MOLECULAR STUDIES OF LOUPING ILL VIRUS

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ABSTRACT

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Molecular Studies of Louping ill Virus

The genomic RNA encoding the structural proteins of louping ill, a tick-borne flavivirus, was cloned and sequenced. Sequence comparisons of louping ill envelope protein showed greater homology with tick-borne than mosquito-borne flaviviruses and greater homology with the western than the far eastern subtype of tick-borne encephalitis virus. Louping ill and tick-borne encephalitis viruses are probably varieties of a common tick-borne ancestral virus. The average amino acid sequence diversity between members of the tick-borne serogroup was significantly lower than that of mosquito-borne serogroups, suggesting that tick-borne flaviviruses have been subjected to different evolutionary immune selection pressure from the mosquito-borne viruses. Using the published model of tick-borne encephalitis envelope protein and the derived sequence data on louping ill virus, three discontinuous peptides (amino acids 81-88, 207-212 and 230-234) which may represent critical molecular determinants within the receptor binding site of tick-borne flaviviruses, were identified. These peptides may provide a specific genetic marker for these viruses.

Recombinant baculoviruses and vaccinia viruses containing cloned DNA, encoding either the envelope protein or the structural proteins of louping ill virus, were constructed. Glycosylated envelope protein, presented both inside and on the surface of insect and mammalian cells, was expressed by all four recombinant viruses. Differences in antigenic presentation of envelope protein were observed between envelope protein and structural protein constructs as well as between insect cell and mammalian cell expression systems. Despite the expression of epitopes known to elicit neutralizing and

protective antibodies when present in authentic antigen, the recombinant envelope protein expressed by either baculovirus or vaccinia virus failed to induce, under the experimental conditions employed, either neutralizing or protective antibodies in both mice and rabbits against louping ill virus. Hence, louping ill envelope protein expressed by baculoviruses and vaccinia viruses was antigenically reactive but immunogenically inert.

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1.1 History of Louping ill, a Tick-Borne Flavivirus

Louping ill (LI) virus is a tick-transmitted arbovirus commonly found in the United Kingdom with a recognized disease association in vertebrates (Reid, 1984). The terms "louping ill" and "trembling", which can be traced back to the 18th century, were used to describe a neurological disease of sheep in Scotland (McFadzean, 1894, 1900). The first demonstration of the transmission of LI disease from sheep to sheep by intracerebral inoculation of infected sheep brain emulsion was made by Pool *et al.* (1930). Subsequently, a laboratory model of LI disease in mice was established by intracerebral inoculation of infectious materials (Alston and Gibson, 1931; Greig *et al.*, 1931). Elford and Galloway (1933) then identified the etiological agent of LI as a filterable virus which could be inactivated by heat (Lepine, 1931), formalin (Gordon *et al.*, 1932), bile salts (Smith, 1939) or ethyl ether (Andrewes and Horstmann, 1949).

Tick-borne transmission of the virus was demonstrated by Macleod and Gordon (1932), who infected mice with LI virus by intracerebral inoculation of a suspension of ticks which had fed on infected sheep. Louping ill virus is transmitted by the three host tick *Ixodes ricinus,* which has a life cycle from egg to adult of at least 3 years, during which only about 3 weeks are spent on vertebrate hosts (Lees and Milne, 1951). Immature ticks will take blood meals indiscriminately from any vertebrate that feeds or nests on the ground whereas adult ticks feed only on larger mammalian species (Arthur, 1963; Milne, 1949). The disease has been reported in sheep (Greig *et al.*, 1931; Doherty and Reid, 1971), goats (Reid *et al.*, 1984; Gray *et al.*, 1988), cattle (Dunn, 1952), pigs (Bannatyne *et al.*, 1980), horses (Fletcher and Galloway, 1937; Timoney, 1980) and dogs (Mackenzie *et al.*, 1973). Man is also susceptible to infection by LI virus with cases reported in laboratory workers (Rivers and Schwentker, 1933; Edward, 1948; Webb *et al.*, 1968), butchers and

sheep handlers (Lawson *et al.*, 1949; Williams and Thorburn, 1962). However, the abundance of sheep farming in the upland grazings of the United Kingdom ensures that sheep are the most common vertebrate host for maintenance of LI virus. Several wild species such as shrew (*Sorex araneus*), wood mouse (*Apodemus sylvaticus*) (Smith *et al.*, 1964), red grouse (*Lagopus scoticus*) (Williams *et al.*, 1963; Reid and Boyce, 1974), red deer (*Cervus elaphus*) (Dunn, 1960; Adam *et al.*, 1977) and roe deer (*Capreolus capreolus*) (Reid *et al.*, 1976) can also be infected. Among the wild species, red grouse, which is capable of developing viraemia sufficient to infect *Ixodes ricinus* ticks (Reid *et al.*, 1980), may act as a temporary amplifying host for LI virus in endemic areas (Reid, 1984).

Louping ill virus belongs to the family Flaviviridae. There are currently 68 registered, antigenically related but distinct, flaviviruses; many of which are important human and veterinary pathogens (Westaway *et al.*, 1985; Porterfield, 1987; Shope, 1980). Based on the arthropod vector of transmission, flaviviruses are grouped into tick-borne and mosquito-borne viruses. Mature flavivirus consists of a 20-30nm icosahedral nucleocapsid surrounded by a lipid envelope that contains membrane (M) and envelope (E) proteins (Heinz, 1986). The membrane protein is non-glycosylated whereas the envelope protein is glycosylated except in the cases of West Nile and Kunjin viruses (Shapiro *et al.*, 1973a,b; Wright *et al.*, 1981; Wright, 1982; Smith and Wright, 1985; Wengler *et al.*, 1985; Coia *et al.*, 1988). In addition, a glycosylated precursor of the membrane protein (prM) is present in immature intracellular virus particles (Shapiro *et al.*, 1972a,b, 1973a,b). These three proteins together with the capsid (C) protein form the structural components of the virus whereas a number of nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) have been identified in infected cells (Rice *et al.*, 1985, 1986a,b). With the help of improved molecular and cell biology

techniques, structure-function studies of viral proteins have increased our understanding of the replication and pathogenicity of flaviviruses.

1.2 Flavivirus Replication: Cellular Uptake of Virus

Virus infection starts with the uptake of the virus by the host cells. The initial step of virus attachment is not clearly defined because no specific receptors for flavivirus have yet been identified. Using electron microscopy, Gollins and Porterfield (1985) established the mode of entry of West Nile virus into the macrophage-like cell line P388D1. Single virus particles were taken up into the cytoplasm in coated pits within 30 seconds of infection at 37°C and appeared, within 2 minutes, in fully or partially coated vesicles. Viral particles in large endocytic vacuoles were soon detected. Although an intraendosomal acid catalyzed fusion process was recently demonstrated for West Nile virus in infection of macrophages (Gollins and Porterfield, 1986; Kimura *et al.*, 1986), further studies are required to define the mechanisms involved in the uncoating and release of viral RNA into the cytoplasm. Viral RNA, once released, is translated in the rough endoplasmic reticulum (Boulton and Westaway, 1976; Westaway and Ng, 1980). Viral envelope protein of Kunjin virus has been detected in cells by immunofluorescence microscopy at 4 hours post-infection (Ng *et al.*, 1983). An early phase of flaviviral RNA synthesis was observed at about 6 hours post-infection with RNA polymerase activity being detected in the cytoplasm (Westaway, 1980; Chu and Westaway, 1985).

1.3 Flavivirus Replication: Viral Proteins Translation and Processing

Once the viral RNA is uncoated, translation of the genome proceeds to generate viral-specific proteins. In recent years, the nucleotide sequences of many flaviviruses have been reported and they show a common architecture of the genome (Castle *et al.*, 1985; Rice *et al.*, 1985; Wengler *et al.*, 1985; Castle *et al.*, 1986; Dalgarno *et al.*, 1986; Deubel *et al.*, 1986; Zhao *et al.*, 1986;

Flavivirus Sequence	References
Louping ill virus	Shiu *et al.* (1991a)
Tick-borne encephalitis virus	Mandl *et al.* (1988, 1989a) Pletnev *et al.* (1990)
Yellow fever virus	Rice *et al.* (1985) Ballinger-Crabtree and Miller (1990)
West Nile virus	Castle *et al.* (1985, 1986) Wengler *et al.* (1985)
Kunjin virus	Coia *et al.* (1988)
Japanese encephalitis virus	Sumiyoshi *et al.* (1987) Hashimoto *et al.*,(1988) Nitayaphan *et al.* (1990)
Murray Valley encephalitis virus	Dalgarno *et al.* (1986)
St Louis encephalitis virus	Trent *et al.* (1987)
Dengue 1 virus	Mason *et al.* (1987a)
Dengue 2 virus	Deubel *et al.* (1986, 1988a) Hahn *et al.* (1988)
Dengue 3 virus	Osatomi and Sumiyoshi (1990)
Dengue 4 virus	Zhao *et al.* (1986) Mackow *et al.* (1987)

Mackow *et al.*, 1987; Mason *et al.*, 1987a; McAda *et al.*, 1987; Sumiyoshi *et al.*, 1987; Trent *et al.*, 1987; Coia *et al.*, 1988; Deubel *et al.*, 1988a; Hahn *et al.*, 1988; Hashimoto *et al.*, 1988; Mandl *et al.*, 1988; Mandl *et al.*, 1989a; Ballinger-Crabtree and Miller, 1990; Nitayaphan *et al.*, 1990; Osatomi and Sumiyoshi, 1990; Pletnev *et al.*, 1990; Shiu *et al.*, 1991a; refer to the reverse of the opposite page for more details). Flaviviruses are positive-sense, single-stranded, type1-capped RNA viruses with all viral proteins encoded within a single open reading frame on the 11 kb genome (Westaway, 1980; Rice *et al.*, 1985). The presence of a long open reading frame in the genome of flaviviruses, the lack of subgenomic mRNA species in infected cells (Boulton and Westaway, 1977) and the detection of proteins larger than NS5 in infected cells (Cleaves, 1985; Ozden and Poirier, 1985; Crawford and Wright, 1987), together suggest that flavivirus proteins are formed by post-translational cleavage of a polyprotein. The N-termini of all the structural and nonstructural proteins of at least one flavivirus have been determined (Boege *et al.*, 1983; Bell *et al.*, 1985; Castle *et al.*, 1985; Wengler *et al.*, 1985; Rice *et al.*, 1986a; Speight *et al.*, 1988; Chambers *et al.*, 1989; Speight and Westaway, 1989). From these sequence data, a general model of translation and co-translational/ post-translational processing of flavivirus proteins was established with the gene order of translation as follows: 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'.

1.3.1 Capsid (C) Protein

Capsid protein has a molecular weight of 13kDa-16kDa as determined by SDS-polyacrylamide gel electrophoresis (Rice *et al.*, 1985). The single open reading frame encoded by genomic RNA starts with capsid protein translation. Alignment of the N-termini of the structural proteins based on nucleotide sequences revealed that capsid protein is enriched in basic amino acid residues which may be necessary for the interaction with negatively charged RNA

during packaging into the nucleocapsid (Sumiyoshi *et al.*, 1987). With the exception of tick-borne encephalitis (TBE) and yellow fever viruses, a comparison of different flavivirus C proteins revealed a conserved sequence box of five amino acids (Asn-Met-Leu-Lys-Arg) (Mandl *et al.*, 1988). The role of this conserved sequence on the function of capsid protein is unknown.

The initiating methionine residue is removed from the capsid protein by cellular aminopeptidase (Rice *et al.*, 1985, 1986b). The putative carboxyl terminus of the protein is composed of a hydrophobic stretch of amino acid residues which form the hydrophobic transmembrane signal sequence for hydrolysis of capsid and premembrane proteins by cellular signalase (Rice *et al.*, 1986b; Markoff, 1989). Endoplasmic signalase liberates the N-terminus of premembrane protein while leaving the capsid protein inserted into membranes. Encapsidation of RNA and envelopment of the completed nucleocapsid by endoplasmic reticulum containing prM and E proteins, with their N-termini oriented into the endoplasmic reticulum lumen, then proceed (Trent *et al.*, 1987). The second cleavage of the capsid protein from the membrane may be effected by the viral protease responsible for the cleavages of the polyprotein precursor to generate the nonstructural proteins. This enzyme recognizes pairs of basic residues followed by a small-side-chain amino acid. Such a sequence is found just N-terminal to the membrane-spanning hydrophobic domain of the capsid protein and is conserved in most flaviviruses (Mandl *et al.*, 1988). Recently, carboxypeptidase sequencing of the virion-associated capsid protein of West Nile virus revealed such cleavage after double basic amino acids (Nowak *et al.*, 1989) in order to detach the carboxyl terminal hydrophobic domain from the virion C protein.

1.3.2 **Premembrane (prM)/ Membrane (M) Proteins**

The membrane protein (M) with molecular weight of 8-8.5 kDa (Rice *et al.*, 1985) is known to be cleaved from a precursor glycoprotein (prM) (Shapiro

et al., 1973a,b). The glycosylated premembrane protein (prM) has a molecular weight about 21 kDa (Rice *et al.*, 1985). The N-glycosylation site (Asn-X-Thr/Ser) (Pless and Lennarz, 1977) of prM protein sequence is conserved among members of the same serological subgroup (Mandl *et al.*, 1988). The cysteine residues of premembrane protein are also conserved among flaviviruses suggesting conservation of the secondary structure of the protein (Nowak and Wengler, 1987).

The N-terminus of prM of several flaviviruses is preceded by the sequence Val-X-Ala. This site conforms to the (-3,-1) rule for a sequence immediately adjacent to the signal peptidase cleavage site (Perlman and Halvorson,1983; von Heijne,1984). However, the prM signal sequences of some flaviviruses do not share this homology. The hydrophobic stretch of amino acids preceding prM serves to initiate the translocation of the polypeptide chain into the lumen of the endoplasmic reticulum with the N-terminus of prM released by cleavage with cellular signal peptidase. On the other hand, cleavage of prM to generate mature protein M is a late event performed by a Golgi protease (Wengler and Wengler, 1989; Randolph *et al.*, 1990) recognizing the consensus sequence Arg-X-Arg/Lys-Arg present in several viral glycoproteins (McCune *et al.*, 1988) and in some hormone precursors (Strauss *et al.*, 1987a,b). The M protein has an internal stop-transfer sequence and a carboxyl terminal signal sequence that anchors the non-glycosylated M protein in the membrane (Rice *et al.*, 1985; Zhao *et al.*, 1986; Coia *et al.*, 1988; Mandl *et al.*, 1988). The M protein is quite hydrophobic and, embedded in the lipid bilayer of the virion envelope, may interact with the hydrophobic carboxyl terminal region of E protein.

1.3.3 **Envelope (E) Protein**

The viral envelope (E) protein, which is antigenically broadly cross-reactive within the family (Porterfield, 1980; Heinz *et al.*, 1983; Stephenson *et*

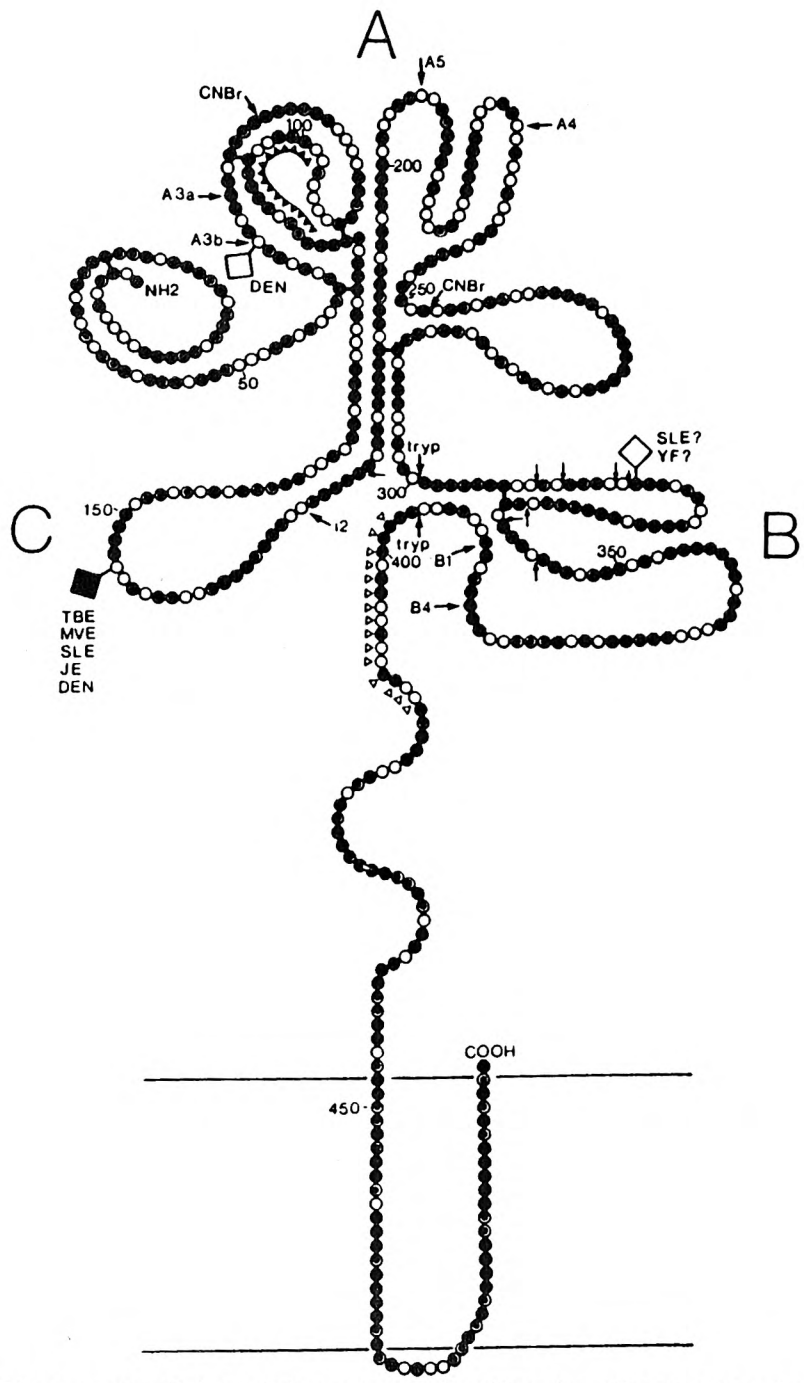
al., 1984; Gould *et al.*, 1985a; Heinz, 1986), has a molecular weight range from 51kDa to 60kDa (Rice *et al.*, 1985). Since both glycosylated and non-glycosylated forms of E protein occur in some flaviviruses (Wright *et al.*, 1981; Wright, 1982; Schlesinger *et al.*, 1983), glycosylation is probably not critical to its fundamental biological functions. This is supported by the observation that complete deglycosylation of tick-borne encephalitis (TBE) virus by endoglycosidase F did not impair infectivity and haemagglutinating activity. Carbohydrate also does not seem to play a major role in the antigenic structure of the TBE virus glycoprotein since the reactivities of both the native and the deglycosylated viruses were identical when analyzed with either monoclonal antibodies or polyclonal antiserum (Winkler *et al.*, 1987). However, these experiments do not exclude the possibility that the formation of certain antigenic determinants may depend on a conformational change induced by the addition of carbohydrate during biosynthesis as has been suggested for other viral envelope proteins (Kaluza *et al.*, 1980; Pierotti *et al.*, 1981; Long *et al.*, 1986).

Similar to the premembrane protein, the envelope protein is preceded by a hydrophobic signal sequence. The N-terminus of the protein is liberated by cleavage with cellular signal peptidase. The carboxyl terminal region of E protein is characterized by two hydrophobic domains: the stop-transfer sequence and signal sequence for nonstructural NS1 protein. The two hydrophobic domains also help to anchor the envelope protein in the lipid bilayer of virion envelope (Rice *et al.*, 1985; Zhao *et al.*, 1986; Coia *et al.*, 1988; Mandl *et al.*, 1988). Alignment of E protein amino acid sequences of different flaviviruses shows that the most conserved region of E protein is located around residue 100 (Mandl *et al.*, 1989b). Two further regions that are significantly conserved in E protein are located at the N-terminus (residues 1 to 42) and close to the carboxyl terminus (residues 410 to 445). Hypervariable regions extend from residues 145 to 170 and from residues 200 to 215. The

primary sequences of E proteins also reveal potential amphipathic α-helix formation by stretches of amino acids from positions 398 to 413 which may represent potential T-cell antigenic determinants (Berzofsky *et al.*, 1987; Mandl *et al.*, 1989b).

The envelope protein represents the viral haemagglutinin which, upon immunization or in the course of natural infection, induces haemagglutination-inhibiting, neutralizing and protective antibodies (Kitano *et al.*, 1974; Della Porta and Westaway, 1977; Trent, 1977; Heinz *et al.*, 1981; Gould *et al.*, 1986; Roehrig, 1986). Hence the structural and functional organization of E protein is important for understanding the biology of flaviviruses and the mechanisms of virus-cell interactions. Based on competitive binding studies of monoclonal antibodies, three non-overlapping antigenic domains (A, B, C) were defined on the envelope protein of TBE virus (Heinz, 1986). The epitopes within each domain vary with respect to their serological specificities and functional activities. Domain A not only contains broadly flavivirus-cross-reactive epitopes but also subtype-specific epitopes. Most of the domain B epitopes are specific for the tick-borne complex of flaviviruses, whereas domain C predominantly contains subtype-specific epitopes (Mandl *et al.*, 1989b). With respect to the induction of neutralizing and haemagglutination-inhibiting antibodies, domain A seems to be the most important since the monoclonal antibodies binding to this domain reveal the highest specific activities. These are lower for domain B antibodies and weakest for domain C antibodies (Guirakhoo *et al.*, 1989).

Sequence comparisons of all flaviviral E proteins showed perfect conservation of 12 cysteine residues which form six disulfide bridges. In addition, they exhibit highly homologous hydrophobicity profiles, suggesting that all members of Flaviviridae share a common architecture of their E proteins. Based on these observations, Nowak and Wengler (1987) proposed that the E protein was made up of three major regions (R1 to R3) separated by



Model of the TBE virus protein E. Open circles represent hydrophilic amino acid residues (Arg, Lys, Asn, Asp, Gln, Glu, His), dotted circles show intermediate amino acid residues (Pro, Tyr, Ser, Trp, Thr, Gly), and solid circles show hydrophobic amino acid residues (Ile, Val, Leu, Phe, Cys, Met, Ala). Amino acids were classified by the scale of Kyte and Doolittle .. Position numbers are shown every 50 amino acids. Cysteine residues forming disulfide bridges are connected by solid lines. Arrows depict cleavage sites that liberate IRF1 (tryp) and IRF3 (CNBr), respectively. Small arrows indicate potential cleavage sites within these fragments that are not utilized. Two solid lines stand for the lipid membrane that is spanned by two transmembrane regions of protein E. The polypeptide chain is folded to indicate the antigenic domains A, B, and C, which are designated by large capital letters. Arrows together with the names of neutralizing MAbs depict the locations of the mutations identified in the respective antigenic variants of TBE virus by sequence analysis. A line of solid triangles indicates the almost perfectly conserved sequence within domain A. A line of open triangles marks the region of a potential T-cell determinant. A solid diamond represents the carbohydrate side chain of TBE virus. The Murray Valley (MVE), St. Louis (SLE) and Japanese (JE) encephalitis viruses and DEN viruses have potential N-glycosylation sites at the homologous position. Yellow fever (YF) and St. Louis encephalitis viruses have such a site within domain B, DEN viruses within domain A. The homologous positions of TBE virus are shown by open diamonds.

two intervening sections (L1 and L2) as well as two transmembrane segments at the carboxyl terminus. The model of Nowak and Wengler also included the locations of potential antigenic sites as deduced from the hydrophobicity profile of the protein. Recently, Mandl *et al.* (1989b) mapped the location of structurally defined antigenic determinants onto the amino acid sequence of envelope protein of TBE virus by analysis of antigenic variants and immunoreactive fragments, and proposed a molecular-epitope model of TBE virus envelope protein (see figure on the reverse of the opposite page), which was a refinement of the original model by Nowak and Wengler (1987).

Domain A of the TBE model is believed to cover amino acids 50 to 125 (containing three disulfide bridges) and also amino acids 200 to 250. The flavivirus-cross-reactive epitopes are mapped onto amino acids 50 to 77 which lie at a protein region maintained by three disulfide bridges. This disulfide-specified region also contains the most conserved sequence (residues 98 to 111) of E protein, suggesting a crucially important function of this sequence element. This highly conserved region may be involved in acid pH-induced membrane fusion because it contains the sequence Gly-Leu-Phe-Gly which also forms the fusion-active N-terminus of the influenza virus HA2 (Wharton *et al.*, 1988). Domain B covers the protein region located between residues 301 and 395. Its structural integrity can be destroyed by opening the disulfide bridge, but antigenic reactivity is fully restored when renaturation of the disulfide bridge is allowed to occur (Winkler *et al.*, 1987). Antigenic domain C is proposed as a single highly variable loop which does not contain any disulfide bridges but carries the single carbohydrate side chain of the TBE virus E protein. Domain C is resistant to SDS denaturation, suggesting that it is not composed of discontinuous regions of the protein like those of domain A. However, this denaturation resistance is lost when the carbohydrate side chain is removed (Guirakhoo *et al.*, 1989), suggesting that glycosylation stabilizes domain C, although domain C epitopes do not directly depend on

the presence of the sugar residues. Subtype-specific monoclonal antibodies directed against domain C epitopes exhibit very little cross-reactivity, suggesting the involvement of hypervariable amino acid residues between positions 145 and 170.

1.3.4 **NS1/NS2A Proteins**

The NS1 glycoprotein sequence is preceded by a stretch of hydrophobic amino acids which acts as a signal sequence for the translocation of NS1 across the endoplasmic reticulum to be cleaved by signal peptidase (Rice *et al.*, 1985, 1986b; Trent *et al.*, 1987; Speight *et al.*, 1988). There is no stop-transfer sequence (Sabatini *et al.*, 1982) or translocation sequence within NS1 before the proposed Val-X-Ala cleavage site before NS2A protein (Coia *et al.*, 1988; Speight *et al.*, 1988; Chambers *et al.*, 1989). The processing and transport of NS1 glycoprotein probably depend on the function of NS2A protein. This stems from the result of recombinant vaccinia expression studies of dengue virus NS1 which showed that the cleavage between the carboxyl terminus of NS1 and the downstream protein NS2A occurred when the complete NS2A was present. It had been suggested that NS2A may be a cis-acting protease that cleaves itself from NS1 or that NS2A may provide sequences for recognition by a specific cellular protease that cleaves at the NS1-NS2A region (Falgout *et al.*, 1989). The importance of NS2A protein in NS1 processing is also reflected in the observation of higher molecular weight polypeptides containing both NS1 and NS2A epitopes (Mason *et al.*, 1987b). This also agrees with the observation of Chambers *et al.* (1990) that production of yellow fever NS1 involves polyproteins of 70 and 64 kDa which presumably represent the entire NS1-NS2A region and a truncated NS1-NS2A polyprotein respectively.

Processing at the carboxyl terminus of NS1 is likely to be a complex event. In addition to a lower molecular weight soluble form (40-46kDa), a higher molecular weight membrane bound form of Japanese encephalitis NS1

(56-60kDa) had also been demonstrated (Mason *et al.*, 1987b). It is not unexpected as multiple carboxyl terminal cleavage sites have been reported in other viral glycoproteins (Chatis and Morrison, 1983; Chen and Huang, 1986). The N-terminus of NS2A follows a signalase cleavage site which lacks an upstream hydrophobic region. A novel protease may be responsible for this cleavage (Speight *et al.*, 1988; Chambers *et al.*, 1989) although delayed cleavage by signalase has not been excluded. Recently, Hori and Lai (1990) have identified an octapeptide sequence at the carboxyl terminus of NS1 which is required for cleavage of the dengue virus NS1-NS2A junction. The processing of different forms of NS1 cannot be resolved until the carboxyl termini of the proteins have been precisely determined. The different forms of NS1 are thought to function in flavivirus assembly and release. In addition to this putative role of NS1, Schlesinger *et al.* (1985, 1986, 1987) have recently demonstrated that vaccination of animals with purified NS1 of dengue virus type 2 or yellow fever virus provides protection against challenge by the respective homologous viruses. Since a small fraction of NS1 is displayed on the surface of infected cells (Cardiff and Lund, 1976; Gould *et al.*, 1985a,1986; Smith and Wright, 1986), this protection is thought to be mediated by cytolysis of infected cells via complement fixing NS1 antibodies.

N-linked glycosylation of NS1 occurs in the lumen of the endoplasmic reticulum. Mandl *et al.* (1989a) described a specific pattern of conservation of the potential N-glycosylation sites of NS1 protein. The perfect conservation of two potential N-glycosylation sites among the NS1 proteins of mosquito-borne flaviviruses and the existence of a third site that is conserved among most members of the Japanese encephalitis virus-subgroup suggest an important role for carbohydrate. One might speculate that the N-glycosylation of NS1 is required for the secretion of virus consistent with the putative role of this protein in virus maturation. On the other hand, the TBE virus NS1 sequence contains one potential N-glycosylation site at a

homologous position and two other unrelated potential N-glycosylation sites as well. The possible structural or functional relevance of these findings remains to be elucidated (Mandl *et al.*, 1989a).

1.3.5 NS2B/NS3/NS4A-NS4B/NS5 Proteins

The molecular weights for the remaining nonstructural proteins are as follows: NS2B(12-15kDa), NS3 (67-76kDa), NS4A(16kDa), NS4B(27kDa) and NS5 (91-98kDa) (Rice *et al.*, 1985; Chambers *et al.*, 1989; Speight and Westaway, 1989). Sequence analysis showed that NS3 and NS5 are highly conserved among flaviviruses (Rice *et al.*, 1985; Castle *et al.*, 1986; Zhao *et al.*, 1986; Sumiyoshi *et al.*, 1987; Coia *et al.*, 1988; Deubel *et al.*, 1988a; Mandl *et al.*, 1989a; Pletnev *et al.*, 1990). Alignment of NS5 with the polymerase genes of various plant and animal RNA viruses (Kamer and Argos, 1984) revealed conservation of the sequence Gly-Asp-Asp (G-D-D), which is thought to be essential for RNA-dependent polymerase activities (Rice *et al.*, 1985; Rice *et al.*, 1986b). Moreover, NS3 also contains sequence motifs characteristic of the D-E-A-D family of RNA helicases (Gorbalenya *et al.*, 1989a). NS3 and NS5, hence, constitute the enzymatic components of the viral replicase.

The cleavage site at the N-terminus for each of NS2B, NS3, NS4A and NS5 includes a pair of basic amino acids (Lys-Arg or Arg-Arg) in the (-2, -1) positions relative to the point of cleavage, followed by a short side chain amino acid (Gly/Ser/Thr/Ala) in the + 1 position. It was proposed that sites of this form are recognized by a virus-encoded protease which is probably located in the cytosol (Rice *et al.*, 1985; Speight *et al.*, 1988; Speight and Westaway, 1989). Based on sequence homology and molecular modelling studies of RNA viruses and known serine proteases, the N-terminal domain of NS3, which is a trypsin-like serine protease (Bazan and Fletterick, 1989; Gorbalenya *et al.*, 1989b), has been suggested to be the candidate enzyme. Preugschat *et al.* (1990) demonstrated that NS3 is indeed the viral protease responsible for cleaving

the NS2A-2B, NS2B-3 and presumably for cleavages generating the termini of NS4A and NS5 as well. Recently, the cleavage site of NS3 of dengue-2 virus was shown to involve only a single basic amino acid (Val-Lys-Lys-Gln-Arg↓Ala) rather than a pair of basic amino acids (Biedrzycka *et al.,* 1987). The enzyme responsible for this monobasic cleavage is also mediated by the same virus-encoded NS3 protease (Rice and Strauss, 1990). Furthermore, the proteolytic activities of NS3 have also recently been found to require the presence of NS2B (Rice and Strauss, 1990).

In contrast, the N-terminus of the hydrophobic NS4B protein immediately follows a predicted signalase cleavage site, suggesting that this protein may be processed in association with microsomal membranes (Speight *et al.*, 1988; Chambers *et al.*, 1989). Processing of the NS2-3-4-5 region is likely to be complex and occurs rapidly. Chambers *et al.* (1990) showed the existence of a precursor-product relationship for processing of NS4A-NS4B to NS4B. NS4A and/or NS4B contain membrane-spanning domains which anchor them in intracellular membranes as well as cytoplasmic domains which may interact with the putative catalytic components of the RNA replicase, NS3 and NS5, to promote assembly of membrane-bound replication complexes (Rice *et al.*, 1985; Grun and Brinton, 1986, 1987).

1.4 Flavivirus Replication: Viral RNA Synthesis

The 5' and 3' ends of the genome are characterized by noncoding sequences. There are significant primary 5' and 3' terminal sequence homologies observed among mosquito-borne flaviviruses (Brinton *et al.,* 1986; Sumiyoshi *et al.,* 1987; Brinton and Dispoto, 1988). Conversely, the 5' and 3' terminal sequences of TBE RNA differ from those reported for terminal genomic structures of mosquito-borne flaviviruses (Pletnev *et al.,* 1990). In particular, the 3' noncoding sequences are much shorter in TBE virus than mosquito-borne flaviviruses (Mandl *et al.,* 1989a; Pletnev *et al.*, 1990). The 3'

terminal untranslated sequence of flaviviruses is non-polyadenylated. It contains one or more oligonucleotide sequences which are repeated (Grange *et al.,* 1985; Takegami *et al.,* 1986) and an extensive stem and loop structure can be formed from the nucleotide sequence at the 3' terminus (Wengler and Castle, 1986). Interestingly, the complement of the 5' terminal sequence [equivalent to the 3' terminal sequence of the (-) strand] is related to the 3' terminal sequence of the (+) strand of yellow fever, West Nile and dengue viruses (Rice *et al.,* 1985; Zhao *et al.,* 1986). Similar to the 3' terminal sequences, a potential secondary structure can be formed by the 5'-noncoding sequence and the 5' half of the capsid (C) protein coding sequence (Pletnev *et al.,* 1990). In view of these characteristic primary and secondary structures of the 5' and 3' terminal sequences, both ends of RNA may play important roles in forming the recognition sites for the viral RNA dependent-RNA polymerase though the composition, form and structure of the polymerase complex remains obscure. In addition, Rice *et al.* (1985) proposed that binding of the capsid protein to the stable 3' terminus structure on the positive (+) strand RNA could prevent replication of minus (-) strand RNA and initiate encapsidation.

Studies of viral RNA replication had shown the existence of single-stranded 44S RNA, double-stranded 20S RNA and 26S RNA in infected cells (Stollar *et al.,* 1967; Westaway, 1980). The 20S RNA, which contains full length strands of 44S RNA of plus and minus polarities, probably acts as a replicative form (RF) whereas the 26S RNA has properties compatible with those of a replicative intermediate (RI) (Cleaves *et al.,* 1981). By comparing the total incorporation of [^{3}H] uridine in 44S RNA, 20S RNA (RF) and 26SRNA (RI), it was deduced that RF of Kunjin virus functions as a recycling template for 44S RNA synthesis. From these results, Chu and Westaway (1985) concluded that initiation of semiconservative replication converts the RF to an RI on which

only one nascent strand of RNA (44S RNA) is asymmetrically replicated per cycle.

1.5 Flavivirus Replication: Virus Maturation and Release

Electron micrographs of infected cells showed proliferation of both smooth and rough endoplasmic reticulum with accumulation of virions within their cisternae by 8-12 hours after infection. The hypertrophied membranes originate in the juxtanuclear region and form smooth membrane structures (SMS), convoluted masses of smooth membranes, lamellae of rough endoplasmic reticulum and distended cisternae bounded by smooth or rough membranes in which virions accumulate. These organelles are often adjacent to each other and interconnected (Murphy, 1980; Westaway, 1980). Leary and Blair (1980) proposed that SMS are formed by budding of envelope protein containing endoplasmic reticulum to enclose the capsid protein and viral RNA genome from the cytoplasm. The immature virions then begin condensation and are transported to the Golgi apparatus where viral maturation takes place. Intracellular virions have been observed to be released from infected cells by at least two routes. The lamellae of smooth membranes in Kunjin-infected cells have been found to open directly to the exterior (Westaway, 1980). Virus-containing vesicles have also been observed to move to the periphery of the cytoplasm and fuse with the plasma membrane (Filshie and Rehacek, 1968). Nevertheless, the different stages of flavivirus morphogenesis are still largely unknown. The role of nonstructural proteins such as NS1 in virus assembly, maturation and release remains to be elucidated.

1.6 Viral Pathogenicity and Immunity

1.6.1 Protective Immunity

Envelope protein is responsible for the induction of haemagglutination-inhibiting, neutralizing and protective antibodies (Kitano *et al.*, 1974; Della

Porta and Westaway, 1977; Trent, 1977; Heinz *et al.*, 1981; Gould *et al.*, 1986; Roehrig, 1986). Extensive antigenic cross-reactivties among flaviviruses had been demonstrated by antibody absorption haemagglutination-inhibition analysis (Clarke, 1960), cross-neutralization tests (De Madrid and Porterfield, 1974) and cross-immunofluorescence microscopy (Gould *et al.*, 1985a). Cross-protection was also observed in mouse challenge experiments and the extent of protection depended on the relatedness of the viruses (Hammon and Sather, 1956; Tarr and Hammon, 1974).

Passive protection against flaviviruses could be conferred on animals by administering E-specific monoclonal antibodies (MAbs) (Heinz *et al.*, 1983; Matthews and Roehrig, 1984; Buckley and Gould, 1985; Brandriss *et al.*, 1986; Gould *et al.*, 1986; Cecilia *et al.*, 1988). Although protection *in vivo* is probably mediated directly via neutralization of virus infectivity in some cases, antibodies that show neutralization *in vitro* may or may not show protection against challenge virus *in vivo*. Furthermore, non-neutralizing E-specific antibodies may show protection via complement-dependent lysis of virus-infected cells (Gould *et al.*, 1990). In addition to E-specific MAbs, antibodies against dengue prM also protect mice against challenge with dengue viruses when administered passively. Some prM-specific MAbs also show low titres of virus neutralization (Kaufman *et al.*, 1989). The mechanisms of protection and neutralization are not clear but steric hindrance of E protein epitopes by added prM antibodies and /or antibody mediated cytolytic activity may explain the functional activities observed with prM antibodies (Gould *et al.*, 1990).

On the other hand, infection by flaviviruses also stimulates production of antibodies against intracellular nonstructural viral proteins. Monoclonal antibodies against 17D yellow fever NS1 protein had been shown to protect mice against lethal 17D yellow fever encephalitis (Schlesinger *et al.*, 1985; Gould *et al.*, 1986). The mechanism of protection is believed to be due to

antibody mediated cytolysis of infected cells displaying NS1 on the cell surface (Cardiff and Lund, 1976; Gould *et al.*, 1985a, 1986; Smith and Wright, 1986). Besides NS1 antibodies, passively administered NS3 MAbs had also been demonstrated to increase the survival time of mice challenged with a lethal dose of dengue virus (Tan *et al.*, 1990). Although the mechanism of protection by NS3 MAbs is unknown, the information adds to the importance of viral nonstructural proteins in the induction of protective immune responses in infected hosts.

1.6.2 Antibody-Dependent Enhancement (ADE)

In addition to neutralization of virus infectivity *in vitro*, E-specific antibodies may also enhance the susceptibility of cells to infection of viruses under certain conditions. This phenomenon is known as antibody-dependent enhancement (ADE) of virus infectivity (Halstead *et al.*, 1973; Porterfield, 1986). In the presence of sub-neutralizing antibodies, ADE of flavivirus replication had been demonstrated *in vitro* in peripheral human blood cells of the monocyte-macrophage lineage as well as in murine and human macrophage-like cell lines (Halstead and O' Rourke, 1977; Peiris and Porterfield, 1979; Schlesinger and Brandriss, 1981; Phillpotts *et al.*, 1985). Enhanced virus infectivity is due to increased binding of the antibody-virus complexes to the Fc and /or complement receptors on the cell surface and increased internalization of the ligand-receptor complexes into the cells (Cardosa *et al.*, 1983; Gollins and Porterfield, 1984).

There is some evidence that an equivalent immune-mediated phenomenon exists *in vivo* with flaviviruses. ADE had been proposed to be an important factor in the development of dengue haemorrhagic fever / dengue shock syndrome in the course of sequential dengue infections or in primary infections of children with passively acquired maternal antibodies (Halstead, 1981). Although there are no reports of a similar situation in nature with

flaviviruses other than the dengue serotypes, *in vivo* immune enhancement of yellow fever and Japanese encephalitis virus neurovirulence for mice was recently demonstrated (Gould *et al.*, 1987; Gould and Buckley, 1989). However, there was no analogy between ADE mechanisms *in vitro* and increased yellow fever neurovirulence *in vivo* after parenteral administration of antibody (Gould *et al.*, 1987). In view of the above evidence on enhancement of flavivirus infectivity by antibody, further investigations on the role played by the host immune status in the pathogenicity of flaviviruses are warranted, particularly in the design of safe and effective new generation vaccines against flavivirus pathogens.

1.6.3 Molecular Basis of Flavivirus Virulence

Nucleotide sequence comparisons of parent (wild type) and attenuated (vaccine) yellow fever (Hahn *et al.*, 1987) and Japanese encephalitis (JE) viruses (Nitayaphan *et al.*, 1990) have shown genetic changes associated with attenuation of virulence. Many of the nucleotide changes resulting in amino acid substitutions were located in the envelope protein genes. Recently, Holzmann *et al.* (1990) and Cecilia and Gould (1991) reported single amino acid substitutions in TBE and JE envelope proteins which led to reduced neurovirulence of neutralization-resistant "escape" mutants for mice. Nevertheless, there is no association between mouse virulence and envelope protein sequences from different strains of Murray Valley encephalitis virus (Lobigs *et al.*, 1988). Sequence analysis of dengue virus envelope protein from strains isolated from mild and severe cases of disease also revealed no correlation between amino acid changes and disease severity (Blok *et al.*, 1989). Therefore, the severity of disease is probably determined by factors other than the envelope protein gene alone. It is likely that genetic changes occurring outside the envelope gene can also alter flavivirus virulence by affecting different stages of flavivirus replication. Apart from viral factors,

host-specific factors such as age and immune status also play important roles in determining the severity of disease caused by flaviviruses, particularly dengue haemorrhagic fever and dengue shock syndrome (Halstead, 1981; Burke *et al.*, 1988; Kliks *et al.*, 1988). Furthermore, since flaviviruses are transmitted by arthropod vectors, the prevalence of mosquitoes or ticks in endemic areas will also determine the risk and level of exposure of hosts to the virulent viruses. In conclusion, flavivirus virulence is multifactorial and depends on a variety of viral, vector and host factors.

1.7 Aims of the Thesis

Apart from TBE virus, molecular studies of other tick-borne flaviviruses are limited as compared with mosquito-borne flaviviruses. In contrast to more widespread distribution of TBE virus, LI virus apparently occupies a unique ecological niche centred on the United Kingdom. The first objective of the project is to clone and sequence the LI virus genome encoding the structural proteins in order to facilitate analysis of the evolutionary relationships between tick-borne flaviviruses, particularly between LI and TBE viruses. The structural protein cDNA clones will then be used to develop recombinant virus expression systems for studying the structure-function relationships of E protein. The second objective of the project is to use these recombinant viruses in experiments designed to analyze the antigenic and immunogenic properties of recombinant LI envelope protein. It is hoped that the information obtained will lead to a more detailed understanding of the properties required for successful immunization against virulent flavivirus infection.

CHAPTER 2

MATERIALS AND METHODS

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2.1 Chemicals

Chemicals and solvents were purchased from Aldrich Chemical Company UK, Sigma Chemical Company UK, British Drug House (BDH) UK, Pharmacia Fine Chemicals and Gibco Ltd. UK. Solutions were sterilized where necessary either by autoclaving or by filter sterilization.

2.2 Radiochemicals

The following radiochemicals were purchased from Amersham International plc:

[α -^{32}P]dATP [6000Ci/mmol; 10μCi/μl]

[γ -^{32}P]ATP [5000Ci/mmol; 10μCi/μl]

[α -^{35}S]dATP [600Ci/mmol; 10μCi/μl]

[^{35}S]methionine [1200-1350Ci/mmol; 10-15μCi/μl]

2.3 Enzymes

Restriction endonucleases, T4 DNA ligase and calf intestinal alkaline phosphatase (CIP) were purchased from Boehringer Mannheim Ltd. *Taq* DNA polymerase was supplied by Promega Corporation. All other enzymes were obtained from Amersham International plc.

2.4 Cells and Growth Media

Continuous lines of porcine kidney (PS) cells, CV-1 TK$^+$ (thymidine kinase$^+$) cells, CV-1 TK$^-$ (thymidine kinase$^-$) cells and *Spodoptera frugiperda* (Sf) cells were used throughout the study. PS cells were routinely grown as monolayers at 37°C and subcultured at intervals of 5 days in Leibovitz L-15 medium containing 5% (v/v) fetal calf serum (FCS), 10% (v/v) tryptose phosphate broth, 100U/ml of penicillin and 100μg/ml of streptomycin. CV-1 (TK$^+$ and TK$^-$) cells were routinely grown as monolayers at 37°C in 5% CO_2 and subcultured at

intervals of 4 days in Eagle's minimum essential medium (EMEM) containing 5% (v/v) FCS, 100U/ ml of penicillin and 100 μg/ ml of streptomycin. CV-1 TK^- cells were grown in EMEM medium as above containing 25μg/ ml of 5-bromo-2'-deoxyuridine for at least 3 days before selection of recombinant vaccinia virus. Maintenance media contained 2.5% FCS instead of 5% FCS. Sf cells were routinely grown as monolayers at 28°C and subcultured at intervals of 3-4 days in TC 100 medium containing 10% (v/v) fetal calf serum and 100U/ ml kanamycin. Sf cells were grown in suspension culture before infection with baculoviruses.

2.5 Viruses

Louping ill (LI) virus (strain 369/T2) and a strain of tick-borne encephalitis (TBE) virus from Central Europe were kindly supplied as freeze-dried preparations from Dr. Leake and Professor Varma (London School of Hygiene and Tropical Medicine, UK). The preparations were inoculated intracerebrally into two litters of newborn TO mice. When the mice were sick, 10% (w/v) infected brain suspensions were prepared in phosphate-buffered saline (PBS) pH 7.4, clarified by centrifugation (2000xg, 10 min) and stored as aliquots at -70°C. Wild type baculovirus [*Autographa californica* nuclear polyhedrosis virus (AcNPV)] and recombinant baculovirus stocks were grown and assayed on confluent monolayers of Sf cells in TC100 medium (Brown and Faulkner, 1977). Wild type vaccinia (strain WR) and recombinant vaccinia virus stocks were grown on CV-1 TK^+ cells in EMEM medium.

2.6 Mice and Rabbits

Random bred (strain TO) white mice 3 to 8 week old were purchased from A. Tuck and Son, Battlesbridge, Essex, UK. Dutch rabbits (2 kg body weight) were supplied by the Ophthalmology Department of Oxford University. Three

week old PO white mice were obtained from the NERC Institute of Virology and Environmental Microbiology.

2.7 Plaque Purification of Louping ill Virus

Louping ill virus (strain 369/T2) was used as a 10% (w/v) suspension of infected suckling mouse brain. The virus was grown in PS cells at a multiplicity of infection (m.o.i.) of 0.01 and the infected supernatant medium harvested 3 days post-infection (pi). The virus was titrated on PS cells and plaque picked under carboxymethylcellulose (CMC) overlay (Gould and Clegg, 1987). Plaque-purified LI virus was propagated once in suckling TO mouse brain to generate virus stocks for subsequent molecular studies.

2.8 Growth and Purification of Louping ill Virus

PS cells were infected at low m.o.i. (0.01-0.1) with the virus stock and the infected supernatant medium was collected from 3 to 5 days pi. Virions were precipitated from infected cell culture fluid with polyethylene glycol (PEG, 70g/l) and sodium chloride (23g/l). The PEG precipitate was resuspended in TNE buffer (10mM Tris-HCl pH 7.4, 100mM NaCl, 1mM EDTA) and the virus was purified by centrifugation through a 30%-50% (w/w) sucrose step gradient (28000rpm, SW28 rotor, 3h). For protein analyses, the interface was harvested and the virus pelleted by ultracentrifugation (38000 rpm, SW41 rotor, 2h) after diluted fourfold with TNE buffer. The virus pellet was then resuspended in radioimmune precipitation assay (RIPA) buffer [1% Triton X-100, 1% (w/v) sodium deoxycholate, 150mM NaCl, 50mM Tris-HCl pH 7.4, 10 mM EDTA and 1% (w/v)SDS] and stored at -70°C. For RNA extraction, the virus was harvested from the interface of a sucrose step gradient, diluted twofold with TNE buffer and then purified again by equilibrium centrifugation through a linear 30%-50% (w/w) sucrose gradient (22000rpm, SW28 rotor, 12-16h). The virus band

was collected and pelleted by ultracentrifugation (38000 rpm, SW41 rotor, 2h).

2.9 Nucleic Acid Manipulations

All enzymatic manipulations of RNA and DNA followed standard protocols as detailed by Sambrook *et al.* (1989) unless otherwise stated.

2.9.1 RNA Extraction and cDNA Synthesis

The purified virus pellet was resuspended in TNE buffer containing 1% SDS. Viral RNA was ethanol precipitated after two phenol and one chloroform extractions. The integrity of viral RNA was confirmed by denaturing 5 mM methylmercury (II) hydroxide gel electrophoresis (Fig. 1). First strand cDNA was obtained by reverse transcription of 0.5 μg viral RNA using random primers. The second DNA strand was synthesized by the RNaseH/ DNA polymerase I method essentially as described by Gubler and Hoffman (1983). cDNAs were also synthesized from LI RNA with LI-specific primer (after some sequence data had been obtained), TBE-E and NS1-specific primers determined from the sequence of TBE western subtype (Mandl *et al.*, 1988, 1989a) [LI-specific primer: 5'TTTGCTTCACAAGCCGC3' (nucleotides 1225-1241); TBE-E-specific primer: 5'GCCTTCCCAATTGAACT3' (nucleotides 2243-2259); TBE-NS1-specific primer: 5'CCTGTGTCATTTTTCAT3' (nucleotides 3053-3069)].

2.9.2 Construction of LI cDNA-Bacteriophage Libraries

The cDNAs were cloned into λgt10 using a λgt10 cloning system marketed by Amersham International plc following the manufacturer's recommended protocols. The cDNAs were methylated by *Eco*RI methylase and *Eco*RI linkers were then ligated onto both ends of the cDNAs. Sticky *Eco*RI ends on each terminus of the cDNA were produced by *Eco*RI digestion. Before the cDNAs were ligated with dephosphorylated *Eco*RI-digested λgt10 vector arms, the

excess linker molecules were removed using prepacked columns. Linear concatameric molecules formed in the ligation reaction were packaged *in vitro* to produce infectious phage. These phage were then grown on the selective high frequency lysogeny (hfl^+) *E. coli* NM514 host which allowed recombinants to grow.

2.9.3 Screening of Recombinant Bacteriophage Plaques

Positive λ plaques were identified by filter hybridization with ^{32}P nick-translated TBE envelope protein cDNA probe [nucleotides 1055-1555, Mandl *et al.* (1988)] kindly provided by Dr. Kunz, Dr. Heinz and Dr. Mandl (Institute of Virology, Vienna).

2.9.4 Polymerase Chain Reaction (PCR)

The 5' coding sequence of the open reading frame was obtained by PCR reaction. First strand cDNA was synthesized from 0.1 µg of viral RNA with LI-specific primer [5' CTCGTATGACAGCGAGTCAT 3' (nucleotides 506-525)]. The RNA-cDNA product was then ethanol precipitated with 5M ammonium acetate and PCR performed with upstream *Hind*III-TBE primer [5' CTCAAGCTTTCGTGAACGTGTTGAGAA 3' (nucleotides 65-82)] and downstream *Bam*HI-LI primer [5' AACGGATCCAGCACTCACTGTCCTCTT 3' (nucleotides 265-282)]. The PCR reaction mixture consisted of 50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 1.5mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100, 250 mM of each dNTP, 50 pmole of each upstream and downstream primer with 2.5 units of *Taq* DNA polymerase (Promega) in a final volume of 100 µl. This mixture was overlaid with 70 µl of paraffin oil and heated to 95°C for 3 min in the first amplication cycle. This was followed by 30 cycles of 1.5 min at 95°C, 1 min at 50°C and 2 min at 72°C. The last cycle was ended with 5 min at 72°C.

2.9.5 Sequence Determination and Computer Analysis

Positive λ clones were subcloned into dephosphorylated *Eco*RI-digested pUC 118 sequencing vector in opposite orientations and the recombinant plasmids transfected into competent *E. coli* XL1-Blue cells. Recombinant plasmids with inserts in opposite orientations were digested with *Xba*I and *Sph*I. Deletion mutants were generated using exonuclease III. Single-stranded DNA templates were produced from overlapping deletion mutants by addition of M13KO7 helper phage (m.o.i. 10). All overlapping clones were sequenced in both directions using the enzymatic dideoxy-sequencing method (Sanger *et al.*, 1977). Similarly, the PCR-product was subcloned into *Bam*HI and *Hind*III-digested pUC118 and pUC119 plasmid vectors which were then sequenced with universal M13 primer. Sequence data were analyzed using the programmes of Staden (1986).

2.9.6 Construction of LI E-Protein DNA Clone and Recombinant Transfer Vectors

Polymerase chain reaction (20 cycles) was used to amplify the segment of DNA (LIE) coding for envelope protein with preceding hydrophobic sequence and ATG start codon (nucleotides 781-2358) from plasmid pUC118 containing cloned LI structural protein cDNA under similar conditions to those as described previously in section 2.9.4. Both the upstream and downstream primers had built-in *Bgl*II restriction enzyme sites to facilitate subcloning into the dephosporylated *Bam*HI-site of pAcYM1 transfer vector (Matsuura *et al.*, 1987) for the baculovirus system (Fig. 2) and the *Bgl*II-site of pSC11-*Bgl*II transfer vector for the vaccinia system (Fig. 3). The upstream primer (5'CTTAGATCTAATATGGTCGCCGTTGTGTCCCTA3') contained the translation start codon whereas the downstream primer (5'TGCAGATCTCTACTACGCTCCCACCCCGAGAGT3') had termination codons

introduced. Recombinant transfer vectors in the sense orientation were selected by restriction mapping.

The transfer plasmid pSC11-*Bgl*II was kindly provided by Dr. J. C. S. Clegg (PHLS Centre for Applied Microbiology & Research, Salisbury, UK). It was derived from pSC11 (Chakrabati *et al.*, 1985) by changing the unique *Sma*I site to a *Bgl*II site as described by Lathe *et al.* (1984) to facilitate insertion of the exogenous gene. The plasmid pSC11 was linearized with *Sma*I and ligated in the presence of a large excess of a 5'-non-phosphorylated synthetic linker containing a *Bgl*II site (HO-5'-CGCAGATCTGCG-3'-OH) which had been previously self-annealed. Plasmid DNA was freed of non-covalently attached linkers using GeneClean (Bio 101 Inc.) and redigested with *Sma*I to remove any remaining unmodified closed circular DNA. The modified termini were annealed by slow cooling from 70°C and a 100ng sample was used to transform competent *E. coli* HB101. Plasmids prepared from the resulting colonies were characterized by restriction enzyme analysis and sequencing to confirm the expected structure.

2.9.7 Construction of LI Structural Proteins-DNA Clone and Recombinant Transfer Vectors

The DNA clone (LIS) encoding LI structural proteins C, prM/M and E (nucleotides 1-2700) was constructed from clones SH12 and SH14 as shown in Fig. 4. The DNA clone SH12, which contains LI nucleotide sequence 1-282, was derived from purified LI viral RNA by PCR reaction as described under section 2.9.4. The DNA clone SH14, which contains LI nucleotide sequence 219-2700, is a deletion mutant generated by exonuclease III treatment of LI cDNA clone SH10 (nucleotides 219-2970). The DNA clone (LIS) was then subcloned into the filled in-*Bam*HI site of pAcYM1 transfer vector (Fig. 5) and the *Sma*I site of pSC11 transfer vector (Fig. 6). Recombinant shuttle vectors in the sense orientation were selected by restriction mapping.

2.10 Preparation of Infectious AcNPV DNA

Suspension culture of Sf cells (200ml, $1.5x10^6$ cells/ml) were infected with wild type baculovirus (m.o.i. 0.1) and grown at 28°C for 4 days. The cells were removed from infected cell culture medium by centrifugation at 3000 rpm for 10 min. Virus particles were then pelleted from the cell culture medium (25000 rpm, SW28 rotor, 2h). The virus pellets were resuspended in 1 ml of TE buffer (10mM Tris-HCl pH 7.4 and 1mM EDTA) and layered onto a 10%-50% (w/w) sucrose step gradient. After centrifugation (25000 rpm, SW28 rotor, 1h), the virus band was harvested from the interface, diluted in TE buffer and pelleted (25000 rpm, SW28 rotor, 2h). The purified virus was resuspended in 800 µl TE buffer and incubated at 60°C for 10 min with 200µl of lysis buffer [10% (w/v) sodium N-lauroylsarcosinate and 10mM EDTA]. The viral DNA was then purified by CsCl gradient centrifugation (45000rpm, Ti50 rotor, 16h, 20°C). Ethidium bromide and CsCl were subsequently removed from the purified DNA by butanol extraction and dialysis against TE buffer respectively. The purified AcNPV DNA was stored at 4°C and remained infectious for at least 3 months.

2.11 Transfection and Selection of Recombinant Baculoviruses

Sf cells (10^6) were incubated with 1 ml of FCS-free TC100 medium just before transfection. A transfection mixture (24µl) containing 200ng purified infectious AcNPV DNA, 2 µg recombinant transfer vector DNA and 8 µl of Lipofectin™ (BRL) was added to Sf cells incubated at 28°C. Five hours later, the supernatant medium was replaced with fresh TC 100 medium containing 10% FCS. The medium was harvested on the second day pi and titrated on confluent monolayers of Sf cells (Matsuura *et al.*, 1986, 1987). Plaques exhibiting no evidence of occlusion bodies, as determined by transmission light microscopy, were recovered and retitrated on Sf cells to obtain

recombinant polyhedrin-negative virus. Following a third plaque picking, high titre recombinant virus stock [10^7-10^8 plaque forming units (pfu)/ml] was produced for protein analyses.

2.12 Transfection and Selection of Recombinant Vaccinia Viruses

CV-1 TK$^+$ ($2x10^6$) cells were infected with wild type vaccinia virus (strain WR) at m.o.i. 0.01-0.05. Two hours pi, the cells were incubated with 2 ml of FCS-free EMEM. A transfection mixture (48µl) containing 2 µg recombinant transfer vector DNA and 16 µl of Lipofectin™ (BRL) was added to CV-1 TK$^+$ cells incubated at 37°C. Two hours later, the supernatant medium was replaced with fresh EMEM medium containing 5% FCS. The medium was changed 24h pi and the monolayer harvested at 48h pi. The infected cells were frozen and thawed 3 times to release the virus which was then titrated on confluent monolayers of CV-1 TK$^-$ cells which had been grown in EMEM containing 5% FCS and 25µg/ml of 5-bromo-2'-deoxyuridine (BUdR) for at least 3 days. After 1 hour adsorption, the virus inoculum was removed and 0.8% agarose containing EMEM, 2.5% FCS and 25 µg/ml BUdR was added. Two days pi, agarose containing 200µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was overlaid onto the plaques. Recombinant blue plaques, which developed after overnight incubation at 37°C, were picked up. The recombinant virus was plaque purified 3 times in selective growth medium before generation of high titre stock (10^7-10^8 pfu/ml) for protein study.

2.13 SDS-Polyacrylamide Gel Electrophoresis and Coomassie Blue Staining of Recombinant Protein

Sf cells, infected with wild type or recombinant baculoviruses (m.o.i. 10), were lysed 72h pi with RIPA (as defined on page 27 section 2.8) buffer. Uninfected and infected Sf cell lysates were boiled with equal volume of 2X

reducing sample buffer [4.6% (w/v) SDS, 0.125M Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 0.001% bomophenol blue and 20% glycerol] for 5 minutes. Aliquots representing 10^5 cells were then electrophoresed on 10% or 12% SDS-polyacrylamide gel (Laemmli, 1970). The protein gel was stained with Coomassie Blue solution (50% methanol, 0.05% Coomassie Brilliant Blue, 10% acetic acid and 40% water) and then immersed in destaining solution (10% methanol, 10% acetic acid and 80% water).

2.14 Western Blot Analysis of Recombinant Protein

Expression of the envelope protein was examined by immunoblotting of Sf cell lysate infected with recombinant baculovirus (m.o.i. 10) and CV-1 cell lysate infected with recombinant vaccinia virus (m.o.i. 10) using LI E-specific MAb 4.2, whereas structural protein expression was examined using rabbit polyclonal antiserum against LI virus. PS cell lysate infected with LI virus or purified LI virus served as the positive control. Purified LI virus for protein analyses was prepared as previously described under section 2.8. PS cell lysate infected with LI virus (m.o.i. 0.5) was harvested 3 days pi in RIPA buffer. Sf cell lysate infected with recombinant or wild type baculovirus was harvested 72h pi while CV-1 cell lysate, infected with recombinant or wild type vaccinia virus, was harvested 24h pi. Proteins were resolved on 10% or 12% SDS-polyacrylamide gels (Laemmli, 1970) with or without the use of reducing agents (Cane and Gould, 1989) and subsequently electroblotted onto nitrocellulose paper. The filter paper was blocked with 5% dried milk powder in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 30 min at room temperature. The blot was then incubated overnight at 4°C with either diluted MAb 4.2 (1:500) or polyclonal antiserum (1:200) in PBS containing 5% milk powder and 0.05% Tween 20. The filter was subsequently washed with PBS-0.05% Tween 20 and incubated for 90 min at room temperature with diluted (1:3000) affinity-purified anti-mouse Ig or anti-rabbit Ig linked to

horse-radish peroxidase (Bio-Rad). The blot was developed using diaminobenzidine and hydrogen peroxide following extensive washing with PBS-0.05% Tween 20.

2.15 Immunoprecipitation of Radiolabelled Envelope Protein With or Without Tunicamycin

Labelled cell lysates were prepared from PS cells, Sf cells and CV-1 cells infected with LI virus (m.o.i. 5), recombinant baculovirus (m.o.i. 10) and vaccinia virus (m.o.i. 10) respectively. Infected PS cells were incubated at 37°C with Leibovitz L15 medium containing 5% FCS. Actinomycin D (5µg/ml) with or without tunicamycin (10µg/ml) was added to the growth medium 24h pi until the time of harvesting the cells with RIPA buffer. At 30h pi, PS cells were incubated with methionine-free and FCS-free medium for 2h and then labelled with ^{35}S-methionine (100µCi/ml) for 4h. Infected Sf cells were incubated at 28°C with TC100 medium containing 10% FCS. Tunicamycin (10µg/ml) was added to the growth medium 18h pi until the time of harvesting the cells with RIPA buffer. At 23h pi, the infected Sf cells were incubated with methionine-free and FCS-free medium for 1h and then labelled with ^{35}S-methionine (30µCi/ml) for 1 (envelope gene construct) or 3 hours (structural gene construct). Infected CV-1 cells were incubated at 37°C with EMEM containing 5% FCS in 5% CO_2. Tunicamycin (10µg/ml) was added to the growth medium 2h pi until the time of harvesting the cells with RIPA buffer. At 4h pi, CV-1 cells were incubated with methionine-free and FCS-free medium for 2h and then labelled with ^{35}S-methionine (100µCi/ml) for 2h. Immune complexes formed between MAb 4.2 and labelled antigens were precipitated using Staphylococcus protein A-Sepharose (Sigma) and washed. Immunoprecipitated proteins were resolved by 10% SDS-polyacrylamide gel (Laemmli, 1970) and visualized by autoradiography.

2.16 Indirect Immunofluorescence Tests of Authentic and Recombinant Envelope Proteins

The antigenic properties of authentic and recombinant LI envelope protein expressed by baculoviruses and vaccinia viruses were compared by indirect immunofluorescence microscopy using a panel of E-specific monoclonal antibodies (MAbs), some of which were kindly provided by Dr. H. W. Reid (Moredun Research Institute, Edinburgh, UK). PS cells, Sf cells and CV-1 cells infected respectively with LI, recombinant baculoviruses and vaccinia viruses were grown on coverslips. Infected Sf cells (m.o.i. 5) and CV-1 cells (m.o.i. 5) were acetone-fixed 24h pi whereas infected PS cells (m.o.i. 0.01) were fixed 48h pi. The coverslips were incubated with MAbs diluted in PBS at 37°C for 40 min. The cells were washed with PBS and incubated with diluted sheep anti-mouse Ig-biotin (1:75 in PBS, Amersham International) at 37°C for 40 min. The cells were washed again with PBS and incubated with diluted fluorescein-streptavidin (1:400 in PBS, Amersham International) at 37°C for 5 min. The coverslips were washed with PBS before mounting in glycerol saline (pH 8.6) containing 1,4-diazabicyclo (2,2,2) octane. In order to localize the expressed E protein on the surface of Sf cells and CV-1 cells, immunofluorescence tests were also performed with non-fixed infected cells (Gould *et al.*, 1985a,b).

2.17 Mice Immunization and Tick-Borne Flavivirus Challenge

2.17.1 Immunization of Mice Against Baculovirus Infected Cell Lysate

Three week old TO mice were each immunized against lysate from 10^6 Sf cells infected with either wild type baculovirus (Baculovirus-W) or recombinant baculovirus containing LI envelope (E) gene (Baculovirus-E). The lysate was injected either with or without adjuvant intramuscularly on day 1, 3, 14, 21 and 28. The mice were bled one week after the last immunization and the pooled antisera were tested for the presence of antibodies against LI virus using western blot and *in vitro* plaque reduction neutralization tests.

The animals were challenged when 8 weeks old intracerebrally with 10^2 pfu LI virus or intraperitoneally with 10^2 pfu TBE virus. The animals were observed for neurological signs and /or death for up to two weeks.

2.17.2 Immunization of Mice Against Vaccinia Virus

Four week old TO mice were each immunized subcutaneously with $5x10^6$ pfu wild type vaccinia virus (Vaccinia-W) or recombinant vaccinia virus containing LI envelope (E) gene (Vaccinia-E). The animals were immunized subcutaneously again two weeks later with 10^6 pfu of the same vaccinia viruses. The mice were bled two weeks after the last immunization and the pooled antisera were tested for the presence of antibodies against LI virus using western blot and *in vitro* plaque reduction neutralization tests. *In vitro* neutralization tests against vaccinia virus were also performed. The animals were challenged when 8 weeks old intracerebrally with 10^2 pfu LI virus or intraperitoneally with 10^2 pfu TBE virus. The animals were observed for neurological signs and /or death for up to two weeks.

2.18 Rabbit Immunization

2.18.1 Immunization Against Baculovirus Infected Cell Lysate

Female Dutch rabbits (2kg body weight) were immunized against lysate from 10^7 Sf cells infected with wild type baculovirus (Baculovirus-W) or recombinant baculoviruses containing LI envelope (Baculovirus-E) or structural genes (Baculovirus-CME) intramuscularly with complete Freund's adjuvant on day 1 and intramuscularly with incomplete Freund's adjuvant on day 14. The animals were boosted with the same dose subcutaneously on day 28. The animals were bled out 1 week after the last immunization and the antisera were tested for the presence of antibodies against LI virus using western blot and *in vitro* plaque reduction neutralization tests. Antiserum from rabbit which had been immunized against cell lysate infected with recombinant

baculovirus containing LI envelope gene (Baculovirus-E) was also tested for protective antibodies against LI and TBE viruses in mouse passive protection assays.

2.18.2 Immunization Against Vaccinia Virus

Female Dutch rabbits (2kg body weight) were immunized against $5x10^7$ pfu wild type vaccinia virus (Vaccinia-W) or recombinant vaccinia viruses containing LI envelope (Vaccinia-E) or structural genes (Vaccinia-CME) intradermally on day 1, $5x10^6$ pfu of the same viruses on day 14 and again on day 28. The animals were bled out 1 week after the last immunization and the antisera were tested for the presence of antibodies against LI virus using western blot and *in vitro* plaque reduction neutralization tests. *In vitro* neutralization tests against vaccinia virus were also performed. Antiserum from rabbit which had been immunized against recombinant vaccinia virus containing LI envelope gene (Vaccinia-E) was also tested for protective antibodies against LI and TBE viruses in mouse passive protection assays.

2.19 Analysis of Antisera

2.19.1 Western Blot Analysis

Purified LI virus for protein analyses was prepared as previously described under section 2.8 and subjected to 10% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) without the use of reducing agents (Cane and Gould, 1989). The protein antigen was subsequently electroblotted onto nitrocellulose paper. The filter was then processed as described in the section on western blot analysis of recombinant protein. The blot was incubated with either rabbit or mice antisera at a dilution of 1:50 in PBS containing 5% milk powder and 0.05% Tween 20. Louping ill E-specific MAb 4.2 (diluted 1:500) served as the positive control.

2.19.2 Plaque Reduction Neutralization Test Against Louping ill Virus

Serial two-fold dilutions of rabbit or mice antisera were incubated with 20 pfu of LI virus (strain 369/T2) in EMEM medium for 1 h at 37°C. PS cell monolayers in 24 well Linbro plates were then incubated with 0.2 ml of virus-antibody mixture for 1 h at 37°C. After adsorption, 1 ml of EMEM containing 2.5% FCS and 1% SeaPlaque agarose was overlaid into each well. The monolayers were fixed with 10% formol saline and stained with 0.1% (w/v) naphthalene black 3 days pi. The titres of the sera were recorded as the reciprocal of the highest dilutions that reduced the plaque numbers by 50%.

2.19.3 Plaque Reduction Neutralization Test Against Vaccinia Virus

Serial dilutions of antisera from mice or rabbits which had been immunized against vaccinia virus were incubated with 25 pfu of wild type vaccinia virus (strain WR) in EMEM medium for 1 h at 37°C. CV-1 cell monolayers in 24 well Linbro plates were then incubated with 0.2 ml of virus-antibody mixture for 1 h at 37°C. After adsorption, 1 ml of EMEM-CMC overlay was added into each well. The monolayers were fixed with 10% formol saline and stained with 0.1% (w/v) naphthalene black 24h pi. The titres of the sera were recorded as the reciprocal of the highest dilutions that reduced the plaque numbers by 50%.

2.19.4 Mouse Passive Protection Assay

Eight week old TO or three week old PO mice were inoculated intraperitoneally with 100µl of immunized rabbit antisera. The mice were challenged intraperitoneally 5 hours later with either 10^4 pfu of LI virus or 10^2 pfu of TBE virus. The animals were then observed for development of neurological signs and /or death for up to 3 weeks.

2.20 **Statistical Analysis**

The average survival times of challenged animals were compared using Student's *t* test. The limits of confidence interval were set at 95%.

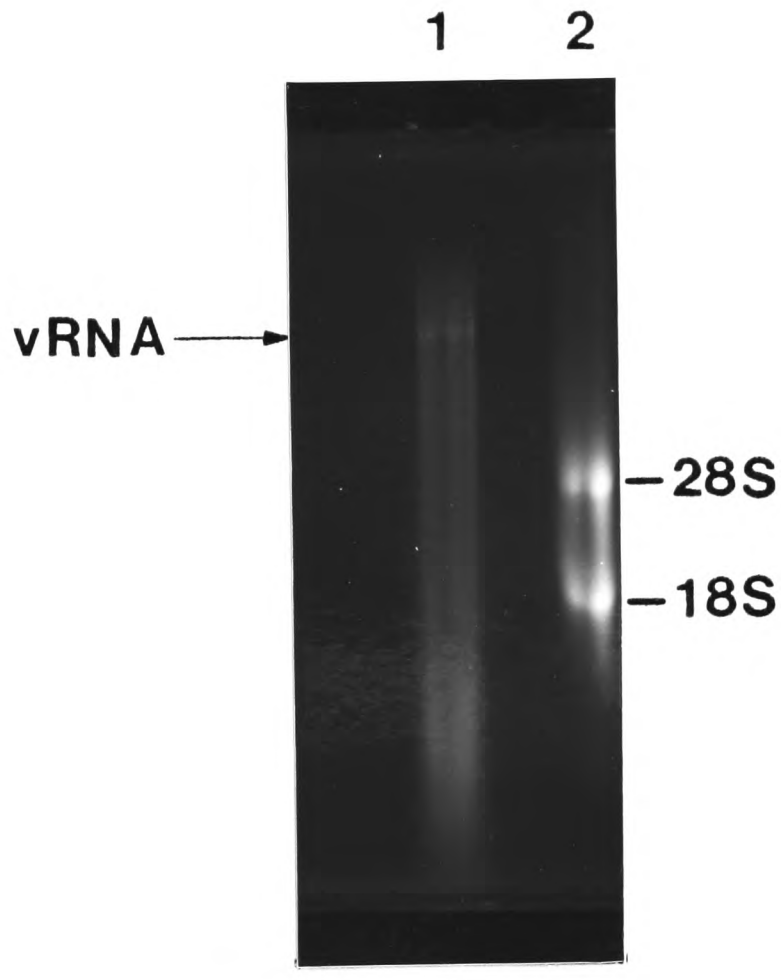


Fig. 1. Denaturing agarose gel of purified louping ill (LI) viral RNA. Track 1, vRNA - purified LI viral RNA; track 2, ribosomal RNA markers.

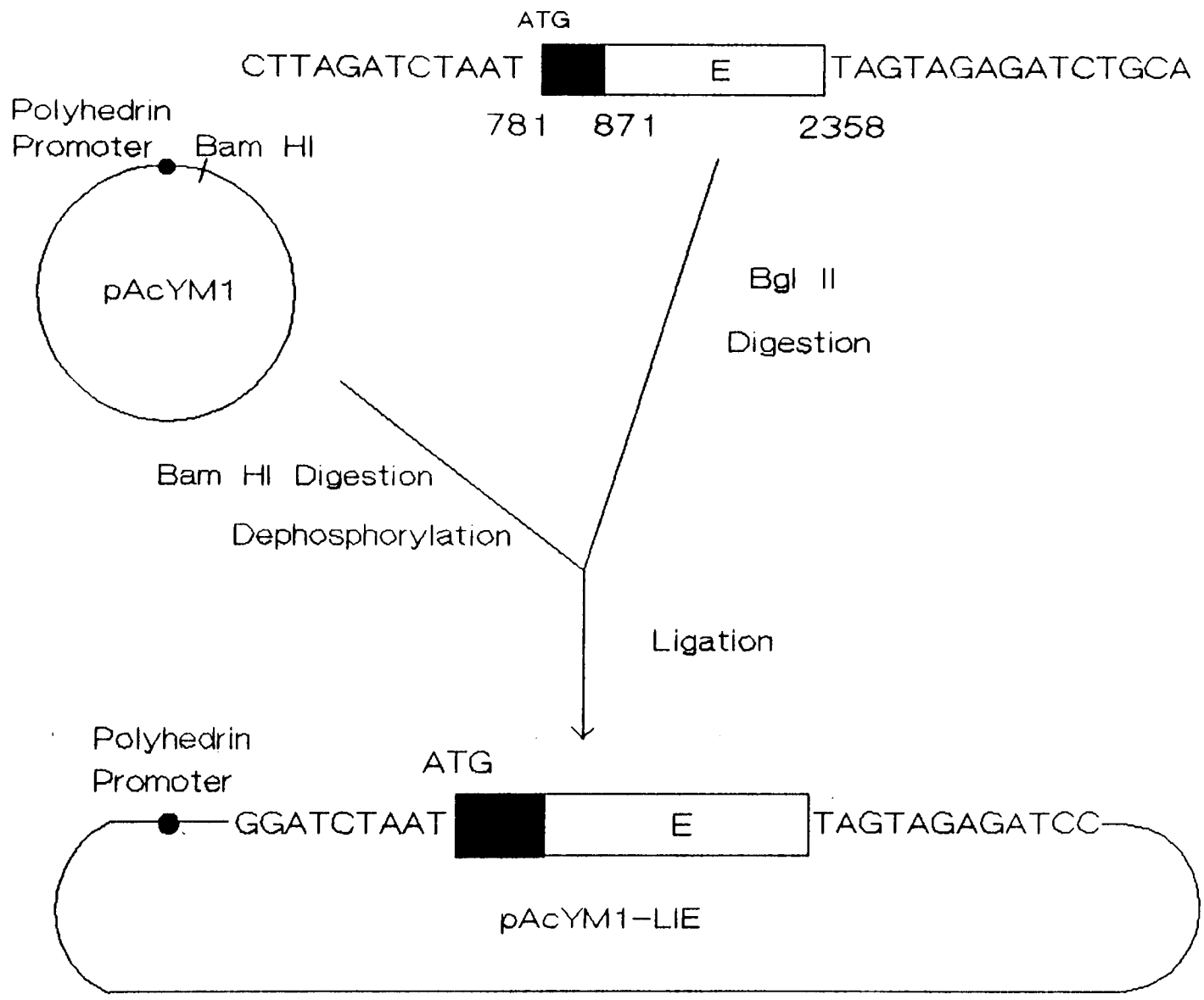


Fig. 2. Construction of pAcYM1-LIE, recombinant baculovirus transfer vector containing louping ill (LI) envelope (E) gene (nucleotides 871-2358).

■	Hydrophobic sequence (nucleotides 781-870) preceding envelope protein, part of which (nucleotides 826-870) acts as the signal sequence.

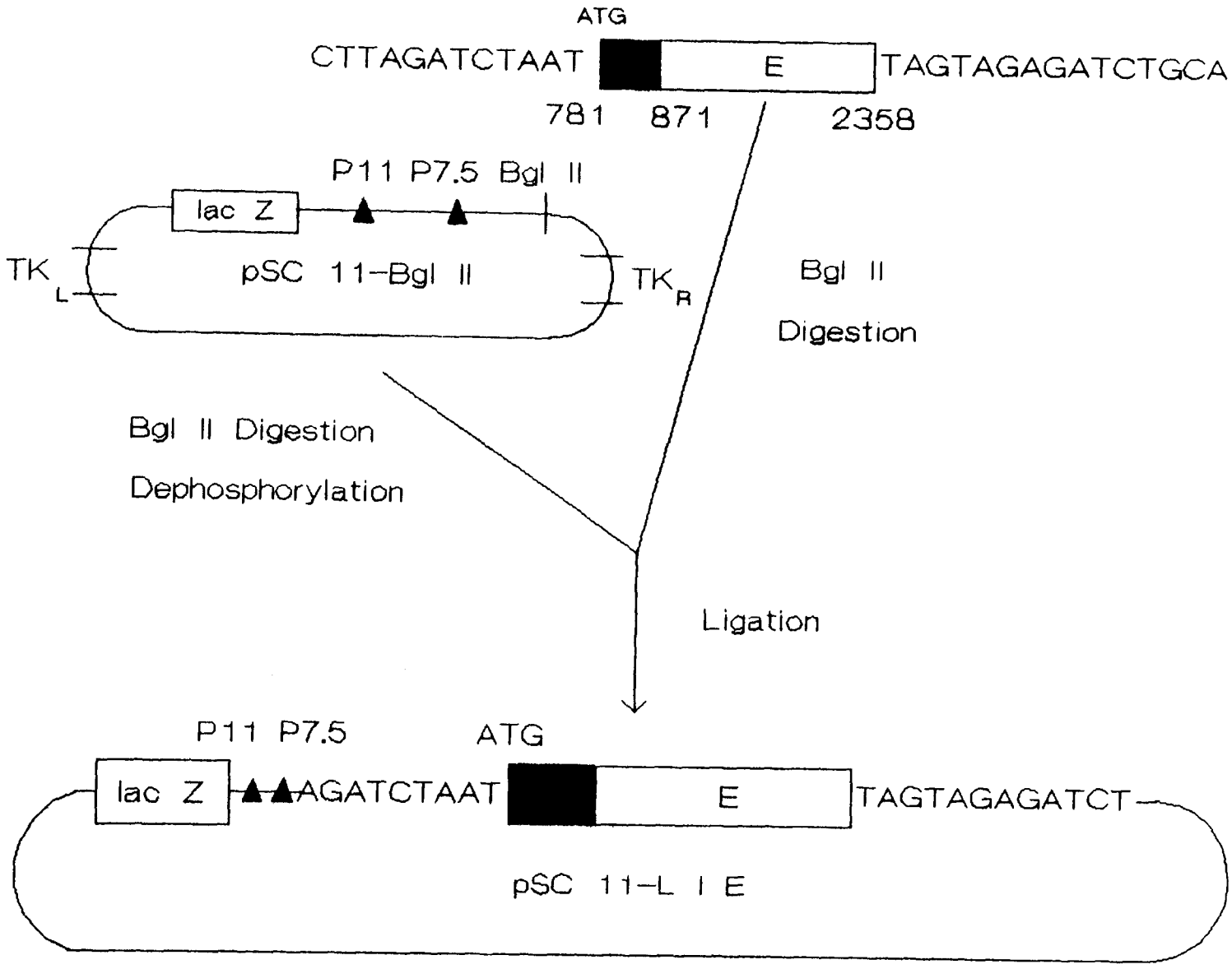


Fig. 3. Construction of pSC11-LIE, recombinant vaccinia virus transfer vector containing louping ill (LI) envelope (E) gene (nucleotides 871-2358).
lac Z: β-galactosidase gene;
TK_L and TK_R: interrupted thymidine kinase gene sequences

■	Hydrophobic sequence (nucleotides 781-870) preceding envelope protein, part of which (nucleotides 826-870) acts as the signal sequence.

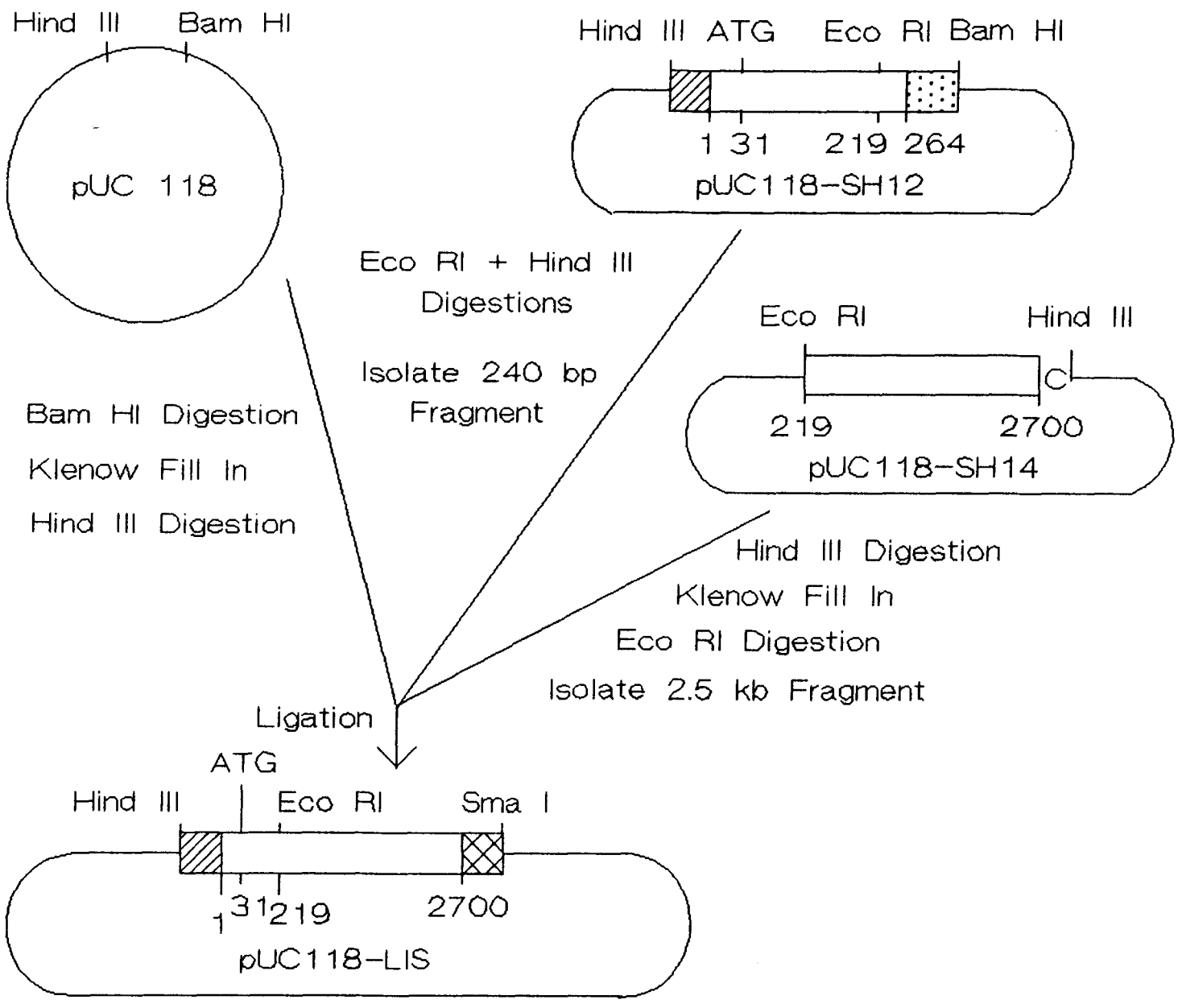


Fig. 4. Construction of DNA clone (LIS) encoding louping ill (LI) structural proteins (nucleotides 1-2700) from clones SH12 (nucleotides 1-282) and SH14 (nucleotides 219-2700).

	5'TCGTGAACGTGTTGAGAA3'
	5'AGCACTCACTGTCCTCTT3'
	5'CAAGCTGATC3'

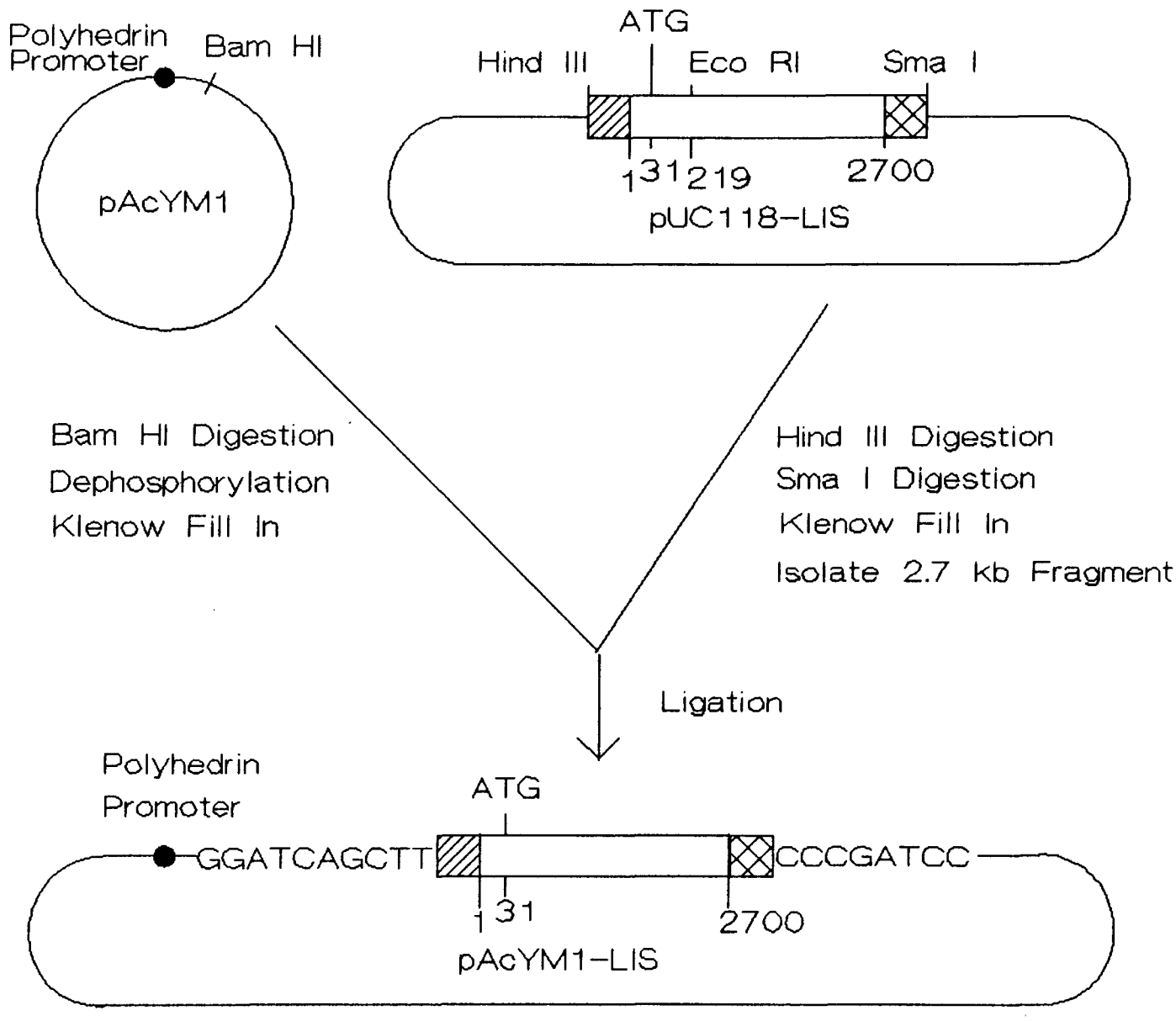


Fig. 5. Construction of pAcYM1-LIS, recombinant baculovirus transfer vector containing louping ill (LI) structural genes (nucleotides 1-2700).

▨	5'TCGTGAACGTGTTGAGAA3'
▩	5'CAAGCTGATC3'

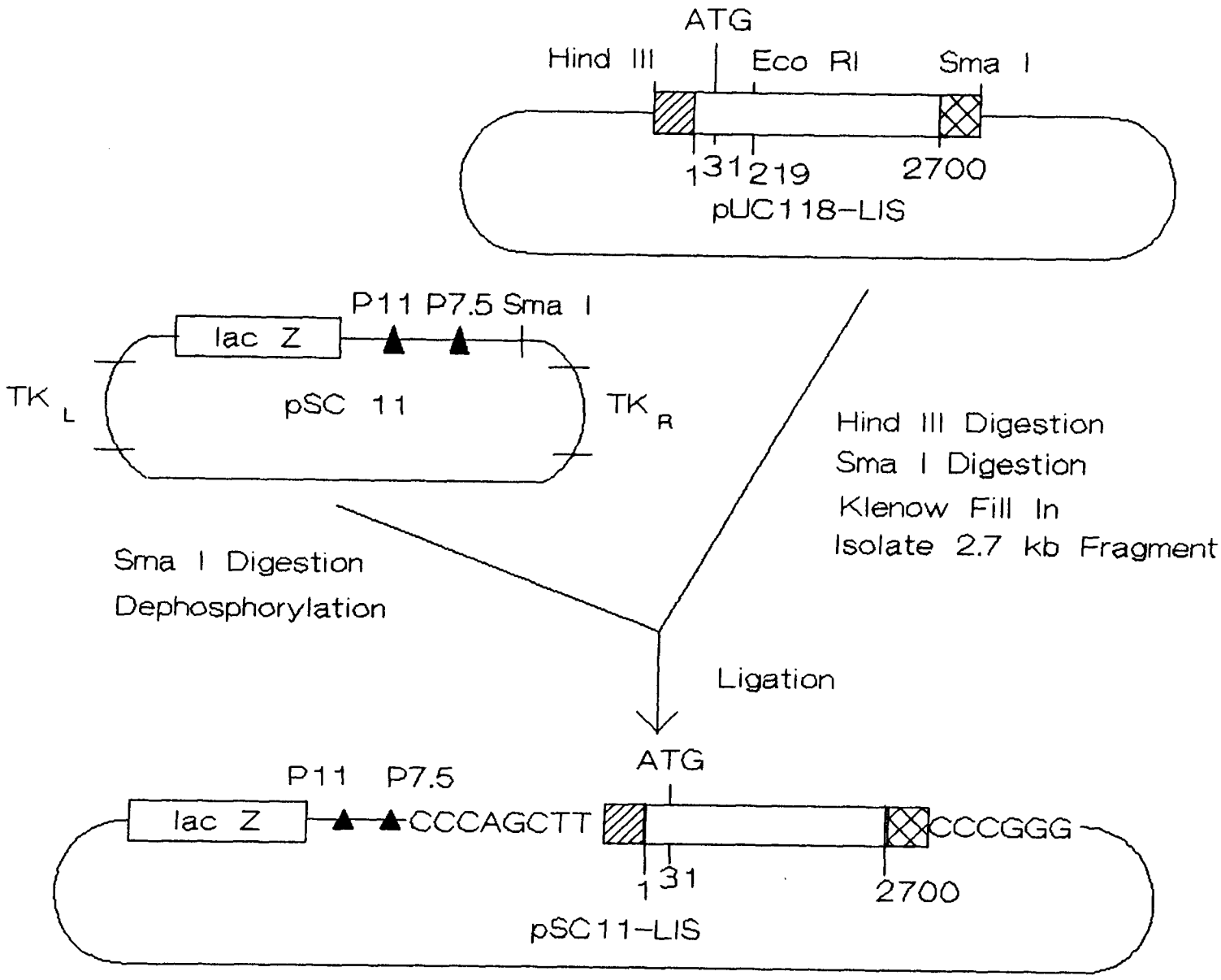


Fig. 6. Construction of pSC11-LIS, recombinant vaccinia virus transfer vector containing louping ill (LI) structural genes (nucleotides 1-2700).
lac Z: β-galactosidase gene;
TK_L and TK_R: interrupted thymidine kinase gene sequences

	5'TCGTGAACGTGTTGAGAA3'
	5'CAAGCTGATC3'

CHAPTER 3

GENOMIC SEQUENCE OF THE STRUCTURAL PROTEINS OF LOUPING ILL VIRUS: COMPARATIVE ANALYSIS WITH TICK-BORNE ENCEPHALITIS VIRUS

CONTENTS PAGE

3.1 Introduction

Based largely on cross-neutralization tests, LI virus is classified in the same antigenic subgroup as other tick-borne encephalitic flaviviruses (De Madrid and Porterfield, 1974; Porterfield, 1980; Calisher *et al.*, 1989). The tick-borne subgroup comprises western subtype of TBE (W-TBE) virus in Europe, Omsk haemorrhgaic fever and far eastern subtype of TBE (FE-TBE) viruses in the USSR, Kyasanur Forest disease virus in India, Langat virus in Malaysia, Negishi virus in Japan and Powassan virus in Canada. Stephenson *et al.* (1984), Gould *et al.* (1985a) and Gaidamovich *et al.* (1986) have demonstrated close antigenic relatedness between LI and W-TBE viruses using monoclonal antibodies. The tick-borne flaviviruses also show similar but distinguishable polypeptide profiles after limited proteolytic digestion of the envelope (E) glycoprotein (Heinz and Kunz, 1981). Nevertheless, among the tick-borne encephalitis serocomplex, LI virus appears to be unique to the United Kingdom (Greig *et al.* 1931; Hughes and Kershaw, 1957; Walton and Kennedy, 1966) and in contrast with other tick-borne flaviviruses, is not considered a major human pathogen. Guirakhoo *et al.* (1989) also provided evidence of antigenic variation among LI, W-TBE and FE-TBE viruses. The possible reasons for these differences and the precise genetic relationships between LI and TBE viruses are not yet known. The genomic RNA of LI virus encoding structural proteins was cloned and sequenced. The sequence data of the LI genome were then compared with those of TBE in order to analyze the evolutionary relationships of tick-borne flaviviruses and to make predictions concerning the characteristics of the envelope protein.

3.2 Results

3.2.1 DNA Clones and Sequence Analysis

Three cDNA clones SH1, SH8 and SH10 with sizes 0.9, 1.9 and 2.7kb respectively and a 300 bp PCR product (SH12) were derived from purified LI (strain 369/T2) viral RNA. Cross-hybridization and restriction mapping of the clones confirmed that the three cDNA clones SH1, SH8 and SH10 overlapped at the 5' region (Fig. 7). Clones SH10 and SH12 were sequenced in both directions. Translation of the DNA nucleotide sequence into amino acids revealed one single open reading frame. This nucleotide sequence with the appropriate encoded amino acids is shown in Fig. 8. Alignment with published nucleotide and amino acid sequences of W-TBE and FE-TBE (Mandl *et al.*, 1988, 1989a; Pletnev *et al.* 1990) showed that the DNA sequence encodes the C, prM, M, E and the N-terminal region of nonstructural NS1 protein of LI virus. These results confirmed the belief that LI and TBE viruses are extremely closely related genetically.

3.2.2 Hydrophobicity Profile

The hydrophobicity plot of the structural proteins and the N-terminal region of NS1 of LI virus (Fig. 9A) was analyzed. It was found to be similar to those of W-TBE and FE-TBE viruses (Fig. 9B) (Mandl *et al.*, 1988, 1989a; Pletnev *et al.*, 1990). The N-termini of prM, E and NS1 are preceded by hydrophobic sequences which exhibit signal sequence characteristics (Perlman and Halvorson, 1983). Hydrophobic sequences followed by single charged basic residues (Arg), that are believed to stop the transfer of the nascent polypeptide into the lumen of the endoplasmic reticulum, were identified at the carboxyl termini of M and E proteins. These hydrophobic signal and stop-transfer sequences were underlined in Fig. 8.

3.2.3 N-glycosylation Sites

Alignment of N-glycosylation sites of premembrane and envelope proteins of LI virus and 12 different flaviviruses was also carried out (Fig. 10). The results confirm previous findings of Mandl *et al.* (1988) that N-glycosylation sites are conserved among members of the same serological subgroup for premembrane protein sequence but the N-glycosylation sites of E protein show a lower degree of conservation.

3.2.4 Sequence Homologies and Variations

The primary amino acid sequences of LI structural proteins were aligned with corresponding published sequences of TBE viruses and 10 mosquito-borne flaviviruses viz. yellow fever (YF), West Nile (WN), Kunjin (KUN), Japanese encephalitis (JE), Murray Valley encephalitis (MVE), St. Louis encephalitis (SLE), dengue type 4 (DEN4), dengue type 2 (DEN2), dengue type 3 (DEN3) and dengue type 1 (DEN1) viruses. Homologies were calculated from aligned amino acid sequences as percentage identical amino acid residues with gaps counted as mismatches. The percentage homology of LI virus with each virus is shown in Table 1. When relative homologies of the envelope protein were compared, there was a close genetic relationship between LI virus and both subtypes of TBE. Interestingly, the degree of homology between LI and TBE envelope proteins is comparable with that which exists between the two subtypes of TBE. On the other hand, for the capsid and membrane proteins, LI virus showed greater homology with W-TBE (C = 91%, M = 95%) than FE-TBE (C = 82%, M = 85%) and these evolutionary relationships are illustrated in Fig. 11. In contrast, individual mosquito-borne flaviviruses were much less closely related to LI virus (14-43% homology).

Alignment of the amino acid sequences of structural proteins of the above viruses (Figs. 12 and 13) revealed conservation of the cysteine residues of premembrane (indicated by asterisks in Fig. 12) and envelope (indicated by

asterisks in Fig. 13) proteins among flaviviruses. Disulfide bridges formed by these conserved cysteine residues are important for conservation of premembrane and E protein secondary structures. Variable amino acid residues of envelope proteins among the three tick-borne flaviviruses and escape mutants of W-TBE at corresponding positions are shown in Table 2. Two hypervariable regions corresponding to amino acids 81-88 and 230-234 encode different peptides in corresponding positions of LI and TBE viruses. A potentially important domain consisting of 6 amino acid residues EHLPTA (207-212) of envelope protein was conserved among the three tick-borne flaviviruses but absent in mosquito-borne flaviviruses. This domain is highlighted in Table 2.

3.3 **Discussion**

The nucleotide sequence of the structural region of LI virus reported above demonstrates the shared molecular organization between LI and other flaviviruses. The existence of a single open reading frame agrees with that reported for the flaviviral RNA genome. As observed with other flaviviral structural proteins, the C, prM/M and E proteins of LI virus are probably formed by co-/post-translational cleavage of a polyprotein. The initiating methionine residue is removed from the capsid protein by cellular aminopeptidase (Rice *et al.*, 1985; 1986b). The mature virion C protein is later cleaved (predicted cleavage sequence for louping ill virus: GKRR↓SVTN) away from the hydrophobic membrane anchor (underlined in Fig. 8) at its carboxyl terminus during virus assembly (Mandl *et al.*, 1988; Rice *et al.*, 1990). On the other hand, both the premembrane and envelope proteins of LI virus are preceded by hydrophobic signal sequences required for translocation of the following protein into the endoplasmic reticulum. The N-termini of these proteins are liberated by cellular signal peptidase (Perlman and Halvorson, 1983; von Heijne, 1984). By analogy with other flaviviruses, processing of LI

premembrane protein to generate mature M protein is likely to involve cleavage of consensus dibasic (Arg-Arg) residues by Golgi protease (Rice *et al.*, 1986b; Strauss *et al.*, 1987a,b). The mature membrane and envelope proteins are anchored in the lipid bilayer of virion envelope by the paired hydrophobic domains formed by the carboxyl terminal regions of the proteins (Trent *et al.*, 1987; Coia *et al.*, 1988).

Comparison of the LI virus envelope protein sequence with other flaviviruses revealed a closer genetic relatedness between LI and TBE than between LI and individual mosquito-borne flaviviruses. Hence, the molecular homology data correlate well with the conventional serological classification of LI virus in the tick-borne serocomplex (De Madrid and Porterfield, 1974; Porterfield, 1980; Calisher *et al.*, 1989). The degree of capsid and membrane protein homologies between LI and W-TBE was significantly higher than either that between LI and FE-TBE or that between W-TBE and FE-TBE. These data imply that LI and TBE are probably varieties of a common tick-borne ancestral virus and that LI is evolutionarily more close to W-TBE than FE-TBE. Thus, homology data of both C and M proteins may be useful in predicting the evolutionary relationships of tick-borne flaviviruses. The high degree of relative homologies which average about 90% among members of the tick-borne serogroup contrast with the relatively lower homologies among members of individual mosquito-borne serogroups which average about 70%-75% (Hahn *et al.*, 1988, Heinz *et al.*, 1990). Therefore tick-borne flaviviruses might have been subjected to different evolutionary immune selection pressure from mosquito-borne flaviviruses. Further investigations are necessary to determine if differences in biology of the arthropod vectors may contribute to the difference in selection pressure.

Envelope protein represents the viral haemagglutinin which, upon immunization or in the course of natural infection, induces haemagglutination-inhibiting, neutralizing and protective antibodies (Kitano

et al., 1974; Della Porta and Westaway, 1977; Trent, 1977; Heinz *et al.*, 1981; Gould *et al.*, 1986; Roehrig *et al.*, 1986). Thus, the structural and functional organization of E protein is important for understanding the biology of flaviviruses and the mechanisms of virus-cell interactions. Based on competitive binding of monoclonal antibodies, three non-overlapping antigenic domains (A, B, C) were defined on TBE envelope protein (Heinz, 1986). Recently, Mandl *et al.* (1989b) mapped the location of structurally defined antigenic determinants onto the amino acid sequence of TBE envelope protein by analysis of escape mutants and immunoreactive fragments. In view of the close homology of amino acid sequences between LI and TBE envelope proteins, the molecular epitope model for TBE should also apply to LI virus. This is supported by the percentage variability of amino acids of tick-borne flaviviruses within each antigenic domain. Domain B which contains tick-borne complex-specific epitopes shows, as expected, the lowest variability of residues (8.4%) when compared with domains A and C (10.2% and 11.5% respectively) as shown in Table 2.

Domain A is the most important in the induction of neutralizing and haemagglutination-inhibiting antibodies because monoclonal antibodies binding to this domain reveal the highest specific activities (Guirakhoo *et al.*, 1989). Comparison of amino acid residues between LI virus and the two serotypes of TBE revealed two hypervariable regions (amino acids 81-88 and 230-234) within domain A which encode different peptides in corresponding positions of LI and TBE viruses. The peptide 230-234 probably forms part of the neutralization sensitive domain since amino acid change has been reported within this region in a TBE neutralization escape mutant (Mandl *et al.*, 1989b) whereas the peptide 81-88 falls within the antigenic domain I defined in dengue type 1 virus envelope protein by bacterial expression studies (Mason *et al.*, 1990). Hence the two hypervariable regions within domain A may contain critical epitopes that determine biological and

serological differences between LI and TBE viruses. This is of particular significance since LI virus appears less virulent for humans than many of the other viruses within the TBE serocomplex.

On the other hand, amino acid sequence analysis of the envelope proteins of both types of TBE and 13 different mosquito-borne flaviviruses by Mandl *et al.* (1989b) revealed a hypervariable region extending from residues 200-215. There are 6 contiguous amino acids EHLPTA (207-212) within this region that are conserved in LI and TBE viruses but absent in the mosquito-borne viruses (Fig. 13 and Table 2) although the amino acid at position 206 adjacent to the hexapeptide is different in LI and TBE viruses. The hexapeptide EHLPTA may be important for two reasons. Firstly, since amino acid substitution has been reported in a TBE neutralization escape mutant in this hexapeptide (Mandl *et al.*, 1989b), these residues, at positions 207-212, may form an important tick-borne flavivirus neutralization sensitive domain. Whether or not this conserved peptide and the two hypervariable regions of envelope protein constitute part of the receptor binding site of tick-borne flaviviruses requires further investigation. Secondly, the recognition of a unique hexapeptide in tick-borne flaviviruses may provide a useful tool for identification of other tick-borne flaviviruses and may indicate whether or not flaviviruses described as "no known vector" (Porterfield, 1980) are likely to be transmitted by ticks. Indeed, the potential usefulness of EHLPTA as a genetic marker for tick-borne flaviviruses is recently supported by nucleotide sequences of Negishi (K. Venugopal, personal communication) and Langat (C. W. Mandl, personal communication) viruses. These tick-borne flaviviruses also have EHLPTA in their E protein sequences.

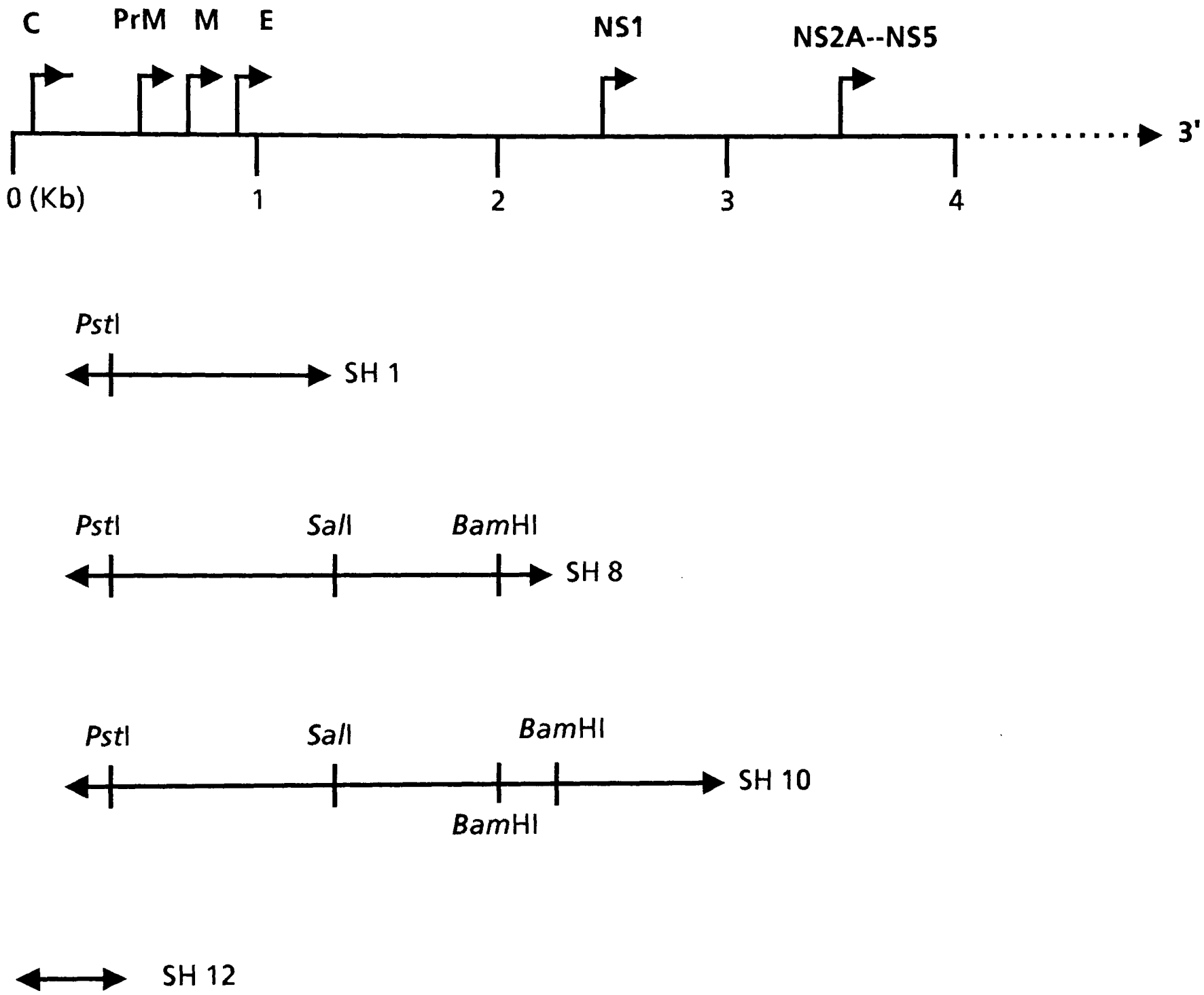


Fig. 7. Location of DNA clones on the genome of louping ill virus.

Fig. 8. Nucleotide and amino acid sequences of louping ill (LI) virus (strain 369/T2). The RNA sequence encoding C, PrM/M, E and part of NS1 is listed. Nucleotide position 1 corresponds to the first nucleotide sequenced (equivalent to nucleotide 83 in W-TBE and nucleotide 97 in FE-TBE) and amino acid residues are numbered from the first methionine of the polyprotein. The expected cleavage sites giving rise to the amino-termini of C, PrM, M, E and NS1, determined by sequence alignment with W-TBE and FE-TBE, are indicated by arrows. N-glycosylation sites are indicated by asterisks. Hydrophobic regions, thought to act as stop transfer sequences, are underlined by solid lines, whereas potential signal sequences are depicted by dashed lines. The single letter amino acid code is used: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan and Y, tyrosine. *Note*. The sequence data reported in this thesis have been deposited with EMBL/GenBank Data Libraries under Accession No. M37687.

```
AAAGACAGCUUAGGAGAACAAGAGCUGGGGAUGGGCAGGAAGACCAUCCUGAAAGGUAAG   60
                                    M | G  R  K  T  I  L  K  G  K   10
                                      └→C

GGGGGCGGUCCCCCUCGACGAGUGUCGAAAGAGACCGCAACGAAGACGCGUCAACCUAGA  120
 G  G  G  P  P  R  R  V  S  K  E  T  A  T  K  T  R  Q  P  R    30

GUCCAAAUGCCAAAUGGACUGGUGUUGAUGCGCAUGAUGGGGAUCUUGUGGCAUGCUGUG  180
 V  Q  M  P  N  G  L  V  L  M  R  M  M  G  I  L  W  H  A  V    50

GCUGGCACUGCGAGAAACCCUGCUUUGAAAGCAUUCUGGAAUUCCGUCCCGUUGAGGCAG  240
 A  G  T  A  R  N  P  A  L  K  A  F  W  N  S  V  P  L  R  Q    70

GCCACUGCAGCACUUCGGAAGAUAAAGAGGACAGUGAGUGCUUUGAUGGUUGGCUUGCAA  300
 A  T  A  A  L  R  K  I  K  R  T  V  S  A  L  M  V  G  L  Q    90

AGGCGCGGAAAAAGGAGGUCAGUGACGAACUGGAUGAACUGGUUGCUGGUCAUUGCUCUG  360
 R  R  G  K  R  R  S  V  T  N  W  M  N  W  L  L  V  I  A  L   110
                   ----------------------------------------

CUGGGGAUGACACUUGCAGCGACAGUAAGGAAAGAAGGGGACGGCACCACUGUGAUCAGG  420
 L  G | M  T  L  A  A  T  V  R  K  E  G  D  G  T  T  V  I  R   130
----- └→PrM

GCUGAAGGAAGGGACGCGGCAACGCAGGUGCGUGUGGAGAACGGCACCUGUGUGAUCCUG  480
 A  E  G  R  D  A  A  T  Q  V  R  V  E  N  G  T  C  V  I  L   150
                                  *

GCCACAGACAUGGGGUCAUGGUGUGAUGACUCGCUGUCAUACGAGUGUGUGACCAUAGAG  540
 A  T  D  M  G  S  W  C  D  D  S  L  S  Y  E  C  V  T  I  E   170

CAAGGGGAAGAGCCUGUUGACGUGGAUUGUUUUUGCCGGAAUGUUGACGGAGUCUAUCUG  600
 Q  G  E  E  P  V  D  V  D  C  F  C  R  N  V  D  G  V  Y  L   190

GAGUAUGGCCGUUGUGGGAAACAGGAGGGCUCACGGACAAGACGCUCGGUGCUGAUCCCA  660
 E  Y  G  R  C  G  K  Q  E  G  S  R  T  R  R | S  V  L  I  P   210
                                               └→M

ACCCAUGCCCAGGGCGAGUUGACGGGGAGGGGACGCAAAUGGUUGGAAGGAGACUCACUG  720
 T  H  A  Q  G  E  L  T  G  R  G  R  K  W  L  E  G  D  S  L   230

CGGACACACCUCACGAGAGUGGAAGGAUGGGUUUGGAAAAACAAACUGCUUGCUCUGGCG  780
 R  T  H  L  T  R  V  E  G  W  V  W  K  N  K  L  L  A  L  A   250
                                               ----------

AUGGUCGCCGUUGUGUGGCUAGCACUGGAGAGUGUGGUGACCAGGGUUGCCGUGCUGGUU  840
 M  V  A  V  V  W  L  A  L  E  S  V  V  T  R  V  A  V  L  V   270
                                               ----------
----------------------------------------

GUGCUCCUGUGUUUGGCCCCGGUCUACGCCUCACGCUGCACACACUUGGAAAACAGGGAC  900
 V  L  L  C  L  A  P  V  Y  A | S  R  C  T  H  L  E  N  R  D   290
------------------------------└→E
```

```
UUUGUCACUGGCACCCAGGGGACUACAAGAGUCACCUUGGUGCUGGAGCUGGGUGGAUGC  960
 F  V  T  G  T  Q  G  T  T  R  V  T  L  V  L  E  L  G  G  C   310

GUAACCAUAACCGCUGAGGGGAAGCCCUCAAUGGAUGUGUGGCUUGACGCCAUCUACCAG 1020
 V  T  I  T  A  E  G  K  P  S  M  D  V  W  L  D  A  I  Y  Q   330

GAAAGCCCUGCCAAGACACGCGAGUACUGUUUGCACGCCAAGUUGUCAGAGACCAAGGUU 1080
 E  S  P  A  K  T  R  E  Y  C  L  H  A  K  L  S  E  T  K  V   350

GCUGCUAGAUGUCCAACGAUGGGACCAGCUGUCCUGACUGAAGAACGCCAGAUUGGCACA 1140
 A  A  R  C  P  T  M  G  P  A  V  L  T  E  E  R  Q  I  G  T   370

GUGUGCAAGAGGGACCAGAGUGAUCGAGGCUGGGGAAAUCACUGUGGUUUGUUUGGAAAG 1200
 V  C  K  R  D  Q  S  D  R  G  W  G  N  H  C  G  L  F  G  K   390

GGCAGCAUUGUGGCCUGCGUCAAGGCGGCUUGUGAAGCAAAGAAGAAAGCUACGGGAUAC 1260
 G  S  I  V  A  C  V  K  A  A  C  E  A  K  K  K  A  T  G  Y   410

GUGUACGAUGCCAACAAAAUAGUGUACACAGUGAAGGUUGAACCACACACGGGAGACUAU 1320
 V  Y  D  A  N  K  I  V  Y  T  V  K  V  E  P  H  T  G  D  Y   430

GUUGCCGCCAAUGAGACGCACAAGGGGAGGAAGACGGCGACUUUCACGGUUUCUUCAGAG 1380
 V  A  A  N  E  T  H  K  G  R  K  T  A  T  F  T  V  S  S  E   450
          *

AAGACCAUCUUGACCCUAGGGGAAUAUGGAGACGUCUCACUGCUAUGUAGGGUAGCCAGU 1440
 K  T  I  L  T  L  G  E  Y  G  D  V  S  L  L  C  R  V  A  S   470
```

```
GGCGUCGACUUGGCUCAGACCAUCAUCCUGGAGCUCGACAAGACAGCGGAGCACCUUCCA 1500
 G  V  D  L  A  Q  T  I  I  L  E  L  D  K  T  A  E  H  L  P   490

ACAGCUUGGCAGGUCCACCGAGACUGGUUUAAUGACCUGGCUCUCCCGUGGAAGCAUGAU 1560
 T  A  W  Q  V  H  R  D  W  F  N  D  L  A  L  P  W  K  H  D   510

GGAAACCCACAUUGGAACAAUGCAGAGAGACUGGUUGAGUUUGGCGCUCCUCAUGCUGUC 1620
 G  N  P  H  W  N  N  A  E  R  L  V  E  F  G  A  P  H  A  V   530

AAGAUGGAUGUGUACAACCUUGGAGAUCAGACUGGAGUGCUGCUGAGGGCACUAGCAGGG 1680
 K  M  D  V  Y  N  L  G  D  Q  T  G  V  L  L  R  A  L  A  G   550

GUUCCUGUGGCACACAUUGAGGGAAACAAGUAUCACCUGAAGAGUGGUCAUGUGACCUGC 1740
 V  P  V  A  H  I  E  G  N  K  Y  H  L  K  S  G  H  V  T  C   570

GAAGUGGGACUGGAAAAACUAAAGAUGAAAGGUCUCACGUACACAAUGUGUGAUAAAUCA 1800
 E  V  G  L  E  K  L  K  M  K  G  L  T  Y  T  M  C  D  K  S   590

AAGUUUGCAUGGAAGAGAACUCCAACAGACAGCGGGCAUGACACAGUGGUUAUGGAAGUC 1860
 K  F  A  W  K  R  T  P  T  D  S  G  H  D  T  V  V  M  E  V   610
```

```
ACAUUCUCCGGGUCAAAGCCAUGCAGGAUCCCGGUGAGAGCAGUGGCACAUGGAUCUCCU 1920
 T  F  S  G  S  K  P  C  R  I  P  V  R  A  V  A  H  G  S  P   630

GAUGUCAAUGUGGCCAUGCUGAUAACGCCAAACCCCACAAUUGAAAAUGAUGGAGGGGGC 1980
 D  V  N  V  A  M  L  I  T  P  N  P  T  I  E  N  D  G  G  G   650

UUCAUAGAGAUGCAGCUUCCCCCAGGGGACAAUAUCAUCUAUGUUGGGGAACUGAGUCAU 2040
 F  I  E  M  Q  L  P  P  G  D  N  I  I  Y  V  G  E  L  S  H   670

CAAUGGUUCCAGACAGGGAGCAGCAUUGGAAGGGUGUUCCAAACAACCAGGAAAGGUAUA 2100
 Q  W  F  Q  T  G  S  S  I  G  R  V  F  Q  T  T  R  K  G  I   690

GAAAGACUGACAGUAAUAGGAGAGCACGCCUGGGACUUUGGAUCCGCUGGUGGCUUCUUU 2160
 E  R  L  T  V  I  G  E  H  A  W  D  F  G  S  A  G  G  F  F   710

GGCUCAAUUGGAAAGGCGGUGCACACGGUUCUUGGUGGUGCCUUCAACAGCAUCUUUGGA 2220
 G  S  I  G  K  A  V  H  T  V  L  G  G  A  F  N  S  I  F  G   730

GGAGUGGGGUUCCUGCCAAAGCUCCUAAUGGGAGUGGCACUGGCCUGGUUGGGACUGAAC 2280
 G  V  G  F  L  P  K  L  L  M  G  V  A  L  A  W  L  G  L  N   750

ACAAGGAACCCCACAAUGUCCAUGAGCUUUCUCUUGGCUGGAGGUCUGGUCUUGGCCAUG 2340
 T  R  N  P  T  M  S  M  S  F  L  L  A  G  G  L  V  L  A  M   770

ACUCUCGGGGUGGGAGCGGAUGUCGGCUGUGCAGUGGACACAGAGCGAAUGGAGCUCCGC 2400
 T  L  G  V  G  A  D  V  G  C  A  V  D  T  E  R  M  E  L  R   790

UGUGGUGAAGGUUUGGUUGUGUGGAGAGAGGUUUCAGAGUGGUAUGACAACUAUGCAUAC 2460
 C  G  E  G  L  V  V  W  R  E  V  S  E  W  Y  D  N  Y  A  Y   810

UACCCGGAGACACCAGGGGCUCUUGCAUCGGCAGUAAAAGAGGCAUUUGAGGAGGGAAGC 2520
 Y  P  E  T  P  G  A  L  A  S  A  V  K  E  A  F  E  E  G  S   830

UGUGGCGUGGUCCCCCAGAACAGACUUGAGAUGGCCAUGUGGAGAAGCUCGGUCACGGAA 2580
 C  G  V  V  P  Q  N  R  L  E  M  A  M  W  R  S  S  V  T  E   850

CUGAAUCUGGCUUUAGUGGAAGGGGAUGCAAAUCUCACAGUGGUGGUAGACAAAAACGAC 2640
 L  N  L  A  L  V  E  G  D  A  N  L  T  V  V  V  D  K  N  D   870

CCUACUGACUACCGAGGUGGAGUUCCUGGCACGCUGAAAAAAGGGAAGGACAUGAAGGUC 2700
 P  T  D  Y  R  G  G  V  P  G  T  L  K  K  G  K  D  M  K  V   890

UCUUGGAGGAGCUGGGGCCACUCAAUGAUCUGGAGUAUCCCUGAGGCUCCUCGGCGUUUC 2760
 S  W  R  S  W  G  H  S  M  I  W  S  I  P  E  A  P  R  R  F   910

AUGGUGGGUACAGAGGGACAAAGUGAGUGCCCACUAGAGAGACGAAAAACAGGUGUGUUC 2820
 M  V  G  T  E  G  Q  S  E  C  P  L  E  R  R  K  T  G  V  F   930
```

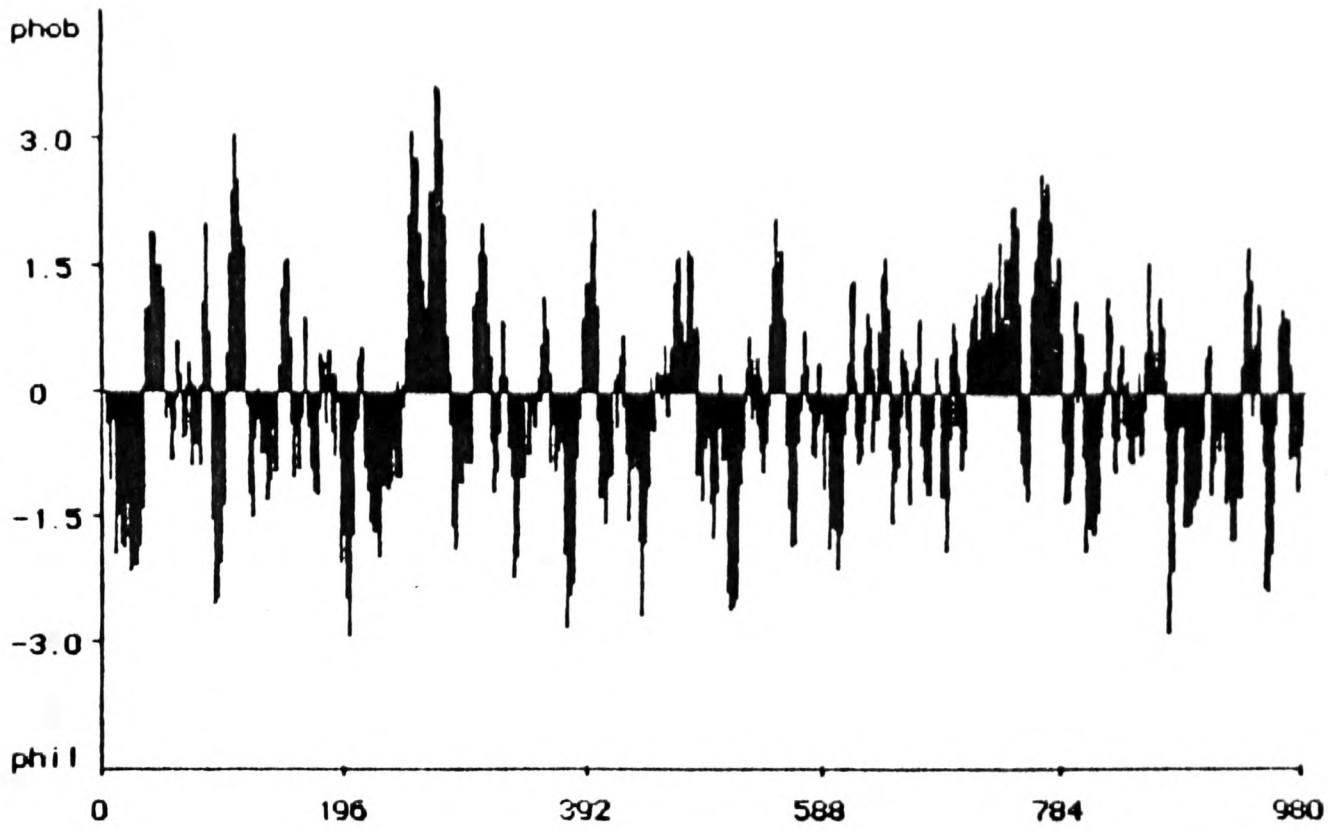
NS1

```
ACGGUUGCAGAGUUUGGGGUCGGUCUGAGAACAAAAGUCUUUCUGGACUUUAGACAGGAA 2880
 T  V  A  E  F  G  V  G  L  R  T  K  V  F  L  D  F  R  Q  E   950

CCAACACACGAGUGUGACACAGGAGUGAUGGGGGCCGCCGUCAAAAACGGCAUGGCGGUC 2940
 P  T  H  E  C  D  T  G  V  M  G  A  A  V  K  N  G  M  A  V   970

CACACAGAUCAAAGCCUCUGGAUGAAAUCG
 H  T  D  Q  S  L  W  M  K  S
```

(A)



(B)

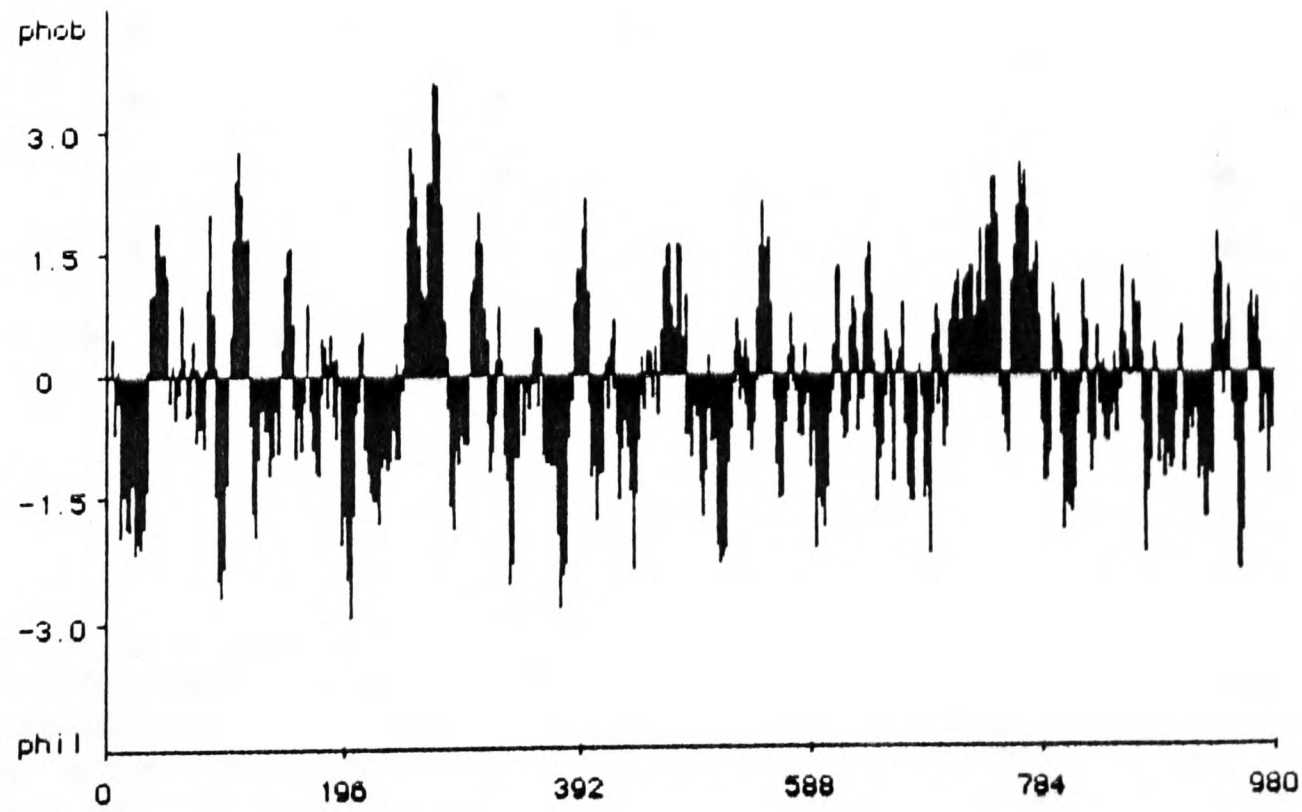


Fig. 9. Hydrophobicity plots of the structural proteins and the N-terminal region of NS1 of (A) louping ill virus and (B) TBE virus. The caspid protein of LI and TBE viruses covers amino acids 2-112, the premembrane protein covers amino acids 113-280, the membrane protein covers amino acids 206-280, the envelope protein covers amino acids 281-776 and the N-terminal region of NS1 covers amino acids 777-980.

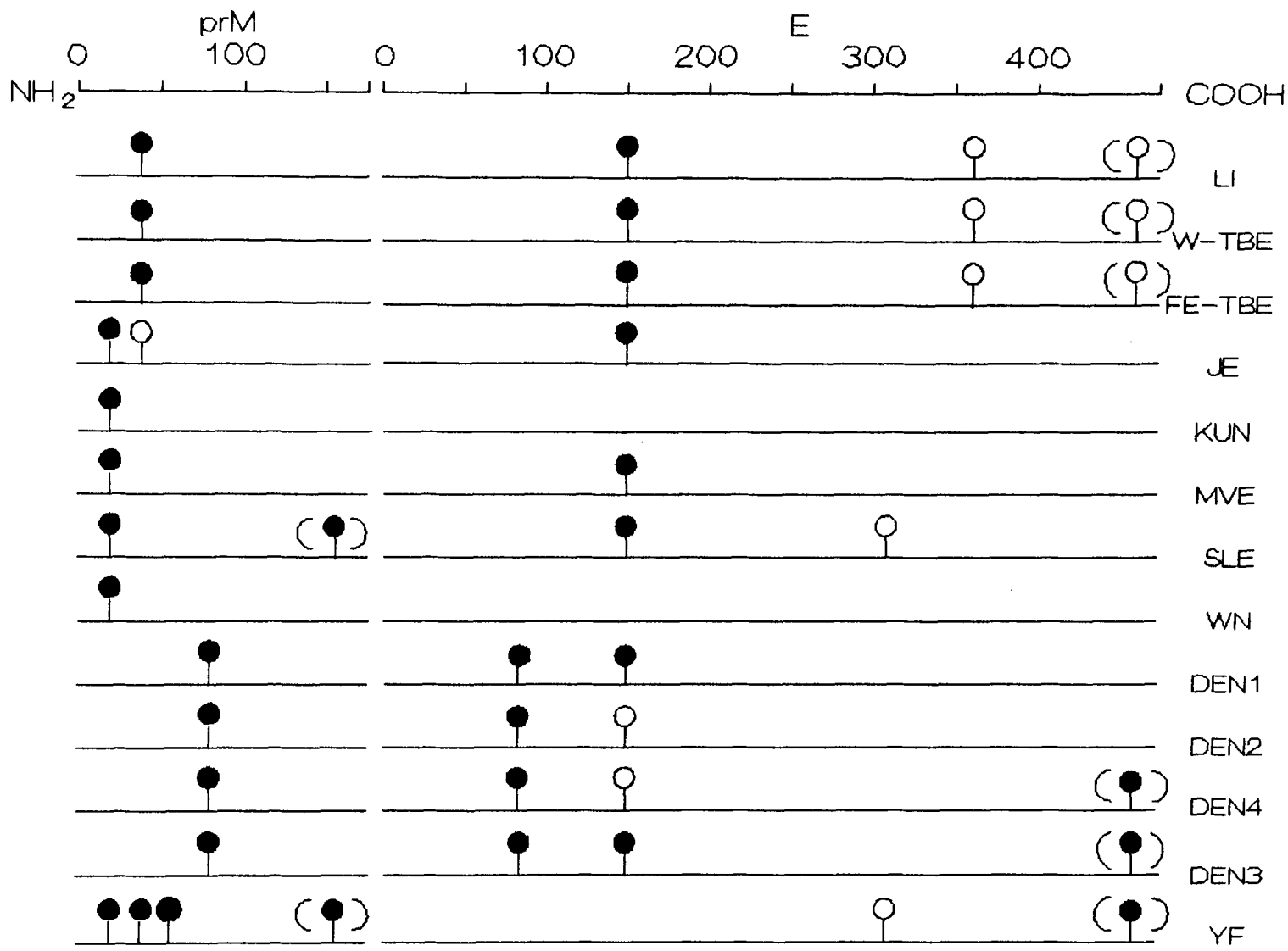


Fig. 10. N-glycosylation sites present in the amino acid sequences of the proteins prM and E of LI and 12 different flaviviruses. Solid circles represent N-glycosylation sites of the consensus sequence Asn-X-Thr/Ser. Open circles are used for "weak" glycosylation sites, i.e., X = Pro or Asp. N-glycosylation sites which are part of transmembrane regions of the proteins are shown in parentheses in order to indicate their inaccessibility to glycosylation. LI, louping ill virus; W-TBE, western subtype of tick-borne encephalitis virus; FE-TBE, far eastern subtype of tick-borne encephalitis virus; JE, Japanese encephalitis virus; KUN, Kunjin virus; MVE, Murray Valley encephalitis virus; SLE, St. Louis encephalitis virus; WN, West Nile virus; DEN1, dengue type 1; DEN2, dengue type 2;DEN4, dengue type 4; DEN3, dengue type 3 and YF, yellow fever virus.

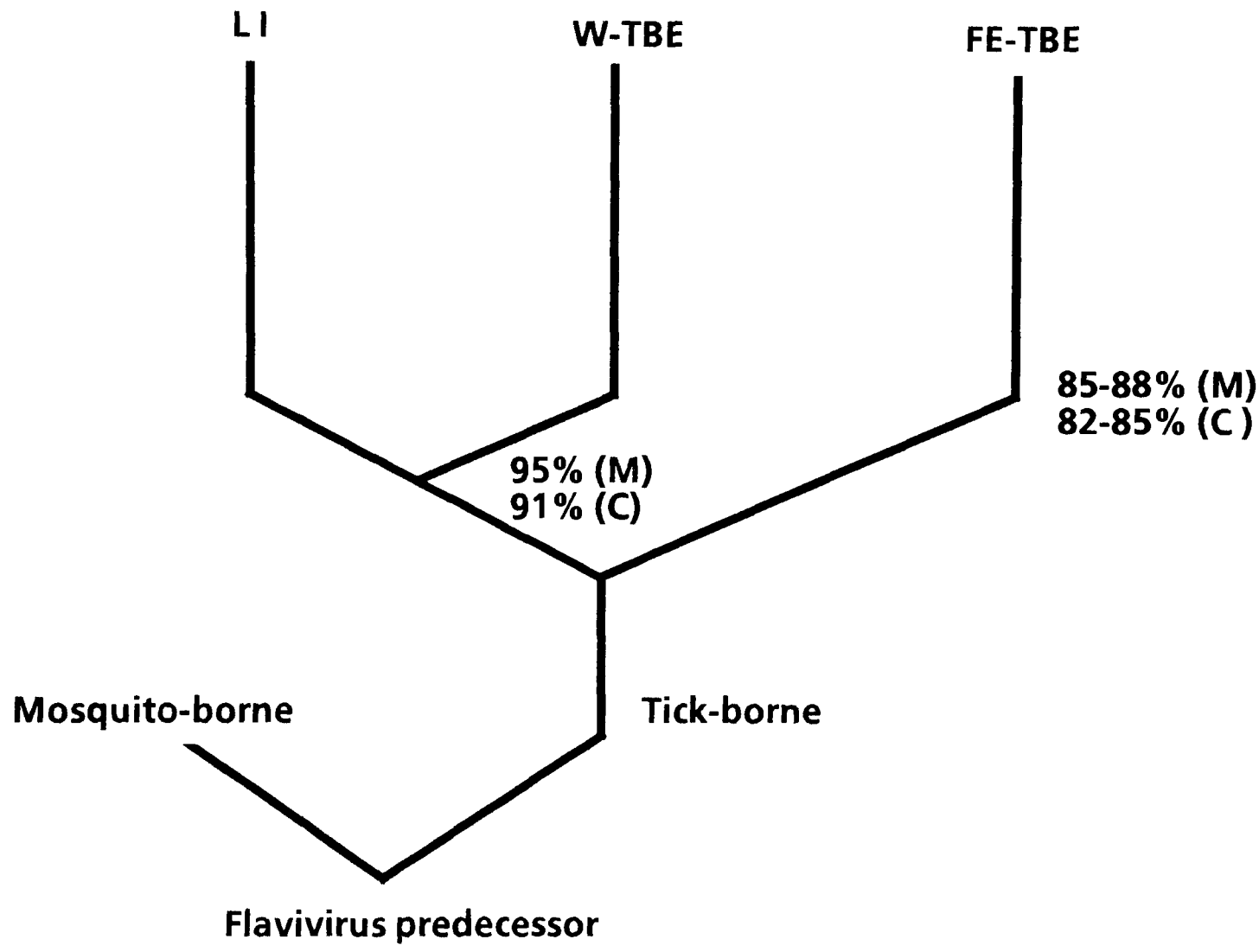


Fig. 11. Schematic representation of the evolutionary relationships among tick-borne flaviviruses drawn on the basis of the homologies of the capsid (C) and membrane (M) proteins. Homologies relate to the viruses segregating at the branching points.

Fig. 12. Alignment of the capsid (C) and premembrane (PrM) / membrane (M) protein sequences of flaviviruses. Amino acid sequences of LI (Shiu *et al.*, 1991a), W-TBE (Mandl *et al.*, 1988), FE-TBE (Pletnev *et al.*, 1990), YF (Rice *et al.*, 1985), WN (Castle *et al.*, 1985), KUN (Coia *et al.*, 1988), JE (Sumiyoshi *et al.*, 1987), MVE (Dalgarno *et al.*, 1986), SLE (Trent *et al.*, 1987), DEN4 (Zhao *et al.*, 1986), DEN2 (Hahn *et al.*, 1988), DEN3 (Osatomi and Sumiyoshi, 1990) and DEN1 (Mason *et al.*, 1987a) are aligned. Gaps are introduced to maximize homology (indicated by dashes). Dots stand for identical amino acid residues as LI. Conserved cysteine residues are indicated by asterisks. The single letter amino acid code is used.

```
                ┌→C
     LI  MGR-KTILKGKGGGP--PRR-VSKETA-TKT-RQPRVQMPNGLVLMR-MMGIL----WHAVAGTARNPALKAFWNSVPLR
   W-TBE  .VK-.A.........--...-......-...-..............-.....----..........V..........K
  FE-TBE  .AG-.A.........--...-......-K..-..S............-.....----.........S.V..S..K....K
     YF  .SGR.A--Q..TL.VNMV..G.-RSLS-N.I-K.KTK.IG.RPGPS.GVQ.FIFFFLFNILT.KKITAH..RL.KMLDP.
     WN  .S-K.PGGP..NRAVNMLK.GMPRGLSLIGL-KRAMLSLID.KGPI.FVLAL.AFFRFT.I.P.R--AV.DR-.RG.N-K
    KUN  .S-K.PGGP..SRAVNMLK.GMPRVLSLTGL-KRAMLSLID.RGPT.FVVAL.AFFRFT.I.P.R--AV.DR-.R..N-K
     JE  .T-K.PGGP..NRAINMLK.GLPRVFPLVGV-KRVVMSLLD.RGPV.FVLALITFFKFT.L.P.K--AL.GR-.KA.E-K
    MVE  .S-K.PGGP..PRVVNMLK.GIPRVFPLVGV-KRVVMNLLD.RGPI.FVLAL.AFFRFT.L.P.K--ALMRR-.K..N-K
    SLE  .S-K.PGKP.RNRVVNMLK.G..RVNPL.GL-KRILGSLLD.RGPV.F.LA..TFFRFT.LQP.E--ALKRR-.RA.D-K
   DEN4  .---NQRK.VVRPPFNMLK.ERNRVSTPQGLVKRFSTGLFS.KGPL.MVLAFITFLRVLSIPP..--GI..R-.GQLK-K
   DEN2  .--NDQRK.ARNTPFNMLK.ERNRVSTVQQLTKRFSLG.LQ.RGPLKLF.ALVAFLRFLTIPP..--GI..R-.GTIK-K
   DEN3  .--NNQRK.TGKPSINMLK.VRNRVSTGSQLAKRFSRGLL..QGP.KLV.AFIAFLRFLAIPP..--GV.AR-.GTFK-K
   DEN1  .--NNQRK.TGRPSFNMLK.ARNRVSTVSQLAKRFSKGLLS.QGP.KLV.AFIAFLRFLAIPP..--GI.AR-.G.FK-K

                                                                  ┌→PrM
     LI  Q-ATAALRKIKRTVSALMVGLQRRG---KRRSVTN------WMNWLLVIALLGMTLAATVRKEGDGTTVIRAEGRDAATQ
   W-TBE  .-.....................K..---....A.D------..S.....T.............R..S.......K.....
  FE-TBE  .-..........KA..T.........---....AVD------.TG....VV...V.........R..........K.....
     YF  .-GL.V...V..V.AS..R..SS.---....HDVL--TVQFLILGMLLMTG.V..---...NR-WLLLNVT-SE.LGKT
     WN  .T.MKH.LSF.KELGT.TSAIN..SSKQ.K.GG.AG----FTILLG.IACAGAV..SNFQG.V--MM..NATDVT.VI.I
    KUN  .T.MKH.LSF.KELGT.TSAIN..SSKQ.K.GGKTG----IAFMIG.IAGVGAV..SNFQG.V--MM..NATDVT.II.I
     JE  SV.MKH.TSF..ELGT.IDAVNK..RKQNK.GGNEGSIMWLASLAVVIAYAGA.K.SNFQG.L--LM.INNTDIA.VIVI
    MVE  TT.MKH.TSF.KELGT.IDV.NK..KKQ.K.GGSETSVLMV-I-FM.IGFAAALK.STFQG.I--MM..NATDIA.VIAI
    SLE  RT.LKH.NGF..DLGSMLDTIN..PSK--K.GGTRSLLGLA-A---.IGLASSLQ.STYQG.V--LMSINKTDAQSAINI
   DEN4  NK.IKI.IGFRKEIGRMLNI.NG.-----K..TIT--------LLC.IPTVMAFS.STRDGEP--LMI.AKH.RGRPLLF
   DEN2  SK.INV..GFRKEIGRMLNI.N..------..TAGM--------IIM.IPTVMAFH.TTRNGEP--HMI.S.Q.KGKSLLF
   DEN3  SG.IKV.KGF.KEI.NMLSIINK.-----KKTSLC--------LMMMLP.T.AFH.TSRDGEP--RMI.GKN.RGKSLLF
   DEN1  NG.IKV..GF.KEI.NMLNIMN..-----K....M--------LLM.LPTA.AFH.TTRGGEP--HMI.SKQ.RGKSLLF
```

```
                 *          *       *             * *            *           →M
    LI   VRV-ENGTCVILATDMGSWCDDSLSYECVTIEQGEEPVDVDCFCRNVDGVYLEYGRCGKQEGSR-TRRSVLIPTHAQGELT
 W-TBE   ...-.......................D..................................-........S.......
FE-TBE   ...-..................T.....D..........S.......................-........S....D..
    YF   FS.-GT.N.TTNILEAKY..P..ME.N.PNLSPR...D.I..W.YG.EN.RVA..K.DSAGR..RS..AIDL...ENHG.K
    WN   PTAAGKNL.IVR.M.V.YL.E.TIT...PVLAA.ND.E.I..W.TK-SS..VR....T.TRH..RS...LTVQ..GEST.A
   KUN   PPAAGKNL.IVR.M.V.HM...TIT...PVLSA.ND.E.I..W.TK-LA..VR....T.TRH..RS...LTVQ..GEST.S
    JE   PTSKGENR.WVR.I.V.YM.E.TIT...PKLTM.ND.E....W.D.-QE..VQ....TRTRH.KRS....SVQ..GESS.V
   MVE   PTPKGPNQ.W.R.I.I.FM...TIT...PKL.S.ND.E.I..W.DK-QA..VN....TRARH.KRS...ITVQ..GEST.V
   SLE   PSANGAN..IVI.L.V.IM.K.DIT.L.PVLSA.ND.E....W.DV-EE.WVH....TRMGH..RS...ISVQH.GDST.A
  DEN4   KTTEGINK.TLI.M.L.EM.E.TVT...PLLV-NT..E.I..W.NL-TSTWVM..T.TQSGER.REK...ALTP.SGMG.E
  DEN2   KTKDGTNM.TLM.M.L.EL.E.TIT.K.PFLK-QN..E.I..W.NS-TSTWVT..T.TTTGEH.REK...ALVP.VGMG.E
  DEN3   KTASGINM.TLI.M.L.EM...TVT.K.PH.T-EV..E.I..W.NL-TSTWVT..T.NQAGEH.RDK...ALAP.VGMG.D
  DEN1   KTSAGVNM.TLI.M.L.EL.E.TMT.K.PR.T-ET..D....W.NA-TETWVT..T.SQTGEH.RDK...ALAPEVGLG.E


    LI   GRGRKWLEGDSLRTHLTRVEGWVWKNKLLALAMVAVVWLALESVVT-RVAVLVVLLCLAPVYA
 W-TBE   ...H..........................T....T......-................
FE-TBE   ...H......................V.T..VI.....TV.....-....V...I.......
    YF   T.QE..MT.RMGERQ.QKI.R.FVR.PFF.VTALTIAY.-.G.NM.Q..VIALLVIAVG.A.S
    WN   NKKGA..DSTKATRY.VKT.S.ILR.PGY..VAAVIG.M-.G.NTMQ..VIAIL.IAV..A.S
   KUN   NKKGA.MDSTKATRY.VKT.S.ILR.PGY..VAAVIG.M-.G.NTMQ..VFA.L.ILV..A.S
    JE   NKKEA..DSTKATRY.MKT.N.IIR.PGY.FLAATLG.M-.G.NNGQ..VFTIL.ILV..A.S
   MVE   NKKDA..DSTKATRY..KT.N.IIR.PGY..VA.VLG.M-.G.NTGQK.IFT.L.LLV..A.S
   SLE   TKNTP..DTVKTTKY..K..N.FCR.PGY..VAL.IG.M-.G.NN.Q..VFVIM.MLI..A.S
  DEN4   T.AET.MSSEGAWK.AQ...S.ILR.PRF..LAGFMAYM-IGQTGIQ.TVFF.LMMLV..S.G
  DEN2   T.TET.MSSEGAWK.AQ.I.T.ILRHPGFTIMAAILAYI-IGTTHFQ..LIFIL.TAI..SMT
  DEN3   T.TQT..SAEGAWRQVEK..T.ALRHPGFTILALFLAHY-IGTSL.QK.VIFIL.MLVT.SMT
  DEN1   T.TET.MSSEGAWKQIQK..T.ALRHPGFTVIALFLAHA-IGTSI.QKGIIFIL.MLVT.SM.
```

Fig. 13. Alignment of the envelope protein sequences of flaviviruses. Amino acid sequences of LI (Shiu *et al.*, 1991a), W-TBE (Mandl *et al.*, 1988), FE-TBE (Pletnev *et al.*, 1990), YF (Rice *et al.*, 1985), WN (Castle *et al.*, 1985), KUN (Coia *et al.*, 1988), JE (Sumiyoshi *et al.*, 1987), MVE (Dalgarno *et al.*, 1986), SLE (Trent *et al.*, 1987), DEN4 (Zhao *et al.*, 1986), DEN2 (Hahn *et al.*, 1988), DEN3 (Osatomi and Sumiyoshi, 1990) and DEN1 (Mason *et al.*, 1987a) are aligned. Gaps are introduced to maximize homology (indicated by dashes). Dots stand for identical amino acid residues as LI. Conserved cysteine residues are indicated by asterisks. The single letter amino acid code is used.

```
                  *                           *                             *              *
    LI  SRCTHLENRDFVTGTQGTTRVTLVLELGGCVTITAEGKPSMDVWLDAIYQESPAKTREYCLHAKLSETKVAARCPTMGPA  80
 W-TBE  ...............................................N.............D.............  80
FE-TBE  ..........................................S....N.............D.............  80
    YF  AH.IGITD...IE.VH.G.W.SAT..QDK...VM.PD...L.IS.ETVAIDR..EV.KV.YANA.THV.INDK..ST.E.  80
    WN  FN.LGMS....LE.VS.A.W.D....GDS....MSKD..TI..KMMNMEAANL.DV.S..YL.SV.DLSTR.A.....E.  80
   KUN  FN.LGMS....LE.VS.A.W.D....GDS....MSKD..TI..KMMNMEAANL.EV.S..YL.TV..LSTK.A.....E.  80
    JE  FN.LGMG....IE.AS.A.W.D....GDS.L..M.ND..TL..RMIN.EASQL.EV.S..Y..SVTDISTV.....T.E.  80
   MVE  FN.LGMSS...IE.AS.A.W.D....GDS.I..M.AD..TL.IRMMN.EATNL.LV.N..YA.TV.DVSTVSN...T.ES  80
   SLE  FN.LGTS.....E.AS.A.WID....G.S...VM.PE..TL.FKVMKMEATEL.TV.K..YE.T.DTLSTV.....T.E.  80
  DEN4  M..VGVG.....E.VS.GAW.D....H.....TM.Q...TL.FE.TKTTAKEV.LL.T..IE.SI.NITT.T....Q.EP  80
  DEN2  M..IGIS.....E.VS.GSW.DI...H.S...DM.KN..TL.FE.ILEEAKQ..TL.K..IE...TN.TTDS....Q.EP  80
  DEN3  M..VGVG.....E.LS.A.W.DV...H.....TM.KN..TL.IE.QKTEATQL.TL.KL.IEG.ITNITTDS....Q.E.  80
  DEN1  M..VGIG.....E.LS.A.W.DV...H.S...TM.KD..TL.IE.LKTEVTN..VL.KL.IE..I.N.TTDS....Q.E.  80


                *        *      *   *
    LI  VLTEERQIGTVCKRDQSDRGWGNHCGLFGKGSIVACVKAACEAKKKATGYVYDANKIVYTVKVEPHTGDYVAANET--HK  158
 W-TBE  T.A..H.G.................................H...........................--.S  158
FE-TBE  T.A..H.S.....................T...S........H...........................--.S  158
    YF  H.A..NEGDNA...TY......G............A.FT.-..SMSLFE.-.QT..Q.VIRAQL.V.AKQE-.WNTDI-  156
    WN  HNEKRADPAF...QGVV.....G.........DT.A.F..TT.ATGWIIQKENI.YEVAIF.HGP.TVESHGK----IG  156
   KUN  HNDKRADPSF...QGVV.....G.........DT.A.F..ST.ATGRIILKENI.YEVAIF.HGP.TVESHG.YFTQTG  160
    JE  HNEKRADSSY...QGFT.....G.........DT.A.FS.TS.AIGRTIQPENI.YEVGIF.HGT.TSENHG.YSAQVG  160
   MVE  HN.KRADHNYL...GVT.....G.........DT.A.FT.SNSAAGRLILPEDI.YEVG.F.HGS.DSTSHG.YSTQIG  160
   SLE  HN.KRSDPTF.....VV.....G.........DT.A.FT.KN.ATGKTILRENI.YEVAIF.HGS.DSTSHG.YSEQIG  160
  DEN4  Y.K..QDQQYI.R..VV.....G.......GV.T.A.FS.SG.ITGNLVRIENLEYTVV.T.HNGDTHA.-G.D.SN.G  159
  DEN2  T.N..QDKRF...HSMV.....G.......G..T.AMFT.KKNMEGKIVQPENLEYTVVITPHSGEEHA.-G.D.GK.G  159
  DEN3  I.P..QDQNY...HTYV.....G........L.T.A.FQ.LESIEGKVVQHENL.YTVIIT.HTGDQHQ.-G...QGVT  159
  DEN1  T.V..QDTNF..R.TFV.....G.......LIT.A.FK.VT.LEGKIVQYENL.YSVI.T.HTGDQHQ.-G...TE.G  159
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                                        *
    LI  GRKTATFTVSSE--KTILTLGEYGDVSLLCRVASGVDLAQTIILELDKTAEHLPTAWQVHRDWFNDLALPWKHDGNP---  233
 W-TBE  .....S..I...--.....M.....................V.......V.....................E.AQ---  233
FE-TBE  .....S......--R....M.D..................V.......S.....................E.AQ---  233
    YF  --..LK.DAL.GAPSQEVEFIG..KAT.E.Q.QTA..FGNSY.A.METES------.I.D.Q.AQ..T...QSGSGG---  225
    WN  ATQAGR.SITPSAPSYT.K.....E.TVD.EPR..I.TSAYYVMSVGEKS------FL...E..M..N...SSA.ST---  227
   KUN  AAQAGR.SITPAAPSYT.K.....E.TVD.EPR..I.TSAYYVMTVGTKT------FL...E..M..N...SSAESN---  231
    JE  ASQA.K..ITPNAPSIT.K..D..E.I.D.EPR..LNTEAFYVMTVGSKS------FL...E..H......TSPSST---  231
   MVE  ANQAVR..I.PNAPAITAKM.D..E.TVE.EPR..LNTEAYYVMTIGTKH------FL...E.....LL..TSPAST---  231
   SLE  KNQA.R..I.PQAPSFTANM....T.TID.EAR..INTEDYYVFTVKEKS------.L.N.D..H..N...TSPATT---  231
  DEN4  VT----AMITPRSPSVEVK.PD..ELT.D.EPGLELYFNEM.LMKMK.KT------.L..KQ..LN.P...TAGADTSEV  229
  DEN2  KE----VKITPQSSI.EAE.TG..T.TME.SPRT.L.FNEMVL.QMKDK.------.L...Q..L..P...LPGADTQGS  229
  DEN3  AE----I.SQASTAEA..P--...TLG.E.SPRT.L.FNEM.L.TMKNK.------.M...Q..F..P...TSGATTKTP  227
  DEN1  TT----A.ITPQAPTSEIQ.TD..ALT.D.SPRT.L.FNEMVL.TME.KS------.L..KQ..L..P...TSGASTSQE  229


                                                                    *                   *
    LI  HWNNAERLVEFGAPHAVKMDVYNLGDQTGVLLRALAG-VPV--AHIEGNKYHLKSGHVTCEVGLEKLKMKGLTYTMCDKS  310
 W-TBE  N.....................................K....-...--.....T.............................T  310
FE-TBE  N.....................................KS...-...--...D.T.............................T  310
    YF  V.REMHH....EP...ATIR.LA..N.E.S.KT..T.AMR.TKDTNDN.L.K.HG...S.R.K.SA.TL..TS.KI.TDK  305
    WN  T.R.R.T.M..EE...T.QS.VA..S.E.A.HQ....AI..--EFSSNTV-K.T...LK.R.KM...QL..T..GV.S.-  303
   KUN  V.R.R.T.M..EE...T.QS.IA..S.E.A.HQ....AI..--EFSSNTV-K.T...LK.R.KM...QL..T..GV.S.-  307
    JE  A.R.R.L.M..EEA..T.QS.VA..S.E.G.HQ....AIV.--EYSSS-V-K.T...LK.RLKMD..AL..T..G..TE-  306
   MVE  E.R.R.I....EE...T.QS.VA..S.E.A.HQ....AI..--EFSSSTL-K.T...LK.R.KM...KL..T..G..TE-  307
   SLE  D.R.R.T....EE...T.QT.VA..S.E.RPAT....AIPA--TVSSSTL-T.Q...LK.RAK.D.V.I..T..G...S-  307
  DEN4  ...YK..M.T.KV...KRQ..TV.ES.E.AMHS....ATE.--DSGD..H--MFA..LK.K.RM...RI..MS....SG-  304
  DEN2  N.IQK.T..T.KN...KNQ..VV..S.E.AHMT..T.ATEI--QMSS..L--.FT..LK.RLRMD..QL..MS.S..TG-  304
  DEN3  T..RK.L..T.KNA..K.QE.VV..S.E.AMHT..T.ATEI--QTSG.TS--IFA..LK.RLKMD...L..MS.A..LN-  302
  DEN1  T..RQDL..T.KTA..K.QE.V...S.E.AMHT..T.ATEI--QTSGTTT--IFA..LK.RLKMD..TL..MS.V..TG-  304
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                                        *
    LI  KFAWKRTPTDSGHDTVVMEVTFSGSK-PCRIPVRAVAHGSPDVNVAMLITPNPTIENDGGG---FIEMQLPPGDNIIYVG  386
 W-TBE  ..T...A.................T.-.............................N...---.................  386
FE-TBE  ..T...I.............A...T.-.......................M........N...---.................  386
    YF  M.-FVKN...T..G....Q.KV.-KGA......IVADDLTAAI.KGI.V.V..IASTNDDE--VL..VNP.F..SY.I..  381
    WN  A.KFA...A.T..G...L.LQYT.TDG..KV.TSS..SLNDLTP.GR.V.V..FVSVATANSKVL..LEP.F..SY.V..  383
   KUN  A.RFLG..A.T..G...L.LQYT.TDG..K..ISS..SLNDLTP.GR.V.V..FVSVSTANAKVL..LEP.F..SY.V..  387
    JE  ..SFAKN.A.T..G...I.LSY...DG..K..IVS..SLNDMTP.GR.V.V..FVATSSANSKVLV..EP.F..SY.V..  386
   MVE  ..TFSKN.A.T..G...L.LQYT..DG..K..ISS..SLNDMTP.GRMV.A..YVASSTANAKVLV.IEP.F..SY.V..  387
   SLE  A.TFSKN...T..G..IV.LQYT..NG...V.ISVT.NLMDLTP.GR.V.V..F.STG.ANNKVM..VEP.F..SY.V..  387
  DEN4  ..SIDKEMAETQ.G.T.VK.KYE.AGA..KV.IEIRDVNKEK.V-GRI.SST.LA..T--NSVTN..LER.L-.SY.VI.  380
  DEN2  ..KVVKEIAETQ.G.I.IR.QYE.DGS..KT.FEIMDLEKRH.L-GR.T.V..IVTKK--DSPVN..AEP.F..SY.II.  381
  DEN3  T.VL.KEVSETQ.G..LIK.EYK.EDA..KI.FSTEDGQGKAH.-GR.I.A..VVTKK--EEPVN..AEP.F.ESN.VI.  379
  DEN1  S.KLEKEVAETQ.G.VLVQ.KYE.TDA..K..FSSQDEKGVTQ.-GR...A..IVTDK--EKPVN..AEP.F.ESY.VV.  381


    LI  ----ELSHQWFQTGSSIGRVFQTTRKGIERLTVIGEHAWDFGSAGGFFGSIGKAVHTVLGGAFNSIFGGVGFLPKLLMGV  462
 W-TBE  ----.........K.........K.K.....................LS.............................L..  462
FE-TBE  ----.........K.........K.....................T...LT.V...L..........L.........I.V..  462
    YF  RGDSR.TY..HKE.....KL.TQ.M..V...A.M.DT....S......T.V..GI...F.S..QGL...LNWIT.V...A  461
    WN  RGEQQIN.H.HKS.....KA.T..LR.AQ..AAL.DT......V..V.T.V...I.Q.F....R.L...MSWITQGIL.A  463
   KUN  RGEQQIN.H.HKS.....KA.TA.L..AQ..AAL.DT......V..V.T.V.....Q.F....R.L...MSWITQG.L.A  467
    JE  RGDKQIN.H.HKA..TL.KA.S..L..AQ..AAL.DT......I..V.N.......Q.F....RTL...MSWITQG...A  466
   MVE  RGDKQIN.H.HKE.....KA.S..L..AQ..AAL.DT......V..V.N.......Q.F....RTL...MSWISQG.L.A  467
   SLE  RGTTQINYH.HKE.....KALAT.W..AQ..A.L.DT......I..V.N.......Q.F....RTL...MSWITQG.L.A  467
  DEN4  VGNSA.TLH..RK.....KM.ES.YR.AK.MAIL..T......V..L.T.L.....Q.F.SVYTTM....SWMIRI.I.F  460
  DEN2  VEPGQ.KLD..KK.....QM.E..MR.AK.MATL.DT......L..V.T.....L.Q.F.AIYGAA.S..SWTM.I.I..  461
  DEN3  IGDKA.KIN.YRK.....KM.EA.AR.AR.MAIL.DT......V..VLN.L..M..QIF.S.YTAL.S..SWIM.IGI..  459
  DEN1  AGEKA.KLS..KK.....KM.EA.AR.AR.MAIL.DT......I..V.T.V..LI.QIF.T.YGVL.S..SWTM.IGI.I  461
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    LI  ALAWLGLNTRNPTMSMSFLLAGGLVLAMTLGVGA  496
 W-TBE  .........M.........................  496
FE-TBE  V.......M.........................  496
    YF  V.I.V.I....M.....MI.V.VIMMFLS.....  495
    WN  L.L.M.I.A.DRSIA.T..AV..VL.F.SVN.H.  497
   KUN  L.L.M.I.A.DRSIALT..AV..VL.FLSVN.H.  501
    JE  L.L.M.V.A.DRSIALA..AT..VLVFLATN.H.  500
   MVE  L.L.M.V.A.DKSIALA..AT..VL.FLATN.H.  501
   SLE  L.L.M..QA.DRSI.LTL.AV..ILIFLATS.Q.  501
  DEN4  LVL.I.T.S..TS.A.TCIAV..TT.FLGFT.Q.  494
  DEN2  IIT.I.M.S.STSL.V.LV.V.IVT.YLGVM.Q.  495
  DEN3  L.T.I...SK.TS..F.CIAI.IIT.YLGVV.Q.  493
  DEN1  L.T.....S.STSL..TCIAV.MVT.YLGVM.Q.  495
```

Table 1. Amino acid sequence homologies between tick-borne flaviviruses and between louping ill and mosquito-borne flaviviruses.

Viruses compared*	Percentage homology			
	C	PrM-M	M	E
LI/W-TBE	91%	96%	95%	94%
LI/FE-TBE	82%	94%	85%	91%
W-TBE/FE-TBE	85%	96%	88%	96%
LI/YF	32%	33%	27%	43%
LI/WN	22%	35%	28%	41%
LI/KUN	22%	36%	28%	41%
LI/JE	18%	33%	28%	41%
LI/MVE	21%	34%	31%	40%
LI/SLE	21%	29%	32%	41%
LI/DEN4	14%	25%	25%	37%
LI/DEN2	17%	25%	20%	38%
LI/DEN3	15%	26%	19%	37%
LI/DEN1	21%	26%	16%	37%

* See text for virus designations.
PrM-M: Part of protein prM that is not contained in protein M

Table 2. Variable and conserved amino acid residues of envelope proteins of tick-borne flaviviruses

Amino acid position	LI	W-TBE	W-TBE Neutralization escape mutants	FE-TBE	Amino acid position	LI	W-TBE	W-TBE Neutralization escape mutants	FE-TBE
47	A	A		S	234	H	N		N
52	S	N		N	266	R	K		K
*67	E	D	(G)	D	267	A	A		S
*71	A	A	(V)	A	277	E	E		D
81	V	T		T	279	N	T		T
83	T	A		A	310	S	T		T
86	R	H		H	313	A	T		T
88	I	G		S	317	T	A		I
115	A	A		T	331	T	T		A
120	A	A		S	335	S	T		T
130	Y	H		H	358	I	I		M
158	K	S		S	367	D	N		N
164	T	S		S	*384	Y	Y	(H)	Y
167	V	I		V	*389	S	S	(R)	S
*171	K	K	(E)	R	395	T	K		K
176	L	M		M	405	T	K		K
178	E	E		D	407	R	K		R
198	I	V		V	426	A	A		T
206	A	V		S	430	F	L		L
*207	E	E	(G)	E	431	G	S		T
208	H	H		H	433	I	I		V
209	L	L		L	437	V	V		L
210	P	P		P	448	I	I		L
211	T	T		T	458	L	L		I
212	A	A		A	460	M	L		V
230	D	E		E	463	A	A		V
232	N	A		A	471	T	M		M
*233	P	Q	(K)	Q					

Residues 207-212 are conserved in LI and TBE but absent in mosquito-borne flaviviruses.
*Amino acid changes detected in neutralization escape mutants of W-TBE as reported by Mandl *et al.* (1989b).
Percentage variability = [No. of amino acid positions coding different amino acids among tick-borne flaviviruses excluding escape mutants X 100%] ÷ [Total No. of amino acids in the antigenc domain].
Percentage variability of amino acid residues of antigenc domains A, B and C are respectively:

$$\text{A: } {}^{13}/_{127} \times 100\% = 10.2\%$$

$$\text{B: } {}^{8}/_{95} \times 100\% = 8.4\%$$

$$\text{C: } {}^{3}/_{26} \times 100\% = 11.5\%$$

Antigenic domain A covers amino acid residues 50 to 125 and 200 to 250
Antigenic domain B covers amino acid rsidues 301 to 395
Antigenic domain C covers amino acid residues 145 to 170 (Mandl *et al.*, 1989b).

CHAPTER 4

ANTIGENIC AND IMMUNOGENIC PROPERTIES OF RECOMBINANT ENVELOPE PROTEIN OF LOUPING ILL VIRUS

CONTENTS PAGE

4.1 Introduction

With the advent of recombinant DNA techniques, production of genetically-engineered protein has facilitated studies on the structure-function relationships of viral proteins. Bacterial, insect cell and mammalian cell expression vectors have been used to express flaviviral proteins. Antigenic fragments of Japanese encephalitis and dengue viruses had been expressed in *Escherichia coli* to map amino acid sequences that form specific antigenic determinants (Mason *et al.*, 1987b; Mason *et al.*, 1989; Mason *et al.*, 1990). While partial protection against challenge with yellow fever virus had been reported in mice immunized against a bacterially synthesized yellow fever NS1 protein fragment (Cane and Gould, 1988), one disadvantage of viral protein expression in *E. coli* is that the recombinant protein is usually not properly modified. Hence both insect cell and mammalian cell expression systems find their applications in expressing proteins, properly modified, in their correct cellular compartment.

The insect baculovirus [*Autographa californica* nuclear polyhedrosis virus (AcNPV)] vector is useful for high level foreign gene expression (Smith *et al.*, 1983, 1985; Matsuura *et al.*, 1986, 1987; Luckow and Summers, 1988) whereas recombinant vaccinia viruses provide a novel method for studying the immune responses to foreign proteins and are useful for immunizing animals against challenge by pathogenic agents (Mackett, 1990). Mice, which had been immunized against either recombinant vaccinia virus or baculovirus infected insect cells expressing Japanese encephalitis (JE) envelope protein, had been shown to develop neutralizing antibodies against JE virus (Matsuura *et al.*, 1989; Yasuda *et al.*, 1990). In addition, mice vaccinated against recombinant vaccinia viruses expressing JE envelope protein or dengue structural proteins, were protected against challenge with the respective homologous viruses (Bray *et al.*, 1989; Yasuda *et al.*, 1990). Similarly, active immunization of mice

against either dengue or JE structural and NS1 proteins expressed by baculovirus recombinants also induced resistance to fatal infection by either virus (Zhang *et al.*, 1988; McCown *et al.*, 1990).

In contrast with mosquito-borne flaviviruses, little protein expression and characterization work had been done on tick-borne flaviviruses. The envelope protein is central to the biology of flaviviruses because it is responsible for virus-cell interactions and the induction of neutralizing and protective antibodies (Kitano *et al.*, 1974; Della Porta and Westaway, 1977; Trent, 1977; Heinz *et al.*, 1981; Gould *et al.*, 1986; Roehrig, 1986). As the nucleotide sequence of LI virus has been obtained, recombinant baculoviruses and vaccinia viruses expressing LI envelope protein were constructed in order to study the antigenic and immunogenic properties of recombinant tick-borne flaviviral E protein.

4.2 Results

4.2.1 Recombinant Baculoviruses containing LI Genes

The recombinant baculovirus transfer vectors containing LI envelope gene (pAcYM1-LIE) and structural genes (pAcYM1-LIS) were constructed as detailed in Fig. 2 (section 2.9.6) and Fig. 5 (section 2.9.7) under Materials and Methods. The LI genes were positioned downstream of the polyhedrin promoter and were flanked, both 5' and 3', by polyhedrin-specific sequences. Homologous recombination, which occurred between transfected recombinant transfer vectors and infectious wild type viral DNA in insect cells, resulted in insertion of LI gene(s) into the baculovirus genome with excision of the polyhedrin gene. Polyhedrin-negative recombinant baculoviruses were selected by absence of occlusion bodies under the light microscope. For the recombinant baculovirus containing LI envelope gene (Baculovirus-E), the first AUG downstream from the polyhedrin promoter corresponded to the methionine residue (amino acid 251, nucleotide 781) in the hydrophobic carboxyl terminal

region of membrane (M) protein. This was followed by the hydrophobic signal sequence and the entire envelope gene coding sequence with stop codons located after the carboxyl terminus (amino acid 776, nucleotide 2358) of E protein. For the recombinant baculovirus containing LI structural genes (Baculovirus-CME), the first AUG downstream from the polyhedrin promoter corresponded to the AUG (amino acid 1, nucleotide 31) initiating synthesis of the capsid (C) protein. This was followed by the entire structural region, N-terminal 114 amino acids of NS1 protein (up to amino acid 890, nucleotide 2700) and 30 vector-specified amino acids (QADPRSGLLVQHLLSARFCALLIYRQLYVF - single letter amino acid code).

4.2.2 Recombinant Vaccinia Viruses containing LI Genes

The recombinant vaccinia virus transfer vectors containing LI envelope gene (pSC11-LIE) and structural genes (pSC11-LIS) were constructed as detailed in Fig. 3 (section 2.9.6) and Fig. 6 (section 2.9.7) under Materials and Methods. The LI genes were positioned downstream of the 7.5K promoter of the co-expression vectors (pSC11 and pSC11-*Bgl*II) which have *lacZ* gene placed under the control of 11K promoter. LI gene(s) and *lacZ* gene were flanked by vaccinia virus thymidine kinase (TK) gene sequences and both genes were inserted into the TK locus of vaccinia virus genome by homologous recombination *in vivo*. TK^- recombinants were selected by plaque assay on TK^- cells in the presence of BUdR and distinguished from spontaneous TK^- mutants by blue coloration of the viral plaques when X-gal was added to the agarose overlay. For the recombinant vaccinia virus containing LI envelope gene (Vaccinia-E), the first AUG downstream from the 7.5K promoter corresponded to the methionine residue (amino acid 251, nucleotide 781) in the hydrophobic carboxyl terminal region of M protein. This was followed by the same construct as that of the corresponding recombinant baculovirus. The recombinant vaccinia virus containing LI structural genes (Vaccinia-CME) had

the same construct as that of the corresponding recombinant baculovirus except for 29 vector-specified amino acids (QADPREFCERMANEGKISYSSRTRWDIST - single letter amino acid code) following the N-terminal 114 amino acids of NS1 protein.

4.2.3 Expression of LI Proteins in Insect Cells

Louping ill-specific protein products produced in Sf cells by recombinant baculoviruses were examined by SDS-polyacrylamide gel electrophoresis and immunoblotting. Coomassie Blue staining of SDS-polyacrylamide gel revealed expression of a 55K protein in Sf cells infected with polyhedrin-negative recombinant baculovirus containing LI envelope gene (Baculovirus-E) (Fig. 14). The expressed 55K (E) protein migrated at the same position as the envelope protein of LI virus as shown by immunoblotting with LI E-specific MAb 4.2 (Fig. 15). In addition to E protein, a 21K (premembrane / prM) protein, immunoreactive with polyclonal antiserum against LI virus, was also detected in Sf cells infected with recombinant baculovirus containing LI structural genes (Baculovirus-CME) (Fig. 16).

4.2.4 Expression of LI Proteins in Mammalian Cells

Louping ill-specific protein products produced in CV-1 cells by recombinant vaccinia viruses containing either LI envelope (Vaccinia-E) or structural genes (Vaccinia-CME) were respectively examined by western blot with LI E- specific MAb 4.2 (Fig. 17) or polyclonal antiserum (Fig. 18). Envelope (E) protein was expressed by both vaccinia viruses. In addition to E protein, a 36K protein, immunoreactive with polyclonal antiserum against LI virus, was also detected in cells infected with recombinant vaccinia virus containing LI structural genes (Vaccinia-CME) (Fig. 18).

4.2.5 Effect of Tunicamycin on Recombinant E Protein

In order to determine whether or not the E protein expressed by the recombinant viruses was glycosylated, the effect of tunicamycin on the mobilities of radiolabelled envelope proteins was studied. The labelling experiments with the incorporation of tunicamycin were carried out as described under Materials and Methods (section 2.15). Authentic and recombinant E proteins expressed by both recombinant baculovirus (Baculovirus-E) and vaccinia virus (Vaccinia-E) shifted in mobility from 55K to 53K in the presence of tunicamycin (10μg/ml) in the cell culture media (Fig.19). A similar shift in mobility was also observed in recombinant E protein expressed by baculovirus containing LI structural genes (Baculovirus-CME) (Fig.20). In addition, a 36K labelled protein, which showed no shift in mobility in the presence of tunicamycin, was also precipitated by LI E-specific MAb 4.2 from Sf cells infected with Baculovirus-CME (Fig. 20)

4.2.6 Antigenic Properties of Recombinant E Protein

The antigenic reactivities of LI envelope protein expressed by recombinant baculoviruses (Fig. 21) and vaccinia viruses (Fig. 22), determined by indirect immunofluorescence microscopy using a panel of E-specific monoclonal antibodies (MAbs) reactive with LI virus (Fig. 23), are shown in Table 3. LI envelope proteins expressed by all four recombinant viruses were reactive with neutralizing MAbs 3.3 and 7.5, and neutralizing / protective MAbs 4.2 and 7.1. However, none of the recombinant E proteins reacted with neutralizing MAb 3.1. The MAbs T7, 813 and 34.1 define epitopes that are broadly cross-reactive among flaviviruses (Gould *et al.*, 1985a, 1990). The epitope represented by MAb T7 could be detected in E protein expressed in mammalian cells but not in insect cells. The E-specific epitope defined by 813 was expressed by recombinant viruses containing LI structural genes but not by viruses containing only the LI envelope gene. MAb 34.1 was reactive with E

proteins expressed by Vaccinia-E and Baculovirus-CME but did not react with E proteins expressed by Vaccinia-CME and Baculovirus-E. Expression of the E protein on the outer surface of infected Sf cells (Fig. 24) and CV-1 cells (Fig. 25) was demonstrated using non-fixed cells and MAb 4.2.

4.2.7 Immunogenic Properties of Recombinant E Protein

4.2.7.1 Characterization of Antisera By Western Blot and *In Vitro* Neutralization Test

The results of analyses of antisera from mice and rabbits immunized against recombinant E protein of LI virus, for binding and neutralizing antibodies against the authentic virus, are summarized in Table 4. Although pooled antisera from mice immunized against cell lysate infected with Baculovirus-E were positive for binding antibodies against LI virus by western blot with purified authentic E antigen (Fig. 26), significant neutralizing antibodies were not detected. Moreover, no binding or neutralizing antibodies against LI virus could be detected in pooled antisera from mice immunized against Vaccinia-E despite significant development of neutralizing antibodies against wild type vaccinia (neutralization titre 1:40) by the mice. On the other hand, louping ill E-binding antibodies could be detected in antisera from rabbits immunized against Vaccinia-E (Fig. 27) or cell lysate infected with Baculovirus-E (Fig. 28) but they were absent in antisera from rabbits immunized against E protein expressed by recombinant viruses containing LI structural genes. Nonetheless, no neutralizing antibodies against LI virus could be detected in any rabbit antisera.

4.2.7.2 Characterization of Rabbit Antisera By Mouse Passive Protection Assay

The results of mouse passive protection assays for antisera from rabbits immunized against Sf cell lysate infected with wild type baculovirus

(Baculovirus-W) or recombinant baculovirus containing LI envelope gene (Baculovirus-E) are shown in Table 5. None of the comparable groups of mice, inoculated passively with antisera from rabbits which had been immunized against Sf cell lysate infected with Baculovirus-E or Baculovirus-W, showed significantly different survival times upon challenge with either LI or TBE virus.

The results of mouse passive protection assays for antisera from rabbits immunized against wild type vaccinia virus (Vaccinia-W) or recombinant vaccinia virus containing LI envelope gene (Vaccinia-E) are shown in Table 6. No significant differences in survival times were detected between comparable groups of mice, which had been passively inoculated with antisera from rabbits immunized against Vaccinia-E or Vaccinia-W, upon challenge with either LI or TBE virus.

4.2.7.3 Challenge of Mice, immunized against recombinant LI envelope protein, with Tick-Borne Flaviviruses

The results of challenge experiments in mice immunized against Sf cell lysate infected with Baculovirus-E or Baculovirus-W with or without adjuvant are shown in Tables 7 and 8. No significant differences in survival times were detected between comparable groups of mice upon challenge with either LI (Table 7) or TBE (Table 8) virus. The results of challenge experiments in mice immunized against Vaccinia-E or Vaccinia-W are shown in Table 9. Survival times of comparable groups of mice were not significantly different from each other upon challenge with either LI or TBE virus.

4.3 Discussion

In this study, the antigenic and immunogenic properties of LI envelope protein expressed by recombinant baculoviruses and vaccinia viruses containing either inserted LI envelope (E) gene or structural genes were analyzed. All four recombinants expressed a 55K protein which migrated at

the same position as authentic E protein and reacted with either LI E-specific monoclonal or polyclonal antiserum. The expressed 55K protein was glycosylated in both insect and mammalian cells and, like the authentic protein, shifted in mobility to 53K in the presence of tunicamycin. The observed molecular weight difference of 2K agreed with a previous report of the presence of a single carbohydrate side chain in the envelope protein of TBE and LI viruses (Winkler *et al.*, 1987). In addition, expression of a glycoprotein which has the same size as the authentic envelope protein suggested proper processing of recombinant tick-borne flaviviral E protein in the endoplasmic reticulum and correct cleavage of envelope protein from the structural polyprotein. This is further supported by the expression of 21K premembrane (prM) protein by recombinant baculovirus containing LI structural genes (Baculovirus-CME) as endoplasmic signalase is responsible for the cleavage of flaviviral premembrane and envelope proteins from polyprotein precursor (Perlman and Halvorson, 1983; von Heijne, 1984; Rice *et al.*, 1985, 1986b). Nevertheless, premembrane protein expression could not be demonstrated in cell lysate infected with recombinant vaccinia virus containing LI structural genes (Vaccinia-CME). This may be explained by the lower level of protein expression by recombinant vaccinia virus than baculovirus. Alternatively, premembrane protein may be rapidly cleaved to membrane protein in mammalian cells. On the other hand, a 36K protein band was also observed in cell lysate infected with Vaccinia-CME. This probably represented a breakdown product of envelope protein as a protein band of the same size was immunoprecipitated from Sf cell lysate infected with Baculovirus-CME using E-specific monoclonal antibody.

Antigenic presentation of the envelope protein expressed by recombinant baculoviruses and vaccinia viruses was examined using a panel of E-specific monoclonal antibodies reactive against authentic LI virus. The epitope reactive with MAb T7 was detected in envelope proteins expressed in

mammalian cells but not in insect cells whereas the epitope defined by MAb 3.1 was not expressed by any of the recombinant E proteins. This contrasts with JE envelope protein expressed by vaccinia virus (Yasuda *et al.*, 1990) which displays epitopes identical to that of the authentic protein. Moreover, 17D YF envelope protein, expressed by recombinant baculovirus, is also antigenically indistinguishable from the authentic viral protein in mammalian cells (Shiu *et al*, 1991b). Hence epitope processing of recombinant tick-borne flaviviral E protein may be distinct from that of mosquito-borne flaviviruses. Differences in epitope processing were also observed between the structural protein (CME) construct and the envelope protein (E) construct. The flavivirus-cross-reactive epitope defined by MAb 813 was expressed in cells infected with recombinant viruses containing LI structural genes but not in cells infected with viruses containing LI envelope gene only. On the other hand, expression of the epitope defined by MAb 34.1 varies with the type of construct as well as the cell in which the envelope protein was expressed. Some of the neutralizing and protective epitopes (MAbs 3.3, 4.2, 7.1 and 7.5) displayed by the authentic E protein were also detected on the recombinant proteins expressed in either insect or mammalian cells.

As the recombinant glycoprotein was expressed on the surface of both infected insect and mammalian cells, it seemed reasonable to assume that the genetically-engineered envelope protein would be a good immunogen. To test this hypothesis, rabbits and mice were immunized against recombinant LI envelope protein expressed in insect or mammalian cells. Rabbits, which had been immunized against recombinant vaccinia virus (Vaccinia-E) or Sf cell lysate infected with recombinant baculovirus (Baculovirus-E), developed antibodies which recognized authentic LI envelope protein on immunoblots. However, the rabbit antisera showed no neutralizing activity against LI virus. Moreover, passive immunization of mice with rabbit antisera conferred no protection on the animals against challenge with LI or TBE viruses.

Immunization of mice against Vaccinia-E failed to induce binding antibodies reactive with authentic LI envelope protein despite the fact that the animals were immunized against vaccinia virus. In contradistinction, mice immunized against Sf cell lysate infected with Baculovirus-E, like their rabbit counterpart, developed binding antibodies against authentic LI envelope protein. This difference in the mouse immune response is probably due to the higher level of envelope protein expression by the recombinant baculovirus. However, despite these differences in antibody response in immunized mice, neutralizing antibodies against LI virus were not detected in mice whether or not they were immunized against Vaccinia-E or LI envelope protein expressed in insect cells. Since many of the previous expression studies on flaviviral E protein demonstrated a poor correlation between neutralizing antibodies and animal protection, mice immunized against recombinant LI envelope protein were also subjected to challenge with either LI or TBE virus. No protection against these viruses was observed. In addition, none of the rabbits, which had been immunized against either recombinant vaccinia containing LI structural genes (Vaccinia-CME) or infected Sf cell lysate expressing LI premembrane and E proteins, developed binding or neutralizing antibodies against authentic LI envelope protein.

The absence of neutralizing and protective immune responses in animals immunized against recombinant LI envelope protein, which expressed epitopes capable of inducing neutralizing and protective antibodies when present in authentic antigen, suggests that these antigenic determinants in the recombinant protein were not immunogenic. The recombinant LI envelope protein was probably not in the correct conformation to induce neutralizing antibodies and protective immune responses in recipient animals. The poor immunogenicity of recombinant LI envelope protein in the induction of neutralizing antibodies correlated with studies on expressed dengue 2 (Deubel *et al.*, 1988b), dengue 4 (Zhao *et al.*, 1987; Zhang *et al.*, 1988; Bray *et*

al., 1989) and yellow fever (Hahn *et al.*, 1990) envelope proteins. In these studies, either low titres of neutralizing antibodies were detected in some but not all immunized animals or no significant neutralizing activities were found. However, in contrast to tick-borne and other mosquito-borne flaviviruses, JE envelope protein expressed by recombinant baculovirus (Matsuura *et al.*, 1989; McCown *et al.*, 1990) or vaccinia virus (Yasuda *et al.*, 1990) was effective in stimulating the immunized animals to develop neutralizing antibodies. It is likely that as distinct from other flaviviruses, recombinant JE envelope protein retained its neutralizing epitopes in a highly immunogenic form as suggested by Yasuda *et al.* (1990). With respect to the induction of protective immune responses by expressed flaviviral envelope protein, LI behaved quite differently from the mosquito-borne flaviviruses. Mice, which had been immunized against recombinant vaccinia viruses expressing dengue 4 (Bray *et al.*, 1989) or JE (Yasuda *et al.*, 1990) envelope proteins in the absence of expressed NS1, were protected against challenge with the respective homologous viruses whereas recombinant LI envelope proteins expressed by baculovirus and vaccinia virus failed to elicit protective immune responses in mice.

One of the possible explanations for the poor immunogenicity of recombinant LI envelope protein despite the expression of epitopes capable of inducing neutralizing and protective antibodies when present in authentic antigen, may be that the genetically-engineered envelope protein was not expressed as a polymeric complex, which had been reported for envelope proteins of TBE (Heinz and Kunz, 1980) and West Nile (Wengler *et al.*, 1987) viruses. Significant difference in immunogenicity between monomeric and multimeric protein complexes had previously been demonstrated for Semliki Forest virus (Morein *et al.*, 1978). Della Porta and Westaway (1977) had also reported failure of monomers or dimers of envelope glycoprotein of Kunjin virus to stimulate a detectable antibody response while large complexes

elicited an antibody response similar to complete virions. In view of the evidence that glycosylated premembrane, envelope and NS1 proteins of flaviviruses were processed by endoplasmic signalase cleavage of polyprotein precursor (Rice *et al.*, 1985, 1986b) and that NS1 was co-retained in an early secretory compartment with immature forms of E in Japanese encephalitis virus infected cells (Mason, 1989), it is tempting to speculate that maturation of the envelope protein of some flaviviruses, which probably involves assembly into an immunogenic polymer, may depend on interactions among glycosylated structural proteins (E and prM) and nonstrucutral NS1. Indeed, the importance of premembrane and NS1 proteins in the maturation of envelope protein was recently substantiated by Mason *et al.* (1991), who showed the induction of high titres of neutralizing antibodies and high levels of protection against lethal JE virus infection in mice which had been immunized against recombinant vaccinia virus expressing premembrane, envelope, NS1 and NS2A proteins of JE virus. These authors also proposed that C protein produced in the absence of viral genomic RNA would interfere with the proper assembly of flaviviral membrane proteins. Hence, the presence of capsid gene in the LI recombinant virus constructs may inhibit the maturation and assembly of E protein to an immunogenic form. In addition, the absence of NS1 in the LI recombinant virus constructs is likely to contribute significantly to the apparent lack of protective immune responses in animals immunized against recombinant E protein. Purified viral NS1 protein alone had been shown effective in protecting animals against both yellow fever (Schlesinger *et al.*, 1986) and dengue viruses (Schlesinger *et al.*, 1987). Moreover, mice which had been immunized against a bacterially synthesized yellow fever NS1 protein fragment were protected against challenge with yellow fever virus (Cane and Gould, 1988). In addition, mice immunized against recombinant vaccinia virus expressing dengue virus NS1 were also protected against lethal dengue virus encephalitis through the mechanism of complement-mediated

lysis of infected cells (Falgout *et al.*, 1990). The importance of NS1 in protection against tick-borne flaviviruses and potential detrimental effect of capsid gene on immunogenicity of envelope protein are recently supported by study on recombinant vaccinia expressing C, prM/M, E and NS1 genes of TBE virus. Mice, immunized against recombinant vaccinia carrying TBE structural and NS1 genes, developed low levels of TBE E-specific antibodies although they were protected from TBE virus challenge (A. S. Belyaev, personal communication). Hence future investigations are needed to clarify the roles of structural and NS1 proteins in the maturation of LI envelope protein and the immune mechanisms involved in the protection of animals against tick-borne flaviviruses. Probably, the development of recombinant virus expression systems containing LI prM, E and NS1 genes would help to answer some of these problems.

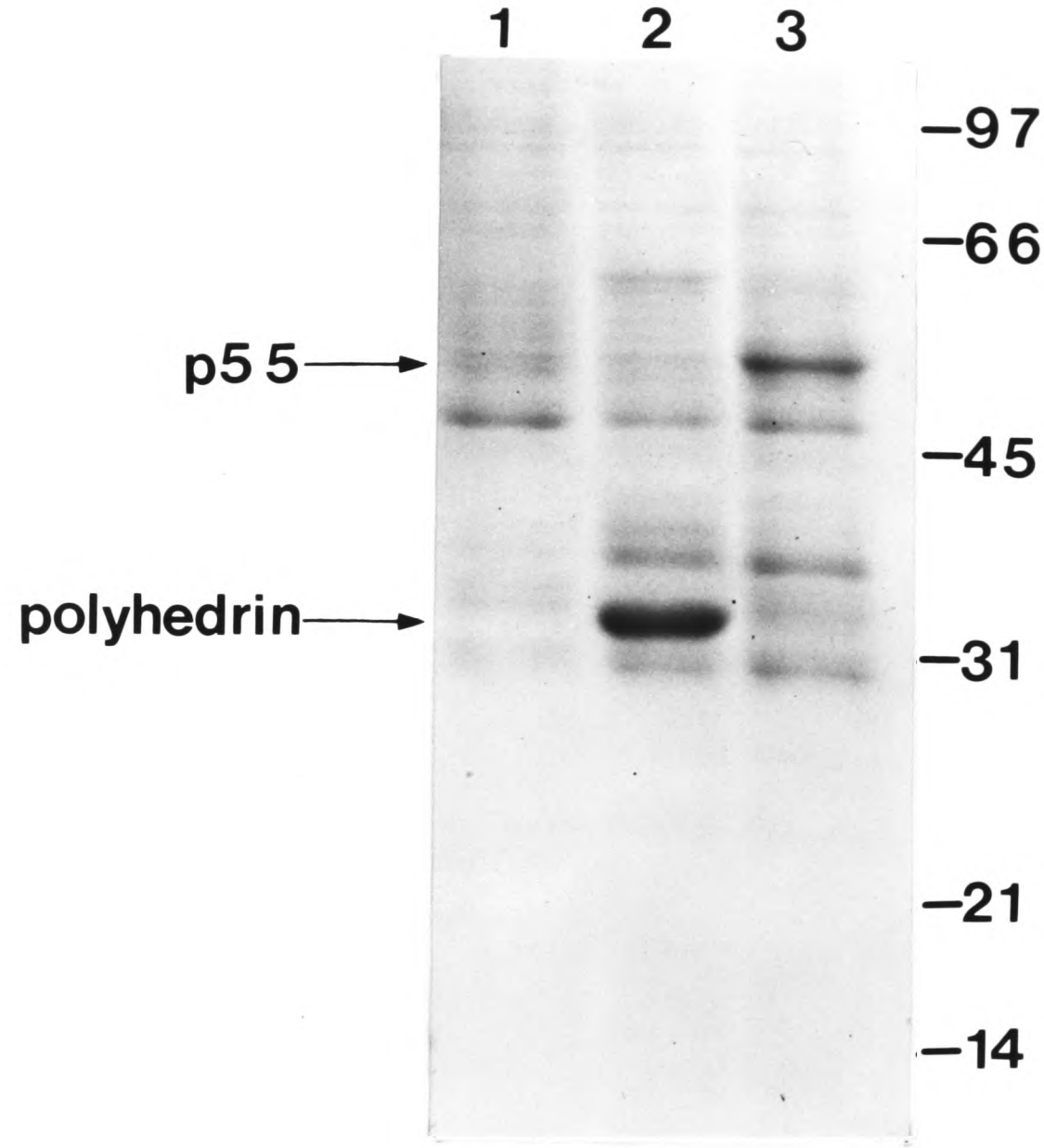

Fig. 14. Coomassie Blue stained protein gel of Sf cell lysate infected with recombinant baculovirus containing LI envelope gene (Baculovirus-E). Track 1, non-infected Sf cell lysate; track 2, Sf cell lysate infected with wild type baculovirus (Baculovirus-W); track 3, Sf cell lysate infected with Baculovirus-E.

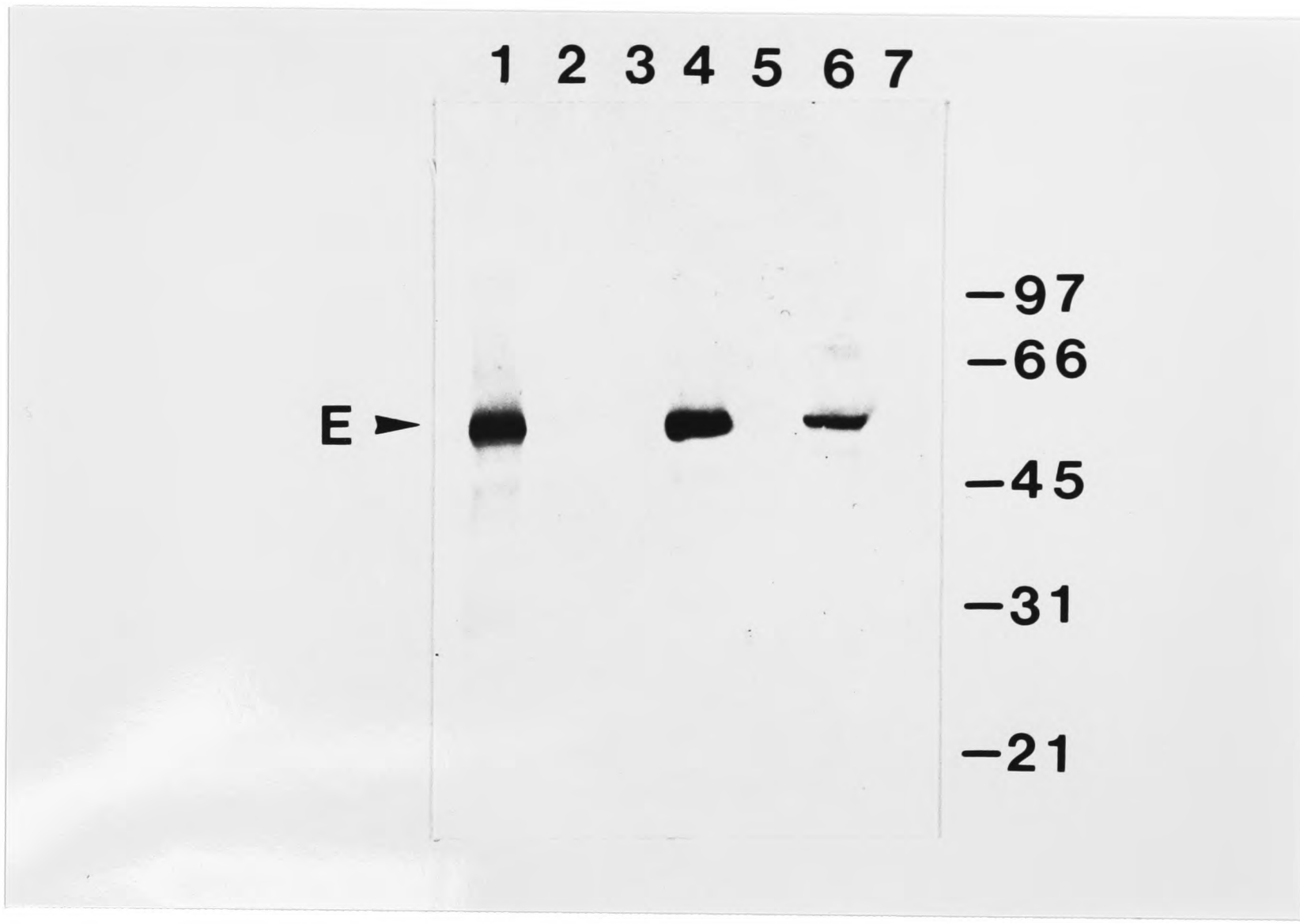

Fig. 15. Immunoblot of recombinant baculovirus (Baculovirus-E) infected Sf cell lysate with LI E-specific MAb 4.2. Track 1, Sf cell lysate infected with recombinant baculovirus containing LI envelope gene (Baculovirus-E); track 2, Sf cell lysate infected with wild type baculovirus (Baculovirus-W); track 3, non-infected Sf cell lysate; track 4, purified LI virus; track 5, tissue culture medium from non-infected PS cells; track 6, PS cell lysate infected with LI virus; track 7, non-infected PS cell lysate; E - envelope protein.

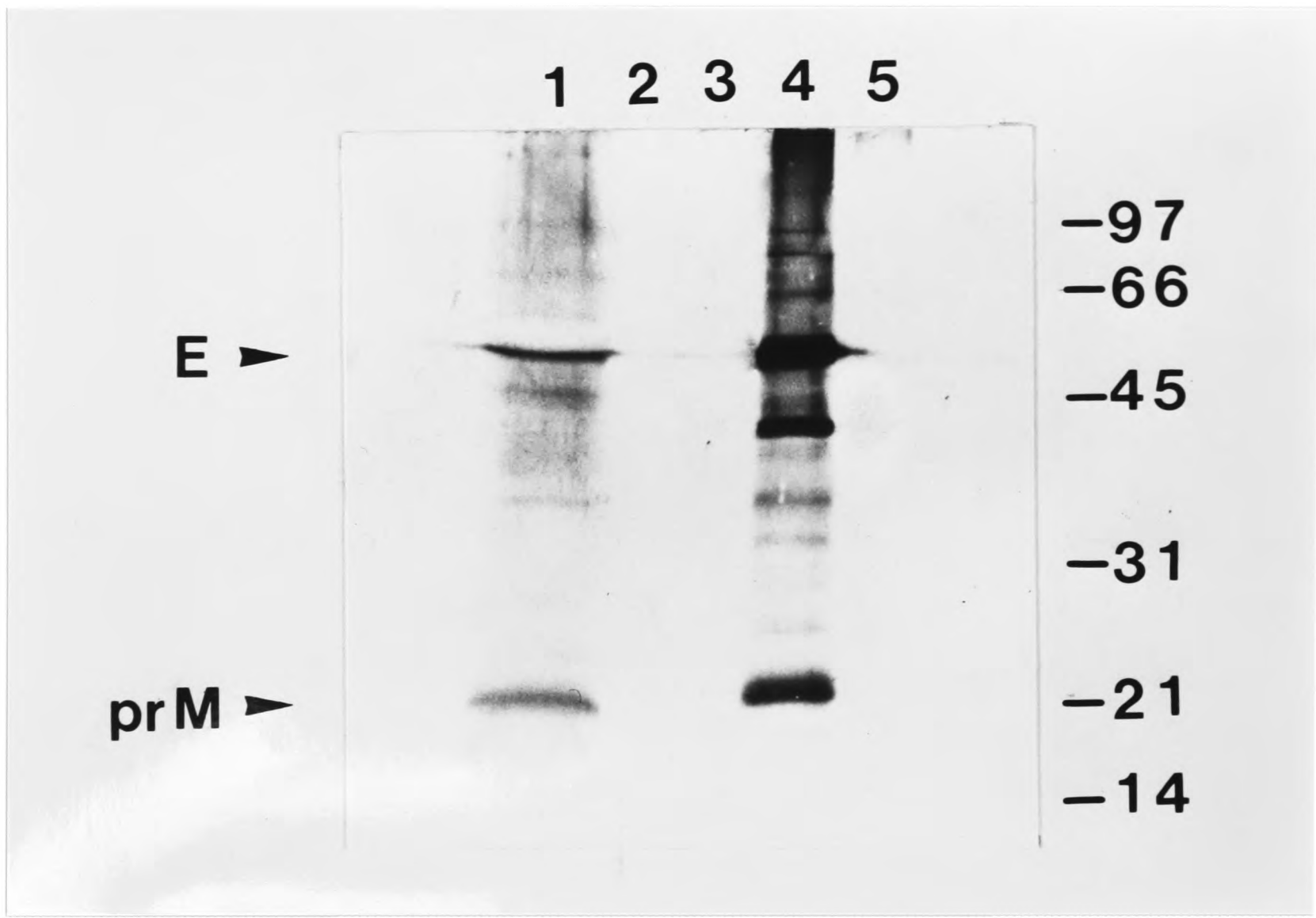

Fig. 16. Immunoblot of recombinant baculovirus (Baculovirus-CME) infected Sf cell lysate with rabbit polyclonal antiserum against LI virus. Track 1, Sf cell lysate infected with recombinant baculovirus containing LI structural genes (Baculovirus-CME); track 2, Sf cell lysate infected with wild type baculovirus (Baculovirus-W); track 3, non-infected Sf cell lysate; track 4, PS cell lysate infected with LI virus; track 5, non-infected PS cell lysate. E - envelope protein; prM - premembrane protein.

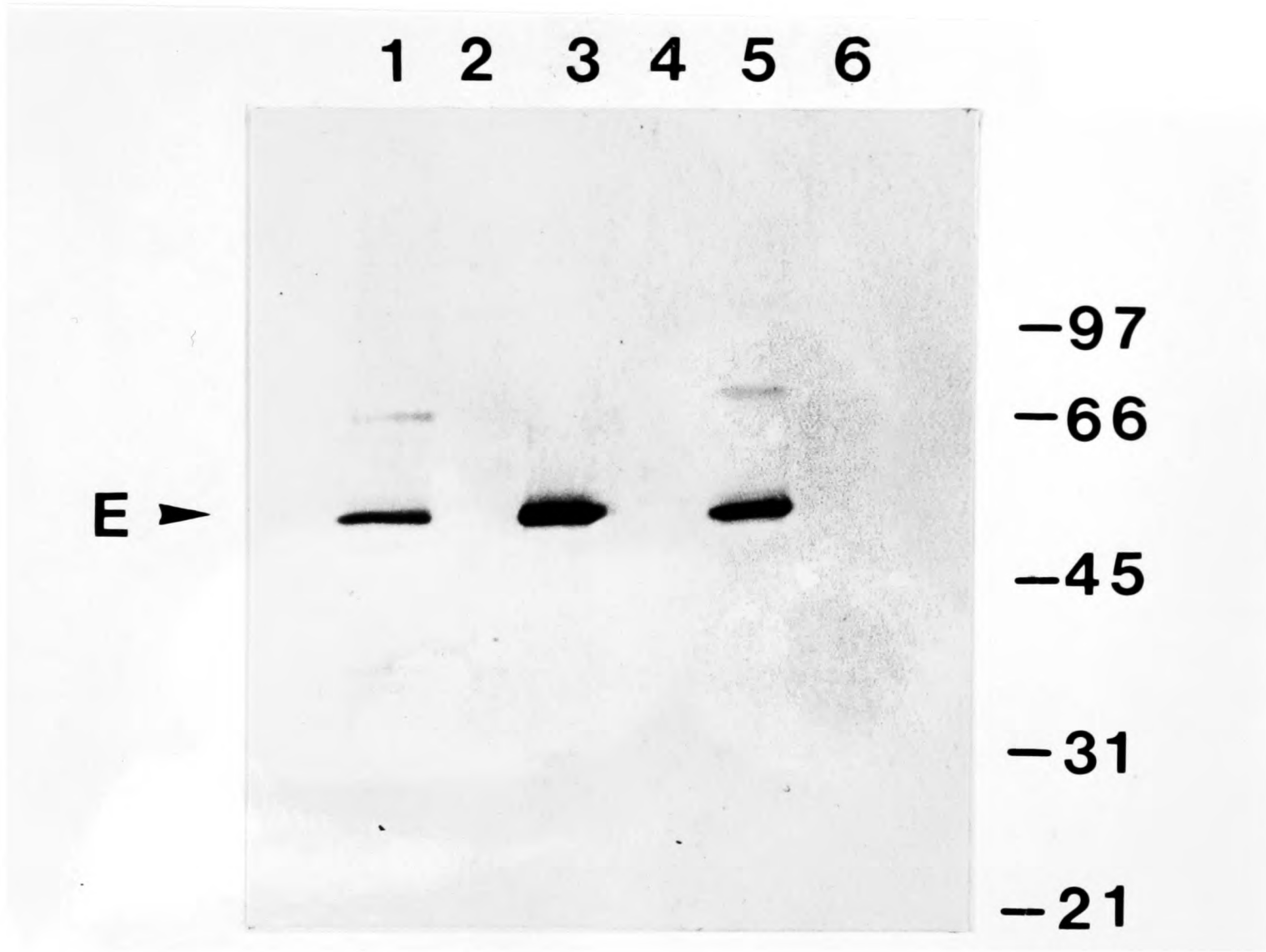

Fig. 17. Immunoblot of recombinant vaccinia virus (Vaccinia-E) infected CV-1 cell lysate with LI E-specific MAb 4.2. Track 1, CV-1 cell lysate infected with recombinant vaccinia virus containing LI envelope gene (Vaccinia-E); track 2, CV-1 cell lysate infected with wild type vaccinia virus (Vaccinia-W); track 3, purified LI virus; track 4, tissue culture medium from non-infected PS cells; track 5, PS cell lysate infected with LI virus; track 6, non-infected PS cell lysate; E - envelope protein.

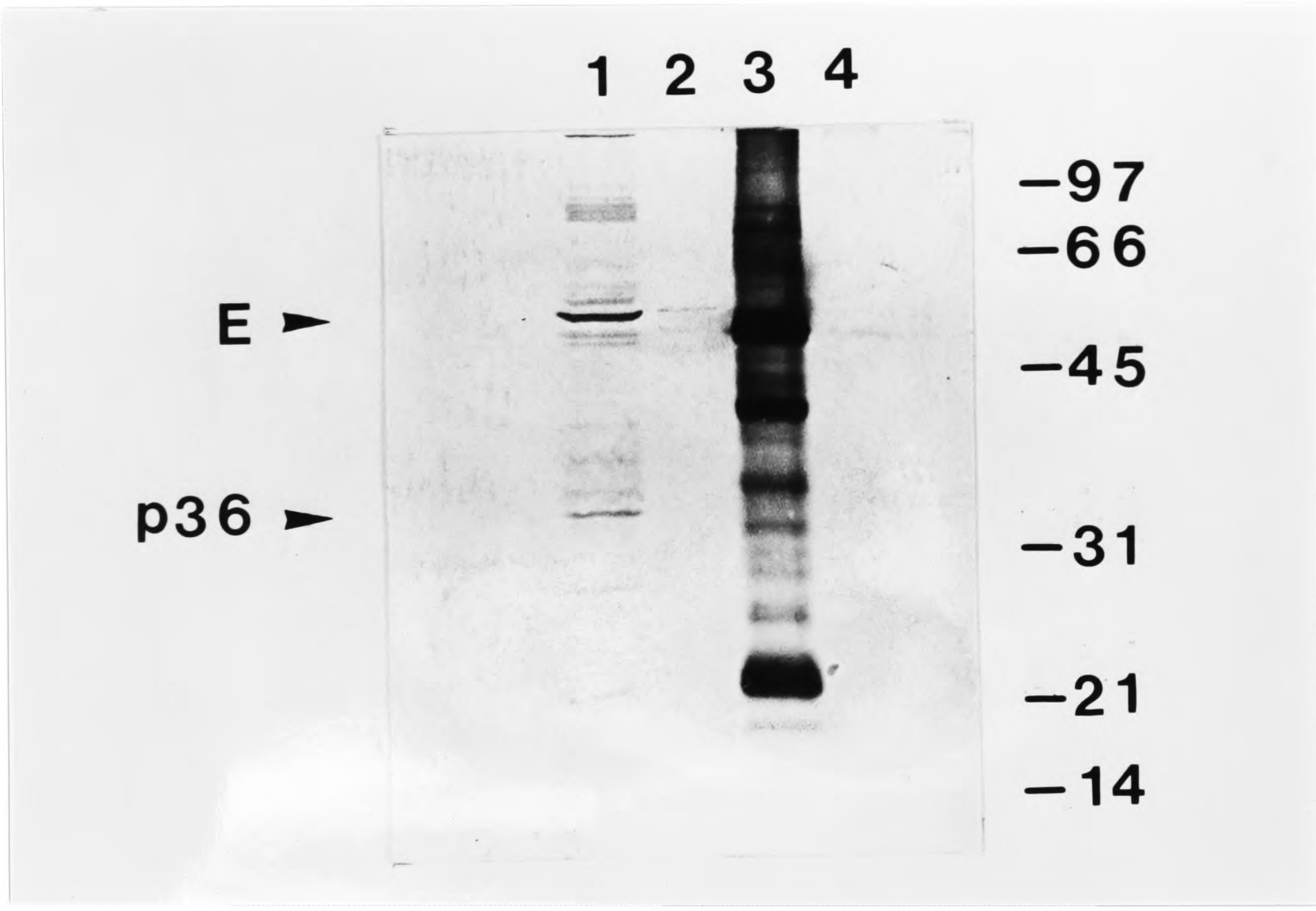

Fig. 18. Immunoblot of recombinant vaccinia virus (Vaccinia-CME) infected CV-1 cell lysate with rabbit polyclonal antiserum against LI virus. Track 1, CV-1 cell lysate infected with recombinant vaccinia virus containing LI structural genes (Vaccinia-CME); track 2, CV-1 cell lysate infected with wild type vaccinia virus (Vaccinia-W); track3, PS cell lysate infected with LI virus; track 4, non-infected PS cell lysate; E - envelope protein.

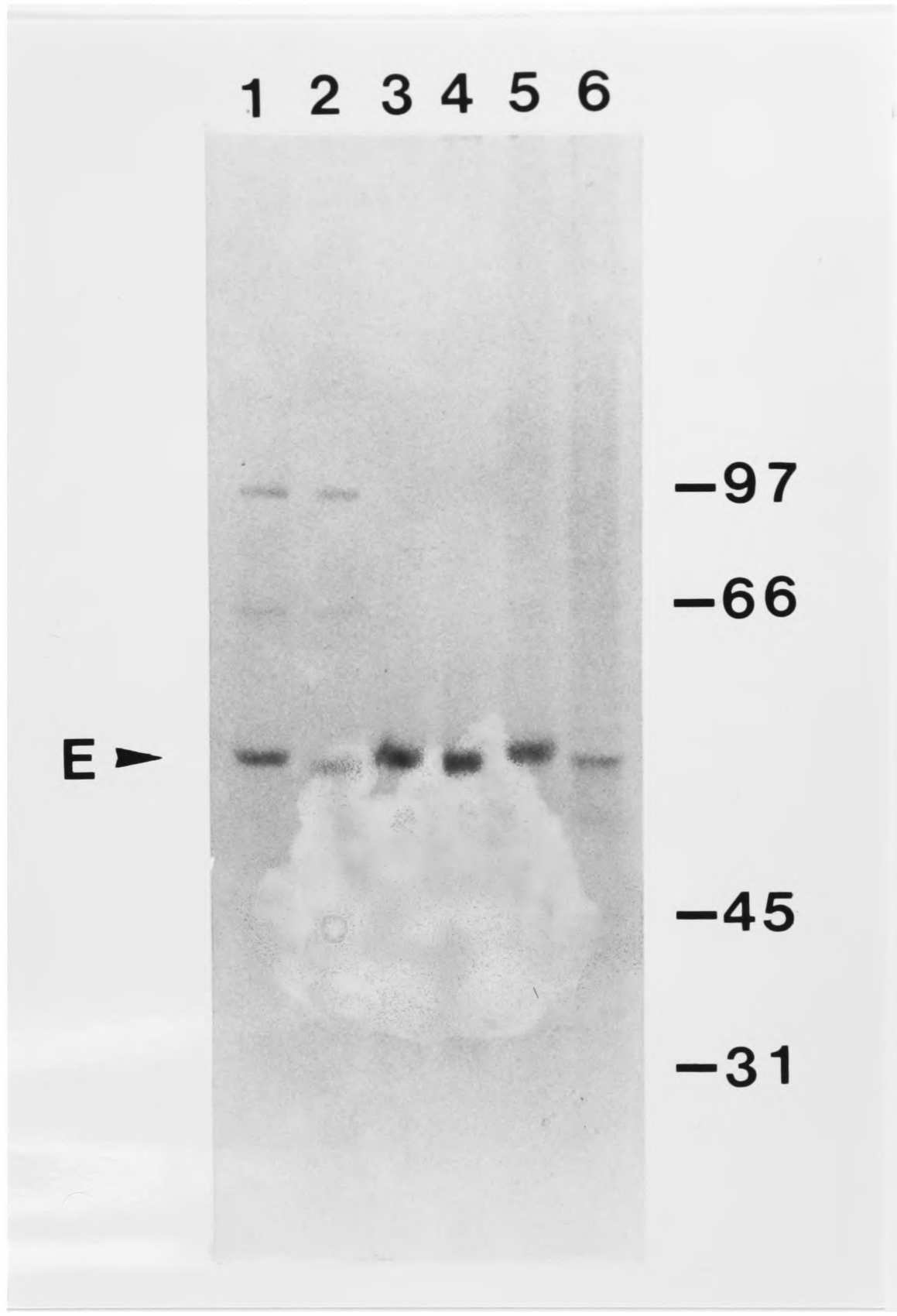

Fig. 19. Effect of tunicamycin on the mobilities of immunoprecipitated radiolabelled envelope proteins expressed by recombinant baculovirus and vaccinia virus containing LI envelope gene. Track 1, labelled PS cell lysate infected with LI virus in the absence of tunicamycin in the cell culture medium; track 2, labelled PS cell lysate infected with LI virus in the presence of tunicamycin in the cell culture medium; track 3, labelled Sf cell lysate infected with recombinant baculovirus containing LI envelope gene (Baculovirus-E) without tunicamycin in the cell culture medium; track 4, labelled Sf cell lysate infected with Baculovirus-E in the presence of tunicamycin in the cell culture medium; track 5, labelled CV-1 cell lysate infected with recombinant vaccinia virus containing LI envelope gene (Vaccinia-E) without tunicamycin in the cell culture medium; track 6, labelled CV-1 cell lysate infected with Vaccinia-E in the presence of tunicamycin in the cell culture medium; E - envelope protein.

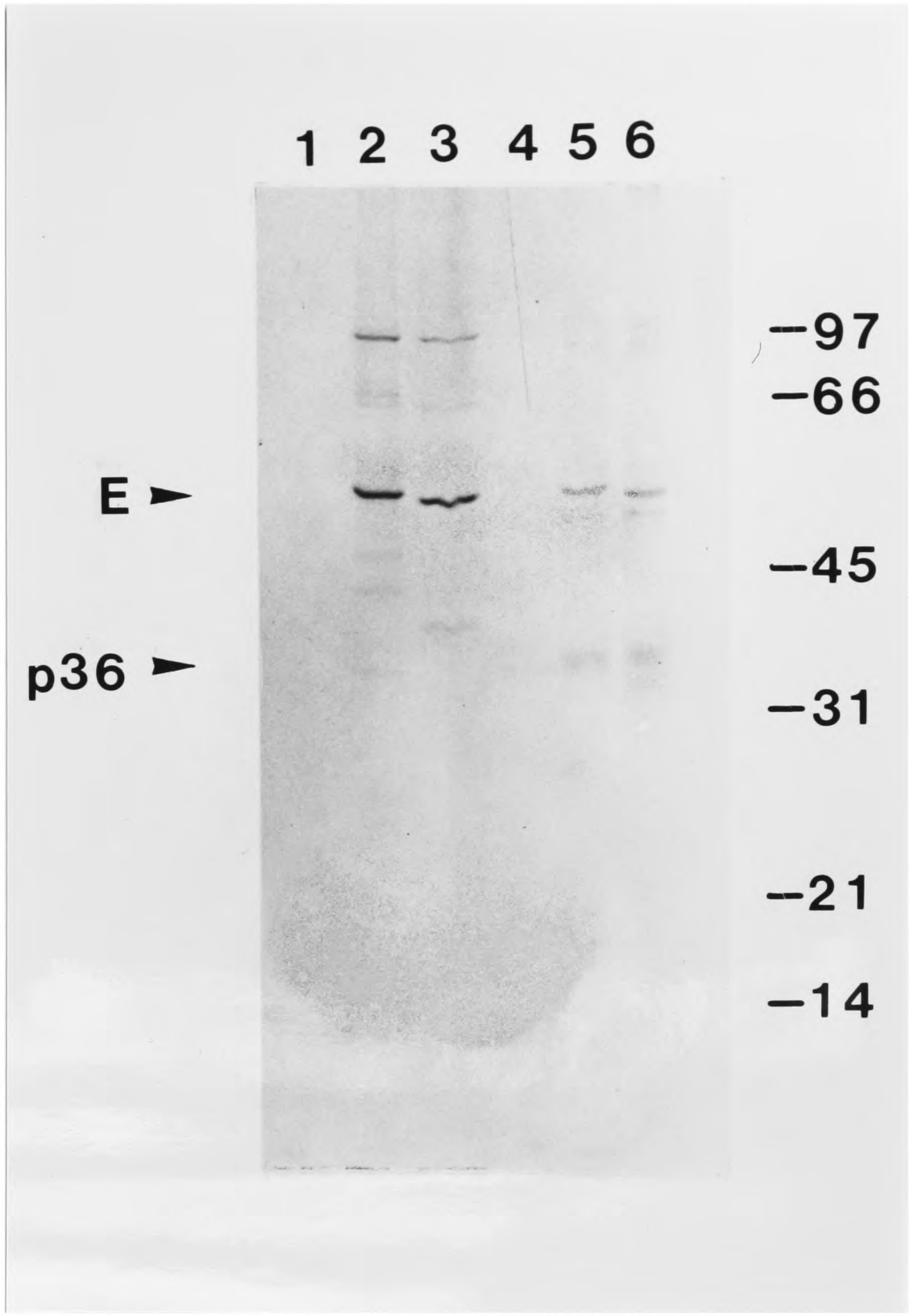

Fig. 20. Effect of tunicamycin on the mobility of immunoprecipitated radiolabelled envelope protein expressed by recombinant baculovirus containing LI structural genes (Baculovirus-CME). Track 1, labelled non-infected PS cell lysate; track 2, labelled PS cell lysate infected with LI virus in the absence of tunicamycin in the cell culture medium; track 3, labelled PS cell lysate infected with LI virus in the presence of tunicamycin in the cell culture medium; track 4, labelled Sf cell lysate infected with wild type baculovirus (Baculovirus-W); track 5, labelled Sf cell lysate infected with Baculovirus-CME in the absence of tunicamycin in the cell culture medium; track 6, labelled Sf cell lysate infected with Baculovirus-CME in the presence of tunicamycin in the cell culture medium;E - envelope protein.

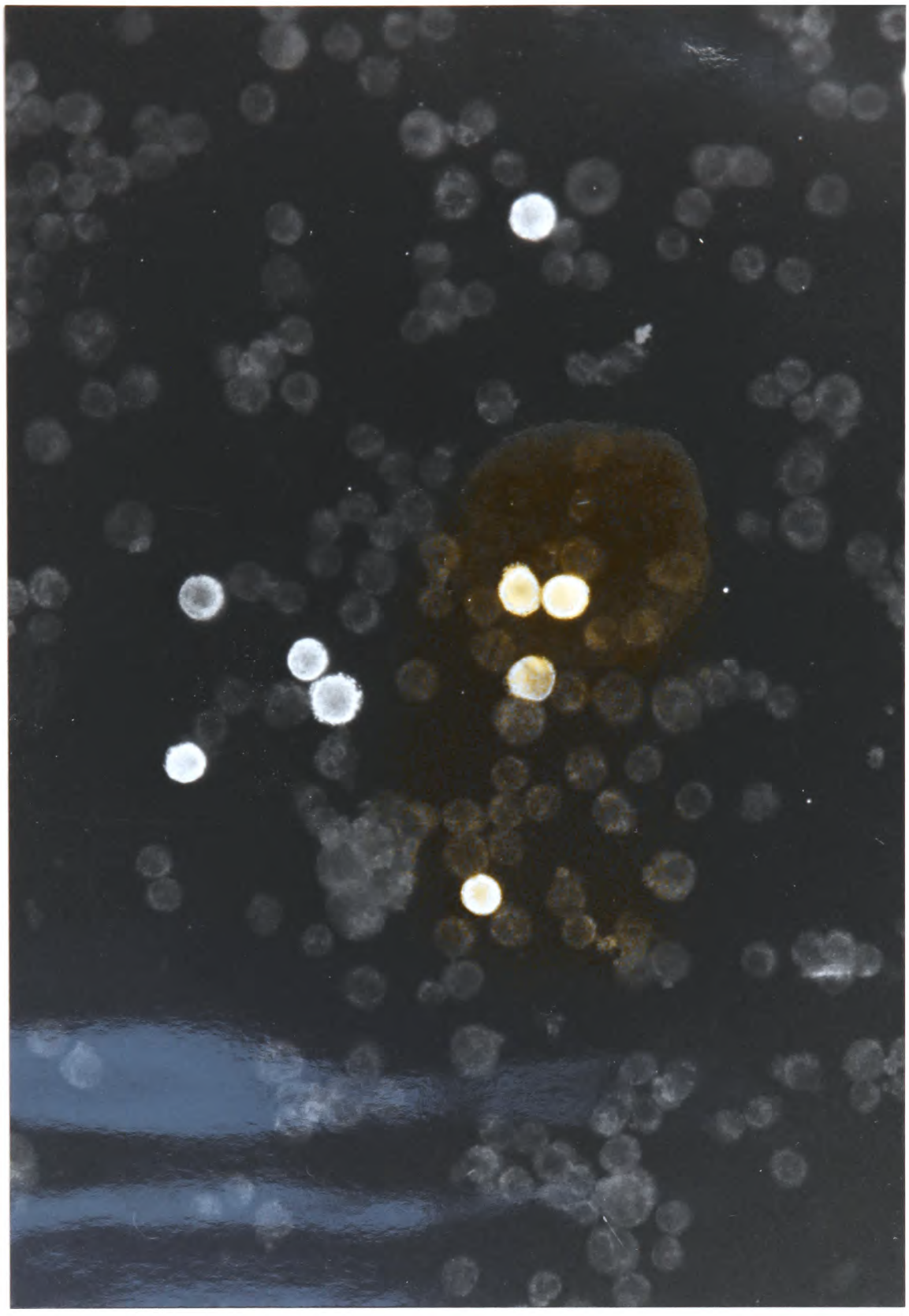

Fig. 21. Indirect immunofluorescence of acetone-fixed Sf cells infected with recombinant baculovirus containing LI envelope gene. Similar pattern was observed for recombinant baculovirus containing LI structural genes.

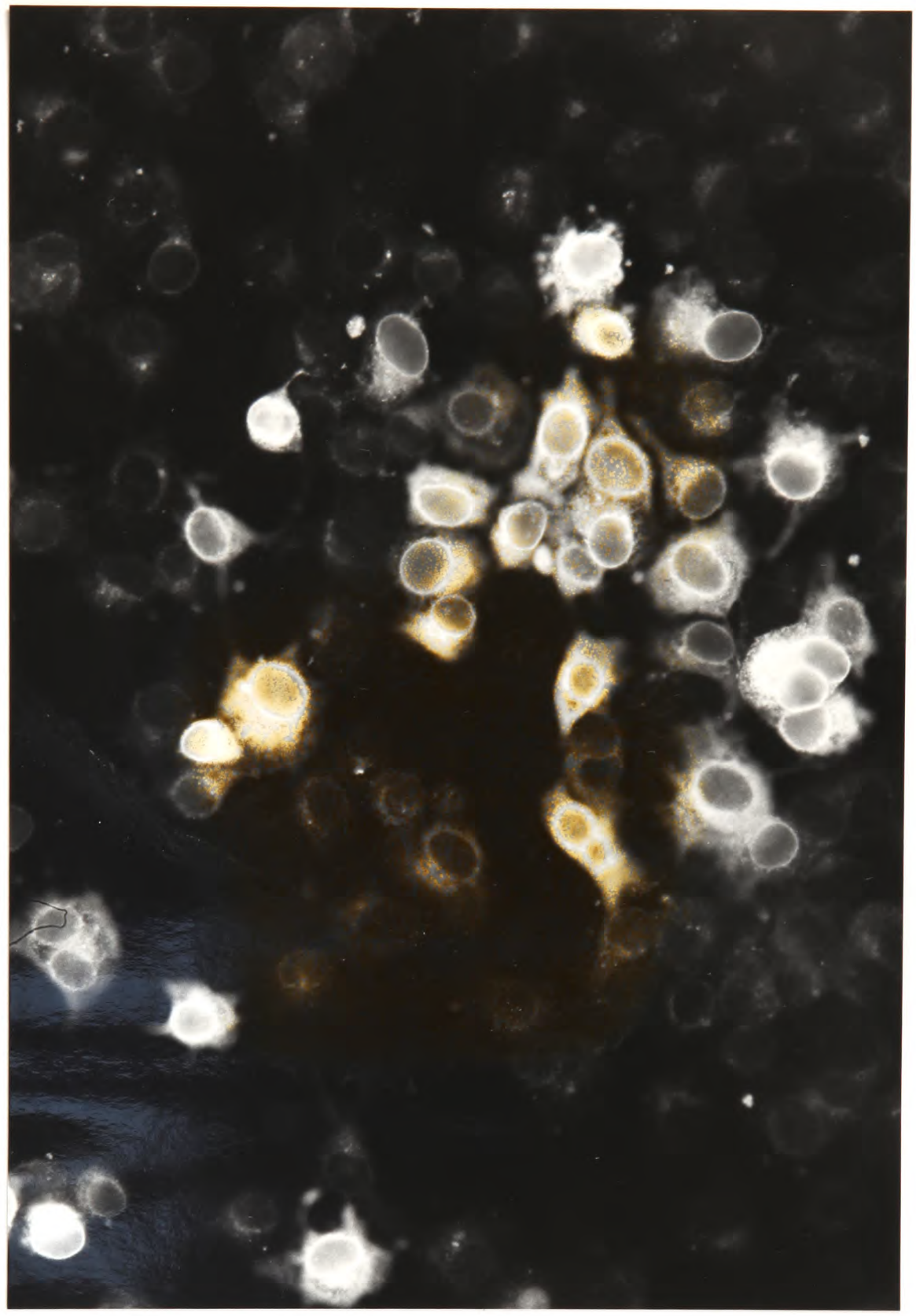

Fig. 22. Indirect immunofluorescence of acetone-fixed CV-1 cells infected with recombinant vaccinia virus containing LI envelope gene. Similar pattern was observed for recombinant vaccinia virus containing LI structural genes.

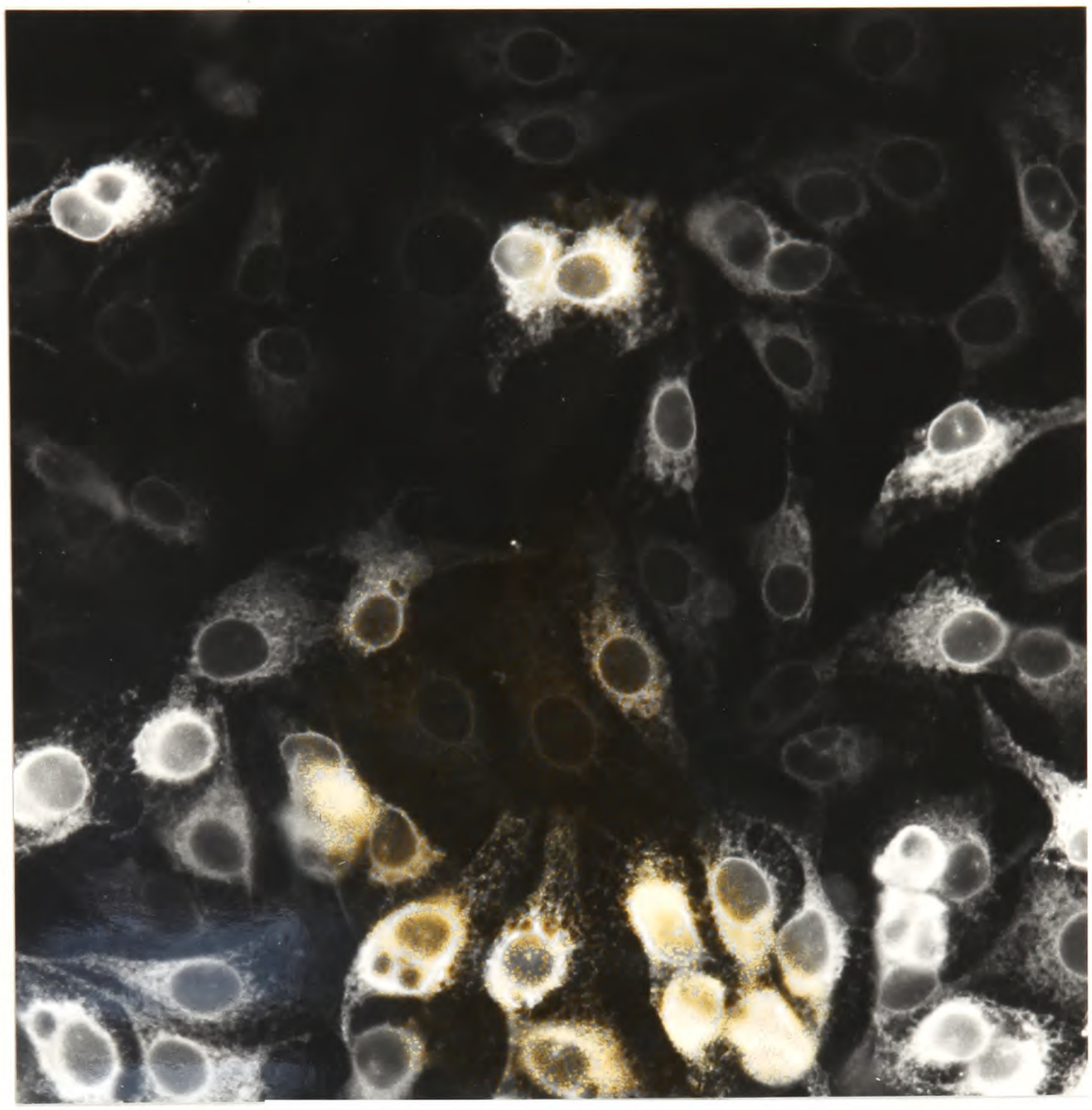

Fig. 23. Indirect immunofluorescence of acetone-fixed PS cells infected with louping ill (LI) virus.

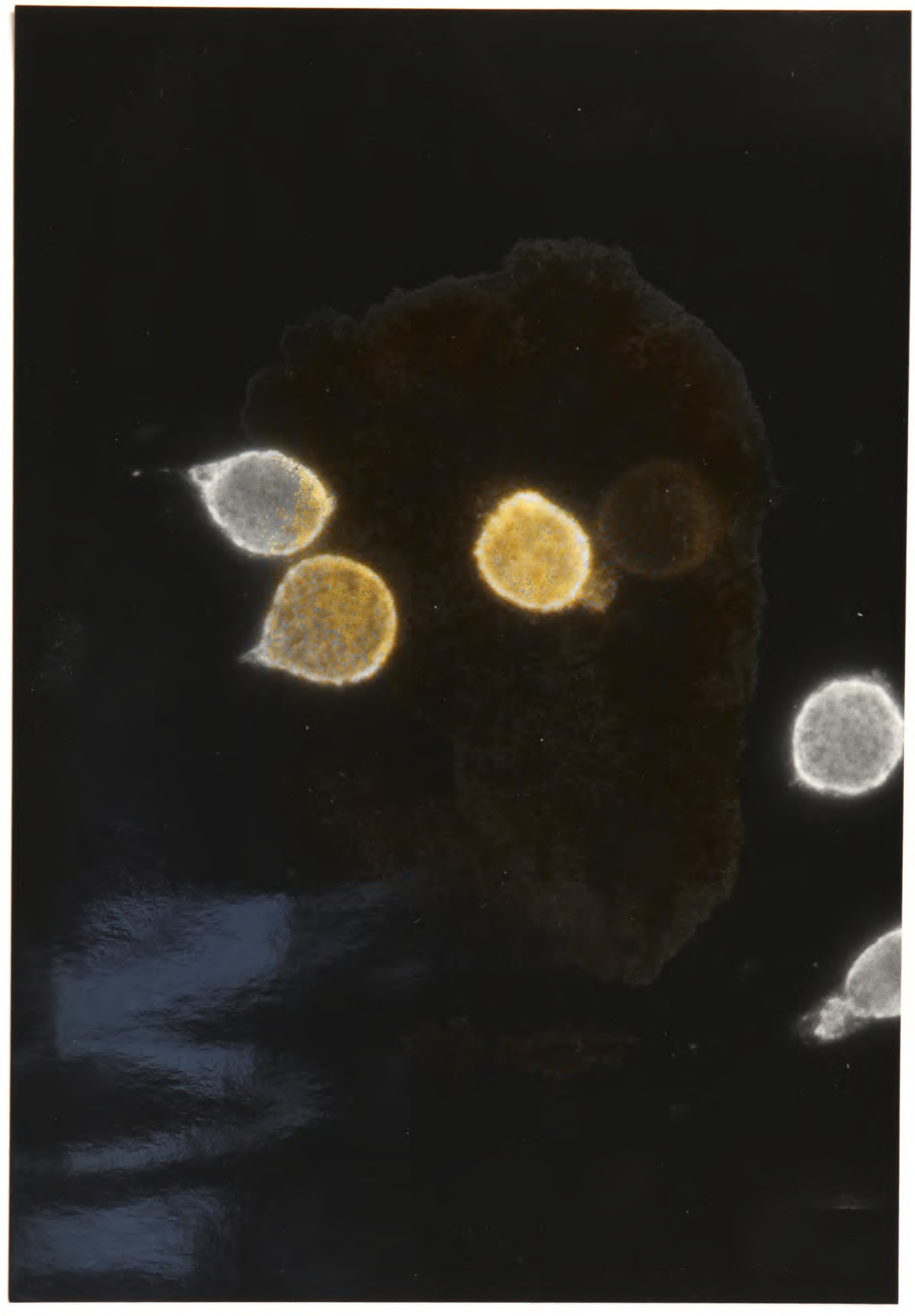

Fig. 24. Indirect immunofluorescence of non-fixed Sf cells infected with recombinant baculovirus containing LI envelope gene. Similar pattern was observed for recombinant baculovirus containing LI structural genes.

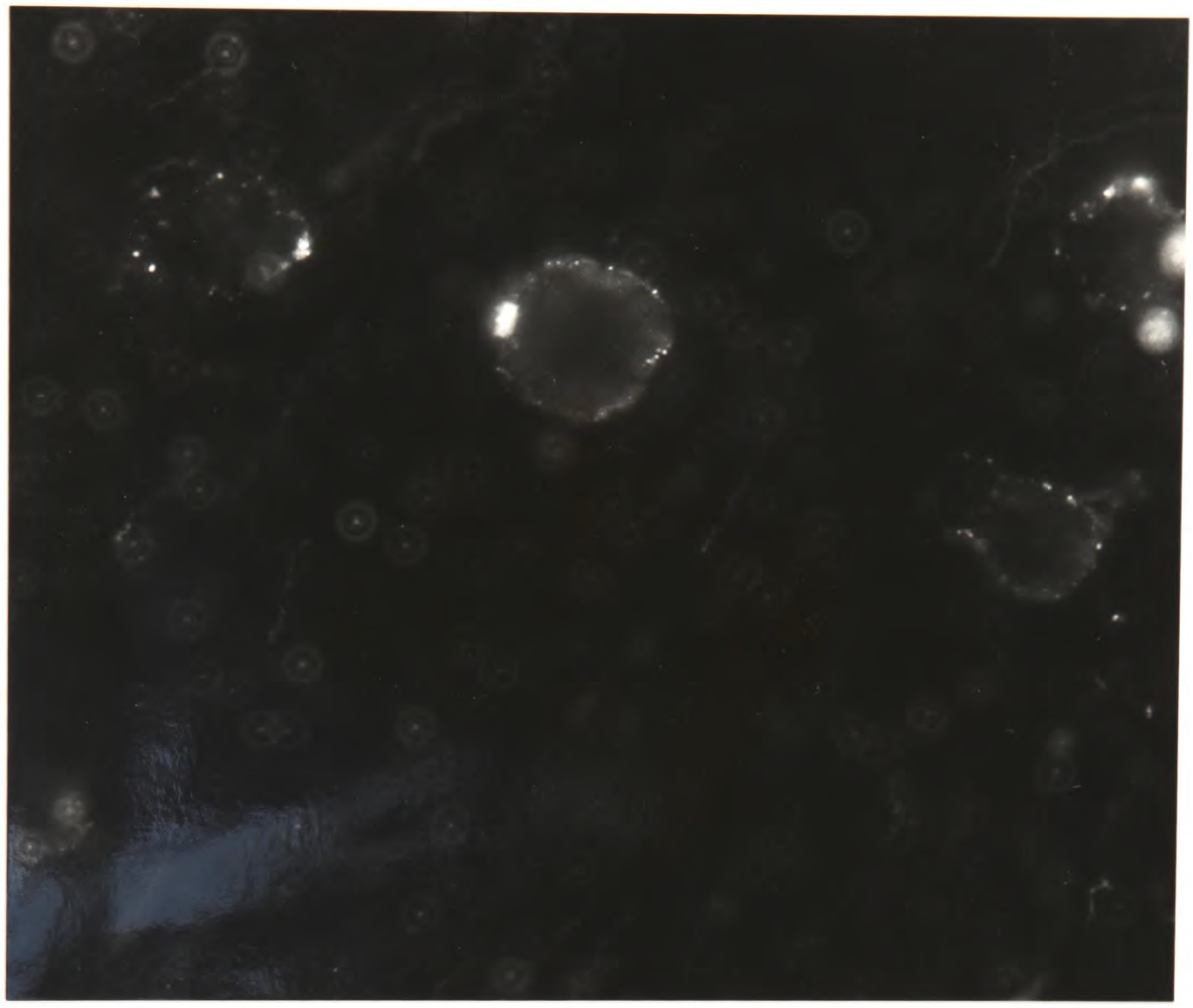

Fig. 25. Indirect immunofluorescence of non-fixed CV-1 cells infected with recombinant vaccinia virus containing LI envelope gene. Similar pattern was observed for recombinant vaccinia virus containing LI structural genes.

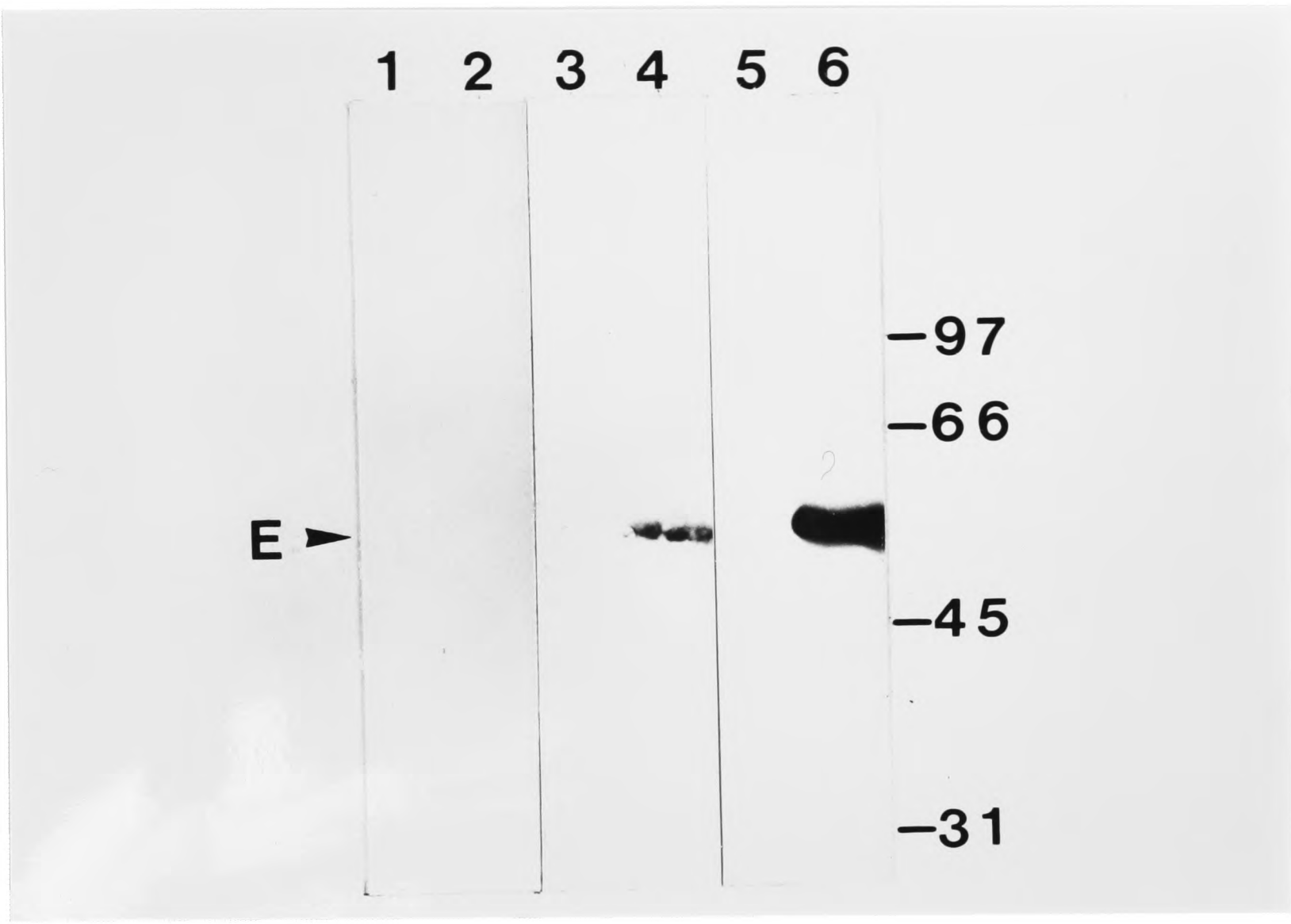

Fig. 26. Western blot analysis of pooled antisera from mice immunized against Sf cell lysate infected with recombinant baculovirus containing LI envelope gene (Baculovirus-E). Track 1, tissue culture medium from non-infected PS cells probed with pooled antisera from mice immunized against Sf cell lysate infected with wild type baculovirus (Baculovirus-W), with or without adjuvant; track 2, purified LI virus probed with pooled antisera from mice immunized against Sf cell lysate infected with Baculovirus-W, with or without adjuvant; track 3, tissue culture medium from non-infected PS cells probed with pooled antisera from mice immunized against Sf cell lysate infected with Baculovirus-E, with or without adjuvant; track 4, purified LI virus probed with pooled antisera from mice immunized against Sf cell lysate infected with Baculovirus-E, with or without adjuvant; track 5, tissue culture medium from non-infected PS cells probed with LI E-specific MAb 4.2; track 6, purified LI virus probed with LI E-specific MAb 4.2; E - envelope protein.

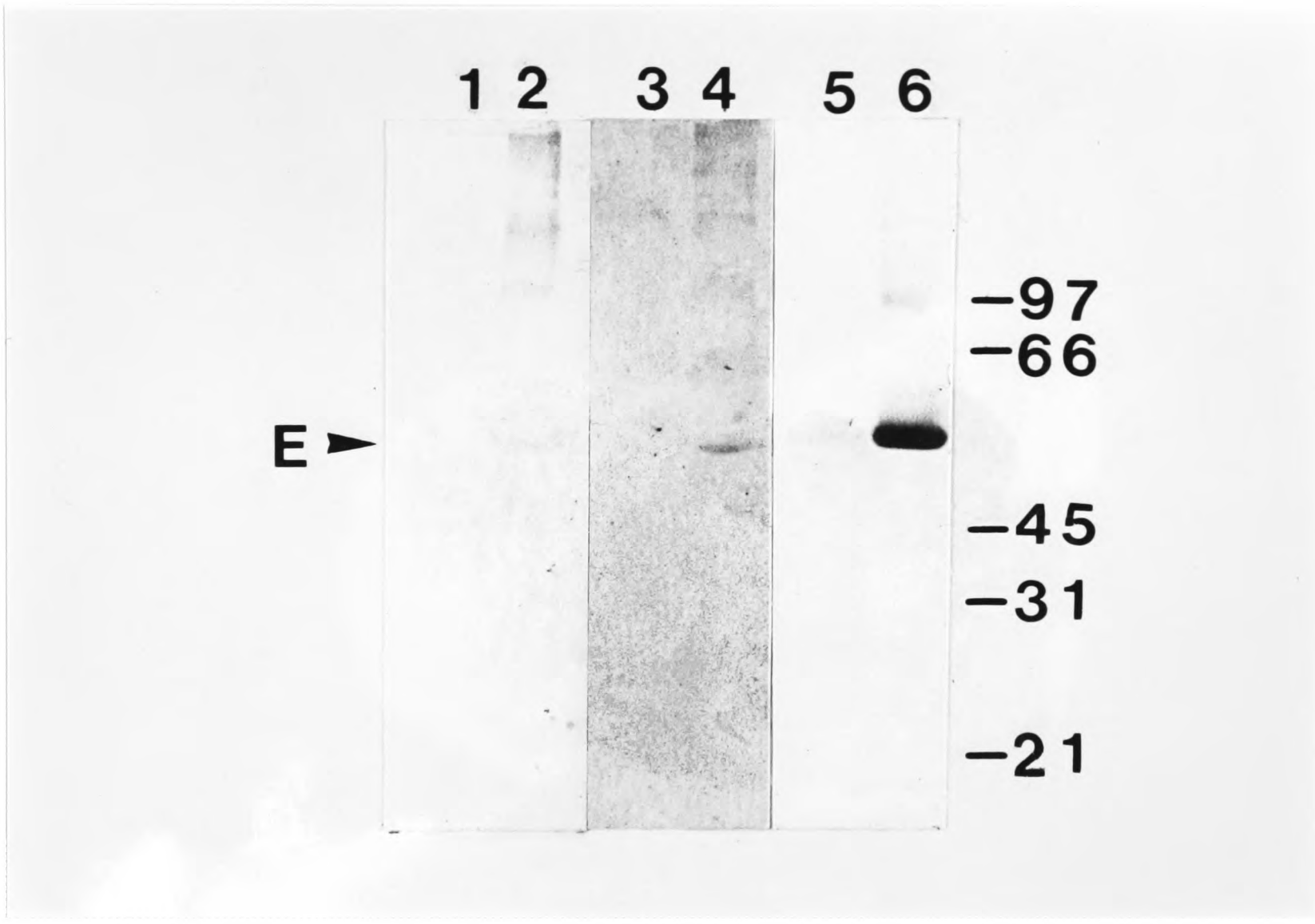

Fig. 27. Western blot analysis of antiserum from rabbit immunized against recombinant vaccinia virus containing LI envelope gene (Vaccinia-E). Track 1, tissue culture medium from non-infected PS cells probed with antiserum from rabbit immunized against wild type vaccinia virus (Vaccinia-W); track 2, purified LI virus probed with antiserum from rabbit immunized against Vaccinia-W; track 3, tissue culture medium from non-infected PS cells probed with antiserum from rabbit immunized against Vaccinia-E; track 4, purified LI virus probed with antiserum from rabbit immunized against Vaccinia-E; track 5, tissue culture medium from non-infected PS cells probed with LI E-specific MAb 4.2; track 6, purified LI virus probed with LI E-specific MAb 4.2; E - envelope protein.

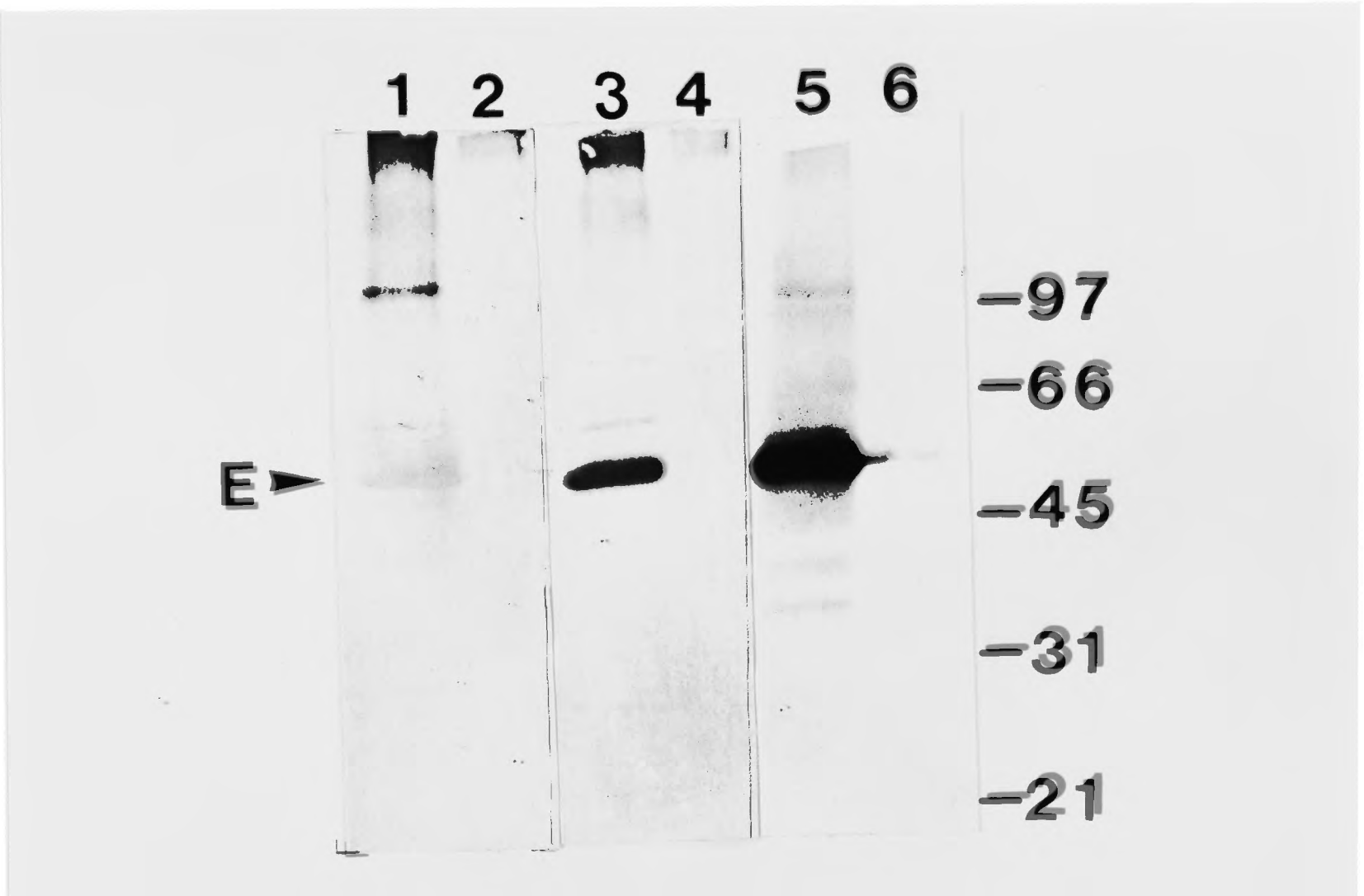

Fig. 28. Western blot analysis of antiserum from rabbit immunized against Sf cell lysate infected with recombinant baculovirus containing LI envelope gene (Baculovirus-E). Track 1, purified LI virus probed with antiserum from rabbit immunized against Sf cell lysate infected with wild type baculovirus (Baculovirus-W); track 2, tissue culture medium from non-infected PS cells probed with antiserum from rabbit immunized against Sf cell lysate infected with Baculovirus-W; track 3, purified LI virus probed with antiserum from rabbit immunized against Sf cell lysate infected with Baculovirus-E; track 4, tissue culture medium from non-infected PS cells probed with antiserum from rabbit immunized against Sf cell lysate infected with Baculovirus-E; track 5, purified LI virus probed with LI E-specific MAb 4.2; track 6, tissue culture medium from non-infected PS cells probed with LI E-specific MAb 4.2; E - envelope protein.

Table 3. Reactivities of E-specific MAbs with recombinant baculoviruses and vaccinia viruses containing louping ill (LI) envelope (E) and structural (CME) genes examined by indirect immunofluorescence (IF) tests

MAbs	*In vitro* Neutralization vs. LI	Mouse Protection vs. LI	Baculovirus- E (IF)	Vaccinia -E (IF)	Baculovirus - CME (IF)	Vaccinia -CME (IF)	Louping ill (IF)
3.1	+	-	-	-	-	-	+
3.3	+	-	+	+	+	+	+
4.2	+	+	+	+	+	+	+
7.1	+	+	+	+	+	+	+
7.5	+	-	+	+	+	+	+
T7	-	-	-	+	-	+	+
813	-	-	-	-	+	+	+
34.1	+	-	-	+	+	-	+

+ = positive reaction; - = no reaction detected

Table 4. Characterization of antisera from immunized rabbits and mice

Antisera	Binding antibodies vs. LI	Neutralization titre vs. LI	Neutralization titre vs. vaccinia (strain WR)
Rabbit (Baculovirus - E)	+	<1:10	N.A.
Rabbit (Baculovirus - CME)	-	<1:10	N.A.
Rabbit (Baculovirus - W)	-	<1:10	N.A.
Rabbit (Vaccinia - E)	+	<1:10	1:5000
Rabbit (Vaccinia - CME)	-	<1:10	1:800
Rabbit (Vaccinia - W)	-	<1:10	1:10000
Mice (Baculovirus - E/with or without adjuvant)	+	<1:10	N.A.
Mice (Baculovirus - W/with or without adjuvant)	-	<1:10	N.A.
Mice (Vaccinia - E)	-	<1:10	1:40
Mice (Vaccinia - W)	-	<1:10	1:80

* Neutralization titres of all preimmune rabbit sera against LI and vaccinia (strain WR) viruses were <1:10
+ = positive reaction; - = no reaction detected
N.A. - Not appropriate

Table 5. Mouse passive protection assays using antisera from rabbits immunized against cell lysate infected with wild type baculovirus (Baculovirus - W) or recombinant baculovirus containing LI envelope gene (Baculovirus - E)

Antisera	Average survival time of TO mice challenged with LI virus (Days ± S.E.M.*)	No. of survivors/ Total (%)
Rabbit-Baculovirus - E	12.25 ± 0.99	8/20 (40%)
Rabbit-Baculovirus - W	10.67 ± 0.67	11/20 (55%)
Antisera	Average survival time of TO mice challenged with TBE virus (Days ± S.E.M.*)	No. of survivors/ Total (%)
Rabbit-Baculovirus - E	8.80 ± 0.25	0/10 (0%)
Rabbit-Baculovirus - W	8.75 ± 0.16	0/8 (0%)

* S.E.M. Standard Error of Mean

Table 6. Mouse passive protection assays using antisera from rabbits immunized against wild type vaccinia virus (Vaccinia-W) or recombinant vaccinia virus containing LI envelope gene (Vaccinia - E)

Antisera	Average survival time of PO mice challenged with LI virus (Days ± S.E.M.*)	No. of survivors/ Total (%)
Rabbit-Vaccinia - E	9.11 ± 0.48	1/10 (10%)
Rabbit-Vaccinia - W	9.18 ± 0.48	1/12 (8%)
Antisera	Average survival time of TO mice challenged with TBE virus (Days ± S.E.M.*)	No. of survivors/ Total (%)
Rabbit-Vaccinia - E	8.40 ± 0.16	0/10 (0%)
Rabbit-Vaccinia - W	8.50 ± 0.22	0/10 (0%)

* S.E.M. Standard Error of Mean

Table 7. Challenge of mice, immunized against cell lysate infected with wild type baculovirus (Baculovirus -W) or recombinant baculovirus containg LI envelope gene (Baculovirus - E), with louping ill (LI) virus

Intracerebral LI challenge in TO mice immunized against	Average Survival Time (Days ± S.E.M.*)	No. of survivors/ Total (%)
1. Sf cell lysate infected with Baculovirus - E and Adjuvant	5.78 ± 0.28	0/9 (0%)
2. Sf cell lysate infected with Baculovirus - E	5.78 ± 0.22	0/9 (0%)
3. Sf cell lysate infected with Baculovirus - W and Adjuvant	6.00 ± 0.17	0/9 (0%)
4. Sf cell lysate infected with Baculovirus - W	5.89 ± 0.26	0/9 (0%)
5. Sf cell lysate and Adjuvant	5.90 ± 0.18	0/10 (0%)
6. Sf cell lysate	5.70 ± 0.15	0/10 (0%)
7. Non-immunized	5.67 ± 0.17	0/9 (0%)

* S.E.M. Standard Error of Mean

Table 8. Challenge of mice, immunized against cell lysate infected with wild type baculovirus (Baculovirus - W) or recombinant baculovirus containing LI envelope gene (Baculovirus - E), with tick-borne encephalitis (TBE) virus

Intraperitoneal TBE challenge in TO mice immunized against	Average Survival Time (Days ± S.E.M.*)	No. of survivors/ Total (%)
1. Sf cell lysate infected with Baculovirus - E and Adjuvant	9.33 ± 0.41	0/9 (0%)
2. Sf cell lysate infected with Baculovirus - E	9.20 ± 0.39	0/10 (0%)
3. Sf cell lysate infected with Baculovirus - W and Adjuvant	9.10 ± 0.28	0/10 (0%)
4. Sf cell lysate infected with Baculovirus - W	8.80 ± 0.20	0/10 (0%)

* S.E.M. Standard Error of Mean

Table 9. Challenge of mice, immunized against wild type vaccinia virus (Vaccinia - W) or recombinant vaccinia virus containing LI envelope gene (Vaccinia - E), with tick-borne flaviviruses

	Average Survival Time (Days ± S.E.M.*)	No. of survivors/ Total (%)
Intracerebral LI challenge in TO mice immunized against		
Vaccinia - E	6.20 ± 0.13	0/10 (0%)
Vaccinia - W	6.10 ± 0.18	0/10 (0%)
PBS	6.00 ± 0.32	0/5 (0%)
Intraperitoneal TBE challenge in TO mice immunized against		
Vaccinia - E	10.00 ± 0.65	0/10 (0%)
Vaccinia - W	10.00 ± 0.56	0/10 (0%)

* S.E.M. Standard Error of Mean

CHAPTER 5

GENERAL DISCUSSION

AND

FUTURE RESEARCH

CONTENTS PAGE

5.1 Genomic Structure and Evolutionary Relationships of Tick-Borne Flaviviruses

The report on the nucleotide sequence of 17D yellow fever virus by Rice *et al.* (1985) was a milestone in studies of flaviviruses. Since then, the rapid accumulation of sequence data on flaviviruses has led to a better understanding of the genomic structure and organization of members of the family. Although multiple internal initiation of viral protein translation may occur for Kunjin virus (Westaway, 1977), it is now more widely accepted that flaviviruses share a common genomic organization of a single open reading frame with the gene order of translation as follows: 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (Rice *et al.*, 1985; 1986b). Moreover, sequence information has facilitated the study of evolutionary relationships among flaviviruses at the genetic level.

In this thesis, the genomic sequence of the structural proteins of louping ill (LI), a tick-borne flavivirus, was reported. The results showed that LI virus shares the same genomic organization as other flaviviruses, at least, in the structural region. Sequence comparisons of LI envelope protein showed greater homology with tick-borne than mosquito-borne flaviviruses and greater homology with the western than the far eastern subtypes of TBE virus. Homology data, therefore, correlate well with the conventional classification of LI virus in the tick-borne serocomplex. With the capsid and membrane proteins, the degree of homology between LI and the western subtype was greater than that between the two TBE subtypes, indicating very close evolutionary relationships between LI virus and the western subtype of TBE virus. Thus, LI and TBE viruses may be varieties of a common tick-borne ancestral virus.

On the other hand, the average amino acid sequence diversity between members of the tick-borne serogroup was significantly lower than that of

mosquito-borne serogroups. Hence a distinctive difference in flavivirus genome stability could be detected between tick-borne and mosquito-borne viruses despite the observed overall stability of the flavivirus genome when compared with the high mutation rate reported for other RNA viruses (Holland *et al.*, 1982). This could be partly related to differences in the biology of different arthropod vectors. The fact that mosquitoes have a much shorter life cycle as compared to ticks (Kettle, 1984) would imply that the transmission of a mosquito-borne virus from an arthropod vector to a mammalian host and back to an arthropod vector would occur much faster than a tick-borne virus. Hence, mosquito-borne virus is then probably exposed to more changes in immune selection pressure imposed by the hosts in the natural environment than tick-borne virus. This may also explain why homology data of the capsid and membrane proteins, which are less exposed to immune surveillance than E protein, are more useful than the envelope protein in predicting the evolutionary relationships between tick-borne flaviviruses.

5.2 Putative Receptor Binding Site and Potential Genetic Marker of Tick-Borne Flaviviruses

Comparative analysis of the envelope sequence data between the tick-borne flaviviruses revealed two hypervariable peptides (amino acids 81-88 and 230-234) in the antigenic domain A of TBE virus envelope protein (Heinz, 1986). In addition, a hexapeptide EHLPTA (amino acids 207-212), which is conserved in TBE and LI viruses but absent in mosquito-borne flaviviruses, was also identified. The two hypervariable peptides are in close proximity to EHLPTA in the conformation-dependent antigenic domain A of the molecular-epitope model of TBE envelope protein (Mandl *et al.*, 1989b). The hypervariable-conserved-hypervariable sequence structure on the tick-borne flavivirus envelope protein reported in this thesis is reminiscent of the receptor binding sites of foot and mouth disease virus (Fox *et al.*, 1989) and on

the haemagglutinin of influenza virus (Weis *et al.*, 1988). The conserved peptide in the viral attachment proteins of these viruses is the binding domain whereas the flanking hypervariable regions represent the binding sites of neutralizing antibodies. Antigenic sites, identified on the basis of amino acid substitutions in different strains of influenza virus (Wiley *et al.*, 1981), do occur in proximity to the receptor-binding pocket of haemagglutinin. Indirect evidence also exists suggesting that the two hypervariable peptides in the putative tick-borne flavivirus receptor binding site may encode antigenic determinants. The hypervariable peptide 81-88 falls within the antigenic domain I defined in the dengue type 1 virus envelope protein by bacterial expression studies (Mason *et al.*, 1990) and an amino acid substitution has been reported in the hypervariable peptide 230-234 in a neutralization-resistant escape mutant of TBE virus. Correlation of sequence diversity and antigenic diversity has also been reported by Roehrig *et al.* (1990) for dengue-2 envelope protein. Hence, it would be interesting to see if synthetic peptides corresponding to these hypervariable regions could induce antibodies that could differentiate between tick-borne flaviviruses. Whether or not these hypervariable and conserved peptides indeed constitute part of the receptor binding site of tick-borne flaviviruses requires future research on the three-dimensional crystallographic structure of E protein, the identification and molecular characterization of tick-borne flavivirus receptor(s) and site-directed mutagenesis studies on the receptor binding properties of the envelope protein. Moreover, the conserved hexapeptide EHLPTA, detected in the envelope sequences of LI and TBE viruses, may provide a useful genetic marker for the identification of other tick-borne flaviviruses and may indicate whether or not flaviviruses described as "no known vector" (Porterfield, 1987) are likely to be transmitted by ticks. The importance of EHLPTA as a genetic marker is recently supported by sequence data from tick-borne flaviviruses like Negishi (K. Venugopal, personal communication) and Langat (C. W.

Mandl, personal communication) viruses, which also show conservation of EHLPTA in their E protein sequences . Comparative analysis of sequence data from other known and suspected tick-borne flaviviruses in future, will prove whether or not EHLPTA is useful as a genetic marker for these viruses.

5.3 Recombinant Envelope Protein of Tick-Borne Flavivirus

Many of the flaviviruses which are important human pathogens still pose significant health problems throughout the world. Hence there is a demand for the development of safe and effective vaccines against these viruses, in particular Japanese encephalitis, dengue and TBE viruses. The production of large quantities of viral antigens safely and efficiently by recombinant DNA technology will provide excellent opportunities for conducting biological studies to evaluate the potential of these antigens as "second generation vaccines". Recombinant protein expression studies also increase our understanding of the functions of many flavivirus encoded proteins and their interactions with each other in the biology of the virus. In this thesis, the envelope protein of LI virus was expressed in both insect and mammalian cells using recombinant baculovirus and vaccinia virus expression systems. The recombinant E protein, which is processed in the endoplasmic reticulum, was transported to the surface of both insect and mammalian cells. Indirect immunofluorescence microscopy with characterized monoclonal antibodies was used to demonstrate that many of the epitopes of the authentic antigen were also expressed by the recombinant protein. However, some of the epitopes were absent on the recombinant E protein. This contrasts with the expressed E proteins of Japanese encephalitis (Yasuda *et al.*, 1990) and yellow fever (Shiu *et al.*, 1991b) viruses which are antigenically indistinguishable from the authentic antigen. In addition, LI recombinant E protein, under the experimental conditions employed, failed to induce neutralizing and protective antibodies in rabbits as well as protective immune responses in

mice against challenge with tick-borne flaviviruses. Hence, it is likely that the expressed protein did not take up the totally correct immungenic and antigenic conformation of the authentic antigen. Comparative analysis of antigenic reactivities between recombinant viruses containing different LI gene constructs also showed that the antigenic conformation of the recombinant LI envelope protein is partly dependent on the processing of other structural proteins in addition to E protein. The recent report by Mason *et al.* (1991) that highly immunogenic Japanese encephalitis E protein is expressed by vaccinia recombinants containing premembrane, envelope and NS1 genes seems to demonstrate the importance of both premembrane and NS1 proteins in the maturation of recombinant flavivirus E protein to its authentic antigenic and immunogenic conformation. These authors also suggest that C protein, produced in the absence of viral genomic RNA, would interfere with the proper assembly of the viral membrane proteins. The absence of NS1 and the presence of capsid gene in the LI recombinant virus constructs may then explain the observed poor immunogenicity of LI envelope proteins expressed by these recombinant viruses despite the fact that MAbs were antigenically reactive with many epitopes present on the recombinant antigen. Construction of recombinant virus expression systems containing LI prM, E and NS1 genes in future will prove whether or not the maturation of recombinant LI envelope protein, like the JE virus, is dependent on the interactions between glycosylated structural and NS1 proteins. This may then form the basis for the development of recombinant vaccine against tick-borne flavivirus pathogens.

5.4 Future Research

The work reported in this thesis on the molecular studies of louping ill virus increase not only our understanding of the genetic relationships of tick-borne flaviviruses but also some of the functional characteristics of recombinant

envelope protein. The identification of potentially important domains of E protein based on sequence analysis will undoubtedly stimulate further detailed investigations on the exact functional roles played by these domains in the biology of tick-borne flaviviruses, in particular, virus-receptor interactions. The use of appropriate synthetic peptides corresponding to these domains will help to define and correlate more precisely the structural and functional properties of E protein. Perhaps, development of the infectious clone of LI or TBE virus in the near future will prove the most useful in the molecular genetic analysis of tick-borne flavivirus replication and in the precise definition of sequence changes responsible for phenotypic variations among different strains of LI or TBE viruses. The information obtained will be invaluable to the design of second generation vaccines against tick-borne and probably other flavivirus pathogens. Finally, continuous efforts on the improvement of expression vectors will soon allow the feasibility and practicability of multivalent vaccines against flaviviruses to be realized.

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