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Assembly of Nucleocapsidlike Structures in Animal Cells Infected with a Vaccinia Virus Recombinant Encoding the Measles Virus Nucleoprotein

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A vaccinia virus recombinant containing the measles virus nucleoprotein gene was shown to induce the synthesis of a 60 kDa phosphorylated nucleoprotein similar to authentic measles virus nucleoprotein. Mammalian or avian cells infected with the recombinant virus displayed tubular structures reminiscent of viral nucleocapsids both in the cytoplasm and in the nucleus. Such structures could be labelled in situ by using an immunogold detection method specific for measles virus proteins. Electron microscopic examination of tubular structures purified from cells infected with the vaccinia virus recombinant indicated that they displayed most of the features of measles virus nucleocapsids, although their length was on the average shorter. These results demonstrate the spontaneous assembly of measles virus nucleocapsids in the absence of viral leader RNA and provide a means for a detailed molecular analysis of the requirements for nucleocapsid assembly. Furthermore, these findings raise the possibility of achieving complete assembly of measles virus particles, devoid of infectious RNA, by using a vaccinia virus vector.

An essential step in the assembly of measles virus particles is the encapsidation of the single-stranded RNA genome (approximately 16 kb) into a flexible rodlike structure designated nucleocapsid (for a review, see reference 8). Measles virus nucleocapsids display helical symmetry and measure approximately 1,000 nm in length and 18 nm in diameter. The nucleoprotein (NP), a 60-kDa phosphorylated protein, is the major constituent of nucleocapsids. Two other proteins, a 72-kDa phosphorylated protein and a 180- to 200-kDa large protein, are essential components of measles virus nucleocapsids required for transcription and replication of encapsidated RNA. Complete maturation of measles virus particles is achieved by migration of the nucleocapsids to the plasma membrane, where they associate with the 37-kDa matrix protein and become enveloped by budding through a cell membrane studded with two viral glycoproteins, an 80-kDa hemagglutinin and a 60-kDa fusion protein. Further understanding of the morphogenesis of measles virus particles could be obtained by isolating each of the steps involved in assembly. Here we report the in vivo assembly of structures which mimic measles virus nucleocapsids in cells infected with a vaccinia virus (VV) recombinant encoding the measles NP.

To synthesize the measles virus NP in animal cells, we inserted cDNA containing the NP coding region (5) into the thymidine kinase locus of the VV genome under the control of the 7.5-kDa VV promoter (14). The recombinant VV, hereafter referred to as VVNP, was then used to infect Vero cells, an African green monkey cell line, or chicken embryo fibroblasts and investigate NP expression, subcellular localization, and assembly. That the recombinant virus indeed encoded NP was checked by labelling VVNP-infected Vero cells with [^{35}S]methionine, lysing the cells with phosphate-buffered saline (PBS) containing 1% Nonidet P-40, and immunoprecipitating the mixture with an NP-specific monoclonal antibody. In agreement with previous reports (3, 14) the ^{35}S-labelled NP from the VVNP infection migrated in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with an apparent molecular size of 60 kDa (Fig. 1B), identical to that of the same protein from measles virus-infected Vero cells. Since authentic NP from measles

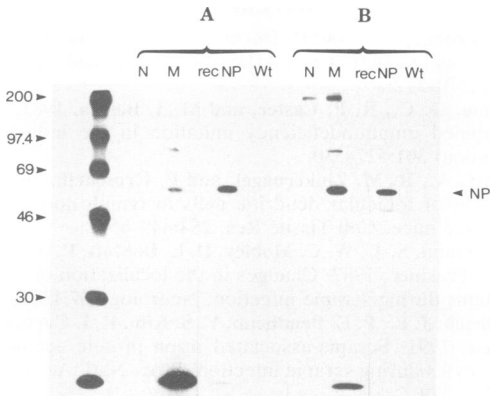

FIG. 1. Radioactive labelling of NP with [^{32}P]phosphate (A) or [^{35}S]methionine (B) in Vero cells infected with VVNP or measles virus. Samples: N, uninfected cells; M, measles virus-infected cells; recNP, cells infected with VVNP; Wt, cells infected with wild-type VV. Positions of molecular weight markers (in kilodaltons) are shown at the left; the position of the 60-kDa NP is shown at the right. Approximately 10^6 Vero cells were infected with measles virus for 24 h or VVNP (1 PFU per cell) for 15 h. At this time, the culture medium was replaced by a medium devoid of phosphate or methionine in order to label cells with [^{32}P]phosphate (100 μCi/ml) or [^{35}S]methionine (50 μCi/ml) for 2 h. Cells were then recovered from the dishes by scraping, pelleted, and lysed by addition of PBS containing 1% Nonidet P-40. After centrifugation, NP was immunoprecipitated from the cytoplasmic extracts, using the monoclonal antibody CL105 directed against NP and protein A-Sepharose as previously described (1). Immunoprecipitated proteins were separated on a 10% SDS-polyacrylamide gel, which was then treated for fluorography.

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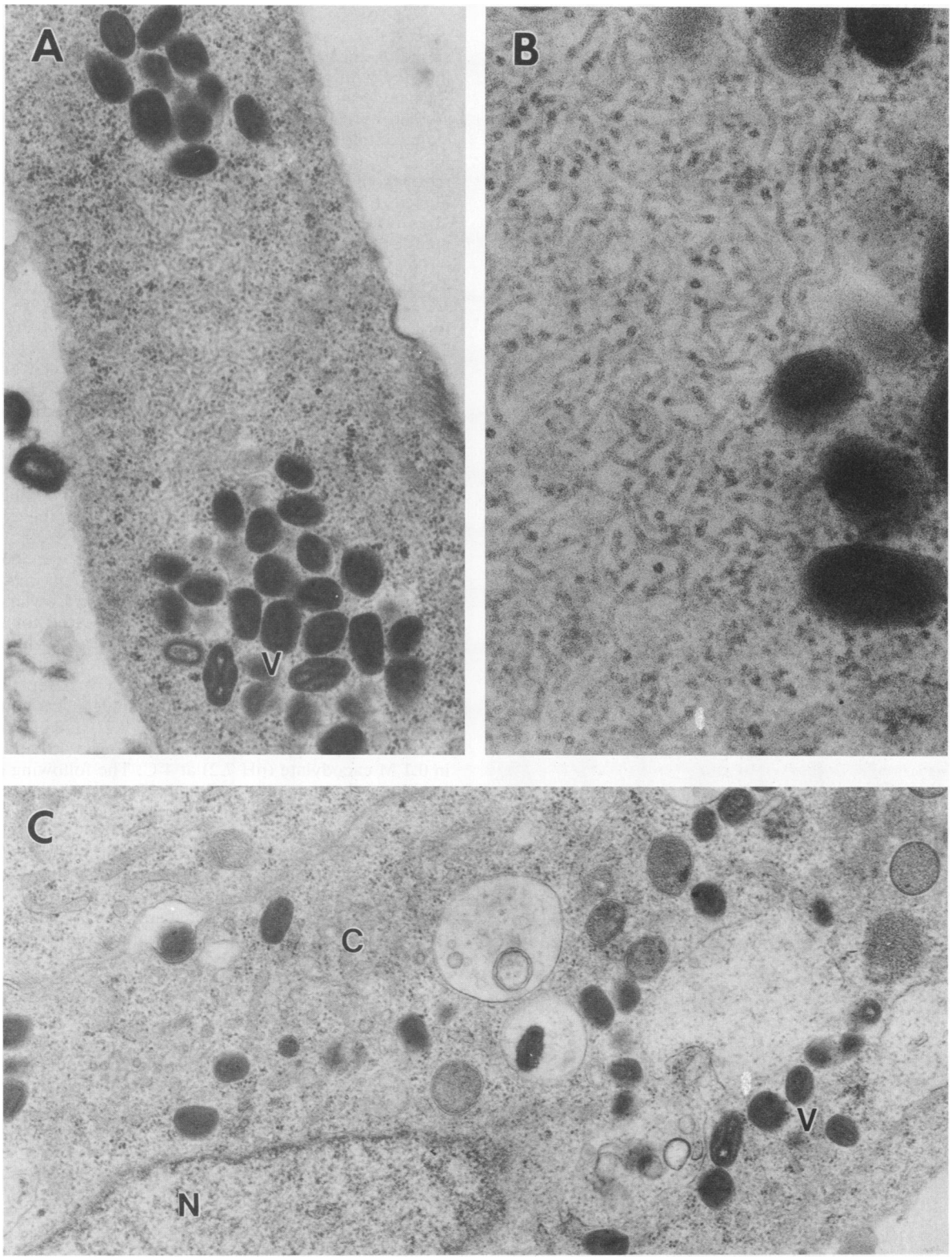

FIG. 2. Observation by transmission electron microscopy of thin sections of a chicken embryo fibroblast infected with VVNP or wild-type VV. (A) Portion of cell cytoplasm containing two distinct groups of VV particles (V) surrounding a dense array of tubular structures (magnification, ×34,000). The area containing tubules appears less electron dense than the surrounding cytoplasm due to the apparent exclusion of other cytoplasmic components. (B) Enlargement of a portion of panel A (magnification, ×80,000). Numerous cross sections and longitudinal sections of intertwining tubules are visible in the vicinity of electron-dense VV particles. (C) Region of a cell infected with wild-type VV containing viral particles at various stages of maturation (magnification, ×34,000). Primary chicken embryo fibroblasts were infected for 24 h with VVNP or wild-type VV at about 2 PFU per cell. Cells were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.2), postfixed in 2% osmium tetroxide, stained with 2% uranyl acetate, dehydrated in alcohol, and embedded in LX112 epoxy resin. Ultrathin sections were observed with a Philips EM 410 microscope at 80 kV.

virus-infected cells is phosphorylated (9), it was of interest to determine whether this posttranslational modification occurred on NP expressed from VV. Immunoprecipitation of NP from cells infected with VVNP and incubated with [^{32}P]phosphate revealed a phosphoprotein of 60 kDa corresponding in size to authentic NP and not found in cells infected with wild-type VV (Fig. 1A).

The overall localization of NP in VVNP-infected cells was studied by using immunofluorescent staining with a monoclonal antibody directed against the NP. The results showed that NP accumulated not only in the cytoplasm, where measles virus replication is known to occur, but also in the nucleus (results not shown). Nuclear localization of measles virus NP is a well-known phenomenon whose relevance to measles virus infection is uncertain.

To examine whether any morphological changes typical of measles virus infection occurred in cells infected with VVNP, primary chicken embryo fibroblasts were infected for 24 h at about 2 PFU per cell. Cells were then recovered by scraping, pelleted, fixed, embedded in LX112 epoxy resin, cut into ultrathin sections, and observed under the electron microscope. Cells infected with VVNP displayed numerous tubular structures (Fig. 2A and B) typically found in measles virus-infected cells, in which case they are known to correspond to viral nucleocapsids (7). Such structures were not found in cells infected with wild-type VV (Fig. 2C). As in measles infections, cross sections and longitudinal sections of the tubules were observed both in the cytoplasm and in the nucleus.

Definitive proof that the tubular structures were composed of NP encoded by VVNP was sought by immunogold labelling of fixed cell sections embedded in Lowicryl K4M. For this experiment, Vero cells were infected for approximately 48 h with 0.02 PFU of VVNP or measles virus per cell at a multiplicity sufficient to induce 50% fusion of the cell monolayer. At this point, the medium was replaced with fixation buffer (2.5% paraformaldehyde–0.1% glutaraldehyde diluted in 0.1 M cacodylate [pH 7.2]) and incubated 1 h at 20°C. Samples were concentrated in agar (13) and washed in 0.1 M cacodylate (pH 7.2) at 4°C. The following day, the samples were stained at 4°C with 2% uranyl acetate diluted in Michaelis buffer, and dehydration and Lowicryl embedding were carried out as previously described (12). For immunogold labelling, Lowicryl thin sections were collected on Formvar carbon-coated nickel grids. The grids were floated on PBS containing 1% normal goat serum (NGS) for 1 h at room temperature. Grids were then floated for 16 h on PBS containing 1% NGS and 0.1% guinea pig serum directed against measles antigens. They were then washed three times in PBS–1% NGS and incubated for 1.5 h in PBS containing 1% NGS and 5% colloidal gold-coupled anti-guinea pig serum. After two washes with PBS–0.5% NGS and two with PBS, the grids were postfixed with 2.5% glutaraldehyde diluted in PBS, washed twice with water, coated with 1.8% uranyl acetate–0.2% methylcellulose (11), and examined in a Philips EM 410 microscope at 80 kV.

In measles virus-infected cells, the gold particles accumulated mostly on cytoplasmic and nuclear inclusions of viral

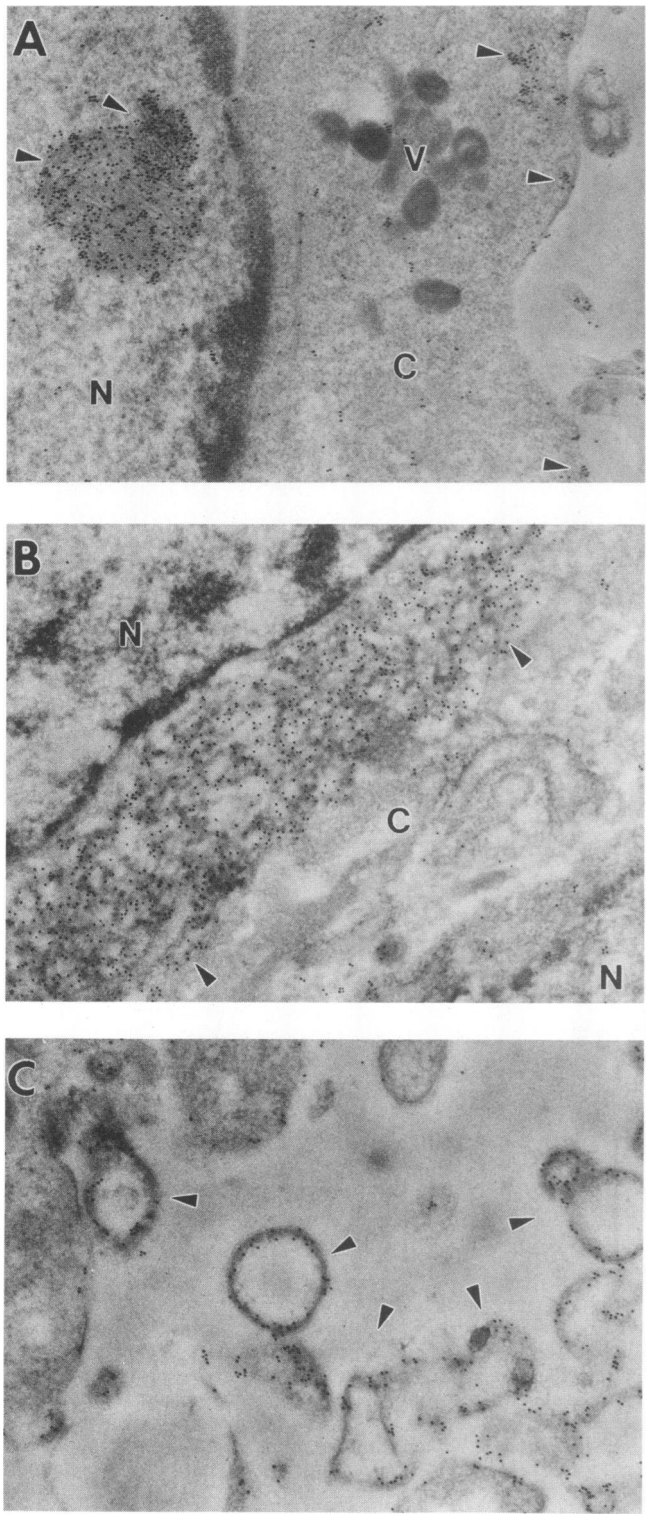

FIG. 3. Immunogold labelling of tubules produced in Vero cells infected with VVNP or measles virus. (A) Portion of a cell infected with VVNP (magnification, ×28,900). In this cell, immunolabelling of the NP with 10-nm gold particles is preferentially concentrated on the inclusion body within the cell nucleus (N). Densely packed tubules cut longitudinally can be noticed within the inclusion body. Sparse labelling (arrows) is also apparent in the cytoplasm (C) and at the inner periphery of the cytoplasmic membrane. V, VV particles. (B and C) Intracellular and extracellular areas, respectively, from cells infected with measles virus. For details of cell preparation, see text.

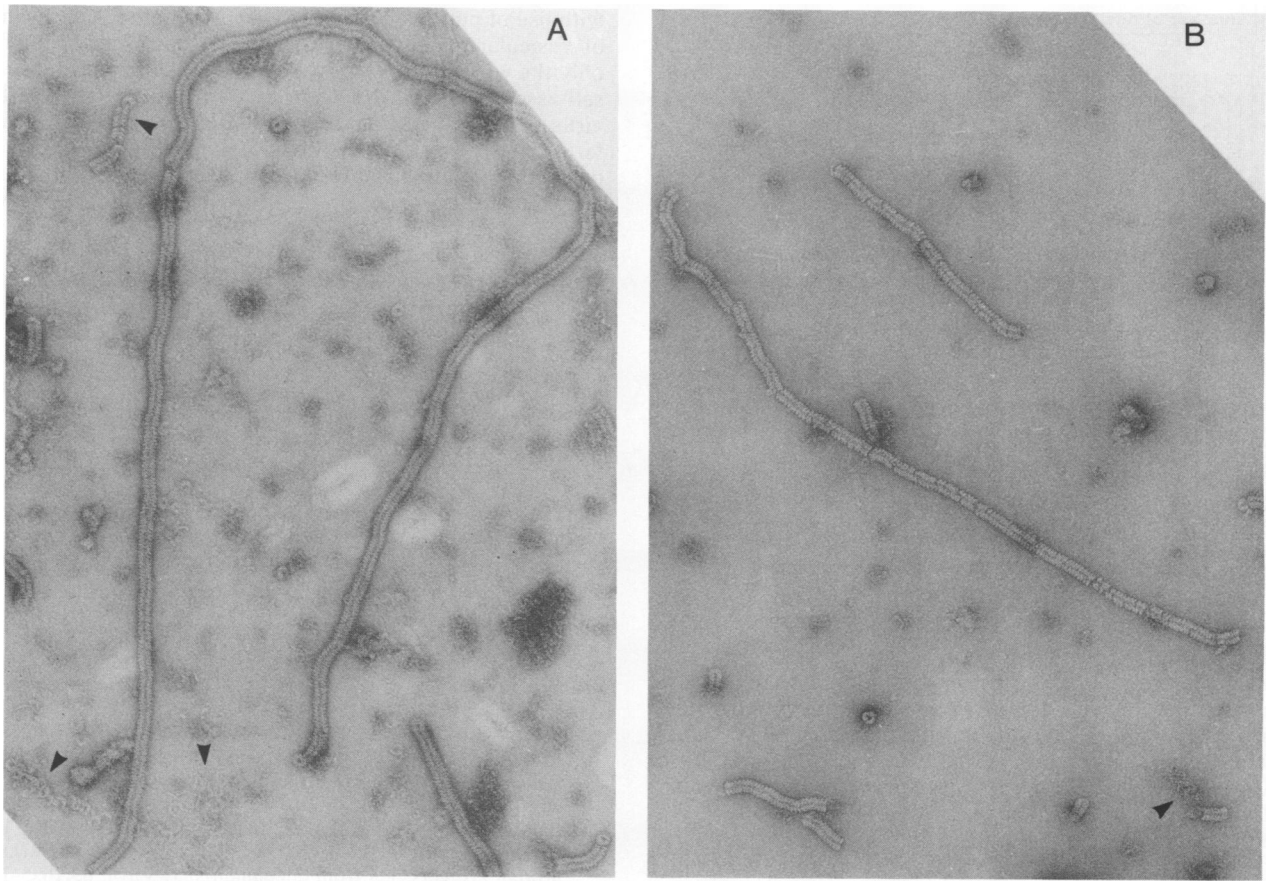

FIG. 4. Electron micrographs of negatively stained nucleocapsids from measles virus- or VVNP-infected cells. (A and B) Nucleocapsids from Vero cells infected with VVNP and measles virus, respectively. The nucleocapsid shown in panel A is larger than the average nucleocapsid from VVNP-infected cells but typical in all other respects. Arrows point to partially uncoiled nucleocapsids. Magnification, ×80,000). For details of nucleocapsid preparation, see text.

tubules, as illustrated for a cytoplasmic inclusion (Fig. 3B), but also on extracellular enveloped particles (Fig. 3C). In cells infected with VVNP, the gold particles accumulated preferentially on the tubular structures both in the cytoplasm and in the nucleus. A typical nuclear inclusion of immunogold-labelled tubules in cells infected with VVNP is shown in Fig. 3A. Sparse labelling in the cytoplasm of the cell could correspond to newly synthesized NP not yet assembled into tubules or to poorly defined tubules. We also noticed immunogold labelling at the cell surface, suggesting that some budding of nucleocapsids might also occur, although this needs to be investigated in more detail.

Further characterization of the tubular structures was obtained by disrupting cells infected with VVNP and examining crude cytoplasmic fractions or purified nucleocapsids under the electron microscope after negative staining. For this experiment, about 4×10^7 Vero cells were infected with VVNP (0.02 PFU per cell) or measles virus for 2 days. Cells were then recovered from the culture flasks by scraping, pelleted, and washed in PBS. Cells were resuspended in 2 ml of 10 mM Tris-HCl (pH 8)–0.1 mM EDTA and lysed by 20 strokes of a Dounce homogenizer. The cell lysates were centrifuged for 5 min to eliminate nuclei at 3,000 rpm in a Chilspin MSE centrifuge. The cytoplasmic fractions were centrifuged for 10 min at 12,000 rpm in a Sigma 202MK centrifuge, and the resulting supernatants were either directly prepared for electron microscopy or submitted to centrifugation for 3 h in a Beckman SW41 rotor on a discontinuous CsCl gradient (1.5 ml of 20%, 2 ml of 25%, 2 ml of 30%, 2 ml of 35%, and 1.5 ml of 40%). The visible bands appearing within the region of 30% CsCl were recovered, dialyzed against 10 mM Tris-HCl–0.1 mM EDTA, spotted on carbon-coated, glow-discharged grids, and negatively stained with 2% uranyl acetate in water.

Examination of the CsCl-purified material or even crude cytoplasmic fractions revealed large numbers of tubular structures which displayed some of the characteristic features of measles virus nucleocapsids (Fig. 4). They appeared as flexible rods 18 nm in diameter with repeated serrations along the edges and a central core approximately 5 nm in diameter. To avoid breakage due to the procedure of preparation, the length of the nucleocapsids was measured by using crude cytoplasmic fractions. The overall length of nucleocapsids produced in cells infected with VVNP was generally smaller than that of nucleocapsids produced in measles virus-infected cells (Fig. 5). Calculation of the average lengths showed that the former were about half the size of the latter (0.56 μm instead of 1.15 μm). This finding suggests that the nucleocapsids from cells infected with VVNP were initially assembled smaller in size and/or more fragile than measles nucleocapsids. The fact that we often observed a large number of partially uncoiled nucleocapsids

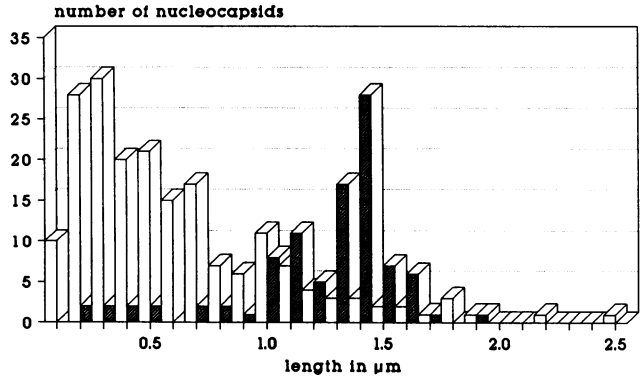

FIG. 5. Histogram of the distribution in size of nucleocapsids from Vero cells infected with VVNP or measles virus. The lengths of nucleocapsids from Vero cells infected with VVNP (▭) or measles virus (▨) were determined on cleared cytoplasmic fractions prepared as described in the text for Fig. 4. A total of 98 and 192 nucleocapsids, respectively, were photographed and measured. The mean lengths calculated for nucleocapsids were 0.56 µm (VVNP infection) and 1.16 µm (measles virus infection).

in samples from cells infected with VVNP suggests that they are indeed more fragile. Close examination of many nucleocapsids indicated that the serrations along the edges which correspond to the structural subunits are more tightly packed and more irregularly oriented in nucleocapsids from cells infected with VVNP than in measles virus nucleocapsids. When nucleocapsids from cells infected with VVNP were centrifuged to equilibrium on CsCl gradients for 18 h at 36,000 rpm in a Beckman SW41 rotor, they sedimented at a buoyant density of 1.30 g/ml, a value identical to that found for the dense nucleocapsids that accumulate in measles virus-infected cells (10).

This study shows that the measles virus NP can assemble into nucleocapsidlike structures in the absence of other measles virus proteins. Nevertheless, structural differences between nucleocapsids produced in cells infected with VVNP and measles virus are apparent and could be explained by the lack of other measles virus proteins or a specific RNA sequence. Previous work has characterized differences in protein composition of two morphologically distinct nucleocapsid species from measles virus-infected cells (10). This aspect could be further investigated by coexpression of the measles virus NP with other measles virus proteins. It is noteworthy that the cDNA used to obtain synthesis of measles virus NP from the VV genome was devoid of the 52-nucleotide leader sequence thought to be required for initiation of encapsidation (6, 8). Preliminary experiments have, however, indicated that RNA is associated with the nucleocapsids (results not shown). Thus, in the absence of a natural nucleation site, provided by measles virus RNA, nucleocapsid assembly can take place. Whether the measles virus NP uses related nucleation sites on cellular or VV-derived RNA will require further investigation. Spontaneous in vitro assembly of a virus with helical symmetry has been well documented and extensively studied in the case of tobacco mosaic virus. Similar assembly of the helical nucleocapsids of animal viruses has not been described despite numerous instances of expression of viral NP. For vesicular stomatitis virus, in vitro assembly of small disks, presumably precursors to nucleocapsids, was found to occur with use of purified NP (4). However, whether full assembly of vesicular stomatitis virus nucleocapsids can occur from only the viral NP is unknown. This raises the possibility that self-assembly of the NP from measles virus or the morbillivirus genus is a unique phenomenon. Assembly of measles virus nucleocapsids devoid of genomic RNA might also occur in a natural measles virus infection and thus correspond to a dead end for the formation of infectious viral particles.

Considerable progress in the investigation of measles virus should be possible with use of a recently developed strategy for the generation of live viral particles from a complete cDNA copy of measles virus RNA (2). Our findings provide another tool for studying the molecular requirements for assembly of nucleocapsids as well as a potential means of obtaining complete maturation of enveloped measles virus particles in the absence of genomic RNA.

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