Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2062–2066, April 1981
Biochemistry

Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins

(membrane glycoproteins/capsid protein/alphavirus/togavirus/protein processing)

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Communicated by Ray D. Owen, December 11, 1980

ABSTRACT The nucleotide sequence of intracellular 26S mRNA of Sindbis virus has been determined by direct sequence analysis of the cDNA made to this RNA with reverse transcriptase. From this, the amino acid sequences of the encoded virus structural proteins, which include a basic capsid protein and two integral membrane glycoproteins, have been deduced. The features of these proteins as related to their functions are discussed. We suggest that three proteases are required to produce these proteins from their polyprotein precursor: a viral protease, which functions in the cytosol to release the capsid protein; signalase, which makes two cleavages to separate the glycoproteins; and a protease of the Golgi complex that cleaves after double basic residues, to process the precursor form of one of the glycoproteins.

Alphaviruses such as Sindbis virus and the closely related Semliki Forest virus are simple enveloped viruses. The icosahedral nucleocapsid is assembled in the cytoplasm, diffuses to the cell surface, and buds through the host cell plasmalemma, acquiring a lipoprotein envelope that contains only virus-encoded glycoproteins. These glycoproteins are synthesized on the rough endoplasmic reticulum and glycosylated; they migrate to the plasma membrane by way of the Golgi apparatus, where the carbohydrates are modified and lipids are covalently attached. The interaction between the alphavirus nucleocapsid and its glycoproteins is much more specific than that of other enveloped viruses; mature virions contain exclusively alphavirus proteins (1).

All three of the virus structural proteins are translated as a continuous polypeptide from a single mRNA molecule—26S RNA (2). We have determined the nucleotide sequence of Sindbis HR 26S RNA to investigate in detail the structure and processing of the viral proteins and to make possible further study of the temperature-sensitive mutants previously characterized genetically and physiologically (3).

MATERIALS AND METHODS

Details of the methods and strategy used for preparation and sequence determination of single-stranded cDNA to Sindbis 26S RNA will be published elsewhere. Briefly, 26S RNA was used as a template for synthesis of cDNA at 42.5°C by using avian myeloblastosis virus reverse transcriptase (kindly provided by J. Beard) primed with either oligo(dT)$_{12-18}$ (Collaborative Research, Waltham, MA) or a mixture of short (≈6–8 nucleotides) random oligonucleotides derived from calf thymus DNA (a gift from J. Casey). The reaction mixture contained 4 mM sodium pyrophosphate to inhibit second-strand synthesis. After 30–60 min, an excess of EDTA (Na form) was added to stop the reaction, and the cDNA-RNA hybrid was isolated by phenol/chloroform extraction followed by ethanol precipitation. The RNA strand was hydrolyzed by incubation in 0.1 M NaOH at 60°C for 30 min. The cDNA was chromatographed on Bio-Gel A-J m (Bio-Rad); the excluded peak fractions were pooled, and the cDNA was ethanol precipitated. The cDNA was then digested with *Hae* III, *Taq* I, *Hha* I, or *Rsa* I (New England Biolabs) and labeled at the 5′ ends with T4 polynucleotide kinase or at the 3′ ends with terminal deoxynucleotidyl transferase [Bethesda Research Laboratories (Rockville, MD), ribosubstitution grade] essentially as described by Maxam and Gilbert (4). Single-stranded, end-labeled restriction fragments were separated on 6% polyacrylamide sequencing gels, excised, and eluted, and their sequences were determined by using the base-specific chemical cleavage procedure (4). The modifications of Smith and Calvo (5) were used for long (>300 nucleotides) fragments. By using four different restriction enzymes and determining the sequences of numerous partial digestion products and of some fragments from both the 5′ and the 3′ direction, virtually all of the sequence was determined more than once and sufficient overlap was obtained to align all of the fragments.

RESULTS AND DISCUSSION

Sequence of 26S RNA. The entire nucleotide sequence of Sindbis virus (HR strain) 26S RNA, except a sequence of ≈150 nucleotides at the 5′ end that has not been unambiguously determined, and the deduced amino acid sequences of the encoded proteins are shown in Fig. 1. From the AUG codon initiating synthesis of the capsid protein, there is an open reading frame for 3735 nucleotides encoding, in order, capsid protein and glycoproteins E3, E2, and E1, followed by a termination codon. The identification of the NH_2 terminus and the COOH terminus of each protein is discussed below. The deduced amino acid sequence is in precise agreement with the NH_2-terminal sequences found by automated sequence analysis of E1 and E2 (6), of PE2 (the precursor to E3 and E2) (J. R. Bell and M. W. Hunkapiller, personal communication), and of the hydrophobic "roots" of E1 and E2 derived by treatment of intact virions with α-chymotrypsin (unpublished data). In addition, sequences of many of the tryptic peptides from the Sindbis (strain Sa-AR-86) capsid protein have been published recently (7); most of these sequence data are consistent with our deduced sequence for the capsid protein.

The experimental amino acid compositions for E1, E2, and the capsid protein are in excellent agreement with the compositions deduced from the nucleotide sequence and furnish additional support for the deduced protein sequences. In addition, our sequence data adjacent to the poly(A) tail is in complete agreement with the sequence obtained for the same region by using a chain termination method (9).

The method we have used requires only a few micrograms of purified RNA and, because molecular cloning is not involved,

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Abbreviation: SFV, Semliki Forest virus.

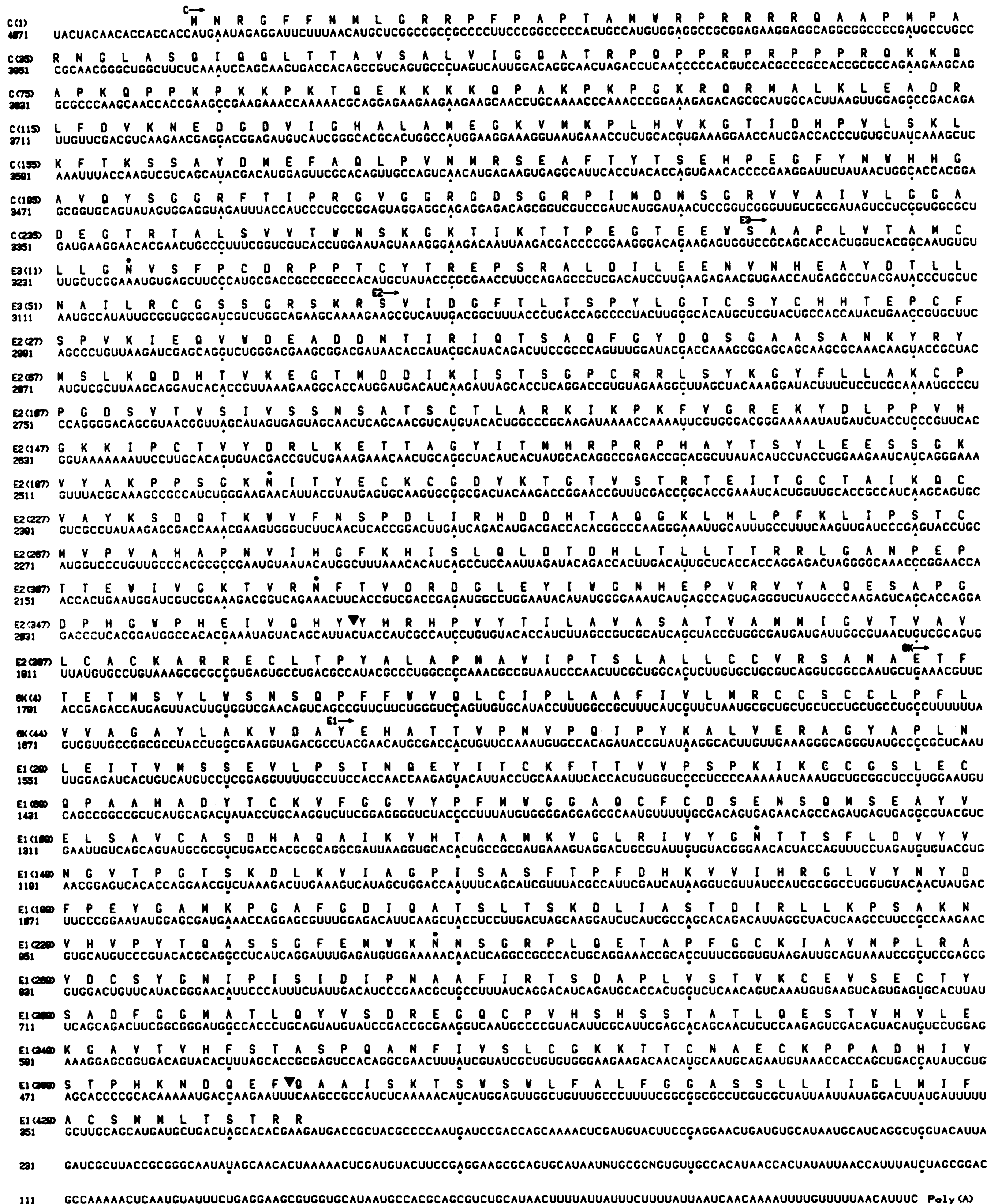

FIG. 1. Nucleotide sequence of Sindbis 26S RNA and amino acid sequence of the encoded proteins. Nucleotides are numbered from the 3′ end. Amino acids are numbered from the NH_2 terminus of each protein [capsid (C), E3, E2, the 6000-dalton one (6K), E1]. The beginning of each protein is labeled, and the NH_2 termini of the polypeptide segments from E2 and E1 isolated from spikeless particles after chymotrypsin treatment are indicated by triangles. Carbohydrate attachment sites are denoted by asterisks. The single letter amino acid code is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

there is no chance of selecting a minor variant in the population. We are currently using this method to locate sequence changes in Sindbis *ts* mutants.

The coding regions of 26S RNA of Semliki Forest virus (SFV) have been sequenced recently (10, 11). The amino acid sequences of Sindbis structural proteins are compared with those of SFV in Fig. 2. Sequences have been aligned to maximize homology. The overall homology between the viruses is striking: 47% of the residues are identical and another 12% represent conservative substitutions. It is also obvious that some areas of the proteins are more highly conserved than others (see Fig. 2), as will be discussed below. The cysteine residues are in general conserved except in certain hydrophobic areas.

Overall Structure of Sindbis 26S RNA. We have identified a *Hae* III fragment of the 26S cDNA that includes the NH_2 terminus of the capsid protein and extends from the AUG codon ≈175 nucleotides toward the 5′ end of the RNA. If we assume that this fragment is derived from the 26S RNA, this brings the overall length of the Sindbis 26S RNA to at least ≈4230 nucleotides, not including the poly(A) tail, which is in good agreement with previous estimates of its size (2). A 5′ untranslated region of 175 nucleotides if fairly long in comparison with most known mRNAs; however, the mRNA for VP1 of SV40 has 240 nucleotides 5′ to the initiation codon (12). The 3′ untranslated sequence is 318 nucleotides long in Sindbis compared with 264 nucleotides in SFV (11). Thus, despite numerous deletions in the coding regions relative to one another (see Fig. 2), the lengths of the two virus 26S RNAs are remarkably similar. From the AUG codon to the beginning of the poly(A) tail is 4023 nucleotides in SFV and 4053 nucleotides in Sindbis.

We have examined the codon usage in Sindbis 26S RNA; for most of the amino acids, the distribution among the possible codons is clearly nonrandom (data not shown). The base composition or nearest-neighbor frequency cannot account for the codon frequency found. In addition, despite the extensive nucleotide sequence divergence between Sindbis and SFV, the two viruses show similar codon usage (data not shown). (As an example of this divergence, regions in which the amino acid sequence is totally conserved between the two viruses show an average of about 0.8 base changes per codon.) We do not find a low frequency of the C-G doublet, as has been found for some DNA viruses such as simian virus 40 (13) or for RNA viruses such as fowl plague virus (14), which have mammalian or avian hosts. Sindbis and SFV have a wide host range that includes insects as well as birds and mammals. The codon preferences found in these two viruses may be due in part to an adaptation for optimal translational efficiency in this wide range of hosts.

Capsid Protein. The capsid protein of Sindbis is 264 amino acids long. Inspection of the sequence shows a striking clustering of lysine, arginine, and proline in the NH_2-terminal half. Seventy percent of these three amino acids, as well as 85% of the glutamine and 60% of the alanine, are within the first 120 residues. This basic region of the capsid protein is probably important in interacting with the virion RNA. Although SFV capsid protein has a similar basic region near the NH_2 terminus, there is relatively little sequence homology between the two proteins in this region (see Fig. 2). The COOH-terminal end (residues 166–264), however, shows remarkable sequence homology between Sindbis and SFV; 76% of the residues are identical and another 6% are conservative substitutions. This conservation suggests that this region could be important in protein-protein interactions, such as those between capsid protein subunits to form the nucleocapsid and those between the nucleocapsid and the COOH-terminal end(s) of the transmembrane viral glycoproteins.

Glycoprotein E3. Sindbis E3 is 64 amino acids long. The first 19 residues are uncharged, highly conserved between Sindbis and SFV (see Fig. 2), and may serve as the signal sequence for the insertion of PE2 into the endoplasmic reticulum during

FIG. 2. Comparison of the amino acid sequence of the Sindbis structural proteins shown in Fig. 1 (upper sequence) with that of the structural proteins of SFV (from refs. 10 and 11) (lower sequence). A dot in the SFV sequence means that the amino acid is identical to that above in the Sindbis sequence. A dot between the amino acids of the two sequences denotes a conservative substitution (R = K, S = T, D = E, Q = N, V = L = I = M, A = G, A = V, Y = F). Possible carbohydrate attachment sites are denoted by asterisks. An attempt was made to keep the number of gaps introduced to a minimum. Nucleotide sequence homology was used to position gaps in some areas where amino acid sequence homology is low. The single letter amino acid code is used as in Fig. 1.

protein synthesis (15). This putative signal sequence is not cleaved from PE2 during protein synthesis; rather, PE2 is cleaved to E2 and E3 during virus maturation (2, 15).

Sindbis E3 is known to be glycosylated (16), and the sequence contains a single glycosylation site of the type Asn-X-Ser/Thr (Asn-14). Also, during amino acid sequence analysis of PE2 and E3, Asn-14 is not recovered, as is characteristic of glycosylated asparagine residues, whereas other amino acids around this site are recovered (M. W. Hunkapiller, J. R. Bell, and J. Mayne, personal communication). Thus, it is virtually certain that Asn-14 is glycosylated. The polysaccharide chain is known to be of the complex type (E. G. Strauss, personal communication). It is interesting that this glycosylated site occurs within the putative signal sequence for PE2 and is conserved between Sindbis and SFV. The E3 polypeptide of SFV has, in addition, a second potential glycosylation site at Asn-60 (11).

Glycoprotein E2. Sindbis E2 is 423 amino acids long and has two potential glycosylation sites. Burke and Keegstra (17) have shown that Sindbis E2 has two carbohydrate units, one of which is a simple oligosaccharide chain containing only mannose and *N*-acetylglucosamine and the other of which is a complex chain that contains, in addition, galactose, fucose, and sialic acid. They isolated glycopeptides after Pronase digestion of E2 and found that the glycopeptide of the complex chain had the amino acid composition $Asn_1Ile_{0.8}Thr_{0.7}$, while the simple chain had the composition $Asn_1Phe_{0.6}Thr_{0.1}$. From Fig. 1, it is apparent that Asn-196 carries a complex chain and Asn-318 has a simple chain. It is of interest that SFV E2 also carries two carbohydrate chains (11), one at Asn-200 and the second at Asn-262.

Sindbis E2 has two long stretches of uncharged amino acids near its COOH terminus. The first of these is 28 amino acids long (residues 363–390) and begins near the NH_2 terminus of the hydrophobic root derived from E2 by treatment of the intact virion with chymotrypsin (unpublished data) (Fig. 1). About 30 residues can be removed from the COOH-terminus of PE2 by chymotrypsin treatment of intact microsomes (18), making it clear that this hydrophobic domain traverses the bilayer. E2 has 5 or 6 residues of fatty acid covalently attached (19), all of which are found in the root (unpublished data). There are three threonines and one serine in this first hydrophobic domain that could serve as attachment sites.

The second hydrophobic domain is 23 amino acids long (residues 396–418) and also appears to contain attached fatty acid (J. R. Bell, personal communication). Its orientation in the virion is unknown. The homology (87%) between the Sindbis and SFV proteins in this region is striking and contrasts with the low homology found in the first hydrophobic region. This homology, together with the many conserved cysteine residues, suggests that this segment may provide the specificity for the interaction between the viral nucleocapsid and the glycoproteins during budding.

55 Amino Acid Peptide (4200- or 6000-Dalton Protein). This peptide is made up of predominantly uncharged amino acids (91%) and probably serves in whole or in part as the signal sequence for E1. Whether it also has other functions is not known. The corresponding Sindbis and SFV peptides diverge widely (see Fig. 2), implying that hydrophobicity is important for the function of this peptide but the exact sequence is not.

Glycoprotein E1. E1, the viral hemagglutinin (20), is the largest of the Sindbis glycoproteins (439 amino acids). Two potential glycosylation sites (Asn-139 and Asn-245) are present, and both appear to be glycosylated. First, E1 of Sindbis grown in primary chicken cells contains both simple and complex polysaccharide chains, although E1 from Sindbis grown in a continuous hamster cell line contains only complex chains (21, 22). Second, the carbohydrate composition of E1 suggests that two chains are present (22). Third, the fact that Sindbis E1 migrates more slowly than E2 in $NaDodSO_4$-containing gels, whereas SFV E1 (which contains a single glycosylation site [ref. 11]) and E2 comigrate, suggests that Sindbis E1 has two chains, as does E2. We hypothesize that Asn-139 has a complex chain and Asn-245 has a simple chain in chicken-grown virus and that both have complex chains in hamster grown virus. The single glycosylation site of SFV E1, Asn-141 (11) is shifted by two residues from the first Sindbis site, a shift that requires mutations in two separate codons (see Fig. 2).

Sindbis E1 has two long sequences of uncharged amino acids. The first is 17 amino acids long and begins at Val-80. In the region of E1 from Asp-75 to Glu-109, which includes this uncharged segment, there are only seven differences between Sindbis and SFV and four of these are highly conservative changes (see Fig. 2). This uncharged region is not present in Sindbis spikeless particles (unpublished data), suggesting that it is not imbedded in the viral membrane. It may play a role in mediating virus fusion with intracellular membranes during penetration (11).

The second uncharged region is located at the COOH terminus of E1 and begins seven residues from the NH_2 terminus of the root of E1 (Fig. 1; unpublished data). A sequence of 33 uncharged amino acids is followed by two arginine residues, suggesting that E1 spans the bilayer with only two (or at most a few) residues exposed on the cytoplasmic side. This uncharged domain contains six serine and three threonine residues, which are potential attachment sites for the one or two palmitic acid molecules located in this region of E1 (ref. 19; unpublished data). Sindbis and SFV show little homology in this region, but both sequences are highly hydrophobic (see Fig. 2).

Sites of Cleavage. The NH_2 terminus of the Sindbis capsid protein is Met-Asn-Arg- (refs. 7 and 15; J. R. Bell, personal communication), thus identifying the start of this protein. The COOH terminus is established by the (capsid) tryptic peptide Thr-Thr-Pro-Glu-Gly-Thr-Glu-Glu-Trp sequenced by Boege *et al.* (7) found at residues 256–264 (see Fig. 1). The NH_2-terminal sequence of PE2 (and E3), beginning Ser-Ala-Ala-Pro- (J. R. Bell, personal communication), follows directly. Thus, tryptophan is the COOH-terminal amino acid of the capsid protein, and the protease responsible for the capsid-PE2 cleavage has a specificity similar to chymotrypsin. It has been suggested that this proteolytic activity resides in the capsid protein itself (23–25). The highly conserved region around the cleavage site (see Fig. 2) may be important for a site-specific, viral-encoded cleavage. This cleavage occurs during translation and apparently must take place if the signal sequence for PE2 is to function (26).

The cleavage site between E3 and E2 has yet to be precisely determined. The amino terminus of E2 is Ser-Val-Ile- (ref. 6; see Fig. 1). E3 is radiolabeled by lysine (J. Mayne, personal communication), therefore, the COOH terminus is either Lys or Lys-Arg. In SFV, the COOH-terminal Arg is apparently removed (11). The cleavage of PE2 to form E3 and E2, in either case, involves a trypsin-like specificity. The origin and localization of the protease responsible for this is at present unknown. It occurs relatively late, approximately 20 min after synthesis of PE2 and is required for virus maturation (2). After cleavage, Sindbis E3 is found in the culture medium (16) while that of SFV remains associated with the mature virion (27). Thus, the cleavage may occur in the plasma membrane, outside the cell, or internally, perhaps in the lumen of Golgi vesicles shortly before or concomitant with their fusion with the plasma membrane. The latter alternative is suggested by analogy to the processing of such proteins as pro-albumin and parathyroid prohormone (11), which are cleaved after double basic amino acids in the

Golgi shortly before secretion of the mature protein (28). The failure of several groups to detect PE2 in the plasma membrane lends further support to this idea.

The COOH terminus of E2 is (Ala, Asn)-Ala, as determined with carboxypeptidase Y (T. Crowley, personal communication). The beginning of E1 is Tyr-Glu-His- (ref. 6; see Fig. 1). There is a sequence of 55 residues between the end of E2 and the beginning of E1. This is probably the peptide isolated by Welch and Sefton (16) from Sindbis virus-infected cells, which they called a 4200 polypeptide. They have located the leucine and methionine residues in the first 20 residues of this polypeptide by microsequence analysis, finding methionine at position 7 and leucine at position 10 (B. Sefton, personal communication), in agreement with the sequence shown in Fig. 1.

The cleavages at the end of PE2 and the beginning of E1 both occur after alanine residues (for SFV as well as Sindbis; see Fig. 2). It is thus tempting to propose that signalase is responsible for both cleavages (the properties of signalase have recently been reviewed; see ref. 29). If so, and if the signalase activity is restricted to the lumen of the rough endoplasmic reticulum, it would predict that the COOH-terminal region of PE2 spans the membrane twice, at least transiently. This could occur if the COOH terminus of PE2 and the 55 amino acid polypeptide form a set of stop transfer sequences and internal signal sequences that leads to multiple crossings of the membrane of the endoplasmic reticulum. After cleavage, the 55 amino acid peptide could be membrane-associated or could be released into the lumen of the endoplasmic reticulum. The COOH terminus of PE2, after cleavage, could remain transmembranous, or it could fold in such a way as to become partly or completely cytoplasmic. This domain contains proline and asparagine, which would be unusual in an intramembranous region, but it also appears to contain covalently attached lipid (J. R. Bell, personal communication), suggesting membrane association. Further work will be required to resolve these questions. It is noteworthy, however, that the two host proteases thought to be involved in processing the Sindbis structural polypeptides are located in the lumen of the endoplasmic reticulum and in the Golgi apparatus, whereas a virus-encoded protease appears to function in the cytosol. Virus-specific proteases that cleave capsid precursors located in the cytosol have also been reported for other viruses (30), including picornaviruses (31), RNA tumor viruses (32), and probably adenovirus (33). We suggest as a general rule that virus-encoded proteases are used for the processing in the cytosol of virus polypeptides.

We would like to thank E. Lenches for preparing the chicken fibroblasts; T. E. Crowley for help with the 26S RNA isolation; H. V. Huang, T. Hunkapiller, and M. and R. Douglas for writing the computer programs and preparing the figures; J. R. Bell, M. W. Hunkapiller, and E. G. Strauss for helpful discussions; and L. Hood and T. Maniatis for their generosity in lending us supplies and equipment. We are especially grateful to H. Garoff, W. J. Welch, and B. M. Sefton for sending us preprints of their work. This work was supported by Grant PCM 77-26728 from the National Science Foundation, Grants GM 06965 and AI 10793 from the National Institutes of Health, and Biomedical Research Support Grant 507 RR 07003 from the National Institutes of Health. C.M.R. was supported by Training Grant GM 00086 from the National Institutes of Health.

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