

Topology of the non-structural rotavirus receptor glycoprotein NS28 in the rough endoplasmic reticulum

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The rotavirus non-structural glycoprotein (NS28), the receptor for the virus core during budding into the lumen of the rough endoplasmic reticulum (RER), is 175 amino acids long and possesses an uncleaved signal sequence and two amino-terminal glycosylation sites. Utilizing one of three potential hydrophobic domains, the protein spans the membrane only once, with the glycosylated amino-terminal region oriented to the luminal side of the ER and the carboxy-terminal region to the cytoplasmic side. To localize sequences involved in translocation of NS28, we constructed a series of mutations in the coding regions for the hydrophobic domains of the protein. Mutant protein products were studied by *in vitro* translation and by transfection *in vivo*. In transfected cells, all mutant forms localize to the ER, and none are secreted. *In vitro*, each of the three hydrophobic domains is able to associate with microsomes. However, glycosylation and proteolysis of wild-type and mutant forms of NS28 indicates that the wild-type protein is anchored in the membrane only by the second hydrophobic domain, leaving ~131 residues exposed on the cytoplasmic side for receptor–ligand interaction.

Key words: rotavirus budding/RER localization/receptor glycoprotein/membrane topology

Introduction

Rotaviruses mature by an unusual pathway involving budding of the inner viral core into the lumen of the RER (Estes *et al.*, 1983). In the series of events surrounding this process, the core first assembles in a viroplasmic inclusion body located in the cytoplasm of the infected cell and then buds into the lumen, becoming transiently enveloped in a membrane vesicle (Petrie *et al.*, 1983; Kabcenell *et al.*, 1988). At some stage during the budding process the outer viral proteins are assembled onto the core, the ephemeral membrane coat is removed and the mature virus accumulates in the luminal space.

The budding of the core and the subsequent envelopment by the membrane is a key step in this series of events and appears to involve the interaction of the core with the non-structural viral protein NS28 (also known as NCVP5; Arias *et al.*, 1982) which acts as a receptor for the core (Au *et al.*, 1989). Topologically, the transfer of the enveloped core into

the lumen of the ER is equivalent to the budding of particles from the cytoplasm to the exterior of the cell—an event for which few well-characterized model systems are available. The interaction between NS28 and the rotavirus core therefore represents an interesting model system for the events that accompany budding, a maturation process that is held in common by many enveloped viruses.

NS28 contains high mannose carbohydrate, a finding consistent with its exclusive RER location (Both *et al.*, 1983). cDNA clones for the gene are available, and the inferred amino acid sequence for the bovine strain reveals three putative hydrophobic sequences (H1, H2, H3) within the N-terminal half of the protein (Baybutt and McCrae, 1984; Powell *et al.*, 1988). There are three in-frame AUG codons, the first having a strong consensus sequence for initiation (Kozak, 1984) from which a protein of 175 amino acids can be translated. Asparagine residues at positions 8 and 18 are glycosylated, indicating that the first hydrophobic domain is both uncleaved (Both *et al.*, 1983; Kabcenell and Atkinson, 1985) and lumenally oriented. The C-terminal region of the protein is protease sensitive (Ericson *et al.*, 1983; Kabcenell and Atkinson, 1985) demonstrating that it is located on the cytoplasmic side of the membrane.

To identify the membrane-spanning domain and to localize the region where the polypeptide emerges from the membrane on the cytoplasmic side, we have modified the protein by site-directed mutagenesis of the cloned gene and studied the products after *in vitro* translation. The same mutant genes have also been transfected and expressed under the direction of the SV40 late promoter in CMT4 cells, in order to provide confirmation *in vivo* of the targeting of the mutants to the ER. We conclude from the results that NS28 is anchored in the ER membrane by H2 and that the protein emerges from the membrane near residue 44, leaving 131 amino acids potentially available for receptor–ligand interaction.

Results

Rationale for the construction of deletion mutants

Figure 1 presents the sequence of the N-terminal 90 amino acids of wt NS28 as deduced from the cDNA sequence of viral genomic segment 10 (Powell *et al.*, 1988). A hydrophobicity analysis (Argos *et al.*, 1982) reveals three putative hydrophobic regions extending from residue 7 to 25 (H1), 30 to 54 (H2) and 63 to 80 (H3). To analyze the functional significance of these regions for membrane translocation, oligonucleotides were designed to loop out sequences of the coding regions to delete specific amino acids from the individual hydrophobic domains. The resulting eight variant proteins (Figure 1) were designed to determine which domain(s) could act as signal sequences, which domain(s) might be transmembrane, and which sequence(s) specify retention in the ER membrane.

The H1 domain contains two sites for N-linked glyco-

sylation, both of which are filled in NS28 isolated from virus-infected cells (Arias *et al.*, 1982). Therefore H1 might have a role as a signal sequence, leaving H2 or H3 as candidates for the transmembrane and anchoring domains. H2 constitutes the longest hydrophobic domain and contains only two charged amino acids. It therefore appeared reasonable to assign the membrane-spanning region provisionally to H2.

To investigate the possible function of H1 as a signal sequence, we designed mutants $\Delta 1-16$, $\Delta 1-29$ and $\Delta 29-84$. Mutant $\Delta 1-16$ lacks half of the H1 domain including the glycosylation site at position 8: glycosylation at the remaining site would constitute evidence for translocation, and identify H1 as an unlikely candidate for the signal sequence. The possible involvement of H1 in translocation is also addressed by mutants $\Delta 1-29$ and $\Delta 29-84$, since the entire H1 domain is deleted in the first mutant, whereas in the latter only H1 is retained.

The possibility of H2 acting as the sole signal and/or membrane spanning sequence was tested by removal of both H1 and H3, yielding mutant $\Delta 1-29/66-83$, and by construction of mutant $\Delta 28-46$ that lacks the majority of H2. Mutant $\Delta 40-48$ was designed to test whether the basic residues at positions 47 and 48 act as possible membrane anchor signals. The ability of H3 to mediate membrane translocation was investigated with mutant $\Delta 66-83$, which lacks the H3 region while retaining both H1 and H2; and with mutant $\Delta 2-64$, which yielded a protein containing H3 as the sole potential signal that controls translocation.

All mutant genes yielded mRNAs of appropriate length when transcribed from the T7 promoter (data not shown) and these were used for functional assays after *in vitro* translation in the presence of canine pancreatic microsomes.

The H2 domain is necessary for translocation and glycosylation

The two sites for N-linked glycosylation within the first 18

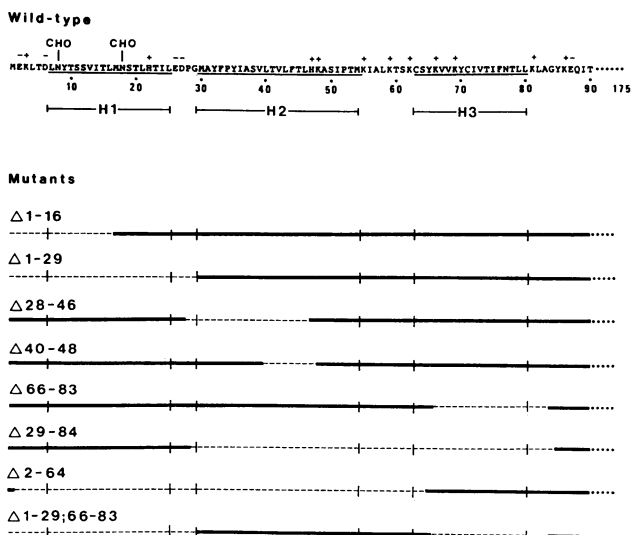


Fig. 1. Partial (amino-terminal) maps for wild-type and mutant forms of NS28. The amino-terminal 90 amino acid residues of wt NS28 are displayed using the single-letter amino acid code. The distribution of charged amino acids is indicated. Two N-linked glycosylation sites at positions 8 and 18 are marked by CHO. Putative hydrophobic regions, designated H1, H2 and H3, are underlined. Mutants are defined by the residues deleted (Δ). Broken lines, deleted regions; solid lines, retained sequences.

residues of wt NS28 provide a convenient and sensitive assay for domain translocation. Translocation of the deleted proteins therefore was initially inferred when these two sites were filled. Figure 2 shows the translation products of both wt and mutant mRNAs in the absence and presence of canine microsomes. The wt primary translation product is a 20 kd protein (lane 3). The presence of microsomes during translation results in addition of N-linked carbohydrate at both sites, thus increasing the apparent mol. wt by 8 kd (Both *et al.*, 1983, lane 4, arrowed) and yielding a glycosylated product with an efficiency of $\sim 90\%$.

Although glycosylation sites remain potentially available on mutants $\Delta 1-16$, $\Delta 28-46$, $\Delta 40-48$, $\Delta 66-83$, an increase in mol. wt due to carbohydrate addition at two sites is only observed in constructs $\Delta 40-48$ (lane 12) and $\Delta 66-83$ (lane 14). Mutant $\Delta 1-16$ is glycosylated at the single remaining site (lane 6); but mutants $\Delta 28-46$ and $\Delta 29-84$ (deleted in H2) do not undergo glycosylation (lanes 10 and 16). As anticipated, polypeptides lacking the glycosylation sites are not glycosylated (lanes 8, 18, 20), and the last of these mutants is inefficiently translated.

Three conclusions can be drawn from these results. (i) The fact that the mutant protein containing only the H1 domain is not glycosylated demonstrates that H1 alone cannot mediate translocation of the protein. It appears, therefore, that H1 is not the signal sequence and that this function should lie within H2 or H3. This finding is further supported by the high efficiency of glycosylation of mutant $\Delta 1-16$ at the single remaining site at residue 18 (lane 6). (ii) H3 is not required for translocation because removing this domain (mutant $\Delta 66-83$) does not abolish glycosylation (lane 14). (iii) The signal sequence function therefore can be provisionally assigned to the H2 domain. Such an assignment is supported by the observation that a major deletion of 16 residues in the H2 region abolishes glycosylation at both sites (lane 10). The shortening of the H2 domain by removing

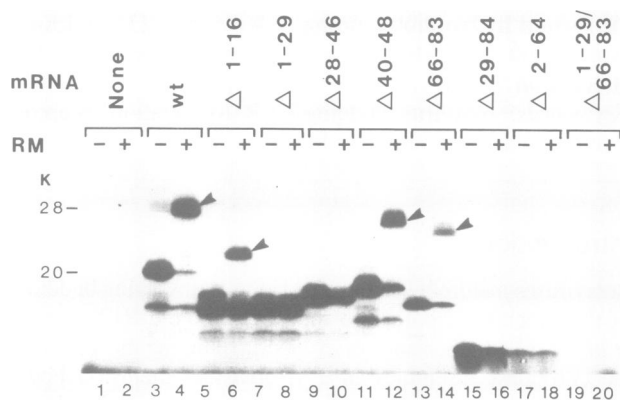


Fig. 2. The H2 domain is essential for glycosylation. mRNA encoding NS28 and variant proteins were transcribed from Bluescript M13+ constructs using T7 RNA polymerase. Protein synthesis was performed in rabbit reticulocyte lysates in the absence and presence of dog pancreatic microsomes (-/+ RM). Lanes 1 and 2 are control translations that lack exogenous mRNA. Lanes 3 and 4 show the precursor and glycosylated form of the wt protein; their corresponding mol. wts are shown on the left. Lanes 5-20 contain the primary and where present the glycosylated forms (arrowed) of the truncated mutant proteins. The lower mol. wt band underneath the primary translation product in lanes 3, 4, 5 and 6 presumably results from initiation at Met 30, since this product has a similar size to the major product of mutant $\Delta 1-29$ (lane 7).

a sequence that includes two basic residues affects glycosylation only minimally (lane 12). The extent of the region essential for insertion and translocation can thus be localized to amino acids 28–40.

The H2 domain acts as both an internal signal sequence and as a transmembrane anchor

To test for membrane association and translocation of all the variant forms of NS28, membranes were treated by the alkaline-mediated-release protocol (Fujiki *et al.*, 1982). Figure 3 shows luminal (S2) and membrane-bound (P2) fractions of all protein variants. In addition, free protein fractions (S1) are shown for proteins that are not glycosylated. Translocation of yeast prepro- α -factor, a glycoprotein known to be secreted, was used as a control in a parallel experiment: it was found in the microsomal fraction after the first centrifugation (data not shown), but transferred to the soluble fraction after alkaline treatment (lane 1, arrowed). In contrast, the integral membrane status of wt NS28 is confirmed in lane 4. Mutant proteins with intact H2 domains (lanes 6, 10, 13, 25), mutant $\Delta 40-48$ (lane 8) and $\Delta 2-64$ (lane 22) were also found to be integral. Mutants $\Delta 28-46$ and $\Delta 29-84$ are inefficiently inserted, as some material is found in the soluble fraction before alkaline extraction of luminal contents (lanes 14 and 17 respectively). However, the material that is found associated with the pellet fraction remains membrane bound even after alkaline treatment (lanes 16 and 19). None of the variant proteins were found in the lumen of the microsomes.

These results support the observations made above with regard to glycosylation. The integral membrane status of $\Delta 1-29$, $\Delta 1-29/66-83$ and $\Delta 66-83$ indicates that H2 must function both as a signal sequence and as a transmembrane anchor. H1 can mediate interaction with the membrane, but it is not sufficient to achieve translocation because mutants $\Delta 28-46$ and $\Delta 29-84$ were not glycosylated (Figure 2) even though both proteins interacted with the membrane (Figure 3). Unexpectedly, H3 alone ($\Delta 2-64$) is sufficient to mediate association with the membrane.

Expression of mutant NS28 proteins in vivo confirms exclusive ER location of the protein

CMT4 cells transfected with either wt NS28 or each of the

variants expressed intracellular products at the mol. wt expected for each deletion, albeit at varying levels (Figure 4). No immunoprecipitable product was detected in the media (data not shown). Wild-type, $\Delta 40-48$ and $\Delta 66-83$ were glycosylated at both of the potential N-linked sites and $\Delta 1-16$ was glycosylated at its only remaining site. These carbohydrates were endoH sensitive, demonstrating the presence of high mannose type glycosylation that was not processed by enzymes located in the medial or trans-Golgi elements. $\Delta 2-64$, $\Delta 1-29$ and $\Delta 1-29/66-83$ have their potential glycosylation sites deleted and thus are not modified. $\Delta 28-46$ and $\Delta 29-84$ have both potential N-linked glycosylation sites available but they are not utilized. Thus expression of both wild-type and mutant forms of NS28 *in vivo* confirms the results obtained *in vitro*.

Immunofluorescent localization of NS28 in transfected cells

The expression of NS28 in CMT4 cells transfected with plasmids containing the coding sequences for wt NS28, or for each of the mutant NS28 sequences, was also evaluated by indirect immunofluorescence localization using a primary polyclonal rabbit antiserum to a β -galactosidase–NS28 fusion protein and a fluorescein-conjugated secondary antibody (Figure 5, panels a, c, e and g). These staining patterns were then compared with those of rhodamine-conjugated wheat germ agglutinin, a marker for the Golgi apparatus (panels b, d, f and h).

Localization of NS28 and the variants $\Delta 1-16$, $\Delta 1-29$, $\Delta 40-48$, $\Delta 2-64$, $\Delta 28-46$, $\Delta 66-83$ and $\Delta 1-29/66-83$ yielded a distinct and extensive arborizing reticular pattern which was predominant immediately surrounding the nucleus (or nuclei) but also extended to the periphery of each cell (data only shown for wt NS28, $\Delta 1-16$, $\Delta 40-48$ and $\Delta 1-29/66-83$). Fluorescence derived from NS28 would thus include the nuclear envelope, the endoplasmic reticulum (ER) and most likely, transitional elements (TE) of the ER that come into close proximity with, but are nevertheless distinct from the Golgi apparatus (Poruchynsky *et al.*, 1985).

This type of localization pattern for NS28 did not coincide with the pattern of staining for the rhodamine-conjugated wheat germ agglutinin (most clearly evident in panels e and f), indicating that wt NS28 and each of the mutant proteins

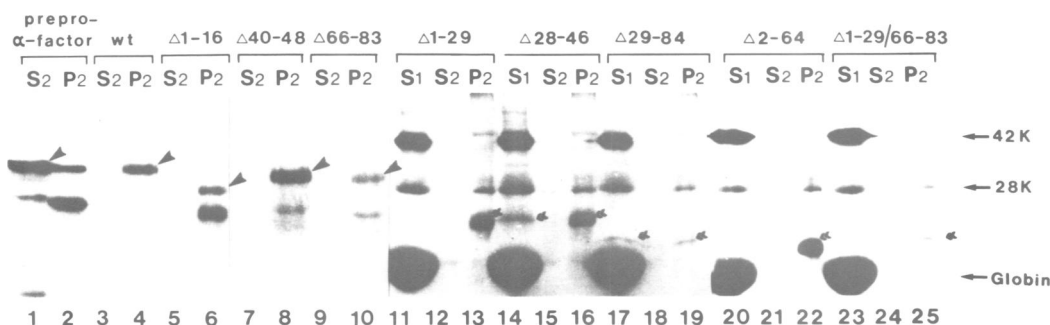


Fig. 3. Both wild-type and variant forms of NS28 associate with membranes. Proteins were synthesized in rabbit reticulocyte lysate supplemented with microsomes as described in Materials and methods. Membrane-bound and free proteins (S1) were separated by centrifugation through a high-salt sucrose cushion. Membrane-associated proteins in the pellet were then resuspended in 100 mM sodium carbonate, pH 11.5. Membranes were recentrifuged, separating integral membrane proteins in the pellet (P2) from the released microsomal contents and peripheral proteins present in the supernatant (S2). S1 fractions are not shown for protein variants known to be translocated and glycosylated (Figure 2). Release of processed yeast prepro- α -factor from the lumen served as a positive control (lanes 1 and 2). Heavy arrows indicate glycosylated proteins; double arrows mark non-glycosylated NS28 variant polypeptides. Heavy bands in S1 fractions at 42, 28 kd and the globin region (as indicated on the right-hand side) derive from endogenous products synthesized in the lysate (Jackson and Hunt, 1983).

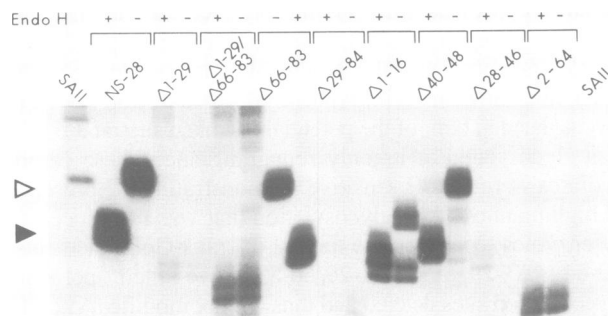


Fig. 4. *In vivo* expression of wild-type and NS28 variants in CMT4 cells. CMT4 cells were transfected with the cDNAs of NS28 variants and the intracellular material was immunoprecipitated with an antibody directed against the *lacZ*-NS28 fusion protein. Half of each sample was treated with endoH (reducing the apparent mol. wt of variants with carbohydrate content by ~8 kd). This is the region showing NS28 of two gels from different experiments. All variants appeared as doublets, presumably as a result of proteolysis of the C terminus, since for each of the glycosylated proteins the smaller product was glycosylated as well. This proteolytic activity could not be inhibited by the presence of protease inhibitors. In addition NS28, Δ 1-16, Δ 40-48, and Δ 1-29/66-83 contain a lower mol. wt band due to ectopic initiation at Met 30 and/or Met 54. This band of NS28 co-migrated with the product of Δ 1-29 which uses Met 30 for initiation. The intermediate band in Δ 40-48 presumably results from partial endoH digestion and indicates the addition of two high mannose carbohydrate groups to both its available sites: this intermediate band comigrates with the glycosylated band in Δ 1-16, demonstrating that this variant utilizes its only potential N-linked carbohydrate site.

probably remained associated with the endoplasmic reticulum. The variant Δ 29-84, which is not translocated, is apparently localized in the nucleus and in a perinuclear region (data not shown). These morphological interpretations are supported by the endoH-sensitive glycosylation of those mutants that retain the glycosylation site because neither the wild-type nor mutant forms of the protein receive complex carbohydrate (see above).

Topology of the wild-type glycoprotein

Previous studies have demonstrated that NS28 is a transmembrane protein with a protease-sensitive C-terminal domain protruding into the cytoplasm (Ericson *et al.*, 1983; Kabcenell and Atkinson, 1985). Although the translocation data presented above for the mutant proteins strongly suggest that H2 spans the membrane, Chan *et al.* (1988) have recently proposed a model for the topology of this protein in the membrane in which H3 (amino acids 63–80) acts as the transmembrane domain.

To further investigate the identity of the transmembrane sequence, we analysed the digestion products of wt NS28 obtained following digestion with five proteolytic enzymes, namely trypsin, α -chymotrypsin, bromelain, proteinase K and protease V8. Figure 6 displays all the potential cleavage sites present in bovine NS28 for trypsin, α -chymotrypsin and protease V8. Whereas the distribution of trypsin sites is more or less random throughout the protein (starting from residue 48), α -chymotrypsin sites are concentrated in the N-terminal region (upstream from residue 86) and protease V8 sites in the C-terminal region (downstream from residue 86). The region of the protein surrounding residue 86 is unique in that it contains sites for all three proteases within a cluster of three amino acids. If H3 spans the membrane,

this cluster should be membrane-proximal and digestion should yield an 18 kd N-terminal-protected fragment (10 kd polypeptide, 8 kd carbohydrate). If, on the other hand, the H3 domain is exposed on the cytoplasmic side, limited proteolysis with all enzymes except protease V8 should yield, in addition, fragments shorter than 18 kd.

As demonstrated in Figure 7, we found this to be the case. Under conditions that completely remove the mature form of the protein (with the exception of α -chymotrypsin), each digestion leads to a series of fragments that have an 18 kd fragment in common (indicated by a double arrow on the left-hand side in Figure 7), resulting from cleavage at the shared sites near residue 86. However, trypsin, protease K and, to a lesser extent, α -chymotrypsin also share a smaller fragment of ~13 kd (indicated by a long arrow on the left-hand side in Figure 7). A fragment of this size can result either from cleavage near residue 44 (theoretical mol. wt 13.1 kd) or 65 (theoretical mol. wt 15.6 kd), as only these clusters contain sites for α -chymotrypsin; the size of the fragment obtained favours cleavage near residue 44. No fragments can be observed between the 13 and 18 kd bands, suggesting that the sites within H3 are protected from proteolytic attack. The relatively low radioactivity in the 13 kd band can be attributed to partial accessibility to protease and the fact that this fragment contains only three of the possible 10 methionines present in the full-length protein. EndoH treatment of the membrane-bound fractions results in a similar pattern of fragments with mol. wts reduced by ~8 kd, confirming that the proteolytic fragments are derived from the N-terminal region of NS28 (data not shown).

To investigate further the origin of the apparent 13 kd fragment, similar digestions were carried out with mutant Δ 40-48. This protein lacks both the α -chymotrypsin site at position 44 and the trypsin site at position 48 (Figure 6). The pattern of proteolytic fragments obtained from this mutant is almost identical to that of wt NS28 (Figure 7, lanes 8–13) with a 17 kd fragment, similar to the 18 kd fragment of wt NS28, evident as the strongest band in all digestions. However, the smaller 13 kd fragment is absent from all digests. This observation can be attributed to the cleavage sites removed by Δ 40-48 and provides strong supporting evidence that these sites indeed are partially accessible in the wt protein.

Proteolysis at 30°C confirms the accessibility of residues 44–48

Enhanced cleavage within residues 44–48 of the wt protein was clearly demonstrated when proteolysis was carried out under more stringent conditions (Figure 8). Digestion at 30°C results in a lower number of fragments for each individual protease and a higher yield of the 13 kd fragment observed after trypsin, α -chymotrypsin and proteinase K treatment (indicated by a long arrow on the left-hand side, lanes 2, 3 and 5). The fragments are all endoH sensitive and migrate with mol. wts reduced by ~8 kd (lanes 8–11). The same conditions of proteolysis were applied to mutant Δ 40-48 (Figure 8, middle picture). Fragments smaller than ~17 kd could not be detected, thus confirming the results obtained for digestions at 0°C. Deletion of the available cleavage sites in the wt protein therefore results in proteolytic protection upstream from residue 85.

To rule out the possibility that the NS28 molecule emerges

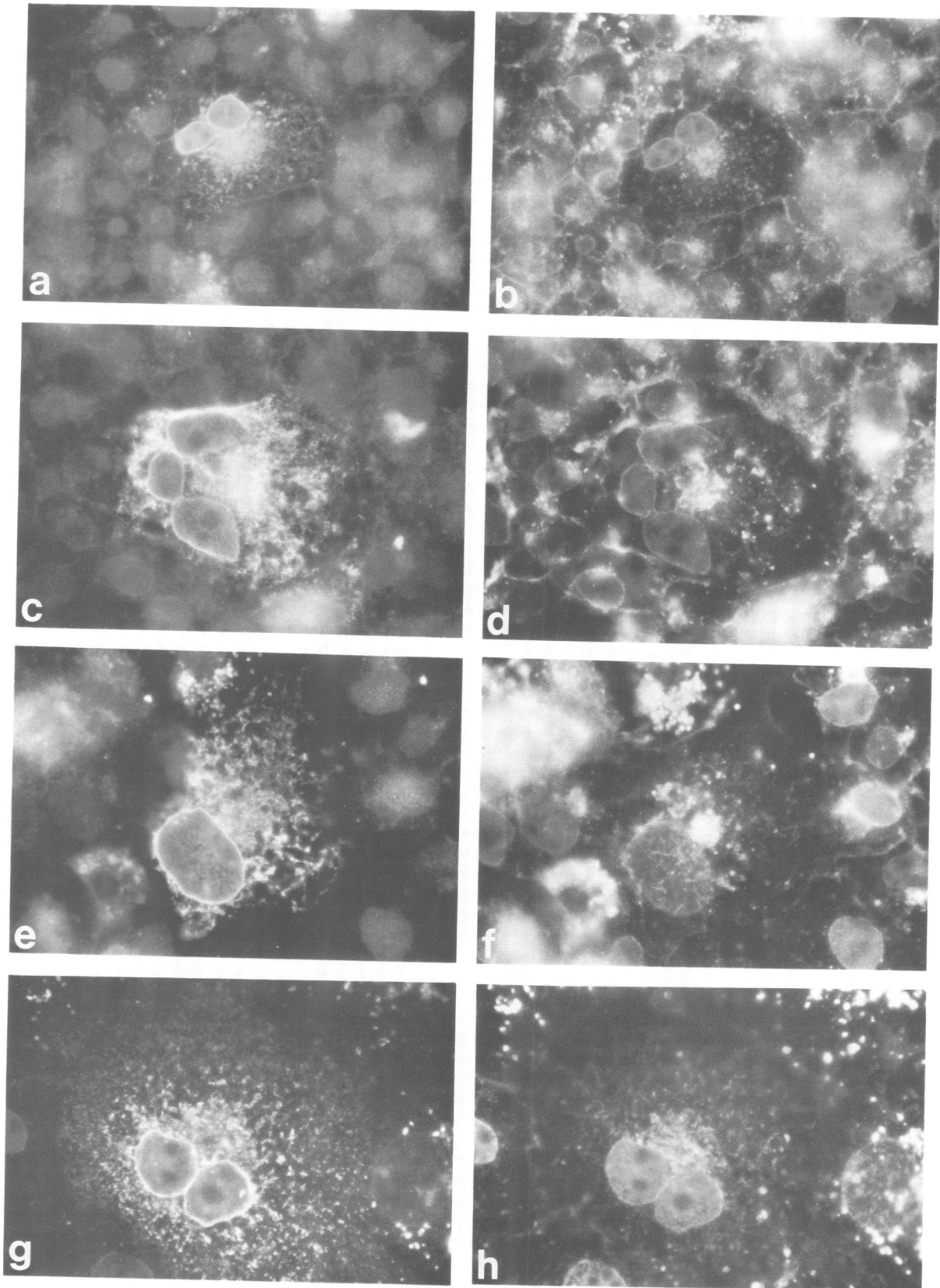


Fig. 5. The intracellular immunofluorescent localization of NS28 in CMT4 cells. Transfections were with wt NS28 (a), $\Delta 1-16$ (c), $\Delta 40-48$ (e) or $\Delta 1-29/66-83$ (g), and detection was with a rabbit polyclonal antiserum to the *lacZ*-NS28 fusion protein and a fluorescein-conjugated secondary antibody. Cells were also double labelled with wheat-germ agglutinin conjugated to rhodamine (b,d,f,h). Magnification 625 \times .

from the membrane downstream of residues 65/66 (the closest cleavage sites for trypsin and α -chymotrypsin downstream of residues 40-48), mutant $\Delta 66-83$ was

subjected to proteolysis at 30°C under otherwise identical conditions to those described above. In this mutant, the α -chymotrypsin site at residue 65 is fused directly to the

multiple protease cleavage region at residues 85–87 and all other cleavage sites in H3 are abolished (Figure 6). Assuming that only residues upstream of residue 44 are protected in the membrane, proteolysis of this mutant protein with trypsin and α -chymotrypsin is expected to produce fragments of lower mol. wt (~ 13 kd) than that obtained from digestion with protease V8 (~ 16 kd), because protease V8 has no sites upstream of residue 87. This prediction is confirmed in Figure 8. Under conditions that completely remove the full-length protein, trypsin, α -chymotrypsin and protease K all yield a single fragment of ~ 13 kd (lanes 2, 3 and 5), which migrates with a mol. wt < 6.5 kd after endoH treatment. In contrast to these results, protease V8 degradation leads to fragments of higher mol. wt (lane 4). It should be noted that mutant $\Delta 66-83$, from which the entire H3 domain has been deleted, now yields essentially a single proteolytic

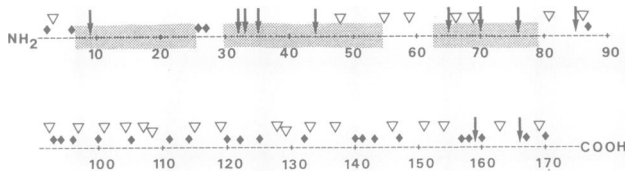


Fig. 6. Location of proteolytic cleavage sites within wt NS28. The dotted line represents the wt amino acid sequence for the bovine NS28 protein (Powell *et al.*, 1988). Potential cleavage sites for trypsin (∇), α -chymotrypsin ($|$) and protease V8 (\blacklozenge) are indicated. Putative hydrophobic regions (Figure 1) are shown as shaded blocks.

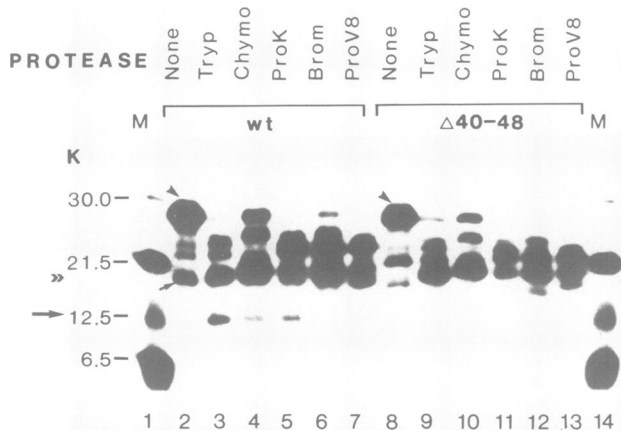


Fig. 7. Protease sensitivity of membrane-inserted wt NS28 and mutant $\Delta 40-48$. Protein mixtures containing microsomes were post-translationally treated at 0°C with trypsin, α -chymotrypsin, proteinase K, bromelain and protease V8 as described in Materials and methods. Proteolysis was terminated by adding an excess of aprotinin and phenylmethylsulphonylfluoride and membrane-bound fragments then purified by sedimentation of microsomes through a high-salt sucrose cushion. Proteolytic fragments were analysed by 14% SDS-PAGE. Lanes 1 and 14 are mol. wt markers. Lanes 2 and 8 show wt and $\Delta 40-48$ protein respectively in the absence of protease (indicated by heavy arrow; minor bands presumably arise from incomplete glycosylation of the wt protein). Lanes 3–7 and 9–13 show membrane-bound fragments after proteolysis with the enzymes as indicated. The arrows on the left-hand side of the figure identify the 18 kd cleavage product (double arrow) and the 13 kd product (single long arrow). Note that the 13 kd fragment is present in wt NS28 but absent in mutant $\Delta 40-48$. Molecular weights were deduced from commercial markers (lanes 1 and 14), wt glycosylated NS28 (28 kd; heavy arrow in lane 2) and an internal translation side product starting with methionine at position 30 (theoretical mol. wt 17.4 kd; light arrow in lane 2).

product, implying that interaction between H3 and the remainder of the protein may be responsible for partial protection of some sites in the wt protein (Figure 8).

Discussion

NS28 is an example of a small group of membrane proteins that are localized to intracellular membranes in an orientation in which the N terminus is exposed on the inner surface of the membrane and whose signal sequences are not cleaved. In this regard, this rotavirus protein resembles cytochrome

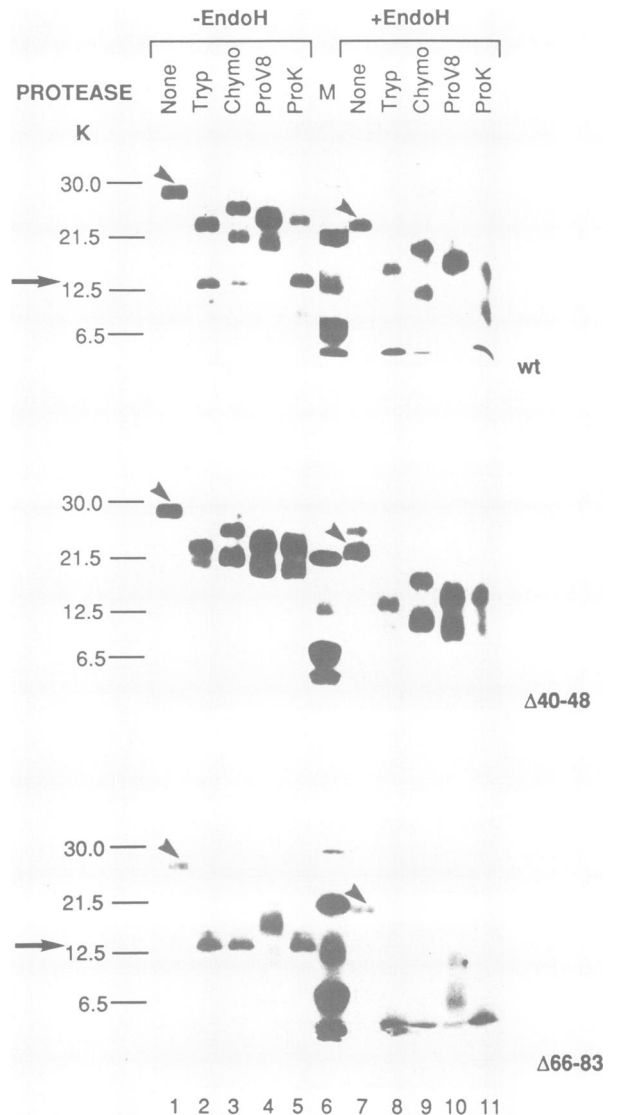


Fig. 8. Proteolytic cleavage sites upstream of the H3 domain are accessible. Proteolysis of membrane-associated wt NS28, mutant $\Delta 40-48$ and mutant $\Delta 66-83$ was carried out 30°C using trypsin, α -chymotrypsin, protease K and protease V8 (see Materials and methods). After addition of protease inhibitors, membrane-associated fragments were sedimented through a high-salt sucrose cushion. Half of the membrane fraction was treated with endoH (reducing the apparent mol. wt of fragments with carbohydrate content by ~ 8 kd) and samples were separated by 13% SDS-PAGE. Lanes 1 and 7 show intact forms of the protein (indicated by heavy arrows) before and after endoH treatment respectively. The proteolytic fragments obtained from the indicated proteases are shown in lanes 2–5 ($-\text{endoH}$) and 8–11 ($+\text{endoH}$). Lane 6 contains mol. wt markers. The glycosylated 13 kd proteolytic fragment present in wt and mutant $\Delta 66-83$ is identified by a long arrow on the left-hand side.

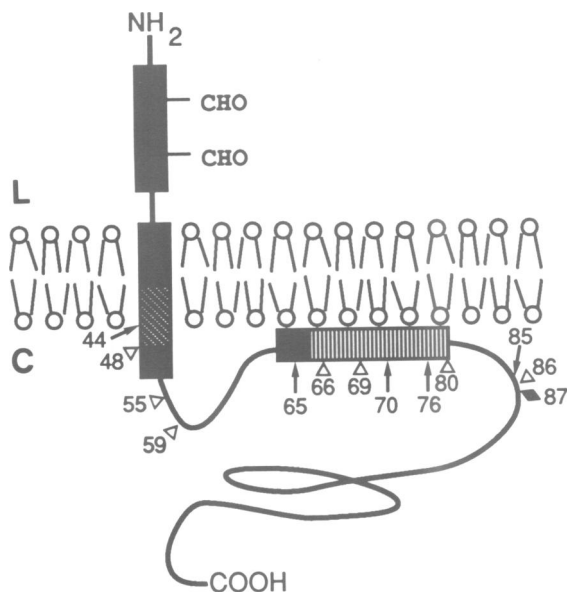


Fig. 9. A model for the topology of NS28 in the ER membrane. The blocks represent the three putative hydrophobic domains. Diagonally and vertically striped regions indicate deletions $\Delta 40-48$ and $\Delta 66-83$ respectively. The position of potential proteolytic cleavage sites within residues 43–88 are shown for trypsin (∇), α -chymotrypsin (\cap) and protease V8 (\blacklozenge). The protein emerges from the membrane near the α -chymotrypsin site at amino acid 44. C, cytoplasm; L, lumen.

P450, which is also ER localized and has a single membrane-spanning domain (Sakaguchi *et al.*, 1987). Comparisons can also be made with the coronavirus E1 protein, a protein targeted to the Golgi but having three putative hydrophobic domains at the N terminus (Machamer and Rose, 1987). However, unlike NS28, the coronavirus E1 protein utilizes all three hydrophobic domains as membrane-spanning regions. Such a situation cannot apply to rotavirus NS28: since H1 contains the glycosylation sites that are utilized, this domain must have a luminal orientation leaving only H2 and H3 as potential membrane-spanning domains. It is unlikely that both of these domains are membrane-spanning, since the C terminus of the protein is protease-sensitive and therefore situated on the cytoplasmic side of the membrane (Ericson *et al.*, 1983).

Glycosylation at the two sites resident within H1 has provided a convenient assay for monitoring the translocation of NS28. The translocation studies demonstrate that amino acids 28–40 in the H2 region are essential for glycosylation. Processing takes place only when H1 is accompanied by H2, but not when H1 is either present alone or is combined with H3. These results, together with the fact that deletion of the first 16 amino acids (mutant $\Delta 1-16$) does not hinder glycosylation at the single remaining site, makes it unlikely that either H1 or H3 function as a signal sequence. We therefore conclude that H2 serves as an internal combined signal and membrane anchor sequence. Although the initial assignment of the three hydrophobic domains was somewhat arbitrary (Argos *et al.*, 1982), it is interesting to note that despite the restricted region of the protein encompassed by H3 (17 amino acids) and the presence of charged residues, this region nevertheless does appear to constitute a potential membrane-spanning domain since the presence of H3 alone is sufficient to mediate association of the protein with the membrane (mutant $\Delta 2-64$). From the combined proteolysis

and translocation data of the wt and mutant forms of NS28, we conclude that NS28 is a transmembrane protein that emerges at the cytoplasmic side of the membrane near residue 44.

Recently Chan *et al.* (1988) investigated the topology of NS28 of the SA11 strain by proteolytic degradation of protein synthesized *in vitro* in the presence of canine pancreatic microsomes. In contrast to our results, these workers identified H3 as the membrane-spanning domain. Although we are able to confirm that the H3 domain is protected from digestion with α -chymotrypsin, trypsin and protease K, we attribute this protection to factors other than transmembrane status of this region. In our experiments trypsin sites upstream of the H3 region at amino acids 48–67 and α -chymotrypsin sites at positions 44 and/or 65 clearly are available to these enzyme(s), suggesting that H3 is merely 'embedded' on the surface of the cytoplasmic side of the membrane; this would leave a protease-sensitive loop of ~ 18 residues between H2 and H3 (Figure 9). Alternatively, protection of H3 might result from the intermolecular disulphide bonding of cysteines at positions 63 and 71. This could be due to a multimeric form of NS28 in the membrane, a likely structure in view of its function as a receptor for the core. Either of these two possibilities would provide preferential cleavage sites and yield more than one digestion product.

Analysis of the proteolytic digestion products of mutant $\Delta 40-48$ (lacking both the α -chymotrypsin site at amino acid 44 and the trypsin site at amino acid 48) has enabled us to confirm the site at which the polypeptide emerges from the membrane as being close to residue 44. This conclusion is supported by deletion of residues 66–83, which results in the juxtaposition of the α -chymotrypsin site at amino acid 65 with that at 85. As expected, the proteolytic fragments of this mutant generated by trypsin, α -chymotrypsin and proteinase K (Figure 8) appear to be of similar size to those of the wt protein (~ 13 kd), indicating that the cleavage sites at positions 44 and 48 are accessible. A model (Figure 9), in which NS28 emerges from the membrane at the α -chymotrypsin site at amino acid 44, implies that only amino acids 30–44 are required for spanning the membrane. Fifteen amino acids seems to be rather short for a transmembrane domain, but the definition of the upstream boundary of the H2 region is somewhat arbitrary and two amino acids further upstream may be included in this region. Previous experiments conducted with the influenza haemagglutinin membrane anchor have demonstrated that 17 hydrophobic amino acids are sufficient to anchor the HA protein stably in the membrane (Doyle *et al.*, 1986).

Emergence of the protein from the membrane at or near position 44 leaves ~ 131 residues potentially available as the receptor domain for interaction with the core. In a receptor–ligand binding assay using membranes derived from cells infected with SA11 rotavirus, Au *et al.* (1989) demonstrated that digestion of the membranes with trypsin totally abolished receptor activity, whereas digestion with α -chymotrypsin only partially reduced binding of the core. This observation suggests that the carboxy-proximal residues (131–175) are not essential for receptor activity (the SA11 NS28 protein possesses an additional α -chymotrypsin site at position 131). Now that the point at which the protein emerges from the membrane has been identified, a better definition of the domains involved in receptor–ligand

interaction should be able to be made using an *in vitro* approach involving site-directed modification of the cytoplasmic domain of the protein. Furthermore, since the ligand involved (the virus core) appears amenable to crystallographic analysis (B.Harris, I.Anthony, A.R.Bellamy and S.Harrison, unpublished observations, 1988), there now appear to be good prospects for reaching a detailed understanding of the nature of the interaction of this unusual receptor protein with its ligand.

Materials and methods

Plasmids

A cDNA clone of dsRNA genomic segment 10 encoding NS28 of the bovine rotavirus strain NCDV was obtained (Powell *et al.*, 1988). The inferred protein sequence exhibits a high degree of homology with the homologous proteins of other rotavirus strains, but lacks the α -chymotrypsin cleavage site at residue 131 found in NS28 of the simian strain SA11 (Chan *et al.*, 1988). For initial transcription experiments, an *AhaIII*-*SalI* fragment containing the entire coding region of the 175-residue protein was subcloned into *SmaI*-*SalI* digested transcription vector pSP65 (Promega). Subsequently the gene was transferred into the transcription vector Bluescript M13+ (Stratagene) as an *EcoRI*-*SalI* fragment, yielding plasmid pBSM13-NS28. Plasmids coding for the N-terminally truncated NS28 proteins Δ 1-16 and Δ 1-29 were constructed by subcloning the *HindIII*-*SalI* and *BamHI*-*SalI* gene fragments into the polylinker region of Bluescript M13+. The resulting mRNAs contain the initiation codons corresponding to residues 17 and 30 respectively. Internal deletions were introduced by oligonucleotide-directed mutagenesis and all truncated genes are designated by the amino acids deleted (inclusive numbering applies).

Site-directed mutagenesis

Oligonucleotides (26mers) designed to introduce deletions were composed of two sequences, each 13 nucleotides in length and complementary to the sequence flanking the desired deletions. Synthesis was carried out on a DNA synthesizer (model 380A, Applied Biosystems). Both the M13mp9 and Bluescript M13+ cloning systems were used to loop out target sequences. Deletions Δ 40-48 and Δ 28-46 were obtained by transferring the *EcoRI*-*SalI* gene fragment from the pSP65 construct into bacteriophage M13mp9. The appropriate oligonucleotide was annealed to the single-stranded form of the vector and extended by DNA polymerase (Klenow) in the presence of T4 ligase (Zoller and Smith, 1982). Mutant RF DNA was prepared from positive colonies and the modified *EcoRI*-*SalI* gene fragment inserted into Bluescript M13+ for transcription. Mutations Δ 66-83, Δ 29-84 and Δ 2-64 were introduced directly into the pBSM13-NS28 clone. Preparation of single-stranded plasmid DNA, annealing and oligonucleotide-primed second strand synthesis were both carried out following protocols recommended by the supplier except that P32 protein was not included in primer extension. The gene coding for mutant Δ 1-29/66-83 was obtained by using the pBSM13+/ Δ 1-29 clone as a template for deletion of the 66-83 sequence.

Colonies carrying the mutated genes were all identified by differential hybridization with the appropriate ³²P-labelled oligonucleotide used to introduce the deletion. The presence of the deletion was confirmed by dideoxynucleotide sequence analysis (Sanger *et al.*, 1977).

In vitro transcription and translation

The Bluescript M13+ derivatives, containing viral cDNA templates downstream from the T7 promoter, were linearized with *SalI* and transcribed according to directions provided by the supplier (Stratagene). Typically, 1 μ g plasmid DNA was incubated for 30 min at 37°C in 40 mM Tris-HCl, pH 8, 8 mM MgCl₂, 2 mM spermidine, 50 mM NaCl, 0.25 mM rNTPs, 30 mM dithiothreitol, 1 U/ μ l RNasin (Promega) and 10 U T7 polymerase (Stratagene) in a total volume of 25 μ l. The DNA template was removed by addition of 1 U RQ1 DNase 1 (Promega) and incubation for 10 min at 37°C. RNA was then purified by phenol/chloroform extraction followed by ethanol precipitation and washing with 70% ethanol. The RNA was stored in water at -20°C for 2-3 months without any evidence of degradation.

Translation reactions were performed using nuclease-treated rabbit reticulocyte lysate according to the supplier's protocol (Promega). Approximately 0.5-1.0 μ g mRNA was translated in a standard 25 μ l reaction containing 40 μ Ci [³⁵S]methionine (Amersham): translocation was studied by supplementing the translation mixture with 4 equivalents of canine pancreatic microsomes (Promega). After incubation at 30°C for 1-1.5 h, the reaction was stopped by adding cycloheximide and RNase A to final

concentrations of 1 mM and 80 μ g/ml respectively. Translation products were analysed by electrophoresis in 12.5% SDS-polyacrylamide gels (Laemmli, 1970). For fluorography, the gels were soaked in 7% acetic acid and then in Amplify (Amersham), dried and exposed to preflashed X-ray film at -80°C for 1-3 days.

Membrane sedimentation and carbonate extraction

Membrane-bound full-length proteins, or proteolytic degradation fragments, were selectively isolated by sedimentation of microsomes through a high-salt 0.5 M sucrose cushion in a Beckman airfuge using the A-100/30 rotor. Translation products (5 μ l) were diluted to 40 μ l with translation salts (Kabacell and Atkinson, 1985) and layered over a 60 μ l sucrose cushion in cellulose propionate airfuge tubes. Centrifugation for 4 min at 20 p.s.i. yielded a pellet fraction (P1) containing membrane bound material, and a supernatant fraction (S1) containing free material. Supernatants were either TCA precipitated or desalted by passage through a Biogel P2 column followed by lyophilization of the eluted proteins. Pellet fractions were either directly prepared for gel analysis, or resuspended in 100 μ l of 0.1 M sodium carbonate, pH 11.5, to release luminal contents (Fujiki *et al.*, 1982).

Microsomes in sodium carbonate were incubated at 0°C for 30 min and then centrifuged at 30 p.s.i. for 10 min to sediment membranes containing integral membrane proteins (P2) and leave released microsomal components in the supernatant (S2). Supernatants were neutralized with 1 N HCl and concentrated as described above. Both membrane-bound and free proteins were solubilized in Laemmli buffer and equal amounts analysed by electrophoresis in 12.5% SDS-polyacrylamide gels and by subsequent fluorography.

Protease sensitivity and endoH digestion

Translation mixtures were diluted 1:1 with translation salts and, when supplemented with microsomes, adjusted to 3 mM tetracaine-HCl. Aliquots (10 μ l) of this reaction mixture were then separately treated with either 100 μ g/ml TMCK-treated trypsin (Serva), 500 μ g/ml α -chymotrypsin (Calbiochem), 50 μ g/ml proteinase K (Boehringer), 200 μ g/ml bromelain (Sigma) or 500 μ g/ml protease V8 (Miles) for 30 min on ice. For more stringent conditions of proteolysis, digestions were carried out at 30°C for 30 min with protease as above, except that the proteinase K concentration was raised to 100 μ g/ml. To permeabilize microsomes in control samples, Triton X-100 was added to 1% prior to the addition of protease. Proteolysis was terminated by adding PMSF and trasylol (Serva) to a final concentration of 1 mM and 0.5 mg/ml respectively. Samples then were either processed directly for SDS-PAGE, or centrifuged through a sucrose cushion to purify membrane-bound fragments as described above. For treatment with endo- β -*N*-acetylglucosaminidase H (endoH) the pellet fraction was solubilized in buffer containing 10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 75 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 0.5% DOC, 0.5% Triton X-100, 0.05% SDS and 50 U/ml trasylol and divided into two aliquots. One sample was treated with 0.2 mU endoH (Boehringer) for 1 h at 37°C; the other served as a control. Both samples were then analysed by SDS-PAGE and mol. wts estimated using markers (Amersham) of carbonic anhydrase (30.0 kd), soybean trypsin inhibitor (21.5 kd), cytochrome c (12.5 kd) and aprotinin (6.5 kd).

Transfection and immunoprecipitation

The cDNAs of NS28 and mutants Δ 40-48, Δ 28-46, Δ 66-83 and Δ 1-29/66-83 were isolated from the corresponding Bluescript M13+ plasmid as *EcoRI*-*SalI* fragments while those of Δ 1-16 and Δ 1-29 were each isolated as *HindIII*-*SalI* fragments. All fragments were blunt-ended using Klenow enzyme, ligated to phosphorylated *XbaI* linkers (New England Biolabs) and transferred to the *XbaI* site of the eukaryotic expression vector pSVL (Pharmacia). All plasmid DNAs used for transfection were isolated by the alkaline lysis procedure (Maniatis *et al.*, 1982), purified on CsCl/ethidium bromide gradients, ethanol precipitated and resuspended in water.

Transfection was performed as described previously (Poruchynsky *et al.*, 1985) except that the CMT4 cell line with the SV40 large T antigen under the control of the inducible metallothioneine promoter was used. Cells were grown to 80% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM glutamine (all from Gibco Laboratories) and the monolayer was then rinsed and transfected in Tris-buffered saline (TBS). DNAs at a concentration of 20 μ g/ml were added, followed by DEAE-dextran (Pharmacia, 2 \times 10⁶ daltons: 500 μ g/ml; Guan and Rose, 1984). After 1.5 h at 37°C, the TBS was removed, 100 μ M chloroquine (Sigma) in DMEM was added, and cells were incubated at 37°C for 3 h. After removal of the chloroquine, DMEM containing 100 μ M ZnCl₂ and 1 μ M CdSO₄ was added and the cells were incubated at 37°C for a further 40 h.

Prior to radiolabelling, monolayers were incubated at 37°C in DME salts lacking serum and methionine but supplemented with all other amino acids and glucose (1 mg/ml) for 10 min. L-[³⁵S]Methionine was then added to the medium at 150 µCi/ml and labelling allowed to proceed for 4 h at 37°C. The medium was collected, non-adherent cells were pelleted by centrifugation for 10 min in a microfuge and the supernatant was analysed by immunoprecipitation. The transfected cells were harvested in 1 × PBS using a rubber policeman following a rinse in phosphate-buffered saline.

Immunoprecipitations were performed using a rabbit polyclonal antiserum raised against a fusion protein of β-galactosidase and NS28. Immune complexes were precipitated using Protein A–Sepharose CL48 (Pharmacia), treated with endoH and processed for SDS–PAGE as described previously (Poruchynsky *et al.*, 1985).

Immunofluorescent localization of wild-type and variant NS28 in transfected cells

CMT4 cells were plated on glass coverslips in 35 mm dishes, transfected as described above and processed for immunofluorescence microscopy as described previously (Poruchynsky *et al.*, 1985). A 1:500 dilution of the polyclonal rabbit antiserum to the β-galactosidase–NS28 fusion protein described above was utilized as the primary antiserum in these localization studies. A fluorescein-conjugated secondary goat anti-rabbit antibody (Cappel Laboratories) was used at a 1:400 dilution and cells were double labelled with wheat-germ agglutinin conjugated to rhodamine (Vector Laboratories) as described previously (Poruchynsky *et al.*, 1985). The specimens were examined using a Zeiss photomicroscope and photographed using Ektachrome ASA400 film.

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