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Location of Sequences within Rotavirus SA11 Glycoprotein VP7 Which Direct It to the Endoplasmic Reticulum

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The Simian 11 rotavirus glycoprotein VP7 is directed to the endoplasmic reticulum (ER) of the cell and retained as an integral membrane protein. The gene coding for VP7 predicts two potential initiation codons, each of which precedes a hydrophobic region of amino acids (H1 and H2) with the characteristics of a signal peptide. Using the techniques of gene mutagenesis and expression, we have determined that either hydrophobic domain alone can direct VP7 to the ER. A protein lacking both hydrophobic regions was not transported to the ER. Some polypeptides were directed across the ER membrane and then into the secretory pathway of the cell. For a variant retaining only the H1 domain, secretion was cleavage dependent, since an amino acid change which prevented cleavage also stopped secretion. However, secretion of two other deletion mutants lacking H1 and expressing truncated H2 domains was unaffected by this mutation, suggesting that these proteins were secreted without cleavage of their NH_2-terminal hydrophobic regions or secreted after cleavage at a site(s) not predicted by current knowledge.

The mechanisms by which proteins are targeted to specific organelles in cells have been intensively studied in recent years. One of the best-characterized transport signals is that which directs proteins across the membrane of the endoplasmic reticulum (ER) (26). This is a peptide of widely varying sequence which is composed of a charged NH_2-region, a central hydrophobic core, and a polar COOH-terminal region. In most cases the signal peptide is located at the NH_2 terminus of a protein and in secreted proteins is removed by signal peptidase (24, 25). Only ovalbumin is known to be secreted without cleavage of its signal peptide, which is located internally in the protein (27). For integral membrane proteins, removal of the signal peptide is optional (27).

We are studying the nature and location of sequences in the rotavirus glycoprotein VP7 which direct its transport in the cell. Rotaviruses, which are major cause of acute gastroenteritis in animals and humans (8, 13), have an unusual morphogenetic pathway which makes it interesting to study the targeting and transient integration of their glycoproteins into the ER. Electron microscopy has shown that rotavirus subviral particles assemble in cytoplasmic inclusions and mature by budding through the membrane of the rough ER, becoming transiently enveloped in the process. The temporary envelope surrounding the particles is later removed, leaving mature viruses within the lumen of the ER (5, 8, 18).

VP7, the major serotype antigen of rotaviruses, is derived from a precursor which is cleaved as it is translocated across the ER membrane (7, 12). The protein is also modified with high-mannose carbohydrate that is sensitive to digestion with endoglycosidase H (Endo H) (8). No complex carbohydrate is detectable on viral VP7 (12), suggesting that the protein is not transported to the Golgi apparatus but remains in the lumen of the ER. Just how VP7 is retained in this organelle and incorporated into maturing virus particles is not yet clear. While it appears to be an integral membrane protein (12), its retention in the ER contrasts with the behavior of many other viral glycoproteins, e. g., influenza hemagglutinin and neuraminidase and vesicular stomatitis virus (VSV) glycoprotein G, which are transported as integral membrane proteins to the cell surface, where these particular viruses mature (11, 19, 20).

The VP7 gene from several rotavirus serotypes has now been cloned and sequenced (9, 10). The single open reading frame in each case predicts a 326-residue polypeptide containing two prominent NH_2-terminal hydrophobic regions, H1 and H2 (residues 6 to 23 and 32 to 48, respectively). The H1 domain is preceded by a potential initiation codon with a weak consensus sequence (14, 15); a strong ATG codon precedes H2, and this configuration is conserved in all the genes studied (9, 10). We have used gene mutagenesis and expression to investigate the role of these domains in directing VP7 to its correct cellular location. Here, using a mutant which has amino acids 23 to 61 deleted and another retaining only the H2 region, we show that the H1 domain as well as the H2 domain can direct the transport of VP7 across the ER membrane in vivo and in vitro.

Earlier studies showed that removal of amino acids 47 to 61 from the polypeptide converted VP7 from a glycoprotein which located in the ER to one which was secreted from the cell and modified with complex (endo H-resistant) carbohydrate (18). Other VP7 variants lacking residues 42 to 61 or 43 to 61 were also modified and secreted. Here we show that the new mutant lacking residues 23 to 61 was similarly glycosylated and secreted. One of the explanations considered for these results was that a cleavage site had been relocated in these proteins to a position suitable for use by signal peptidase. Using empirical rules formulated by von Heijne (24), we identified a potential signal peptidase cleavage site in the secreted VP7 variants, which, if used, could account for their secretion. A single amino acid substitution adjacent to this cleavage site prevented secretion of a variant lacking residues 23 to 61. However, the same mutation had no effect on the secretion of two other variants lacking residues 42 to 61 and 47 to 61, suggesting that fortuitous use of this cleavage site does not explain their secretion.

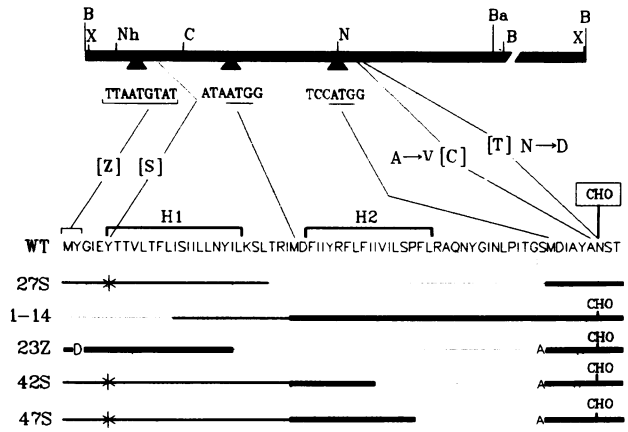

FIG. 1. Partial maps for SA11 wild-type and variant VP7 genes and proteins. The top of the figure shows the positions of the first three in-frame initiation codons, and the location of mutations Z, S, C, and T relative to key restriction endonuclease sites within the wild-type VP7 gene (B, *Bam*HI; Ba, *Bal*I; C, *Cla*I; N, *Nco*I; Nh, *Nhe*I; X, *Xho*I). The amino acid sequence of the NH_2-terminal 71 residues of wild-type VP7 (WT) are shown in the single-letter code. Hydrophobic domains H1 and H2 are bracketed. For the VP7 mutants, bold lines indicated amino acid sequence translated from the gene with amino acid substitutions as indicated. Coding regions present but not translated because of an in-frame termination codon (*) are shown as thin lines. (This region in Δ1-14 is not translated due to the deletion of the first initiation codon.) Regions of DNA deleted are shown as dotted thin lines. CHO, N-linked carbohydrate attached to Asn-69.

MATERIALS AND METHODS

Transfection of DNA and detection of VP7 proteins. The construction of wild-type and deleted VP7 genes in a simian virus 40 (SV40) expression vector has been described previously (18). Transfection of these plasmid DNAs into COS cells and analysis of the expressed VP7 proteins were carried out essentially as described (18), with the following modifications. Supercoiled plasmid DNA (20 μg per 100-mm plate) was added to cells (20 to 60% confluent) in Dulbecco modified Eagle medium (DMEM) plus 300 μg of DEAE-dextran per ml for 6 to 14 h at 37°C. Cell monolayers were then treated with 100 μM chloroquine in DMEM plus 10% fetal calf serum for 2 to 3 h. Tunicamycin (5 μg/ml) was added at least 4 h prior to radiolabeling, usually overnight. Cells were radiolabeled for 4 h with 50 to 75 μCi of [^{35}S]methionine (specific activity, >800 Ci/mmol) (Amersham Corp.) per ml, beginning 40 to 44 h after removal of the DNA and DEAE-dextran. Radiolabeled VP7 expressed in these cells was immunoprecipitated from both cell lysates and culture medium. Cell monolayers were harvested and lysed in buffer (18) containing 0.5% sodium dodecyl sulfate (SDS). Polyclonal antiserum to denatured VP7 was used to immunoprecipitate expressed VP7 proteins from the postnuclear supernatant of COS cell lysates. For detection of secreted VP7 in the culture medium, we used polyclonal antiserum against native VP7, prepared by inoculating rabbits with a recombinant vaccinia virus carrying the wild-type SA11 VP7 gene under the control of a vaccinia virus promoter (1a). Antisera were added overnight at 4°C, and then preswollen protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals) were added for 2 h without preincubation. Pelleted beads were washed extensively in buffer containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 25 mM Tris (pH 7.4), and 5 mM EDTA. Antibody-VP7 complexes were released from the beads by boiling and analyzed on 10% polyacrylamide-SDS gels (18).

Mutations in the VP7 gene. The complete VP7 gene clone used in this work differed at three positions from the previously published sequence derived from a partial clone (3). Thus, Cys-32, Leu-37 and Thr-65 were changed to Phe, Phe, and Ile, respectively (Fig. 1). In addition, mutants which had an internal deletion also had Ala substituted for Ser at residue 62 (Fig. 1). (Deletion mutants are named according to the codons deleted from the open reading frame of the VP7 gene). To simplify nomenclature, we will refer to these mutants as Δ42, Δ47, etc. Further modifications to these genes are described below (summarized in Table 1). Details of the construction of the wild-type and deleted VP7 genes Δ42-61, Δ47-61, and Δ1-14 in the expression vector pJC119 have been described (18). Additional mutants with deletions of residues 23 to 61 (Δ23) and 27 to 61 (Δ27) were also produced by Bal 31 digestion in the same way.

(i) Z and S mutations. The Z mutation changes the weak consensus sequence at the first ATG codon in VP7 to a strong one (14), at the same time changing Tyr-2 to Asp. The S mutation introduces a stop codon in place of Tyr at residue 6. These mutations were made as follows. Complementary synthetic oligonucleotides corresponding to bases 29 to 91 (coding strand) and 33 to 93 (noncoding strand) of the SA11 VP7 gene were synthesized (Applied Biosystems DNA synthesizer, model 380A), except that the sequence 5′-TTAATGT-3′ at the first initiation codon was changed to 5′-ATAATGG-3′. In addition, for each oligonucleotide, at the position corresponding to base 66 in the coding strand, a mixture of all four nucleotides was used. Since this was the third nucleotide of a Tyr codon, half of the molecules retained the wild-type amino acid sequence, and half acquired a stop codon. The Z and S mutations were incorporated into the VP7 gene in the SV40 expression vector pJC9N (Fig. 2). (This was derived from pJC9 [18] by trimming DNA cut at the *Nhe*I site [base 5660] with S1 nuclease and religating it.) The annealed synthetic oligonucleotides had *Cla*I- and *Nhe*I-compatible ends and were ligated to the 5.0-kilobase (kb) *Pvu*I-*Nhe*I fragment from pJC9N (left half of vector, Fig. 2) and the 3.2-kb *Pvu*I-*Cla*I fragment (right half of vector, Fig. 2) from pJC9 or one of the deletion mutants (Δ42, Δ47, etc.) derived from it (18). Correct assembly of the VP7 genes in the vector was monitored by restriction endonuclease digestion. Plasmids of the desired genotype, i.e., Δ47S, Δ42S, Δ27S, and Δ23Z, were identified by nucleotide sequencing.

(ii) C mutation. The C mutation changes the VP7 codon GCT (Ala-68) to GTT (Val), removing a potential cleavage site for signal peptidase (24). It was introduced into the VP7 gene by site-directed mutagenesis. The 5′- terminal 394-base-pair (bp) *Bam*HI fragment of the gene from mutant Δ42 was subcloned into bacteriophage M13mp10. A synthetic

TABLE 1. Summary of VP7 point mutations

Mutation	Genotype	Phenotype
Z	TTAATGT → ATAATGG	Weak to strong first ATG, plus Tyr-2 → Asp
S	TAC → TA(A/G)	Tyr-6 → stop codon
C	GCT → GCA	Ala-68 → Val; deletes cleavage site for signal peptidase
T	AAT → GAT	Asn-69 → Asp; removes glycosylation site

oligonucleotide complementary to bases 241 to 260 of the coding strand of the VP7 gene, with A substituted for G at base 251, was annealed to the single-stranded M13 template and extended with *Escherichia coli* DNA polymerase (Klenow fragment) in the presence of T4 DNA ligase (17). After transformation into *E. coli* TG1, colonies containing mutant phage were identified by hybridization to the radiolabeled oligonucleotide, and the presence of the mutation was confirmed by nucleotide sequencing (22). Mutant double-stranded M13 DNA was prepared, and the 5'-terminal 379-bp *Xho*I-*Bal*I fragment carrying the C mutation was obtained. This fragment was incorporated into the SV40 vector by ligation with the 4.4-kb *Eco*RI-*Xho*I (left part) and 3.6-kb *Eco*RI-*Bal*I (right part) fragments prepared from pJC9N (Fig. 2), giving mutant Δ42C. This plasmid was then used to prepare the 3.7-kb *Eco*RI-*Nco*I fragment so that the C mutation could be combined with other mutations in the VP7 gene by ligation with the 4.4-kb *Eco*RI-*Xho*I fragment and a ≈0.2-kb *Xho*I-*Nco*I fragment from the desired deletion mutant (18). In this way, variants Δ42C, Δ47SC, Δ42SC, and Δ23ZC were also constructed.

(iii) T mutation. The T mutation converts AAT (Asn-69) to GAT (Asp), removing the only site for N-linked glycosylation in SA11 VP7 (3). The sequence complementary to bases 244 to 262 of the gene was synthesized with C substituted for T at position 253. The 5'-terminal *Bam*HI fragment from mutant Δ1-14 was subcloned into M13, and the oligonucleotide was annealed and extended as described for the C mutation. From one mutant, the 5'-terminal *Xho*I-*Bal*I fragment was prepared and incorporated into the SV40 vector as described above. Δ23ZT was formed by combining the T mutation in the 3.7-kb *Eco*RI-*Nco*I fragment from this plasmid with the appropriate DNA fragments from Δ23Z.

The presence of the correct mutation(s) in DNAs used for transfections was confirmed by nucleotide sequencing (16).

In vitro transcription and translation. The VP7 gene in pJC9N and derived mutants (Fig. 1 and 2). was excised with *Xho*I and ligated into the *Sal*I site of the polylinker region of the vector Bluescribe M13$^+$ (Vector Cloning Systems) under the control of the T7 transcription promoter. Prior to transcription, the vector was made linear by digestion with *Hin*dIII, and transcripts were synthesized as directed by the supplier, except that 500 μM m7G(5')ppp(5')G (Pharmacia) was included and GTP was reduced to 40 μM. In vitro translation reactions were carried out in reticulocyte lysates (Promega Biotec) as recommended by the supplier, in the presence of [^{35}S]methionine (Amersham). Typically, 1 μl of a 25-μl transcription reaction mixture was used in each translation reaction. Reaction mixtures (12.5 μl) were incubated for 60 min at 30°C in the presence of 5 μl of canine pancreatic microsomes or buffer (Amersham). When appropriate, translation products (4 μl) were digested on ice with 500 μg of trypsin per ml for 1 h. Translation mix (2 μl) was digested with Endo H (Boehringer Mannheim) as described previously (7). Translation products (2 μl) were boiled in buffer containing 10% sucrose, 10% SDS, 1 M dithiothreitol, 2 mM EDTA, and 50 mM Tris (pH 6.8) and analyzed on 10% polyacrylamide–SDS gels (18).

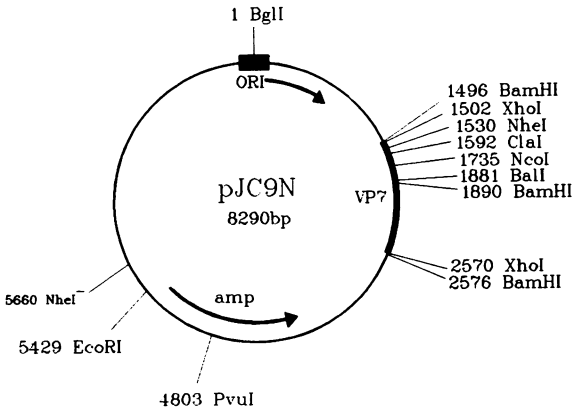

FIG. 2. Partial restriction map of pJC9N. The wild-type SA11 VP7 gene (bold line) was inserted into the unique *Xho*I site of the SV40-based expression vector pJC119 (18). Relevant restriction endonuclease sites, the approximate location of the ampicillin resistance gene (amp), the direction of transcription of the VP7 gene from the SV40 late promoter, and the SV40 origin or replication (ORI) are indicated. The *Nhe*I site at position 5660 was eliminated as described in the text.

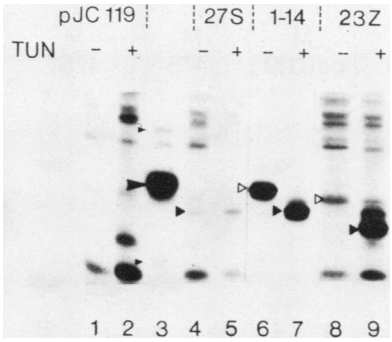

FIG. 3. Ability of variant VP7 genes to direct the synthesis of glycoproteins. COS cells, untreated or treated with tunicamycin (TUN) as indicated, were transfected with SV40 vector pJC119 alone (tracks 1 and 2) or with the vector carrying variant VP7 genes (tracks 4 to 9). VP7 proteins were recovered from radiolabeled cell lysates by immunoprecipitation and analyzed by polyacrylamide gel electrophoresis. The large solid arrowhead indicates ^{125}I-labeled SA11 VP7 marker, track 3; the small open arrowheads indicate glycosylated VP7 proteins; the medium-sized solid arrowheads indicate nonglycosylated proteins; and the small solid arrowheads in track 3 indicate the positions of the 46-kDa (upper) and 30-kDa (lower) standard protein markers. Tracks 6 and 7 are briefer autoradiographic exposures of the same gel.

RESULTS

H1 and H2 domains direct VP7 transport. SA11 VP7 is an integral membrane protein which is completely translocated into the ER and modified with tunicamycin-sensitive, high-mannose carbohydrate attached at Asn-69 (12) (Fig. 1). The location in VP7 of a sequence(s) mediating transport was analyzed by determining the ability of proteins derived from VP7 genes carrying deletions of either the H1 or H2 domain or both to be transported and glycosylated in transfected COS cells. Mutant Δ27S, which lacked residues 1 to 62 (i. e., both the H1 and H2 regions) (Fig. 1), directed the synthesis of a protein whose size was not changed when tunicamycin, an inhibitor of N-linked glycosylation, was added to the culture medium (Fig. 3, tracks 4 and 5). This indicated that the protein was not glycosylated, implying that amino acids 63 to 326 of VP7 contained no region which could direct the transport of the protein across the ER membrane. These results were confirmed by showing that the Δ27S protein was neither glycosylated nor protected from digestion with trypsin after being translated in a cell-free system in the presence

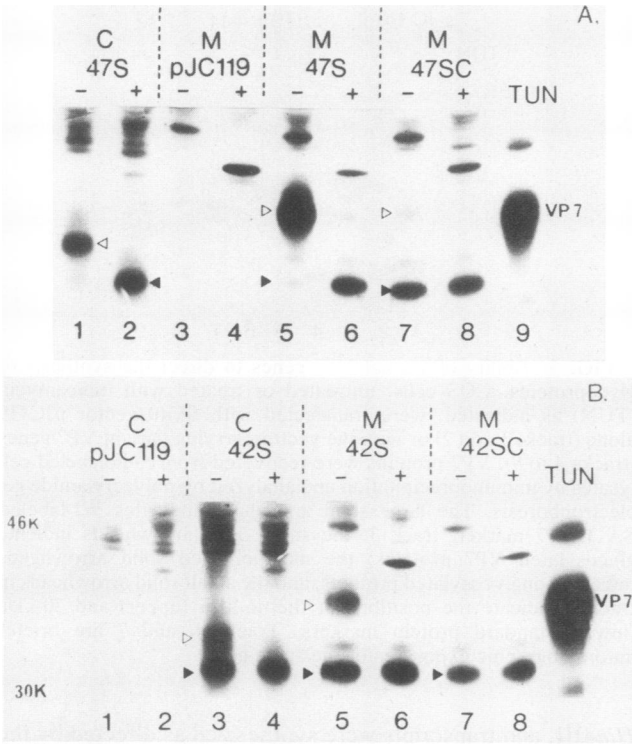

FIG. 4. Glycosylation and secretion of VP7 variants with truncated H2 hydrophobic domains. COS cells, untreated or treated with tunicamycin (TUN) as indicated, were transfected with (A) Δ47S (tracks 1, 2, 5, and 6) or Δ47SC (tracks 7 and 8) and (B) Δ42S (tracks 3 to 6) or Δ42SC (tracks 7 and 8) DNAs or pJC119 vector alone. Proteins were recovered from radiolabeled cell lysates (C) or culture medium (M) by immunoprecipitation and analyzed by polyacrylamide gel electrophoresis. In panel A, track 9 contains ^{125}I-labeled SA11 marker proteins. Other symbols are as described in the legend to Fig. 3. The positions of 46- and 30-kDa standard marker proteins are also indicated.

of pancreatic microsomes (see below). In contrast, mutants Δ1-14 (which had only the H2 domain) and Δ23Z (which had only the H1 domain) (Fig. 1) both gave rise to polypeptides in COS cells that were sensitive to tunicamycin, i. e., they were glycosylated (Fig. 3, compare tracks 6 through 9). Therefore, the H1 and H2 regions were each able to direct the transport of VP7 variants across the ER membrane.

Secreted variants of VP7. It was previously shown that deletion of residues 42 to 61, 43 to 61, or 47 to 61 resulted in secretion of VP7 from transfected COS cells (18). However, in these mutants translation could have initiated at either the first or the second ATG codon. To determine whether the shortened H2 domain in these mutants was sufficient to direct VP7 to the ER, mutants for which initiation at the second ATG was obligatory were constructed by placing a termination codon between the first and second ATG codons in place of Tyr-6 (S mutation; Table 1). Transfection of COS cells with mutant Δ47S gave rise to a protein which was glycosylated (Fig. 4A, compare tracks 1 and 2) and secreted (tracks 5 and 6), with a half-life of approximately 2 h (data not shown). Mutant Δ42S directed the synthesis of glycosylated and nonglycosylated products (Fig. 4B, tracks 3 and 4, respectively), both of which were secreted (tracks 5 and 6), even though the Δ42S H2 region retained only seven consecutive hydrophobic amino acids (Phe-37 to Ile-41 plus Ala-62 and Met-63 brought into juxtaposition by the deletion [Fig. 1]). The secreted Δ47S and Δ42S glycoproteins, like Δ47 and Δ42 (18), were also resistant to digestion with Endo H (data not shown), presumably as a consequence of their passage through the Golgi apparatus and modification with complex carbohydrate.

In addition to these secreted variants, a new mutant, Δ23Z, which retained only the H1 domain (Fig. 1), also produced a glycosylated protein (Fig. 5A, compare tracks 2 and 3), which was secreted (Fig. 5B, tracks 6 and 7). Extracellular Δ23Z also had a higher molecular weight than its intracellular counterpart and was modified with Endo H-resistant carbohydrate (data not shown), as observed for Δ42S and Δ47S.

Location of potential signal peptidase cleavage sites. Most secreted proteins have an NH_2-terminal signal peptide which is cleaved during translocation across the ER membrane (24, 26). Cleavage occurs on the C-terminal side of a tripeptide sequence in which certain amino acids are preferred. Empirical rules formulated from the study of known cleavage sites allow the probability of cleavage at a particular location to be calculated (24). In the wild-type and Δ1-14 proteins, which are retained in the ER (18), several potential cleavage sites were identified following the H2 domain (Fig. 6). Two of these sites were also present following the hydrophobic domain in Δ23Z, Δ42S, and Δ47S. Therefore, one explanation considered for the secretion of VP7 variants was that during transport they were cleaved by signal peptidase at a site unused in wild-type VP7, thereby losing any potential anchor domain. Since these variants were all secreted as glycoproteins, cleavage cannot have occurred at the site between Ser and Thr because this lies within the only glycosylation site in SA11 VP7, Asn-Ser-Thr (Fig. 6). To test whether use of the high-probability cleavage site Ala-Tyr-Ala ↓ (the arrow indicates the cleavage site) could account for secretion, we changed this to Ala-Tyr-Val ↓ (C mutation; Fig. 1 and Table 1). Apparently the presence of a hydrophobic amino acid at the −1 position prevents cleavage by signal peptidase (24).

Δ23Z is cleaved in vitro but Δ23ZC is not. We first examined the effect of the C mutation on cleavage of the secreted variant Δ23Z in an in vitro transcription and translation system. To study processing without the problem of glycosylation, the T mutation (Fig. 1 and Table 1) was also included in the gene. This changes Asn-69 to Asp, removing

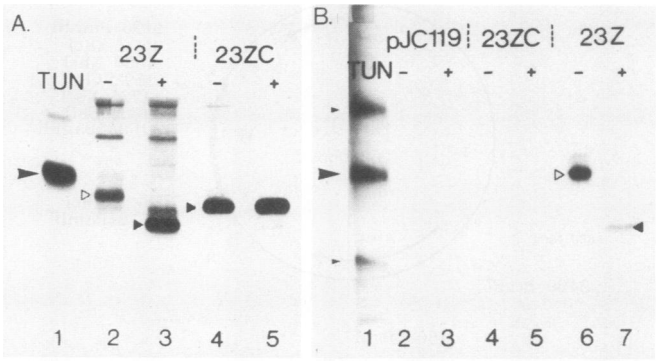

FIG. 5. Glycosylation and secretion of VP7 variants retaining only the H1 hydrophobic domain. COS cells were transfected with Δ23Z or Δ23ZC DNAs and untreated or treated with tunicamycin (TUN) as indicated. VP7 proteins were recovered by immunoprecipitation from cell lysates (A) or culture medium (B) and analyzed by gel electrophoresis. Symbols are as for Fig. 3. In panel A, tracks 2 and 3 and tracks 4 and 5 are from the same gel exposed for different times.

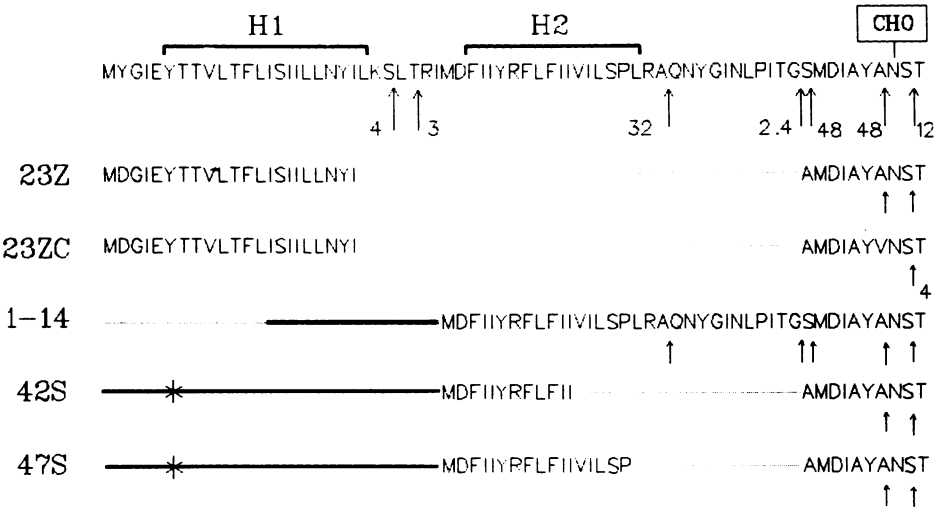

FIG. 6. Location of predicted cleavage sites for signal peptidase in the NH_2-terminal region of wild-type and variant VP7 proteins. The single-letter amino acid code indicates the sequence of each protein. Thin dotted lines show deleted regions. Arrows show the location of predicted cleavage sites, and the probability of cleavage, calculated from the five amino acids to the left of each site (24), is given at the base of each arrow (maximum score is 48). Arrows without numbers indicate that the site score is the same as in wild-type VP7. In Δ23ZC one cleavage site is lost when Ala-68 is changed to Val. Other symbols are as for Fig. 1.

the only glycosylation site. It unexpectedly transpired that the C mutation had the effect of reducing glycosylation efficiency (see below), and therefore we were able to compare processing of Δ23ZT and Δ23ZC (Fig. 7). The genes were transcribed in vitro, and the RNAs were translated in rabbit reticulocyte lysates in the presence or absence of canine pancreatic microsomes. Transport of the protein into the microsomes was assayed by the appearance of a trypsin-resistant product (12, 23). When no RNA was added to the translation system, no protein products were seen between the 30- and 46-kilodalton (kDa) markers (data not shown). RNA transcripts derived from Δ23ZT translated in vitro in the absence of microsomes yielded two products at ≈35kDa (Fig. 7A, track 3), the lower of which was identical in size to that derived from Δ27S (track 2). Both products were digested with trypsin in the absence of microsomes (track 5). In the presence of microsomes, a new, lower-molecular-weight band appeared (track 4) which was protected from trypsin digestion (track 6); the Δ27S product was not. The appearance of this lower-molecular-weight band was consistent with processing of the H1 domain from this variant by signal peptidase. In contrast, the Δ23ZC protein translated with or without microsomes did not change in size (Fig. 7B, tracks 2 and 3), and that made with microsomes was unaffected by Endo H digestion (track 4), showing that it was not glycosylated. (In other experiments, Endo H clearly affected the size of glycosylated VP7 proteins [manuscript in preparation].) In the absence of microsomes Δ23ZC was trypsin sensitive. However, in their presence, the protein was trypsin resistant (compare tracks 5 and 6). Together, the data indicate that Δ23ZC protein crossed the microsomal membrane without glycosylation or cleavage.

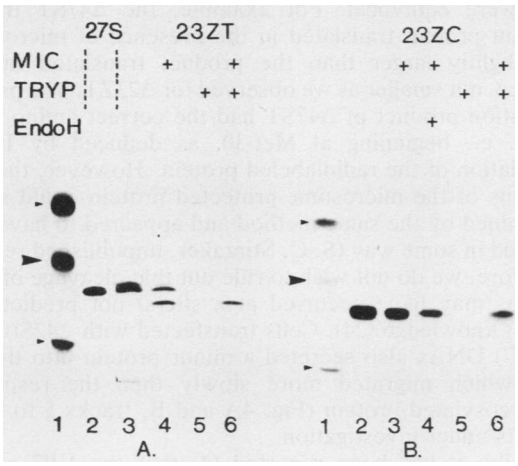

FIG. 7. Inhibition of microsome-dependent processing by a mutation in the proposed cleavage site. The Δ27S (A, track 2), Δ23ZT (A, tracks 3 to 6), and Δ23ZC (B, tracks 2 to 6) genes were transcribed in Bluescribe M13$^+$ and translated in the presence (+) or absence of microsomes (MIC) and treated with trypsin (TRYP) or Endo H as indicated. Products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Tracks 1 (A and B) contain VP7 (large solid arrowhead) and the standard 46- and 30-kDa marker proteins (small arrowheads). Panels A and B are separate gels.

C mutation prevented secretion of Δ23Z but not of Δ42S and Δ47S. To determine whether the Ala → Val substitution which prevented cleavage in Δ23ZC also had an effect on secretion of this variant, the gene was incorporated into the SV40 expression vector pJC119 and used to transfect COS cells. Three effects were observed: (i) the size of the intracellular nonglycosylated Δ23ZC protein increased (Fig. 5A, track 5) compared with its counterpart from Δ23Z (track 3); (ii) glycosylation of the Δ23ZC polypeptide was inhibited (compare Fig. 5A, tracks 4 and 5); and (iii) secretion was prevented (Fig. 5B, tracks 4 and 5) compared with Δ23Z (tracks 6 and 7).

Similarly, the Ala → Val change was included in the variants Δ42S and Δ47S, and its effect on their secretion from COS cells was examined. The intracellular products of Δ47SC and Δ42SC were indistinguishable from those of Δ47S and Δ42S, respectively (data not shown). In contrast to Δ23Z, the C mutation had no effect on the secretion of these variants (Fig. 4A and B, tracks 7 and 8), although a reduction in the efficiency of glycosylation of these proteins was still

observed (Fig. 4A and B, compare tracks 5 and 7). The slight difference in migration between the nonglycosylated bands of Δ47S and Δ47SC (Fig. 4A, tracks 6 and 8) was not reproducible when they were run together in the same track (data not shown). Therefore, since the C mutation did not prevent their secretion, the Δ42S and Δ47S proteins may have been secreted without cleavage. Alternatively, they may have been cleaved at another, unpredicted site(s).

DISCUSSION

Proteins that are transported across the ER membrane and secreted from the cell have a signal sequence, generally at the NH_2 terminus, which is cleaved from the protein (24, 25). In a few instances, the signal peptide is not removed and the protein remains anchored in the membrane by its NH_2 terminus. These are known as class II membrane proteins (27), examples of which are influenza neuraminidase (2), the asialoglycoprotein receptor (23), and human transferrin receptor (28).

The rotavirus glycoprotein VP7 is completely transported across the ER membrane and retained as an integral membrane protein (12). Since there is no significant hydrophobic region at its COOH terminus (10), one of the hydrophobic domains at the NH_2 terminus of the protein could conceivably serve to anchor the protein. Transport of VP7 is also accompanied by cleavage of its precursor, and this event presumably represents the removal of a signal peptide (4, 7), although VP7 transport has not been shown to be dependent on interaction with the signal recognition particle (26).

Location of signal peptides in VP7. In this work, the location of signal peptides in VP7 was examined by gene mutagenesis and expression. Mutant Δ1-14 (18), in which the H1 domain was deleted, and two new mutants, Δ23Z (in which H2 was removed) and Δ27S (from which both H1 and H2 were deleted), defined their location. Studies with Δ27S showed that residues 63 to 326 of VP7 were not capable of directing the protein into the ER. In contrast, residues 30 to 62 in Δ1-14 (incorporating the H2 domain) and 1 to 23 in Δ23Z (the H1 domain) both directed the transport of VP7 across the microsomal membrane in vivo and in vitro. However, it is not possible to say which hydrophobic domain functions as the signal peptide when both are present in a protein which initiates at the first ATG codon.

Deletion of amino acids adjacent to the H2 domain in VP7 converted the protein from one with an ER location to one which was secreted from the cell (18). In the present work, mutants Δ42S and Δ47S were constructed so that a terminator was inserted between the first and second ATG codons (Table 1) and translation began at Met-30. These genes also expressed glycosylated and secreted proteins at levels very similar to those obtained for Δ42 and Δ47 (unpublished results). In Δ42S, the protein had a hydrophobic core of only seven consecutive residues, probably the minimum acceptable for a eucaryotic signal peptide (25), and residues C-terminal to the remaining H2 region may assist in spanning the membrane in a manner similar to that proposed for the truncated COOH-terminal transmembrane region of the VSV G protein (1). In the Δ42S protein, this may place a conformational restraint on the attachment of carbohydrate at the nearby Asn-Ser-Thr site and account for the observed reduced efficiency of glycosylation.

Utilization of a new cleavage site. One explanation considered for the secretion of VP7 variants was that a site previously unused by signal peptidase had been relocated, as a result of the deletion, to a favorable position, so that cleavage removed the potential hydrophobic anchor region. The Ala-Tyr-Ala↓ sequence following the residual hydrophobic domain in each secreted variant represents such a site (24). However, only in mutant Δ23Z did it appear to be used. Cleavage of nonglycosylated Δ23ZC protein in vitro was prevented by the C mutation, which was designed to inhibit processing by signal peptidase at that site (Table 1) (25). In transfected COS cells, the C mutation both increased the size of the intracellular Δ23ZC polypeptide and prevented its secretion. (The minor Δ23Z product [Fig. 3, track 9; Fig. 5A, track 3], which runs above the major nonglycosylated product, may therefore represent uncleaved, unglycosylated Δ23Z protein.) The results are consistent with the idea that protein Δ23ZC is anchored in the cell by its H1 hydrophobic domain in a manner described for other class II proteins (6, 21, 27). The substitution of Val for Ala immediately next to the glycosylation site in Δ23Z, Δ42S, and Δ47S (see below) also had the unexpected effect of greatly decreasing the efficiency of carbohydrate attachment, although the reason for this is not clear.

Secretion without cleavage? By analogy with the results obtained for Δ23Z, it was also anticipated that signal peptide processing might account for secretion of the Δ42S and Δ47S proteins since the high-probability cleavage site Ala-Tyr-Ala↓ was also present in these polypeptides. However, the C mutation had no effect on the secretion of Δ42SC and Δ47SC from COS cells, although it did affect their glycosylation efficiency. At least for Δ47SC, secretion was not explained by cleavage at the alternative site, Val-Asn-Ser↓ (Fig. 6), since a small fraction of protein in the medium was still glycosylated (Fig. 4A, compare tracks 7 and 8). However, for Δ42SC, no glycosylated protein was detectable in the medium, probably because Δ42S was poorly glycosylated to begin with. Therefore, we cannot rule out the use of this alternative cleavage site in Δ42SC, although we think it unlikely since the site was not used in Δ23ZC.

We did attempt to determine directly whether the Δ42S and Δ47S proteins were cleaved by using the same in vitro translation approach employed for Δ23Z; however, the results were equivocal. For example, the Δ47ST trypsin-resistant protein translated in the presence of microsomes was slightly larger than the product translated in their absence, not smaller as we observed for Δ23ZT. The primary translation product of Δ47ST had the correct amino terminus, i. e., beginning at Met-30, as deduced by Edman degradation of the radiolabeled protein. However, the NH_2 terminus of the microsome-protected protein could not be determined by the same method and appeared to have been modified in some way (S. C. Stirzaker, unpublished results). Therefore, we do not wish to rule out that cleavage of these proteins may have occurred at a site(s) not predicted by current knowledge (24). Cells transfected with Δ42S(C) and Δ47S(C) DNAs also secreted a minor protein into the medium which migrated more slowly than the respective nonglycosylated protein (Fig. 4A and B, tracks 5 to 8). Its origin is under investigation.

Finally, it has been reported (4) that the VP7 gene is bicistronic, coding for two proteins of similar size, one being derived by initiation at the first ATG, followed by cleavage of a signal peptide (the H1 region), and the second by initiation at the second ATG, with no cleavage of the protein. However, we have observed that some variant VP7 proteins migrate anomalously in acrylamide-SDS gels. For example, protein Δ27S comprises 264 amino acids (expected size, 29 kDa), but runs with an apparent size of 35.5 kDa (Fig. 7A). Therefore, cleavage sites predicted on the basis of

TABLE 2. Phenotypes of VP7 variants

Protein	Cleaved in vitro	Glycosylated	Secreted from COS cells (reference)
Wild type	ND[a]	+	− (18)
Δ27S	−	−	−
Δ1-14	ND	+	− (18)
Δ23Z	+	+	+
Δ23ZC	−	−[b]	−
Δ42S	?	±	+
Δ42SC	?	−[b]	+
Δ47S	?	+	+
Δ47SC	?	−[b]	+

[a] ND, Not determined.
[b] The attachment site was present but glycosylation was inhibited by the C mutation.

the size of mature VP7 may not be correct. Processing of the wild-type and Δ1-14 proteins will need to be compared to assess whether signal peptide cleavage can occur after the H1 or H2 domain or both.

The data presented here (summarized in Table 2) indicate that either hydrophobic domain alone can direct VP7 to the ER and suggest that VP7 mutant Δ23Z is secreted as a result of fortuitous cleavage of its hydrophobic domain. Mutants Δ42S and Δ47S were not cleaved at the same site as Δ23Z but were still secreted, perhaps because their residual hydrophobic domains were too short to anchor the protein in the ER membrane or because they were cleaved at an unpredicted site. Alternatively, if residues deleted in these variants are in some way modified to provide anchor function in wild-type VP7, then a positive signal for ER location may exist. The location of the signal peptide cleavage site and the nature of the anchor and targeting signal in VP7 still remain to be elucidated.

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LITERATURE CITED

1. **Adams, G. A., and J. K. Rose.** 1985. Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. Cell **41**:1007–1015.
1a. **Andrew, M. E., D. B. Boyle, B. E. H. Coupar, P. L. Whitefeld, G. W. Both, and A. Richard Bellamy.** 1987. Vaccinia virus recombinants expressing the SA11 rotavirus VP7 glycoprotein gene induce serotype-specific neutralizing antibodies. J. Virol. **61**:1054–1060.
2. **Bos, T. J., A. R. Davis, and D. P. Nayak.** 1984. NH_2-terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation. Proc. Natl. Acad. Sci. USA **81**:2327–2331.
3. **Both, G. W., J. S. Mattick, and A. R. Bellamy.** 1983. Serotype-specific glycoprotein of Simian 11 rotavirus: coding assignment and gene sequence. Proc. Natl. Acad. Sci. USA **80**:3091–3095.
4. **Chan, W.-K., M. E. Penaranda, S. E. Crawford, and M. K. Estes.** 1986. Two glycoproteins are produced from the rotavirus neutralization gene. Virology **151**:243–252.
5. **Dubois-Dalq, M., K. V. Holmes, and B. Rentier (ed.).** 1984. Assembly of enveloped viruses, p. 171–182. Springer-Verlag, New York.
6. **Engleman, D. M., and T. A. Steitz.** 1981. The spontaneous insertion of proteins into and across membranes: the helical hairpin hypothesis. Cell **23**:411–422.
7. **Ericson, B. L., D. Y. Graham, B. B. Mason, H. H. Hanssen, and M. K. Estes.** 1983. Two types of glycoprotein precursors are produced by the simian rotavirus SA11. Virology **127**:320–332.
8. **Estes, M. K., E. L. Palmer, and J. F. Obijeski.** 1983. Rotaviruses: a review. Curr. Top. Microbiol. Immunol. **105**:123–184.
9. **Glass, R. I., J. Keith, O. Nakagomi, T. Nakagomi, J. Askua, A. Z. Kapikian, R. M. Chanock, and J. Flores.** 1985. Nucleotide sequence of the structural glycoprotein VP7 gene of Nebraska calf diarrhea virus: comparison with homologous genes from four strains of human and animal rotaviruses. Virology **141**:292–298.
10. **Gunn, P. R., F. Sato, K. F. Powell, A. R. Bellamy, J. R. Napier, D. R. Harding, W. S. Hancock, L. J. Siegman, and G. W. Both.** 1985. Rotavirus neutralizing protein VP7: antigenic determinants investigated by sequence analysis and peptide synthesis. J. Virol. **54**:791–797.
11. **Jones, L. V., R. W. Compans, A. R. Davis, T. Bos, and D. P. Nayak.** 1985. Surface expression of influenza virus neuraminidase, an amino-terminally anchored viral membrane glycoprotein, in polarized epithelial cells. Mol. Cell. Biol. **5**:2181–2189.
12. **Kabcenell, A. K., and P. A. Atkinson.** 1985. Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11. J. Cell Biol. **101**:1270–1280.
13. **Kapikian, A. Z., and R. M. Chanock.** 1985. Rotaviruses, p. 863–906. *In* B. N. Fields (ed.), Virology. Raven Press, New York.
14. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. **12**:857–874.
15. **Kozak, M.** 1986. Point mutations that define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
16. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
17. **Nisbet, I. T., and M. W. Beilharz.** 1985. Simplified DNA manipulations based on *in vitro* mutagenesis. Gene Anal. Techniques **2**:23–29.
18. **Poruchynsky, M. S., C. Tyndall, G. W. Both, F. Sato, A. R. Bellamy, and P. A. Atkinson.** 1985. Deletions into an NH_2-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum membrane glycoprotein. J. Cell Biol. **101**:2199–2209.
19. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513–524.
20. **Roth, M. G., R. W. Compans, L. Giusti, A. R. Davis, D. P. Nayak, M. J. Gething, and J. Sambrook.** 1983. Influenza virus hemagglutinin expression is polarized in cells infected with recombinant SV40 viruses carrying cloned hemagglutinin DNA. Cell **33**:435–443.
21. **Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik.** 1982. Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. **92**:1–22.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
23. **Spiess, M., and H. F. Lodish.** 1986. An internal signal sequence: the asialoglycoprotein receptor membrane anchor. Cell **44**:177–185.
24. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133**:17–21.
25. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184**:99–105.
26. **Walter, P., R. Gilmore, and G. Blobel.** 1984. Protein translocation across the endoplasmic reticulum. cell **38**:5–8.
27. **Wickner, W. T., and H. F. Lodish.** 1985. Multiple mechanisms of protein insertion into and across membranes. Science **230**:400–407.
28. **Zerial, M., P. Melancon, G. Schneider, and H. Garoff.** 1986. The transmembrane segment of the human transferrin receptor functions as a signal peptide. EMBO J. **5**:1543–1550.