CHARACTERISATION AND ANALYSIS OF THE NS1 GENE OF TICK-BORNE ENCEPHALITIS VIRUS

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A thesis submitted in partial fulfilment of the requirements of the Oxford Brookes University for the degree of Doctor of Philosophy.

This research programme was carried out in collaboration

With

The Centre for Applied Microbiology and Research,

Porton Down.

November 1992

Infectious disease is one of the few genuine adventures left in the world. The dragons are all dead and the lance grows rusty in the chimney corner.....About the only sporting proposition that remains unimpaired by the relentless domestication of a once free-living human species is the war against those ferocious little fellow creatures, which lurk in the dark corners and stalk us in the bodies of rats, mice, and all kinds of domestic animals; which fly and crawl with the insects, and waylay us in our food and drink and even our love.

Hans Zinsser, 1935

ABSTRACT

Tick-borne encephalitis virus (TBEV) encodes a highly immunogenic non-structural glycoprotein, NS1. The proposed NS1 gene from the TBEV strains Neudörfl and K23 was identified, cloned and sequenced. The NS1 gene from the Neudorfl strain of TBEV was then cloned under the powerful constitutive cytomegalovirus (CMV) major immediate-early promoter (IE) and the CMV IEP/NS1 fragment used as the basis of an adenovirus E1a deletion mutant. The novel combination of the cytomegalovirus immediate- early promoter and the adenovirus vector produced extremely high levels of NS1 expression in cells which do not support the replication of the adenovirus deletion mutant. The recombinant protein was shown to be indistinguishable from authentic TBEV NS1 in its (i) apparent molecular weight by polyacrylamide gel electrophoresis, (ii) glycosylation pattern, (iii) ability to form high molecular weight complexes, and (iv) ability to be secreted from cells. Furthermore, appropriate processing of NS1 expressed by the adenovirus recombinant occurred independently of any additional TBEV-encoded gene function.

When inoculated directly into mice, the recombinant adenovirus RAd51 was shown to elicit an antibody response to the TBEV NS1 antigen. Immunization of mice with RAd51 conferred both protection from disease and death when challenged with a lethal dose of TBEV. The capability of RAd51 to elicit an immune response following inoculation into mice was shown to result from de novo synthesised NS1 and not co-inoculated NS1. It has been proposed that protection elicited against TBEV challenge by NS1 is due to complement mediated cytolysis of infected cells. The ability of NS1 to protect mice deficient in the terminal lytic pathway of the complement cascade, implied that complement mediated cytolysis is not the major mechanism by which protection is elicited.

ACKNOWLEDGEMENTS

I am indebted to my mentor Dr G.W.G. Wilkinson and to my other supervisors Dr J.R. Stephenson and Dr L. King. Helpful additional guidance was given by Dr M. Cranage. Mr J. Lee and Miss L. Bradley provided the initial TBEV stock used in this work and the monoclonal antibodies described. Mr M. Dennis arranged the importation of the B10 D2 mice from the USA while Miss J. Sinclare cared for the mice. I wish to thank the PHLS for allowing the work described in this thesis to be performed whilst I worked at the Centre for Applied Microbiology and Research at Porton Down. As ever, I am truly indebted to my husband Andy.

DEDICATION

This thesis is dedicated to Dr P.A. Nuttall, without whom I would never have started, and to my husband Andy without whom I may never have finished.

PUBLICATIONS

Material presented in this thesis has been, or is in the preparation for publication:-

Jacobs, Stephenson and Wilkinson. (1992). High level expression of the tick-borne virus NS1 protein by using an adenovirus-based vector: Protection elicited in a murine model. J. Virol. 66: 2086-2095.

Jacobs, Stephenson, Wilkinson. Sequence of the NS1 gene of the K23 isolate of Tick-Borne Encephalitis Virus: Conservation of Motifs. (1992) in press Arch Virol.

Jacobs, Stephenson, Wilkinson. Characterisation of the Protective Immune Response Elicited in Mice. in Prep.

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ABBREVIATIONS:

ACGM : Advisory Committee on Genetic Manipulations

ADE : Antibody Dependent Enhancement

ATP : Adenosine-5'-Triphosphate

b : Base

ß-Gal : ß-Galactosidase

bp : Base-pair

BSA : Bovine Serum Albumin

C : Core Protein

CAMR : Centre for Applied Microbiology and Research

cDNA : Complementary DNA

CE : Central European (TBEV)

CEF : Chick Embryo Fibroblasts

CIS : Commonwealth of Independent States

CMC : Complement Mediated Cytolysis

CMV : Cytomegalovirus

CNS : Central Nervous System

cpe : Cytopathic Effect

C-terminus : Carboxyl-terminus

CTL : Cytotoxic T Lymphocyte

dATP : Deoxyadenosine Triphosphate

dCTP : Deoxycytosine Triphosphate

ddATP : Dideoxyadenosine Triphosphate

ddCTP : Dideoxycytosine Triphosphate

ddGTP : Dideoxyguanosine Triphosphate

ddNTP : Dideoxyribonucleoside Triphosphate

ddTTP : Dideoxythymidine Triphosphate

dGTP : Deoxyguanosine Triphosphate

DHF : Dengue Heamorrhagic Fever

DMSO : Dimethyl Sulphoxide

dNTP : Deoxyribonucleoside Triphosphate

DSS : Dengue Shock Syndrome

DTT : Dithiothreitol

dTTP : Deoxythymidine Triphosphate

E : Envelope Protein

EDTA : Ethylenediaminetetraacetic Acid [di-sodium] Salt

EndoH : Endoglycosidase H

ER : Endoplasmic Reticulum

FCS : Foetal Calf Serum

FE : Far Eastern (TBEV)

GlycoF : Glycosidase F

GMEM : Glasgows Minimal Essential Media

h : Hour

HBS Buffer : Hepes Buffered Saline Buffer

hpi : Hours Post Infection

ICSP : Intr-Cellular Specific Protein

IE : Immediate Early

IgG : Immunoglobulin G

IgM : Immunoglobulin M

IMS : Industrial methylated Spirit

IP : Intra-Peritoneum

IPTG : Iso-propyl-3-D-thio -galactopyranoside

JEV : Japanese Encephalitis Virus

KFDV : Kyasanur Forest Disease Virus

L15 : Leibovitz 15 media

LIV : Louping Ill Virus

M : Membrane Protein

MEVE : Muray Valley Encephalitis Virus

min : Minute

moi : Multiplicity of Infection

MOPS Buffer : 3-N-Morpholino-Propane-Sulphonic Acid Buffer

NP40 : Nonidet 40

NS1 : Non-Structural Protein 1

NS2a : Non-Structural Protein 2a

NS2b : Non-structural Protein 2b

NS3 : Non-Structural Protein 3

NS4a : Non-Structural Protein 4a

NS4b : Non-Structural Protein 4b

NS5 : Non-Structural Protein 5

N-terminus : Amino-Terminus

OHFV : Omsk Haemorrhagic Encephalitis Virus

ORF : Open Reading Frame

PAGE : Polyacrylamide Gel Electrophoresis

PCR : Polymerase Chain Reaction

pfu : Plaque forming Units

pi : Post Infection

PMSF : Phenylmethylsulphonylfluoride

PrM : Pre-Membrane Protein

RF : Replicative Form

RI : Replicative intermediate

RNA : Ribonucleic Acid

rRNA : Ribosomal RNA

RSSEV : Russian Spring Summer Encephalitis Virus

SDS : Sodium Dodecyl Sulphate

sec : Second

SLEV : St Louis Encephalitis Virus

SMB : Suckling Mouse Brain

TE Buffer : Tris EDTA Buffer

TBE Buffer : Tris-Borate EDTA Buffer

TBEV : Tick Borne-Encephalitis Virus

TEMED : N,N,N′,N′-Tetramethylethylenediamine

TGN : Trans Golgi Network

UV : Ultra Violet

WHO : World Health Organisation

WNV : West Nile Virus

w/v : Weight/Volume

w/w : Weight/Weight

YFV : Yellow Fever Virus

CHAPTER 1. INTRODUCTION.

1.1 TICK-BORNE ENCEPHALITIS VIRUS

1.1.1. Historical Perspective:

Tick-borne encephalitis virus (TBEV) is classified as a member of the flavivirus genus within the virus family Flaviviridae (Westaway et al., 1985). Flavivirus research truly began with Major Walter Reed's experiments which described for the first time a filterable agent, yellow fever virus (YFV), capable of inducing disease in man, and demonstrated that transmission of YFV was via an arthropod vector (Reed et al., 1901; Reed and Carroll, 1902). Since the identification of YFV, a large number of arboviruses including Russian Spring Summer Encephalitis virus (RSSEV), louping Ill virus (LIV), dengue virus, Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Murray Valley encephalitis virus (MVEV) and Kunjin virus have been isolated and classified within the Flaviviridae.

Some of the earliest records of a tick-borne flavivirus infection were made in 19th century Scotland. These reports described the characteristic louping or leaping associated with LIV-infected sheep and hill cattle (Williams, 1897). LIV remained a veterinary problem, until an effective vaccine was developed in the 1930's (reviewed Edward, 1947). While LIV has never been considered clinically important (reviewed Davidson et al., 1991), the TBEV infections of the far eastern (FE) and central European (CE) virus subtypes are recognised as major public health problems within endemic regions.

TBEV has been recognised as a clinically important pathogen since the 1930's. TBEV was first isolated during the early 1930's following an epidemic of neurological disorders amongst forest workers from the eastern region of the Commonwealth of Independent States (CIS)

(Reviewed Silber and Soloviev, 1946; Smorodintseff, 1939). In searching for its causative agent, a team lead by Silber isolated a previously unknown tick-borne virus which, because of it's seasonal incidence, was designated RSSEV. Responding to this outbreak of RSSEV, the Russians developed the first TBEV vaccine (reviewed Smorodintsev et al., 1940).

Outside the CIS, the first epidemic for which TBEV was recognised as the causative agent was recorded during 1949 in Czechoslovakia (reviewed Blaskovic, 1958; Hloucal, 1960). As the virus responsible for this outbreak was very similar to that of FE TBEV and like LIV, it was designated Russian louping Ill Virus, later re- classified as CE TBEV.

Until the 1950's only the four distinct virus types within the TBEV had been identified; LIV, Omsk haemorrhagic fever virus (OHFV), the FE TBEVs, and CE TBEVs. OHFV stands out from the other three TBEV isolated because rather than inducing a encephalitic type pathology, OHFV infections are of a haemorrhagic nature. It is interesting that two antigenicaly related viruses, FE TBEV and OHFV isolated in areas of near geographical proximity and transmitted by similar mechanisms should cause such different diseases. Since 1950, the number of viruses identified as being antigenically related to the original TBEV isolates has risen to include viruses such as Kyasanur Forest disease virus (KFDV, Work, 1958), Langat virus (Gordon-Smith, 1956), Powassan virus (McLean and Donohue, 1959), Negishi virus (Ando et al., 1952), Karski virus (Lvov et al, 1976) and Royal Farm virus (Williams et al., 1972).

Table 1.1. Flaviviridae Complexes and Examples of Viruses Contained Within Them (including the major human pathogens):

COMPLEX/ Vector	VIRUS	DISEASE	DISTRIBUTION
TBEV/ Tick	Louping Ill		UK
	CE	Encephalitis	Europe
	OHF	Haemorrhage/fever	USSR
	KFD	Haemorrhage/Fever	India
	FE	Encephalitis	USSR
	Langat		Malaya
	Powassan	Encephalitis	Canada, USA
	Negishi		Japan
Modoc/ Unknown	Modoc		
	Others		
JE/ Mosquito	JE	Encephalitis	Japan,SE Asia W Pacific
	SLE	Encephalitis	USA
	MVE	Encephalitis	Australia
	WN	Encephalits/fever	Africa, Asia, Europe
	Others		
Ntaya/ Mosquito	Ntaya		
	Others		
Uganda S/ Mosquito	Uganda S		
	Others		
Dengue/ Unknown	Dengue 1	Fever/Haemorrhage	Asia, Caribbean, S.America
	Dengue 2	Fever/Haemorrhage	Asia, Caribbean S.America
	Dengue 3	Fever/Haemorrhage	Asia, Caribbean, S.America
	Dengue 4	Fever/Haemorrhage	Asia, Caribbean, S.America
Rio Bravo/ Mosquito	Rio Bravo		
	Others		
Tyuleniy/ Tick	Tyuleniy		
	Others		
Antigenically isolated includes	Yellow Fever	Fever	Africa, Tropical America

Based on Calisher *et al*., 1989.

1.1.2. Classification of TBEV:

The Flaviviridae contains over 60 characterised viruses (Table 1.1) all classified within the genus Flavivirus. The presence of neutralizing, complement-fixing and haemagglutinating antigens on the surface of flavivirus particles has allowed their antigenic relationships to be examined. Classification of the flaviviruses began in 1944, when the complement fixation and neutralisation assays were used to established the antigenic relationship between two tick-borne flaviviruses, (LIV and RSSEV) and between both LIV and RSSEV and the three mosquito-borne flaviviruses (WNV, JEV and SLEV) (Casals, 1944; Casals and Webster, 1944).

The Flaviviruses as a group were first demonstrated in 1954 when a systematic study of arboviruses using the haemaglutination assay identified two distinct virus groups (Casals and Brown, 1954). Originally designated the Group A and Group B viruses, the two groups were later renamed the Alphaviruses and Flaviviruses. In 1974, on the basis of their non-helical nuclear capsids, viral envelope and RNA genome, the Alpha- and Flaviviruses were classified together in the new virus family, the Togaviridae (Fenner et al., 1974). Later two more genera, the Pestiviruses and Rubiviruses were added to the Togaviridae (Porterfield et al., 1978).

A closer inspection of the Alpha- and Flaviviruses however, revealed that although morphologically similar, significant differences in their genetic organisation and replication strategy existed. In fact, the Flavivirus genomic organisation was shown to be more like that of the picornaviruses than the alphaviruses with the mode and site of Flavivirus assembly having more in common with bunyaviruses than alphaviruses (Boulton and Westaway, 1972; Brawner et al., 1977). These differences persuaded the 1984 International Committee for the

Nomenclature of Viruses to vote the Flaviviruses into a separate family (reviewed Westaway et al., 1985). The application of new molecular biological technology has recently confirmed the 1984 classification and highlighted sufficient similarities between the Flaviviruses and the Pestiviruses for inclusion of the Pestiviruses into the Flaviviridae as a second genus (Collett et al., 1988; Horzinek, 1991).

Serological analysis of the Flavivirus genus by cross-neutralization, showed that the tick-borne flaviviruses formed antigenically distinct complexes distinct from those of the other flaviviruses (Madrid and Porterfield, 1974). Interestingly, each flavivirus antigenic complex identified by the 1974 study, reflected an ecological feature, such as their vector, common to the viruses within it. The tick-borne viruses comprising the newly identified complex, TBEV, had been shown in earlier studies using haemagglutination-inhibition and immuno-diffusion techniques, to form seven closely related subgroups; the LIV, OHFV, KFDV, Langat virus, Powassan virus, Negishi virus and the TBEV CE and FE subtypes (Clark, 1964). Initially, it was not clear whether the FE and CE subtypes were distinguishable but tryptic peptide mapping of the virus encoded proteins and, more recently, nucleotide sequencing has succeeded in differentiating the two subtypes at a molecular level (Heinz and Kunz, 1981 & 1982; Mandl et al., 1988 & 1989a; Pletnev et al., 1990). In the most recent classification of the flaviviruses, the tick-borne strains of Karshi virus and Royal Farm virus along with the Phnom Phen bat virus and Carey Island virus were classified as additional members of the previously defined TBEV complex (Table 1.1) (Calisher et al., 1989).

1.1.3. Transmission of TBEV:

As arboviruses, (with the exception of milk-borne and transovarial transmission) the tick-borne flaviviruses require continuous cycling between their tick vectors and primary vertebrate hosts. The success of this transmission is directly dependent on a number of specific conditions being met which, by their very nature, define the geographical distribution of viruses. In order, therefore, to understand the epidemiology of TBEV, one must appreciate its natural history.

Two types of vertebrate host have been identified for the tick-borne flaviviruses, the primary hosts and the dead-end hosts. Primary hosts of TBEV include a number of small mammals and birds which, when infected, are capable of supporting a viremia sufficient to infect feeding ticks (Cerny, 1975; review Chamberlain et al., 1954). Dead-end hosts, although susceptible to infection, are unable to support a level of viremia sufficient to ensure continued transmission of the virus. Dead-end hosts of viruses in the TBEV complex (except LIV) include the larger vertebrates such as goats, cows, sheep and man.

In order to maintain the virus transmission cycle uninfected ticks and primary hosts must become infected. Irregular transmission of virus between ticks occurs transtadially and transovarially, with venereal transmission between the sexes having also been identified (Burgdorfer and Verma, 1967; Rao, 1963 Rehacek, 1962; Singh et al., 1968). These modes of transmission have traditionally been considered of little significance to the maintenance of the viral reservoir. In general, transmission of TBEV to uninfected ticks is believed to occur while they feed on an infected primary host. For transmission to occur, the level of viremia in the host must be sufficient to infect the midgut cells of the feeding tick and the cells of the tick's

midgut must be susceptible to infection by the virus being transmitted (reviewed Casels and Reeves, 1959). Once an infection of the ticks midgut cells has been established, the virus spreads via the hemocoele to the ticks salivary glands. The virus can then be transmitted from ticks into the blood stream of a host during tick engorgement via its "saliva". Ticks are thought to remain infected for life with apparently little ill effect.

Although members of the TBEV complex may be carried by a wide variety of tick vectors, they appear to be preferentially transmitted by specific tick species. While CE and LI viruses are usually transmitted by Ixodes ricinus, and FE TBEV by Ixodes persulcatus, isolation of these viruses from ticks of the genus Dermacentor and Haemophysalis collected in habitats unfavorable to the Ixodes has been recorded (reviewed Blaskovic, 1958). Powassan virus, OHFV and KFDV are most frequently isolated from Ixodes cookei, Dermacentor pictus and Haemophysalis spinigera respectively. On rare occasions, TBEV has been isolated from the Aedes mosquito although the infrequency of these isolations implies that this form of transmission is of little epidemiological importance (Przesmycki et al., 1960). Because of its ability to withstand low pH, TBEV can also transmitted via milk. Transmission to human populations via this route has posed a significant public health problem and been responsible for a number of TBEV outbreaks (Gresikova, 1958).

1.1.4. Epidemiology of TBEV:

Since arboviruses occur only where climate and conditions support sufficient numbers of closely associated primary hosts and suitable vectors, the epidemiology of TBEV infections is defined by the ecology of the virus-host relationship. While these factors restrict KFDV in

India to Karnataka provence and parts of the adjacent states, OHFV to Omsk and Novosibirsk and LIV mainly to upland sheep grazing areas of the UK (LIV having also been isolated in Norway and Spain), these factors have allowed the viruses of the CE and FE TBEV subtypes to spread across large areas of the temperate northern hemisphere. In reflecting the distribution of their main tick-vector, the FE TBEVs have been identified throughout the CIS, while those of the CE subtype have been located in most countries of Europe and Scandinavia (Balaskovic, 1958). LIV is the only member of the TBEV complex to be found in the UK; the geographical isolation of the UK has probably prevented infection of the native tick population by other viruses of the TBEV complex.

As the natural habitat of the tick grows and recedes in response to changing climate and land use, the geographical limits of human infection alter. TBEV was initially limited to forest workers and hunters. Today, however, more extensive and frequent incursions by the public into forested and grassland areas and the expansion of domestic animal production has meant that the incidence of both FE and CE TBE is on the increase. The influence of man over the spread of the TBEV foci has been apparent since the beginning of the 20th century when the development of sheep husbandry in the heather moorlands of Scotland resulted in an invasion of LIV infected Ixodes ricinus into the region. Reported cases of FE TBEV across the USSR have doubled between the years 1985 and 1990 (Dr. A.Karavanov, IPEV CIS. personal communication). In 1977, the Times newspaper reported that serological data collected in Austria, Czechoslovakia, Hungary and south-west Germany suggested that between 1000 and 1300 cases of TBEV were occuring annually, five years later this figure had doubled

(Times, November 14, 1977). In recognising this increasing danger, the World Health Organisation (WHO) recently made TBEV a special, notifiable disease in 15 European states.

The clinical reports of TBEV infection tend to be seasonal and climate dependent, with the designation of 'Russian Spring-Summer encephalitis virus for viruses of the FE subtype reflects this. In Europe, tick activity occurs later and lasts longer than in the CIS, with outbreaks not being reported until the summer months and continuing into early autumn (reviewed Smorodintseff, 1939). The increased activity of questing ticks, stimulated by the warm months of summer, accounts for the increase in incidence of TBEV, while the reduction of incidences during winter months corresponds with the tick's diapause.

Members of the TBEV are expressed in man as either encephalitis or haemorrhagic fever and they are responsible for a range of illnesses. The diversity of the clinical symptoms associated with TBEV infections may be accounted for by the ability of the various TBEVs to operate through different vectors. Human TBEV infections are generally bi-phasic in nature and can be either so mild as to escape detection; moderately severe, with or without permanent residua; or fatal. The first phase of a TBEV infection, which frequently escapes diagnosis, is systemic and characterised by fever, headache and general malaise, often with gastrointestinal involvement. The second phase of the encephalitic disease is marked by the return of fever and involves the central nervous system (CNS). Haemorrhagic forms of TBEV lack a CNS involvement and are characterised by vomiting, diarrhoea and internal haemorrhage. The FE TBEV's although antigenically and clinically very similar to those of the CE subtype, are associated with a more severe disease and a fatality rate as high as 20% compared to that of between

1 and 5% for the CE TBEV's. Furthermore, survivors of FE TBEV infection, unlike those of the CE subtype, often experience a protracted convalescence which is frequently accompanied by residual paralysis of the upper limbs.

1.1.5. Control of TBEV:

Eradication of any arbovirus disease depends on the interruption of the virus transmission cycle. The cycle can be broken by removing either the tick or primary host. Although both these objectives are difficult to achieve, the control of vectors and host population numbers has been reasonably successful in limiting the incidence of disease.

Methods available to reduce tick population in a circumscribed area are neither economical nor suitable for continual application. Temporary control of tick vectors following the predictions of epidemics by surveillance programmes which include vector trapping, antibody screening of primary hosts and monitoring of climatic conditions does, however, appear to be a more practicable solution. Prediction of epidemics allows for the limited use of effective but expensive and often environmentally damaging control measures such as the pasteurization of milk and spraying of uninhabited forest areas with DDT within endemic regions to be used (reviewed Blaskovic, 1959). These control measures, when combined with public education and legal requirements, have proved to be of use in controlling TBEV outbreaks. Personal protection from tick bites by wearing suitable clothing and the use of insect repellants is only really practicable for the transient visitor.

While the temporary removal of ticks from the transmission cycle is feasible, unless specific conditions apply the successful removal of the primary host is not. Vaccination of a primary host population which consists of small, wild mammals is clearly not feasible. The vaccination of a 'captive' primary host population, such as the introduction of the human yellow fever vaccine 17D and the immunization of sheep against LIV has, however, been successful. The LIV vaccines have reduced the incidence of disease amongst grazing animal of the UK. The first LIV vaccine was a formalin-inactivated virus prepared from infected sheep brain, spinal cord and spleen (reviewed Gorden et al., 1962). Although successful, this vaccine was withdrawn because of the risk it posed to those involved in its manufacture and the possibility of its contamination with Scrapie. A second LIV vaccine, also a formalin-inactivated, was prepared in secondary sheep kidney cells (Brotherston and Boyce, 1970). A modified form of this cell grown vaccine is still available.

Vaccination of domestic animals including cows, sheep and goats against both FE and CE TBEV has been attempted (Blaskovic et al., 1960). Although these animals play little role in the transmission cycle of FE and CE TBEV, their vaccination is an important step in the prevention of human infection resulting from virus transmission via infected milk. The implication of primary host vaccination on the natural virus reservoir has yet to be examined. Since grouse and deer are being killed by LIV at this moment the overall effect of primary host vaccination on the natural reservoir may prove to be negligible and therefore be of little use in controlling the emergence of arboviruses.

1.1.6. Human TBEV Vaccines:

TBEV vaccines, although able to reduce the incidence of infection, do not address the problem of virus transmission nor, presumably, affect the virus reservoir. Since both the economical impact and price in terms of human life and misery of TBEV, especially in endemic regions, is so very high, the development of TBEV vaccines has been a priority.

In addition to the LIV vaccines, a large number of candidate TBEV vaccines has been prepared, the success of which has been variable (reviewed Stephenson, 1988). The first flavivirus vaccine available for human use was a 1% formalin-inactivated suspension of infected mouse brain developed in Russia against the original RSSE TBEV isolate (Smorodintsev et al., 1940; Smorodintsev and Ilyenko, 1962). Unfortunately this vaccine contained myelin which produced an unacceptably high level of allergic reaction in vaccinees. Attempts to remove the contaminant resulted in a reduction in the antigenicity of the vaccine and consequently it was withdrawn.

Development of cell culture systems for vaccine production initiated an extensive FE TBEV vaccine research programme in the CIS (Ilyenko, 1959; Smorodinstev and Ilyenkov, 1960). An RSSEV based vaccine prepared in primary avian fibroblasts successfully protected in laboratory experiments, but when used to vaccinate populations in endemic regions failed to reduce the incidence of human disease and was withdrawn. More recently the programme has included the development of an FE TBEV candidate vaccine based on virus passaged in green monkey kidney cells (Chumakov et al., 1990; Grachev et al., 1985) or the naturally occurring attenuated TBEV strain Yelantsev. In addition to the development of vaccines against the FE strains of TBEV, the CIS programme has made improvements in vaccine production

methods (Elbert et al., 1980, 1981 & 1985). At the present time, the two FE TBEV vaccines available in the CIS are both based on inactivated virus prepared in chick cells (reviewed Chumakov et al., 1990). An assessment of their efficacy however is difficult to obtain in the West.

The first CE TBEV vaccine was based on infected, trypsinised chick embryo cultures inactivated with formaldehyde (Danes and Benda, 1960 & 1960a). This inactivated vaccine was used in an immunization programme by the Czechoslovakians. Further epidemics of TBEV in eastern Europe during the 1960's, however, led to the production of a more efficient CE TBEV vaccine which is still in use today. A collaboration between the Centre for Applied Microbiology and Research (CAMR) and the Institute of Virology, Vienna produced this formalin-inactivated vaccine based on the CE Neudörfl strain of TBEV. Over several million doses of a highly effective, purified form of the Neudorfl vaccine have been administered, with only rare and temporary side effects being reported (Heinz et al., 1980; Kunz et al., 1980). Because of the growing concern surrounding CE TBEV and the commercial benefits to be gained from it, a new CE TBEV vaccine based on the CE TBEV strain K23 is now under going trials (Bock et al., 1990; Klockmann et al., 1991).

1.1.7. TBEV Vaccine Development:

The current approach to the development of flavivirus vaccines has been reviewed extensively (Brandt, 1988; Gibson et al., 1988; Stephenson, 1985 & 1988). A future generation TBEV vaccine should ideally be capable of protecting against viruses from several of the

TBEV complexes, and if possible, it should protect against viruses from other flavivirus antigenic complexes without presenting a risk to vaccinees.

Immunization or prior infection with a flavivirus can afford a degree of protection against closely related viruses (Price, 1968; Price et al., 1963 & 1969; Sather and Hammon, 1970; Smithburn, 1942). The precise immunological relationships between the flaviviruses appear to be important since monkeys were protected against 28 different flaviviruses when immunized first against YFV, then Langat virus, dengue virus, and finally JEV but not when the vaccines were administered in a different order (Price et al., 1969). This cross-protection does, however, suggest that sequential immunization with several flavivirus vaccines may afford protection against a broad range of flaviviruses.

An inherent risk in immunization against flaviviruses is the stimulation of antibody dependent enhancement (ADE) of subsequent infections (reviewed Halstead., 1982; Porterfield, 1986). The phenomenon of ADE in man is well documented for dengue virus. Four subtypes of dengue virus exist and exposure to each offers a reasonable level of protection against re-infection by the homologous serotype but only limited protection against the other three. Subsequent infection by a heterologous serotype can cause dengue haemorrhagic fever/shock syndrome (DHF/DSS) (reviewed Halstead, 1980; Halstead et al., 1973). Serological and epidemiological data suggest these more severe forms of dengue fever result from subneutralizing levels of circulating anti-dengue antibodies mediating the enhancement of subsequent dengue virus infections.

The mechanism of ADE may involve the infection of cell by virus complexed with an IgG or IgM antibody which binds to either the Fc receptor or complement receptor C3 of a cell (Cardosa et al., 1983; Daughaday et al., 1981; Halstead and O' Rourke, 1977; Schlesinger and Brandriss, 1981). The cell is able to internalise the complexed virus more efficiently than non-complexed virus thus enhancing infection (Gollins and Porterfield, 1984). ADE has, however, only been demonstrated with other flaviviruses in experimental models and thus its importance to flavivirus vaccines in general is unclear (Barrett and Gould, 1986; Gould et al., 1987; Gould and Buckley, 1989., Hawkes, 1964; Hawkes and Lafferty, 1967; Kayser et al., 1985; Phillpotts et al., 1985).

The potentially dangerous implications of ADE have prompted investigations into the development of future flavivirus vaccines based on non-virion antigens. The use of non-virion antigens as flavivirus vaccines would remove the possibility of raising antibodies able to bind the virion and to mediate ADE. Identification of an extra-cellular, non-virion protein (NS1) encoded by flaviviruses, which has the potential to protect mice against homologous challenge has raised the prospect of its use in new vaccines (Cane and Gould, 1988; Gould et al., 1986; Henchal et al., 1988; Schlesinger et al., 1985, 1986 & 1987). Although B-cell epitopes between NS1 of the flaviviruses appear to be poorly conserved, a high degree of homology at the genetic and phenotypic level suggests that a broadly reactive vaccine based on the T-cell response against NS1 antigen may be possible (Mandl et al., 1989a; Pletnev et al., 1990; Iacono-Conners and Schmaljohn, 1992; Stephenson et al., 1984). Furthermore, the significance of a B-cell response to the NS1 antigen in vaccine mediated protection has yet to be investigated.

1.2. THE TBEV VIRION:

1.2.1 The TBEV Genome:

TBEV contains a single-stranded, positive-sense, RNA genome of approximately 10.5Kb. This RNA molecule is infectious when inoculated intracerebrally into mice (Sokol et al., 1959) and since subgenomic flavivirus RNA has not been identified, is presumed to act, in a manner similar to that of the picornaviruses, that is as the sole viral mRNA.

The RNA molecule of TBEV encodes a single translated open reading frame (ORF), flanked by non-coding regions which are presumed to carry signals for RNA synthesis and translation (Fig. 1.1) (Mandl et al., 1988 & 1989a). The 5′ non-coding region of the TBEV genome is approximately 100 nucleotides, and computer analysis predicts the presence of a RNA stem-loop structure (Pletnev et al., 1990). Similar structures have been described for a number of other positive strand RNA viruses such as the mosquito-borne flaviviruses, picornaviruses and alphaviruses (Brinton and Dispoto, 1988). Evidence from these RNA viruses and in particular YFV supports the involvement of the secondary structures in viral replication and translation of the flavivirus genome (Ruiz-Linares et al., 1989). The similarities between the tick-borne and mosquito-borne flaviviruses 5′ non-coding regions imply a replication strategy common to the flaviviruses.

Downstream of the 5′ non-coding region is the major ORF which accounts for approximately 90% of the coding capacity of the genome. In common with the mosquito-borne flaviviruses and picornaviruses, the TBEV genome encodes the TBEV structural proteins before the TBEV non-structural proteins. The TBEV gene order from the translation initiation codon is; the (anchored) core protein (C), the pre-membrane

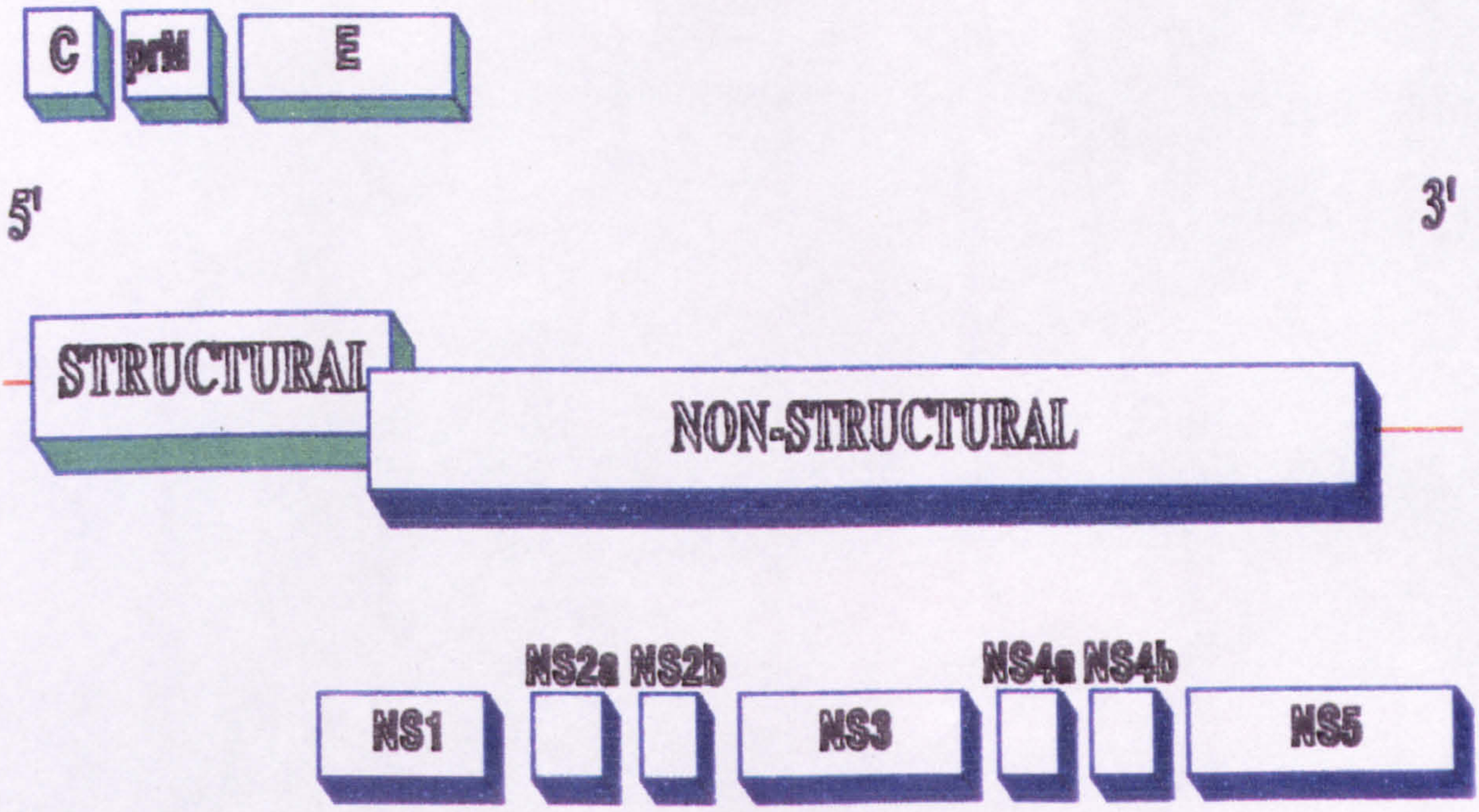


Fig. 1.1 The TBEV genome
The 3' and 5' non-coding regions are indicated by a red line at the ends of the open reading frame.

protein (PrM), the envelope protein (E), and the non-structural proteins (NS) NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 respectively (Fig. 1.1). A second ORF, absent from the genome of the mosquito-borne viruses, is a feature of the TBEV 5′ non-coding region. The significance of this small and apparently untranslated ORF is not known (Mandl et al., 1988; Pletnev et al., 1990).

Following the long ORF of the TBEV genome is a 3′ non-coding region. This region has been found in at least two forms (Mandl et al.,1991). The first form, typical of the TBEV strain Neudörfl, contains approximately 100 nucleotides, and carries a 3′ terminal polyadenylation signal preceded by the di-nucleotide AC. The 3′ terminus of the TBEV strain Hypr, however, like those of the mosquito-borne flaviviruses, consists of over 450 nucleotides and terminates with the di-nucleotide CU. The absence of a polyadenylation signal makes the genome of the mosquito-borne flaviviruses and TBEV strain Hypr unusual amongst the positive-strand RNA animal viruses. Unlike the shorter form of the TBEV 3′ non-coding regions the longer flavivirus 3′ non-coding region has the potential to form a stem-loop structure which appears to protect the 3′ terminus from endonuclease digestion by hydrogen bonding the terminal nucleotide (Brinton et al., 1986; Grange et al., 1985; Hahn et al., 1987; Wengler and Castle, 1986). The stem loop structure(s) of the 3′ non-coding regions of some plant viruses have been shown to be involved in the regulation of RNA synthesis. This suggests a similar role for the equivalent region in TBEV (Strauss and Strauss, 1983).

A degree of homology between the two forms of TBEV non-coding region has been demonstrated (Mandl et al., 1991). From the limited sequence data available for TBEV (both FE and CE subtypes), a high level of homology exists between nucleotides of the short form of 3′ non-coding

region and the nucleotides immediately downstream of the large ORF in the longer forms. As with the alphaviruses, repeating sequence elements with serogroup specific patterns have been identified in the 3′ non-coding region of the mosquito-borne flaviviruses (reviewed Chambers et al., 1990a). In both forms of TBEV 3′ non-coding region, however, these repeating sequence elements are absent. Alternatively, a sequence of approximately 20 nucleotides near the 3′ end of the TBEV NS5 gene is repeated immediately upstream of the polyadenylation signal in the shorter 3′ non-coding region and a similar distance upstream of the translational stop codon in the larger 3′ terminal region (Mandl et al., 1991). The importance of these elements has yet to be determined, although it has been suggested that they may be recognition sequences for RNA replicases.

1.2.2. TBEV Virion Proteins:

The three structural TBEV proteins form a spherical virion with a diameter of approximately 50nM (Fig. 1.2) (Slavik et al., 1970). Although morphologically indistinguishable, two virion forms have been characterised, the intra-cellular form which contains PrM and the extra-cellular form which contains the mature membrane protein Mb (Guirakhoo et al., 1991). The TBEV genome is packaged within a nucleocapsid, composed of C, which exhibits cubic symmetry and which is surrounded by a bi-lipid envelope (Abdelwahab et al., 1964; Heinz and Kunz, 1979). The components of the TBEV and SLEV bi-lipid envelope are consistent with nascent virions budding through internal host cell membranes (Heinz and Kunz, 1979; Trent and Neaves, 1980). Anchored through the bi-lipid envelope by its hydrophobic C-terminus is E in association with PrM/M, both of which form spiked projections on the virion's surface (Heinz and Kunz, 1979a & 1980; Slavik et al.,

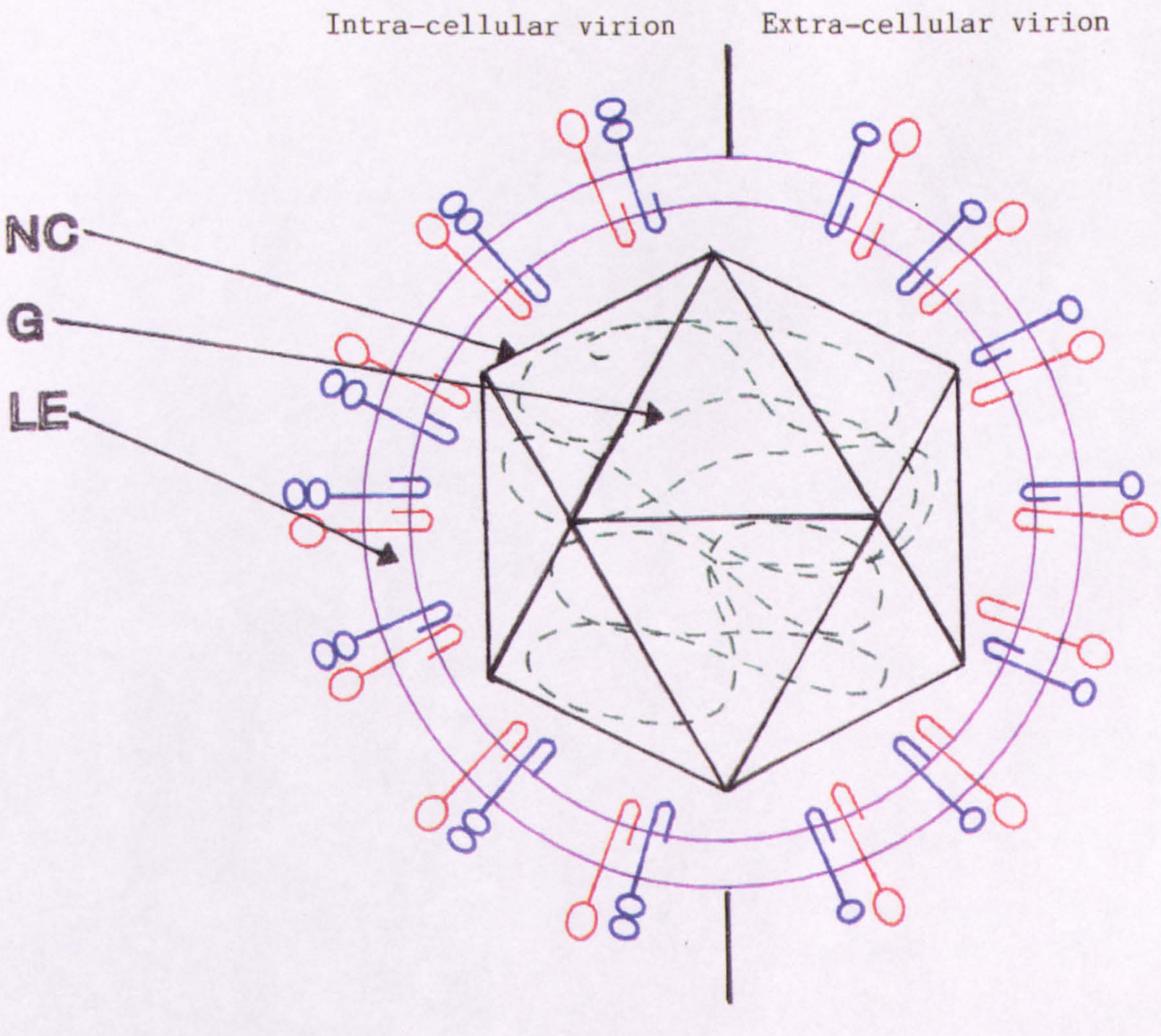


Fig. 1.2 Schematic Representation of the TBEV Virion.
Two forms of the virion are depicted: on the right is the extra-cellular virion; and on the left is the intra-cellular virion.

NC Nucleocapsid
G Genome
LE Bi-lipid envelope

Envelope protein

Pre membrane protein

Membrane protein

1970). The E-PrM association probably arises from interactions between their hydrophobic tails. The mechanism by which E is anchored may involve either a dual transmembrane hook structure as depicted in Fig. 1.2, or a single transmembrane element. Evidence has been presented which suggests that there is no or little interaction between the capsid and the proteins of the lipid envelope (Heinz and Kunz, 1979a & 1980).

The structural components of the TBEV nucleocapsid are small (15KDa) basic (lysine rich) proteins (Heinz and Kunz, 1979 & 1979a, Krasilnikov et al., 1984). The positive charge carried by C may assist associations between C and the genomic RNA (Pletnev et al., 1986; Rice et al., 1985). C is found in two forms, anchored C and C. As is characteristic for other flaviviruses, the TBEV encoded anchored C has a hydrophilic N-terminus and an internal hydrophilic domain separated by an internal hydrophobic region. However, TBEV C lacks both a hydrophobic region and second translational start codon located in the N-terminal hydrophobic region of the mosquito-borne viruses anchored C (Mandl et al., 1988). The C-terminus of anchored C consists of a hydrophobic domain, which has been shown to direct translocation of the downstream protein, prM, in dengue virus and YFV (Markoff, 1989; Ruiz-Linares et al., 1989a). The C-terminal and internal hydrophobic domains may assist in the assembly of capsids by 'anchoring' anchored C to cellular membranes, and stabilize interactions between aggregating Cs (Nowak et al., 1989; Rice et al., 1985). The hydrophobic C-terminal or 'anchor' of C encoded by WNV, Kunjin virus and YFV, is removed during maturation to generate C may account for the release of nascent nucleocapsids from membranes (Chambers et al., 1990; Nowak et al., 1989; Speight and Westaway, 1989). Since the TBEV C gene product synthesised in a cell-free

translation system migrates more slowly on acrylamide gels than virion associated proteins and since sequence alignment with WNV has identified a conserved proteolytic cleavage site within TBEV anchored C, TBEV anchored C is probably also modified in this way (Mandl et al., 1988; Svitkin et al., 1984). The removal of this putative membrane anchor in TBEV, however, will remain speculative until the C-terminal of TBEV C has been determined directly.

PrM (14.5KDa) is the glycosylated precursor of M (7.5KDa) and is produced when proteolytic cleavage removes the glycosylated hydrophobic N-terminal domain from PrM (Nowak et al., 1989). This modification is necessary for the generation of fusion competent, infectious virions (Guirakhoo et al .,1991; Randolf et al., 1990). Like C, PrM contains a hydrophobic C-terminus which, in YFV, has been shown to function as the translocation signal sequence for E and may anchor M into the bi-lipid envelope of the virion (Despres et al., 1990; Ruiz-Linares et al., 1989). It has also been suggested that PrM/M plays a role in the protective humoral response (Heinz and Kunz, 1977; Heinz et al., 1981; Kaufman et al., 1989).

The TBEV surface protein E (55KDA) contains haemagglutination, complement fixation and neutralization antigenic determinants and directs the major humoral protective immunological response in a host (Heinz et al., 1981). Analysis of disulphide bonding within E of TBEV has helped elucidate its secondary structure, while topographical epitope analysis has allowed the construction of an antigenic map (reviewed Heinz, 1986; Heinz et al., 1983 & 1983a). Three antigenic domains A, B and C within TBEV E have been identified which correspond to the antigenic domains R1, R2 and R3 of WNV E and domains I, II and III of dengue virus E (reviewed Heinz, 1986 & 1990; Mandl et al., 1989; Mason et al., 1990; Megret et al., 1992; Nowak and Wengler,

1987). In addition to their antigenicity, the three domains of E have been linked with specific roles (Guirakhoo et al., 1989; reviewed Heinz, 1990; Heinz et al., 1983; Holzmann et al., 1990). A point mutation in domain B leads to virus attenuation, while domain A, both contains a tetra-peptide found in the fusion-active amino terminal of influenza virus HA2 and is able to undergo both antibody- and pH-mediated conformational changes.

Of the three N-linked glycosylation motifs identified within TBEV E, only the N-terminal site appears to be available for modification (Winkler et al., 1987). While conserved amongst the characterised tick-borne flaviviruses, the position of the oligosaccharide in the mosquito-borne viruses is not conserved. Although encoding the glycosylation motifs, E encoded by Kunjin virus, WNV and at least one strain of YFV, is unglycosylated, the oligosaccharide side chain having been added appears to be removed during virion maturation (Deubel et al., 1987; Wengler et al., 1985; Wright, 1982). The importance of the glycan side chain has yet to be determined.

1.2.3. TBEV Non-Structural Proteins (excluding NS1):

NS5, the largest of the TBEV encoded processed proteins (91Kd), and NS3 are both positively charged and hydrophilic proteins. NS5 contains the tri-peptide Gly-Asp-Asp of the RNA-dependent-RNA polymerase motif found in many plant and animal positive-stranded RNA viruses (Iacono-Connors and Schmaljohn, 1992; Kamer and Argos, 1984; Mandl et al., 1989a). This is, however, the only evidence for the flavivirus polymerase being provided by NS5 (Grun and Brinton, 1987). Amongst the positive-strand RNA animal viruses, only picornaviruses and viruses of the Flaviviridae encode the RNase polymerase gene at the 3′ end of their genome.

The consensus sequence of an RNA helicase motif and of a nucleotide triphosphate binding site have been identified in the C-terminus of TBEV NS3 (64KDa), implying a role in the viral replicase complex (Gorbalenya et al., 1989). The N-terminus region of NS3 exhibits homology with a functional trypsin-like serine protease (reviewed Bazan and Fletterick, 1990; Iacono-Conners and Schmaljohn, 1992; Timofeev et al., 1990). The protease domain is contained within the 181 residues at the N-terminus of NS3 where three spatially conserved amino acids of the catalytic triad are located. In addition to the triad, a substrate binding pocket which interacts with the cleavage sites and on which efficient processing is dependent has also been identified in the N-terminal of NS3. To date, NS3 associated protease activity has been demonstrated for the mosquito-borne dengue virus, WNV and YFV (Cahour et al., 1992; Chambers et al., 1991; Falgout et al., 1991; Preugschat et al., 1990; Wengler et al., 1991).

The small non-structural proteins, NS2a, NS2b, NS4a and NS4b have only recently been demonstrated for the mosquito-borne flaviviruses (Speight and Westaway, 1989a), while in TBEV their existence remains theoretical. These four small non-structural proteins of TBEV have been predicted from nucleotide sequence analysis and are apparently rich in hydrophobic residues (Mandl et al., 1989). If, as with other positive-strand RNA viruses such as the coronaviruses and alphaviruses, the flavivirus replication complex is membrane bound as suggested by Grun and Brinton (1988) these hydrophobic, small non-structural proteins may direct the association between the viral replication complex or hydrophilic NS3 and NS5 and the cellular membranes (reviewed Chambers et al., 1990a).

Recent work with dengue virus has suggested a role for NS2a in mediating cleavage of the NS1:NS2a junction. The protease requirement is in the N-terminal 2/3 of NS2a which may either act directly or provide an obligatory sequence for a specific cellular protease (Falgout and Lai, 1990; Falgout et al., 1989). In the dengue and yellow fever viruses, NS2b has been shown to be a necessary component in the cleavage mechanism of the NS3 encoded protease (Cahour et al., 1992; Chambers et al., 1991; Falgout et al., 1991). Little is known about NS4a and NS4b.

1.2.4. TBEV RNA Synthesis:

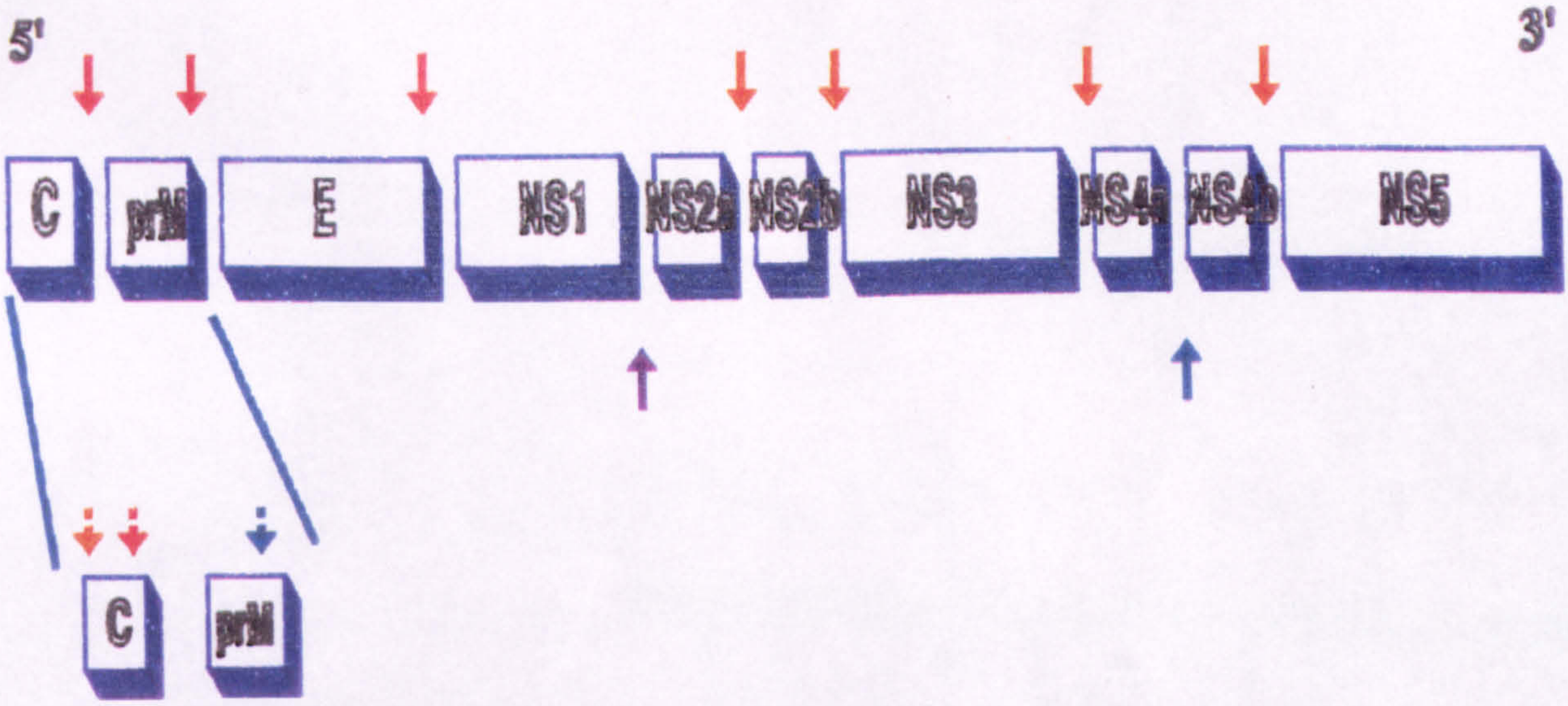
RNA synthesis of the tick-borne flaviviruses is believed to be associated with the endoplasmic reticulum in the perinuclear region of the infected cell (Lubiniecki and Henry, 1974). Although identification of the replicative forms of TBEV RNA has yet to be made, they have been demonstrated in cells infected with mosquito-borne flaviviruses (Chu and Westaway, 1985; Cleaves et al., 1981; Stollar et al., 1967). For dengue virus and Kunjin virus, two forms of the replicative complex have been identified: the replicative form (RF) and the replicative intermediate (RI). The RF is an RNA duplex consisting of positive and negative sense viral RNA copies, while the RIs appear to be semi-denatured RFs with the nascent RNA strands in the process of being transcribed. The relationship between the two types of replicative complex is unclear. RNA dependent RNA polymerase activity has been detected in cells infected with dengue virus, WNV and Kunjin virus (Cardiff et al., 1973; Chu and Westaway, 1987; Grun and Brinton, 1986). This polymerase may be encoded by the NS5 gene.

On the basis of the data gathered so far, several strategies for flavivirus RNA synthesis have been proposed (reviewed Brinton, 1986; Chu and Westaway, 1985; Westaway, 1987). The basic strategy requires that the genomic RNA provides the template for negative strand synthesis which in turn becomes the template for progeny RNA. The resultant progeny RNA molecules then act as mRNA, as further templates for negative strand RNA synthesis, or are encapsulated into virions. Virus replication is however still poorly understood partly because of the lack of data on the mechanism regulating RNA synthesis.

1.2.5. Processing of the TBEV Primary Translation Product:

The TBEV genome is polycistronic, translation initiates at the N-terminal methionine of C, generation a single polyprotein precursor from which the viral proteins are co- and post-translationally processed (Fig. 1.3) (Lyapustin et al., 1986). A scheme involving multiple initiation of flaviviruses and similar to that of the alphaviruses, has been proposed (Westaway et al., 1977; reviewed Westaway, 1980; Westaway et al., 1984). The alternative and more convincing interpretation of current data which argues for the single initiation theory is now, however, generally accepted (reviewed Westaway, 1987; Rice, 1986).

The mosquito-borne flaviviruses gene order has been identified by direct N- and C- terminal sequencing of the polyprotein cleavage products and by alignment the predicted cleavage sites on primary amino acid sequences from the long ORF (Bell et al 1985, Biedrzycka et al 1987, Chambers et al 1989 & 1990, Coia et al., 1988; Nowak et al., 1989; Speight et al., 1988; Speight and Westaway., 1989; Wengler et al., 1990; Wright et al., 1989). Analysis of these data has revealed the existence of logical and consistent processing signals and



Cellular signalase located in the lumen of the ER.

Viral protease located in the cytoplasm, which recognises di-basic amino acids.

Unknown viral protease.

Possible cellular signalase located in the lumen of the ER.

Cellular methionine amino pepsidase located in the cytoplasm.

Protease recognising di-basic amino acids located in the cytoplasm.

Protease recognising di-basic amino acids in Post-Golgi vesicles.

Fig. 1.3 Schematic Representation of the Predicted Event which Process the TBEV Translation Product.

cleavage sites (reviewed Chambers et al., 1990a; Rice and Strauss, 1990). Direct N-terminal sequencing of the TBEV structural proteins and alignment of the TBEV amino acid sequence with that of the mosquito-borne viruses has confirmed the presence of the putative processing elements in the TBEV polyprotein (Boege et al., 1983; Iacono-Connors and Schmaljohn, 1992; Mandle et al., 1988, 1989a & 1991a; Pletnev et al., 1990).

Processing of individual TBEV proteins from the nascent polyprotein precursor is presumed to be by cellular signalases and virus-encoded proteases. A signalase located within the lumen of the endoplasmic reticulum (ER) cleaves following a recognition signal defined by the -3-1 rule (Val-X-Ala where X is uncharged) (von Heijne, 1983). A potential -3,-1 site is located at the C-terminal of each of the TBEV structural proteins, and cleavage following these motifs liberates anchored C, PrM and E from the polyprotein precursor (Mandl et al ., 1988 & 1991a; Pletnev et al., 1990). In addition to liberating the structural proteins from the polyprotein, this signalase cleavage also generates the N-terminal of PrM, E and NS1 and the C-terminal of anchored C, PrM and E. To permit access of the signalase to the cleavage sites, PrM, E and NS1 are translocated across the ER.

The translocation signal sequences, located immediately upstream of the -3,-1 signalase recognition motif in anchored C, PrM and E, are comprised of three characteristic regions: a basic N-terminal domain; a central hydrophobic domain and a more polar C-terminal domain. The amino acid preceding each signal domain is charged, and probably acts as the translocation stop-transfer signal (Mandl et al., 1988 & 1991a; Pletnev et al., 1990; von Heijne 1983 & 1985). Following cleavage, PrM, E and NS1 are held in the lumen of the ER for a limited period to allow for virus assembly. The signalase responsible for generating

the structural proteins may also cleave the NS4a-NS4b junction, since the cleavage has no viral requirement and the amino acid sequence preceding the N-terminal of NS4b is consistent with a signalase cleavage site and translocation signal (Cahour et al., 1992; Mandl et al., 1989a). Furthermore, generation of the NS4b N-terminal in the ER is consistent with NS4a and NS4b containing membrane spanning domains.

The second type of cleavage event involves a virus encoded protease located in the cytoplasm, which cleaves between two basic amino acids (reviewed Krausslich and Wimmer, 1988; Wellink and von Kammen, 1988). Di-basic residues form the C-terminus of NS2a, NS2b, NS3 and NS4b, and cleavage between these residues generates the N-terminus of NS2b, NS3, NS4a, and NS5 (Iacono-Conner and Schmaljohn ,1992; Mandl et al.,1989a; Pletnev et al, 1990; reviewed Rice and Strauss, 1990). The trypsin-like serine protease of NS3, in association with NS2b, has been implicated in this processing (Cahour et al., 1992; Chambers et al., 1991; Falgout et al., 1991; Preugschat et al., 1990; Wengler et al., 1991). A second protease recognising di-basic amino acids may be responsible for the removal of the C-terminal hydrophobic domain of anchored C.

Three additional cleavage events, necessary for TBEV maturation, are the removal of a methionine residue from the N-terminus of the C protein (Boege et al., 1983), the maturation of PrM to M and cleavage between NS1 and NS2a. It has been proposed that a cellular protease with an acidic requirement and which recognises di-basic residues removes the hydrophobic N-portion domain from PrM since trimming of PrM occurs in the acidic post-Golgi vesicles (Pletnev et al., 1990; Randolf et al., 1990). Removal of the N-terminal methionine from C,

is probably carried out by a cellular methionine amino peptidase while the source and specificity of the cleavage between NS1 and NS2a region has yet to be determined.

1.2.6. TBEV Virion Entry and Uncoating:

Although specific host cell receptors have not yet been identified for any of the flaviviruses, there is considerable evidence to indicate that mosquito-borne viruses are taken into cells by receptor mediated endocytosis via coated pits which transport the virus to internal endosomes (Gollins and Porterfield, 1985 & 1986; Ng and Lau, 1988). Flaviviruses are also able to gain entry into a cell, via antibody bound to Fc or C3 cell receptors, (the mechanism of ADE).

Fusion of TBEV with the endosomal membranes and the release of the viral genome into the cytoplasm probably results from pH dependent conformational changes. Consistent with this proposed mechanism of virus entry is the ability of weak bases to inhibit dengue virus, WNV, SLEV and YFV virus infections (Brandriss and Schlesinger, 1984; Randolf and Stollar, 1990) and to inhibit the release of WNV from the endosomes into the cytosol (Gollins and Porterfield, 1985). Observations that SLEV and JEV is maintained more successfully in an alkaline environment than an acidic one (Duffy and Stanley., 1945; Duffy, 1946) and that an acidic environment stimulates the uncoating of extra-cellular WNV are also consistent with this mechanism of entry (Gollins and Porterfield, 1986 & 1986a). pH dependent changes within antigenic domain A of TBEV E have been identified as possibly directing virion-cell fusion (Guirakhoo et al., 1989). In the light of the work by Guirakhoo et al on TBEV, the ability of TBEV to remain

infectious at pH values below those required to inactivate other flaviviruses and the transmission of TBEV in the acidic environment of milk are unexpected (Gresikova, 1958 & 1959; Guirakhoo et al., 1989).

1.2.7. TBEV Virion Maturation:

TBEV assembly occurs in association with cell membranes (Lubiniecki and Henry, 1974). Once translated, PrM, E and NS1 are translocated through the ER, cleaved from the polyprotein translation product, glycosylated and oligomerised, (where appropriate). The glycoproteins are then transported to the Golgi complex, where further modifications to their nascent oligosaccharide side chains take place. Virion assembly is presumed to be rapid, since the identification of intermediate steps including the mechanisms of nucleocapsid assembly and the characterisation of budding intermediates, has not been made (Demsey et al., 1974; Deubel et al., 1981; Filshie and Rehacek, 1968). It is proposed that the maturation of flavivirus virions consists of several, probably concurrent, events. Nucleocapsids are believed to assemble on the cytoplasmic face of cellular membranes, stabilized by the hydrophobic C-terminal anchor of C. The C anchor is cleaved to release nascent nucleocapsids, which are encapsulated by E and PrM budding through the internal cellular membranes into the post-Golgi vesicles.

1.2.8. TBEV Virion Egress:

The passage of virions out of the cell is through acidic post-Golgi vesicles. A mechanism by which nascent exiting TBEV virions are prevented from undergoing pH-mediated fusion with the intracellular membranes of these vesicle has been identified. The mechanism suggests that the PrM/E heterodimers identified in fusion incompetent,

immature virions inhibit the conformational changes in E required for fusion (Wengler and Wengler, 1989). Immediately prior to egress, however, PrM and E are disassociated allowing generation of fusion competent virions by trimming of PrM to M (Randolf et al., 1990) and mature virions are released at the cell surface by vacuole discharge.

1.3. TBEV NON-STRUCTURAL PROTEIN NS1:

Although little is known about the TBEV non-structural glycoprotein NS1 (47-51KDa), primary sequence comparisons with the mosquito-borne flaviviruses have identified several conserved features. These conserved features include the N- and C-terminal proteolytic cleavage motifs, potential N-linked glycosylation sites and the putative NS1 translocation signal sequence located in the C-terminal of E. A high degree of structural conformity between the tick-borne and mosquito-borne flaviviruses is implied by 11 spatially conserved cysteine residues (Iacono-Connors and Schmaljohn, 1992; Mandl et al., 1989a; Pletnev et al., 1990).

The 24 amino acid sequence preceding the N-terminus of NS1 in the TBEV translation product (between residues 752 and 776 for the Neudorfl strain of TBEV) are thought to direct translocation of NS1 across the ER and are consistent with the amino acid composition of translocation signal sequences preceding the N-terminus of NS1 in the mosquito-borne flaviviruses (Putnak et al., 1988; Falgout et al., 1989). Translocation of NS1 across the ER positions the E/NS1 junction for cleavage by a cellular signalase located in the ER. At the N-terminal of the putative NS1 translocation signal in TBEV is an arginine residue with the potential to act as the stop-transfer signal for translocation, while an amino acid sequence, (Val-Gly-Ala), characteristic of the -3-1 signalase recognition motif forms the C-terminal of the translocation signal sequence (von Heijne, 1983 & 1985; Mandl et al., 1989a & 1992a; Pletnev et al., 1990).

The three amino acids forming the C-terminus of TBEV NS1 (Val-Val-Ala) are also consistent with a -3-1 signalase motif, however, the signalase located in the ER and responsible for generating the C-

terminus of anchored C, PrM and E is not thought to mediate NS1:NS2a cleavage unless the translocation signal directing the E:NS1 junction into the ER, also directs translocation of NS2a. An indication as to the specificity of this novel enzyme comes from dengue virus for which the eight amino acids immediately preceding the C-terminal of NS1 have been implicated in the NS1:NS2a cleavage (Hori and Lai, 1990). The TBEV NS1 C-terminus octapeptide is consistent with signal sequence identified in dengue virus NS1 and may therefore provide a similar function. In addition to the octapeptide, deletion analysis has shown that efficient proteolytic cleavage at the NS1:NS2a junction of dengue virus also requires the presence of at least 70% of the NS2a protein (Falgout and Lai, 1990; Falgout et al., 1989).

An alternative C-terminal NS1 cleavage product, a fusion protein of NS1 and a portion of the adjacent N terminus of NS2a, have been identified in YFV, JEV and possibly MVEV, but not in TBEV- or dengue virus-infected cells (Chambers et al., 1990; Hall et al., 1990; Lee et al., 1989; Mason et al., 1987). The significance of this additional polyprotein cleavage product is unknown.

The primary sequence of TBEV NS1 has revealed three potential N-linked glycosylation motifs (Asn-X-Thr/Ser) at amino acids 861-863, 983-985 and 998-1000 of the Neudörfl strain (reviewed Kornfeld and Kornfeld ,1985; Mandl et al.,1989a). The position of the TBEV N-linked oligosaccharide side chain at amino acid 983-985 is conserved with one of the N-linked glycosylation sites found on the mosquito-borne flavivirus NS1. At least two oligosaccharide side-chains have been characterised for the NS1 protein of YFV, dengue virus and JEV while the ability of TBEV NS1 to incorporate radiolabelled sugars is consistent with it being glycosylated (Mason, 1989; Post et al., 1990; Stephenson et al., 1987; Winkler et al., 1988).

A secreted, particulate, form of the TBEV NS1, which is approximately 4 kDa larger than the intra-cellular form, has been identified (Lee et al., 1989). The glycosylation studies of YFV, dengue virus and JEV suggest that the size increase associated with the extra-cellular form resulted from modification of the glycan side-chains during export of NS1 from the cell (Lee et al., 1989; Mason, 1989; Post et al., 1990; Winkler et al., 1988). Both the intra- and extra-cellular forms of NS1 encoded by the tick-borne Powassan virus and several of the mosquito-borne viruses, have the ability to form heat labile dimers and possibly higher oligomers (Despres et al., 1991; Parish et al., 1991; Putnak and Schlesinger, 1990; Winkler et al., 1988; Schlesinger et al., 1990). Bonds which stabilize dengue virus dimers are sensitive to both heat and acid pH but resistant to reduction by 2-mercaptoethanol (Winkler et al., 1988), but while the JEV intra-monomer bonds are also heat and acid labile they appear to be partially sensitive to reduction (Fan and Mason, 1990). The importance of NS1 dimers is unknown however, although dengue virus NS1 dimers do appear to be more immunogenic than the monomers (Falconer and Young, 1990).

Antibodies raised to extra-cellular TBEV NS1 failed to neutralise viral plaque formation (Phillpotts et al., 1987). This result is consistent with biochemical data which indicate that in dengue virus NS1 does not form part of the virion (Brandt et al., 1970). NS1 of dengue virus, JEV, Kunjin virus and YFV has been detected on the surface of cells and shown to have properties consistent with cell association via hydrophobic domains even though the NS1 primary sequence contains no obvious hydrophobic regions (Cardiff and Lund,

1976; Despres et al., 1991; Fan and Mason, 1990; Mason, 1989; Putnak and Schlesinger, 1990; Schlesinger et al., 1990; Westaway and Goodman, 1987; Winkler et al., 1989).

During the course of natural infections, NS1 evokes a strong immune response. Experimental data from dengue virus- and YFV-infected mice and monkeys has shown that protection from lethal challenge may be afforded by immunization with purified NS1 protein (Cane and Gould, 1988; Schlesinger et al., 1986 &1987). Mice have also been protected from virus challenge by the passive transfer of anti-NS1 monoclonal antibodies (Gould et al., 1986; Henchal et al., 1986; Schlesinger et al., 1985). Although unable to neutralise virus infection, anti-NS1 antibodies can direct complement mediated lysis of virus-infected cells which has been proposed as a mechanism by which the protective immune response may arise (Schlesinger et al., 1985, 1986, 1987 & 1990). Initial work with YFV suggested that a correlation existed between the ability of anti-NS1 monoclonal antibodies to elicit protection and to direct complement mediated cytolysis (CMC) of infected cells. More recent studies involving other flaviviruses including TBEV have not supported this finding (Despres et al., 1991a; Henchal et al., 1988; Phillpotts et al., 1987; Putnak and Schlesinger, 1990). The role and importance of the humoral protective humoral response has only been implied in these protection studies, while the importance of an anti-NS1 MHC class I cytotoxic T lyphocyte (CTL) response is unknown. As new vaccines based on the TBEV NS1 protein are being considered the important implications of the immune response to NS1 merits further investigation.

1.4. AIMS:

The main aims of this study are to firstly express recombinant TBEV NS1 in the absence of other TBEV encoded proteins using an adenovirus vector, and secondly to examine the potential of the recombinant NS1 to protect mice against lethal TBEV challenge. In order to express TBEV NS1 gene it will be necessary to determine the factors encoded within the TBEV ORF which are required for synthesis of NS1. This will be achieved by examining the potential of several TBEV cDNA fragments when placed under the control of the powerful constitutive cytomegalovirus (CMV) major immediate-early IE promoter to express recombinant NS1 in transient expression experiments. Each cDNA fragment will contain a different form of the NS1 gene encoded by the Neudörfl and K23 strains of TBEV and will be generated by PCR, using either reverse transcripts of the TBEV genome or large TBEV cDNA fragments as a template. A recombinant adenovirus based on a TBEV cDNA fragment with the potential to express high levels of NS1 will then be constructed to allow the possible bio-physical characterisation of the TBEV gene product can be undertaken. Since the role of NS1 in virus replication is unknown, expression of TBEV NS1 as described above should lead to a better understanding of its significance. Furthermore the expression system will be a suitable tool with which to investigate the immune response elicited by NS1 from both an encephalitic and tick-borne flavivirus *in vivo*.

CHAPTER 2. Materials and Methods

2.1. Cell Culture:

PS cells, a line derived from pig kidneys, were used to propagate both the Neudörfl and K23 strains of TBEV. 293 cell, an adenovirus-transformed human embryonic cell line constitutively expresses the adenovirus E1 gene product, supported efficient replication of E1a-recombinant adenovirus mutants (Graham et al., 1977). Efficient expression from the cytomegalovirus (CMV) immediate early (IE) promoter expression cassette in the adenovirus vector was obtained in the absence of adenovirus gene expression when propagated in human embryo lung fibroblasts, MRC5 cells (Wilkinson and Akrigg, 1992). Since adenovirus gene expression was absent in MRC5 cell infected with the adenovirus vector, adenovirus replication was also inhibited.

Cell were passaged after being washed with pre-warmed phosphate buffered saline pH 7.5 (PBS: NaCl 8% (w/v); KCl 0.2% (w/v); 0.12%; KH_2PO_4 0.12% (w/v); 0.19% (w/v) Na_2HPO_4 (anhydrous)) and removed from the solid support with 1% trypsin/ethylenediaminetetraacetic acid, [di-sodium] salt solution (EDTA) (GibcoBRL, Paisley, Scotland). Further flasks (Nunc, GibcoBRL) were routinely seeded at a ratio of 1:3 and, when required, cells were enumerated using a haemocytometer (Weber Scientific, Lancing, UK) following dilution in trypan blue vital stain (Flow, ICN, High Wycombe, Bucks) at a final concentration of 0.02%.

Each cell line was propagated under specific conditions: PS monolayers were grown at 37°C, in Leibovitz 15 media (L15; Imperial Laboratories, Andover, UK) supplemented with 5% (v/v) foetal calf serum (FCS, Imperial Laboratories), 0.1% tryptone phosphate broth (Difco, East Molesly, Surrey, UK) and appropriate antibiotics, while 293 and MRC5 monolayer were grown in Glasgow Minimal Essential

Media (GMEM) (Imperial Laboratories) supplemented with 5% FCS and 2mM glutamine under 5% CO_2/air at 37°C. When infected with virus, cell monolayers were maintained under the appropriate conditions in media containing only 2% FCS (maintenance media). For stimulation of the CMV IE promoter, 0.01 mg/ml forskolin (Sigma, Poole, UK) was added to infected MRC5 cells. In one experiment the culture medium was replaced with protein free medium (PAA, Linz, Austria).

2.2. TBEV Strains:

The two Central European TBEV virus strains used in this study were Neudörfl and K23. The Neudörfl strain of TBEV was originally isolated from an infected Ixodes ricinus tick collected in Austria (Heinz and Kunz, 1981). Plaque purified Neudörfl strain of TBEV was obtained from the progeny of a single viral plaque, following three sequential passages in chick embryo fibroblasts (CEF) grown in an agar suspension at the CAMR, Porton Down, UK. A further passage, in suckling mouse brain (SMB), generated the seed stock from which working stocks were derived after a second SMB passage. The K23 strain was isolated from an Ixodes ricinus tick collected in the Karlsruhr region of Germany (Rehse-Kupper et al., 1978). Following three passages in SMB, the virus was purified in tissue culture by limited end point dilution. A purified stock of this virus was kindly supplied by Dr Hilfenhous (Behringwerke AG, Marburg, Germany) and on its receipt at CAMR, the virus was passaged once in SMB prior to use.

2.3. Propagation of TBEV:

High-titred stocks of both Neudörfl and K23 strains of TBEV were kindly provided by Mr J.Lee. For working purposes, 70% confluent PS cells monolayers (unless specified) were infected with virus at a multiplicity of infection (moi) of between 0.1 and 1 plaque forming units per cell (pfu/cell) in a minimal volume of PBS. After 4 h incubation at 37°C, the cells were washed with pre-warmed PBS and re-incubated in maintenance media until required (2 to 4 days).

2.4. Titration of TBEV:

TBEV strains were titrated in 24 multiwell plates (Nunc). To each well, 0.5 ml PS cells at $3X10^5$ cells/ml and 0.1 ml of virus diluted in PBS, from a series of 10-fold dilutions, were added. Following a 4 h incubation at 37°C, the infected cells were gently overlayed with 0.5 ml L15 carboxy methyl cellulose overlay (Meridick, Birmingham, UK) suspended in 2X L15 media supplemented with 3% (v/v) FCS. The cells were incubated at 37°C for 4 days before being fixed in formal saline (10% formalin in PBS) and stained with 0.1% crystal violet (Meridick) in 20% industrial methylated spirit (Merick).

2.5. TBEV Plaque Reduction Neutralization Assay:

TBEV plaque reduction neutralization assays were performed as titrations (section 2.4) with 100 µl aliquots of 2-fold dilutions of antisera in PBS being reacted with an equal volume of PBS containing 20 virus pfu for 4 h at 37°C prior to being mixed with the cells. The neutralisation titre was calculated as being the reciprocal of the antisera dilution which produced a 50% reduction in the number of plaques, in comparison with the control sample of virus reacted with PBS.

2.6. Production of Recombinant Adenovirus Stocks:

In addition to those E1a- recombinant adenoviruses constructed in this study, a defective adenovirus expressing ß-gal (RAd35) kindly supplied by Dr GWG Wilkinson of CAMR, was used as a control virus. Recombinant adenovirus stocks were prepared from 293 cells which had been infected with an moi of 0.1 pfu/cell when 80% confluent. The virus, diluted in a minimal volume of media, was left to adsorb to the cells overnight at 37°C. Next day, the cells were washed with pre-warmed PBS and returned to the incubator in maintenance media until the cytopathic effect (cpe) was seen in 100% of the monolayer. Virus was harvested from the infected cells that had been washed in PBS and recovered by a 5 min centrifugation in a MSE minstral (Fisons, Loughborough, Leics) at 2000 rpm. The cells were resuspended in PBS (2 ml per $2X10^6$ cell) and the virus extracted by mixing the cells vigorously with an equal volume of Arklone P (ICI, Runcorn, Cheshire, UK). The virus containing aqueous phase was separated by centrifugation at 2,000 rpm in an MSE minstral, harvested, aliquoted and stored at -70°C.

2.7. Titration and Plaque Purification of Recombinant Adenovirus Stocks:

Recombinant adenoviruses were titrated in 293 cells grown in flat bottomed, 96 well, microtitre plates, (Nunc). Monolayers (80% confluent) were infected with 0.1 ml from a 10-fold serial dilution of the virus in culture medium and incubated overnight at 37°C. Next day, the inoculum was replaced with 0.1 ml of fresh medium and the plates were returned to the incubator for 8 days. Media was

replaced after 4 days and again if acidic. Cells were examined for the presence of a cpe and viral titre was calculated by the Spearman-Karber endpoint determination (Finney, 1971). Plaque-purified recombinant adenoviruses were obtained from wells containing virus diluted to the end-point. The media from an appropriate well was harvested, diluted and re-plated. This procedure was repeated twice before the virus was deemed to be plaque purified.

2.8. Purification of Adenovirus by Gradient Centrifugation:

The aqueous phase of the Arklone P extracted virus (section 2.6) was carefully pipetted on top of a CsCl solution (1.6 ml of density 1.45 g/ml and 3.0 ml of density 1.33 g/ml in 5mM Tris/HCl, 1 mM EDTA, pH 7.8) in a 15 ml Beckman (Beckman Instruments, High Wycombe, Bucks) centrifuge tubes and centrifuged at 90,000g in a Beckman L5-6513 Ultracentrifuge, for 2 h. The opalescent virus layer, located at the interface between the higher and lower density solutions, was removed by aspiration and diluted 1:2 with the Tris/EDTA buffer before being layered onto a second CsCl gradient of 3 ml of density 1.33 g/ml and 2.0 ml of density 1.45 g/ml. This gradient was centrifuged overnight (16 hrs) at 100,000g, and a band of opalescent virus formed at a density of 1.34-1.35 g/ml. After harvesting, the virus was dialysed overnight against 10% glycerol in the buffer used to make up the gradients.

2.9. Infection of PS Cell Monolayers With Infected Mouse Brain Homogenate and Sera:

Sera was harvested from Balb/C mice as described in section 2.38, while brains were removed from the decapitated heads of anesthetised mice and placed into universal tubes containing 1 ml ice cold sterile PBS. The brains were chopped up, rinsed in fresh, ice cold PBS and placed into a second sterile glass universal containing 1 ml/brain of ice cold PBS and 1/2 inch depth of sterile 0.3 cm sterile glass spheres (Sigma). The brains were vortexed for 1 min and stored at -70°C. When required, both the brains and sera were rapidly thawed at 37°C and placed on ice. 50 µl sera and 0.5 ml brain homogenate aliquots were incubated at 37°C for 4 h on PBS washed cells growing on glass coverslips. The inoculum was removed, the cells rinsed with PBS and reincubated at 37°C in maintenance media for a further 7 days. The presence of infectious virus was assessed by an immunofluorescence antibody test (section 2.28).

Work TBEV took place in category 3 facilities under the appropriate regulations. All recombinant adenoviruses were worked with in category 2S containment in accordance with the Advisory Committee on Genetic Manipulations (ACGM) recommendations.

2.10. Plasmids Strains:

Plasmid pMTL23 (Fig. 3.1) was kindly given by S.Chambers of CAMR (Chambers et al., 1989), while the transfection vector, pMV100 (Fig. 3.2), and the recombinant adenovirus transfer vector pMV60 (Fig. 3.20) were kind gifts from Dr. Wilkinson of CAMR (Wilkinson and Akrigg, 1992). Plasmid pJM17 (Fig. 3.21), containing the genome of adenovirus type 5 dl309 with the prokaryotic vector pBRX inserted

into the E1a gene and a deletion in the E3 gene, was a gift from Professor Frank Graham at McMaster University Canada (McGrory et al., 1988).

2.11. Bacterial Strains:

The two bacterial strains used in this study were obtained from either GibcoBRL or the Promega Corporation (Southampton,UK.). The E. coli K12 JM109 endA1, recA1, gyrA96, thi, hasR17, (r^{-k}, m^{+k}),relA1, supE44, (lac-proAB), [F′, traD36, proAB, $lac^{I}qZ$ M15] was preferentially used to propagate plasmids. JM109 contains an F′ episome carrying the $lacI^{q}$ mutation responsible for the over production of the lac repressor which allows expression of the lac operon to be regulated by the lac inducer IPTG.

E. coli MAX efficiency DH5 competent cells F-, Ø80d lacZ M15, (lacZYA-argF), U169, recA1, endA1, hsdR17 (r^{-k},M^{+k}), supE44, -, thi-1, gyrA, relA1, were used only for high efficiency transformations. This strain is F-, the F′ episome being replaced with the 80d lac Z M15 marker which provides α complementation of the ß-gal gene provided by the plasmid. Induction of the ß-gal gene permitted positive identification of recombinant colonies.

2.12. Propagation of Bacterial Strains:

Bacterial strains were grown in sterile L-broth (10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl per litre, pH 7.5), or on L-agar (15 g sterile bacto-agar/Ltr L-broth). In antibiotic selection experiments, ampicillin (Sigma) was used at a final concentration of 50 µg/ml and the chromogenic substrate 5-bromo-4-chloro-3-indoyl-3-galactosidase (x-gal), which turns blue when

hydrolysed by ß-galactosidase, was used at a final concentration of 0.02% (w/v) together with 50 µM iso-propyl-3-D-thio-galactopyranoside (IPTG).

Long term storage of transformed bacterial colonies was achieved at -80°C, when bacterial cultures, grown overnight at 37°C in L-broth supplemented with 50µg/ml ampicillin, were mixed with an equal volume of sterile glycerol and frozen in 1 ml aliquots. To recover the bacteria from -80°C, a loopful of the thawed suspension was streaked out onto L-agar, or inoculated directly into nutrient broth and incubated overnight at 37°C.

2.13. RNA Extraction From TBEV-Infected PS Cells:

Total cellular RNA was extracted from two 225 cm^2 flasks of TBEV-infected PS cells. At 48 h post infection (hpi), cell monolayers were washed twice in PBS at room temperature and detached from their solid support using 0.3 cm diameter sterile glass spheres (Sigma). The cells were recovered by 10 min centrifugation in an MSE minstral centrifuge at 3000 rpm, resuspended in 0.9 ml of 0.15 M Nacl; 0.1 M Tris/HCl pH 7.5; and 1 mM EDTA containing 25 U/ml placental RNase inhibitor (Sigma) and allowed to swell. After 10 min, the cells were lysed by the addition of Nonidet P40 (NP40: 10% (v/v) stock solution) to a final concentration of 1%, and vigorously vortexed for 30 sec. The cell nuclei and debris were removed by a 2 min centrifugation (12,500g) using an MSE micro-centrifuge and the RNA extracted from the supernatant 3 times with phenol, and twice with chloroform. The RNA was recovered by ethanol precipitation, washed, and dissolved in TE buffer (TE Buffer: 0.01 M Tris/HCl pH 8.0; 1 mM EDTA) containing 25 U/ml placental RNase inhibitor. The concentration of RNA was assessed by spectrophotometry (Pye Unican

SP 6-550 spectrophotometer) at 260 nm where an optical density of 1.0 is equivalent to RNA at 40 µg/ml. During this procedure certain precautions were taken to minimise the risk of contamination by ribonucleases, these involved; good aseptic technique; the use of double distilled or a similar high quality water; heat sterilisation of glass wear and autoclaving of buffers and reagents where practicable.

2.14. Denaturing Formaldehyde Gel Electrophoresis of RNA:

Samples were prepared by mixing 4.5 µl of RNA in a MSE micro-centrifuge tube with 2.0 µl 1X MOPS buffer (10X MOPS buffer: 0.2 M 3-N-Morpholino-propane-sulphonic acid, pH 7.0; 0.05 mM sodium acetate; 0.01 mM EDTA); 3.5 µl 40% formaldehyde; 10 µl re-crystalised formamide (Casey and Davidson, 1977) and denaturing at 55°C for 15 min. After chilling on ice, the samples were mixed at a ratio of 5:1 with sample buffer (50% (w/v) ficol; 1 mM EDTA; 0.25% (w/v) bromophenol blue) and applied to a pre-run horizontal denaturing formaldehyde agarose gel formed in the BRL mini gel apparatus (Lehrich et al., 1977). The 1% high melting point agarose gel was prepared in water by cooling a melted agarose solution to 60°C before adding 5X MOPS buffer and formaldehyde to give a final concentration of 1X and 2.2 M, respectively. Electrophoresis was carried out in 1X MOPS buffer, at 50 V for 4 h , after which the nucleic acids were stained by immersing the gel for 1 h in 1 µg/ml ethidium bromide diluted in the tank buffer, and destained in fresh tank buffer for a further hour. The stained bands were visualised by transillumination with longwave UV light (365 nm) and photographed (polaroid film 667) using a red filter. E. coli 16S and 23S r RNA molecular weight markers were included on the gel.

2.15. Reverse Transcription of Purified RNA:

cDNA copies of total infected cell RNA were prepared in 20 µl volume reverse transcription reactions. RNA (5 µg) and (0.5µg) random hexonucleotide primers (Boehringer Mannheim, Lewes, UK) were incubated in the presence of 0.1% dimethyl sulphoxide (DMSO) at room temperature for 3 min, before being added to pre-warmed reverse transcription mix containing 1X PCR buffer (Promega Corporation), (10X PCR buffer: 100 mM Tris/HCl pH 8.3; 15 mM KCl, 500 mM $MgCl_2$; 1% Triton X100: gelatin 0.1% (w/v)); 20 U placental RNase inhibitor (Sigma); 500 µM of each of dATP, dTTP, dCTP and dGTP (Perkin-Elmer, Beaconsfield, Bucks.); and 2.5 mM $MgCl_2$. 20U of Moloney murine luekaemia virus reverse transcriptase (Pharmacia Biosystem Ltd, Milton Keynes, UK) was added and the reaction was incubated at 37°C for 40 min.

2.16. Polymerase Chain Reaction:

The polymerase chain reaction was used to amplify specific regions of cDNA containing the NS1 gene generated by reverse transcription (section 2.15). The limits of the amplified fragments were defined by 2 oligonucleotides which hybridised to opposite strands of the target sequence so that their 3′ ends were towards each other. The 20 µl PCR mix contained 4 µl of the reverse transcription product; 1X PCR buffer (Promega Corporation); 1 µM of each of both primer; 1 U Taq DNA polymerase (Promega Corporation) and was overlayed with white paraffin oil (Sigma). When the cDNA template was provided from the reverse transcription reaction, the dNTP mix present in that volume was sufficient for the PCR, however, when the template was provided from an alternative source, a 0.2 mM dNTP mix (Promega Corporation) was supplied. During each PCR cycle, the cDNA template

was denatured at 94°C for 45sec, and primers allowed to anneal for 2 min at a temperature calculate by assigning each A and T base of the primer 2°C and each G and C base 4°C and subtracting 10°C from the sum. Once annealed, extension from the primers was performed for 4 min at 72°C. This three step cycle was repeated 30 times, after which the oil overlay was removed by chloroform extraction, and the DNA recovered by ethanol precipitation. PCR primers, sequencing primers and single stranded adaptors were kindly synthesised by J.M.Chamberlain using an Automatic DNA Synthesiser BT8500,(Biotech Instrumentation Ltd, Luton, Beds, UK).

2.17. Restriction Endonuclease Digestion:

Restriction endonucleases and their buffers were supplied by the Promega Corporation and used according to the manufacturer's instructions.

2.18. Agarose Gel Electrophoresis:

High melting point agarose (0.8% (w/v)) was melted in 1X Tris-borate-EDTA (TBE) buffer (TBE buffer 10X: 90 mM Tris; 90 mM boric acid; 2.5 mM EDTA pH8.3) and poured in a horizontal mini gel apparatus (BRL) at a temperature of between 50°C and 60°C. DNA samples were mixed with 1/10 volume loading buffer (10% ficol (v/v); 0.05% bromophenol blue (w/v)) prior to electrophoresis. Following electrophoresis at 80 V for 1 h, the nucleic acids were stained with ethidium bromide staining and visualised under a UV light source as described in section 2.14.

2.19. Elution of DNA fragments from Agarose gels:

cDNA was recovered from agarose gels by electroelution. Following ethidium bromide staining, the required DNA fragments were excised precisely from the gel and placed into a dialysis bag with 1/2X TBE buffer. The bag was immersed in 1/2X TBE buffer so that it was perpendicular to the electrodes. Electroelution was achieved following the application of 50 V for 30 min, with the current's polarity being reversed for the last 30 sec to detach any cDNA adhering to the inner wall of the bag. The buffer and eluted cDNA were removed from the bag and the DNA recovered by ethanol precipitation.

2.20. Ligation of DNA Molecules:

Following the linearisation of plasmid vectors, they were treated with bacterial alkaline phosphatase (GibcoBRL) according to the manufacturer's instructions to prevent self-ligation. The cDNA inserts and linearised vectors were then mixed at a concentration of 5:50 µg/ml, respectively, in 25 µl volume of ligase buffer (Promega Corporation) (10X ligase buffer: 300 mM Tris/HCl pH 7.8; 100 mM $MgCl_2$; 100 mM DTT; 10 mM ATP) and using two units T4 DNA Ligase (GibcoBRL). The ligation reaction was allowed to proceed at 15^{oC} temperature overnight.

2.21. Preparation of Adaptors:

Adaptors were supplied as single-stranded DNA molecules. The palindromic nature of these oligonuceotides allowed molecules to self-anneal to form double-stranded molecules. In a two step reaction, 1 µg of adaptor in 1X kinase buffer (kinase buffer 10X: 500 mM Tris/HCl pH 9.0; 100 mM $MgCl_2$; 50 mM DTT; 1 mM spermidine)

containing 1 mM ATP and 200 U T4 polynucleotide kinase were first phosphorylated when the reaction was incubated at 37°C for 30min. Followed 5 min in a water bath at 95°C, the water bath was turned off and the oligonucleotides allowed to anneal overnight.

2.22. Transformation of Competent Cells:

Circular recombinant plasmid DNA was used to transform competent E. coli, according to instruction supplied by the appropriate manufacturer (GibcoBRL or Promega Corporation). Briefly, 5 µl of the ligation reaction, diluted 5-fold in TE buffer, was mixed with 100 µl of competent cells and placed on ice for 30 min. Following heat shock at 42°C for 45 sec, the cells were returned to ice and after 2 min the cells were gently added to 0.9 mls warmed L broth which was shaken at 37°C for 1 h. The cells were then plated onto L agar containing 50 µg/ml ampicillin to select for colonies containing plasmids.

2.23. Small Scale Plasmid Preparation:

Rapid, small scale plasmid preparations were obtained from a single host bacterial colony based on the method of Birnboim and Doly (1979). Single colonies were picked and overnight cultures grown in L broth containing 100 µg/ml ampicillin. Cells from 1.5 ml of each culture were recovered by centrifugation in a MSE micro-centrifuge (12,000 rpm), resuspended in 100 µl 25 mM Tris/HCl pH 8.0; 500 mM EDTA; 50mM glucose and placed on ice. After 5 min, 200 µl 1% SDS (w/v) in 0.2 M NaOH was added, followed by 150 µl 3 M Sodium acetate pH 4.8, 5 min later. After 10 min the supernatant was clarified by centrifugation (12,000 rpm, 5 min), and harvested.

The DNA was recovered from the clarified supernatant by ethanol precipitation and the resulting DNA resuspended in TE buffer for analysis.

2.24. Large Scale Plasmid Preparation:

Large amounts of purified plasmid DNA were prepared using a protocol which followed that of Chewell and Helinski (1969). Individual cloned bacterial colonies were picked and grown overnight in 3 ml L. broth (containing 100 µg/ml ampicillin) in a shaking incubator at 37°C. Next day, each overnight culture was made up to 500 ml with L. broth (plus ampicillin) and further incubated with shaking until the OD_{650} reached 0.9, when chloroamphenicol (Sigma) (75 mg/ml in ethanol) was added to a final concentration of 75 µg/ml. The cultures were incubated for another 12 h before being harvested by centrifugation at 8000 rpm, 4°C for 15 min in a Sorval GSA rotor (DuPont, Stevenage, Herts) and resuspended in 2 ml of 25% (w/v) sucrose and 50 mM Tris/HCl (pH 8.0). After 5 min 0.3 ml of 20 mg/ml lysozyme (Sigma) in 0.25 M EDTA was added. The suspension was intermittently agitated on ice over a period of 5 min after which 2 ml of 0.25 M EDTA was added. Cells were lysed after 5 min by the addition of 3 ml lysis solution (1% Brij 58 (w/v); 0.4% Na deoxycholate (w/v): 10 mM Tris; 1 mM EDTA), followed by vigorous mixing and incubated on ice for 20 min. Cell debris was removed by centrifugation at 15,000 rpm at 4°C for 45 min (Sorval SM24 rotor) and the clarified lysate decanted. Solid $CsCl_2$, to a final concentration of 0.95 g/ml was added together with 100 µl of ethidium bromide (200 µg/ml) to the lysates. Samples were then transferred to Ti56 polymer tube (Beckman, High Wycombe, Bucks, UK). The tubes were heat sealed and centrifuged at 38000 rpm for 60 h.

Following centrifugation, the tubes were examined under a UV light (366 nm wavelength) and the plasmid containing lower, fluorescent band aspirated.

Plasmid DNA was extracted 3 times with propan-2-ol (Meridick) saturated with $CsCl_2$ from this band and dialysed 3X against 200 volumes of TE buffer. To determine DNA concentration the absorbance of the plasmid preparation was measured at 260 nm and 280 nm.

2.25. DNA Sequencing:

TBEV cDNA within plasmids were sequenced by overlapping primer extension reactions which between them spanned the entire length of the cDNA insert (Fig. 3.11). Apart from the primers, reagents were supplied in the United States Biochemical Corporation's (Cleveland, Ohio, USA) Sequenase version 2.0 kit. For each template reaction, 3–5 µg of the plasmid DNA was denatured by incubation in 0.2 M NaOH at 37°C for 30min. The reaction was neutralised by a 0.1 volume of 2 M ammonium acetate pH 4.5 and the denatured DNA recovered by ethanol precipitation. After washing with ethanol, the DNA was redissolved in 7 µl water to which 2 µl 5X Sequenase reaction buffer (5x Sequenase reaction buffer: 200 mM Tris/HCl pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl) and 1 µl sequencing primer (0.5 pM) were added. The primer annealed to the denatured plasmid during a 2 min incubation at 65°C. The reaction was then allowed to cool before 1 µl of 0.1 M DTT; 2 µl of 1X labelling mix (labelling mix: 7.5 µM dATP; 7.5 µM dCTP; 7.5 µM dTTP; 7.5 µM dGTP); 0.5 µl [35S]-dATP (5 µCi) (Amersham international PLC, Aylesbury, Bucks) and 2 µl Sequenase diluted 1/8 with enzyme buffer (Enzyme buffer: 10 mM Tris/HCl pH 7.5; 5 mM DTT; 0.5 µl/ml BSA) were added. The concentration of labelling mix was varied to allow sequencing near to the primer (0.05–0.1X) or further

from it (5X). Primer extension proceeded at room temperature for 3-5 min, before 2.5 µl of the reaction was added to each of 4 prewarmed tubes containing 80 µM of 1 of the 4 termination mixes (ddATP, ddCTP, ddGTP, ddTTP). The termination reactions were incubated for 5 min at 37°C and were stopped with 4 µl of stop solution (95% formamide; 20 mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol FF).

Prior to analysis on a pre-heated sequencing gel each sample was boiled for 2 min. Electrophoresis of the sequencing reactions was carried out in a 376 cm X 17 cm X 0.2 cm vertical polyacrylamide gel apparatus (Raven). Gels were prepared from 50 ml of a 6% (w/v) acrylamide/bis acrylamide stock solution containing 8 M urea and TBE buffer, and polymerised by the addition of 150 µl of 10% (w/v) ammonium persulphate and 50 µl N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma). An inverted sharks tooth comb was inserted into the top of the gel. Once set, electrophoresis took place in TBE buffer at 1100 V for 4-6 h (Pharmacia 3000/150 power supply). The electrophoresed sequencing gels were fixed in methanol 10% (v/v); acetic acid (10% v/v) solution for 10 min, before being placed onto 3MM paper (Whatmann, Maidstone, Kent) covered with Saran Wrap (Genetic Research Instrumentation, Dunmow, Essex) and dried under vacuum at 80°C. The dried gels were compressed and sealed in a film cassette holder, which immobilised them under X-ray film (Amersham International PLC). Autoradiographs were developed using Kodak DX80 developer and FX40 fixer.

2.26. DNA Transfection of Eukayotic Cells:

The calcium phosphate DNA transfection methodology was developed by Graham and Van der Eb (1973). 10 µg of plasmid DNA was dissolved in 210 µl 0.5 mM EDTA; 1 mM Tris pH 7.5 and pipetted into a 10 ml polypropylene tube (Falcon). The tube was gently vortexed as 30 µl of 2 M $CaCl_2$ was added dropwise to the DNA solution. This mixture was in turn, added dropwise to a tube containing 240 µl of 2X HBS buffer (280 mM NaCl; 50 mM Hepes: 1.5 mM Na_2HPO_4 pH 7.12) during vigorous vortexing. A precipitate was allowed to form at room temperature and after 30min the transfection mixture was added directly to the culture media of cells which had been seeded onto a 50 mm petri dish 24 h before to produce an 80% confluent monolayer. The transfection mixture was removed following an overnight incubation at 37°C and the cells washed once with PBS, and once with complete media. Fresh media was then added and the cells incubated a further 24 h before being analysed for expression.

2.27. Production of Infected Cell Lysates:

Cells were washed with PBS before being removed from their solid support with a rubber policeman into PBS. The suspended cells were recovered from PBS by a 2 min gentle centrifuged in an MSE Micro-centrifuge at 6500g and resuspended in 100 µl of buffer (0.15 mM NaCl; 0.1 M Tris pH 7.5; 1 mM EDTA) to which 20 µg/ml of the protease inhibitor phenylmethylsulphonylfluoride (PMSF) and NP40 (final concentration of 1% (w/v)) had been added. The cells were lysed by vigorous vortexing for 30sec, the debris removed by centrifugation, and supernatant stored at -20°C.

2.28. Indirect Immunofluorescence:

Cells grown on glass coverslip were washed once in PBS and fixed by immersion in acetone for 30 sec. Fixed cells were first incubated for 1 h with the appropriate anti-NS1 monoclonal antibodies prepared from tissue culture supernatant (diluted 1:50 in PBS), before being washed in PBS (30 min) and then incubated for 1 h with fluorochrome-conjugated goat anti-mouse IgG (Sigma) diluted 1:500 in PBS. The cells were washed once more and the coverslips mounted in 95% glycerol. All incubations and washes were performed at 37°C in a moist chamber. The cells were examined by UV light microscopy. Photographs were taken using Leitz Dialux 20EB microscope with appropriate filters and attachments.

Immunofluorescence was also used to demonstrate the presence of antigens on the cell suface. PBS washed cells were fixed for 15-30 min with freshly prepared 2% solution of fixative (10% (w/v) methanol free paraformaldehyde solution made up in a corning plastic tube was gently heated in a water bath, NaOH was added until the paraformaldehyde dissolved after which 10 ml of the paraformaldehyde was added to 9.7 ml of water and 30.3 ml of PBS and keeped on ice until required). The fixed cells were rinsed well with PBS and covered with 1% BSA in PBS for 15 min (blocking buffer). The blocking buffer was gently aspirated and antibody (1:50 in PBS) added to the cells for 45 min. The cells were again washed in PBS and 1% BSA in PBS before being incubated for 45 min in fluorochrome-conjugated goat anti-mouse IgG diluted 1:500 in PBS. The conjugate was then rinsed off and the cells washed well in PBS before being mounted in 95% glycerol and examined by UV microscopy.

2.29. Radio-Labeling of Virus-Infected Cells:

Uninfected and RAd51-infected cells were grown in methionine-free GMEM (Imperial Laboratories) supplemented with 2% FCS, and 2 mM glutamine, under 5% CO_2/air prior to the addition 250 µCi of [^{35}S] methionine (Amersham International PLC) in a minimal amount of fresh methionine free medium. After the pulse, cells were rewashed and returned to the incubator in fresh maintenance media.

2.30. Immunoprecipitation of NS1 From Radiolablled Cells:

Immunoprecipitation experiments were performed on [^{35}S] methionine radiolabelled infected-cell lysates (section 2.29) and directly on labelled cell culture supernatants. Cell lysates (100 µl) or supernatants (0.5 ml) are reacted for 1 h on ice with an anti-NS1 monoclonal antibody pool containing the monoclonal antibodies; T12, T33/1, and T33/3 (Stephenson et al., 1984). The antibody:antigen complexes were captured on protein A- and protein G- Sepharose beads (Pharmacia) during a 1 h incubation at 4°C. The beads were then washed three times with PBS, and recovered by 5 min centrifugation in an MSE micro-centrifuge (12,000 g) before being resuspended in PAGE sample buffer (section 2.23) containing 0.5% 2-mercaptoethanol. The protein were released from the beads by boiling for 10 min prior to SDS PAGE (section 2.33). Following electrophoresis, the gel was dried down under vacuum and subjected to autoradiography (section 2.25).

2.31. Endoglycosidase Treatment of NS1:

Glycosidase F (glycoF; endoF [EC 3.2.1.96] and N-glycosidase F [EC 3.2.2.18) and endoglycosidase H (endoH; [EC 3.2.1.96] reactions were carried out according to the manufacturer's instructions

(Boehringer). Briefly, immunoprecipitated radiolabelled NS1 (100 µl) released and separated from the Sepharose beads by boiling samples for 10 min in 400 µl of SDS (0.1% (w/v)) followed by centrifugation in an MSE micro-centrifuge. 100 µl of the recovered protein was treated with 0.1 U of glycoF in 0.25 M sodium acetate; 20 mM EDTA, 10mM 2-mercaptoethanol, (pH 6.7) or with 0.03 U of endoH in 50 mM sodium acetate (pH 5.5); 1 mg/ml of BSA, (pH 5.5), 20 µg/ml PMFS and 25 µg/ml aproteinine were added and the reaction incubated for 24 h at 37°C before analysis by SDS PAGE (section 2.35).

2.32. Ultra-Violet Light Treatment of Virus Stocks:

UV irradiation of RAd51 stocks was performed using a purpose built apparatus. 10 ml of virus was pipetted into a 100 cm diameter petri dish which was placed on a rocking table situated 30 cm from the UV light source. The gentle rocking motion of the table ensured that the virus suspension was evenly irradiated. The UV light source delivered 1.5 mW/cm_2 which is equal to 1.5 $\mu J/mm\underline{2}$ per sec. Samples from the same RAd51 stock (titrated at 10^7 pfu/ml) was irradiated for 0s, 60 sec (0.9 mJ/mm^2), 120sec (1.8 mJ/mm^2), 5min (4.5 mJ/mm^2), 10min (9.0 mJ/mm^2) and 15min (13.5 mJ/mm^2). 2 ml of each radiated sample was inoculated into 293 cells grown on glass coverslip and test by immunofluorescence for expression of the NS1 antigen or titrated in 293 cells as a test of virus infectivity.

2.33. Poly-Acrylamide Gel Electrophoresis (PAGE):

A SDS polyacrylamide resolving gel (10% (w/v); acrylamide; 0.3% (w/v) bis acrylamide; 375 mM Tris/HCl pH 8.8; 0.1% (w/v) SDS) was polymerised by the addition of 0.1% (w/v) ammonium persulphate and 0.0445% (v/v) TEMED, and poured into the required size mould. The

gel was carefully overlayed with isobutanol (Meridick) to ensure a level surface and allowed to set. The isobutanol was then poured off and a stacking gel (5% (w/v) acrylamide; 0.15% (w/v) bis acrylamide; 130 mM Tris/HCl pH 6.8; 0.1% (w/v) SDS) polymerised by the addition of 0.1% (w/v) ammonium persulphate and 0.01% (v/v) TEMED, added. The gels were run in buffer contained Tris-glycine buffer (25 mM Tris pH 8.3; 250 mM glycine; 0.1% SDS) and the samples prepared in 1X sample buffer (10X sample buffer: 1% (w/v) SDS; 50% (v/v) glycerol; 0.15% (w/v) bromophenol blue). Samples were not boiled in reducing agent prior to electrophoresis, since this treatment destroys epitopes recognised by the anti-NS1 monoclonal antibodies T12, T33/1 and T33/3 and suggesting that these antibodies recognise conformational dependent epitopes. The apparent molecular weights of the sample proteins was established relative to prestained markers proteins (BioRad, Hemal Hempsted, Herts). A gradient SDS PAGE comprising of an acrylamide gradient from 5% to 15% was prepared using a Buchler gradient maker (Buchler Industrial Inc, Fort Lee, New Jersey, USA). A 5% gel (5% (w/v) acrylamide; 0.15% (w/v) bis acrylamide; 375 mM Tris/Hcl pH 8.8; 0.1% SDS) and a 15% gel (15% (w/v) acylamide; 0.45% (w/v) bis acrylamide; 375 mM Tris/Hcl pH 8.8; 0.1% SDS) were mixed in the gradient maker immediately following polymerisation with 0.1% (w/v) ammonium persulphate and 0.008% or 0.004% TEMED respectively, and pumped into the gel former. The gel was overlayed with isobutanol and a stacking gel added as described above.

2.34. Coomassie staining of SDS Poly-Acrylamide Gels:

When required the presence of proteins on SDS polyacylamide gel was demonstrated by stained with coomassie blue R-250 (Sigma). Following electrophoresis the stacking gel was removed and the resolving gel fixed and stained by immersion in 0.25% coomassie blue R-250 in methanol, acetic acid and water (3:1:6) for 2 h at room temperature. After overnight destaining in the stain solution without coomaassie blue, the gel was dried under vacuum for storage.

2.35. Western Transfer Experiments:

Proteins separated by SDS PAGE were electrophoretically transferred to a nitrocellulose membrane (Hybond C plus, Amersham International PLC) essentially according to Burnette and Dunn (Burnette and Dunn 1986). The nitrocellulose filter was immersed in transfer buffer (25 mM Tris; 190 mM glycine; 20% methanol (v/v)) for 2 min before construction of a 'sandwich' of scotchbrite pad; filter paper (2 sheets); gel; nitrocellulose filter; filter paper (2 sheets); scotchbrite pad. The 'sandwhich' was placed into a BioRad Transblot apparatus with the nitrocellulose filter between the gel and anode and immersed completely in transfer buffer. Transfer was achieved by the application of 30 V (0.12 amp) overnight at room temperature.

Western blots were washed in washing buffer (PBS containing 0.3% (v/v) Tween 20; 3% (w/v) and solid milk powder (Cadbury's) at room temperature for 1 h. The blots were immersed for a second h in washing buffer containing 10 µg/ml of the appropriate antibody after which they were washed 3 times in blocking buffer, and incubated for 1 h in washing buffer containing anti-mouse IgG horseradish peroxidase conjugate (Amersham International PLC). Finally, the filter was washed once in washing buffer and 4 times in PBS, and

drained well. Equal volumes of the Chemiluminescent detection agents A and B (Amersham International PLC) were mixed and applied to the nitrocellulose. After 1 min agitation, the agents were poured off and the filter wrapped in Saran Wrap before exposure to X-ray film which was developed as in section 2.25.

2.36. Mice Strains:

Female Balb/C mice were obtained from Harlan Olac, (Bicester, Oxon.) and used at 4-6 weeks old. Haemolytic complement positive mice (sufficient) and mice lacking the complement C5 receptor therefore devoid of haemolytic complement activity (deficient) (reviewed in Rosenberg and Tichibana, 1986) were provided as the congenic mouse pair B10 D2 sufficient (NSN) and B10 D2 deficient (OSN) by Jackson's Laboratory, Bar Harbour, Maine, USA.

2.37. In Vivo LD_{50} Assay of TBEV:

A group of 10 mice was injected subcutaneously with 200 µl of virus from a single dilution in a 10-fold series. The mice were maintained until a hind leg paralysis occurred and death was imminent, when they were sacrificed. One LD50 dose was calculated using a modification of Reed and Meunch method (Reed and Meunch, 1938).

2.38. Recombinant Adenovirus Immunization Schedule:

Mice were immunised with recombinant adenoviruses by inoculating 10^7 pfu of virus diluted to 100 µl with PBS into the intra-peritoneal cavity (IP). This inoculation protocol was repeated after 1 and 3 weeks. Serum was obtained from blood samples

collected from terminally anesthetised mice by cardiac puncture. The serum was separated by centrifugation (3000 rpm) in serum separation tube (Becton Dickinson).

CHAPTER 3. Results

3.1. Cloning, Sequencing and Transient Expression of the TBEV NS1 gene:

3.1.1 Overview:

In order to identify the TBEV NS1 it was necessary to generate a cDNA construct containing the NS1 gene. Total cytoplasmic RNA was purified from TBEV-infected cells, and reverse transcribed using random primers. From the TBEV genomic RNA, a cDNA fragment of 1,891 bp, containing the complete NS1 gene together with part of the E and NS2a genes (FENS12a) was generated by PCR. Initially, PCR generated a truncated fragment with a 858 bp deletion which mapped to a region with potential to encode a hairpin loop in the corresponding RNA molecule. The treatment of RNA with DMSO in the presence of random primers prior to the reverse transcription reaction was found to be necessary for efficient generation of the full-size cDNA fragment. Restriction endonuclease cleavage sites within the PCR primers facilitated cloning of the PCR-amplified fragment into the vector pMTL23 (Fig. 3.1). pMTL23 is based on the pUC series of vectors, but contains a different polylinker region providing alternative cloning sites (Chambers et al., 1988). The fragment FENS12a was cloned into pMTL23 generating plasmid pMV40. The sequence of the cloned TBEV fragment was determined and found to be in agreement with the published results (Mandl et al., 1989), except for a translationally silent change from T to A as base 3265.

To ensure that the NS1 coding sequence was correctly identified five additional viral DNA constructs in which the putative NS1 ORF was presented in a different context were generated by PCR using FENS12a as the template, including; the NS1 gene embedded within a long ORF, the gene without additional 3′ coding sequence, the gene without

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additional 5′ coding sequence, the gene without a translocation signal sequence, and the predicted coding region. The inclusion of appropriate translational start and stop codons on each NS1 construct to be expressed was achieved by encoding them within the PCR primers or by addition of the adaptor L1. The generation of these various constructs permitted the identification of elements required for appropriate expression of NS1 in a transient expression system before going on to construct a recombinant adenovirus. Each fragment was checked by DNA sequencing to ensure PCR had not generated a mutant before being cloned under the control of the CMV major IE promoter in the transient-expression vector pMV100 (Wilkinson and Akrigg, 1992) (Fig. 3.2). In pMV100, the polylinker of pUC19 has been replaced by a fragment containing the CMV major IE promoter (-299 to +69) and its associated polyadenylation signal (+2757 to +3053). Nucleotide sequence numbering of the CMV major IE gene is as described by Akrigg et al., (1985). Two BamHI sites and an XbaI site are situated between the IE promoter and polyadenylation signal to facilitate cloning into the expression vector. Each TBEV cDNA fragment was cloned into pMV100 at the XbaI restriction site and the resultant plasmids transfected into 239 cells. Expression of the NS1 gene product was assayed by immunofluorescence.

The CMV major IE promoter was chosen to drive expression for it is an extremely powerful constitutive promoter. The strength of the promoter lies in its enhancer element which contains an array of imperfect 16, 18, 19 and 21 imperfect bp repeats (Reviewed in Wilkinson and Akrigg, 1991). The 18 and 19 bp repeats bind transcription factors, this binding can be stimulated by various agents including forskolin and significantly enhances yields of

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proteins under its control (Wilkinson and Akrigg, 1992). Furthermore the CMV IE promoter can function in a wide range of cell types, although the host range of CMV is extremely limited.

3.1.2. Extraction of Total RNA From TBEV-Infected Cells:

PS cells were infected with the TBEV Neudörfl strain at 0.1 pfu/cell. Total cytoplasmic RNA purified from infected PS cells as described in section 2.13, and was examined by formaldehyde gel electrophoresis (Fig. 3.3). The presence of discrete bands corresponding to 28S and 18S cellular ribosomal (r)RNA purified from mock-infected PS cells, suggested that the viral RNA in the sample may also be intact.

3.1.3. Production of TBEV cDNA:

First strand synthesis of cDNA was generated by a reverse transcription reaction in which total cytoplasmic RNA was treated with DMSO as described in 2.15. It was not necessary to directly assess the efficiency of this reaction as a successful PCR would imply a efficent reverse transcription reaction.

3.1.4. PCR Amplification of the TBEV NS1 Gene:

Since the PCR primers used in this study were annealed at temperatures higher than the annealing temperature required by random primers, it was not necessary to remove the random primers from the reverse transcription reaction, before it was used in PCR. Using the DNA sequence data (and the co-ordinates) of the Neudörfl strain genome given by Mandl and co-workers (Mandl et al., 1988 & 1989), a 1,891 bp cDNA fragment containing 33% of the 3′ end of the E gene, the complete

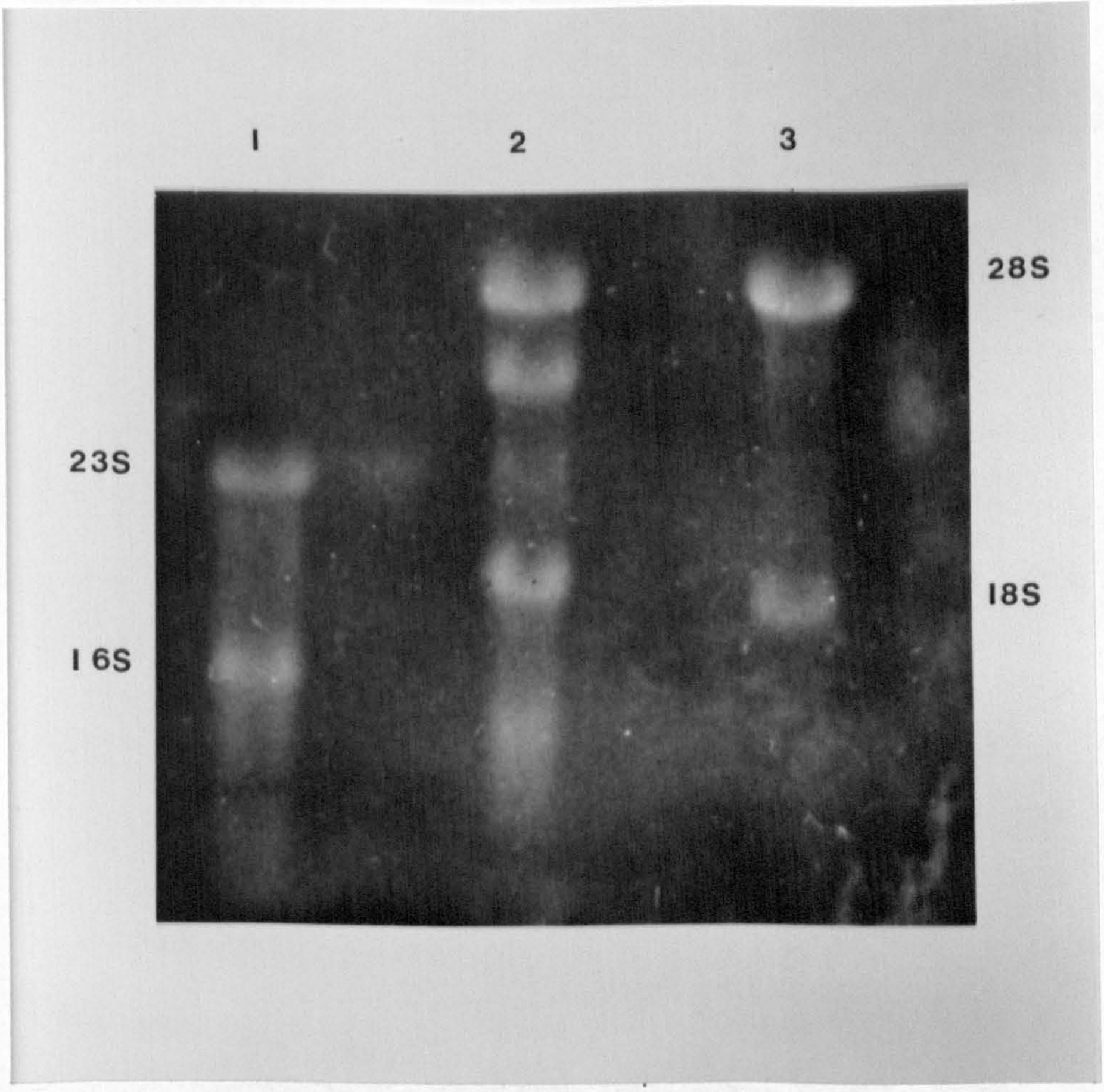
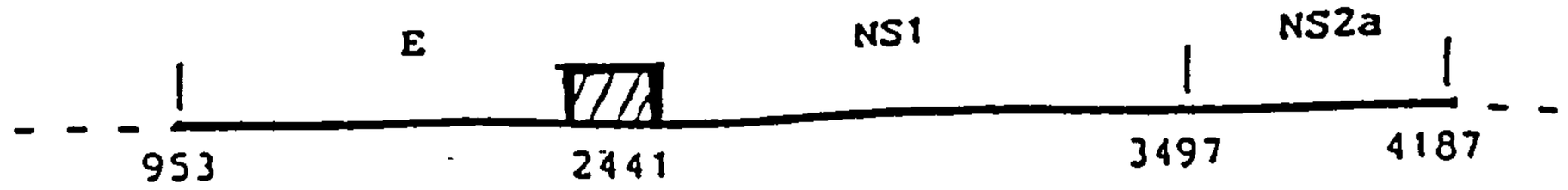


Fig. 3.3. Formaldehyde Gel Electrophoresis of Total Cytoplasmic RNA: RNA purified from TBEV-infected PS cells was subjected to formaldehyde gel electrophoresis (Lane 2). 28S and 18S rRNA markers obtained from mock infected PS cells are shown on the right (Lane 3) and E coli 23S and 16S rRNAs are shown on the left (Lane 1).

NS1 gene, and 79% of the NS2a gene (FENS12a) was identified as encoding all the elements required for efficient expression of NS1 (Fig. 3.4). Fragment FENS12a was amplified by PCR using primers P1 and P2 (Fig. 3.4) and conditions described in section 2.16. Primer P1 is homologous to the 26 bases downstream of the nucleotide 2096 on the positive cDNA strand of the E gene, while primer P2 is complementary to 25 bases on the positive cDNA strand ending at nucleotide 3987 in the NS2a gene. Primers P1 and P2 also contain additional 5′ sequence elements designed to provide the PCR-generated DNA fragment FENS12A with flanking BglII restriction endonuclease cleavage sites. To assess the product from each PCR reaction a 2 µl aliquot was examined by electrophoresis on a 1% agarose gel (Fig. 3.5, Lane 6).

3.1.5. Cloning of FENS12a into pMTL23:

The 1,891 bp DNA PCR fragment generated using primers P1 or P2 lacked translational signals and the XbaI restriction endonuclease sites necessary for cloning into pMTL23. This deficiency was remedied by the ligation of the palindromic adaptor L1 to FENS12a (Fig. 3.4). The adaptor L1 contained pre-formed BglII restriction site at each end and an XbaI site at the centre. Upstream of the XbaI site was a nucleotide sequence encoding a translational stop codon (TAA) and 3′ to the XbaI restriction site was a translational start codon (ATG). Following PCR amplification the TBEV cDNA fragment generated, FENS12a, was recovered from PCR by ethanol precipitation. The dried precipitates were resuspended in BglII digestion buffer and treated with the endonuclease BglII. The digested cDNA fragments were purified by agarose gel electrophoresis and electro-elution. Double



FENS12a 2096 L1/P1 ——— P2/L1 3987

FENS1 2096 L1/P1 ——— P4 3496

FNS12a 2441 P3 ——— P2/L1 3987

FNS1 2441 P3 ——— P4 3496

FSNS12a 2366 P5 ——— P2/L1 3987

FSNS1 2366 P5 ——— P4 3496

Oligonucleotides used:

P1 GGGCC{AGATCT}ATCATCTATGTTGGGGAACTGAGTCA

P2 GCGCGC{AGATCT}GCTGTGTCATGAGAGCCATGAGCA

P3 GGCC[TCTAGA]ATGGATGTTGGTTGCGCTGTGG

P4 GCGCC[TCTAGA]TTACGCAACCACCATTGAGCGAAC

P5 GGGCAGA[TCTAGA]ATGAGAAACCCTACAATGTCC

L1 GATCT}CATAA[TCTAGA]TTATG{A

[ ] *Xba*I endonuclease cleavage site.
{ } *Bgl*II endonuclease cleavage site, in L1 sticky end only.
ATG/TAA Initiation/termination codons, TTA is the complementary form of the termination codon.
——— TBEV nucleotide sequence.

Fig. 3.4. Map of the TBEV NS1 cDNA Fragments Generated by PCR: At the top of the figure is shown the location of the TBEV gene products encoded by this portion of the genome (E, NS1 and NS2a). Vertical lines denote putative cleavage sites, while numbers below indicate the first nucleotide of each gene. The hatched region represents the 25 amino acid translocation signal sequence. PCR DNA fragment designations are listed on the left and on the right are maps of the sequence encoded by the fragment. The numbers adjacent to the fragments indicate its first and last nucleotide. The DNA fragments FENS12a, FENS1, FNS1, FSNS12a and FSNS1 were generated using the oligonucleotide primers P1 to P5, as indicated. The adaptor L1 was self-annealed and ligated to the PCR-generated fragments as indicated to provide either a translational start or stop codon, as appropriate.

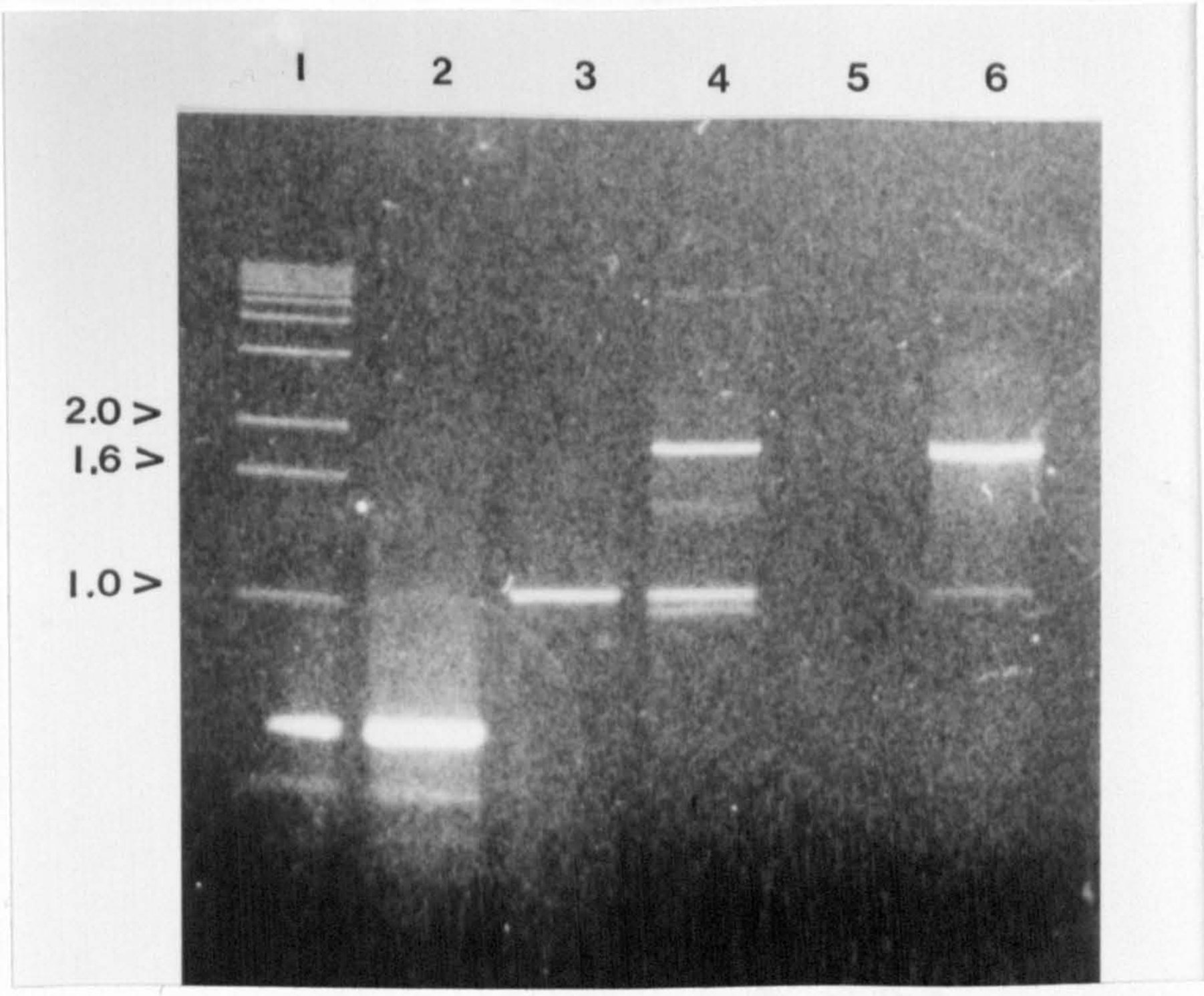
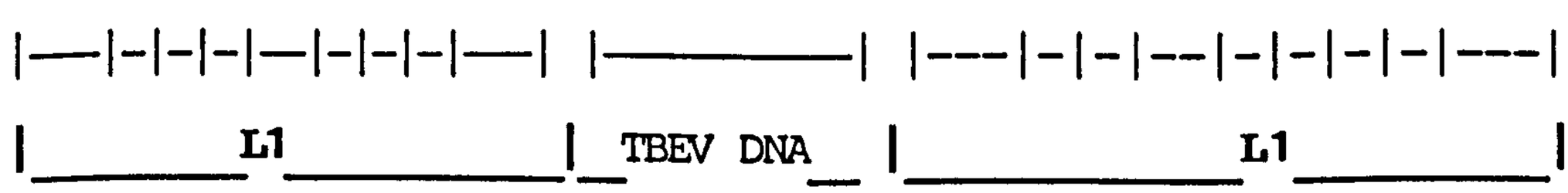


Fig. 3.5. Assessment of PCR Amplified TBEV cDNA by Agarose Gel electrophoresis: Following amplification, DNA was separated on a 1% agarose gel and strained with ethidium bromide. A control DNA template from dengue virus genomic cDNA and appropriate primers is shown in Lane 2. An approximately 1.0 kb DNA fragment (Lane 3) was generated by PCR in which the template was provided from a reverse transcription reaction primed by the primer P2. No DNA fragment was generated by PCR when the reverse transcription reaction providing the PCR template was primed with P2 but not treated with DMSO (Lane 5). When the TBEV-infected cell RNA was treated with 0.1% DMSO for 5 min at room temperature prior to the reverse transcription reaction, and random primers were included in the reaction, the full 1.891 bp viral DNA fragments (Lane 6) was generated by PCR with this template. When in addition to the condition used to generate the cDNA fragment in Lane 6, P2 had been added to the reverse transcription reaction generating a 1.0 kb and 1891 bp fragment (Lane 4). Molecular weight markers are shown on the left (Lane 1) and all sizes are in kb.

stranded, phosphorylated L1 adaptors, prepared as described in 2.21, were ligated to the viral cDNA at a ratio of 2 µg of adaptor per 2 µg of viral cDNA in order to generated the following DNA molecule:



The viral cDNA ligated to the adaptor was recovered from the ligation reaction by ethanol precipitation, and digested with XbaI. Digestion with XbaI removed the translational stop codon from the 5' of L1/FENS12a/L1 and the translational start codon from the 3' end of L1/FENS12a/L1, and facilitated cloning of the fragment into the plasmid vector pMTL23 at the plasmids unique XbaI restriction site (Fig. 3.6).

Competent E. coli cells, transformed with pMTL23 containing the TBEV cDNA fragment L1/FENS12a/L1, grew as white colonies when plated onto L-agar containing ß-gal and IPTG. White colonies resulted from disruption of the lac Z gene by the insertion of the viral construct and permitted positive selection of recombinant plasmids. Individual white colonies were harvested and amplified. A plasmid, pMV40, was recovered from the transformed cells in a small scale plasmid DNA preparation and the presence of L1/FENS12a/L1 insert in pMV40 was demonstrated by examination of XbaI digested pMV40 on a 1% agarose gel (Fig. 3.7, Lane 1).

In addition to cloning the NS1 gene of the Neudorfl strain, a similar DNA fragment FKENS12a, containing the NS1 gene from the TBEV K23 strain was also generated. FKENS12a was generated by reverse

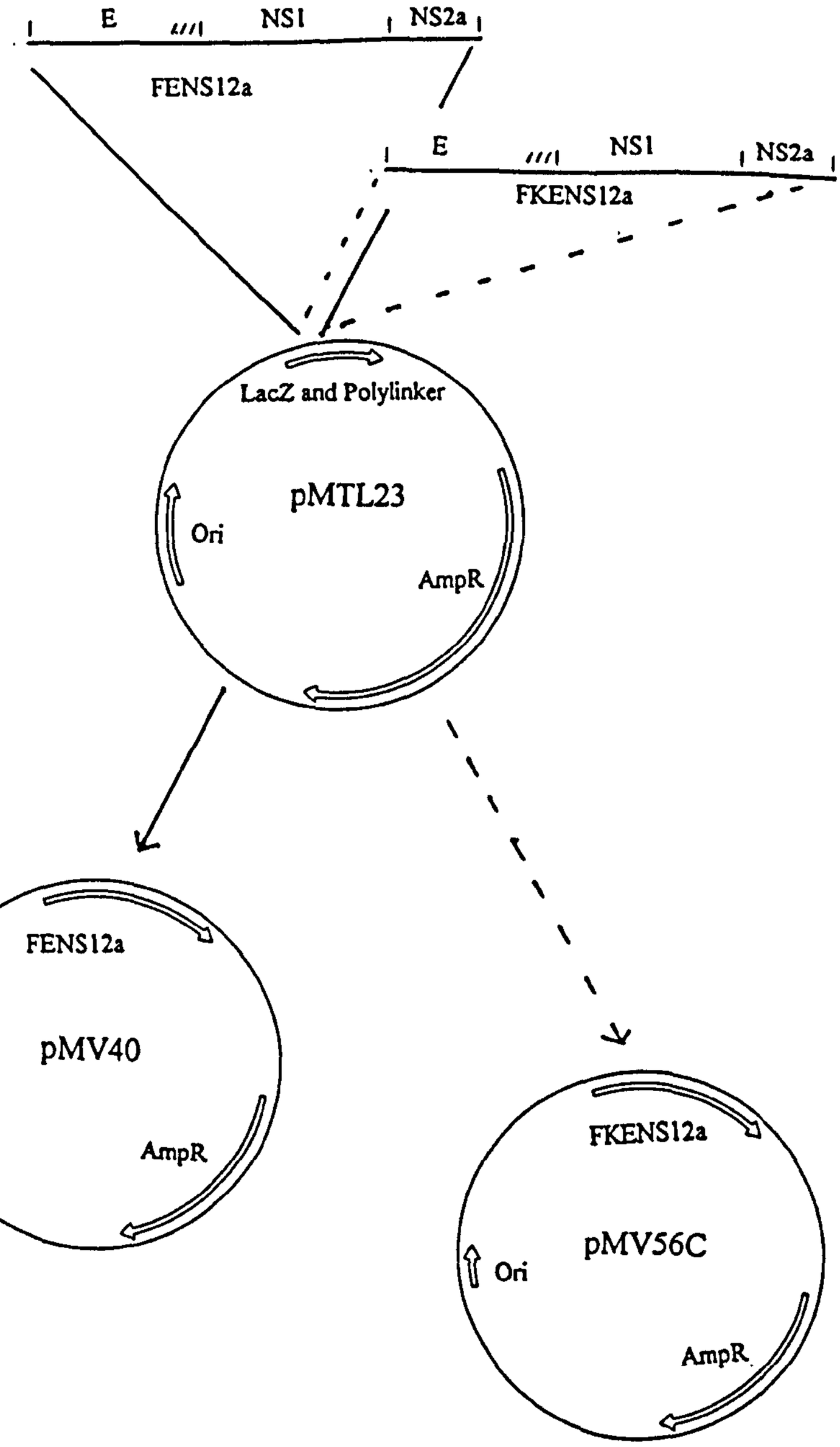


Fig. 3.6. Schematic Representation of the Cloning of L1/FENS12a/L1 and L1/FKENS12a/L1 into pMTL23: Cloning of L1/FENS12a into the XbaI site of pMV23 generating pMV40, is indicated by the continuous line on the left. Cloning of L1/FKENS12a/L1 into pMTL23 at the XbaI restriction site, generating pMV56C, is indicated by the broken line on the right.

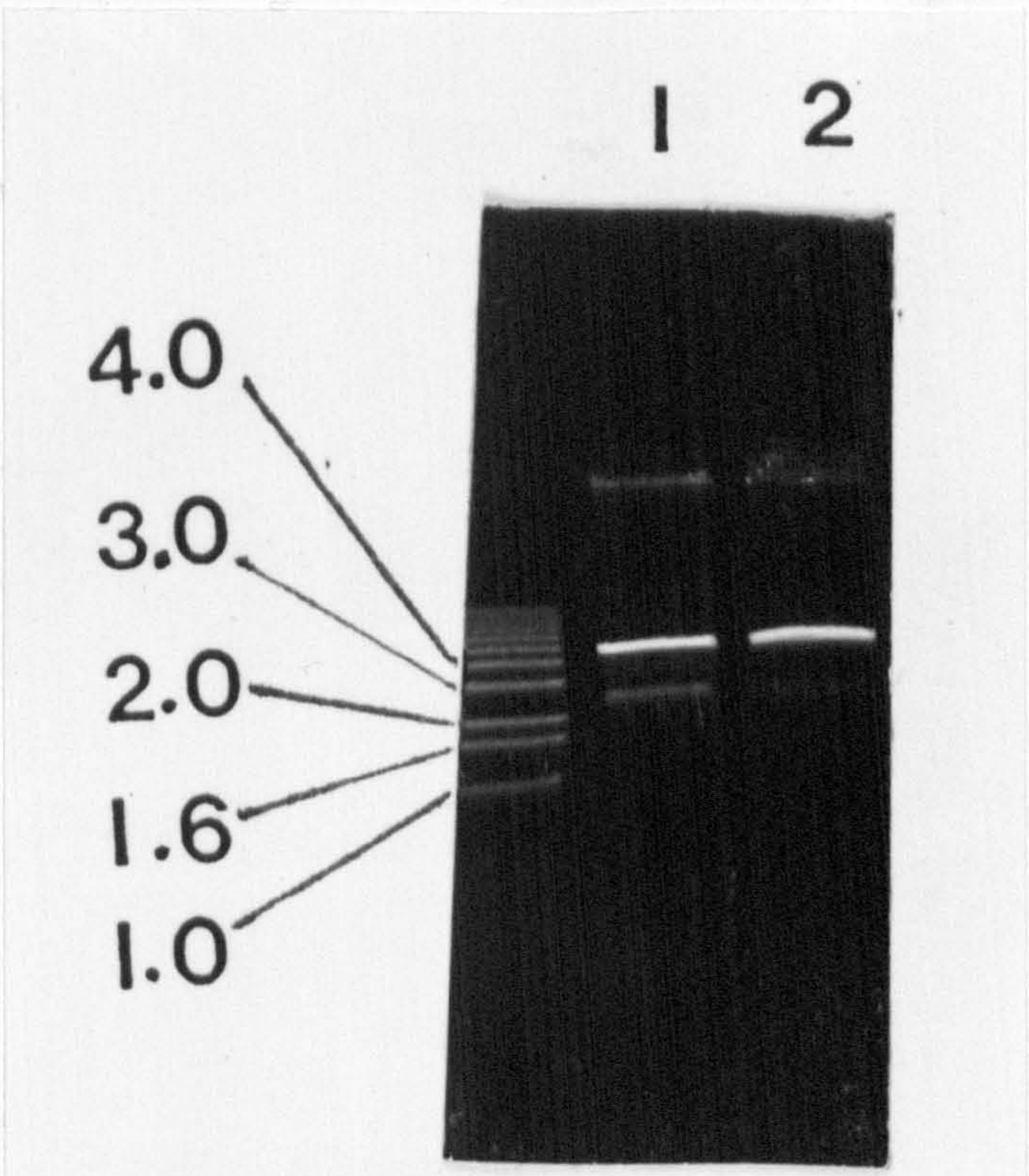


Fig. 3.7. XbaI Digest of pMV40 and pMV56C: XbaI-digested pMV40 and pMV56C electrophoresed through a 1% agarose gel demonstrated the presence of the 1891bp DNA fragment FENS12a in pMV40 (Lane 1) and the 1891bp fragment FKENS12a in pMV56C (Lane 2). Molecular weight markers shown in the left hand lane. The size of several markers, in kb, is indicated by numbers to the left of the figure.

transcription and PCR from TBEV strain K23-infected cell RNA using the same primers P1 and P2. Adaptors L1 were ligated to FKENS12a as described for the fragment FENS12a allowing L1/KFENS12a/L1 to also be cloned into pMTL23 at its unique XbaI restriction endonuclease cleavage site, generating pMV56C (Fig. 3.6, Fig. 3.7, Lane 2). The nucleotide sequence of the K23 cDNA fragment in pMV56C was then determined.

3.1.6. Nuclectide Sequence Analysis of the NS1 Gene From the Neudörfl and K23 TBEV Strains:

pMV40 was denatured with NaOH prior to sequencing to allow the sequencing primer access to its complementary sequence. After the primer had annealed and the sequencing reaction had been completed, samples were boiled and subjected to electrophoresis. The gels were then dried down and visualised by autoradiography. Reading of the sequence was performed manually. The nucleotide sequence of viral fragment cloned into pMV40 was obtained from a number of primer extension reactions, the strategy for which is illustrated in Fig. 3.8 and Fig. 3.9. Since the nucleotide sequence of the Neudörfl strain had been published previously, only one strand of FENS12a was sequenced. The nucleotide sequence of both the positive and negative strand of the K23 strain NS1 gene in FKENS12a was determined as the K23 sequence had not been determined previously. The strategy used to sequence the NS1 gene from the K23 strain was the same as that for the Neudörfl strain.

The nucleotide sequence of the cloned Neudörfl cDNA fragment FENS12a in pMV40 was found to be in agreement with the published results (Mandl et al., 1988 & 1989a), except for a translationally silent

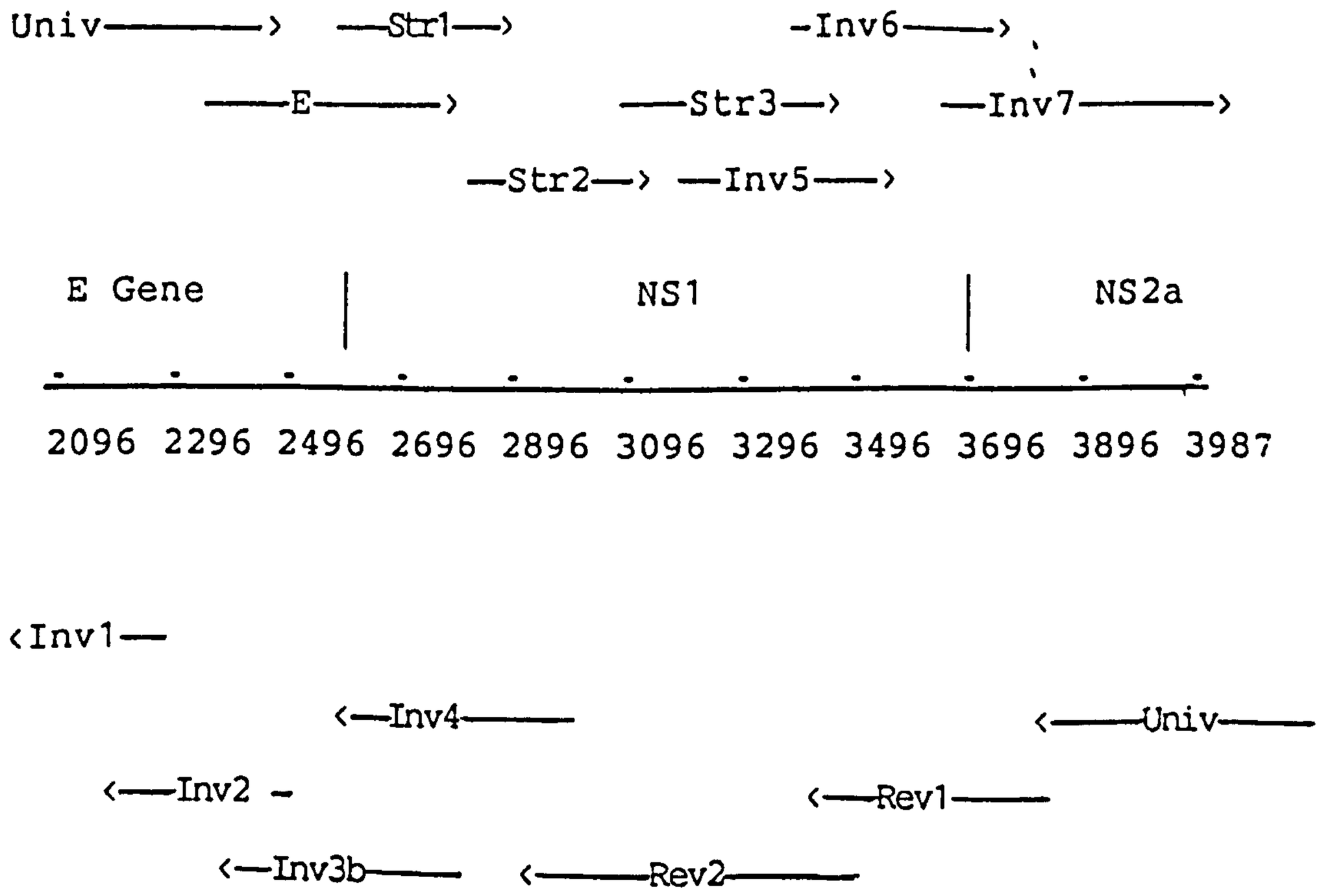


Fig. 3.8. Strategy for Sequencing NS1: All sequencing primers were complementary to both the Neudörfl and K23 strains. Individual primers are denoted by the lettering shown over the arrows while the length of each arrow indicates the region of sequence obtained by primer extension from that primer. The sequence of each primer is shown in Fig. 3.9. The flavivirus genome is depicted by the continuous line. The location of the gene products encoded by this portion of the genome (E, NS1 and NS2a) are shown, while the vertical line (|) denotes putative cleavage sites. The numbering below the genome is that of the nucleotide sequence from the Neudörfl strain of TBEV.

E	5′–	GGAGTGGGGTTTCTACCAAA –3′	2323
Str1		CATCAGCCATAAAGGAG	2584
Str2		CTTCATGGTGGGCACGGAAG	2858
Str3		AATGACACAGGCAC	3072
Inv5		CGATAATGCTGACG	3149
Inv6		CCAGAATGGTGCTG	3381
Inv7		GATAGAGAGCCTGGT	3667
Rev1		GACGGTGAGGCTTCTG	3790
Rev2		GTAATTCACCGTTGTCC	3496
Inv4		GATCTGTGTGGATTGC	3017
Inv3b		GAATGGCCCCAGC	2793
Inv2		CCATTCTGAGACCTC	2510
Inv1		CAGAAAGCCTCCAGC	2227

Sequencing Primer, M13 reverse 5′–AACAGCTATGACCATG– 3′

Sequencing Primer, M13 universal 5′–TTTTCCCAGTCACGAC– 3′

Fig. 3.9. The Sequencing Primers: The number to the right of each primer sequence is the position of that primers 5′ nucleotide within the Neudörf1 strain genome (Mandl et al., 1989a). The M13 sequencing primers were supplied by United States Biochemical Corporation, and are complementary to sequences adjacent to the cloning site in pMTL23 permitting primer extension into the cloned DNA fragment.

change from T to A at base 3265 (Fig. 3.10). The nucleotide sequence obtained for the NS1 gene of K23 strain encoded by FKENS12a is also shown in Fig. 3.10.

The amino acid sequence of NS1 encoded by the Neudörfl strain of TBEV and by the K23 strain fragment cloned into pMV56C was derived by the 'TRANS' subroutine in 'DNASTAR' software from the nucleotide sequence Fig. 3.11. Despite 37 nucleotide substitutions, the amino acid sequence of K23 strain NS1 encoded by pMV56C differed from that of the NS1 encoded by the TBEV Neudörfl strain by only eight residues. The NS1 nucleotide sequence of these two CE TBEV strains show a lower degree of homology with the published sequence of the FE Sofin strain of TBEV and Langat strain of TBEV. The position of the N-linked glycosylation motifs within the primary sequence of all four TBEV NS1 genes so far sequenced are absolutely conserved, one of which is also spatially conserved with one of the motifs identified within the primary sequence of the mosquito-borne viruses. The homology profiles of TBEV NS1, as determined by 'Dot-plot' analysis, between K23 strain and the other TBEV NS1 sequences available revealed two specific patterns (Fig. 3.12), one common to comparisons between the K23 strain and the viruses of the CE subtypes represented by Neudörfl strain of TBEV; the other pattern common to comparisons between K23 strain and the viruses of the FE subtypes represented by the Sofin strain and 205 strain of TBEV. This homology pattern may represent a means of typing the FE and CE isolates

In addition to the general sequence homology observed between the NS1 primary sequence of tick-borne viruses, 11 cysteine residues were identified as being conserved between the tick-borne and mosquito-borne viruses so far sequenced (Fig. 3.11). The conservation of the cysteine residues implies a common protein structure.

```
                                                                    2410
K23   AGA AAC CCT ACA ATG TCC ATG AGC TTT CTC CTG GCC GGA GGT CTG
N     ... ... ... ... ... ... ... ... ... ... T.. ..T ... ... ...
      ____________________________________________________________

                                                          *         2455
K23   GTC TTG GCC ATG ACC CTT GGA GTG GGG GCG GAT GTT GGC TGC GCT
N     ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
      ________________________________________________

                                                                    2500
K23   GTG GAC ACA GAA CGA ATG GAG CTC CGC TGT GGC GAG GGC CTG GTT
N     ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ..C

                                                                    2545
K23   GTG TGG AGA GAG GTC TCA GAA TGG TAT GAC AAC TAT GCC TAC TAC
N     ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...

                                                                    2590
K23   CCG GAG ACA CCG GGG GCC CTT GCA TCA GCC ATA AAG GAG ACA TTT
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    2635
K23   GAA GAG GGA AGC TGT GGT GTA GTC CCC CAG AAC AGG CTC GAG ATG
N     ..G ... ... ... ... ..C ... ... ... ... ... ... ... ... ...

                                                                    2680
K23   GCC ATG TGG AGA AGC TCA GTC ACA GTG CTG AAT TCG GCT CTG GCG
N     ... ... ... ... ... ..G ... ... .A. ... ... .T. ... ... ...

                                                                    2725
K23   GAA GGG GAG GCA AAT CTC ACA GTG GTG GTG GAC AAG TTT GAC CCC
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    2770
K23   ACT GAC TAC CGA GGT GGT GTC TCC GGT TTA CTG AGA AAA GGA AAG
N     ... ... ... ... ... ... ... C.U ... ... ... .A. ... ... ...

                                                                    2815
K23   GAC ATA AAA GCC TCC TGG AAA AGC TGG GCC CAT TCA ATC ATC TGG
N     ... ... ... .U. ... ... ... ... ... ... ... ... ..G ... ...

                                                                    2860
K23   AGT ACT CCT GAG GCC CCC CGT CGC TTC ATG GTG GGC ACG GAA GGA
N     ..C .T. ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    2905
K23   CAA AGT GAG TGT CCC CTA GAG AGA CGG AAG ACA GGT GTT TTC ACG
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    2950
K23   GTG GCA GAA TTC GGG GTT GCC CTG AGA ACA AAG GTT TTC TTG GAT
N     ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...

                                                                    2995
K23   TTC AGA CAG GAA CCA ACA CAT GAG TGT GAC ACA GGA GTG ATG GGA
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
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                                                                    3040
K23   GCT GCC GTC AAG AAT GGC ATG GCA ATC CAC ACA GAT CAA AGT CTC
N     ... ..A ... ... ..C ... ... ... ... ... ... ... ... ... ...

                                                                    3085
K23   TGG ATG AGA TCA ATG AAA AAT GAC ACA GGC ACT TAC ATA GTT GAA
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    3130
K23   CTT TTG GTC ACT GAC CTG AGG AAC TGC TCA TGG CCT GCT AGC CAC
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    3175
K23   ACT ATC GAT AAT GCT GAC GTG GTG GAC TCG GAG TTA TTG CTT CCG
N     ... ... ... ... ... ... ... ... ... ..A ... ... C.. ... ...

                                                                    3220
K23   GCG AGC CTG GCA GGA CCC AGA TCC TGG TAC AAC AGG ATA CCC GGT
N     ... ... ... ... ... ... ... ... ... ... ... ... ... .T. .C.

                                                                    3265
K23   TAT TCA GAA CAG GTG AAA GGG CCA TGG AAG TAC ACG CCT ATC CGA
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    3310
K23   GTC ATC AGA GAG GAG TGT CCC GGC ACG ACC GTT ACT ATC AAC GCC
N     ..T ... ... ... ..C ... ... ... ... ... ... ..C ... ... ...

                                                                    3355
K23   AAG TGT GAC AAA AGA GGA GCA TCT GTG ACG AGT ACC ACA GAG AGT
N     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                    3400
K23   GGC AAG GTT ATC CCA GAA TGG TGC TGC CGA GCC TGC ACA ATG CCA
N     ... ... ..A ... ... ... ... ... ... ... ... ..G ... ... ...

                                                                    3445
K23   CCA GTG ACG TTC CGG ACA GGA GCT GAT TGT TGG TAT GCC ATG GAA
N     ... ... ... ... ... ... ..T ... A.. ... ..C ... ... ... ...

                                                                    3491
K23   ATA CGG CCA GTC CAT GAC CAG GGA GGG CTT GTT CGC TCA ATG GTG
N     ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ...

                3497
K23   GTT GCG
N     ... ...
```

Fig. 3.10. Nucliotide sequence of K23 and Neudörfl strain of TBEV in cDNA fragments FENS12a: The translocation signal coding sequence is underlines and the N- terminus of the putative NS1 gene is indicated by *. Numbers on the right and above each row indicates the number of the last nucleotide in each row as determined by Mandl et al 1988, 1989a).

```
   ______________________________________ *
N  RNPIM SMSFL LAGGL VLAMT LGVGA DVGCA VDTER MELRC GEGLV VWREV 801
K  ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S  ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
L  ....L ..G.. .S... ..... ..... ..... ..... ..... ..... .....

N  SEWYD NYAYY PETPG ALASA IKETF EEGSC GVVPQ NRLEM AMWRS SVTEL 851
K  ..... ..... ..... ..... ..... ..... ..... ..... ..... ...V.
S  ..... ..... ....L ..... ..... ...T. .I... ..... ..... .A...
L  ..... ...FH ....A V.... VQRAY ..EI. .I... ..... ..... .VL..

              # ##
N  NLALA EGEAN LTVVV DKFDP TDYRG GVPGL LKKGK DIKVS WKSWG HSMIW 901
K  ..... ..... ..... ..... ..... ..S.. .R... ..A.. ..... ..I..
S  ....V ..D.. ..... ..L.. ..... .I.S. ..... ..... ..... .....
L  ..... ..... ..... ..A.. S.... ..... .N... ..... .R... R..L.

N  SIPEA PRRFM VGTEG QSECP LERRK TGVFT VAEFG VGLRT KVFLD FRQEP 951
K  .T... ..... ..... ..... ..... ..... ..... ..... ..... .....
S  .V... ..L.. ..... S.... ..... ..... ..... ..... ..... ....S
L  .V... ..... I.V.. GR... FA... ...M. ..... I.... ...M. L...L

                                    ###               ###
N  THECD TGVMG AAVKN GMAIH TDQSL WMRSM KNDTG TYIVE LLVTD LRNCS 1001
K  ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S  ..... ..... ..... ...V. ..... ..K.V R.... ..... ..... .....
L  .T... ..... ..... ...V. ..... ..K.I ....T VT... .I... ....T

N  WPASH TIDNA DVVDS ELFLP ASLAG PRSWY NRIPG YSEQV KGPWK YTPIR 1051
K  ..... ..... ..... ..L.. ..... ..... ..... ..... ..... .....
S  ..... ..... E.... ..... ..... ..... ..... ..... ..... .S...
L  ..... ..... G..N. K.... ..... ...T. .V... .A... R...A HT.V.

N  VIREE CPGTT VTINA KCDKR GASVR STTES GKVIP EWCCR ACIMP PVTFR 1101
K  ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S  .T... ....R ..... D.... ..... ..... ..... ..... T..L. .....
L  IK... ....R ...DK A.... ..... ..... ..... ..... T.EL. ...Y.

N  TGTDC WYAME IRPVH DQGGL VRSMV VA 1128
K  ..... ..... ..... ..... ..... ..
S  ..... ..... ..... ..... ..... ..
L  ..... ..... ..... T.... ..... ..
```

<u>Fig. 3.11. Amino acid sequence Comparisons of NS1 from the TBEV Strains (N) neudörfl, (K) K23, (S) Sofin and (L) Langat:</u> Three potential N-linked glycosylation sites are indicated by ###, and the potential translocation signal sequence is overlined. The number to the right of each line identifies the left hand amino acid of that line with respect to the Neudörfl sequence as reported by Mandl <u>et al</u>., (1988, 1989a & 1991a). The sequence of the strain Sofin is from Pletnev <u>et</u> <u>al</u>., (1990), and the Langat strain is from Iacono-Connors and Schmaljohn, (1992).

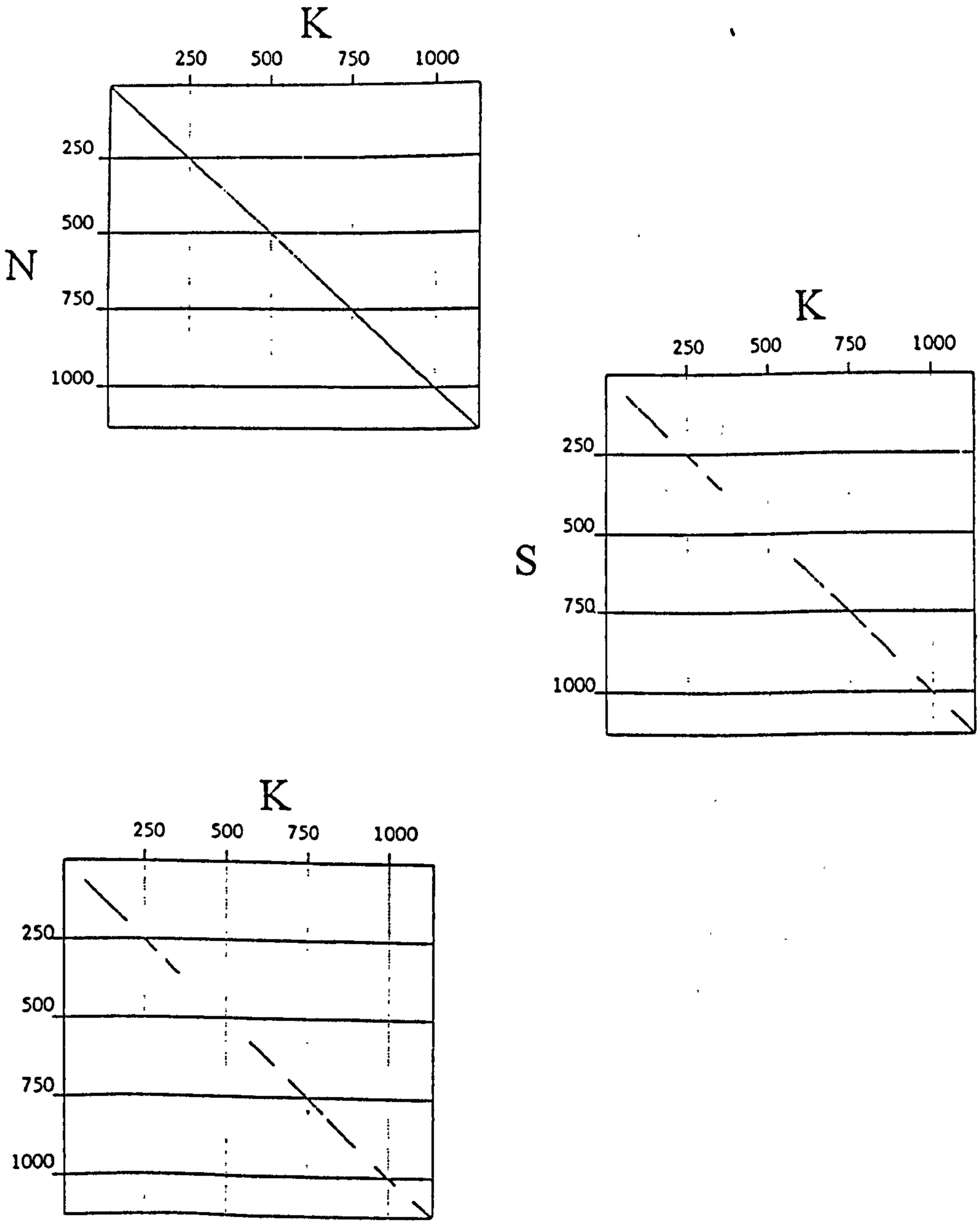


Fig. 3.12. Dot Matrix Analysis of the Homology Between NS1 of the TBEV K23 (K) Strain and the Neudörfl (N), Sofin (S) or 205 Strains: The matrices were created by the DNASTAR programme, 'DOTPLOT' using 90% match with 30 nucleotide segments. The numbers to the left and above each plot indicates the nucleotide number when the first base of NS1 is 1.

3.1.7. Characterisation of the Truncated 1 kb PCR Fragment:

In the initial reverse transcription reactions carried out using primer P2 and not treating the RNA template with DMSO, a truncated fragment of approximately 1 kb was amplified by the PCR (Fig. 3.5, Lane 3). Restriction cleavage of the aberrant 1Kb fragment indicated that it contained the first 1kb (approximately) of the 1891bp viral DNA fragment with 0.9kb of the 3′ end having been deleted. Analysis of the TBEV Neudorfl strain nucleotide sequence between the region described by the primers P1 and P2 revealed a near perfect inverted repeat of the 25 nucleotide sequence between bases 3157 and 3181 (IR1) occurring between nucleotides 3930 and 3954 (IR2) (Fig. 3.13). This inverted repeat conferred the potential for a stem-loop structure within the genomic RNA which would involve approximately 0.9 kb from the 3′ of the region defined by the PCR primers P1 and P2 ie 3′ of fragment FENS12a. The reverse transcriptase was therefore assumed to have 'jumped' the stem-loop structure during the reverse transcription, generating a truncated cDNA copy of the genome which, in turn, was used as the PCR template, and passing the error on to the amplified fragments.

To test whether the proposed stem-loop structure was responsible for the amplification, by PCR, of the truncated DNA fragment, the truncated fragment was sequenced. Following its amplification by PCR the truncated fragment was purified and the adaptor L1 ligated to allow insertion of the fragment into pMTL23. Nucleotide sequencing of the truncated fragment revealed it to contain the region between nucleotide 2096 to 3119 of the genome from the TBEV Neudörfl strain. This region formed the 5′ 1033 bp of the fragment FENS12a stretching

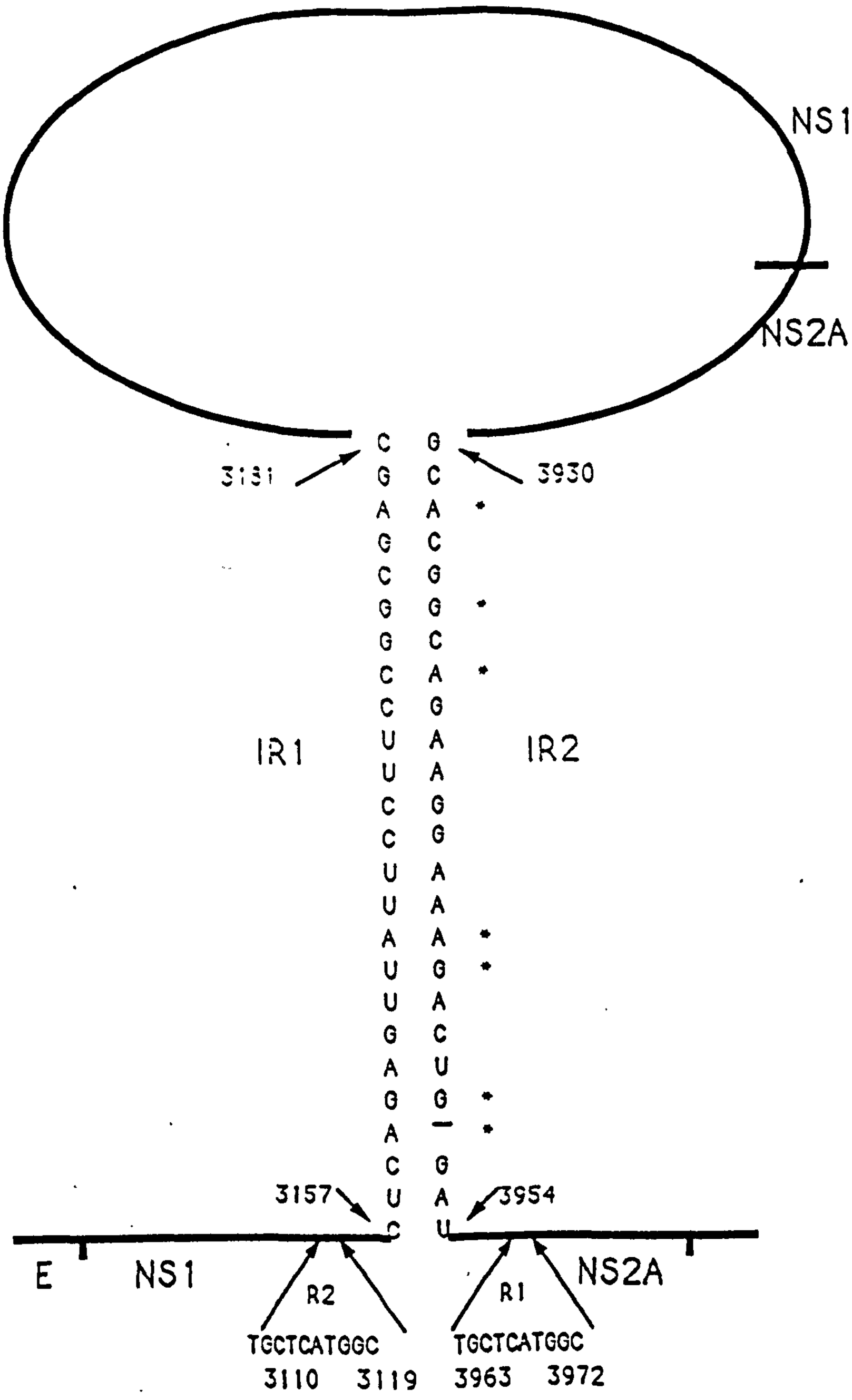


Fig. 3.13. The Nucleotide Sequences Involved in the Generation of the Truncated PCR Product: The primer P2 was complementary to the sequence between nucleotides 3110-3119 (R2) and to the sequence between 3963-3972 (R1). The nucleotides between 3157-3181 (IR1) and between 3930-3954 (IR2) form the putative stem-loop structure. * indicates mismatched base pairs in the putative stem-loop structure.

from the 5′ of primer P1 to a position 38 nucleotides upstream from the first nucleotide of the inverted repeat (IR1) sequence at position 3157 (Fig. 3.13).

The 10 nucleotides forming the 3′ end of the truncated fragment (between bases 3110-3119) were found to be complementary to the 10 nucleotides of the 3′ end of the primer P2 and therefore represented a second region of homology (R2) in FENS12a to the primer P2 (Fig.3.13). It was concluded that the primer P2 had preferentially recognised the 10 bases of R2, possibly because the secondary structure had made R1 inaccessible to P2. In an attempt to denature this structure prior to reverse transcription the RNA template was treated with DMSO in the presence of random primers (2.15). This method resulted in the successful generation of the full 1891 bp PCR DNA fragment FENS12a (Fig. 3.5, Lane 6). It was surprising that treatment of the genome with DMSO allowed binding of P2 to 3963-3972, as when RNA templates were treated with DMSO in the presence of P2 prior to the reverse transcription reaction the resultant cDNA template could only generate a truncated fragment in PCR (not shown). When random primers were included in the reverse transcription but the RNA was not treated with DMSO however, no PCR product could be detected (Fig. 3.5, Lane 5).

The need to treat genomic RNA with DMSO prior to its inclusion in the reverse transcription reaction in order to generate the complete fragment FENS12a provides evidence that secondary structure may impede the reaction. A comparison identified a repeat and inverted repeat sequence to be present in the genomes of the CE TBEV strains of Langat (Iacono-Connors et al., 1992) and K23 (this Thesis), and the Far Eastern strains Sofin (Pletnev et al., 1990) and 205 (Safronov et al., 1991), but not in the avaliable sequences of the mosquito-borne viruses.

3.1.8. Generation of Additional Constructs Containing the NS1 Gene:

Five additional DNA fragments in which the NS1 gene of the Neudörf1 strain was presented in a different context were amplified by PCR in which the template was provided by the sequenced fragment FENS12a excised from pMV40 on a XbaI fragment. Three additional PCR primers P3, P4 and P5 were designed to be used in conjunction with P1 and P2 in PCR to generate the additional viral cDNA fragments (Fig. 3.4).

Primers P3 and P5 are homologous to 18 bp on the positive strand from nucleotide 2441 and 2366 respectively. Primer P3 defines the putative 5′ terminal of the NS1 gene, while P5 defines the 5′ end of the putative translocation signal sequence 78 nucleotides upstream from the putative 5′ terminal of the NS1 gene. These primers were also designed to generate an upstream XbaI site to facilitate cloning and an inframe translation initiation codon (AUG). Primer P4, is complementary to 21 bp on the positive strand ending at nucleotide 3496 and defines the putative 3′ terminus of the NS1 gene, and also contains both an additional upstream element that generates a translation termination codon (TAA) and an XbaI site during PCR. The five additional Neudörf1 strain DNA fragments containing the NS1 gene generated and described in Fig. 3.4, were designated: FENS1 containing the 33% of E gene and the putative NS1 gene in FENS12a but not the NS2a sequence; FNS12a containing the putative NS1 gene and NS2a sequence of FENS12a but not the E gene portion; FNS1 containing only the putative NS1 gene from FENS12a; and FSNS1 and FSNS12a which were identical to FNS1 and FNS12a but with the addition of the 78 nucleotides located immediately upstream from the putative 5′ terminal of NS1 at the 3′ terminal of the E gene expected to encode the translocation signal. Since the fragments FENS1, FNS12a, and FSNS12a were generated using primers P1 or P2, the adaptor L1 was ligated as

for fragment FENS12a, to facilitate cloning and translation. As only one terminal of the fragment required this modification, only 1 µg of adaptor was reacted with 2 µg of fragment in the ligation reaction.

The remaining fragments, FNS1 and FSNS1, which did not require the addition of adaptors were inserted directly into the pMTL23 following digestion with XbaI. Each additional cloned viral fragment was sequenced using the primers described in Fig 3.8 and Fig. 3.9. The sequences of the additional fragments were found to agree with the nucleotide sequence of the template FENS12a. Each construct contained an in-frame translation initiation codon derived from either the PCR primers or, in the case of fragment FENS12a and FENS1, by the addition of the adaptor L1. An inframe stop codon, present in either primer P4 or the adaptor L1, was also incorporated into all the fragments.

3.1.9. Cloning of TBEV NS1 cDNA Constructs into the Transient Eukaryotic Expression Vector:

Following sequencing of the TBEV cDNA fragments, the fragments were excised from pMTL23 using XbaI and isolated by electrophoresis and electro-elution before being inserted into pMV100 at a unique XbaI restriction site of pMV100. The resultant plasmids were used to transform competent E. coli. The plasmids were recovered from the cells of a single colony generated and their cDNA inserts examined. Plasmids were designated; pMV39 containing FENS12a, pMV43 containing FENS1, pMV41 containing FNS1, pMV53 containing FNS12a, pMV45 containing FSNS1 and pMV54 containing FSNS12a (Fig. 3.14). Restriction digestion of the plasmid with XbaI confirmed the insertion of the TBEV cDNA fragments at the appropriate site (Fig. 3.15), while HindIII digestion demonstrated that the IEP/TBEV cDNA expression cassettes could be excised from the plasmid (Fig. 3.15). SacI

Fragment						Plasmid
FENS12a	L1/P1	E		NS1	NS2a	P2/L1 pMV39
FENS1	L1/P1				P4	pMV43
FNS12a		P3				P2/L1 pMV53
FNS1		P3			P4	pMV41
FSNS12a		P5				P2/L1 pMV54
FSNS1		P5			P4	pMV45

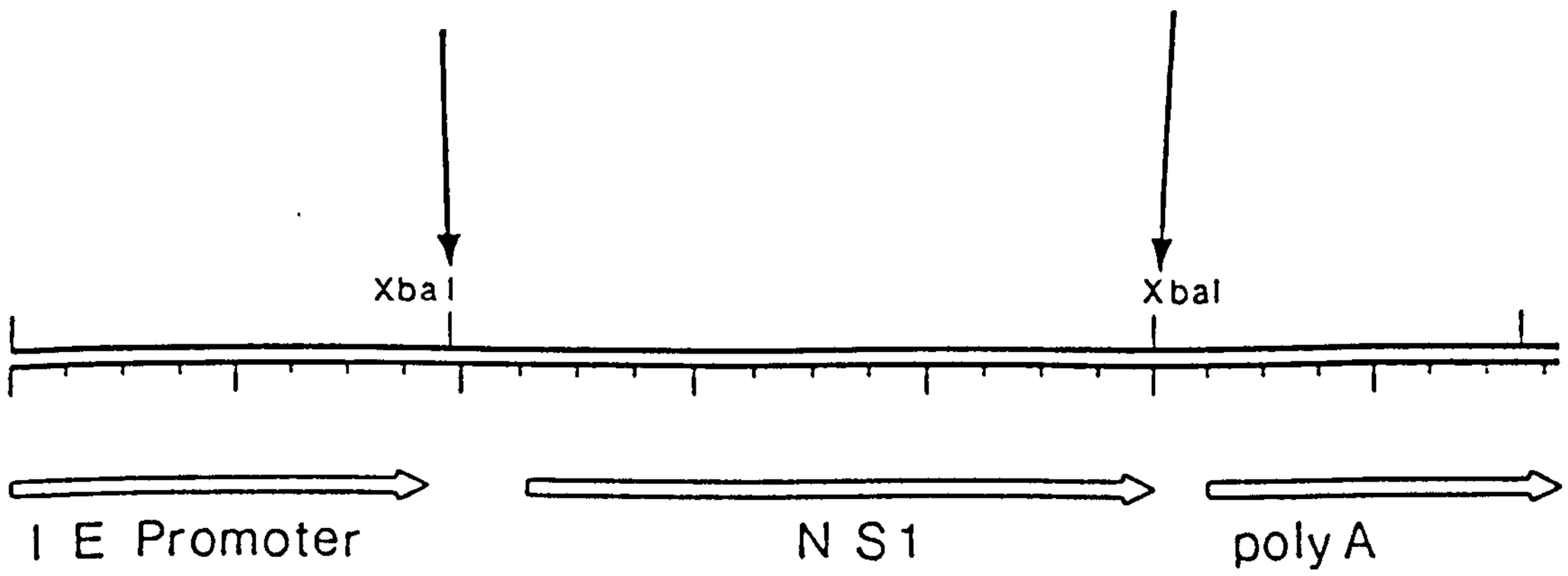


Fig. 3.14. Cloning of the NS1 Gene Under the Control of the IE Promoter: Insertion of the XbaI-digested PCR fragments containing the TBEV NS1 gene into the XbaI site of pMV100 placed the gene under the control of the CMV major IE promoter. pMV100 contains the CMV major IE promoter (-299 to +69) upstream of a multiple cloning site in a pUC base plasmid. Downstream of the multiple cloning site in pMV100 is located the polyadenylation signal sequence derived from the CMV major IE gene (+2757 to +3053). The name given to each plasmid generated in the cloning experiment is indicated on the right hand side.

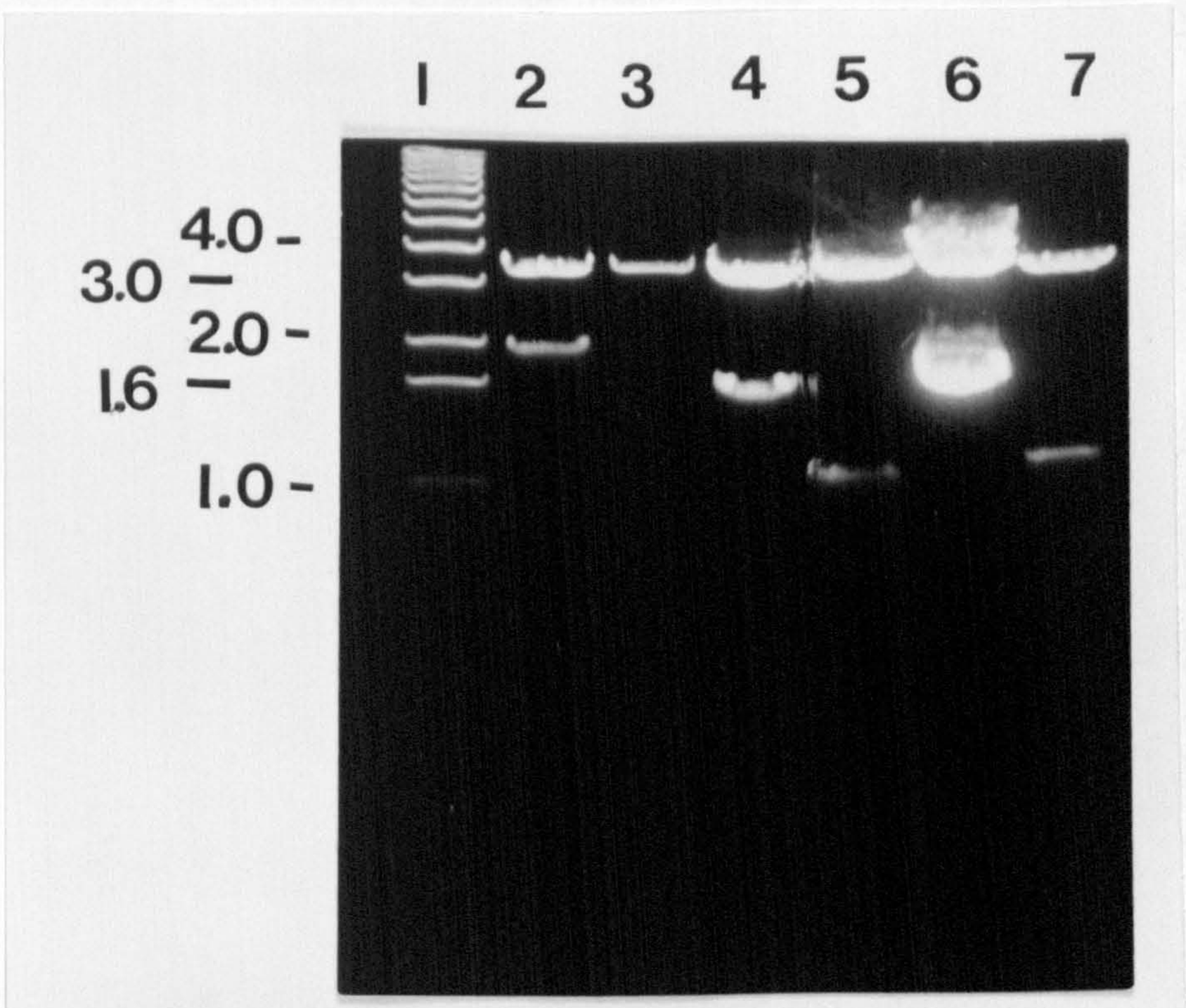


Fig. 3.15. XbaI Digest of pMV39, pMV43, pMV53, pMV41, pMV54, and pMV45: XbaI digested plasmids were electrophoresed through a 1% agarose gel and stained with ethidium bromide. XbaI digestion demonstrates the presence of FENS12a in pMV39 (Lane 2); of FENS1 in pMV43 (Lane 3); of FNS12a in pMV53 (Lane 4); of FNS1 in pMV41 (Lane 5); of FSNS12a in pMV54 (Lane 6); and of FSNS1 in pMV45 (Lane 7). The size (in kb) of several molecular weight markers (lane 1) are indicated on the left.

restriction digestion cleaves pMV100 and the NS1 gene asymmetrically which allowed the orientation of the TBEV cDNA fragment to be ascertained with respect to the CMV IE promoter (Fig. 3.17).

3.1.10. Transient Expression of NS1:

Using the transfection technique described in section 2.26, plasmids containing the CMV IE promoter/NS1 expression cassettes were introduced into 293 cells and expression of the NS1 gene product was assayed by immunofluorescence (Fig. 3.18). NS1 expression was detected in transfected cell using the anti-NS1 monoclonal antibodies, as the first antibody and fluorescein-conjugated goat anti-mouse IgG as the second antibody. A strong green cytoplasmic immunofluorescence was observed only in cells infected with TBEV or transfected with plasmids containing the translocation signal sequence immediately upstream of the NS1 gene, i.e., with pMV45 (fragment FSNS1) and pMV54 (fragment FSNS12A) (Fig. 3.18, 2 and 3). The expressed protein was recognised by three different monoclonal antibodies (T12, T33/1 and T33/3) raised against the NS1 protein, with the cytoplasmic pattern of fluorescence produced being identical to that observed in TBEV-infected cell. Significant levels of expression could not be detected in cells transfected with constructs containing the additional upstream E gene sequences, plasmids pMV39 (fragment FENS12A) (Fig. 3.18, 4) and pMV43 (fragment FENS1), or those lacking the signal sequence altogether, pMV41 (fragment FNS1) and pMV53 (fragment FNS12A). No significant levels of fluorescence could be detected in mock-transfected or mock-infected cells.

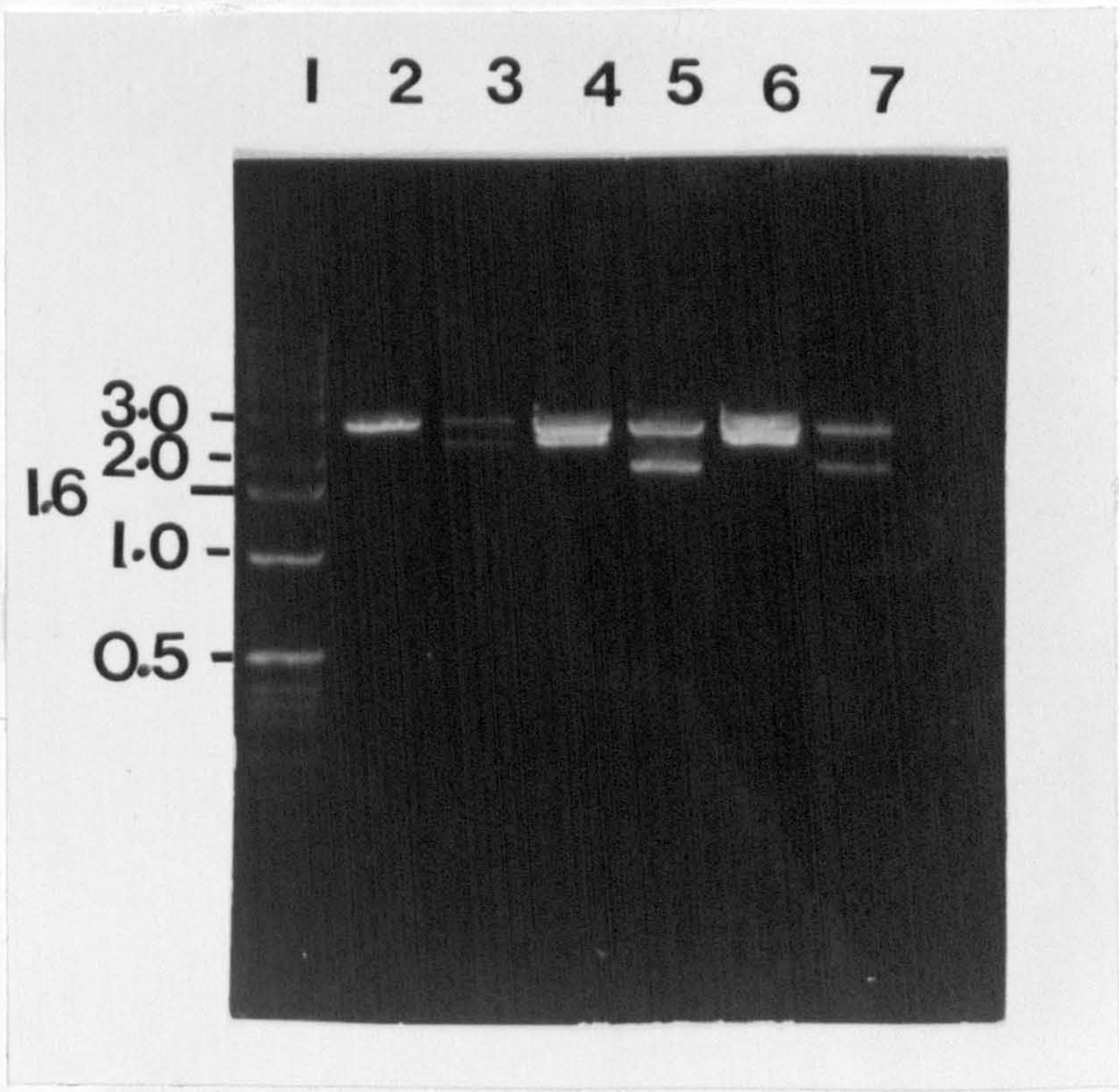


Fig. 3.16. HindIII Digest of pMV39, pMV43, pMV53, pMV41, pMV54, and pMV45: HindIII digested plasmids were electrophoresed through a 1% agarose gel and stained with ethidium bromide. HindIII digestion demonstrates the presence of FENS12a in pMV39 (Lane 2); of FENS1 in pMV43 (Lane 3); of FNS12a in pMV53 (Lane 4); of FNS1 in pMV41 (Lane 5); of FSNS12a in pMV54 (Lane 6); of FSNS1 in pMV45 (Lane 7). The size (in kb) of several molecular weight markers (lane 1) are indicated on the left.

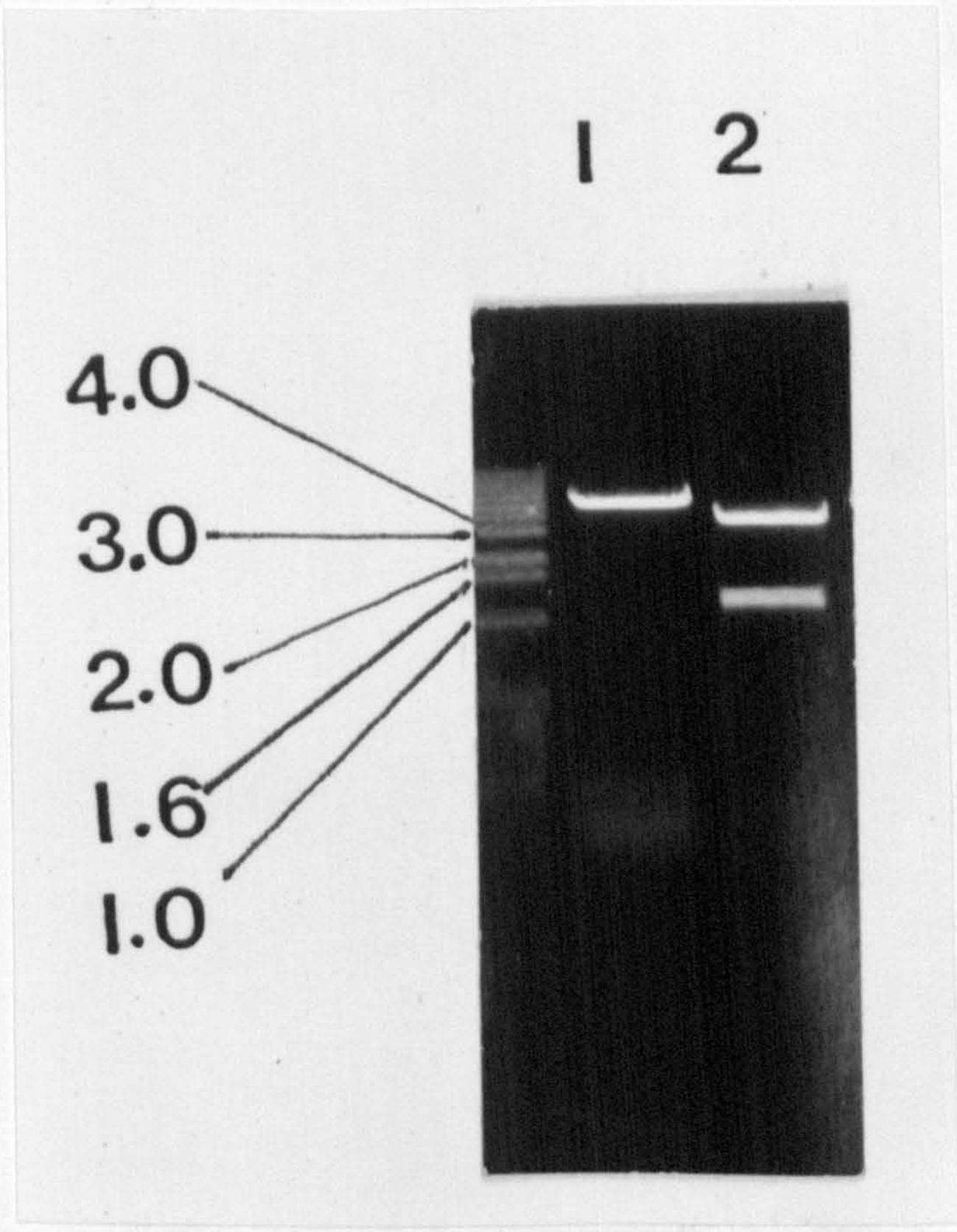
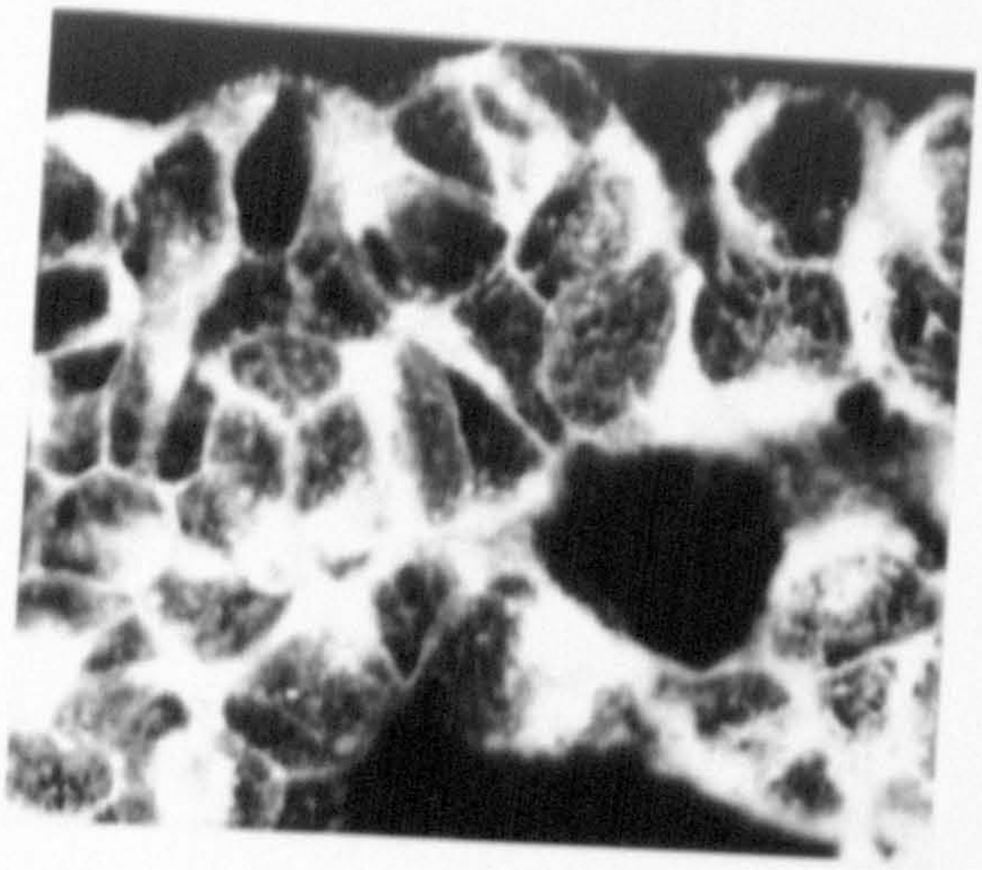


Fig. 3.17. Orientation of FSNS1 in pMV100 by SacI Digestion: SacI cleaves at the 5′ end of the IE promoter in pMV100 and 38 bp into the 5′ end of FSNS1. Following cloning of FSNS1 into pMV100, the orientation of FSNS1 with respect to the IE promoter in pMV100 was determined by SacI digestion. The FSNS1 is correctly orientated in pMV100 with respect to the IE promoter as SacI digestion produces one fragment of approximately 3200 bp and one fragment of approximately 500 bp (not visible) (Lane 1). SacI digestion of a plasmid in which FSNS1 is incorrectly orientated with respect to the IE promoter in pMV100 generates a fragment of approximately 1500 bp and one of approximately 2200 bp (Lane 2). The size, in kb, of several molecular weight markers in the left hand lane are indicated on the left of the figure.



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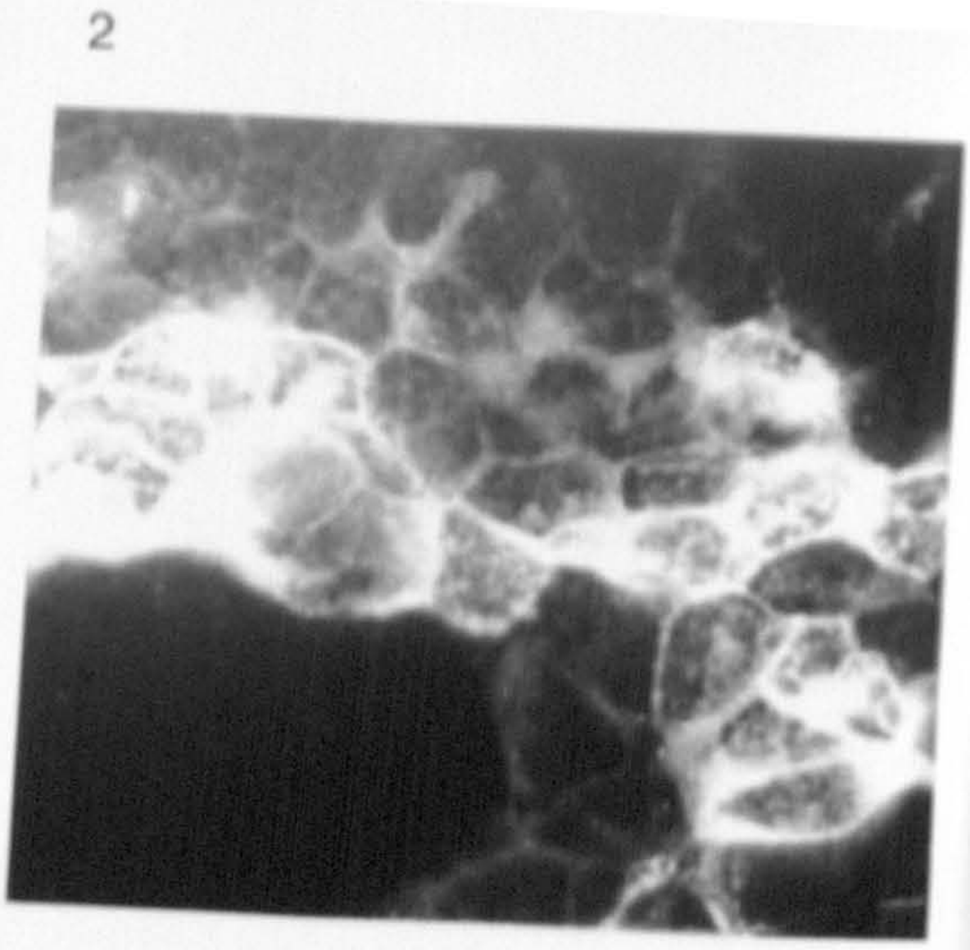

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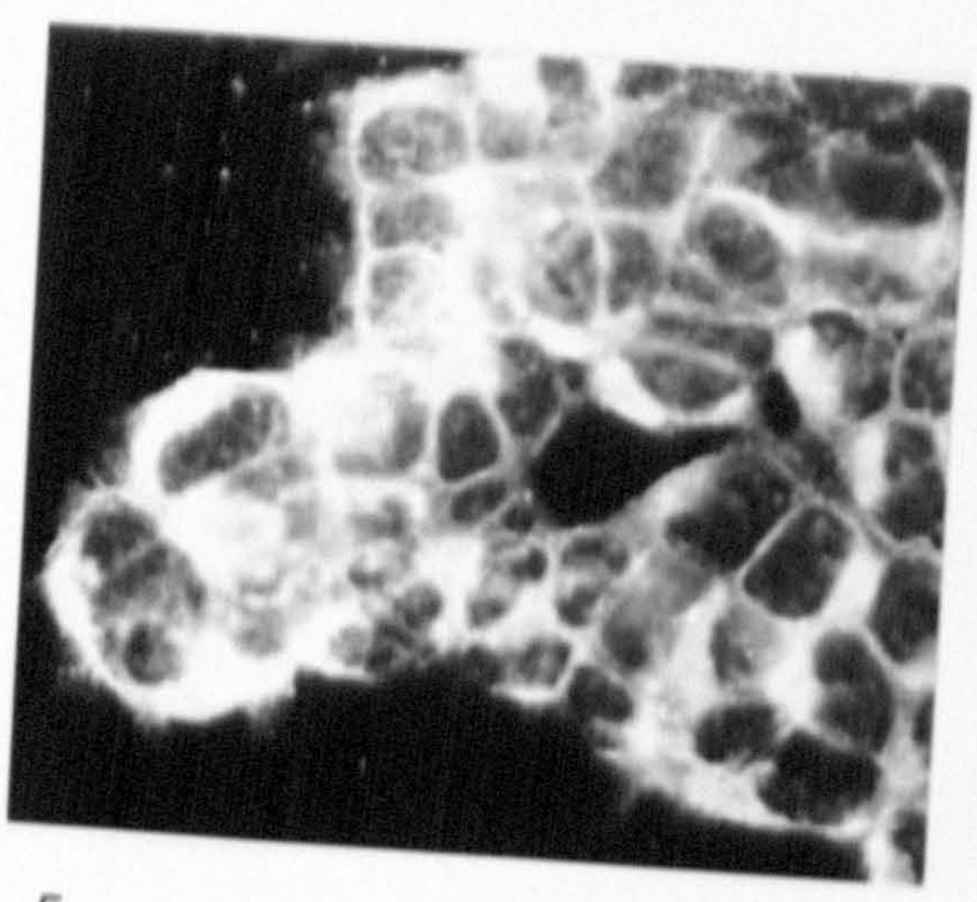

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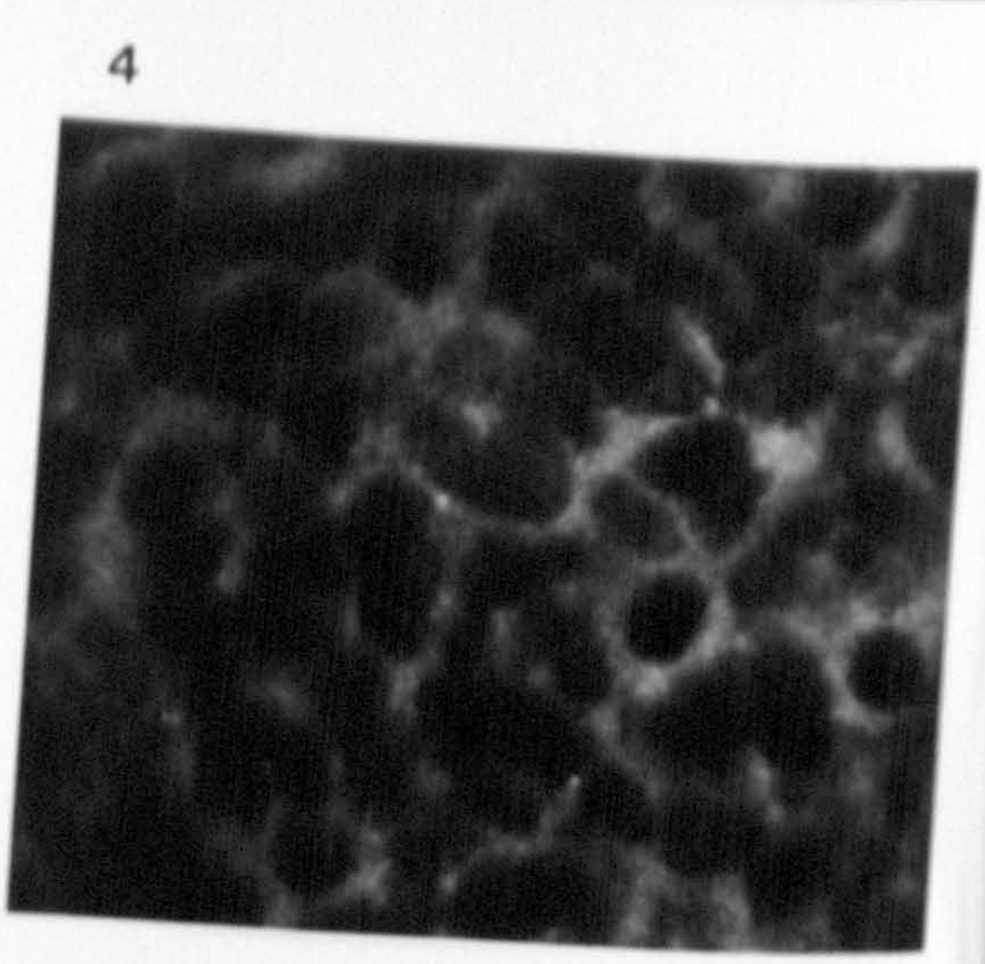

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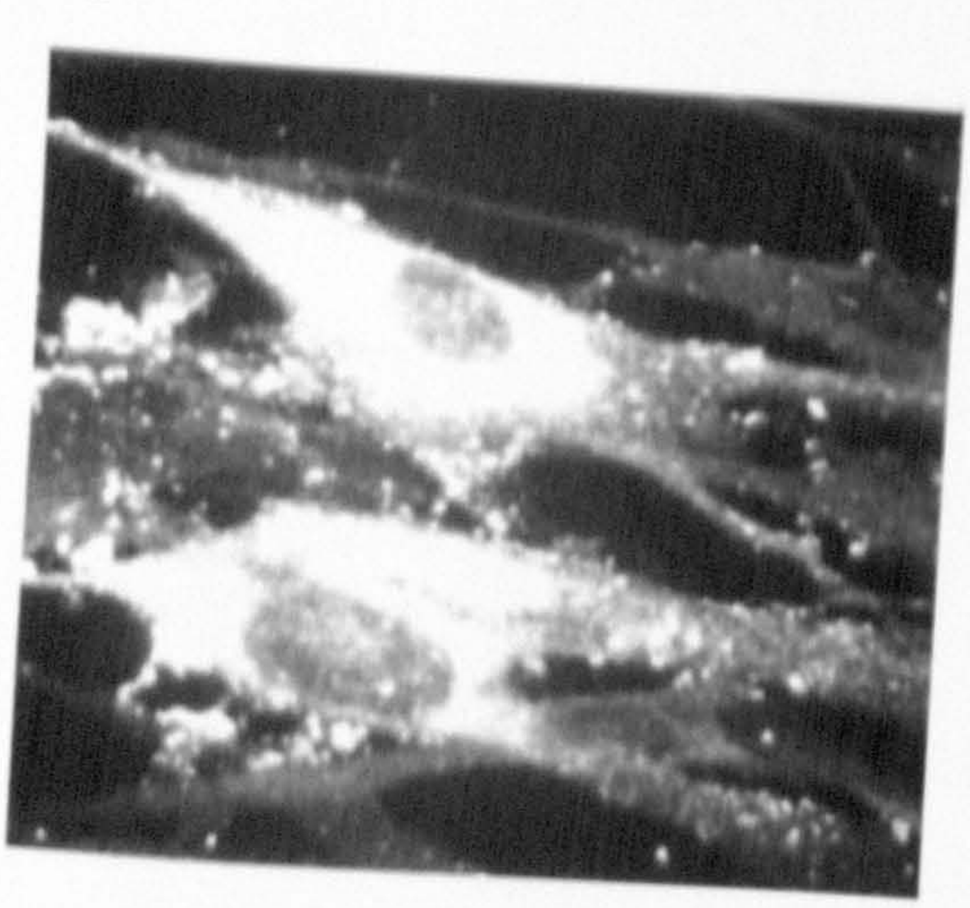

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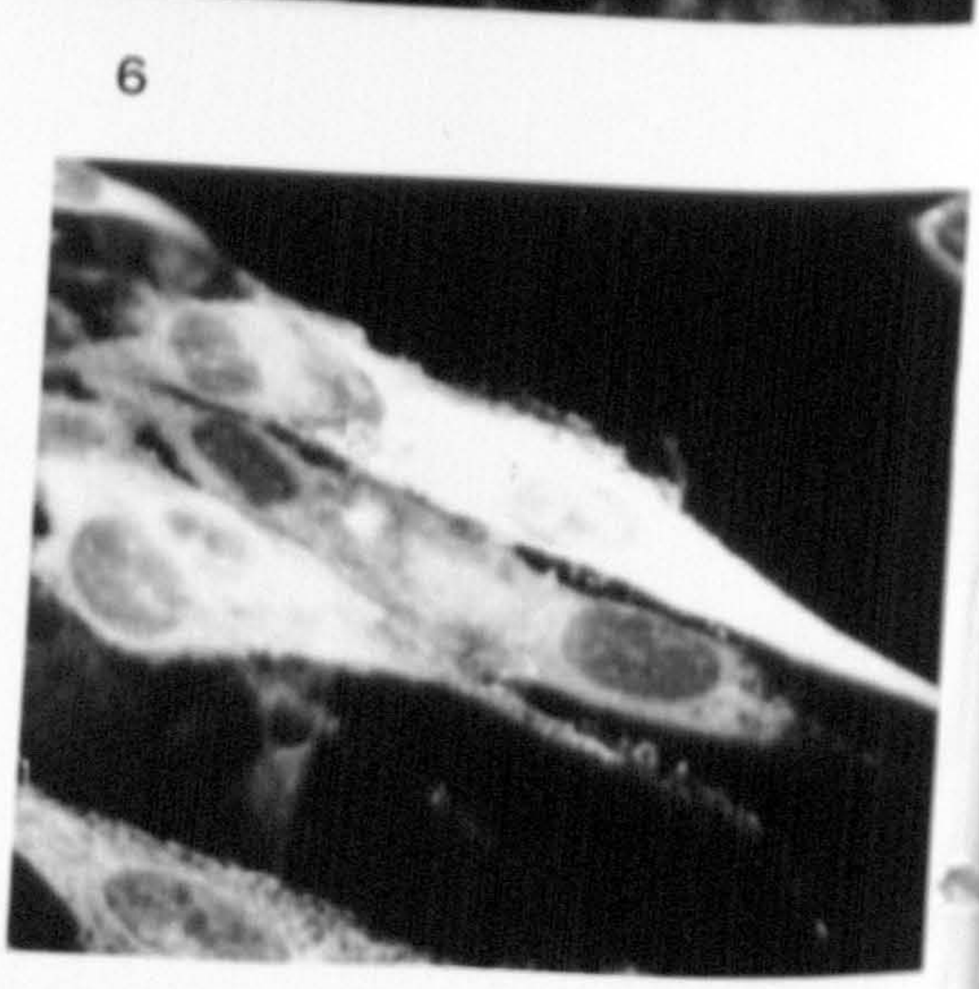

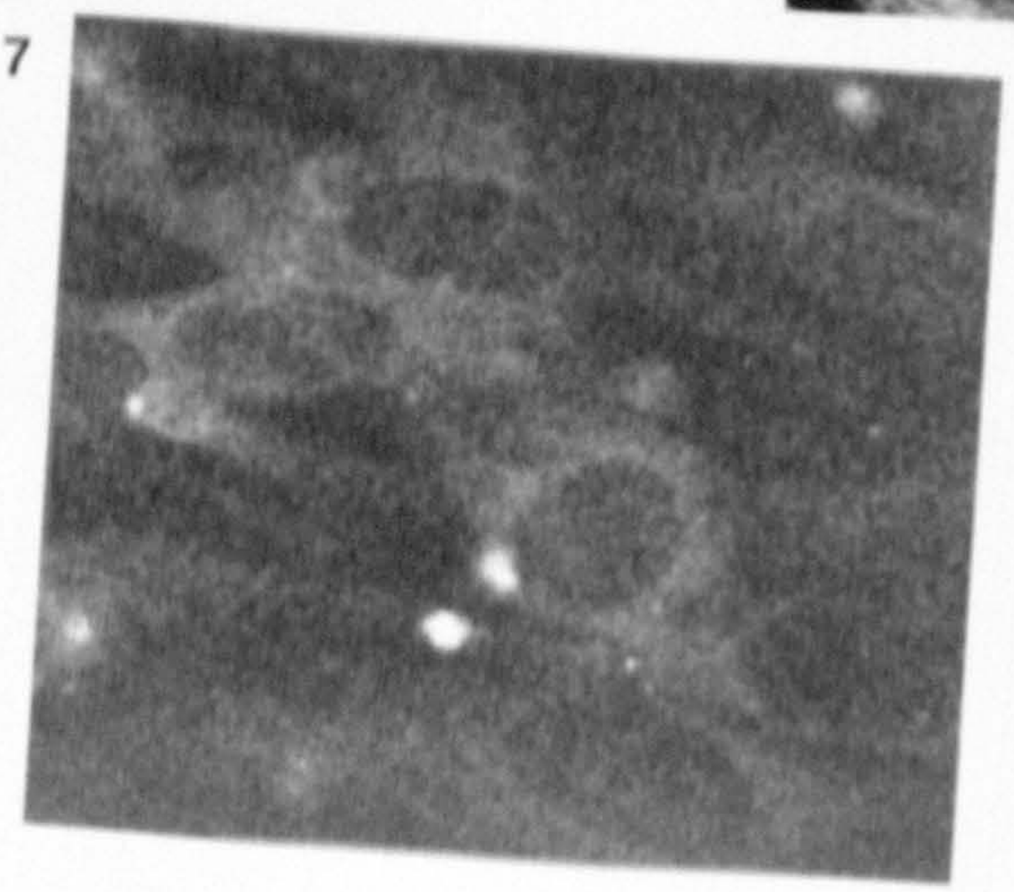

7

Appropriate presentation of the translocation signal sequence and therefore translocation of NS1 to the ER, appears to be critical for efficient expression of the NS1 antigen. What is not clear, however, is why the internal translocation signal of fragment FENS1 and FENS12a was unable to permit expression of a protein recognised by anti-NS1 monoclonal antibodies, it is possible that the signal was masked by chain folding rendering it non-functional.

The proteins expressed by pMV45 and pMV54 and recognised by the anti-NS1 monoclonal antibodies were examined further by Western-blot (Fig. 3.19). Lysates of cells harvested 48 h post-transfection with pMV45, pMV54, and cells infected with TBEV, were prepared for separation by SDS PAGE in a non-reducing SDS loading buffer. Reducing conditions were not used and samples were not boiled prior to electrophoresis. Following electrophoresis, the separated viral proteins were transferred from the gel to Hybond membranes and probed with an anti-NS1 monoclonal antibody pool (T12, T33/1, T33/3).

Fig. 3.18. Detection of NS1 Expressed in Cells by Immunofluorescence: Expression of TBEV NS1 detected by immunofluorescence with the anti-NS1 monoclonal antibody pool (T12, T33/1, T33/3) in TBEV-infected 293 cells (1); pMV45-transfected 293 cells (2); pMV54-transfected 293 cells (3); pMV39-transfected 293 cells (4); TBEV-infected MRC5 cells (5); RAd51-infected MRC5 cells (6) and mock-infected MRC5 cells (7).

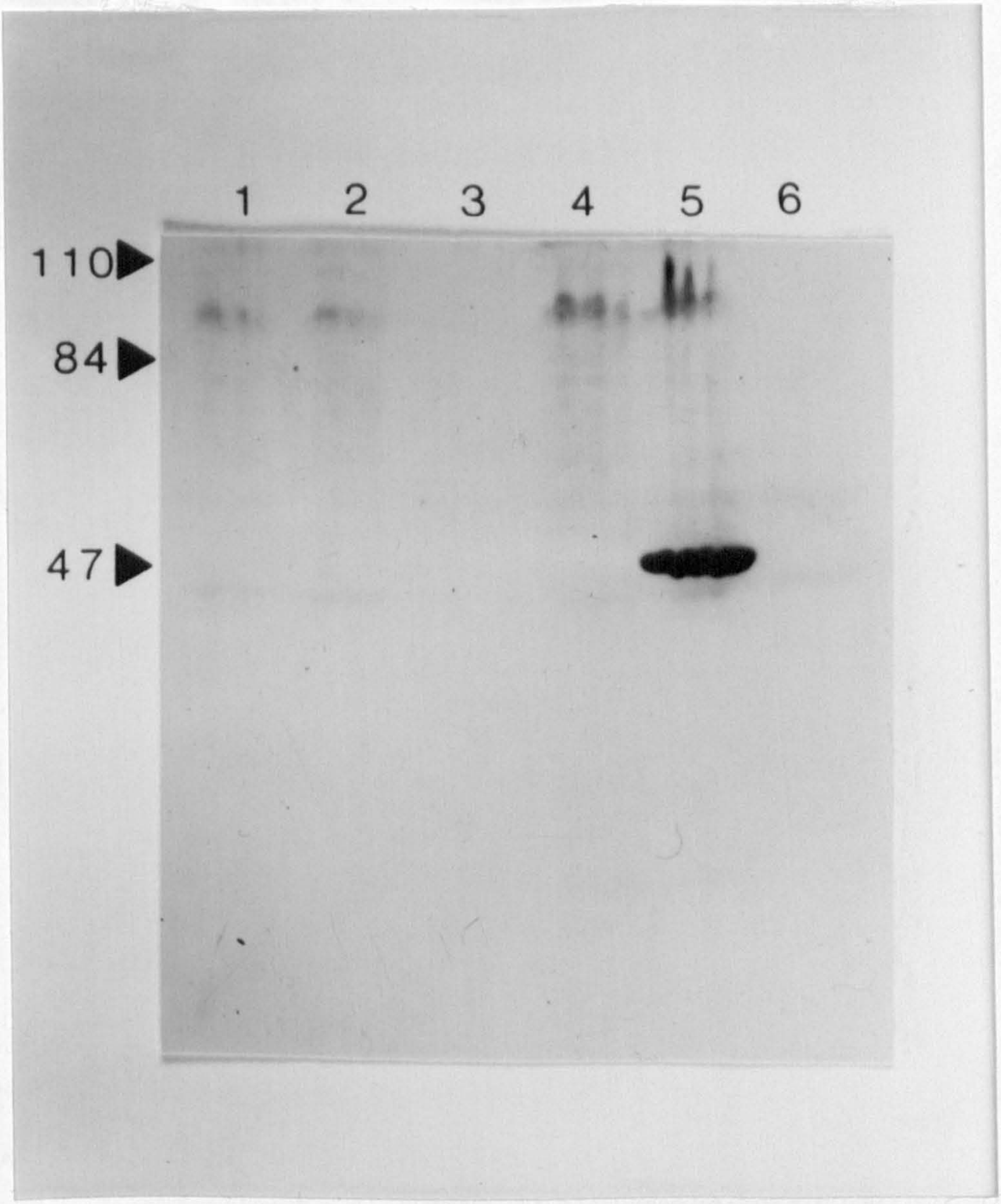

Fig. 3.19. Detection of NS1 by Western Blot: Lysates prepared from 293 cells were separated on a 10% SDS-PAGE before being electrophoretically transferred to a nitrocellulose filter. The proteins transferred to the membrane were probed with the anti-NS1 monoclonal antibody pool (T12, T33/1, T33/3). NS1 (Mr of 47 kDa) was only detected in TBEV-infected 293 cell monolayers (Lane 5). A larger NS1 complex of approximately 92 kDa was detected in TBEV-infected cells (Lane 5), RAd51 infected 293 cells (Lane 4), pMV45 tranfected 293 cells (Lane 2) and pMV54 transfected 293 cells (Lane 1). NS1 specific proteins were not detected in the mock transfected (Lane 3) or mock infected 293 cells (Lane 6). Sizes in kDa are indicated on the left.

The location of NS1 bound antibody was visualised by chemiluminescence reagents which recognised the anti-mouse antibody and recorded on radiographic film. The monomeric form of the NS1 (47 kDa) could readily be demonstrated in TBEV-infected (Fig. 3.19, Lane 5) but not in pMV45- or pMV54-transfected (Fig. 3.19, Lane 2 and Lane 1) 293 cell extracts. A diffuse, slower-migrating band corresponding to a protein with an apparent molecular mass of approximately 92KDa, however, gave a positive signal with the infected cell extract and the pMV45- and pMV54-transfected cell extracts. Neither the NS1 specific 47 kDa or 92KDa band were seen in mock-transfected or mock-infected samples (Fig. 3.19, Lane 3 and Lane 6).

This result demonstrated that TBEV and plasmids pMV45 and pMV54 encode a protein which participates in high molecular weight complexes of approximately 92 kDa. The 92 kDa protein may be homodimers of NS1 or NS1 complexed with cellular encoded components.

The co-migration of the NS1 specific product detected in cells transfected with pMV54 with cells transfected with pMV45 implies that the fragment FSNS12a contained sufficient information to permit the removal of the NS2a-encoded peptide from the FENS12a translation product. If the NS2a portion had not been removed the protein encoded by the fragment in pMV54 (FSNS12a) was expected to migrate more slowly than that encoded by pMV45, on SDS PAGE.

It was found that the 75 nucleotides from the C-terminal of the E gene needed to be retained immediately upstream of the NS1 gene within the ORF in order to generate a protein recognised by anti-NS1 monoclonal antibodies. The requirement for appropriate processing of the C-terminus of NS1 was circumvented in the construct FSNS1 by the insertion of a translational stop codon at the predicted cleavage site.

3.2. Expression of TBEV NS1 Encoded by a Defective Adenovirus Expression Vector:

3.2.1. Overview:

Plasmids pMV45 and pMV54 were identified in transient DNA transfection experiments as being able to induce the expression of TBEV NS1 antigens. There was no obvious difference in the level of expression of NS1, distribution of immunofluorescence, apparent molecular weight or their ability to form large molecular weight complexes between the pMV45- and pMV54-encoded NS1. Since it was envisaged that co-expression of NS2a would complicate the analysis of animal protection experiments, plasmid pMV45, which contained only the NS1 coding sequence, was used as the basis for expression in the adenovirus vector.

The expression cassette was excised from pMV45 on a HindIII fragment and inserted into the adenovirus transfer vector pMV60 generating pMV51. pMV60 is identical to pXCX2 (Spessot et al., 1989) except that a linker (containing the HindIII cloning site) has been inserted at the unique XbaI cleavage site (Wilkinson and Akrigg, 1992) (Fig. 3.20). The HindIII linker in pMV60 is located between regions with homology from either side of the E1a gene which map to the region between nucleotide 30 and 420, and between 3328 and 5788 of the human adenovirus type 5 genome and are able to direct homologous recombination with the adenovirus genome provided by pJM17. pJM17 contains the entire human adenovirus type 5 (Ad5dl309) genome with the prokayotic vector pBRX inserted into the E1a gene (McGrory et al., 1987) (Fig. 3.21). The prokaryotic vector insertion in pJM17 makes the adenovirus genome too large to package into the adenovirus nucleocapsid. Furthermore the adenovirus genome contained within

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pJM17 has a deletion in the E3 gene region. The products encoded by the E3 gene are not essential for virus replication in tissue culture but is potentially important in <u>in vivo</u> study since it is thought to protect adenovirus-infected cells against lysis by adenovirus-specific cytotoxic-T lymphocytes (CTL) (reviewed Wold and Gooding, 1991).

Following co-transfection of pMV51 and pJM17 into 293 cells, a recombination event led to the generation of the defective recombinant adenovirus RAd51. During the recombination event, the IEP/NS1 expression cassette replaced both the prokaryotic vector and Ad E1a gene region. Replacement of this region by the CMV IE/NS1 expression cassette reduced the size of the adenovirus genome (pJM17) sufficiently to enable viral packaging of the viral genome generating the recombinant adenovirus RAd51. RAd51 was harvested by Arklone P extraction and plaque purified by limiting end point dilution titration.

A significant feature of the Ad vector is that it is defective in its E1a function. E1a is a trans-activator of early gene expression and like other adenovirus E1 deletion mutants, RAd51 can only replicate in a helper cell line, i.e. a cell line providing the E1a function such as 293 cells. Infection of cells lacking the E1 helper function results in constitutive expression from the CMV major IE promoter without activation of adenovirus early phase gene expression. Infection of non-permissive cells with recombinant defective adenoviruses at a high moi can be accomplished without inducing detrimental effects (e.g., cpe), and allows the detection of the expressed protein without any <u>de novo</u> synthesised adenovirus-encoded proteins being present.

Since recombinant TBEV NS1 was to be used in examining the protection elicited in mice by the protein it was necessary to investigate whether the recombinant proteins were processed by the infected cell in a manner similar to that of the TBEV-encoded protein. During its synthesis TBEV NS1 is translated to the lumen of the ER, where it is folded and glycosylated. Further modification occurs within the Golgi complex before NS1 is secreted from the infected cell. Detailed comparisons were made between NS1 produced by TBEV and RAd51-infected cells to demonstrate the ability of the NS1, synthesised from the recombinant adenovirus, to complete these post translational modifications.

3.2.2. Construction of Recombinant Adenovirus:

The CMV IEP/NS1 expression cassette was excised from pMV45 on a HindIII fragment (Fig. 3.16, Lane 7), and inserted into pMV60 at a unique HindIII restriction site located between the two regions of adenovirus sequence. The resultant plasmid was designated pMV51 (Fig. 3.22). The presence of the CMV IE/NS1 expression cassette was confirmed by XbaI digestion and its orientation in relation to the flanking sequences established by digestion with SacI which cleaves the plasmid and CMV IE/NS1 expression cassette asymmetrically.

Plasmids pJM17 (5 µg) and pMV51 (5 µg) were co-transfected into a monolayer of 293 cells (Fig. 3.23). The cells were incubated for seven days at which time the formation of viral plaques in the monolayer was observed. Co-transfection had enabled homologous recombination between pMV51 and pMJ17 to occur resulting in the incorporation of the expression cassette CMV IE/NS1 into the adenovirus genome, replacing both the adenovirus E1 gene region from nucleotide 402 to 3328 and the intervening prokaryotic vector.

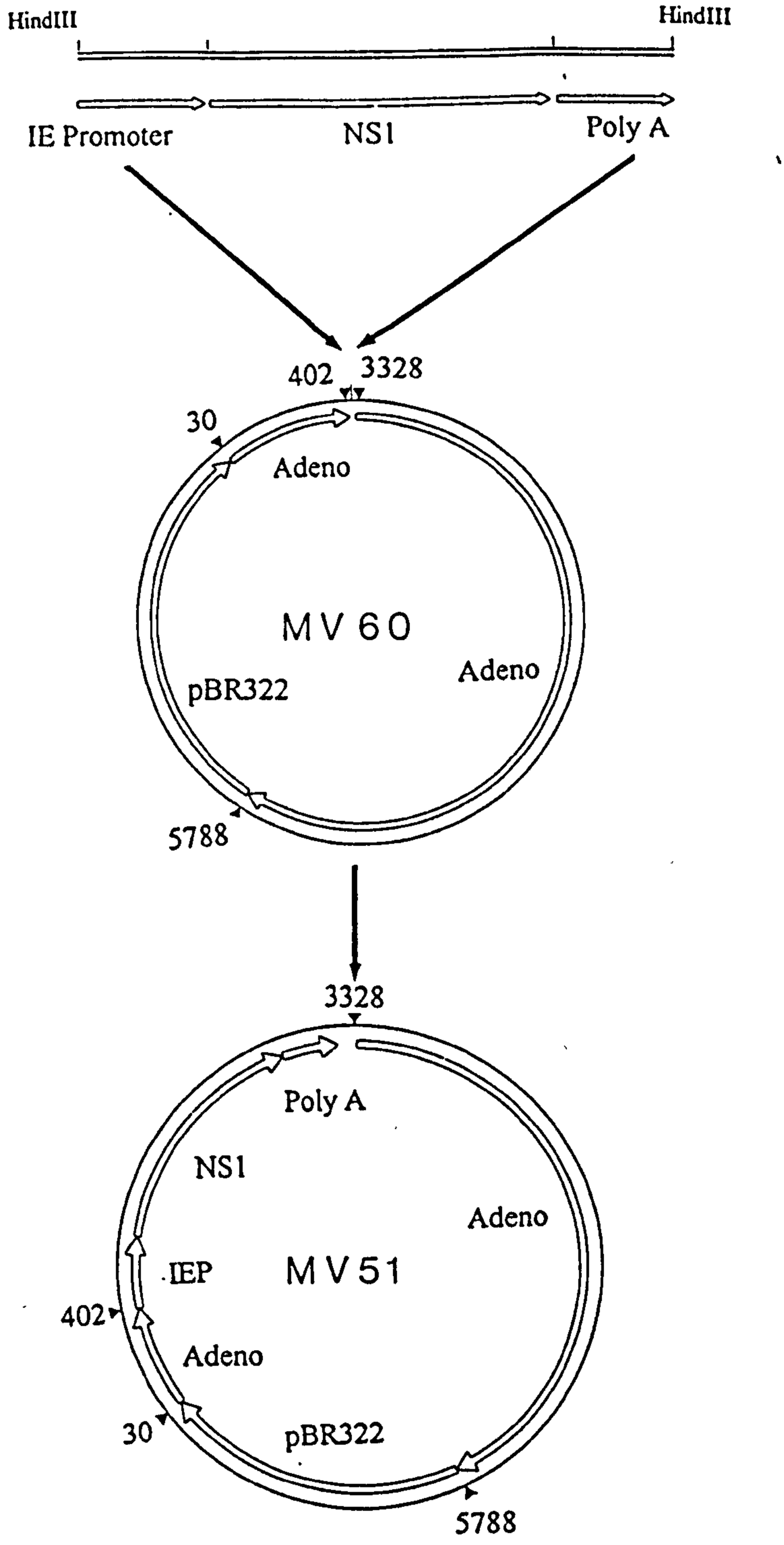

Fig. 3.22. Cloning of the IEP/NS1 Expression Cassette into the Adenovirus Transfer vector pMV60: The IE/NS1 expression cassette was excised from pMV45 on a HindIII fragment and inserted into the transfer vector pMV60 to generate pMV51. DNA sequence numbers shown on the plasmids correspond to the adenotype 5 genome.

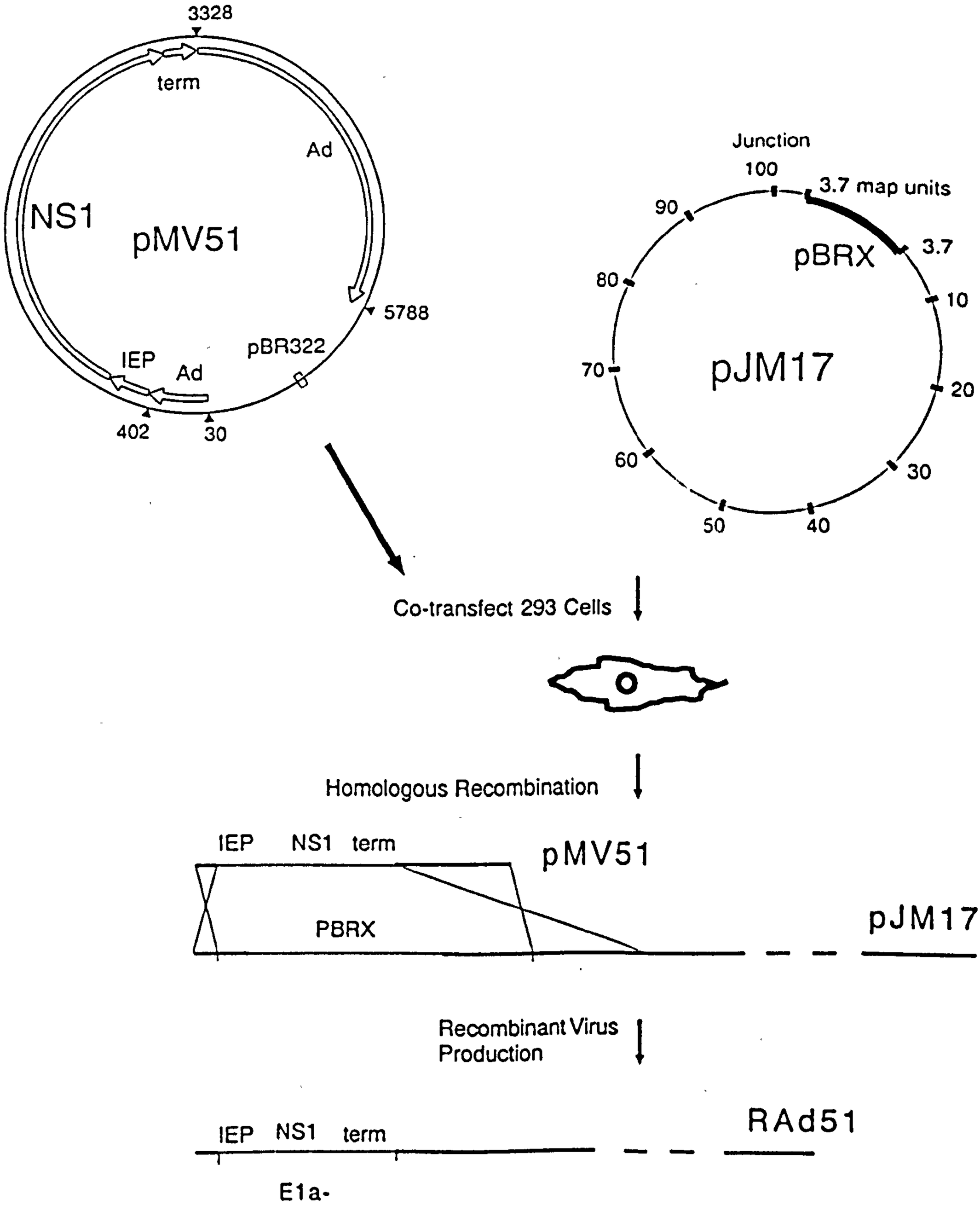

Fig. 3.23 Generation of Adenovirus Recombinant RAd51: Plasmids pJM17 and pMV51 were co-transfected into 293 cells. Homologous recombination between the two plasmids results in the EI gene (bp 452-3328) and the prokaryotic vector pBRX in pMJ17 being replaced with the IE promoter/NS1 expression cassette. Homologous recombination reduced the overall size of the adenovirus genome in pMJ17, and the resultant recombinant adenovirus RAd51 was package within an adenovirus particle.

Following recombination the size of the genome was decreased sufficiently to enable packaging of the recombinant genome into an adenovirus particle. The recombinant virus, designated RAd51, was harvested by Arklone P extraction. The purified RAd51 was used to infect a 175 cm^2 flask of 80% confluent 293 cells. When the monolayer had been destroyed, the virus was harvested with Arklone P and plaque purified by limiting end point dilution titration.

3.2.3. EXPRESSION NS1 FROM RAd51:

Expression of NS1 in RAd51-infected cells was initially investigated by immunofluorescence. Monolayers of both permissive 293 cells and non-permissive MRC5 cells were infected with RAd51 at 30 pfu/cell as determined on 293 cells. 293 and MRC5 monolayers were also infected with TBEV at 1 pfu/ml or PBS. Two days post infection the monolayers were examined for the expression of NS1 by immunofluorescence (Fig. 3.18). The cytoplasmic pattern of fluorescence observed following RAd51 infection of MRC5 cells (Fig. 1.18, 6) was similar to that observed with TBEV-infected cells (Fig. 1.18, 5), while no fluorescence was detected in mock-infected cells (Fig. 1.18, 7). Expression of NS1 in MRC5 cells with RAd51 at a moi of up to 30 pfu/cell produced no obvious cytopathic effect in the monolayer.

3.2.4. Analysis of NS1 Expressed From RAd51:

The formation of larger molecular weight forms of NS1 in RAd51-infected cells lysates was demonstrated by Western blot analysis (Fig. 3.19). Cell lysates prepared 24 hpi from RAd51- and TBEV- infected 293 cells were separated by SDS PAGE and electrophoretically transferred to Hybond membranes. The membranes were probed with the anti-NS1 monoclonal antibody pool (T12, T33/1, T33/3) and the

antibodies detected by chemiluminescent reagents. The samples were not boiled or reduced prior to electrophoresis, since the large molecular weight forms of NS1 were believed to be heat labile and because reduction prevents recognition by the anti-NS1 monoclonal antibodies used in this study. A diffuse protein band of 94 kDa, which co-migrated with a NS1 specific protein from both pMV45- (Fig. 3.19, Lane 2) and pMV54- (Fig. 3.19, Lane 1) transfected cell lysates and from TBEV-infected (Fig 3.19, Lane 5) cell lysates, was detected in RAd51-infected cells (Fig. 3.19, Lane 4). The inability to detect monomeric NS1 encoded by the DNA fragment FSNS1 has been discussed in section 3.1.9.

The monomeric form of RAd51-encoded NS1 was, however, demonstrated when RAd51-infected MRC5 cell lysates were subjected to SDS PAGE stained with coomassie blue (Fig. 3.26). Both the recombinant and the wild type TBEV extra-cellular forms of NS1 were consistently found to co-migrate on polyacylamide gels and to be of a higher apparent molecular weight than their intracellular counterparts.

3.2.5. Glycosylation of NS1.

During its synthesis in infected cells, NS1 of TBEV is translocated to the lumen of the ER, where it is glycosylated and then transported to the Golgi complex where there is further modification of its oligosaccharide side chains. The ability of recombinant NS1 to complete these post-translational modifications was examined by comparing the susceptibility of the recombinant and the TBEV-encoded NS1 to glycosidase digestion with endoH and glycoF (Fig. 3.24). EndoH removes N-linked high mannose or hybrid oligosaccharide side chains but is ineffective against complex glycans, whereas endoF cleaves all three type of N-linked glycan.

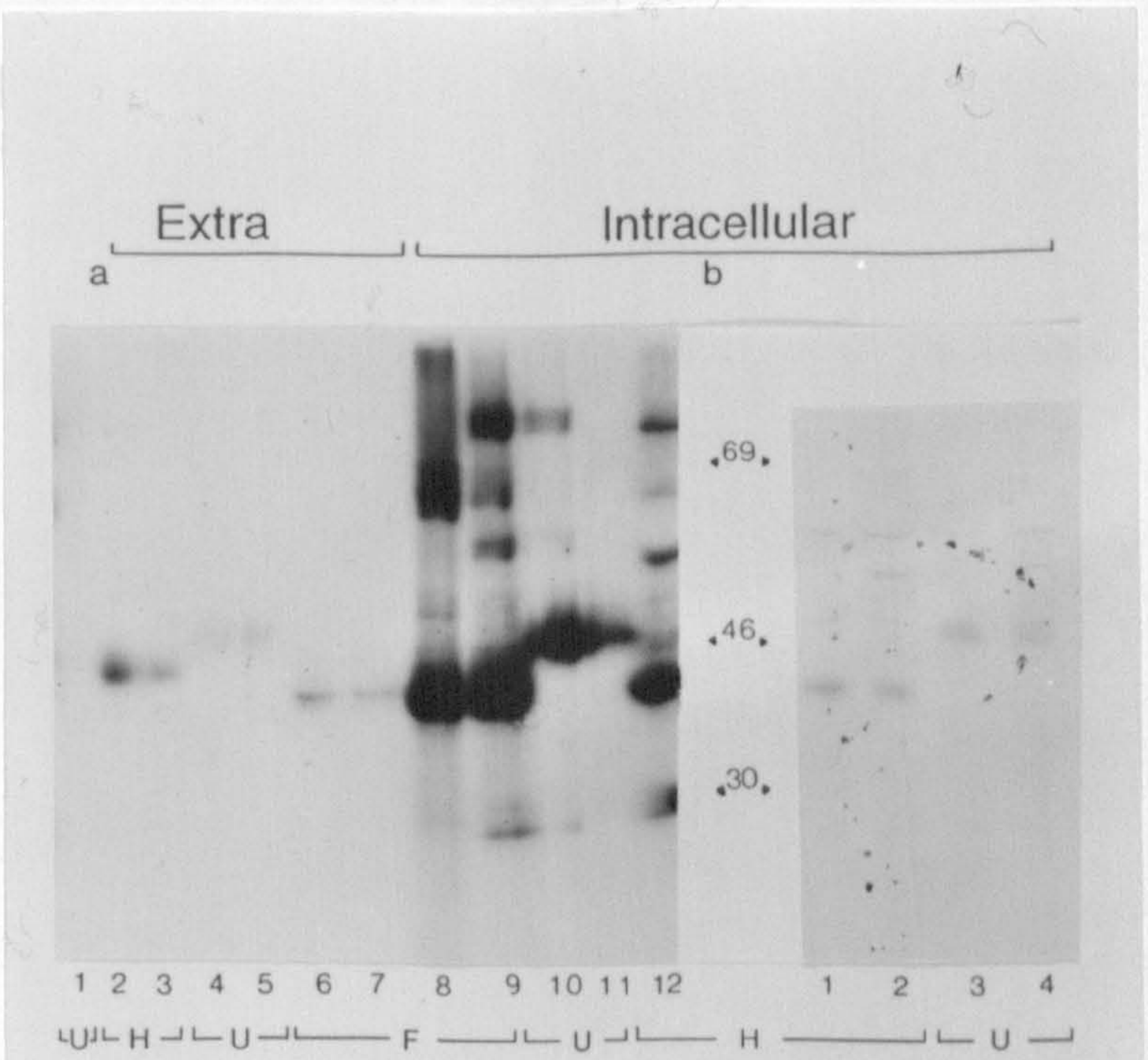

Fig 3.24. Effect of Glycosidase Treatment on NS1: Radiolabelled NS1 from RAd51 and TBEV infected 293 cells was immunoprecipitated with anti-NS1 monoclonal antibody pool (T12, T33/1, T33/3) and examined by SDS-PAGE on a 10% gel and detected by autoradiography. TBEV-encoded NS1 is shown in (a) Lanes 2, 4, 6, 8, 10, 12 and (b) Lanes 1 and 3, Rad51-encoded NS1 is shown in (a) Lanes 3, 5, 7, 9,11 and (b) Lanes 2 and 4, following treatment with glycoF (F) or endoH (H) or no treatment (U). (a) Lane 1 containes mock infected, intra-cellular samples not subjected to glycosidase treatment. Sizes on the left are shown in kDa.

80% confluent, 293 cells were infected with RAd51 at a moi of 10 pfu/cell. The infected cells were pulse labelled 24 pi with ^{35}S methionine for one hour. Cell lysates and culture supernatant from the infected cells were obtained three or four h following the pulse label and NS1 immunoprecipitated by an anti-NS1 monoclonal antibody pool (T12, T33/1, T33/3). NS1 was released from the immune complex by boiling for ten minutes and the sepharose beads were removed by centrifugation. Aliquots of the released antigen were then treated with either endoH or glycoF as described in section 2.31. The digested NS1 samples were boiled before being subjected to electrophoresis on a 10% SDS PAGE followed by autoradiography.

Treatment with both glycosidases was shown to have a similar effect on the recombinant and TBEV-encoded NS1 protein as analysed by SDS PAGE. The presence of N-linked high mannose or hybrid oligosaccharides side chains on the intracellular forms of the NS1 protein was revealed by endoH digestion, which reduced the apparent size of NS1 by 8 kDa from 47 to 39 kDa (FIG. 3.24(a), lane 12; Fig. 3.24(b), Lanes 1 and 2). Treatment of intracellular TBEV and RAd51 NS1 with glycoF produced a band which co-migrated with the endoH treated form indicating that no complex or biantennary side chains were present on the intracellular form of the protein (Fig. 3.24(a), Lanes 8 and 9). The molecular mass of the de-glycosylated form of NS1, 39 kDa, is in agreement with the value for the primary amino acid sequence of 39,162 Da predicted from DNA sequence analysis.(Pletnev et al., 1989).

Unlike the intracellular form of NS1, treatment of extra-cellular NS1 (TBEV or RAd51-encoded) with endoH did not generate protein of similar apparent molecular weight to that generated by glycoF digestion (Fig. 3.24(a), Lanes 2,3,6 and7). Untreated extra-cellular

NS1 (Fig. 3.24(a), Lane 4 and 5) migrates more slowly than the corresponding intracellular form on SDS PAGE (Fig. 3.24(a), Lanes 10 and 11; Fig. 3.24(b), lanes 3 and 4), with an apparent molecular mass of 51KDa. Following treatment of extra-cellular NS1 with glycoF, however, the protein co-migrated with the treated intracellular form described above. EndoH treatment of extra-cellular NS1 generated a protein which migrated with an apparent molecular mass of 47 kDa, not 39 kDa, implying that in going from the intra-cellular form, both the recombinant and the wild type NS1 proteins acquired additional complex or biantennary oligosaccharide side chains.

3.2.6. Kinetics of NS1 Expression and Secretion:

The study of the kinetics of NS1 synthesis and secretion from infected cells was also investigated by immunoprecipitation of pulse labelled infected cell monolayers (Fig. 3.25). At 80% confluence, MRC5 monolayers were infected with an moi of 30 pfu/cell of RAd51 and treated with the CMV IE promoter stimulant forskolin. Following 24 h incubation the infected cells were labelled with ^{35}S methionine for one hour. Cytoplasmic lysates and culture supernatant were obtained 0 h (Fig. 3.25, Lanes 1 & 2), 1 h (3 & 4), 2 h (5 & 6), 3 hours (7 & 8), 4 h (9 & 10), and 5 h (11 & 12) after the addition of the pulse. Samples 13 and 14 are cell lysates and tissue culture supernatant respectively, from mock-infected cells pulse-labelled and then incubated for 4 h. All samples were immunoprecipitated with the monoclonal antibody T12 and subjected to electrophoresis on a 10% SDS polyacrylamide gel, followed by autoradiography.

The autoradiograph was overexposed to show the temporal expression of intracellular NS1 (Fig. 3.25); in shorter exposures the difference in migration of the intra- and extra- cellular forms of NS1 could be

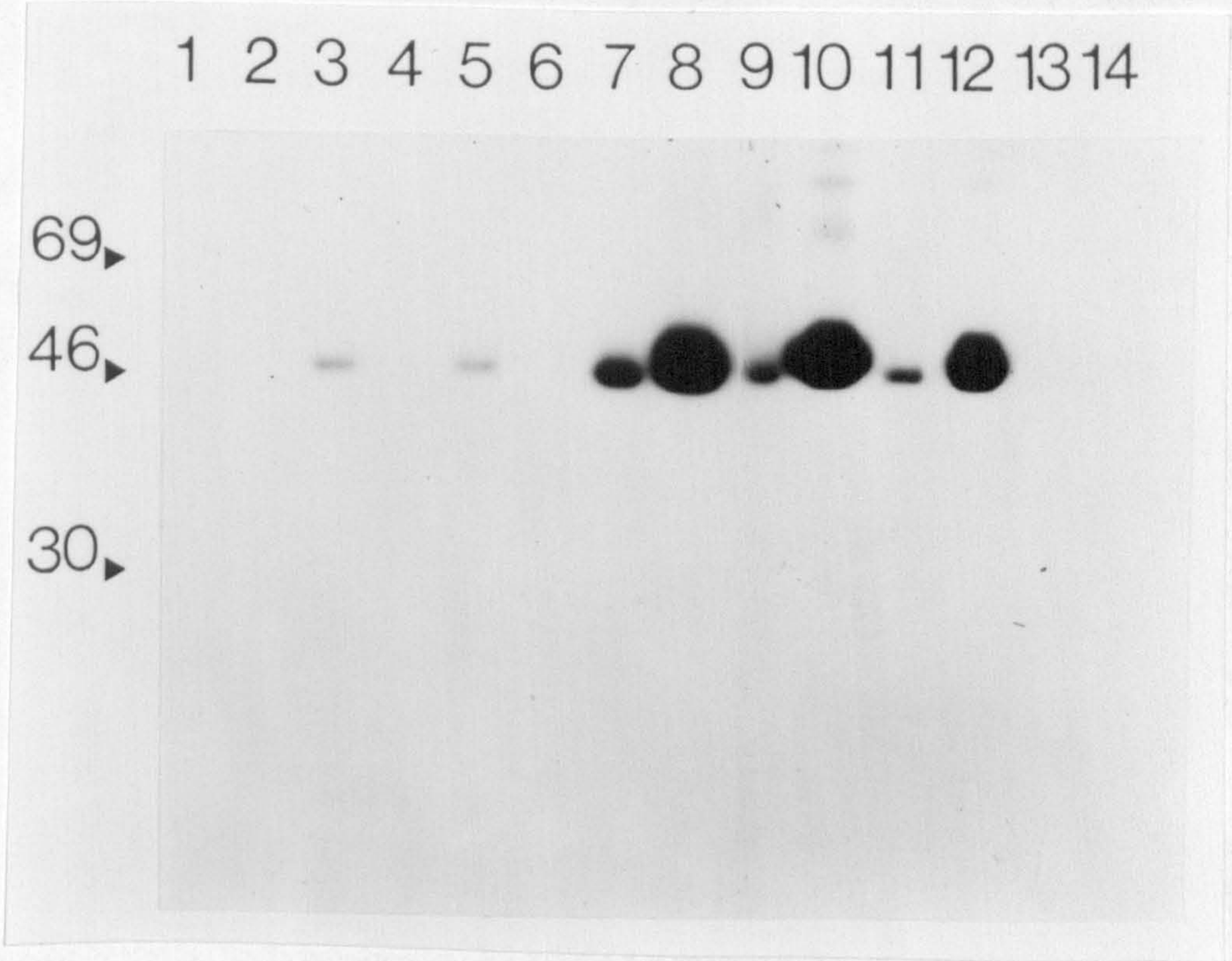

Fig. 3.25. Time Course of NS1 Secretion From RAd51 Infected MRC5 Cells: Cell lysates (Lanes 1, 3, 5, 7, 9, 11 and 13) and culture supernatants (Lanes 2, 4, 6, 8, 10, 12 and 14) were obtained at 0 (Lane 1 and 2), 1 h (3 and 4), 2 h (5 and 6), 3 h (7 and 8), 4 h (9 and 10) and 5 h (11 and 12) following the addition of a 1 h pulse with ^{35}S methionine. Lanes 13 and 14 are cell lysates and tissue culture supernatants, respectivly, from mock infected cells pulsed for 1h with ^{35}S methionine and then incubated for 5 h. All samples were immunoprecipitated with the monoclonal antibody T12 and subjected to SDS-PAGE, followed by autoradiography. Sizes are shown in kDa.

seen (data not shown). The intracellular forms of NS1 was detectable one hour after addition of the isotope, reaching a maximum by 3 h, after which its level declined. Extra-cellular NS1 protein was detected in RAd51-infected tissue culture supernatant at high levels from three hours onward. Detection of intra-cellular prior to the extra-cellular NS1 is consistent with there being a product-precursor relationship between the two forms of the protein.

3.2.7. Level of NS1 Expressed in RAD51-Infected Cells:

Experiments performed using the expression vector based on a combination of the CMV major IE promoter and the adenovirus vector indicated that the system was capable of producing surprisingly high levels of expression (Wilkinson and Akrigg, 1992). To assess the level of expression of NS1 from RAd51-infected cell lysates and culture supernatants were separated by SDS PAGE and visualised using the protein stain coomassie blue (Fig. 3.26).

In order to maximise expression levels, MRC5 cells grown in 175 cm^2 flasks were infected with RAd51 or the control adenovirus E1- deletion mutant expressing ß-galactosidase, RAd35, at a very high moi of 100 pfu/cell and incubated for six days in protein free medium containing forskolin. The protein free media was obtained from PAA, Linz, Austria. Tissue culture supernatant (1/125 of total sample) and cytoplasmic cell lysates (1/50 of the total sample) were subjected to SDS PAGE on a 5%-15% polyacrylamide gradient gel (Fig. 3.26). In samples heat treated prior to electrophoresis, the presence of an infected-cell specific protein (ICSP) was clearly shown. The ICSP had an apparent molecular mass of 47 kDa in cell extracts (Fig. 3.26, Lane 5), and co-migrated with a less abundant cellular protein and a slightly bigger ICSP of approximately 51 kDa present in the

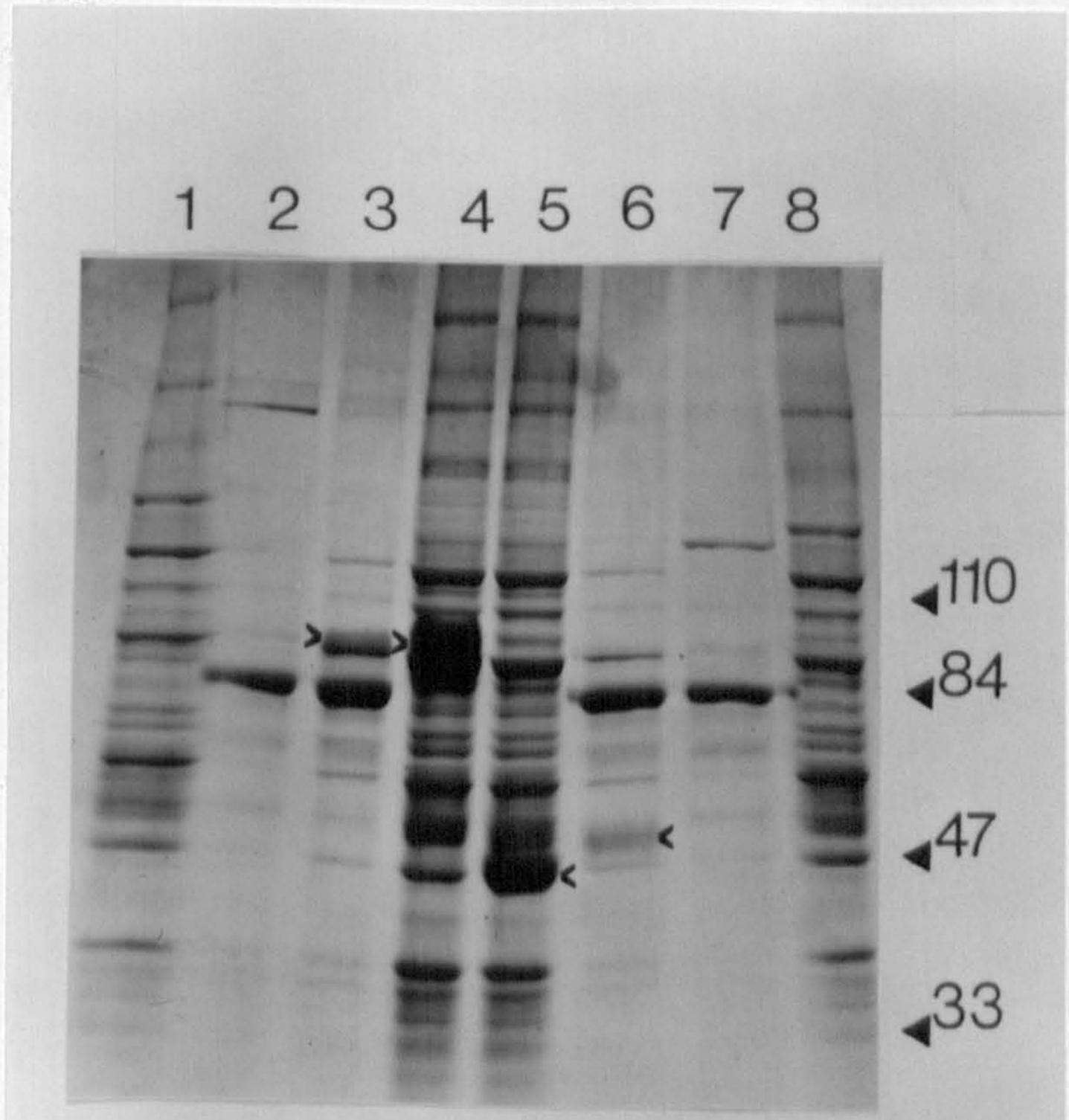

Fig. 3.26. Detection of Recombinant NS1 on a Coomassie Stained Polyacrylamide Gel: RAd51-infected MRC5 culture supernatants and MRC5 cell lysates were subjected to SDS-PAGE on a 5 to 15% gradient polyacrylamide gel. Lanes 1 and 8 show E1- adenovirus-infected cell lysates; 2 and 7 show E1- adenovirus-infected cell supernatants; 3 and 6 show RAd51-infected cell supernatants; and 4 and 5 show RAd51 infected cell lysates. Only Lanes 5 to 8 were heat denatured prior to electrophoresis. Bands attributed to recombinant NS1 are indicated with arrowheads. Sizes are shown in kDa.

supernatant (Fig. 3.26, Lane 6). These ICSPs are interpreted as representing the intra- and extra-cellular forms of NS1. In intra- and extra- cellular samples that were not boiled prior to electrophoresis, neither protein band was visible, but additional slower migrating bands are apparent, which presumably correspond to NS1 protein complexes (Fig. 3.26, Lane 3 and 4). Absence of the high molecular weight complex in the boiled samples implies that they are heat sensitive. Surprisingly, in the unboiled samples, the extra-cellular protein complex migrated faster than the intracellular protein complex. This anomaly may be due to some conformational property of the oligomerised protein.

The NS1 protein was identified as the predominant cytoplasmic protein in RAd51-infected cells, whereas NS1 can not be visualised on similarly stained gels in extracts of TBEV-infected cells. The very high levels of NS1 expression observed by using the adenovirus recombinant clearly exceeded the yield obtained from TBEV-infected cultures.

3.3. In Vivo Studies of NS1:

3.3.1. Overview:

It has been established that, in mice, an immune response to NS1 of the mosquito-borne flaviviruses YF and dengue, both causative agents of haemorrhagic diseases, confers a degree of protection against lethal challenge with homologous virus (Gould et al., 1986; Henchal et al., 1988; Schlesinger et al., 1985, 1986 & 1987). To investigate the possibility of a similar role for the NS1 antigen of an encephalitic and of a tick-borne flavivirus, vaccination with the recombinant adenovirus RAd51 was assessed in a mouse protection model. Preliminary experiments had demonstrated that the adenovirus recombinant RAd35 could express ß-galactosidase, under the control of the CMV IE promoter, when infected into primary murine macrophage culture. It was therefore decided to attempt to inoculate mice directly with RAd51 rather than to try and purify the recombinant NS1 protein. Once it had been established that mice could raise anti-NS1 antibodies when immunized with RAd51, experiments were undertaken to examine the potential of this response to protect mice against both death and disease caused by TBEV.

Further experiments were carried out to demonstrate the importance of the terminal lytic pathway of the complement cascade. Since the level of NS1 expressed in vitro was so very high it could not be ruled out that small amounts of NS1 contaminating the RAd51 inoculum opposed to de novo synthesised protein were responsible for the protection reported. An experiment in which mice were immunized with RAd51 treated with UV radiation and/or purified RAd51 was undertaken in order to demonstrate that protection was elicited by de novo synthesised protein.

3.3.2. Serological analysis of NS1 expressed From RAd51:

Four Balb/C mice were inoculated with RAd51 according to the set schedule (section 2.38). One week following the third inoculation with RAd51, sera were obtained from blood collected from the immunized mice by terminal cardiac puncture. The presence of anti-NS1 antibodies in the pooled RAd51 antisera from the mice was determined by analysis of the ability of the sera to recognise NS1 on western transfers (Fig. 3.27).

293 cells were infected with RAd51 and TBEV at a moi of 1.0 pfu/cell. Cell lysates were prepared from the infected cell 48 hpi and a parallel lysate of mock infected-cells. Duplicate samples of each cell lysate were subjected to SDS PAGE on a 10% polyacrylamide gel. Samples were not boiled or treated with reducing agent prior to electrophoresis. Following electrophoresis the proteins were electrophoretically transferred to Hybond membranes. One sample of each lysate was probed with anti-NS1 monoclonal antibody T12 (Fig 3.27(a)). This demonstrated the presence of the 94 kDa "dimer" in samples from TBEV - and NS1-infected cells (Fig. 3.27(a) Lane 2 and 3). The 47 kDa NS1 band was demonstrated in the TBEV-infected cell sample (Fig. 3.27(a) Lane 2), as expected, but was not detected in the RAd51-infected cell sample (Fig. 3.27(a) Lane 3). The second sample from each lysate was probed with RAd51 antisera (Fig. 3.27(b)). The presence of anti-NS1 antibody in the RAd51 antisera that recognised the ''dimeric" complexes in TBEV- and RAd51-infected cells was demonstrated (Fig. 3.27(b)., Lanes 2 and 3). The 47 kDa protein in the TBEV-infected sample appeared to be absent, probably as a result of there being no anti-47 kDa specific antibodies in the anti-RAd51 antisera (previously demonstrated in section 3.2.4). The concentration of recombinant adenovirus inoculated into the mice

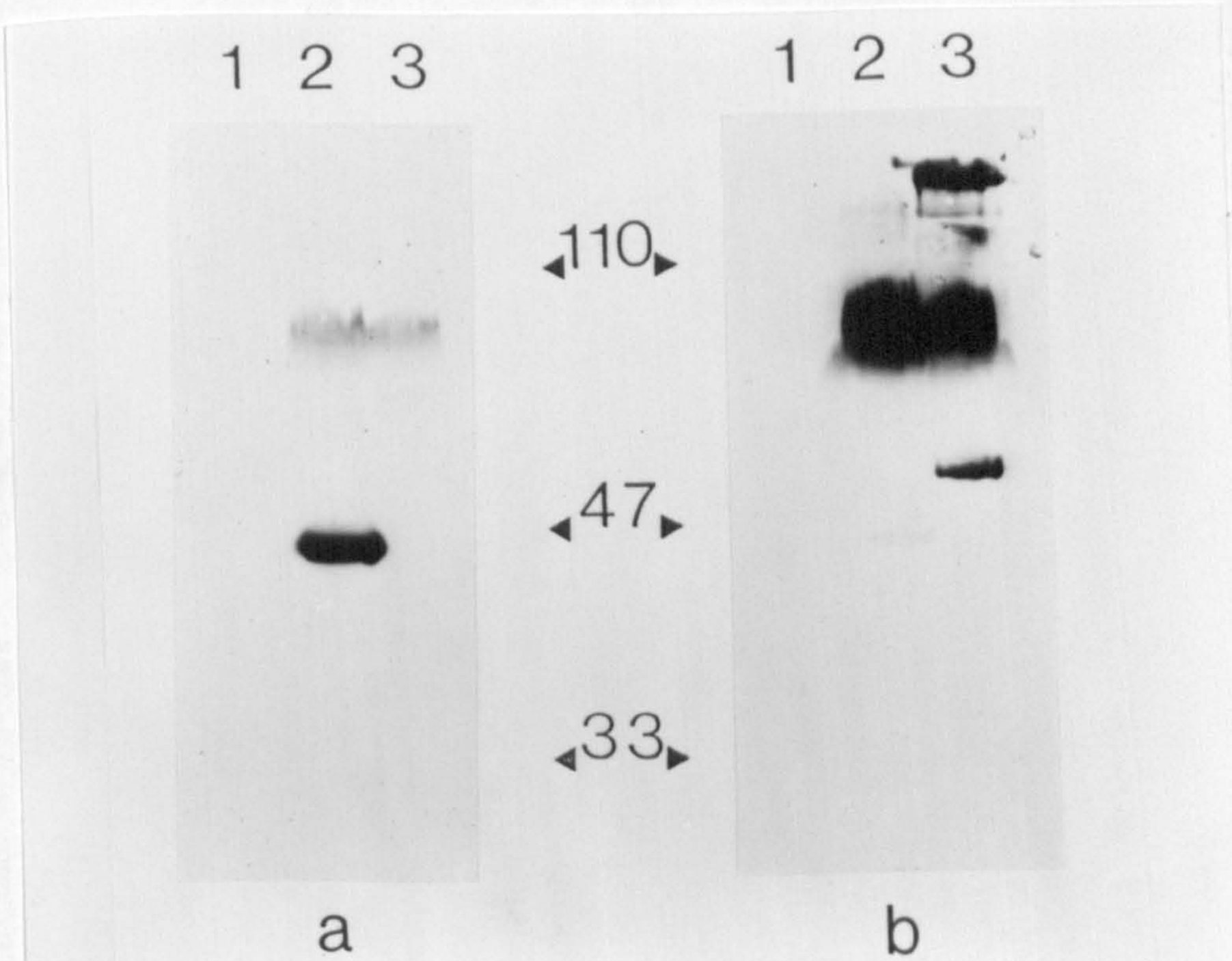

Fig. 3.27. Demonstration of the Reactivity of anti sera raised to RAd51: Cytoplasmic extracts from 293 cells that were uninfected (Lane 1), TBEV infected (Lane 2) and RAd51 infected (Lane 3) were subjected to SDS-PAGE on a 10% polyacrylamide gel. Samples were not denatured or treated with reducing agents prior to electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with (a) anti-NS1 monoclonal antibody T12 and (b) serum from RAd51 vaccinated mice. The 62KDa and >110KDa bands specific to lane 3b can be attributed to adenovirus fibre and capsid proteins respectively. Sizes are shown in kDa.

during the study was high enough to elicit an anti-adenovirus immune response as the 62 kDa and >110 kDa bands specific to Fig. 3.27(b), Lane 3, can be attributed to the adenovirus fibre and capsid proteins respectively.

The ability of the RAD51 antisera to neutralise homologous virus infections was tested by plaque reduction neutralisation assay. Five duplicate volumes of PS cells were infected with 20 pfu of Neudörfl strain of TBEV that had been reacted with RAd51 antisera at dilutions of either 1/2, or 1/4, or 1/8, or 1/16 or 1/32 in PBS. No reduction in the number of plaque was seen in monolayer infected with virus previously treated with RAd51 antisera when compare to the number of plaques in the monolayers infected with virus reacted with PBS. This indicated that anti-RAD51 antisera did not contain antibodies capable of neutralising TBEV infections. The anti-NS1 recombinant antibodies in the RAd51 antisera were also found to fix complement (not shown).

Localisation of NS1 on the surface of paraformaldehyde fixed PS cells expressing NS1 was demonstrated by immunofluorescence using the method in section 2.28 (Fig. 3.28). The ability of NS1 to localise to the infected cell surface is consistent with a mechanism of protection involving complement mediated lysis of infected cells.

3.3.3. Protection Elicited Against Lethal TBEV Challenge by Immunization with by RAd51:

The dose of the Neudorfl strain of TBEV required to kill 50% of Balb/C mice (1 LD_{50} dose) when introduced subcutaneously was calculated prior to the protection study as described in section 2.37. RAd51 was inoculated as prescribed by the immunization schedule (section 2.38) into 22 Balb/C mice. At the same time 16 mice were inoculated with the E1- recombinant adenovirus expressing ß-gal

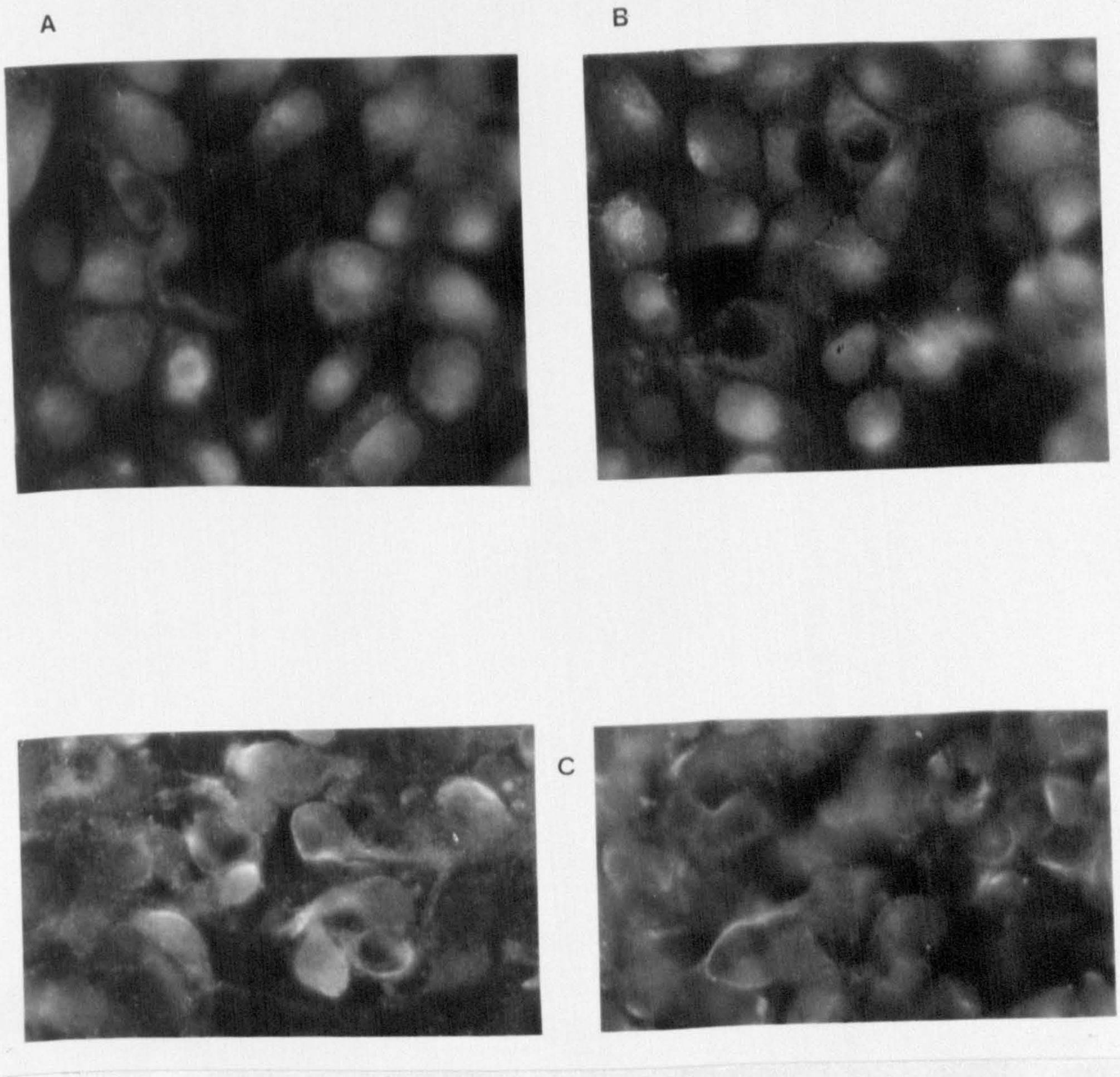

Fig. 3.28. Location of RAd51 Encoded NS1 on the Cell Surface: The presence of RAd51 encoded NS1 was demonstrated on the suface of PS cells infected with RAD51 and fixed with paraformaldehyde fixed (c) by indirect immunofluorescence with monoclonal antibodies T12, T33/1 and T33/3, but not on the surface of similarly fixed RAd35-infected PS cell (b) or mock-infected PS cells (a).

(RAd35) and 12 mice were inoculated with PBS. One week following the third inoculation of the immunization schedule, sixteen of the RAd51 inoculated mice were challenged subcutaneously with the Neudörfl strain of TBEV; eight of the mice received 100 LD_{50} and eight received 1000 LD_{50}. The remaining six RAd51 inoculated mice were challenged with PBS. At the same time eight of the RAd35 inoculated mice and six of the PBS inoculated mice were challenged with 100 LD_{50} of TBEV, the remaining mice received 1000 LD_{50}.

No mouse challenged with a lethal dose of TBEV following vaccination with PBS or RAd35 survived longer than 15 days post challenge, the majority dying at, or around, day 10 (Table 3.1). Eleven out of 16 animals vaccinated with RAd51, however, not only survived severe virus challenge, but also failed to exhibited any symptoms of disease. The five mice vaccinated with RAd51 which did die, although exhibited symptoms of disease similar to the control group, still survived, on average, six days longer than their RAd35 and PBS counterparts. There was no significant difference in survival time between the controls inoculated with PBS or RAd35. The size of the TBEV challenge dose did not significantly affect survival time of the mice which died or the survival rate of the protected mice.

3.3.4. Protection Against Disease Following TBEV Challenge in Mice by Immunized With RAd51:

In an attempt to understand the mechanism by which NS1 protects mice from TBEV an experiment was performed to identify at which point NS1 interrupts TBEV spread following infection. To examine the potential of the immune response to NS1 to inhibit the spread of a productive TBEV infection in mice it was necessary to established the pattern of virus spread in the absence of immunizing NS1.

TABLE 3.1. Protection of RAd51 Vaccinated Mice Against TBEV Challenge:

Immunization	TBEV Challenge Dose (LD_{50})	Mice Survived/ Total N° (%)	Average Survival Time (days)
RAd51	100	5/8 (62.5)	18
RAd51	1000	6/8 (75)	15
RAd51	0 (PBS)	6/6 (100)	–
RAd35	100	0/8 (0)	10
RAd35	1000	0/8	11
PBS	100	0/6	10
PBS	1000	0/6	9

Thirty Balb/C mice were inoculated subcutaneously with 10 LD_{50} of the Neudörfl strain of TBEV. A challenge dose of 10 LD_{50} was used, as in the previous protection experiment RAd51 immunization failed to protect 100% of the mice which may have been due to the high TBEV challenge dose overwhelming the protective immunity in a proportion of the mice (section 3.15). Each day post challenge serum samples were obtained from two mice by terminal cardiac puncture (section 2.38) and the brain was removed from each mouse, washed in ice cold PBS before being pooled, homogenised (section 2.9) and stored with the sera at -70°C. Sample collection ceased 10 days post challenge when all of the remaining mice had died. When all samples had been collected they were used to infect monolayers of PS cells. Seven days pi the cells were examined for the presence of a productive TBEV infection by immunofluorescence using the TBEV anti-E monoclonal antibodies (T7, T9, S8 and S12) at 5 µg/30 ml blocking buffer (Table 3.2). The TBEV E antigen was detected in cells inoculated with serum harvested from mice on days 3, 4 and 5 post challenge. TBEV E antigen was only detected in the cells inoculated with brain samples taken after day 5 following challenge and persisted until death.

Following this study, a further 54 mice were immunized according to the prescribed schedule with RAd51-infected cell lysates while 30 mice were infected with RAd51 and 30 with PBS. One week following the third inoculation of the immunization schedule, all the mice were challenged with TBEV Neudörfl strain at 10 LD_{50}.

From the PBS and RAd35 vaccinated groups, two mice were sacrificed each day post challenge and serum and brain samples were obtained. The samples from the RAd35 immunized mice were pooled each day as were those from the PBS immunized mice. On day one post challenge, and from day 14 to the end of the experiment at day 19 post challenge, two

Table 3.2. Recovery of Infectious TBEV From Mice Immunized with RAd51 Prior to TBEV Challenge:

Days Post Challenge	Mice Immunized with RAd51 Prior to Challenge With $10LD_{50}$ TBEV				Mice Immunized with RAd35/PBS Prior to Challenge with $10LD_{50}$ TBEV			
	SERA		Brains		Sera		Brains	
	NV	V	NV	V	NV	V	NV	V
1	+ +		+ +		++		++	
2	+ + +		+ + +		++		++	
3	+ + +		+ + +			++	++	
4	+ + +		+ + +			++	++	
5	+ + +		+ + +			++		++
6	+ +	+	+ +	+	++			++
7	+ + +		+ + +		++			++
8	+ + +		+ + +		++			++
9	+ + +		+ + +		++			++
10	+ + +		+ + +		++			++
11	+ + +		+ +	+	All mice had died or			
12	+ + +		+ + +		been sacrificed on or			
13	+ + +		+ +	+	before day 10			
14	+ + +		+ + +					
15	+ + +		+ + +					
16	+ + +		+ + +					
17	+ + +		+ + +					
18	+ + +		+ + +					
19	+ + +		+ + +					

KEY

V	Infectious virus recovered.
NV	No infectious virus recovered.
+ +	Two mice samples.
++	Two mice samples pooled.

mice were sacrificed from the RAd51-immunized mice group. Between day two and 13, three mice inoculated with RAd51 were sacrificed. Serum and brain samples were obtained from the RAd51-immunized mice as for the RAd35 mice, however, the sample were not pooled, but labelled so that the serum and brains from individual mice could be identified.

The samples collected were used to infect PS cells and a productive infection in the cells assessed by immunofluorescence using the anti-E monoclonal antibody pool (Table 3.2). The spread of virus in the RAd35-immunized mice mirrored that in the PBS immunized mice, with an apparent viremia lasting from day two to day five post challenge and infectious TBEV appearing in the brain on day five and persisting until death. Again similar results were obtained with the PBS immunized group and the RAd35-immunized group in that all the mice had all been either sacrificed or had died by day 11.

The mice treated with RAd51, with the exceptions described below, had neither an apparent viremia or infection of the brain following challenge with TBEV. The exceptions included a mouse taken on day six which in both a viremia and virus in the brain were detected; and two mice, one taken at 11 and one 13 which had an apparent TBEV infection of the brain. These infected RAd51-vaccinated mice would probably have succumbed to the TBEV infection and died. In addition to the three infected mice, two RAd51 vaccinated animals died on day 13 post challenge. Statistically, the group of 54 mice inoculated with RAd51-infected cell lysates in this experiment had an apparent survival rate of approximately 90%, a slightly higher level of protection than seen in the previous experiment.

3.3.5. Examination of TBEV Recovered From Mice Immunized With RAd51:

The possibility that TBEV mutants capable of evading the anti-NS1 immune surveillance were responsible for the death of the mice not protected when immunized with RAd51 was examined. Sera and blood samples collected on day 6, 11 and 13 post challenge (section 3.17) that contain TBEV E antigen were detected by the anti-NS1 monoclonal antibodies (T12, 33/1, 33/3) in immunofluorescence assays (data not shown). These data suggests that the death of the mice was not due to RAd51 immune selection of a NS1 mutant TBEV capable of evading the immune response raised to the recombinant NS1.

3.3.6. Protection Elicited Against Lethal TBEV Challenge in Complement Deficient Mice to TBEV:

The mechanism by which NS1 elicits protection of mice is unknown. Antibody dependent complement mediated lysis of infected cells is currently accepted as the most satisfactory explanation. This model is based primarily on an observation that complement-fixing monoclonal antibodies to YFV could partially protect mice against virus challenge (Schlesinger et al 1985., 1986 & 1987). In other flavivirus animal models (including TBEV), however, a correlation between the ability of an anti-NS1 monoclonal antibody to mediate cytolysis of infected cell and to protect mice against virus challenge has not been shown (Despres et al., 1991a; Henchal et al., 1988; Phillpotts et al., 1987; Putnak and Schlesinger, 1990). In order to study the importance of complement mediated lysis directed by anti-NS1 antibodies in the protection of mice against TBEV, a congenic strain of B10 D2 mice unable to express the complement C5 receptor on the cell surface was employed in a protection study.

It was necessary to perform a preliminary experiment to establish the LD_{50} of TBEV for both the C5 deficient (OSN) and sufficient (NSN) B10 D2 mice. This was established using the protocol described in section 2.37. Groups of 10 OSN and 10 NSN mice were challenged with 200µl virus 10^{-3}, 10^{-4}, 1^{0-5}, 10^{-6} and 10^{-7} in PBS and observed. Mice from both strains began to died on day eight post challenge, and all of the mice which succumbed to virus challenge had died by day 18. All NSN mice inoculated with virus diluted to 10^{-4} and 10^{-5} died, and of the 10 mice NSN mice which survived TBEV challenge, one had been inoculated with virus diluted at 10^{-3}, three inoculated with virus at 10^{-6} and six mice with virus diluted to 10^{-7}. Nine OSN mice survived challenge with TBEV. Two OSN mice challenged with virus diluted to 10^{-3} survived as did one mouse challenged with virus diluted to 10^{-6} and six mice challenged with virus diluted to 10^{-7}. All the OSN mice inoculated with TBEV diluted to 10^{-4} or 10^{-5} died. Since both strains of mice appeared to be equally susceptible to the virus challenge permitting a similar amount of virus to be used as the challenge doses in the actual experiment.

For the experiment 36 OSN mice and 34 NSN mice were immunized with RAd51 as in the prescribed schedule (Table 3.3). In addition 11 OSN and 11 NSN mice were immunized with RAd35 and eight OSN and eight NSN mice inoculated with PBS. 30 mice from each group immunized with RAd51 were challenged with 10 LD_{50} of Neudörfl strain of TBEV as were eight RAd35 immunized mice and four PBS immunized mice. The remaining mice in each group were challenged with PBS.

No mouse died from the effect of immunization with RAd51, RAd35 or PBS. All mice immunized with the control virus RAd35 succumbed to TBEV challenge, while one mouse immunized with PBS survived TBEV challenge. The survival of the PBS-immunized mouse is not unexpected

Table 3.3. Protection Afforded by RAd51 Against TBEV Challenge in Mice Deficient in the Terminal Lytic Pathway of the Complement Cascade:

	Immunization	TBEV Challenge Dose (LD_{50})	Mice Survived/ Total N° (%)
NSN	RAd51	10	15/30 (50)
OSN	RAd51	10	18/32 (56)
NSN	RAd51	0 (PBS)	3/3 (100)
OSN	RAd51	0	4/4 (100)
NSN	RAd35	10	0/7 (0)
OSN	RAd35	10	0/8 (0)
NSN	RAd35	0	3/3 (100)
OSN	RAd35	0	4/4 (100)
NSN	PBS	10	0/4 (0)
OSN	PBS	10	1/4 (25)
NSN	PBS	0	4/4 (100)
OSN	PBS	0	4/4 (100)

KEY
NSN B10 D2 mice sufficient in the complement C5 receptor.
OSN B10 D2 mice deficient in the complement C5 receptor.

since not all mice receiving TBEV challenge in the LD_{50} analysis died. With the exception of the OSN mouse which survived TBEV challenge following immunization with PBS, results obtained from the OSN and NSN control groups were identical. Although 56% of the OSN mice immunized with RAd51 survived TBEV challenge, compared to 50% of the NSN mice, using the Exact Fisher Test significant protection was found to have been obtained in both groups when compared with the mice immunized with Rad35 ($P<0.1$). The terminal lytic pathway of the complement cascade does not appear to be necessary for the protection elicited by NS1.

3.3.7. Protection Elicited by RAd51 Treated With UV-irradiated and/or Gradient Purified:

To determine whether the protection observed in mice was elicited by the NS1 antigen contaminating the RAd51 inoculum or by de novo synthesised RAd51-encoded NS1, mice were immunized with RAd51 that had been purified on CsCl gradients and/or irradiated. By either removing contaminating NS1 or inactivating recombinant adenovirus expressing NS1 in the inoculum the role of each component in the protection of mice against lethal challenge with TBEV could be assessed. Virus was extracted with Arklone-P from monolayers of 293 cell infected with RAd51 at 1 pfu/cell. The virus extracts were divided into two, one portion being stored at -70°C while the remainder was purified from NS1 through two CsCl gradients as described in section 2.8. Following dialysis the 'purified' virus was stored at -70°C. Both the purified and unpurified virus stocks were titrated in 293 cells.

Virus was Arklone-P extracted from monolayers of 293 cell infected with RAd51 at 1 pfu/cell. The virus extracts were divided into two, one portion being stored at -70°C while the remainder was purified

Table 3.4. The Titre of RAd51 Following Shortwave UV Irradiation:

Source of RAd51	UV Exposure (mJ/mm^2)	Titre of RAd51 pfu/ml
Infected cell lysates	0	10^7
	0.9	$4x10^4$
	1.8	$2x10^2$
	4.5	0
	9.0	0
	13.5	0
CsCl purified infected cell lysates	0	10^7
	0.9	$2x10^6$
	1.8	$1x10^2$
	4.5	0
	9.0	0
	13.5	0

Table 3.5. The ability of the CMV IE Promoter to Drive Expression Following Shortwave UV Irradiation

Source of RAd51	UV Exposure (mJ/mm^2)	% of Cell Monolayer Infected
Infected cell lysates	0	100
	1.062	25
	3.124	5
	5.31	0
	10.62	0
	21.24	0
CsCl purified infected cell lysates	0	100
	1.062	75
	3.124	25
	5.31	0
	10.62	0
	21.24	0

from NS1 through two CsCl gradients as described in section 2.8. Following dialysis the 'purified' virus was stored at -70°C. Both the purified and unpurified virus stocks were titrated in 293 cells. As described in section 2.32, 5 ml aliquots of the purified and unpurified virus stocks diluted to 10^7 pfu/ml were treated in parallel with UV light at 1.5 mW/cm^2 for 0 sec, 60 sec (0.9 mJ/mm^2), 120 sec (1.8 mJ/mm^2), 5 min (4.5 mJ/mm^2), 10 min (9.0 mJ/mm^2) and 15 min (13.5 mJ/mm^2). The irradiated virus was then stored at -70°C. Virus samples not exposed to irradiation were titrated and shown to have maintained an infectivity of 10^7 pfu/ml. Each irradiated sample was assayed for adenovirus infectivity by titration in 293 cells and for expression from the CMV IE promoter by immunofluorescence staining. Virus samples receiving 4.5 mJ/mm^2 or more were unable to induce plaque formation (Table 3.4). Expression of NS1 was assayed 24 hpi by immunofluorescence using the anti-NS1 antibody T12. In no cell monolayer infected with virus exposed to 4.5 mJ/mm^2 of UV light or more could NS1 expression be detected (Table 3.5). Virus stock in which expression from the CMV IE promoter and plaque formation had been inhibited by the minimum amount to irradiation (4.5 mJ/mm^2) in an attempt to minimise any denaturing effect on the inoculum of prolonged UV treatment.

Balb/C mice were divided into three groups of 12 mice, one of 11 mice and a group of 6 mice. Each mouse was immunized according to the prescribed schedule (section 2.38). The mice in one of the groups of 12 receive CsCl purified RAD51, while a second group of 12 mice received the irradiated infected cell lysate and the third group UV irradiated, CsCl purified RAd51. The mice in the group of 11 received untreated RAd51-infected cell lysate and the mice in the group of six were inoculated with PBS (Table 3.6). One week following the third

Table 3.6. Capability of Treated RAd51 to Protect Mice Against Lethal TBEV Challenge:

Immunization (treatment)	TBEV Challenge Dose (LD_{50})	Mice Survived/ Total N° (%)	Average Survival Time (days)
RAd51	10	5/8 (62.5)	17
	0 (PBS)	3/3 (100)	-
RAd51 #	10	7/8 (82.5)	17
	0	4/4 (100)	-
RAd51 *	10	0/8 (0)	13
	0	4/4 (100)	-
RAd51 #*	10	0/8 (0)	11
	0	4/4 (100)	-
PBS	10	0/4 (0)	12
	0	2/2 (100)	-

KEY

\# Virus had been purified on a CsCl gradient prior to inoculation

* Virus had been subjected to 5.31 mJ/mm^2 UV radiation prior to inoculation.

inoculation of the immunization schedule, eight mice from each of the RAd51 groups and six from the PBS inoculated group were challenged subcutaneously with 10 LD_{50} of the TBEV Neudörfl strain. The remaining mice were challenged with PBS.

In addition to those mice given PBS prior to challenge, the mice given UV-treated RAd51, whether CsCl purified or not, failed to survive the virus challenge (Table 3.6). The average survival time of the mice in the 'unprotected' groups was 12 days. The majority of mice receiving non-irradiated RAd51, CsCl purified or not, survived the lethal challenge. The average survival rate of the mice receiving non-irradiated RAd51 prior to challenge (17 days) was not significantly different from those mice challenged with either 100 LD_{50} or 1000 LD_{50} (16 days) in the original protection study immunized with RAd51. It is notable that while 62.5 % of the mice receiving RAd51-infected lysates were protected against TBEV challenge, 82.5% were protected when the virus had been purified on a CsCl gradient prior to inoculation, the significance of this result, however, is unclear as the number of mice used in this study was too small to be of statistical value.

The presence of contaminating NS1 in the RAd51 virus lysate injected into mice is not responsible for the protection of the mice against lethal TBEV challenge. In the defective adenovirus expression system used throughout this work, protection of mice from a lethal virus infection appears to be afforded by de novo synthesis of NS1.

CHAPTER 4. DISCUSSION.

4.1. Discussion.

Expression of TBEV NS1 was dependent on correctly identifying its gene sequence and associated processing signals within the long ORF. A cDNA fragment, encoding the NS1 gene with additional sequence from the upstream E gene and downstream NS2a gene, was originally generated by PCR using reverse transcripts of the TBEV genome as templates. Initially, these reactions generated a truncated cDNA fragment with a 0.9kb deletion which map to a region with potential to encode secondary structure in the corresponding RNA molecule. In order to efficiently generate full-size cDNA fragments it was necessary to denature this structure by treating the RNA molecule with DMSO prior to reverse transcription. Since TBEV genomic RNA contained a 24 bp inverted repeat with the potential to form a hairpin structure it was assumed that this structure was preventing primer binding in that region. This structure may play a role in either RNA synthesis or transcription of the TBEV genome. Its location at the beginning of the non-structural proteins is intriguing since in this position the structure has the potential to downregulate expression of the TBEV non-structural proteins.

Except for a translationally silent change from T to A at base 3265, the sequence of the cloned Neudörfl NS1 gene was found to be in agreement with published results (Mandl et al., 1989a). The amino acid sequence of K23 encoded NS1, as derived from its nucleotide sequence, showed a high degree of homology with the Neudörfl strain. Furthermore, the position of the three potential N-linked glycosylation motifs and all 12 cysteines residues identified in the NS1 sequence of TBEV strain K23 were absolutely conserved with those of the Neudörfl strain, 11 of the cysteine residues being further

conserved with those in NS1 of all flaviviruses so far sequenced. Since cysteine residues are involved in the formation of di-sulphide bonds their extensive spatial conservation implies a common tertiary structure which may be important in the functioning of NS1.

With a view to determining the NS1 coding region, 5 additional cDNA fragments (Fig. 3.4) were generated by PCR using the original TBEV clone as a template. Each TBEV cDNA fragment was cloned under the control of the CMV IE promoter and their ability to encode NS1 tested in DNA transfection experiments. Of the plasmids tested, only the two were found to synthesise NS1. Since these two cDNA were the only ones to contain just the putative translocation signal sequence upstream of the NS1 gene it was concluded the expression of recombinant NS1 is dependent on this signal. What could not be determined by this work, however, was whether the arginine proposed to act as a translocation stop signal, interrupts translocation of E into the ER and therefore necessitate the use of this translocation signal in the TBEV translation product.

In the absence of a translocation signal, expression of NS1 could not be detected. If NS1 is not translocated it is possible that the protein was degraded in the cytosol by proteases or not recognised by anti-NS1 antibodies. Why NS1 could not be detected in cells transfected with the NS1 gene preceded by translocation signal sequence and additional sequence from E, was not clear. Since translocation of NS1 within the primary translocation product is directed by an internal signal sequence and it has been reported that internalisation of a signal sequence does not interfere with its functioning, this result was unexpected (Bangs et al., 1986; Rottier et al., 1987). It has been reported however that for the signal to function an alpha helix formed by the signal's central and hydrophobic

domains must be maintained (Emr and Silhavy, 1983). It may be therefore, that the additional amino acids from E had a destabilizing effect on this structure, inactivating the signal. Alternatively, the E:NS1 cleavage site may have been masked by the additional E sequence. Inhibition of this cleavage could, therefore , have generated an inappropriately processed NS1 fusion protein which was not detected.

Each of the two cDNA fragment capable of inducing the synthesis of NS1 had a translational stop codon specifically inserted. In pMV45 (from which RAd51 was derived) the stop codon had been placed at the proposed C-terminal of NS1, while in the pMV54, the stop codon followed 79% of the coding sequence for NS2a. With pMV54 recombinant TBEV NS1 was expressed with 79% of NS2a, for which it appeared that appropriate cleavage of the NS1:NS2a junction had occurred. Although this result does not implicate NS2a in this cleavage, it is consistent with reports that NS1:NS2a cleavage is inhibited when NS1 is expressed in the presence of less than 75% of NS2a (Falgout et al., 1989; Falgout and Lai, 1990). Since there was no apparent difference in the level of expression of NS1 or the distribution of NS1 specific immunofluorescence when co-expressed with part of NS2a, the requirement for NS1:NS2a cleavage was assumed to have been circumvented in the shorter fragment by the stop signal. This strategy therefore permitted the expression of NS1 without NS2a and has also been used in the expression of JEV and YFV NS1 (Despres et al 1991; Fan and Mason, 1990). It was decided to use the cDNA fragment containing just the NS1 coding region rather than that with additional NS2a sequence for insertion into a recombinant adenovirus, since to express NS2a may have interfered with the interpretation of data obtained from in vitro and in vivo experiment.

A detailed comparison between NS1 encoded by TBEV and by the recombinant adenovirus RAd51 demonstrated that both NS1 antigens were indistinguishable in (i) apparent molecular weight by SDS PAGE, (ii) ability to recognise a number of anti-NS1 monoclonal antibodies, (iii) glycosylation pattern, (iv) ability to form high molecular weight complexes and (v) ability to be secreted from the cell. Furthermore, as both the intra- and extra- cellular NS1 antigens encoded by RAd51 co-migrated with their TBEV encoded counterparts, the ends of the NS1 gene were assumed be either identical or extremely close to the coding sequence of the wild type TBEV NS1.

Endoglycosidase treatment demonstrated the presence of N-linked mannose rich (or hybrid) oligosaccharide side chains on both the recombinant and TBEV encoded intra-cellular forms of NS1. Whether all three potential glycosylation sites identified in the primary sequence of TBEV NS1 were glycosylated is not yet known. For the mosquito-borne flaviviruses dengue, JEV and YFV only two N-linked glycosylation motifs have been identified in the primary sequence of NS1 both of which are known to be used (Despres et al., 1991; Falgout et al., 1989; Mason, 1989; Post et al., 1990; Winkler et al 1988).

The larger extra-cellular forms of NS1 (both TBEV and RAd51 encoded) were found to contain at least one mannose rich (or hybrid) and one complex side chain. Modification of a mannose rich oligosaccharide side chain to a complex type is consistent with maturation of intra-cellular NS1 to the extra-cellular form occuring in the median Golgi prior to secretion (Dunphy 1985 cell 40 463-72). This proposal was further supported by evidence which showed the co-migration of the de-glycosylated backbone of intra- and extra-cellular NS1 (both recombinant and authentic).

A pattern of glycosylation similar to that of extra-cellular TBEV NS1 has been reported for dengue virus and JEV NS1, although both side chains of the YFV NS1 appear to be modified. The difference in the glycosylation patterns of the extra-cellular forms of NS1 encoded by YFV compared to the other flaviviruses so far investigated probably reflects divergent amino acid sequences rather than the cell type in which they were propagated since both the JEV and dengue virus studies were performed in Vero cells (Despres et al., 1991; Mason, 1989; Post et al., 1990).

The significance of the TBEV NS1 oligosaccharide side chains is unknown. The presence of side chains is not thought, however, to influence processing or transportation of NS1 through the cell as it has been reported that YFV, JEV and dengue virus encoded NS1 is processed by, and secreted from, tunicamycin treated cells (Despres et al., 1991; Mason, 1989; Winkler et al., 1989). The apparent absence of a role for NS1 glycosylation in the transportation of NS1 is consistent with a more general proposal that in tissue culture, at least, oligosaccharide side chains do not signal processing or transportation of proteins (Keller and Swank, 1978; Olden et al., 1978; Weiland et al., 1987).

The study reported here clearly demonstrated that the intra-cellular and extra-cellular forms of NS1, from both TBEV- and RAd51-infected cells, participated in the formation of higher molecular weight complexes which survived non-reducing SDS-PAGE but not boiling in SDS. In view of the fact that the high level of expression from the recombinant NS1 may be expected to saturate an interaction with a specific cellular component, it is proposed that these higher-molecular-weight complexes represent NS1 homodimers. TBEV NS1

homodimers have also been identified in Powassan virus infected cells, although, like TBEV NS1 dimers, characterisation of these dimers is awaited (Winkle et al., 1988).

The apparent absence of monomeric NS1 in RAd51-infected cells was unexpected. If, however, dimerisation is a concentration dependent process then dimerisation of NS1 in RAd51 infected cells is probably rapid. Alternatively it is possible that dimerisation of NS1 in TBEV infected cells is delayed by some requirement of virion maturation. The significance of TBEV NS1 dimers, and whether they are mature functional complexes in themselves or are, precursor of a homodimers, has also to be determined.

The ease with which intra-cellular TBEV NS1 was identified in cells suggests that it is allowed to accumulate in the cell. While mechanisms exist for the retention of specific proteins within cells, it unlikely than NS1 is actively retained as no known signal sequences, such as the KDEL signal, could be identified in the primary sequence of either the Neudorfl or K23 strains of TBEV (Munro and Pelham, 1986). Specific retention of NS1 prior to the median Golgi can not, however, be ruled out. Alternatively, accumulation of NS1 within the cell may be determined by some aspect of its own assembly or role. Accumulation of NS1 prior to the median Golgi is consistent with the proposal that NS1 has a role in virion assembly within the ER or early Golgi complex(Hase et al., 1987 & 1990; Ishak et al., 1988; Leary and Blair, 1980; Ng, 1987).

After leaving the ER, NS1 is assumed to be transported to the Golgi complex. Proteins move sequentially through the Golgi complex to the trans-Golgi network (TGN) where they are sorted into either the membrane, secretory or lysosome traffic (Griffiths and Simons, 1986). As in mammalian cells, the oligosaccharide side chains of NS1 are

trimmed in the Golgi complex of insect and probably arthropod cells. In insect/arthropod cell, however, the final stages of glycan modification associated with mammalian cells, are not completed (Jarvis and Summers 1899). While this altered processing appeared to have little effect on the cellular transportation and properties of YFV NS1, NS1 secretion from insect/arthropod cells was inhibited (Depres et al., 1991; Mason, 1989). Whether this inhibition of secretion resulted from glycan modification or not remains unclear Differences in the way proteins are processed by insect/arthropod cell compared to mammalian cell are important and of particular relevance to arboviruses. The ability of an arbovirus to encode a protein which is processed differently in the arthropod vector compared to the mammalian host has implications in aspects of arbovirology including the etiology of arbovirus disease.

An undefined screening event within the TGN determines whether proteins destined for the cell surface are constitutively delivered or enter the regulated exocytic pathway (reviewed Burgess and Kelly, 1987; Griffiths and Simons, 1986; Gumbiner and Kelly, 1982). While passage along the constitutive secretory pathway is non-specific, the passage of proteins via regulated pathway is receptor mediated (clathrin coated vesicles) and involves the concentration of proteins in acidified secretory vesicle. Since the larger, extra-cellular form of NS1 does not appear to accumulate in either TBEV or RAd51-infected cells it is unlikely that NS1 is secreted via the regulatory pathway. The presence of NS1 in the regulated pathway could be determined by demonstrating a build up of recombinant NS1 in exocytotic vesicles using immuno-electronmicroscopy or by studying the effect of acidotropic drugs on NS1 secretion. A demonstration of NS1 egress via

the constitutive pathway would be intriguing since it would suggest that NS1 plays no role in those stages of virion maturation occurring in acidified vesicles.

It is unclear whether, on reaching the cell surface, NS1 associates with the membrane directly or reattaches following secretion. Since NS1 expressed in insect cells appears not to be secreted but remains located at the cell surface, surface expression of NS1 appears not to require reattachment (Despres et al., 1991). By using two different fluorochromes one conjugated to anti-TBEV E monoclonal antibodies and the other to anti-TBEV NS1 monoclonal antibodies in immuno-fluroescence studies it could be shown whether cells not infected with TBEV display NS1 on their surface.

If NS1 was found on the surface of uninfected cells, it would implicate the involvement of constitutively expressed cellular ligands in this association. This would be an interesting result as a mechanism by which NS1 associates with the cell surface has yet to be determined. Several potential mechanisms for NS1:cell association were considered briefly in this study. Sequence analysis of the NS1 gene from the Neudörfl and K23 TBEV strains revealed neither regions of hydrophobicity or homology with phophatidylinostol (PI) anchors (Furguson and Williams, 1988). It has recently been demonstrated directly that PI linkers are not involved in YFV NS1:cell surface interaction since YFV NS1 was found to be insensitive to phospholipase C (Post et al., 1990). The potential for NS1 oligosaccharide chains to direct cell association also appears unlikely since baculovirus expressed YFV NS1 was located to the cell surface (Despres et al., 1991). One positive note in the ongoing investigations into NS1:cell surface interaction is work which has suggested that the dimeric form of NS1 is less hydrophilic than the monomeric form, and thus the

process of dimerisation may lead NS1 to become membrane associated (Winkler et al., 1989). Consistent with this proposals is the demonstration that NS1 dimers are present on the cell surface (Schlesinger et al 1990).

Since recombinant NS1 was found to be indistinguishable from the authentic protein in glycosylation, folding, assembly, transportation and secretion, it is proposed that all the signals required for these processes are contained within the NS1 gene identified in this study. Moreover, it was assumed that cellular processing of the authentic and recombinant proteins were fundamentally the same. It should be noted however, that appropriate processing and functioning of NS1 during TBEV infections may have requirements additional to those of the recombinant protein.

In addition to investigations into the TBEV NS1 gene and its encoded product, this study has demonstrated the potential of a novel adenovirus vector/CMV IE promoter expression vector. This vector is based on a mammalian system and therefore was expected to process NS1 in a manner similar to that seen in a clinical TBEV infection. Expression of TBEV NS1 was not undertaken using an insect based vector as it was felt that altered processing of glycoproteins associated with baculovirus may affect protection elicited by NS1. This concern appears to have been borne-out recently when it was reported that immunization with recombinant baculovirus encoded YFV NS1 failed to protect mice against a lethal virus challenge (Despres et al., 1991a). The major advantage of a baculovirus system over available mammalian expression system is its potential to express high levels of the recombinant protein. During the course of this study, however, it became apparent that recombinant NS1 encoded by RAd51 constituted the major protein in infected cells. This unexpectedly high level of NS1

expression appears to be a property of the adenovirus vector system since similar high levels of expression of ß-gal have recently been achieved with this vector (Wilkinson and Akrigg, 1992).

A significant advantage that the adenovirus system presented here has over comparable viral vectors, is the absence of vector-encoded proteins expression in non-permissive cells. The absence of adenovirus encoded proteins not only enabled the identification and purification of NS1 to be undertaken easily and efficiently but also allowed for a more accurate interpretation of data, particularly that generated in immunological studies. Of particular importance to the animal studies was that infection with E1- recombinant adenovirus induced no discernable effects in the mice. The inability of the defective adenovirus to replicate in non-permissive cells also provides a degree of biological containment. In summary, therefore, the data presented here has indicated that the novel defective adenovirus vector system used in this study has distinct advantages over both mammalian and baculovirus expression systems.

The data presented in this thesis also demonstrated for the first time that the NS1 antigen could elicit an immune response sufficient to protect mice against either a tick-borne flavivirus or an encephalitic flavivirus. Furthermore, this is the first report of protection being elicited using a cloned cDNA fragment containing only the NS1 gene sequence, thereby providing definitive evidence that flavivirus NS1 free of other virus encoded proteins is able to elicit a protective immune response.

Since immunization of RAd51 appeared to protect mice against death it was assumed the infection of the target organ (brain) had been inhibited. In addition to demonstrating that infectious virus could not be recovered from brains of mice challenged with TBEV following

RAd51 immunization, this study further demonstrated protection against viremia. Similar results have been reported for mice challenged with the Hypr strain of TBEV following immunization with formalin inactivated virus (Malkova, 1960a; Malkova and Kolman 1964). The capacity of NS1 to inhibit disease seems therefore to be similar to the capacity of the whole virus.

Although not tested, it was expected that RAd51 immunization would not inhibit replication of TBEV immediately following immunization. This prediction was based on reports in which the sera from mice protected against lethal challenge, following immunization with YFV and dengue virus NS1 antigens, contained antibodies capable of neutralizing the challenge virus Schlesinger et al., 1985, 1986 & 1987). The prediction is further supported by reports of infectious virus being recovered from regional lymph nodes (only) close to the site of virus injection in mice immunized against the Hypr strain of TBEV (Malkova, 1960; Malkova and Frankova, 1959). Although, immunization with RAd51 protects mice against a viremia and death, one would not expect protection against infection since it is unlikely that any vaccine totally protects against infection. Although a non-specific immune response based on natural killer T cells and cytokines is elicited at the time of infection, the activation of a B- and memory based T-cell immunity is not so immediate.

Since protection against death has been observed following immunization with YFV or dengue virus purified NS1 or anti-NS1 monoclonal antibodies, it is presumed that the anti-NS1 humoral response (in association with MHC class II restricted T cells) is sufficient to protect mice and monkeys against lethal virus challenge (Cane and Gould, 1988; Gould et al., 1986; Henchal et al., 1986; Schlesinger et al., 1985, 1986 & 1987), although the importance of a

humoral based immunity in protection elicited with RAd51 was not defined a strong antibody response to NS1 was clearly elicited. The Importance of a humeral response to RAd51 in protect mice could be assessed by investigating whether anti-serum from RAd51-immunized mice could protedct non-immune mice against virus challenge. Although protection of mice by the passive transfer of anti-serum raised to a vaccinia virus vector expressing dengue virus NS1 and NS2a has been reported, the precise role of NS1 was unclear since either NS2a or vaccinia virus encoded protein may have accounted for the protective response (Falgout et al., 1990). This result does, however, demonstrate the importance of the adenovirus expression system used here, in immunological studies, as the absence of vector encoded proteins ensured that protection is afforded solely by antibodies raised to recombinant protein.

If anti-NS1 antibodies were responsible for protection obtained in RAd51 immunized mice, the mechanism by which virus is cleared may be similar to that activated in mice protected following immunization with YFV or dengue virus NS1 antigens. Neutralization of a TBEV infection in vivo by anti-NS1 antibodies is not generally considered a mechanism of protection, since NS1 appears not to form part of the TBEV virion. The absence of antibodies in serum raised to RAd51 capable of neutralizing TBEV, was confirmed in this study by plaque reduction neutralisation assay. CMC has been suggested as a mechanism by which NS1 antibodies are able to elicit protection (Schlesinger et al., 1985, 1986 & 1987). This study reports, however, that CMC appears to play, at the most, only a minor role in the protection of mice. The implication that mechanism/s other that CMC are important in the NS1 induced immunity, is consistent with reports of anti- TBEV, YFV and JEV NS1 antibodies capable of protecting mice against lethal

virus challenge but unable to direct CMC in vitro (Despres et al., 1991a; Henchal et al.,1988; Phillpotts et al., 1987; Putnak and Schlesinger, 1990). It is possible, however, that CMC could have played a minor role in the protection against TBEV challenge since a correlation between the ability of individual YFV and dengue virus antibodies to protect mice against lethal virus challenge and to direct CMC when assayed in vitro has been reported (Schlesinger et al., 1985, 1986 & 1987). To determine the precise extent to which CMC is involved in the humoral based protective response elicited following NS1 immunization, the protection experiment using C5 deficient mice could be repeated but instead of monitoring death, the ability of the challenge virus to disseminated throught complement deficient animals could be assessed.

A third potential mechanism of virus clearance is by antibody dependent cell cytolysis (ADCC). ADCC occurs when antibodies capable of directed phagocytic cells against cells expressing the NS1 antigen, are elicited. While it is possible to assay for the ability of antibodies to direct complement mediated cytolysis of infected cells, the ability of antibodies to direct ADCC could be assayed in vitro, It should be noted however that activity in vitro is not necessary an indication as to its role in vitro.

Although a humeral based immune response does appear to be essential for the clearence of some viral infections, such as measles and Herpes simplex, it is possible that protection elicited by NS1 could involve a CTL response. Evidence for an anti-NS1 CTL response is consistent with reports of an anti-NS1 CTL epitope having been mapped in dengue virus immune individuals and least one murine T cell epitope which maps to NS1 (Kurane et al., 1990; Rothman et al., 1990). Since the raising of an MHC class I CTL response requires a de novo synthesised

antigens (reviewed Braciale and Braciale, 1991), it was presumed that immunization with RAd51 would elicit an MHC class I restricted CTL response as RAd51 and de novo synthesis of NS1 had been demonstrated. Activation of a CTL response could be demonstrated by the transfer of MHC class I restricted CTLs from mice immunzed with RAd51 into non-immune mice prior to challenge (adoptive treanfer). Since survival of mice into which cell has been transfered would implicate a CTL response in the protection.

It is not known whether TBEV NS1 is an essential gene for virus replication. If non-essential then incomplete protection following vaccination with RAd51 may have resulted from immunization positively selecting for TBEV NS1 deletion mutants capable of evading immune surveillance. Virus recovered from mice immunized with NS1 but had succumbed to TBEV challenge was therefore tested and shown to be recognised by several anti-NS1 monoclonal antibodies, discrediting this hypothesis.

Since the potential of the recombinant adenovirus vector used in this study to elicit an MHC class I restricted CTL response has not been investigated, the possibility that it is inappropriate for use in this type of study must be considered. Both the E1 and E3 gene products of adenovirus are able to downregulate an MHC class I CTL response (reviewed Wold and Gooding, 1991). It might be, therefore, that breakthrough expression of these gene products has caused a downregulation of the CTL response. This is unlikely, however, as the adenovirus vector reported here has deletions in both genes. Alternatively, the protective immunity elicited may have been downregulated as a result of the associated CTL lysis, which, unlike CMC lysis that permeablized the plasma membranes allowing osmotic swelling and release of the cytoplasm, fragments DNA in the nucleus of

the cell under attack prior to apoptosis (Martz and Howell, 1989). This is of importance, probably to adenovirus based vector as the adenovirus genome consists of a DNA molecule which functions in the nucleus. Evidence from Reovirus type 3 infections on the other hand suggests that the CTL DNA fragmentation mechanism does not affect RNA molecules in the cytoplasm and would therefore spare the TBEV genome (Martz and Howell, 1987; Martz and Howell, 1989). This highlight a more general point which must be considered in the used of recombinant virus vector when trying to elicit an MHC class I restricted CTL response. In order to ensure an appropriate MHC class I restricted CTL response, the mechanism of infection used by the virus from which the recombinant protein originated should wherever possible be reflected in the mechanisms used by the vector (reviewed Long and Jacobson, 1989).

This study was able to demonstrate that de novo synthesised NS1 from RAd51 whether by a humoral or cellular response (or both) provided protection against viral challenge. The novel combination of defective adenovirus and strong constitutive CMV IE promoter has not only proved to be an excellent high level expression system for the investigation of proteins in vitro, it has also proved a significant potential for used in vaccine development where a non-replicative system has a distinct advantage over currently available vectors.

Although the NS1 gene sequence has been identified and its product characterised many question remain to be answered before the processing and function of NS1 is fully understood. The recombinant adenovirus RAd51 will facilitate future studies into these question and can be used to investigate more thoroughly the immunological basis for the protection elicited by TBEV NS1. The protection provided by the recombinant TBEV NS1 protein has clear implications for flavivirus

vaccine development programs, since it demonstrates that NS1 can elicit protection without inducing antibodies capable of enhancing subsequent infections.

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