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A Second Form of Infectious Bursal Disease Virus-Associated Tubule Contains VP4

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Preparations of density gradient-purified infectious bursal disease virus (IBDV) were found to contain full and empty icosahedral virions, type I tubules with a diameter of about 60 nm, and type II tubules 24 to 26 nm in diameter. By immunoelectron microscopy we demonstrate that virions and both types of tubular structures specifically react with anti-IBDV serum. In infected cells intracytoplasmic and intranuclear type II tubules reacted exclusively with an anti-VP4 monoclonal antibody, as did type II tubules in virion preparations. The immunofluorescence pattern with the anti-VP4 antibody correlated with electron microscopical findings. Neither purified extracellular nor intracellular virions were labeled with the anti-VP4 MAb. Our data show that the type II tubules contain VP4 and suggest that VP4 is not part of the virus particle.

Infectious bursal disease, also designated Gumboro disease, is a highly contagious viral disease of young chickens which is of major importance for the poultry industry (5). Virulent infectious bursal disease virus (IBDV) strains specifically affect the bursa of Fabricius, initially causing lysis of B cells followed by bursal atrophy. Ultrastructural analyses have indicated that virus replication primarily occurs in lymphoid cells (17, 24, 25, 36). IBDV is a member of the *Birnaviridae* (32). Birnaviruses contain two segments, A and B, of genomic double-stranded RNA within a nonenveloped single-shelled icosahedral virion, about 60 nm in diameter, which consists of 132 morphological subunits (3, 32). Segment A encodes a precursor polyprotein of 110 kDa, which is processed cotranslationally into three mature viral proteins, VP2, VP3, and VP4 (13). VP2 and VP3 are major structural proteins of IBDV, whereas VP4 most likely represents a virus-encoded protease (1, 16). A second open reading frame, preceding and partially overlapping the polyprotein gene, encodes VP5 (28). The smaller segment B encodes VP1, a 97-kDa multifunctional protein with polymerase activity (40, 41).

Electron microscopy of density gradient-purified virus preparations revealed the presence of full and empty virions of typical icosahedral structure (3, 11, 12, 26, 34, 35). Type I tubules, made up of regularly repeated subunits and having diameters of 60 nm, similar to those of virions, are also frequently observed (11, 35). In addition, Harkness et al. (11) detected a second form of tubular structure (24 to 26 nm in diameter) in IBDV preparations by adsorption to anti-IBDV antibody-coated grids. Whereas type I tubules were observed in cells infected by members of all three genera of *Birnaviridae* (10, 35, 38, 42), type II tubules were only detected in IBDV-infected cells (reference 11 and this study) as well as in purified virus preparations. However, the composition and function of such tubules are still unknown.

Published reports about the ultrastructure of IBDV morphogenesis in cells and tissues mainly deal with virus localization inside cells and the kinetics of virion formation (5, 17, 24, 25, 36, 39). Only Käufer and Weiss (17) reported the presence of tubular structures within the nuclei of IBDV-infected bursal cells. To analyze IBDV-associated structures in detail, we first established hybridoma lines secreting monoclonal antibodies (MAbs) against IBDV proteins. To this end, chicken embryo fibroblasts (CEF) were infected with attenuated IBDV serotype 1 strain P2 (37) and virus progeny were purified by ultracentrifugation (27). Thereafter, BALB/c mice (Moellegard, Skensved, Denmark) were repeatedly injected intramuscularly with purified virus. Supernatants of fused spleen cells of the immunized BALB/c mice were first screened by enzyme-linked immunosorbent assay (ELISA) (29). Eight hybridoma lines producing MAbs against viral proteins VP2, VP3, and VP4 of IBDV were established (Table 1). Five MAbs recognized VP2 (IBDV 1, 3 to 5, and 8), one MAb reacted with VP3 (IBDV 2), and two MAbs detected VP4 (IBDV 6 and 7). Four MAbs showed reactivity in ELISA, the indirect immunofluorescence test (IIFT), Western blotting, and radioimmunoprecipitation assay (RIPA), and the remaining four MAbs reacted only in ELISA, IIFT, and RIPA. One MAb each against VP2, -3, and -4 was selected for these studies.

To analyze the specificity of the anti-VP4 MAb, confluent CEF were infected with IBDV strain P2, labeled with Tran^{35}S-label (ICN, Costa Mesa, Calif.), incubated, and analyzed as previously described (2, 23, 29). After incubation of the labeled cell lysates with anti-VP4 MAb (Fig. 1A, lane 1) a protein with a molecular mass of approximately 28 kDa could be precipitated. A protein with the same molecular mass was precipitated by the rabbit anti-IBDV serum, in addition to viral proteins of 97, 48, and 32 kDa (Fig. 1A, lane 3), which correspond to VP1, VP2, and VP3, respectively. Following prior incubation of the cell lysate with anti-VP4 MAb and removal of the antibody-antigen complexes, no 28-kDa VP4 could be precipitated by the rabbit anti-IBDV serum (Fig. 1A, lane 2). Similar results were obtained after immunoprecipitation of in vitro translation products of in vitro-transcribed segment A RNA (Fig. 1B, lanes 1 to 3) by using a full-length cDNA clone of segment A of strain P2 constructed according to the method of Mundt and Vakharia (30). Figure 1B, lane 4, shows the total in vitro translation products of in vitro-transcribed segment A RNA.

To determine the localization of VP4 in IBDV-infected

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TABLE 1. Reactivities of MAbs directed against IBDV in different immunological tests[a]

MAb[b]	Protein recognized	ELISA	IIFT	Western blotting	RIPA
IBDV 1	VP2	+	+	−	+
IBDV 2	VP3	+	+	+	+
IBDV 3	VP2	+	+	+	+
IBDV 4	VP2	+	+	−	+
IBDV 5	VP2	+	+	−	+
IBDV 6	VP4	+	+	+	+
IBDV 7	VP4	+	+	+	+
IBDV 8	VP2	+	+	−	+

[a] +, positive reaction; −, no reaction.
[b] MAbs used for IFT and immunoelectron microscopy in this study are in boldface.

CEF, and to discriminate it from chicken tubulin, immunofluorescence studies were performed (28). Cells were reacted with polyclonal rabbit anti-IBDV serum (preadsorbed on noninfected cells) (kindly provided by H. Becht, Institute of Virology, Justus-Liebig-University, Giessen, Germany), anti-VP4 MAb, or polyclonal rabbit anti-chicken tubulin serum (Sigma, Deisenhofen, Germany). No specific fluorescence was detected in noninfected cells after incubation with either rabbit anti-IBDV serum or the anti-VP4 MAb (data not shown). Rabbit anti-chicken tubulin serum revealed network-like structures within the cells (data not shown). For double labeling, fixed cells were first incubated with a mixture of anti-IBDV serum and anti-VP4 MAb or anti-tubulin serum and anti-VP4 MAb. IBDV-infected cells showed intensively stained granular aggregates in the cytoplasm labeled by the anti-IBDV serum (Fig. 2A). VP4-specific fluorescence within the same cell, as demonstrated by tetramethyl rhodamine b isothiocyanate-labeled anti-mouse antibodies, showed the presence of granular as well as needle-shaped structures (Fig. 2B). After double labeling with anti-tubulin serum and anti-VP4 MAb, all cells exhibited a filamentous web-like fluorescence pattern typical of cellular tubulin (Fig. 2C). In addition, in IBDV-infected cells the typical needle-shaped fluorescence caused by anti-VP4 MAb immunoreaction was observed (Fig. 2D).

In all stocks of purified virions, we observed intact genome-containing IBDV particles (Fig. 3A); empty or partially disrupted IBDV particles; large (type I) tubules of different lengths made up of regularly repeated subunits and characterized by diameters similar to those of virions (Fig. 3B); and small (type II) helical tubules, about 24 to 26 nm in diameter, displaying regular cross striations with a periodicity of about 5 nm (Fig. 3C). Similar preparations of noninfected cells did not reveal any tubular structures.

Infected cell cultures, embedded in epoxy resin glycid ether 100 (Serva, Heidelberg, Germany) by the usual methods, contained intracytoplasmic empty or full virions either singly or in pseudocrystalline arrays (Fig. 3D). In close proximity to these pseudocrystals, bundles of type II tubules could be detected (Fig. 3D). Similar type II tubules were also found within the nuclei of infected cells (Fig. 3E). They were particularly prominent in cells showing advanced stages of virus replication (Fig. 3E). In addition, adjacent to viral pseudocrystals, local aggregations of fine fibrillar material were visible only in cells embedded in acrylic resin (Fig. 4A).

For immunolabeling, purified virus preparations were incubated with antibodies directed against chicken tubulin or purified IBDV or with MAbs directed against VP2, VP3, or VP4 and marked by anti-species antibodies (GAR_{10} and GAM_{10}; British BioCell International, Cardiff, United Kingdom) tagged with 10-nm-diameter gold particles. Virions, type I tubules (Fig. 3F), and type II tubules (Fig. 3G) were all recognized by the anti-IBDV serum, although relative reactivity varied with different dilutions of the antiserum. At higher serum dilutions only virions and type I tubules were tagged, whereas type II tubules were not marked (data not shown). This result suggests that in the anti-IBDV serum antibodies against the type II tubules were less prominent than those against virions or type I tubules, possibly because of variation in the ratio of VP4 to VP1, -2, and -3 in the virus preparations.

Since type II tubules superficially resemble cellular microtubules (22), we carefully assessed their reactivity with an antiserum directed against chicken tubulin. Neither virions nor type II tubules (Fig. 3H) reacted with the anti-tubulin antibodies, nor did type I tubules (data not shown). In contrast, type II tubules were specifically labeled with the anti-VP4 MAb (Fig. 3I), which failed to mark virions or type I tubules (see below). Lack of reactivity with this antiserum in immunoelectron microscopy, as well as a labeling pattern in immunofluorescence tests strikingly different from that of the anti-VP4 MAb, clearly showed that type II tubules were not related to microtubules.

For intracellular labeling of viral proteins, noninfected and infected cells were fixed with 0.5% glutaraldehyde in phosphate-buffered saline, pH 7.2, for 30 min and embedded in the acrylic resin Lowicryl K4M (Lowi, Waldkraiburg, Germany) (4). Ultrathin sections were incubated with different antibodies, and results are summarized in Table 2. The specificity of postembedding labeling of ultrathin sections was monitored by incubating noninfected cells, by using gold conjugate without primary antibody or with non-IBDV-specific antibodies, and by

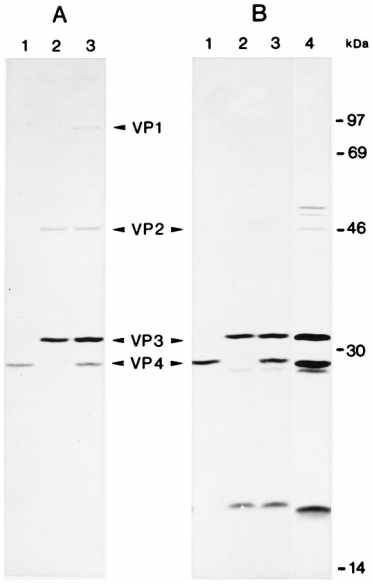

FIG. 1. RIPA of VP4. (A) CEF infected with IBDV strain P2 were labeled 8 h postinfection with Tran^{35}S-label, lysed, and immunoprecipitated with an anti-VP4 MAb (lane 1) or rabbit anti-IBDV serum (lane 3). To demonstrate specificity of the anti-VP4 MAb, cell lysate was preincubated with the anti-VP4 MAb, antigen-antibody complexes were removed by centrifugation, and the remaining lysate was precipitated with the rabbit anti-IBDV serum (lane 2). The locations of viral proteins VP1, VP2, VP3, and VP4 are marked. (B) In vitro translation products of in vitro-transcribed segment A RNA were immunoprecipitated with an anti-VP4 antibody (lane 1) or rabbit anti-IBDV serum (lane 3). Lane 2 shows precipitation with anti-IBDV serum after VP4 depletion by the anti-VP4 MAb. Total translation products are shown in lane 4. Positions of molecular mass markers are indicated.

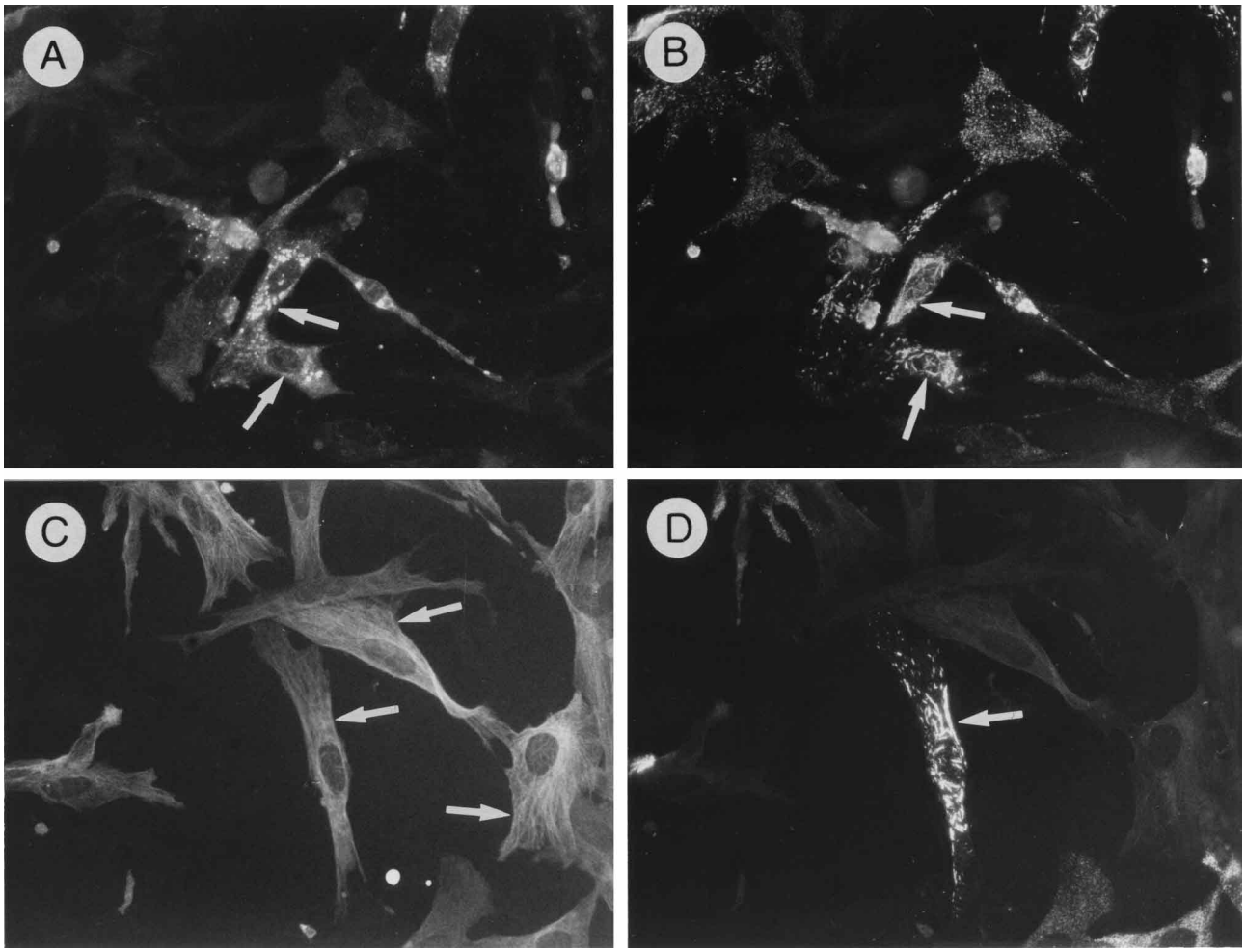

FIG. 2. Immunofluorescence of infected monolayer cells (arrows). Fluorescence of IBDV-infected CEF with polyclonal anti-IBDV serum (A) or anti-VP4 MAb (B) is shown. Double labeling with anti-tubulin serum (C) or anti-VP4 MAb (D) of IBDV-infected CEF highlights differences in the fluorescence patterns. Magnification, ×337.

incubation with normal rabbit serum. No reaction with viral or cellular structures was observed (data not shown).

Immunolabeling with the anti-IBDV serum showed specific reactivity with viral pseudocrystals, type II tubules, and fibrillar material (Fig. 4A). In contrast, intracytoplasmic or intranuclear type II tubules did not react with the anti-tubulin antibodies (Fig. 4B). MAbs directed against VP2 (Fig. 4C) or VP3 (Fig. 4D) marked viral pseudocrystals but failed to react with the type II tubules. It is notable that reactivity with the anti-VP2 MAb was significantly stronger than that with the anti-VP3 MAb. The anti-VP4 MAb reacted exclusively with cytoplasmic and intranuclear type II tubules (Fig. 4E and F) and not with virions (Fig. 4G). No other intracellular structures were labeled by this antibody. Thus, the fibrillar inclusions could represent virus factories or could be the result of deposition of overproduced and condensed viral proteins. Following this reasoning, overexpression of VP4 could then account for its aggregation into type II tubules.

An association between fibrillar material of a nonstructural viral protein, NS1, and intracellular virions has also been observed in bluetongue virus, a member of the *Reoviridae* family of viruses containing double-stranded RNA (7). The authors postulated that virus-fibril complexes were intermediates in virion morphogenesis. The appearance of tubular structures during virus morphogenesis has been observed in various members of the *Reoviridae* (6–8, 14, 15, 19–21, 31, 33). In orbiviruses, virus development occurs in association with the cytoplasmic granular matrix and is accompanied by the formation of regular substructured filaments and tubules consisting of the nonstructural protein NS1. In rotaviruses, tubular structures were detected in the nuclei of infected cells in bundles (21), which are probably formed by the aberrant assembly of inner capsid material (19). Three different intracellular tubular forms associated with the replication of bovine rotavirus also appeared to be assembled from structural virus proteins. However, the importance of these tubules is still unclear. It is important to emphasize in this context that the type II tubules described in this study did not appear to consist of proteins present in virions.

We describe here a novel form of IBDV-associated tubules, designated type II, which are detectable in the cytoplasm, the cellular compartment where IBDV replication actually takes place, as well as in the nucleus. By means of a MAb specific for VP4, the presumptive viral protease, we demonstrated that type II tubules contain VP4. Immunofluorescence studies of IBDV-infected cells showed striking needle-like structures, highlighted by the anti-VP4 MAb, which correlated with the location of type II tubules as observed in ultrastructural anal-

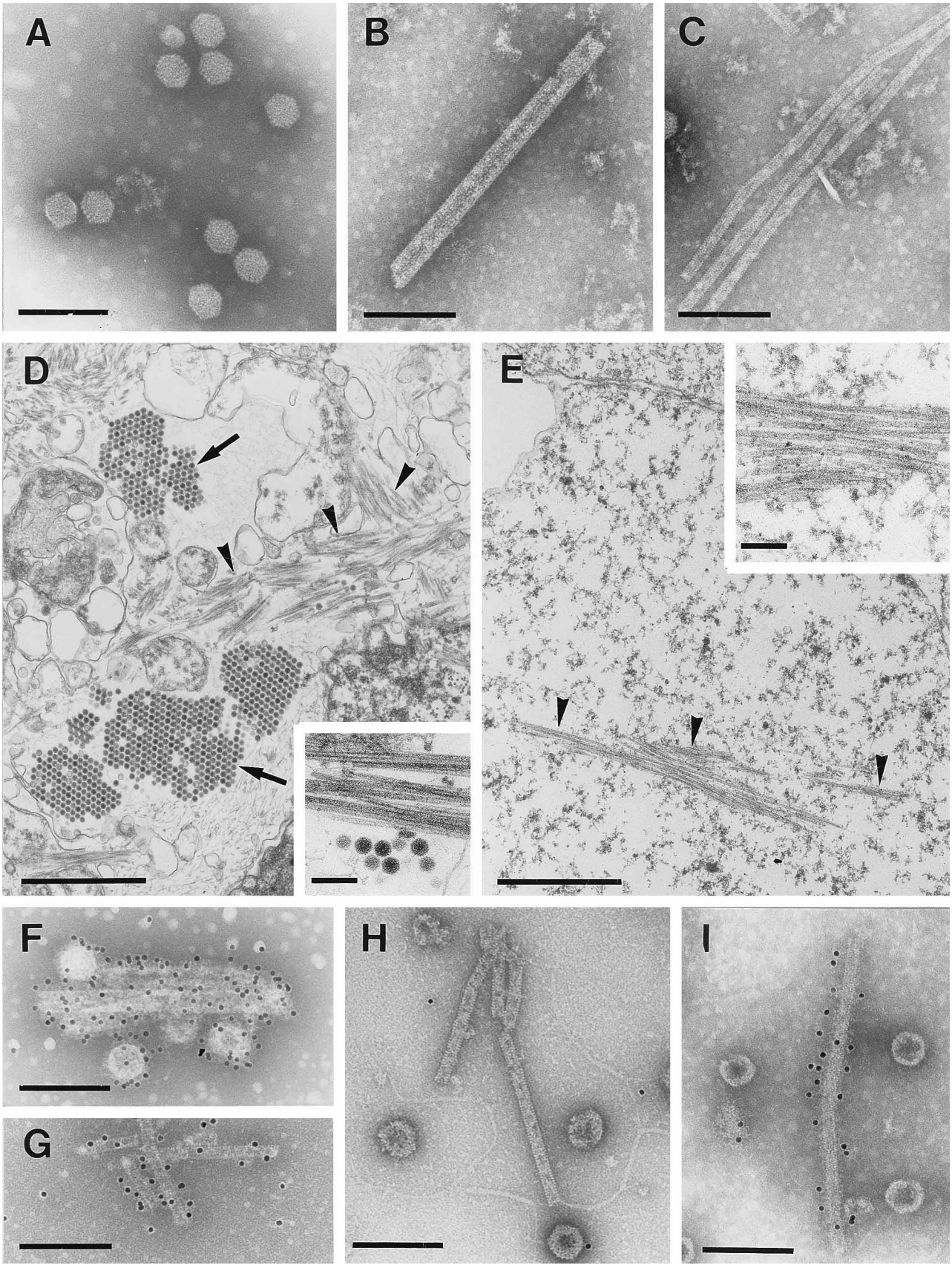

FIG. 3. IBDV and virus-associated structures in purified virion preparations and infected cells. Purified virus preparations regularly contained virions (A), type I tubules of virion diameter (B), and type II tubules (C). Cytoplasmic pseudocrystalline virion arrays (D and E, arrows) as well as needle-shaped cytoplasmic (D) and intranuclear (E) type II tubules (arrowheads) are shown in ultrathin sections of infected CEF. Insets in panels D and E show intracytoplasmic and intranuclear type II tubules, respectively, at a higher magnification. Reactivity of virions (F), type I tubules (F), and type II tubules (G) with anti-IBDV serum and marking of type II tubules with anti-VP4 MAb (I) are shown. There was no staining with the anti-tubulin serum (H). Bars: 150 nm (A to C and F to I), 1 μm (D and E), and 150 nm (insets).

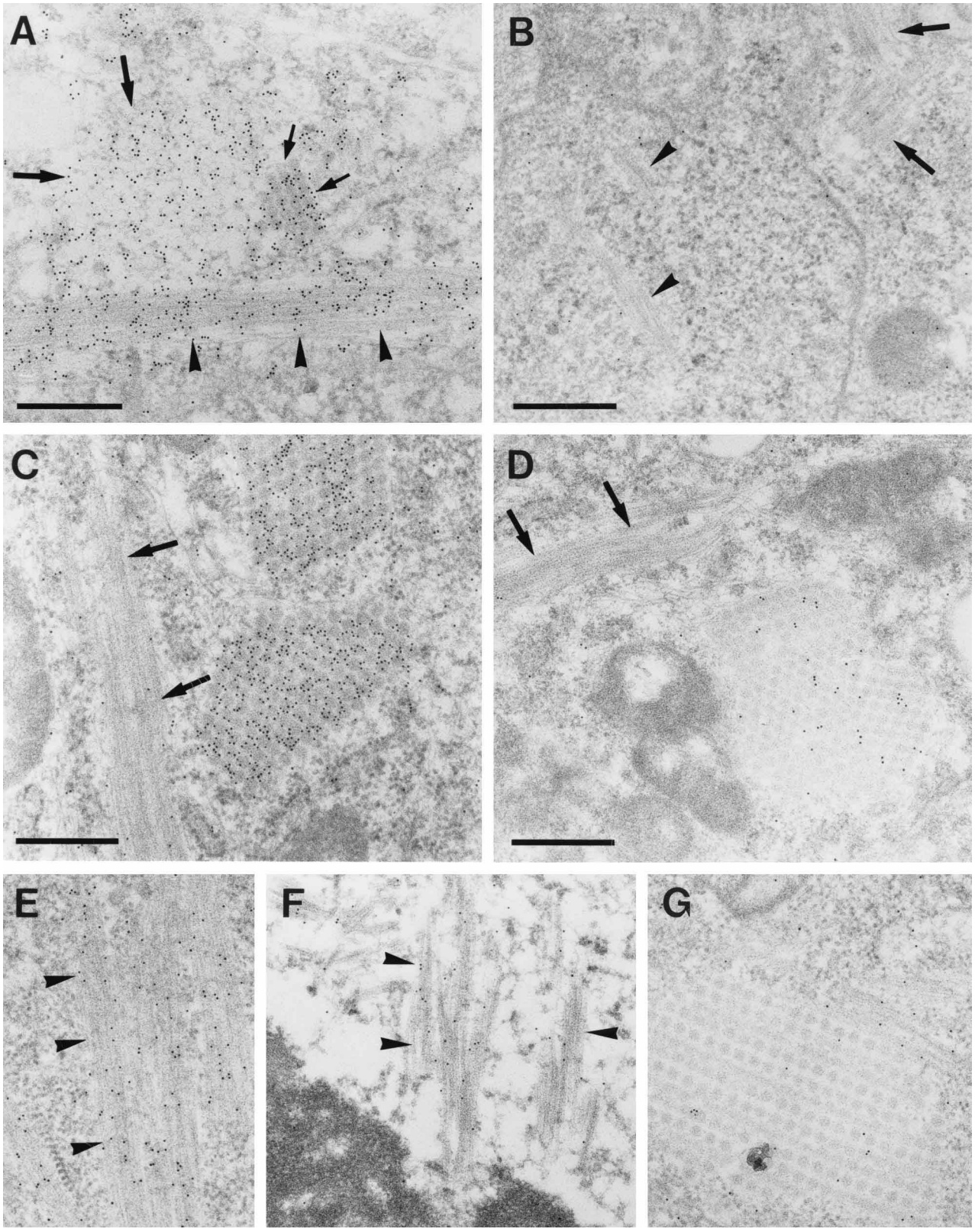

FIG. 4. Localization of IBDV antigens in Lowicryl-embedded cells by immunogold labeling. (A) Fine fibrillar inclusions (large arrows), virion pseudocrystals (small arrows), and needle-shaped bundles of type II tubules (arrowheads) were labeled by the anti-IBDV serum. (B) Intracytoplasmic (arrows) and intranuclear (arrowheads) type II tubules failed to react with anti-tubulin antibodies. (C to G) Anti-VP2 (C) and anti-VP3 (D) MAbs marked only virion pseudocrystals, not cytoplasmic type II tubules (arrows). In contrast, anti-VP4 MAb specifically reacted with intracytoplasmic (E) and intranuclear (F) type II tubules (arrowheads) but failed to react with virions (G). Bars, 500 nm.

TABLE 2. Labeling pattern on ultrathin sections of IBDV virions and associated structures in infected CEF 24 h postinfection[a]

Antibody	Virus-containing pseudocrystals	Fine fibrillar cytoplasmic inclusions	Bundles of cytoplasmic type II tubules	Bundles of intranuclear type II tubules
Anti-IBDV HIS	XXX	XXX	XX	XX
Anti-tubulin HIS	0	0	0	0
Anti-VP2 MAb	XX	XX	0	0
Anti-VP3 MAb	X	X	0	0
Anti-VP4 MAb	0	0	XX	XX

[a] XXX, intense labeling; XX, distinct labeling; X, weak labeling; 0, no labeling; HIS, hyperimmune serum.

yses. In contrast, the anti-VP4 MAb did not react with mature virions, virions in pseudocrystal arrays, or type I tubules. This indicates that VP4 may not be a component of the virion. Previously, this protein has been described as a minor virion component, since it was present in purified virions prepared by a variety of methods (18). However, as we show here, VP4-containing type II tubules were invariably present in every purified virus preparation. This could explain why virions, type I tubules, and type II tubules were recognized by a rabbit antiserum prepared against purified IBDV.

The function of the type II tubules in IBDV infection is still unclear. If VP4 is indeed the protease, aggregation might serve to inactivate excess protease to prevent lethal damage to the virus. Surprisingly, although IBDV replicates in the cytoplasm of infected cells, these VP4-containing type II tubules were detected in intracytoplasmic and intranuclear locations. So far, it is unclear how VP4-containing tubules or their immature forms reach the nucleus. No obvious nuclear localization consensus signal has been found (9).

In summary, our data show the following: (i) type II tubules present in purified IBDV virion preparations as well as in virus-infected cells contain VP4, (ii) VP4 does not appear to be a constituent of mature virions, and (iii) fibrillar cytoplasmic inclusions localized adjacent to virus pseudocrystals contain VP2 and VP3. Comparative studies with other birnaviruses will be initiated to search for similar structures in other virus-host systems.

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