Theta Particles: a Structure Found in Hamster Sarcoma Virus

ANTHONY P. ALBINO, ETIENNE DEHARVEN, AND F. KINGSLEY SANDERS

Sloan-Kettering Institute for Cancer Research, New York, New York 10021

Received for publication 19 May 1972

Hamster sarcoma virus (HaSV), a ribonucleic acid tumor virus, pelleted from tissue culture fluid manifests type C morphology by electron microscopy. However, if virus is first concentrated by polyethylene glycol or ammonium sulfate followed by density gradient banding, the virus shows a dramatically atypical barred core structure, termed "theta particles." This structure suggests a condensation of the ribonucleoprotein into a flat disc. Atypical particles are found with HaSV and not in similarly treated feline leukemia virus or Rauscher-murine leukemia virus. Differences in the composition of HaSV as compared with these other viruses may be responsible for the production of such particles.

All ribonucleic acid (RNA) tumor viruses of the type C morphology (1, 3, 5, 8, 10) exhibit a typical enveloped spheroid structure with a central core. Based on its increased affinity for uranyl acetate (7), the central core is assumed to contain RNA and is presumably complexed with basic proteins. However, the exact architecture of the ribonucleoprotein in the core is still a subject of much investigation (15, 18).

We have attempted similar morphological studies of hamster sarcoma virus (HaSV), a newly isolated RNA tumor virus (2) that has recently been shown to be a hamster-specific virus by immunological methods (22), although it has not yet been studied in any detail by electron microscopy techniques.

Electron micrographs of virus concentrated from tissue culture fluid with polyethylene glycol (PEG) or ammonium sulfate reveal very atypical core structures. The viral envelope plus its internal condensed core resemble the Greek letter θ when viewed in thin sections; therefore, we propose the term "theta" for such particles. Our evidence suggests that the theta particle is not the natural form of the virus, but is formed during concentration and subsequent gradient centrifugation. Theta formation indicates a structural property peculiar to the HaSV since similar particles are not produced when samples of (feline leukemia virus (FeLV) and Rauscher-murine leukemia virus (R-MuLV) are treated in the same way.

MATERIALS AND METHODS

Virus production. HaSV is produced by an established line of hamster cells, B-34 (2), that continuously

sheds virus into the tissue culture medium. Virus produced in monolayers or in a continuously maintained Spinner culture (22) was used.

FeLV was produced by a virus-shedding monolayer cell line initiated from a lymphosarcoma of a cat (14).

FeLV and HaSV monolayer cultures were grown in F-10 medium (concentrated powder from Gibco) with 10% fetal calf serum and buffered with NaHCO_3 . Spinner cultures were grown in F-10 Spinner medium (free of calcium with 2 g of glucose added per 5-liter package) in two-liter flasks (Bellco). All B-34 cell cultures used were shown to contain mycoplasma by Jorgen Fogh of this Institute (12), but this contamination was assumed to be negligible as mycoplasma could not be seen with the electron microscope either associated with the cells or in virus preparations. Furthermore, HaSV was harvested from cells in log phase growth, which suggests that the limited amount of mycoplasma present was not depleting essential biochemical pools (e.g., arginine) needed by the cells as well as the virus.

R-MuLV was purchased from Electro-Nucleonics Laboratories, Inc., Bethesda, Maryland (lot no 104-49-9). It had been purified from large volumes of tissue culture fluid from JLS-V9 mouse cells (BALB/c bone marrow cells infected with R-MuLV [27]) by zonal centrifugation, followed by two cycles of sucrose density centrifugation (26). The purified virus was suspended in 0.1 M NaCl -0.01 M tris(hydroxymethyl)-aminomethane (Tris)-0.001 M ethylenediaminetetraacetic acid (EDTA) pH 7.0 buffer and delivered frozen at 10^{10} to 10^{11} virus particles/ml (20).

Samples of FeLV and HaSV were prepared in the following way. Two-liter samples of tissue culture fluid were centrifuged (Sorvall, model RC2B) for 20 min at $7,000 \times g$ at 4°C to remove the cells. Some samples of cell-free fluid were used directly, whereas others were frozen at -70°C for 48 hr, then thawed. The virus was then precipitated from the supernatant fluid using ammonium sulfate (ultrapure, Schwarz/Mann,

300 g of dry salt/1,000 ml of supernatant fluid) or PEG (Carbowax, Schwarz/Mann, 6,000 100-g dry flakes/1,000 ml of supernatant fluid), adjusted to 0.5 M NaCl final concentration (19). The preparations were then stirred for 30 min at 4 C and the precipitates were collected by reentrifuging at $7,000 \times g$ (Sorvall rotor Hs4) for 30 min. The pellets obtained were resuspended in 0.1 M Tris-NaCl-0.001 M EDTA pH 7.4 buffer (this buffer was used throughout) to a final volume of 38 ml. The virus was then pelleted further for 75 min at $110,000 \times g$ (Beckman L265-B ultracentrifuge, SW27 rotor), resuspended in 1.5 ml of buffer, layered on top of a 15 to 60% (w/w) sucrose gradient (ultrapure sucrose, ribonuclease free, Schwarz/Mann), and centrifuged for 180 min at $110,000 \times g$ (SW27 rotor). This procedure gave a visible band of virus at an isopycnic density of 1.15 g/ml. The viral band was pipetted off and used for subsequent electron microscopy.

Electron microscopy: method 1. Varying proportions (5 to 50%) of the total virus band collected from a gradient were diluted to 3.0 ml with buffer and pelleted into a BEEM (Better Equipment for Electron Microscopy, Inc., Bronx, N.Y.; reference 25) capsule at $42,000 \times g$ for 120 min (SW27 rotor), then were fixed with 1% glutaraldehyde and osmium tetroxide. The fixed sample was then treated with 0.5% uranyl acetate prior to dehydration with ethanol. Fixation and treat-

ment with uranyl acetate was done in the BEEM capsule at 4 C. Thin sections were double stained by the procedure of Frasca and Parks (13) and coated with carbon film.

Method 2. HaSV from tissue culture fluids was filtered through a 0.22- μ m Nalgene filter, pelleted directly into a BEEM capsule without any prior use of PEG, ammonium sulfate, or density gradient centrifugation, and then fixed and stained the same way.

A Siemens 101 electron microscope with a double condenser and a liquid nitrogen-cooled anticontamination device was used. Micrographs were taken at direct magnifications ranging from $\times 16,000$ to $\times 110,000$.

RESULTS

HaSV prepared by method 2 (see Materials and Methods) consisted of type C particles approximately 100 nm in diameter with central cores of approximately 55 nm (Fig. 1). HaSV-infected cells exhibit the typical budding process with the formation of enveloped A particles (6) upon release from the cell membrane (Fig. 2). Type C particles were also seen associated with the cells.

Examination of HaSV prepared by method 1 (Fig. 3) showed that 30 to 40% of the particles

FIG. 1. Typical field of HaSV pellet from tissue culture fluid showing type C and enveloped A particles, $\times 68,800$. Arrows show particles with dense material at the periphery of the core.

FIG. 2. Type C virion budding from B-34 cell. Inset: Enveloped A particle after release from cell membrane. Both $\times 84,710$.

in each field did not consist of the type C morphology but rather of enveloped structures, each containing a flattened core which, in favorable cross sections, resembled the Greek letter θ (Fig. 4). Close examination showed these theta particles to have a diameter of 100 nm but to lack typical cores. Instead of a central core, electron-dense bands extend the entire diameter of the particle. The structures seen in particles sectioned in various planes are consistent with the interpretation that the cores of theta particles are in the form of one or more flattened discs, which by reason of their affinity for uranyl acetate are assumed to contain RNA. Within these dense bands there appears to be a further structural periodicity of 8 nm (Fig. 5). The width of the bands is approximately 12 nm.

Theta particles have only been found so far in HaSV whether the viral containing fluid was frozen or not. To simulate the conditions of their production, a frozen sample of R-MuLV was thawed and injected into a Spinner flask containing 1 liter of F-10 Spinner media and allowed to incubate at 37 C for 48 hr. The fluid containing virus was then frozen at -70 C for 48 hr, after

which it was thawed, precipitated with PEG (see Materials and Methods), and banded on 15 to 60% sucrose. The viral band was located by optical density readings at 280 nm.

Upon subsequent fixation and staining, R-MuLV did not show any theta particles, only type C plus an assortment of breakdown products. These virions maintained typical 100-nm envelopes and 55-nm core diameters.

FeLV prepared by method 1 also showed type C particles with a 100-nm diameter but no theta structures. The diameter of the cores of FeLV seemed slightly swollen (75 nm) but was still within the accepted range for RNA tumor virus cores (1, 17).

The peculiar condensation of the core in theta particles does not seem to result solely from density gradient centrifugation in sucrose. HaSV preparations banded on potassium tartrate (15 to 60%; w/w) and minimally osmotic Ficoll (Pharmacia, Uppsala, Sweden, 15 to 50%; w/w) gradients contained the same proportion of theta particles.

It also seemed unlikely that PEG was uniquely responsible for this effect since HaSV precipi-

FIG. 3. Equilibrium density centrifugation in sucrose showing viral peak at 1.15 g/ml. A 200-ml amount of fresh media containing 5 μ Ci of 3 H-uridine/ml (Schwarz/Mann Radiochemicals) was added to two Blake bottles of B-34 cells and incubated for 24 hr. HaSV harvested by method 1 or 2 gave same results. Fractions (1.3 ml) were collected by a needle inserted into the bottom of the tube. Samples (50 μ liters) were assayed for radioactivity and refractive indices were determined on every other fraction with a Zeiss refractometer.

tated with ammonium sulfate also contained theta particles, regardless of the type of gradient later used. Samples of PEG-precipitated pellets, before gradient centrifugation, showed less than 1% theta particles.

Although virions of type A and C morphology are seen associated with cells and in cell-free pellets (prepared by method 2), it was necessary to analyze virions from tissue culture without any previous exposure to a centrifugal field. Tissue culture fluid from Spinner and monolayer cultures was filtered through a 0.22- μ m Nalgene filter to remove the cells, and then through a 0.1- μ m filter (Millipore Corporation) which does not permit virions to pass. These filters were then fixed, stained, embedded, and thin-sectioned. Electron micrographs showed that the virions trapped in the filter were type C. Various grids examined failed to show any theta particles. Accordingly, theta particles do not represent a native form of the virus.

There remained the remote possibility that the B-34 cells used for our studies produced an HaSV that responds abnormally to concentration

procedures. Therefore, samples of HaSV were prepared in the same manner as above from the following sources: (i) 55th passage B-34 cells (both monolayer and Spinner cultures), (ii) 8th passage B-34 stock culture (kindly provided by I. M. Macpherson), (iii) tissue from a tumor, B-34-T-1, initiated in a newborn hamster by B-34 cells (38th passage), and (iv) tissue from a tumor, B-34-T-2, initiated in a newborn hamster by injecting HaSV subcutaneously. HaSV produced in all the above cases showed theta particles following our concentration procedure.

Therefore, it seems that the tendency to produce theta particles is a phenomenon peculiar to this hamster sarcoma virus and not the result of an atypical virus produced by one cell line. Although hamster cells are chronically infected with the R-type particle (4, 9), none were seen in fluids prepared either by method 1 or method 2 and, therefore, it is assumed that these particles are not released by the cells.

DISCUSSION

From the preceding evidence it seems that theta particles arise during precipitation and density gradient centrifugation since they do not appear in virus filtered or pelleted from tissue culture directly. Theta forms can, therefore, be considered artifactual but are, nevertheless, indicative of the aberrant composition of HaSV, for other RNA tumor viruses (FeLV and R-MuLV) do not undergo similar condensation. The core in these other viruses seems sufficiently stable to maintain their type C configuration. One interpretation is that the protein "structure" stabilizing the core of HaSV (and therefore the RNA) is defective in some respect and will collapse when stressed, thereby displacing the RNA both from its location and normal mode of coiling. Figure 1 shows that the ribonucleoprotein in the cores of some virions prepared by method 2 has started to condense and is compressed along the periphery of the inner membrane. But the cores are still centrally located. Although we call the particles in Fig. 1 type C, it is recognized that these virions do not have a classical type C morphology as many do not have a typically electron-dense core. Their type C nomenclature is based on: (i) the morphological similarity with other RNA tumor viruses, (ii) the fact that HaSV has the gs-1 antigen specific for hamster viruses (22), (iii) the finding that HaSV has six major proteins analogous in molecular weight to all other RNA tumor viruses examined so far (11, 21), and (iv) the banding of HaSV at a density in sucrose of 1.15 g/ml which is approximately where other RNA tumor viruses band (1.16 g/ml; reference 21).

FIG. 4. *HaSV* pellet after precipitation and gradient centrifugation showing theta and type C particles. $\times 68,800$.

Several recent theories concerning the mode of RNA coiling in the core assert that the RNA is wound in a three-dimensional configuration even in its condensed type C state (15, 16, 18, 23).

These theories can also apply to HaSV, since we do observe type C and enveloped A particles under appropriate conditions. But upon condensation to a theta configuration, the RNA could flatten into a disc, tightly coiling around itself into a spiral, as a rope on a flat plane. Sections cut through a randomly oriented population of these particles would show some virions with internal bar-like structures as well as particles with an unusually large core that appeared to fill up most if not all of the virus (Fig. 6), which we interpret as tangential sections of the disc-like core.

Figure 6 shows particles with two bars. These structures could consist of two or more discs in different planes which may or may not be attached to each other. They could not correspond to actual rods since such structures must occasionally be seen in transverse sections as dots which were never observed. Within the bars of theta particles, there appears to be a periodicity

suggesting some sort of ordered, possibly helical, structure. At present it is difficult to reconcile the concept of an ordered structure with the idea that type C particles contain a disorganized single-stranded coil of RNA. But the electron-dense material seen at the periphery (Fig. 6, arrow a) of virions containing bar structures may be this disorganized RNA that has not condensed into a disc.

Preliminary evidence suggests that the protein content of HaSV is atypical. A major structural arginine-rich protein of the avian tumor virus group has been shown to be associated with viral cores (and thus presumably the RNA; reference 11). Studies using guanidine-hydrochloride columns show that the amount of analogous protein in HaSV appears to be reduced when compared with other RNA tumor viruses (21). This protein may stabilize the RNA and restrain it in its type C morphology or at least share in its stabilization with other viral proteins not as yet identified. A reduced quantity of this protein may allow a structural weakness that causes the core structure to alter under stress. It will be interesting to discover whether the theta configuration is

FIG. 5. High magnification of theta particles. Arrows show average distance measured to determine periodicity of 8 nm. $\times 308,000$.

FIG. 6. Arrow b shows a theta particle cut tangential to the disc. Arrow a shows ribonucleoprotein that is not in the disc structure, but rather the disorganized three-dimensional structure associated with type C particles. Note also the theta particle with two bars. $\times 220,000$.

restricted to HaSV or will be found in other hamster sarcoma and leukemia viruses, and if theta particles are as infective as particles prepared by method 2. In addition, it must be noted that theta particles could not be identified when virions prepared by either method were negatively stained with phosphotungstic acid.

Further work is being conducted to answer these questions and to determine the cause of instability in this virus which results in the theta configuration.

ACKNOWLEDGMENTS

We would like to thank Jorgen Fogh for testing the cells for mycoplasma contamination and to acknowledge the excellent technical assistance of Janet Mengo, Juan Marchese, and the secretarial assistance of Patricia Potenza.

This investigation was supported in part by Public Health Service grant CA-08748 from the National Cancer Institute.

LITERATURE CITED

1. Bader, J. P. 1969. RNA tumor viruses, p. 293-327. In H. B. Levy (ed.), *The biochemistry of viruses*. Dekker, New York.
2. Bassin, R. N., P. J. Simmons, F. C. Chesterman, and J. D. Harvey. 1968. Murine sarcoma virus (Harvey): characteristics of focus formation in mouse embryo cell cultures and virus production by hamster tumor cells. *Int. J. Cancer* 3:265-272.
3. Bernhard, W. 1960. The detection and study of tumor viruses with the electron microscope. *Cancer Res.* 20:712-727.
4. Bernhard, W., and P. C. Tournier. 1964. Infection virale inapparente de cellules de hamsters décelée par la microscope électronique. *Ann. Inst. Pasteur (Paris)* 107:447-452.
5. Bonar, R. A., U. Heine, and J. W. Beard. 1964. Structure of BAI strain A (Myeloblastosis) avian tumor virus. *Nat. Cancer Inst. Monogr.* 17:589-614.
6. deHarven, E. 1968. Morphology of murine leukemia viruses, p. 97-129. In M. Rich (ed.), *Experimental leukemia*. Appleton-Century-Crofts, Inc., New York.
7. deHarven, E., and C. Friend. 1962. Electron microscope studies on mouse lymphomas. *Proc. 5th Int. Congr. Electron Microsc.* 2:MM-5.
8. deHarven, E., and C. Friend. 1964. Structure of virus particles partially purified from the blood of leukemic mice. *Virology* 23:119-124.
9. dePetris, S., and J. J. Harvey. 1969. Presence of unusual virus particles in two hamster tumor tissue culture cell lines induced by murine sarcoma virus. *J. Gen. Virol.* 5:561-564.
10. deThe, G., and T. E. O'Connor. 1966. Structure of a murine leukemia virus after disruption with Tween-ether and comparison with two myxoviruses. *Virology* 28:713-728.
11. Fleissner, E. 1971. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. I. Avian leukemia-sarcoma viruses. *J. Virol.* 8:778-785.
12. Fogh, J., H. Fogh. 1969. Procedures for control of mycoplasma contamination of tissue cultures. *Ann. N. Y. Acad. Sci.* 172:15-30.
13. Frasca, J. M., V. R. Parks. 1965. A routine technique for double-staining ultrathin sections using uranyl and lead salts. *J. Cell Biol.* 25:157-159.
14. Jarrett, O., H. M. Laird, D. Hay, and G. W. Crighton. 1968. Replication of cat leukemia virus in cell cultures. *Nature (London)* 219:562-568.
15. Kakefuda, T., J. P. Bader. 1969. Electron microscopic observations on the ribonucleic acid of murine leukemia virus. *J. Virol.* 4:460-474.
16. Lacour, F., A. Fourcade, C. Verger, and E. Delain. 1970. Coiled structure of the nucleocapsid of avian myeloblastosis virus. *J. Gen. Virol.* 9:89-92.
17. Laird, H. M., O. Jarrett, G. W. Crighton, and W. F. H. Jarrett. 1968. An electron microscopic study of virus particles in spontaneous leukemia in the cat. *J. Nat. Cancer Inst.* 41:867-871.
18. Luftig, R. B., and S. Kilham. 1971. An electron microscope study of Rauscher leukemia virus. *Virology* 46:277-279.
19. McSharry, J., and R. Benzinger. 1970. Concentration and purification of vesicular stomatitis virus by polyethylene glycol "precipitation." *Virology* 40:745-746.
20. Monroe, J. H., and P. M. Brandt. 1970. Rapid semiquantitative method for screening large numbers of virus samples by negative staining electron microscopy. *Appl. Microbiol.* 20:259-262.
21. Nowinski, R. C., E. Fleissner, N. H. Sarkar, and T. Aok. 1972. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. *J. Virol.* 9:359-366.
22. Nowinski, R. C., L. J. Old, P. V. O'Donnel, and F. K. Sanders. 1971. Serological identification of hamster oncornaviruses. *Nature N. Biol.* 230:282-284.
23. Nowinski, R. C., L. J. Old, N. H. Sarkar, and D. H. Moore. 1970. Common properties of the oncogenic RNA viruses (oncornaviruses). *Virology* 42:1152-1157.
24. Sarkar, N. H., R. C. Nowinski, and D. H. Moore. 1971. Characteristics of the structural components of the mouse mammary tumor virus. *Virology* 46:1-20.
25. Smith, K. O., and W. D. Genle. 1969. Pelleting virus and virus infected cells for thin-section electron microscopy. *Proc. Soc. Exp. Biol. Med.* 130:1117-1119.
26. Toplin, I. 1967. Purification of the Moloney and Rauscher murine leukemia viruses by use of zonal ultracentrifuge systems. *Appl. Microbiol.* 15:582-589.
27. Wright, B. S., P. A. O'Brien, G. P. Shibley, S. A. Mayyasi, and J. C. LasFargues. 1967. Infection of an established mouse bone marrow cell line (JLS-V9) with Rauscher and Moloney murine leukemia viruses. *Cancer Res.* 27:1672-1677.