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Fusion protein of the paramyxovirus simian virus 5: Nucleotide sequence of mRNA predicts a highly hydrophobic glycoprotein

(parainfluenza virus/cDNA molecular cloning/membrane protein/cell fusion)

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ABSTRACT **The nucleotide sequence of the mRNA coding for the fusion glycoprotein (F) of the paramyxovirus, simian virus 5, has been obtained. There is a single large open reading frame on the mRNA that encodes a protein of 529 amino acids with a molecular weight of 56,531. The proteolytic cleavage/activation site of F, to yield F_2 and F_1, contains five arginine residues. Six potential glycosylation sites were identified in the protein, two on F_2 and four on F_1. The deduced amino acid sequence indicates that F is extensively hydrophobic over the length of the polypeptide chain. Three regions are very hydrophobic and could interact directly with membranes: these are the NH_2-terminal putative signal peptide, the COOH-terminal putative membrane anchorage domain, and the NH_2-terminal region of F_1.**

The parainfluenza virus, simian virus 5 (SV5), is a prototype of the paramyxovirus family of negative-strand RNA viruses, which are widely known for their ability to cause cell fusion and hemolysis. The SV5 virion contains an envelope consisting of a membrane with a nonglycosylated protein (M) associated with its inner surface and two integral membrane glycoproteins (HN and F) that form spike-like projections on the outer surface (1). Inside the envelope is the ribonucleoprotein, which consists of the viral 50S RNA, a major structural protein subunit (NP), and two minor protein components (P and L) that are associated with an RNA polymerase activity that transcribes the 50S RNA into mRNAs (1–3).

Extensive studies on the surface glycoproteins have shown that HN has both receptor-binding (hemagglutinating) and neuraminidase activity and that F is involved in virus penetration, hemolysis, and cell fusion and is also required for the intracellular spread of virus (4–6). The F glycoprotein is synthesized as an inactive precursor, F_0, that is cleaved by a host proteolytic enzyme to form the biologically active protein consisting of the disulfide-linked chains, F_1 and F_2 (2, 7). Cleavage of F_0 releases a free NH_2 terminus of F_1, and in the case of Sendai virus, it has been shown that cleavage causes a conformational change in the molecule with exposure of new hydrophobic regions (8). Direct amino acid sequencing of the NH_2 terminus of F_1 indicates that it is highly hydrophobic and the sequence is conserved between three paramyxoviruses (SV5, Sendai, and Newscastle disease virus), and it has been suggested that this hydrophobic region may be directly involved in membrane fusion (9–11). Further evidence in support of the importance of this region of the molecule in causing cell fusion comes from the finding that treatment of cells with oligopeptides that mimic the NH_2-terminal hydrophobic region of F_1 prevents cell fusion and virus penetration by SV5 and Sendai viruses (11, 12).

Membrane fusion is of major importance in cell biology (e.g., it is involved in endocytosis, secretion, myogenesis, and fertilization). At present, the mechanisms involved in membrane fusion are not understood. Studies involving the paramyxovirus F protein, which can cause fusion at physiological pH (13), may provide a means of elucidating the biochemical and biophysical events involved in the process.

In this paper, we present the nucleotide sequence of the SV5 F mRNA, derived from cDNA cloning of SV5 mRNAs (14) and the predicted amino acid sequence of the fusion protein.

MATERIALS AND METHODS

Nucleotide Sequencing. The clone Fc (14) was used for nucleotide sequence analysis, which was done as described (15).

Nuclease S1 Analysis and Primer Extension. Poly(A)-containing mRNAs from SV5-infected CV1 cells were isolated as described (14). To determine the 5′ end of the SV5 F mRNA, nuclease S1 analysis was done as described (16) using a *Pst* I/*Hpa* II DNA fragment of the Fc clone (nucleotides −181 to +136) and 5′ uniquely labeled at nucleotide 136. Primer extension sequence analysis of the 5′ end of the SV5 F mRNA was done as described (16) using as a primer a *Rsa* I/*Sau*3AI DNA fragment (nucleotides 36–93) and 5′ uniquely labeled at nucleotide 93.

Computer-Assisted Analysis. DNA sequence analysis programs of Lagrimini *et al.* (17) were used. Secondary structure predictions were done according to Chou and Fasman (18) using the program of Stevens (19). Relative hydrophobicity was calculated using the program of Kyte and Doolittle (20).

RESULTS

Nucleotide Sequence of the F Gene and Its Predicted Protein Sequence. A cDNA clone derived from SV5-specific mRNAs, designated Fc, has been shown (14) to prevent the *in vitro* synthesis of both F and M proteins in hybrid-arrested translation experiments. In addition, clone Fc was used to select SV5-specific mRNAs which, when translated *in vitro*, yielded the F and M proteins. Analysis of SV5-specific poly(A)-containing mRNAs, by RNA blot analysis, showed that clone Fc hybridized to the mRNAs for F (≈1800 nucleotides) and M (≈1400 nucleotides) (14). Thus, clone Fc is considered to have been derived from a polycistronic transcript (5′-M-F-3′) and such transcripts have been shown to exist in SV5-infected cells (14).

Clone Fc was chosen for sequencing because it was considered likely to contain a complete copy of the F mRNA. The 5′ end-labeled restriction fragments were prepared and sequenced using the strategy shown in Fig. 1. All restriction sites used were overlapped, and the sequence of both DNA

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Abbreviations: SV5, simian virus 5; F, fusion protein; HA, influenza virus hemagglutinin.

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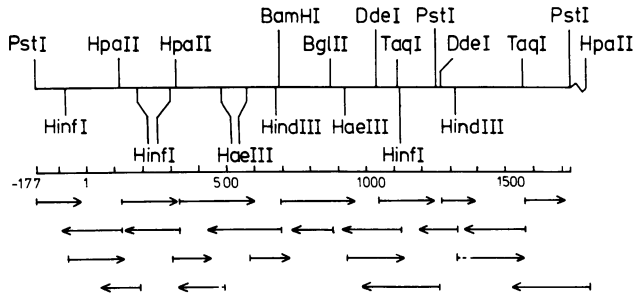

FIG. 1. Restriction endonuclease cleavage map and sequence determination strategy used for clone Fc. Restriction endonuclease cleavage sites used in sequencing are shown. Nucleotide 1 corresponds to the 5'-terminal nucleotide of the F mRNA. Nucleotides −1 to −177 correspond to the intergenic region and the 3'-terminal portion of the M mRNA. Base of each arrow denotes the restriction site that was 5' end-labeled for sequence determination; body of arrow indicates the direction and extent of the analysis performed. Dashed line in arrow indicates region not analyzed. Zigzag line represents pBR322 sequences.

strands was determined for >96% of the F protein coding region.

The nucleotide sequence of clone Fc, in the mRNA sense, is shown in Fig. 2. The clone contains 1887 base pairs, excluding G·C tails. One hundred six nucleotides precede a poly(A) tract of 37 nucleotides. Following this region, the first ATG codon (nucleotides 30–32) is followed by the only large open reading frame in the sequence, which extends to a termination signal at nucleotides 1617–1619. Translation beginning at this initiation codon would yield a protein of 529 amino acids (M_r, 56,531). This sequence contains the NH_2-terminal 20 amino acids of F_1 (residues 103–122) determined by direct protein sequencing (9, 11).

The NH_2-terminal 20 amino acids of F are uncharged, consistent with this region acting as a signal sequence for translocation of the F protein across the rough endoplasmic reticulum (21). The NH_2 terminus of F_2 has been shown to be a leucine residue (9), but because leucine is predicted to occur at residues 8, 13, 14, and 20, the site of cleavage of the signal peptide cannot be determined. Five arginine residues (98–102) precede the 26 uncharged amino acids of the NH_2 terminus of F_1 (residues 103–128) and form the cleavage/activation site of F. Assuming the NH_2 terminus of mature F_2 is the leucine at residue 20 and the COOH terminus is the threonine at residue 96 (see *Discussion*), then F_2 consists of 78 amino acids (M_r, 8489). F_1 is predicted to consist of 427 amino acids (residues 103–529) (M_r, 45,438). The predicted COOH-terminal sequence of F_1 contains a hydrophobic domain of 38 amino acids (residues 472–509), and this region is likely to anchor the F protein in the membrane, leaving a hydrophilic domain of 20 amino acids (residues 510–529) as a cytoplasmic tail. In the case of Sendai virus, there is direct evidence, derived from proteolysis of inside-out vesicles, that 20 amino acids of the F protein reside on the cytoplasmic side of the membrane (22). There are six potential sites for the *N*-glycosidic linkage of oligosaccharides (i.e., an asparagine followed by an unspecified amino acid, followed by serine or threonine). Two of these are in F_2 (residues 65–67 and 73–75) and four are in F_1 (residues 352–354, 427–429, 431–433, and 461–463). Secondary structure predictions of the F protein (18) indicate that four of the six potential glycosylation sites occur on or immediately after β turns (residues 61–64, 349–352, 429–432, and 457–460).

Determination of the 5' End of Monocistronic F mRNA. As discussed above, clone Fc was thought to be derived from a polycistronic mRNA containing part of M and all of the F gene. As shown in Fig. 2, clone Fc contains 203 nucleotides preceding the translation initiation codon for F, and this region contains a stretch of poly(A) for 37 nucleotides.

To determine the 5' end of monocistronic F mRNAs, an Fc DNA fragment (*Pst* I/*Hpa* II) 5' uniquely labeled at nucleotide 136 was hybridized to virus-specific mRNAs and the hybrids were digested with nuclease S1. As shown in Fig. 3, two protected fragments of 136 and 158 nucleotides were obtained: the ends of these fragments map on the sequence of clone Fc (Fig. 2) to nucleotides 1 and 22, respectively. It was considered likely that the shorter fragment was derived by protection of the 5' end of monocistronic F mRNAs and the longer fragment was derived from protection of polycistronic transcripts up to the poly(A) tract (Fig. 2, nucleotide −23). These data do not establish either the absence or presence of poly(A) in a population of polycistronic transcripts, because under the conditions used, nuclease S1 digests nucleotides in long A·T hybrids (23). To provide further information about the 5' end of the mRNAs, a small Fc DNA fragment (*Rsa* I/*Sau*3AI, nucleotides 36–93), 5' uniquely labeled at nucleotide 93, was hybridized to virus-specific mRNAs and the primer was extended with reverse transcriptase. As shown in Fig. 4, a major extended species of 93 nucleotides and a minor species of ≈1500 nucleotides were observed. The size of the small extended product maps the 5' end of the F monocistronic mRNA to nucleotide 1 (Fig. 2) and the larger ≈1500-nucleotide species is compatible in size with a cDNA copy of a M–F polycistronic mRNA extending from the primer at the 5' end of the F region to the 5' end of the M mRNA. The DNA sequence of the 5'-terminal region of the 93-nucleotide extended product (Fig. 4) is complementary to the mRNA sense sequence of clone Fc (Fig. 2) and confirms that the 5' ends of monocistronic F mRNAs map to nucleotide 1 (Fig. 2).

At the 3' end of clone Fc there are 13 adenosine residues (nucleotides 1710–1722) and these probably represent part of the poly(A) tail in the original mRNA before it was cloned. Thus, the above data suggest that a monocistronic F mRNA is 1709 nucleotides long, excluding poly(A) residues, and contains a 5' noncoding region of 29 nucleotides and a 3' noncoding region of 90 nucleotides. The nuclease S1 mapping data and the sequence of clone Fc also indicate, but do not prove, that the intergenic region (i.e., nucleotides found in the 50S virion RNA but lacking from the complementary monocistronic mRNAs) between M and F of SV5 is 22 nucleotides long. In two other negative strand viruses, VSV and Sendai virus, the intergenic regions are two and three nucleotides long, respectively (24–26).

DISCUSSION

The deduced amino acid sequence of the SV5 F protein contains the 20 amino acids sequenced directly from the NH_2 terminus of F_1 (9, 11) and the unprocessed precursor is 529 amino acids long (M_r, 56,531). The unglycosylated F protein synthesized *in vitro* (i.e., including signal peptide) was found to have a M_r of 48,000 (14), and this indicates that F has an aberrant mobility on polyacrylamide gels, which is probably due to the hydrophobic nature of the protein.

The most striking feature of the amino acid sequence of the F protein is the overall hydrophobicity: 54% of the amino acids are nonpolar, 34.6% are polar, and 11.4% are charged; many stretches of uncharged amino acids can be identified (e.g., residues 1–20, 22–35, 59–77, 103–128, 154–169, 172–185, 209–224, 275–287, 301–333, 388–405, 420–434, and 472–509). Three regions of F have a hydropathic index (20) of >20 (Fig. 5), a value normally found for regions of proteins that interact with membranes. These regions are the presumed signal peptide, the presumed COOH-terminal membrane anchorage domain, and the NH_2 terminus of F_1. It has previously been suggested that the NH_2 terminus of F_1 can interact with the membrane of a target cell, mediating the biological activities of F (i.e., viral penetration, cell fusion,

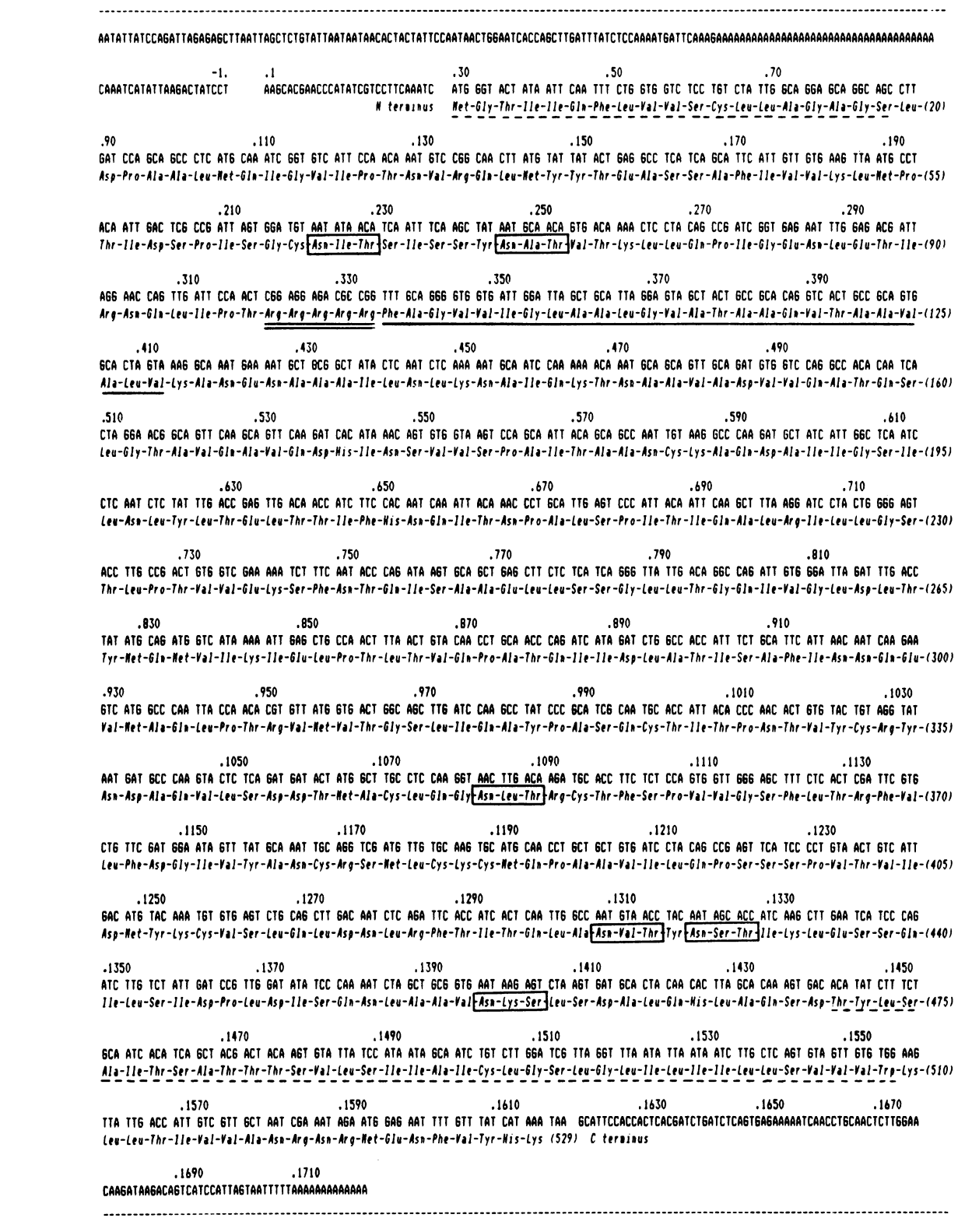

FIG. 2. Nucleotide sequence of clone Fc in mRNA sense and predicted amino acid sequence of F protein. Nucleotide 1 is the 5′-terminal nucleotide of the F mRNA. Presumptive signal sequence and membrane anchorage region are underlined with a broken line. The cleavage site is indicated by a double line, and the NH_2-terminal sequence of F_1 determined previously by direct amino acid sequencing is underlined. Potential glycosylation sites are boxed.

and hemolysis; refs. 9 and 10, reviewed in ref. 28). For a surface spike glycoprotein, the whole sequence of F is remarkably hydrophobic [cf. influenza virus hemagglutinin (HA); Fig. 5], and it is likely that in the three-dimensional structure of the F protein the few charged amino acids will be strategically located on the outside of the molecule.

There are two potential glycosylation sites on F_2 and four on F_1, which is consistent with the ratios of glycosylation of F_2 and F_1 found previously (7). Analysis of the carbohydrates of the SV5 F protein indicated that F_2 and F_1 contained one and three carbohydrate chains, respectively (29). However, the molar chain value for F_2 was calculated on a

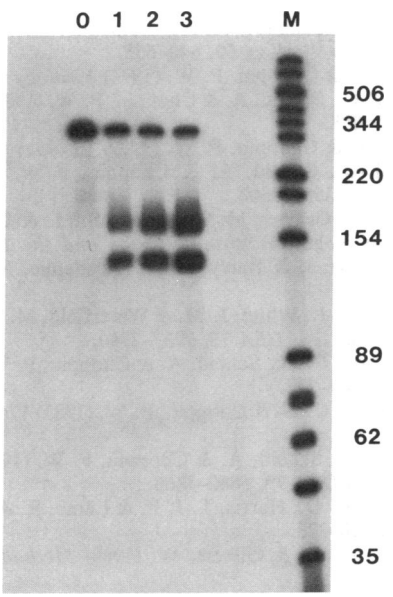

FIG. 3. Nuclease S1 analysis to determine the 5′ end of the F mRNA. A *Pst* I/*Hpa* II fragment (nucleotides −181 to +136) 5′ labeled at nucleotide 136 was hybridized to poly(A)-containing RNA from SV5-infected CV1 cells, treated with nuclease S1, and analyzed on 6% polyacrylamide gels containing 9 M urea. Lanes: 0, no added mRNA; 1–3, increasing mRNA concentrations in the ratio 1:2:4; M, ^{32}P-labeled size markers from *Hin*fI-digested pFVM45 DNA (16). Numbers on right represent nucleotides.

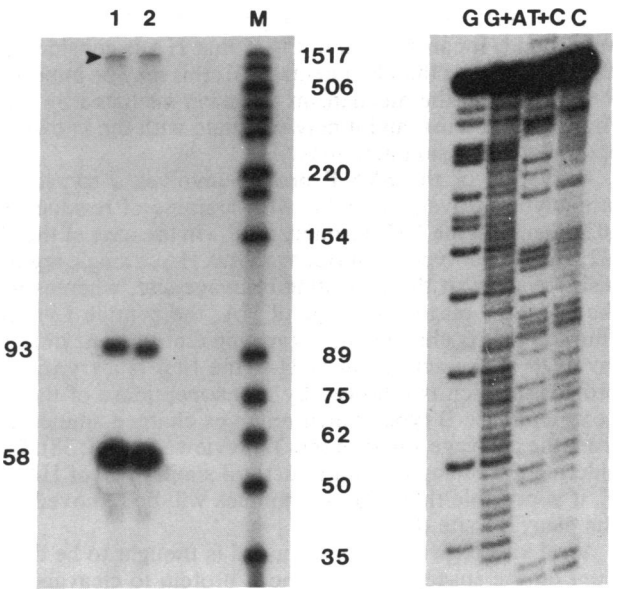

FIG. 4. Primer extension and sequence analysis of the SV5 F mRNA. An *Rsa* I/*Sau*3AI DNA fragment derived from clone Fc (nucleotides 36–93) 5′ uniquely labeled at nucleotide 93 was hybridized to poly(A)-containing mRNAs from SV5-infected CV1 cells, and the primer was extended with reverse transcriptase. (*Left*) cDNAs were analyzed on a 6% polyacrylamide gel containing 9 M urea (lanes 1 and 2): 58, 58-nucleotide primer; 93, 93-nucleotide-extended product; arrow indicates the M_r ≈1500 nucleotide-extended product; M, marker as in Fig. 3. (*Right*) Small (93 nucleotides) extended product was sequenced and the 5′-terminal region is shown. Base-specific chemical cleavages are indicated above each lane of the gel.

M_r of 14,000 for F_2 rather than the predicted M_r of ≈8500 found here. Two potential glycosylation sites in F_1 have their asparagine residues four amino acids apart (residues 427 and 431), and it is possible that the second of these sites does not contain a carbohydrate chain because of steric hindrance, resulting from addition of the first chain.

Both the paramyxovirus F proteins and the influenza virus HA protein share some common biological and structural features: (*i*) they are both cleaved by a cellular protease to yield two disulfide-linked subunits F_2–F_1 and HA_1–HA_2 (5, 30, 31); (*ii*) cleavage activates the infectivity of the virus (5, 32, 33); (*iii*) cleavage causes a conformational change in the molecule (8, 34, 35); (*iv*) the cleaved proteins mediate cell fusion (with paramyxoviruses this occurs at neutral pH; ref. 13), whereas influenza virus HA only causes cell fusion *in vitro* at pH 5.0–5.5 (reviewed in ref. 36); and (*v*) the NH_2 termini of F_1 of paramyxoviruses and HA_2 of influenza virus that are generated by proteolytic cleavage are very hydrophobic and show some degree of homology (9, 10).

It was of interest to examine the nucleotide and amino acid sequences of SV5 F and influenza HA [strain A/Aichi/68 (H3)] (27) to search for regions of homology. Computer-assisted comparison of the sequences for regions of 5–7 identical nucleotides, using a matrix program (17), detected only a single 12-nucleotide region of SV5 F (nucleotides 534–545) that was identical to that found in HA (nucleotides 975–986), but for HA these nucleotides are out of the translational reading frame. Other matches of 5–7 nucleotides were scattered over the mRNAs, and no obvious domains of close homology could be found (data not shown). Similarly, at the

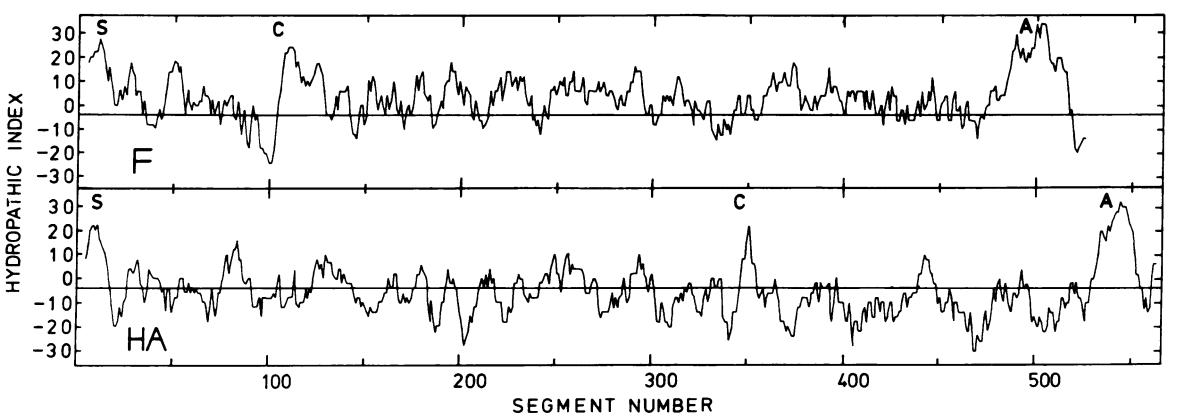

FIG. 5. Hydropathy plot of the SV5 F protein and influenza virus HA. Relative hydrophobicity and hydrophilicity of the proteins along their amino acid sequence were calculated as described (20), using a segment length of 9 amino acids. The consecutive scores are plotted from the NH_2 to the COOH terminus of the proteins, and midpoint line corresponds to the grand average of the hydropathy of the amino acid compositions found in most sequenced proteins (20). (*Upper*) SV5 F protein. (*Lower*) Influenza virus HA (strain A/Aichi/68) (27). Signal sequence (S), cleavage/activation site (C), and membrane anchorage domain (A) are indicated.

protein level, only scattered matches of 2–4 amino acids were found. It can be seen in Fig. 5 that HA is considerably less hydrophobic than F at neutral pH; this may be important with respect to the mechanism of fusion mediated by these viral glycoproteins, and it may correlate with the known pH optima of the fusion activities.

Activation of the SV5 F protein involves a trypsin-like proteolytic cleavage after the fifth arginine of residues 98–102, liberating the NH_2 terminus of F_1. In the case of the H1, H2, and H3 subtypes of influenza virus HA, a single arginine residue is lost at the activating cleavage site, whereas with the H7 (fowl plague) subtype of HA, the peptide Lys-Arg-Glu-Lys-Arg is eliminated, suggesting that two enzymes are involved in the activation of HA: the first is a trypsin-like protease, which is followed by an exopeptidase of the carboxypeptidase B type, which removes charged amino acids from the cleavage site (see ref. 37; reviewed in ref. 38). Considering the biological and structural similarities of HA and F, it is possible that all five arginines will be removed from the cleavage site of SV5 F.

Viral virulence in the host animal is thought to be dependent on the susceptibility of the F protein to cleavage by a host protease (5), and evidence for this has been provided in the case of virulent and avirulent strains of the paramyxovirus Newcastle disease virus (39). A similar correlation has been made and extended with influenza virus HA, where it has been found that strains virulent for chickens have a "highly cleavable" HA that contains a long basic connecting peptide, whereas avirulent strains have a "noncleavable" HA that contains only a single arginine at the cleavage site (37, 40). SV5 virions grown in tissue culture cells (e.g., CV1, MDBK) always contain a cleaved F protein (2, 4), whereas Sendai virus grown in most tissue culture cells contains mostly uncleaved F (5). As SV5 possesses a highly cleavable F that contains five arginine residues at the cleavage site, it is possible that Sendai virus F protein contains only a single arginine or lysine residue at the cleavage site.

To understand how the hydrophobic F protein is folded to form the membrane spike and to help in elucidating the mechanism by which this protein causes cell fusion, it would be of great interest to obtain its crystallographic structure. However, because of the overall hydrophobic character of the molecule, the protein may prove difficult to crystallize.

The F protein, when reconstituted into lipid vesicles, is capable of causing cell fusion if a means of binding it to the cell is provided (41). Now that a cDNA clone to the F protein has been obtained and sequenced it should be possible, by genetic manipulation, to investigate the domains of the protein essential for mediating cell fusion.

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