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Invited Review

Visualization of viral assembly in the infected cell

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Summary. The study of the virus life cycle in infected cells is a methodological challenge due to the small size and diversity of the viral components. Recent developments on preservation of fine structure and molecular localization have provided a group of powerful methods with wide applications in cell biology and virology. Among the different electron microscopy (EM) techniques available to visualize viral assembly at the intracellular level, we will focus on conventional ultrathin sections, cryosections, and freeze-substitution. For obtaining molecular information associated to ultrastructure we have now a group of methods to detect viral proteins (immunogold labeling), as well as the viral genome, through the different techniques for detection of nucleic acids (the enzyme-gold approach, *in situ* hybridization, and elemental mapping). We will illustrate the applications of these methods with examples of viruses that exhibit different levels of structural complexity. These new approaches help to detect and identify viruses in clinical samples and to characterize the virus life cycle and the cellular components involved, to obtain data that could help for a therapeutic intervention, and to characterize virus-like particles that can be the basis of new and safe vaccines.

Key words: Viral morphogenesis, Electron microscopy methods

1. Introduction

The study of viral morphogenesis needs the contribution of different fields in biology: cellular biology, structural biology and molecular biology contribute to build the knowledge on how viruses assemble in the cells they infect. In fact, the simplicity of viruses make them very adequate and attractive models to understand how macromolecular assemblies work and develop particular functions. At the cellular level, the study of the viral life cycle is closely related to the characterization of the cellular systems involved, their function, and the signals that govern communication between them. During the last two decades the considerable development of methods for ultrastructural analysis and specific detection of particular components at the electron microscopy level has provided very adequate tools to study microbial pathogens (and in particular to visualize viruses) within the cellular environment (Carrascosa, 1986, 1988; Sodeik et al., 1993; Risco and Pinto da Silva, 1995; Nermut and Hockley, 1996). In this review we have mainly focused on animal viruses, since, for obvious reasons, most of the interest for ultrastructural analysis of cells and viruses have been centered on animal systems. The same approaches can also be applied to viruses that affect plants, yeast, fungi or bacteria introducing the adequate technical modifications imposed by the existence of thick cell walls in these organisms (Tomenius and Oxefelt, 1982; Martelli and Russo, 1985, Hayat, 1986; Baron-Epel et al., 1988; Rodríguez-Cerezo et al., 1997).

Reviewing the data available for different viral families, it can be said that almost any cellular organelle can be used by viral components to start the assembly of new viral particles (Fig. 1). We will focus on animal viruses, that, as summarized in Fig. 1A, can exhibit different levels of structural complexity (Nermut, 1987; Harrison et al., 1996). The simplest of these viruses would consist of a filamentous ribonucleoprotein (a). One or more capsids of protein (usually of icosahedral symmetry) can enclose the ribonucleoprotein (b, c). Enveloped viruses are more complex. They incorporate one or more membranes from the cell, membranes that have been previously modified by the insertion of viral proteins (d-f). Some enveloped viruses contain a layer of matrix protein interacting with the internal face of the membranous envelope (f). Finally, poxviruses are very large viruses whose detailed internal organization has not been defined yet (g). The different components of the mature virion can be put together in or several steps (Fig. 1B).

Viruses can assemble in the cytoplasm or nucleoplasm, in association with cellular membranes (plasma membrane, nuclear envelope, endoplasmic reticulum, intermediate compartment, Golgi apparatus)

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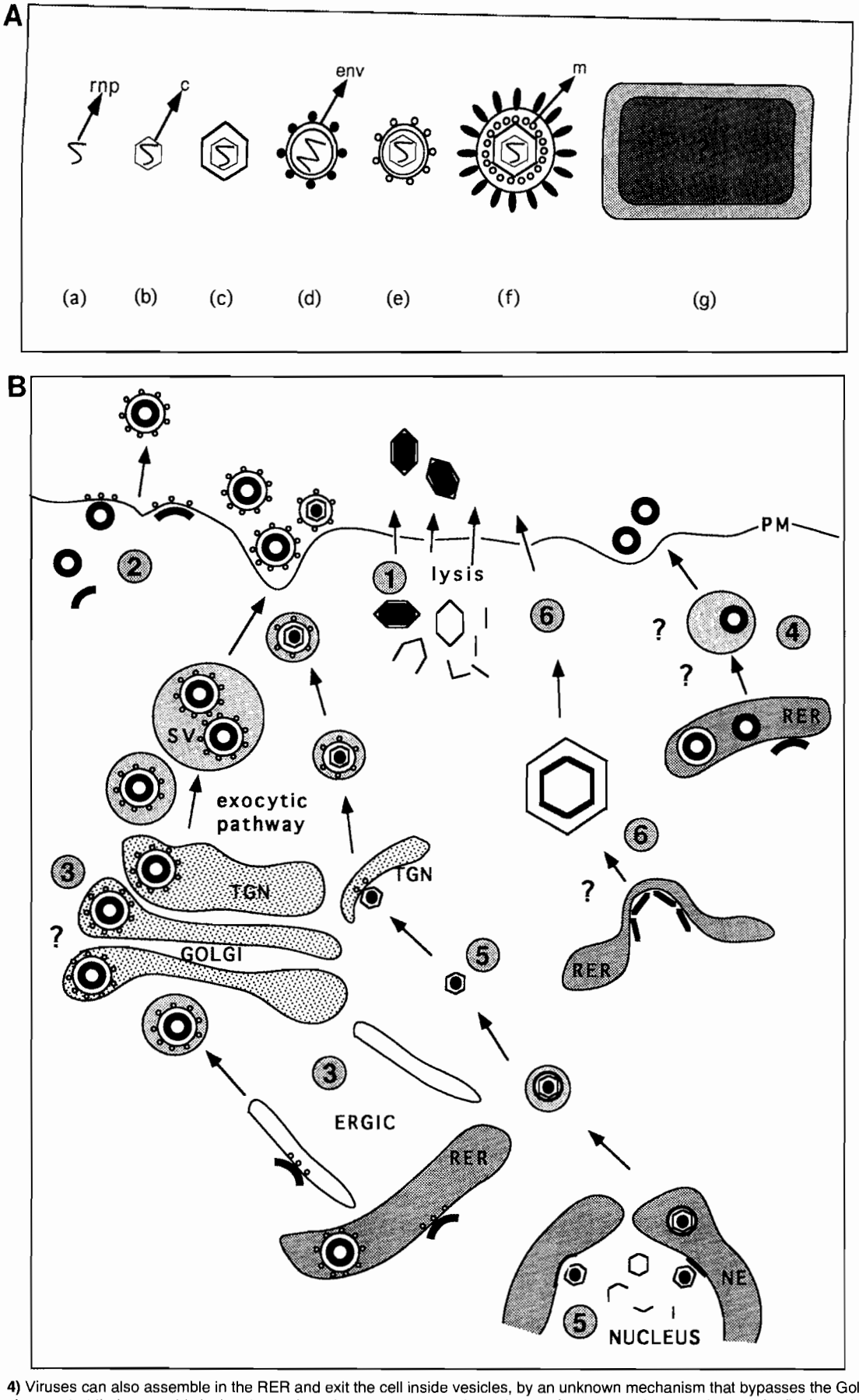


Fig. 1. Basic structural elements of animal viruses (A) and morphogenetic pathways for their incorporation in mature virions (B). **A.** Viruses can exhibit different levels of structural complexity, and combine a variable number of components: **a)** filamentous structures, constituted by the viral genome complexed with viral proteins; **b and c)** one or more proteic capsids (c), that usually exhibit an icosahedral symmetry; **d and e)** one or more lipidic envelopes (env), derived from cellular membranes modified by the insertion of viral proteins; (f) a combination of all these components and an additional layer of "matrix protein" (m); **g)** very large and complex viruses, such as poxviruses, contain many proteins (more than one hundred) and an organization still under study since its detailed characterization has not been possible to date. **B.** Basic morphogenetic pathways for animal viruses. **1)** The simplest one consists of an assembly of all viral components within the cytoplasm and exit by cell lysis. **2)** Some enveloped viruses assemble part of their components within the cytoplasm or on the cytoplasmic face of the plasma membrane, location of the final assembly and exit by budding. **3)** Some enveloped viruses bud in the rough endoplasmic reticulum (RER) or subsequent components of the exocytic pathway (ERGIC or Golgi complex), go through the Golgi complex by a transport system between cisternae still to be defined, and exit the cell inside secretory vesicles (SV). **4)** Viruses can also assemble in the RER and exit the cell inside vesicles, by an unknown mechanism that bypasses the Golgi complex. **5.** Some viruses start their assembly inside the nucleus, incorporate a transitory cisterna from the nuclear envelope and a final envelope from the trans-Golgi network (TGN). **6.** ASF-like viruses apparently use a whole RER cisterna to start their assembly, by a mechanism not completely understood.

or in complex organelles such as mitochondria. Some viruses assemble as immature particles that undergo molecular modifications and structural reorganization to render the final mature forms (Hunter, 1994; Trus et al., 1996). Once the basic assembly is completed, the new virions can use different pathways to exit the cell. Some viruses, for example, picornaviruses or adenoviruses, are released by cell lysis (Stanway, 1990; Chen et al., 1993) (Fig. 1B-1) while others (orthomyxoviruses, filoviruses) exit the cell by budding at the plasma membrane (Fig. 1B-2) (Jin et al., 1994; Geisbert and Jahrling, 1995). Corona-viruses, or bunyaviruses, make use of the transport system provided by the constitutive secretory pathway (Fig.1B-3) (Tooze et al., 1987; Jäntti et al., 1997). There are also some poorly defined transport mechanisms that allow the virions to go from the endoplasmic reticulum to the plasma membrane bypassing the constitutive secretory pathway (Fig. 1B-4). This is the case of rotaviruses, that acquire a transient envelope during this process (Tian et al., 1996; Jourdan et al., 1997). Retroviruses bud at the plasma membrane or at intracellular membranes. Their structure and morphogenesis have been extensively studied (Gelderblom et al., 1989; Gonda et al., 1989; Coffin, 1992; Nermut and Hockley, 1996; Gelderblom, 1997) but the structural features that underlie the different morphologies of retroviral particles and the apparently different assembly pathways are not well understood (Vogt, 1997). Herpesviruses exhibit complex morphogenetic pathways (Roizman and Sears, 1996; Granzow et al., 1997) that start inside the nucleus (Fig. 1B-5). Virions are later released into the cytoplasm and pass through the trans-Golgi network (TGN) before being released from the cell. African swine fever (ASF)-like viruses (Fig. 1B-6) and Poxviruses exhibit very complex morphogenetic pathways (Sodeik et al., 1993; Andrés et al., 1998; Rouiller et al., 1998). In particular, Poxviruses follow a long and complex assembly process, in which several intracellular compartments participate (Schmelz et al., 1994; Rodríguez et al., 1997). This process has been compared to the formation of cellular organelles. Thus, when facing the structural study of the morphogenetic pathway of a virus, an adequate preservation of cellular compartments and viral macromolecular assemblies is fundamental. In this sense, considerable advances have been made in recent years.

Among the different methods for visualizing viruses we will focus on the techniques that allow the obtention of detailed images of the viruses within the intracellular environment. Some of these methods could be considered routine or "conventional" while some others have been developed or improved in recent years. Ultrathin sections of resin-embedded material constitute the most direct way for visualizing virions in infected cells at the electron microscopy (EM) level. This method has provided most of the structural information available on the intracellular viral assembly for many viral groups. Methods based on freeze-fracture have been shown to be very useful for studying cellular membranes (Pinto da Silva, 1987) but they are less adequate for visualizing viruses within the intracellular environment due to the complex interpretation of images. Specific methods that combine freeze-fracture with resin-embedding and thin-sectioning can be applied, however, to study particular aspects of viral particles that associate with cellular membranes (Torrisi et al., 1992).

The combination of optimal ultrastructural preservation with specific detection of components is essential to obtain molecular information on the ultrastructure and to go beyond the mere morphological description. In this sense, the development of cryo-methods for electron microscopy has provided a significant improvement in both goals. We will review the different possibilities offered by this group of methods with particular examples on different viral groups.

2. Basic procedures

2.1. Conventional methods for ultrastructural analysis

Sample preparation needs to cover a number of steps to stabilize the biological material under the conditions for observation in the electron microscope. These steps include a fixation of the components (using chemical fixatives), dehydration, (since observation will be done under high vacuum) and building a physical support for the material, that usually consists of the infiltration with a plastic resin, that is transparent to electrons and gets hard when it polymerizes. The type of resin to use depends on the information we look for, either ultrastructure or specific localization of components. Ultrathin sectioning in an ultramicrotome completes the procedure, since samples must be very thin in order to allow the electrons to get trough.

The same basic procedures can be applied to any kind of cell or tissue, although, as we mentioned before, when dealing with plants, bacteria, yeast, and fungi, particular modifications must be included in order to allow an adequate infiltration of the different media through the rigid and rather impermeable cell walls present in these organisms.

When analyzing ultrastructure, an adequate preservation of molecular components is fundamental. Chemical fixation for EM usually includes protein fixatives such as paraformaldehyde and glutaraldehyde together with lipid fixatives, such as tannic acid and osmium tetroxide (Bullock, 1984; Hayat 1989a). Uranyl acetate, the most common staining agent for EM, can also be included as an additional fixative, since it stabilizes phospholipids prior to dehydration which increases membrane preservation and contrast (Erickson et al., 1987; Grief et al., 1994). In particular, tannic acid has been reported as a very good contrast enhancer for cellular and viral membranes (Olesen, 1979; Gelderblom 1991; Berryman et al., 1992; Hart et al., 1993). It improves the quality of fixation, particularly when aldehydes are used. It also acts as a ligand between

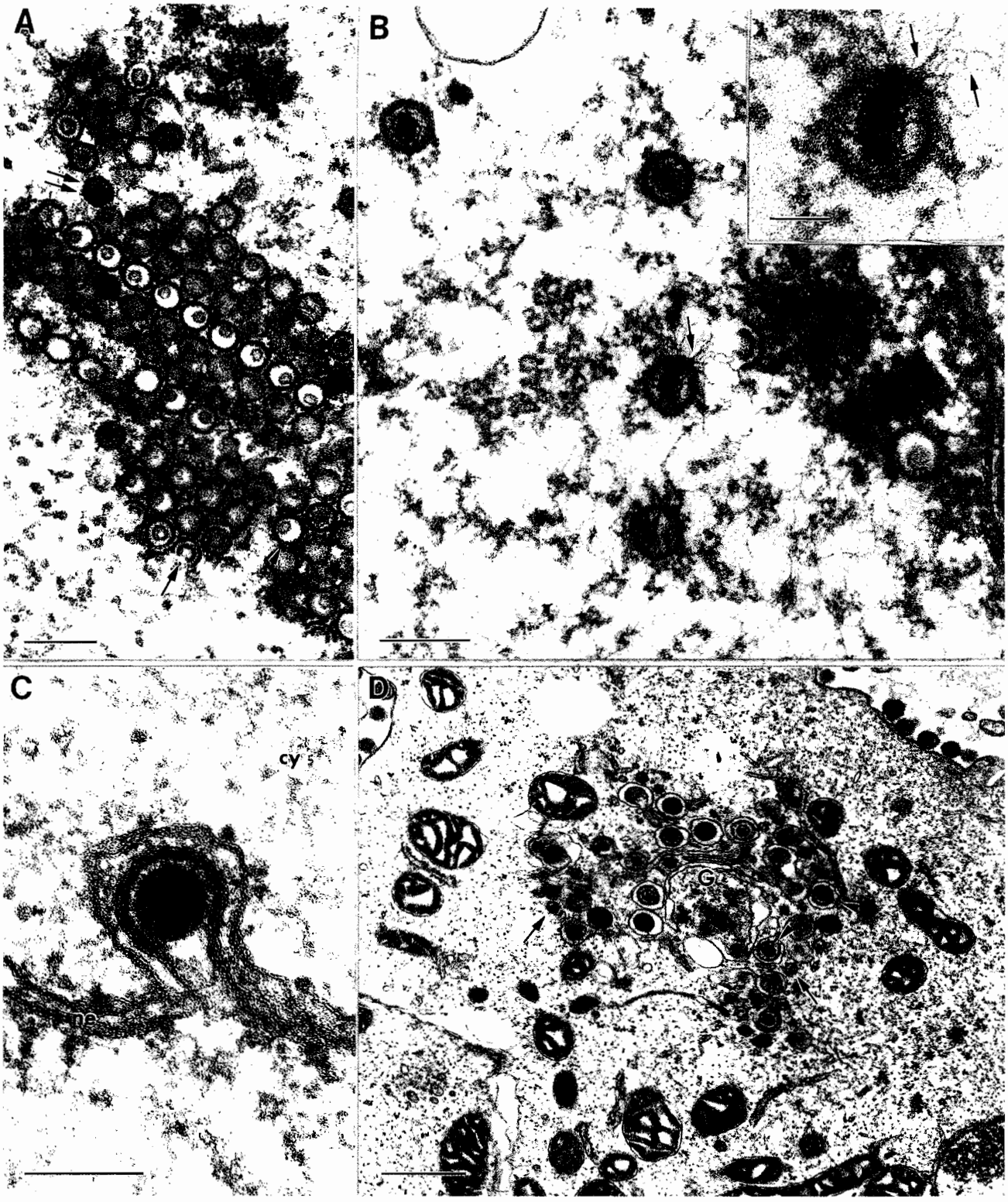


Fig. 2. Assembly of a herpesvirus (pseudorabies virus, PrV) studied by conventional embedding methods (fixation with glutaraldehyde and osmium tetroxide and embedding in Epon). The fine definition of both cellular and viral structure allows the definition of the different steps of the complex morphogenetic pathway (only a few are reproduced here). (A) and (B) show the intranuclear maturation of nucleocapsids. **A.** Crescent-shaped capsids with an annular protein structure (arrow), empty capsids (arrowhead), and DNA-containing nucleocapsids (double arrows) form large groups arranged as pseudocrystals. **B.** Structures resembling nucleic acid threads are found in contact with vertices of capsids (arrows in mainfield and inset). **C.** Budding through the nuclear envelope (ne) allows the viral particle to exit the nucleus into the cytoplasm (cy). **D.** Naked nucleocapsids (arrows) were detected in the Golgi area (G), where virions acquire their definitive envelope (arrowheads), probably from the trans-Golgi network. Bars represent 250 nm for A, 150 nm for B and C, 50 nm for inset in B, and 750 nm for D. Reproduced from Granzow et al., 1997, with copyright permission from the American Society for Microbiology.

heavy metals and membranes enhancing, for example, the absorption of osmium tetroxide. Potassium ferricyanide is also used in combination with osmium tetroxide for increasing membrane contrast, since it has proved to reveal cellular membrane systems that are "invisible" in its absence (Forbes et al., 1977; Pimenta and de Souza, 1985; Risco et al., 1994). A combination of some or all these fixatives usually provides an outstanding contrast and definition of both viral assemblies and cellular organelles, helping to define associations between them. When working with cells infected in culture, fixation of cells in situ (exchanging the culture medium by the fixative and collecting the cells in the fixative) improves overall structural preservation.

How to perform dehydration is very important since the main damage to fine structure and loss of components takes place during this process. It is recommended that dehydration in increasing concentrations of a solvent such as acetone, ethanol, or methanol must be as short as possible and with the samples in ice. When working with isolated or cultured cells, the dehydration steps can be as short as 5 min each at 4 °C, conditions that approach the structural preservation of fine details to the results of freeze-substitution (see section 4.2 and Fig. 9). Acetone is reported to cause less shrinkage than ethanol, while methanol apparently provides better results concerning the preservation of nucleic acids within viral particles.

Finally, infiltration in a plastic resin will give the physical support to the biological material under the conditions of observation in the electron microscope (high vacuum, irradiation with an electron beam). The two main classes of resins in use today are the epoxides (Epon-812, Araldite, Spurr's) and the acrylics (LRWhite, LR Gold, and the Lowicryls) (Spector et al., 1998). Epoxides are the resins of choice for ultrastructural studies, while acrylics are suitable for ultrastructural cytochemistry (see section 2.2). Epoxide resins are more stable under the electron beam and in combination with adequate staining methods, a high contrast and definition of fine details (viral envelopes and capsids, spikes and peplomers) can be obtained (Hayat 1993). Conventional staining methods for sections (saturated uranyl acetate and lead citrate) can be modified to improve contrast. For example, when using saturated uranyl acetate in 70% aqueous ethanol, the contrast of membranes improves considerably. Very thin sections (20-30 nm) are usually necessary for visualizing fine details of small virions (20-100 nm in diameter) while thicker sections (around 100 nm) offer better possibilities to study larger and more complex enveloped viruses.

The work on herpesviruses (Nii, 1992; Granzow et al., 1997) and retroviruses (Gonda et al., 1989; Nermut and Hockley, 1996; Gelderblom, 1997) illustrate well the results that can be obtained with these basic procedures, that are always the mandatory first step when studying the morphogenesis of a particular virus. Both retroviruses and herpesviruses are enveloped virions exhibiting morphogenetic pathways that involve cellular endomembranes. An optimal preservation of membranes is then important when visualizing these viruses. Small protein aggregates, such as viral spikes and cores, must also be well preserved. Fig. 2 shows some of the steps in the complex morphogenesis of herpesviruses. High contrast and definition of viral components were obtained in this work by Granzow et al. (1997). In particular, note the outstanding definition of the viral nucleic acid strands (Fig. 2B) and the good contrast of cellular membranes (Fig. 2C,D). Fig. 3 shows part of a classical work on the structure of the human immunodeficiency virus (HIV). Its mature and immature forms were characterized by Gelderblom (1991). Optimal results in preservation of fine details were obtained in this study. Note, for example, the fine definition of the small viral spikes.

2.2. Immunolocalization of viral proteins

Low-temperature embedding in acrylic-type resins after a mild chemical fixation allows an optimal preservation and presentation of macromolecules on the surface of ultrathin sections to be available for specific detection. The London resins (LR White and LR Gold) and the low-temperature Lowicryl resins are the most common acrylic resins in use today (Hayat, 1989a). These resins are less stable under the electron beam than epoxide resins, but they are less viscous and more hydrophilic. They do not copolymerize with the biological material, as epoxy-resins do. This makes numerous macromolecules literally "get off" from the section surface, being accessible for specific detection (Kellenberger et al., 1987). Embedding at low temperatures (up to -80 °C) also helps to preserve the three-dimensional structure of proteins, and reduce the damage caused by dehydration (Newman and Hobot, 1993). Postembedding labeling methods are performed on thin-sections of embedded cells and tissues on EM grids. In recent years this has become the most popular method for immunoelectron microscopy studies. In the case of pre-embedding immunolabeling methods, antibody labeling is performed on fixed and permeabilized cells prior to embedding.

Immunolabeling and enzymatic methods in combination with colloidal gold probes of different sizes are used to localize specific viral components within the complex intracellular environment, which then helps to identify the assemblies of viral origin, and the existence of intermediate or immature viral forms. Since their incorporation in microscopical methods more than 25 years ago, gold conjugates have became a fundamental tool in electron microscopy (Faulk and Taylor, 1971; Hayat 1989b; Raska et al., 1990; Spector et al., 1991). Conjugates of primary or secondary antibodies with highly electron-dense spheres of colloidal gold (commercially available in a convenient range of sizes) are widely used to localize proteins on sections, with high sensitivity and specificity. Multiple labeling is also

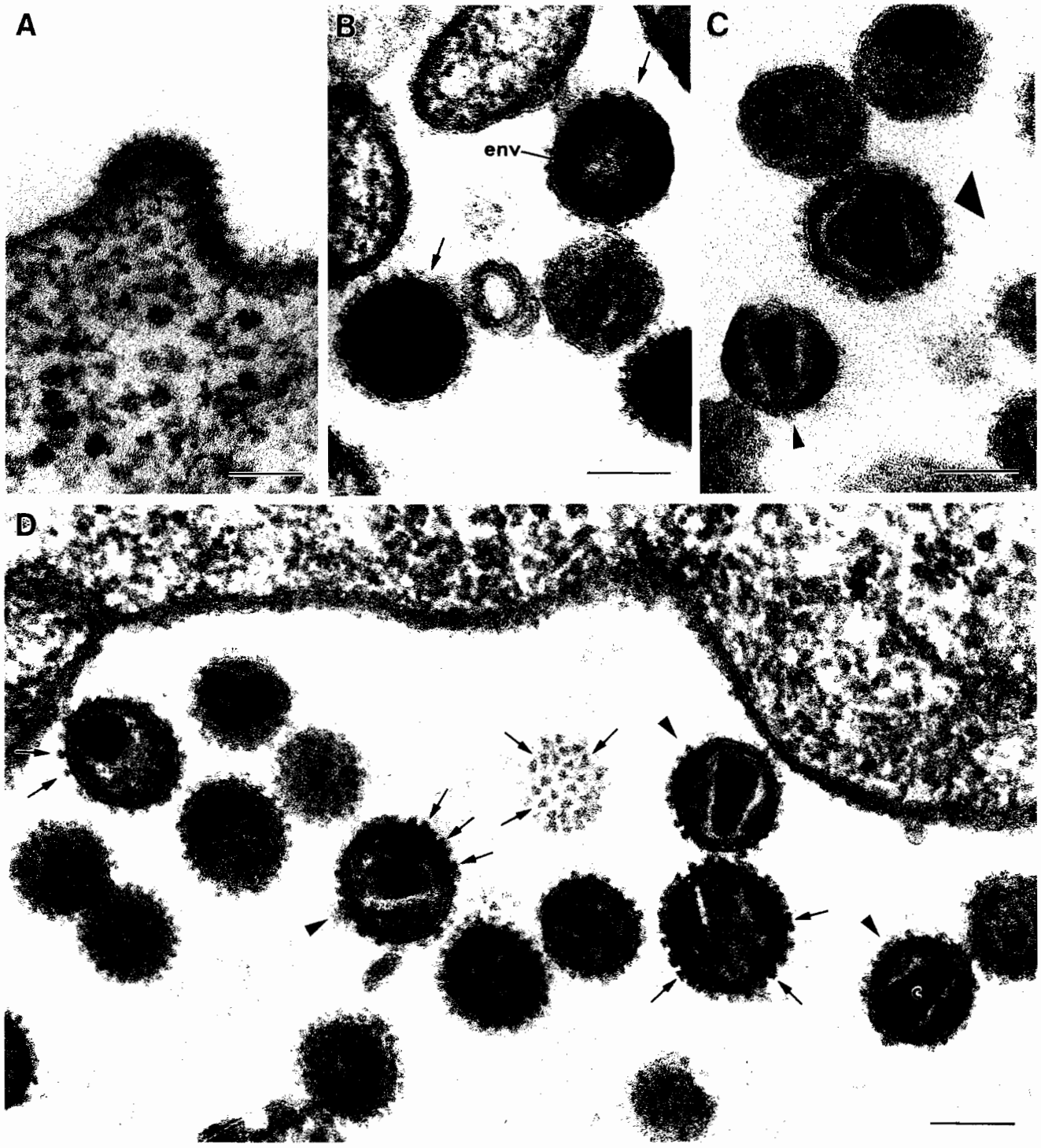


Fig. 3. Assembly of the human immunodeficiency virus (HIV) as studied by conventional embedding procedures (fixation with glutaraldehyde, tannic acid and osmium tetroxide, and embedding in Epon). The fine definition of the viral components allows the identification of different stages in the assembly process. Budding profiles **(A)** originate immature virions (arrows in **B**), whose electron-dense ribonucleoprotein shell is attached to the viral envelope (env). Intermediate stages in the morphological transition from immature to mature viral particles are occasionally observed (large arrowhead in **C**). Mature virions (small arrowheads in C and D) have already condensed and organized the cone-shaped internal core (c). In these samples tannic acid has provided a high contrast of the viral envelopes. Note the fine definition of viral spikes (arrows in D). Bars: 100 nm. (A-C, reproduced from Gelderblom, 1991, with copyright permission from Lippincott-Raven; D reproduced from Gelderblom, 1997, with copyright permission from Los Alamos National Laboratory).

possible using gold particles of different sizes. Colloidal gold probes as small as 1nm are available today (Slot and Geuze, 1985) and the 1.4 nm Nanogold particles (which consist of a non-colloidal gold particle covalently bound to the protein) provide increased label intensity and better penetration, and have been successfully applied in preembedding studies (Ochs et al., 1994). Immunogold labeling in electron microscopy follows the same basic principles of any immunoreaction. These procedures start with a saturation with blocking buffers and sequential incubations with the specific antibodies and gold markers (Hayat, 1989b; Risco et al., 1995c, 1996). Enzymes are also used for detection of specific components since they also preserve their capacity for recognizing their substrates once complexed with the gold particle. These conjugates have been successfully used to localize proteins, lipids, sugars, and nucleic acids (Bendayan, 1984, 1989; Londoño and Bendayan, 1987) and they can be particularly useful for localizing viral genomes in viral particles (see section 5).

With the molecular data provided by these localization techniques we can identify the viral-related aggregates within the complex cellular environment (Sodeik et al., 1993; Risco et al., 1995a,b, 1998) which considerably helps to follow the sequential incorporation of different viral components during morphogenesis and their final organization within the viral particle. Fig. 4 shows the immunolocalization of proteins in different retroviruses: the murine leukemia virus (MuLV), the mouse mammary tumor virus (MMTV), and the human immunodeficiency virus (HIV). Immunodetection of MuLV proteins (Fig. 4A) allowed the identification and following of the virus during the early phase of infection (Risco et al., 1995b), once the virions had entered the cells and suffered structural changes (note the significant reduction in virion diameter associated to entry). Figs. 4B-D show that when using small gold probes (≤ 5 nm), different domains within a polypeptide can be mapped in the viral particle, even in relatively small virions. The information obtained indicated a radial arrangement of these polypeptides within the viral particle (Menéndez-Arias et al., 1992). The specific localization of different proteins in HIV particles is the first step for a posterior detailed characterization of the intracellular distribution of the viral proteins during the viral life cycle.

3. Modifications of basic procedures

3.1. Immunolabeling in sections of epoxide-resins

Acrylic resins are the most adequate for specific detection of components, but the contrast and definition of membranes obtained in these materials is poor when compared with epoxy-resins. As a consequence, the correlation between the labeling signal and the structure associated to it can be difficult, mainly in the case of membranes. Embedding in an epoxy-resin after a strong chemical fixation usually does not allow a later detection of specific molecular components of the sample. There are, however, exceptions to this, since some proteins resist rather drastic fixations and are still detectable on epoxy-sections. Since osmium tetroxide is the most damaging fixative for posterior immunodetection of proteins, the use of low concentrations of this agent (0.05-0.1%, p/v) can provide an optimal compromise between structural definition of membranes and immunodetection. Fig. 5 shows a direct comparison of immunogold labeling on Lowicryl K4M and Epon sections, from a study on the assembly of vaccinia virus (VV, a member of the *Poxviridae* family) by Rodríguez et al. (1997). The assembly of VV starts in the viral factories (F) whose detailed organization and origin are still unknown. Lowicryl sections allow an optimal detection of the p15 viral protein (Fig. 5A,B) both on the periphery of the viral factories (Fig. 5A) and around the electron-dense masses that form when viral assembly is blocked (Fig. 5B). In both cases it is not possible to distinguish the structures that support the strong labeling signals. Conventional sections of an epoxy-resin (Fig. 5C,D) clearly showed the membranous elements recruited on the periphery of the viral factories (membranes organized as viral crescents) or around the electron-dense masses (numerous tubular and vesicular membranes). Labeling on sections of an epoxy-resin (EML-812), after a treatment with a low concentration of osmium tetroxide, allowed a direct correlation between labeling and structure (Fig. 5E,F) to establish that p15 reaches the centers of viral assembly as part of the tubulo-vesicular elements recruited there.

3.2. Characterizing the intracellular environment of viral assembly: use of markers for cellular compartments and controlled permeabilization of cells

Cellular markers can be very useful when dealing with viruses that assemble in association with endomembranes. Table 1 summarizes some of the markers most widely used by cell biologists for specific identification of cellular compartments (most of them have been shown to work both in immunofluorescence and immunoelectron microscopy). Some viral proteins are also used as markers for a particular cellular compartment, once their accumulation in the mentioned compartment has been demonstrated. That is the case for the membrane (M) glycoprotein of coronaviruses used as a specific marker for the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Machamer and Rose, 1987)

In particular cases it is also interesting to apply some mild permeabilization treatments for a better visualization of the connections between membranous compartments, and between cellular and viral membranes, difficult to visualize in intact cells. These images can help to define some of the steps of assembly, in particular for complex viruses such as VPPA-like viruses (Rouiller et al., 1998) and Poxviruses (Sodeik et al., 1993).

Fig. 6A,B show the information obtained by

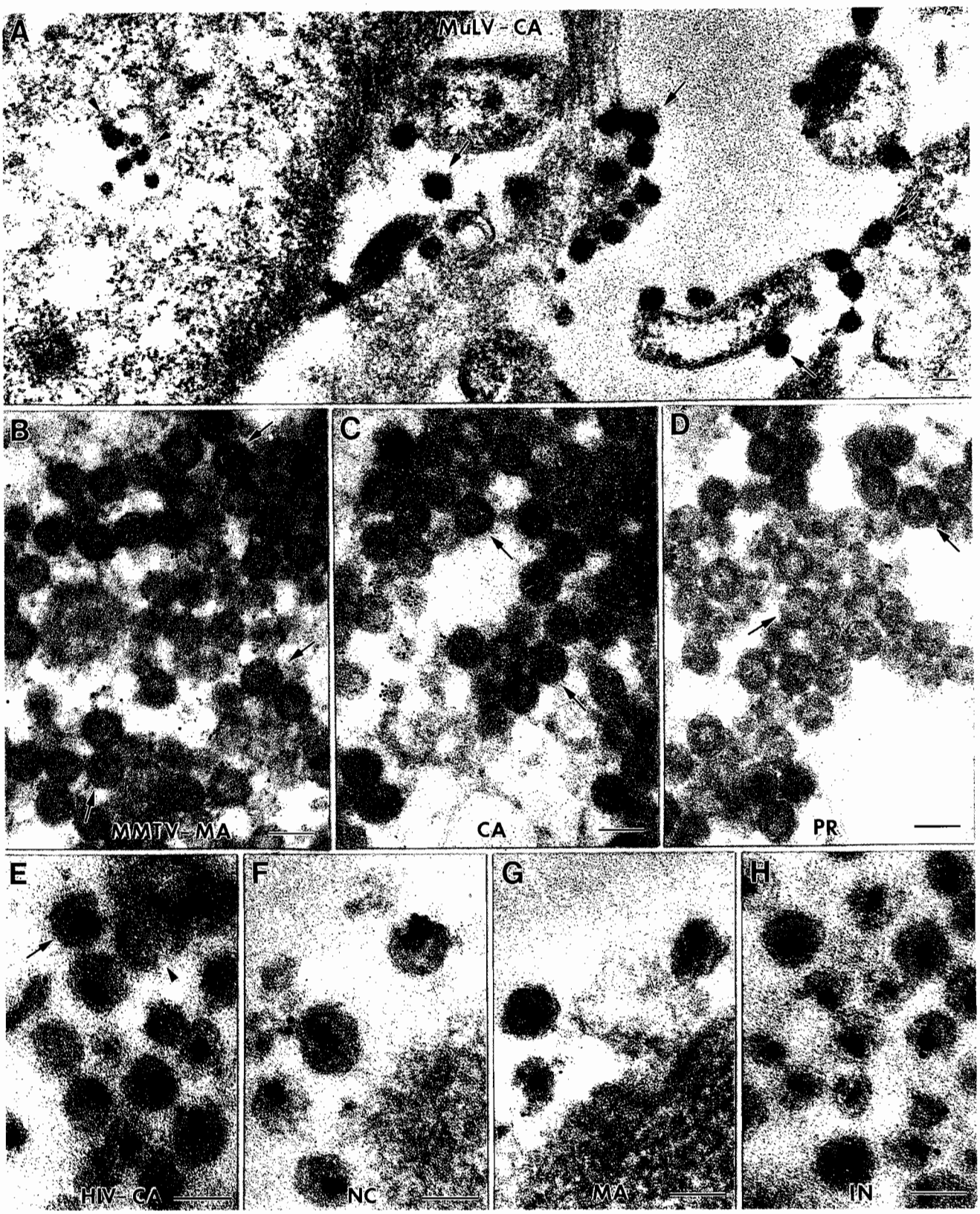

A
MuLV - CA
B
MMTV - MA
C
CA
D
PR
E
HIV - CA
F
NC
G
MA
H
IN

Table 1. Some antigenic markers used in cell biology .

ANTIGEN	CELLULAR LOCALIZATION	REFERENCE
ER-p34	Endoplasmic reticulum	Prehn et al., 1990
Protein disulfide isomerase (PDI)	Endoplasmic reticulum	Tooze et al., 1989
BiP	Endoplasmic reticulum	Pelham, 1989
p62	Endoplasmic reticulum	Mundy and Warren, 1992
rab-2	Intermediate compartment	Chavrier et al., 1990
p53	Intermediate compartment	Schweizer et al., 1988
p58	Intermediate compartment	Saraste et al., 1987
Giantin	Periphery of Golgi stacks	Seelig et al., 1994
p115	Cis-Golgi	Waters et al., 1992
MG-160	Cis/middle Golgi	Gonatas et al., 1989
Mannosidase II	Middle/trans-Golgi	Moremen et al., 1991
Galactosyl-transferase	Trans-Golgi	Roth and Berger, 1982
TGN46	Trans-Golgi network (TGN)	Ponnambalam et al., 1996
Mannose 6-phosphate receptor (MPR)	Pre-lysosomal compartment	Griffiths et al., 1988
LIMP	Lysosomes	Barriocanal et al., 1986
Fibrillarin	Nucleolus	Christensen et al., 1986
RNA	Nucleolus, ribosomes	Eilat and Fischel, 1991
DNA	Chromatin, viral particles	Scheer et al., 1987

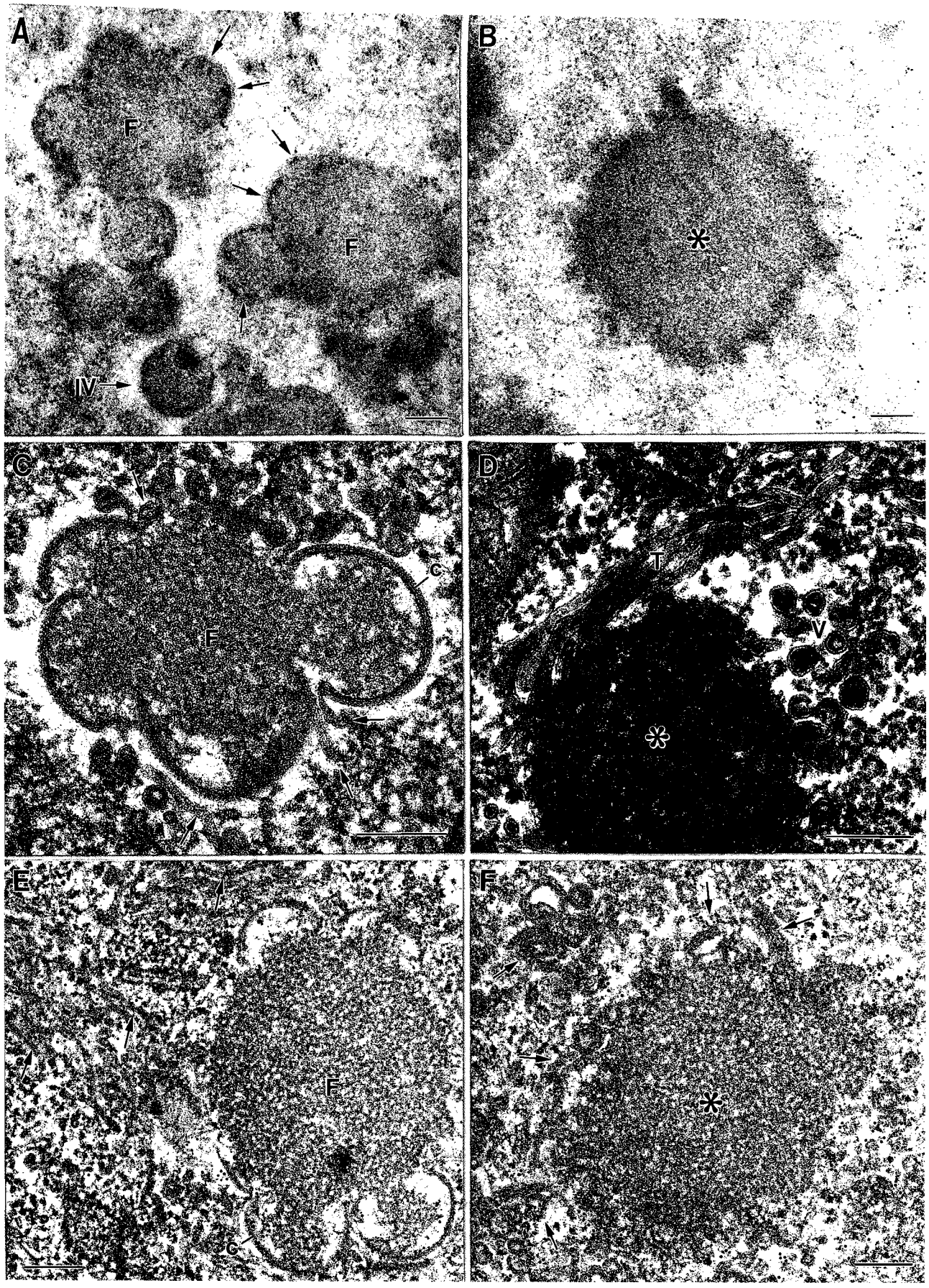
permeabilization with streptolysin O (SLO) in defining the connections of the VV viral membranes with cellular tubular membranes, whose identification as ERGIC-components was established by immunogold detection of a specific marker protein in cryosections (Sodeik et al., 1993). Figs. 6C to 6F are part of the work by Rodríguez et al. (1997) and show that the tubular and vesicular membranes recruited on the periphery of the viral factories, as well as the electron-dense masses formed when VV assembly is blocked, are related to the ERGIC, as indicated by the specific labeling obtained with the anti-ERGIC-53, that localizes a resident protein of this compartment (Schindler et al., 1993).

Considering that constitutive cellular proteins are present in cells in much lower amounts than the viral proteins produced during an infection, an adequate preservation of most of the epitopes is critical for a successful detection of a cellular marker. Cryomethods, described in the following sections, usually improve this preservation and help in the identification of membranous organelles.

4. Cryomicroscopy for cells

During the last 15 years, considerable advances in ultrastructural analysis have been provided by the development of cryomethods at the EM level. Although studies of conventionally fixed specimens have greatly increased our knowledge of cell and viral structure, the possibility of fixing biological samples by rapid freezing in a cryogen (such as liquid ethane, propane or freon) has boosted the study of macromolecular assemblies, viruses, and cells in a state closer than ever before to the "native" hydrated organization (Dubochet et al., 1988). The reason for these improvements is that cryofixation physically immobilizes cellular structure and stops activity within milliseconds, compared to seconds to minutes needed by chemical fixatives to penetrate and react with intracellular structures. This often leads to artifactual changes in cellular organization. An adequate vitrification of the sample always constitutes the most critical step of these procedures, and important differences exist depending on the size of the material to be frozen (Dahl and Staehelin, 1989; Ryan et al., 1990; Kellenberger, 1991). When vitrifying cells, which are considerably large structures, a good vitrification can be obtained in a limited depth from the surface that first contacts the cryogen. For material not treated with

Fig. 4. Immunolocalization of viral proteins in Lowicryl K4M sections of different retroviruses. **(A)** Shows the immunogold localization of capsid protein (CA or p30) of the murine leukemia virus (MuLV, a type C oncoretrovirus) in thin-sections of NIH 3T3 cells at short post-infection times. The intense immunolabeling signal allows the identification of virions within the cellular environment, although they suffer major structural changes (arrows point to extracellular virions, while arrowheads mark intracellular viral particles). **(B)** to **(D)** show the detection of protein domains in immature capsids (known as intracytoplasmic A particles) of the mouse mammary tumor virus (MMTV, a type B oncoretrovirus). Specific antibodies for different regions of the precursor gag-related polypeptides that form the A particles and a 5 nm protein A-gold conjugate were used. (B) Immunolocalization of the domain corresponding to the matrix protein (MA or p10), (C) the capsid protein (CA or p27), and (D) the MMTV protease (PR). Thin sections of murine L1210 leukemia cells were used. Considering the sequential order of the protein domains in the Gag and Gag-Pro precursors, the immunogold data show that the arrangement of polypeptides in the intracytoplasmic A particles is radial, with MA forming the outer surface of the immature capsids and PR being located closer to the center of the particle. **(E)** to **(H)** Immunogold detection of different HIV proteins in released extracellular virions. Capsid (E), nucleocapsid (NC), matrix (MA) and integrase (IN, present in smaller amount than the other three proteins) were specifically immunolabeled, as a previous step to follow the viral proteins during the virus life cycle. The arrow in E points to a mature HIV virion, while the arrowhead points to an immature HIV particle. Gold conjugates of 5 nm were used in A, B, C, D, and G, while 10 nm gold particles were used in E, F, and H. Bars: 100 nm. B to D, reproduced from Menéndez-Arias et al., 1992, with copyright permission from the American Society for Microbiology.


A
F
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IV
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V
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F

cryoprotectants, 5-10 μm is the realistic size limit for most cells prepared by plunge or slam freezing. Using cryoprotectants, such as glycerol, this depth can increase up to approximately 30 μm (Grief et al., 1994). Freezing at high pressure brings this size limit to 500-600 μm (Moor, 1987). The cooling rate must be of at least 10,000°C/second in order to avoid the formation of large ice crystals (>2-5 nm) that would damage the fine ultrastructural details (Heuser et al., 1979; Knoll et al., 1991; Ryan, 1992). Subsequent processing of the frozen cells can be directed to basically two different procedures: cryosections or freeze-substitution, each allowing different unique applications.

4.1. Cryosections: visualizing cellular and viral membranes

Cryosections for electron microscopy are mechanically less stable than resin-embedded sections, but generally several times better in the sensitivity of antigen detection. The preparation of cryosections does not involve dehydration of specimens as does the preparation of resin sections (reviewed by Griffiths et al., 1983; Tokuyasu, 1986; Griffiths, 1993). Cryosections have been shown to be the most adequate way to study the diversity of cellular endomembranes (McDowall et al., 1989). They are, then, an important tool when dealing with large complex virions that have more than one envelope, such as poxviruses, or viruses that assemble in association with intracellular membranous organelles, since individual membranes are clearly delineated in cryosections. In addition, cryosections usually offer better sensitivity for detection of molecules due to improvements in preservation. The chemically fixed and cryoprotected frozen sample is sectioned in a cryoultramicrotome, and later thawed (no dehydration or embedding in plastic resin is applied). Membranes look "white" in these preparations, and their particular aspect has allowed the adequate characterization of particular membranous components belonging to different compartments, such as the endosomal-lysosomal system and the secretory pathway (McDowall et al., 1989). In Fig. 7 Sodeik et al. (1993) show us the information that excellent cryosections can provide. Intracellular poxvirions can contain up to four distinct membranes, clearly distinguished in cryosections (Fig. 7A, inset), but not in conventional Epon-type sections. Very good immunolabeling signals for simultaneous detection of viral and cellular proteins are illustrated in Fig. 7A.

Immunogold detection of viral proteins has also been combined with cryomicroscopy of whole virions. In the work by Ross et al. (1996) the advantages of immunogold labeling with the high resolution structural preservation provided by cryo-EM were combined. With this novel approach, they studied the structure and topology of some key proteins in the poxvirus vaccinia (Fig. 7B). The data obtained by this method are complementary to the study of sectioned viral particles and can be very useful when studying the organization and assembly of complex viruses.

Depending on the particular virus under study, cryosections may need to be complemented with the information provided by ultra-thin sections of conventionally epoxide-embedded material, since some organelles and small viral particles present a poor contrast and are difficult to identify in cryosections. An alternative that combines the main advantages of cryomicroscopy, epoxy-resins, and conventional thin-sectioning is provided by freeze-substitution.

4.2. Freeze-substitution

Freeze-substitution following ultra-rapid freezing is a superior method for preserving cell ultrastructure (Harvey, 1982; Hippe-Sanwald, 1993; Spector et al., 1998). This method is applied on frozen specimens, and basically consists in a sophisticated dehydration technique performed in freeze-substitution equipments. Fast-frozen samples (that can also be previously chemically fixed) are submitted to dehydration in pure (water-free) solvent at the lowest temperature that allows the solvent to remain liquid. Under these conditions the water of the sample is substituted by the solvent in a very mild process, with a minimal distortion of fine structural details or extraction of soluble components. If fixatives are included in the organic solvent, they penetrate the samples at low temperature and react with the material when the temperature rises. The following steps are common to a conventional embedding in a resin and ultrathin sectioning. According to the experience accumulated by numerous studies discussed in the literature, extraction of cellular components or artifacts related to changes in shape and size of structures are greatly minimized, which renders a very good preservation of fine structural details (Monaghan and Robertson, 1990; Kellenberger, 1991). This might be critical for the correct identification of intermediate viral assemblies during morphogenesis, and for the study of small viruses whose structure is not well defined in cryosections. Two examples of freeze-substitution for

Fig. 5. Direct comparison of the possibilities offered by Lowicryl and Epon sections for studying ultrastructure and localization of specific proteins. **(A)** and **(B)** show the localization of a membrane VV protein (p15) on the periphery of viral factories (F) or the equivalent structures (asterisks) formed when viral assembly is blocked (see Rodríguez et al., 1997 for details). Samples were submitted to mild fixation and embedding in Lowicryl K4M. **(C)** and **(D)** show the same structures after strong fixation and embedding in Epon. Numerous membranous elements (tubules and vesicles) are detected around the factories and dense masses, and in continuity (arrows) with the viral crescents (c). **(E)** and **(F)** show immunogold detection on Epon sections using a fixation with a low OsO_4 concentration. In these images it can be seen that the viral protein is actually incorporated to the tubulo-vesicular membranous elements recruited to the periphery of masses and factories (arrows). Bars: 200 nm. Reproduced from Rodríguez et al., 1997, with copyright permission from the American Society for Microbiology.

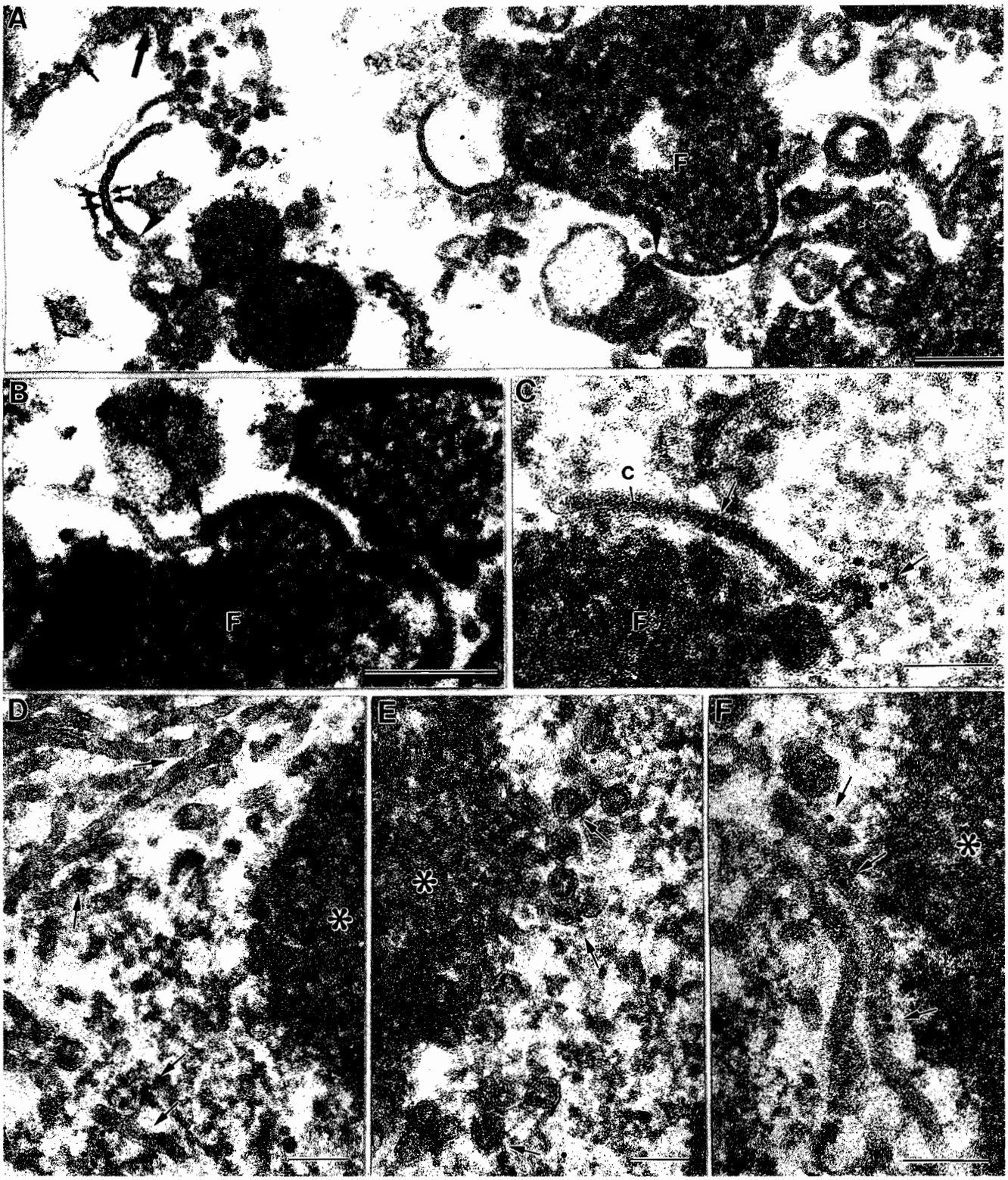


Fig. 6. Controlled permeabilization of cells help to define connections between cellular and viral membranes. **A and B.** Epon sections of VV-infected HeLa cells in which the plasma membrane has been permeabilized at 12 h after infection with the bacterial toxin streptolysin O (SLO). Continuities between the rough endoplasmic reticulum (RER) and membrane tubules (T) are seen (large arrows). A ribosome on the RER is indicated (small arrow in A). The membrane continuities between the cellular membranes and the viral crescents (c) are also evident under these conditions (arrowheads). The small double arrows in B indicate a forming crescent where the two membranes are distinct and in continuity (large arrowhead) with other membranes. **C to F.** Immunogold labeling on Epon sections showing the localization (arrows) of ERGIC-53 (a marker of the intermediate compartment) on the cellular tubulovesicular membranous elements recruited on the periphery of the viral factories (F) and the masses (asterisks) formed in the absence of viral assembly. Bars: 200 nm for A and B; 100 nm for C-F. A and B, reproduced from Sodeik et al., 1993, with copyright permission of the Rockefeller University Press; C to F, modified from Rodríguez et al., 1997, with copyright permission from the American Society for Microbiology.

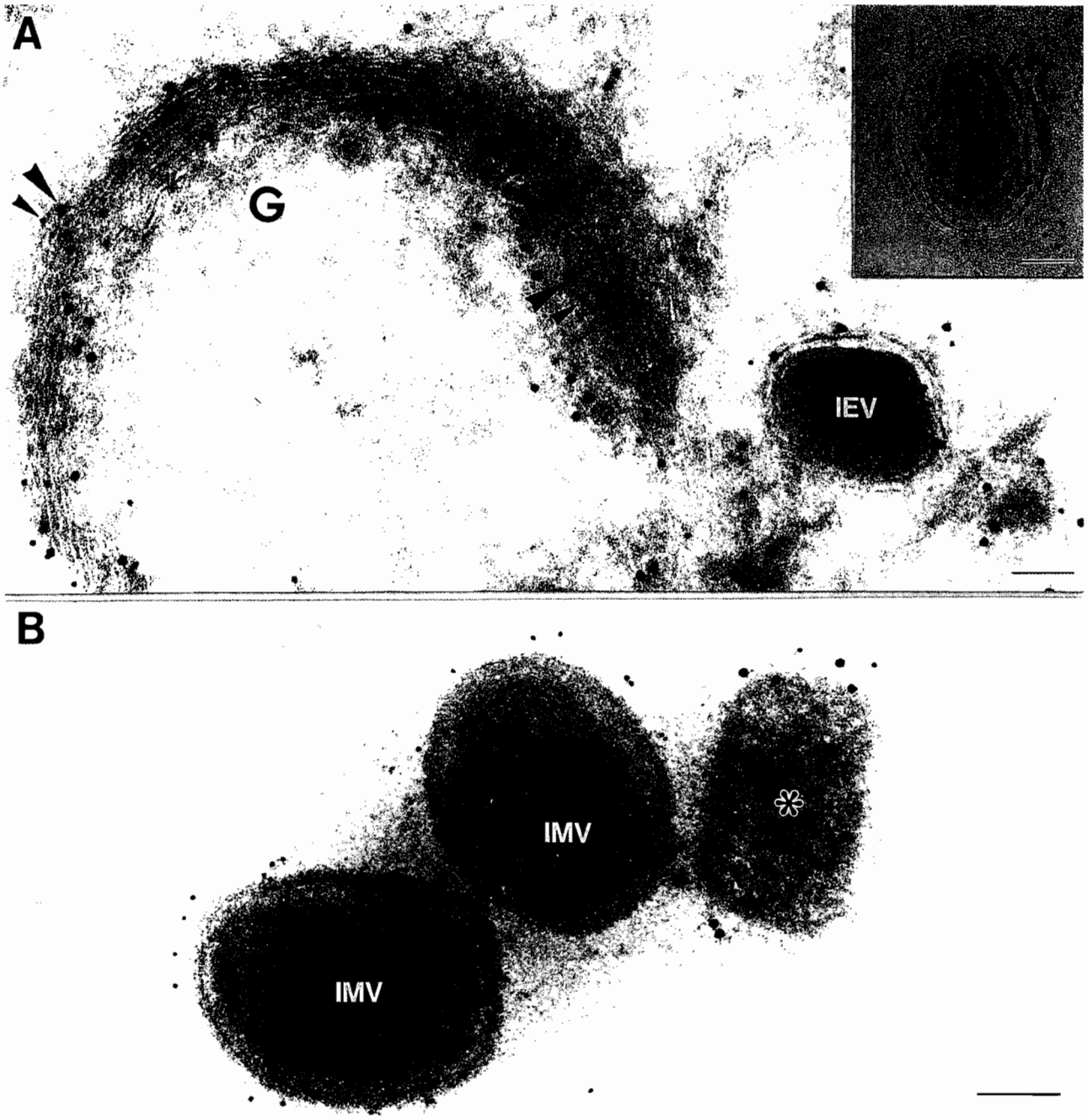


Fig. 7. Thawed cryosections of VV-infected HeLa cells show a clear definition of cellular and viral membranes as well as optimal immunolabeling signals. **A.** Double immunogold labeling for detecting the VV protein p37 (15 nm gold particles, large arrowheads) and the M protein of the coronavirus MHV (9 nm gold particles, small arrowheads), that contains a Golgi-retention motif. Both proteins colocalize in the Golgi complex (G) and a small amount of MHV-M colocalizes with VV-p37 on the outer of the two membranes of the intracellular enveloped virus (IEV). The four membranes of the IEV, very difficult to distinguish in conventional resin sections, are clearly seen in cryosections (inset, arrowheads). **B).** Combination of immunogold labeling and cryomicroscopy of vitrified vaccinia virions. In this double-labeling experiment the two intact intracellular mature virions (IMV) label strongly for the membrane protein p32, while a released viral core (asterisk) labels strongly for the core protein p39. Bars: 100 nm. A, Modified with permission from Schmelz et al., 1994, with copyright permission from the American Society for Microbiology; B, reproduced from Ross et al., 1996, with copyright permission from Oxford University Press.

the study of viral assembly are provided in Fig. 8. The existence of a dramatic morphological transformation between immature and mature virions during the morphogenesis of coronaviruses has been detected by freeze-substitution (Risco et al., 1998). This process was not detected in previous studies using conventional embedding procedures and cryosections (Klumperman et al., 1994). Comparison of conventional with freeze-substituted coronavirus-infected cells demonstrates that freeze-substitution provides a considerably better preservation of virion size and shape (compare Fig. 8A,B) which gives solid ground to confirm the existence of two morphologically distinct viral particles. Structure and immunogold detection of proteins in HIV virions is also significantly improved by freeze-substitution (Fig. 8C,D) in a work by Grief et al. (1994). Size of immature (arrowheads) and mature (arrows) viral particles is very well preserved. Immunolabeling signals using monoclonal antibodies specific for p24 and p6 HIV proteins, are also particularly intense. Heterogeneous preservation of shape and immunogold signals (Fig. 8E) are observed in Lowicryl sections after conventional low-temperature embedding of HIV virions (Risco, unpublished results).

Fig. 9 is a summary of the evolution of embedding procedures over the last years, from conventional methods, (with extensive dehydration) and improved embedding procedures (chemical fixation with multiple fixatives and very short dehydration steps), to freeze-substitution. In all three cases the same structure is shown: a viral factory from VV-infected cells (Risco and Carrascosa, unpublished results). While extraction of components and collapse of the structure is evident after conventional embedding (Fig. 9A), a much better preservation of cytoplasmic elements and the interior of the viral factory is obtained when fixation is more complete and dehydration steps are reduced to a minimum (Fig. 9B). In addition, freeze-substitution provides an improved preservation of fine structural details, such as the small, regularly-spaced spikes of the viral crescents (Fig. 9C).

5. Localization of viral nucleic acid in cells

These methods are of great interest for the study of the incorporation of the viral genome into viral particles, probably the most difficult step to study, from an structural point of view, in the morphogenesis of a virus. The different possibilities for nucleic acid detection on ultrathin sections are basically the following:

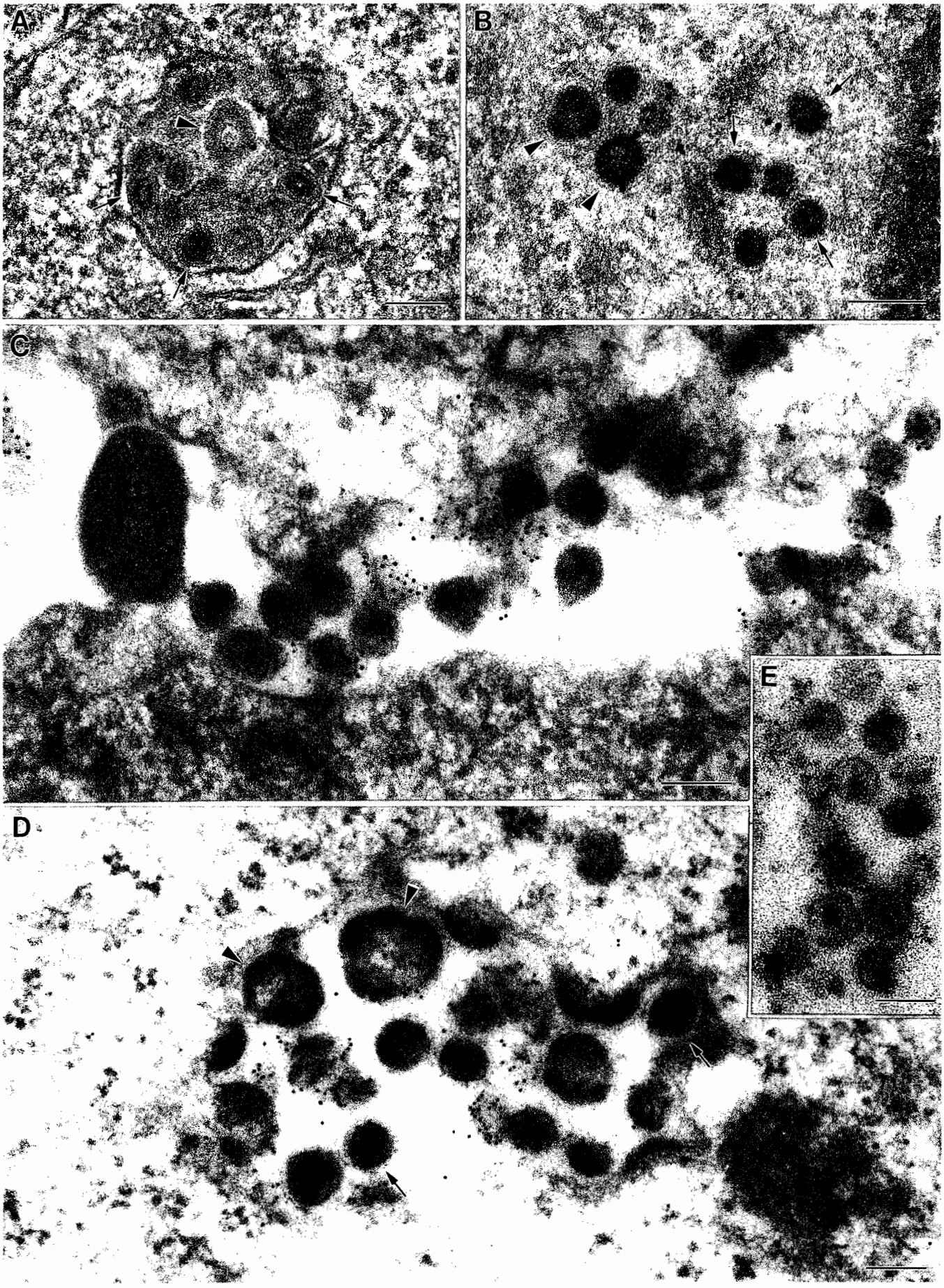
- Immunolabeling methods, based on the same principles as the immunogold detection of proteins.

- The enzymatic approach, developed by Bendayan (1989) for detecting cellular nucleic acids, can be applied to detect viral nucleic acids within viral particles. RNase- and DNase-gold complexes are commercially available and can be used to label RNA and DNA both in acrylic and epoxide sections (Fig. 10A-C).

- *In situ* hybridization with complementary probes, which allows the localization of specific sequences. Molecules of biotin or digoxigenin are incorporated to the probes by nick translation, to be later detected by immunogold (for technical details see Puvion-Dutilleul et al., 1998). The efficiency of hybridization is usually low, although it can be considerably increased by pretreating the sections with a protease (protease + RNase when detecting DNA) prior to hybridization. Excellent results were obtained in extense and elaborated works on adenoviruses and herpesviruses (Fig. 10D-F) developed by Puvion-Dutilleul and Puvion (1989, 1990, 1991) and Puvion-Dutilleul et al. (1998).

- Phosphorus maps. Elemental mapping using X-ray microanalysis or energy filtering, can be applied to the structural study of viruses. In particular, it can be used to selectively detect nucleic acids through the elaboration of phosphorus maps (P-maps), due to the higher content of this element in nucleic acids when compared to proteins (Özel et al., 1990; Harauz et al., 1995; Quintana et al., 1998). Electron Energy Loss Spectroscopy (EELS) possesses higher sensitivity and spatial resolution than X-ray microanalysis for light elements (Leapman and Hunt, 1991; Krivanek et al., 1992) and it has been used to obtain P-maps of nucleic acid-containing structures since 1980 (Ottensmeyer and Andrew, 1980). Although this application of elemental mapping does not allow the localization of specific sequences, it solves the inherent sensitivity problem of the previous three methods described, since the whole amount of nucleic acid contained in the sectioned structure is detected in the P-map. This offers an important advantage when studying, for example, which viral assemblies have already incorporated the genome and which remain empty. It can also be very useful in the characterization of virus-like particles and in the study of major rearrangements that take place in virions during processes of structural maturation (Fig. 10G,H). Special care must be taken to

Fig. 8. Freeze-substitution of infected cells allows a more precise characterization of viral - related assemblies. **(A) and (B)** show Epon sections of intracellular viral particles of a coronavirus (the transmissible gastroenteritis virus, or TGEV) as visualized by conventional embedding procedures (A) or after freeze-substitution in osmium-acetone (B). Preservation of virion size and shape, as well as immunogold signal (a polyclonal anti-TGEV antiserum and a 10 nm gold conjugate were applied in both cases) significantly improved in freeze-substituted samples. Large (arrowheads) and small (arrows) virions are better preserved by freeze-substitution. **(C) and (D)** show HIV-1 viral particles after freeze-substitution in methanol-uranyl acetate and immunogold detection of p24 (C) or p6 (D) viral proteins. In (C) a group of extracellular virions exhibit a strong immunolabeling signal associated to the core protein p24. In (D) immature (arrowheads) and mature (arrows) virions show clear differences in size, structure and distribution of p6 protein. **(E)** shows a group of HIV particles after immunolabeling of p24 on Lowicryl sections after conventional low-temperature embedding. Structural preservation and labeling intensity do not match freeze-substitution results. Bars: 100 nm. A and B, modified from Risco et al., 1998, with copyright permission from the American Society of Microbiology; C and D, reproduced from Grief et al., 1994, with copyright permission from Elsevier Science.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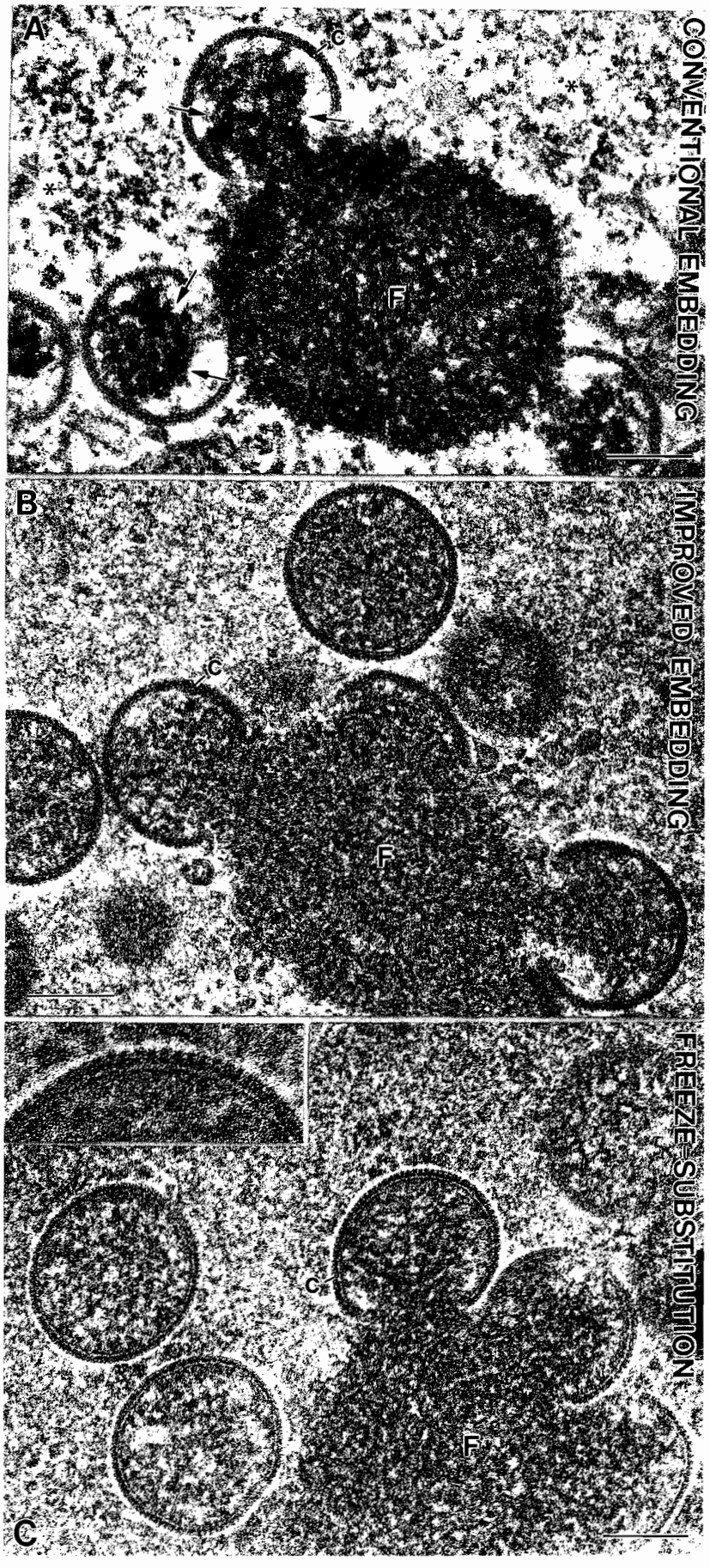
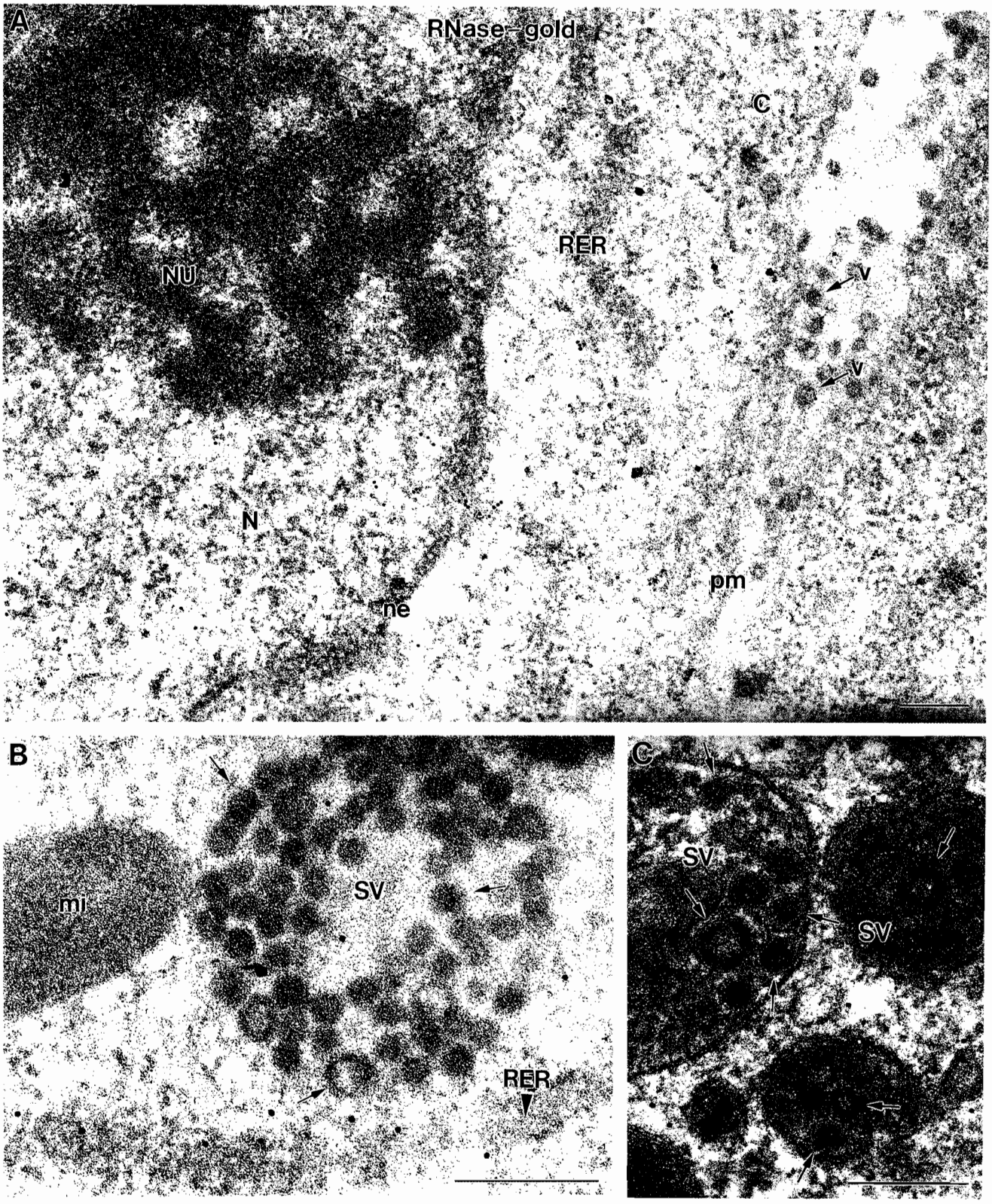


Fig. 9. Epon sections showing the structure of a VV viral factory with characteristic viral crescents (c) as visualize by **(A)** conventional embedding procedures **(B)** improved embedding protocols, and **(C)** embedding after freeze-substitution. In **(A)** dehydration has caused extensive extraction of cytoplasmic components (asterisks) and collapse (arrows) of the content of the viral factory **(F)**. In (B) the short dehydration steps after applying a complete set of fixatives for proteins and lipids (glutaraldehyde, tannic acid, osmium tetroxide, potassium ferricyanide, and uranyl acetate) has allowed a better preservation of cytoplasmic components and the content of the viral factory. In addition, freeze-substitution **(C)** recovers fine structural details, such as the small spikes of the viral crescents (inset), occasionally observed after conventional embedding procedures but very common after freeze- substitution. Bars: 200 nm.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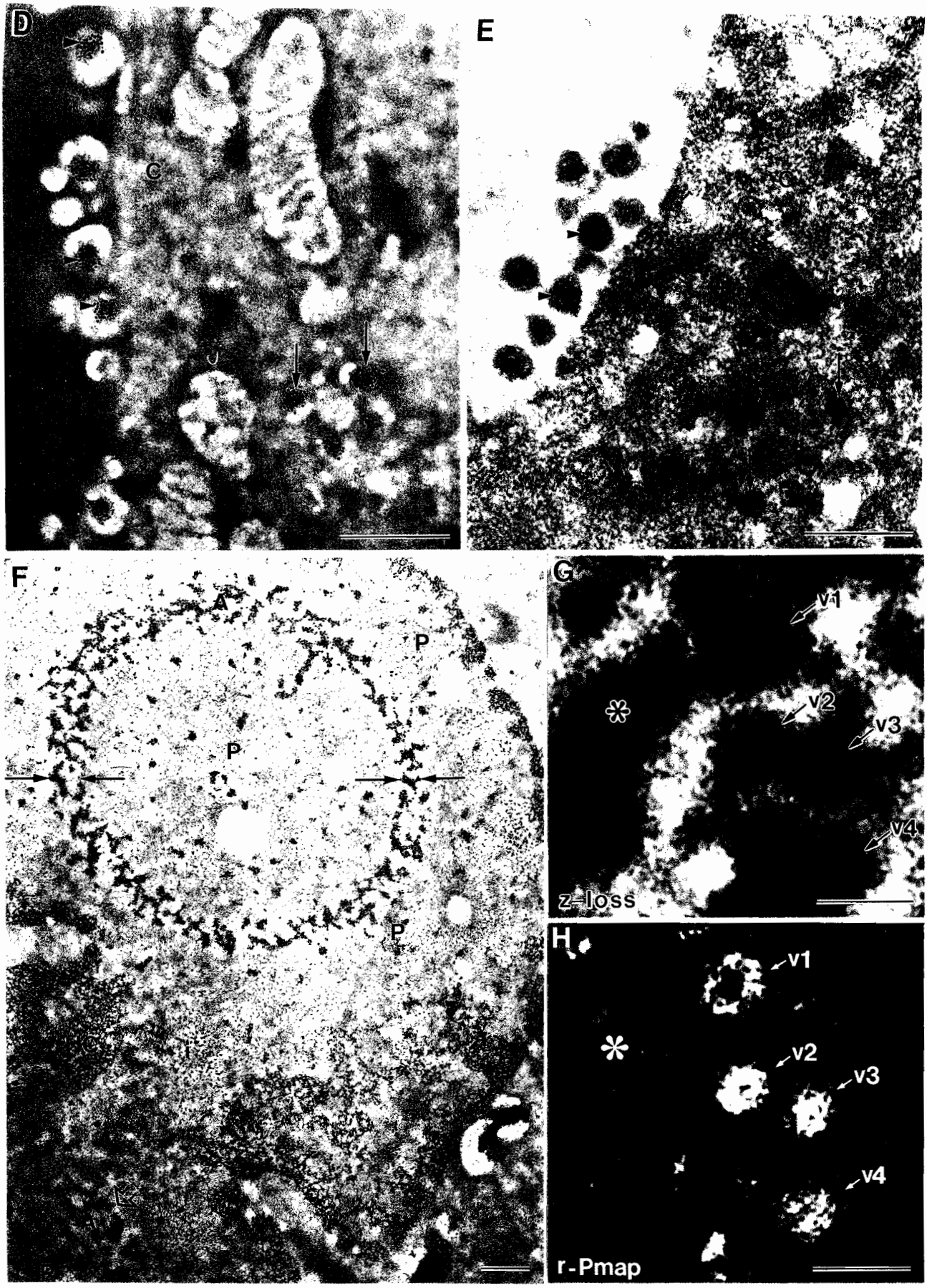
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Fig. 10. Different methods for viral nucleic acid localization at the EM level. **(A) to (C)** show the enzymatic approach, in which an RNase-gold complex was used to localize RNA in immature and mature coronavirions. (A) and (B) correspond to Lowicryl K4M sections, while (C) is an Epon section. The RNase-gold conjugate reacts with RNA-containing cellular structures such as the nucleolus (NU) and the rough endoplasmic reticulum (RER). Both large (immature) and small (mature) coronavirions also specifically react with the conjugate, mainly at the periphery of the viral particles (arrows). **(D) and (E)** show the excellent results of in situ hybridization procedures applied to herpesvirus HSV-1 virions. In this case signals were equally intense with protease-RNase digestion (D) or RNase digestion alone (E) before hybridization with the complementary probe. In both cases, clusters of gold particles decorate nucleoids of intracytoplasmic (arrows) and extracellular (arrowheads) virions. **(F)** Shows the localization of the genome of the adenovirus Ad5 DNA inside the nucleus of an infected cell. Gold particles are most abundant over the clusters of fibrils which constitute the large and pleomorphic ssDNA accumulation sites (A) which here are in the form of rings (arrows) or crescent (empty arrow). Slight labeling is observed at the peripheral replicative zone (P). Double digestion with protease and RNase prior to hybridization allowed a considerable increase in the sensitivity of Ad5 DNA detection. **(G) and (H)** correspond to a filtered (z-loss) image and a reconstituted phosphorus map (rPmap) of the same field (from an unstained Lowicryl HM23 section) containing four TGEV viral particles (marked as v1 to v4). (F) According to the P-map the large viral particle (v1), which corresponds to an immature virion, exhibits a peripheral RNA distribution, an arrangement that changes completely in small mature virions (v2, v3, and v4). The latter exhibit a homogeneous distribution of the P signal inside the smaller core. A segment of contiguous cytoplasm disappears in the P-map (asterisks). c: cytoplasm; N: nucleus; ne: nuclear envelope; pm: plasma membrane; v: virions; SV: secretory vesicle; mi: mitochondrion; HC: host chromatin. Bars represent 200 nm for A-C; 0.5 μm for D-F; 100 nm for G and H. C, reproduced from Risco et al., 1998, with copyright permission from the American Society of Microbiology; D and E are reproduced from Puvion et al., 1990 with copyright permission from Academic Press, Inc.; F from Puvion-Dutilleul and Puvion, 1989 with copyright permission from Gustav Fischer Verlag; G and H from Quintana et al., 1998, with copyright permission from Elsevier Science.

optimize the conditions for sample preparation and to obtain "true P-maps" (Quintana et al., 1998).

In the near future energy filtering transmission electron microscopy (EFTEM) will most probably provide new contributions to the detailed structural analysis of viral assemblies. EFTEM allows ultrastructural analysis to be carried out by selectively using the different types of electrons generated in a transmission electron microscope. For example, zero-loss images are produced by electrons that have not lost any energy and, when using unstained specimens, the images are formed by the mass of the different structural components. These images have a good contrast due to an increase in the signal-to-noise ratio obtained when eliminating the contribution of inelastically scattered electrons, and are obtained in the absence of staining agents, whose interaction with the biological material is not completely understood as yet (Krivanek et al., 1995; Quintana et al., 1998). In combination with cryomethods, these new imaging techniques will get us closer to the native structures than ever before.

6. Concluding remarks

The study of viral infection has had an obvious main interest in pathology and the search for effective therapeutic treatments. But at the same time, the study of the multiple aspects related to the interaction between viruses and the cells they parasite has provided unique contributions to the knowledge of the cell itself: the mechanisms of protein processing and modification, the pathways of intracellular transport and signaling, the processing of messenger RNAs, among others, have obtained major benefits from the study of viruses. Today, as we get to know about how viruses are built and work, new tools are emerging to use them in our benefit. New vectors and virus-like particles of defined composition can be used for specific delivery of genes, as safe and efficient vaccines, or as protein cages for the controlled polymerization of materials (Jolly, 1994; Roy, 1996; Douglas and Young, 1998). To make all this possible, the contribution of the structural analysis is fundamental. In some cases, the definition of the basic structure and assembly pathway of a virus have been followed by high-resolution structural studies through cryoelectron microscopy, electron crystallography and X-ray diffraction, to reach atomic or near atomic resolution. Viruses help to understand how macromolecular assemblies work, and this has wide applications in many areas of biology.

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