JOURNAL OF VIROLOGY, Nov. 1973, p. 1164-1172

Vol. 12, No. 5
Printed in U.S.A.

Isolation and Preliminary Characterization of the RNA-Containing R-Type, Virus-Like Particle of BHK-21 Cells

EVELYN ALBU[1] AND KATHRYN V. HOLMES[2]

Department of Microbiology, Georgetown University Schools of Medicine and Dentistry, Washington, D.C. 20007

Received for publication 14 June 1973

An R-type virus-like particle (VLP) has been isolated from the medium of BHK-21-F cells by ultracentrifugation and polyethylene glycol precipitation. The R-type VLP contains RNA which sediments at 60 to 70*S* in sucrose density gradients and has a molecular weight of approximately 10^7, as estimated by gel electrophoresis. The R-type VLP can be labeled with ^{3}H-uridine in the presence of actinomycin D. On the basis of morphology, site of maturation, and preliminary biochemical characterization, the R-type VLP does not appear to fit into any of the major groups of animal viruses.

A morphologically unique type of virus-like particle (VLP) was observed in thin sections of the BHK-21 clone 13 line of baby hamster kidney cells (23) by Bernhard and Tournier (2) and Magee-Russell et al. (26). Because of the spokelike structures radiating from the dense core to the periphery of the VLP, Shipman et al. (32) termed it an R-type virus-like particle. VLP with the same morphology have been observed in thin sections of hamster tumors, both spontaneous (35, 36) and induced by polyoma, simian virus 40 (36), Rous sarcoma virus (45), and murine sarcoma virus (8). This type of VLP has been implicated as the causative agent in hamster melanoma (10). Vertical transmission of the VLP in the LSH strain of inbred hamsters has been demonstrated (43).

Because the R-type VLP, which is often observed in tumor cells, is morphologically distinct from known oncogenic viruses, it may represent a new type of oncogenic virus. Alternatively, it may be a virus which is a secondary invader of tumor cells. It is important to characterize the R-type VLP and to determine its biological activity and its role in oncogenesis. It is possible that some of the characteristics for which BHK-21 cells are so widely used in cell biology and virology may be the result of chronic infection with the R-type VLP. The VLP may, for instance, play a role in determining the ability of the cells to support the replication of a wide variety of other viruses (37, 42).

In this publication we report a method for isolating and purifying the R-type VLP from the medium of BHK-21-F cells and describe the physical properties of the intact VLP and the biochemistry of its nucleic acid. These studies provide a basis for preliminary classification of the R-type VLP.

(A preliminary report of a portion of this research was presented at the 12th Annual Meeting of the American Society for Cell Biology, November 1972; E. Albu and K. V. Holmes, J. Cell Biol. **55**:1a, 1972.

MATERIALS AND METHODS

Cell cultures. Monolayers of the BHK-21-F subline (15) of the BHK-21 clone 13 cell line derived from baby hamster kidney by Macpherson and Stoker (23) were used for these studies. Repeated cultures show that the cells are free of contamination with bacteria, fungi, and mycoplasmas. No cytopathic effects were observed in the cells, and intensive electron microscope investigations of thin sections of BHK-21-F cells consistently showed large numbers of R-type VLP but failed to detect any other virus or virus-like particles in the cells.

Preparation of isotopically labeled VLP. Monolayers of BHK-21-F cells were grown in 75-cm^2 Falcon tissue culture flasks containing 20 ml of Dulbecco modified Eagle medium (GIBCO) supplemented with 10% heat-inactivated calf serum and 10% tryptose phosphate broth. Just before the monolayers of cells were confluent, ^{3}H-uridine (Amersham Searle; >20,000 mCi/mmol) or ^{14}C-thymidine (Amersham Searle; >520 mCi/mmol) was added to give a final

[1] Present address: Schering Corporation, Galloping Hill Rd., Kenilworth, N.J. 07033.

[2] Present address: Department of Microbiology, The University of Texas Southwestern Medical School, Dallas, Tex. 75235.

activity in the medium of 10 μCi/ml or 2 μCi/ml, respectively. The cells were incubated in the presence of the isotope for 10 to 24 h.

Isolation of the VLP. Medium in which BHK-21-F cells had grown for 80 to 96 h was centrifuged at 900 × *g* for 10 min at 4 C to remove cells and debris. Because the VLP appears to be heat labile, all subsequent procedures were carried out at 4 C. A 35-ml sample of medium was layered onto a 3-ml cushion of 50% sucrose in Tris buffer (0.01 M Tris, pH 7.4, and 0.1 M NaCl) with 1% bovine serum albumin (BSA) and centrifuged for 2 h at 27,000 rpm in a Beckman SW27 rotor. The visible band above the sucrose cushion was collected with a Pasteur pipette and then precipitated with 7.5% polyethylene glycol (PEG) in 1 M NaCl by stirring for 4 to 12 h. The precipitate was pelleted at 10,000 rpm for 30 min in a Sorvall centrifuge, resuspended in 0.1 of the original volume in 0.01 M Tris buffer (pH 7.4) with 0.1 M NaCl, layered onto 20 to 40% or 20 to 45% sucrose density gradients, and centrifuged for 4 h at 27,000 rpm in an SW27 rotor. The density of each gradient fraction was determined by using a Bausch and Lomb refractometer. Samples of each fraction were spotted onto 4.25-cm filter paper disks, dried, precipitated three times with cold 5% trichloroacetic acid, washed twice with cold 95% ethanol and once with ethyl ether, and dried. The disks were counted in a Packard Tricarb scintillation counter in vials containing 10 ml of Liquifluor. Samples of each fraction were examined by electron microscopy.

Electron microscopy. A drop of each gradient fraction was placed on a carbon-coated, Formvar-covered grid, which was washed with one drop of distilled water and then stained with 1% phosphotungstic acid, pH 7.4.

R-type VLP which had been purified by banding in sucrose density gradients was pelleted in 0.8-ml cellulose nitrate tubes in a Beckman SW50 rotor at 40,000 rpm for 3 h and fixed in 1% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon resin as described previously (6). Sections across the VLP-pellet were stained with uranyl acetate and lead citrate and examined with a Phillips 300 electron microscope.

RNA extraction. Two methods were used to extract RNA from the VLP. Some samples containing R-type VLP were treated with 500 μg of Pronase (Sigma) per ml for 15 min at 37 C and then 0.5% or 1% sodium dodecyl sulfate (SDS) for 15 min at 37 C, and layered directly onto 15 to 30% sucrose gradients in TES buffer (0.1 M NaCl, 0.001 M EDTA, and 0.05 M Tris, pH 7.4) with 0.5% SDS. Gradients were centrifuged for 2 h in an SW50.1 Beckman rotor at 40,000 rpm at 23 C. Gradient fractions were collected and analyzed as described above. RNA was extracted from other samples of R-type VLPs by a modified phenol method described elsewhere (T. Sreevalsan; in Methods in Molecular Biology, Vol. IV, in press).

RESULTS

Structure and maturation of the VLP. In thin sections of BHK-21 clone 13 cells, the characteristic R-type VLP is observed only within the cisternae of the rough endoplasmic reticulum (RER) (2, 6, 26, 32, 40) or within the space of the nuclear envelope (6). When the cells are poorly fixed or subjected to osmotic shock, VLP can be seen budding from the membrane of the endoplasmic reticulum (6, 32, 41). Within the RER cisternae in well-fixed cells, the VLP are embedded in an amorphous dense material which often makes them difficult to distinguish. The VLP has never been observed budding from the plasma membrane. Figure 1 shows a portion of a BHK-21-F cell, with a dilated sac of RER, containing more than 20 VLP. The endoplasmic reticulum is closely opposed to the underside of the plasma membrane. Although VLP, like corona viruses (1, 4, 7, 12, 27), appears in the endoplasmic reticulum and can be released from apparently intact cell monolayers, little is known about the mechanism of virus release. It is possible that some of the VLP may be released from intact BHK-21-F cells directly from the RER into the extracellular space by fusion of the plasma membrane with the endoplasmic reticulum.

The ultrastructure of the R-type VLP has been described previously (2, 6, 8, 10, 18, 26, 32, 35, 36, 40, 41, 43, 45). It is about 100 nm in diameter and contains a dense central core about 45 nm in diameter. We have observed in favorable sections (Fig. 2) that a denser ring structure is sometimes visible within the core. This narrow dark ring, about 5-nm wide, is 20 to 25 nm in diameter. The fine dense lines radiating from the core to the rim of the particle give the VLP its characteristic radial appearance.

Isolation of the R-type VLP. We decided to use the BHK-21-F subline (15) for the isolation of the R-type VLP for several reasons. First, it was our impression that these cells contained more VLP per cell than did other sublines of BHK cells which we examined. Second, we had done extensive work on the susceptibility of the BHK-21-F cell line to fusion induced by the simian paramyxovirus SV5 (6, 15, 16), and we were interested in the role which the R-type VLP might play in predisposing cells to fusion by SV5. Finally, the lipids of the plasma membrane of BHK-21-F cells have been well characterized biochemically (19–21). Our approach to the isolation of the R-type VLP was to correlate electron microscope identification of the VLP with the presence of radioisotopic label within the VLP. We have studied R-type VLP that was released into the medium over monolayers of intact BHK-21-F cells rather than intracellular VLP in order to start with a preparation relatively free of cell debris and to maximize the possibility that we were studying the mature form of the VLP.

To determine whether we could label any

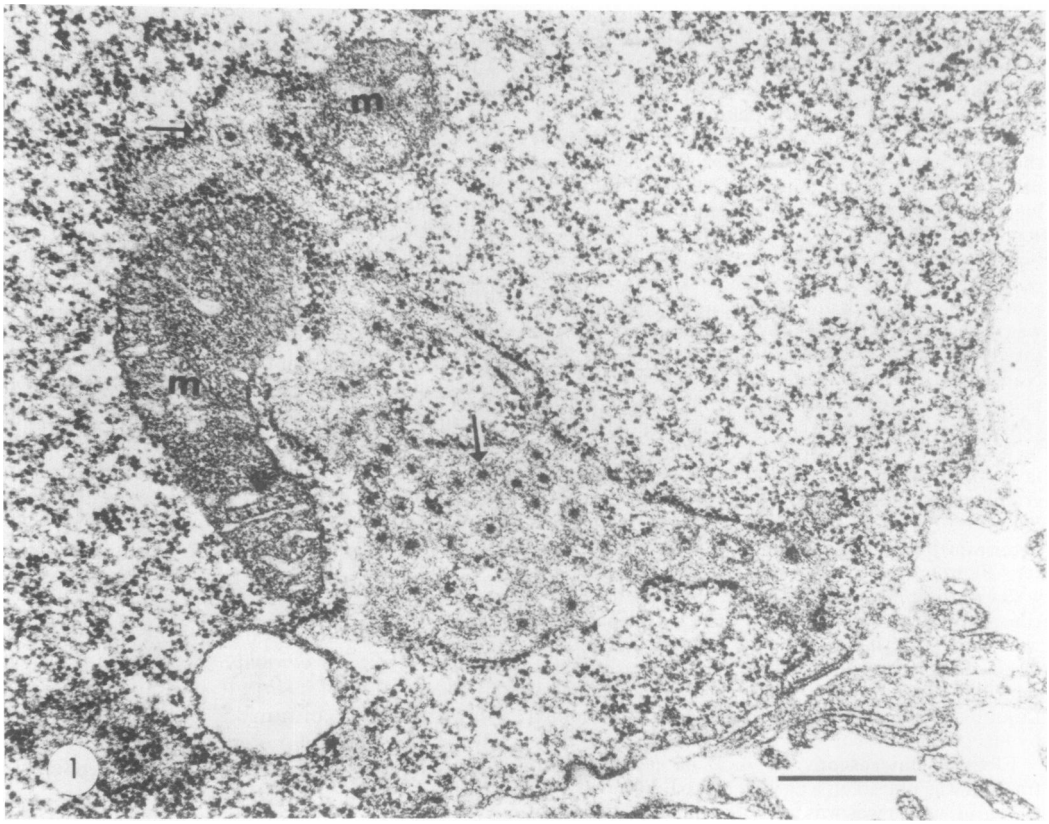

FIG. 1. *Thin section of a BHK-21-F cell released from a monolayer with trypsin and EDTA. In the cytoplasm of the cell near several mitochondria (m) is a large sac of RER filled with a homogeneous dense material. Embedded in this dense material are more than 20 R-type VLP (arrow indicates one). The RER is closely opposed to the plasma membrane. At sites such as this, the fusion of RER with plasma membrane could permit release of R-type VLP from an intact cell. Bar is 500 nm.*

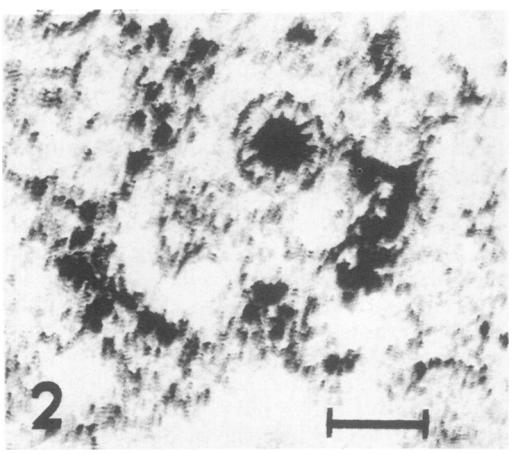

FIG. 2. *Portion of the RER of a BHK-21-F cell containing an R-type VLP. The characteristic radial spokes extending from the dense core to the periphery of the particle are clearly defined. Within the dense core is a ring of very dense material which can often be observed in favorably section VLP. Bar is 100 nm.*

nucleic acid in the VLP with radioisotopes, subconfluent monolayers of BHK-21-F cells were labeled with ^{3}H-uridine or ^{14}C-thymidine for 12 h. The supernatant medium was clarified, pelleted onto a 50% sucrose cushion, PEG precipitated, and centrifuged on a 20 to 45% sucrose gradient. Samples of the gradient fractions were precipitated with cold 5% trichloroacetic acid, and counted in a scintillation counter. Alternate fractions of the gradients were examined by electron microscopy of negatively stained preparations. Under these conditions of centrifugation, the ^{3}H-uridine-labeled medium yielded two well-separated peaks of radioactivity, one at 1.08 to 1.10 g/cm^{3} and one at 1.12 to 1.14 g/cm^{3}. Electron microscopy showed that the material in the upper band was primarily cell debris, such as small granules, clumps of amorphous material, and membrane fragments of various sizes. The heavier band contained many regular round particles, 85 to 100 nm in diameter, as well as a small amount of cellular debris like that seen in the upper

band. These spherical particles were not seen in other fractions. The particles occurred singly, in pairs, or held together in clumps of finely fibrillar material. The coincidence in this lower band of 100-nm spherical particles with a peak of ^{3}H-uridine in trichloroacetic acid-insoluble form suggests that the particles present in this peak contain RNA. In double label experiments, no incorporation of ^{14}C-thymidine was detected in this 1.12- to 1.14-g/cm^{3} band, although a small amount of ^{14}C-thymidine label was found in the upper band containing cellular debris. These experiments suggest that the R-type VLP does not contain appreciable amounts of DNA.

The fractions under the 1.12- to 1.14-g/cm^{3} peak containing the 100-nm spherical particles were pooled and precipitated again with PEG. Half of the sample was incubated with 5 μg of pancreatic RNase per ml (RNase A, Worthington Biochemicals Corp.) at 37 C for 30 min, and the other half served as a control. The samples were then layered onto sucrose density gradients, centrifuged, collected, and analyzed as before. Figure 3 shows that there was now a single peak of material containing acid-precipitable ^{3}H-uridine, and that this material is present in a form which is resistant to RNase. By electron microscopy the fractions under this peak contained only the spherical 100-nm particles and little or no cellular debris. The spherical particles were observed only in the fractions under the peak, suggesting that the RNA in this peak is protected from the RNase by being inside the R-type VLP.

Because the VLP did not show its morphologically unique features in the negatively stained preparations from sucrose gradients, we took R-type VLP from the above experiment, pelleted it, prepared the pellets for thin sectioning, and examined a cross section through the center of the pellet. The pellet (Fig. 4) contained large numbers of characteristic R-type VLP and occasional small bits of membrane. The VLP in this double-banded material are well separated from each other and are not bound to membranes. Although the VLP were somewhat distorted by the extensive isolation procedures, the characteristic spokelike structures radiating from the cores of some of the particles are visible at the arrows. Therefore, we believe we have developed a method for isolating and purifying the R-type VLP.

Properties of the intact VLP. To determine whether synthesis of the R-type VLP was inhibited by actinomycin D, we added 5 μg of actinomycin D per ml to the medium of BHK-21-F cells and incubated it for 2 h at 37 C. ^{3}H-uridine (10 μCi/ml) was then added to the

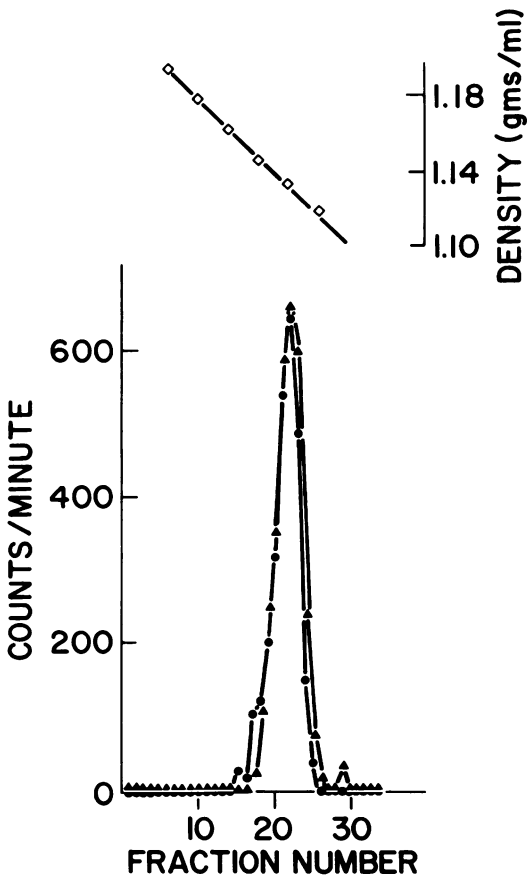

FIG. 3. *Isolation and RNAse resistance of R-type VLP. R-type VLP labeled with ^{3}H-uridine was partially purified by banding once in a sucrose density gradient. Fractions containing ^{3}H-uridine label and 100-nm particles were precipitated with 7.5% PEG in 1 M NaCl and resuspended. The half of the sample treated with 5 μg* of RNase per ml (▲) *and the control half* (●) *were incubated at 37 C for 30 min and then layered onto 20 to 45% sucrose density gradients and centrifuged at 27,000 rpm for 4 h at 4 C in an SW27 rotor. Fractions collected from the bottom were precipitated with trichloracetic acid, and radioactivity was determined in a liquid scintillation counter.*

medium. After an additional 10-h incubation at 37 C, the medium from the actinomycin D-treated cells was harvested and its R-type VLP was purified as described above. The purified R-type VLP was centrifuged for 17 h at 22,000 rpm on a 20 to 60% sucrose density gradient in Tris buffer. Table 1 shows the trichloroacetic acid-precipitable counts in control and actinomycin D-treated cells, media, and purified VLP. Although the amount of label incorporated into BHK-21-F cells treated with actinomycin D was only 1.3% of the control, more counts were incorporated into the isolated VLP by the actinomycin D-treated cells than by the

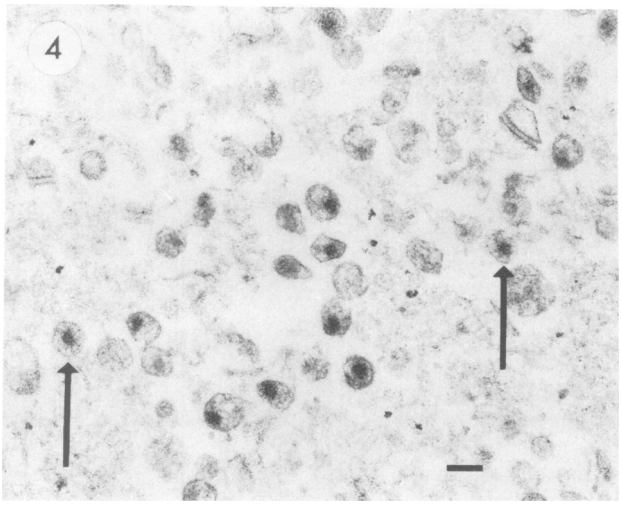

FIG. 4. *R-type VLP from fractions 19 to 23 of Fig. 3 pelleted at 40,000 rpm for 3 h at 4 C in an SW50 rotor. Arrows point to VLP in which the characteristic radial spokes of the R-type VLP can be distinguished. Bar represents 100 nm.*

TABLE 1. *Purification of R-type VLP from control and actinomycin D-treated BHK-21-F cells*[a]

BHK-21-F cells	Trichloroacetic acid-precipitable counts			
	Homogenized cells[b]	Medium[c]	Sucrose interface	1.12 to 1.14 g/cm³ peak from sucrose density gradient
Control	117,689,000	38,940,000	6,121,750	16,750
Actinomycin D-treated	1,525,000	3,134,000	154,280	21,525

[a] BHK-21-F cells, control or pretreated for 2 h with 5 μg of actinomycin D per ml, were labeled with 10 μCi of ^{3}H-uridine per ml for 10 h. Supernatant medium above intact cells was used for isolation of R-type VLP by pelleting onto 50% sucrose cushion, PEG precipitating, and centrifuging in a 20 to 60% sucrose density gradient in 0.1 M Tris buffer, pH 7.4, with 0.1 M NaCl for 17 h at 22,000 rpm in an SW27.1 Beckman rotor. Cells were homogenized after removal of medium.

[b] From 20 monolayers of BHK-21 cells in 75-cm² Falcon flasks.

[c] From 400 ml of medium overlying these cells.

control cells. The greatest purification of the R-type VLP was achieved during density gradient centrifugation, when large amounts of labeled cell debris were found at the top of the gradient or in the 1.08- to 1.10-g/cm³ band. Figure 5 shows that ^{3}H-uridine is incorporated into the R-type VLP even in actinomycin D-treated cells, and that the equilibrium density of the virus-like particle is 1.12 to 1.13 g/cm³. For comparison, the reported equilibrium density for avian myeloblastosis virus (AMV) and other oncorna viruses is 1.16 to 1.18 g/cm³ (26). Under these conditions of centrifugation, there is a broad shoulder toward the heavier side of the peak, possibly indicating disruption of the VLP during centrifugation. Occasionally a small minor peak appeared with a density of 1.18 to 1.19 g/cm³.

Sedimentation of the R-type VLP in cesium chloride density gradients led to degradation of the particles. The VLP also appeared to be disrupted by storage at 37 C and by freezing and thawing. For this reason, in all of our experiments the VLP was held at 4 C, 1% BSA was added to help stabilize the virus, and freezing and thawing of the VLP was avoided whenever possible.

RNA of the R-type VLP. Since the R-type VLP could be labeled with ^{3}H-uridine but not with ^{14}C-thymidine and ^{3}H-uridine is incorporated into the VLP in the presence of actinomycin D, it appears that the VLP is an RNA-containing particle which replicates from an RNA template.

RNA was extracted by Pronase and SDS treatment from banded and PEG-precipitated

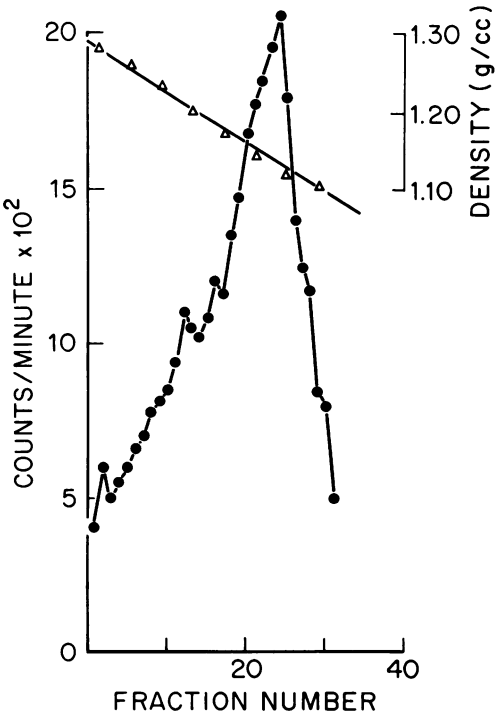

FIG. 5. *Isopycnic centrifugation of R-type VLP labeled with ³H-uridine in the presence of actinomycin D. BHK-21-F cells were pretreated with 5 μg of actinomycin D per ml for 2 h and then ³H-uridine was added. R-type VLP was isolated from the medium after 10 h. The R-type VLP was layered onto a 20 to 60% sucrose density gradient and centrifuged for 17 h at 27,000 rpm at 4 C in an SW27 rotor. Background, <20 counts/min.*

R-type VLP, labeled with ³H-uridine, and then centrifuged on a 15 to 30% sucrose gradient in TES with 0.5% SDS. ¹⁴C- or ³H-labeled 18 and 28*S* rRNA supplied by T. Sreevalsan were used as markers. The RNA isolated from the VLP peak (Fig. 6) was calculated to have a sedimentation coefficient of 60 to 70*S* by the method of Martin and Ames (24). A small amount of label is found near the top of the gradient in some experiments. The 70*S* RNA was degraded by incubation for 15 min at 37 C with 1 μg of pancreatic RNase per ml in TES with 0.1 M NaCl. The RNA is, therefore, believed to be single stranded. The highest yields of 60 to 70*S* R-type VLP RNA were isolated from medium which had never been stored or frozen and thawed. VLP which had been stored under suboptimal conditions yielded RNA which sedimented at 30 to 35*S* and 4 to 9*S*. The 60 to 70*S* VLP RNA was degraded by heating at 95 C for 3 min and by treatment with 20 volumes of 95% DMSO for 5 min at 37 C after the method of Duesberg (9) and Erickson (22).

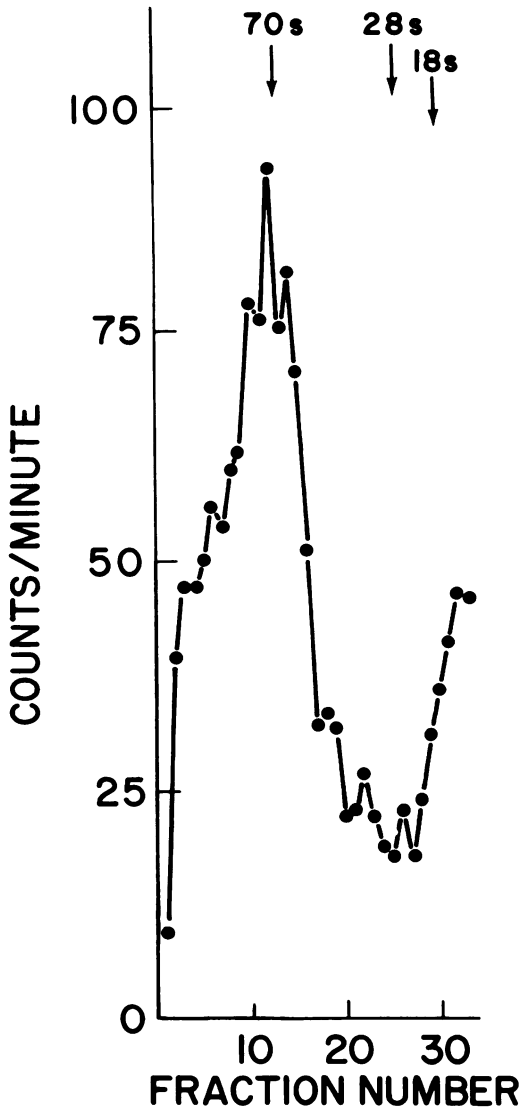

FIG. 6. *Sedimentation of RNA isolated from purified R-type VLP. ³H-uridine-labeled RNA was extracted with Pronase and SDS from R-type VLP partially purified in a sucrose density gradient. The VLP RNA was centrifuged in a 15 to 30% sucrose gradient in TES with 0.5% SDS for 2 h at 23 C at 40,000 in an SW50.1 rotor. rRNAs (28 and 18S) were used as markers.*

The size of the VLP RNA was also estimated by polyacrylamide gel electrophoresis by the method of Peacock and Dingman (28). ³H-uridine-labeled RNA was extracted from purified R-type VLP with SDS and Pronase and run on 2.2% polyacrylamide gels with 0.5% agarose with ¹⁴C-uridine-labeled AMV RNA run in the same gel as a marker. The results (Fig. 7) indicate that the RNA of the R-type VLP is very

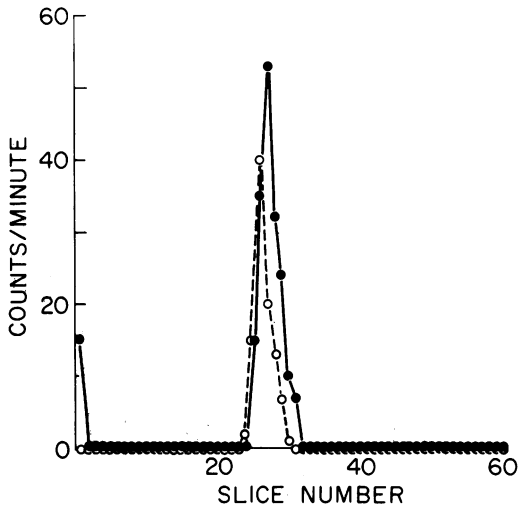

FIG. 7. *Polyacrylamide gel electrophoresis of RNA from R-type VLP (●) and AMV (○). RNA was extracted by Pronase and SDS from ^{3}H-uridine-labeled R-type VLP and from ^{14}C-uridine-labeled AMV and was co-electrophoresed on 2.2% polyacrylamide, 0.5% agarose gels. Radioactivity in gel slices was assayed in a liquid scintillation counter.*

close to the size of AMV RNA. A separate gel containing 42*S* Sindbis RNA, supplied by T. Sreevalsan, was run as a marker to assure that we were detecting the 70*S* RNA rather than the 30 to 35*S* degradation product of AMV RNA. Based on this data, the 70*S* RNA of the R-type VLP, like AMV (30), has a molecular weight of approximately 10^7.

DISCUSSION

By using a combination of electron microscopy and radioisotopic labeling techniques, we have been able to isolate the R-type VLP found in BHK-21 cells. A preliminary characterization of the VLP and its RNA is reported in this paper. It is important to note, however, that although this method results in good isolation of the R-type VLP, free of cellular material, only very small amounts of VLP are recovered from the medium of the BHK-21-F cells and relatively little ^{3}H-uridine is incorporated into the VLP. These problems may be inherent in the nature of working with an unknown VLP. There is not a suitable control cell which is susceptible to infection with the R-type VLP. Consequently, studies done only on cells carrying the R-type VLP are difficult to interpret. To circumvent this problem, we have studied VLP released into the medium over intact monolayers of BHK-21-F cells. Except for the observation that the VLP develops by budding into the RER (32, 40, 41), the replicative cycle of the R-type VLP is not understood. The VLP may be released directly into the extracellular space from intact cells by fusion of the RER with the plasma membrane, or it may be released only during lysis of a small number of cells in an otherwise intact monolayer. In a chronic infection with a VLP, cellular metabolism and division may be only minimally altered and the synthesis of the VLP components may occur at a much lower level than that in some lytic viral infections. This appears to be true for the chronic infection of BHK-21 cells of the R-type VLP.

The difficulties of handling the R-type VLP from BHK-21 cells have been overcome sufficiently to permit its preliminary characterization. The VLP contains labile, single-stranded RNA with a molecular weight of about 10^7 and a sedimentation constant of 60 to 70*S* in sucrose density gradients. Most viruses that replicate in the cytoplasm are RNA-containing viruses with the exceptions of the pox viruses and certain herpesviruses. Therefore, it is not surprising to find that the R-type VLP which matures in the cytoplasm contains RNA rather than DNA. In contrast to our findings, however, Kaaden and Dietzschold (17) have reported the isolation of a DNA-containing VLP from disrupted BHK-21 cells. The isolated VLP in their publication were not examined in thin sections where their characteristic radial spokes could be identified, and their negatively stained VLP do not show radial spokes. Since, in our system, we can identify large numbers of R-type VLP by their characteristic morphology in thin sections of purified material, and these appear exclusively in the fractions containing high-molecular-weight, radioactively labeled RNase-resistant RNA, we believe that we are indeed studying the RNA of the R-type VLP.

Although in the absence of suitable control cells it is difficult to study the replicative process of the R-type VLP, several experiments suggest that the ongoing synthesis of VLP RNA in BHK-21-F cells may not require a DNA intermediate. ^{3}H-uridine can be incorporated into the RNA of the R-type VLP in the presence of actinomycin D. No RNA-dependent DNA polymerase (reverse transcriptase) activity (34, 39) was detected in BHK-21-F cells or in concentrated R-type VLP preparations using either endogenous or synthetic templates (W. P. Parks and M. Myers, personal communications). However, the interaction between R-type VLP and BHK-21-F cells is a chronic infection and could be analagous to chronic infections with oncorna viruses in which the levels of reverse transcriptase are greatly reduced (13, 14, 29).

After the work described in this paper was completed, Birkmayer et al. (3) reported the isolation from amelanotic hamster melanoma cells of membraneous sacs containing three to five R-type VLP. The sacs had a density of 1.213 g/cm^3, which is much heavier than the 1.12- to 1.14-g/cm^3 density of the free, well-separated R-type VLP which we report here. Birkmayer et al. have isolated single-stranded RNA with a sedimentation constant of 70*S* from these membrane-bound R-type VLP. In marked contrast to our findings with the R-type VLP from BHK-21-F cells, however, Birkmayer et al. (3) found RNA-dependent DNA polymerase activity associated with the sacs of R-type VLP from the hamster melanoma cells. This activity was stimulated either by the 70*S* RNA or by poly rA-dT.

One of the goals of our research is to characterize the R-type VLP and to compare it to the known types of viruses in the standard virus classification schema. In this publication we compared the R-type VLP with AMV, a member of the oncorna virus group. Although the virions of R-type VLP and AMV are of similar size, their appearances in thin sections are quite different. The site of replication of the R-type VLP and AMV are also different. Whereas AMV matures by budding at the plasma membrane, the R-type VLP is formed by budding into the cisternae of the RER. The RNA isolated from the R-type VLP and AMV has approximately the same electrophoretic mobility in polyacrylamide agarose gels, and the same sedimentation velocity, 60 to 70*S*, in sucrose density gradients. The RNA of the R-type VLP is extremely labile to heat or DMSO treatment, as are the RNAs of oncorna viruses, progressive pneumonia virus (38), and the intracisternal A-type VLP (22, 44). A recent report by Cheung et al (5) indicates that the 70*S* RNA of Rous sarcoma virus may be formed by association of smaller RNA units shortly after Rous sarcoma virus virions are released from cells. The same type of phenomenon could also be occurring during the development of the R-type VLP, and this may be related to the instability of the VLP RNA. In spite of the similarity of the size and instability of the RNA isolated from the R-type VLP and AMV, we believe that the differences in morphology, site of maturation, and density of the R-type VLP and AMV are so significant that the R-type VLP should not be classified with the oncorna virus group. Preliminary studies in our laboratory indicate that the R-type VLP also differs significantly from the corona virus group. Further studies which will lead to classification of the R-type VLP are now under way.

The findings reported in this paper are important to all studies which utilize BHK-21 cells for biological and virological investigations. The R-type VLP is present in varying amounts in all BHK-21 sublines which have been carefully studied by electron microscopy. Although BHK cells show no cytopathic effect and replicate well in tissue culture, the production of the VLP may have significant effects on the cells. The VLP genome may confer malignant potential on some or all of the chronically infected BHK-21 cells. The VLP genome may direct the synthesis of specific enzymes and/or capsid proteins which aid the synthesis and assembly of other types of virions. The presence of the VLP genome within the BHK-21 cell could be responsible for changes in the cell surface antigens of the hamster cells.

The presence of actinomycin-resistant RNA synthesis in these cells (25) must be interpreted with caution.

The recent development for use in domestic animals of live attenuated rabies virus vaccine prepared in BHK-21 cells (33) is a matter of particular concern to us in view of the possibility that preparations of the live virus vaccine may contain the R-type VLP. Because uninactivated R-type VLP may be administered to domestic animals in the near future, the need to understand the biological significance, host range, and oncogenic potential of the R-type VLP is particularly urgent.

ACKNOWLEDGMENTS

We thank Susan J. Eberhard, Patricia M. LeMaire, and Patricia Wiesner for their excellent technical assistance, and K. Takemoto, L. Sturman, and E. C. Pirtle for helpful discussions.

This research was supported by grants from The Research Corporation, The Anna Fuller Fund, Public Health Service training grant T1-AI-298 from the National Institute of Allergy and Infectious Diseases, and Public Health Service grant CA-12798 from the National Cancer Institute.

LITERATURE CITED

1. Becker, W. B., K. McIntosh, J. H. Dees, and R. M. Chanock. 1967. Morphogenesis of avian infectious bronchitis virus and a related human virus (strain 229 E). J. Virol. **1**:1019–26.
2. Bernhard, W., and P. Tournier. 1964. Infection virale inapparante de cellules de hamsters décelée par la microscopie electronique. Ann. Inst. Pasteur **107**:447–452.
3. Birkmayer, G., F. Miller, and B. R. Balda. 1972. Inhibition of high molecular weight RNA synthesis in a hamster melanoma by ethidium bromide *in vivo*. Hoppe-Seyler's Z. Physiol. Chem. **355**:1749–1754.
4. Chaney, C., and F. Robbe-Maridor. 1970. Enhancing effect of the murine sarcoma virus (MSV) on the replication of the mouse hepatitis virus (MHV) *in vitro*. Proc. Soc. Exp. Biol. Med. **131**:302–305.
5. Cheung, K. S., R. Smith, M. Stone, and W. Joklik. 1972. Comparison of immature (rapid harvest) and mature Rous sarcoma virus particles. Virology **50**:851–864.
6. Compans, R. W., K. V. Holmes, S. Dales, and P.

Choppin. 1966. An electron microscopic study of moderate and virulent virus cell interactions of the parainfluenza virus SV5. Virology **30**:411-426.
7. David-Ferreira, J. F., and R. H. Manker. 1965. An electron microscopic study of the development of a mouse hepatitis virus in tissue culture cells. J. Cell Biol. **24**:57-77.
8. dePetris, S., and J. J. Harvey. 1969. Presence of unusual virus particles in two hamster tumor tissue culture cell lines induced by murine sarcoma viruses. J. Gen. Virol. **5**:561-564.
9. Duesberg, P. H. 1968. Physical properties of Rous sarcoma virus RNA. Proc. Nat. Acad. Sci. U.S.A. **60**:1511-1518.
10. Epstein, W., K. Fukuyama, and M. Benn. 1968. Transmission of a pigmented melanoma in golden hamsters by a cell free ultrafiltrate. Nature (London) **219**:979-980.
11. Erickson, R. L. 1969. Studies on the RNA from avian myeloblastosis virus. Virology **37**:124-131.
12. Hamre, D., D. A. Kindig, and J. Mann. 1967. Growth and intracellular development of a new respiratory virus. J. Virol. **1**:821-882.
13. Hanafusa, H., D. Baltimore, D. Smoler, K. F. Watson, A. Yaniv, and S. Spiegelman. 1972. Absence of polymerase protein in virions of alpha type RSV. Science **177**:1188-1191.
14. Hanafusa, H., and T. Hanafusa. 1971. Noninfectious RSV deficient in DNA polymerase. Virology **43**:313-316.
15. Holmes, K. V., and P. Choppin. 1966. On the role of the response of the cell membrane in determining virus virulence. J. Exp. Med. **124**:501-520.
16. Holmes, K. V., and P. Choppin. 1968. On the role of microtubules in movement and alignment of nuclei in virus-induced syncytia. J. Cell Biol. **39**:526-543.
17. Kaaden, O., and B. Dietzschold. 1971. Isolation and characterization of extracellular virus-like particles from BHK cells. Arch. Gesamte Virusforsch. **35**:317-321.
18. Kisch, A., R. Kelley, and B. Eberle. 1972. Differential enhancement of R-type virus particles in polyoma-transformed BHK-21 cells by dimethyl sulfoxide. J. Nat. Cancer Inst. **49**:911-913.
19. Klenk, H.-D., and P. W. Choppin. 1969. Lipids of plasma membrane of monkey and hamster kidney cells and of parainfluenza virions grown in these cells. Virology **38**:255-268.
20. Klenk, H.-D., and P. W. Choppin. 1970. Plasma membrane lipids and parainfluenza virus assembly. Virology **40**:939-947.
21. Klenk, H.-D., and P. W. Choppin. 1970. Glycosphingolipids of plasma membranes of cultured cells and an enveloped virus (SV5) grown in these cells. Proc. Nat. Acad. Sci. U.S.A. **66**:57-64.
22. Kuff, E. L., K. K. Lueders, A. L. Ozer, and N. A. Wivel. 1972. Some structural and antigenic properties of intracisternal A particles occurring in mouse tumors. Proc. Nat. Acad. Sci. U.S.A. **69**:218-222.
23. Macpherson, I., and M. Stoker. 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. Virology **16**:147-151.
24. Martin, R. G., and D. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372-1379.
25. Martin, S. J. and F. Brown. 1967. Synthesis of RNA in BHK cells in the presence of actinomycin D. Biochem. J. **105**:979-86.
26. McGee-Russell, S. M., A. D. Vizoso, and F. K. Sanders 1965. Proceedings of the Third European Regional Conference on Electron Microscopy, p. 367. Publishing House of Czechoslovak Academy of Science, Prague.
27. Oshiro, L. S., J. H. Schieble, and E. H. Lennette. 1971. Electron microscopic studies of coronavirus. J. Gen. Virol. **12**:161-168.
28. Peacock, A. C., and C. W. Dingman. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry **1**:668-674.
29. Peebles, P. T., D. K. Haapala, and A. F. Gazdar. 1972. Deficiency of viral ribonucleic acid-dependent deoxyribonucleic acid polymerase in noninfectious virus-like particles released from murine sarcoma virus-transformed hamster cells. J. Virol. **9**:488-493.
30. Robinson, W. S., A. Pitkanen, and H. Rubin. 1965. The nucleic acid of the Bryan strain of Rous sarcoma virus: purification of the virus and isolation of the nucleic acid. Proc. Nat. Acad. Sci. U.S.A. **54**:137-144.
31. Sharp, D. G., and J. W. Beard. 1954. Virus of avian erythromyeloblastic leukosis. IV. Sedimentation, density and hydration. Biochim. Biophys. Acta **14**:12-17.
32. Shipman, C., G. C. V. Weide, and B. I. Ma. 1969. Prevalence of type R virus-like particles in clones of BHK-21 cells. Virology **38**:707-710.
33. Sikes, R. K., G. V. Peacock, P. Acha, R. J. Arko, and R. Diercks. 1971. Rabies vaccines: duration of immunity study in dogs. J. Amer. Vet. Med. Ass. **159**:1491-1499.
34. Spiegelman, S., A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek, and K. Watson. 1970. Synthetic DNA-RNA hybrids and RNA-RNA duplexes as templates for the polymerases of the oncogenic RNA viruses. Nature (London) **228**:430-432.
35. Stenback, W. A., A. L. Van Hoosier, D. B. Ferguson, and J. J. Trentin. 1970. Significance of virus particles observed in spontaneous and induced hamster tumors. Comp. Leuk. Virus Res. **36**:559-565.
36. Stenback, W. A., G. L. Van Hoosier, and J. J. Trentin. 1966. Virus particles in hamster tumors as revealed by electron microscopy. Proc. Soc. Exp. Biol. Med. **122**:1219-1223.
37. Stoker, M., and I. Macpherson. 1964. Syrian hamster fibroblastic cells like BHK-21 and its derivatives. Nature (London) **203**:1355-1357.
38. Stone, L. B., K. K. Takemoto, and M. A. Martin. 1971. Physical and biochemical properties of progressive pneumonia virus. J. Virol. **8**:573-578.
39. Temin, H., and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226**:1211-1213.
40. Thomas, A. J., E. Delain, and E. Hollande. 1967. Morphogenese d'un virus du hamster associé a la souche BHK, ou à des tumeurs. C.R. Acad. Sci. **264**:785-788.
41. Thomas, A. J., M. Henry, E. Hollande, and C. Vilain. 1968. Nouvelles observations sur le virus du hamster associé aux cellules BHK et aux tumeurs. C.R. Acad. Sci. **266**:2129-2132.
42. Vaheri, A., W. D. Sedwick, and S. A. Plotkin. 1967. Growth of rubella virus in BHK 21 cells. Proc. Soc. Exp. Biol. Med. **125**: 1086-1092.
43. Van Hoosier, G. L., W. A. Stenback, J. C. Parker, J. A. Burke, and J. J. Trentin. 1970. The effects of cesarean derivation of foster nursing procedures on enzootic viruses of the LSH strain of inbred hamsters. Lab. Anim. Care **20**:232-237.
44. Yang, S. S., and N. A. Wivel. 1973. Analysis of high-molecular-weight ribonucleic acid associated with intracisternal A particles. J. Virol. **11**:287-298.
45. Zeigel, R. F., G. Rabotti, and M. V. A. Smith. 1969. EM observations on the association of viruses with membrane systems in hamster tumor cells propagated in tissue culture. J. Nat. Cancer Inst. **43**:653-657.