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Aura Alphavirus Subgenomic RNA Is Packaged into Virions of Two Sizes

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The alphavirus genome is 11.8 kb in size. During infection, a 4.2-kb subgenomic RNA is also produced. Most alphaviruses package only the genomic RNA into virions, which are enveloped particles with icosahedral symmetry, having a triangulation number (T)=4. Aura virus, however, packages both the genomic RNA and the subgenomic RNA into virions. The genomic RNA is primarily packaged into a virion that has a diameter of 72 nm and which appears to be identical to the virions produced by other alphaviruses. The subgenomic RNA is packaged into two major, regular particles with diameters of 72 and 62 nm. The 72-nm-diameter particle appears to be identical in construction to virions containing genomic RNA. The 62-nm-diameter particle probably has T=3. The large and small Aura virions can be partially separated in sucrose gradients. In addition to these two major classes of particles, there are other particles produced that appear to arise from abortive assembly. From these results and from previous studies of alphavirus assembly, we suggest that during assembly of alphavirus nucleocapsids in the infected cell there is a specific initiation event followed by recruitment of additional capsid subunits into the complex, that the triangulation number of the complex is not predetermined but depends upon the size of the RNA and interactions that occur during assembly, and that budding of assembled nucleocapsids results in the acquisition of an envelope containing glycoproteins arranged in a manner determined by the nucleocapsid.

The alphaviruses are a group of 26 viruses, many of which cause important human or veterinary diseases (23). The alphavirus genome consists of plus-sense RNA of about 11.8 kb, including a 5′ cap and a 3′ poly(A) tract (28). The genomic RNA is assembled with 240 molecules of a basic capsid protein of 30 kDa into a T=4 icosahedral nucleocapsid (7, 8, 22). During virus assembly, the T=4 nucleocapsid buds through the cell plasma membrane and acquires a lipoprotein envelope in which two virus-encoded glycoproteins, E1 and E2 (each about 50 kDa), are embedded. The glycoproteins also exhibit T=4 icosahedral symmetry and are present as 80 spikes, each of which consists of a trimer of E1-E2 heterodimers (1, 7, 9, 22, 24, 30). The equimolar ratio of the virus structural proteins and the obvious icosahedral symmetry exhibited by the glycoproteins at the surface of the virus led to the hypothesis that virus assembly requires a specific interaction between the cytoplasmic domains of the glycoproteins and the nucleocapsid protein present in the nucleocapsid (10). Direct binding of the envelope proteins to the nucleocapsid has been demonstrated (13, 21), and molecular genetic studies with chimeric alphaviruses have demonstrated that there is a sequence-specific interaction between the cytoplasmic domain of glycoprotein E2 and the nucleocapsid (20). Lateral interactions between the glycoproteins are also known to be of importance during virus assembly (12, 17, 31). The assembled virion has a diameter of about 70 nm, while the nucleocapsid has a diameter of about 40 nm.

We recently reported that whereas most alphaviruses package only the 11.8-kb genomic RNA into virus particles, Aura virions contained both genomic RNA and 4.2-kb subgenomic RNA (25). The subgenomic RNA is transcribed from minus-strand copies of alphavirus genomic RNA during the process of infection and serves as mRNA for the three structural proteins (28). Recent evidence that there is a packaging signal in alphavirus RNAs that is required for efficient assembly of nucleocapsids within the infected cell has accumulated (3, 4, 16, 32), and our results with Aura virus led us to suggest that the Aura virus subgenomic mRNA contains an encapsidation signal, unlike the subgenomic mRNAs of other alphaviruses. In this communication, we report that preparations of Aura virions contain particles of two different sizes. Particles in the first group are the standard size (72-nm diameter), appear to have T=4 icosahedral symmetry, and contain both genomic RNA and subgenomic RNA (presumably present in different virions). The second class of particles is smaller (62-nm diameter), may possess T=3 symmetry, and contains only the subgenomic mRNA. In addition, there are small numbers of larger particles present in Aura virus preparations, many of which appear to arise by aberrant assembly. Thus, packaging of Aura virus RNA into particles appears to be less precise than for other alphaviruses, and particles of different sizes appear to be formed by different combinations of the same viral components.

MATERIALS AND METHODS

Propagation of Aura virus. Aura virus strain BeAR 10315 was propagated as previously described (25).

Electron microscopy. Electron microscopy of Aura virus preparations was performed both in Austin, Tex., and in Tübingen, Federal Republic of Germany. In Austin, samples were mounted on 400-mesh carbon-coated grids and were negatively stained with 1% uranyl acetate. Images were photographed in a JEOL 100CX electron microscope at an instrumental magnification of ×50,000. In Tübingen, piloform- and carbon-coated, glow-discharged copper grids were floated on droplets of buffered gluteraldehyde (0.1% [vol/vol])-fixed virus solutions for 10 min. Preparations were subsequently stained by floating on 1%

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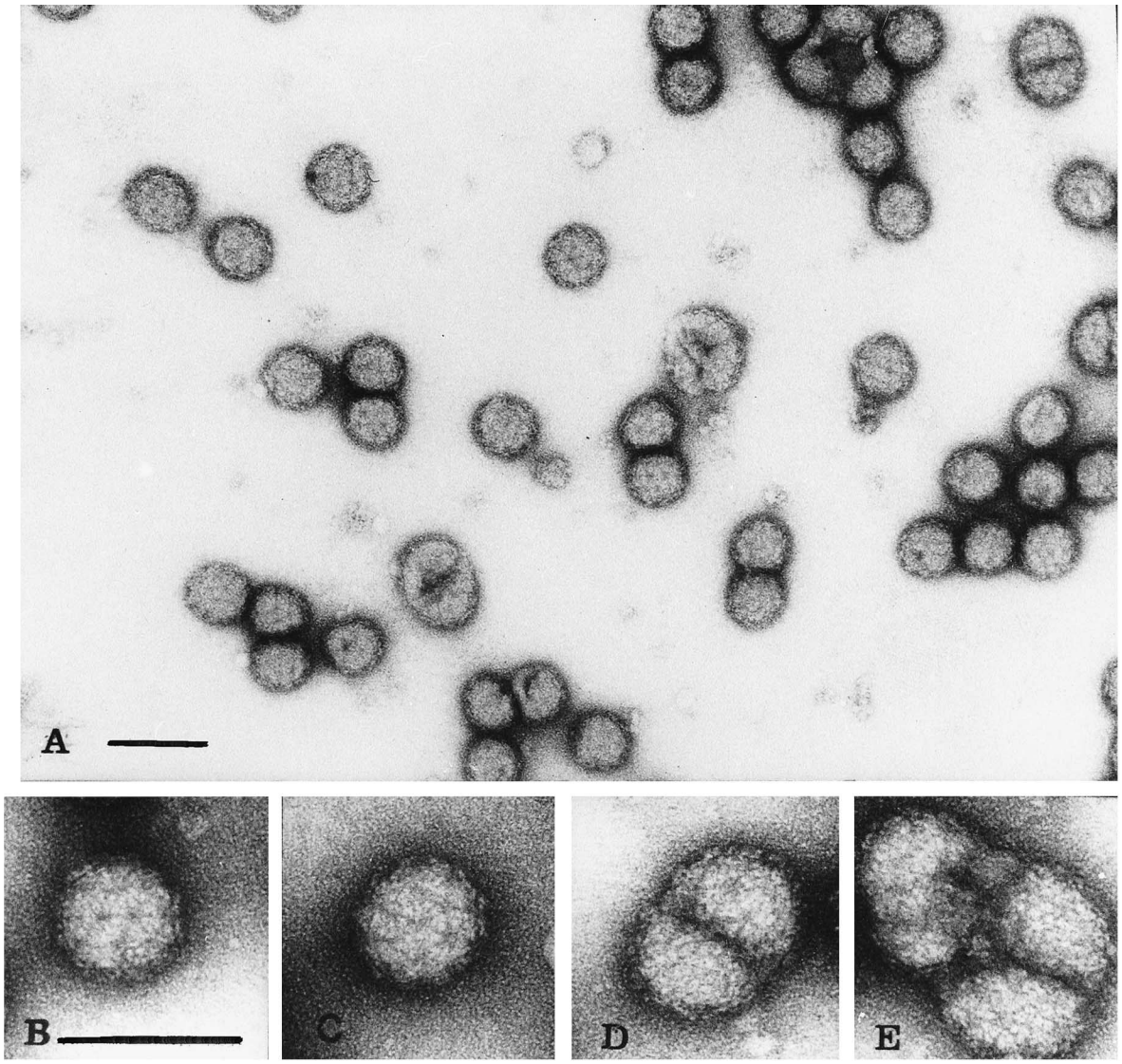

FIG. 1. Electron microscopy of Aura virus. (A) A field of Aura particles that illustrates the different types of particles seen. (B) A high-magnification view of a smaller, regular particle. (C) A high-magnification view of a larger, regular particle. (D) A monster particle that may contain two hemicapsids fused together. (E) A monster particle that appears to contain three partly formed nucleocapsids. The scale bars indicate 100 nm (the magnification in panels B to E is the same).

uranyl acetate solution (pH 4.3), air dried, and examined in a Zeiss EM 910 electron microscope. Photographs were taken on Agfa Scientia film at an instrumental magnification of ×50,000 or ×100,000. In either case, the diameters of the virions were measured on negatives or on contact prints. Each particle was measured on two perpendicular axes, and only particles having identical diameters on the two axes were included in the data set.

RESULTS

Electron microscopy of Aura virions. We have routinely observed that Aura virus preparations grown in BHK cells contain regular particles of two predominant sizes plus small numbers of other particles. Electron micrographs of a collection of Aura virions that illustrate the different sizes and shapes of particles seen are shown in Fig. 1. Figure 1A shows a field that illustrates the various particles seen and clearly contains regular particles of two different sizes. Figure 1B illustrates a smaller particle at higher magnification, and Fig. 1C shows a larger particle at higher magnification. Both the smaller and larger particles appear to exhibit regular icosahedral symmetry.

Regular particles in unfractionated preparations of Aura virus were measured, and a frequency distribution of particle sizes is shown in Fig. 2. There are two predominant sizes of such regular particles with diameters of 62 and 72 nm. The distribution of the sizes of regular particles in these two classes is very sharp (note that the scale in Fig. 2 is broken in order to display the size distribution more clearly), and the standard deviations in the measured sizes were only about 0.5 nm. The 72-nm-diameter particles are the same size as has been reported for other alphaviruses, and we assume that these are normal Aura virions having T=4 symmetry. In a few cases, the resolution of the structures on the surface of the virus particle was good enough to show T=4 symmetry, confirming this assignment. The smaller particles are 86% the size of the normal particles, consistent with T=3 symmetry. Careful searching of small particles revealed a few examples in which T=3 symme-

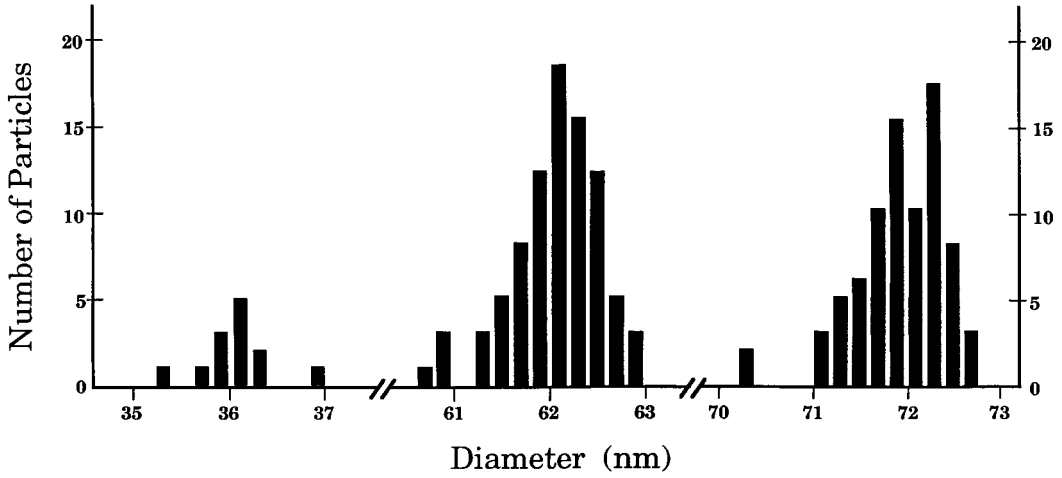

FIG. 2. Size distribution of Aura virus particles. The diameters of regular particles selected at random were measured, and the results were plotted as a frequency distribution. Only particles that were well separated and that had the identical diameter in two orthogonal directions were included in the data set.

try could be assigned with some confidence. We conclude that Aura virus RNA can assemble into regular particles of at least two sizes; the larger particles appear to be normal T=4 alphavirus virions, while the smaller particles are probably T=3 virions, although the possibility that they are an altered form of T=4 virions cannot be ruled out.

In addition to these two predominant classes of particles, other types of particles were also observed in small numbers. These particles include particles with a mean diameter of 36 nm, consistent with T=1 symmetry (Fig. 2); particles that appear to be regular icosahedral virions but which are larger than the 72-nm-diameter particles; particles that appear to have more than one nucleocapsid in a single envelope, a phenomenon that has been described for other alphaviruses (27); monsters that appear to contain prolate ellipsoids or two half-icosahedral nucleocapsids (Fig. 1D), discussed in more detail below; and a number of other oddly shaped particles (e.g., Fig. 1E).

Aura virions with different sedimentation coefficients. We have routinely observed the presence of two bands of Aura virus upon sedimentation in sucrose gradients when the virus was propagated in BHK cells but not when the virus was grown in mosquito cells. A sucrose gradient illustrating this phenomenon is shown in Fig. 3. The single band of mosquito-grown virus and the two bands of the BHK-grown virus appear homogeneous, but it is easy to overload such gradients with virus such that in the case of the BHK-grown virus only a single broad band may be visible.

The two bands of virus from the gradient in Fig. 3, labeled a and b, were isolated separately. The densities of the particles in the two bands were examined by isopycnic centrifugation and were not detectably different (1.223 g/ml).

Virus bands a and b were next examined by electron microscopy. The more rapidly sedimenting band, band a, contained only 72-nm-diameter particles, whereas the more slowly sedimenting band contained only 62-nm-diameter particles (data not shown). Thus, Aura virus particles of the two different sizes were separable on sucrose gradients in this experiment.

RNA present in Aura virus particles. The two bands of BHK virus, a and b, were collected, RNA was extracted, and the RNA was examined by gel electrophoresis (Fig. 4). The particles in the faster-sedimenting band, band a, contained both 49S genomic RNA and 26S subgenomic RNA. The amount of 49S RNA relative to 26S RNA in particles is greater than the amount of 49S RNA relative to 26S RNA found within the infected cell (Fig. 4, compare lanes 1 and 2). The more slowly sedimenting particles, band b, contained only 26S RNA.

Thus, this Aura virus preparation contained predominantly two classes of virions, a faster-sedimenting class of particles with a diameter of 72 nm which contained both 49S and 26S RNA, presumably present in different virions, and a more slowly sedimenting class of 62-nm-diameter particles which contained only 26S RNA. Each class appeared to be homogeneous upon sedimentation in sucrose gradients.

Distribution of 26S RNA-containing virions. In the Aura virus preparation shown in Fig. 3 and 4, Aura virions of the two predominant sizes separated cleanly in sucrose gradients. In other preparations of BHK-grown virus, however, we have been unable to cleanly separate the two size classes, although two viral bands are always seen in sucrose gradients and the slower band contains only 26S RNA. One such preparation is

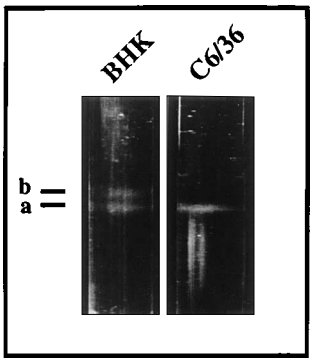

FIG. 3. Velocity sedimentation of Aura virus grown in BHK cells or in mosquito cells. BHK cells or C6/36 mosquito cells were infected with Aura virus, and the culture fluid was harvested 24 h after infection. Virus was pelleted by centrifugation at 23,000 rpm for 4 h at 4°C in a Beckman SW27 rotor. The virus pellet was resuspended in TNE buffer (50 mM Tris-Cl [pH 7.4], 200 mM NaCl, 5 mM EDTA), and the virus was sedimented in a linear 10 to 30% sucrose gradient in TNE buffer at 32,000 rpm for 1.25 h at 4°C in a Beckman SW41 rotor. A photograph of the resulting gradients is shown. Two virus bands, labeled a and b, are seen in the BHK-grown virus preparation, but only one band, corresponding to the lower BHK band, is visible in the mosquito cell-grown virus preparation.

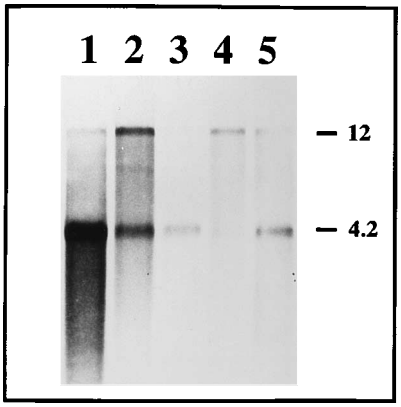

FIG. 4. Distribution of genomic and subgenomic RNAs in the two virus particles. BHK cells were infected with Aura virus (lanes 1, 2, and 3) or with Sindbis virus (lanes 4 and 5) and labeled with [^{3}H]uridine for 12 h in the presence of 20 μg of dactinomycin per ml. RNA was extracted from purified virus (lanes 2, 3, and 4) or from infected cells (lanes 1 and 5) and subjected to electrophoresis in denaturing agarose gels. Lane 2 is from the faster-migrating Aura virus band (band a) in Fig. 1, and lane 3 is RNA from the slower Aura virus band (band b). The numbers to the right of the gel are the sizes of the two Aura virus RNAs (in kilobases) as determined by comparison with the known molecular sizes of Sindbis virus RNAs.

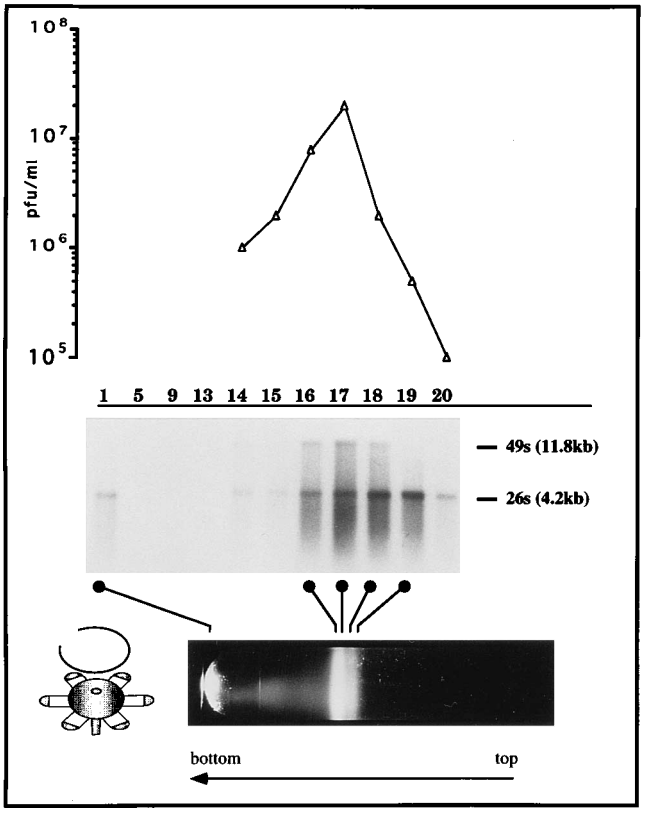

FIG. 5. Aura virus grown in BHK cells. Aura virus was grown in BHK clone 15 cells and harvested at 24 h after infection. The virus was concentrated by pelleting in a Kontron TST 28.1 rotor at 24,000 rpm for 2 h. The pellet was resuspended in 200 μl of TNE buffer, layered over a continuous 10 to 30% sucrose gradient in the same buffer in a 35-ml TST 28.1 tube, and centrifuged for 2.5 h at 24,000 rpm at 4°C in a Kontron TST centrifuge. Fractions were collected by puncturing the bottom of the tube, and drops were collected in TNE buffer. Samples were removed for plaque assay on BHK cells, and RNA was prepared from the remainder of the sample after pelleting the virus in a Beckman TLA 45 rotor at 45,000 rpm for 1 h. Equal volumes of RNA from the different fractions were separated by gel electrophoresis in a nondenaturing agarose gel in Tris-acetate buffer. The RNA was transferred to a nylon membrane and hybridized with a nick-translated cDNA probe encompassing the subgenomic RNA region of the genome. A photograph of the sucrose gradient tube (bottom), the infectivity titers of selected fractions (top), and the RNA patterns in different fractions (middle) are shown.

shown in Fig. 5. The sucrose gradient in Fig. 5 shows the two major virus bands and a light smear of particles down to the bottom of the gradient; the two major bands appear more heterogeneous and less well resolved than the two bands in Fig. 3, perhaps in part because of overloading. This gradient was fractionated by dripping from the bottom of the tube, and individual fractions were examined for RNA content, for particle content by electron microscopy, and for infectivity in a plaque assay. Of the major bands, the upper band contained only 26S RNA and the lower band contained both 26S and 49S RNA, confirming the results in Fig. 3 and 4. 26S RNA was also present in the smear of material present down to the bottom of the tube and in the pellet. The infectivity assays demonstrated that the faster band contained infectious virus but that the upper band was essentially noninfectious. Electron microscopic examination of the different fractions showed, however, that the separation of particles by size was incomplete (data not shown). It appears that the composition of the Aura virions is sufficiently variable from experiment to experiment that clear separation of the two size classes is not always possible. This variability does not detract from the major finding that Aura virus preparations contain two major size classes of regular particles, however, which is reproducible.

DISCUSSION

Two sizes of Aura virions. Our results clearly show that virus particles of two sizes are produced during infection of BHK cells by Aura virus. The larger particles are approximately the size that has been reported for other alphaviruses and contain both 49S RNA and 26S RNA. We assume that the 49S RNA-containing particles are equivalent to other alphavirus virions and contain one molecule of 49S RNA encapsidated in a T=4 icosahedral nucleocapsid and surrounded by a glycoprotein-containing envelope whose glycoproteins are organized into a T=4 lattice. The composition of the large particles that contain subgenomic RNA is less clear. They may contain three molecules of RNA such that their RNA content is approximately the same as that of the 49S RNA-containing virions and thus organized into a structure with the same properties as the 49S RNA-containing virions. It is also possible that only one or two molecules of 26S RNA are packaged into a T=4 structure, resulting in heterogeneity in the properties of the larger particles. Variability in packaging, including formation of different abortive particles, could depend upon the exact cell culture conditions and might explain the differences in separation of large and small particles on sucrose gradients observed in the course of these experiments. It is also noteworthy that we previously found that the particle/PFU ratio for Aura virus was $\sim 10^3$ (25), whereas for other alphaviruses, such as Sindbis virus, this ratio is ≤ 10 (27). The presence of subgenomic RNA-containing Aura virus particles can account for only a small part of the 100-fold difference in specific infectivity, and the low specific infectivity of Aura virus particles suggests that most Aura virus particles are defective because of aberrant assembly processes.

The smaller particles contain only 26S RNA and are 86% the diameter of the larger particles. The size of these particles suggests that they contain a T=3 icosahedral nucleocapsid in

which 180 molecules of capsid protein encapsidate a single 26S RNA molecule and are surrounded by an envelope in which 180 glycoprotein heterodimers are also organized with T=3 symmetry. Presumably the size of a 26S RNA molecule is such that a T=3 nucleocapsid can accommodate it, but 49S RNA cannot be packaged into such a particle because of volume constraints.

Although we believe it likely that the small particles possess T=3 symmetry, we cannot rule out the possibility that they represent a condensed form of a T=4 particle. Contracted forms of alphavirus nucleocapsids have been reported after various treatments (for a recent review, see reference 28), and furthermore, contracted forms of the Semliki Forest alphavirus that appear to retain icosahedral symmetry have been observed (34). If a contracted T=4 Aura virus nucleocapsid were to be produced within infected BHK cells, budding could subsequently lead to the production of a smaller virion. This possibility appears to be unlikely because of our observation that a few 62-nm-diameter Aura virus particles appear to possess five- and sixfold axes that are related by T=3 symmetry and because of the findings of Cheng et al. (7) that in the Ross River alphavirus virion the packing of glycoproteins is so tight that it seems unlikely that a full complement of 240 glycoproteins could be accommodated in a smaller particle. However, detailed studies of the structures of Aura virions of the two sizes using cryoelectron microscopic reconstructions will be required to firmly establish the nature of the smaller particles.

It is of interest that the two sizes of Aura virus particles were observed only in BHK-grown virus and not in mosquito-grown virus. We previously showed that about threefold-more 26S RNA than 49S RNA was incorporated into Aura virus when the virus was grown in BHK cells than when it was grown in mosquito cells (25). There are two possibilities to explain these observations. In one model, the smaller nucleocapsids do not form or are not stable in mosquito cells or if formed are unable to bud efficiently from mosquito cells because of a different composition of the plasma membrane, leading to a failure to form the smaller virus particles and to the incorporation of smaller amounts of 26S RNA into virions. In the second model, smaller amounts of 26S RNA are incorporated into virions for some unrelated reason, such as the sequestration of 26S RNA in ribosomes in mosquito cells, and this leads to production of lesser amounts of the smaller particles.

Smaller particles found in other alphaviruses. In the case of other alphaviruses, the 4-kb subgenomic RNA apparently lacks a packaging signal and is not packaged so that smaller 26S RNA-containing particles are not seen. However, smaller particles have been reported in the case of alphavirus preparations that contain defective interfering (DI) particles. Johnston et al. (14) reported that passaging of Sindbis virus at high multiplicities of infection led to the appearance of smaller virions whose diameter was 80% that of standard virions at the same time that DI particles arose in the stock. Similarly, Barrett et al. (2) reported the occurrence of DI particles of Semliki Forest virus that were 84% the diameter of standard virus. In both cases, these 80 to 84% particles are close to the value of 86% found for the smaller class of Aura virions. We suggest that these DI particles are equivalent to the smaller Aura virions and arose through packaging of the smaller DI RNAs, which contain packaging signals. Brown and Gliedman (5) also found that two classes of smaller particles of Sindbis virus could be seen following infection of mosquito cells, one class 80% the size of normal particles and the second 59% the size of normal particles. The 80% particles are similar in size to the putative T=3 virions, and the 59% particles could be T=1 virions. The ability to form smaller particles thus appears to be general in alphaviruses and not particular to Aura virus. We suggest that these smaller particles form whenever a suitable-size RNA molecule with the appropriate packaging signal is available within the cell.

Construction of alphavirus virions. If our hypothesis that alphavirus virions can form using different triangulation numbers is correct, it raises interesting questions with regard to the subunit interactions that occur during virus assembly. The nucleocapsid of the virus is assembled in the cytoplasm of the infected cell and nucleocapsids can be readily visualized with the electron microscope or isolated from infected cells (6, 27). No empty capsids have ever been reported for alphaviruses, and it is believed that capsid protein requires RNA for assembly into nucleocapsids. Further, there is evidence that an encapsidation sequence in the viral RNA is required for efficient assembly of the nucleocapsid within the cell (16, 25, 32). We suggest that one or more molecules of capsid protein interacts with the encapsidation sequence in a specific fashion to initiate nucleocapsid formation. Additional capsid protein molecules are recruited into the complex both through capsid protein-capsid protein interactions and capsid protein-RNA interactions, the latter being nonspecific, presumably electrostatic, interactions at this stage of assembly. We postulate that both protein-protein and protein-RNA interactions are required for continued assembly. The protein-protein interactions during assembly may favor construction of a T=4 structure, and encapsidation of 49S RNA requires a capsid of at least T=4, but if the RNA is small, a side pathway leading to T=3 or even T=1 may ensue. Small RNAs may also be encapsidated into a T=4 structure; whether such assembly requires that more than one RNA molecule be recruited into the initiated complex is unclear. This model is also consistent with the formation of the larger particles having T=7 that were observed by Lee and Brown (15) in preparations of Sindbis virus containing mutations in the nucleocapsid protein.

This model for the construction of alphavirus nucleocapsids is similar in concept to that proposed for the assembly of the turnip crinkle virion (26). Turnip crinkle virus has been shown to initiate assembly by formation of a stable and specific initiation complex consisting of six capsid protein subunits bound to a specific domain in the viral RNA, followed by sequential recruitment of capsid protein dimers into the initiated complex. Although virus particles will form with nonviral RNAs, the virion RNA is greatly favored because it can form a specific initiation complex and nonviral RNAs are largely excluded during assembly. Similarly, alphavirus capsids have been found to assemble in vitro with nonviral RNAs (33), but in the infected cell the virion RNA is greatly favored and little nonviral RNA (or subgenomic RNA in the case of most alphaviruses) is found in virions. It is also of interest that in the case of turnip crinkle virus, abortive particles that appear to arise from multiple nucleation events have been described upon assembly of particles in vitro (26). The monster Aura virus particles that appear to contain two hemicapsids fused together could result from two separate initiation events, e.g., initiation on two subgenomic RNAs followed by merger of these two initiated complexes into a single particle during attempted assembly of a T=4 particle or initiation on two different encapsidation signals in Aura virus genomic RNA if more than one signal exists.

Following assembly of the nucleocapsid within the cell, it buds through the cell plasma membrane and acquires an envelope containing viral glycoproteins. Specific interactions between the C-terminal cytoplasmic domain of glycoprotein E2 and the nucleocapsid (10, 11, 15, 18–20) are postulated to lead to the incorporation of 240 glycoprotein heterodimers assembled into the observed T=4 array (9, 22, 29, 30). Lateral in-

teractions between the glycoproteins are also important for assembly and the glycoproteins will assemble into a regular array that is isomorphic with the array in the virion even in the absence of nucleocapsids (31). The relative contributions of the E2-capsid protein interactions and the lateral interactions between the glycoproteins to the free energy of virus formation are not known. We assume that the E2-capsid interaction is dominant and that when smaller virions are assembled, a T=3 nucleocapsid would assemble a T=3 array of glycoproteins. Refined structural analysis of these particles is clearly important in order to establish the triangulation numbers of the nucleocapsid and the envelope of the smaller particles and to further define the interactions that occur during assembly of the virion and of the morphologically variant particles.

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