Post-translational glycosylation of coronavirus glycoprotein E1: inhibition by monensin

H. Niemann*, B. Boschek, D. Evans†, M. Rosing, T. Tamura, and H.-D. Klenk

Institut für Virologie, Fachbereich Humanmedizin der Justus-Liebig-Universität Gießen, Frankfurter Str. 107, D-6300 Gießen, FRG

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The intracellular sites of biosynthesis of the structural proteins of murine hepatitis virus A59 have been analyzed using cell fractionation techniques. The nucleocapsid protein N is synthesized on free polysomes, whereas the envelope glycoproteins E1 and E2 are translated on the rough endoplasmic reticulum (RER). Glycoprotein E2 present in the RER contains N-glycosidically linked oligosaccharides of the mannose-rich type, supporting the concept that glycosylation of this protein is initiated at the co-translational level. In contrast, O-glycosylation of E1 occurs after transfer of the protein to smooth intracellular membranes. Monensin does not interfere with virus budding from the membranes of the endoplasmic reticulum, but it inhibits virus release and fusion of infected cells. The oligosaccharide side chains of E2 obtained under these conditions are resistant to endoglycosidase H and lack fucose suggesting that transport of this glycoprotein is inhibited between the trans Golgi cisternae and the cell surface. Glycoprotein E1 synthesized in the presence of monensin is completely carbohydrate-free. This observation suggests that the intracellular transport of this glycoprotein is also blocked by monensin.

Key words: O-glycosylation/coronavirus/membrane glycoproteins/monensin

Introduction

Most of the viral glycoproteins that have been analyzed in detail, e.g., those of influenza virus, vesicular stomatitis virus, and alphaviruses, contain N-glycosidic carbohydrate-protein linkages. Studies of these virus systems have contributed to our understanding of the biosynthesis of this class of glycoproteins (for review, see Klenk and Rott, 1980). Some characteristic features are co-translational glycosylation in the rough endoplasmic reticulum (RER), transport through the Golgi apparatus to the budding site which is usually the plasma membrane, and trimming of the mannose-rich side chains and readdition of sugar moieties in the Golgi apparatus (Struck and Lennarz, 1980).

Only a few viral glycoproteins with O-glycosidic linkages are known. These are the vaccinia virus hemagglutinin (Shida and Dales, 1981), herpes virus glycoproteins (Olofsson et al., 1981), the penton fiber of adenoviruses (Ishibashi and Maizel, 1974), and glycoprotein E1 of coronaviruses. The O-glycosidic nature of the carbohydrate-protein linkages in the latter glycoprotein has been established by analyzing the sugar composition and the alkali lability of the oligosaccharide side chains and by the observation that glycosylation of E1 is not

*Present address: John Innes Institute, Colney Lane, Norwich NR4 7LU, UK.
†To whom reprint requests should be sent.

inhibited by tunicamycin (Niemann and Klenk, 1981a, 1981b; Holmes et al., 1981; Rottier et al., 1981). Despite the fact that a large variety of secretory and membrane proteins of great biological interest are glycoproteins with glycosidic linkages, intracellular transport and processing of these glycoproteins are still only poorly understood. Coronaviruses are therefore a useful system to study the biosynthesis of this type of membrane glycoproteins.

There is yet another aspect that makes coronaviruses an interesting model for membrane studies. Whereas most enveloped viruses mature at the plasma membrane of the host cell, coronaviruses are assembled by budding from intracellular membranes of the RER and of cytoplasmic cisternae (Becker et al., 1967). On the basis of similar observations Holmes and Behnke (1981) suggested that the virions are transported from the RER through the Golgi apparatus to smooth walled vesicles that fuse with the plasma membrane, thereby releasing the virus into the extracellular space. Thus, there are analogies in coronavirus maturation and in secretory processes, and the coronavirus envelope may become useful as a molecular probe for mechanisms involved in secretion.

The present study was undertaken to throw light on the intracellular sites of translation and processing of the structural proteins of the murine hepatitis virus (MHV) A59. Particular emphasis was given to the analysis of the glycosylation site of glycoprotein E1.

Results

Translation sites of virus-specific proteins

Three structural polypeptides have been identified with MHV A59 (Sturman et al., 1980). The phosphorylated core protein N (50 K) (Stohlman and Lai, 1979), the plenomorphic spike glycoprotein E2, which exists in the mature virus in two forms designated E2 (180 K) and E2 (90 K), and the glycosylated matrix protein E1 (23 K).

To study the site of biosynthesis of these structural components, sister cultures of MHV A59-infected 17Cl1 cells (multiplicity of infection: 50 p.f.u./cell) were pulse-labeled with [3H]leucine or [3H]uridine for 1 h or 5 h, respectively, prior to cell fractionation at 16 h post-infection. Cell fractionation was carried out according to Friedman et al. (1972). After removal of the cell nuclei, eight fractions were obtained by two consecutive isopycnic centrifugation steps. Electron microscopic inspection (data not shown) in combination with the determination of 5'-nucleotidase, glucose-6-phosphatase, galactosyl-transferase, and β-glucuronidase as marker enzymes for plasma membranes, endoplasmic reticulum, Golgi apparatus, and lysosomes, respectively, revealed that fractions 2, 3, and 4 are derived from the smooth endoplasmic reticulum and from the plasma membrane with fraction 4 being enriched in Golgi apparatus and lysosomes. Fractions 6 and 7 contain RER, and fraction 8-free polysomes. Fraction 5 contains a mixture of both smooth and rough membranes. Fraction 1 consists predominantly of fat droplets.

mRNA was isolated from the free ribosomal fraction 8 and the rough microsomai fraction 7 and translated in reticulocyte
lysates in the presence of [35S]methionine (850 Ci/mmol). Virus-specific products were immunoprecipitated using a hyperimmune rabbit serum raised against purified virus (Niemann and Klenk, 1981b). These samples were analyzed on SDS-polyacrylamide gels together with products which were obtained by direct immunoprecipitation of the corresponding [3H]leucine-labeled cell fractions after lysis in RIPA buffer (see Materials and methods). Figure 1, lanes B and F, shows that the only coronavirus-specific polypeptide translated on free ribosomes is the core protein N. Both the spike glycoprotein E2 and the matrix glycoprotein E1 are synthesized on membrane-associated ribosomes (lanes C and G). The in vitro translation product of E2 (lane C) co-migrates with the non-glycosylated form of E2 (lane D), that was obtained from infected cell lysates pulse-chase-labelled in the presence of tunicamycin. The in vitro translation (lane C) as well as the immunoprecipitation of the rough microsomal fraction (lane G) yielded the 20-K species of E1 but not the 23-K species, which is also formed in the presence of tunicamycin (lane D) (Niemann and Klenk, 1981b).

Post-translational modifications of coronavirus-glycoproteins E1 and E2

To verify that glycosylation of E1 is a post-translational event and to ascribe this process to a specific intracellular compartment we pulse-chase-labeled infected 17Cl1 cells (10 min pulse, various lengths of chase) with [3H]leucine prior to cell fractionation. The data obtained by immunoprecipitation of the lysed cell fractions indicated that labeled glycoproteins E1 and E2 were detected in smooth membranes 10–15 min after the pulse. After 60 min of chase most of the labeled material was present in the smooth membrane fraction. By this time the 20-K species of E1 was processed into the 23-K species (data not shown).

Figure 2 demonstrates that this increase in mol. wt. is due to glycosylation. In this experiment we labeled MHV A59-infected cells for the times indicated with tritiated leucine or with the tritiated sugars mannose, glucosamine, and galactose prior to cell fractionation. A comparison of the rough microsomal fraction 7 (left side) with the smooth membrane fraction 4 (right side) shows that both glucosamine and galactose were incorporated into E1 (23 K) only in the latter membrane preparation. The absence of any mannose in E1 in either of the two fractions is in agreement with the previous carbohydrate consistent analysis of glycoprotein E1 isolated from purified coronavirus preparations (Niemann and Klenk, 1981a).

Recent permethylation analyses of carbohydrate side chains released from glycoprotein E1 by β-elimination have shown that the first sugar residue being added to the protein backbone is N-acetylgalactosamine (Niemann, Geyer, and Klenk, in preparation). When the various cell fractions were analyzed for N-acetylgalactosaminyl transferase activity (see Materials and methods) it was found that only fraction 4 was able to convert 14C-labeled UDP-N-acetylgalactosamine into an immunoprecipitable form (Table I). This experiment together with the pulse-chase labeling studies supports the notion that the entire sugar moiety of E1 is attached after the transfer of the polypeptide to smooth internal membranes.

Table I. Characterization of cell fractions obtained from A59-infected 17Cl1 cells

| Fraction number | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
|-----------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Density (g/ml)  | 1.020 | 1.054 | 1.081 | 1.130 | 1.158 | 1.190 | 1.297 | 1.316 |
| Protein (%)     | 1.1   | 2.8   | 16.7  | 12.4  | 13.5  | 20.4  | 22.5  | 1.4   |
| Poly(A)* RNA (c.p.m.) (%) | n.d. | n.d.  | 216   | 1236  | 452   | 14597 | 172614 | 13609 |
| 5'-Nucleotidase (µM P1/h/mg protein) | n.d. | 0.293 | 0.682 | 0.421 | 0.404 | 0.321 | 0.246 | n.d. |
| Glucose-6-phosphatase (µM P1/h/mg protein) | n.d. | 0.082 | 0.250 | 0.105 | 0.355 | 0.520 | 0.635 | n.d. |
| β-Glucuronidase (Δ OD450/h/mg protein) | n.d. | 0.024 | 0.036 | 0.260 | 0.039 | 0.007 | 0.007 | n.d. |
| Galactosyl transferase (c.p.m./mg protein) | n.d. | 7.5 x 10^3 | 1.0 x 10^4 | 2.2 x 10^8 | 2.7 x 10^8 | 1.4 x 10^8 | 1.3 x 10^8 | n.d. |
| N-Acetylgalactosaminyl transferase (c.p.m./mg protein) | n.d. | 104   | 287   | 1276  | 257   | 291   | 181   | n.d. |
The data shown in Figure 2 demonstrate also that the 90-K form of E2 is found predominantly in smooth membranes and that it is only poorly labeled with tritiated mannose, although this sugar is readily incorporated into the 180-K precursor. These observations are compatible with the general concept that N-glycosidic oligosaccharides are trimmed in the Golgi apparatus and they support the notion that proteolytic cleavage of E2 is also a post-translational event (Sturman, 1981).

**Effect of monensin on the biosynthesis of E1 and E2**

To further substantiate the essential role of intracellular transport for the processing of both coronavirus glycoproteins we have analyzed the effects of the ionophore monensin. Evidence has been obtained with some glycoproteins that this compound interferes with the transfer in the Golgi apparatus (Tartakoff and Vassalli, 1979). Figure 3 shows that, in the presence of 5 μM monensin, glycosylation of E1 is inhibited. Since the energy charge of monensin-treated cells was not significantly reduced (90% of control cells) and cell fractionation experiments performed on monensin-treated infected cells indicated that E1 was transported into smooth membranes under such conditions (data not shown), inhibition of glycosylation of E1 was not due to an arrest of this polypeptide in the RER.

Figure 3 also demonstrates that, in the presence of monensin, cleavage of E2 is inhibited, whereas incorporation of sugars, with the exception of fucose, still occurs. The incorporation of galactose was further substantiated by acid hydrolysis (1 N HCl, 4 h, 100°C) of [3H]galactose-labeled material isolated by immunoprecipitation and preparative SDS-PAGE. About 90% of the label applied for paper chromatography co-migrated with a galactose standard (data not shown).

To study the influence of monensin on the processing of the carbohydrate side chains of glycoprotein E2, we prepared glycopeptides of material isolated by immunoprecipitation from monensin-treated infected cells and compared it to material obtained in a similar manner from the rough microsomal fraction of untreated infected cells as well as from mature virus particles (Figure 4). Essentially all the [3H]-mannose-labeled E2 glycopeptides derived from the RER were sensitive to treatment with endo-β-N-acetylgalactosaminidase H (endoglycosidase H) (panel A). The majority of released oligosaccharides eluted in the position of Man₆GlcNAc and Man₆GlcNAc. In the presence of monensin, ~60% of the E2 side chains had gained endoglycosidase H-resistance indicating a partial conversion of the mannosereich side chains added co-translationally in the RER into complex type side chains (panel B). Treatment of these complex type glycopeptides with neuraminidase from *Vibrio cholerae* did not alter the mobility of the peak fraction on the calibrated Biogel P4 column (data not shown). In glycoprotein E2 derived from mature coronaviruses, ~75% of mannoselabeled carbohydrate side chains were resistant to treatment with endoglycosidase H (Figure 4, panel C). The mannosereich side chains were heterogeneous when sized on the Biogel column, eluting in positions of Man₆GlcNAc to Man₆GlcNAc.

To find out whether monensin interferes with coronavirus maturation, infected cells were inspected with the electron microscope. Figure 5 demonstrates that the budding process is not inhibited by monensin. In fact, if compared to untreated cells (Figure 5A), in the presence of monensin, virions appear to accumulate in the canaliculari of the RER (Figure 5B). Whereas monensin had little effect on the structure of the RER, the regular stacks of the Golgi apparatus had disappeared to give rise to the large vacuoles that are commonly

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**Fig. 2.** Sugar incorporation into viral proteins in different cell fractions. Cultures of 17C11 cells were infected with MHV A59 and grown in medium containing 10 mM sodium pyruvate instead of glucose to enhance the uptake of radiolabeled sugars. 16 h post-infection, [3H]Uricine (Leu, 10 μCi/ml, 65 Ci/mmol), 6-[3H]glucosamine (GlcN, 50 μCi/ml, 36 Ci/mmol), 2-[3H]mannose (Man, 50 μCi/ml, 18 Ci/mmol) or 1-[3H]galactose (Gal, 50 μCi/ml, 35 Ci/mmol) were added to the growth medium for either 15 or 60 min. Cells were fractionated and virus-specific glycoproteins from the rough microsomal fraction 7 (left side) and smooth membrane fraction 4 (right side) analyzed as described in the legend to Figure 2.

**Fig. 3.** Effects of monensin on the glycosylation of coronavirus polypeptides E1 and E2. Monolayers of 17C11 cells were infected with MHV A59. After the 1 h adsorption period of the virus, glucose-depleted pyruvate medium (10 mM sodium pyruvate) containing monensin (5 μM) was added and cells were kept in the presence of the ionophore throughout the experiment. 16 h post-infection, cultures were pulse-labeled for 3 h as described in the legend to Figure 3. In addition, 1-[3H]fucose (Fuc) was applied at 50 μCi/ml (8 Ci/mmol). Cells were then lysed and samples prepared for SDS-PAGE by immunoprecipitation as described before.
observed in cells treated in this way. These vacuoles contained relatively little released virus, but virus budding in various stages of completeness could frequently be observed (Figure 5C). Experiments with the aim of reversing the effects of monensin were unsuccessful since virus was not released from cells when the inhibitor was washed out 16 h post-infection. However, a discharge of virus particles from the RER was observed paralleled by an accumulation of particles into large vacuoles. Many of these particles had lost their characteristic structure suggesting that degradation had started (Figure 5D).

Discussion

We have analyzed the glycosylation of the envelope proteins of MHV A59 at various stages of co-translational and post-translational processing. Previous studies (Niemann and Klenk, 1981a, 1981b), have shown that E1 and E2 belong to different classes of glycoproteins which can be distinguished by several structural and biosynthetic aspects. E2 carries exclusively N-glycosidic carbohydrate protein linkages, it undergoes post-translational proteolytic cleavage and acylation. The oligosaccharide side chains of E1, in contrast, are linked by O-glycosidic bonds. E1 is not acetylated, and post-translational proteolytic cleavage has not been observed. Cells infected with MHV A59 offer, therefore, an ideal system to study the temporal and spatial separation of N- and O-glycosylation. The data presented here demonstrate that the nucleocapsid protein N is translated on free ribosomes as is the case with many other virus systems, while both glycoproteins E1 and E2 are synthesized on membrane-associated ribosomes.

We have previously reported that E2 has N-glycosidic carbohydrate-protein linkages (Niemann and Klenk, 1981a). It was, therefore, not surprising to find that E2 present in RER contains oligosaccharide side chains of the mannose-rich type indicating that glycosylation of this glycoprotein is a co-translational event.

Evidence has been presented in the literature that glycosylation of proteins with O-glycosidic bonds is also initiated on the nascent polypeptide (Strous, 1979; Sugahara et al., 1981). Our data, however, demonstrates that O-glycosylation of coronavirus glycoprotein E1 is a post-translational event occurring after the transfer of the polypeptide to the Golgi apparatus. This is indicated by the finding that the glycosylated form of E1 is found only in the smooth membrane fraction. Furthermore, N-acetylgalactosaminyl transferase, the enzyme responsible for linking the first sugar of the side chain to the polypeptide, is present only in the smooth membrane fraction. No activity was found in the rough microsomal fraction.

Monensin has found wide application in recent years as an inhibitor of glycoprotein transport. Although there is increasing evidence that this compound has multiple sites of action, with some glycoproteins, such as the G protein of vesicular stomatitis virus (Johnson and Schlesinger, 1980) and the glycoproteins of Semliki Forest virus (Kääriäinen et al., 1980) and Sindbis virus (Strous and Lodish, 1980), the block in transport appears to be localized somewhere within the Golgi apparatus. Since the glycoproteins of these viruses do not reach the plasma membrane in the presence of the drug, virus assembly is inhibited. Coronavirus buds from the membrane of the RER. It is therefore not surprising that their budding is not inhibited through monensin. Virions are present in large amounts in the canaliculi of the RER. It is interesting to note that infected cells did not fuse in the presence of the inhibitor. This indicates that glycoprotein E2 carrying the fusion capacity of the virus (Holmes et al., 1981; Collins et al., 1982) does not reach the cell surface.

The transport block by monensin has consequences for the glycosylation of both coronavirus glycoproteins. The observations that co-translational glycosylation of glycoprotein E2 is not inhibited and that the majority of the oligosaccharide side chains obtained under these conditions have lost their sensitivity to endoglycosidase H further support the concept that the transport of E2 is arrested between the trans Golgi cisternae, where complex oligosaccharides are constructed (Roth and Berger, 1982) and the cell surface. Incomplete synthesis of N-glycosidic carbohydrate side chains was also observed, when the effects of monensin on the glycoproteins of Semliki Forest virus were analyzed (Pesonen and Kääriäinen, 1982). Interestingly, glycoprotein E1 is completely carbohydrate-free when synthesized in the presence of monensin. This finding is compatible with the view that the inhibitor prevents the transport of E1 to its glycosylation site. We cannot, however, exclude the possibility that enzymes responsible for adding O-linked oligosaccharides may be inhibited by monensin. Since all evidence suggests that monensin acts on Golgi not endoplasmic reticulum membranes this would support our cell fractionation results suggesting that O-linked sugars are added in the Golgi. It is not yet possible to localize exactly this glycosylation site.

Although monensin does not inhibit budding of coronavirus, it interferes with virus release from the cell. Our data suggest that virions are trapped within the RER and that they are degraded even after removal of the inhibitor. The block in

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Fig. 4. Gel chromatography profiles of endoglycosidase H-treated glycopeptides of coronavirus glycoproteins E2 isolated from the rough microsomal fractions (panel A), monensin-treated cells (B), and mature virus (C). Infected cultures were labeled with 2-[^3]H]mannose as described for Figures 2 and 3. Immunoprecipitated glycoprotein E2 was isolated by preparative SDS-PAGE, eluted from the gels and digested with pronase, desalted and digested with endoglycosidase H. Samples were analyzed on a calibrated Biogel P4 (minus 400 mesh) column (200 x 1 cm). Elution was carried out with water containing 0.02% NaN3. Fractions of 0.5 ml were taken and assayed by liquid scintillation counting. I: complex type glycopeptides. II: endoglycosidase H-sensitive oligosaccharides. Bovine serum albumin was used to detect the void volume (V0), and arrows indicate the positions of marker oligosaccharides Man5GlcNAc (1) and Man5GlcNAc (2) kindly provided by R. Datema.
Post-translational glycosylation of coronavirus glycoprotein E1

Virus release could be the result of the substantial destruction of the cell architecture that occurs in the presence of monensin. Alternatively, virions may not be secreted from the cells, because their glycoproteins are either not or incompletely glycosylated. The carbohydrates would then have a signal function for the exocytosis of the virus. In any case, the data indicate that the Golgi apparatus plays an important role in the release of the virus from the cell.

Fig. 5. Electron micrographs of MHV A59-infected 17C11 cells. (A) Cells were infected at a multiplicity of infection of 50 p.f.u./cell and fixed in situ 16 h post-infection (for details see Koennecke et al., 1981). Virus particles are seen in the RER, in inclusions of the nuclear membrane (NM), in transient elements of the Golgi apparatus (G), and occasionally in coated vesicles (CV). (Magnification x 26 000). (B) Cells 16 h after infection to which monensin was added after the 1 h adsorption period of the virus. The Golgi apparatus is drastically dilated and mitochondria (M) have a more electron-dense appearance. While no virus particles were found outside the cell and within small smooth walled vesicles, the RER is loaded with particles. (Magnification x 18 000). (C) Cells were treated as in (B), but monensin was washed out of the culture medium 16 h post-infection. Cells were fixed 40 min after release of the monensin block. Various stages of virus budding (arrow heads) into smooth walled vacuoles derived from the Golgi apparatus can be seen. (Magnification x 27 000). (D) The same cultures as shown in (C). A time-dependent discharge of virus particles from the RER was observed into vacuoles (Vac), which was almost completed at 40 min after the release of the monensin block. Note the reduced diameter and diffuse appearance of virus particles in these vacuoles. (Magnification x 17 000). Bars represent 500 nm.
Materials and methods

Materials
Radionucleotides compounds used in these studies were purchased from Amer- sham and applied at concentrations as described previously (Niemann and Klenk, 1981b). Monensin was from Calbiochem (San Diego, CA). Tunicamycin was obtained from Eli Lilly (Indianapolis, IN). Adenosine-5'-monophosphate and glucose-6-phosphate were from Boehringer (Mannheim, FRG). Triton X-100 and phenolphthalein-β-D-glucuronic acid from Sigma. Endo-β-N-acetylglucosaminidase H (Streptomyces griseus) was purchased from Seikagaku Kogyo (Tokyo, Japan), and trypsin was obtained from Wor- thington. Neuraminidase (Vibrio cholerae) was purchased from Behringwerke (Marburg, FRG). Oligo(dT)\_20\_m cells was from Bethesda Research Laboratories.

Viruses and cells
Mouse hepatitis virus A59 was grown in 17Cl1 cells as described previously (Niemann and Klenk, 1981b).

Cell fractionation
Fractionations of infected cells (multiplicity of infection: 50 p.f.u./cell) were performed according to Caligiuri and Tammi (1970). Washed cells were pre-swollen for 20 min at 0°C in reticulocyte standard buffer (RSB) (10 mM Tris/HCl, pH 7.4; 10 mM KCl; 1.5 mM MgCl₂ and disrupted by 20 strokes in a tight-fitting Dounce homogenizer. The post-nuclear fraction was adjusted to 0.2% sodium deoxycholate buffer, pH 6.7, containing 2% (v/v) Triton X-100 and 20 mM MnCl₂. To these mixtures 50 μl aliquots (200 μg protein) of cell fraction number 7 (rough microsomal fraction) of infected cells were added in 0.1 M cacodylate buffer, pH 6.7, containing 1% Triton X-100 and 10 mM MnCl₂ and 20 μCi/ml [35S]cysteine (61 Ci/mmol). The reaction was carried out for 3 h at 37°C. Samples were then mixed with the same volume of RIPA buffer and subjected to immunoprecipitation. Transferase activity was expressed in immunoprecipitable c.p.m./mg protein in the cell fraction from non-infected cells.

Immunoprecipitation
For immunoprecipitation, cells or cell fractions were lysed at 0°C in RIPA buffer (50 mM Tris/HCl, pH 7.2; 10 mM EDTA; 1% (v/v) Triton X-100; 1% (w/w) sodium deoxycholate; 0.1% (w/v) SDS) and 5% (v/v) trisylol and processed as previously described (Niemann and Klenk, 1981b).

Determination of the energy charge of infected cells
The concentrations of ATP, ADP, and AMP of cells were determined after metabolic labeling with tritiated adenine, extraction with perchloric acid, and paper chromatography according to Cali-Lazar et al. (1978).

Isolation and in vitro translation of mRNA
mRNA was isolated from [3H]uridine-labeled cell fractions by extraction with hot phenol followed by ethanol precipitation and purification on oligo(dT)\_25\_m cellulose. mRNA preparations with an absorption ratio OD_260/OD_280 > 1.9 were used for the in vitro translation in the messenger-dependent reticulocyte lysate (Amersham). Usually the translation mixture (20 μl) contained 1–2 μg of poly(A)\_x RNA and 10 μCi [35S]methionine (Amersham, 1280 Ci/mmol). The reaction was performed at 30°C for 30 min stopped by the addition of 60 μl of RIPA buffer and samples were subjected to immunoprecipitation.

Electron microscopy
Thin sections of infected 17Cl1 cells or of pellets of cell fractions were prepared as previously described (Koennecke et al., 1981).

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