tris[hydroxymethyl]-aminomethane [Tris], 0.5 M NaCl, 0.05 M MgCl_2 , pH 7.4) containing 6% polyethylene glycol (PEG). The viral precipitate appearing after 24 hr at 5 C was pelleted at $2,000 \times g$ for 10 min and was resuspended in RSB (11).

Extraction of RNA from virions, cytoplasm, and nucleocapsids. The procedure for extraction of RNA from virions, cytoplasm, and nucleocapsids is described in a previous paper (21). Virions, cytoplasmic extract, or viral nucleocapsids suspended in RSB were made 1% with respect to sodium dodecyl sulfate (SDS), and to this was added an equal volume of 90% redistilled phenol in RSB. The resulting two phases were separated by centrifugation at $2,000 \times g$ for 10 min at 4 C, the aqueous phase was drawn off, and the RNA was re-extracted with one-third volume of 90% phenol. The RNA was precipitated from the aqueous phase by the addition of two volumes of cold ethanol. After 12 hr at -20 C , the RNA precipitate was pelleted at $10,000 \times g$ for 10 min, washed once with ice-cold 70% ethanol, and resuspended in the appropriate buffer, usually NTE (0.1 M NaCl, 1 mM EDTA, and 0.01 M Tris, pH 7.4) containing 0.5% SDS and 15% sucrose.

Polyacrylamide gel electrophoresis. The procedure for electrophoresis in gels composed of 2.3% acrylamide and 0.12% N,N' -bis-methylene acrylamide has been described (21). Samples of ^3H -RNA extracted from virions, cytoplasm, or nucleocapsids were mixed with marker ribosomal 28S and 18S ^{14}C -RNA in 0.5% SDS and 15% sucrose in NTE buffer. Samples of 5 to 50 μg of RNA in 50 to 200 μl were subjected to electrophoresis for 170 min at 5 ma/gel. Gels frozen at -70 C were sectioned into lengths of 1.5 mm, depolymerized in Nuclear-Chicago solubilizer, and counted in a scintillation spectrometer with the aid of a double-labeling computer program. Analysis of proteins dissolved in boiling 1% SDS by electrophoresis in neutral SDS 7.5% acrylamide gels has been described in detail (22, 23).

RESULTS

Virion RNA species analyzed by electrophoresis on polyacrylamide gels. Infectious B virions were

produced by infecting CE cells at a multiplicity of ~ 0.001 , and defective T virions were produced by infecting CE cells with the third undiluted passage of stock VS virus. Infected cells were labeled with ^3H -uridine ($10 \mu\text{Ci/ml}$) throughout the course of infection. Virions released into the medium of each culture and collected at 20 hr postinfection were individually concentrated and purified by PEG precipitation and rate zonal centrifugation in gradients of 0 to 40% sucrose (6, 11). A predominant light-scattering band of B particles appeared in one gradient and a predominant, more slowly sedimenting band of T particles in the other (6). The RNA species of B and T virions collected separately from each gradient were extracted by the phenol-SDS method and analyzed by electrophoresis on 2.3% acrylamide gels along with marker 28S and 18S ribosomal ^{14}C -RNA.

Figure 1 shows the characteristic electrophoretic migration of the RNA species extracted from purified B and T virions. The T RNA migrated as a homogeneous peak, whereas the banded B

FIG. 1. Electropherograms of VS viral ^3H -RNA species extracted from purified infectious B virions (B) or purified defective T virions (T). RNA was extracted by the phenol-SDS method and was subjected to electrophoresis on 2.3% polyacrylamide gels with chicken cell ribosomal ^{14}C -RNA for 170 min at 5 ma/gel. Arrows indicate from left to right the peak positions of 28S and 18S ^{14}C -RNA subjected to coelectrophoresis.

virions contained at least two other minor peaks in addition to a predominant slowly migrating peak. Assuming that electrophoretic mobility in this system is governed largely by molecular weight of the naked RNA species (3), and using values of 1.75×10^6 and 0.7×10^6 for the molecular weights of marker ribosomal 28S and 18S RNA species, respectively (10), the molecular weight of T virion RNA can be calculated as $\sim 0.95 \times 10^6$ and that of the predominant B virion RNA as 3.2×10^6 . The more rapidly migrating minor RNA peaks isolated from banded B virions have approximate molecular weights of 2.0×10^6 and 1.6×10^6 , which are equivalent to the LT particles described by Petric and Prevec (16) and Bishop and Roy (2).

Boiling for 3 min, followed by rapid cooling in ice, resulted in no dissociation or any other significant change in the electrophoretic mobility of B or T virion RNA. These data and those of Mudd and Summers (13), who studied formaldehyde denaturation of VS viral RNA, indicate that the RNA species in VS virions are covalently linked, single polynucleotide chains and are not hydrogen-bonded segments.

Optimal times of VS viral RNA synthesis. Baseline studies were performed to delineate the cycle of total viral RNA synthesis in actinomycin-treated CE cells productively infected with VS virus. Cultures infected at a multiplicity of ~ 5 PFU of purified B virions/cell were pulse labeled with ^3H -uridine ($20 \mu\text{Ci/ml}$) at hourly intervals. Uninfected cultures of CE cells likewise exposed to actinomycin ($5 \mu\text{g/ml}$) were labeled in parallel; actinomycin reduced cellular RNA synthesis by 99% or greater. RNA extracted with SDS from the cytoplasm of actinomycin-treated infected and uninfected cells was precipitated onto membrane filters with 10% trichloroacetic acid, and the radioactivity was counted.

Figure 2 shows the increment above the baseline uninfected cell level of acid-precipitable VS viral ^3H -RNA extracted at hourly intervals after 15-min pulse labeling of infected cells. When plotted on a logarithmic scale, the rate of viral RNA synthesis was exponential from 2 to 5 hr after infection. In other studies performed at this multiplicity or higher, the rate of viral RNA synthesis began to decline at 6 to 7 hr after infection.

Diversity of intracellular VS viral RNA species. Studies reported to date indicate the presence in infected cells of a large variety of VS viral RNA species as well as considerable variability from one experiment to another (5, 13, 24). This variability and heterogeneity appears to be dependent, at least in part, on multiplicity of infection and autointerference (5). Despite limitations

FIG. 2. Increased rate of total VS viral RNA synthesis with progressive time of infection. CE monolayers containing $\sim 2 \times 10^7$ cells were infected with purified B virions at a multiplicity of ~ 5 . Each group of three cultures was pulse labeled with ^3H -uridine ($20 \mu\text{Ci/ml}$) at hourly intervals after infection and 30 min after exposure to actinomycin D ($5 \mu\text{g/ml}$). The cytoplasm of cells disrupted by Dounce homogenization was made 0.5% with SDS, one-fifth of each portion (0.3 ml) was precipitated with 10% cold trichloroacetic acid onto membrane filters, and the radioactivity was counted. The values plotted represent net increases in precipitable radioactivity after subtraction of ^3H counts incorporated into RNA of parallel uninfected cells treated with actinomycin in the same way.

inherent in the system, we have attempted to characterize the VS viral RNA species produced in this system by polyacrylamide gel electrophoresis.

Figure 3 illustrates the electrophoretic profile of VS viral RNA species extracted from CE cells infected at a multiplicity of ~ 20 and labeled with ^3H -uridine from 2.5 to 4.5 hr (Fig. 3A) or from 4 to 6 hr (Fig. 3B) postinfection. At least five resolvable RNA species are evident if one considers the major peaks and ignores the very rapidly migrating components, which probably are digested RNA. Under these conditions of infection and labeling, the major intracellular RNA species peaked between the 18S and 28S ribosomal RNA markers and can be calculated to have a molecular weight of $\sim 10^6$, which is equivalent to that of 23S RNA extracted from T virions (1, 8). The more rapidly migrating species of RNA probably represent heterogeneous messengers described as 13S to 16S (5, 7, 24). The more slowly migrating species probably consist of the large 28S messenger RNA (7) as well as progeny virion RNA of 28 to 32S and of 42S (5, 16).

Several other factors must be considered to explain the diversity of the RNA species.

FIG. 3. Polyacrylamide gel profiles of ^3H -RNA extracted from CE cells infected with VS virus at a multiplicity of ~ 20 and labeled with ^3H -uridine ($20 \mu\text{Ci/ml}$) for A, 2.5 to 4.5 hr postinfection or B, 4 to 6 hr postinfection. The RNA extracted with SDS-phenol from infected cells after the 2-hr labeling period was subjected to electrophoresis on 2.3% polyacrylamide gels for 170 min at 5 ma/gel with 28S and 18S ribosomal ^{14}C -RNA, the peak activity of which is marked by arrows.

Secondary structure may play a role in electrophoretic mobility. We found, for example, that 28S RNA eluted from acrylamide gels migrated as 32S RNA when heated to 70 C for 30 min and slow-cooled in 0.1 M NaCl before re-electrophoresis. In addition, one must consider the presence of complementary RNA species of approximately the same molecular weight and hence of equivalent electrophoretic mobility. This likelihood is illustrated by the data shown in Table 1 in which 8 to 16% of VS viral RNA extracted from cells was ribonuclease-resistant and presumably double-stranded. Markedly increased resistance to ribonuclease of 26 to 70% was produced by melting and slow annealing of total viral RNA extracted from infected cells (Table 1). Similar data indicating relatively small amounts of replicative forms of double-stranded RNA have been reported by others (20, 24).

Characterization of intracellular VS viral nucleocapsids. Ribonucleocapsids of VS virus can be extracted from virions by exposure to detergents, or can be recovered without detergents from disrupted infected cells. The nucleocapsids

TABLE 1. *Percentage of ribonuclease-resistant intracellular viral RNA before and after melting and self-annealing^a*

Period of ³ H-uridine labeling (hr)	Acid insoluble counts/min after ribonuclease (%)	
	No self-annealing	Self-annealing
0 to 2	8	26
2 to 4	16	70

^a CE cells were infected with purified VS-B virus at a multiplicity of 20, exposed to actinomycin D (5 µg/ml), and labeled with ³H-uridine (20 µCi/ml) from 0 to 2 hr or 2 to 4 hr. RNA was extracted from cytoplasm by the phenol-SDS method and, after ethanol precipitation, was suspended in NTE buffer (0.1 M NaCl, 1 mM EDTA, and 0.01 M Tris, pH 7.4). Half of each preparation was left untreated and the other half was boiled for 3 min, then heated at 80°C for 30 min and slowly cooled overnight. Half of each of the heated and unheated samples were treated with 50 µg of pancreatic ribonuclease per ml for 30 min at 37°C. All samples were then precipitated onto membrane filters with 10% trichloroacetic acid, and the radioactivity was counted. Samples which were boiled and cooled quickly in ice water showed less than 0.1% ribonuclease resistance. Uninfected cells contained about 6% ribonuclease-resistant RNA.

can be purified by equilibrium centrifugation in CsCl (22) or by rate zonal centrifugation in sucrose gradients (5, 16). The sedimentation coefficients of the respective VS viral nucleocapsids have been estimated to be 140S for B nucleocapsids, 100S for LT nucleocapsids, and 80S for T nucleocapsids (16, 24). The following experiments were designed to compare the protein and RNA composition of three classes of VS viral nucleocapsids.

In the first series of experiments, CE cells were productively infected with purified B virions at a multiplicity of ~20 PFU/cell; the cells were exposed to actinomycin D, labeled with ³H-uridine and ¹⁴C-amino acids from 3 to 6.5 hr postinfection, and then were disrupted by Dounce homogenization. The cytoplasmic contents of the cells along with marker 80S chicken ribosomes were fractionated by rate zonal centrifugation in 15 to 30% sucrose gradients prepared either in RSB (0.01 M NaCl) or HSB (0.5 M NaCl).

Figure 4 shows the similar sedimentation profiles of the cytoplasmic content of VS viral-infected CE cells centrifuged in sucrose gradients in RSB (Fig. 4A) and HSB (Fig. 4B). In each gradient, the peak fractions designated 2 and 3 for both ³H-RNA and ¹⁴C-protein, when compared with 80S ribosomes, were estimated to have

FIG. 4. *Velocity sedimentation analysis of cytoplasmic extracts of CE cells infected with VS-B virions at a multiplicity of 20; actinomycin D (5 µg/ml) was added at 1.5 hr postinfection, and cells were labeled with ³H-uridine (20 µCi/ml) and ¹⁴C-amino acids (1.1 µCi/ml) from 3 to 6.5 hr postinfection, at which time cells were harvested and disrupted by Dounce homogenization. The resulting cytoplasmic extract mixed with marker 80S chicken ribosomes was halved; one half was layered on a continuous gradient of 15 to 30% sucrose in RSB, whereas the other half was layered on a continuous gradient of 15 to 30% sucrose in HSB. Both gradients were centrifuged at 4°C and 95,000 × g for 3 hr, and fractions of 1.3 ml each were collected while absorbancy at 260 nm was continuously monitored. The arrows mark the peak absorbancy of 80S ribosomes. Distribution of acid-insoluble ³H-RNA and ¹⁴C-protein is shown for A, RSB and B, HSB sucrose gradients. The peak fractions numbered 1, 2, and 3 were collected for analysis of viral proteins (see Fig. 5 and Fig. 6).*

sedimentation coefficients of 110S and 140S, respectively, presumably representing nucleocapsids of LT and B (16). Examination by electron microscopy of negatively stained material from these regions of similar gradients revealed the presence of typical coils of VS viral nucleocapsids (14, 16).

The polyacrylamide gel profiles (Fig. 5) reveal the ¹⁴C-protein composition of the nucleocapsids recovered from regions 2 and 3 of the sucrose gradient shown in Fig. 4A, as well as the ¹⁴C-protein composition of the diffuse region 1 recovered from the top of the same sucrose gradient. The region 1 electropherogram is

FIG. 5. Electropherograms of VS viral ^{14}C -proteins extracted from the three regions of the RSB sucrose gradient analysis of VS viral-infected cytoplasm shown in Fig. 4A. Each fraction collected was made 1% with respect to SDS and 2-mercaptoethanol and then was boiled for 2 min. Twenty-five microliters of fraction 1 and 100 μl iters each of fractions 2 and 3 mixed with marker ^3H -proteins of purified VS virions were subjected to electrophoresis on 7.5% SDS acrylamide gels. Panel 2 shows the protein profile of 110S viral nucleocapsids and panel 3 shows that of 140S viral nucleocapsids. The arrows mark the peak regions of VS virion ^3H -protein migration in each of the three gels. Note the minor peaks of nonstructural protein (NS_1) in fraction 31 of gels 2 and 3 and the major NS_1 peak in fraction 32 of gel 1 (23).

obscured by considerable amorphous cell protein, but the position to which VS viral proteins G, N, and S have migrated can be identified and, in particular, the large peak of soluble intracellular viral protein NS_1 (23). In contrast, the 110S and 140S nucleocapsids recovered from regions 2 and 3 of the sucrose gradient in Fig. 4A consist primarily of the nucleocapsid N protein as well as protein L and trace amounts of NS_1 ; the envelope proteins G and S are virtually missing or represent minor contamination.

Figure 6 illustrates the electrophoretic profiles of the ^{14}C -proteins extracted from the top com-

FIG. 6. Electropherogram of VS viral ^{14}C -proteins extracted from the three regions of the HSB (0.5 M NaCl) sucrose gradient analysis of the cytoplasmic content of VS viral-infected cells shown in Fig. 4B. Analysis of the viral proteins was identical to that described in Fig. 5; the only difference in this experiment was that the 110S nucleocapsids (region 2) and 140S nucleocapsids (region 3) were collected from a high salt gradient. Note the absence of proteins L and NS_1 in the nucleocapsid extracts.

ponent in region 1 and the nucleocapsids in regions 2 and 3 of the sucrose gradient depicted in Fig. 4B. Under these conditions of centrifugation in a sucrose gradient prepared in HSB (0.5 M NaCl), essentially only the nucleoprotein (N) can be detected in the 110S and 140S nucleocapsids. These data indicate that proteins L and NS_1 are more loosely associated with VS viral nucleocapsids than is the structural protein N.

Similar results were obtained by analysis of the proteins of 80S T nucleocapsids (*see below*) which also contain proteins N, L, and NS_1 in the same proportions and the same associations as in the 110S and 140S nucleocapsids.

RNA composition of intracellular nucleocapsids. The intracellular nucleocapsids recovered from

CE cells infected with purified VS-B virions at a multiplicity of ~ 20 were compared with the nucleocapsids recovered from CE cells infected with undiluted passage VS virus. Each set of cells was labeled with ^3H -uridine plus ^3H -adenosine at different stages of infection, and the nucleocapsids were identified by fractionation of cytoplasm treated with BRIJ-58 and deoxycholate by centrifugation in 15 to 30% sucrose gradients. The ^3H -RNA in the nucleocapsids recovered in the peak fraction of each gradient was extracted with phenol and SDS and analyzed by electrophoresis on 2.3% polyacrylamide gels.

Figure 7 demonstrates that under these conditions of infection and labeling, a single nucleocapsid species can be identified in cells productively infected with purified B virions as well as the cells infected with B virions contaminated with auto-interfering T virions from the third undiluted passage of VS virus. Productive infection with B virions gave rise to nucleocapsids with a mean sedi-

mentation coefficient of 110S (Fig. 7A), whereas infection under conditions of autointerference by T particles gave rise to nucleocapsids with a mean sedimentation coefficient of 80S. RNA was extracted from the fractions shown in Fig. 7A and C and was analyzed by electrophoresis on 2.3% polyacrylamide gels. As noted in Fig. 7B and D, the 110S nucleocapsids contained RNA which comigrated with 28S ribosomal RNA equivalent to 1.75×10^6 , whereas the 80S nucleocapsids contained RNA which migrated to a position equivalent to 23S RNA or a molecular weight in the range of 10^6 .

These experiments reveal that the 110S nucleocapsid with 28S LT RNA is the predominant species of intracellular nucleocapsid formed under conditions productive of infectious virions, whereas the major nucleocapsid formed in cells undergoing autointerference is the 80S variety containing 23S T RNA.

RNA species present in virions progressively re-

FIG. 7. Comparative sedimentation coefficients and RNA content of VS viral nucleocapsids recovered from CE cells productively infected (A, B) and under conditions of autointerference (C, D). One set of five CE cultures ($\sim 10^8$ cells) was productively infected with VS-B virions at a multiplicity of ~ 20 and labeled with ^3H -uridine ($20 \mu\text{Ci}/\text{ml}$) from 2 to 5 hr postinfection, whereas another set of five CE cultures ($\sim 1 \times 10^8$ cells) was infected with a third undiluted passage of VS virus and labeled with ^3H -uridine plus ^3H -adenosine ($15 \mu\text{Ci}/\text{ml}$ of each) from 1.5 to 3.75 hr postinfection. The cells from each set of cultures were ruptured by Dounce homogenization, and the cytoplasm (mixed with 0.5% deoxycholate and 0.5% BRIJ-58) was centrifuged through a 15 to 30% sucrose gradient for 3 hr at $95,000 \times g$. The peak fractions of ^3H -RNA (marked by a bar) were extracted with phenol and SDS and were analyzed by electrophoresis on 2.3% polyacrylamide gels. A, Sedimentation in 15 to 30% sucrose of nucleocapsids ($\sim 110\text{S}$) extracted from productively infected cells; the arrow marks the peak A_{260} of 80S ribosomes. B, Migration on acrylamide gel of ^3H -RNA extracted from 110S nucleocapsids; the arrows mark 28S and 18S ribosomal ^{14}C -RNA. C, Sedimentation in 15 to 30% sucrose of nucleocapsids ($\sim 80\text{S}$) extracted from CE cells infected under conditions of autointerference; the arrow marks the peak A_{260} of 80S ribosomes. D, Migration on acrylamide gel of ^3H -RNA extracted from 80S nucleocapsids; the arrows mark 28S and 18S ribosomal RNA.

leased from productively infected cells. Experiments were designed to determine which of the three classes of intracellular nucleocapsids were packaged and released as virions at various stages of productive infection. To this end, parallel experiments were performed with CE cells infected with purified VS-B virions at a multiplicity of ~ 20 and were labeled either under simulated conditions of pulse-chase or of prolonged labeling at consecutive periods of infection. The pulse-chase experiments were performed by adding actinomycin D at 1.5 hr postinfection and then by pulsing with ^3H -uridine for 3.5 min at 2.5 hr postinfection. The cells were washed after the pulse, and the medium was replaced with a large excess of unlabeled uridine. Released virions were harvested at 4 hr (90-min chase) or 5.5 hr (180-min chase) postinfection. In a parallel experiment performed under identical conditions except for prolonged labeling and no chase, infected cells were labeled with ^3H -uridine for 1 hr, after which the released virions were collected at 4 hr or 5.5 hr postinfection. In both experiments, the released virions in each harvest were not separated on the basis of size but were partially purified from cell debris by differential centrifugation and PEG precipitation before extraction of virion ^3H -RNA by the phenol-SDS method for analysis on 2.3% polyacrylamide gels.

Figure 8 reveals progressively greater release of B virions containing 42S RNA from productively infected cells. However, as shown in Fig. 8A, 28S RNA was the predominant species (presumably in the form of LT virions) after a 3.5-min pulse followed by a 90-min chase. When the same cells were chased for 180 min, the amount of released 42S RNA virions increased greater than sixfold, whereas the amount of released 28S RNA virions remained relatively constant (Fig. 8B). Similar results were obtained by longer continuous labeling of 1 hr without chase (Fig. 8C, D). The amount of 28S RNA in released virions was about the same at 4 hr and 5.5 hr after infection, suggesting a steady level of production of LT virions throughout the cycle of infection. However, as in the pulse-chase experiment, the amount of 42S virion ^3H -RNA released over a period of 1 hr increased significantly between 4 and 5.5 hr (Fig. 8C, D). Conversely and quite strikingly, the large amount of T-particle 23S RNA produced and released was considerably greater during the period from 3 to 4 hr (Fig. 8C) than during the 4.5- to 5.5-hr period (Fig. 8D).

Also, T virions were not produced after a short pulse followed by chase, whereas LT virions were (Fig. 8A, B), as compared with the early release of a considerable amount of T virions during prolonged labeling and despite conditions designed

FIG. 8. Progressive release of B virions containing 42S RNA under conditions of simulated pulse-chase or of continuous labeling with ^3H -uridine. Two sets of six monolayer cultures each containing about 2×10^7 cells were infected with purified B virions at a multiplicity of ~ 20 and were exposed to actinomycin D ($5 \mu\text{g}/\text{ml}$) at 1.5 hr. Two conditions of labeling were employed. Half the cultures were pulsed for 3.5 min with ^3H -uridine ($20 \mu\text{Ci}/\text{ml}$) at 2.5 hr postinfection. These cultures were then washed twice with 10 ml of PBS containing 4.2 mg/liter of unlabeled uridine and were reincubated in Eagle minimal essential medium with 5% fetal calf serum and the same amount of unlabeled uridine. Released virions were harvested at 4 hr (90-min chase) and 5.5 hr (180-min chase) after infection. The other set of six cultures was labeled with ^3H -uridine ($20 \mu\text{Ci}/\text{ml}$) for 1 hr prior to harvesting virions at 4 hr and 5.5 hr postinfection. Virions harvested at each time were partially purified by differential centrifugation and polyethylene glycol (PEG) precipitation; the RNA extracted from these virions with phenol and SDS was analyzed with marker 28S and 18S ^{14}C -RNA (arrows) by electrophoresis on 2.3% polyacrylamide gels. A, 90-min chase after 3.5-min pulse. B, 180-min chase after 3.5-min pulse. C, labeled from 4 to 5 hr. D, labeled from 5.5 to 6.5 hr.

to produce optimal yields of infectious B virions (Fig. 8C). In additional experiments which are not reported here in detail, it was found that only LT virions with 28S RNA were released for 6 hr by cells infected with VS-B virions at a multiplicity of 0.001 PFU/cell, whereas about two-thirds of the virions released in 6 hr by cells infected with auto-interfering undiluted passage VS virus were T particles containing 23S RNA. These data not only confirm in greater detail earlier observations on the variability in proportions of the three classes of VS virions produced by infected cells, but also suggest a precursor-product relationship of the 28S and 23S RNA on one hand and the 42S RNA in infectious virions on the other.

Nucleocapsid RNA compared with released virion RNA from the same cells. In an attempt to explore further the possible precursor-product relationship of cellular nucleocapsids to released virions, we compared the RNA species in nucleocapsids and virions produced by the same cells.

CE cells productively infected with purified B virions at a multiplicity of ~ 20 PFU/cell were exposed to actinomycin at 30 min postinfection and labeled with ^3H -uridine plus ^3H -adenosine ($15 \mu\text{Ci/ml}$ of each) from 1.5 to 3.75 hr postinfection. Virions released into the medium at 3.75 hr were harvested and were partially purified by differential centrifugation and PEG precipitation. The same cells were disrupted at 3.75 hr postinfection and the nuclei removed. The specific gravity of the infected cytoplasm was then made 1.3 g/ml with CsCl , and the mixture was centrifuged for 48 hr at $160,000 \times g$ in the SW65 rotor at 4 C. The nucleocapsids formed a light scattering band at a density of 1.3 g/ml and were collected. RNA extracted with phenol-SDS from virions and cellular nucleocapsids was analyzed by polyacrylamide gel electrophoresis.

The electropherograms shown in Fig. 9 compare the distribution of 23S, 28S, and 42S RNA species extracted from cellular nucleocapsids and virions produced by the same cells. As noted, 42S RNA was by far the predominant species in the virions released from these cells, but the nucleocapsids recovered from the same cells contained 23S RNA as the principal species as well as large amounts of 28S, 32S, and 42S RNA.

These data suggest that 23S and 28S RNA, perhaps in the form of 80S and 110S nucleocapsids, could be precursors of the 42S RNA incorporated into infectious virions. Presumably, the process of assembly is inefficient so that 23S and 28S RNA can be packaged individually and released as defective T or LT virions.

Proof of a precursor-product relationship requires reliable pulse-chase experiments. Despite numerous attempts, we have not been able to obtain convincing data by this technique. Part of the problem relates to the large pool of intracellular RNA precursors which precludes an adequate chase. The difficulty encountered can be illustrated by briefly recounting an experiment performed under conditions similar to those shown in Fig. 9 but in which the infected cells were pulse labeled and in which the nucleocapsids and virion RNA were analyzed later in infection.

Figure 10 illustrates the results of an attempt to pulse RNA precursors into nucleocapsid RNA and to chase the labeled nucleocapsids into released virions. Figure 10A shows the rather amorphous labeling of nucleocapsid RNA with ^3H -uridine when cells were pulsed for 4 min at 4 hr

FIG. 9. Comparison of the RNA content of intracellular nucleocapsids ($\circ$) and virions ($\bullet$) released from the same productively infected cells. Three monolayer cultures of CE cells ($\sim 6 \times 10^6$ cells total) were infected with purified B virions at a multiplicity of ~ 20 . Actinomycin D ($5 \mu\text{g/ml}$) was added at 30 min postinfection, and the cells were labeled with ^3H -uridine plus ^3H -adenosine ($15 \mu\text{Ci/ml}$ each) from 1.5 to 3.75 hr postinfection. At this time, released virions were harvested and partially purified by differential centrifugation and PEG precipitation; nucleocapsids were isolated from the same cells disrupted by Dounce homogenization and banded by equilibrium centrifugation for 48 hr in CsCl of starting specific gravity 1.3 g/ml. RNA species extracted from virions and nucleocapsids were individually analyzed by polyacrylamide gel electrophoresis along with 28S and 18S ribosomal ^{14}C -RNA markers (arrows).

after infection with purified B virions; most of the label seems to be in the region of 23 to 28S RNA. After chasing with cold uridine for 90 min (5.5 hr postinfection), virtually all the nucleocapsid RNA was 28S and considerable amounts of 42S and 28S RNA were recovered from released virions (Fig. 10B). After a further 90-min chase (7 hr postinfection), the distribution of RNA species in nucleocapsids and virions was quite similar to what it was at 5.5 hr (Fig. 10C). We have no explanation for the large amount of 28S RNA in virions and nucleocapsids. The complete absence of 23S RNA in nucleocapsids and virions could be a function of more efficient ligation of 23S RNA with 28S RNA to form a 42S nucleocapsid that is released in the form of 42S virions.

FIG. 10. Attempt to pulse label RNA in nucleocapsids (○) and chase the RNA into virions (●) released from actinomycin-treated CE cells infected with purified B virions at a multiplicity of ~ 20 . The RNA from purified released virions and cellular nucleocapsids isolated in CsCl gradients was analyzed by electrophoresis on 2.3% acrylamide gels as described in the legend to Fig. 9. Panel A, 4-min pulse at 4 hr postinfection. Panel B, 90-min chase by washing and incubating pulsed cells in 4.2 mg/liter cold uridine and by harvesting virions and nucleocapsids at 5.5 hr postinfection. Panel C, 180-min chase as in Panel B. Arrows designate peaks of 28S and 18S ^{14}C -RNA.

DISCUSSION

The genome of VS virus transcribes a series of messenger RNA species that are associated with polyribosomes and are complementary to 42S virion RNA (2, 5, 13, 24). The exact sizes of these complementary messengers have not been determined, but there is one very large messenger (about 28S) and a series of smaller ones which appear, by various estimates, to range in size from 10S to 18S (7). It seems reasonable to assume that there are at least five VS viral messengers to account for the five identifiable VS viral proteins (23) and the five (or six) genetic complementation groups (17). The aggregate molecular weight of the recognized proteins of VS virus accounts for virtually the entire coding potential of the infectious viral genome (23). The input parental RNA of infectious B virions has an associated tran-

scriptase which appears to have the capacity to transcribe *in vivo* all the messenger RNA species (7, 11). The input RNA species of T and LT virions appear to lack this function (2, 7). Although it seems likely that the RNA species in T and LT defective virions represent two of the segments of the infectious B genome, no one has yet been able to identify their coding potentials. Moreover, the estimated combined molecular weights of T and LT RNA do not quite equal that of B RNA. However, with some refinement, the available techniques should be adaptable to solve the problems of VS viral transcription. Exposure of infected cells to cycloheximide blocks the formation of intracellular VS viral nucleocapsids (7) but allows unimpeded synthesis of complementary messenger RNA species (7, 12). This technique provides a basis for dissecting the transcriptional and replicative events in VS viral RNA synthesis.

More difficult than transcription is the problem of replication of VS virus that results in production of progeny RNA. The studies reported here confirm and extend the observations (16, 24) that cells infected with VS virus can synthesize at least three size classes of viral RNA which are anticomplementary to virion RNA and which associate with viral proteins to form progeny nucleocapsids of at least three different lengths. Moreover, the progeny nucleocapsids appear to get wrapped up in different virions, each of which contains one of the nucleocapsid RNA species. The nucleocapsids which contain 42S, 28S, or 23S RNA each contain the same three proteins, designated N for the structural nucleoprotein and L and NS₁ for two minor nucleocapsid-associated proteins which are readily dissociated in high salt. One or both of the dissociable nucleocapsid proteins could be the transcriptase (S. Emerson and R. R. Wagner, unpublished data). The three nucleocapsids were found to have sedimentation coefficients of approximately 140S, 110S, and 80S, which contain RNA species that migrate on polyacrylamide gels quite similarly to the RNA species of B, LT, and T virions, respectively. The proportionate synthesis of each type of nucleocapsid and the corresponding release of each virion type probably depend on many factors, but of principal importance perhaps is the multiplicity of infection and the purity of stocks of infecting virus (5, 7). Conditions can be arranged by which infected cells produce mainly B virions, but contamination of input B virions with defective T virus results in far greater yield of T nucleocapsids and T virions.

Of considerable interest is the fact that cells which were productively infected, i.e., producing primarily B virions with 42S RNA, contained large amounts of nucleocapsids with 28S RNA. As infection proceeded, or under conditions of a

simulated pulse-chase in which progressively more B virions were produced, the proportion of released T virions declined, whereas released LT virions remained fairly stable. Another related observation was that productively infected cells released far more B virions than T and LT virions, but the same cells contained as much or more nucleocapsids with 28S and 23S RNA than nucleocapsids with 42S RNA. The failure to detect T virions or nucleocapsids with 23S RNA following periods of chase after short pulse labeling suggests that 23S RNA is rapidly synthesized and rapidly released in the form of T virions or that the 23S RNA is chased into B nucleocapsids and virions in productively infected cells.

Several alternative hypotheses concerning the mechanism of VS viral RNA replication arise from these studies and those of other laboratories (1, 7, 13, 24). The least likely, perhaps, is that the input parental B RNA is immediately cleaved, after which the two or more segments are transcribed, and the transcribed RNA species are translated and replicated. This hypothesis also demands that the replicated pieces be ligated with varying effectiveness to make three classes of progeny virion RNA. The failure to demonstrate transcriptional functions for short RNA segments, such as those in T and LT virions (2) as well as evidence for conservation of input B RNA (1), makes this hypothesis unlikely. A somewhat more tenable hypothesis is that input 42S RNA uses its transcriptase to transcribe 28S RNA and smaller messengers (7) and is alternately transcribed to yield 42S complementary RNA which is, in turn, completely replicated to yield 42S B progeny RNA or is partially replicated to produce 28S LT and 23S T progeny RNA. Failure to detect significantly large amounts of complementary or anticomplementary 42S RNA in productively infected cells makes this hypothesis unsatisfactory. A third alternative is that input parental 42S RNA is transcribed to produce complementary 28S RNA as well as complementary smaller messenger species (7). The 28S RNA could serve as messenger for the L protein or all three nucleocapsid proteins, the combined molecular weight of which is about 280,000 (23). The smaller messenger RNA species could be translated into envelope proteins G and S (21), which have a combined molecular weight of ~100,000 exclusive of the sugar residues of the glycoprotein. The complementary strands of 28S and smaller RNA species could be joined by a ligase and then replicated or, more likely, could be replicated individually to produce 28S and 23S nucleocapsid RNA species, which are then ligated with variable efficiency to form 42S nucleocapsids which could be rapidly released as B virions. Deficiency or malfunction of the ligase

would result in release of progeny T and LT virions in addition to or rather than B virions.

The design of future experiments on replication of VS virus must include a search for the putative replicase and ligase that would seem to be essential for producing 42S nucleocapsid RNA.

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