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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 263, No. 29, Issue of October 15, pp. 14956-14963, 1988
Printed in U.S.A.

Membrane Integration and Intracellular Transport of the Coronavirus Glycoprotein E1, a Class III Membrane Glycoprotein*

(Received for publication, February 23, 1988)

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The E1-glycoprotein (M_r = 26,014; 228 amino acids) of mouse hepatitis virus A59 is a class III membrane glycoprotein which has been used in this study as a model system in the study of membrane integration and protein transport. The protein lacks an NH_2-terminal cleavable signal sequence and spans the viral membrane three times. Hydrophobic domains I and III could serve as signal sequences for cotranslational membrane integration. Domain I alone was sufficient to translocate the hydrophilic NH_2 terminus of E1 across the membranes as evidenced by glycosylation of a newly introduced *N*-glycosylation site. The COOH-terminal part of E1 involving amino acids Leu^{124} to Thr^{228} was found to associate tightly with membranes at the post-translational level, although this part of the molecule lacks pronounced hydrophobic sequences. Membrane protection assays with proteinase K showed that a 2-kDa hydrophilic fragment was removed from the COOH terminus of E1 indicating that the protein is largely embedded into the membrane. Microinjection of *in vitro* transcribed capped and polyadenylated mRNA into CV-1 cells or into secretory AtT20 pituitary tumor cells showed that the E1-protein accumulated in the Golgi but was not detectable at the plasma membrane or in secretory granules. The 28 NH_2-terminal hydrophilic amino acid residues play no role in membrane assembly or in intracellular targeting.

Various NH_2-terminal portions of E1 were fused to Ile^{145} of the cytoplasmic N-protein of mouse hepatitis virus. The resulting hybrid proteins were shown to assemble into membranes *in vitro* and were detected either in the rough endoplasmic reticulum or transient vesicles of microinjected cells.

Membrane proteins have been divided into three groups based on their specific orientation in the membrane (Wickner and Lodish, 1985; Garoff, 1985). According to this classification of E1-glycoprotein of MHV[1] A59 belongs to the group III proteins which span a membrane several times (Armstrong *et al.*, 1984; Rottier *et al.*, 1986). The E1-protein has three functional domains. The ectodomain representing the 28 NH_2-terminal amino acids is hydrophilic and carries exclusively *O*-linked oligosaccharides which exhibit, in conjunction with the terminal amino acid sequence Ser-Ser-Thr-Thr-, blood group M activity (Niemann *et al.*, 1984b). A hydrophilicity analysis of E1 according to Kyte and Doolittle (1982) reveals four internal hydrophobic stretches (Fig. 1) that span the viral membrane three times and presumably contribute to the rigidity of the viral membrane. The carboxyl-terminal part of E1 interacts with the viral nucleocapsid and thus plays an important role in the stages of virus formation (Sturman *et al.*, 1980).

Cell fractionation studies of MHV A59-infected cells indicated that the E1 protein was synthesized on membrane-associated polysomes (Niemann *et al.*, 1982). In contrast to most other viral glycoproteins the E1 protein could not be detected at the plasma membrane of infected cells other than in the form of virus particles. The intracellular distribution of E1 was restricted to perinuclear regions (Doller and Holmes, 1980) and thus paralleled the sites at which budding of coronavirus particles was observed at early stages of infection (Becker *et al.*, 1967; Holmes *et al.*, 1981; Tooze *et al.*, 1984). Recent studies showed that this intracellular accumulation of the E1-protein in smooth vesicles is not due to an interaction of E1 with other coronavirus proteins but is an integral feature of the E1-protein itself (Machamer and Rose, 1987; Rottier and Rose, 1987; Niemann *et al.*, 1987).

In this study we have used *in vitro* transcription/translation and microinjection techniques in combination with indirect immunofluorescence to study the membrane assembly process and the transport properties of the E1-polypeptide in more detail. We show that the E1-protein accumulates in perinuclear regions of fibroblasts and secretory cells. Based on the expression of various E1-mutants we show that deletions or additional *N*-glycosylation of the amino-terminal domain of E1 do not effect the Golgi-specific transport block. Internal hydrophobic domains I and III could mediate cotranslational integration of the polypeptide into microsomal membranes. An E1-mutant lacking all three hydrophobic domains associates with membranes also post-translationally. We show that fusion proteins between various parts of the E1 and a cytoplasmic protein integrate into membranes cotranslationally and accumulate in membranes of the RER and perinuclear vesicles.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Experimental Strategy and Construction of Mutants of the E1-gene of MHV A59—To study the membrane assembly

* This work was supported by Grant Nie 175/5 from the Deutsche Forschungsgemeinschaft and by Fonds der Chemischen Industrie. This work is part of the Ph.D. thesis of T. M. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MHV, mouse hepatitis virus; RER, rough endoplasmic reticulum; ACTH, adrenocorticotropic hormone; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate.

[2] Portions of this paper (including "Experimental Procedures," Fig. 9, and one table) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

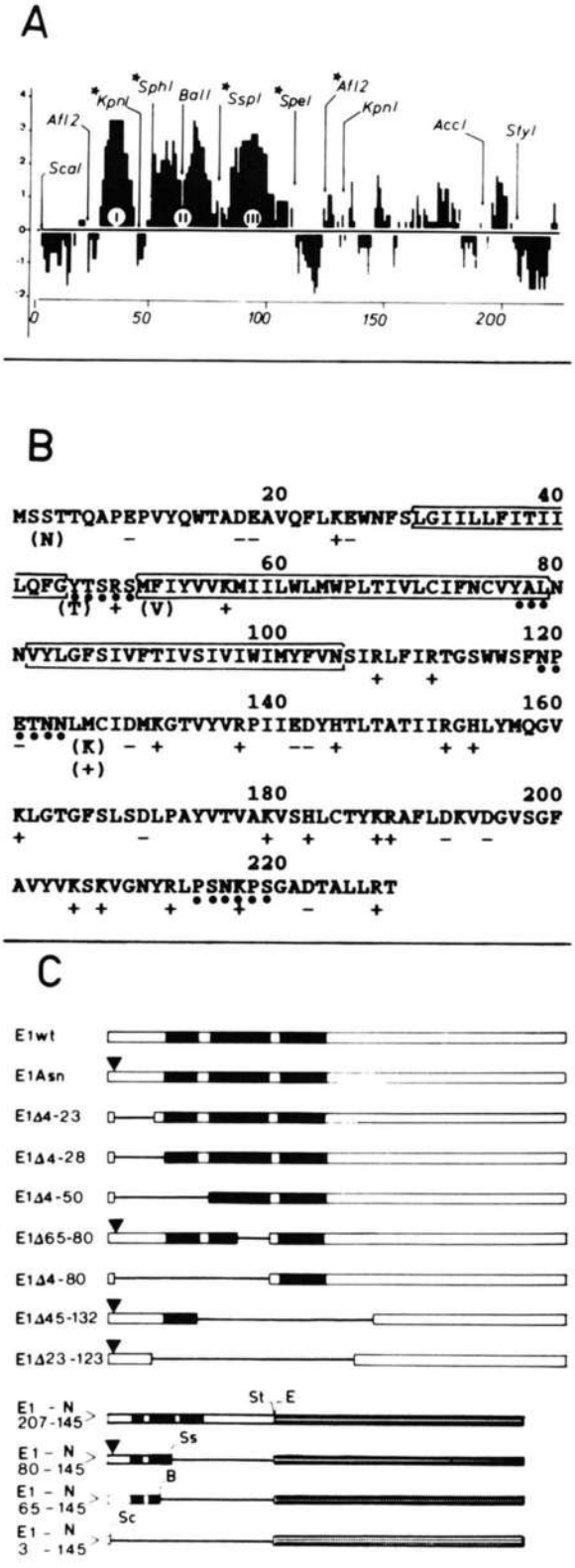

FIG. 1. **Mutants of the E1-protein.** *Panel A*, hydropathy plot of the E1-polypeptide according to the program of Kyte and Doolittle (1982). The positions of restriction sites used for the construction of mutants are indicated. Restriction sites marked with a *star* were introduced by site-directed mutagenesis. *Panel B*, amino acid sequence of the E1-polypeptide shown in the single letter code. Amino acid changes resulting from the generation of restriction sites are indicated. Charged residues are indicated by + or − underneath the sequence. *Open boxes* show sequences with α-helical probability according to Eisenberg *et al.* (1984). *Dots* indicate the location of β-bends determined by the programs of Chou and Fasman (1978) and Cid *et al.* (1982). *Panel C*, construction of E1 deletion mutants and E1-N fusion genes. *Black boxes* indicate regions encoding hydrophobic domains. *Dotted areas* correspond to sequences encoding the MHV JHM nucleoprotein. Restriction sites used for the construction of fusions are depicted. *Filled triangles* symbolize mutations made to introduce *N*-glycosylation sites.

process of the E1-protein of MHV A59 we used *in vitro* synthesis of capped and polyadenylated E1-specific mRNA from pSP65 vectors (Krieg and Melton, 1984) and its subsequent translation in the presence of translocation-competent microsomal membranes. To obtain polyadenylated transcripts, an oligo(dA-dT) fragment derived from pSVa970 (Min Jou *et al.*, 1980) was inserted downstream from the E1 coding sequences (Niemann *et al.*, 1984a) as detailed in the Miniprint Section. Membrane translocation was assessed (i) by protection of the translocated domains from attack of exogenous proteinase K; (ii) by cosedimentation of the translated products with the microsomal fraction at neutral or alkaline pH; (iii) by glycosylation of a newly introduced *N*-glycosylation site at the NH_2 terminus of the E1-protein. Based on predictions of the secondary structure of the E1-protein (Fig. 1*B*; Rottier *et al.*, 1986) and on the hydrophobicity (Fig. 1*A*; Kyte and Doolittle, 1982) we introduced additional restriction sites into the E1-gene by site-directed mutagenesis. These sites were used to construct a set of deletion mutants and fusion proteins as indicated in Fig. 1*C*. To analyze the intracellular distribution of the individual proteins, the corresponding mRNA was microinjected into various cell types and the proteins were visualized by indirect immunofluorescence.

The Hydrophilic NH_2-terminal Domain of the E1-protein Plays No Role in Membrane Integration nor in Establishing the Topology—Fig. 2 shows the results obtained by *in vitro* translations of E1-specific mRNA carrying mutations within the hydrophilic NH_2-terminal region. In agreement with published data (Niemann and Klenk, 1981) the wild type E1-protein (M_r 26,014) is not glycosylated when microsomal membranes are added to the translation mixture. This observation supports the structural model of the E1-protein which suggests that the potential sequon (-Asn[27]-Phe-Ser-) is embedded in the membrane and thus not accessible for the glycosyl transferase. Proteinase K treatment of the transla-

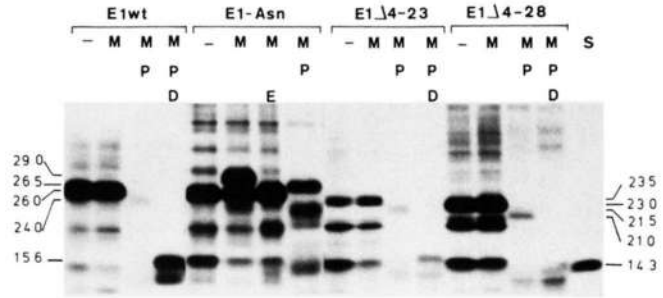

FIG. 2. **The NH_2-terminal domain of the E1-protein is not required for membrane integration *in vitro*.** Capped and polyadenylated mRNA was translated in reticulocyte lysate in the absence (−) or presence of translocation-competent microsomal membranes (*M*). Aliquots were treated with proteinase K (*P*) in the absence or presence of 1% Triton X-100 (*D*). Numbers characterizing individual mutants represent the first and the last amino acid of the deletion within the E1-protein. [^{35}S]Methionine-labeled samples were analyzed on a 15% SDS-polyacrylamide slab gel and visualized by autoradiography. Molecular weights are indicated in kilodaltons and were estimated with the following molecular mass standards: myosin (200,000 Da), phosphorylase *b* (93,500 Da), bovine serum albumin (69,000 Da), ovalbumin (46,000 Da) carbonic anhydrase (30,000 Da), lysozyme (14,300 Da), and aprotinin (6,500 Da) and cyanogen bromide fragments prepared from myoglobin (yielding fragments in the range from 1,700 to 17,200 Da). *E*, endo-β-*N*-acetylglucosaminidase; *S*, globin standard.

tion products obtained in the presence of membranes yielded a truncated 24,000-dalton form. Rottier *et al.* (1985) have shown that this species represents the E1-protein lacking a 2,000-dalton fragment from the carboxyl-terminal end. To assess lumenal exposure of the NH_2-terminal domain, an *N*-glycosylation site (-Asn[3]-Thr-Thr-) was introduced into this region by site-directed mutagenesis. The resulting polypeptide, designated E1Asn, was indeed glycosylated in the presence of membranes, as indicated by the formation of a 29,000-dalton species. The proteolytic cleavage product from this glycosylated species was larger (M_r 26,500) than that of E1wt, again demonstrating that in the absence of detergent the proteolytic attack occurred exclusively within the carboxyl-terminal part of the E1-molecule. In the presence of detergent the E1Asn-species was degraded to a 15,600-dalton fragment as also obtained from E1wt, indicating that the *N*-glycosylation site was removed (data not shown).

Consistent with the size of the deletions, the two mutants E1Δ4–23 and E1Δ4–28 generated integral membrane proteins that were about 2,500 or 3,000 daltons smaller than the E1wt-peptide. Both peptides were efficiently integrated into the membranes. Proteolysis gave products that were again about 2,000 daltons smaller than the original peptides indicating that their overall structure in the lipid bilayer remained unaltered. In the presence of detergent all E1-mutants were degraded to the 15,600-dalton species indicating that the NH_2 terminus was removed under such conditions. As indicated by the size of this fragment and further evidence below, additional cleavage in detergent also removed parts of the COOH-terminal tail.

The E1-protein Contains More than One Signal Sequence—To analyze which of the internal hydrophobic domains was essential for membrane integration, we produced mutants in which one or more of these domains were deleted. The results are summarized in Fig. 3. A deletion of the first hydrophobic domain, as present in E1Δ4/50 (M_r 20,100), neither prevented membrane integration nor did it alter the orientation of the protein in the membrane, as indicated by the proteolytic removal of the typical 2,000-dalton fragment. Analysis of E1Δ45–132 (M_r 15,000), retaining solely the first hydrophobic sequence, did not yield any detectable protected fragment. The results obtained with preprolactin control mRNA (Fig. 3*C*) indicated that the membrane preparation was not leaky for the protease. The E1Δ45–132-Asn molecule, carrying the newly created *N*-glycosylation site, yielded a glycosylated 18,500-dalton species. Treatment with endo-β-*N*-acetylglucosaminidase H created a third molecular species which was somewhat larger than the nonglycosylated form. The cotranslational addition of increasing amounts of an acceptor peptide for *N*-glycosylation (benzoyl-Asn-Leu-Thr-methylamide; Bause, 1983) revealed that only one of the two sites was glycosylated (data not shown).

The E1Δ23–123-Asn (Fig. 3*A*) lacked all three hydrophobic domains and provided the NH_2-terminal glycosylation site as a reporter group for lumenal exposure. This peptide was not glycosylated and was completely degraded by the protease even in the absence of detergent. This finding excludes the possibility that smaller E1-peptides could diffuse through the membrane and provides further evidence that the COOH-terminal hydrophilic part of E1 was not intrinsically resistant to the protease.

Mutant E1Δ4–80 (Fig. 3*C*), retaining hydrophobic domain III, was inserted into the membranes. Treatment with the protease revealed that it was not secreted but remained anchored in the membranes. This domain seemed to be sufficient to stabilize the carboxyl-terminal part of the molecule within

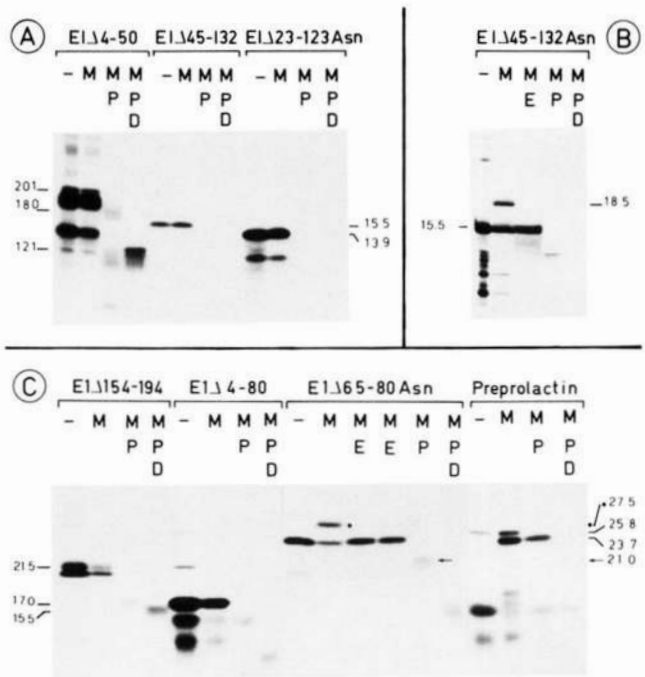

FIG. 3. **Membrane translocation of E1-mutants lacking individual hydrophobic domains.** For details of translation and abbreviations see the legend to Fig. 2. *A*, E1Δ4–50 lacks the first, E1Δ45–132 the second and third, and E1Δ23–123 lacks all three transmembrane domains, respectively. Samples were analyzed on a 15% SDS-polyacrylamide slab gel. *B*, E1Δ45–132-Asn contained a newly created *N*-glycosylation site within the amino-terminal part. *E* indicates the treatment of the sample with endoglycosidase H prior to gel electrophoresis. Samples were analyzed on a 22% SDS-slab gel containing 6 M urea. *C*, E1Δ154–194 was analyzed to demonstrate that the deleted part of the molecule is not responsible for a translocation block of the COOH-terminal domain. E1Δ4–80 retains the third transmembrane domain; E1Δ65–80 lacks the first part of the second hydrophobic domain. The translation of preprolactin mRNA was included to assess the quality of the membranes in terms of translocation activity, tightness against proteinase K, and signal peptidase activity.

the membrane, since protease treatment removed only the COOH-terminal 2,000-dalton fragment from E1Δ4–80. In the presence of detergent, however, the E1Δ4–80 molecule was degraded to an 8,500-dalton species. The size of this product in comparison to that obtained from E1Δ154–194 under detergent conditions (15,500 daltons) indicates that in both instances the resistant fragments contained hydrophobic sequences and parts from the COOH-terminal part of the E1-molecule. The deletion of amino acids 154–194 made the COOH-terminal region susceptible to proteinase K even in the absence of detergent, as evidenced by the release of a 4,500-dalton fragment yielding a peptide of almost the same size as the product obtained in the presence of detergent.

When part of the hydrophobic domain II was deleted, as shown in Fig. 3*C* for E1Δ65–80-Asn, the overall topology of the mutant protein remained unaltered. Protease cleavage removed a 6500-dalton fragment and thus did not occur at the original site around amino acid 205, but about 40 amino acids displaced toward the NH_2 terminus yielding a protected fragment of about 21 kilodaltons. We interpret these findings to mean that part of the domain II helps to stabilize the COOH-terminal tail of E1 in the membranes.

Co- and Post-translational Interaction of the E1-mutants with Microsomal Membranes—To analyze whether membrane integration of the individual mutants was coupled to translation, we examined peptides, to which membranes had been

added before or after their synthesis, for cosedimentation with the membranes at neutral or alkaline pH. The results of Fig. 4 show that all the molecular species retaining one of the hydrophobic domains integrated exclusively at the cotranslational level and were present in the pellet fraction. The finding that the peptides E1Δ45–132 and E1Δ4–80 were not released at alkaline pH further supports our conclusion that the hydrophobic domains I and III function simultaneously as signal and stop transfer sequences.

In contrast, peptide E1Δ23–123, although lacking all three internal hydrophobic domains, clearly associated with the membranes at the co- and post-translational level at either pH.

Membrane Assembly of E1-N Fusion Proteins—We have constructed four E1-N fusion proteins containing NH_2-terminal E1-specific sequences fused via the amino acid indicated to Ile^{145} of the nucleoprotein of MHV JHM (Fig. 1*C*). The results summarized in Fig. 5 revealed that all peptides with the exception of E1-N(3–145) were integrated and anchored in the membranes.

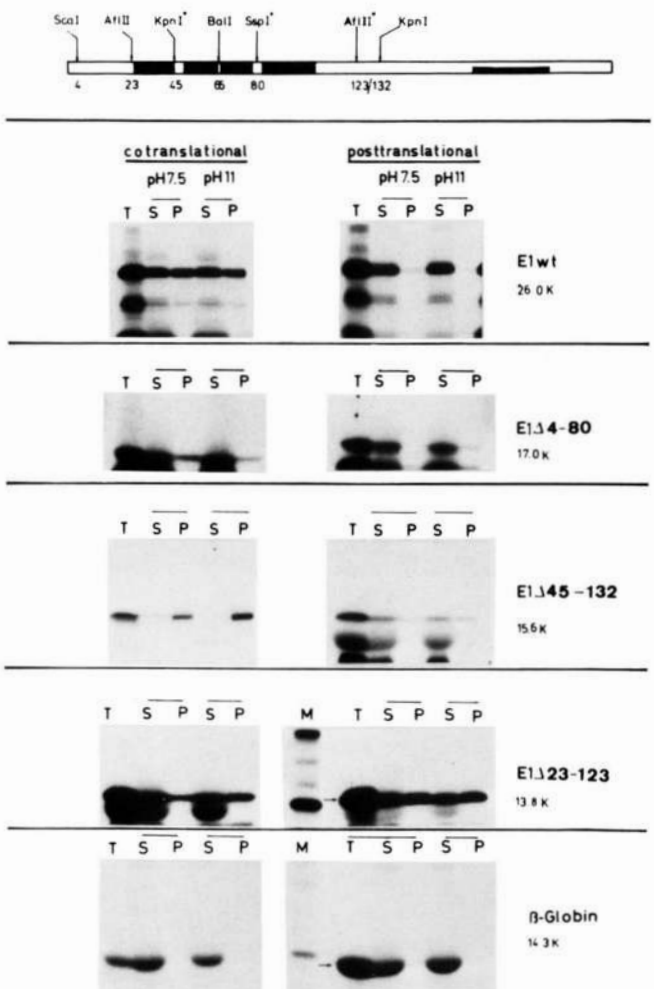

FIG. 4. **Cotranslational and post-translational interaction of E1-mutants with microsomal membranes.** Capped and polyadenylated mRNA was translated in reticulocyte lysate in the presence (cotranslational) or absence (post-translational) of membranes. Translation reactions were terminated after 45 min by the addition of cycloheximide. After 5 min at room temperature membranes were added to the post-translational assays. Incubation was continued for another 30 min for both series. Membrane association was determined by centrifugation of the samples through neutral or alkaline sucrose cushions as detailed in the Miniprint Section. Supernatant (*S*) and pellet (*P*) fractions were collected and analyzed on 15% SDS-polyacrylamide gels as above. Globin was included as a negative control. *T* indicates the total translation mixture, and *M* stands for molecular mass markers (lysozyme, 14,300 daltons; carbonic anhydrase, 30,000 daltons).

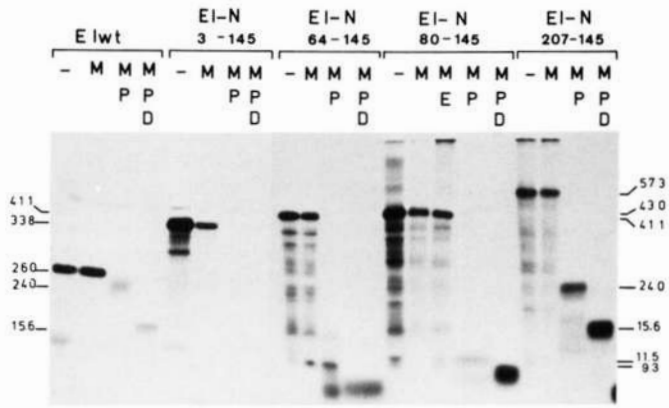

FIG. 5. **Membrane translocation of various E1-N fusion proteins *in vitro*.** For experimental details see the legend to Fig. 1 or the Miniprint Section. Numbers specifying the mutants indicate the last amino acid of the E1-protein fused in-frame to isoleucine residue (145) of the nucleoprotein. Samples were analyzed on a 15% SDS-polyacrylamide gel. *M*, microsomal membrane; *P*, aliquots treated with proteinase K; *D*, aliquots treated with proteinase K in presence of 1% Triton X-100.

As demonstrated by the analyses of E1-N(64–145) and E1-N(80–145), the second hydrophobic domain or the remainder of it was also embedded into the membranes and thus protected against proteolytic attack yielding products of 10,400 and 11,200 daltons, respectively. Fragments of this size could not be derived from the nucleoprotein, since no proteolytic degradation products could be identified from E1-N(3–145). E1-N(207–145) yielded fragments in the protease protection assay that were indistinguishable from the corresponding fragment derived from E1wt, indicating the identical membrane topology of the fusion protein. The topology of the NH_2 termini was verified by analyzing the corresponding variants carrying the newly created *N*-glycosylation site (data not shown).

Intracellular Transport Properties of the E1-protein and Its Mutants—The *in vitro* synthesized mRNA was capped and polyadenylated in order to increase its half-life after microinjection into eucaryotic cells (Huez *et al.*, 1981; Drummond *et al.*, 1985). The intracellular targeting of the E1-proteins was studied by indirect immunofluorescence as detailed under "Experimental Procedures."

In agreement with published data (Machamer and Rose, 1987; Niemann *et al.*, 1987; Rottier and Rose, 1987), the E1-protein accumulated in perinuclear regions of the injected cells (Fig. 6*B*). In double-labeling experiments these regions could not be distinguished from those recognized by the Golgi-specific rhodamine-labeled wheat germ agglutinin (Fig. 6*A*). The specific distribution of E1 was observed in about 50% of the injected cells while the remaining cells did not respond with any synthesis of E1-protein.

No E1 could be detected on the surface of injected cells as judged by the failure of staining with polyclonal E1-specific antibodies against virus particles and purified by elution from Western blots. In addition, no staining was obtained with antibodies directed against a synthetic peptide consisting of the eight NH_2-terminal amino acids (Ser-Ser-Thr-Thr-Gln-Ala-Pro-Glu) of E1 (data not shown). Even at late stages after injection or when 3-fold larger amounts of RNA (3 μg/ml) were injected, E1 was absent from the plasma membrane. In such instances also the nuclear membrane and the RER of

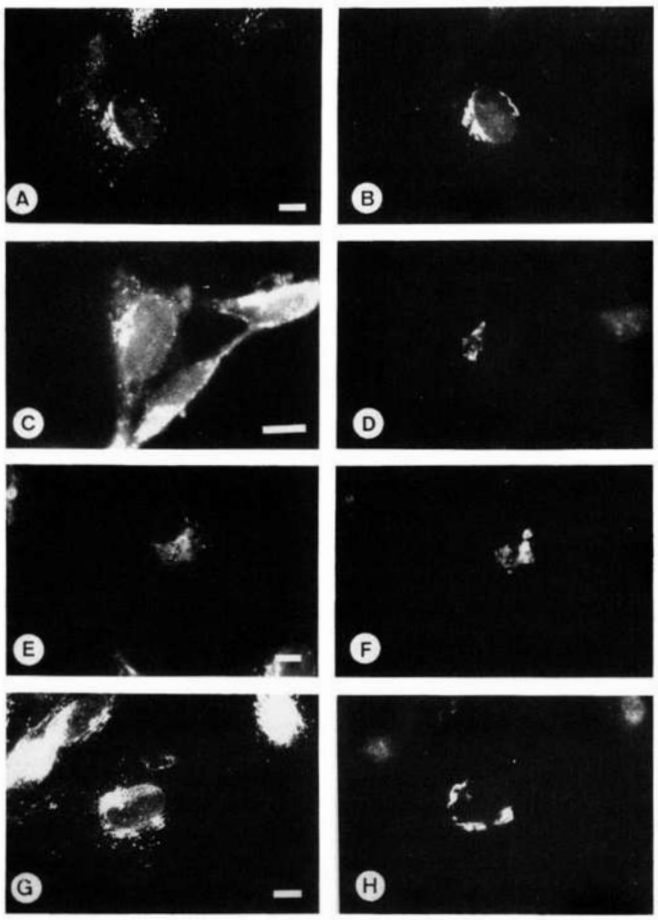

FIG. 6. **Transport properties of mutants of the E1-protein.** Capped and polyadenylated mRNA was microinjected into CV-1 cells or secretory AtT20 cells. The distribution of the E1-protein was determined by indirect immunofluorescence 6 h after injection. *A*, E1wt mRNA injected into CV-1 cells were stained with rhodamine-conjugated Golgi-specific WGA. *B*, same cells as in *A* but stained with polyclonal E1-specific antibodies and fluorescamine-labeled goat anti-rabbit IgG. *C*, AtT20 cells injected with E1wt mRNA were stained with anti-ACTH rabbit serum and rhodamine-labeled goat anti-rabbit IgG. *D*, same cells as in *C* but stained with a monoclonal E1-specific antibody and fluorescamine-conjugated goat anti-mouse IgG. *E*, CV-1 cells microinjected with E1-Asn mRNA and stained with WGA. *F*, same cells as in *E* but stained for E1 as in *D*. *G*, CV-1 cells injected with E1Δ4–28-specific mRNA and visualized by staining with WGA. *H*, same cells as in *G*, but stained for the presence of E1 as in *D*. The *bars* represent 20 μm.

the injected cells contained E1-protein (data not shown). This observation indicates that the E1-protein is accumulating rapidly in membranes of the Golgi and piles up in the RER only after the former membranes are saturated (Tooze *et al.*, 1984).

To determine whether the perinuclear accumulation of E1-protein was a phenomenon restricted to fibroblasts, we injected mRNA into AtT20-cells, a transformed mouse pituitary gland cell line secreting ACTH. Again, the E1-protein was present in the Golgi region of the injected cells (Fig. 6*D*). No E1 was detectable at the cell surface (not shown), and no E1 was present in peripheral secretory granules that were labeled with antibodies against ACTH (Fig. 6*C*).

Fig. 6, *E* and *F*, show that the E1Asn mutant protein also accumulated in Golgi-like compartments. Since the E1Asn species was efficiently glycosylated in the *in vitro* assay, it is highly likely that this glycosylation also occurs *in vivo*. This would then allow the conclusion that cotranslation *N*-glycosylation of the amino-terminal domain of E1 does not alter its intracellular transport properties.

In addition, a deletion of most of the hydrophilic NH_2-terminal domain had no influences on the intracellular targeting as shown for the E1Δ4–28-protein in Fig. 6, *G* and *H*.

Intracellular Transport of E1-N Fusion Proteins—Fig. 7 shows the intracellular distribution of newly synthesized E1-N-proteins. A monoclonal antibody directed against the nucleoprotein was used to detect the fusion proteins. In agreement with the observation that E1-N(3–145) did not integrate into the membranes *in vitro* (Fig. 5), the polypeptide was found dispersed throughout the cytoplasm of the injected cell (Fig. 7*A*). In contrast, E1-N(64–145) containing the first and part of the second hydrophobic domain accumulated in membranes of the RER (Fig. 7*C*) as indicated by double-labeling with a polyclonal antibody binding to the carboxyl-terminal domain of canine ribophorin I (Fig. 7*D*). Therefore, both antibodies bound to epitopes that were located at the cytoplasmic face of the RER. The E1-N(80–145)-protein containing the first two membrane-spanning domains accumulated

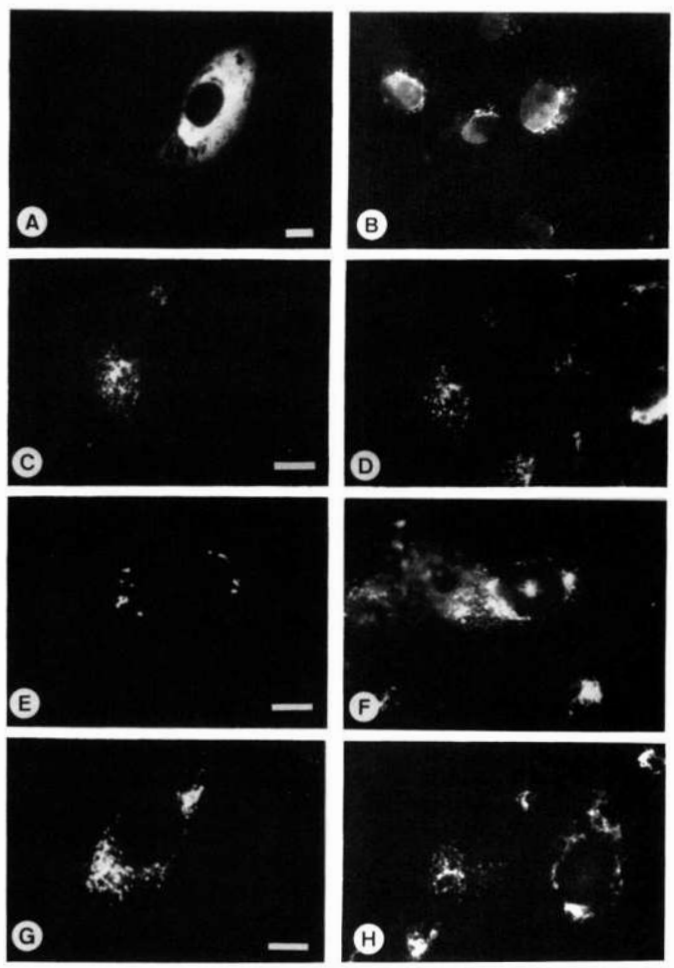

FIG. 7. **Transport properties of various E1-N fusion proteins.** CV-1 cells were microinjected with capped and polyadenylated mRNA encoding various E21-N fusion proteins. The N-specific distribution in *A*, *C*, *E*, and *G* was visualized 6 h after injection with a monoclonal antibody directed against the nucleoprotein of coronavirus JHM (Wege *et al.*, 1984). *B*, same injected cells as in *A* using E1-N(3–145)-specific mRNA and stained with WGA. *D*, cells injected as in *C* with E1-N(64–145) but stained with a rabbit antiserum against the cytoplasmic domain of canine ribophorin I to specify the rough endoplasmic reticulum. *F*, cells injected as in *E* with E1-N(80–145)-specific mRNA and stained with WGA. *H*, cells injected as in *G* with E1-N(207–145)-specific mRNA. The cells in *H* were labeled with the anti-ribophorin antibody. The *bars* represent 20 μm.

in perinuclear membranes (Fig. 7*E*) which were not labeled with the ribophorin-specific antibody (not shown). Some of the E1-N containing compartments were stained by the Golgi-specific lectin (Fig. 7*F*). The intracellular distribution E1-N(207–145) followed basically the pattern specific for the RER. The labeled structures, however, seemed to have a more vesicular character. By using WGA in similar double-labeling experiments it became obvious that these vesicles were not closely associated with Golgi compartments. It is feasible to assume that these vesicular structures represent transient vesicles which are derived from the RER and constitute the primary sites of virus maturation in the infected cell (Becker *et al.*, 1967; Tooze *et al.*, 1984).

DISCUSSION

We have analyzed the topogenic signals and the intracellular transport properties of the glycoprotein E1 of MHV A59, a class III membrane glycoprotein.

One of the models for the biosynthesis of polytopic membrane proteins suggests that these multispanning proteins are translocated into the endoplasmic reticulum membrane by alternating signal and stop transfer sequences (Friedlander and Blobel, 1985; Kopito and Lodish, 1985). Recently Zerial *et al.* (1987) have demonstrated that foreign peptides could replace the internal signal and anchor sequence of the human transferrin receptor. These studies suggested that the hydrophobic character and the position in the molecule rather than the actual amino acid composition determine the character of a transmembrane sequence. In light of these findings we did not attempt to take the internal hydrophobic domains of the E1-protein of MHV A59 completely out of their context by transferring them into different proteins. Instead, we have constructed deletion mutants and fusion proteins which retained authentic E1-sequences either from the NH_2 terminus or from the COOH terminus.

We show here that the domains I and III could function as signal and stop transfer sequences determining the topology of the E1-molecule (Fig. 8).

(i). The NH_2-terminal hydrophilic domain of E1 does not play a role in the membrane integration process or in determining the topology of the E1-protein. No cleavable signal sequence is uncovered by the removal of this part of the E1-molecule which notably shows the largest degree of heterogeneity among different strains of coronaviruses (Lapps *et al.*, 1987; Rasschaert *et al.*, 1987; Boursnell *et al.*, 1984).

(ii). Hydrophobic domain I alone was sufficient to translocate the amino-terminal part of the E1-molecule to the lumenal side as demonstrated by the glycosylation of the newly created *N*-glycosylation site in E1Δ45–132-Asn. No glycosylation was observed when membranes were added post-translationally. The orientation of the E1Δ45–132 molecules is identical to that of the M2-protein of influenza virus (Lamb *et al.*, 1985) but differs from that of other glycoproteins with internal uncleavable signal sequences such as the asialoglycoprotein receptor (Spiess and Lodish, 1986), the human transferrin receptor (Zerial *et al.*, 1986), or the human glucose transporter (Mueckler and Lodish, 1986). At present we do not know whether domain I can translocate only NH_2-terminal sequences of a limited size. While the E1-proteins from the bovine and the avian coronaviruses have hydrophilic ectodomains containing 28 and 22 amino acids, respectively, the corresponding ectodomain of the E1-preprotein from transmissible gastroenteritis virus is 46 amino acids in length. Interestingly, this polypeptide is synthesized with an additional NH_2-terminal cleavable signal sequence of 17 amino acid residues (Laude *et al.*, 1987).

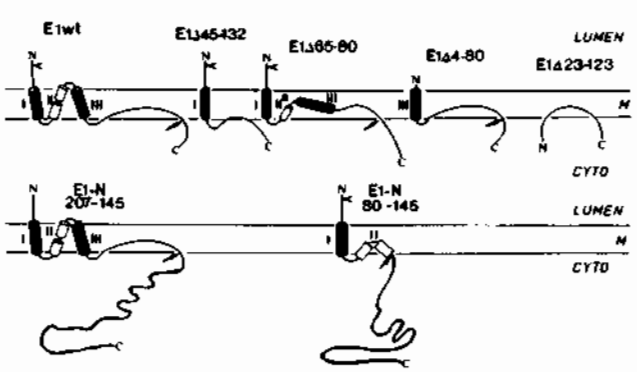

FIG. 8. **Postulated topology of individual E1-mutants in the membrane as evidenced by *N*-glycosylation and protease protection assays.** The estimated cleavage sites are indicated by *arrows*. For further details see the text.

(iii). The transmembrane domain I functioned as a stop transfer sequence, even though basic amino acid residues present in the cytoplasmic loop between domains I and II were removed together with domains II and III. Clearly, E1Δ45–312 was not secreted into the lumen since the native glycosylation site (Asn^{27}-Phe-Ser) was not glycosylated in this deletion mutant or in a corresponding E1-N fusion protein.

(iv). The presence of a second signal sequence within the third hydrophobic domain was demonstrated by the analysis of E1Δ4–80. This protein was inserted into the membrane exclusively at the cotranslational level, and the peptide exhibited the authentic orientation (Fig. 8). It has been shown previously that signal recognition particles exert a translational block as late as up to a point in the translation when two-thirds of the E1-molecule (150 amino acids) have been synthesized (Rottier *et al.*, 1985). These data are in agreement with our observation that the third domain indeed functions as a signal sequence.

Our conclusion that the hydrophobic domain II of the E1-protein is not actively involved in the membrane insertion process is based on indirect evidence. First, the two polypeptides containing either a combination of domains I and II (present in mutant E1-N(80–145)) or II and III (present in E1Δ4–50) assembled in the membrane in the original orientation. Second, E1Δ65–80 which lacked the first half of domain II was integrated efficiently into membranes with the authentic topology, as indicated by *N*-glycosylation of the NH_2 terminus. We interpret these findings to mean that membrane integration and orientation of domain II are predetermined by the presence of domains I and III. However, our data do not exclude the possibility that domain II could function independently as a signal sequence.

The capability of the COOH-terminal tail of E1 to associate with membranes post-translationally was unexpected. This behavior may reflect the natural role of E1 as a matrix protein guiding the viral nucleocapsid to the place of virus budding (Sturman *et al.*, 1980).

Our microinjection experiments indicated that the E1-protein has an intrinsic signal for a retention in Golgi-like compartments in fibroblasts and secretory AtT20 cells. This retention signal of the E1-protein is functional in the absence of other viral proteins. Similar results have been obtained previously for the E1-protein of avian infectious bronchitis virus (IBV) (Machamer and Rose, 1987) and for the E1-protein of MHV A59 using DNA expression vectors (Niemann *et al.*, 1987; Rottier and Rose, 1987). In MHV A59-infected AtT20 cells virus particles were shown to bud into pre-Golgi compartments and then share the secretory pathway with the secretory protein ACTH through the same Golgi stacks into

the trans-Golgi network. At this site the constitutive secretory pathway for the virus and the regulated secretory pathway for the hormone diverged (Tooze *et al.*, 1987). We show here that this transport property was also shared by the isolated E1-protein, since it was not detected in secretory post-Golgi vesicles filled with ATCH.

Studies by Machamer and Rose (1987) demonstrated that the first transmembrane domain of the protein from the infectious bronchitis virus was responsible for its retention in the Golgi while a protein retaining only the third transmembrane domain was transported to the plasma membrane. Unfortunately, we were unable to detect E1-peptides after microinjection of mRNA encoding E1Δ45–132 and E1Δ4–80. At present we do not know whether this is due to an instability of the corresponding mRNA, whether the protein synthesized *in vivo* was degraded, or whether it was too dispersed throughout the cells to be detected with the antibodies.

The described modifications of the ectodomain of the E1-molecule had no influence on the E1-specific transport properties. To assess the applicability of parts of the E1-molecule to direct fusion proteins into the Golgi, we have microinjected mRNA encoding various parts of the E1-protein fused in frame to a carboxyl-terminal part of the cytoplasmic N-protein of MHV JHM. Each of the fusion proteins containing one or more of the hydrophobic domains of E1 was detected in perinuclear membranes. The fusion proteins E1-N(64–145) and E1-N(80–145) were not transported into the Golgi indicating that particular nucleoprotein-specific sequences added to the cytoplasmic COOH terminus prevented release from the RER. Only in very few cells the intracellular distribution of E1-N(207–145) overlapped with the Golgi pattern as stained by WGA, and it was also different than the pattern obtained with RER-specific antibodies. We suggest that the compartments harboring the E1-N(207–145) are transient vesicles which in the virus-infected cells are the sites of particle formation. Experiments involving immunoelectron microscopy on cells infected with recombinant vaccinia virus are currently in progress.

Acknowledgments—We thank David Meyer (UCLA, Los Angeles) and John Tooze (EMBL, Heidelberg) for antibodies directed against ribophorin and ACTH, respectively. We are indebted to Bernhard Dobberstein (EMBL, Heidelberg) for providing dog pancreatic membranes and for fruitful discussions. We thank Juan Ortin (Universitad Autónoma, Madrid) and Carl Blobel (University of California, San Francisco) for plasmids pSVa970 and pB4.

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Supplement to:
Membrane Integration and Intracellular Transport of the Coronavirus Glycoprotein E1, a Class III Membrane Glycoprotein.
T. Mayer, T. Tamura, M. Falk, and H. Niemann

Experimental Procedures

Cells and Virus - The A59 strain of mouse hepatitis virus was grown in sac(-)-cells, a nonproducer Moloney sarcoma line. For microinjection of mRNA, mouse L tk⁻-cells, HeLa- or CV-1 primate cells were used. AtT20-cells, descendents of a mouse LAF1 pituitary tumor, were kindly provided by J. Tooze, (EMBL, Heidelberg). All cells with the exception of AtT20 cells were grown in DMEM in the presence of 10 % fetal calf serum. For AtT20-cells the medium was supplemented with 10 % horse serum.

Plasmids - The construction of E1-specific cDNA in pUR250 and sequencing of the E1-gene of MHV A59 has been described previously (Niemann et al., 1984a; Armstrong et al., 1984). Plasmid pS538, harboring the gene for the nucleocapsid protein of the JHM strain of MHV, was constructed by Skinner and Siddell (1983). The expression vector pSVA970 which contained the hemagglutinin gene of the influenza A virus (strain Victoria 3/75; H3N2) was used as a source of a poly(A) block. It was kindly provided by Dr. J. Ortin (University of Madrid, Spain). The construction of the cDNA clone and has been described by Min Jou et al. (1980). The vector pSP65 was purchased from Progema (Heidelberg).

Enzymes, Chemicals and radiochemicals - Restriction endonucleases FokI and MnlI were from New England Biolabs (Schwalbach/Ts., FRG). All other restriction enzymes, DNA-polymerase (Klenow fragment), SP6-RNA-polymerase, T4-DNA-ligase, and ⁷'-mGpppG, oligo(dT)-cellulose were from Boehringer (Mannheim, FRG). Wheat germ extract, reticulocyte lysate, and nuclease-free bovine serum albumine were from Bethesda Research Laboratories. Nonlabelled nucleotides were obtained from PL-Pharmacia (Freiburg, FRG). [γ-³²P]-ATP (> 400 Ci/mmol), and [³⁵S]-methionine (800 Ci/mmol) were from Amersham-Buchler (Braunschweig, FRG).

Construction of E1 mutant genes. - Standard cloning techniques were applied according to Maniatis et al., (1982). The dut⁻, ung⁻ *E. coli* strain RZ1032 (Kunkel 1985) was used to produce single stranded M13-templates containing uridine residues. The table lists the oligonucleotides which were used to create specific mutations of the E1-gene. For the construction of pSP65E1Δ4/23 recombinant E1M13mp19-DNA was digested with ScaI and AflII, partially filled in with Klenow polymerase and dTTP, treated with mung bean nuclease and religated. PSP65E1Δ54/28 was obtained by treating ScaI-digested E1M13mp19-DNA with Bal31 and subsequent ligation of the truncated E1-sequences into E1M13mp19 that was cleaved with ScaI and HindIII. Clone pSP65E1Δ65/80 was obtained by insertion of the 750 bp SspI-HindIII-fragment from pSP65E1(Ile61) into pSP65E1 previously cleaved with BalI and HindIII. To delete coding sequences corresponding to the first and second membrane spanning domain, pSP65E1(Ile81) was linearized with SspI and partially digested with ScaI. The 3799 bp fragment was isolated and religated to yield pSP65E1Δ4/80. The deletion mutant pSP65E1Δ65/132, lacking the sequences corresponding to the second and third hydrophobic domain, was produced by cleaving pSP65E1(Thr45) with KpnI, deletion of the 244 bp fragment and religation. For the construction of pSP65E1Δ23/123, encoding an E1-protein lacking all the internal hydrophobic domains, pSP65E1(Lys126) was treated with AflII, the 3766 bp-fragment was isolated and religated. PSP65E1Δ154/194 was constructed in the following manner: pSP65E1Δ65/132 was cleaved with BalI and AccI, the 5'-protruding ends were filled with Klenow polymerase and the vector-fragment was religated to yield pSP65E1Δ65/132;Δ154/194. A 367 bp fragment carrying the desired deletion was isolated after digestion with KpnI and HindIII and was used to replace the corresponding fragment in the wild type E1-gene. Mutants pSP65E1AsnΔ65/132 and pSP65E1AsnΔ23/123 were obtained by inserting the AflII-HindIII-fragments from pSP65E1Δ65/132 and pSP65E1Δ23/123, respectively, into pSP65E1Asn digested with AflII and HindIII. Fig. 9 A shows the properties of the pSP65E1 transcription vectors.

Designation of mutant[a]	Mutation	Sequence of synthetic oligonucleotide[b]
E1Asn	Ser(3) to Asn(3)	5'-TATGAGTAATACTACTC-3'
E1Δ4/50	deletion of Thr(4) to Met(50)	5'-AATATGAGTAGTATGTTTATTTAT-3'
E1(Thr45) (KpnI)	Tyr(45) to Thr(45)	5'-CTACAGTTCGGTACCACGAGCCGTAG-3'
E1(Leu51) (SphI)	Phe(51) to Leu(51)	5'-GCCGTAGCATGCTTATTATTTATGGTGG-3'
E1(Ile81) (SspI)	Asn(81) to Ile(81)	5'-TGCGCTAAATATTGTGTATCTTGG-3'
E1(Ser113) (SpeI)	Gly(113) to Ser(113)	5'-GTTTATCAGGACTAGTAGCTGGTGG-3'
E1(Lys126) (AflII)	Met(126) to Lys(126)	5'-CAAACAACCTTAAGTGTATAGAT-3'

[a] Newly introduced restriction sites are denoted below the designation.
[b] Mutated nucleotides are underlined.

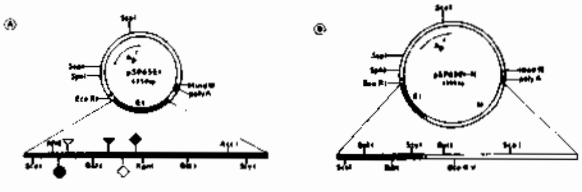

Fig. 9. Properties of the *in vitro* transcription vectors used in this study. *A:* pSP65E1 carrying a poly(dA/dT) segment (dotted area) to allow for the synthesis of polyadenylated mRNA. Naturally occuring restriction sites are indicated. The symbols represent restriction sites introduced by site directed mutagenesis as described above (●KpnI; ▽SphI; ▼SspI; ◇SpeI; ◆AflIII). *B:* pSP65E1-N containing the structural genes for the E1-protein of MHV A59 (Niemann et al., 1987) and the N-gene of coronavirus JHM (Skinner and Siddell, 1983). The E1-gene still carries the TAA-codon for termination of translation. Sites used for the in-frame fusions between E1 and N are indicated.

Construction of E1-N fusion genes. - The construction of pSP65E1-N (Fig. 9) harboring the E1-gene upstream from the nucleoprotein gene in the authentic sequential arrangement as present in mRNA number 6 from MHV A59 infected cells has been reported previously (Niemann et al., 1987). This construct contained the termination codon for translation of the E1-protein and 17 nucleotides of the intergenic noncoding region upstream from the ATG translation start codon of the nucleoprotein gene of MHV JHM. Four different constructs were made in which E1-specific sequences of various lengths were fused in frame to the EcoRV site of N-gene as indicated in Fig. 1C. In these constructs the numbers in brackets refer to the numbers of amino acids which constitute the border between the E1- and N-specific sequence.

Preparation of cover slips for microinjection: - Glass-cover slips (15 mm in diameter) were defattened in acetone for 3 h, dried with Kim wipes and treated overnight with 0.2 % (w/w) Na_2CO_3. After 5 washes with distilled water coverslips were transferred onto filter paper and were air dried. To a suspension of the white of an egg in 500 ml 20 mM NaOH, 4 ml polyvinylpyrrolidone-solution (25 % w/vol in distilled water) was added and this mixture was stirred at room temperature for 2 h. The mixture was then cleared by filtration. The cover slips were placed into the filtrate and incubated for 2 h at 65° C. After a quick rinse with distilled water the cover slips were fixed at room temperature for 20 min in ethanol/glacial acetic acid (3:1) and air-dried. The cover slips were then incubated in 0.1 M triethanolamine/HCl (pH 8.0) containing 0.25 % (vol/vol) acetic anhydride. Subsequently they were washed with ethanol-water mixtures ranging from 60 % (vol/vol) ethanol to 100 % ethanol. Cover slips were dried and sterilized by overnight exposure to UV-light. Several 200 mesh copper alphabet finder grids used in electron microscopy were placed onto the coated cover slips in a vacuum chamber. A gold layer of about 500 nm thickness was evaporated onto the surface resulting in a visible shadow image of the grid bars.

Microinjection of capped and polyadenylated mRNA - For microinjection of capped and polyadenylated mRNA cells were grown over night on pretreated glass cover slips. Prior to microinjection the medium was replaced by DMEM containing 10 % fetal calf serum and 20 mM HEPES, pH 7.2. Microinjections were performed essentially as described by Ansorge (1982), controlling the injection pressure by an Eppendorf microinjector 5242. In general an RNA concentration of 1 µg/µl was used.

Antisera - The production of a polyclonal anti-MHV A59-antiserum in rabbits has been described previously (Niemann and Klenk, 1981). Polyclonal E1-specific antibodies were prepared from the crude rabbit serum as described by Burke et al., (1982) and Olmsted (1981). Briefly, viral polypeptides from 2 x 10^{9} plaque forming particles were separated on a 5 to 15 % SDS-polyacrylamide gel. After transfer of the proteins onto nitrocellulose the position of the E1-protein was determined by staining with Ponceau S (Sigma, 0.2 % (w/v) in 3 % (w/v) aqueous trichloroacetic acid). The E1-containing stripe (0.5 x 7 cm) was excised and washed for 90 min with washing buffer (phosphate buffered saline containing 10 % horse serum and 0.2 % (w/v) Triton X100). The stripe was incubated over night under agitation at 4 °C with 5 ml of crude rabbit anti MHV A59 antiserum (diluted 1:50 in washing buffer). After 3 washes (15 min each) with washing buffer and a quick rinse with phosphate buffered saline alone, bound antibodies were eluted with 1.5 ml of 0.2 M Tris(hydroxymethyl)aminomethan / glycine, pH 2.8, containing 0.2 % (w/v) gelatine. After 2 min incubation at room temperature the solution was neutralized by the addition of an appropriate volume of 1 M Tris-base. For indirect immunofluorescence studies the eluted antibodies could be used without further concentration. For the production of the peptide-specific H-2 antiserum which recognizes the nonglycosylated aminoterminus of the E1-polypeptide, 10 mg of peptide were coupled covalently to keyhole limpet hemocyanine. Rabbits were immunized with the complex in the presence of complete Freund's adjuvant and antibodies were purified essentially as described by Tamura et al. (1983). Monoclonal anti-N serum was kindly provided by Dr. Helmut Wege (Würzburg).

Indirect immunofluorescence - Visualization of E1- and E1-N-polypeptides by indirect immunofluorescence was done according to the techniques described by Kepp et al. (1985). Rhodamine conjugated wheat germ lectin was from E.Y. Laboratories Inc. (San Mateo, USA). A polyclonal rabbit antiserum raised against the carboxyterminal domain of canine ribophorin I was kindly provided by Dr. David Meyer (Dept. of Biological Chemistry, UCLA, Los Angeles, USA).

***In vitro* Transcription of recombinant pSP65-vectors** - Recombinant pSP65-DNA was linearized with HindIII and dissolved at 0.1 µg/µl in 100 µl 40 mM Tris-HCl, pH 7.5, containing 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 100 µg/ml BSA, 10 mM DTT, 0.5 mM ATP, CTP, UTP, 0.05 mM GTP, 0.5 mM ⁷'-mGpppG, and 1.0 U/µl RNasin. The reaction was started by the addition of 30 U SP6-polymerase. After 1 h at 40° C, another 30 U of polymerase were added and the reaction was continued for 30 min. The reaction mixture was extracted with phenol/chloroform and precipitated with ethanol.

***In vitro* translations.** - Capped and polyadenylated RNA synthesized *in vitro* was translated in the presence of [³⁵S]methionine (1 mCi/ml final concentration) in reticulocyte lysate as described previously (Jackson and Hunt, 1983). To demonstrate membrane integration of newly synthesized proteins, a typical translation assay in reticulocyte lysate contained 5 µl lysate, 0.5 µl microsomal membranes, 1.5 µl [³⁵S]methionine, 2 µl E1-specific mRNA (250 ng/µl distilled water pretreated with diethylpyrocarbonate, 1 µl water, and 5 µl salt solution (100 mM potassium acetate, 2 mM magnesium acetate). After incubation for 1 h at 30°C 45 µl 50 mM Tris/HCl pH 7.4 containing 100 mM NaCl were added. 20 µl aliquots were taken and either boiled in sample buffer or treated with proteinase K (100 µg/ml final concentration) in the presence or absence 1 % (w/v) Triton X100. After treatment for 10 min at 25 °C phenylmethylsulfonylfluoride (40 mg/ml ethanol) was added and incubation was continued for another 10 min at 0 °C. To demonstrate posttranslational association with membranes the translation was terminated after 45 min by the addition of cycloheximide (250 µM final concentration) prior to the addition of membranes. Incubation was continued for 30 min. The samples were analyzed according to Gilmore and Blobel (1985). The translation mixtures were placed onto a neutral (50 µl) or alkaline (100 µl) sucrose cushion in an 18° A100 airfuge rotor (Beckman instruments). The neutral sucrose solution contained 500 mM sucrose, 150 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mM dithiothreitol in 50 mM Tris(hydroxymethyl)aminomethane / acetate, pH 7.5. Centrifugations were performed at 20 psi for 2 min, allowing additional 10 s for acceleration and 2 min for breaking. The alkaline sucrose cushion consisted of 200 mM sucrose, 150 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mM dithiothreitol in 30 mM HEPES, pH 11.0. In this instance centrifugation was for 6.5 min at 30 psi.

Digestion with endo-N-acetylglucosaminidase H. - Aliquots of the translation mixture (1.25 µl) were diluted with 3.75 µl gel loading buffer (Laemmli, 1970) and boiled for 1 min. After addition of 40 µl Tris/HCl buffer (250 mM, pH 6.8 and 0.2 % SDS) samples were treated for 20 h with 15 µU endo-β-N-acetylglucosaminidase H (Miles, Frankfurt, FRG) at 37° C.