# Current Topics in Microbiology and Immunology

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Editors

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# Structure and Assembly of Alphaviruses

H. GAROFF*, C. KONDOR-KOCH* AND H. RIEDEL*



* European Molecular Biology Laboratory, Postfach 102209, 6900 Heidelberg, Federal Republic of Germany

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* European Molecular Biology Laboratory, Postfach 102209, 6900 Heidelberg, Federal Republic of Germany

## 1 The Life Cycle of Alphaviruses

The alphaviruses are enveloped animal viruses that belong to the family of Toga viruses. The molecular biology of this virus group is almost entirely based on studies with two members of this virus group, the Semliki Forest Virus (SFV) and the Sindbis virus. Like all other viruses the alphaviruses are completely dependent on their host cell for their replication. The virus particles themselves can be regarded simply as a piece of nucleic acid, which represents their genome, wrapped in a protective coat. In the case of the alphaviruses this coat is represented by the nucleocapsid structure and the surrounding membrane or envelope. The virus coat must, in addition to its protective function, also provide the means whereby the virus particle can get into the host cell and release its genome into the cell cytoplasm to start virus infection. This function is carried out by the virus-coded glycoproteins that form the spike-like projections on the surface of the virions.

The entry of the virus particle into the host cell starts with binding of the virus through its spikes to a receptor molecule present on the surface of the cell (see Fig. 1, *Helenius* et al. 1980a, b; *White* and *Helenius* 1980). After this initial binding event the virus is taken up into coated pits and then routed inside coated vesicles to the lysosomes where the acidic pH is thought to induce a change in the conformation of the spike glycoproteins such that these cause a fusion between the viral and the lysosomal membrane. As a result the viral nucleocapsid enters the cell cytoplasm and releases the RNA genome (a 42S RNA molecule). The viral RNA functions as a mRNA molecule for synthesis of an RNA-dependent RNA polymerase which will transcribe more viral genomes as well as a subgenomic RNA molecule (26S RNA) which corresponds to about one-third of the 42S RNA molecule at its 3′ end. This smaller RNA molecule serves as a messenger RNA for all structural proteins of the virus particle, that is the capsid protein ($3 \times 10^4$ daltons), and the three-membrane proteins E3 ($10^4$ daltons), E2 ($5 \times 10^4$ daltons), and E1 ($5 \times 10^4$ daltons). The translation starts from a single initiation site close to the 5′ end of the 26S RNA molecule and the proteins are synthesized sequentially in the order, capsid protein, p62, and E1; the p62 protein is an intracellular precursor protein for E3 and E2. As shown in the Fig. 1, the capsid proteins assemble with the viral genome into nucleocapsid structures in the cell cytoplasm, whereas the p62 and E1 proteins are inserted into the rough endoplasmic reticulum membrane (RER) to become integral membrane proteins, which together will form the spike glycoprotein complex of the virus. This complex is transferred from its initial site of synthesis in the RER through the Golgi complex to the plasma membrane (PM) of the host cell, and here they will be specifically incorporated into a viral envelope during the budding process.

The simple nature of the alphaviruses and the fact that their replication in the animal cell is so heavily dependent on normal cellular functions have made these viruses very important tools for research in cellular biology. They are excellent model systems, for example, in the study of adsorptive endocytosis in animal cells (recently reviewed by *Helenius* et al. 1980b) and in studies on the structure and biosynthesis of the plasma membrane. With the two latter examples in mind we will discuss in this review the structure

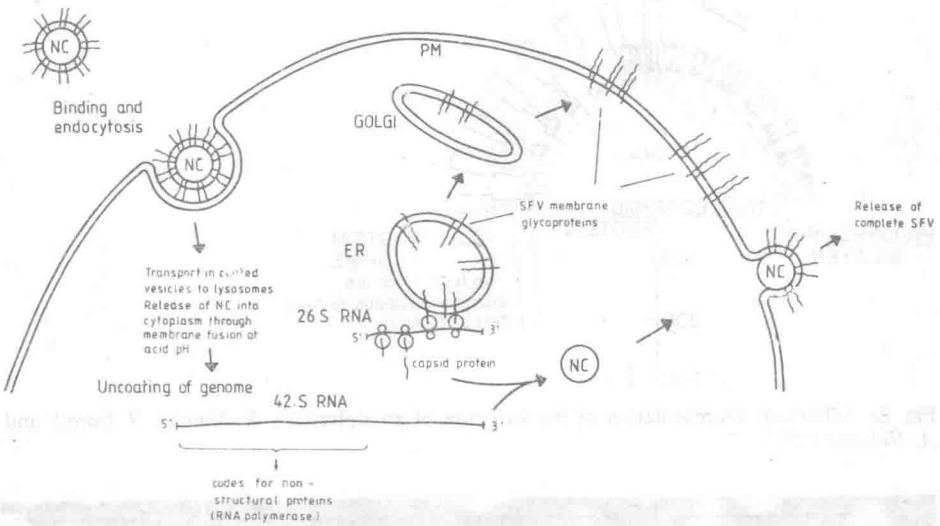


Fig. 1. Replication of an alphavirus. See text for explanation

and assembly of alphaviruses. Several excellent reviews on this matter have been written during the last few years (*Kääriäinen* and *Renkonen* 1977; *Strauss* and *Strauss* 1977; *Simons* and *Garoff* 1980), but recent progress in this area has been considerable and we therefore feel it appropriate to discuss the new findings.

We will start our discussion with some general features of the virus particle and then deal with the structure of the membrane glycoproteins in detail. The amino acid sequences for all structural proteins have recently been elucidated for both SFV and Sindbis virus, and there are many functional implications that can be drawn from these data. We will then discuss the biosynthesis of the viral membrane, a problem which has now been worked out in some detail using in vitro translation systems. In the last part we shall follow the intracellular pathway of the newly made spike glycoproteins from the RER to the PM where the budding takes place. In our discussion we will not limit ourselves strictly to the alphaviruses but we will also discuss important findings that have been obtained for other enveloped viruses, such as vesicular stomatitis virus (VSV) and influenza virus. This is particularly necessary where the data on alphaviruses are not extensive.

## 2 General Features of the Alphavirus Particle

Alphaviruses are spherical particles with a diameter of approximately 65 nm when analyzed by negative contrast electron microscopy (Fig. 2b, see also *von Bonsdorff* 1973). The spike glycoproteins are seen as a 6- to 10-nm-thick fuzzy layer on the surface of the virus particles. Careful examinations of the Sindbis virus and SFV surfaces have shown that the spikes form an icosahedral lattice (*von Bonsdorff* and *Harrison* 1975; *Brown* et al.

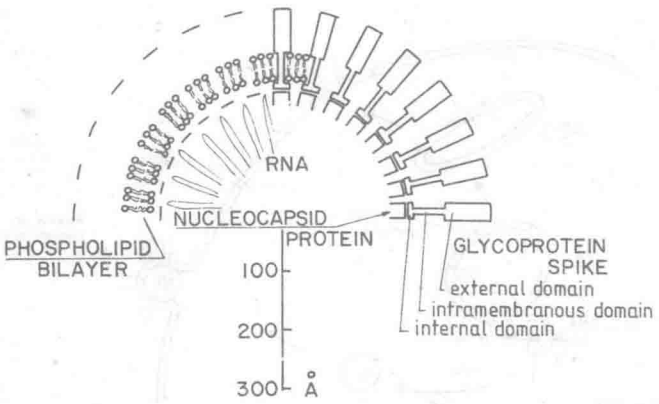


Fig. 2a. Schematic representation of the structure of an alphavirus. *K. Simons, H. Garoff,* and *A. Helenius* (1977)

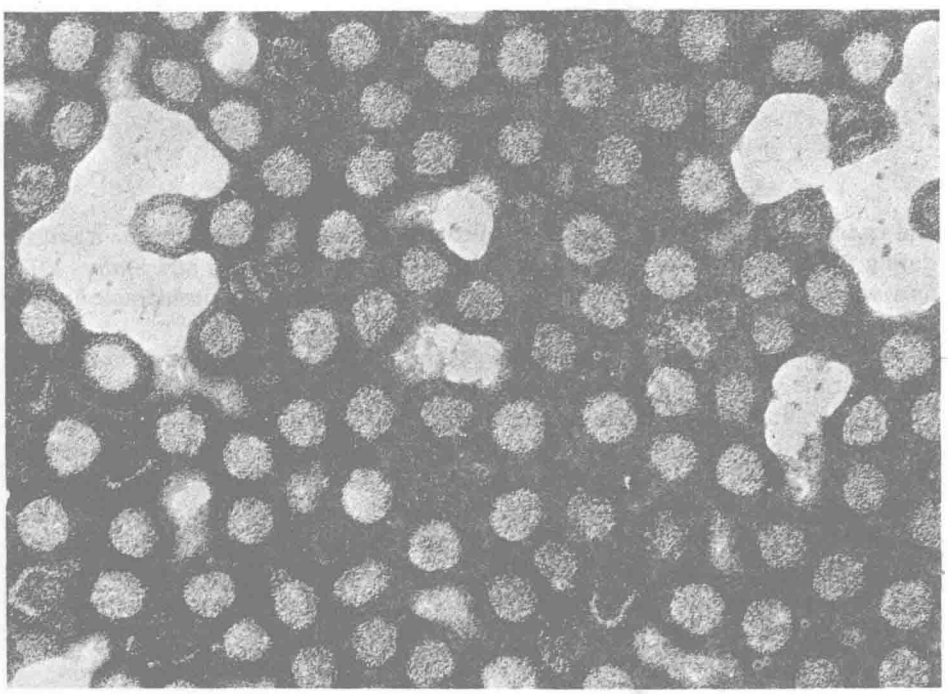

Fig. 2b. Electron microscopy picture of SFV. Negative-contrast with potassium phosphotungstate, X 136.000. Courtesy of *C.-H. von Bonsdorf*

1972; *Horzinek* 1973). Symmetrically arranged subunits have also been demonstrated on the viral nucleocapsids, which can easily be isolated after solubilization and removal of the membrane with a mild detergent such as Triton X-100 (see p. 9) (*Osterrieth* 1968; *Horzinek* and *Mussgay* 1969; *Brown* et al. 1972; *von Bonsdorff* 1973). The exact icosahedral arrangement of the subunits on the membrane and the nucleocapsid is not clear, but a

similar arrangement would be expected since there is a direct interaction between the nucleocapsid and the spike glycoproteins in the virus particle (see p. 39 and Fig. 2a). Electron density distribution analyses of Sindbis virus have shown that the lipids of the alphaviruses are arranged in a bilayer between the spikes and the nucleocapsid (*Harrison* et al. 1971).

The molecular wheight of the SFV particle has been calculated to be $42 \times 10^6$ daltons, using data from neutron diffraction analyses of SFV and its chemical composition (*Jacrot B, Cuillec M* and *Söderlund H*, unpublished results; *Laine* et al. 1973). SFV contains four structural polypeptides, that is the capsid, E1, E2, and E3 polypeptides, whereas Sindbis virus lacks E3 (*Simons* and *Kääriäinen* 1970; *Garoff* et al. 1978; *Strauss* et al. 1968; *Schlesinger* et al. 1972). The proteins are present in an equimolar ratio in the virus particle and they are all specified by the virus genome. *Strauss* (1978) has estimated that less than 0.5% of the protein in Sindbis virus is derived from the host cell. In contrast to the protein composition, the oligosaccharide units of the spike glycoproteins and the lipids of the bilayer appear to be specified by the host cell. The lipid composition of the virus and, to a certain extent, the carbohydrate composition of the viral membrane proteins can, therefore, vary considerably depending on which cell type has been used for virus production (*Keegstra* et al. 1975; *Gottlieb* et al. 1979; *Renkonen* et al. 1971, see also *Kääriäinen* and *Renkonen* 1977). For instance, SFV grown in baby hamster kidney cells has a very different lipid composition from SFV grown in *Aedes albopictus* cells (*Luukkonen* et al. 1976). In each case the lipid composition of the virus reflects that of the PM of the respective host.

## 3. The Spike Glycoprotein Complex of Alphavirus Particles

### 3.1 The Subunit Structure of the Spike

In electron microscopic pictures of alphaviruses individual spikes cannot usually be seen clearly (see Fig. 2b). This is in contrast to the spikes of many other enveloped viruses, e.g., myxoviruses where two types of spikes, the hemagglutinin and the neuraminidase, can easily be differentiated on a morphological basis. The spikes of alphaviruses appear to be very closely packed on the virus surface and it is also possible that their structure is more flexible than those of many other viruses. In good, negative-contrast electron microscopic pictures of SFV the spikes have been shown to be 3–3.5 nm thick and 6–10 nm long, the distance between spikes being 4–6 nm (*von Bonsdorff* 1973).

The subunit structure of the alphavirus spikes has been studied by us, using two biochemical approaches (*Simons* et al. 1973a; *Ziemiecki* and *Garoff* 1978). In one approach we solubilized the spike glycoproteins with Triton X-100, a detergent which does not affect the interactions between protein subunits, yet solubilizes membrane proteins (see p. 9), and determined the protein composition of the membrane protein-detergent complexes (see Fig. 3). The solubilized complexes were physically homogeneous and contained approximately $5 \times 10^4$ daltons of detergent and $10^5$ daltons of protein. All three membrane proteins of SFV were represented in the complex fraction at an equimolar ratio. These results thus suggested that the spike glycoprotein of SFV is an oligomeric structure, but they did not answer the basic question whether one or more types of spikes existed on the surface of SFV. The solubilized complexes could represent only E1-E2-E3

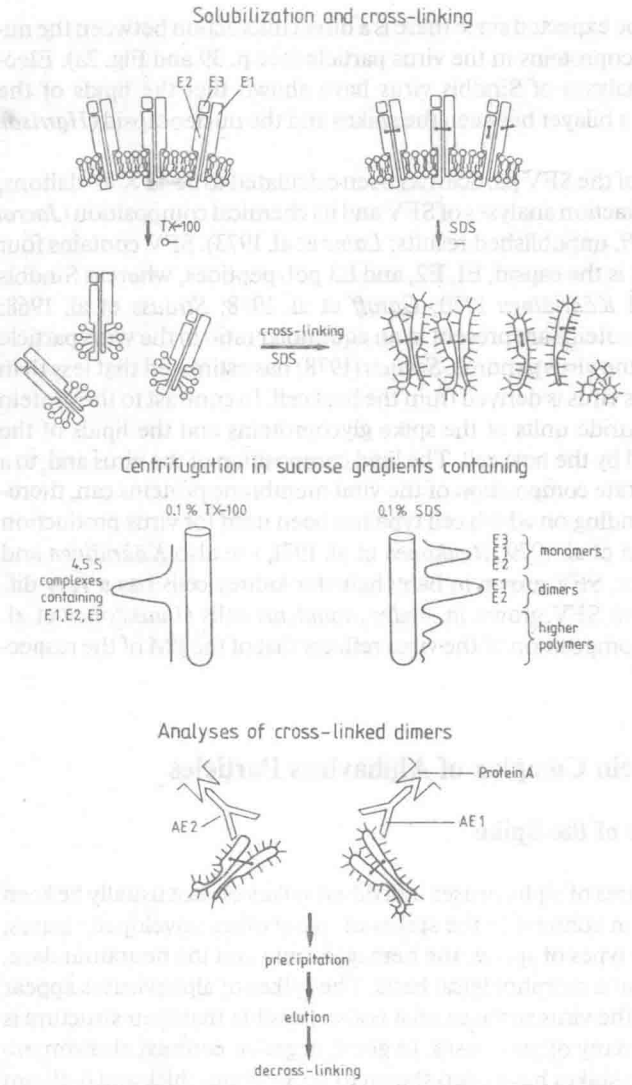


Fig. 3. Investigation of the subunit structure of the spike glycoprotein complex in SFV. See text for details

trimers, or a mixture of E1-E1-E3 and E2-E2-E3 trimers, or possibly other combinations of polypeptides which together give a molecular weight of approximately $10^5$ daltons.

In the other approach we treated intact virus particles with a cleavable protein-protein cross-linking reagent, dithiobispropionimidate, and characterized the cross-linked glycoprotein complexes formed after solubilization in SDS (see Fig. 3). The complexes formed should represent true subunit interactions in the virus particle as SDS effectively dissociates all proteins which are not covalently bound together. (The Triton X-100 solubilized complexes could be formed through artificial aggregation). On the other hand,

this approach might not show all the subunit interactions in the viral spike since it is strictly dependent on the distances between reactive amino groups in the proteins. Separation of solubilized, cross-linked complexes by centrifugation in sucrose gradients showed one major, cross-linked complex with an apparent molecular weight of $10^5$ daltons when analyzed in SDS polyacrylamide gel electrophoresis (PAGE). The subunit structure of the cross-linked complex was determined using specific antibodies against E1 and E2 as shown in Fig. 3. The complex was precipitated using either antibody, in the presence of 0.1% SDS to prevent any nonspecific aggregation, and the precipitates were analyzed by SDS-PAGE after cleavage of the cross-linking reagent. Both antibodies were shown to precipitate E1-E2 cross-linked complexes, showing that the spikes on the viral surface are heterodimers of E1 and E2. The E3 protein was not found in the cross-linked complex but most probably the SFV spike also includes this protein since it is associated with E1 and E2 in the Triton X-100 solubilized complex. In Sindbis virus, which lacks E3, the spike glycoprotein is probably an E1-E2 dimer (*Schlesinger* and *Schlesinger* 1972; *Bracha* and *Schlesinger* 1976; *Jones* et al. 1977).

## 3.2 The Spike Glycoprotein Complex is a Transmembrane Protein

One of the outstanding features of the spike glycoprotein complex is that it spans the lipid bilayer, thereby dividing the spike protein into three topologically distinct domains: a large external hydrophilic domain, an intramembranous domain, and a small internal hydrophilic domain (see Fig. 2a, 4). The spanning of the spike glycoprotein complex has been shown in experiments using protease digestion, surface labeling, and protein-protein cross-linking. When intact virus particles were treated with protease, the external hydrophilic portions were digested, and in the membranes of the spikeless particles only small stubs of the E1 and the E2 proteins remained (*Utermann* and *Simons,* 1974). These represented the intramembranous and internal domains of the spikes. The internal

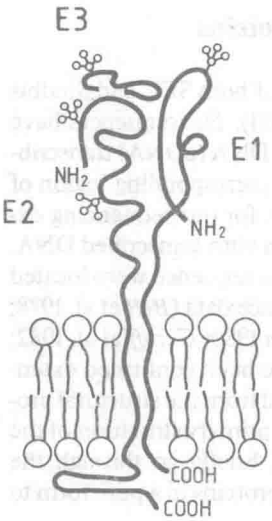


Fig. 4. Orientation of the *E1* and *E2* polypeptide chains in the lipid membrane. *H. Garoff* (1979)

domain has been directly demonstrated by protease treatment of microsomal vesicles derived from the RER of infected cells (see p. 25). In the virus particle itself it has been possible to show that an additional part of the glycoprotein (the internal one) could be labeled with [$^{35}$S]formylmethionylsulphatemethylphosphate, after the viral membrane had been made permeable to the reagent by adding small amounts of Triton X-100 (*Garoff* and *Simons* 1974; *Simons* et al., 1980).

In the cross-linking experiment we treated virus particles with dimethylsuberimidate and found extensive cross-linking of the spike glycoproteins to the underlying nucleocapsid (*Garoff* and *Simons* 1974). Considering the short distance over which the cross-linking reagent can react, we could only explain the result by suggesting that internal parts of the spike glycoproteins were being linked to the nucleocapsid. Additional evidence for the spanning nature of the spike glycoproteins includes several findings suggesting a direct protein-protein interaction between the spikes and the nucleocapsid, both at the PM of the infected cell and in the virus particle. These findings will be dealt with in the section on virus budding (see p. 39).

### 3.3 The Orientation of the E1 and E2 Polypeptide Chains in the Viral Envelope

In order to understand the assembly of the membrane proteins of alphaviruses in the RER membrane (see p. 25), it is important to know the orientation of the membrane polypeptide chains with respect to the lipid bilayer. We have isolated the intramembranous segments of SFV after removal of the external portions of the spikes with a protease and mapped these on the polypeptide chains of E1 and E2 using a gradient of radioactivity which had been generated in the viral proteins (*Garoff* and *Söderlund* 1978). The results showed that the intramembranous segments were derived from the carboxyterminal regions of both E1 and E2. Therefore, the E1 and E2 polypeptides are oriented in the membrane with the aminoterminus on the outside and the carboxyterminus on the inside of the membrane as shown in Fig. 4.

## 4 Amino Acid Sequence of Alphavirus Structural Proteins

The complete amino acid sequences of all structural proteins of both SFV and Sindbis virus are now known (*Garoff* et al. 1980a, b; *Rice* and *Strauss* 1981). The sequences have been established by rapid DNA sequencing of complementary DNA (cDNA) transcribed either from the 26S messenger RNA molecule or from the corresponding region of the viral 42S RNA genome (see p. 11). We used cloned cDNA for our sequencing experiments whereas *Rice* and *Strauss* worked directly with the in vitro transcribed DNA. The coding regions for the different proteins on the nucleotide sequence were located using available amino- and carboxyterminal amino acid sequence data (*Bell* et al. 1978; *Bonatti* and *Blobel* 1979; *Kalkkinen* et al. 1980, 1981; *Kalkkinen* 1980; *Garoff* et al. 1982; *Welch* et al. 1981), and the deduced amino acid sequences have been confirmed extensively through amino acid sequence analyses of peptides derived from the structural proteins (*Boege* et al. 1980; *Garoff* et al. 1982). Before describing the primary structures of the proteins and their functional implications we will, however, briefly go through the different approaches which have been used to isolate the viral proteins in a pure form to

make them suitable for chemical analyses. We shall also deal with the different steps involved in the molecular cloning of the viral mRNA sequences.

## 4.1 Isolation of the Structural Proteins of Alphaviruses

All isolation procedures start from purified virus particles, which is an enormous advantage when compared with the purification of proteins in most other biological systems. Before the viral proteins can be isolated these must be obtained in a dissociated form so that all protein-protein, protein-RNA, and protein-lipid interactions must be broken. This has usually been accomplished using various detergents. They all solubilize the viral membrane from the nucleocapsid under proper conditions but their effect on the protein-protein interactions in the subviral components varies, depending on the denaturing properties of the detergent used. For example, Triton X-100, which is a mild detergent, leaves the spike glycoproteins and the nucleocapsid intact; SDS; which is a harsh detergent, dissociates very effectively all viral proteins but denatures them completely so that all biological activity is lost. The solubilization of the alphavirus membrane has been studied in great detail by *Helenius* and *Simons* and the reader is referred to their review *(Helenius* and *Simons* 1975). After dissociation and solubilization of the viral proteins, these can be separated from each other and isolated using normal techniques of protein purification. Table 1 is a compilation of procedures used to isolate the alphavirus proteins. The most difficult task has been to separate the two integral membrane proteins E1 and E2, largely because they are of similar size. In most procedures the viral membrane is first solubilized with Triton X-100 into spike glycoprotein-detergent complexes (E3-E2-E1 in SFV and E2-E1 in Sindbis virus, which lacks E3) and nucleocapsids. The latter is removed from the mixture by centrifugation and the spike glycoproteins in the supernatant are used for further purification of the membrane proteins. For Sindbis virus it has been possible to separate E1 and E2 in the presence of Triton X-100 by using chromatography on diethylaminoethanol or glass wool under appropriate ionic conditions, isoelectric focusing, or simply by solubilizing E1 specifically from a virus pellet in low salt (see Table 1 for details and references). Similar separation approaches using spike glycoprotein complexes of SFV have, however, failed completely (cf. *Helenius* and *Kartenbeck* 1980; *Garoff,* unpublished observation), suggesting that the E1-E2 association in SFV is much stronger than in Sindbis virus. The only way to separate E1 and E2 of SFV in an undenatured form at present is to use sodium deoxycholate. This detergent first dissociates the spike subunits from each other and then allows the E2 protein to reaggregate with itself, such that it can be separated from the monomeric E1 by, for example, sedimentation in density gradients. The nucleocapsid remains intact in the presence of sodium deoxycholate and is pelleted during the centrifugation, whereas the small E3 protein stays at the very top of the gradient. In this procedure all SFV proteins are thus separated from each other by a single solubilization and separation step (*Helenius* et al. 1976).

The isolation of the membrane glycoproteins in the presence of Triton X-100 or deoxycholate has been important for studying the different biological properties of the membrane proteins, such as the hemagglutinating activity of E1 (*Dalrymple* et al. 1976), and also for in vitro reconstitutions of viral membranes (*Helenius* et al. 1977; *Helenius* et al. 1981). For chemical analyses, however, SDS solubilized and denatured proteins can be

Table 1. Isolation of proteins from alphavirus particles

| Sample | Solubilization | Subviral components | Separation of subviral components | Dissociation and isolation of protein subunits | Biological activity (hemagglutination) | References |
|---|---|---|---|---|---|---|
| Sindbis virus solution | TX-100 1% neutral pH isotonic salt | (E1E2) spikes? + nucleocapsids | Centrifugation | Chromatography of (E1E2) on DEAE Sephadex in 0.004 *M* Tris/pH 8.0/0.5% TX-100: E1 bound, E2 not, E1 elutes in 0.2 *M* NaCl | Present (?) | *Burke* and *Keegstra* (1976) |
| Sindbis virus solution | TX-100 1% neutral pH isotonic salt | (E1E2) spikes? + nucleocapsids | Centrifugation | Chromatography of (E1E2) on glass wool at neutral pH and isotonic salt (?): E2 bound E1 not, E2 elutes in high salt and pH | Present (?) | *Bell* et al. (1978) |
| Sindbis virus solution | TX-100 1% neutral pH isotonic salt | (E1E2) spikes? + nucleocapsids | Centrifugation | Isoelectric focusing pH 3–10.5 1% TX-100 separates E1 (pI=6) E2 (pI=9) and nucleocapsid (pI=3) | Present | *Dalrymple* et al. (1976) |
| Sindbis virus pellet | TX-100 1% 0.01 *M* phosphate pH 6.0 | – | – | E1 elutes from pellet | Present (?) | *Burke* and *Keegstra* (1976) |
| SFV solution | Intact virions layered on a 10%–50% (w/w) sucrose gradient containing 7.5 m*M* sodiumdeoxycholate 50m*M* sodium phosphate pH 8.0 100 m*M* NaCl | E3 and E1 monomers + E2 octamers? + nucleocapsids | Centrifugation separates all components | – | Present | *Helenius* et al. (1976) |
| SFV and Sindbis solutions | SDS | Polypeptide monomers | – | SDS-PAGE or chromatography on hydroxyl apatite or concanavaline A Sepharose | Not present | *Kalkkinen* et al. (1980) *Garoff* et al. (1974) *Mattila* (1979) |

Questionmarks in table indicate facts that are only assumed and not shown

used, and these can easily be separated through chromatography on hydroxylapatite, or concanavaline A Sepharose, or by preparative SDS-PAGE (see Table 1 for references).

## 4.2 Cloning of cDNA from SFV 26S RNA and 42S RNA

There are approximately 1250 amino acids in the alphavirus structural proteins and their sequence determination would have been a tremendous task using conventional amino acid sequencing. Fortunately we were able to use the newly developed techniques of transcription of RNA into cDNA, molecular cloning, and DNA sequencing. The most important steps in this process are shown schematically in Fig. 5 (see also *Garoff* et al. 1980a, b). The 26S RNA molecule (or the 42S RNA) is first transcribed from its 3′ end into single-stranded cDNA using reverse transcriptase and an oligo (dT) primer. After a second DNA strand has been synthesized with DNA polymerase (from *Escherichia coli*)

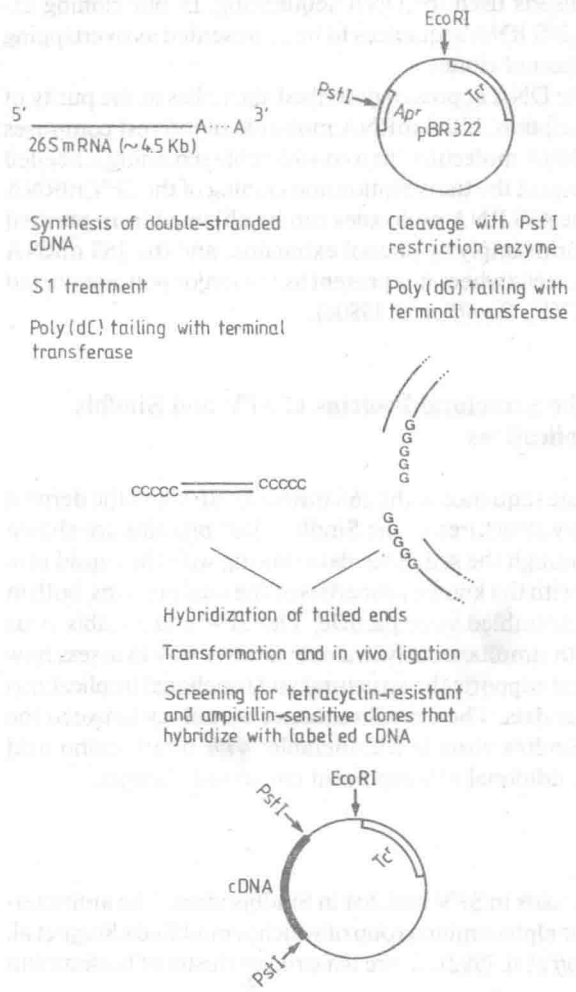


Fig. 5. In vitro transcription of 26S mRNA into cDNA followed by molecular cloning. For explanation see text

the loop of the double-stranded DNA molecule is opened with a single strand-specific nuclease (S1, from *E. coli*). This treatment also makes both ends of the DNA molecule even or "flush" by removing all single-stranded DNA. The DNA molecule is then inserted into a unique restriction endonuclease cleavage site (Pst I) in the *E. coli* plasmid pBR 322 (the vector) using the (dG-dC) tailing procedure described by *Röwekamp* and *Firtel* (1980). In this procedure a poly (dC) stretch is tailed onto the 3′ ends of the cDNA molecule (the insert) and a poly (dG) stretch is added to the 3′ ends of the vector molecule which has been cleaved with the restriction endonuclease Pst I. Insertion of the cDNA into the vector occurs through G-C base pairing when both components are mixed together. The hybrid plasmid molecules are used for transformation of *E. coli* (the host) and bacterial clones containing hybrid plasmids are selected by screening the clones in two ways. Firstly, we take advantage of the tetracycline resistance gene present on pBR 322 but not in the host and simply grow the bacteria on plates containing this antibiotic, and, secondly, we screen the clones by colony hybridization with $^{32}$P-labeled single-stranded cDNA (*Grunstein* and *Hogness* 1975). Positive clones are grown up, the hybrid plasmids are isolated, and their inserts used for DNA sequencing. In our cloning experiments we found the complete 26S RNA sequences to be represented as overlapping DNA segments in three hybrid plasmid clones.

The major problem in using the DNA approach described above lies in the purity of the mRNA used for in vitro transcription. If the mRNA molecule of interest comprises only a small fraction of the total mRNA molecules then considerable screening is needed to find the correct clones. In this respect the transcription and cloning of the SFV mRNA sequences was straightforward; the 42S RNA molecules can be obtained from purified virus particles in an almost pure form simply by phenol extraction, and the 26S mRNA can easily be isolated from infected cells where it is present as the major polyadenylated mRNA species (*Glanville* et al. 1976b; *Garoff* et al. 1980a).

## 4.3 Amino Acid Sequences of the Structural Proteins of SFV and Sindbis Virus and Their Functional Implications

Fig. 6 shows the complete nucleotide sequence of the 26S mRNA of SFV and the derived amino acid sequences. The primary structures of the Sindbis virus proteins are shown below those of SFV. We will go through the sequence data starting with the capsid protein and try to correlate these data with the known properties of the viral proteins, both in the infected host cell and in the assembled virus particle. The SFV and Sindbis virus amino acid sequences are dealt with simultaneously in a comparative way to assess how the extent of sequence conservation supports the structural and functional implications we are deducing from the sequence data. The overall sequence homology between the structural proteins of SFV and Sindbis virus is considerable: 47% of all amino acid residues are homologous and an additional 12% represent conserved changes.

### 4.3.1 Capsid Protein

The capsid protein has 267 amino acids in SFV and 264 in Sindbis virus. The aminoterminal amino acid is methionine, the alpha-amino group of which is modified (*Boege* et al. 1980; *Bonatti* and *Blobel* 1979; *Garoff* et al. 1982). There is a striking cluster of basic amino

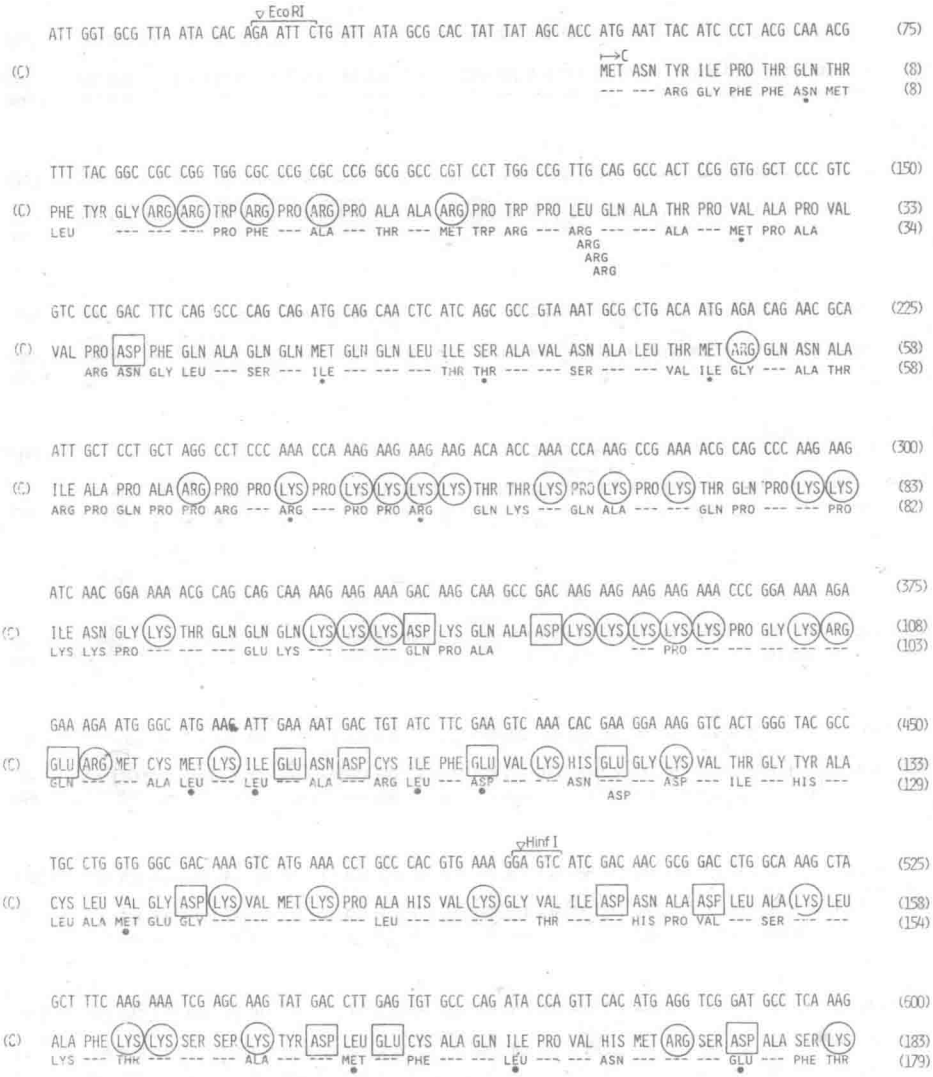


Fig. 6. Nucleotide sequence of the SFV 26S RNA (*top row*), the corresponding amino acid sequence (*middle row*), and the amino acid sequence of the Sindbis virus structural proteins (*bottom row*). Nucleotides are numbered from the 5′ end of the RNA molecule and all amino acids from the aminoterminus of each protein. The amino- and the carboxyterminal ends of each protein are indicated by *arrows*, glycosylation sites are shown by *triangles*, and membrane-spanning regions of the viral glycoproteins are *underlined* for Sindbis virus and *overlined* for SFV. Amino acids in *boxes* are negatively charged (Asp and Glu), and those *circled* are positively charged (Lys and Arg). Some restriction endonuclease cleavage sites are shown on the nucleotide sequence. The alignment of the amino acid sequences of the two alpha-viruses has been made to maximize homology and therefore numerous small deletions (*empty spaces*) and insertions (*amino acids below each other*) are present. A *line* in the position of an amino acid in the Sindbis virus sequences indicates homology with the SFV sequence. A *dot* under an amino acid in the Sindbis virus sequences indicates a conserved change (see text)