NUCLEOTIDE SEQUENCE ANALYSIS OF THE NS GENES OF WILD-TYPE AND THREE COMPLEMENTATION GROUP E MUTANTS OF VESICULAR STOMATITIS VIRUS NEW JERSEY

by

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SUMMARY

Vesicular stomatitis virus (VSV) is the prototype of the family Rhabdoviridae. There are two serotypes of VSV - Indiana (IND) and New Jersey (NJ) which can be differentiated from one another on the basis of little or no cross neutralisation using antibody raised against the virion glycoprotein. VSV has a genome consisting of a non-segmented single strand of negative sense RNA approximately 11,000 bases in length. The genome codes for five proteins: L (large), M (matrix), NS (non-structural), N (nucleocapsid) and G (glycoprotein). The L, NS and N proteins associate with the genomic RNA and form the ribonucleoprotein core. N protein is the major structural component of the ribonucleoprotein core, the L and NS proteins function in viral transcription and replication; G and M comprise the viral envelope. M protein is believed to be located on the inner side of the envelope and is the group-specific antigen. G is the transmembrane glycoprotein and forms the spikes that project from the virion.

The NS protein of VSV is a multifunctional protein with a proposed role in transcription, replication and virion maturation. It is a phosphoprotein and evidence indicates that the degree of phosphorylation of NS affects its function. On SDS-polyacrylamide gels the NS protein migrates with an apparent molecular weight of 40-50,000. However, the molecular weight calculated from sequence data is approximately 30,000. Three ts mutants of VSV New Jersey, Missouri strain have been shown to possess lesions in the NS gene and these have been classed in

complementation group E. The mutants (tsE1, tsE2, tsE3) are a heterogenous group. They exhibit a variety of phenotypes and on SDS-polyacrylamide gels the NS proteins of these mutants show a differential migration pattern both in relation to wild-type virus and to one another. It was therefore of interest to determine the sequence of the NS mRNA of wild-type virus and complementation group E mutants in order to precisely map the position of the mutations and perhaps propose a model to explain the differential electrophoretic migration pattern of their NS proteins.

The complete nucleotide sequences of the NS mRNAs of wild-type, complementation group E mutants (tsE1, tsE2, tsE3) and two revertant viruses (tsE1/R1 and tsE3/R1) were determined from cloned full-length cDNAs. cDNA was synthesised using RNA prepared from infected cells as template and NS-specific recombinant plasmids were identified by a combination of Northern blot analysis and mRNA selection/in vitro translation.

The mRNA of wild-type VSV NJ, Missouri strain is 856 nucleotides long excluding poly(A) and encodes a protein of 274 amino acids (molecular weight 31,000). The protein initiates at AUG (position 11-13) and terminates at UAA (position 833-835). The NS protein is highly acidic especially in the amino terminal half of the molecule and has an overall charge of -18.5 at pH7.0. There are 46 potential phosphorylation sites (13 threonines, 33 serines) in the NS protein.

The sequence of the NS gene of wild-type VSV NJ, Missouri strain was compared with the sequences of the NS genes of three other strains of VSV; together the four

sequences represent two strains from each of the two VSV serotypes. Comparison showed that between serotypes there is approximately 50% nucleotide and 33% amino acid homology. Within the IND serotype there is 97% nucleotide and 95% amino acid homology; within the NJ serotype there is 85% nucleotide and 90% amino acid homology. Two regions are highly conserved between the four proteins, in particular the carboxy terminus 21 amino acids exhibit 90% homology when conservative changes are taken into consideration. The hydropathic profiles of the four proteins are similar and the proteins are predominantly hydrophilic.

In total there are 18 potential phosphorylation sites conserved bewteen the four proteins. Hsu and Kingsbury (1985) identified five amino acids between residues 35 and 78 of the NS protein of VSV IND San-Juan strain as the constitutively phosphorylated residues of the NS protein. They proposed that phosphorylation of these residues was necessary for a basal level of NS activity. These same five residues were found to be strictly conserved between the four strains of VSV.

Analysis of the sequences of cloned cDNAs of the NS mRNAs of the tsE1, tsE2 and tsE3 viruses showed that tsE1 and tsE3 each have a single mutation whereas tsE2 has a double mutation. Three of the four mutations occur in a region of 18 nucleotides (bases 92-109) indicating a possible 'hot-spot' of mutation. Interestingly, this region encodes the most hydrophilic portion of the NS protein.

None of the mutations generate a stop codon. The NS mRNAs of the three complementation group E mutants are the same length as that of the wild-type virus and encode a

protein of the same size. All of the mutations however change the charge of the amino acid they affect. In tsE1 a glutamic acid residue is changed to a lysine; in tsE2 an aspartic acid residue is changed to a glycine and a lysine residue is changed to a glutamic acid; in tsE3 a glutamic acid residue is changed to a glycine.

Secondary structure predictions of the NS proteins of wild-type and complementation group E mutants by the method of Chou and Fasman (1978) predict substantial alterations to secondary structure in the area of the mutations in all three mutants.

A number of revertants of tsE1 and tsE3 have been isolated but none have been obtained from tsE2 which has a double mutation. The NS mRNAs of two revertant viruses - one of tsE1 (tsE1/R1) and one of tsE3 (tsE3/R1) were cloned and sequenced. It was found that at the site of mutation in tsE1 and tsE3 the revertant sequence corresponded to that of the wild-type. However, there were two further changes to the nucleotide sequence. The first change, at position 472, did not affect an amino acid; the second, at position 513, caused a methionine to arginine change. This is an example of a charge change (neutral to positive) which does not affect the proteins mobility on SDS-polyacrylamide gels. Furthermore, this change causes a minimal alteration to the predicted secondary structure of the revertant NS protein.

The exploitation of these full-length cloned cDNAs of the NS mRNAs in the study of the function and role of the NS protein is discussed.

ABBREVIATIONS

Abbreviations for buffers and media are given in the Materials and Methods section.

A	adenine
bp	base pair(s)
BHK	baby hamster kidney
C	cytosine
CPE	cytopathic effect
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	any 2'-deoxyribonucleoside-5'-triphosphate (dATP, dCTP, dGTP, dTTP)
ddATP	2',3'-dideoxyadenosine-5'-triphosphate
ddNTP	2',3'-dideoxyribonucleoside-5'-triphosphate
DI	defective interfering particle
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EM	electron microscope
ER	endoplasmic reticulum
5 FU	5-fluorouracil
g	gravity
G	guanine
IND	Indiana
Kb	kilobase
ul	microlitre
ml	millilitre

MIS	Missouri
M	molar
mol wt	molecular weight
M-S	Mudd-Summers
NJ	New Jersey
OGD	Ogden
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming unit
RNA	ribonucleic acid
RNase	ribonulease
RNP	ribonucleoprotein
RPM	revolutions per minute
S-J	San Juan
SDS	sodium dodecyl sulphate
T	thymidine
ts	temperature sensitive
U	uracil
UV	ultra-violet
VSV	vesicular stomatitis virus

Chapter 1

INTRODUCTION

The Rhabdoviridae are a diverse family of enveloped RNA viruses which can infect a wide variety of vertebrates, invertebrates and plants. The family is of importance because many of its members cause disease in man and his domestic animals. The most important member of the family from this point of view is the causative agent of rabies, a disease which is still a great problem in many areas of the world. As a pathogen in man rabies virus has a long history. Democritus is thought to have made the first recorded description of canine rabies some 500 years BC (Steele, 1975). Although the disease in man can now be treated, rabid vampire bats are still responsible for the deaths of hundreds of thousands of cattle annually in the Americas (Steele, 1975). Economically important diseases caused by rhabdoviruses have been well documented over the years. Bovine ephemeral fever virus causes a three to four day illness in cattle which involves sudden fever with temperatures up to 107°F, shivering, stiffness, loss of appetite and lameness which can sometimes be permanent (Heuschele, 1970). Vesicular stomatitis virus (VSV), the prototype rhabdovirus, causes vesicular stomatitis disease which was first reported by Theiler (1901) as a disease of horses and mules in the Transvaal, South Africa. VSV can naturally infect a wide variety of mammals including man, but disease is most common in cattle. The symptoms of VS disease closely resemble those of foot and mouth disease; however, VSV infection is usually milder and the recovery more rapid and complete (Howatson, 1970). The vesicles found on the mouths and hooves of infected animals are usually accompanied by drooling saliva. Foot lesions are

more common in pigs and lesions are also found on the teats and udders of cows. Other symptoms can include elevated temperature, loss of appetite, loss of weight and in cows there is cessation of milk production (Hanson, 1952; Howatson, 1970; Bishop and Smith, 1977). An outbreak of VSV in California's San Joaquim Valley highlights the economic importance of the disease. In a two month period two dairies sustained losses valued at $225,000. These losses amounted to $202/cow in one dairy and $97/cow in the other dairy (Goodger et al, 1985). In humans the disease can be inapparent or resemble influenza, with a sore throat, fever and general malaise lasting several days (Howatson 1970; Andrewes et al, 1978).

Lettuce necrotic yellow virus (in Australia), potato yellow dwarf virus (in North America) and strawberry crinkle virus (in USA, Britain, Australia, South Africa and New Zealand) all produce disease in dicotyledonous plants and have an insect vector (Francki, 1973). There are six rhabdoviruses which infect monocotyledonous plants and five of these cause serious field disease in cereals (Francki, 1973; Francki and Randles, 1980).

As man spreads his commercial interests it seems that members of the Rhabdoviridae will always be able to cause problems. Many members of the family are capable of causing widespread and fatal disease in fish eg. infectious hematopoietic necrosis virus (IHNV), a pathogen of salmon and young trout (Amend, 1975; Pilcher and Fryer, 1978) which is responsible for great losses of young fish at hatcheries in western North America (Groberg, 1983). As the fish farming industry continues to grow rhabdoviruses like IHNV

and the others capable of causing severe disease in fish will obviously be of even greater importance.

However, not all rhabdoviruses cause disease. The most interesting of the rhabdoviruses which are completely innocuous to their host is the sigma virus of Drosophila. Sigma virus is transmitted in the germ lines of male and female Drosophila and confers a sensitivity to CO_2 (L'Heritier, 1958; Prinz, 1973; Teninges et al, 1980). The enhanced CO_2 sensitivity was thought to be a unique property of sigma virus; however, some of the other rhabdoviruses have been found to have this effect on adult insects (Bussereau, 1971, 1972, 1973, 1975; Bussereau et al, 1975; Teninges et al, 1980). Although sigma virus is unable to multiply in mammalian cells Brun (1984) suggested that sigma virus is a rhabdovirus which has become trapped in a non-biting dipteran and in order to ensure its survival has come to behave as a hereditary factor.

As already mentioned VSV is the prototype rhabdovirus. VSV has been more extensively studied than any other member of the family because it is relatively safe to work with; it can grow to a high titre in a number of cell lines and is relatively easily purified; the virion-associated RNA polymerase is a stable enzyme capable of synthesising large quantities of mRNA in vitro (Banerjee et al, 1977). The virus has also become an important tool in answering a variety of problems in molecular biology eg. glycoprotein metabolism and transport (Chatis and Morrison, 1979; Irving et al, 1979; Lingappa et al, 1978; Rothman and Lodish, 1977; Rose et al, 1980; Toneguzzo and Ghosh, 1978; Hubbard and Ivatt, 1981).

After some general discussion of the taxonomy of the Rhabdoviridae the review will be concentrated almost entirely on VSV.

1. TAXONOMY

The Rhabdoviridae are a family of bullet-shaped or bacilliform RNA viruses which have been isolated from vertebrates, invertebrates and plants. These viruses show considerable variation in size, ranging between 130nm and 380nm in length and 60nm to 95nm in width. The viruses that infect vertebrates are generally bullet-shaped and those that infect plants are bacilliform (Brown et al, 1979).

Although a large number of viruses which share the rhabdovirus morphology have been isolated, few have been well enough characterised to be classed as members of the family Rhabdoviridae and are termed 'possible members' (Brown et al, 1979). Viruses are considered as members only when morphologic studies are comprehensive and when viral RNA and proteins have been shown to have the same general characteristics as those of VSV or rabies virus (for vertebrate and invertebrate viruses) or lettuce necrotic yellow virus (for plant viruses) (Brown et al, 1979).

There are 75 members of the family Rhabdoviridae but very few of these are included in the two recognised genera - Lyssavirus and Vesiculovirus (see Table 1.1) (Brown et al, 1978). The genus Lyssavirus contains the rabies-related viruses and the genus Vesiculovirus the VSV-related viruses. The subgroup A plant viruses are four plant viruses that resemble VSV; the subgroup B plant viruses are plant viruses

that resemble rabies virus (Brown et al, 1979).

In general, there is no evidence for relatedness between plant and vertebrate viruses. Indeed there are very few similarities between the individual plant viruses which makes subdivision into genera impossible (Brown et al, 1979).

2. VESICULOVIRUSES

The nine viruses which comprise the genus Vesiculovirus are shown in Table 1.1.

The Indiana (IND) serotype of VSV was isolated in 1925 in Richmond, Indiana from vesicles on the tongues of cattle (Cotton, 1927). The New Jersey (NJ) serotype was isolated in 1926 following an outbreak of the disease in New Jersey. All subsequent isolates have belonged to one or other of these serotypes. The two serotypes are differentiated on the basis of little or no cross-neutralisation using antibody raised against the virion glycoprotein (Cartwright and Brown, 1972; Kelley et al, 1972).

Table 1.2 shows the subdivisions of the IND and NJ serotypes. The IND serotype was divided into the four subtypes after examination of cross-relationships by complement fixing and neutralisation tests (Cartwright and Brown, 1972). The subclassification of the five strains of VSV NJ in two subtypes is based on reciprocal differences in antibody neutralisation of virion infectivity, oligonucleotide maps of virion RNA, base sequence homology and heterologous DI particle-mediated interference (Reichmann et al, 1978). There is evidence that the two

subtypes of VSV NJ differ in their biological properties (Khan and Lazzarini, 1977; Reichmann et al, 1978; Prevec and Kang, 1970). Until recently, for example, the Concan subtype had only been isolated from outbreaks involving horses and cattle while Hazelhurst isolates were from outbreaks involving swine. However, isolates from an outbreak of VSV in Colorado in 1982 were identified as members of the Hazelhurst subtype. These isolates were from horses and black flies (Simuliidae) and were similar but not identical to the swine isolates (Schnitzlein and Reichmann, 1985).

3. MORPHOLOGY

Over the last 20 to 30 years, electron microscopy of negatively stained preparations of VSV have established its basic features (Howatson and Whitmore, 1962; Simpson and Hauser, 1966; Nakai and Howatson, 1968; Cartwright and Brown, 1972; Murphy and Harrison, 1974; Thomas et al, 1985). A model of the virus structure is shown in Figure 1.1 together with an electron microscope picture of a negatively stained virus particle. The virion is cylindrical with a hemispherical cap at one end which gives the particle its characteristic bullet shape. Intact virions are approximately 70nm in diameter and 180nm long (Murphy and Harrison, 1974). They consist of an internal ribonucleoprotein (RNP) core surrounded by a lipid envelope with glycoprotein spikes (6-10nm) projecting from it (Lenard and Compans, 1974; Lenard, 1978; McSharry, 1980). The RNP core is coiled 30 to 35 times to form a helix with almost

the same length as the virion (Newcomb et al, 1982). Four different microscopy procedures were used to calculate the periodicity of the nucleocapsid with the conclusion that it was 3.5-3.7nm (Thomas et al, 1985). Nucleocapsid length was determined by scanning transmission electron microscopy (STEM) measurements of mass per unit length and total mass; contour length of extended negatively stained nucleocapsids; characterisation of the structural repeat along the nucleocapsid strand; calculation of nucleocapsid length from in situ measurements (Thomas et al, 1985). An axial channel is often seen when the particle is penetrated by stain. Some controversy exists as to whether the flattened end is an artefact caused by the preparative technique and the virion is in actual fact bacilliform as are the plant rhabdoviruses (Orenstein et al, 1976).

4. VIRION STRUCTURE AND COMPOSITION

A. Proteins

Wagner et al (1972) introduced a standard nomenclature for VSV proteins in order to clarify the confusion which had arisen through the use of different classification systems in different laboratories. There are five recognised VS virion proteins. These are L (large), NS (non-structural), N (nucleocapsid) which together are associated with the genomic RNA and form the internal RNP core; and G (glycoprotein) and M (matrix) which are associated with the virus outer membrane (Kang and Prevec, 1969; Wagner et al, 1969; Cartwright et al, 1970; Wagner et al, 1970; Bishop and Roy, 1972; Emerson and Wagner, 1972). At present no one has

provided conclusive evidence for the existence of any virus coded protein which is present in infected cells but not in mature virions (Mudd and Summers, 1970; Wagner et al, 1970; Kang and Prevec, 1971; Wagner et al, 1972; Wunner and Pringle, 1972a, 1972b). However, R. Hermann (personal communication, 1985) has suggested that the NS gene encodes a second protein.

Thomas et al (1985) used dark field scanning transmission electron microscopy to perform mass analysis of purified virions of VSV IND, Mudd-Summers strain, pronase-treated virions and nucleocapsids. Their results are summarised in Table 1.3. M protein is the most abundant protein (1,826 copies) in the virion, whereas the G protein makes the largest contribution to its mass (Thomas et al, 1985).

The L protein of VSV IND serotype, Mudd-Summers strain has a molecular weight (mol wt) of 241,012 (Schubert et al, 1984). It is the largest of the VSV proteins but is present in only minor amounts in the virion and is located in the nucleocapsid (Bishop and Roy, 1972; Emerson and Wagner, 1972; Szilagyi and Uryvayev, 1973). The L protein has been shown to form at least part of the transcriptase complex (Naito and Ishihama, 1976; Emerson and Wagner, 1972, 1973; Emerson and Yu, 1975; Szilagyi, 1975; Mellon and Emerson, 1978; Kingsford and Emerson, 1980) and recently Ongradi et al (1985b) suggested that L protein of VSV NJ is the transcriptase itself.

The L mRNA is 6,380 nucleotides long (57.13% of the genome) (Schubert et al, 1984). Sequence data show there to be some short open reading frames in the genome-sense strand

of the L gene with the longest of these capable of encoding a protein of mol wt 10,000. However, there is no evidence for other functional transcripts (Schubert et al, 1984).

The G protein is a major virion protein. The G protein of
VSV IND, San Juan strain has a mol wt of 75,416 (Rose and Gallione, 1981). It is the only glycosylated virus protein and constitutes the spikes which project from the virion envelope giving rise to type specific neutralising antibody (Cartwright and Brown, 1972; Kelley et al, 1972). The G protein appears to be the main target of the antiviral immune system because only antibodies to G protein neutralise virus infectivity (Kelley et al, 1972) and cytotoxic T cells recognise mainly G proteins on the surface of virus infected cells (Zinkernagel et al, 1978). The G protein serves as the virus ligand receptor (Cartwright et al, 1970), mediates cell to cell fusion in vitro (Florkiewicz and Rose, 1984) and B cell mitogenicity (Goodman-Smitkoff et al, 1981).

The G mRNAs of VSV IND and NJ serotypes have both been sequenced (Rose and Gallione, 1981; Gallione and Rose, 1983). They are 1,672 and 1,573 nucleotides long respectively making G mRNA the second longest VSV mRNA.

The N protein is the second major virion protein. Gallione et al (1981) sequenced the N mRNA of VSV IND, San Juan strain and calculated a mol wt of 47,355 for the N protein. The N mRNA is 1,326 nucleotides long (Gallione et al, 1981).

The N Protein is found closely associated with the genomic RNA in virions, and both positive-sense genomic RNA

(so called replicative intermediate) and mRNAs in infected cells (Soria et al, 1974; Grubman and Shafritz, 1977; Rubio et al, 1980; Naeve and Summers, 1980; Adam et al, 1986). It is thought that N protein confers resistance to ribonculease digestion on the viral RNA (Cartwright et al, 1970). The N protein has been shown by complement fixation to function as the group specific antigen (Cartwright and Brown, 1972).

Thomas et al (1985) using STEM proposed that the N protein subunit in the RNP has a wedge-shaped, bilobed structure and this property can explain the characteristic undulating configuration assumed by the nucleocapsid even when the RNA strand is well extended.

The M protein is the third major virion protein. Rose and Gallione (1981) sequenced the M mRNA of VSV IND, San Juan strain. The M mRNA is 831 nucleotides long and encodes a protein of 229 amino acids with a calculated mol wt of 26,064 (Rose and Gallione, 1981).

The M protein is thought to be a peripheral membrane protein. It lines the inner surface of the virion envelope perhaps interacting with the lipid bilayer, the internal portion of G protein and the nucleocapsid core (Wagner, 1975). In addition to its proposed structural role M protein has a role in directing virus budding from infected cells (Knipe et al, 1977). There have been reports that M protein has a regulatory role in virus directed RNA synthesis (Clinton et al, 1978) and functions as an inhibitor of viral transcription in vitro (Carroll and Wagner, 1979; Lombard and Printz-Ane, 1979; De et al, 1982; Pinney and Emerson, 1982; Wilson and Lenard, 1981). The proposed role in RNA synthesis and transcription will be

discussed in more detail later.

The complete nucleotide sequences of cDNA clones of the NS mRNA from VSV IND, San Juan strain and VSV NJ, Ogden strain have been determined (Gallione et al, 1981; Gill and Banerjee, 1985). The NS mRNAs are 822 and 856 nucleotides in length respectively and code for proteins of mol wt 25,000 and 31,000. The NS protein runs anomalously on SDS polyacrylamide gels with a mol wt ranging between 39,000 and 50,000 (Knipe et al, 1977). It is a phosphorylated protein but dephosphorylation decreases the electrophoretic mobility and so this is not responsible for the aberrant mobility (Gallione et al, 1981). Although there is no direct evidence, the likeliest explanation for the aberrant mobility is a deficiency in SDS binding caused by the large proportion of the negatively charged amino acid residues in the NS protein (Gallione et al, 1981).

The NS protein is found associated with the genomic RNA, L and N proteins (Wagner, 1975). It exists in infected cells in a variety of phosphorylated forms and it has been suggested that the extent of phosphorylation regulates transcription (Hsu and Kingsbury, 1980). It is a multifunctional protein as evidenced by the variety of phenotypes exhibited by the complementation group E mutants which have been shown to have ts lesion(s) in the NS gene (Evans et al, 1979). The NS protein is the subject of this thesis and so it will be discussed in more detail later.

B. Lipids

The lipid composition of VSV has been shown to consist predominantly of phospholipids (61%) and neutral lipids

(39%) with a cholesterol-to-phospholipid molar ratio of 0.6 (McSharry and Wagner, 1971). The lipids contribute 21.1% of the total mass of the virion (Thomas et al, 1985). The lipids are derived from the host cell and vary according to the cell type, reflecting the composition of the host cell plasma membrane (McSharry and Wagner, 1971).

C. RNA

The VSV genome consists of one linear, single-stranded molecule of negative sense RNA (Wagner, 1975). The RNA is responsible for 1.4% of the total mass of the virion (Thomas et al, 1985) and is particularly AU rich. The structure and organisation of the genome will be discussed in some detail in the next section.

5. VSV GENOME ORGANISATION

A. General

The basic features of the VSV genome are shown in Figure 1.2. If the sequences of the leader region (Colonno and Banerjee, 1978a,b); L gene of VSV IND, Mudd-Summers strain (Schubert et al, 1984); N, NS, G and M mRNAs of VSV IND, San Juan strain (Gallione et al, 1981; Rose and Gallione, 1981; Rose et al, 1980) are taken together with the sequences of four inter-cistronic junctions (McGeoch, 1979; Schubert et al, 1980; Rose, 1980) and the terminal tail region (Schubert et al, 1980) then some basic facts about the organisation of the VSV genome can be established. It is unfortunate that Schubert et al (1984) chose the L gene of the Mudd-Summers strain of the IND serotype and not

the San Juan strain since most of the sequence data available are from the San Juan strain.

The genome is 11,162 nucleotides in length. Analysis reveals a remarkably simple but efficient use of the genome - 99.37% of the nucleotides are translated into mRNA; 93.88% encodes polypeptide specifying information. The leader RNA and the five genes are arranged in a linear array without overlaps and with the minimum of untranscribed intervening or terminal sequences (McGeoch, 1979; Rowlands, 1979; Keene et al, 1980; Rose, 1980; Schubert et al, 1980). The virion associated RNA-dependent RNA polymerase has been shown by UV-inactivation studies to transcribe the genome in the order 3'-leader-N-NS-M-G-L-5' (Abraham and Banerjee, 1976; Ball and White, 1976; Ball and Wertz, 1981). Villarreal et al (1976) reported that the molar ratios of the mRNA species made in infected cells were N:(NS+M):G:L:100:107:36:5.6 and the five viral proteins were synthesised in almost identical amounts. The partial termination that occurs at or near intercistronic boundaries has been termed attenuation (Iverson and Rose, 1981). There is no firm evidence for the expansion of coding capacity by the use of multiple reading frames, and no consensus RNA splicing signals which might indicate processing of the mRNA are found in the nucleotide sequence.

B. Leader RNA

Plus strand leader RNA is 47-50 nucleotides long and transcribed from the precise 3' end of the genome (Banerjee et al, 1977). Plus strand leader RNA has been implicated in the shut-off of host macromolecular synthesis (Weck and

Wagner, 1978; McGowan and Wagner, 1979; McGowan et al, 1982; Grinnell and Wagner, 1983) but its function in viral replication is unknown. The full-length plus sense RNA synthesised from the genome during VSV replication serves as template not only for the synthesis of additional progeny minus sense genomes (Wagner, 1975) but also for minus strand leader RNA similar in size to the plus strand leader. Leader RNA, like virion RNA, is neither capped, methylated nor polyadenylated (Colonno and Banerjee, 1976, 1978a,b).

It has been proposed that plus and minus strand leader RNAs may function as decision points in the switch from viral transcription to replication (Leppert et al, 1979). The precise mechanism of this switch is unknown but it has been suggested that replication is mediated by the encapsidation of newly replicated viral RNA by N protein (Blumberg et al, 1983; Kingsbury, 1974). Blumberg et al (1983) proposed that the nascent plus and minus strand leader RNAs contain an encapsidation nucleation site which could be represented by a five-times-repeated A residue at every third base from the 5' end (see Figure 1.3). Thomas et al (1985) calculated there were nine nucleotides per N monomer in the RNP and this result is in agreement with the figure reached by P. Taylor (personal communication) who reached the same conclusion by analysing sequence data for preferential periodicity of certain bases or types of base.

The plus strand leader RNA can be divided into three functional domains shown in Figure 1.4 (Kurilla et al, 1982). The highly conserved proximal third is thought to play a role in initiation of RNA synthesis and in nucleation of N protein assembly (see above). The middle third

contains a TATA-like sequence (Rose and Iverson, 1979) and is thought to interact with virion polymerase components (Keene et al, 1981). The 3' proximal third may be involved in leader RNA termination and it is this region that has been proposed to interact with cellular La protein (Wilusz et al, 1983; Kurilla and Keene, 1983). La protein is a 45KD protein which is normally found transiently bound to unprocessed cellular RNAs that are products of RNA polymerase III (Rinke and Steitz, 1982). The various roles of leader RNA will be discussed in greater detail in later sections.

C. Intercistronic Junctions

The sequence 3'-AUAC(U)7-5' has been described at the 5' terminus of each of the five genes of VSV (McGeoch, 1979; Schubert et al, 1980; Rose, 1980). It is thought that the polyadenylation signal is contained within this sequence (McGeoch, 1979; Schubert et al, 1980; Rose, 1980). The sequence is followed by the dinucleotide GA (or CA at the NS:M junction) (Rose and Iverson, 1979; Rose, 1980). The dinucleotide is untranscribed and marks the intercistronic junction.

The intercistronic junctions are followed by sequences with the general form 5'-AACAGNNAUC-3' (N represents a variable nucleotide) which is complementary to the 5' end of the mRNA (McGeoch, 1979; 1980). At the intercistronic junctions there is no indication of potential secondary structure that could account for the phenomenon of attenuation.

D. Gene Products

The five mRNAs transcribed from the VSV genome are capped and methylated at the 5' terminus and polyadenylated at the 3' terminus (Abraham et al, 1975; Rose, 1975; Rose and Knipe, 1975). In this section the processes of capping and methylation and polyadenylation will be discussed.

(i) Capping and Methylation

There is a good deal of evidence indicating the 5' capped and methylated structure is not only necessary for efficient translation of mRNAs but also confers stability on some mRNAs. Both et al (1975) were the first to show this for mRNAs of VSV and reovirus. Later it was shown that capped mRNAs are more efficient at binding to ribosomes to form initiation complexes than their uncapped counterparts (Rose and Lodish, 1976; Lodish and Rose, 1977). It is possible that the cap structure is involved in, or required for, VSV mRNA synthesis because it has been reported that VSV mRNAs synthesised in vitro are always capped (Banerjee et al, 1977).

The terminal cap structures in a number of viruses have been studied. The cap structure of VSV mRNAs contains the α phosphate of the penultimate adenosine residue and the α and β phosphates of GTP, ie. $G^{\alpha\beta}_{pp}/^{\alpha}_{p}A...$ and so they differ from the $G^{\alpha}_{p}/^{\beta\gamma}_{pp}N$ found on the mRNAs of reovirus, vaccinia virus and cytoplsmic polyhedrosis virus (Abraham et al, 1975; Moss et al, 1976; Furuichi and Shatkin, 1977; Furuichi, 1978). The three phosphate bridge structure of VSV mRNAs can arise in two ways. It could be synthesised by a mechanism involving $G^{\alpha\beta\alpha}_{ppp}$ and $5'^{\alpha}_{p}A$ ends from newly

synthesised mRNA or it might be initiated with $_{ppp}$A ends and capping occurs by the removal of the γ phosphate of guanosine and the β, γ phosphates of adenosine (Banerjee, 1980).

Testa and Banerjee (1977) showed that in vitro the methylation of the 5' cap of VSV mRNAs using the methyl group donor S-Adenosyl-L-methylmethionine occurs in the following manner:

GpppA ⟶ GpppA^{m} ⟶ 7mGpppA^{m} to give the dimethylated product RNA. However, VSV mRNAs isolated from infected cells are more extensively methylated, probably by cellular enzymes, and so the cap structures are a heterogenous group usually in the form 7mGppp(m)A^{m}p(m)A$^{(m)}$p where (m) indicates positions of heterogenous non-obligatory methylation (Rose, 1975; Moyer and Banerjee, 1976). Although methylation of the N7 position has been shown to be essential for efficient translation in vitro the function of the other methylations is unknown (Both et al, 1975; Muthukrishnan et al, 1975; Lodish and Rose, 1977). Horikami et al (1984) utilised two host range mutants of VSV, hr1 and hr8, which have a mRNA methylation defect and direct the synthesis in vitro of full-length capped but unmethylated viral mRNAs (Horikami and Moyer, 1982), to test the requirements for mRNA function in mammalian cells under physiological conditions. Methylation of the G residue of the cap was a strict requirement for translation in vivo (Horikami et al, 1984).

(ii) Polyadenylation

VSV mRNAs synthesised in vivo and in vitro are polyadenylated at their 3' termini with stretches of poly(A)

residues ranging between 64 and 200 bases in length (Banerjee et al, 1974; Rose and Knipe, 1975). Studies of the products of in vitro VSV transcription have shown there to be a small fraction of polycistronic viral transcripts - two or more messages encoded by adjacent genes connected by tracts of 200 polyadenylate residues plus the dinucleotide encoded by the intergenic junction (Herman et al, 1978;1980; Rose et al, 1977). A further in vivo study by Schubert and Lazzarini (1981) demonstrated that, at the same frequency, readthrough even occurs distal to the L gene giving rise to L mRNA linked to a 59 base sequence which is complementary to the extreme 5' end of the genome.

Examination of sequence data shows that the long poly(A) stretches are not encoded by the genome. This has led to the proposal that polyadenylation occurs by a 'chattering' mechanism at a sequence of the seven U residues which occur at each intergenic junction (Herman et al, 1980; Banerjee et al, 1974; McGeoch, 1979; Schubert et al, 1980). The idea of oligo(U) sequences being used to initiate poly(A) synthesis is not without precedent since a comparable situation seems to exist with the segmented negative strand influenza A viruses (Hay et al, 1977; Skehel and Hay, 1978) and paramyxoviruses (Gupta and Kingsbury, 1982).

Masters and Samuel (1984) detected the in vivo synthesis of polycistronic VSV transcripts by RNA blot hybridisation. They observed all the possible combinations of sequentially linked messages as identified by their apparent molecular weights and patterns of hybridisation. The NS-M dicistronic mRNA was synthesised more frequently

than the other polycistronic transcripts which were synthesised in a roughly uniform distribution (Masters and Samuels, 1984). Although analysis of the RNA following RNase H treatment in the presence of oligo(dT) indicated that in vivo-synthesised poly(A)$^+$ polycistronic species NS-M, M-G and N-NS-M had poly(A) tracts at their 3' termini but not internally at the intercistronic boundaries (Masters and Samuels, 1984) direct sequence analysis is required to confirm this observation. If the observation is correct and there are no poly(A) tracts internally this represents a major difference between in vivo and in vitro transcription.

It is important that the polycistronic transcript synthesised in vitro and in vivo be identified as products of normal or aberrant transcription if we are to elucidate the mechanism of VSV transcription.

6. SEQUENCE INFORMATION

A. Codon Usage

In all of the VSV mRNAs sequenced to date there is non-random usage of codons with a deficiency overall in the dinucleotide CG. Thus the codon usage for five amino acids - serine, proline, threonine, alanine and arginine - is affected (Rose and Gallione, 1981). The CG deficiency is seen between adjacent codons as well as within codons and so it is probably not an adaptation of the virus to a relative shortage of cellular tRNAs recognising CG-containing codons. The reason for the CG deficiency in viral mRNA and vertebrate DNA in general is still unknown (Subak-Sharpe, 1967, 1969).

B. Comparative Sequence Analysis

McGeoch et al (1980) compared the terminal sequences of the N mRNA of VSV IND (Glasgow strain) and VSV NJ (Missouri strain, Hazelhurst subtype) with a view to examining the relationship between the two genomes. The two sequences were readily aligned showing an overall homology of 70.8% at the protein level (McGeoch et al, 1980). When this figure was compared to the 4-20% homology estimated from hybridisation experiments between the two serotypes (Repik et al, 1974) it was concluded that although the overall homology between the genomes of the two serotypes was greater than previously estimated there is little exact homology over extended regions(McGeoch et al, 1980).

More recently N, NS and G mRNAs from both serotypes of VSV have been sequenced thus allowing more detailed comparison of the two genomes (Banerjee et al, 1984; Gallione and Rose, 1983; Rose and Gallione, 1981; Gallione et al, 1981; Gill and Banerjee, 1985).

Gallione and Rose (1983) determined the sequence of the G mRNA of VSV NJ and compared it with their earlier sequence of the G mRNA from VSV IND (Rose and Gallione, 1981). The positions and sizes of the transmembrane domains, the signal sequences, and the glycosylation sites are identical in both VSV serotypes (Gallione and Rose, 1983). An alignment of the two amino acid sequences shows an overall homology of 50.9%. It seems significant that the homology between the G proteins was less than that obtained for the N proteins (McGeoch et al, 1980). Since G protein is the type specific VSV antigen and N the group specific

antigen this is what we might have expected. Although the signal sequence and transmembrane domains of both G proteins are among the least conserved regions they have retained their hydrophobic character. This suggests that exact amino acid sequence is relatively unimportant as long as hydrophobicity is maintained (Gallione and Rose, 1983).

Banerjee et al (1984) sequenced the N mRNA from VSV NJ serotype, Ogden strain and compared it with the corresponding sequence of the IND serotype previously determined by Gallione et al (1981). The N mRNA from VSV NJ is three nucleotides longer than the N mRNA from VSV IND but both code for proteins of 422 amino acids (Banerjee et al, 1984). At the nucleotide level 67.7% homology was calculated and at the protein level 80% homology was calculated when conservative replacements of amino acids were considered (Banerjee et al, 1984).

An extensive comparison of NS sequence data from both serotypes will be presented in a later section.

Thus analysis of nucleotide sequences, even of small regions of the genome, can be an accurate method of determining relationships within the family Rhabdoviridae. Certainly it is more sensitive and definitive than peptide mapping, hybridisation or oligonucleotide fingerprinting which have been used previously.

7. VIRION ASSOCIATED ENZYME ACTIVITIES

In addition to the RNA-dependent RNA polymerase (Baltimore et al, 1970) there are two methyltransferase activities - 7-methyl G transferase and 2-O methyl A

transferase - (Testa and Banerjee, 1977; Banerjee *et al*, 1977), a guanylyl transferase activity (Banerjee, 1980) and a polyadenylation activity associated with purified virions of VSV. It is possible that the polymerase enzyme complex carries out all of the enzyme activities involved in virus specific RNA synthesis. There have been reports by several groups that there is also a protein kinase activity (Imblum and Wagner, 1974; Moyer and Summers, 1974; Witt and Summers, 1980). The enzyme was thought to be host derived, but Sanchez *et al* (1985) provided some evidence to indicate that the L protein has a kinase activity and is capable of phosphorylating NS protein *in vitro*.

Two other enzyme activities, nucleoside diphosphate kinase and nucleoside triphosphatase (Roy and Bishop, 1971), are lost on removal of the viral membrane and so it has been suggested they are host derived (Hefti *et al*, 1975). Other virion-associated enzyme activities which may be host derived are a proteinase (Holland *et al*, 1972) and a ribonuclease (Szilagyi and Uryvayev, 1973; Kolakofsky and Altman, 1978).

8. VSV REPLICATIVE CYCLE

The processes by which VSV enters the cell and then replicates are not fully understood. There are still gaps in our understanding of some areas while conflicting results in others make them controversial. In studies of the VSV replicative cycle the IND serotype has been extensively used. It is assumed the NJ serotype is very similar. Much of the work has been carried out using *in vitro* systems and

very often their relevance to the situation *in vivo* is open to question.

A. Viral Attachment, Penetration and Uncoating

Like other enveloped viruses the glycoprotein of VSV is the viral component that mediates attachment to the host cell. Selective proteolytic removal of the VSV glycoprotein reduces viral infectivity by five orders of magnitude (Bussereau *et al*, 1975) and antibody to the glycoprotein can efficiently neutralise the virus (Kelley *et al*, 1972). The oligosaccharide chains on the G protein do not seem to be responsible for viral attachment (Gibson *et al*, 1978). VSV grown in the presence of tunicamycin, an antibiotic that inhibits glycosylation, has a specific infectivity comparable to its glycosylated counterpart (Gibson *et al*, 1978). Vandepol *et al* (1986) showed in a recent study that the sites at which neutralising antibodies attach to the glycoprotein correspond to the sites of carbohydrate attachment in both the glycoproteins of VSV and rabies virus. Thus it could be that alteration of glycosylation may be important in escape from antibody recognition and this might play an important role in the evolution of rhabdoviruses and indeed other viruses (Vandepol *et al*, 1986).

VSV cell attachment represents a specific interaction between VSV G protein and a limited number (approximately 4000) of saturable 'receptors' as well as non-saturable binding sites (Schlegel *et al*, 1982). Schlegel *et al* (1983) solubilised membrane constituents and determined which components were able to bind VSV, thus inhibiting virus-cell

interactions. It was found VSV binds preferentially to the acidic phospholipid phosphatidylserine (Schlegel et al, 1983).

A number of studies indicate that VSV enters the cell mainly by endocytosis. Electron microscopy has shown that cell-bound VSV concentrates in clathrin-coated regions of the plasma membrane and appears in cytoplasmic vesicles that result from endocytosis of the coated pits (Simpson et al, 1969; Dahlberg, 1974). Dansylcadaverine and amantadine both inhibit receptor-mediated endocytosis. Although neither drug interferes with cell surface binding both were shown to greatly decrease internalisation of the virus and RNA synthesis (Schlegel et al, 1982).

It is thought that acidification of the endosomes by an ATP-dependent proton pump is responsible for initiating the fusion of viral and cellular membranes that leads eventually to the release of the viral nucleocapsid into the cell cytoplasm (Schlegel and Wade, 1985). Evidence from a number of studies indicates that under certain conditions VSV G proten is involved in membrane fusion. Mammalian cells can be fused by exogenous VSV under acidic conditions (White et al, 1981). Cells expressing cloned G protein are fusogenic at low pH (Florkiewicz and Rose, 1984; Riedel et al, 1984). Using fluorescence energy transfer and electron microscopy, purified G protein can be shown to affect liposome membrane fusion (Eidelman et al, 1984). When incorporated into liposomes G protein can haemolyse erythrocytes under acidic conditions (Bailey et al, 1984). Schlegel and Wade (1985) showed that peptides corresponding to the amino terminus of G protein can also function as a pH

dependent haemolysin. The haemolytic domain was later localised to the six amino-terminal amino acids (Schlegel and Wade, 1985). Conservation of the amino terminus of G protein from several strains of VSV and the closely related rabies virus indicates that the membrane-destabilising properties of this domain might be important for glycoprotein function (Schlegel and Wade, 1985).

These data support the conclusion that prior to initiating RNA synthesis, infectious VSV must pass through an endosomal compartment, and so follows a route similar to that of the alphavirus Semliki forest virus and the influenza virus fowl plague virus (Helenius et al, 1980; Marsh et al, 1984; Matlin et al, 1982).

B. RNA Synthesis

The first step in the infectious cycle of any negative strand RNA virus is mRNA synthesis. When the virion envelope has been removed the infecting virus initiates transcription using the RNA-dependent-RNA polymerase which has been packaged in the mature virus particle. Figure 1.5 is a summary of VSV RNA synthesis. The products of standard virus transcription, both in vivo and in vitro, consist of five monocistronic RNAs and a leader RNA (Moyer et al, 1975; Moyer and Banerjee, 1975; Freeman et al, 1977; Rhodes et al, 1977). Recently, however, Kurath and Leong (1985) isolated six viral mRNA species from salmon cells infected with the piscine rhabdovirus IHNV. The sixth RNA coded for a previously unrecognised viral protein induced in infected cells but not present in mature virions. The non-virion protein was designated the NV protein (Kurath and Leong,

1985). It is the first report of a non-virion rhabdovirus protein in infected cells. If an analagous protein were found in VSV and other rhabdoviruses it could have a general role in the rhabdovirus replication cycle. Alternatively, the protein might be specific to replication in fish cells or replication at colder temperatures. Therefore, it would be of interest to see if an NV protein is coded by other piscine rhabdoviruses eg. spring viraemia of carp virus, pike fry rhabdovirus, or viral hemorrhagic septicaemia virus (Pilcher and Fryer, 1978).

(i) Viral Polypeptides Involved in VSV Transcription

Three of the five virus-specified proteins are involved in transcription. The G and M proteins can be removed from the virion leaving the viral subparticle which consists of RNA and L, NS and N proteins. The subparticle is both infectious and transcriptionally active (Brown *et al*, 1967; Cartwright *et al*, 1970; Bishop and Roy, 1972; Emerson and Wagner, 1972; Szilagyi and Uryvayev, 1973).

Dissociation-reconstitution experiments in which virions are dissociated into the individual proteins and then transcriptase activity assayed *in vitro* after reconstitution of various fractions have been performed in order to analyse the role of the various proteins in transcription.

Emerson and Wagner (1972) showed the RNA-N complex was essential for transcription. A transcriptionally active complex was not formed when L+NS proteins were added to deproteinised RNA (Emerson and Wagner, 1972). Further evidence of N protein involvement came from the observation

that antibody directed against the RNA-N complex inhibits transcription (Carroll and Wagner, 1978).

The dissociation-reconstitution experiments strongly suggested that the L protein is at least part of the transcriptase (Naito and Ishihama, 1976; Emerson and Wagner, 1972, 1973; Emerson and Yu, 1975; Abraham and Banerjee, 1976; Szilagyi, 1975; Mellon and Emerson, 1978; Kingsford and Emerson, 1980; Ongradi *et al*, 1985a, 1985b). The laboratories of both Emerson and Banerjee have provided evidence that the L and NS proteins are required for transcription - using VSV IND it has been shown that L on its own does not synthesise RNA when reconstituted with the RNA-N template; however, addition of NS protein leads to RNA synthesis (Emerson and Yu, 1975; Banerjee *et al*, 1974; Mellon and Emerson, 1978; Kingsford and Emerson, 1980; De and Banerjee, 1985). De and Banerjee (1985) using dissociation-reconstitution studies concluded that the requirements for L and NS proteins were catalytic and stoichometric respectively. Their results suggest that NS protein is involved in the elongation step of RNA synthesis whereas L protein is involved in initiation of the RNA chains (De and Banerjee, 1985). However, Szilagyi and his coworkers using *in vitro* reconstitution experiments with *ts* mutants of VSV NJ have suggested that L alone is the transcriptase while the NS protein exerts some controlling role over RNA synthesis by L protein (Ongradi and Szilagyi, 1981; Ongradi *et al*, 1985a, 1985b). Direct evidence for the involvement of both L and NS proteins in transcription comes from the observation that antibody against L and NS proteins has the ability to inhibit transcription *in vitro* by RNPs of

VSV IND (Harmon and Summers, 1982). The conflicting results as to the requirement for NS in transcription may represent greater differences between the two serotypes than previously expected since Szilagyi used VSV NJ in his experiments and the other workers used VSV IND.

A major criticism of the in vitro reconstitution assays and a factor vital to any meaningful interpretation of the results they generate is the lack of characterisation of the RNA synthesised in the reactions. The controversy over the roles of L and NS proteins in transcription might be resolved if the product RNA was characterised by gel electrophoresis, hybridisation analysis or direct sequencing.

(ii) Transcription

Two models have been proposed to account for transcription of the VSV genome and these are summarised in Figure 1.6. Dissociation-reconstitution experiments have been used to study transcription and so all the work has been done in vitro. The interpretation of the results of these experiments and their relevance to the situation in vivo as well as biological significance of the product of the reactions are all open to question. Even today the exact mechanism of transcription has not been elucidated and different laboratories using slightly different techniques still generate conflicting data.

In the multiple initiation model the virion associated RNA polymerase initiates transcription at five distinct promoter sequences distal to the 3' terminus of the genome (Chanda and Banerjee, 1981; Naeve and Summers, 1981). The

model is based on experiments that demonstrate that solubilised virions initiate transcription at internal genes *in vitro* under limiting reaction conditions (Chanda and Banerjee, 1981; Naeve and Summers, 1981). In addition, Pinney and Emerson (1982) reported conditions of transcription *in vitro* in which initiations at the N gene occur in great molar excess over initiations at the 3' leader gene. Thornton *et al* (1984) reported that, when they used a UV irradiated RNA-N_{Λ} protein template reconstituted with L and NS proteins full-length mRNA synthesis decreased by 90% whereas synthesis of leader RNA and other small initiated mRNA sequences continued; this observation, combined with the fact that under partial reaction conditions the reconstituted complex synthesised polyphosphorylated oligonucleotides, some of which corresponded to 5' terminal sequences transcribed from leader RNA and genes coding for mRNAs, led them to support the multiple entry model.

In two sets of experiments supporting the multiple entry model high proportions of additional short oligonucleotides, of unknown origin, have been detected (Chanda and Banerjee, 1981; Thornton *et al*, 1984). These oligonucleotides could be merely the products of aberrant transcriptive events brought about by the treatment of template and/or polymerase. The fact that some correspond to the sequences at the 5' end of leader RNA and mRNAs could be fortuitous. Direct experimental evidence against the multiple entry model is the observation by Keene *et al* (1981) that a DI particle, HR-LT2, is unable to trancribe its internal genes.

Much of the work supporting the multiple entry model

assumes that the oligonucleotides synthesised by virions under limiting reaction conditions reflect the initial steps in transcription. It does not rule out the possibility that during viral maturation virions are stopped at various stages of transcription and so in the subsequent *in vitro* reactions they are completing rather than beginning transcription (Chanda and Banerjee, 1981; Naeve and Summers, 1981). In a reconstitution assay Emerson (1982) recombined template completely devoid of enzyme with polymerase so that transcription had to start *de novo*. The data suggest that the polymerase begins transcription at the 3' end of the genome, and reaches internal genes, only by sequentially transcribing the 3' preceeding sequences (Emerson, 1982). This led to the formulation of the single initiation model of transcription in which the polymerase has a single entry site at the 3' terminus of the genome RNA (Emerson, 1982; Iverson and Rose, 1982). Iverson and Rose (1981) examined the kinetics of synthesis *in vitro* of the 5' ends of mRNAs. From their data they too concluded that there is a single polymerase entry site at the 3' end of the genome (Iverson and Rose, 1981). For the first time RNA products were characterised by sequence analysis - partial sequence analysis of two small RNA species whose synthesis was highly resistant to UV irradiation showed them to be a 5' capped fragment of the N mRNA and a 5' fragment of leader RNA (Iverson and Rose, 1981).

The single entry promoter model is consistent with the generation of monocistronic mRNAs by nuclease cleavage of a full-length precursor (Colonno and Banerjee, 1976) or by a stop/start mechanism whereby the polymerase moves

sequentially along the genome and initiates and terminates transcription at each gene boundary (Banerjee *et al*, 1977). The mechanism at present is controversial. Polycistronic transcripts have been detected both *in vitro* (Rose *et al*, 1977; Herman *et al*, 1980) and *in vivo* (Masters and Samuel, 1984). However, it is not clear at present if the transcripts are the result of aberrant or normal transcription. Certainly the transcripts detected *in vitro* and *in vivo* seem to differ from one another in the extent of polyadenylation. Polycistronic transcripts synthesised *in vitro* are polyadenylated internally at intercistronic junctions whereas those synthesised *in vivo* are polyadenylated at the 3' molecular termini but not internally (Masters and Samuels, 1984). The difference in polyadenylation could represent a major difference between *in vivo* and *in vitro* transcription which would mean that not all results from studies *in vitro* are a true reflection of the situation *in vivo*. Thus new approaches in elucidating the mechanisms involved in many aspects of the VSV life cycle must be sought if we are to build a clear picture of VSV replication.

Transcription by VSV can be divided into primary transcription which occurs on release of nucleocapsids into the cell cytoplasm and secondary transcription which occurs after replication ie. synthesis of full-length genomic RNA.

(iii) Primary Transcription

Flamand and Bishop (1975) estimated that only one out of every five virus particles absorbed actually participates in primary transcription. Primary transcription gives rise

to the five species of mRNA (Morrison et al, 1974; Both et al, 1975; Knipe et al, 1975) in addition to the leader RNA (Leppert et al, 1979; Blumberg et al, 1981; Kurilla et al, 1982) and a sequence complementary to the 5' terminal extracistronic region of the virus genomic RNA (Schubert and Lazzarini, 1981). It has been reported that primary transcription can continue at a linear rate for up to 6 hr post infection (at 31-39^{o}C) (Flamand and Bishop, 1973, 1974). At the optimum temperature the maximum activity corresponds to one genome mass equivalent copy per 90 seconds per active genome (Flamand and Bishop, 1974).

(iv) Protein Synthesis

The mRNAs synthesised during primary transcription become associated with ribosomes and protein synthesis begins. In order for replication, the next stage in RNA synthesis, to occur protein must be synthesised (Bishop and Smith, 1977). If protein synthesis is inhibited, by use of cycloheximide for example, then only primary transcription occurs and no genome sized RNA can be detected (Perlman and Huang, 1973; Wertz and Levine, 1973). Protein synthesis must be continuous because replication ceases if protein synthesis is inhibited after its onset (Wertz and Levine, 1973; Rubio et al, 1980).

(v) Replication

VSV can be thought to replicate in two stages. The negative sense RNP first of all serves as the template for the synthesis of full-length complementary RNA (positive sense RNA). The positive sense strand RNA complexed with N

protein is then able to act as the template for the synthesis of negative sense virion RNA in the second stage of replication (Emerson, 1985). Both postive and negative sense RNA complexed with N protein have been detected in infected cells (Soria et al, 1974). The problem fundamental to an understanding of VSV replication is what controls the switch from mRNA to genome RNA synthesis.

Results from a number of studies indicate that the viral RNA polymerase, perhaps in conjunction with certain host factors, carries out both transcription and replication (Emerson and Yu, 1975; Perlman and Huang, 1973; Pringle, 1978). Studies by several workers have led to the proposal that the availability of N protein, necessary for the encapsidation of genomic RNA, is the switch from transcription to replication (Kingsbury, 1974; Blumberg et al, 1981; Lazzarini et al, 1981).

Coupled transcription-translation systems capable of synthesising full-length genomic RNA are available and have been used to study VSV replication in vitro (Ghosh and Ghosh, 1982; Hill et al, 1981; Davis and Wertz, 1982; Patton et al, 1983). Patton et al (1984) used a transcription-translation system to test the requirement for protein synthesis in replication. In the transcription-translation system nucleocapsids of a VSV DI particle which contained only the 5' 25% of the standard genome were used as templates so that replication could be studied in the absence of mRNA synthesis (Stamminger and Lazzarini, 1979). Viral mRNAs purified by hybridisation to cDNA clones were used to direct the synthesis of individual proteins. Using the system it was demonstrated that the

synthesis of N protein alone resulted in the replication of genome length RNA by both DI intracellular nucleocapsids and virion derived nucleocapsids (Patton et al, 1984). Blumberg et al (1983) reported that the 5' end of leader RNA may serve as a specific binding site for N protein. Thus it is conceivable from the studies of Patton and Blumberg that N protein binds to the 5' end of nascent leader RNA initiating encapsidation of the growing RNA strand. Encapsidation might protect the RNA from processing or cause readthrough of the transcriptional signals and so lead to the synthesis of genome size RNA.

Arnheiter et al (1985) used two monoclonal antibodies directed against different epitopes on N protein as molecular probes to gain some insight into the mechanisms involved in the regulation of RNA synthesis in vivo and in vitro. The results of the study indicate that VSV genome replication is controlled by the availability of N protein even when the polymerase has access to the host factors and multiple phosphorylated forms of the NS protein thought to be involved in genomic RNA synthesis (Arnheiter et al, 1985).

The two antibodies described by Arnheiter et al (1985) are interesting in light of the very different effects they have on replication. Antibody 1 binds to nucleocapsids and probably the pool of free (unbound) N protein, it inhibits transcription and when microinjected into the cell protects it against infection by VSV. Antibody 2 only binds poorly to nucleocapsids and does not inhibit transcription. However, when it is microinjected into the cell it binds selectively to free N and delays the appearance of progeny

virus (Arnheiter et al, 1985). The antibodies distinguished two distinct pools of N protein - a diffusely staining, dynamic pool (N protein not associated with RNA) and a punctuate, staining stable pool (N protein assembled into nucleocapsids). The existence of a pool of free N protein had previously been suspected from pulse-chase experiments (Knipe et al, 1977; Hsu et al, 1979; Rubio et al, 1980).

It seems that the NS protein has a role in replication quite apart from its proposed role as part of the polymerase. The N protein has a tendency to self-aggregate under physiological conditions (Blumberg et al, 1983; Sprague et al, 1983). However, N-NS protein complexes have been isolated from infected cells (Peluso and Moyer, 1984; Bell et al, 1984) and some monoclonal antibodies directed against N or NS protein can immunoprecipitate N-NS complexes in vitro (Arnheiter et al, 1985). Thus it is possible that one of the roles of replication for NS protein is to complex with free N protein in order to prevent N protein self-aggregation (Arnheiter et al, 1985).

A number of genetical and biochemical studies have estabished that replication of VSV in permissive cells requires unidentified host factors (Simpson et al, 1979; Morrongiello and Simpson, 1979; Mukherjee and Simpson, 1984). Mukherjee and Simpson (1984) recently used non-steroidal anti-inflammatory inhibitors known to compromise prostaglandin biosynthesis, cyclic nucleotides and various enzyme systems to demonstrate the dependence of VSV replication on pre-existing host factors.

There are differences in the relative amounts of positive and negative sense RNA present in infected cells at

different times during the infectious cycle (Simonsen et al, 1979). Early in replication the ratio of plus to minus strands is 1:1; however, later the minus strands are preferentially synthesised (Simonsen et al, 1979). It seems the key to regulating the synthesis of plus and minus strands lies in the sequences at the ends of the genome because plus and minus strands of DI particles are synthesised in roughly equal amounts throughout infection (Schubert et al, 1979). The first 46-48 bases at either end of the DI particle genome are highly conserved (Moyer et al, 1977; Emerson et al, 1977) and are the same as those at the 3' end of the standard virus genome.

In summary, replication of genome length RNA by VSV is dependent upon several, as yet, unidentified host factors. One of the main controls over RNA synthesis seems to be the availability of N protein. If there is insufficient N or N synthesis is restricted in some way then replication is restricted and this restiction is presumably accompanied by increased transcription. The evidence indicates that during N protein deficiency all forms of genome length RNA synthesis are restricted (Arnheiter et al, 1985; Patton et al, 1984). By including small differences at the end of its genome it seems that the virus has developed a means to regulate the replication of plus and minus strand RNA.

(vi) Secondary Transcription

It has been reported that at the time when replication occurs there is a very marked rise in the rate of viral mRNA synthesis (Flamand and Bishop, 1974). The increased mRNA synthesis is due to secondary transcription which uses

progeny genomic RNA as template. The synthesis of viral mRNA switches from a linear to an exponential rate because of this secondary transcription although it does eventually decrease (Flamand and Bishop, 1974). There is a limit to the amount of viral RNA that can be synthesisied in infected cells because there are similar amounts of viral RNA in cells infected with high or low multiplicities of infection (Flamand and Bishop, 1974). There seems to be a host cell involvement in regulation of the infectious cycle because the amount of viral RNA synthesised and the yield of virus have been shown to be dependent on the host cell type (Flamand and Bishop, 1973, 1974).

C. Viral Protein Synthesis and Maturation

VSV mRNAs are translated efficiently on host cell ribosomes (Rose, 1980). Rose (1977, 1978) isolated and sequenced translation initiation sites from VSV mRNAs and the sequences determined from the protected sites from each of the five VSV mRNAs are shown in Figure 1.7. Each site contains a single AUG initiation codon and the capped 5' end of the mRNA only appears in the initiation site when it is close to the initiation codon. The sequences do not share common features indicating that a variety of structures can result in equal translation efficiencies *in vivo* (Villarreal *et al*, 1976). The significance of the homologies (shown by dashed boxes) between the sites is unknown. Results from several workers indicate all five VSV mRNAs are translated with equal efficiency (Villarreal *et al*, 1976; Lodish and Froshauer, 1977). Thus the synthesis of each protein is regulated by transcriptional as opposed to translational

control. The known VSV proteins are structural components of the virus and each one may play a role in the final assembly process which results in budding and virus release (Odenwald et al, 1986).

The N protein is synthesised as a soluble protein and assembled into nucleocapsids that accumulate in the cytoplasm (Knipe et al, 1977). As already discussed, the NS protein can form a complex with free N protein in the cytoplasm of infected cells (Bell et al, 1984; Peluso and Moyer, 1984) possibly to prevent the self-aggregation of N protein which occurs extensively in the absence of viral proteins (Blumberg et al, 1983; Sprague et al, 1983). The nucleocapsids remain in the cytoplasm and serve as templates for new negative strand RNA synthesis or take part in secondary transcription.

The NS protein of VSV exists in a variety of phosphorylated forms in infected cells (Kingsford and Emerson, 1980; Hsu and Kingsbury, 1982). Electrophoresis of the various forms of NS separates them into two classes (NS1 and NS2) which differ markedly in their degree of phosphorylation and in their degree of transcriptase activity in vitro and ability to bind to nucleocapsids (Clinton et al, 1979; Kingsford and Emerson, 1980; Kingsbury et al, 1981; Hsu and Kingsbury, 1982). Pulse-chase experiments indicate that there is a very large pool of NS protein in the cytoplasm of the infected cell (Hsu et al, 1979; Rubio et al, 1980). The NS protein has been shown to be phosphorylated at multiple serine and threonine residues (Clinton and Huang, 1981; Hsu et al, 1982). A number of studies which have analysed NS phosphorylation by chemical

and enzymic cleavage of NS protein support the hypothesis that all NS molecules, no matter what their extent of phosphorylation, are phosphorylated at a number of specific residues which have been termed the 'constitutive' phosphorylation sites. The 'constitutive' phosphorylation sites, which all seem to lie in the amino half of the protein, must be phosphorylated for any basal level of NS activity (Hsu and Kingsbury, 1985; Bell and Prevec, 1985). Results from Sanchez et al (1985) suggest the L protein may be responsible for the phosphorylation of NS protein.

The L protein is not found in a cytoplasmic pool but enters RNPs immediately after synthesis (Hsu et al, 1979). NS protein is capable of binding directly to the RNA-N protein complex; however, binding of L is dependent on the presence of NS protein (Mellon and Emerson, 1978). It is known that L can bind to NS (Naito and Ishihama, 1976) so L may bind indirectly to the RNA-N complex by binding to NS. Alternatively, NS binding might expose sites on the RNA which allow L to bind (Mellon and Emerson, 1978).

The G protein can be divided into four major domains: the N-terminal signal sequence, the main body of the protein, the transmembrane domain, and the cytoplasmic C-terminal region (Rose and Gallione, 1981). The VSV G protein has proved to be an excellent model for studying biosynthesis, processing and transport of integral membrane proteins (Lodish and Rothman, 1979). Studies carried out using VSV infected cells have demonstrated that the nascent viral glycoprotein is inserted into the membrane of the rough endoplasmic reticulum (ER) and the signal sequence is proteolytically removed (Chatis and Morrison, 1979; Irving

et al, 1979; Lingappa et al, 1978). As translation proceeds the nascent polypeptide is continuously inserted through the membrane into the lumen. Glycosylation occurs cotranslationally (Kruppa, 1979; Rothman and Lodish, 1977). Two identical oligosaccharide chains are linked to the peptide chain at asparagine residues (Reading et al, 1978). The two glycosylation sites in VSV IND G protein are Asn-Ser-Thr at position 178-180 and Asn-Gly-Thr at position 335-357 (Neuberger et al, 1972; Rose and Gallione, 1981). The oligosaccharide chains have a high mannose content and are preformed on dolicholpyrophosphate molecules (Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1978). The hydrophobic transmembrane domain is assumed to stop the transfer of the growing G protein by anchoring it in the lipid bilayer of the ER. The highly charged C-terminal region of the G protein remains on the cytoplasmic side of the ER membrane (Chatis and Morrison, 1979; Rose et al, 1980; Toneguzzo and Ghosh, 1978). The N linked high-mannose oligosaccharides of the G protein are trimmed and modified in the Golgi complex (Hubbard and Ivatt, 1981; Bergman et al, 1981). Specific sugars are removed and new 'terminal sugars', usually neuraminic acid, are added (Bergman et al, 1978). A fatty acid chain (palmitate) is linked to the single cysteine residue in the cytoplasmic domain in VSV IND but not in VSV NJ (Magee et al, 1984; Rose et al, 1984). The G protein is transported to the plasma membrane and normally leaves it as an integral part of budding virus particles. The G protein forms spikes, which are probably monomeric (Crimmins et al, 1983) and more than 95% of the protein is exposed on the exterior of the virion (Rose and

Gallione, 1981).

Several workers have described a soluble G protein (Gs) present in the culture medium of VSV infected cells (Kang and Prevec, 1970; Little and Huang, 1977). It has been suggested that Gs arises by proteolytic cleavage of the G protein at the cell surface thus causing the protein to be shed into the medium (Chatis and Morrison, 1982; Irving and Ghosh, 1982; Little and Huang, 1978). Shedding of soluble viral proteins is not a phenomenon unique to VSV. It has been reported in a number of other systems like rabies virus infected cells (Dietzschold et al, 1983), murine leukaemia virus infected cells (Bolognesi et al, 1975) and tumour cell lines (Racevski and Sarkar, 1978). Garrais-Wabnitz and Kruppa (1984) showed that in BHK cells there is a short form of G protein found intracellularly which lacks the membrane-anchoring oligopeptide and so is secreted into the medium. Graeve et al (1986) extended the observations to other cell lines and different VSV isolates. Synthesis of Gs in a cell free system combined with kinetic studies indicate that Gs is formed during or shortly after the translation process rather than at the cell surface by proteolytic fragmentation of the membrane-spanning G protein (Graeve et al, 1986). The biological role of the soluble antigens produced by VSV, rabies virus and different tumour viruses remains obscure. It is possible that the soluble antigens neutralise antibodies or block cytotoxic immune cells thus aiding virus infected cells to escape from the immunological defence of the host (Irving and Ghosh, 1982; Little and Huang, 1978).

The M protein of VSV is synthesised on free polysomes

and released into the soluble fraction of infected cells (Knipe et al, 1977). Although initially a large proportion of M protein is non-membrane associated, the protein appears to accumulate in the membrane fractions of infected cells as the infection proceeds (David, 1973; Knipe et al, 1977). It is interesting that in vitro M can accumulate in all classes of membranes, including those of uninfected cells, while in infected cells, association of M with cellular membranes seems to be only transient (Morrison, 1980). It is not clear whether there is any direct association of M with the lipid bilayer of the plasma membrane. Inspection of the predicted M protein sequence does not reveal any long hydrophobic or non-polar domains that might be inserted into the membrane (Rose and Gallione, 1981).

It has been proposed that the M protein of VSV plays a regulatory role in virus-directed RNA synthesis in infected cells (Clinton et al, 1978). Work has suggested that M protein serves as an inhibitor in vitro of viral transcription (Carroll and Wagner, 1979; Lombard and Printz-Ane, 1979; De et al, 1982; Pinney and Emerson, 1982; Wilson and Lenard, 1981). The role of M protein in regulation of transcription has been studied using monoclonal antibodies directed against M protein (Pal et al, 1985; Ye et al, 1985). Pal et al (1985) reported that monoclonal antibodies directed against three antigenic determinants of M protein affected in vitro transcription in very different ways. The results indicated that exposure of epitope 1 on the surface of M protein is essential for inhibiting transcription by VSV (Pal et al, 1985). It has also been reported that VSV can induce cell fusion to a

certain level with G still on the cell surface (Storey and Yong-Kang, 1985).

D. Virion Assembly and Virus Budding

Budding of a mature virus particle from the plasma membrane of an infected cell is dependent upon the assembly of the membrane-associated G protein, the soluble M protein and the RNP at a discrete site on the membrane (Knipe et al, 1977). The budding process is selective in that only RNPs containing negative stranded RNA seem to be recognised (Roy et al, 1973; Wagner, 1975). The mechanisms which govern the sorting and assembly events are for the most part unknown but it seems that the five VSV structural proteins play an important part.

The formation of particles lacking the RNP is very rare in infections with wild-type virus although they have been reported after infection with certain ts mutants (Schnitzer and Lodish, 1979). The RNP seems to be essential in determining the morphology of the budding particle (Odenwald et al, 1986). The amount of G protein incorporated into virions varies depending mainly on the time of harvest (Lodish and Porter, 1980). It is possible that G protein determines the site of budding because in polarised cell lines G protein is transported mainly to the basolateral membrane and it is from this site that budding takes place (Rodriguez-Boulan and Pendergast, 1980; Rodriguez-Boulan and Sabatini, 1978). However, studies using ts045, a spontaneous mutant isolated by Flamand (1970) which has a block in G protein transport (Bergman et al, 1981), found that virus particles devoid of G protein bud

from the cell membrane albeit with low efficiency (Schnitzer et al, 1979).

All the available evidence points to the involvement of M protein being crucial to the budding process. A number of methods (protease digestion, labelling and cross-linking experiments) indicate that M protein is localised on the inside of the envelope and may form a bridge between G protein and the RNP (Dubovi and Wagner, 1977; Zakowski and Wagner, 1980). The M protein may also interact with membrane phospholipid (Zakowski et al, 1981). The association of M protein with the RNP has been shown by experiments with complete virions. Non-ionic detergents, eg. Triton-X and octylglucoside, remove G protein and viral membranes leaving particles termed skeletons. Newcomb and Brown (1981) have shown that in skeletons the M protein remains associated with a tightly coiled RNP. If M protein is removed from the skeleton by varying salt concentration then the RNPs lose their tightly-coiled formation (Newcomb et al, 1982). Addition of M protein to extended RNPs converts them to tightly-coiled forms (Thornton et al, 1982).

Odenwald et al (1986) gained some insight into the assembly processes at the cytoplasmic and outer surfaces of the plasma membrane by preparing platinum replicas of these areas and examining them under the electron microscope (Heuser and Evans, 1980; Hirokawa and Heuser, 1982). They combined the technique with immunolabelling of the viral N, M and G proteins in order to view the events involved in viral morphogenesis.

The M protein is not present in the membrane and is

found associated only with tightly coiled RNPs in contrast to N protein which is part of the loose and tightly coiled RNPs (Odenwald et al, 1986). It seems that a necessary step in the initiation of viral assembly is the interaction between the cytoplasmic domain of G protein and the loosely coiled RNP. When the contact between the two has been made, coiling of the RNP occurs and the particle takes on its characteristic morphology. The M protein might function to alter the conformation of the RNP such that it is amenable to coiling or it might cross-link adjacent turns in the tight coil. The two possibilities of M function are not mutually exclusive and so it may be involved in both processes (Odenwald et al, 1986).

9. THE EFFECT OF VSV INFECTION ON HOST CELL METABOLISM

Infection of many cell types with VSV results in a drastic reduction in the ability of the host cell to synthesise RNA (Baxt and Bablanian, 1976; Weck and Wagner, 1978, 1979; Yaoi et al, 1970). This effect, in parallel with the reduced ability to synthesise DNA (McGowan and Wagner, 1981; Yaoi and Amano, 1970) and protein (Baxt and Bablanian, 1976; Marcus and Sekellick, 1975; McAllister and Wagner, 1976; Wertz and Youngner, 1972) eventually leads to cell death (Marcus and Sekellick, 1974; 1975; Marcus et al, 1977).

Cell killing by VSV is a subject on which much work has been concentrated. However, it is still not clear whether cell death reflects a cumulative effect of viral interference with host metabolism or whether there is a

crucial target. Any understanding of the reasons why VSV infected cells die so rapidly is complicated by the facts that three different major biosynthetic pathways are affected and that different cell lines show different degrees of sensitivity to killing. As yet there is no integrated picture of how the virus kills cells but details of the factors required for shut-off of the individual pathways have been obtained. Viral transcription but not replication is essential for cell killing and is required for shut-off of all three pathways (Marcus and Sekellick, 1975; Marcus et al, 1977).

A. RNA Synthesis

The striking effect of VSV infection on host nuclear function is surprising in view of the facts that VSV replication occurs in the cell cytoplasm and that the virus can grow in enucleated cells (Follett et al, 1974).

The rapid and marked inhibition of cellular RNA synthesis on infection with VSV does not seem to be due to any modification of post-transcriptional events such as polyadenylation or transport of RNA (Weck and Wagner, 1978). Similarly an enhanced degradation of cellular mRNA in infected cells has never been observed (Nishioka and Silverstein, 1978). The ability of VSV to shut-off cellular transcription appears to be at the level of binding of the RNA polymerase to its template (Weck and Wagner, 1979). Weck and Wagner (1979) carried out reconstitution experiments with solubilised RNA polymerases dissociated from chromatin of infected and uninfected cells. These experiments showed that infection neither altered the

template capability of the chromatin nor the ability of the polymerases to function on endogenous or exogenous templates. Measurements of the number of active RNA polymerases, however, clearly indicated there was a substantial loss in the number of RNA polymerase II (polII) molecules engaged in chain elongation. In contrast RNA polymerases I and III (polI, polIII) were found to be inhibited later in infection.

Primary transcription of the VSV genome has been shown to be necessary for the inhibition of cellular RNA synthesis (Weck and Wagner, 1979a; Wu and Lucas-Lenard, 1980). However, studies with UV inactivated virus, carried out by Weck et al (1979), demonstrated that transcription of only a small portion at the 3' end of the genome is required for inhibition of host RNA synthesis. The only viral product they detected was a non-adenylated low molecular weight RNA species and so they proposed that leader RNA is the factor responsible for shut-off of host cell RNA synthesis. The results from several studies support this idea.

McGowan et al (1982) using the in vitro transcription system of Manley (1980) demonstrated that although all VSV mRNAs could inhibit transcription in vitro at high concentrations only the small VSV plus strand leader was inhibitory to both polII and polIII at low concentrations. Kurilla et al (1982) showed that leader RNA is transiently associated with the nucleus early in infection. It is in the nucleus that an inhibitor of transcription initiation would be expected to act. In addition, Grinnell and Wagner (1983) examined the kinetics of leader RNA production in cells infected with UV inactivated virus. They showed that

leader RNA is the only viral product whose level correlated with the degree of RNA synthesis inhibition.

The question of how leader RNA shuts off host RNA synthesis is unanswered. The leader RNAs of both serotypes and five strains of VSV have been sequenced (Colonno and Banerjee, 1978a, 1978b; Keene et al, 1980; Giorgi et al, 1983). In terms of regulatory sequences there are several regions of interest. Comparison of the nucleotide sequences of the leader RNAs of VSV NJ and VSV IND shows that there is an AU-rich region of approximately 16 nucleotides beginning 18 nucleotides from the 5' end. This region has been suggested to resemble the TATA or Goldberg-Hogness box (Baker and Ziff, 1981; Gruss et al, 1981; Hu and Manley, 1981) and is presumably involved in RNA polymerase II initiation. There is a purine-rich region at the 3' end of the leader RNA and McGowan et al (1982) suggested that it resembles the deoxynucleotide sequence implicated in RNA polymerase binding (Breathnach et al, 1978; Jelinek et al, 1980). Kurilla and Keene (1983) have found that VSV leader RNA can be immunoprecipitated with antisera against the La protein. It is a possible polIII transcription factor which is bound to polIII precursor RNA (Rinke and Steitz, 1982). The association of leader with La protein requires the presence of the 3' purine-rich nucleotides.

Grinnell and Wagner (1984) demonstrated the presence of an apparent stem-loop structure that contains the AU-rich region of VSV leader RNA which appears to be important in the efficiency of transcription inhibition. Using a cDNA recombinant of the leader RNA and defined synthetic

oligodeoxynucleotides they showed that the AU-rich region alone was capable of inhibiting adenovirus transcription directed by both polII and polIII.

Grinnell and Wagner (1983) compared the ability of the leader RNAs from VSV NJ and VSV IND to inhibit cellular transcription in vivo and in vitro. The leader RNA of VSV NJ was found to be significantly more effective in this respect. Although the nucleotide sequences from the two leader RNAs are very similar (75% homology) most nucleotide differences occur in the AU-rich region. It could be that the leader RNA of VSV NJ is capable of forming a more stable stem-loop structure and so it is more efficient. The available evidence all points to this AU-rich region being very important.

In summary, inhibition of cellular transcription by VSV is caused by decreased initiation by cellular RNA polymerases. This effect is mediated by leader RNA which appears to be a small nuclear RNA capable of regulating transcription in a sequence specific manner.

B. DNA Synthesis

The ability of VSV to inhibit cellular DNA synthesis has not been studied in great depth. By measuring the rates of host DNA, RNA and protein synthesis in the same infected cells it seems unlikely that inhibition of host DNA synthesis is secondary to the other two processes (McGowan and Wagner, 1981). Studies reported by McGowan and Wagner (1981) and elsewhere (Weck et al, 1979; Weck and Wagner, 1978, 1979a, 1979b) strongly suggest that the same VSV function is involved in host DNA and RNA shut-off.

Experiments with ts mutants and DI particles suggest that at least primary transcription of the VSV genome is required for inhibition of host DNA synthesis and UV inactivation studies showed that the UV target size of the region on the VSV genome responsible for inhibition of host DNA and RNA synthesis is very similar (McGowan and Wagner, 1981).

The cellular target for VSV inhibition of DNA synthesis has not yet been located. The studies of McGowan and Wagner (1981) rule out the possibility of a viral effect on nucleoside transport, DNA degradation, inactivation of DNA polymerase and thymidine kinase, and premature termination of already initiated DNA chains. By exclusion they hypothesise that DNA synthesis is inhibited by the blocking of DNA replication in a manner similar to VSV inhibition of transcription (Weck and Wagner, 1979a).

This is an area which requires more work to test if indeed the same VSV function inhibits both processes and then to identify the specific cellular target involved.

C. Protein Synthesis

Although inhibition of host RNA and DNA synthesis might share a common route it is clear that the mechanism of host protein synthesis inhibition is different. The UV inactivation experiments of Marvaldi et al (1977) showed that transcription of the VSV genome as in the other two processes, was required for inhibition of host protein synthesis. However, similar studies by Marvaldi et al (1978) indicated that the region of the VSV genome responsible for host protein synthesis inhibition was bigger than that required for the other two processes. Marvaldi et

al (1978) showed that newly synthesised N protein (and possibly NS) was required for the inhibition of host protein synthesis.

Host protein shut-off must be selective because the cellular translational machinery cannot be totally abolished. The virus must alter the specificity so that viral mRNA is translated in preference to cellular mRNA. It has been shown that VSV inhibition of host protein synthesis takes place at the level of translation (Lodish and Porter, 1980). Lodish and Porter (1980) showed that after infection cellular mRNA remains intact and fully functional in in vitro translation systems. However, it is translated in the cell at a decreasing rate. Several different mechanisms have been proposed to explain this translational control. Nuss et al (1975) suggested that VSV mRNA might be able to initiate protein synthesis more efficiently than cellular mRNA thus out-competing mRNA for ribosomes. Stanners et al (1977) proposed that a specific viral gene product (which they termed P) is required for the inhibition. Their evidence was that infection of L cells with a mutant of the HR (Winnipeg) wild-type isolate of VSV IND did not result in the inhibition of protein synthesis as observed with wild-type virus (Stanners et al, 1977).

Lodish and Porter (1980, 1981) concluded that host protein synthesis inhibition was due primarily to competition for ribosomes by a large excess of viral mRNAs. Schnitzlein et al (1985) tested this hypothesis by regulating the extent of VSV mRNA synthesis by the use of DI particles. The data indicated that competition for cellular ribosomes was not the major factor in host protein synthesis

inhibition. Using the tsG22 mutant (defective in replication but not primary transcription) Schnitzlein et al (1983) showed that tsG22 inhibits host cell protein synthesis to the same extent as wild-type virus even though it generates only 30-50% of the amount of viral mRNA of wild-type virus.

Recently Adam et al (1986) studied the interaction of mRNA with proteins in VSV infected cells by photochemical cross-linking in intact cells. Photochemical cross-linking and immunoblotting of these proteins showed that in addition to host proteins VSV mRNAs also became cross-linked to the N protein (Adam et al, 1986). The UV cross-linking data taken together with work by Rosen et al (1982) and Grubman and Shafritz (1977) who had previously demonstrated that VSV mRNAs are associated with N protein, strongly suggest that N protein mRNA complexes do exist in the infected cell. The N protein associated with VSV mRNA has been shown to inhibit protein synthesis in a rabbit reticulocyte lysate and wheat germ extract (Rosen et al, 1984). The inhibition appears to be at the first step of protein synthesis, the formation of the complex eIF-2.GTP.Met-tRNA (Rosen et al, 1984). The N protein may therefore be involved in the shut-off of total protein synthesis in VSV infected cells (Adam et al, 1986).

10. PSEUDOTYPES AND PHENOTYPIC MIXING

Double infection of one cell with two different viruses often results in the formation of pseudotypes - these are viruses which have the genomes of one virus and the coat proteins of the other. The phenomenon was first

demonstrated for VSV using the IND serotype and the paramyxovirus SV5 (Choppin and Compans, 1970). Phenotypic mixing, in one direction at least, has been recorded between VSV and nine out of the ten families of enveloped viruses. Pseudotype-like particles have been produced *in vitro* by adding VSV NJ serotype envelope components to either bromelain- or pronase-treated VSV IND serotype particles (Bishop *et al*, 1975).

All of the methods for detecting pseudotypes and phenotypically mixed particles depend on selective assays for genomes and envelopes. Pseudotypes containing the VSV genome can be recognised by their resistance to neutralisation by anti-VSV serum, sensitivity to antiserum specific for the virus donating the envelope antigens, complement mediated virolysis, immunoprecipitation, acrylamide gel electrophoresis analysis of constituent proteins, immunoelectron microscopy and various other tests depending on the properties of the envelope proteins (Zavada, 1972; Zavada and Rosenbergova, 1972; Zavada *et al*, 1975; Boettiger *et al*, 1975; Weiss and Bennett, 1980; Witte and Baltimore, 1977; McSharry *et al*, 1971; Choppin and Compans, 1970; Chan *et al*, 1978). Pseudotypes containing the VSV glycoprotein and the genome of other viruses can be analysed by similar means.

Pseudotypes are valuable tools to answer experimentally a variety of questions which arise in the study of viruses. Indeed the screening for pseudotypes with the genome of VSV and the envelope of a heterologous virus has proved to be an extremely sensitive method for detecting cryptic or latent viruses eg. chick helper factor in avian

cells (Love and Weiss, 1974); or as a rapid quantitation assay for slow or non-cytopathogenic viruses eg. Wild et al (1975) demonstrated VSV pseudotype formation in Vero cells infected productively or latently with measles virus strains isolated from patients suffering subacute sclerosing panencephalitis. The sensitivity of the method can be increased using immunoprecipitation with Staphylococcus aureus rather than neutralisation as a detection system (Zavada et al, 1983). Analysis of pseudotype production has been used as a quantitative procedure for detection assays of bovine leukaemia virus, mouse mammary tumour virus and human T lymphotropic virus types I and II (Zavada et al, 1979; Bruck et al, 1982; Chan et al, 1982; Clapham et al, 1984). Reciprocal formation of pseudotypes between VSV and lymphocytic choriomeningitis virus (LCMV) were used to study acute and persistent infections of cell cultures and mice with LCMV (Bruns and Lehmann-Grube, 1984).

11. VIRAL PERSISTENCE

Persistent infections by RNA and DNA viruses have been implicated as important factors in a number of chronic or recurring diseases of man and animals (Stevens et al, 1978; Fields et al, 1980). The mechanisms by which RNA viruses are able to establish and maintain persistent infections are not understood but a number of factors seem to be important eg. the appearance early in such infections of ts mutants; interferon regulation of ts mutant growth; small plaque mutations; DI particles (Preble and Younger, 1973; Fields and Raine, 1974; Younger et al, 1976; Ramseur and Friedman,

1977; Nishiyama et al, 1978; Wagner et al, 1963; Holland and Villarreal, 1974; Kawai et al, 1975; Ahmed and Graham, 1977; Popescu and Lehmann-Grube, 1977; Elliott and Wilkie, 1986).

Extensive genetic changes occur in VSV maintained in persistently infected cells or passaged for a period at high multiplicity. Holland and Villarreal (1974) established a persistent infection of BHK-21 cells using a ts mutant of VSV IND. They analysed the infection by oligonucleotide mapping and nucleotide sequencing over a number of years (Holland et al, 1979; Rowlands et al, 1980; Semler and Holland, 1979). They found that the highly mutated virus clones they isolated following years of persistence exhibited considerable genome stability upon repeated lytic passages in cell culture or in vivo in mice (Holland et al, 1979; Rowlands et al, 1980). There are other virus-host cell systems which have been shown to involve the evolution of viruses eg. VSV in L cells (Youngner et al, 1978) and Semliki forest virus in L cells (Meinkoth and Kennedy, 1980).

Spindler et al (1982) demonstrated that rapid genome evolution of VSV clones can occur equally well during lytic high-multiplicity passages as during long term persistence. They also demonstrated that this genome evolution is random and not determined by selective pressures.

A number of interesting and potentially useful mutants have been isolated from persistent infections. Virus stocks isolated from two persistently infected mouse L cell lines expressed an altered phenotype of RNA synthesis (rt^-) from the viruses used to initiate the infection (rt^+) (Fray and Youngner, 1982). At 34°C and 37°C in L cells productively

infected with the stocks derived from the two cell lines, mRNA transcription was significantly reduced whereas replication of genomic RNA was enhanced compared to the viruses used to initiate the infection. The results from further analysis of these persistent infections demonstrated that a specific viral phenotype (ts$^-$, rt$^-$) evolved and was stable over a period of at least three years, which was the longest period tested (Frey and Youngner, 1984).

Reid et al (1979) isolated a mutant of VSV (VSV-P) from a persistent infection of tumour cells in nude mice. BHK-21 cells infected with VSV-P are resistant to natural killer (NK) cells in vitro and form tumours in nude mice, while BHK-21 cells persistently infected with wild-type VSV are sensitive to NK cells in vitro and do not form tumours in nude mice (Minato et al, 1979). T_1 oligonucleotide maps showed that VSV-P underwent extensive mutation during its selection in vivo in persistently infected BHK-21 cells, and subsequent nude mouse passaging of BHK-21 cells persistently infected with VSV-P revealed continuing rapid evolution of VSV-P in vivo (Jones et al, 1980; Spindler et al, 1982). There has been extensive nucleotide sequence analysis of antibody-selected variants of a variety of RNA viruses and these have shown that the mutations are associated with antibody-binding epitopes in the viral glycoproteins (Wiley et al, 1981; Seif et al, 1985; Skehel et al, 1984; Dietzschold et al, 1983; Hovenac and Air, 1985). Vandepol and Holland (1986) sequenced the RNA of three independently derived VSV-P mutants. The results show that these NK cell resistant mutants selected in vivo accumulated extensive mutations in the surface G protein but only relatively few

changes in the M and NS proteins. This suggests, but does not prove, that G protein plays an important role in NK cell lysis.

A problem in the study of persistent viral infections *in vivo* when *ts* viruses are involved has been the development of procedures for reisolating viruses after infection. However, Hughes *et al* (1985) isolated *ts* mutants of VSV after passage of the mutant *ts*G31 by a series of intracerebral inoculations in mice by inducing hypothermia in the animals. They injected the neuropeptide bombasin to unbalance the normally stable thermoregulatory system of the animal. If this procedure proves useful for the isolation of other viruses then it could provide a valuable and quite unique method for studying various aspects of persistence.

12. VSV AND INTERFERON

Rhabdoviruses are very sensitive to the action of interferon. They rank close to the highly sensitive togaviruses and are on par with vaccinia virus in many test systems (Lockart, 1973). VSV is often used as the test agent in biological assays of interferon (Finter, 1973). Indeed, this sensitivity of VSV to interferon action, its broad host range and the knowledge of certain aspects of its molecular biology have made it an excellent model for the study of interferon mediated interference.

In cells pretreated with interferon there is no detectable accumulation of primary transcripts. In these same cells neither amplified RNA synthesis nor virus protein synthesis can be detected (Marcus and Sekellick, 1978;

Thacore, 1978; Marcus, 1980). The virus transcripts found in interferon-treated cells are equal in size and have the same half-life as in untreated cells which suggest that once transcription is initiated in interferon-treated cells it is completed as normal (Marcus and Sekellick, 1978). It has been reported that the host cell might influence sensitivity to interferon. Wagner et al (1963) demonstrated that two strains of VSV were inhibited to different degrees by interferon acting in mouse L cells but showed the same sensitivity in chicken cells. Thacore (1978) came to similar conclusions from his experiments in which he used different interferons and cell types.

VSV is not considered to be a very efficient inducer of interferon. It could be that an interferon inducing factor is not produced in sufficient quantities as to be sensed by the cell or the characteristic efficient shut-off of cellular macromolecular synthesis precludes interferon mRNA transcription and/or translation (Wertz and Youngner, 1970; Wagner and Huang, 1966; Yamazaki and Wagner, 1970; Weck and Wagner, 1978; Marvaldi et al, 1977, 1978; Stanners et al, 1977). Studies using DI particles and ts mutants have shown that under non-replicating conditions when inhibition of cellular protein synthesis and cell killing are delayed or prevented, VSV is an excellent inducer of interferon (Marcus and Sekellick, 1977; Marcus, 1980). Work by Sekellick and Marcus (1979) has indicated that transcription of a small part of the genome is required for interferon induction. This is presumably required to form double-stranded RNA complexes.

Interferon action is the singularly most effective

means of preventing cell killing by viruses and recent work has implicated interferon induction as a means of cell sparing in persistent infection with VSV (Marcus, 1980).

13. DEFECTIVE INTERFERING PARTICLES

Defective interfering (DI) particles of VSV are deletion mutants which contain only a portion of the parental genome and interfere specifically with standard virus replication (Huang and Baltimore, 1970). They are not capable of self-replication in host cells but require coinfection with competent helper virus which supplies the missing gene products. Under these conditions the replication of helper virus is greatly suppressed - this phenomenon is termed autointerference or the von Magnus phenomenon (von Magnus, 1954). Available evidence strongly suggests that DI particles interfere with replication of helper virus by favourably competing for the limited amount of polymerase or nucleocapsid protein supplied by the helper virus.

Four classes of DI particle genome can be distinguished and these are shown in Figure 1.8. The four classes have been designated fusion, panhandle, snapback and compound (Lazzarini et al, 1981; Perrault, 1981). The four types share certain features - variable portions of the L gene are deleted; the 5' terminal regions of the deleted genomes are identical in sense and sequence to the 5' terminus of the minus strand of the complete genome; the 3' terminal regions are identical to the 5' terminus of either the minus or plus strand region of the complete genome.

(i) Fusion

The fusion DI particles are simple deletion mutants. The best characterised example of this type of DI particle in VSV contains the genetic information from the 3' half of the parental genome (Leamnson and Reichmann, 1974; Stamminger and Lazzarini, 1974). The DI particle appears to be a true deletion mutant in which most of the L gene has been deleted. Hybridisation studies and direct sequencing have established the presence of both parental viral RNA termini on this DI particle RNA (Perrault and Semler, 1979; Epstein et al, 1980). Because the parental 3' terminus is preserved the viral polymerase can transcribe the DI particle genome yielding four functional mRNAs (Colonno et al, 1977; Johnson et al, 1979). The ability to transcribe its genome is unique amongst DI particles and may confer on it special biological properties (Prevec and Kang, 1970; Marcus et al, 1977).

(ii) Panhandle

The genome of the panhandle DI particle contains information from the 5' half of the parental VSV genome but terminates at the 3' end with a short complementary sequence. The first evidence for this type of configuration came from electron microscope studies which showed that the major class of VSV and Sendai virus DI particle appeared as unit length circles suggesting that the RNA had complementary termini (Kolakofsky, 1976; Perrault and Leavitt, 1977). Hybridisation studies subsequently showed that the parental 5' terminus was preserved in this class of

DI particle RNA indicating that the 3' terminus of the DI particle was different from that of the parent virus (Leppert et al, 1977; Perrault et al, 1978). RNA sequencing studies confirmed not only the presence of a complementary sequence at the 3' end but also the structure (Keene et al, 1978; Schubert et al, 1978; 1979; Semler et al, 1978).

(iii) Snapback

Like the panhandle class of DI particle the genome of the snapback class contains information from the 5' half of the parental genome but instead of terminating in a short complementary sequence at its 3' terminus it is linked to an exact complement so that every base has the potential of entering into a duplex. The prototype of this class of molecule is the VSV DI-1011 (Lazzarini et al, 1975). When the molecule is deproteinised the RNA has been shown to snap into a perfect duplex (Schubert and Lazzarini, 1981). The snapback class of DI particle has frequently been found among the progeny of VSV infections (Lazzarini et al, 1975; Perrault, 1976; Perrault and Leavitt, 1977; Gillies and Stollar, 1980).

(iv) Compound

The genome of the compound class of DI particle contains multiple initiation sites at the 3' terminus of its RNA. The prototype of this class is again a VSV DI particle - DI-LT_2. DI-LT_2 was discovered in the original seed stocks of the fusion DI particle, DI-LT. Evidence has suggested that DI-LT_2 was derived from DI-LT (Epstein et al, 1980; Perrault and Semler, 1979). Keene et al (1981) determined

the 3' terminal base sequence of the DI-LT_2 RNA. They found that the 3' end of the RNA was the exact complement of the 5' end of the VSV RNA for 70 nucleotides. After base 71 they found the 3' end of the non-defective genome was present. The non-defective sequence includes the entire leader gene, the intervening sequence and the beginning of the N gene. Although sequencing data do not extend beyond this point hybridisation studies indicate that the DI-LT_2 RNA contains most, if not all, of the genetic information from the 3' half of the VSV genome (Epstein et al, 1980; Perrault and Semler, 1979). Unlike its presumed parent, DI-LT_2 is not transcriptionally active and the internal leader gene is muted (Keene et al, 1981). The difference in transcriptional abilities between DI-LT and DI-LT_2 is paralleled by a difference in their ability to interfere with wild-type VSV (Perrault and Semler, 1979).

There are a number of factors which affect the generation of DI particles. Most important amongst these is the nature of the host cell, time of harvest and multiplicity of infection. Holland et al (1976) showed that some cell types produce more DI particles than others. When they infected cells with a mixed population of DI particles they found that the predominant type in the progeny varied between cell types. The biochemical basis for the host effect is unknown but a number of studies, as already mentioned, have pointed to the involvement of host factors in VSV replication (Nowakowski et al, 1973; Obijeski and Simpson, 1974; Szilagyi et al, 1977; Szilagyi and Pringle, 1978).

DI particles are released from cells at a slower rate

than standard virus and so harvesting early after infection selects against DI particles (Khan and Lazzarini, 1977). This slow release of DI particles does not appear to result from restricted replication of the RNA since DI nucleocapsids are abundantly present in cells infected with a mixture of viruses. It suggests, however, that the assembly of the DI particle might be the limiting factor.

Generally it is true that infecting at high multiplicity favours DI particle generation. DI particles are most often observed in virus stocks that have been passaged at high multiplicity. Faulkner and Lazzarini (1980) reported that infecting at multiplicities below 1pfu/cell usually selects against DI particles.

The exact mechanism by which DI particles interfere with the replication of standard virus is unclear. Data suggest that the presence of DI particles does not affect viral transcription and translation directly but rather interferes with the synthesis of nucleocapsid template for secondary transcription (Perrault and Holland, 1972; Khan and Lazzarini, 1977; Stamminger and Lazzarini, 1977). It was found that although the total amount of intracellular nucleocapsid RNA was almost unaffected by autointerference, the composition of the nucleocapsid material changed dramatically with increased levels of autointerference. Higher proportions of DI nucleocapsids were found in cells exhibiting high levels of autointerference (Khan and Lazzarini, 1977; Stamminger and Lazzarini, 1977).

Although the study of DI particles has limited the number of possible mechanisms of autointerference it has not, as yet, pointed to a particular mechanism. It is

possible that all the mechanisms contribute in varying degrees to autointerference (Huang, 1982). The copy choice model for the generation of DI particles in VSV suggests that during replication the polymerase prematurely terminates, moves with the nascent daughter strand to another site on the same or different template molecule, and resumes elongation of the nascent strand (Lazzarini et al, 1981). Meier et al (1984) analysed the sites where premature termination or resumption of replication had occured during the generation of representatives from the fusion, snapback and panhandle classes of DI particle. The sequence of the relevant regions of the DI particle RNAs were compared with that of the VSV L gene (Schubert et al, 1984) in order to identify the sites of recombination. Although homology between these sites was not detected (ruling out the existence of a general terminator or promoter sequence involved in copy-choice replication) it was found that in many cases premature termination or resumption of RNA replication might be favoured by specific sequences. It has been proposed that specific sequences modulate DI particle generation in a quantitative way and favour the generation of certain types of DI particle genome eg. those of the panhandle type.

In general DI particles can efficiently interfere with the replication of virions of the homologous serotype but are incapable of heterotypic interference (Schnitzlein and Reichmann, 1977). However, Prevec and Kang (1980) reported that the HR-DI particle of VSV IND serotype (which contains sequences from the 3' terminus of the parental genome) can interfere as efficiently heterotypically as homotypically.

The study of the structure and origin of DI particles is important not only because of their proposed importance in the establishment and maintenance of persistent infection (Holland et al, 1980; Holland and Villarreal, 1974; Horodyski and Holland, 1984) but also because they arise from aberrant replicative events. Their study might therefore reveal some much needed details on the mechanism of replication.

14. VSV GENETICS

In the study of virus growth and development the isolation of mutants has proved an invaluable tool. Much of the study on rhabdoviruses has concentrated on this area with the result that there are now hundreds of mutants available for study. However, the field of VSV genetics has become both confused and confusing and since much has already been written (reviews by Flamand, 1980; Pringle, 1975, 1977, 1982, 1985; Pringle and Szilagyi, 1980) I intend to write only briefly on the subject concentrating mainly on the characterised mutants of VSV NJ serotype (see Table 1.4).

Pringle (1977) divided the known mutants of VSV into two broad categories: those with specific phenotypes and conditional lethal mutants where a common phenotype embraces many if not all indispensable genome functions. The tl mutants of VSV IND where the phenotype is due to the thermosensitivity of the virion envelope (Zavada, 1972) or the Rif^+ mutants where the drug rifampicin appears to directly inhibit transcriptase activity (Moreau, 1974) are

examples of the former. The conditional lethal mutants of rhabdoviruses are either temperature sensitive (ts), host restrictive (hr) or temperature dependent host range (tdCE) mutants. The majority of the conditional lethal mutants of VSV appear to arise from 'missense' mutations. However, recently a new class of mutant, the supressor sensitive 'nonsense' mutants have been isolated (B. White, unpublished results). Wild-type VSV IND serotype was mutagenised using the base analogue 5-fluorouracil (5-FU) and 'nonsense' mutants were detected by assaying growth on suppressing and non-suppressing cell lines.

Due to the ease by which they can be assayed the ts mutants have been extensively used in the study of many aspects of the rhabdovirus lifecycle.

With the exception of 71 spontaneous ts mutants isolated from VSV IND by Flamand (1970) the ts mutants of VSV have been derived using one of several mutagens - 5-FU, 5-azacytidine and ethylmethane sulphonate. 5-FU was the most efficient mutagen producing 105 of the 175 ts mutants. The 436 mutants of VSV IND are classified into six complementation groups I-VI. Unambiguous gene assignments have been completed for five of the complementation groups and each of the five viral proteins is represented (Pringle and Szilagyi, 1980). It is thought that the sixth complementation group, which comprises mutants isolated by Flamand (1980) and Rettenmier et al (1975), probably represent intragenic complementation (Pringle and Szilagyi, 1980).

The 48 ts mutants isolated from VSV NJ, Hazelhurst subtype have been classified into six non-overlapping

complementation groups designated A-F. The sixth complementation group has been postulated to represent a sixth, non-structural, virus encoded protein involved in replication (Lesnaw and Reichmann, 1975). However, analysis of the functional properties of ts mutants from three rhabdoviruses (Chandipura virus, VSV IND and NJ) and the complete sequencing of the genome of VSV IND has borne out the prediction by Gadkari and Pringle (1980) that the presence of a sixth complementation group does not imply the existence of a sixth genome product.

Analysis of the functions of representative mutants of VSV NJ has suggested the mutants classified in complementation groups B and F are L protein mutants (Ongradi et al, 1985b). The four remaining viral proteins can be assigned to complementation groups A, C, D and E. Conclusive gene assignments have been made using techniques such as gel electrophoresis of virion proteins, partial proteolysis, tryptic peptide mapping, in vitro dissociation and reconstitution of transcriptase assay, and heteroduplex mapping.

A. Complementation Groups of the VSV NJ Mutants

(i) Group A

The ts lesion in complementation group A has been assigned to the N protein. The assignment was made on the basis of the thermolability of the template activity of the A1 N-RNA complex in in vitro transcription reactions and the covariance of the phenotype with the ts phenotype in a spontaneous A1 revertant (Marks et al, 1985).

(ii) Groups B and F

The mutation in complementation groups B and F was thought to be in the L gene (Bell-Isle and Emerson, 1980; Szilagyi and Pringle, 1979; Pringle and Szilagyi, 1980). Conclusive proof that the mutation was in the L gene was provided by Ongradi et al (1985b). Ongradi et al (1985b) used reconstitution assays to positively identify the L gene as the location of the mutation.

Since the gene encoding the L protein occupies approximately half of the VSV genome it is not surprising that in both the IND and NJ serotypes the complementation group with the largest number of members are those with mutations in this protein.

(iii) Group C

Initially the mutation in complementation group C was tentatively assigned to the M protein on the basis of the similar cytopathology of the mutants to the complementation group III mutants (lesion in M gene) of VSV IND (Unger and Reichmann, 1973). Positive identification of the gene in which the mutation lay was made by analysis of in vivo degradation and peptide mapping (Kennedy-Morrow and Lesnaw, 1984).

(iv) Group D

There is, as yet, no positive assignment made for complementation group D. Pringle et al (1981) observed that one member of the group, tsD1, exhibited an enhanced mutability whereby clones showed a variety of phenotypes

Assignment of the complementation group D mutants to the G gene (Table 1.4) is based on exclusion only.

involving N, G and NS proteins. When tsD1 is incubated at the restrictive temperature an absence of nucleocapsid assembly and an abnormally high ratio of genome length to mRNA is observed (Pringle et al, 1981). Because the two processes appear to be coupled the major phenotypic changes could be accounted for by mutations in the L or N genes.

(v) Group E

The mutations affecting mutants of complementation group E have been shown to be located on the NS gene by gel electrophoresis of virion proteins (Evans et al, 1979; Lesnaw et al, 1979); in vitro reconstitution studies (Ongradi and Szilagyi, 1981; Ongradi et al, 1985a); and tryptic peptide mapping data (Maack and Penhoet, 1980). There are three mutants in this group (tsE1, tsE2, tsE3) and these are of interest not only because of their aberrant migration on SDS polyacrylamide gels but also because of their very different phenotypes. They will be discussed in more detail in later sections.

B. Mapping of Mutations

The efficient techniques for cDNA cloning of viral RNA and the methods of rapid sequencing of DNA now available have made more definitive studies of the well characterised mutants possible. The mutant ts045, a spontaneous mutant isolated by Flamand (1970), is probably the best characterised mutant of the complementation group V of VSV IND and one which has been extensively used in the study of the viral glycoprotein. The mutant has a reversible block in transport of the G protein from the rough ER to the Golgi

apparatus (Bergman *et al*, 1981). By firstly sequencing cDNA clones, then recombining them *in vitro* and expressing the recombinants in COS-1 cells, Gallione and Rose (1985) were able to localise the critical lesion in *ts*045 to a single substitution of a polar amino acid (serine) for a hydrophobic amino acid (phenylalanine) in a hydrophobic domain. It is important that the analysis be extended to other mutants so that the lesions can be localised and the functional domains of the proteins identified.

15. RECOMBINANT DNA TECHNOLOGY AND RNA VIROLOGY

The bulk of our current knowledge of the molecular biology of RNA viruses has come from the application of recombinant DNA technology in their study. Techniques such as cDNA cloning, sequence determination and the ability to express the cDNA clones in eukaryotic and prokaryotic cells have allowed workers to make detailed studies on such diverse topics as genome evolution and protein function.

The discovery of the retroviral enzyme, reverse transcriptase, capable of synthesising DNA (copy DNA or cDNA) from an RNA template (Baltimore, 1970; Temin and Mizutani, 1970) and the steady advances in sequence technology over the last 15-20 years (Sanger *et al*, 1983; Maxam and Gilbert, 1980) have led to the generation of the great amount of sequence data now available. Table 1.5 lists some of the genomes that have been completely sequenced. The sequence data alone have proved invaluable in the analysis of many aspects of the virus lifecycle.

Sequence data have highlighted the heterogenous nature

of the RNA virus genome. Schubert et al (1984) found a number of variants in cDNA clones of the VSV L gene derived from plaque-purified virus. Variants were also detected in a cDNA library derived from the tobacco mosaic virus (TMV) RNA, indeed variants were detected in related TMV stocks obtained from different laboratories (Goelet et al, 1982). Recently, Buonogurio et al (1986) used sequence analysis to study the evolution over a 50 year period of the NS gene of influenza A virus.

The first report of an RNA virus using overlapping reading frames was made with influenza virus (Lamb and Lai, 1980; Lamb et al, 1981). The phenomenon has since been shown to occur in Sendai virus (Giorgio et al, 1983); bunyaviruses (Cabradilla et al, 1983) and arenaviruses (Akashi and Bishop, 1983). Members of the family Arenaviridae and the Phlebovirus genus of the Bunyaviridae have been shown to possess 'ambisense' RNA ie. genes are encoded in both negative and positive senses (Auperin et al, 1984; Romanowski et al, 1985; Ihara et al, 1985).

A number of glycoprotein genes have been sequenced and the data have been useful in identifying regions important to transport and surface expression. Lees et al (1986) sequenced Bunyamwera virus M RNA which encodes the virion glycoproteins and a non-structural protein. They found a single open reading frame sufficient to encode a polyprotein. Comparison of the sequence with the M RNA of snowshoe hare virus (Eshita and Bishop, 1984) revealed quite marked sequence and structural similarities between the genes and gene products of these related but antigenically distinct bunyaviruses (Lees et al, 1986). Satake et al

(1985) calculated a mol wt of 32,588 from their sequence data on the G protein of respiratory syncytial virus. The previously estimated mol wt of 90,000 from the proteins mobility on gels can be attributed to the fact that it is heavily glycosylated with a number of the sugars attached by O-glycosylations.

In many cases sequence data serve to emphasise the differences between viruses even in the same family. The fish rhabdovirus, IHNV, was recently shown to encode six mRNAs as opposed to five mRNAs as in the prototype rhabdovirus, VSV (Kurath and Leong, 1985; Leong et al, 1985). Four viruses which cause acquired immune deficiency syndrome (AIDS) have now been completely sequenced (Muesing et al, 1985; Ratner et al, 1985; Sanchez-Pescador et al, 1985; Wain-Hobson et al, 1985). Analysis of the sequence data not only emphasises the variability between related viruses but has also led to the proposal that AIDS virus be reclassified as a lentivirus due to its similarity to visna virus, the prototype member of the family Lentiviridae (Sonigo et al, 1985).

The genome of yellow fever virus (YFV) was recently sequenced (Rice et al, 1985). This was the first report of the complete nucleotide sequence of a member of the family Flaviviridae. Flavivirus transcription/translation was an area of some controversy. Two models had been proposed - one involving protein processing; the other multiple initiation of mRNAs. The sequence data strongly support the idea that the RNA is translated as a large polyprotein which is subsequently cleaved (Rice et al, 1985).

The Picornaviridae is a large family of viruses which

includes human hepatitis A, many enteroviruses and foot and mouth disease virus. The 3-dimensional (3-D) structure of two viruses from this group [poliovirus, type 1, Mahoney strain and human rhinovirus, type 14 (Hogle et al, 1985; Rossmann et al, 1985)] have now been determined. The large amount of sequence data available on these two viruses and their mutants will now enable workers to relate nucleotide changes to alterations in structure. These types of analyses were first employed when the 3-D structure of the influenza virus haemagglutinin was elucidated (Wilson et al, 1981) eg. Daniels et al (1985) were able to relate the nucleotide sequence changes in the haemagglutinins of amantadine-resistant mutants of influenza virus to structural changes in the haemagglutinin of the wild-type protein.

From the few examples quoted here it is quite clear that the use of recombinant DNA technology has been invaluable in the study of RNA viruses. However, there have also been practical benefits from its application eg. Hu et al (1986) expressed the envelope (env) gene of one of the AIDS viruses in recombinant vaccinia virus in the search for a vaccine. They reported that the recombinant viruses elicit antibodies that specifically recognise AIDS virus env proteins. Denniston et al (1986) reported that a cloned cDNA fragment of hepatitis delta virus (HDV) had been used as a sensitive radioactive probe for the detection of HDV RNA in the serum of patients with either acute or chronic HDV infections.

It is quite apparent that recombinant DNA technology will become increasingly important in the study of RNA

viruses as the problems encountered in their study become more complex.

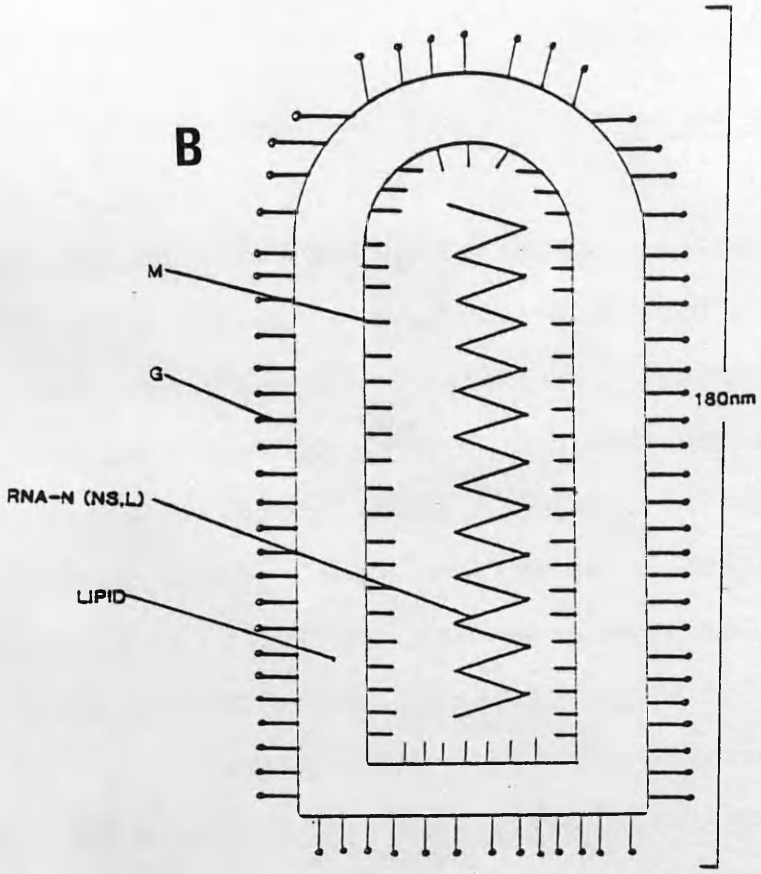
AIMS OF THIS PROJECT

The complementation group E mutants of VSV NJ, strain Missouri have been shown to have a lesion in the NS gene. They are of interest because of the different phenotypes they possess and the fact that on SDS-polyacrylamide gels their NS proteins exhibit a differential migration pattern both in relation to wild-type virus and one another. Recombinant DNA technology was applied to the analysis of the NS genes of wild-type virus and complementation group E mutants. The aims of this project were:

(a) to obtain full-length cDNA copies of the NS mRNAs of wild-type, complementation group E mutant and revertant viruses

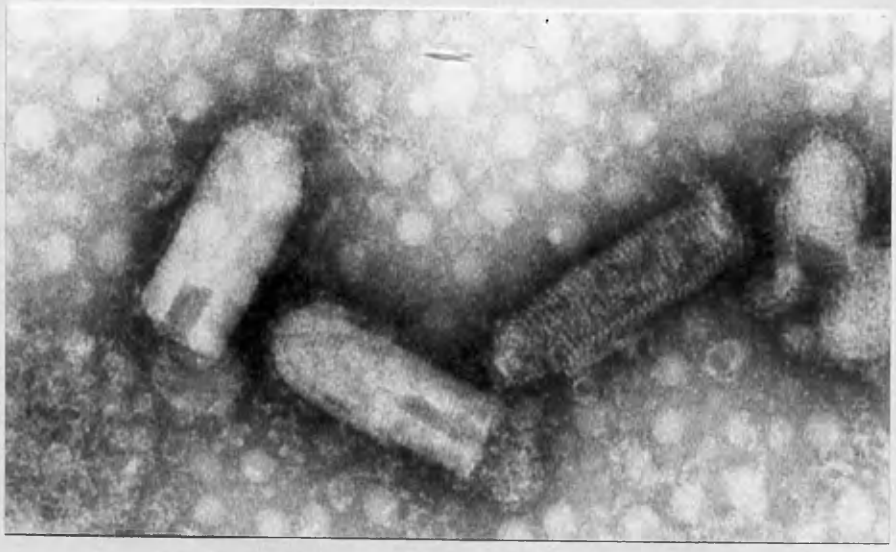
(b) to determine the nucleotide sequence of the cDNAs

(c) to map exactly the position of the mutation(s) in tsE1, tsE2 and tsE3



A MODEL OF THE VSV STRUCTURE

A

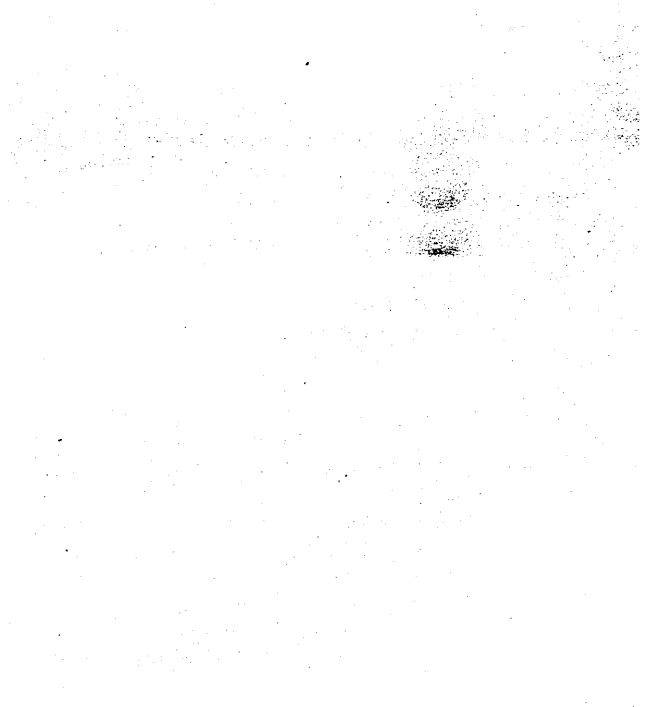


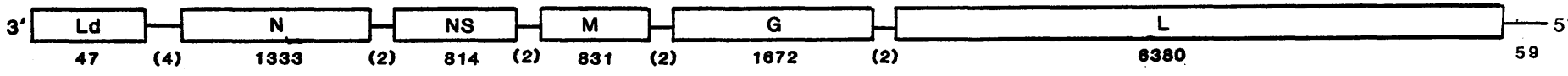
0·1 µm

Figure 1.1

(A) Electron micrograph of negatively stained VSV particles (magnification 180,000).

(B) Schematic model of the VSV structure. The spiral ribonucleocapsid is enclosed within the lipid envelope. Spikes consisting of G protein project from the envelope.

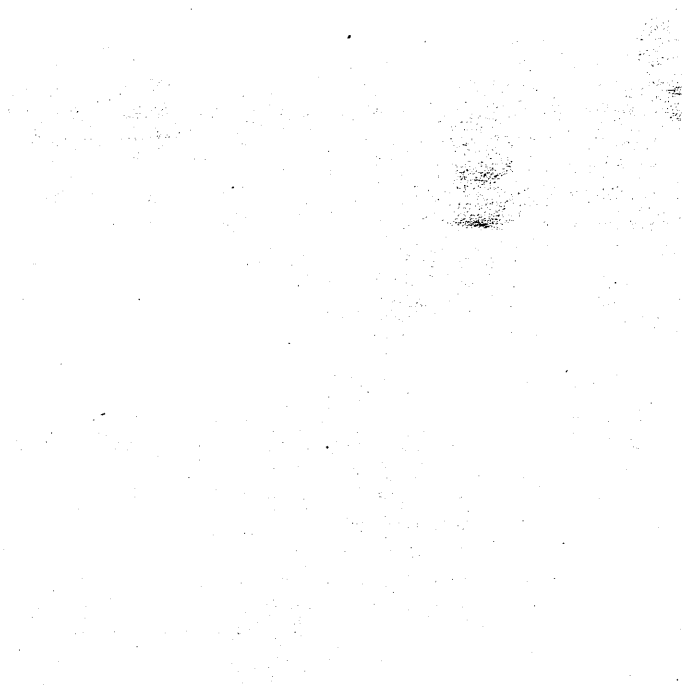




Basic Organisation of the VSV Genome

Figure 1.2

Physical map showing the basic organisation of the VSV genome. The size in nucleotides of the 3' and 5' terminal regions together with the five genes is indicated. The numbers in parenthesis represent the number of nucleotides not found in the transcription products (not drawn to scale).
(From Pringle, 1986 using data of McGeoch, 1979; Rose, 1980; Schubert et al, 1984; Gallione et al, 1981; Rose and Gallione, 1981).

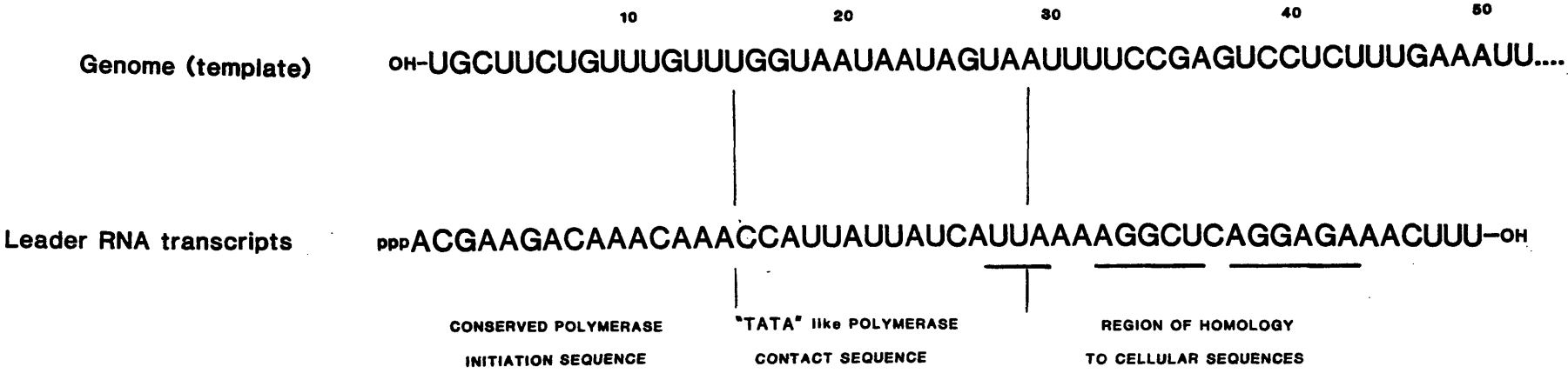


5′–pppACGAANACNANNAAACCA...–3′

N = A, C or G

Figure 1.3

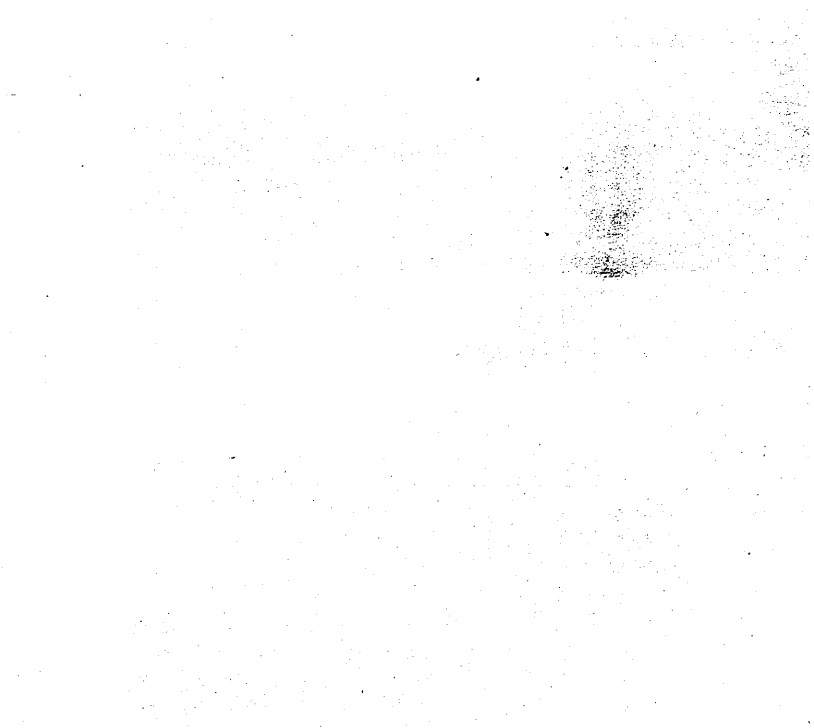
Examination of sequence data from five plus and minus strand leader RNAs revealed that 14 of the first 18 bases were conserved and the consensus sequence is shown opposite. There is a five times repeated A residue every three bases shown opposite. It was suggested that this pattern of A residues is important to encapsidation.
(Blumberg, 1983).



REGULATORY SEQUENCE DOMAINS OF THE VSV LEADER REGION

Figure 1.4

Functional domains of the leader region. The leader sequence is divided approximately into thirds by three sequences - the polymerase initiation and N protein sequence, the polymerase contact sequence and the region which is homologous to cellular 7S and Alu sequences.



SUMMARY OF VSV REPLICATION

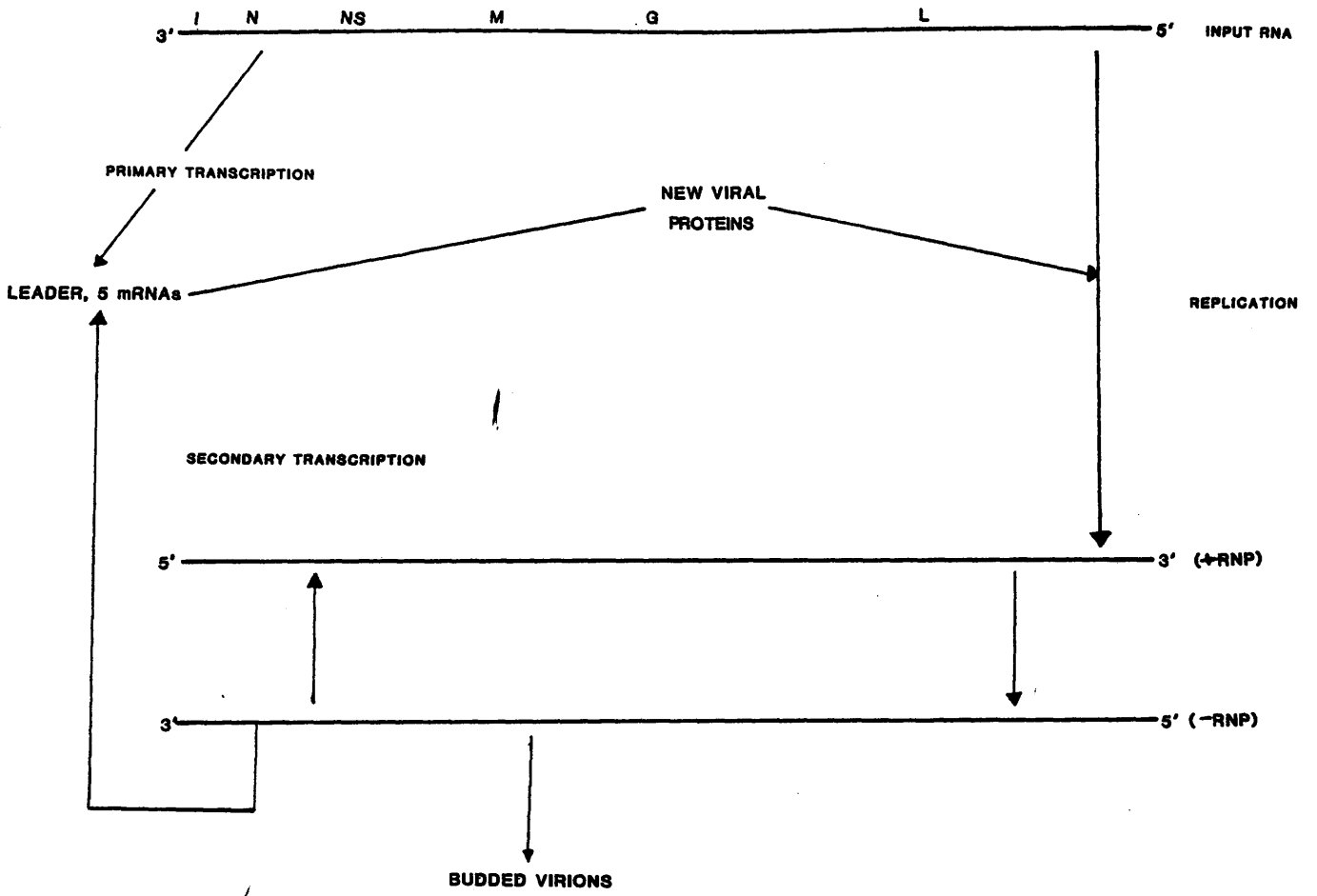


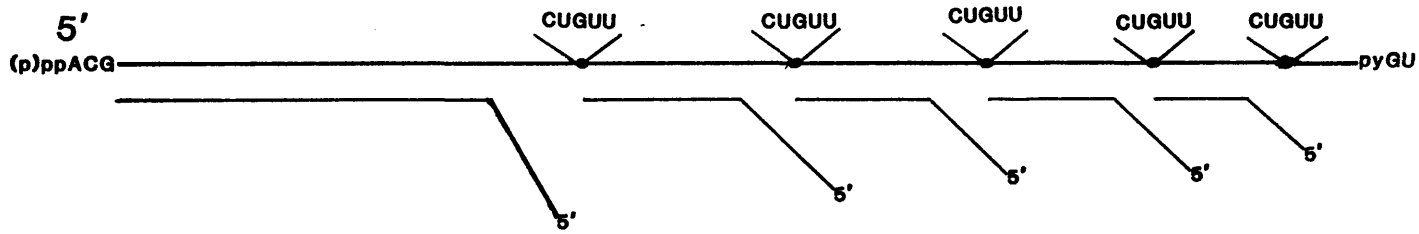
Figure 1.5

Schematic diagram of the VSV replication cycle (Emerson, 1985).

Rhabdovirus replication occurs in two stages. The negative sense genomic RNA is transcribed (primary transcription) from the extreme 3' end to generate the five viral mRNAs which are subsequently translated. When viral protein synthesis decreases and full length, plus sense RNA is synthesised. The plus sense RNA then acts as template for synthesis of full length negative strand RNA which is packaged into budding virions or used as template for subsequent mRNA synthesis (secondary transcription).

MODELS FOR VSV mRNA SYNTHESIS

1. MULTIPLE INITIATIONS



2. SINGLE INITIATION FOLLOWED BY CLEAVAGE

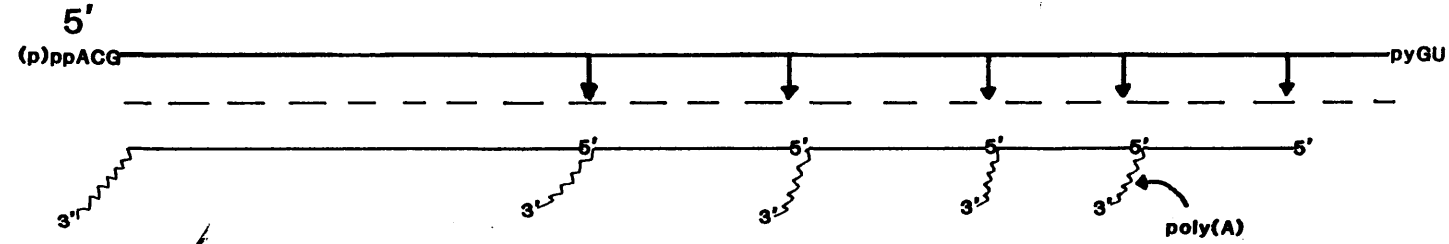


Figure 1.6

Models of VSV mRNA synthesis. Two models have been proposed to explain the monocistronic and sequential transcription of the mRNAs. In the multiple entry model individual promoters and polymerase entry sites are postulated for each gene.

The single promoter model proposes that polymerase enters the genome only at the 3' end. Monocistronic mRNAs are then generated by cleavage of a full-length precursor or by a stop/start mechanism which requires the polymerase to initiate and terminate transcription at gene boundaries. (Based on Banerjee, 1980).

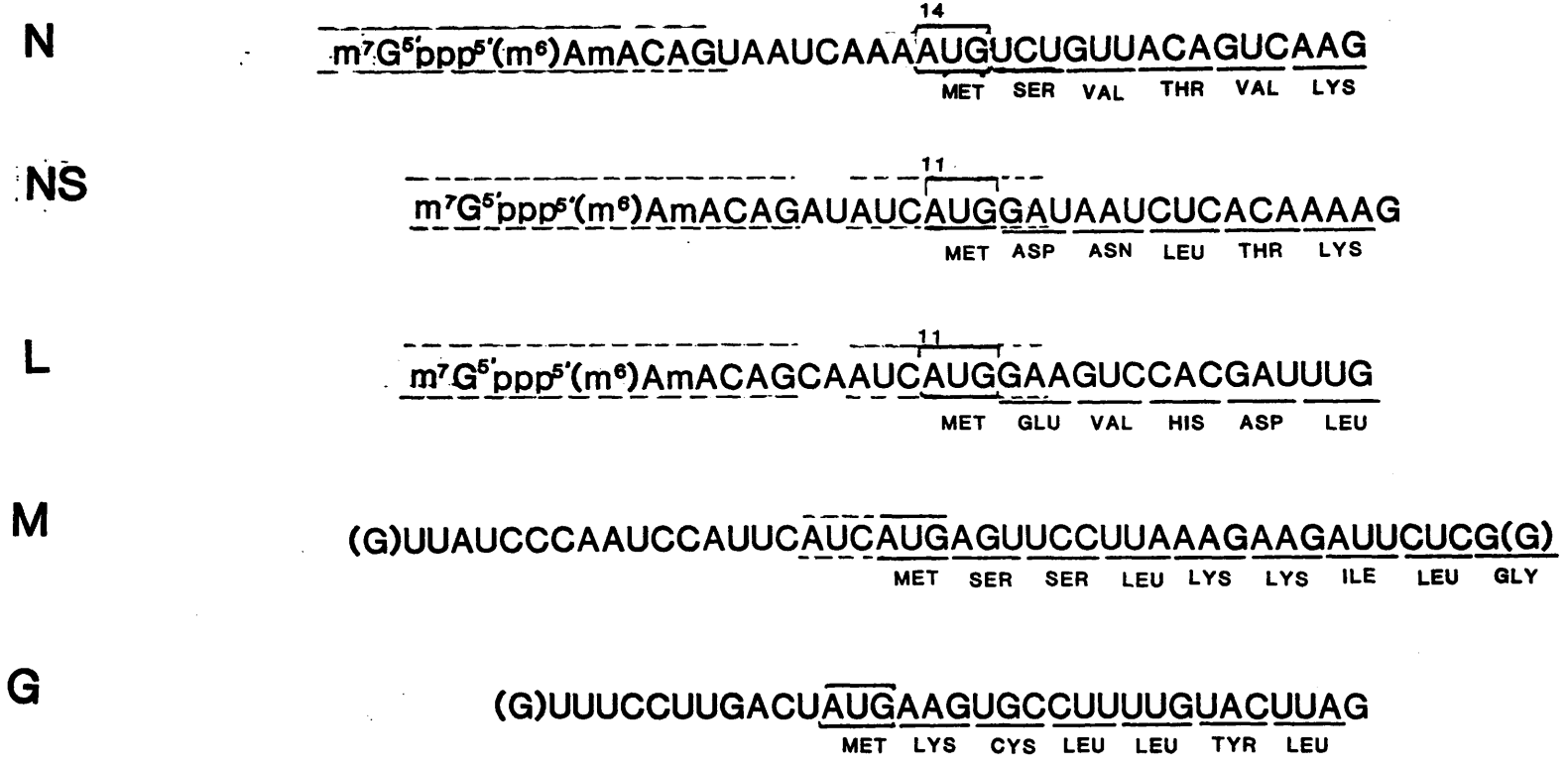

N
14
m7G5'ppp5'(m6)AmACAGUAAUCAAAAUGUCUGUUACAGUCAAG
MET SER VAL THR VAL LYS
NS
11
m7G5'ppp5'(m6)AmACAGAUAUCAUGGAUAAUCUCACAAAAG
MET ASP ASN LEU THR LYS
L
11
m7G5'ppp5'(m6)AmACAGCAAUCAUGGAAGUCCACGAUUUG
MET GLU VAL HIS ASP LEU
M
(G)UUAUCCCAAUCCAUUCAUCAUGAGUUCCUUAAAGAAGAUUCUCG(G)
MET SER SER LEU LYS LYS ILE LEU GLY
G
(G)UUUCCUUGACUAUGAAGUGCCUUUUGUACUUAG
MET LYS CYS LEU LEU TYR LEU

Figure 1.7

The complete sequences of the ribosome recognition sites from the five VSV mRNAs. Regions of homology are indicated by dashed boxes.

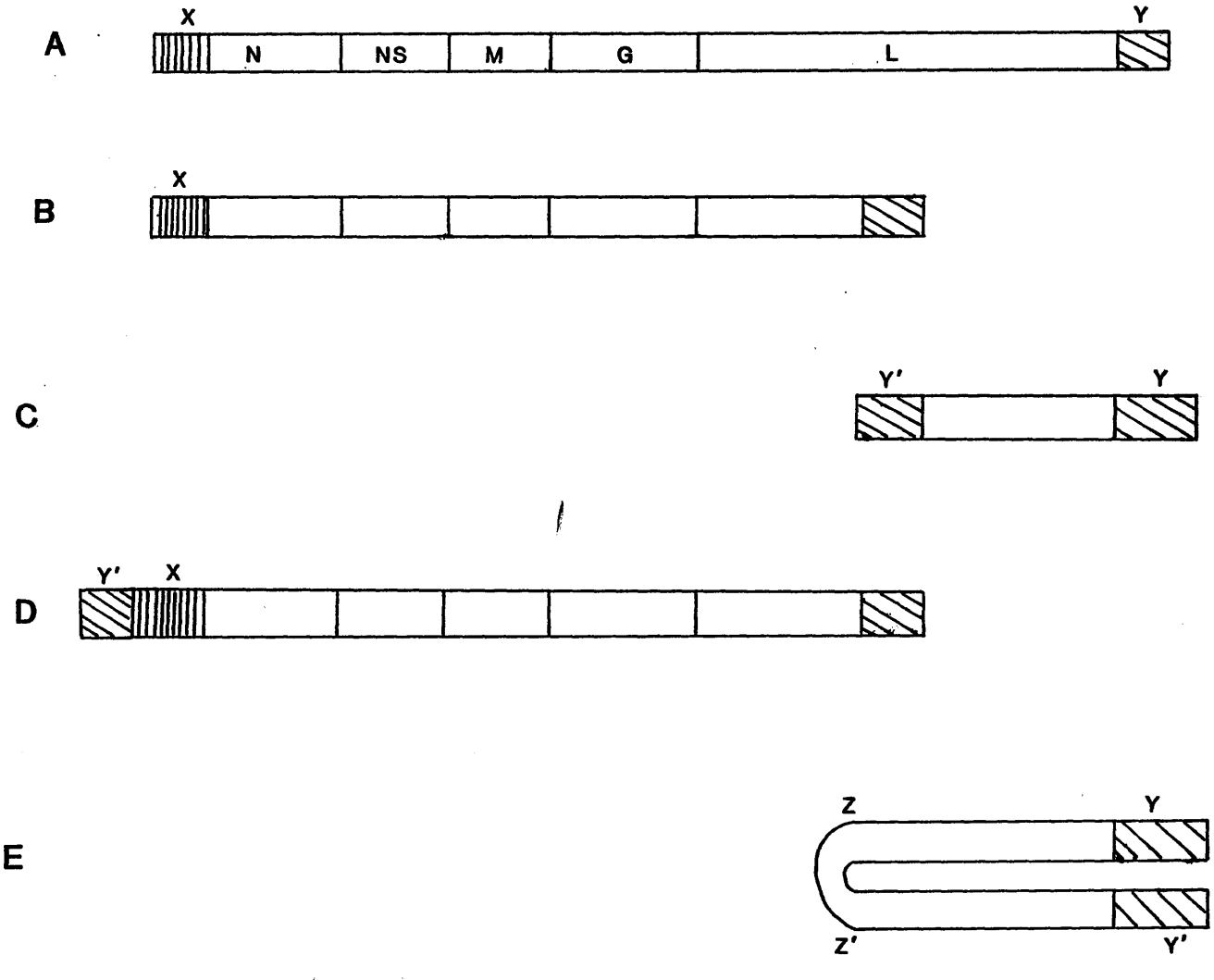

A
X
Y
N
NS
M
G
L
B
X
C
Y'
Y
D
Y'
X
E
Z
Y
Z'
Y'

Figure 1.8

Physical maps of VSV genomic RNA and the four classes of DI particle RNA.

A = VSV genome RNA

B = a simple deletion DI particle

C = panhandle DI particle

D = compound DI particle

E = snap-back DI particle

X and Y' = 3' ends

Y = 5' end

Y, Y' and Z, Z' = regions complementary to each other

N, NS, M, G and L = the 5 VSV mRNAs

The four classes of DI particle are shown in relation to the parental virus genome. The VSV genome is not drawn to scale. (Based on Lazzarini et al, 1981).

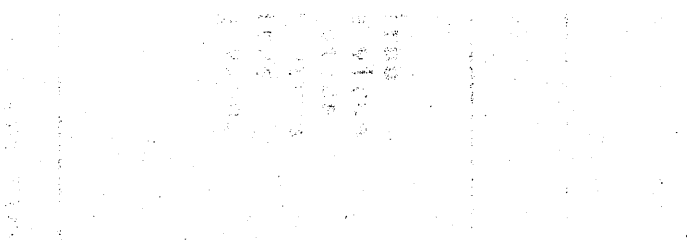


Table 1.1 Classification of the Family Rhabdoviridae

Family	Rhabdoviridae	
Genus	Vesiculovirus	Lyssavirus
Member viruses	VSV (Indiana and New Jersey) Chandipura virus Isfahan virus Piry virus Yug Bugdanovac virus La Joya virus Keuralibu virus Jurona virus	rabies virus Duvenhage virus Kotonkan virus Lagos bat virus Mokola virus Obodhiang virus
	Subgroup A Plant Viruses	Subgroup B Plant Viruses
	lettuce necrotic yellow virus broccoli necrotic yellow virus sonchus virus wheat striate mosiac virus	potato yellow dwarf virus eggplant mottled dwarf virus sonchus yellow net virus sowthistle yellow vein virus

Table 1.2 Subclassification of VSV

Virus	Vesicular stomatitis virus					
Serotype	Indiana				New Jersey	
Subtype	Indiana	Argentina	Brazil	Cocal	Concan	Hazelhurst
Strain	Mudd-Summers San-Juan Glasgow Orsey				Concan Ogden Guatemala	Missouri Hazelhurst

Table 1.3 Protein Composition of the VS Virion

Protein	Mass of Protein Present in Virion (Megadaltons)	Number of Molecules/ Virion
G	75.2	1205
N	59.6	1258
L*	12.1	50
NS*	11.7	466
M*	47.6	1826

* Calibration relative to N protein

Thomas et al (1985)

Table 1.4 Gene Assignments of the Complementation Groups of VSV Indiana and New Jersey Serotypes

Virus	Complementation Group	Assignment
VSV IND	I	L
	II	NS
	III	M
	IV	N
	V	G
	VI	(NS)
VSV NJ	A	N
	B	L
	C	M
	D	(G)
	E	NS
	F	L

( ) indicates that positive gene assignment has not been made

Pringle (1986)

Table 1.5 Animal RNA Containing Viruses for which the Complete Nucleotide Sequence of the Genome has been Determined

Virus Family	Virus	References
Rhabdoviridae	VSV	previously cited
Picornaviridae	poliovirus	Kitamura et al (1981) Racaniello & Baltimore (1981)
	human rhinovirus types 2 and 14	Callahan et al (1985) Skem et al (1985)
	hepatitis A virus	Najarian et al (1985)
Orthomyxoviridae	influenza virus A/PR/8/34	see Winter and Fields (1982)
Flaviviridae	yellow fever virus	Rice et al (1985)
Retroviridae	AIDS retrovirus	Muesing et al (1985) Ratner et al (1985) Sanchez-Pescador et al (1985) Wain-Hobson et al (1985)
	visna virus	Sonigo et al (1985)
	HTLV-II	Shimotohno et al (1985)
	bovine leukaemia virus	Sagata et al (1985)

Chapter 2

MATERIALS AND METHODS

1. MATERIALS

A. Chemicals, Reagents and Enzymes

Agarose, Dithiothreitol (DTT), Bovine serum albumin (BSA), Tris [hydroxymethyl]-aminoethane (Tris), tetracycline, chloramphenicol, lysozyme, 5-chloro-4-bromo-3-indolyl-β-D-galactose (XGal), isopropyl-D-thiogalactoside (IPTG) were all obtained from Sigma Chemical Company.

Caesium chloride, boric acid and sodium hydroxide were purchased from Koch-Light Laboratories.

Formamide was purchased from Fluka AG. Deoxyribonucleoside triphosphates, dideoxyribonucleoside triphosphates, E.coli DNA ligase were obtained from Pharmacia P-L Biochemicals.

Sephadex G-50 was purchased from Pharmacia Fine Chemicals A-B.

Ammonium persulphate and TEMED were supplied by Bio-Rad Laboratories.

PstI-cleaved G-tailed pBR322 DNA, RNase H, DNA polymerase 1, BSA (for use in cDNA synthesis) were supplied by Bethesda Research Laboratories.

Terminal transferase was obtained from Amersham. RNasin was purchased from Anglian Biotechnology. Reverse transcriptase was supplied by NBL enzymes. Ampicillin was purchased from Beecham Research Laboratories. Ethanol was supplied by James Burroughs Ltd. Nitrocellulose was supplied by Schleicher and Schuell GmbH.

All tissue culture reagents were supplied by Gibco-Biocult except calf serum which was prepared in the Institute of Virology.

All other reagents were of analytical grade and were obtained from BDH Chemicals LTd (Acrylamide and Methylenebisacrylamide were 'Electran' grade).

B. Solutions

The following solutions were used in the various experimental procedures:

NTE 100mM NaCl, 10mM Tris-HCl pH7.5, 1mM EDTA

PBS 170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4 pH7.2 (Dulbecco and Vogt, 1954)

SSC 150mM NaCl, 15mM tri-sodium citrate

TBE 90mM Tris-HCl, 90mM boric acid, 1mM EDTA

TE 10mM Tris-HCl pH7.5, 1mM EDTA

50 x Denhardt 1% Ficoll, 1% polyvinylpyrolidine, 1% BSA in 3xSSC

Agarose Gel Buffers: 0.5M Sørenson's citrate buffer (A) 0.5M disodium citrate; 40g NaOH, 105g citric acid for 1,000ml (B) 0.5N HCl.

To make 1,000ml 0.5M Sørenson's citrate buffer pH3.4, combine 460ml solution A and 540ml solution B when this 0.5M solution is diluted to 0.025M it renders a solution of pH3.5.

Running Buffer: 0.025M citrate buffer pH3.5.

Sample Buffer: 0.025M citrate buffer pH3.5, 6M urea, 20% (w/v) sucrose, bromophenol blue.

10M urea stock solution heated, deionised using Amberlite MB-1 resin and filtered immediately before use.

[^{35}S]-methionine (specific activity 900Ci/mmol)

C. Radiochemicals

All radioisotopes were obtained from Amersham International Ltd. The specific activities of those used were:

[γ-^{32}P]	ATP	5,000Ci/mmol
[α-^{32}P]	dATP	2,000-3,000Ci/mmol
	dCTP	2,000-3,000Ci/mmol
	dGTP	2,000-3,000Ci/mmol
	dTTP	2,000-3,000Ci/mmol

[^{32}P]-inorganic orthophosphate (carrier free) was supplied by the Western Infirmary, Glasgow.

D. Plasmid Vector and Bacteria

The vector used in all experiments was pBR322 an Escherichia coli (E.coli) plasmid which carries genes conferring resistance to tetracycline and ampicillin (Sutcliffe, 1978).

The host bacteria used for plasmid propagation were E.coli strain MC1061 (araD, Δ (ara,leu) 7697, galV$^-$, galK$^-$, h$^-$v$^-$, hsm$^+$, strA) (Casadaban and Cohen, 1980).

E. Bacterial Culture Medium

Bacteria were propagated in L-broth. This comprises 177mM NaCl, 10g/l bacto-peptone and 5g/l yeast extract pH7.5 (pre-sterilisation). Bacterial culture plates were L-broth containing 1.5% (w/v) agar. Both liquid media and agar were supplemented with ampicillin (50ug/ml) or tetracycline (15ug/ml) where appropriate.

2. METHODS

A. Growth of Cells and Virus

BHK-C13 cells are a continuous cell line derived from baby hamster kidneys (MacPherson and Stoker, 1962) and were supplied by the Cytology Department, Institute of Virology, Glasgow. The cells were grown in the Glasgow modification of minimal essential medium (MEM; Stoker and MacPherson, 1961) which was supplied as a 10x liquid concentrate. The concentrate was diluted in distilled water and supplemented with 100units/ml penicillin, 100units/ml streptomycin, 5mM L-glutamine and 2.75g/l $NaHCO_3$. Foetal calf serum was added to a final concentration of 10%.

MEM deficient in phosphate was supplied by the Media Department, Institute of Virology, Glasgow.

Vesicular stomatitis virus (VSV), New Jersey serotype (NJ) Missouri strain was first isolated from swine in 1943. Seed stocks were obtained from Dr J.F. Szilagyi. Virus stocks were grown in 2 litre burler bottles ($1-2x10^8$ cells).

Monolayers were infected at low multiplicity with a 10ml inoculum of MEM containing 2.5% calf serum. The virus was allowed to absorb for 1 hr at 31°C when a further 25ml of MEM, containing 2.5% calf serum, was added. Incubation was continued until a well advanced cytopathic effect was observed. This was usually 24 hr post-infection.

The three complementation group E mutants tsE1, tsE2 and tsE3 were originally isolated from wt VSV NJ strain Missouri after exposure to the base analogue mutagen 5-fluorouracil (5-FU) (Pringle et al, 1971). Stocks of these viruses as well as the revertant viruses tsE1R1

(Szilagyi and Pringle, 1979) and tsE3R1 (Evans et al, 1979) were also obtained from Dr J.F. Szilagyi.

B. Virus Purification

The medium from infected cells was decanted and clarified by centrifugation for 30 min at 2,500rpm in a Sorval RC-5B centrifuge using a GSA rotor. The virus was pelleted from the clarified medium through a 9ml glycerol cushion consisting of 30% (v/v) glycerol, 20mM Tris-HCl pH8.0, 1mM EDTA and 100mM NaCl. Centrifugation was for 90 min at 21,000rpm using the Du Pont 10x100 rotor in the Sorval OTD-50 ultracentrifuge. All centrifugation steps were carried out at 4°C. The virus pellets were resuspended in an appropriate volume of 20mM Tris-HCl pH8.0, 1mM EDTA, 0.1m NaCl. The virus was aliquoted and stored at -70°C.

The virus stocks were titrated using standard procedures. Monolayers of BHK-C13 cells in 50mm tissue culture dishes were inoculated with serial dilutions of virus in MEM containing 10% calf serum. Absorption was carried out at 31°C for 1 hr. The inoculum was removed and 5ml of an agar overlay (310ml Eagle's A, 60ml Eagle's B without phenol red, 125ml 3.6% Difco agar, 7ml calf serum). The plates were incubated for 2 days at 31°C. The agar overlay was removed and the cells fixed with 10% cidex for 2 hr. Clear plaques were counted after staining with Giemsa for 1 hr and the concentration of the virus calculated.

The titre of the stock of wt VSV NJ used in this work was 3×10^{11}pfu/ml.

C. Purification of Genomic RNA

VSV genomic RNA was purified using a modification of the method described by Palese and Schulman (1976) for influenza virus RNA. A suspension of VSV was incubated for 15 min at 56°C in 10mM Tris-HCl pH7.4, 10mM KCl, 1.5mM $MgCl_2$ (RSB), 0.2% SDS and 500ug/ml proteinase K. The solution was then made up to a final concentration of 0.5% (w/v) SDS, 140mM LiCl, 10mM Na-acetate pH4.9 and an equal volume of phenol equilibriated with RSB was added and incubated at 56°C for 5 min with continual mixing. The mixture was vigorously shaken for 15 min after the addition of an equal volume of chloroform and then centrifuged for 15 min at 3,000rpm in an MSE Coolspin centrifuge. The aqueous phase was removed and the phenol-chloroform extraction repeated. The RNA in the aqueous phase was precipitated with 2.5 volumes of ethanol. The RNA was recovered by centrifugation at 10,000rpm for 15 min in a Sorval SS34 rotor and the pellet, which formed a film on the side of the tube, resuspended in 0.4ml 0.3M Na-acetate. The RNA was reprecipitated with ethanol in a 1.5ml Eppendorf tube, centrifuged at 12,000g for 10 min in an Eppendorf centrifuge and the pellet washed with 70% (w/v) ethanol. The RNA was lyophylised and stored at -70°C.

D. Extraction of Total Cellular RNA

Monolayers of cells grown in 2 litre burler bottles were infected with virus as previously described. However, the medium contained 10ug/ml Actinomycin D. At 6 hr post-absorption the cells were removed from the burlers by trypsin treatment and centrifuged at 3,000rpm for 5 min at

4°C. A modification of the method first described by Chirgwin et al (1979) was used to extract total cellular RNA. The cell pellet was resuspended in cold PBS and pelleted at 3,000rpm for 5 min at 4°C. The cell pellet was lysed in 5 volumes of 6M guanidinium isothiocyanate, 5mM Na-citrate pH7.0, 100mM β-mercaptoethanol, 0.5% sarcosyl. One gram caesium chloride was added to each 2.5ml of cell lysate. The mixture was layered onto a 1.2ml cushion of 5.7M caesium chloride in 100mM EDTA and centrifuged at 35,000rpm for 12 hr at 20°C in a Beckman SW50.1 rotor. The supernatant was discarded and the RNA, which formed a pellet, resuspended in 10mM Tris-HCl pH7.4, 5mM EDTA, 1% (v/v) SDS and extracted once with an equal volume of a 4:1 mixture of chloroform and 1-butanol. The organic phase was re-extracted once more with an equal volume of the Tris/EDTA/SDS buffer. The aqueous phases were then combined and the RNA precipitated in the presence of 0.3M Na-acetate and 2.5 volumes of ethanol. After centrifugation for 10 min at 12,000g the RNA was resuspended in water and reprecipitated with ethanol. The RNA was stored at -70°C.

E. cDNA Synthesis

cDNA was synthesised using a method based on that of Gubler and Hoffman (1983). Synthesis of first strand cDNA was carried out in a reaction volume of 40ul containing 50mM Tris-HCl pH8.3, 10mM $MgCl_2$, 10mM DTT, 20mM dNTPs, 10uCi [α-^{32}P] dNTPs, 1ug oligo $(dT)_{12-18}$, 50ug RNA and 40 units reverse transcriptase. The reaction was incubated at 42°C for 60 min. Prior to being added to the reaction the RNA and primer were boiled together for 90 sec and then chilled

on ice in order to denature the RNA. The reaction was stopped by the addition of EDTA to 20mM. The products were extracted with phenol/chloroform and twice precipitated with ethanol out of 2M ammonium acetate as described by Okayama and Berg (1982). This procedure removes most of the unincorporated triphosphate. Half of the single stranded cDNA was used in the second strand reaction. At this stage the single strand cDNA existed in the form of a cDNA:RNA hybrid. In the second strand reaction RNase H, which has a specificity for RNA that is part of an RNA:DNA hybrid, was used to digest the RNA. RNase digestion leaves small fragments which can be used by DNA polymerase I as primers for second strand cDNA synthesis. DNA ligase was included in this reaction but is has been reported that it is not absolutely necessary (Gubler and Hoffman, 1983). The second strand reaction consisted of half of the product of first strand synthesis, 20mM Tris-HCl pH7.5, 5mM $MgCl_2$, 10mM $(NH_4)_2$ SO_4, 100mM KCl, 0.15mM β-NAD, 50ug/ml BSA, 40uM dNTPs, 8.5 units/ml of E.coli RNase H, 230 units/ml DNA polymerase I and 0.4ug/100ul E.coli DNA ligase. Incubations were sequentially 60 min at 12°C and 60 min at room temperature. EDTA was again added to 20mM in order to stop the reaction. The product was extracted with phenol/chloroform and precipitated twice from ammonium acetate as previously described.

F. Insertion of cDNA into pBR322

The double stranded cDNA was tailed with dC residues and then annealed to G-tailed PstI-cut pBR322. The conditions for the C-tailing reaction had been established

by Dr Richard M. Elliott such that the particular batch of terminal transferase used would add 20-25 dC residues. The C-tailing reaction was carried out at 37°C for 1 hr in a 50ul volume containing 5 x terminal transferase buffer (BRL), 10mM dCTP and 30 units terminal transferase. The reaction was terminated by heating at 70°C for 10 min. The G-tailed pBR322 was commercially produced (BRL) and had an average of 24 G-residues per 3' end (Lot Number 41111).

In order to find the maximum number of clones obtainable from the input cDNA, a series of 50ul reactions were set up in which varying amounts of C-tailed cDNA were annealed to 25ng G-tailed vector in 150mM NaCl, 10mM Tris-HCl pH7.5, 1mM EDTA for 90 min at 58°C. The annealing mixtures were each used to transform E.coli MC1061 cells directly and the number of tetracycline resistant colonies was noted. The remaining cDNA was annealed under optimal conditions.

G. Transformation of Bacterial Cells with Plasmid DNA

Competent cells were produced by the method of Dagent and Ehrlich (1979). Fifty millilitres of L-broth was seeded with 0.5ml of an overnight culture of E.coli MC1061 and grown at 37°C with vigorous shaking until the OD_{590} reached a value of 0.2 (usually 2 hr). The cells were chilled on ice for 10 min, pelleted at 3,000rpm for 10 min and resuspended in 25ml ice cold 100mM $CaCl_2$. The cell suspension was incubated on ice for 1 hr and the cells centrifuged as above. The resultant pellet was resuspended in 0.5ml 100mM $CaCl_2$ and stored at 4°C overnight before use. The number of transformants decrease with time due to the

decrease in cell viability (Dagent and Ehrlich, 1979).

The plasmid DNA (in a maximum volume of 50ul in TE) was mixed with 100ul of competent cells, stored on ice for 30 min and then heat shocked at 42°C for 2 min; typically 400ul L-broth was added to the DNA/cell suspension and the mixture incubated at 37°C for 1 hr. It was then plated onto agar containing tetracycline (15ug/ml). The plates were incubated at 37°C overnight.

H. Preparation of Plasmid DNA

Plasmid DNA was isolated using the alkali lysis method described by Ish-Horowicz and Burke (1981). Bacterial cells were harvested by centrifugation at 6,000rpm for 10 min at 4°C in a Sorval GSA rotor. In order to disrupt the cell walls the pellet obtained from 500ml of culture was resuspended in 10ml 50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA, 5mg/ml lysozyme, and incubated at room temperature for 5 min. The cells were then lysed with 20ml 0.2N NaOH, 1% (w/v) SDS, which was added with gentle mixing. The samples were placed on ice for 10 min. Fifteen millilitres of pre-cooled 5M K-acetate pH4.8 were then added, the samples mixed vigorously and incubated for a further 10 min on ice (5M K-acetate pH4.8 had a final concentration of 3M K-acetate plus 2M acetic acid; Ish-Horowicz and Burke, 1981; Maniatis et al, 1982). The crude lysate was centrifuged at 18,000rpm for 20 min at 4°C in a Sorval SS34 rotor to pellet chromosomal DNA and cell debris. Plasmid DNA was precipitated from the supernatant by the addition of 0.6 volumes of isopropanol for 15 min at room temperature and recovered by centrifugation at 18,000rpm in a Sorval SS34

rotor for 30 min at 15°C. The resulting pellet was washed with 70% (v/v) ethanol and dried in a vacuum dessicator.

Supercoiled plasmid DNA was purified away from RNA, host cell DNA, linear and open forms of plasmid DNA by isopycnic banding in caesium chloride gradients: the plasmid DNA pellet was resuspended in 8ml TE buffer to which was added 8g solid caesium chloride and 0.8ml ethidium bromide solution (10mg/ml in water). Centrifugation was carried out at 45,000rpm for 36 hr at 15°C in a Beckman Ti50 rotor. The lower band, as visualised under long wave (365nm) UV illumination, containing the supercoiled plasmid DNA was removed and the ethidium bromide extracted with isopropanol saturated aqueous 5M NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA. Caesium chloride was removed from the preparation by dialysis against TE buffer and DNA recovered by ethanol precipitation out of 0.3M Na-acetate. Plasmid DNA was resuspended in sterile distilled water as appropriate.

I. Analytical DNA Extraction ('Miniprep')

Analytical DNA extractions were carried out as described by Ish-Horowicz and Burke (1981): 1.5ml of an overnight culture was harvested by centrifugation for 1 min at 12,000g in a microfuge tube. The pellet was resuspended in 100ul (cold) 50mM glucose, 25mM Tris-HCl pH8.0 EDTA, 4mg/ml lysozyme and incubated for 5 min at room temperature. Two hundred microlitres 0.2N NaOH, 1% (w/v)SDS was added, mixed gently and the sample placed on ice for 5 min. One hundred and fifty microlitres of 5M K-acetate pH4.8 was then added, the sample mixed thoroughly and incubated on ice for a further 5 min. After centrifugation at 12,000g for 5 min

the supernatant, containing the plasmid DNA, was extracted with an equal volume phenol/chloroform (1:1 v/v). The DNA was precipitated with ethanol for 2-5 min at room temperature and centrifuged at 12,000g for 5 min. The DNA pellet was washed with 70% (v/v) ethanol, dried and resuspended in 50ul TE buffer containing RNase A (20ug/ml): 10ul aliquots were subsequently digested with restriction endonucleases and analysed by gel electrophoresis.

J. Bacterial Cell Culture

(i) Seed Stocks

Seed stocks of bacteria were prepared from 10ml cultures which had been inoculated with a single bacterial colony and grown overnight at 37°C with shaking to saturation density. One millilitre of the overnight culture was removed, diluted with an equal volume of 80% (v/v) glycerol and stored at -20°C.

(ii) Growth of Large Scale Cultures

The following method was used to propagate one litre cultures of plasmid carrying bacteria: 10-20ul of glycerol stock was inoculated into 10ml L-broth containing tetracycline (15ug/ml) and grown overnight with shaking.

Five millilitres of this culture was inoculated into one litre of tetracycline containing L-broth (15ug/ml) and the bacteria grown at 37°C with shaking for 24 hr.

Usually after 9 hr chloramphenicol was added (170ug/ml final concentration) to promote amplification of the plasmid (Clewell, 1972).

K. DNA Sequencing

DNA was sequenced using the chain terminator/dideoxy method (Sanger et al, 1977, 1980). There have been many improvements made in the last 5 years in this field so that now the main rate limiting step to the amount of sequence data generated in one day is the number of gels which can be run. Because the cDNA to be sequenced was relatively short (less than 900 bases) restriction enzyme cleavage was used to generate DNA fragments. It must be stated that this method gives an uneven distribution of fragments for cloning and sequencing and that DNase digestion (Anderson, 1981) or more recently sonication (Deininger, 1983) are more efficient methods for generating the random DNA fragments required for this method.

The various steps involved in this method of sequencing are outlined below.

(i) Ligation

The replicative form of M13mp9 or M13mp18 (Messing and Vieira, 1982; Yanisch-Perron et al, 1985) was used in all cloning experiments. Linearised vector (20ng) was incubated with various concentrations of DNA insert (10-30ng) in a final volume of 10ul at 15°C overnight in 10mM Tris-HCl pH7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP. For ligations involving flush or complementary ends 2 units of T4 DNA ligase were used. M13mp9 and M13mp18 were linearised with SmaI and dephosphorylated using calf intestinal alkaline phosphatase (BRL).

Purified DNA insert was digested with an appropriate restriction enzyme. When ligating insert fragments into

vector without complementary ends the digest was made flush-ended by incubating the fragments with 0.4mM dNTPs, 33mM Tris-acetate pH7.5, 66mM K-acetate, 10mM Mg-acetate, 0.5mM DTT and one unit of T4 DNA polymerase at 37°C for 1 hr.

(ii) Transfection

The transfection method was essentially that of Cohen et al (1972). E.coli JM101 (Messing et al, 1977) grown to an optical density (OD) of 0.3 at 630nm was centrifuged at 6,000rpm for 10 min at 4°C. The pellet was resuspended in 0.5 volumes 75mM $CaCl_2$ and the cells left in ice for 30 min. The cells were again centrifuged at 6,000rpm for 10 min at 4°C. They were then resuspended in 0.1 volumes 75mM $CaCl_2$. Aliquots of 0.2ml $CaCl_2$ treated cells were added to the ligation mix and left on ice for 40 min. The suspension was then incubated at 42°C for 2 min and added to 3ml top agar at a similar temperature containing 20ul 100mM IPTG and 20ul 2.5% XGal (dissolved in dimethyl formamide). The mixture was added directly to 90mm L-broth agar plates, the top agar allowed to set and the plates incubated overnight at 37°C.

(iii) Growth of Recombinant M13 Clones for Sequencing

The bacteriophage M13 has been genetically engineered to provide a variety of restriction enzyme sites for cloning and also to provide a detection marker for identifying phage with inserted DNA. The M13 intergenic region contains part of the β-galactosidase (Z) gene and its operator and promoter. The Z gene fragment contains the coding region for the first 145 amino acids of β-galactosidase

interrupted, in frame, by the insertion of synthetic DNA containing restriction enzyme cloning sites. Induction with IPTG causes the peptide produced to complement the defective gene on the F episome of the E.coli JM101. XGal is a lactose substrate analogue and, once added, it is hydrolysed by the functional β-galactosidase to the blue compound bromochloroindole which confers a blue colour to the perimeter of the phage plaques. However, if the peptide coding region is interrupted, by cloning DNA fragments into the restriction enzyme sites then a functional β-galactosidase is not produced and the plaques are colourless. Thus plaques containing inserts can easily be identified and grown up.

One millilitre of a standing culture of E.coli JM101 was added to 100ml 2YT broth (for 1 litre add 10g Bacto tryptone, 10g yeast extract and 5g NaCl). This was dispensed in 1.5ml aliquots into 20ml universal bottles. White plaques were picked using a toothpick into these bottles and incubated at 37°C for 5-6 hr with shaking. The broth was transferred to 1.5ml Eppendorf tubes and cells pelleted by centrifugation at 12,000g for 5 min. The supernatant was poured into another 1.5ml tube and 150ul of 2.5m NaCl, 20% PEG 6,000 added to precipitate the phage. After incubating the mixture at 12,000g for 5 min. The supernatant was completely removed. The pellet was resuspended in 100ul TE buffer and extracted with 0.5 volume of phenol/chloroform and ethanol precipitated. The pellet was resuspended in 20ul TE buffer and stored at -20°C.

(iv) Sequence Analysis of M13 Clones

Three microlitres of the single stranded DNA template was annealed to 2.5ng of a commercial oligonucleotide primer (GTAAAACGACGGCCAGT; Collaborative Research) in 10mM Tris-HCl pH8.5, 10mM $MgCl_2$ in a final volume of 10ul at 37°C for 30 min. One unit of the large fragments of DNA polymerase I (Klenow polymerase) was added to each clone (the concentrated Klenow polymerase stock was diluted in 1mM Tris-HCl pH8.5, 1mM $MgCl_2$) and the DNA aliquoted in 2ul amounts into four 1.5ml eppendorf tubes corresponding to the specific A, G, C and T reactions of each clone. An equal volume of nucleotide mix (Figure 2.1) was added and the reaction continued for 20 min at room temperature. The reaction was chased by the addition of 2ul of 11.5uM dATP for 45 min at room temperature. The reaction was stopped by the addition of formamide dyes and the samples electrophoresed on the same day. Prior to loading on the gel, all samples were boiled for 1 min.

(v) Sequence Gel Electrophoresis

The in vitro synthesised complementary strands formed during the sequence reactions have a common 5' DNA sequence (the synthetic oligonucleotide primer). After denaturation from the parent template strand they can be separated by virtue of their differing chain length by polyacrylamide gel electrophoresis.

Two types of gel were used to electrophorese samples - single strength and buffer gradient gels. The single strength gels were run for 2 hr or 4 hr and were used to clearly resolve bases which ran near the bottom of the gel.

Approximately 100-150 bases could be read from these single strength gels whereas 150-200 could routinely be read from the buffer gradient gels.

High resolution sequencing gels were used as outlined by Sanger and Coulsen (1978). Electrophoresis was carried out in vertical gels of 40x20x0.3cm. Spacers and gel combs were cut from Plastikard. The teeth were 2.5mm wide, 1.5mm apart and 3mm deep. Later sharks-tooth combs (obtained from BRL) were used. Plastikard was initially washed in mild detergent to prevent inhibition of polymerisation of the gel. The notched plate was treated with siliconising agent (2% dimethylide chlorosilane in 1,1,1-trichloroethane). The plain plate was treated with 0.05% siliconising agent (Wackersilicone) and 0.3% acetic acid in ethanol (Garoff and Ansorge, 1981). This treatment encourages sticking of the gel to only one plate when dismantling on completion of electrophoresis.

The gel contained 9M urea, 6% (w/v) acrylamide crosslinked with bis-acrylamide in the ratio 20:1. Gels were polymerised in the presence of 0.25% (w/v) ammonium persulphate, 0.01% (v/v) TEMED and electrophoresed in either 0.5X or 1xTBE (pH8.3 or 8.8) at a constant power of 40 watts for 2-4 hr.

TBE gradient gel electrophoresis was essentially as outlined by Biggin et al (1983). A gradient of 0.5% to 2.5% TBE was used. The top mix was 0.5xTBE, 6% acrylamide, 9M urea and the bottom mix was 2.5xTBE, 6% acrylamide, 9M urea, 5% sucrose (w/v) and 0.1% bromophenol blue (w/v). When preparing the gel 7ml of bottom mix and 50ml of top mix were polymerised in the presence of ammonium persulphate and

TEMED as described. Six millilitres of bottom mix were taken up in a 10ml pipette followed by 6ml of top mix. A gradient was prepared by allowing the introduction of air bubbles into the mixture. The gradient was allowed to settle in the gel sandwich and the remainder of the top gel mix added. The gel was electrophoresed at a constant power of 40 watts.

(vi) Autoradiography

After electrophoresis the urea was removed from the gel and the DNA fixed in the gel by soaking in 10% acetic acid for 30 min. The gel was then dried down on the plain plate by heating at 125°C for 1.5 hr. Autoradiography was carried out against dried down sequencing gels using X-Omat S-film at room temperature typically overnight. The autoradiographs were developed automatically using a Kodak X-Omat Processor Model ME-1.

L. Visualisation of Nucleic Acid

A number of techniques were used for the visualisation of unlabelled nucleic acid.

(i) Ethidium Bromide Staining

Because ethidium bromide intercalates with DNA the DNA can be visualised by transmitted UV light (long wave 365nm or short wave 280nm). Short wave illumination although more sensitive (2ng double stranded DNA can be detected as opposed to 10ng with long wave) results in cleavage of DNA molecules and can therefore be used solely for analytical procedures. Polyacrylamide gels were soaked in a solution

of ethidium bromide (0.5ug/ml) for 30-60 min after electrophoresis. Gels were photographed on a Polaroid camera through a red filter.

(ii) Silver Staining

The method of Whitton et al (1983) was used for the silver staining of RNA. A polyacrylamide gel prepared on a sand-blasted plate was soaked for 30 min (minimum time) in 10% (v/v) acetic acid, 25% (v/v) ethanol and for a further 30 min (minimum) in 0.5% (v/v) acetic acid, 10% (v/v) ethanol. The gel was placed in a solution of 10mM silver nitrate for 2 hr then washed three times in deionised water. The gel was then soaked in 0.75M NaOH containing 0.3% (v/v) formaldehyde and 87.5mg/l sodium borohydride (reducing solution) until the DNA bands were stained. The silver ions are reduced to their elemental form and bind to the nucleic acid. The reducing reaction was stopped before the background became too dark by placing the gel in 5% (v/v) acetic acid.

M. Recovery of DNA from Gel Slices

(i) Agarose

DNA fragments were recovered from agarose by electroelution. The gel slice was placed in a dialysis bag with 800ul 1xTBE and electroeluted for 2-3 hr at 100v or overnight at 50v in 1xTBE. The polarity was then reversed for 2 min to release DNA from the wall of the dialysis tubing and the buffer removed. The eluate was passed through a disposable cellulose acetate filter (Schleicher and Schuell, BA85) to remove any fragments of agarose. The

DNA was ethanol precipitated. The pellet was resuspended in sterile distilled water and extracted once with phenol/chloroform, twice with an excess of diethylether before being precipitated with 0.1 volume 3M Na-acetate and 2.5 volumes ethanol.

(ii) Polyacrylamide

DNA was purified from polyacrylamide gels by the crush-soak method: a hole was punched in the bottom of a 0.75ml microfuge tube using a syringe needle and the gel slice placed inside the vial which was in turn placed inside an intact 1.5ml microfuge tube. The tubes were centrifuged at 12,000g for 10 sec and the gel slice exuded through the hole to form a slurry at the base of the 1.5ml tube. The slurry was resuspended in 400ul of elution buffer (500mM NH_4-acetate, 1mM EDTA) and incubated at 37°C overnight. Residual polyacrylamide fragments were removed by passing the eluent through a siliconised glass wool plug at the base of a 1ml pipette tip. The DNA was recovered by ethanol precipitation and purified by extraction once with phenol/chloroform and twice with diethylether before being precipitated with ethanol.

N. Identification of Recombinant Plasmids with Viral Specific Inserts

The plasmid vector pBR322 contains two antibiotic resistance genes, ampicillin (amp^R) and tetracycline (tet^R) (Sutcliffe, 1978). The PstI cleavage site is located within the ampicillin resistance gene and therefore an Amp^S, Tet^R phenotype is indicative of a DNA insert in pBR322 at that

site. Colonies were replica-plated onto ampicillin and tetracycline containing agar to screen for those with recombinant plasmids.

Identification of viral specific recombinant plasmids was by in situ colony hybridisation (Grunstein and Hogness, 1975) using [α-^{32}P] labelled, alkali-treated, genomic RNA as a probe. In later experiments gene specific inserts from recombinant plasmids were used as hybridisation probes.

Colonies with Amp^S, Tet^R phenotype were replica-plated onto agar plates containing the appropriate antibiotic, grown at 37°C overnight and transferred to a nitrocellulose membrane filter (Schleicher and Schuell, BA85) by placing the filter onto the plate and peeling back gently. The bacteria were prepared for colony hybridisation in the following way:

(a) the filter was placed (colony side up) in turn onto two layers of Whatman 3MM paper saturated with 0.5M NaOH (10 min), 1M Tris-HCl pH8.0 (10 min, repeated twice) and 10xSSC (10 min), to lyse the cells and denature the DNA.

(b) the filter was air dried, submerged in chloroform (twice), air dried, washed in 10xSSC and baked at 80°C for 2 hr in a vaccum oven.

Prehybridisation was performed at 42°C for at least 2 hr in 20ml/3 filters of 5xSSC, 1xDenhardt 0.02% (w/v) Ficoll, 0.02% (w/v) BSA, 0.02% polyvinylpyrolidone, 50% formamide, 100ug/ml sheared herring sperm DNA. The solution was removed and hybridisation to an appropriate ^{32}P-labelled probe carried out in 5ml/3 filters of 50% (v/v) formamide, 5xSSC, 1 x Denhardt, 100ug/ml sheared herring sperm DNA. The DNA probes were heat denatured at 100°C for 5 min and

quick-chilled prior to hybridisation. Non-specifically bound radioactivity was removed from the filter by washing sequentially in 100ml 3xSSC at 42°C for 1 hr and 1xSSC, 0.1% (w/v) SDS for 1 hr at room temperature. The filter was dried and colonies containing plasmids with viral inserts detected by autoradiography. Appropriate controls were included on each filter.

Genomic RNA extracted from VSV was incubated in 0.1M NaOH for 1 hr on ice after which the alkali was neutralised with 0.1 volume of 1M acetic acid. The RNA was recovered by ethanol precipitation in the presence of 0.3M Na-acetate and the RNA 5'-end labelled in a 20ul reaction containing 60mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 15mM DTT, 1mM spermidine, 400ug/ml BSA, 30uCi [α^{32}P] ATP and 2 units T4-polynucleotide kinase. The reaction mixture was incubated at 37°C for 1 hr and the unincorporated [α^{32}P] ATP separated from the labelled RNA by Sephadex G-50 chromatography.

O. Northern Blot Analysis of Intracellular RNA

(i) Gel Electrophoresis of Total Cellular RNA

Unfractionated RNA from infected and uninfected cells was separated on 1.5% agarose horizontal slab gels containing 6M urea and 0.025M citrate buffer pH3.5 adapted from the method of Wertz and Davis (1979).

Each gel consisted of 3g agarose, 70ml distilled water, 10ml 0.5M citrate buffer pH3.5 and 120ml 10M urea and was prepared by melting the agarose in the water in a microwave oven, then adding the citrate buffer and finally the urea which had been heated, deionised and filtered

immediately prior to use. Gels were poured at 4°C on 26.5x16.5cm glass plates in a horizontal slab gel apparatus and left to solidify for 1 hr.

Samples were made up to 50ul with buffer. Electrophoresis was for 18 hr in the cold at 100v with rapid recirculation of the running buffer.

Urea was removed from the gel by three 30 min washes in running water at room temperature with gentle agitation.

(ii) Transfer of RNA to Nitrocellulose

Transfer of RNA to nitrocellulose was essentially by the method described by Thomas (1980). After washing, the gel was soaked in 20xSSC for 30 min and placed onto two sheets of Whatman 3MM paper saturated with 10xSSC. The gel was then covered with a sheet of nitrocellulose (Schleicher and Schuell, BA85) which had been equilibriated with 20xSSC, followed by two sheets of Whatman 3MM paper, a 10cm stack of paper towels and a 1kg weight. Transfer was complete in 12 hr. The nitrocellulose filter was washed in 6xSSC and baked at 80°C for 2 hr in a vacuum oven.

(iii) Hybridisation to Nick-translated ^{32}P-labelled DNA

The filter was cut into strips as required and pre-hybridised for at least 3 hr in 100ml solution of 50% formamide, 5 x Denhardt buffer, 5xSSC. Hybridisation to an appropriate ^{32}P-labelled nick-translated probe was performed in 5ml 50% formamide, 5 x Denhardt, 5xSSC. The strips were washed in 100ml 3xSSC, 0.1% (w/v) SDS at 42°C for 1 hr, then 1xSSC, 0.1% (w/v) SDS at room temperature. The filters were dried and autoradiographed.

(iv) In vivo synthesis of ^{32}P-labelled RNA

^{32}P-labelled RNA synthesised in vivo was used as marker. BHK-C13 cells grown in 80cm^2 tissue culture flasks (approximately 1x10^{7}cells/flask) were infected at a multiplicity of 10pfu/cell at 4°C and allowed to adsorb at this temperature for 1 hr. The medium contained 10ug/ml actinomycin D (actinomycin D remained at this concentration throughout the experiment). The medium was removed and replaced with phosphate deficient medium containing 1mCi [^{32}P]-orthophosphate. Incubation at 31°C was continued for 6 hr. The cells were harvested by the method of Collins et al (1978): after removal of the radioactive solution the cell monolayers were washed once with ice cold 0.1M NaCl, 0.01M Tris-HCl pH8.8, and lysed by adding 1ml cold solubilising buffer (0.1M NaCl, 0.01M Tris-HCl pH8.8, 2mM EDTA, 1% Triton X100, 0.5% sodium deoxycholate, 1% β-mercaptoethanol). The cell extract was removed, centrifuged at 15,000rpm for 5 min at 4°C to remove nuclei and cell debris. The supernatant was adjusted to 0.4M NaCl, 0.5% SDS. An equal volume phenol/chloroform was added, vortexed for 1 min and centrifuged at 3,000rpm for 10 min at 20°C. The aqueous phase was removed and precipitated with 0.1 volume 3M Na-acetate and 2.5 volumes ethanol.

P. mRNA Selection and In Vitro Translation

mRNA selection and in vitro translation was carried out using the method of Elliott (1985).

Approximately 20ug of plasmid DNA was dissolved in 20ul of water and heated at 100°C for 10 min, followed by

chilling on ice. An equal volume of 1M sodium hydroxide was added and the mixture incubated at room temperature for 20 min. The DNA sample was neutralised by adding 0.5 volume of a solution of 1M NaCl, 0.3M Na citrate, 0.5M Tris-HCl pH8.0 and 1M HCl. The sample was mixed and immediately chilled on ice. The DNA was spotted 5ul at a time onto a nitrocellulose disc (Schleicher and Schuell, BA85). The discs were allowed to air dry and then baked for 2 hr at 80°C in a vacuum oven.

Four hundred micrograms of RNA extracted from virus infected BHK-C13 cells were added to the hybridisation solution (10mM 1,4-piperazinediethanesulfonic acid [PIPES pH6.4], 400mM NaCl, 4mM EDTA, 50% formamide) and incubated at 45°C overnight. The hybridisation solution was removed by aspiration and the discs washed 10 times with wash solution (10mM Tris-HCl pH7.6, 0.15M NaCl, 1mM EDTA, 0.5% SDS) and twice with wash solution without SDS. The wash solutions were preheated to 65°C and maintained at 65°C during the wash procedure.

The discs were transferred to individual tubes containing 300ul of water and 500ng calf liver tRNA. The tubes were boiled for 1 min and then snap frozen in a dry-ice/ethanol bath. The samples were thawed on the bench. The RNA was extracted with an equal volume of phenol/chloroform and precipitated with ethanol in the usual way. The RNA was washed with 70% ethanol, dried and resuspended in 5ul sterile water. It was used to programme an *in vitro* translation system (New England Nuclear Company) and the translation products analysed by polyacrylamide gel electrophoresis.

Preparation of Radiolabelled VS Virion Proteins

BHK C13 cells were grown in 30mm petri dishes until confluent and infected at a multiplicity of 4-5pfu/cell. After allowing virus to absorb for 1 hr at 31°C, 2ml of medium containing 30uCi, [^{35}S]-methionine was added. The cells were incubated overnight at 31°C. The medium was decanted and centrifuged at 3,000rpm for 5 min. The supernatant was then poured into a 15ml Beckman quick seal tube and centrifuged at 40,000rpm for 1 hr in a Sorval Ti65 rotor. The supernatant was poured off and the pellet resuspended in 100ul protein dissociation mix (0.125M Tris HCl pH6.8, 10% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue). After boiling for 2 min the sample was stored at -20°C.

The in vitro translation reactions were assembled on ice and initiated by transfer to 37°C where incubation was continued for 1 hr. A typical reaction comprised: 2ul RNA, 2.5ul [^{35}s]-methionine, 5ul rabbit reticulocyte lysate, 2.75ul in vitro translation cocktail, 1ul K-acetate, 0.25ul Mg-acetate. The reaction was terminated by adding 15ul protein dissociation mix (0.125M Tris-HCl pH6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue). Samples were boiled for 2 min and stored at -20°C. Before loading on the gel, samples were boiled for 5 min.

Proteins were fractionated on polyacrylamide gels in the presence of 0.1% SDS using the discontinuous buffer system of Laemmli (1970). The ratio of acrylamide to bisacrylamide was 75:1. Separating gels contained 10%, 12% or 15% acrylamide and stacking gels contained 3% acrylamide. See Table 2.4 Electrophoresis was at 30mA at 4°C until the bromophenol blue was 10mm from the bottom of the separating gel.

To increase the sensitivity of detection of [^{35}S]-labelled proteins the gels were fixed in 45% methanol, 5% acetic acid overnight. They were then soaked in En^{3}Hance (New England Nuclear) for 1 hr and washed in water for 1 hr, before being dried down under vacuum using a Bio-Rad gel drier. They were exposed against X-Omat S film and stored at -70°C.

Q. Nick Translation of DNA

Nucleic acid hybridisation is one of the most powerful techniques for detecting and quantitating specific RNA and DNA sequences. There are a variety of ways by which complementary strands can form duplex structures. However,

no matter what annealing procedure is used and no matter what means are used to quantitate the hybridisation one of the strands of nucleic acid must be labelled. The concept of radiolabelling DNA molecules internally *in vitro* to a high specific activity was first introduced by Rigby *et al* (1977).

DNA was labelled in a 50ul reaction containing 50mM Tris-HCl pH7.5, 10mM $MgSO_4$, 0.1mM DTT, 50ug/ml BSA, 5uM of each of the unlabelled dNTPs and 10uCi of each [α-^{32}P] dNTPs, 0.004ug/ml DNasel and 2 units DNA polymerase. After incubation at room temperature for 1 hr unincorporated [α-^{32}P] dNTPs were removed from the DNA preparation by passage over a Sephadex G-50 column.

R. Quantitation of Nucleic Acid

In order to determine the concentration of nucleic acid DNA or RNA in a particular preparation optical density measurements were read at 260nm and 280nm. Quartz microcuvettes and a Cecil 595 double beam digital UV spectrophotometer were used. The pathlength of the light was 1cm and it was assumed that 1 OD unit was equivalent to 50ug/ml of nucleic acid.

S. Restriction Endonuclease Cleavage

All restriction endonuclease digests were performed in Tris-acetate buffer (33mM Tris-acetate pH7.9, 66mM K-acetate, 10mM Mg-acetate, 0.5mM DTT, 100ug/ml BSA) at 37°C. Smal digests were performed in 15mM Tris-HCl pH8.0, 6mM $MgCl_2$, 15mM KCl, at 37°C. Various concentrations of DNA were digested using the simple rule that 1 unit of a

restriction enzyme would digest 10ug of DNA in 1 hr. The concentration of enzyme in the reaction never exceeded 10% due to the inhibitory effects of glycerol in the enzyme storage buffer. Typically reaction volumes were between 20ul and 60ul.

T. Digestion of DNA with Nuclease BAL31

Bal31 is an enzyme which has two activities (Lau and Gray, 1979): (a) it is a highly specific, single stranded deoxyribonuclease and exonuclease that catalyses the removal of small oligonucleotides or mononucleotides from both 5' and 3' termini of double stranded DNA. Both strands of DNA are degraded at the same rate; (b) it is a single stranded specific endonuclease similar to nuclease S1.

Bal31 was used because of its ability to degrade the 3' and 5' strands of DNA in order to remove the C-tails from insert DNA. One unit of Bal31 was added to a 30ul reaction containing 0.5ug DNA, 12mM $MgCl_2$, 12mM $CaCl_2$, 20mM Tris-HCl pH8.0, 1mM EDTA, 200mM NaCl. The rate at which Bal31 removes nucleotides from the 5' and 3' termini of duplex DNA is variable and depends on a number of factors eg. batch of enzyme. Therefore, reactions were usually about 30 sec in duration at 37°C with 10ul aliquots removed at 10 sec intervals. The aliquots were frozen on dry ice to stop the reaction or EGTA was added to a final concentration of 20mM. Bal31 activity is completely dependent on calcium and so it can be inactivated by EGTA which binds calcium ions.

U. Computing

The nucleotide and amino acid sequences were stored, manipulated, and analysed on a PDP 11/44 computer using the following programs:

(a) TRANS - This program allows the user to translate a DNA sequence in any or all reading frames (Pustell and Kafatos, 1982a).

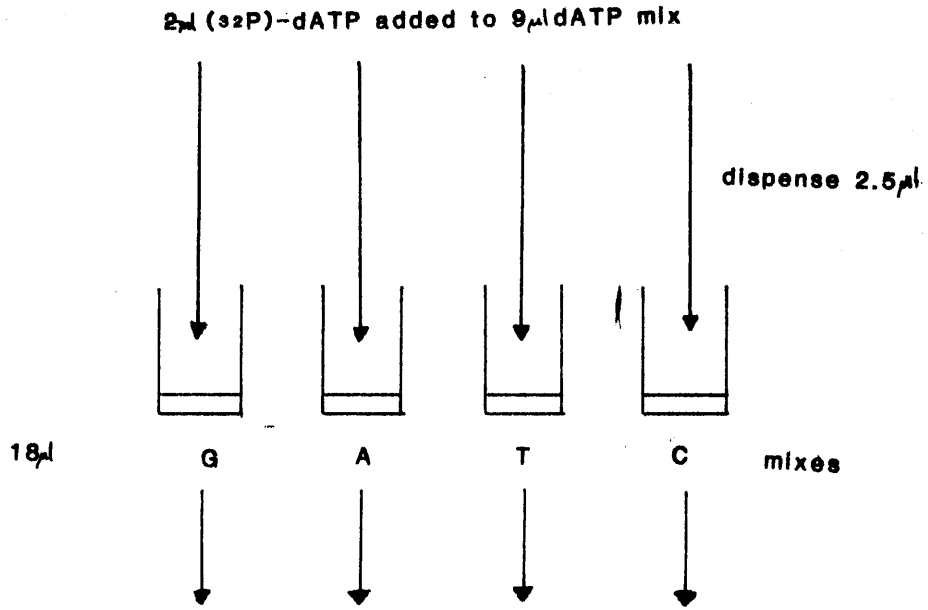
(b) PROFIL - Evaluates the hydrophilicity and hydrophobicity of an amino acid sequence using the SOAP algorithm of Kyte and Doolittle (1982). The result of the prediction is illustrated in graphical form where peaks represent hydrophobic regions.

(c) CINTHOM - This is a dot-matrix plot which aligns two sequences, whether nucleic acid or amino acid, on the X and Y axis of a 2D co-ordinate system. The user defines the parameters of homology (Pustell and Kafatos, 1982b).

(d) HOMOL - This aligns two sequences such that their homology is maximised. The program works by inserting gaps to minimise mismatch (Taylor, 1984).

(e) CHOU and FASMAN - A program which allows the prediction of protein secondary structure (Chou and Fasman, 1978).

The results were graphically displayed using the PLOTCHOU program contained in the University of Wisconsin Genetics Computer Group software package and outputted to a Hewlett-Packard model 7475A plotter.



SEQUENCE REACTIONS

dATP mix is 11.5μl dATP

Figure 2.1

Diagram showing the composition of the nucleotide mix. 2ul [^{32}P]-dATP was added to 9ul dATP mix. 2.5ul of this mixture was then added to 18ul of the G, A, T and C mixes.

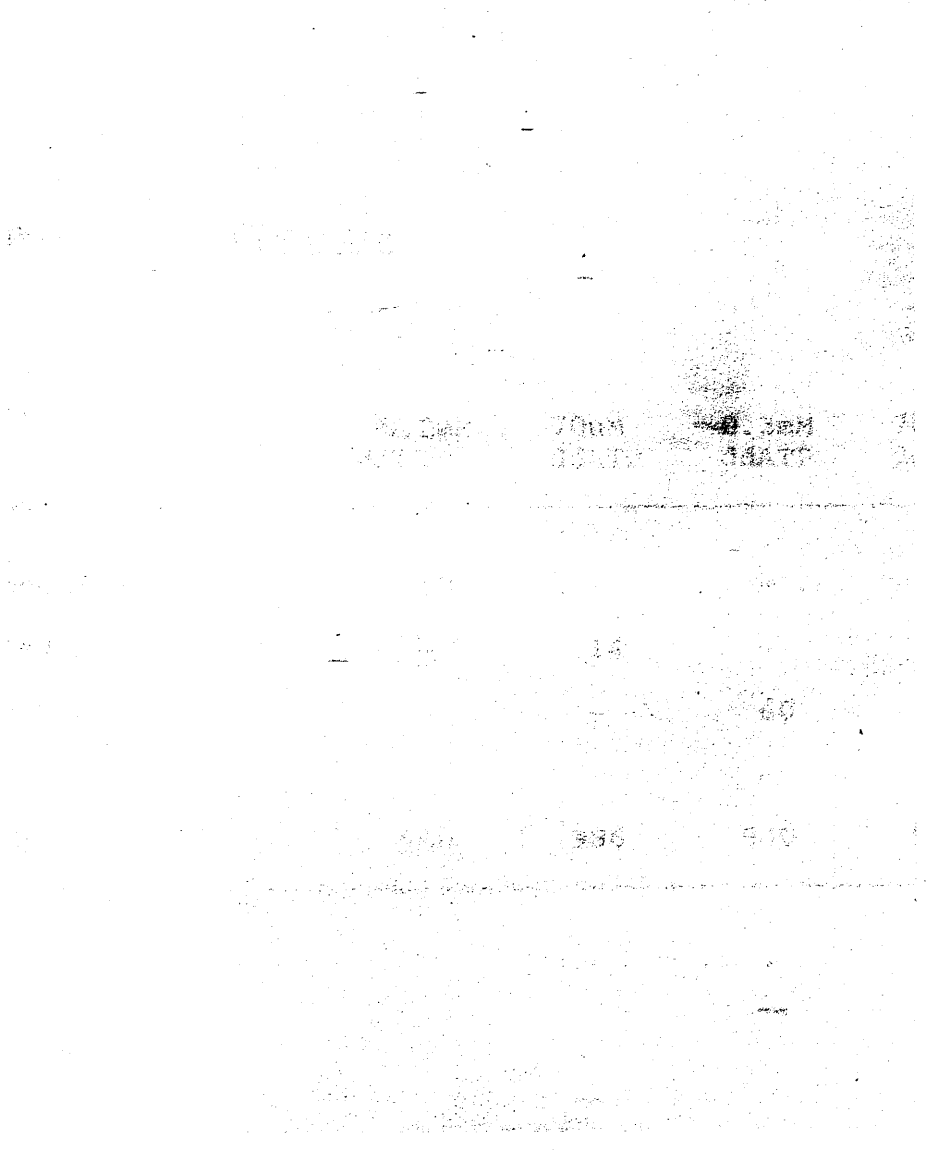


Table 2.1 dNTP Mixes

	dG mix	dA mix	dT mix	dC mix
0.5mM dT	200	200	10	200
0.5mM dC	200	200	200	10
0.5mM dG	10	200	200	200
dNTP-*-mix buffer	50	50	50	50

Table 2.2 ddNTP Mix

	0.2mM ddGTP	70uM ddATP	0.3mM ddATP	70uM ddCTP
5mM ddG	40	-	-	-
5mM ddA	-	14	-	-
5mM ddT	-	-	60	-
5mM ddC	-	-	-	14
H_2O	960	986	940	986

Table of Protein Gel Mixes Table 2.4

	10%	12%	15%
acryl:bis 75:1	8.00	10.30	12.75
1M Tris pH8.8	12.30	12.30	12.30
H_2O	11.70	10.00	7.55
10% SDS	0.33	0.33	0.33
10% AP	0.33	0.33	0.33
TEMED	30ul	30ul	30ul

Stacking Gel	acryl:bis	1.00
	1M Tris pH6.8	0.625
	H_2O	8.00
	10% SDS	0.10
	10% AP	0.10
	TEMED	5ul

Table 2.3 dNTP: ddNTP Mix

Sequencing Mix	dNTP	ddNTP	H_2O
G	400	300	100
A	400	150	250
T	400	400	-
C	400	400	-

dNTP-*-mix buffer = 0.05m Tris-HCl pH8.0
1mM EDTA

Chapter 3

CLONING AND IDENTIFICATION OF THE WILD-TYPE, MUTANT AND REVERTANT VIRUS NS mRNAS

1. RNA PRODUCTION AND cDNA SYNTHESIS

Actinomycin D-treated BHK C13 cells were infected with each of the viruses at low multiplicity (1pfu/cell). Six hours post-adsorbtion the cells were harvested and total cellular RNA was extracted using the guanidinium/CsCl method of Chirgwin et al (1979). Solutions of guanidinium chloride and guanidinium isothiocyanate are potent chaotropic agents which readily dissolve proteins (Cox, 1968). Cellular structures disintegrate and nucleoproteins dissociate rapidly from nucleic acid as ordered secondary structure is lost. Even RNase, an enzyme which is resistant to many forms of physical abuse, eg. boiling, is completely inactive in the presence of 4M guanidinium isothiocyanate and reducing agents like β-mercaptoethanol (Sela et al, 1957). This combination of reagents as used in the method of Chirgwin et al (1979) allows the efficient recovery of large quantities of intact, pure RNA from even those cells which are rich in RNase eg. the pancreatic cells.

Total cellular RNA extracted from infected cells was used as template for cDNA synthesis without being poly (A) or size selected. cDNA was synthesised using oligo $(dT)_{12-18}$ primer and the protocol of Gubler and Hoffman (1983) as outlined in Figure 3.1.

All of the recombinant plasmids had the general form shown in Figure 3.2. Double-stranded cDNA was C-tailed and annealed to PstI digested G-tailed pBR322. The unique PstI site in pBR322 is located in the ampicillin resistance gene and so cDNA inserted at this site inactivates the gene. Therefore recombinant plasmids have a tetracycline resistant, ampicillin sensitive phenotype.

2. IDENTIFICATION OF WILD-TYPE NS GENE-SPECIFIC RECOMBINANT PLASMIDS

The procedure used to identify firstly virus-specific recombinant plasmids and then those containing NS gene-derived inserts is outlined in Figure 3.3.

Recombinant plasmids containing virus-specific inserts were identified by colony hybridisation using ^{32}P-labelled, partially alkali digested, VSV genomic RNA as a probe. VSV genomic RNA was extracted from purified virions using a modification of the method described by Palese and Schulman (1976) for influenza virus RNA. After partial hydrolysis with alkali (to increase the number of free 5' ends) the RNA was 5' end labelled using T4-polynucleotide kinase.

It was calculated that 15% of the recombinant plasmids contained virus-derived inserts. Because the template RNA for the cloning reactions was unselected this high proportion probably reflects both the inhibitory action of actinomycin D and VSV infection on cellular mRNA synthesis and the efficient production of VSV mRNAs, combined with the efficiency of the cloning method.

NS gene-specific recombinant plasmids were identified using a combination of Northern blot analysis and mRNA selection/*in vitro* translation.

For the Northern blot (Figure 3.4) RNA was extracted from infected cells, separated by agarose gel electrophoresis and blotted onto Biodyne membrane. The Biodyne membrane was then probed with nick translated recombinant plasmid DNA. VSV mRNAs, ^{32}-labelled *in vivo* and electrophoresed on the same gel, were used as markers.

The gel system did not resolve the M and NS mRNAs and so when they were transferred to the Biodyne membrane the NS and M mRNAs appeared as a single band (Figure 3.4). In order to positively identify NS gene-specific recombinant plasmids, those hybridising in the NS/M mRNA position on Northern blots were used in mRNA selection/*in vitro* translation experiments (Figure 3.5).

Recombinant plasmids were bound to nitrocellulose and then hybridised with RNA extracted from infected cells. After a stringent wash procedure, bound RNA was eluted and used to program an *in vitro* translation system. Electrophoresis clearly resolved the M and NS proteins thus allowing the positive identification of NS gene-specific recombinant plasmids. For the sake of comparison a wild-type N-gene-specific recombinant plasmid has been included in Figures 3.4 and 3.5.

Using the procedure outlined in Figure 3.3 several recombinant plasmids were identified as containing NS gene-specific inserts. In the library the frequency of plasmids containing inserts derived from the NS mRNA was calculated to be 0.7%.

3. CHARACTERISATION OF THE WILD-TYPE NS GENE-SPECIFIC RECOMBINANT PLASMIDS

Digestion of the NS gene-specific recombinant plasmids with *Pst*1 was performed in order to excise the insert and size it. All of the NS gene-specific recombinant plasmids identified contained a characteristic internal *Pst*1 site (Figure 3.6). The recombinant plasmid digested with *Pst*1 in

Figure 3.6 has an insert of approximately 900 bases and this was thought to be about the size of a full-length copy of the NS mRNA. This recombinant plasmid was designated pNJNS1 and was used for sequence determination.

4. IDENTIFICATION OF RECOMBINANT PLASMIDS CONTAINING MUTANT AND REVERTANT VIRUS NS-SPECIFIC INSERTS

The insert from pNJNS1 was purified from an agarose gel, ^{32}P-labelled by nick translation and used as a specific probe in colony hybridisation experiments to detect NS-specific recombinant plasmids derived from the three complementation group E mutants (tsE1, tsE2 and tsE3) and the two revertant viruses (tsE1/R1 and tsE3/R1).

Figure 3.7 shows a typical result of a colony hybridisation experiment. The frequency of NS gene-specific recombinant plasmids had been calculated to be 0.7% and so low density screening was possible.

NS gene-specific recombinant plasmids from the three complementation group E mutants and the two revertant viruses were sized by PstI digestion and agarose gel electrophoresis. Figure 3.8 shows a PstI digest of pNJNS1 electrophoresed with PstI digests of the three recombinant plasmids which were used for sequence determination of the complementation group E mutants. Since they seemed to be approximately the same size as pNJNS1 (Figure 3.8) it was assumed that they contained full-length copies of the NS mRNA. The plasmids were designated pNJNSE1, pNJNSE2 and pNJNSE3.

CLONING STRATEGY

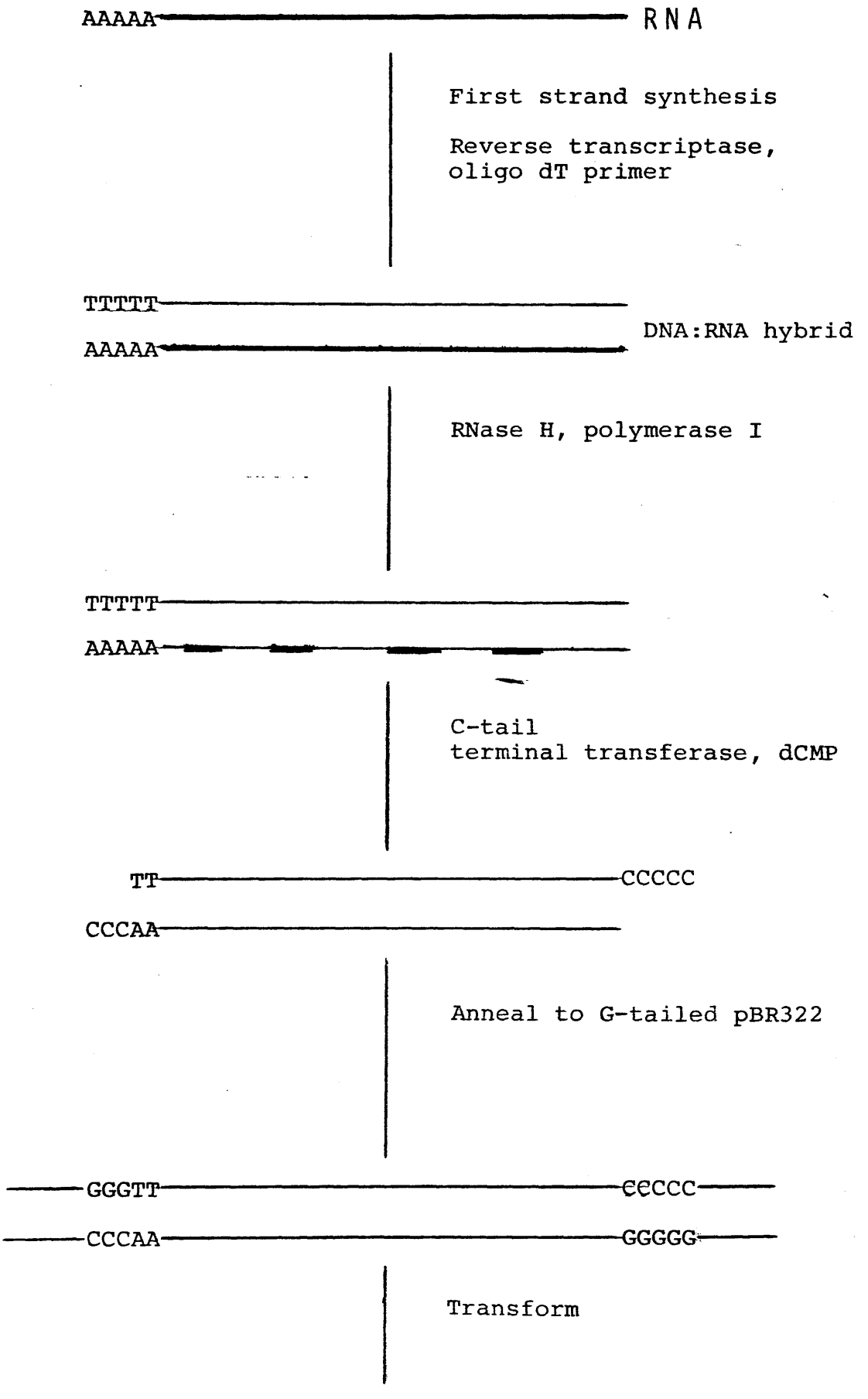


Figure 3.1

A schematic representation of the method used to clone cDNA copies of the VSV mRNAs. The RNA:DNA hybrid produced after the first strand cDNA synthesis reaction was treated with RNase H and DNA polymerase 1. Rnase H specifically digests RNA which is part of an RNA:DNA hybrid. Thus RNase H treatment leaves small RNA fragments which can be used as primers by the DNA polymerase 1 to make double-stranded cDNA. The double-stranded cDNA was C-tailed and annealed to Pstl cut, dG-tailed pBR322. The recombinant plasmids were used to transform competent E.coli MC1061 cells.

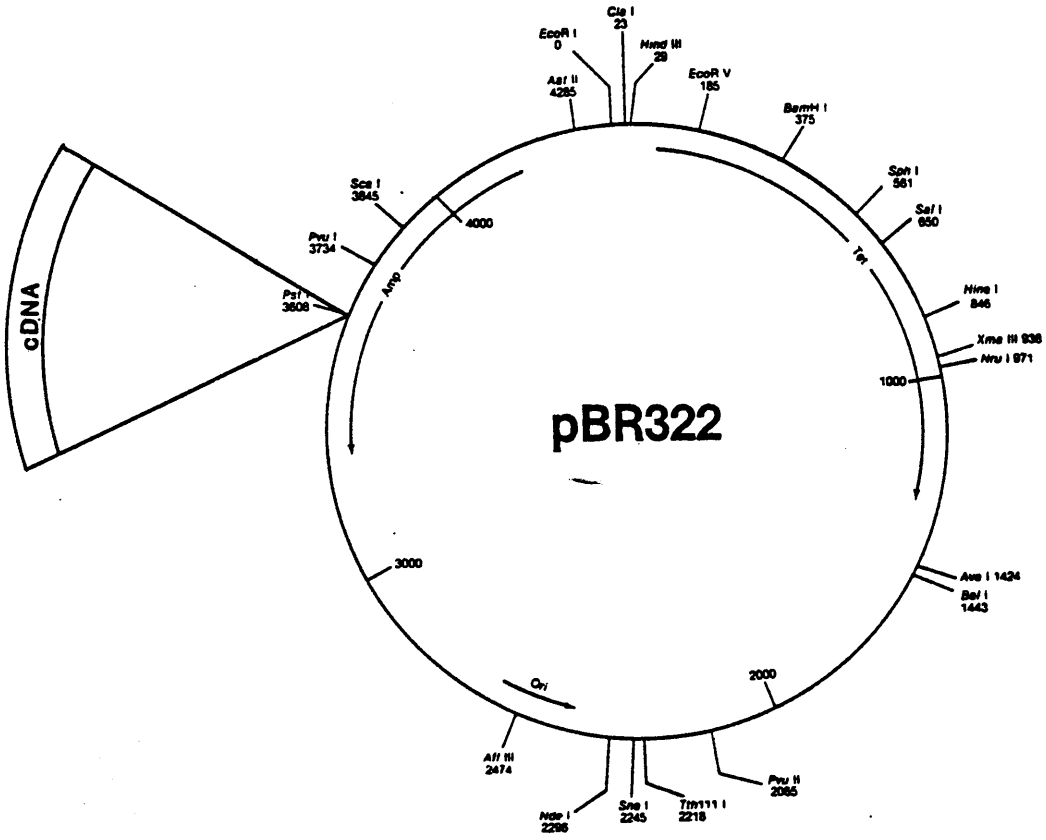

pBR322
cDNA
Cla I
23
EcoR I
0
Hind III
29
Ase II
4295
EcoR V
185
BamH I
375
Sph I
561
Sal I
650
Hinc I
846
Xma III 938
Nru I 971
1000
Ava I 1424
Bal I
1443
2000
Pru II
2065
Tth111 I
2218
Sna I
2245
Nde I
2296
Aff III
2474
3000
Ori
Tet
Amp
4000
Sca I
3845
Pvu I
3734
Pst I
3608

Figure 3.2

Diagramatic representation of the general form of the recombinant plasmids. cDNA was inserted into the unique PstI site of pBR322.

IDENTIFICATION PROCEDURES

1. Colony hybridisation with ^{32}P-labelled VSV genomic RNA partially hydrolysed with alkali

>15% of colonies contained viral specific inserts

2. Northern blot analysis of viral specific plasmids

3. Confirm ID with mRNA selection/*in vitro* translation

0.7% of colonies in the library contained NS-specific inserts

Figure 3.3

An outline of the procedure used to detect NS gene-specific recombinant plasmids.

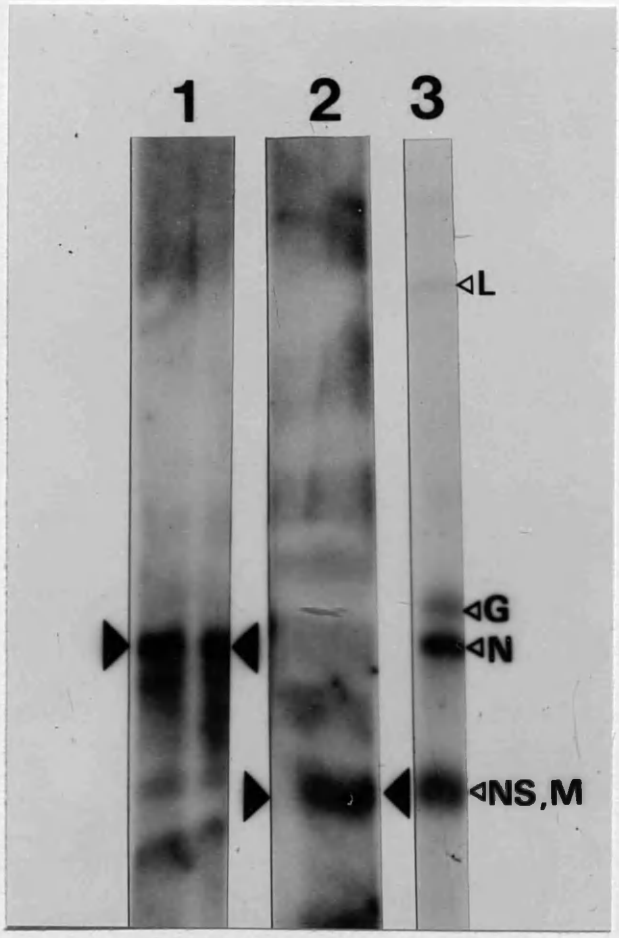

1
2
3
L
G
N
NS,M

Figure 3.4

Northern blot analysis: total cellular RNA was extracted from VSV infected cells, separated by agarose gel electrophoresis and blotted onto Biodyne membrane. The filter was then hybridised with recombinant plasmid DNAs ^{32}P-labelled by nick translation.

(1) pNJN1 (a cDNA clone of the N gene of wild-type VSV NJ, Missouri strain) reacting with the N mRNA.

(2) pNJNS1, reacting with either the NS or M mRNA.

(3) A marker track of VSV mRNAs ^{32}P-labelled *in vivo*, separated by gel electrophoresis and blotted onto Biodyne membrane.

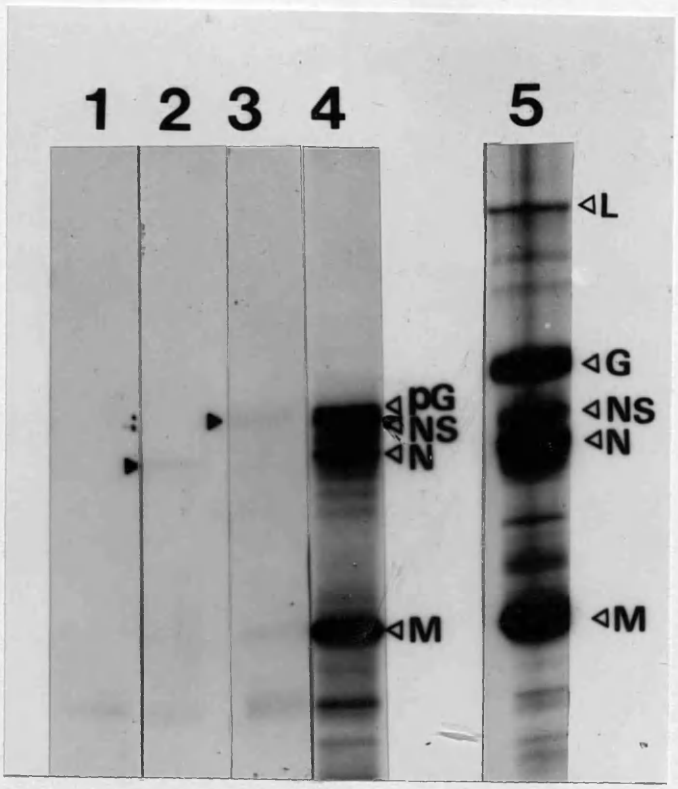

1
2
3
4
5
PG
NS
N
M
L
G
NS
N
M

Figure 3.5

Message selection/*in vitro* translation: recombinant plasmid DNA was bound to nitrocellulose filters and hybridised with total cellular RNA extracted from VSV infected cells. Bound RNA was eluted and used to program a rabbit reticulocyte *in vitro* translation system. The translation products were analysed by polyacrylamide gel electrophoresis.

(1) Proteins translated from mRNA selected with pBR322.
(2) Proteins translated from mRNA selected with pNJN1 .
(3) Proteins translated from mRNA selected with pNJNS1
(4) Translation products of total infected cell RNA.
(5) Marker track of [^{35}S] methionine-labelled VSV proteins.

VSV proteins are indicated at the right. pG is the *in vitro* equivalent of G which has a greater electrophoretic mobility because it is unglycosylated. The solid arrowheads indicate the translation products of the selected mRNAs. A minor band comigrating with the M protein consistently appeared among the proteins translated from mRNA selected using either pNJNS1 or pNJN1.

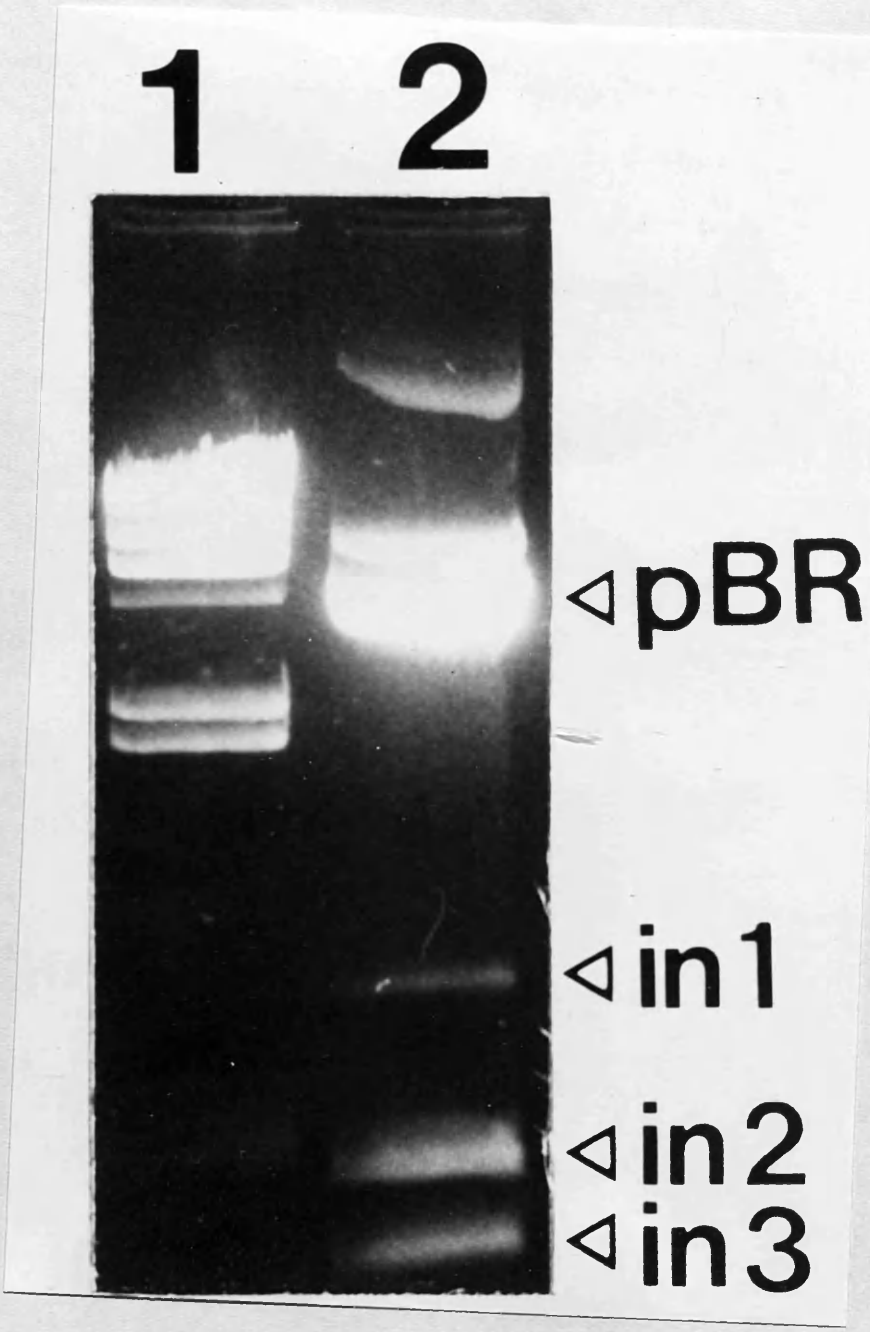
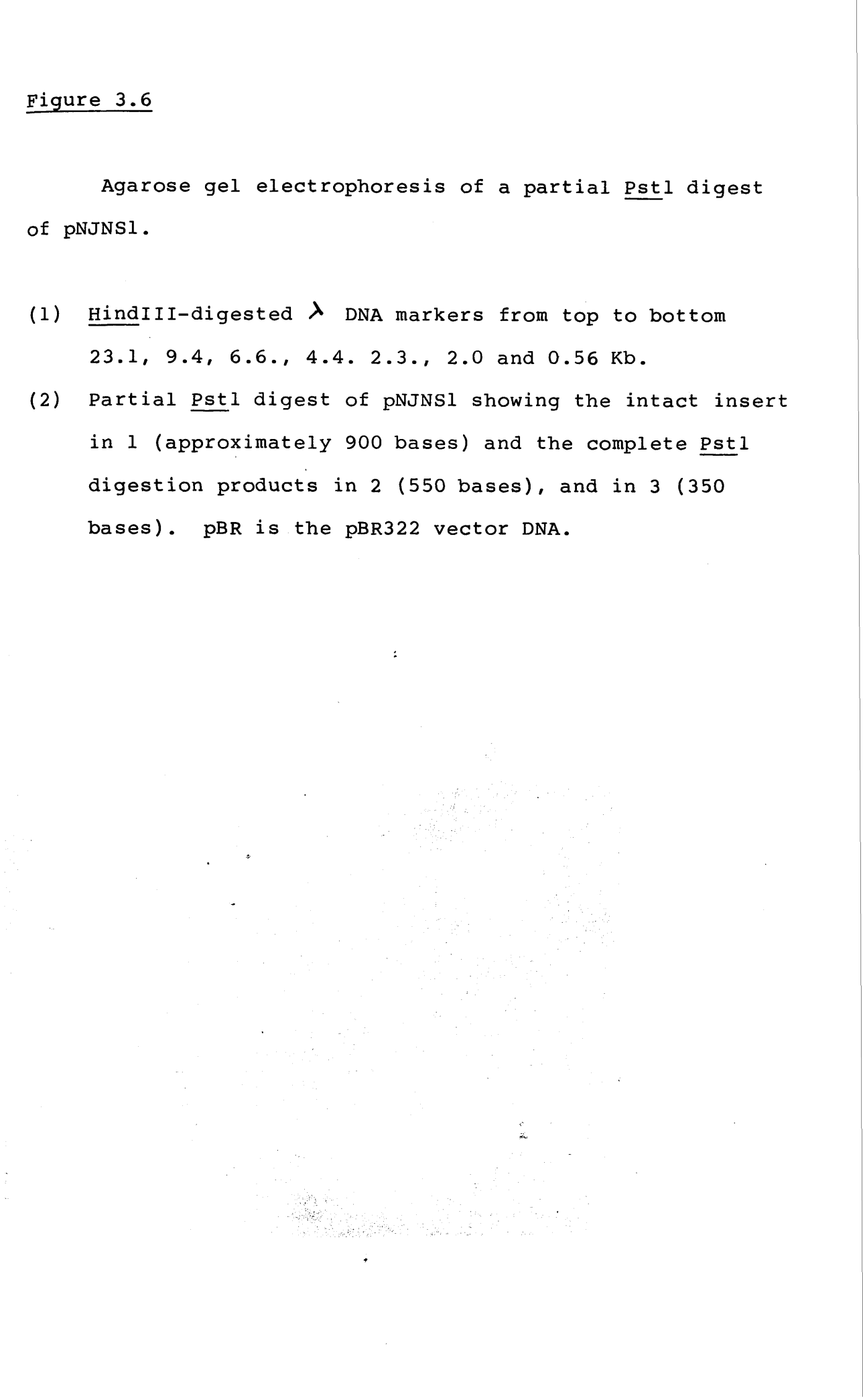

1
2
pBR
in1
in2
in3

Figure 3.6

Agarose gel electrophoresis of a partial Pstl digest of pNJNS1.

(1) HindIII-digested λ DNA markers from top to bottom 23.1, 9.4, 6.6., 4.4. 2.3., 2.0 and 0.56 Kb.

(2) Partial Pstl digest of pNJNS1 showing the intact insert in 1 (approximately 900 bases) and the complete Pstl digestion products in 2 (550 bases), and in 3 (350 bases). pBR is the pBR322 vector DNA.



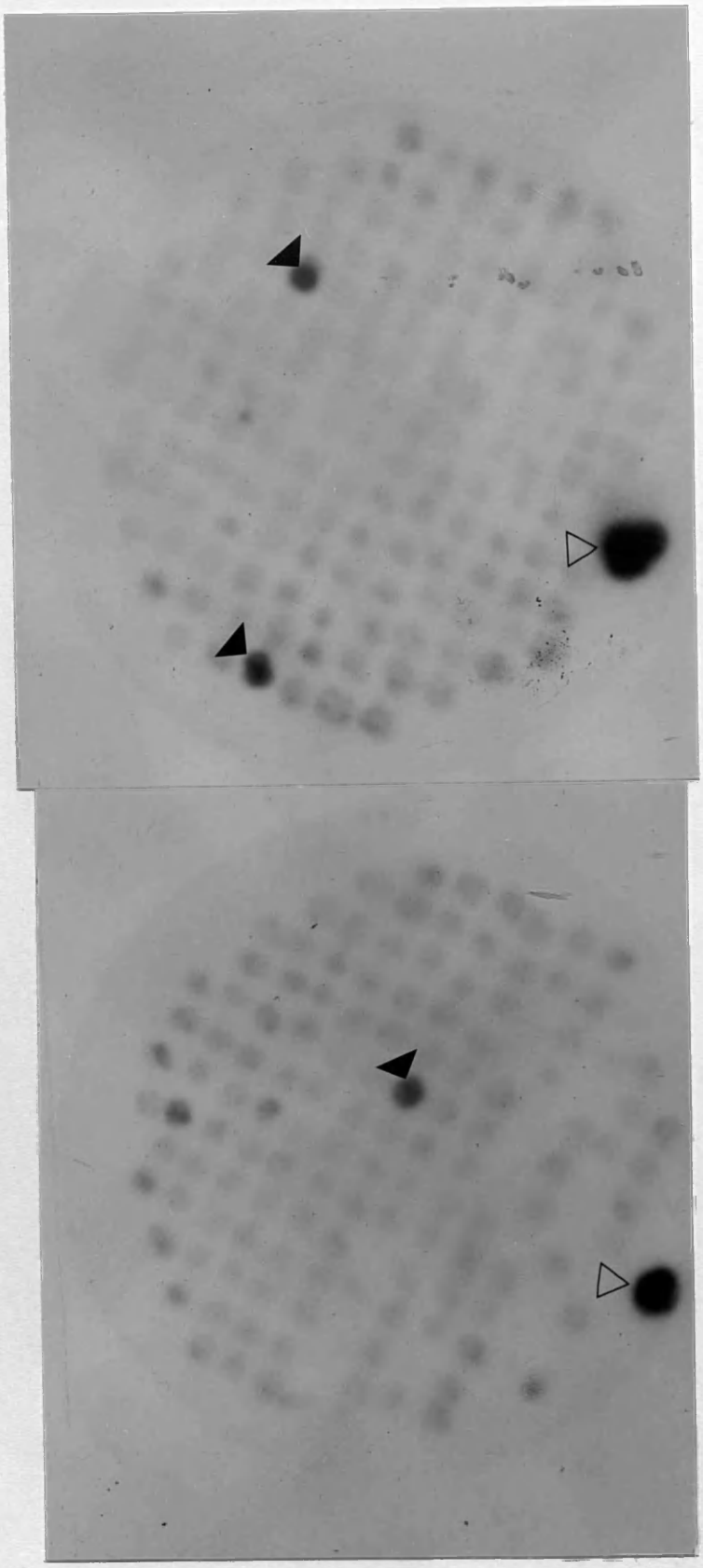


Figure 3.7

Examples of *in situ* colony hybridisation to detect mutant NS gene-specific recombinant plasmids. Nick translated insert from pNJNS1 was used as a probe.

▶ indicates mutant NS gene-specific recombinant plasmids

▷ indicates control colony of pNJNS1 containing *E.coli*

The control is more intense in each case probably due to the fact that the colony was grown from a glycerol stock and the bacteria may have had a higher plasmid copy number.

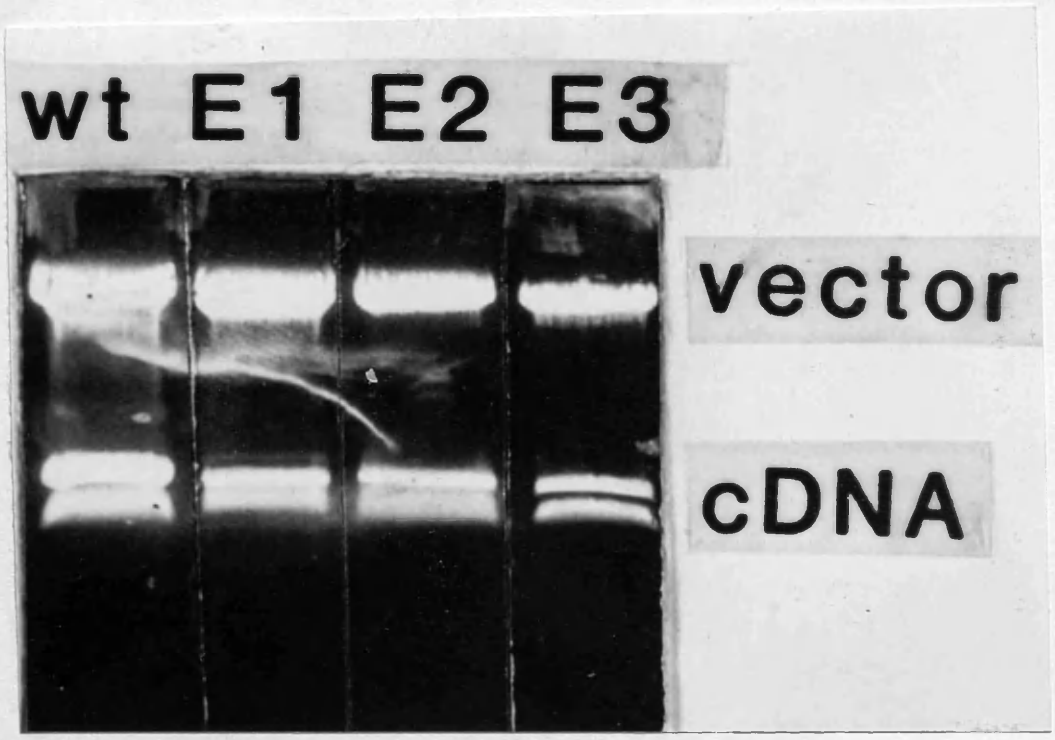
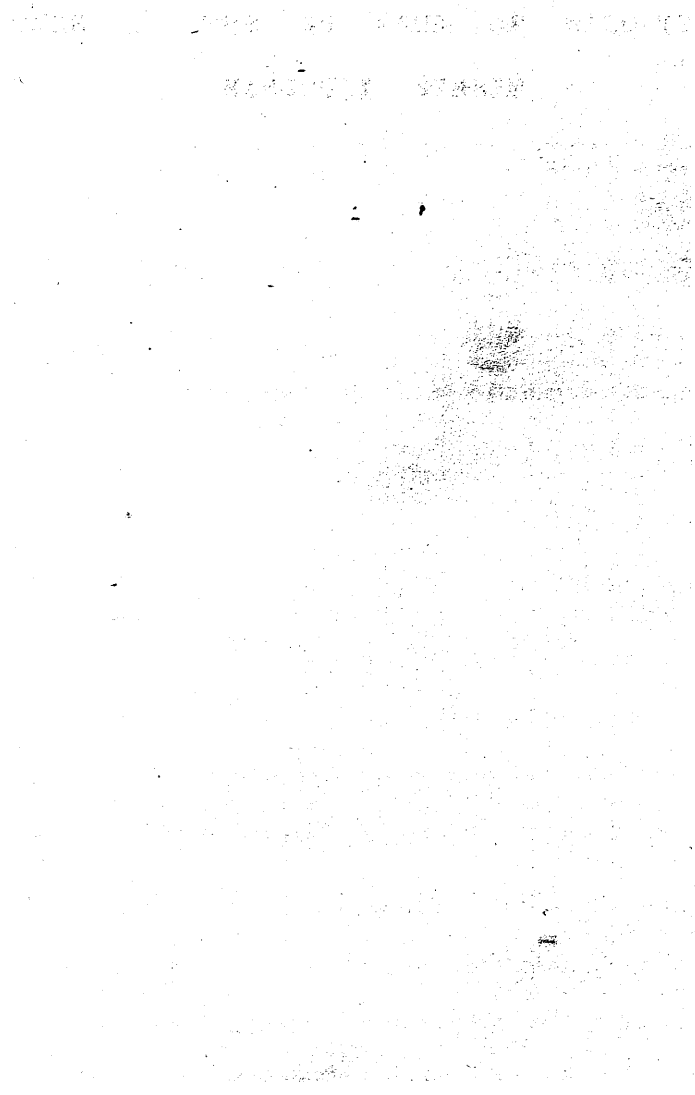

wt E1 E2 E3
vector
cDNA

Figure 3.8

Agarose gel electrophoresis of Pstl digest of pNJNS1, pNJNSE1, pNJNSE2 and pNJNSE3. All inserts are approximately the same size and contain the characteristic internal Pstl site.



Chapter 4

THE SEQUENCE OF THE NS mRNA OF WILD-TYPE VSV NJ MISSOURI STRAIN

The precise role of the NS protein in the VSV infectious cycle is not known. It has been shown that NS protein is involved in transcription, replication and virion maturation (Emerson and Yu, 1975; Evans et al, 1979; Isaac and Keene, 1982; Keene et al, 1981; Kingsford and Emerson, 1980; Mellon and Emerson, 1978; reviewed by Pringle, 1986). Kinetic studies of VSV protein synthesis have shown that 75% of newly synthesised NS protein enters a soluble pool in the cell cytoplasm; the remaining 25% bypasses the soluble pool and enters nucleocapsids directly (Hsu et al, 1979). The results of the kinetic studies indicate that VSV RNP assembly in vivo is a stepwise process analagous to the stepwise assembly of Sendai virus nucleocapsids (Kingsbury et al, 1978).

Evidence suggests that the balance between phosphorylation and dephosphorylation of proteins is a fundamental method of controlling certain processes in viral infection (Clinton et al, 1978; Kamata and Watanabe, 1977; Mellon and Emerson, 1978; Sen et al; 1977; Smith et al, 1979; Sokol and Clark, 1973). For example, the degree of phosphorylation of the p12 protein of mouse mammary tumour virus determines the extent of its binding to homologous viral RNA (Privalsky and Penhoet, 1977) and viral transcription is stimulated by phosphorylation of the influenza virus nucleocapsid protein (Kamata and Watanabe, 1977). The NS protein of VSV is the major phosphoprotein present in infected cells and an integral component of the viral transcriptional complex (Moyer and Summers, 1974; Naito and Ishihama, 1976; Privalsky and Penhoet, 1977; Watanabe et al, 1974). Much work has been concentrated on

finding how phosphorylation affects NS protein activity.

It has been reported that NS phosphorylation is required for *in vitro* transcription (Hsu *et al*, 1982). The degree of phosphorylation of NS protein *in vivo* has been correlated with its ability to bind to nucleocapsids (Clinton *et al*, 1978) and its ability to function as part of the transcriptase (Kingsford and Emerson, 1980). In the infected cell NS protein exists in a variety of phosphorylated forms (Kingsford and Emerson, 1980; Hsu and Kingsbury, 1982) which have been separated on the basis of electrophoretic mobility into two classes, NS1 and NS2. These two classes differ markedly in their degree of phosphorylation and in their abilities to activate transcription (Clinton *et al*, 1979; Kingsford and Emerson, 1980; Kingsbury *et al*, 1981; Hsu and Kingsbury, 1982). The NS1 class has the lower electrophoretic mobility and phosphate concentration. NS1 molecules are found in the intracellular soluble pool of NS protein and intracellular or virion nucleocapsids. In contrast NS2 molecules are more highly phosphorylated and are found mainly in virions (Hsu *et al*, 1982).

There are at least three kinase activities associated with the VS virion. Imblum and Wagner (1974) identified a virion protein kinase that they concluded was associated with the viral envelope and probably acquired from the cellular membrane during the budding process. A protein kinase precipitable with anti-c-src antibody has also been identified. This protein is again thought to be acquired as a consequence of budding (Clinton *et al*, 1982). The NS protein is extensively phosphorylated at serine and

threonine residues; however, c-src is known to be a tyrosine specific kinase (Clinton and Huang, 1981).

Evidence for the involvement of a third type of kinase in the infectious process has accumulated over a number of years. Imblum and Wagner (1974) observed that although their virion kinase activity was sensitive to actinomycin D the level of phosphorylation of intracellular NS was unaffected. Hsu et al (1979) showed that dephosphorylated NS, inactive in in vitro transcription assays, could not be reactivated by phosphorylation with virion-bound kinases. Sinacore and Lucas-Lenard (1982) demonstrated that the virion-bound protein kinases may not be essential for transcriptional activity in vitro. Evidence that the L protein is capable of specifically phosphorylating NS protein has recently been presented (Sanchez et al, 1985). The L fraction of purified nucleocapsids was shown to be capable of phosphorylating exogenous phosphate acceptor proteins like phosvitin and casein. However, an excess of phosvitin was unable to compete with phosphorylation of NS protein by L indicating that the protein kinase activity has an affinity for NS protein (Sanchez et al, 1985).

In this chapter sequence data from the NS mRNA of wild-type VSV New Jersey, strain Missouri is presented and discussed.

1. SEQUENCE ANALYSIS OF THE NS MRNA OF WILD-TYPE VSV NJ, MISSOURI STRAIN

The nucleotide sequence of the insert of pNJNS1 was determined by the dideoxy chain terminator method of Sanger

et al (1977, 1980). Approximately 75% of the sequence was determined on both strands though each base was sequenced an average of five times. Figure 4.1 shows a restriction map of the insert from pNJNS1 with the sites of the restriction enzymes used in the sequence analysis shown. Because the DNA was relatively short (approximately 900bp) restriction enzyme cleavage was used to generate DNA fragments as opposed to DNase digestion (Anderson, 1981) or sonication (Deininger, 1983).

The sequence spanning the internal PstI site was obtained by digesting intact pNJNS1 with HpaII, purifying the appropriate fragment (approximately 175bp) from a polyacrylamide gel and ligating the fragment into M13mp18 DNA.

Due to the method used to clone the double-stranded cDNA into pBR322 there were 20-25 dC or dG residues at either end of the insert fragment when it was excised from the plasmid by PstI digestion. It has been reported that DNA polymerase finds certain sequences, like stretches of dC or dG residues, difficult to copy (Smith, 1980) resulting in unreadable sequence tracks after the polymerase has encountered the homopolymer tracts. Two methods were used with varying success to overcome the problem. Firstly, Ba131 nuclease digestion of purified insert from pNJNS1 removed bases from either end of the DNA molecules. However, due to variation in the enzyme preparations it was difficult to remove the same number of bases consistently. Alternatively, pNJNS1 was digested with HaeIII and electrophoresed with HaeIII-digested pBR322. The appropriate fragment was then purified from the gel and

ligated into HaeIII-digested M13mp18. In this way sequence was read from the HaeIII site in the vector towards the tails.

Figure 4.2 shows the nucleotide and predicted protein sequence of the NS mRNA of wild-type VSV NJ, Missouri strain. The NS mRNA is 856 nucleotides long (excluding polyadenylic acid) and codes for a protein of 274 amino acids (mol wt 31,400). The protein initiates at AUG (position 11-13) and terminates at UAA (position 833-835). Thus there is very efficient utilisation of the mRNA with only the minimum untranslated region.

The sequence shows that pNJNS1 contains a full-length cDNA copy of the NS mRNA because (a) at the 5' end there is the sequence AACAGAGATC which corresponds with the consensus sequence (5')AACAGNNAUC(3') reported to be at the 5' end of all VSV mRNAs (McGeoch, 1979; Rose, 1980; Franze-Fernandez and Banerjee, 1978) and (b) at the 3' end there is the sequence ...UAUG(A_7)Cn. It has been reported that the sequence ...UAUG(U_7) occurs at the mRNA-poly(A) junction in each mRNA (Rose, 1980). The presence of the 12 A residues at the 3' end arises from the oligo$(dT)_{12-18}$ used to prime cDNA synthesis and the C residues from the 'tailing' reactions.

Klapper (1977) examined the frequency of amino acid pairs in proteins and from this work calculated the composition of an 'average' protein. Compared to the 'average' protein of Klapper (1977) the NS protein is relatively rich in aspartic and methionine but deficient in alanine and glycine (Table 4.1).

Figure 4.3 shows a translation of the NS protein with

N-chlorosuccinimide (NCS) cleaves protein molecules at tryptophan residues. The efficiency of the cleavage is dependant upon the local amino acid environment.

acidic and basic amino acids highlighted. The NS protein is highly acidic especially in the amino-terminal half. If we assume the values for the charge of amino acids shown in Table 4.2 then at pH7.0 the NS protein has an overall charge of -18.5.

The acidity is probably the reason for the aberrant mobility of the NS protein on SDS-polyacrylamide gels. Early estimates of the mol wt of NS protein determined from its relative mobility on these gels were usually too large, being between 40-60,000 (Kang and Prevec, 1971; Knipe et al, 1975; Mudd and Summers, 1970; Obijeski et al, 1974). The high concentration of acidic residues especially in the amino-terminal half might be responsible for a deficiency in SDS binding which results in the low electrophoretic mobility of the NS protein. Bell and Prevec (1985) by cleavage of NS protein with N-chlorosuccinimide (NCS), showed that the region responsible for the anomalous migration of NS protein on SDS polyacrylamide gels does reside in the amino-terminal half of the protein.

The aberrant migration of the NS protein on SDS gels has previously been attributed to the fact the NS is a phosphoprotein; however, dephosphorylation of NS protein by alkaline phosphatase results in decreased mobility (Hsu and Kingsbury, 1980). A number of workers have proposed that NS protein has an unusual monomeric structure or it migrates as a dimer (Evans et al, 1979; Gallione et al, 1981). Bell and Prevec (1985) showed that incomplete NCS cleavage of NS protein produces no more than four cleavage products. Since even the simplest dimer of NS protein ie. one cross-linked in the carboxy-terminal fragment, would be expected to

generate eight new partial cleavage products when subjected to incomplete NCS cleavage, the dimer theory must be ruled out.

Figure 4.4 shows there are 46 potential phosphorylated amino acids (ie. serine or threonine) in the NS protein of VSV NJ, strain Missouri: 13 threonines and 33 serines. The majority of the potential phosphorylated amino acids lie in the amino half of the molecule. These data support the work of Bell and Prevec (1985) who showed that most of the phosphate groups are located on residues in the amino-terminal portion of the molecule in the New Jersey and Indiana serotypes of VSV and Piry virus. This consistency supports the idea that phosphorylation in the amino-terminal half of the protein is structurally significant to the molecule.

Figure 4.5 shows a hydropathic plot (Kyte and Doolittle, 1982) of the NS protein. The program of Kyte and Doolittle (1982) uses a hydropathy scale in which each amino acid has been assigned a value reflecting its relative hydrophilicity and hydrophobicity. The program moves along a sequence from the amino to the carboxy terminus calculating, as it does, the average hydropathy. In Figure 4.5 the residues above the line are hydrophobic and those below the line are hydrophilic. As one would expect from such a highly charged protein it is predominantly hydrophilic. The most hydrophilic portions lie in the amino-terminal half of the molecule.

The sequence data of the NS mRNA of wild-type VSV NJ, Missouri strain combined with the results of physical studies on the NS protein of other strains of VSV all point

to the amino-terminal half of the protein being important to its function. Comparison of the sequence of the NS mRNA of the Missouri strain with that of other strains of VSV combined with the comparison of the proteins they encode might now be useful in trying to define the precise areas of the protein that are important to its function.

2. COMPARISON OF THE NS GENES AND PROTEINS

The NS genes of four strains of VSV have now been fully sequenced. The four strains represent two subtypes from each of the two serotypes of VSV. Table 4.3 is a comparison of the four strains at the nucleotide and amino acid levels.

Table 4.3 shows that viruses from the same serotype have the same length of mRNA and code for proteins of the same size. There is high homology between viruses of the same serotype both at the nucleotide and amino acid level. If nucleotide homology is taken as a measure of the relatedness of two viruses then the two viruses from the IND subtype (97%) are more closely related than the two viruses from the NJ serotype (85%).

The two strains of the NJ serotype were isolated six years apart. The Ogden strain representing the Concan subtype was isolated from cattle in Utah in 1949; the Missouri strain representing the Hazelhurst subtype from swine in 1943 (C.R. Pringle, personal communication). I have not been able to find any information on the origin of the two IND strains but the greater sequence divergence in the NJ serotype is probably due to the fact that one of the

strains belongs to the Hazelhurst subtype. The Hazelhurst subtype is regarded as an unrepresentative form of the NJ serotype due to its apparently limited host range (Schnitzlein and Reichmann, 1985). Until 1982 when it was isolated from horses and black flies (Simuliidae) in an outbreak near Loveland, Colorado, the Hazelhurst subtype had only been isolated from swine (Schnitzlein and Reichmann, 1985).

Figure 4.6 shows the nucleotide sequences of the NS mRNAs of the Ogden and Missouri strains of the NJ serotype aligned for maximum homology using the HOMOL program of Taylor (1984). The majority of the differences occur in the 3' half of the mRNA. A limited comparison of 151 bases at the 5' end and 208 bases at the 3' end of the N mRNA of the Missouri and Ogden strains shows a similar degree of homology (D.J. McGeoch, personal communication; Pringle, 1986).

Figure 4.7 shows that there are 27 amino acid differences between the two NS proteins of the Ogden and Missouri strains. In particular, there are six contiguous mismatched residues at position 200-205. However, 13 of the 27 amino acid differences are the result of conservative changes and so one can say that there is only 5% difference in the amino acid content of the two proteins. On SDS-polyacrylamide gels the difference in amino acid composition manifests itself as a difference in the electrophoretic mobility of the NS proteins (Schnitzlein and Reichmann, 1985). Gopalkrishna and Lenard compared the M genes of three strains of VSV IND (San Juan, Glasgow, Orsay) and reported 1.8% nucleotide and 2.6% amino acid differences

between the three strains. The values for the nucleotide and amino acid differences between the NS genes of the San Juan and Mudd-Summers strain of VSV IND (3% and 5% respectively) are approximately double those obtained for the M gene by Gopalkrishna and Lenard (1985). Thus it seems that the M gene is more highly conserved than the NS gene.

Between serotypes there is approximately 50% nucleotide homology and 33% amino acid homology (Table 4.3). In Figure 4.8 the distribution of homology between the NS genes of the IND and NJ serotypes at the amino acid and nucleotide level is shown as a computer generated dot matrix. The computer compares sequences of a certain size and, depending on the degree of homology, prints a dot of variable intensity. Thus regions of homology are denoted by a diagonal line and the level of homology by the thickness of the line. At the nucleotide level the homology is quite scattered; however, at the amino acid level it is apparent that the two proteins are related.

When one examines the amino acid composition of the four NS proteins (See Table 4.4) it can be seen that overall their composition is very similar. However, the serotypes do differ in the relative amounts of valine, methionine, alanine and cysteine. The overall similarity in amino acid composition is reflected in the hydropathicity profiles of the proteins. Figure 4.9 is a comparison of the hydropathic plots of the four NS proteins. The profiles are very similar, the four proteins being predominantly hydrophilic especially in the amino-terminal half.

Figure 4.10 shows the predicted secondary structure of VSV NJ, Missouri strain and VSV IND, San Juan strain. The

secondary structures were predicted following the method of Chou and Fasman (1978). It can be seen from Figure 4.10 that the proteins share a number of features in their predicted secondary structures. The degree of secondary structure predicted for the first 190 amino acids of each protein is very similar. The majority of the potential phosphorylated residues lie in this region. It is unclear how the areas of complex secondary structure and phosphorylation are related to one another. The shaded areas are regions of hydrophobicty. Predictions show that the areas of hydrophilicity are predominantly in the amino-terminal half of the proteins.

As mentioned earlier comparisons of related proteins can give some insight into structurally important regions. If a region is conserved between related proteins then that region is likely to be functionally important. Likewise a rapidly changing region is probably not important to function (Dover and Flavell, 1984). Figure 4.11 is a comparison of the four NS proteins aligned for maximum homology using the HOMOL program of Taylor (1984). The overall homology between the four NS proteins is 33%. If conservative changes are considered then the homology is increased to 52% (see Table 4.5).

In Figure 4.11 there are four domains designated I, II III and IV

Domains I, II and III are regions of high conservation and so might be important to the function of NS protein. In these areas there is approximately double the overall homology. When conservative changes are taken into consideration the homologies are considerably greater being

over 90% for domain III (see Table 4.5).

Domain IV is the area where there is the most difference between the two NS proteins of the NJ serotype and the area where a deletion has been made in the NS protein sequences of the IND serotypes in order to align the proteins. This suggests that this domain is a non-essential or dispensable portion of the protein.

There are 18 potential phosphorylated residues conserved between the four NS proteins and these are highlighted in Figure 4.12. The Mudd-Summers strain of VSV has the least number of serine and threonine residues (38) and of those 48% are conserved between the four proteins. Interestingly, none of the conserved serine or threonine residues appear in the highly conserved carboxy-terminal region (domain III).

Kingsbury and co-workers have studied NS protein phosphorylation by examining the products of chemical or enzymic cleavage of the NS protein of VSV IND. The NS1 class of molecules are phosphorylated in two relatively acidic clusters of chymotryptic peptides and molecules of the NS2 class are phosphorylated at several additional peptides (Hsu et al, 1982). Hsu and Kingsbury (1985) proposed that every molecule of NS protein was phosphorylated at a set of common residues they termed the constitutive phophorylation sites. In their hypothesis phosphorylation of the constitutive sites was essential for a basal level of NS protein activity (Hsu and Kingsbury, 1985).

Further analysis of the NS protein by chemical and enzymic means localised the constitutive sites to a peptide

between amino acids 38 and 75 (Hsu and Kingsbury, 1985). Bell and Prevec (1985) observed the same major phosphorylation patterns in all forms of cytoplasmic NS protein (whether the NS proteins were derived from the soluble cytoplasmic pool or attached to nucleocapsids) and so their results support the hypothesis of Hsu and Kingsbury (1985) that a common set of residues are phosphorylated in all forms of NS protein.

Inspection of the deduced amino acid sequence of the NS protein of the IND serotype (Gallione et al, 1981, with corrections by J.K. Rose, 1985) reveals there are five residues in the peptide between amino acids 38 and 75 with the ability to accept a phosphate group. Hsu and Kingsbury (1985) proposed these five residues were the constitutively phosphorylated residues.

Figure 4.12 shows that the five residues identified by Hsu and Kingsbury (1985) as being the constitutively phosphorylated residues in the NS protein of VSV IND are conserved between the four NS proteins. These data in addition to those of other workers (Hsu and Kingsbury, 1985; Bell and Prevec, 1985) points to the five residues highlighted in Figure 4.12 being important in NS protein activity.

The overall conclusion to be drawn from the comparison of the NS proteins of the four different strains of VSV is that structurally the proteins are very similar although there is only limited sequence homology.

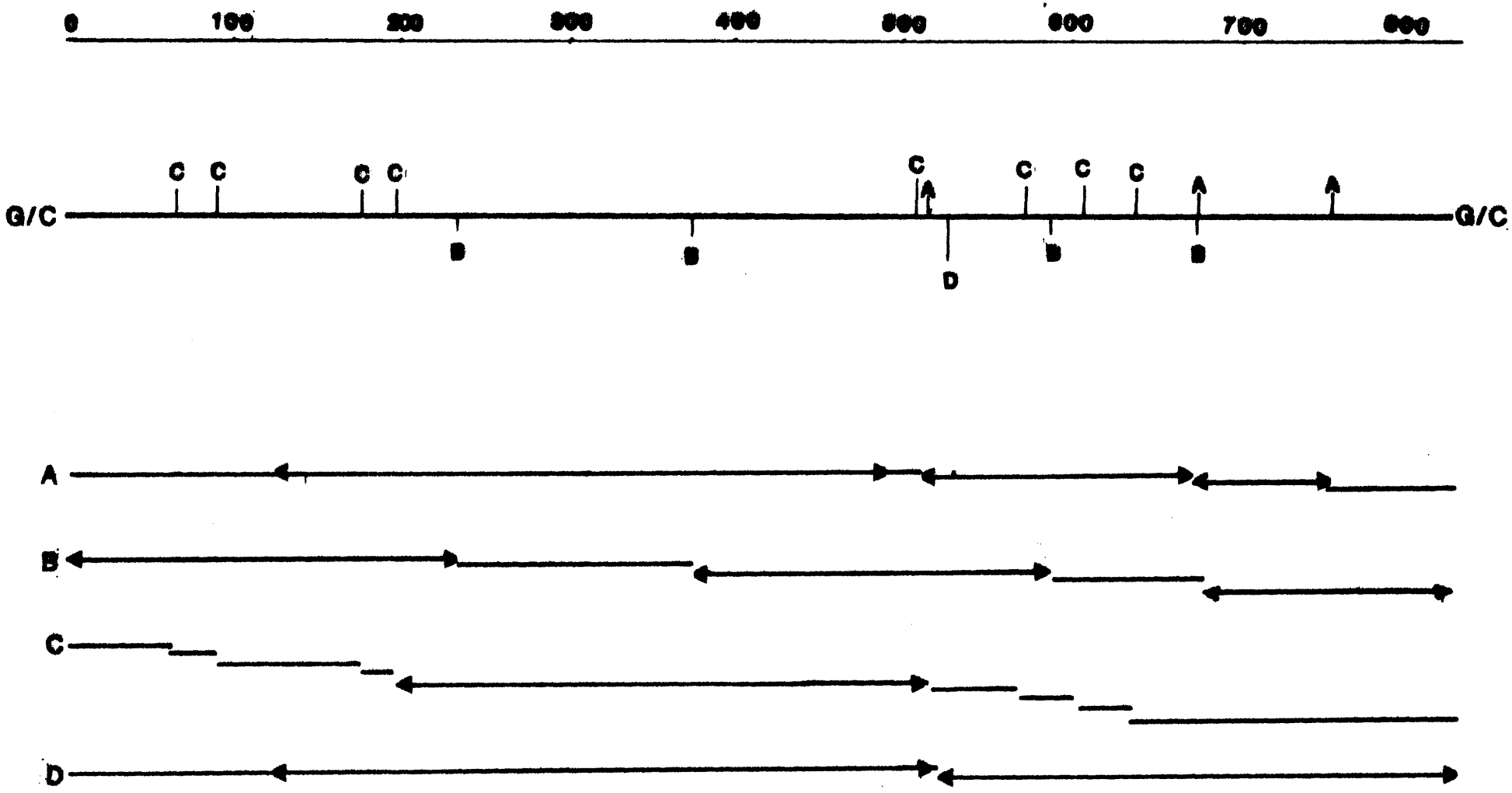

0
100
200
300
400
500
600
700
800
G/C
C
C
C
C
C
C
C
C
A
A
A
B
B
B
B
D
G/C
A
B
C
D

Figure 4.1

Restriction enzyme map of the insert from pNJNS1 showing the cloning and sequencing strategy. The insert of pNJNS1 represents a full-length cDNA copy of the NS mRNA of VSV NJ, Missouri strain. G/C at the ends indicate PstI sites generated by the G/C cloning procedure. The scale measures distances from the 5' end of the NS mRNA in hundreds of nucleotides. Restriction enzyme fragments used to derive the sequences are shown in the lower part of the figure and the arrows represent the limits of the sequence data from each fragment.

A = HpaII

B = HinfI

C = HaeIII

D = PstI

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            Met Asp Ser Ile Asp Arg Leu Lys Thr Tyr Leu Ala Thr Tyr Asp Asn    16
AACAGAGATC ATG GAC AGT ATT GAT CGG CTC AAA ACT TAC TTG GCT ACT TAT GAT AAT    58

Leu Asp Ser Ala Leu Gln Asp Ala Asn Glu Ser Glu Glu Arg Arg Glu Asp Lys Tyr   35
TTG GAT TCT GCC TTG CAG GAT GCA AAT GAG TCT GAA GAA AGA AGA GAG GAT AAA TAT  115

Leu Gln Asp Leu Phe Ile Glu Asp Gln Gly Asp Lys Pro Thr Pro Ser Tyr Tyr Gln   54
CTC CAA GAC CTC TTC ATC GAA GAT CAA GGA GAT AAA CCA ACT CCG TCA TAT TAT CAG  172

Glu Glu Glu Ser Ser Asp Ser Asp Thr Asp Tyr Asn Ala Glu His Leu Thr Met Leu   73
GAA GAA GAA TCG TCA GAT TCA GAT ACT GAC TAT AAT GCT GAA CAT CTT ACG ATG TTG  229

Ser Pro Asp Glu Arg Ile Asp Lys Trp Glu Glu Asp Leu Pro Glu Leu Glu Lys Ile   92
TCG CCG GAT GAA AGA ATA GAC AAA TGG GAA GAA GAT TTG CCC GAA TTG GAA AAG ATT  286

Asp Asp Asp Ile Pro Val Thr Phe Ser Asp Trp Thr Gln Pro Val Met Lys Glu Asn  111
GAT GAT GAT ATA CCA GTA ACT TTT TCT GAC TGG ACA CAA CCT GTA ATG AAG GAA AAC  343

Gly Gly Glu Lys Ser Leu Ser Leu Phe Pro Pro Val Gly Leu Thr Lys Val Gln Thr  130
GGA GGG GAG AAA TCA CTA TCT CTG TTT CCT CCG GTT GGA TTA ACA AAA GTT CAG ACA  400

Asp Gln Trp Arg Lys Thr Ile Glu Ala Val Cys Glu Ser Ser Lys Tyr Trp Asn Leu  149
GAC CAA TGG AGG AAG ACA ATT GAG GCA GTC TGT GAG AGC TCT AAA TAT TGG AAT TTG  457

Ser Glu Cys Gln Ile Met Asn Ser Glu Asp Arg Leu Ile Leu Lys Gly Arg Ile Met  168
TCA GAA TGT CAG ATT ATG AAC TCA GAA GAT CGT CTT ATC CTC AAA GGC CGA ATT ATG  514

Thr Pro Asp Cys Ser Ser Ser Ile Lys Ser Gln Asn Ser Ile Gln Ser Ser Glu Ser  187
ACT CCT GAC TGC AGT TCA TCA ATC AAA TCT CAG AAC TCC ATA CAG AGT TCC GAA TCT  571

Leu Ser Ser Ser His Ser Pro Gly Pro Ala Pro Lys Ser Arg Asn Gln Leu Gly Leu  206
CTC TCC TCC TCG CAT TCA CCC GGT CCA GCA CCA AAG TCA CGG AAT CAA CTA GGT CTA  628

Trp Asp Ser Lys Ser Thr Glu Val Gln Leu Ile Ser Lys Arg Ala Gly Val Lys Asp  225
TGG GAT TCA AAA TCA ACA GAA GTA CAA CTG ATC TCG AAG AGG GCC GGA GTG AAG GAC  685

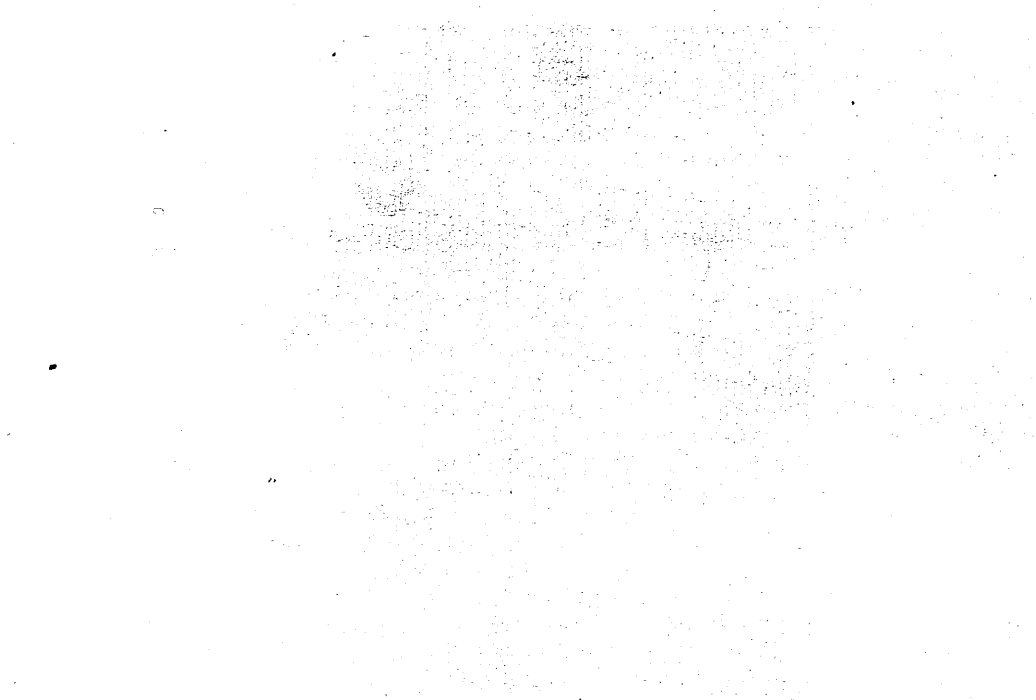
Met Met Val Lys Leu Thr Asp Phe Phe Gly Ser Glu Glu Glu Tyr Tyr Ser Val Cys  244
ATG ATG GTG AAA TTG ACA GAC TTT TTT GGA AGT GAA GAG GAA TAT TAT TCA GTA TGC  742

Pro Glu Gly Ala Pro Asp Leu Met Gly Ala Ile Ile Met Gly Leu Lys His Lys Lys  263
CCA GAA GGG GCC CCA GAT TTG ATG GGA GCA ATC ATC ATG GGA CTA AAA CAT AAA AAG  799

Leu Phe Asn Gln Ala Arg Met Lys Tyr Arg Ile ---                              274
CTT TTT AAC CAA GCA AGA ATG AAG TAT CGT ATC TAA TTAATTCCGATGATCAATATG        856
```

Figure 4.2

Nucleotide sequence and deduced amino acid sequence of the NS mRNA of VSV NJ, Missouri strain. The 5' terminal methylated cap structure and 3' terminal poly(A) tail are not shown. The 5' and 3' consensus sequences are highlighted. The NS mRNA is 856 nucleotides long and codes for a protein of 274 amino acids (mol wt 31,000).



```
                Met Asp Ser Ile Asp Arg Leu Lys Thr Tyr Leu Ala Thr Tyr Asp Asn    16
AACAGAGATC ATG GAC AGT ATT GAT CGG CTC AAA ACT TAC TTG GCT ACT TAT GAT AAT    58

Leu Asp Ser Ala Leu Gln Asp Ala Asn Glu Ser Glu Glu Arg Arg Glu Asp Lys Tyr    35
TTG GAT TCT GCC TTG CAG GAT GCA AAT GAG TCT GAA GAA AGA AGA GAG GAT AAA TAT   115

Leu Gln Asp Leu Phe Ile Glu Asp Gln Gly Asp Lys Pro Thr Pro Ser Tyr Tyr Gln    54
CTC CAA GAC CTC TTC ATC GAA GAT CAA GGA GAT AAA CCA ACT CCG TCA TAT TAT CAG   172

Glu Glu Glu Ser Ser Asp Ser Asp Thr Asp Tyr Asn Ala Glu His Leu Thr Met Leu    73
GAA GAA GAA TCG TCA GAT TCA GAT ACT GAC TAT AAT GCT GAA CAT CTT ACG ATG TTG   229

Ser Pro Asp Glu Arg Ile Asp Lys Trp Glu Glu Asp Leu Pro Glu Leu Glu Lys Ile    92
TCG CCG GAT GAA AGA ATA GAC AAA TGG GAA GAA GAT TTG CCC GAA TTG GAA AAG ATT   286

Asp Asp Asp Ile Pro Val Thr Phe Ser Asp Trp Thr Gln Pro Val Met Lys Glu Asn   111
GAT GAT GAT ATA CCA GTA ACT TTT TCT GAC TGG ACA CAA CCT GTA ATG AAG GAA AAC   343

Gly Gly Glu Lys Ser Leu Ser Leu Phe Pro Pro Val Gly Leu Thr Lys Val Gln Thr   130
GGA GGG GAG AAA TCA CTA TCT CTG TTT CCT CCG GTT GGA TTA ACA AAA GTT CAG ACA   400

Asp Gln Trp Arg Lys Thr Ile Glu Ala Val Cys Glu Ser Ser Lys Tyr Trp Asn Leu   149
GAC CAA TGG AGG AAG ACA ATT GAG GCA GTC TGT GAG AGC TCT AAA TAT TGG AAT TTG   457

Ser Glu Cys Gln Ile Met Asn Ser Glu Asp Arg Leu Ile Leu Lys Gly Arg Ile Met   168
TCA GAA TGT CAG ATT ATG AAC TCA GAA GAT CGT CTT ATC CTC AAA GGC CGA ATT ATG   514

Thr Pro Asp Cys Ser Ser Ser Ile Lys Ser Gln Asn Ser Ile Gln Ser Ser Glu Ser   187
ACT CCT GAC TGC AGT TCA TCA ATC AAA TCT CAG AAC TCC ATA CAG AGT TCC GAA TCT   571

Leu Ser Ser Ser His Ser Pro Gly Pro Ala Pro Lys Ser Arg Asn Gln Leu Gly Leu   206
CTC TCC TCC TCG CAT TCA CCC GGT CCA GCA CCA AAG TCA CGG AAT CAA CTA GGT CTA   628

Trp Asp Ser Lys Ser Thr Glu Val Gln Leu Ile Ser Lys Arg Ala Gly Val Lys Asp   225
TGG GAT TCA AAA TCA ACA GAA GTA CAA CTG ATC TCG AAG AGG GCC GGA GTG AAG GAC   685

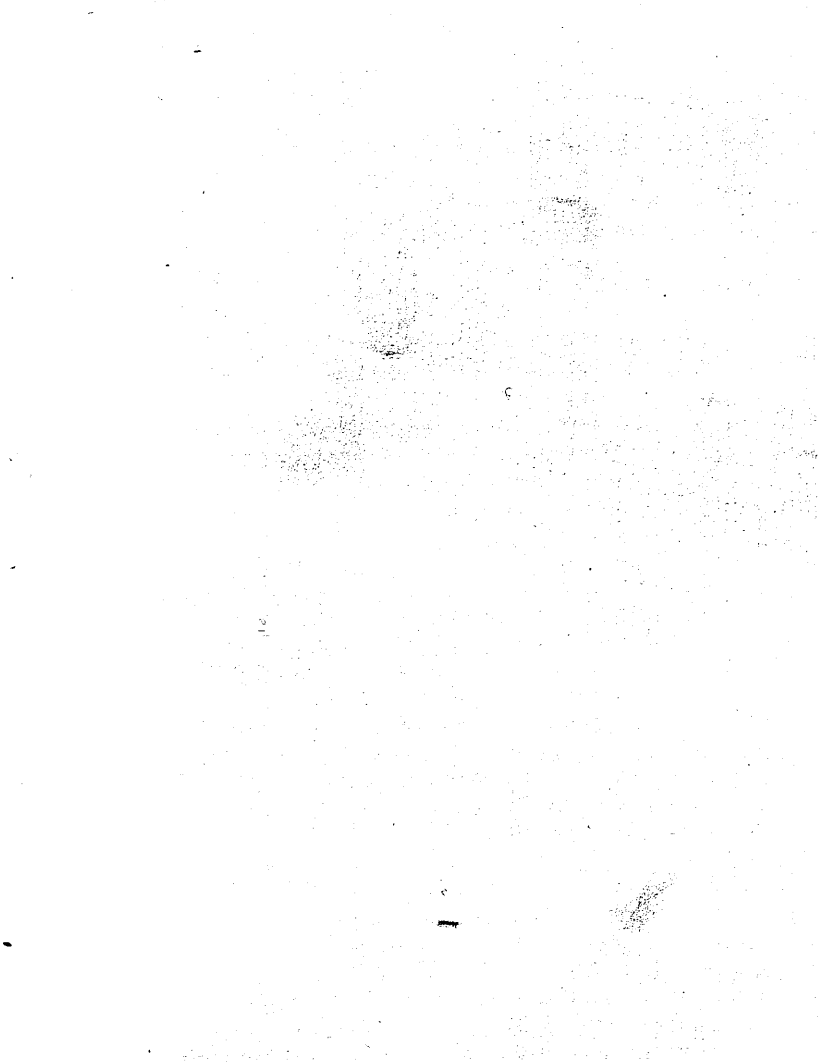
Met Met Val Lys Leu Thr Asp Phe Phe Gly Ser Glu Glu Glu Tyr Tyr Ser Val Cys   244
ATG ATG GTG AAA TTG ACA GAC TTT TTT GGA AGT GAA GAG GAA TAT TAT TCA GTA TGC   742

Pro Glu Gly Ala Pro Asp Leu Met Gly Ala Ile Ile Met Gly Leu Lys His Lys Lys   263
CCA GAA GGG GCC CCA GAT TTG ATG GGA GCA ATC ATC ATG GGA CTA AAA CAT AAA AAG   799

Leu Phe Asn Gln Ala Arg Met Lys Tyr Arg Ile ---                              274
CTT TTT AAC CAA GCA AGA ATG AAG TAT CGT ATC TAA TTAATTCCGATGATCAATATG        856
```

Figure 4.3

Nucleotide and predicted amino acid sequence of the NS mRNA of VSV NJ, Missouri strain. The acidic ( ) and basic ( ) amino acids are highlighted. The NS protein is highly acidic especially in the amino terminal half.



```
                Met Asp Ser Ile Asp Arg Leu Lys Thr Tyr Leu Ala Thr Tyr Asp Asn    16
AACAGAGATC ATG GAC AGT ATT GAT CGG CTC AAA ACT TAC TTG GCT ACT TAT GAT AAT     58

Leu Asp Ser Ala Leu Gln Asp Ala Asn Glu Ser Glu Glu Arg Arg Glu Asp Lys Tyr    35
TTG GAT TCT GCC TTG CAG GAT GCA AAT GAG TCT GAA GAA AGA AGA GAG GAT AAA TAT   115

Leu Gln Asp Leu Phe Ile Glu Asp Gln Gly Asp Lys Pro Thr Pro Ser Tyr Tyr Gln    54
CTC CAA GAC CTC TTC ATC GAA GAT CAA GGA GAT AAA CCA ACT CCG TCA TAT TAT CAG   172

Glu Glu Glu Ser Ser Asp Ser Asp Thr Asp Tyr Asn Ala Glu His Leu Thr Met Leu    73
GAA GAA GAA TCG TCA GAT TCA GAT ACT GAC TAT AAT GCT GAA CAT CTT ACG ATG TTG   229

Ser Pro Asp Glu Arg Ile Asp Lys Trp Glu Glu Asp Leu Pro Glu Leu Glu Lys Ile    92
TCG CCG GAT GAA AGA ATA GAC AAA TGG GAA GAA GAT TTG CCC GAA TTG GAA AAG ATT   286

Asp Asp Asp Ile Pro Val Thr Phe Ser Asp Trp Thr Gln Pro Val Met Lys Glu Asn   111
GAT GAT GAT ATA CCA GTA ACT TTT TCT GAC TGG ACA CAA CCT GTA ATG AAG GAA AAC   343

Gly Gly Glu Lys Ser Leu Ser Leu Phe Pro Pro Val Gly Leu Thr Lys Val Gln Thr   130
GGA GGG GAG AAA TCA CTA TCT CTG TTT CCT CCG GTT GGA TTA ACA AAA GTT CAG ACA   400

Asp Gln Trp Arg Lys Thr Ile Glu Ala Val Cys Glu Ser Ser Lys Tyr Trp Asn Leu   149
GAC CAA TGG AGG AAG ACA ATT GAG GCA GTC TGT GAG AGC TCT AAA TAT TGG AAT TTG   457

Ser Glu Cys Gln Ile Met Asn Ser Glu Asp Arg Leu Ile Leu Lys Gly Arg Ile Met   168
TCA GAA TGT CAG ATT ATG AAC TCA GAA GAT CGT CTT ATC CTC AAA GGC CGA ATT ATG   514

Thr Pro Asp Cys Ser Ser Ser Ile Lys Ser Gln Asn Ser Ile Gln Ser Ser Glu Ser   187
ACT CCT GAC TGC AGT TCA TCA ATC AAA TCT CAG AAC TCC ATA CAG AGT TCC GAA TCT   571

Leu Ser Ser Ser His Ser Pro Gly Pro Ala Pro Lys Ser Arg Asn Gln Leu Gly Leu   206
CTC TCC TCC TCG CAT TCA CCC GGT CCA GCA CCA AAG TCA CGG AAT CAA CTA GGT CTA   628

Trp Asp Ser Lys Ser Thr Glu Val Gln Leu Ile Ser Lys Arg Ala Gly Val Lys Asp   225
TGG GAT TCA AAA TCA ACA GAA GTA CAA CTG ATC TCG AAG AGG GCC GGA GTG AAG GAC   685

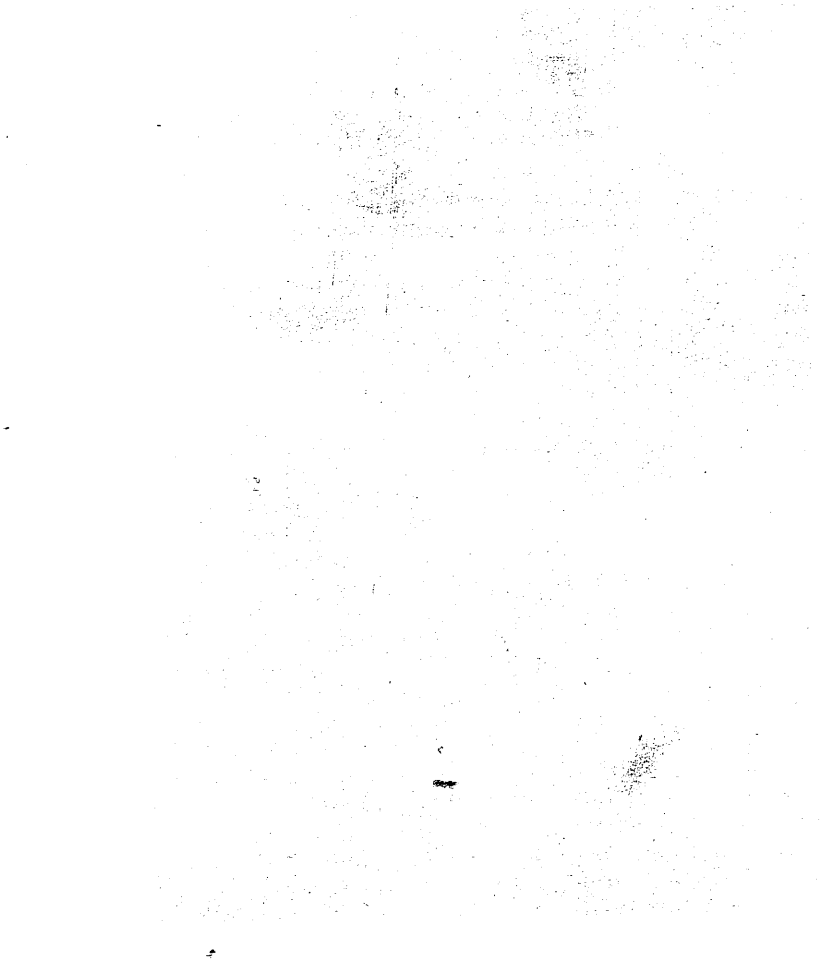
Met Met Val Lys Leu Thr Asp Phe Phe Gly Ser Glu Glu Glu Tyr Tyr Ser Val Cys   244
ATG ATG GTG AAA TTG ACA GAC TTT TTT GGA AGT GAA GAG GAA TAT TAT TCA GTA TGC   742

Pro Glu Gly Ala Pro Asp Leu Met Gly Ala Ile Ile Met Gly Leu Lys His Lys Lys   263
CCA GAA GGG GCC CCA GAT TTG ATG GGA GCA ATC ATC ATG GGA CTA AAA CAT AAA AAG   799

Leu Phe Asn Gln Ala Arg Met Lys Tyr Arg Ile ---                              274
CTT TTT AAC CAA GCA AGA ATG AAG TAT CGT ATC TAA TTAATTCCGATGATCAATATG        856
```

Figure 4.4

Nucleotide and predicted amino acid sequence of the NS mRNA of VSV NJ, Missouri strain with the potential phosphorylated residues (ie. serines and threonines) highlighted. There are 46 potential phosphorylation sites in the protein: 13 threonines, 33 serines.



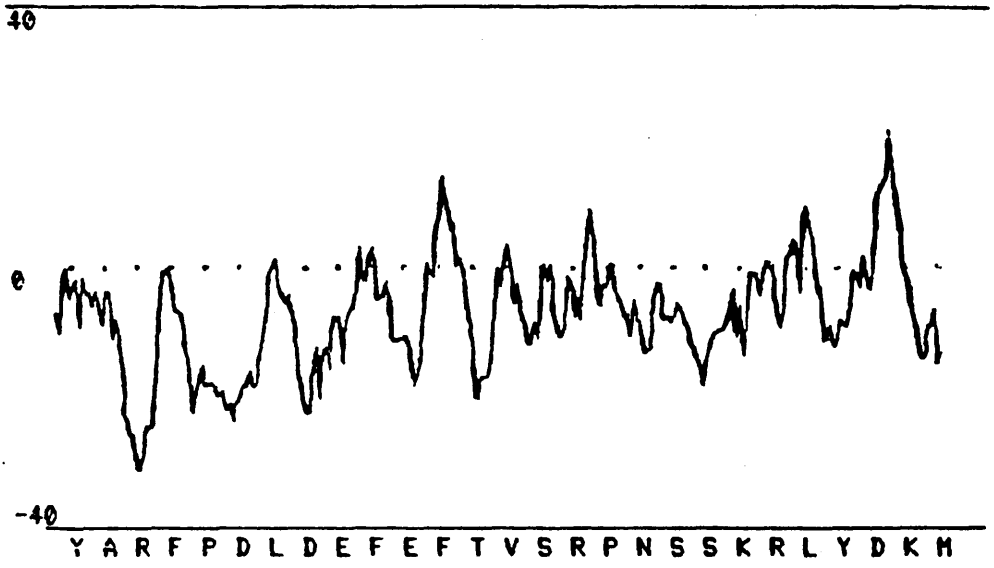

40
0
-40
Y A R F P D L D E F E F T V S R P N S S K R L Y D K M

Figure 4.5

Hydropathy plot of the NS protein of VSV NJ, Missouri strain using the program of Kyte and Doolittle (1982). The degree of hydrophobicity increases with the distance above the horizontal line; hydrophilicity increases with distance below the horizontal line. The letters below the plot refer to the amino acid residues. The NS protein is predominantly a hydrophilic protein.

Parameters used were: group length = 9
shift = 1
spacing between plotted points = 2

A window size of nine amino acids was used in the plot and so the letters below the plot refer to each ninth amino acid.

First sequence: NJNS.RAE
Second sequence: NJNS.BAN

```
  1  AACAGAGATCATGGACAGTATTGATCGGCTCAAAACTTACTTGGCTACTTATGATAATTTGGATTCTGCCTTGC
     ****** ************ ***** ******* ******** ** ****************************
  1  AACAGATATCATGGACAGTGTTGATAGGCTCAAGACTTACTTAGCCACTTATGATAATTTGGATTCTGCCTTGC

 75  AGGATGCAAATGAGTCTGAAGAAAGAAGAGAGGATAAATATCTCCAAGACCTCTTCATCGAAGATCAAGGAGAT
     ******* ***** ***** ****** ************************************************
 75  AGGATGCCAATGAATCTGAGGAAAGACGAGAGGATAAATATCTCCAAGACCTCTTCATCGAAGATCAAGGAGAT

149  AAACCAACTCCGTCATATTATCAGGAAGAAGAATCGTCAGATTCAGATACTGACTATAATGCTGAACATCTTAC
     ************************************************** ********************
149  AAACCAACTCCGTCATATTATCAGGAAGAAGAATCGTCAGATTCAGATACTGATTATAATGCTGAACATCTTAC

223  GATGTTGTCGCCGGATGAAAGAATAGACAAATGGGAAGAAGATTTGCCCGAATTGGAAAAGATTGATGATGATA
     **** **** ******************** ***************** ***** ********************
223  GATGCTGTCACCGGATGAAAGAATAGACAAGTGGGAAGAAGATTTGCCTGAATTAGAAAAGATTGATGATGATA

297  TACCAGTAACTTTTTCTGACTGGACACAACCTGTAATGAAGGAAAACGGAGGGGAGAAATCACTATCTCTGTTT
     **** ** ** ******** ******** ***************** ** ** ********* * ********
297  TACCGGTGACCTTTTCTGATTGGACACAGCCTGTAATGAAGGAAAATGGGGGAGAGAAATCATTGTCTCTGTTC

371  CCTCCGGTTGGATTAACAAAAGTTCAGACAGACCAATGGAGGAAGACAATTGAGGCAGTCTGTGAGAGCTCTAA
     ***** ** ** ********  **** ***** *******  ** ** ********* ** ******** ** **
371  CCTCCAGTCGGGTTAACAAAGATTCAAACAGAACAATGGAAAAAAACCATTGAGGCGGTTTGTGAGAGTTCAAA

445  ATATTGGAATTTGTCAGAATGTCAGATTATGAACTCAGAAGATCGTCTTATCCTCAAAGGCCGAATTATGACTC
     ************ ******** ** *** * ****  *****  * ** *  ************ * ********
445  ATATTGGAATTTATCAGAATGCCAAATTCTTAACTTGGAAGACAGCCTCACTCTCAAAGGCCGATTGATGACTC

519  CTGACTGCAGTTCATCAATCAAATCTCAGAACTCCATACAGAGTTCCGAATCTCTCTCCTCCTCGCATTCACCC
     **** ** ***** *** * ******** ** **  * * *** ** *** ****** ****** ***** **
519  CTGATTGTAGTTCTTCAGTAAAATCTCAAAATTCTGTCCGGAGGTCAGAACCTCTCTACTCCTCTCATTCTCCA

593  GGTCCAGCAC CAAAGTCACGGAATCAACTAGGTCTATGGGATTCAAAATCAACAGAAGTACAACTGATCTCGA
     *****  *** *** **  * ** ** * *   * ********** *** ** ** ********* ******* *
593  GGTCCCCCACTCAAGGTATCAGAGTCCA TCAATTTATGGGATTTAAAGTCCACTGAAGTACAATTGATCTCCA

666  AGAGGGCCGGAGTGAAGGACATGATGGTGAAATTGACAGACTTTTTTGGAAGTGAAGAGGAATATTATTCAGTA
     **** ******** **********  ** ************** ************ ** ** ************
666  AGAGAGCCGGAGTTAAGGACATGACAGTCAAATTGACAGACTTCTTTGGAAGTGAGGAAGAGTATTATTCAGTA

740  TGCCCAGAAGGGGCCCCAGATTTGATGGGAGCAATCATCATGGGACTAAAACATAAAAAGCTTTTTAACCAAGC
     ************** ***** *********** ************** **  * ** ** ** ** ** ** **
740  TGCCCAGAAGGGGCGCCAGACTTGATGGGAGCTATCATCATGGGACTGAAGTACAAGAAACTCTTCAATCAGGC

814  AAGAATGAAGTATCGTATCTAATTAATTCCGATGATCAATATG
     ********** ****** * *****  **   ************
814  AAGAATGAAATATCGTCTTTAATTCCTTTTCATGATCAATATG
```

Matched = 732 out of 857 percent 85
Mutation weight = 5 Gap weights = 3k + 7, k up to 2000
Evolutionary distance = 635

Figure 4.6

Comparison of the nucleotide sequences of the NS mRNAs of the Ogden and Missouri strains of VSV NJ. The sequences are aligned for maximum homology by the program of Taylor (1984).

NJNS.RAE = sequence of NS mRNA of VSV NJ, Missouri strain

NJNS.BAN = sequence of NS mRNA of VSV NJ, Ogden strain

Matched residues are indicated by *

First sequence: OP.RAE
Second sequence: OP.BAN

```
   1 MDSIDRLKTYLATYDNLDSALQDANESEERREDKYLQDLFIEDQGDKPTPSYYQEEESSDSDTDYNAEHLTMLS
       *
   1 MDSVDRLKTYLATYDNLDSALQDANESEERREDKYLQDLFIEDQGDKPTPSYYQEEESSDSDTDYNAEHLTMLS

  75 PDERIDKWEEDLPELEKIDDDIPVTFSDWTQPVMKENGGEKSLSLFPPVGLTKVQTDQWRKTIEAVCESSKYWN
                                                              *  *  *
  75 PDERIDKWEEDLPELEKIDDDIPVTFSDWTQPVMKENGGEKSLSLFPPVGLTKIQTEQWKKTIEAVCESSKYWN

 149 LSECQIMNSEDRLILKGRIMTPDCSSSIKSQNSIQSSESLSSSHSPGPAPKSRNQLGLWDSKSTEVQLISKRAG
           * *  * *    *         *     **   * *           ******   *
 149 LSECQILNLEDSLTLKGRLMTPDCSSSVKSQNSVRRSEPLYSSHSPGPPLKVSESINLWDLKSTEVQLISKRAG

 223 VKDMMVKLTDFFGSEEEYYSVCPEGAPDLMGAIIMGLKHKKLFNQARMKYRI
       *                                  *          *
 223 VKDMTVKLTDFFGSEEEYYSVCPEGAPDLMGAIIMGLKYKKLFNQARMKYRL
```

Matched = 247 out of 274 percent 90
Mutation weight = 5 Gap weights = 3k + 7, k up to 2000
Evolutionary distance = 135

Figure 4.7

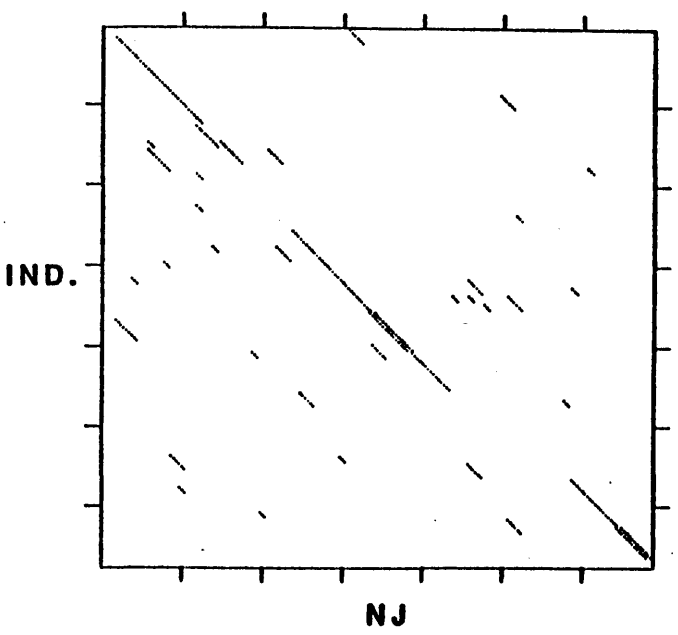
Comparison of the NS proteins of the Ogden and Missouri strains of VSV NJ. The two sequences have been aligned for maximum homology by the HOMOL program of Taylor (1984).

OP.RAE = sequence of NS protein of VSV NJ, Missouri strain

OP.BAN = sequence of NS protein of VSV NJ, Ogden strain

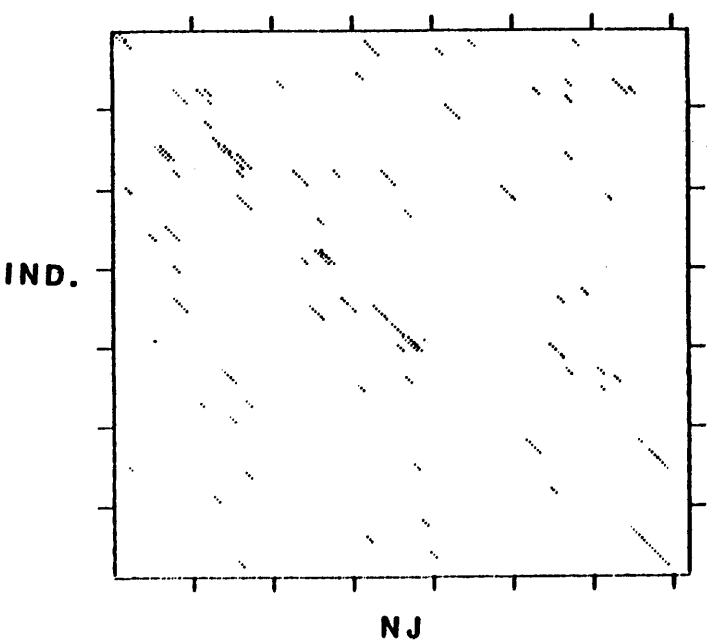
Mismatched residues are indicated by an *.

A



AMINO ACIDS

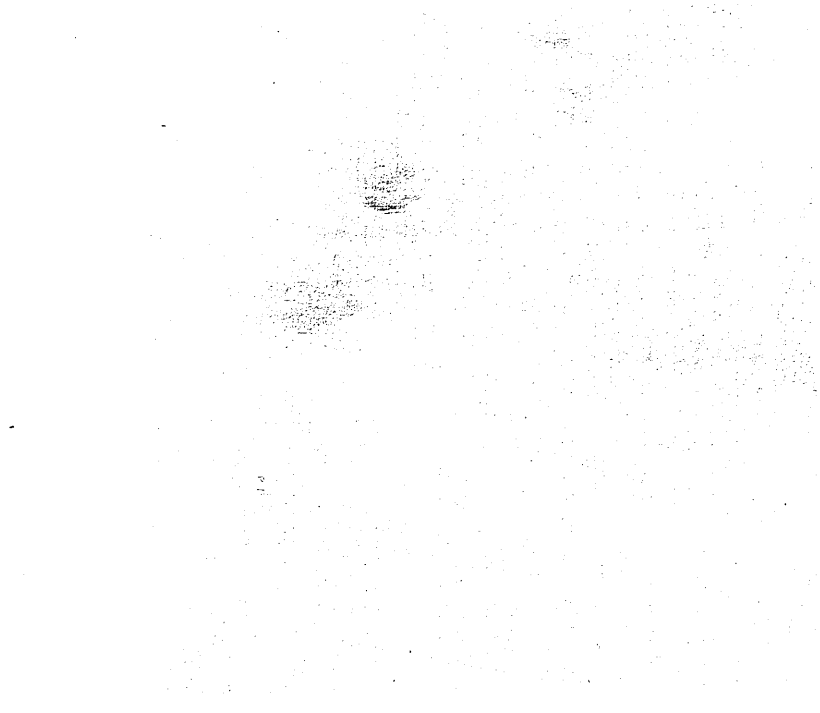
B



NUCLEOTIDES

Figure 4.8

Computer generated dot matrix comparisons of (A) the amino acid sequences and (B) the nucleotide sequences of the NS genes of VSV IND, San-Juan strain and VSV NJ, Missouri strain. In (A) a window of seven amino acid residues was used; in (B) a window of twelve nucleotides was used. Regions of homology are denoted by a diagonal line, and the level of homology by the thickness of the line.



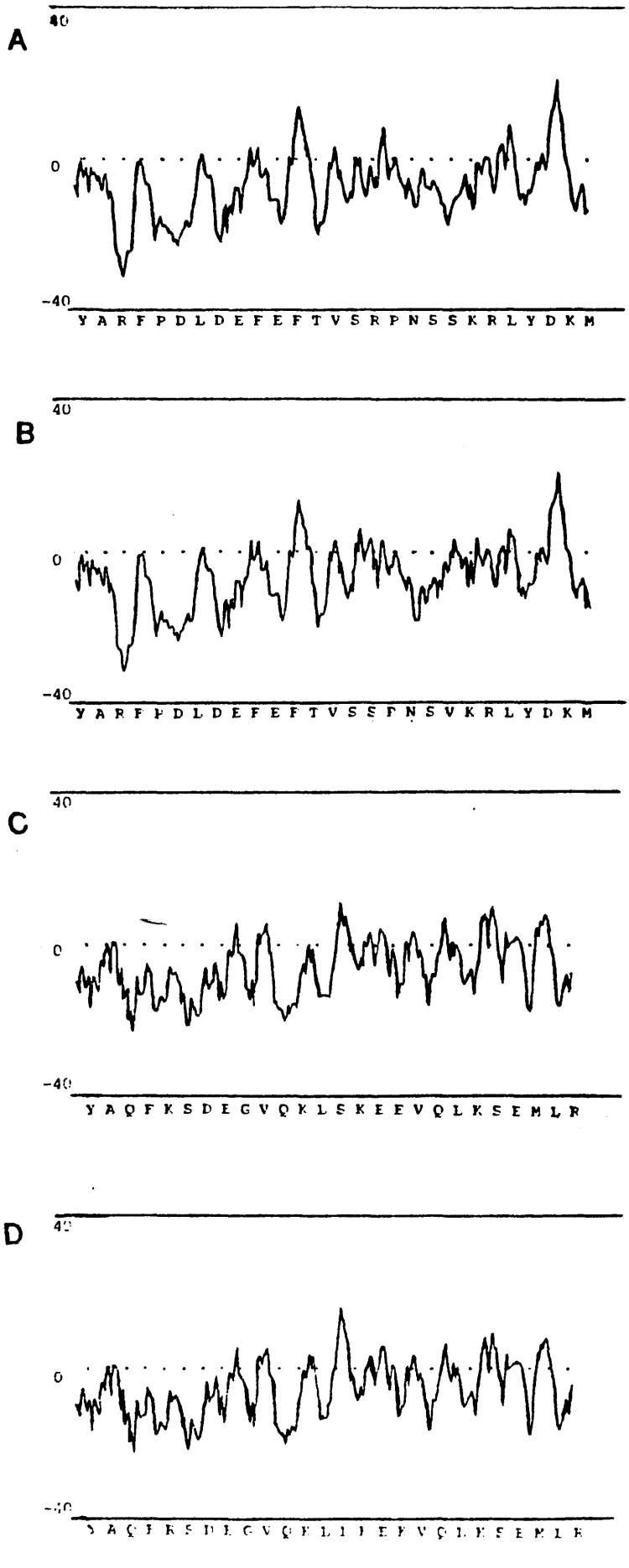

A
40
0
-40
Y A R F P D L D E F E F T V S R P N S S K R L Y D K M
B
40
0
-40
Y A R F P D L D E F E F T V S S F N S V K R L Y D K M
C
40
0
-40
Y A Q F K S D E G V Q K L S K E E V Q L K S E M L R
D
0

Figure 4.9

Comparison of the hydropathic profiles of the NS proteins from four strains of VSV as predicted by the program of Kyte and Doolittle (1982). The degree of hydrophobicity increases with distance above the mid-line; the degree of hydrophilicity increases with distance below the mid-line. Letters beneath the plots refer to amino acid residues.

A = VSV NJ, Missouri strain
B = VSV NJ, Ogden strain

C = VSV IND, San Juan strain

D = VSV IND, Mudd-Summers strain

Parameters: group length = 9

shift = 1

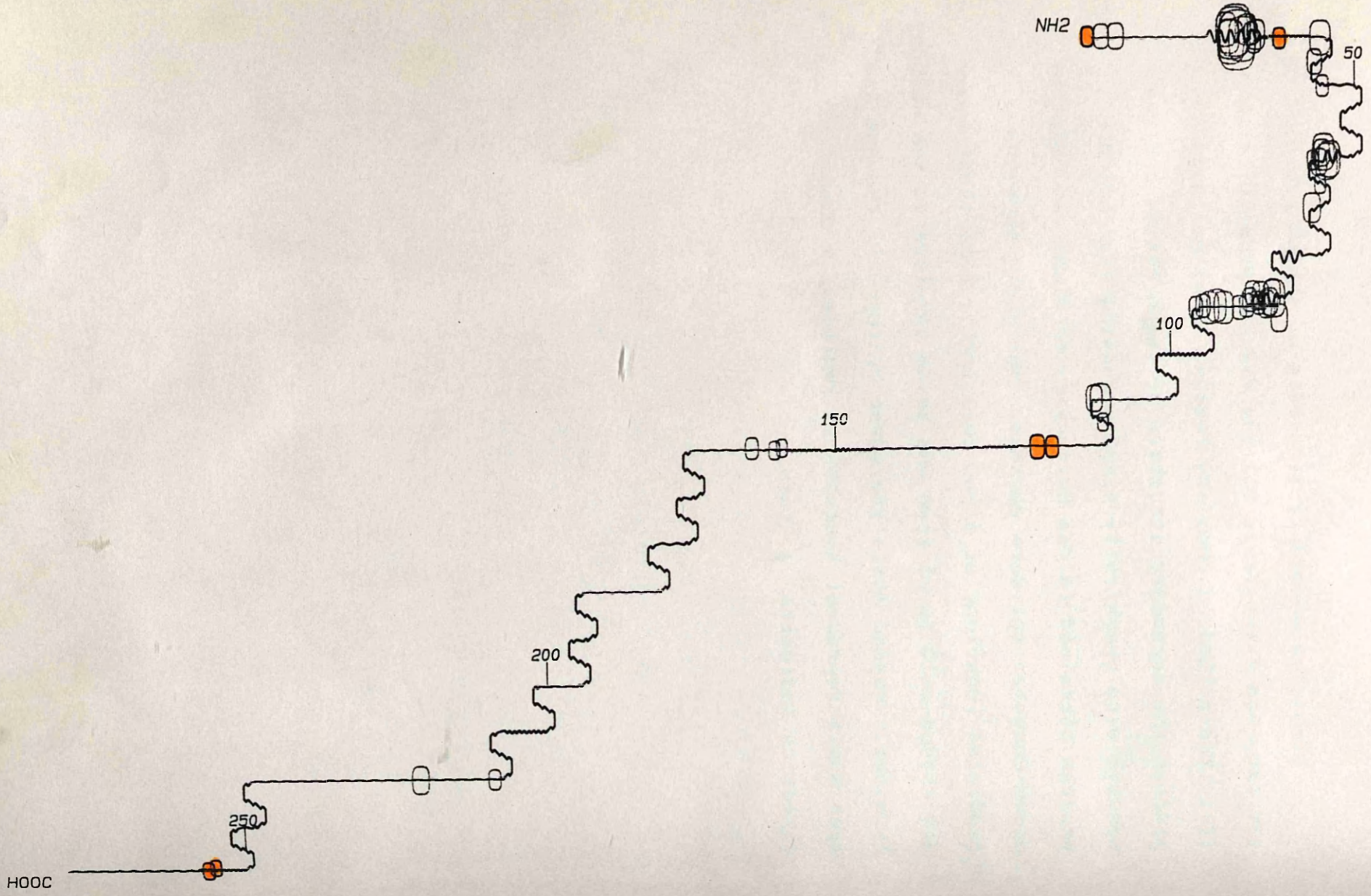
spacing betwen plotted points = 2

The four NS proteins from the different strains of VSV are broadly similar in that they are predominantly hydrophilic proteins.

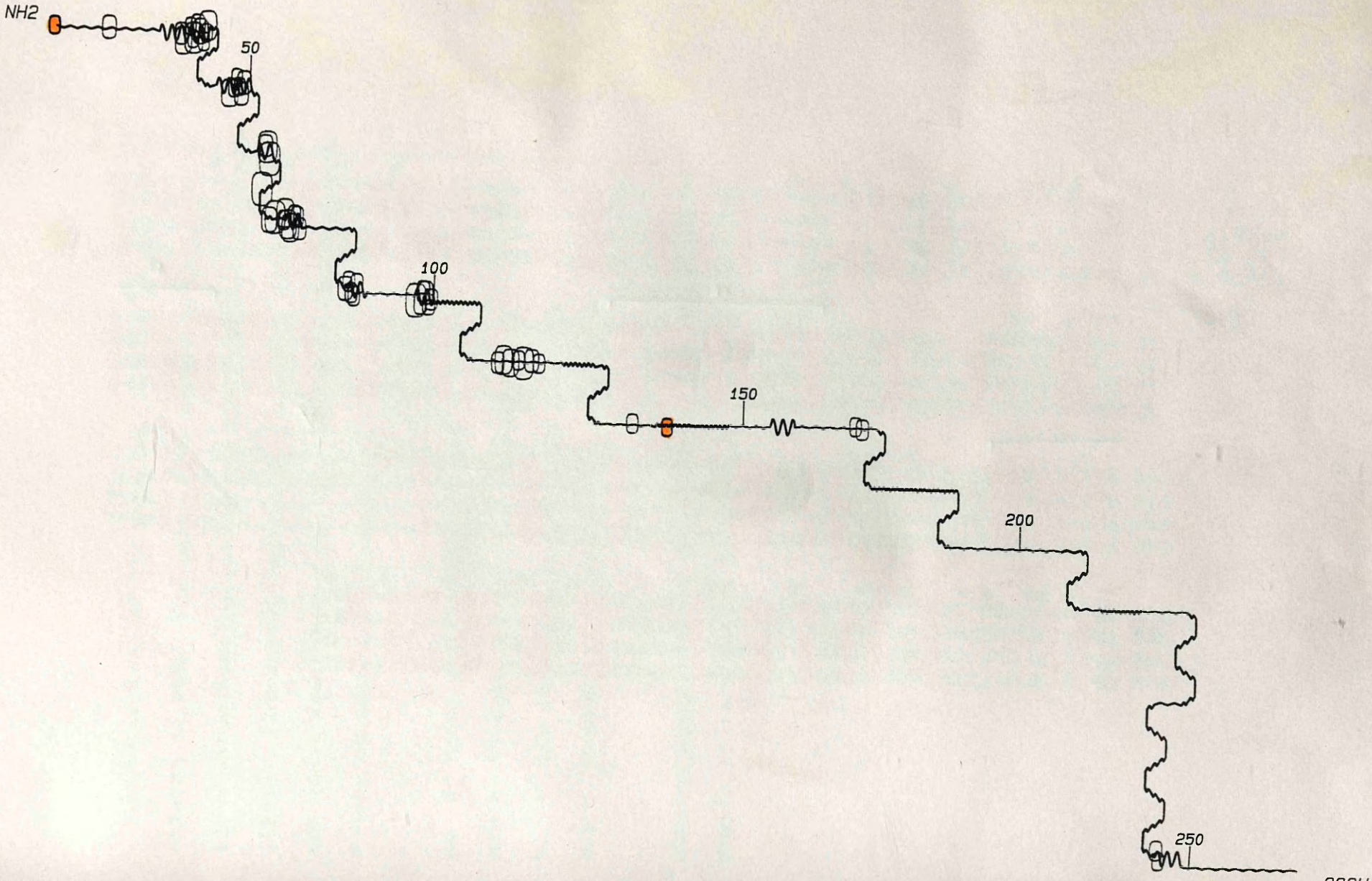
Figure 4.10

Predicted secondary structure of the NS proteins of VSV IND, San Juan strain (A) and VSV NJ, Missouri strain (B). The method of Chou and Fasman (1978) was used to predict the secondary structure of the proteins and the results were graphically displayed using the PLOTCHOU program contained in the University of Wisconsin Genetics Group Computer software package. The plots show the predicted locations of alpha coil (); beta sheet () and random coil () from the amino terminus to the carboxy terminus. Shaded ovals represent hydrophobic regions and open ovals represent hydrophilic regions. A change of direction indicates β -turn.

OP . RAE



OP . ROS



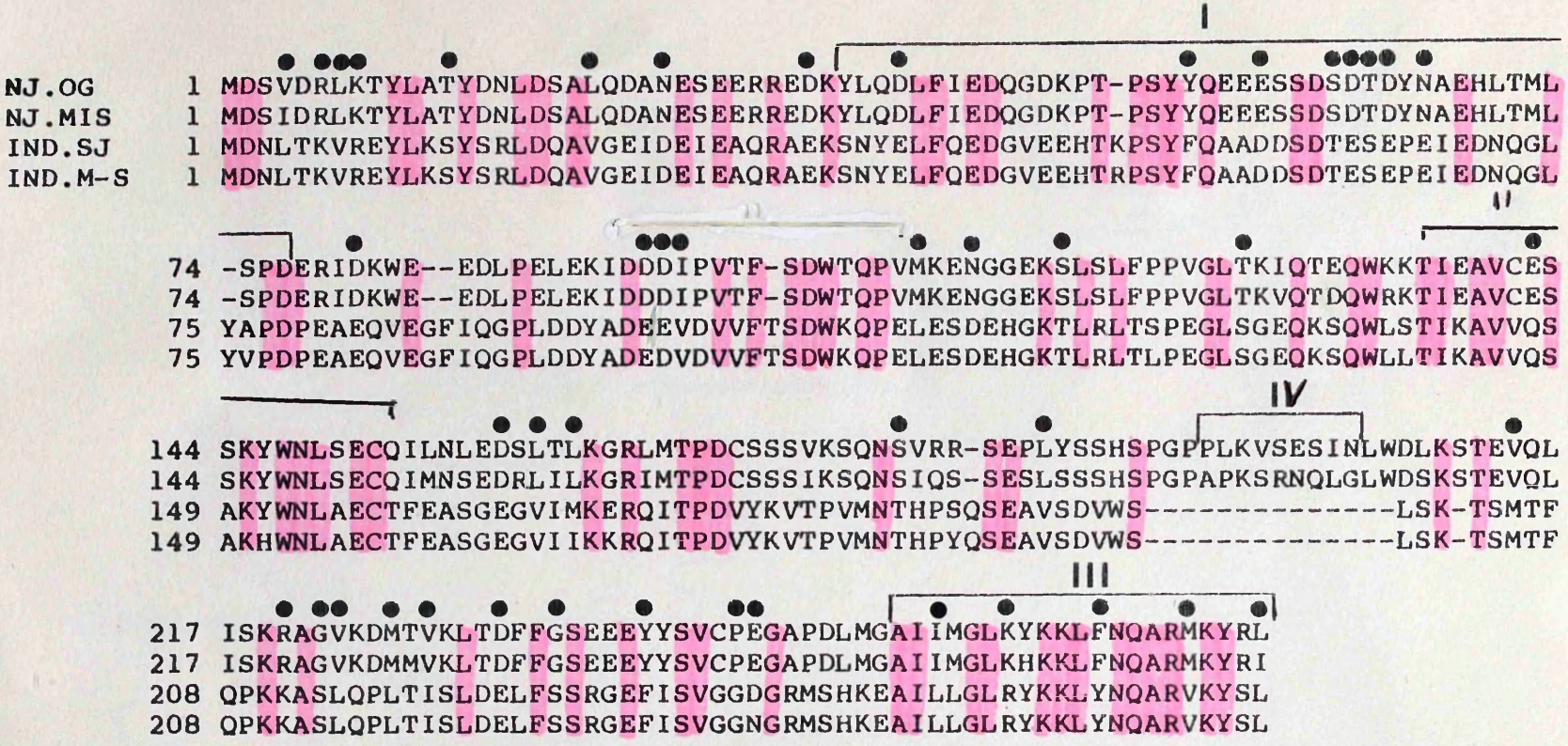

I
II
III
IV
NJ.OG 1 MDSVDRLKTYLATYDNLDSALQDANESEERREDKYLQDLFIEDQGDKPT-PSYYQEEESSDSDTDYNAEHLTML
NJ.MIS 1 MDSIDRLKTYLATYDNLDSALQDANESEERREDKYLQDLFIEDQGDKPT-PSYYQEEESSDSDTDYNAEHLTML
IND.SJ 1 MDNLTKVREYLKSYSRLDQAVGEIDEIEAQRAEKSNYELFQEDGVEEHTKPSYFQAADDSDTESEPEIEDNQGL
IND.M-S 1 MDNLTKVREYLKSYSRLDQAVGEIDEIEAQRAEKSNYELFQEDGVEEHTRPSYFQAADDSDTESEPEIEDNQGL
74 -SPDERIDKWE--EDLPELEKIDDDIPVTF-SDWTQPVMKENGGEKSLSLFPPVGLTKIQTEQWKKTIEAVCES
74 -SPDERIDKWE--EDLPELEKIDDDIPVTF-SDWTQPVMKENGGEKSLSLFPPVGLTKVQTDQWRKTIEAVCES
75 YAPDPEAEQVEGFIQGPLDDYADEEVDVVFTSDWKQPELESDEHGKTLRLTSPEGLSGEQKSQWLSTIKAVVQS
75 YVPDPEAEQVEGFIQGPLDDYADEDVDVVFTSDWKQPELESDEHGKTLRLTLPEGLSGEQKSQWLLTIKAVVQS
144 SKYWNLSECQILNLEDSLTLKGRLMTPDCSSSVKSQNSVRR-SEPLYSSHSPGPPLKVSESINLWDLKSTEVQL
144 SKYWNLSECQIMNSEDRLILKGRIMTPDCSSSIKSQNSIQS-SESLSSSHSPGPAPKSRNQLGLWDSKSTEVQL
149 AKYWNLAECTFEASGEGVIMKERQITPDVYKVTPVMNTHPSQSEAVSDVWS--------------LSK-TSMTF
149 AKHWNLAECTFEASGEGVIIKKRQITPDVYKVTPVMNTHPYQSEAVSDVWS--------------LSK-TSMTF
217 ISKRAGVKDMTVKLTDFFGSEEEYYSVCPEGAPDLMGAIIMGLKYKKLFNQARMKYRL
217 ISKRAGVKDMMVKLTDFFGSEEEYYSVCPEGAPDLMGAIIMGLKHKKLFNQARMKYRI
208 QPKKASLQPLTISLDELFSSRGEFISVGGDGRMSHKEAILLGLRYKKLYNQARVKYSL
208 QPKKASLQPLTISLDELFSSRGEFISVGGNGRMSHKEAILLGLRYKKLYNQARVKYSL

Figure 4.11

Comparison of the four NS proteins of different strains of VSV. The amino acid sequences have been aligned for maximum homology using the HOMOL program of Taylor (1984). The shaded regions ( ) denote amino acids that are conserved between the four strains. The filled circles denote conservative changes.

OG = Ogden strain, NJ serotype

MIS = Missouri strain, NJ serotype

SJ = San Juan strain, IND serotype

M-S = Mudd-Summers strain, IND serotype

Domains I and III are regions of higher homology

Domain II is a region where there is no exact homology


A
NJ.OG 1 MDSVDRLKTYLATYDNLDSALQDANESEERREDKYLQDLFIEDQGDKPT-PSYYQEEESSDSDTDYNAEHLTML
NJ.MIS 1 MDSIDRLKTYLATYDNLDSALQDANESEERREDKYLQDLFIEDQGDKPT-PSYYQEEESSDSDTDYNAEHLTML
IND.SJ 1 MDNLTKVREYLKSYSRLDQAVGEIDEIEAQRAEKSNYELFQEDGVEEHTKPSYFQAADDSDTESEPEIEDNQGL
IND.M-S 1 MDNLTKVREYLKSYSRLDQAVGEIDEIEAQRAEKSNYELFQEDGVEEHTRPSYFQAADDSDTESEPEIEDNQGL
74 -SPDERIDKWE--EDLPELEKIDDDIPVTF-SDWTQPVMKENGGEKSLSLFPPVGLTKIQTEQWKKTIEAVCES
74 -SPDERIDKWE--EDLPELEKIDDDIPVTF-SDWTQPVMKENGGEKSLSLFPPVGLTKVQTDQWRKTIEAVCES
75 YAPDPEAEQVEGFIQGPLDDYADEEVDVVFTSDWKQPELESDEHGKTLRLTSPEGLSGEQKSQWLSTIKAVVQS
75 YVPDPEAEQVEGFIQGPLDDYADEDVDVVFTSDWKQPELESDEHGKTLRLTLPEGLSGEQKSQWLLTIKAVVQS
144 SKYWNLSECQILNLEDSLTLKGRLMTPDCSSSVKSQNSVRR-SEPLYSSHSPGPPLKVSESINLWDLKSTEVQL
144 SKYWNLSECQIMNSEDRLILKGRIMTPDCSSSIKSQNSIQS-SESLSSSHSPGPAPKSRNQLGLWDSKSTEVQL
149 AKYWNLAECTFEASGEGVIMKERQITPDVYKVTPVMNTHPSQSEAVSDVWS--------------LSK-TSMTF
149 AKHWNLAECTFEASGEGVIIKKRQITPDVYKVTPVMNTHPYQSEAVSDVWS--------------LSK-TSMTF
217 ISKRAGVKDMTVKLTDFFGSEEEYYSVCPEGAPDLMGAIIMGLKYKKLFNQARMKYRL
217 ISKRAGVKDMMVKLTDFFGSEEEYYSVCPEGAPDLMGAIIMGLKHKKLFNQARMKYRI
208 QPKKASLQPLTISLDELFSSRGEFISVGGDGRMSHKEAILLGLRYKKLYNQARVKYSL
208 QPKKASLQPLTISLDELFSSRGEFISVGGNGRMSHKEAILLGLRYKKLYNQARVKYSL

Figure 4.12

Alignment of the NS proteins of four strains of VSV. Conserved phosphorylation sites are highlighted. Eighteen potential phosphorylation sites are conserved between the four proteins. The five potential phosphorylated residues conserved in the domain marked (A) are the proposed sites of constitutive phosphorylation.

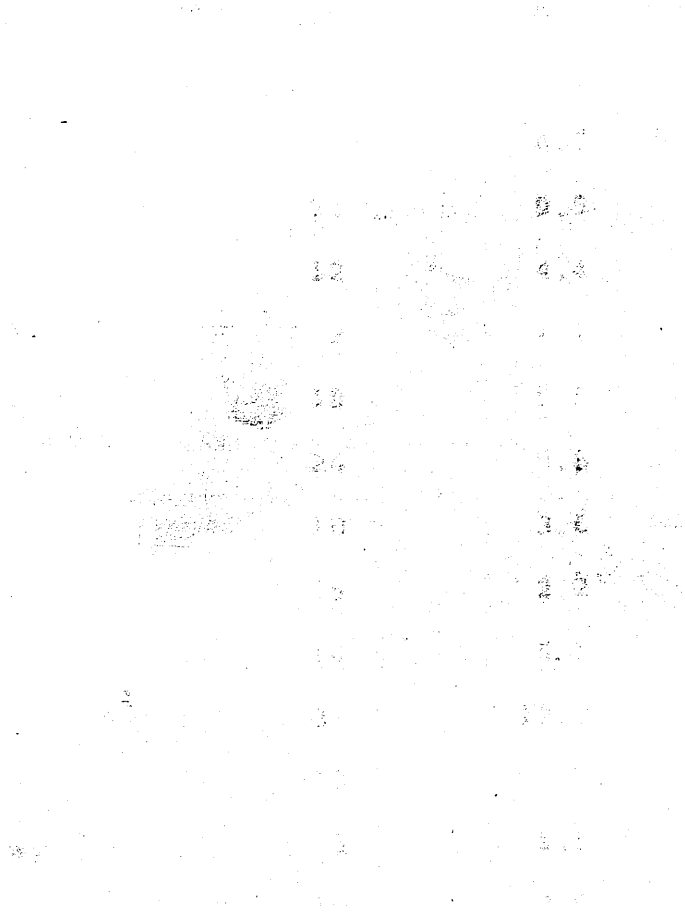


Table 4.1 Amino Acid Composition of the NS Protein of VSV New Jersey, Missouri strain Compared with that of an 'Average' Protein

Amino Acid	NS Protein VSV NJ Missouri Strain		'Average' Protein*
	No of Residues	%	%
Alanine (A)	10	3.6	9.0
Arginine (R)	11	4.0	4.7
Asparagine (N)	9	3.6	4.4
Aspartic Acid (D)	26	9.5	5.5
Cysteine (C)	4	1.5	2.8
Glutamine (Q)	13	4.7	3.9
Glutamic Acid (E)	26	9.5	6.2
Glycine (G)	12	4.4	7.5
Histidine (H)	3	1.1	2.1
Isoleucine (I)	15	5.5	4.6
Leucine (L)	24	8.8	7.5
Methionine (M)	10	3.6	1.7
Phenylalanine (F)	6	2.2	3.5
Proline (P)	14	5.1	4.6
Serine (S)	33	12.0	7.1
Threonine (T)	13	4.7	6.0
Tryptophan (W)	5	1.8	1.1
Tyrosine (Y)	10	3.6	3.5
Valine (V)	9	3.3	6.9

* Klapper (1977)

Table 4.2 Assumed Charges of Amino Acids at pH7.0

Amino Acid	Charge
Lysine	+1
Arginine	+1
Histidine	+0.5
Aspartic Acid	-1
Glutamic Acid	-1

Table 4.3 Comparison of the NS Genes and Proteins of Four Strains of VSV

Serotype	Subtype	Strain	Length of mRNA (no of nucleotides)	Size of Protein (no of amino acids)	% Homology								Reference
					Nucleotide				Amino Acid				
					MIS	OG	SJ	M-S	MIS	OG	SJ	M-S	
NEW JERSEY	Hazelhurst	Missouri (MIS)	856	274	100	85	50	50	100	90	34	33	This paper
	Concan	Ogden (OG)	856	274		100	51	50		100	34	34	Gill and Banerjee, 1985
INDIANA	Indiana	San Juan (SJ)	814	265			100	97			100	95	Gallione et al, 1981 with corrections reported in Gill and Banerjee, 1985
	Cocal	Mudd-Summers (M-S)	814	265				100				100	L D Hudson, C Condra and R A Lazzarini, personal communication

Table 4.4 Comparison of the Amino Acid Composition of the NS Proteins of Four Strains of VSV

Amino Acid	% Amino Acids in NS Protein of VSV			
	NJ Serotype		IND Serotype	
	Hazelhurst	Missouri	Mudd-Summers	San-Juan
Phe	2.2	2.2	3.0	3.0
Val	4.4	3.3	6.0	6.4
Ala	3.3	3.6	5.7	6.0
Asn	3.3	3.3	2.6	2.3
Cys	1.5	1.5	0.4	0.4
Leu	10.6	8.8	9.1	8.3
Ser	10.9	12.0	9.1	10.2
Tyr	4.4	3.6	4.2	4.2
Lys	8.0	7.7	6.8	6.8
Trp	1.8	1.8	1.5	1.5
Ile	4.0	5.5	4.2	3.8
Pro	5.5	5.1	4.5	4.5
His	0.7	1.1	1.9	1.5
Asp	9.1	9.5	7.2	7.2
Arg	3.6	4.0	3.8	3.4
Met	2.9	3.6	1.5	1.9
Thr	5.5	4.7	5.3	5.3
Glu	4.0	4.7	6.0	6.0
Gln	10.2	9.5	10.9	11.7
Gly	4.0	4.4	5.7	5.7

Table 4.5 Actual Homology and Homology Including Conservative Changes Between the Four NS Proteins of VSV

	% Homology	
	Actual	Including Conservative Changes
Overall	33	52
Domain I	62	76
Domain II	67	79
Domain III	68	90

Domains I, II and III are defined in Figure 4.11

Chapter 5

SEQUENCE DETERMINATION AND ANALYSIS OF THE COMPLEMENTATION GROUP E MUTANTS AND THEIR REVERTANTS

The site of the ts lesions in the complementation group E mutants of VSV NJ, Missouri strain have been shown to be in the NS gene (Evans et al, 1979; Lesnaw et al, 1979). The three mutants (tsE1, tsE2 and tsE3) classified in group E form a heterogenous group. They exhibit a variety of phenotypes and their NS proteins migrate differently from both wild-type virus and one another on SDS-polyacrylamide gels. Figure 5.1 shows an in vitro translation of RNA isolated from cells infected with wild-type and mutant viruses. The 10%, 12% and 15% SDS-polyacrylamide gels show the characteristic migration pattern of the wild-type and mutant NS proteins. In our gel system the NS proteins of wild-type, tsE1, tsE2 and tsE3 viruses migrate with apparent molecular weight of 59,000, 50,000, 52,000 and 54,000 respectively.

The complementation group E mutants were isolated from wild-type VSV NJ, strain Missouri after exposure to the base analogue 5-FU (Pringle et al, 1971). Revertants of tsE1 and tsE3 are quite readily isolated; however, revertants of tsE2 have never been detected (Pringle, 1986). The NS proteins of all of the revertant viruses isolated by Pringle co-migrate with the NS protein of wild-type virus on SDS-polyacrylamide gels (Evans et al, 1979; Lesnaw et al, 1979). The mutants of complementation group E and their revertants have proved useful in the study of the role of NS protein in the replicative cycle of VSV (Lesnaw et al, 1979; Ongradi and Szilagyi, 1981; Ongradi et al, 1985a).

The variety of phenotypes exhibited by the complementation group E mutants has led to the proposal of a multifunctional role for the NS protein in the VSV

lifecycle. Because tsE1 was found to be defective in the RNA replication pathway at the restrictive temperature, Lesnaw et al (1979) concluded the NS protein has a role in replication. The role of the NS and L proteins in transcription was studied by reconstitution-dissociation experiments using wild-type virus, tsE1 and tsE1/R1 (Ongradi and Szilagyi, 1981; Ongradi et al, 1985a,b).

A number of workers have studied the nature of the mutations affecting the complementation group E mutants and attempted to correlate their findings with the pattern of NS protein migration seen on SDS-polyacrylamide gels (Lesnaw et al, 1979; Maack and Penhoet, 1980). Maack and Penhoet (1980) reported that the NS protein of tsE1 migrated faster than the NS protein of wild-type virus on a number of gel systems not containing SDS, and concluded that differential SDS binding was not responsible for the aberrant mobility of the complementation group E mutants. They isolated revertant viruses of tsE1 which contained NS proteins which were identical to or larger than the NS of tsE1. Analysis of tryptic peptides of NS protein labelled with inorganic ^{32}P showed that these revertants of tsE1 contained one more non-phosphorylated peptide than tsE1 (Maack and Penhoet, 1980). Maack and Penhoet (1980) proposed that the differential migration pattern exhibited by the complementation group E mutants was due to differences in amino acid chain length.

Lesnaw et al (1979), however, proposed that the aberrant migration pattern of the complementation group E mutants on SDS-polyacrylamide gels was due to conformational rather than size differences since the wild-type and mutant

NS proteins migrated at the same rate when electrophoresed in polyacrylamide gels containing 4M urea in addition to SDS. Peptide maps obtained by limited proteolysis of the NS protein from wild-type and mutant viruses with V8 protease and papain suggested that the mutation would involve an aspartic or glutamic acid residue (Lesnaw et al, 1979).

In order to precisely map the position of the mutation(s) in the NS genes of each virus of complementation group E, it was decided to clone and sequence the cDNA of the NS mRNAs of tsE1, tsE2, tsE3 and the revertant viruses tsE1/R1 and tsE3/R1.

1. SEQUENCE DETERMINATION OF THE NS mRNAS OF THE COMPLEMENTATION GROUP E MUTANTS

The nucleotide sequences of the insert fragments of pNJNSE1, pNJNSE2, pNJNSE3, pNJNSR1 and pNJNSR3 were determined by the dideoxy-chain terminator method as previously described for the insert in pNJNS1.

The inserts were sequenced and analysis of the data revealed that all three plasmids contained the 5' and 3' consensus sequences (already described in Chapter 4) known to be at the ends of the VSV mRNAs. Thus the five plasmids contain full-length cDNA copies of the NS mRNAs from each of the five viruses.

Figure 5.2(a) shows the nucleotide and predicted protein sequence of the NS mRNA of wild-type VSV NJ, Missouri strain, with the positions of the mutations marked. Both tsE1 and tsE3 have a single mutation; tsE2 has two mutations. Figure 5.2(b) compares the sequence tracks in

the region where the mutations occur with the wild-type sequence. The gels show quite clearly the single nucleotide changes summarised in Figure 5.2(a).

Three out of the four mutations lie in a small region of 18 nucleotides and interestingly this region encodes the most hydrophilic region of the protein as indicated in Figure 5.3. This region is possibly a 'hot-spot' of mutation. All of the mutations cause a change in the charge of the amino acid affected.

The fact that tsE2 is a double mutant might explain why revertants have never been isolated. The possibility of viruses reverting to wild-type at both of these positions is so small that detection would require the screening of many more plaques than have been analysed already (C.R. Pringle, personal communication).

2. SEQUENCE DETERMINATION OF NON-ts REVERTANTS OF tsE1 AND tsE3

Figure 5.4 shows the nucleotide and predicted protein sequence of the NS mRNA of wild-type VSV NJ, Missouri strain with the changes found in tsE1/R1 and tsE3/R1 marked.

At the sites of the mutations of tsE1 and tsE3 the revertant viruses have both reverted to the wild-type virus sequence. However, the NS mRNAs of tsE1/R1 and tsE3/R1 were found to have two further changes later in their nucleotide sequence at exactly the same position. Only one of the changes actually alters an amino acid.

I am confident that the sequence changes detected in the two revertant viruses are not the result of cloning

artefacts. The cDNA cloning and sequence determination of inserts from the resultant recombinant plasmids were done at separate times. Furthermore, when the changes were detected inserts from three other plasmids identified as being tsE1/R1 NS gene-specific were sequenced and yielded the same altered sequence at the positions shown in Figure 5.4.

3. PROPOSED MECHANISMS BY WHICH THE MUTATIONS IN COMPLEMENTATION GROUP E MUTANTS MIGHT AFFECT PROTEIN MOBILITY ON SDS-POLYACRYLAMIDE GELS

Table 5.1 is a summary of the sequence data from the three complementation group E mutants. All of the mutations change the charge of the amino acid they affect. None of the mutations generate a stop codon which would lead to premature termination of protein synthesis. Thus the conclusion reached by Maack and Penhoet (1980) that the aberrant electrophoretic mobility of the group E mutants was due to the synthesis of a short NS protein must be discounted. The sequence data show that, for the most part, single base changes are responsible for the dramatic alterations in mobility observed on SDS-polyacrylamide gels.

Lesnaw et al (1979) concluded that the aberrant mobility of the NS proteins of the complementation group E mutants is due to a conformational change in the protein brought about by the mutation(s). Their conclusion is, indeed, supported by the sequence data. The change in charge of a single amino acid in the very small region where most of the mutations lie could be sufficient to alter protein conformation such that there would be differential

binding of SDS. Alterations in the charge of amino acids occurring in other regions of the protein do not seem to be as important to mobility as those in the region of the three mutations of tsE1, tsE2 and tsE3. Both tsE1/R1 and tsE3/R1 possess NS proteins with the same mobility as wild-type NS protein yet both have a change at position 168 (see Figure 5.4) which alters the charge of the amino acid. In gels the presence of 4M urea in addition to SDS might be sufficient to relieve the conformational change so that the NS proteins from wild-type and mutant viruses have the same mobility (Lesnaw et al, 1979).

The predicted secondary structure of the NS proteins of tsE1, tsE2, tsE3 and a revertant virus were determined as previously described. Figure 5.5(a) and 5.5(b) show the results of the analysis. Substantial alterations are predicted in protein structure in all three complementation group E mutants when compared to wild-type virus (see Figure 5.5(a)). In tsE1 there is a new area of β-turn and a region of α-helix is lost; the first mutation in tsE2 causes an area of β-turn to be lost and the second mutation a region of α-helix to be inserted; in tsE3 an area of α-helix is lost. The predicted secondary structure of the NS protein of the revertant virus (Figure 5.5(b)) is exactly the same as the wild-type but for a very minor alteration around the position of the changed amino acid (position 150-175). The change has been predicted to cause a small hydrophilic area in this region.

The overall conclusion from the analysis of the NS proteins of wild-type virus and virus classified in complementation group E is that quite minor changes, for

most part single base changes, in a certain region can affect the protein such that it migrates in an anomalous manner on SDS-polyacrylamide gels. Since the proteins are all the same length the minor changes must have caused quite marked conformational changes in the protein.

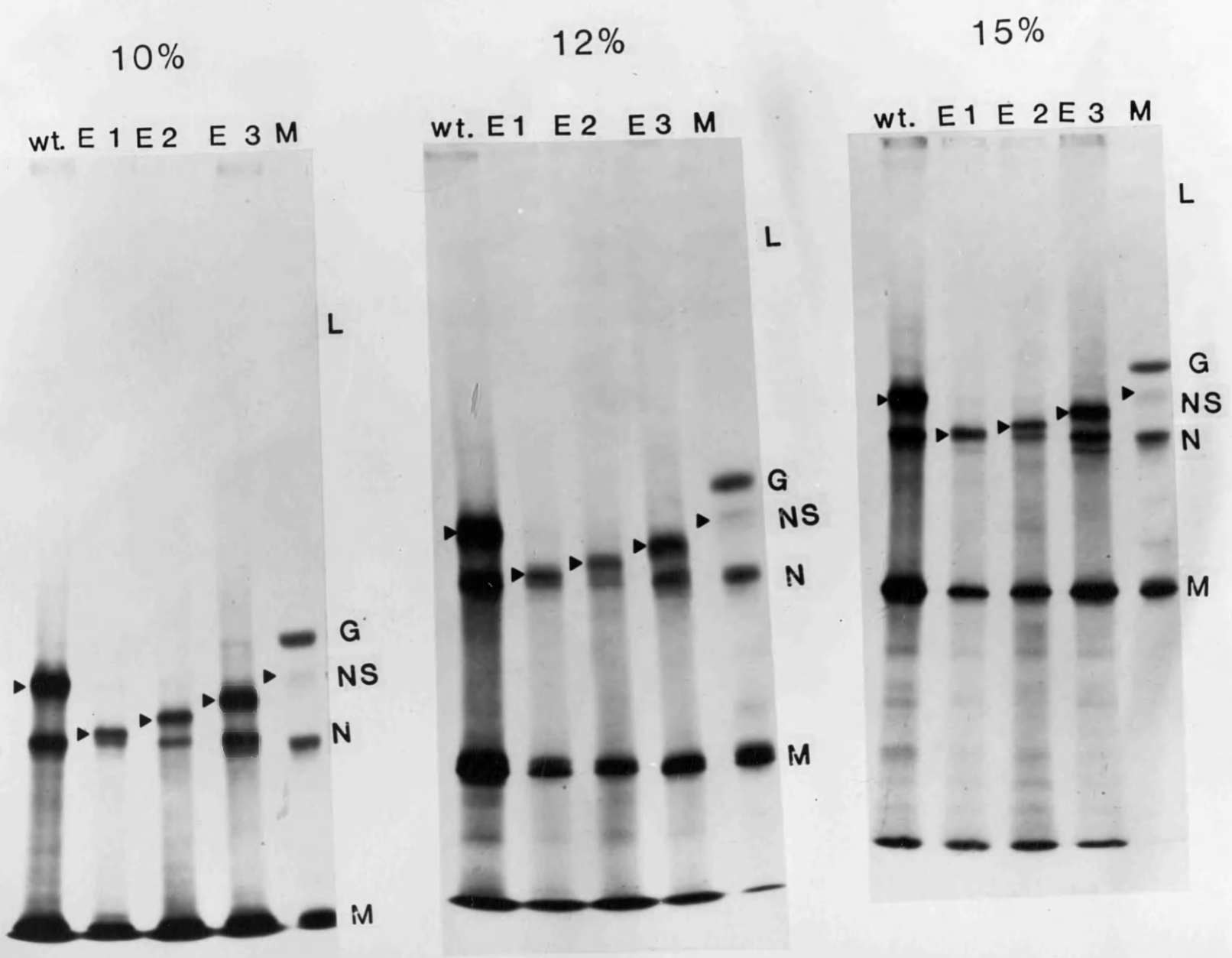
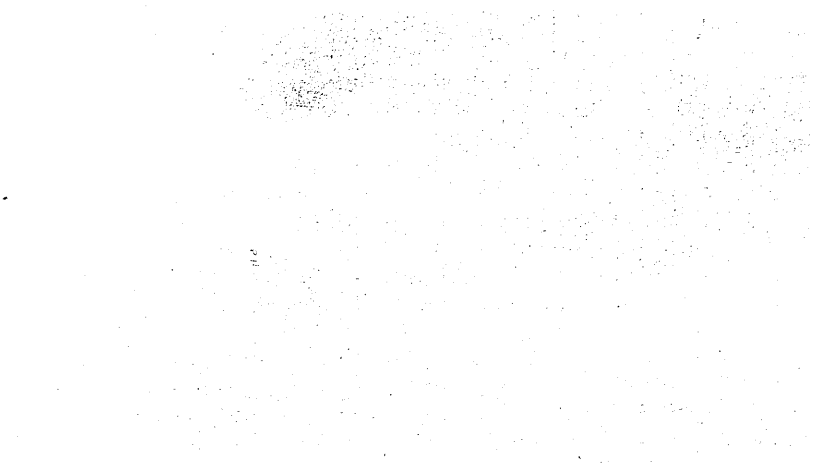

10%
wt. E 1 E 2 E 3 M
L
G
NS
N
M
12%
wt. E1 E2 E3 M
L
G
NS
N
M
15%
wt. E1 E 2 E 3 M
L
G
NS
N
M

Figure 5.1

10%, 12% and 15% polyacrylamide gels showing the electrophoretic mobility differences between the NS proteins of wild-type and the three complementation group E mutants - tsE1, tsE2 and tsE3. RNA was extracted from infected cells and translated in vitro. The marker track (M) consists of viral proteins extracted from infected cells. The solid arrows indicate the positions of the NS proteins translated from total infected cell RNA. The mol wt of the NS protein of each virus was calculated as the average mol wt obtained from the relative migration of the NS protein to the G (62,000), N (47,800) and M (26,000) proteins on each gel.



```
                Met Asp Ser Ile Asp Arg Leu Lys Thr Tyr Leu Ala Thr Tyr Asp Asn   16
AACAGAGATC ATG GAC AGT ATT GAT CGG CTC AAA ACT TAC TTG GCT ACT TAT GAT AAT   58

                                                Gly                 Lys Gly
Leu Asp Ser Ala Leu Gln Asp Ala Asn Glu Ser Glu Glu Arg Arg Glu Asp Lys Tyr   35
TTG GAT TCT GCC TTG CAG GAT GCA AAT GAG TCT GAA GAA AGA AGA GAG GAT AAA TAT  115
                                       E3  G          E1  A   G  E2

Leu Gln Asp Leu Phe Ile Glu Asp Gln Gly Asp Lys Pro Thr Pro Ser Tyr Tyr Gln   54
CTC CAA GAC CTC TTC ATC GAA GAT CAA GGA GAT AAA CCA ACT CCG TCA TAT TAT CAG  172

Glu Glu Glu Ser Ser Asp Ser Asp Thr Asp Tyr Asn Ala Glu His Leu Thr Met Leu   73
GAA GAA GAA TCG TCA GAT TCA GAT ACT GAC TAT AAT GCT GAA CAT CTT ACG ATG TTG  229

Ser Pro Asp Glu Arg Ile Asp Lys Trp Glu Glu Asp Leu Pro Glu Leu Glu Lys Ile   92
TCG CCG GAT GAA AGA ATA GAC AAA TGG GAA GAA GAT TTG CCC GAA TTG GAA AAG ATT  286

Asp Asp Asp Ile Pro Val Thr Phe Ser Asp Trp Thr Gln Pro Val Met Lys Glu Asn  111
GAT GAT GAT ATA CCA GTA ACT TTT TCT GAC TGG ACA CAA CCT GTA ATG AAG GAA AAC  343

                Glu
Gly Gly Glu Lys Ser Leu Ser Leu Phe Pro Pro Val Gly Leu Thr Lys Val Gln Thr  130
GGA GGG GAG AAA TCA CTA TCT CTG TTT CCT CCG GTT GGA TTA ACA AAA GTT CAG ACA  400
          G  E2

Asp Gln Trp Arg Lys Thr Ile Glu Ala Val Cys Glu Ser Ser Lys Tyr Trp Asn Leu  149
GAC CAA TGG AGG AAG ACA ATT GAG GCA GTC TGT GAG AGC TCT AAA TAT TGG AAT TTG  457

Ser Glu Cys Gln Ile Met Asn Ser Glu Asp Arg Leu Ile Leu Lys Gly Arg Ile Met  168
TCA GAA TGT CAG ATT ATG AAC TCA GAA GAT CGT CTT ATC CTC AAA GGC CGA ATT ATG  514

Thr Pro Asp Cys Ser Ser Ser Ile Lys Ser Gln Asn Ser Ile Gln Ser Ser Glu Ser  187
ACT CCT GAC TGC AGT TCA TCA ATC AAA TCT CAG AAC TCC ATA CAG AGT TCC GAA TCT  571

Leu Ser Ser Ser His Ser Pro Gly Pro Ala Pro Lys Ser Arg Asn Gln Leu Gly Leu  206
CTC TCC TCC TCG CAT TCA CCC GGT CCA GCA CCA AAG TCA CGG AAT CAA CTA GGT CTA  628

Trp Asp Ser Lys Ser Thr Glu Val Gln Leu Ile Ser Lys Arg Ala Gly Val Lys Asp  225
TGG GAT TCA AAA TCA ACA GAA GTA CAA CTG ATC TCG AAG AGG GCC GGA GTG AAG GAC  685

Met Met Val Lys Leu Thr Asp Phe Phe Gly Ser Glu Glu Glu Tyr Tyr Ser Val Cys  244
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Pro Glu Gly Ala Pro Asp Leu Met Gly Ala Ile Ile Met Gly Leu Lys His Lys Lys  263
CCA GAA GGG GCC CCA GAT TTG ATG GGA GCA ATC ATC ATG GGA CTA AAA CAT AAA AAG  799

Leu Phe Asn Gln Ala Arg Met Lys Tyr Arg Ile ---                             274
CTT TTT AAC CAA GCA AGA ATG AAG TAT CGT ATC TAA TTAATTCCGATGATCAATATG       856
```

Figure 5.2(a)

Nucleotide and predicted amino acid sequence of the NS protein of VSV NJ, Missouri strain with the positions of the mutations in tsE1, tsE2 and tsE3 marked. Three out of the four mutations lie in an area of 18 nucleotides. All of the mutations change the charge of the amino acid they affect.

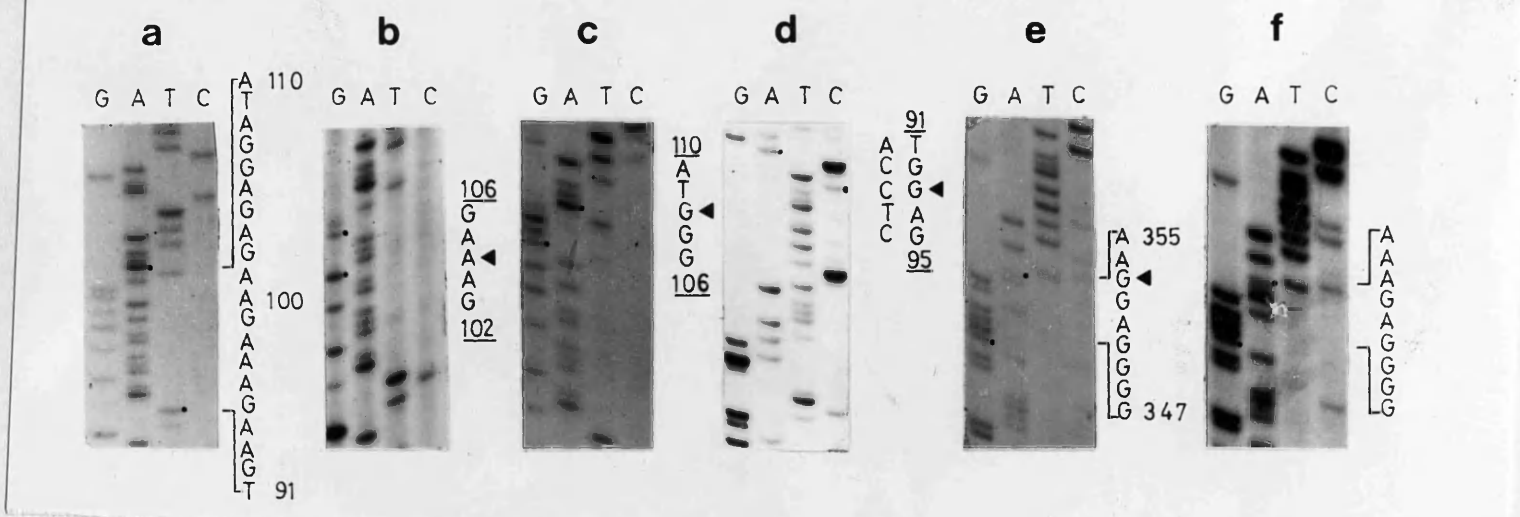

a
b
c
d
e
f
G A T C
110
100
91
106
102
110
106
91
95
355
347

Figure 5.2(b)

Sequence gels showing the nucleotide changes in the tsE1, tsE2 and tsE3 mutants compared to the wild-type sequence.

(A) Position 91-100 - wild-type sequence.

(B) Position 102-106 - tsE1 sequence.

(C) Position 106-110 - tsE2 sequence.

(D) Position 95-91 (minus strand) - tsE3 sequence. For direct comparison with wild-type the complementary sequence is written.

(E) Position 347-355 showing the second mutation in tsE2 compared to (F) showing the wild-type sequence in the same region.

The gels show clearly the A-G changes in tsE2 and tsE3 and the G-A change in tsE1.

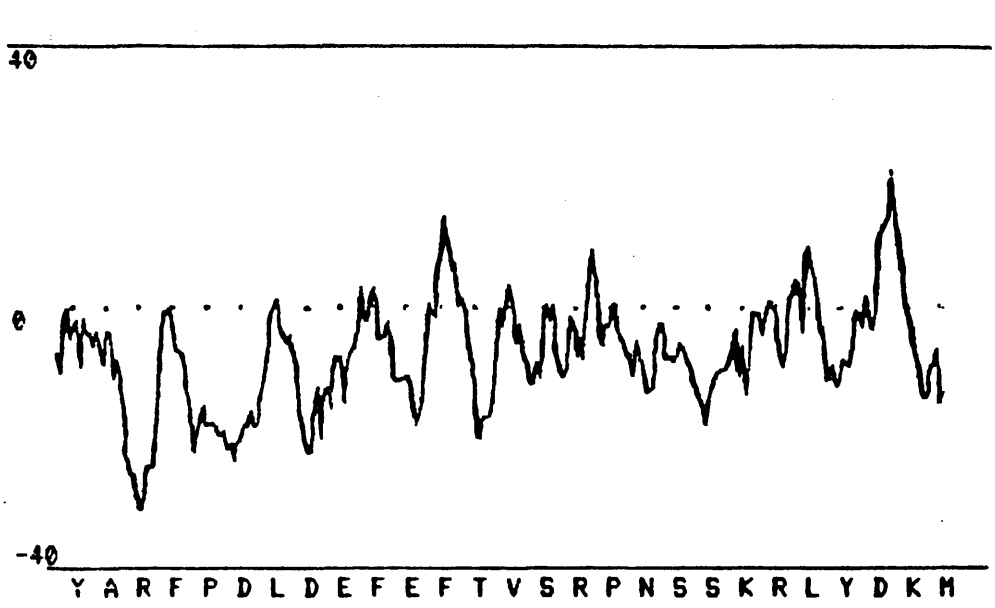
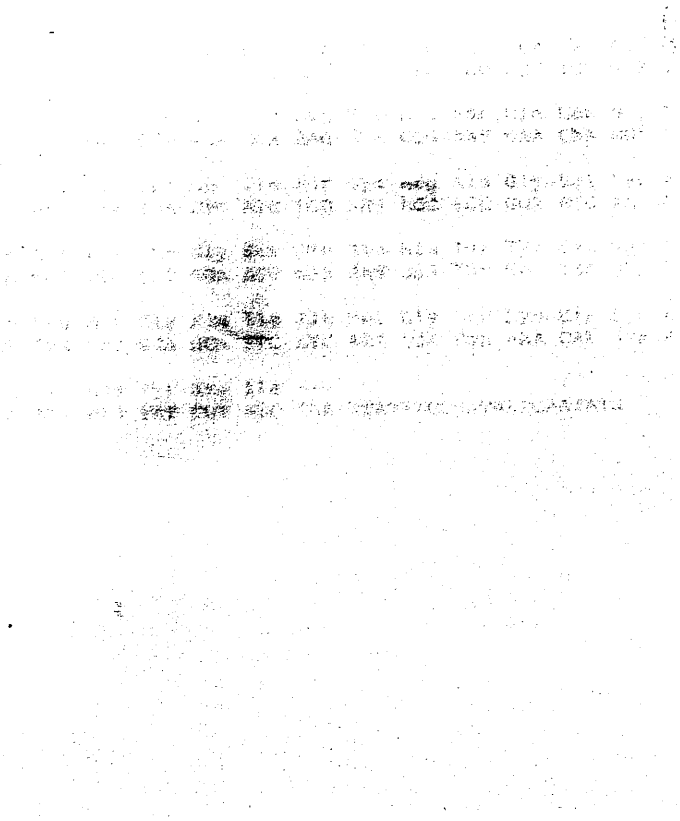

40
0
-40
Y A R F P D L D E F E F T V S R P N S S K R L Y D K M

Figure 5.3

Hydropathic profile of the NS protein of VSV NJ, Missouri strain as predicted by the program of Kyte and Doolittle (1982). The area where three out of the four mutations detected in the complementation group E mutants lie is highlighted. This region represents the most hydrophilic region in the molecule.



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AACAGAGATC ATG GAC AGT ATT GAT CGG CTC AAA ACT TAC TTG GCT ACT TAT GAT AAT    58

Leu Asp Ser Ala Leu Gln Asp Ala Asn Glu Ser Glu Glu Arg Arg Glu Asp Lys Tyr   35
TTG GAT TCT GCC TTG CAG GAT GCA AAT GAG TCT GAA GAA AGA AGA GAG GAT AAA TAT  115

Leu Gln Asp Leu Phe Ile Glu Asp Gln Gly Asp Lys Pro Thr Pro Ser Tyr Tyr Gln   54
CTC CAA GAC CTC TTC ATC GAA GAT CAA GGA GAT AAA CCA ACT CCG TCA TAT TAT CAG  172

Glu Glu Glu Ser Ser Asp Ser Asp Thr Asp Tyr Asn Ala Glu His Leu Thr Met Leu   73
GAA GAA GAA TCG TCA GAT TCA GAT ACT GAC TAT AAT GCT GAA CAT CTT ACG ATG TTG  229

Ser Pro Asp Glu Arg Ile Asp Lys Trp Glu Glu Asp Leu Pro Glu Leu Glu Lys Ile   92
TCG CCG GAT GAA AGA ATA GAC AAA TGG GAA GAA GAT TTG CCC GAA TTG GAA AAG ATT  286

Asp Asp Asp Ile Pro Val Thr Phe Ser Asp Trp Thr Gln Pro Val Met Lys Glu Asn  111
GAT GAT GAT ATA CCA GTA ACT TTT TCT GAC TGG ACA CAA CCT GTA ATG AAG GAA AAC  343

Gly Gly Glu Lys Ser Leu Ser Leu Phe Pro Pro Val Gly Leu Thr Lys Val Gln Thr  130
GGA GGG GAG AAA TCA CTA TCT CTG TTT CCT CCG GTT GGA TTA ACA AAA GTT CAG ACA  400

Asp Gln Trp Arg Lys Thr Ile Glu Ala Val Cys Glu Ser Ser Lys Tyr Trp Asn Leu  149
GAC CAA TGG AGG AAG ACA ATT GAG GCA GTC TGT GAG AGC TCT AAA TAT TGG AAT TTG  457

                                                                        Arg
Ser Glu Cys Gln Ile Met Asn Ser Glu Asp Arg Leu Ile Leu Lys Gly Arg Ile Met  168
TCA GAA TGT CAG ATT ATG AAC TCA GAA GAT CGT CTT ATC CTC AAA GGC CGA ATT ATG  514
                 C                                                      G

Thr Pro Asp Cys Ser Ser Ser Ile Lys Ser Gln Asn Ser Ile Gln Ser Ser Glu Ser  187
ACT CCT GAC TGC AGT TCA TCA ATC AAA TCT CAG AAC TCC ATA CAG AGT TCC GAA TCT  571

Leu Ser Ser Ser His Ser Pro Gly Pro Ala Pro Lys Ser Arg Asn Gln Leu Gly Leu  206
CTC TCC TCC TCG CAT TCA CCC GGT CCA GCA CCA AAG TCA CGG AAT CAA CTA GGT CTA  628

Trp Asp Ser Lys Ser Thr Glu Val Gln Leu Ile Ser Lys Arg Ala Gly Val Lys Asp  225
TGG GAT TCA AAA TCA ACA GAA GTA CAA CTG ATC TCG AAG AGG GCC GGA GTG AAG GAC  685

Met Met Val Lys Leu Thr Asp Phe Phe Gly Ser Glu Glu Glu Tyr Tyr Ser Val Cys  244
ATG ATG GTG AAA TTG ACA GAC TTT TTT GGA AGT GAA GAG GAA TAT TAT TCA GTA TGC  742

Pro Glu Gly Ala Pro Asp Leu Met Gly Ala Ile Ile Met Gly Leu Lys His Lys Lys  263
CCA GAA GGG GCC CCA GAT TTG ATG GGA GCA ATC ATC ATG GGA CTA AAA CAT AAA AAG  799

Leu Phe Asn Gln Ala Arg Met Lys Tyr Arg Ile ---                             274
CTT TTT AAC CAA GCA AGA ATG AAG TAT CGT ATC TAA TTAATTCCGATGATCAATATG       856
```

Figure 5.4

Nucleotide and predicted amino acid sequence of the NS mRNA of VSV NJ, strain Missouri with the positions of the two changes in the revertant viruses tsE1/R1 and tsE3/R1 marked. One of the nucleotide changes is silent in that the amino acid (position 154) is not affected. Whereas the other causes a methionine to arginine change (position 168).

Figure 5.5

Predicted secondary structure of the NS proteins of (A) wild-type VSV NJ, strain Missouri, the three complementation group E mutants (tsE1, tsE2 and tsE3) and (B) revertant virus tsE1/R1. The method of Chou and Fasman (1978) was used to predict secondary structure and the results were graphically displayed using the PLOTCHOU program contained in the University of Wisconsin Genetics Group Computer software package. The whole of the wild-type and revertant structures are shown but only the regions where the mutations affect the predicted structures in tsE1, tsE2 and tsE3 are displayed. The arrows indicate the differences in predicted secondary structure.

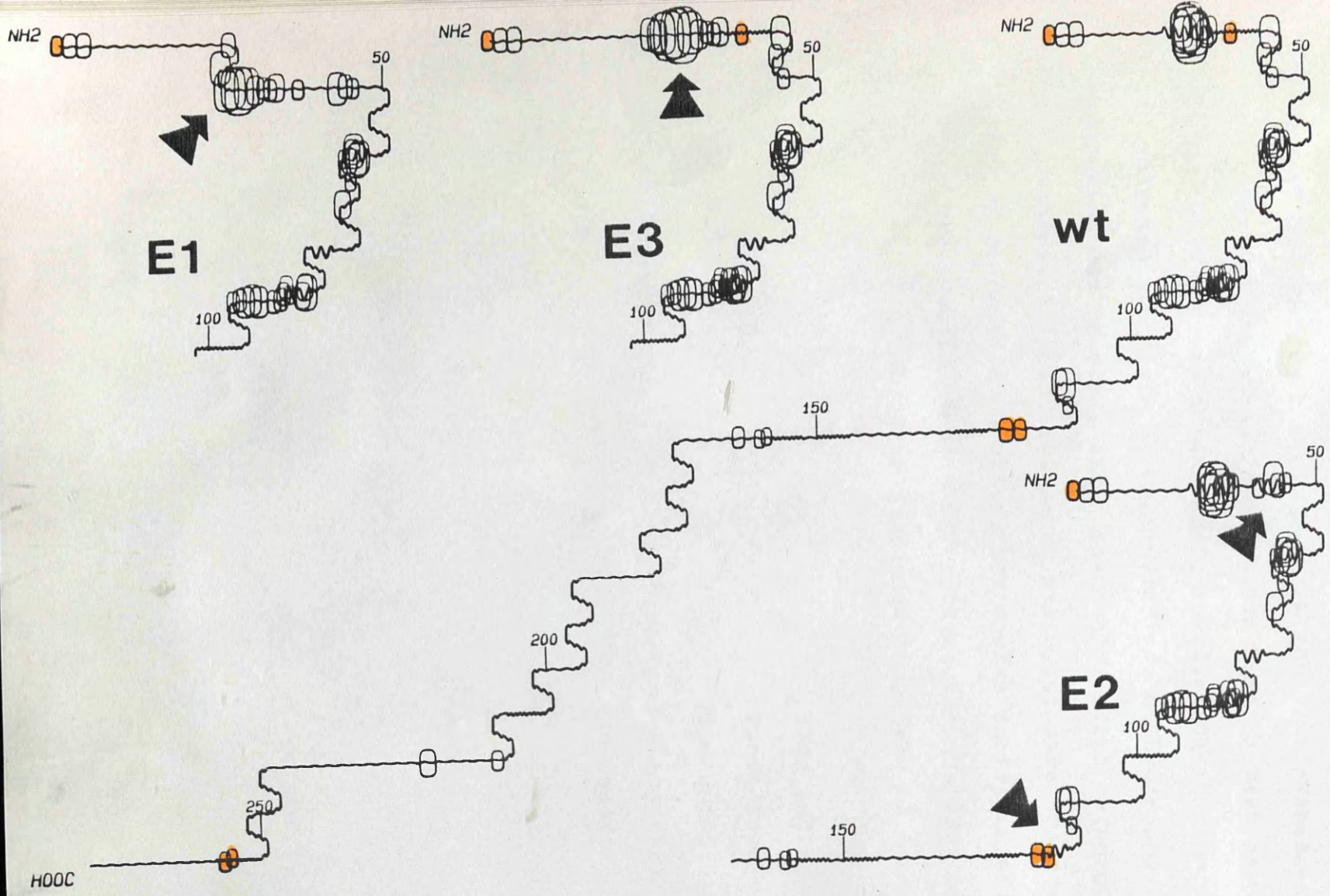
(∿∿∿) = alpha coil

(∿∿∿) = beta sheet

(∿∿) = random coil

Shaded ovals are hydrophobic; open ovals are hydrophilic.

Change of directions indicates β -turn.


NH2
50
E1
100
NH2
50
E3
100
NH2
50
wt
100
150
NH2
50
E2
100
150
200
250
HOOC

OP. REV A

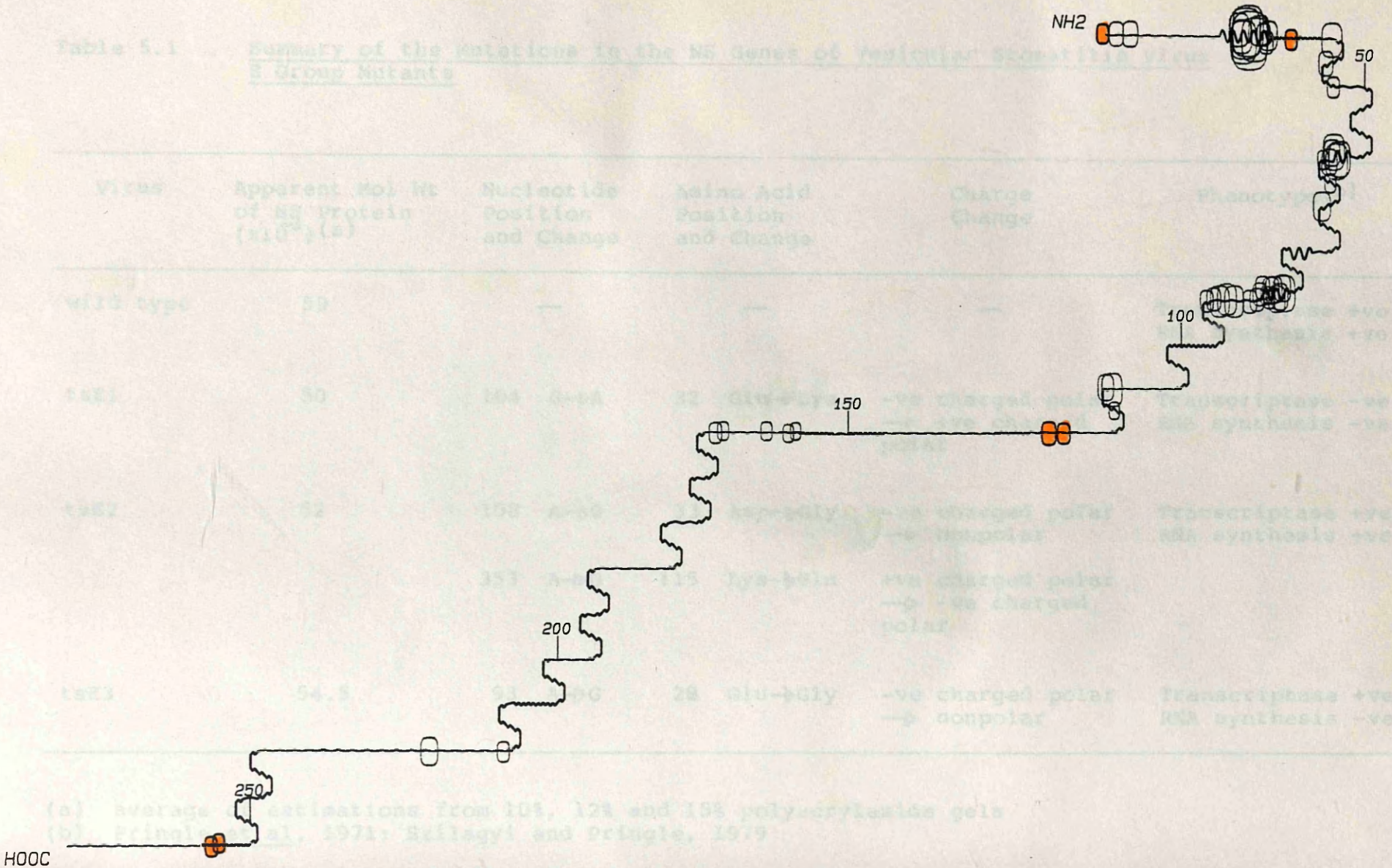


Table 5.1 Summary of the Mutations in the NS Genes of Vesicular Stomatitis Virus E Group Mutants

Virus	Apparent Mol Wt of NS Protein ($x10^{-3}$)(a)	Nucleotide Position and Change	Amino Acid Position and Change	Charge Change	Phenotype(b)
wild type	59	—	—	—	Transcriptase +ve RNA synthesis +ve
tsE1	50	104 G→A	32 Glu→Lys	-ve charged polar → +ve charged polar	Transcriptase -ve RNA synthesis -ve
tsE2	52	108 A→G	33 Asp→Gly	-ve charged polar → nonpolar	Transcriptase +ve RNA synthesis +ve
		353 A→G	115 Lys→Glu	+ve charged polar → -ve charged polar	
tsE3	54.5	93 A→G	28 Glu→Gly	-ve charged polar → nonpolar	Transcriptase +ve RNA synthesis -ve

(a) average of estimations from 10%, 12% and 15% polyacrylamide gels
(b) Pringle et al, 1971; Szilagyi and Pringle, 1979

Chapter 6

REFLECTIONS

1. cDNA CLONING

The RNA template in the cDNA synthesis reactions was purified from infected cells and was fairly 'crude' in that it was not poly(A) selected to enrich for mRNA. It was calculated that the frequency of NS gene containing plasmids in our library was 0.7%. This figure of 0.7% meant that low density screening for NS gene-specific plasmids was quite feasible. Previously many workers have synthesised their template RNA in very large reactions (typically between 10ml and 20ml) *in vitro* and then poly(A) selected full-length mRNA (Gallione *et al*, 1981; Gill and Banerjee, 1985; Rose and Gallione, 1981). The large volume of the *in vitro* mRNA synthesis reactions makes them very expensive (in terms of the amounts of reagents required) and difficult to handle in the latter stages of RNA purification. The work described here has shown that RNA synthesised *in vivo* and easily purified from the infected cells is an efficient template for cDNA synthesis even when it is not poly(A) or size selected.

The method used to synthesise cDNA was described by Gubler and Hoffman (1983). It is both rapid and efficient since it eliminates the need for the S1 nuclease mediated cleavage step of the hairpin loop which is formed between first and second strands in 'classical' cDNA synthesis (Efstradiatis *et al*, 1976). The cleavage step not only lowers the efficiency of the reaction but also often removes 5' terminal sequences from the cloned mRNAs. Gubler and Hoffman (1983) reported that, in their hands, the method was 10- to 50-fold more efficient than methods which used the S1

nuclease cleavage. The method enabled the construction of plasmids which contained full-length copies of the NS mRNA from wild-type, mutant and revertant viruses from five different cloning experiments.

2. SEQUENCE DATA FROM cDNA CLONES

Schubert et al (1984) discovered extensive heterogeneity in cDNA clones prepared from the VSV L gene. There is no evidence for proofreading mechanisms in RNA viruses and this fact might explain why these viruses exhibit the very high mutation rate which contributes to their rapid evolution and general variability (Holland et al, 1982). The L gene is 6,380 nucleotides in length and Schubert et al (1984) detected over 20 nucleotide differences between cDNA clones of the same regions. If expressed, these changes would have created stop codons resulting in incomplete L proteins. When determining the sequence of a cDNA clone one is essentially looking at an individual which has been derived from a diverse population and this was demonstrated by work of Domingo et al (1978) who estimated that wild-type populations of the RNA phage Qβ contained 15% of the most abundant or 'master sequence', the remaining 85% being a multitude of different variants. Virion RNA is a mixture of all the viral genomic species and so when used as a template for sequence determination it yields a consensus sequence directly.

However, I am confident that there is no reason to question the sequence presented here because it was derived from cDNA clones. No variation was found in the sequence of two cDNA

clones of the wild-type NS gene and four cDNA clones of the NS gene of tsE1/R1. Furthermore, the sequence agrees with limited sequence data from D. McGeoch and A. Dolan (unpublished results). They determined part of the NS gene sequence of wild-type, mutant and revertant viruses from virion RNA by primer extension synthesis using a synthetic primer corresponding to a terminal sequence of the N gene. The aim of this work was to lay the foundation for future study of the NS protein by providing full-length cDNA clones which could be expressed in eukaryotic cells.

3. COMPARISON OF THE NS PROTEINS

The four NS gene sequences now available have allowed detailed comparisons at the nucleotide and amino acid level which have given some insight into the structurally important domains on the NS protein and ideas on the possible evolution of the protein.

Although there is generally only limited sequence homology between the four NS proteins, three domains are quite clearly conserved and these have been designated I — III (Figure 4.11). When conservative amino acid changes are considered the carboxy-terminus region (domain III) is over 90% homologous. The high degree of conservation in domain III might mean that this region is of fundamental importance to NS protein function (Dover and Flavell, 1984). Domain III is a very basic region and it shares limited sequence homology, shown in Figure 6.1 (43% when conservative amino acids are considered), with both the L gene (amino acids 784-804) of VSV IND (Schubert et al, 1984) and the sigma

subunit (amino acis 365-388) of the RNA-dependent-RNA polymerase of E.coli (Burton et al, 1981). The L gene is thought to be the viral polymerase (Schubert et al, 1984). The sigma subunit of the E.coli RNA polymerase has been shown to play an important role both in selective binding of polymerase to promoters and in the efficient initiation of transcription (Burgess et al, 1969; Chamberlin, 1974).

Interestingly, the NS protein of VSV and the sigma subunit of the E.coli RNA polymerase share a number of similarities. The sigma subunit, although much larger than the NS protein, has an overall negative charge with the acidic residues situated predominantly in the amino terminal portion of the molecule. When compared to the 'average' protein of Klapper (1977) the sigma subunit like NS protein is relatively rich in aspartic acid but low in glycine (Burton et al, 1981). The sigma protein also exhibits an aberrant mobility on SDS-polyacrylamide gels. The mol wt of 70,263 calculated from sequence data (Burton et al, 1981) is significantly lower than the values of 82,000 and 90,000 estimated by the protein's migration on SDS-polyacrylamide gels (Lowe et al, 1979). Burton et al (1981) suggested that the anomalous behaviour of the sigma protein on SDS-polyacrylamide gels could be due to its unusually high negative charge.

4. ROLE OF NS PROTEIN IN VSV TRANSCRIPTION AND REPLICATION

The information from the comparison of the four NS gene sequences and their homology with other proteins has given some clues as to the possible role of NS protein in

VSV transcription and replication.

Mellon and Emerson (1978) showed by dissociation-reconstitution experiments that the L protein only binds to template in combination with NS protein whereas NS protein alone binds to purified template. Thus they suggested that the NS protein directs binding of the L protein. The results of methylation protection experiments have shown that NS protein binds to an A+U-rich sequence in the middle of the leader gene in plus or minus sense genomic RNA (Isaac and Keene, 1982; Keene et al, 1981). The part of the NS protein that could conceivably function in an interaction between the L protein and template is the carboxy terminus (domain III). The carboxy terminus is highly conserved (90% homology between strains) and basic as one would expect of a region that interacts with nucleic acid (Hudson et al, 1986). The sigma protein which shares some similarity and homology with the NS protein has a similar function (Burgess et al, 1979; Chamberlin, 1974) and is known to interact with promoter DNA and short nascent RNA (Hillel and Wu, 1977; Simpson, 1977; Sverdler et al, 1980).

Once transcription or replication is initiated dissociation of the N protein from the RNA is required so that the L protein can continue polymerisation. De and Banerjee (1985) proposed that the NS protein performs an 'RNA unwinding' function which displaces N protein from the genome RNA thus allowing the L protein access. There is evidence supporting the idea that highly phosphorylated forms of NS are capable of mimicking RNA thus causing transient dissociation of the N protein from the RNA (Hudson et al, 1986). It is known that hyperphosphorylated forms of

NS protein have increased activity in transcription assays (Hsu et al, 1982; Kingsford and Emerson, 1980) and Keene and co-workers have suggested that the nucleocapsid binds RNA mainly through the phosphate groups (Isaac and Keene, 1982; Keene et al, 1981). The idea of a highly charged molecule being able to mimic nucleic acid is not without precedent since it has been reported that heparin (a highly negatively charged molecule) can be used as a nucleic acid analogue in DNA binding studies (Zillig et al, 1970; Yaun et al, 1975; Bickle et al, 1977).

It is known from the results of several workers that purified N protein or N protein synthesised in the absence of other viral proteins tends to form large aggregates which do not seem suitable for assembly of the RNPs (Blumberg et al, 1983; Blumberg et al, 1981; Sprague et al, 1983). The fact that complexes of N and NS have been detected both in the cytoplasm of infected cells and in vitro using monoclonal antibodies directed against N or NS proteins (Bell et al; Peluso and Moyer, 1984; Arnheiter et al, 1985; Wertz et al, 1985) suggests that NS binds to N protein (probably via the phosphate groups as suggested by Keene and co-workers) and so ensures there is N protein available for encapsidation.

5. EVOLUTION OF THE NS PROTEIN

The various isolates of VSV have been passaged a number of times in laboratories throughout the world (see Clewley et al, 1977; Reichmann et al, 1978) and so sequence comparisons may not be directly applicable to the original

isolates. However, the sequence data do give some idea about the evolution of the various genes. The extended structural homology coupled with the lack of sequence homology between the NS genes implies that the gene has undergone quite divergent evolution from a common ancestor.

A number of workers have compared the sequence of analagous genes from both serotypes (Colonno and Banerjee, 1978; Banerjee et al, 1984; Gallione and Rose, 1983; Gill and Banerjee, 1986). A summary of their results is shown in Table 6.1.

The homology between the G genes is surprising since they code for the glycoproteins which are the major antigenic determinants on the virus. One might expect that the G protein of the two serotypes be more different. Interestingly, the NS gene is flanked by two homologous genes (the N and M). The group II (NS) mutants of VSV IND have higher reversion rates than mutants in most other genes (Pringle, 1977) indicating that a number of mutational changes can restore NS gene function. It seems that apart from the conserved regions (domain I — III) the other regions of the NS protein can evolve quite rapidly. As long as the mutations do not disrupt the overall structure of the region it seems that they do not affect the function of the protein.

6. THE COMPLEMENTATION GROUP E MUTANTS AND REVERTANTS

The sequence determination of the three complementation group E mutants and the two revertant viruses revealed that three out of the four mutations lie in

a region of 18 nucleotides (Figure 5.2a) indicating that the region is potentially a 'hot spot' for mutation. Secondary structure predictions of the mutant proteins (Figure 5.5a) indicate quite extensive alterations in the area of the mutation in all three complementation group E mutants. The idea that a single amino acid substitution can have dramatic effects on the folding and mobility of a protein on SDS polyacrylamide gels has support from work in other areas. de Jong (1978) compared the mobilities of the α-crystallin chains of a number of mammalian species and showed that single amino acid changes were responsible for the differing electrophoretic mobilities of these proteins on SDS polyacrylamide gels. The amino acid changes involved alterations in side chain hydrophobicity or proline residues. Recently, Carstens et al (1986) compared the DNA sequence of the Autographa californica nuclear polyhedrosis virus polyhedrin gene with that of the polyhedrin gene from M5 (a morphology mutant). They found that a single point mutation (which caused a leucine to proline change) was responsible for the cubic polyhedron and altered mobility on SDS polyacrylamide gels of the mutant protein. Comparison of predicted secondary structures of the mutant and wild-type proteins revealed extensive changes caused by the single mutation.

The complementation group E mutants were isolated from wild-type VSV NJ, strain Missouri after exposure to the base analogue 5-FU (Pringle et al, 1971). 5-fluorouracil is an analogue of uracil which was first synthesised by Duchinsky et al (1957). It can exist in two forms as shown in Figure 6.2. In the more common keto form 5-FU can take the place

of uracil in RNA, however, unlike uracil, it has the ability to form hydrogen bonds with guanine. Thus 5-FU mutagenesis usually results in uracil to cytosine transitions. When 5-FU exists in the rare enol form it can take the place of cytosine.

Table 5.1 shows that three of the four mutations induced by 5-FU mutagenesis are adenine to guanine changes. The adenine to guanine changes have occurred in the mRNA and so uracil to cytosine changes have occurred in the genome (see Figure 6.3). However, one change is the opposite - guanine to adenine ie. there has been a cytosine to uracil change in the genome. For this change to have occurred it is possible that 5-FU was incorporated into the RNA, in its less common enol form (see Figure 6.2), in place of a cytosine. Having been incorporated into the RNA the 5-FU might then have undergone a tautomeric shift to exist in the keto form and so base-pair with adenine (see Figure 6.3). The majority of mutations induced by 5-FU are lethal and so it is possible that 5-FU acts to increase the relative number of naturally occurring spontaneous mutants in a population. It could be that the mutant with the guanine to adenine change in the mRNA is a spontaneous mutant which was detected by chance.

Sequence analysis of the revertant viruses tsE1/R1 and tsE3/R1 showed that the two viruses had reverted to the wild-type sequence at the positions of the mutations (Figure 5.4). However, two further changes were detected in the nucleotide sequence of the revertant viruses when compared to the wild-type virus. One of the nucleotide changes caused a change in the amino acid sequence.

Apart from the possibility that the revertant stocks have been mixed up in the laboratory (which cannot be excluded), there are two ways to explain this

The two viruses, tsE1/R1 and tsE3/R1, might represent a variant of wild-type virus present in low numbers in the wild-type population which was somehow able to contaminate the revertant selection procedure. However, this explanation is unlikely in view of the fact that the two revertant viruses were isolated in separate experiments (C.R. Pringle, personal communication).

The two viruses could indeed be true revertant viruses that have changed at two extra positions. Evidence that reversion to wild-type can be accompanied by a variety of other changes comes from the work of Maack and Penhoet (1980) and Gallione and Rose (1985).

Maack and Penhoet (1980) isolated a number of revertants of tsE1 whose NS proteins had different migration patterns on SDS-polyacrylamide gels from wild-type and the mutant NS proteins. These revertants could have arisen in a number of ways. They could be true revertants in which reversion has been accompanied by other mutations. They could be pseudorevertants ie. the mutations were replaced by other changes which restored function but affected the migration. Alternatively, the altered migration could be due to the presence of suppressor mutations. Gallione and Rose (1985) determined the sequence of three cDNA clones encoding the G proteins of ts045 (a spontaneous mutant isolated by Flamand (1970) with a reversible block in G transport), parent and revertant viruses and found that reversion had been accompanied by two further changes. The reversion events in tsE1/R1 and tsE3/R1 have been accompanied by the same changes in both viruses. It might

be expected that the accompanying changes be more random but it is possible that the conditions used for selecting the revertant viruses favoured these two extra changes. It would be of interest to isolate and sequence other revertants of tsE1 and tsE3 in order to test this hypothesis.

7. EVIDENCE FOR A SECOND OPEN READING FRAME IN THE NS PROTEIN OF VSV

A number of negative strand viruses employ overlapping reading frames in their replicative strategy. The phenomenon was originally described for influenza virus (Lamb and Lai, 1980; Lamb et al, 1981) and has been found in Sendai virus (Giorgi et al, 1983); measles virus (Bellini et al, 1985); bunyaviruses (Akashi and Bishop, 1983; Ihara et al, 1984, 1985; Cabradilla et al, 1983); and possibly in the Arenaviruses (Akashi and Bishop, 1983). In measles and Sendai virus the second open reading frame (ORF) is found in the genes coding for the phosphoprotein. The group of proteins coded by the internal ORF has been designated the C class (Bellini et al, 1985). The function of the C class proteins is unknown although Bellini et al (1985) have proposed a role in replication or transcription for the measles C protein due to its colocalisation with the nucleocapsid protein.

Examination of the four NS gene sequences available reveals the potential of all four to encode a small (65 or 67 amino acid), basic protein in a second ORF (see appendix). Figure 6.4 shows the potential second ORF of the NS gene of wild-type

VSV NJ, strain Missouri.

At the moment, the evidence for a second protein encoded by the NS gene of VSV is circumstantial. Table 6.2 compares the amino acid composition of the proteins encoded by the second ORF in the four NS genes. With the exception of the relative proportions of acidic and basic amino acids there is little similarity between serotypes in the composition of the four proteins.

Like the NS proteins, those encoded by the second ORF show a high degree of homology within subtypes (92% for IND serotypes, 70% for NJ serotypes) and a low degree of homology between serotypes (16%). The predicted proteins are small (mol wt 7500) and so would not have been detected on the gels previously used to visualise the proteins. However, preliminary experiments using high percentage gels to analyse infected cell extracts radiolabelled with a variety of different amino acids have failed to detect a second NS protein (R.M. Elliott, personal communication).

All initiation codons in the VSV genes so far sequenced are preceded directly by the triplet ATC. Hence, one point of evidence against there being a second protein coded by the NS gene is that in the Missouri strain of the NJ serotype the initiating methionine is preceded by CTT and in the Mudd-Summers strain of the IND serotype it is preceded by TAG.

The NS protein although relatively small is an important protein and serves a number of functions in the virus lifecycle. More study is required so that its precise role and mode of action can be elucidated. Expression of

full-length cDNA clones now available under the control of an appropriate promoter allied to the advanced techniques of site-directed mutagenesis will make the NS protein more amenable for study in the near future.

8. FUTURE PROSPECTS

The work presented in this thesis provides the foundation of future work on the analysis of the structure and function of the NS gene of VSV. The future work can be broadly divided into firstly, the expression of the cDNA clones of the wild-type and mutant virus mRNAs, in eukaryotic cells and then, detailed analysis of the NS gene by site-directed mutagenesis of these cDNA clones.

(i) Expression of cDNA Clones

The full-length cDNA clones of the NS genes of wild-type, mutant and revertant viruses can be expressed in eukaryotic cells under the control of an appropriate promoter eg. the cDNA clones could be ligated into the pJC119 expression vector as used by Sprague *et al* (1983) for the expression of the VSV N gene. pJC119 is a derivative of pML2 which employs the SV40 late promoter to initiate transcription of inserted sequences and also supplies the SV40 splicing and polyadenylation signals (Sprague *et al*, 1983).

The recombinant plasmids can then be transfected into COS-1 cells, a simian line that constitutively expresses SV40 T antigen (Gluzman, 1981). Because the plasmid sequences interrupt the early region of the SV40 T antigen must be supplied in order that the recombinant be replicated.

There are a number of ways by which expression of the NS protein can be monitored eg. immunofluorescence or immunoprecipitation. The biological activity of the

expressed protein can be monitored by its ability to complement the complementation group E mutants at the non-permissive temperature.

(ii) Site-directed Mutagenesis

Oligonucleotide-directed mutagenesis (Zoller and Smith, 1982) could be used to introduce single nucleotide changes, insertions or deletions into the cDNA. The ease by which oligonucleotides can be synthesised means there are no constraints on the types of mutation that can be introduced.

The biological activity of the mutated cDNAs can be tested by transfecting them into COS-1 cells and testing their ability to complement the complementation group E mutants.

It would be of immediate interest to assess the results of:

(a) mutating one or more of the conserved phosphorylation sites in the wild-type gene especially the constitutive phosphorylation sites of Hsu and Kingsbury (1985)

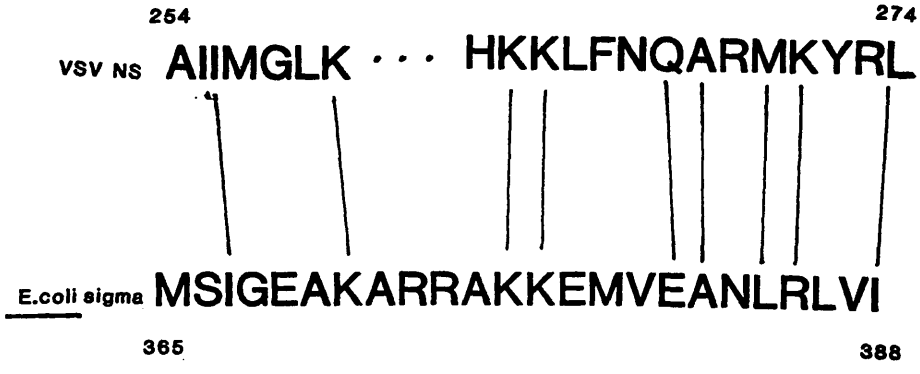
(b) changing amino acid residues in the conserved domains (I and III) of the wild-type gene eg. insert acidic residues in the basic carboxy terminus (domain III)

(c) deleting domain II in the NS gene of the NJ serotype or inserting this region into the NS gene of the IND serotype

(d) changing one or other of the mutations of tsE2 to wild-type sequence

(e) deleting the putative second ORF

Future studies of the type described will help answer many questions concerning the role of the NS protein in VSV transcription and replication.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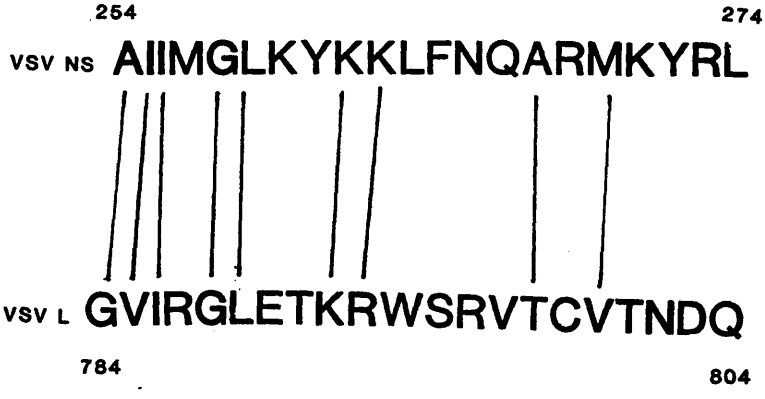
274
VSV NS AIIMGLK · · · HKKLFNQARMKYRL
E.coli sigma MSIGEAKARRAKKEMVEANLRLVI
365
388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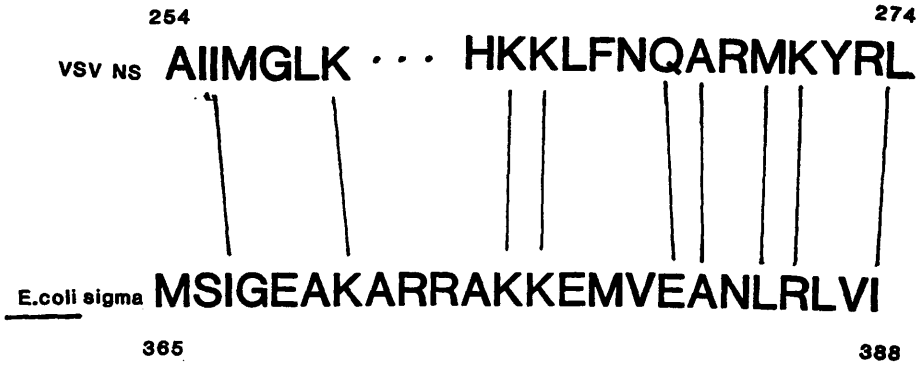
VSV L GVIRGLETKRWSRVTCVTNDQ
784
804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E.coli sigma MSIGEAKARRAKKEMVEANLRLVI
365
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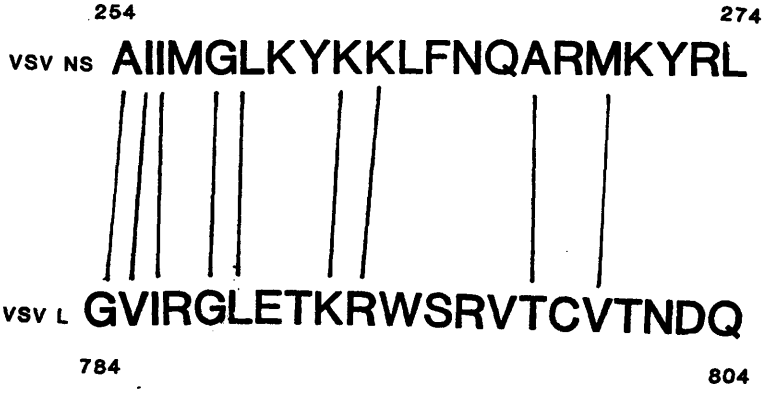
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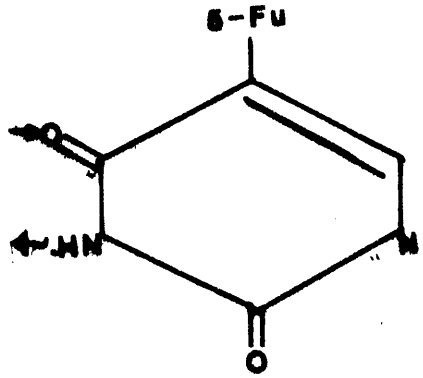
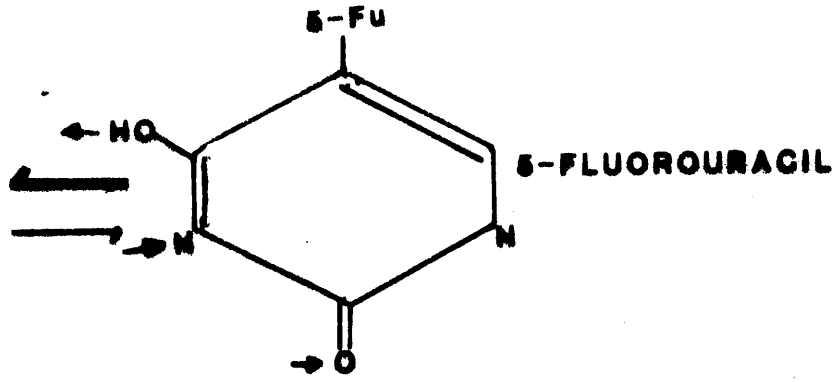
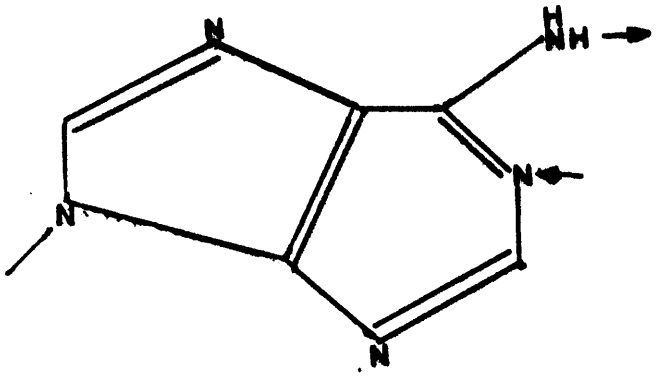
VSV L GVIRGLETKRWSRVTCVTNDQ
784
804



KETO

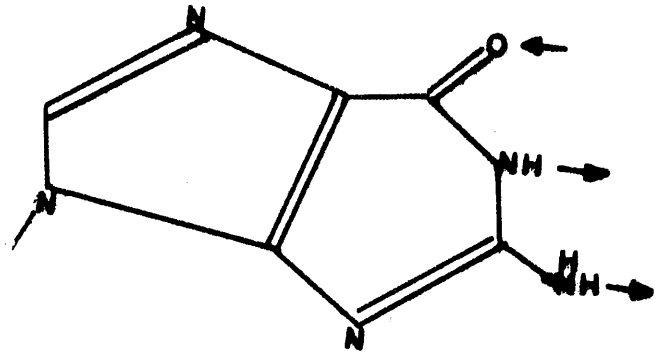


ENOL



adenine

In common keto form 5-Fu pairs with A

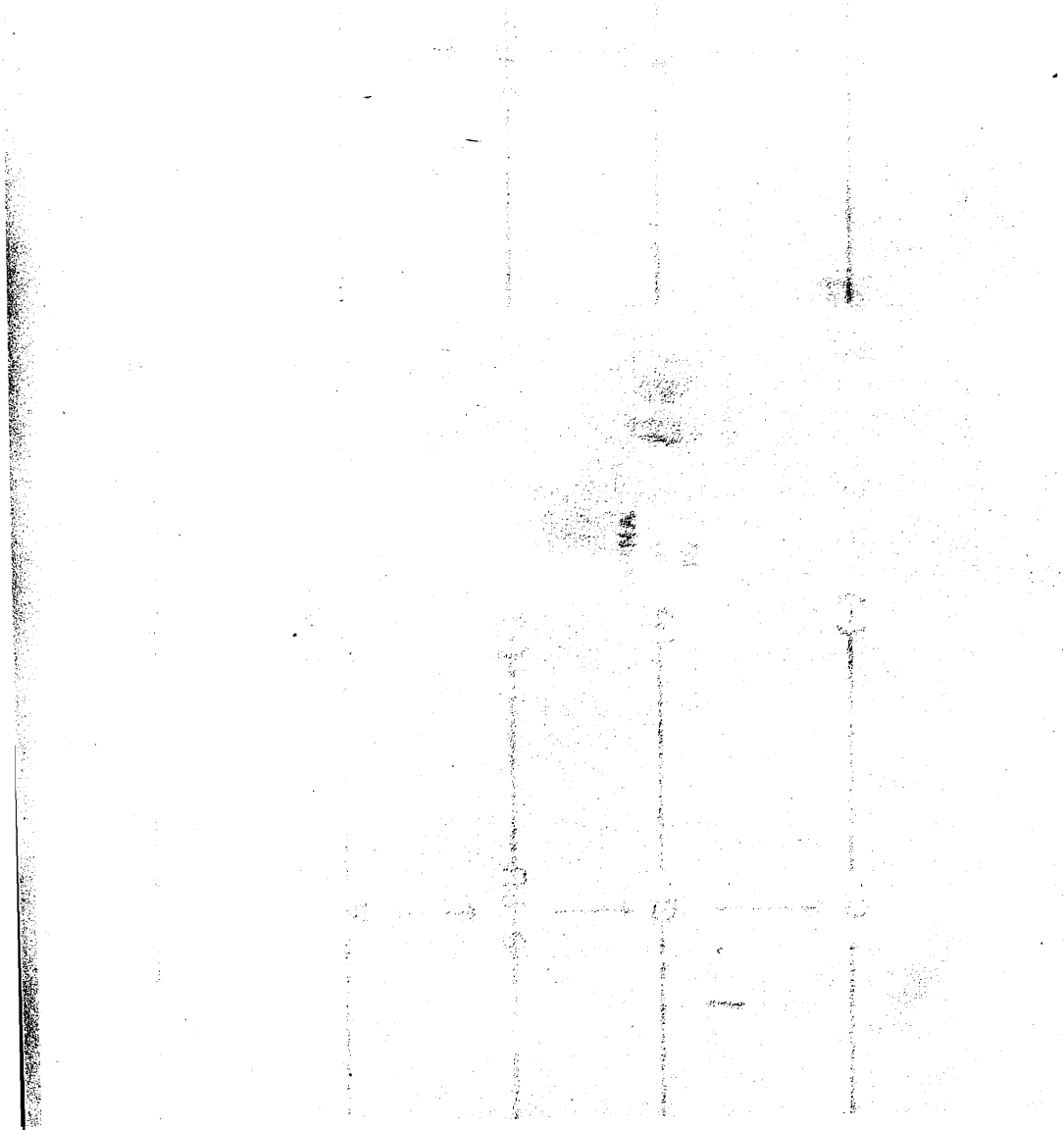


guanine

In rare enol form 5-Fu pairs with G

Figure 6.2

Tautomeric forms of 5-fluorouracil. In the keto form can take the place of uracil in RNA and is capable of base pairing with guanine if it exists in the less common enol form as shown opposite.



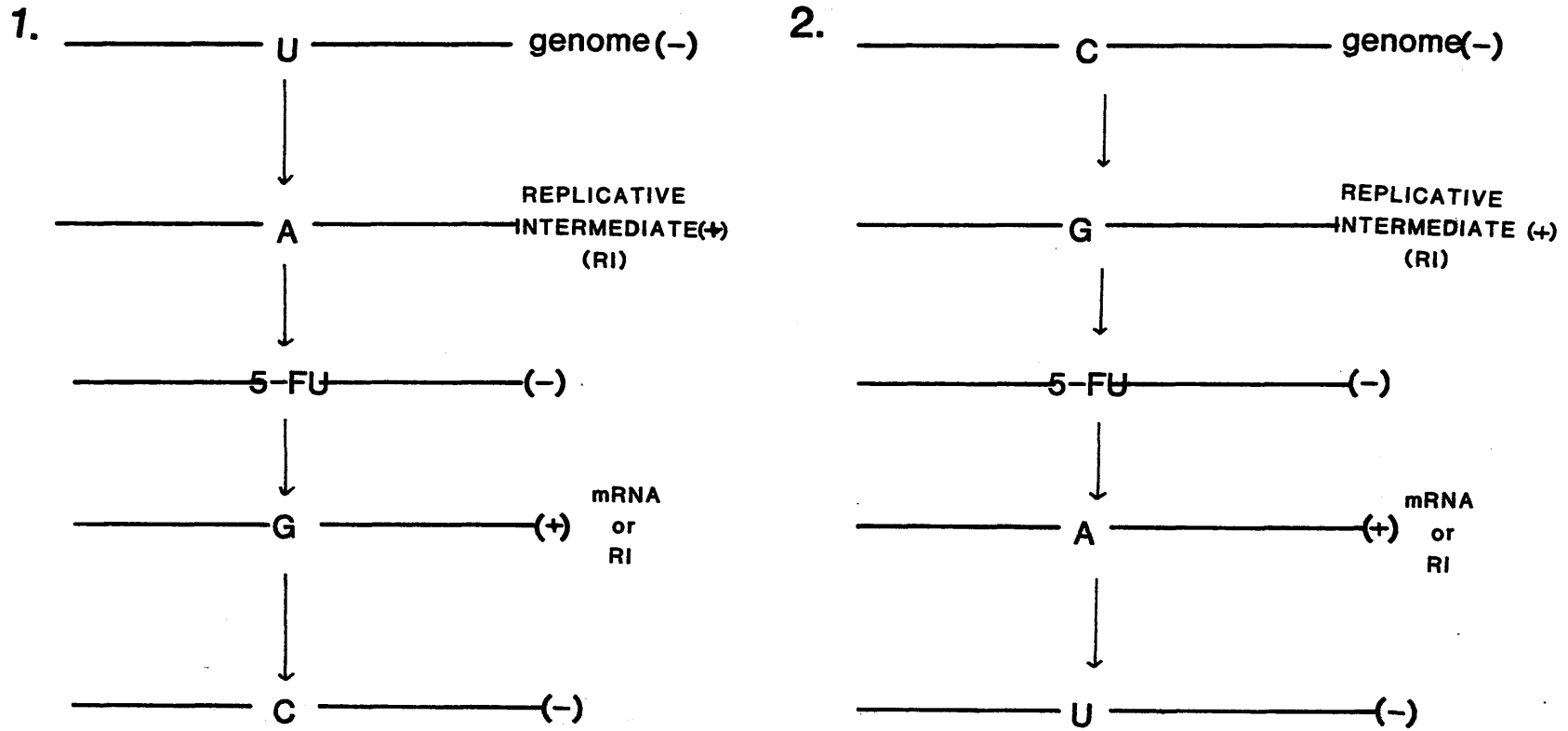
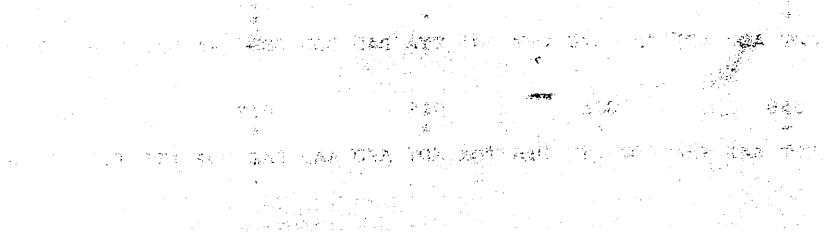

1.
U
genome (–)
A
REPLICATIVE INTERMEDIATE (+) (RI)
5-FU
(–)
G
(+)
mRNA or RI
C
(–)
2.
C
genome (–)
G
REPLICATIVE INTERMEDIATE (+) (RI)
5-FU
(–)
A
(+)
mRNA or RI
U
(–)

Figure 6.3

Two possible modes of action of 5-fluorouracil.

(1) 5-FU takes the place of a uracil in genomic RNA (- sense) and subsequently base pairs with guanine during synthesis of replicative intermediate RNA (+ sense). Thus incorporation of 5-FU causes a U→C change in genomic RNA (- sense) or an A→G change in mRNA (+ sense) and replicative intermediate RNA (+ sense).

(2) 5-FU in its less common enol form takes the place of cytosine in genomic RNA (- sense). However, it undergoes a tautomeric shift and base pairs with adenine in subsequent rounds of replicative intermediate (+ sense) synthesis. Thus in this case 5-FU action leads to a C→U change in genomic RNA (- sense) and a G→A change in mRNA (+ sense) and the replicative intermediate (+ sense).



```
        10          20          30          40          50          60
        *           *           *           *           *           *
CAG AGA TCA TGG ACA GTA TTG ATC GGC TCA AAA CTT ACT TGG CTA CTT ATG ATA ATT TGG
                                                                Met Ile Ile Trp
        70          80          90          100         110         120
        *           *           *           *           *           *
ATT CTG CCT TGC AGG ATG CAA ATG AGT CTG AAG AAA GAA GAG AGG ATA AAT ATC TCC AAG
Ile Leu Pro Cys Lys Met Gln Met Ser Leu Lys Lys Glu Glu Arg Ile Asn Ile Ser Lys

       130         140         150         160         170         180
        *           *           *           *           *           *
ACC TCT TCA TCG AAG ATC AAG GAG ATA AAC CAA CTC CGT CAT ATT ATC AGG AAG AAG AAT
Thr Ser Ser Ser Lys Ile Lys Glu Ile Asn Gln Leu Arg His Ile Ile Arg Lys Lys Asn

       190         200         210         220         230         240
        *           *           *           *           *           *
CGT CAG ATT CAG ATA CTG ACT ATA ATG CTG AAC ATC TTA CGA TGT TGT CGC CGG ATG AAA
Arg Gln Ile Gln Ile Leu Thr Ile Met Leu Asn Ile Leu Arg Cys Cys Arg Arg Met Lys

       250         260         270         280         290         300
        *           *           *           *           *           *
GAA TAG ACA AAT GGG AAG AAG ATT TGC CCG AAT TGG AAA AGA TTG ATG ATG ATA TAC CAG
Glu ---

       310         320         330         340         350         360
        *           *           *           *           *           *
TAA CTT TTT CTG ACT GGA CAC AAC CTG TAA TGA AGG AAA ACG GAG GGG AGA AAT CAC TAT

       370         380         390         400         410         420
        *           *           *           *           *           *
CTC TGT TTC CTC CGG TTG GAT TAA CAA AAG TTC AGA CAG ACC AAT GGA GGA AGA CAA TTG

       430         440         450         460         470         480
        *           *           *           *           *           *
AGG CAG TCT GTG AGA GCT CTA AAT ATT GGA ATT TGT CAG AAT GTC AGA TTA TGA ACT CAG

       490         500         510         520         530         540
        *           *           *           *           *           *
AAG ATC GTC TTA TCC TCA AAG GCC GAA TTA TGA CTC CTG ACT GCA GTT CAT CAA TCA AAT

       550         560         570         580         590         600
        *           *           *           *           *           *
CTC AGA ACT CCA TAC AGA GTT CCG AAT CTC TCT CCT CCT CGC ATT CAC CCG GTC CAG CAC

       610         620         630         640         650         660
        *           *           *           *           *           *
CAA AGT CAC GGA ATC AAC TAG GTC TAT GGG ATT CAA AAT CAA CAG AAG TAC AAC TGA TCT

       670         680         690         700         710         720
        *           *           *           *           *           *
CGA AGA GGG CCG GAG TGA AGG ACA TGA TGG TGA AAT TGA CAG ACT TTT TTG GAA GTG AAG

       730         740         750         760         770         780
        *           *           *           *           *           *
AGG AAT ATT ATT CAG TAT GCC CAG AAG GGG CCC CAG ATT TGA TGG GAG CAA TCA TCA TGG

       790         800         810         820         830         840
        *           *           *           *           *           *
GAC TAA AAC ATA AAA AGC TTT TTA ACC AAG CAA GAA TGA AGT ATC GTA TCT AAT TAA TTC

       850
        *
CGA TGA TCA ATA TG
```

Translation begun with base number 84
Translated to base number 248
Sequence printed from base number 1 to base number 856
Sequence numbered beginning with base number 1

Figure 6.4

Nucleotide sequence of the NS mRNA of VSV NJ, Missouri strain with the translation of the protein encoded by the second open reading frame.

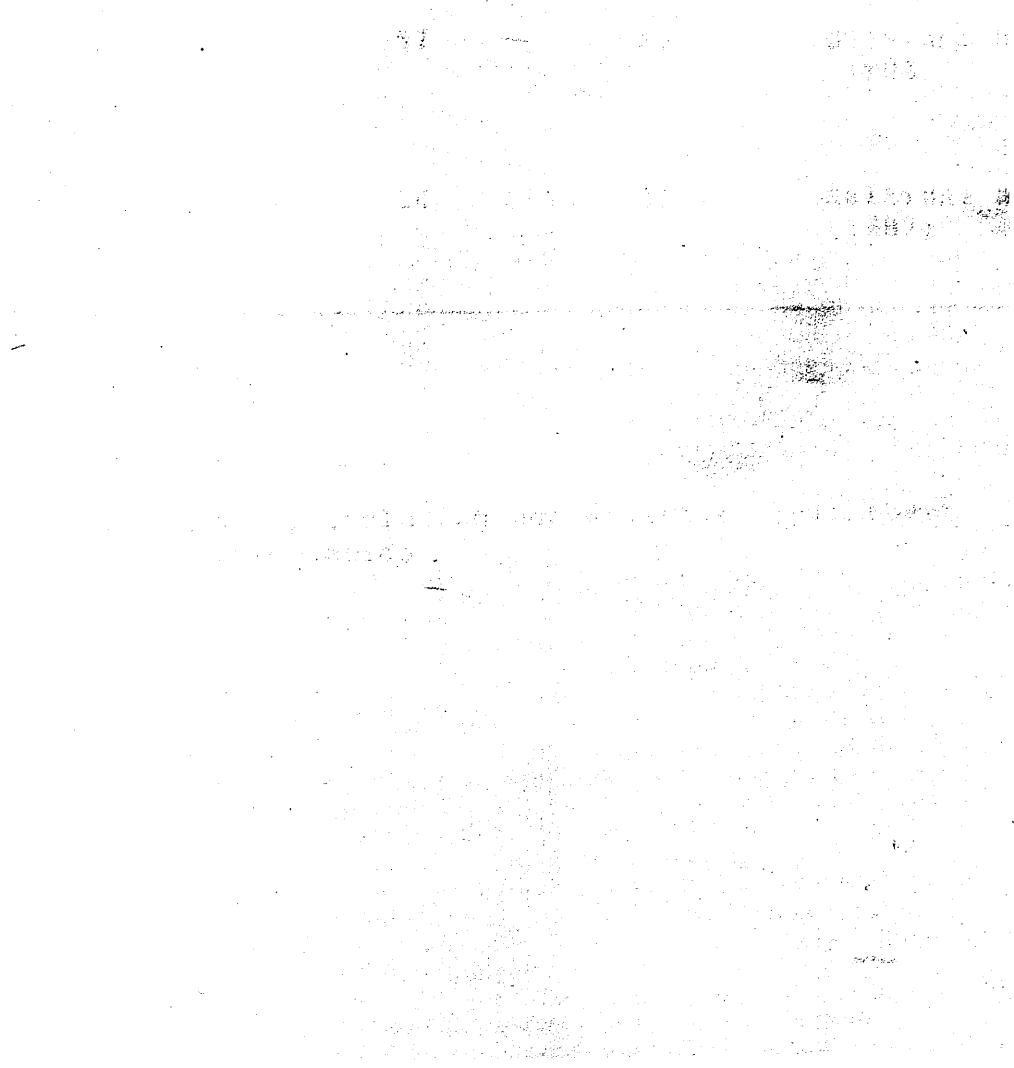


Table 6.1 Nucleotide and Amino Acid Homology between the Genes of VSV Indiana and New Jersey Serotypes

Genes	Nucleotide Homology %	Amino Acid* Homology %	Reference
Leader Template	80	-	Colonno and Banerjee (1978b)
N	68	68	Banerjee et al (1984)
M	57	62	Gill and Banerjee (1986)
G	54	51	Gallione and Rose (1983)

* Homology excluding conservative replacement of amino acids

Table 6.2 Amino Acid Composition of the Proteins Encoded by the Second Open Reading Frame in the NS Protein of VSV

Amino Acid	New Jersey Serotype		Indiana Serotype	
	Ogden %	Hazelhurst %	Mudd-Summers %	San-Juan %
Phe	0.0	0.0	1.5	1.5
Val	0.0	0.0	0.0	1.5
Ala	0.0	0.0	3.0	1.5
Asn	7.7	6.2	4.5	4.5
Cys	4.6	4.6	3.0	3.0
Leu	9.2	9.2	14.9	14.9
Ser	6.2	7.7	9.0	10.4
Tyr	0.0	0.0	3.0	1.5
Lys	10.8	12.3	14.9	14.9
Trp	1.5	1.5	1.5	1.5
Ile	21.5	20.0	9.0	9.0
Pro	3.1	1.5	3.0	3.0
His	3.1	1.5	1.5	3.0
Asp	1.5	0.0	0.0	0.0
Arg	12.3	12.3	7.5	6.0
Met	7.7	7.7	6.0	7.5
Thr	1.5	3.1	4.5	3.0
Glu	4.6	6.2	7.5	9.0
Gln	4.6	6.2	3.0	3.0
Gly	0.0	0.0	3.0	1.5
Basic	26.2	26.4	23.9	23.9
Acidic	6.1	6.2	7.5	9.0
Ser + Thr	7.7	10.8	13.5	13.4

REFERENCES

REFERENCES

Abraham, G. and Banerjee, A.K. (1976). The nature of the RNA products synthesised *in vitro* by subviral components of vesicular stomatitis virus. Virology 71, 230-241.

Abraham, G., Rhodes, D.P. and Banerjee, A.K. (1975). The 5' terminal structure of the methylated mRNA synthesised *in vitro* by vesicular stomatitis virus. Cell 5, 51-58.

Adam, S.A., Choi, Y.D. and Dreyfuss, G. (1986). Interaction of mRNA with proteins in vesicular stomatitis-infected cells. J. Virol. 57, 614-622.

Ahmed, K. and Graham, A.F. (1977). Persistent infections in L cells with temperature-sensitive mutants of reovirus. J. Virol. 23, 250-262.

Akashi, H. and Bishop, D.H.L. (1983). Comparison of the sequences and coding of LaCrosse and snow showe hare bunyaviruses S RNA species. J. Virol. 45 1155-1158.

Altstail, L.D. and Landsberger, F.R. (1981). Lipid-protein interactions between the surface glycoprotein of VSV and the lipid bilayer. Virology 115, 1-9.

Amend, D.F. (1975). Detection and transmission of infectious hematopoietic necrosis virus in rainbow trout. J. Wildlife Dis. 11, 471-480.

Anderson, S. (1981). Shotgun DNA sequencing using cloned DNasel-generated fragments. Nucl. Acids Res. 9, 3015-3027.

Andrewes, C., Pereira, H.G. and Wildy, P. (1978). Viruses of Vertebrates. In: Rhabdoviridae, p187-202, 4th edition. London, Bailliere Tindall, a division of Cassell & Company Ltd.

Arnheiter, H., Davis, N.L., Wertz, G., Schubert, M. and Lazzarini, R.A. (1985). Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. Cell 41, 259-267.

Auperin, D.D., Romanowski, V., Galinski, M. and Bishop, D.H.L. (1984). Sequencing studies of Pichinde arenavirus S RNA indicate a novel coding strategy, an ambisense viral S RNA. J. Virol. 52, 897-904.

Bailey, C., Miller, D. and Lenard, J. (1984). Effects of DEAE-dextran on infection and hemolysis by VSV. Evidence that non-specific electrostatic interactions mediate effective binding of VSV to cells. Virology 133, 111-118.

Baker, C.C. and Ziff, E.B. (1981). Promoters and heterogenous 5' termini of the messenger RNAs of adenovirus serotype 2. J. Mol. Biol. 149, 189-221.

Ball, L.A. and Wertz, G.W. (1981). VSV RNA synthesis: How can you be positive? Cell 26, 143-144.

Ball, L.A. and White, C.N. (1976). Order of transcription of genes of vesicular stomatis virus. Proc. Natl. Acad. Sci. (USA) 73, 442-446.

Baltimore, D. (1970). RNA-dependant DNA polymerase. Nature 226, 1209-1211.

Baltimore, D., Huang, A.S. and Stampfer, M. (1970). Ribonucleic acid synthesis of vesicular stomatitis. II. An RNA polymerase in the virion. Proc. Natl. Acad. Sci. (USA) 66, 572-576.

Banerjee, A.K. (1980). 5' termini cap structure in eukaryotic messenger ribonucleic acids. Microbiol. Rev. 44, 175-205.

Bell, J.C. and Prevec, L. (1985). Phosphorylation sites on phosphoprotein NS of vesicular stomatitis virus. J. Virol. 54, 697-702.

Banerjee, A.K., Abraham, G. and Colonno, R.J. (1977). Review. Vesicular stomatitis virus: Mode of transcription. J. Gen. Virol. 34, 1-8.

Banerjee, A.K., Moyer, S.A. and Rhodes, D.P. (1974). Studies on the in vitro adenylation of RNA by vesicular stomatitis virus. Virology 61, 547-555.

Banerjee, A.K., Rhodes, D.P. and Gill, D.S. (1984). Complete sequence of the mRNA coding for the N protein of vesicular stomatitis virus (New Jersey serotype). Virology 137, 432-438.

Baxt, B. and Bablanian, R. (1976). Mechanism of vesicular stomatitis virus-induced cytopathic effects. II. Inhibition of macromolecular synthesis induced by infectious and defective-interfering particles. Virology 72, 383-392.

Bell, J.C., Brown, E.G., Takayesu, D. and Prevec, L. (1984). Protein kinase activity associated with immunoprecipitates of the vesicular stomatitis virus phosphoprotein NS. Virology 132, 229-238.

Belle Isle, H.D. and Emerson, S.U. (1982). Use of a hybrid infectivity assay to analyse primary transcription of temperature-sensitive mutants of the New Jersey serotype of VSV. J. Virol. 43, 37-40.

Bellini, W.J., Englund, G., Rozenblatt, S., Arnheiter, H. and Richardson, C.D. (1985). Measles virus P gene codes for two proteins. J. Virol. 53, 908-919.

Bellini, W.J., Englund, G., rozenblatt, S., Arnheiter, H. and richardson, C.D. (1985). Measles virus P gene codes for two proteins. J. Virol. 53, 908-919.

Bergmann, J.E., Tokuyasu, K.T. and Singer, S.J. (1981).

Passage of an integral membrane protein, the VSV glycoprotein, through the Golgi apparatus en route to the plasma membrane. Proc. Natl. Acad. Sci. (USA) 78, 1746-1750.

Bickle, T.A., Pirrotta, V. and Imber, R. (1977). A simple generation procedure for purifying restriction endonucleases. Nucl. Acids. Res. 4, 2561-2572.

Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. (USA) 80, 3963-3965.

Bishop, D.H.L. and Flamand, A. (1975). Transcription Processes of Animal RNA Viruses. In: 25th Symposium of the Society for General Microbiology, p95-152. Eds. D.C. Burke and W.C. Russell. Cambridge University Press, Cambridge.

Bishop, D.H.L., Repik, P., Obijeski, J.F., Moore, N.F. and Wagner, R.R. (1975). Restoration of infectivity to spikeless VSV by solubilised viral components. J. Virol. 16, 75-84.

Bishop, D.H.L. and Roy, P. (1972). Dissociation of vesicular stomatitis virus and relation of the virion proteins to the viral transcriptase. J. Virol. 10, 234-243.

Bishop, D.H.L. and Smith, M.S. (1977). The Molecular Biology of Animal Viruses, Vol. 1, p167-280. Ed. D.P. Nayak. Marcel Dekker Inc., New York and Basel.

Blumberg, B.M., Giorgi, C., Kolakofsky, D. (1983). N protein of VSV selectively encapsidates leader RNA *in vitro*. Cell 32, 559-567.

Blumberg, B.M., Kolakofsky, D. (1981). Intracellular VSV leader RNAs are found in nucleocapsid structures. J. Virol. 40, 568-576.

Blumberg, B.M., Leppert, M. and Kolakofsky, D. (1981). Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. Cell 23, 837-845.

Boettiger, D., Love, D.N. and Weiss, R.A. (1975). Virus envelope markers in mammalian tropism of avian RNA tumour viruses. J. Virol. 15, 108-114.

Bolognesi, D.P., Langlois, A.J. and Schafer, W. (1975). Polypeptides of mammalian oncorna viruses. IV. Structural components of murine leukaemia virus released as soluble antigens in cell culture. Virology 68, 550-555.

Both, G., Moyer, S. and Banerjee, A. (1975). Translation and identification of the viral mRNA species isolated from subcellular fractions of VSV infected cells. J. Virol. 15, 1012-1019.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. and Chambon, P. (1978). Ovalbumin gene: Evidence for a leader sequence in mRNA and DNA sequences at the exon-intron boundaries. Proc. Natl. Acad. Sci. (USA) 75, 4853-4857.

Brown, F., Bishop, D.H.L., Crick, J., Francki, R.I.B., Holland, J.J., Hull, R., Johnson, K., Martelli, G., Murphy, F.A., Obijeski, J.F., Peters, D., Pringle, C.R., Reichmann, M.E., Schneider, L.G., Shope, R.E., Simpson, D.I.H., Summers, D.F. and Wagner, R.R. (1979). Rhabdoviridae. Intervirology 12, 1-7.

Brown, F., Cartwright, B., Crick, J. and Smale, C.J. (1967).

Brun, G. (1984). Host Range Mutants of Piry Virus: A New Type of Mutant in Drosophila. In: Negative Strand Viruses: Paramyxoviruses and Rhabdoviruses, p413-420. Eds. D.H.L. Bishop and R.W. Compans. Academic Press Inc.

Infective virus substructure from vesicular stomatitis virus. J. Virol. 1, 368-373.

Brown, D.T. and Riedel, B. (1977). Morphogenesis of VSV: Electron microscope observations with freeze-fracture techniques. J. Virol. 21, 601-609.

Bruck, C., Pontetelle, D., Burny, A. and Zavada, J. (1982). Topographical analysis by monoclonal antibodies of BLV-gp51 epitopes involved in viral functions. Virology 122, 353-362.

Bruns, M. and Lehmann-Grube, F. (1984). Lymphocytic choriomeningitis virus. VIII. Reciprocal formation of pseudotypes with vesicular stomatitis virus. Virology 137, 49-57.

Buongurio, D.A., Nakada, S., Parvin, J.D., Krystal, M., Palese, P. and Fitch, W.M. (1986). Evolution of human influenza A viruses over 50 years: Rapid, uniform rate of change in NS gene. Science 232, 980-982.

Burgess, R.R., Travers, A.A., Dunn, J.J. and Bautz, E.K.F. (1969). Factor stimulating transcription by RNA polymerase. Nature 221, 43-46.

Burton, Z., Burgess, R.R., Moore, D., Holder, S. and Gross, C.A. (1981). The nucleotide sequence of the cloned rpoD gene for the RNA polymerase sigma subunit from E.coli K12. Nucl. Acids Res. 9, 2893-2901.

Bussereau, F. (1971). Etude du symptome de la sensibilite au CO_2 produit par le virus de la stomatite vesiculaire chez Drosophila melanogaster. II. VSV de serotype Indiana. Ann. Microbiol. (Inst. Pasteur) 121, 223-239.

Bussereau, F. (1972). Etude du symptome de la sensibilite au CO_2 produit par le virus de la stomatite vesiculaire

chez Drosophila melanogaster. II. VSV de serotype Indiana. Ann. Microbiol. (Inst. Pasteur) 122, 1029-1058.

Bussereau, F. (1973). Etude du symptome de la sensibilite au CO_2 produit par le virus de la stomatite vesiculaire chez Drosophila melanogaster. III. Souches de differents serotype. Ann. Microbiol. (Inst. Pasteur) 124a, 535-554.

Bussereau, F. (1975). The CO_2 sensitivity induced by two rhabdoviruses, Piry and Chandipura in Drosophila melanogaster. Ann. Microbiol. (Inst. Pasteur) 126b, 389-403.

Bussereau, F., de Kinkelin, P. and Le Berre, M. (1975). Infectivity of fish rhabdoviruses for Drosophila melanogaster. Ann. Microbiol. (Inst. Pasteur) 126a, 389-395.

Byrd, A.D., Kennedy-Morrow, J., Marks, M.D. and Lesnaw, J.A. (1984). Functional relationships within the New Jersey serotype of VSV: Genetic and physiological comparisons of the Hazelhurst and Concan subtypes. J. Gen. Virol. 65, 1769-1779.

Cabradilla, C., Holloway, B.P. and Obijeski, J.F. (1983). The sequence of the SmaI genome of LaCrosse virus. Virology 128, 463-468.

Callahan, P.L., Mizutani, S. and Colonno, R.J. (1985). Molecular cloning and complete nucleotide determination of RNA genome of human rhinovirus type 14. Proc. Natl. Acad. Sci. (USA) 81, 2040-2044.

Capone, J., Toneguzzo, F. and Ghosh, H.P. (1982). Synthesis and assembly of membrane glycoproteins: Membrane anchoring COOH-terminal domain of VSV envelope glycoprotein G contains fatty acids. J. Biol. Chem. 257,

16-19.

Carroll, A.R. and Wagner, R.R. (1978). Inhibition of transcription by immunoglobulins directed against the ribonucleoprotein of homotypic and heterotypic vesicular stomatitis viruses. J. Virol. 25, 675-684.

Carroll, A.R. and Wagner, R.R. (1979). Role of the membrane protein in endogenous inhibition of in vitro transcription by vesicular stomatitis virus. J. Virol. 29, 134-142.

Carstens, E.B., Krebs, A. and Gallerneault, C.E. (1986). Identification of an amino acid essential to the normal assembly of Autographa calafornica polyhedrosis virus polyhedra. Journal of Virology 58, 684-688.

Cartwright, B. (1977). Effect of Concanavelin A on VSV maturation. J. Gen. Virol. 34, 249-256.

Cartwright, B. and Brown, F. (1972). Serological relationships between different strains of vesicular stomatitis virus. J. Gen. Virol. 16, 391-398.

Cartwright, B., Talbot, P. and Brown, F. (1970). The proteins of biologically active subunits of VSV. J. Gen. Virol. 7, 267-272.

Casadaban, M.J. and Cohen, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in E.coli. J. Mol. Biol. 138, 179-207.

Centrella, M. and Lucas-Lenard, J. (1982). Regulation of protein synthesis in VSV-infected mouse L-929 cells by decreased protein synthesis initiation factor 2 activity. J. Virol. 41, 781-791.

Chamberlin, M. (1974). The selectivity of transcription. Ann. Rev. Biochem. 43, 721-775.

Chan, C. and Crouch, N.A. (1978). Shope fibroma virus facilitation of VSV adsorbtion and replication in non-permissive cells. Virology 85, 43-62.

Chan, J.C., East, J.L., Bowen, J.M., Massey, R. and Schochetman, G. (1982). Monoclonal and polyclonal antibody studies of VSV (hr MMTV) pseudotypes. Virology 120, 54-64.

Chan, J.C., Hixson, D.C. and Bowen, J.M. (1978). Detection of vesicular stomatitis virus (murine leukaemia virus) pseudotypes by immunoelection microscopy. Virology 88, 171-176.

Chanda, P.K. and Banerjee, A.K. (1981). Identification of promoter-proximal oligonucleotides and a unique dinucleotide, pppGpC, from in vitro transcription products of vesicular stomatitis virus. J. Virol. 39, 93-103.

Chatis, P.A. and Morrison, T.G. (1979). Vesicular stomatitis virus glycoprotein is anchored to intracellular membranes near its carboxyl end and is proteolytically cleaved at its amino terminus. J. Virol. 29, 957-963.

Chatis, P.A. and Morrison, T.G. (1982). Characterisation of the soluble glycoprotein released from vesicular stomatitis virus-infected cells. J. Virol. 45, 80-90.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.

Choppin, P.W. and Compans, R.W. (1970). Phenotypic mixing of envelope proteins of the parainfluenza virus SV5 and

vesicular stomatitis virus. J. Virol. 5, 609-616.

Chou, P.Y. and Fasman, G.D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. 47, 54-147.

Clapham, P., Nagy, K. and Weiss, R.A. (1984). Pseudotypes of human T-cell leukaemia virus types 1 and 2: Neutralisation by patient's sera. Proc. Natl. Acad. Sci. (USA) 81, 2886-2889.

Clewell, D.B. (1972). Nature of ColE, plasmid replication in E.coli in the presence of chloramphenicol. J. Bacteriol. 110, 667-676.

Clewley, J.P., Bishop, D.H.L., Kang, C-Y., Coffin, J., Schnitzlein, W.M., Reichmann, M.E. and Shope, R.E. (1977). Oligonucleotide fingerprints of RNA species obtained from rhabdoviruses belonging to the vesicular stomatitis virus subgroup. Journal of Virology 23, 152-166.

Clinton, G.M., Burge, B.W. and Huang, A.S. (1978). Effects of phosphorylation and pH on the associateion of NS protein with the VSV cores. J. Virol. 27, 340-346.

Clinton, G.M., Burge, B.W. and Huang, A.S. (1979). Phosphoproteins of VSV: Identity and interconversion of phosphorylated forms. Virology 99, 84-94.

Clinton, G.M. and Huang, A.S. (1981). Distribution of phosphoserine, phosphotyrosine and phosphothreonine in proteins of vesicular stomatitis virus. Virology 108, 510-514.

Cohen, S.N., Chong, A.C.Y. and Hsu, L. (1972). Non-chromosomal antibiotic resistance in bacteria: Genetic transformation by R factor RNA. Proc. Natl.

Acad. Sci. (USA) 69, 2110-2114.

Collins,, P.L., Hightower, L.E. and Ball, L.A. (1978). Transcription and translation of Newcastle disease virus mRNAs in vitro. J. Virol. 28, 324-336.

Colonno, R.J. and Banerjee, A.K. (1976). A unique RNA species involved in initiation of vesicular stomatitis virus RNA transcription in vitro. Cell 8, 197-204.

Colonno, R.J. and Banerjee, A.K. (1978a). Complete nucleotide sequence of the leader RNA synthesised in vitro by vesicular stomatitis virus. Cell 15, 93-101.

Colonno, R.J. and Banerjee, A.K. (1978b). Nucleotide sequence of the leader RNA of the New Jersey serotype of vesicular stomatitis virus. Nucl. Acids Res. 51, 4165-4175.

Colonno, R.J., Lazzarini, R.A., Keene, J.D. and Banerjee, A.K. (1977). In vitro synthesis of messenger RNA by a defective interfering particle of vesicular stomatitis virus. Proc. Natl. Acad. Sci. (USA) 74, 1884-1888.

Combard, A. and Printz-Ane, C. (1979). Inhibition of vesicular stomatitis transcriptase complex by the virion envelope M protein. Biochem. Biophys. Res. Commun. 88, 117-123.

Cotton, W.E. (1927). Vesicular stomatitis. Vet. Med. 22, 169-175.

Cox, R.A. (1968). The use of guanidinium chloride in the isolation of nucleic acids. Meth. Enzymol. 12B, 120.

Crimmins, D.L., Mehard, W.B. and Schlesinger, S. (1983). Physical properties of a soluble form of vesicular stomatitis virus at neutral and acidic pH. Biochemistry 22, 5790-5796.

Crouch, N.A. (1978). Replication of VSV facilitated in non-permissive cells by early functions of shope fibroma virus (40026). Proc. Soc. Exptl. Biol. Med. 157, 225-230.

Dagent, M. and Ehrlich, S.D. (1979). Prolonged incubation in calcium chloride improves the competent of E.coli cells. Gene 6, 23-28.

Daniels, R.S., Downie, J.C., Hay, A.J., Knossow, M., Skehel, J.J., Wang, M.L. and Wiley, D.C. (1985). Fusion mutants of the influenza virus haemagglutinin glycoprotein. Cell 40, 431-439.

David, A.E. (1973). Assembly of the vesicular stomatitis virus envelope: Incorporation of viral polypeptides into the host plasma membrane. J. Mol. Biol. 76, 135-148.

Dahlberg, J.E. (1974). Quantitative electron microscopic analysis of the penetration of VSV into L cells. Virology 58, 250-262.

Davis, N.L. and Wertz, G.W. (1982). Synthesis of vesicular stomatitis virus negative-strand RNA in vitro: Dependence on viral protein synthesis. J. Virol. 41, 821-832.

De, B.P. and Banerjee, A.K. (1984). Specific interactions of VSV L and NS proteins with heterologous genome RNP template lead to mRNA synthesis in vitro. J. Virol. 51, 628-634.

De, B.P. and Banerjee, A.K. (1985). Requirements and functions of vesicular stomatitis virus L and NS proteins in the transcription process in vitro. Biochem. Biophys. Res. Commun. 126, 40-49.

De, B.P., Tahara, S.M. and Banerjee, A.K. (1982). Production and characterisation of a monoclonal antibody

to the N protein of vesicular stomatitis virus. Virology 122, 510-514.

Deininger, P.L. (1983). Random subcloning of sonicated DNA: Application to shot-gun DNA sequence analysis. Anal. Biochem. 129, 216-223.

De Jong, W.W., Zweers, A. and Cohen, L.H. (1978). Influence of single amino acid substitutions on electrophoretic ability of sodium dodecyl sulphate protein complexes. Biochem. Biophys. Res. Commun. 82, 532-539.

Denniston, K.J., Hoyer, B.H., Smedile, A., Wells, F.V., Nelson, J. and Gerin, J.L. (1986). Cloned fragments of the hepatitis delta virus RNA genome: Sequence and diagnostic application. Science 232, 873-875.

Dietzshold, B., Wiktor, T.J., Wunner, W.H. and Varnichis, A. (1983). Chemical and immunological analysis of the rabies soluble glycoprotein. Virology 124, 330-337.

Dietzshold, B., Wunner, W.H., Wiktor, T.J., Lopes, T.J., Lafon, M., Smith, C.L. and Kaprowski, H. (1983). Characterisation of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. (USA) 80, 70-74.

Domingo, E., Sabo, D., Taniguichi, T. and Weissmann, C. (1978). Nucleotide sequence heterogeneity of an RNA phage population. Cell 13, 735-744.

Dover, G.A. and Flavell, R.B. (1984). Molecular co-evolution: DNA diversions and maintenance of function. Cell 38, 622-623.

Dubois-Dalcq, M., Narayan, O. and Griffin, D.E. (1979). Cell surface changes associated with mutation of visna virus in antibody-treated cell cultures. Virology 92,

353-366.

Dubovi, E.J. and Wagner, R.R. (1977). Spatial relationships of the proteins of vesicular stomatitis virus: Induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J. Virol. 22, 500-509.

Dulbecco, R. and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis. J. Exptl. Med. 99, 167-182.

Duschinsky, R., Pleven, E. and Heidelberger, C. (1957). The synthesis of 5-fluoropyrimidines. J. Am. Chem. Soc. 79, 4559-4564.

Efstratiadis, A., Kafatos, F.C., Maxam, A.M. and Maniatis, T. (1976). Enzymatic in vitro synthesis of globin genes. Cell 7, 279-284.

Eidelman, O., Schlegel, R., Tralka, T. and Blumenthal, R. (1984). pH-dependant fusion induced by vesicular stomatitis virus glycoprotein reconstituted into phospholipid vesicles. J. Biol. Chem. 259, 4622-4628.

Elliott, R.M. (1985). Identification of non-structural proteins encoded by viruses of the Bunyamwera serogroup (family Bunyaviridae). Virology 143, 119-126.

Elliott, R.M. and Wilkie, M. (1986). Persistant infection of Aedes albopictus C6/36 cells by Bunyamwera virus. Virology 150, 21-32.

Emerson, S.U. (1982). Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. Cell 31, 635-642.

Emerson, S.U. (1985). Rhabdoviruses. In: Virology, p1119-1132. Eds. B.N. Fields et al. Raven Press, New York.

Emerson, S.U., Dierks, P.M. and Parsons, J.T. (1977). In vitro synthesis of a unique RNA species by a T particle of vesicular stomatitis virus. J. Virol. 23, 708-716.

Emerson, S.U. and Wagner, R.R. (1972). Dissociation and reassociation of the transcriptase and template activities of vesicular stomatitis B and T virions. J. Virol. 10, 297-309.

Emerson, S.U. and Wagner, R.R. (1973). L protein requirement for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. 12, 1325-1335.

Emerson, S.U. and Yu, Y-H. (1975). Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. 15, 1348-1356.

Epstein, D.A., Herman, R.C., Chien, I. and Lazzarini, R.A. (1980). Defective interfering particle generated by internal deletion of the vesicular stomatitis virus genome. J. Virol. 33, 818-829.

Eshita, Y. and Bishop, D.H.L. (1984). The complete sequence of the mRNA of snow-shoe hare bunyavirus reveals the presence of internal hydrophobic domains in the viral glycoprotein. Virology 137, 227-240.

Evans, D., Pringle, C.R. and Szilagyi, J.F. (1979). Temperature-sensitive mutants of complementation group E of VSV New Jersey serotype possess altered NS polypeptides. J. Virol. 31, 325-333.

Faulkner, G.P. and Lazzarini, R.A. (1980). Rhabdovirus Genetics. In: Rhabdoviruses, Vol. II, p163-176. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Fields, B.N. and Raine, C.S. (1974). Altered Neurologic Disease Induced by Reovirus Mutants. In: Mechanisms of

Viral Diseases, p161-167. Ed. C.F. Fox. Benjamin, Meulo Park, California.

Fields, B.N., Jaenisch, R. and Fox, C. (1980). Animal Virus Genetics. Academic Press Inc., New York.

Finter, N.B. (1973). The Assay and Standardisation of Interferon and Interferon Inducers. In: Interferon and Interferon Inducers, p135-176. Ed. N.B. Finter. North-Holland, Amsterdam.

Flamand, A. (1970). Etude genetique du virus de la stomatite vesiculaire: classement de mutants thermosensibles spontanes en groupes de complementation. J. Gen. Virol. 8, 187-193.

Flamand, A. (1980). Rhabdovirus Genetics. In: Rhabdoviruses, Vol. II, p115-140. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Florkiewicz, R. and Rose, J.K. (1984). A cell line expressing vesicular stomatitis virus glycoprotein fuses at low pH. Science 225, 721-723.

Follett, E.A.C., Pringle, C.R., Wunner, W.H. and Skehel, J.J. (1974). Virus replication in enucleate cells: VSV and influenza virus. J. Virol. 13, 394-399.

Francki, R.I.B. (1973). Plant rhabdoviruses. Adv. Virus Res. 18, 257-346.

Francki, R.I.B. and Randles, J.W. (1980). Rhabdoviruses Infecting Plants. In: Rhabdoviruses, Vol. II, p135-165. CRC Press Inc., Florida.

Franz-Fernandez, M.T. and Banerjee, A.K. (1978). In vitro RNA transcription by the New Jersey serotype of vesicular stomatitis virus. I. Characterisation of the mRNA species. J. Virol. 26, 179-187.

Freeman, G.J., Rose, J.K., Clinton, G.M. and Huang, A.S. (1977). RNA synthesis of vesicular stomatitis virus. VII. Complete separation of the mRNAs of vesicular stomatitis virus. J. Virol. 21, 1094-1104.

Frey, T.K. and Youngner, J.S. (1982). Novel phenotype of RNA synthesis expressed by vesicular stomatitis virus isolated from persistent infection. J. Virol. 44, 167-174.

Frey, T.K. and Youngner, J.S (1984). Further studies of the RNA synthesis phenotype seleted during persistent infection with vesicular stomatitis virus. Virology 136, 211-220.

Furuichi, Y. (1978). 'Pretranscriptional capping' in the biosynthesis of cytoplasmic polyhedrosis virus mRNA. Proc. Natl. Acad. Sci. (USA) 75, 1086-1090.

Furuichi, Y. and Shatkin, A.J. (1977). 5'-termini of reovirus mRNA. Ability of viral cores to form post-transcriptionally. Virology 77, 566-578.

Gadkari, D.A. and Pringle, C.R. (1980). Temperature-sensitive mutants of Chandipura virus. II. Phenotypic characteristics of the six complementation groups. J. Virol. 33, 100-106.

Gallione, C.J., Greene, J.R., Iverson, L.E. and Rose, J.K. (1981). Nucleotide sequences of the mRNAs encoding the vesicular virus N and NS proteins. J. Virol. 39, 529-535.

Gallione, C.J. and Rose, J.K. (1983). Nucleotide sequence of a cDNA clone encoding the entire glycoprotein from the New Jersey serotype of vesicular stomatitis virus. J. Virol. 46, 162-169.

Gallione, C.J. and Rose, J.K. (1985). A single amino acid substitution domain causes temperature-sensitive cell-surface transport of a mutant viral glycoprotein. J. Virol. 54, 374-382.

Garoff, H. and Ansorge, W. (1981). Improvements of DNA sequencing gels. Anal. Biochem. 115, 450-457.

Garreis-Wabnitz, C. and Kruppa, J. (1984). Intracellular appearance of a glycoprotein in VSV-infected BHK cells lacking the membrane-anchoring oligopeptide of the viral G protein. EMBO J. 3, 1469-1476.

Genty, N. and Bussereau, F. (1980). Is cytoskeleton involved in VSV reproduction? J. Virol. 34, 777-781.

Ghosh, K. and Ghosh, H.P. (1982). Synthesis *in vitro* of full length genomic RNA and assembly of the nucleocapsid of vesicular stomatitis virus in a coupled transcription-translation system. Nucl. Acids Res. 10, 8341-8351.

Gibson, R., Leavitt, R., Kornfield, S. and Schlesinger, S. (1978). Synthesis and infectivity of vesicular stomatitis virus containing non-glycosylated G protein. Cell 13, 671-679.

Gill, D.S. and Banerjee, A.K. (1985). Vesicular stomatitis virus NS proteins: Structural similarity without extensive sequence homology. J. Virol. 55, 60-66.

Gill, D.S. and Banerjee, A.K. (1986). Complete nucleotide sequence of the matrix protein mRNA of vesicular stomatitis virus (New Jersey serotype). Virology 150, 308-312.

Gillies, S. and Stollar, V. (1980). Generation of defective interfering particles of vesicular stomatitis virus in

Aedes albopictus cells. Virology 107, 497-508.

Giorgi, C., Blumberg, B.M. and Kolakofsky, D. (1983). Sendai virus contains overlapping genes expressed from a single mRNA. Cell 35, 829-836.

Giorgi, C., Blumberg, B.M. and Kolakofsky, D. (1983). Sequence determination of the (+) strand leader RNA regions of the vesicular stomatitis virus Chandipura, Cocal and Piry serotype-genomes. J. Virol. 46, 125-130.

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23, 175-182.

Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J. and Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus. Proc. Natl. Acad. Sci. (USA) 79, 5818-5822.

Goodger, W.J., Thurmond, M., Mahay, J., Mitchell, J. and Smith, P. (1985). Economic impact of an epizootic of bovine vesicular stomatitis virus in California. J. Am. Vet. Med. Assoc. 186, 370-373.

Goodman-Smitkoff, G., Mannino, R.J. and McSharry, J.J. (1981). The glycoprotein isolated from vesicular stomatitis virus is mitogenic for mouse B-lymphocytes. J. Exptl. Med. 153, 1489-1502.

Gopalakrishna, Y. and Lenard, J. (1985). Sequence alterations in temperature-sensitive M-protein mutants (complementation group III) of vesicular stomatitis virus. J. Virol. 56, 655-659.

Graeve, L., Garries-Wabnitz, C., Zauka, M., Breindl, M. and Kruppa, J. (1986). The soluble glycoprotein of vesicular stomatitis virus is formed during or shortly after the translation process. J. Virol. 57, 968-975.

Griffin, J.A. and Compans, R.W. (1979). Effect of cytochalusin on the maturation of enveloped viruses. J. Exptl. Med. 150, 379-391.

Grinnell, B.W. and Wagner, R.R. (1983). Comparitive inhibition of cellular transcription by vesicular stomatitis virus serotypes New Jersey and Indiana: Role of each viral RNA. J. Virol. 48, 88-101.

Grinnell, B.W. and Wagner, R.R. (1984). Nucleotide sequence and secondary structure of VSV leader RNA and homologous DNA involved in inhibition of DNA-dependant transcription. Cell 36, 533-543.

Groberg, W.J. (1983). The Status of Viral Fish Disease in the Columbia River Basin. Proceedings of a Workshop on Viral Diseases of Salmonid Fishes in the Columbia River Basin. Bonneville Power Administration, Portland, Oregon, p1-22.

Grubman, M., Moyer, S., Banerjee, A.K. and Ehranfeld, E. (1975). Subcellular localisation of VSV mRNAs. Biochem. Biophys. Res. Commun. 62, 531-537.

Grubman, M. and Shafritz, D.A. (1977). Identification and characterisation of messenger ribonucleoprotein complexes from vesicular stomatitis virus-infected cells. Virology 81, 1-16.

Grunstein, M. and Hogress, D.S. (1975). Colony hybridisation: A method for the isolation of cloned DNA that contain a specific gene. Proc. Natl. Acad. Sci. (USA) 72, 3961-3965.

Gruss, P., Dhar, R. and Khoury, G. (1981). Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. (USA) 78, 943-947.

Gubler, U. and Hoffman, J.B. (1983). A simple and very efficient method for generating cDNA libraries. Gene 25, 263-269.

Gupta, K.C. and Kingsbury, D.W. (1982). Conserved polyadenylation signals in two negative strand RNA virus families. Virology 120, 518-523.

Hamilton, D.H., Moyer, R.W. and Moyer, S.A. (1980). Characterisation of the non-permissive infection of rabbit cornea cells by VSV. J. Gen. Virol. 49, 273-287.

Hanson, R.P. (1952). The natural history of vesicular stomatitis virus. Bacteriol. Rev. 16, 179-204.

Harmon S.A. and Summers, D.F. (1982). Characterisation of monospecific antisera against all five vesicular stomatitis virus-specific proteins: Anti-L and anti-NS inhibit transcription in vitro. Virology 120, 194-204.

Hefti, E., Roy, P. and Bishop, D.H.L. (1975). The Initiation of Transcription by Influenza Virus Virion Transcriptase. Negative Strand Viruses, Vol. 1, p307-326. Ed. D.W.J. Mahy and R.D. Barry. Academic Press Inc., London.

Heggeress, M.H., Scheid, A. and Choppin, P.W. (1980). Conformation of the helical nucleocapsids of paramyxoviruses and VSV: Reversible coiling and uncoiling induced by changes in salt concentration. Proc. Natl. Acad. Sci. (USA) 77, 2631-2635.

Helenius, A., Kartenbeck, J., Simons, K. and Fries, E. (1980). On the entry of Semliki Forest Virus into BHK-21 cells. J. Cell Biol. 84, 404-420.

Herman, R.C., Adler, S., Lazzarini, R.A., Colonno, R.J., Banerjee, A.K. and Westphal, H. (1978). Intervening

polyadenylic sequences in RNA transcripts of vesicular stomatitis virus. Cell 15, 587-596.

Herman, R.C., Schubert, M., Keene, J.D. and Lazzarini, R.A. (1980). Polycistronic vesicular stomatitis virus RNA transcripts. Proc. Natl. Acad. Sci. (USA) 77, 4662-4665.

L'Heritier, P.H. (1958). The hereditary virus of Drosophila. Adv. Virus Res. 5, 195-245.

Heuser, J. and Evans, L. (1980). Three dimensional visualisation of coated vesicle formation in fibroblasts. J. Cell Biol. 84, 560-583.

Heuschele, W.P. (1970). Bovine ephemeral fever. I. Characteristics of the causative virus. Arch. Gesamte Virusforsch. 30, 195-202.

Hill, V.M., Marnell, L. and Summers, D.F. (1981). In vitro replication and assembly of vesicular stomatitis virus nucleocapsids. J. Virol. 113, 109-118.

Hillel, Z. and Wu, C-W. (1977). Subunit topography of RNA polymerase from E.coli. A cross-linking study with bifunctional reagents. Biochemistry 16, 3334-3342.

Hogle, J.M., Chow, M. and Filman, D.J. (1985). Three-dimensional structure of poliovirus at 2.9 resolution. Science 229, 1358-1365.

Holland, J.J., Doyle, M., Perrault, J., Kingsbury, D.T. and Etchison, J. (1972). Proteinase activity in purified animal viruses. Biochem. Biophys. Res. Commun. 46, 634-639.

Holland, J.J., Kennedy, S.I.T., Semler, B.L., Jones, C.L., Roux, L. and Grabeau, E. (1980). Defective interfering RNA viruses and host cell response. Comprehensive Virology, Vol. 16, p137-192. Ed. H. Fraenkel-Conrat and

R.R. Wagner. Plenum Press, New York.

Holland, J.J. and Villarreal, L.P. (1974). Persistent non-cytocidal vesicular stomatitis virus infections mediated by defective T particles that suppress virion transcriptase. Proc. Natl. Acad. Sci. (USA) 71, 2956-2960.

Holland, J.J., Villarreal, L.P. and Breindl, M. (1976). Factors involved in the generation and replication of rhabdovirus defective T particles. J. Virol. 17, 805-815.

Holland, J.J., Villarreal, L.P., Welsh, R.M., Oldstone, M.B.A., Kohne, D., Lazzarini, R.A. and Scolnick, E. (1976). Long-term persistent vesicular stomatitis virus and rabies virus infection of cells in vitro. J. Gen. virol. 33, 193-211.

Horikami, S.M., Ferra, F.D. and Moyer, S.A. (1984). Characterisation of the infections of permissive and non-permissive cells by host range mutants of vesicular stomatitis virus defective in RNA methylation. Virology 138, 1-15.

Horikami, S.M. and Moyer, S.A. (1982). Host range mutants of vesicular stomatitis virus defective in in vitro RNA methylation. Proc. Natl. Acad. Sci. (USA) 79, 7694-7698.

Hirokawa, N. and Heuser, J. (1982). The inside and outside of gap-junction membranes visualised by deep etching. Cell 30, 395-406.

Horodyski, F.M. and Holland, J.J. (1980). Viruses isolated from cells persistently infected with vesicular stomatitis virus show altered interactions with defective interfering particles. J. Virol. 36, 627-631.

Hovanec, D.L. and Air, G.M. (1985). Antigenic structure of the hemagglutinin of influenza virus B/Hong Kong/8/73 as determined from gene sequence analysis of variants selected with monoclonal antibodies. Virology 139, 384-392.

Howatson, A.F. (1970). Vesicular stomatitis and related viruses. Adv. Virus Res. 16, 195-256.

Howatson, A.F. and Whitmore, G.F. (1962). The development and structure of vesicular stomatitis virus. Virology 16, 466-478.

Hsu, C-H. and Kingsbury, D.W. (1980). Animal Virus Genetics, p1613-1622. Ed. B. Fields, R. Jaenisch and C.F. Fox. Academic Press Inc., New York.

Hsu, C-H. and Kingsbury, D.W. (1982). NS phosphoprotein of VSV: Subspecies separated by electrophoresis and isoelectric focusing. J. Virol. 42, 342-345.

Hsu, C-H. and Kingsbury, D.W. (1985). Constitutively phosphorylated residues in the NS protein of VSV. J. Biol. Chem. 260, 8990-8995.

Hsu, C-H., Kingsbury, D.W. and Murti, K.G. (1979). Assembly of VSV nucleocapsids *in vivo*: A kinetic analysis. J. Virol. 32, 304-313.

Hsu, C-H., Morgan, E.M. and Kingsbury, D.W. (1982). Site-specific phosphorylation regulates the transcriptase activity of VSV NS protein. J. Virol. 43, 101-112.

Hu, S-L. and Manley, J.L. (1981). DNA sequence required for initiation of transcriptase *in vitro* from the major late promoter of adenovirus type 2. Proc. Natl. Acad. Sci. (USA) 78, 820-824.

Huang, A.S. (1982). Significance of sequence rearrangements

in a rhabdovirus. Am. Soc. Microbiol. News 48, 148-151.

Huang, A.S. and Baltimore, A. (1970). Defective viral particles and viral disease processes. Nature 226, 325-327.

Huang, A.S., Palma, E.L., Hewlett, N. and Roizman, B. (1974). Pseudotype formation between enveloped RNA and DNA viruses. Nature (London) 252, 743.

Hubbard, S.C. and Ivatt, R.J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 50, 555-583.

Hudson, L.D., Condra, C. and Lazzarini, R.A. (1986). Cloning and expression of a viral phosphoprotein: Structure suggests NS may function by mimicking an RNA template. J. Gen. Virol. (in press).

Hughes, J.V., Doll, S.C. and Johnson, T.C. (1985). Hypothermia-inducing peptide promotes recovery of vesicular stomatitis virus from persistent animal infections. J. Virol. 53, 781-785.

Hunt, L. and Summers, D. (1976). Association of VSV proteins with Hela cell membranes and released virus. J. Virol. 20, 637-645.

Ihara, T., Akashi, H. and Bishop, D.H.L. (1984). Novel coding strategy (ambisense genomic RNA) revealed by sequence analysis of Punta Toro phlebovirus S RNA. Virology 136, 293-306.

Ihara, T., Matsuura, Y. and Bishop, D.H.L. (1985). Analyses of the mRNA transcription processes of Punta Toro phlebovirus (Bunyaviridae). Virology 147, 317-325.

Imblum, R.L. and Wagner, R.R. (1974). Protein kinase and phosphoproteins of vesicular stomatitis virus. J. Virol.

13, 113-124.

Ish-Horowicz, D. and Burke, J.F. (1981). Rapid and efficient cosmid cloning. Nucl. Acids Res. 9, 2989-2998.

Irving, R.A. and Ghosh, H.P. (1982). Shedding of vesicular stomatitis virus soluble glycoprotein by removal of carboxy-terminal peptide. J. Virol. 42, 322-325.

Irving, R.A., Toneguzzo, F., Rhee, S.H., Holmann, T. and Ghosh, H.P. (1979). Synthesis and assembly of membrane glycoproteins: Presence of leader peptide in non-glycosylated precursor of membrane glycoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. (USA) 76, 570-574.

Iverson, L.E. and Rose, J.K. (1981). Localised attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. Cell 23, 477-484.

Iverson, L.E. and Rose, J.K. (1982). Sequential synthesis of 5'-proximal vesicular stomatitis virus mRNA sequences. J. Virol. 44, 356-365.

Jacobs, B.L. and Penhoet, E.E. (1982). Assembly of VSV: Distribution of the glycoprotein on the surface of infected cells. J. Virol. 44, 1047-1055.

Jelinek, W.R., Toomey, T.P., Leinwand, L., Duncan, C.H., Biro, P.A., Choudary, P.V., Weissmann, S.M., Rubin, C.M., Houck, C.M., Deininger, P.L. and Schmid, C.W. (1980). Ubiquitous interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. (USA) 77, 1398-1402.

Johnson, L.D., Binder, M. and Lazzarini, R.A. (1979). A defective interfering vesicular stomatitis virus that directs the synthesis of functional proteins in the absence of helper virus. Virology 99, 203-206.

Johnson, D.C., Schlesinger, M.J. and Elson, E.L. (1981). Fluorescence photobleaching recovery measurements reveal differences in envelopment of Sindbis and VSV. Cell 23, 423-431.

Jones, C., Spindler, K.R. and Holland, J.J. (1980). Studies on tumourigenicity of cells persistently infected with vesicular stomatitis virus in athymic nude mice. Virology 103, 158-166.

Kamata, T. and Watanabe, Y. (1977). Role for nucleocapsid protein phosphorylation in the transcription of influenza virus genome. Nature (London) 267, 460-462.

Kang, C.Y. and Prevec, L. (1969). Proteins of vesicular stomatitis virus. I. Polyacrylamide gel analysis of viral antigens. J. Virol. 3, 404-413.

Kang, C.Y. and Prevec, L. (1970). Proteins of vesicular stomatitis virus. II. Immunological comparisons of viral antigens. J. Virol. 6, 20-27.

Kang, C.Y. and Prevec, L. (1971). Proteins of VSV. III. Intracellular synthesis and extracellular appearance of virus specific proteins. Virology 46, 678-685.

Kawai, A., Matsumoto, S. and Tanabe, K. (1975). Characterisation of rabies viruses recovered from persistently infected BHK cells. Virology 67, 520-523.

Keene, J.D., Schubert, M. and Lazzarini, R.A. (1980). Intervening sequence between the leader region and the nucleocapsid gene of vesicular stomatitis virus RNA. J. Virol. 33, 789-794.

Keene, J.D., Schubert, M., Lazzarini, R.A. and Rosenberg, M. (1978). Nucleotide sequence homology at the 3' termini of RNA from vesicular stomatitis virus and its defective

interfering particles. Proc. Natl. Acad. Sci. (USA) 75, 3225-3229.

Keene, J.D., Thornton, B.J. and Emerson, S.U. (1981). Sequence specific contacts between the RNA polymerase of VSV and the leader gene. Proc. Natl. Acad. Sci. (USA) 78, 6191-6195.

Kelley, J.M., Emerson, S.U. and Wagner, R.R. (1972). The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralising antibodies. J. Virol. 10, 1231-1235.

Kennedy-Morrow, J. and Lesnaw, J.A. (1984). Structural and functional characterisation of the RNA-positive complementation groups, C and D, of the New Jersey serotype of vesicular stomatitis virus: Assignment of the M gene to the C complementation group. Virology 132, 38-52.

Khan, S.R. and Lazzarini, R.A. (1977). The relationship between autointerference and the replication of a defective interfering particle. Virology 77, 189-201.

Kingsbury, D.W. (1974). The molecular biology of paramyxoviruses. Med. Microbiol. Immunol. 160, 73-83.

Kingsbury, D.W., Hsu, C-H. and Murti, K.G. (1978). Intracellular metabolism of Sendai virus nucleocapsids. Virology 91, 86-94.

Kingsbury, D.W., Hsu, C-H. and Morgan, E.M. (1981). A role for NS protein phosphorylation in VSV virus transcription. The Replication of Negative Strand Viruses. Developments in Cell Biology, Vol.7, p821-827. Elsevier, North Holland/New York.

Kingsford, L. and Emerson, S.U. (1980). Transcriptional

interfering particles. Proc. Natl. Acad. Sci. (USA) 75, 3225-3229.

Keene, J.D., Thornton, B.J. and Emerson, S.U. (1981). Sequence specific contacts between the RNA polymerase of VSV and the leader gene. Proc. Natl. Acad. Sci. (USA) 78, 6191-6195.

Kelley, J.M., Emerson, S.U. and Wagner, R.R. (1972). The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralising antibodies. J. Virol. 10, 1231-1235.

Kennedy-Morrow, J. and Lesnaw, J.A. (1984). Structural and functional characterisation of the RNA-positive complementation groups, C and D, of the New Jersey serotype of vesicular stomatitis virus: Assignment of the M gene to the C complementation group. Virology 132, 38-52.

Khan, S.R. and Lazzarini, R.A. (1977). The relationship between autointerference and the replication of a defective interfering particle. Virology 77, 189-201.

Kingsbury, D.W. (1974). The molecular biology of paramyxoviruses. Med. Microbiol. Immunol. 160, 73-83.

Kingsbury, D.W., Hsu, C-H. and Murti, K.G. (1978). Intracellular metabolism of Sendai virus nucleocapsids. Virology 91, 86-94.

Kingsbury, D.W., Hsu, C-H. and Morgan, E.M. (1981). A role for NS protein phosphorylation in VSV virus transcription. The Replication of Negative Strand Viruses. Developments in Cell Biology, Vol.7, p821-827. Elsevier, North Holland/New York.

Kingsford, L. and Emerson, S.U. (1980). Transcriptional

activities of different phosphorylated species of NS protein purified from VSV virions and cytoplasm of infected cells. J. Virol. 33, 1097-1105.

Kitamura, M., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler, C.H., Dorner, A.J., Emini, E.A., Hanecak, R., Lee, J.J., Van der Werf, S., Anderson, C.W. and Wimmer, E. (1981). Primary structure, gene organisation and polypeptide expression of poliovirus RNA. Nature (London) 291, 547-553.

Knipe, D., Baltimore, D. and Lodish, H.F. (1977). Separate pathways of maturation of the major structural proteins of VSV. J. Virol. 21, 1128-1139.

Knipe, D.M., Baltimore, D. and Lodish, H.F. (1977). Maturation of viral proteins in cells infected with ts mutants of VSV. J. Virol. 21, 1149-1158.

Knipe, D., Rose, J.K. and Lodish, H.F. (1975). Translation of individual species of vesicular stomatitis virus mRNA. J. Virol. 15, 1004-1011.

Kolakofsky, D. (1976). Isolation and characterisation of Sendai virus Dl-RNAs. Cell 8, 547-555.

Kolakofsky, D. and Altman, S. (1978). Endoribonuclease activity associated with animal RNA viruses. J. Virol. 25, 274-284.

Kruppa, J. (1979). Transfer of carbohydrates on to nascent glycoprotein of vesicular stomatitis virus. Biochem. J. 181, 295-300.

Kurath, G. and Leong, J.C. (1985). Characterisation of infectious hematopoietic necrosis virus mRNA species reveals a non-virion rhabdovirus protein. J. Virol. 53, 462-468.

Kurilla, M.G. and Keene, J.D. (1983). The leader RNA of VSV is bound by a cellular protein reactive with Anti-La lupus antibodies. Cell 34, 837-845.

Kurilla, M.G., Pirnica-Worms, H. and Keene, J.D. (1982). Rapid and transient location of the leader RNA of VSV in the nuclei of infected cells. Proc. Natl. Acad. Sci. (USA) 79, 5240-5244.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105-132.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685.

Lamb, R.A. and Lai, C-J. (1980). Sequence of interupted and uninterupted mRNAs and cloned DNA coding for the two overlapping non-structural proteins of influenza virus. Cell 21, 475-485.

Lamb, R.A., Lai, C-J. and Choppin, P.W. (1981). Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: Co-linear and interupted mRNAs code for overlapping proteins. Proc. Natl. Acad. Sci. (USA) 78, 4170-4174.

Lau, P.P. and Gray, B.H. (Jr.) (1979). Extracellular nucleases of Alteromonas espejiana Ba131. IV. The single strand specific deoxyriboendonuclease activity as a probe for regions of altered secondary structure in negatively and positively supercoiled closed circular DNA. Nucl. Acids Res. 331-334.

Lazzarini, R.A., Keene, J.D. and Schubert, M. (1981). The origins of defective interfering particles of the

negative strand RNA viruses. Cell 26, 145-154.

Lazzarini, R.A., Weber, G.H., Johnson, L.D. and Stamminger, G.M. (1975). Covalently linked message and anti-message (genomic) RNA from a defective vesicular stomatitis virus particle. J. Mol. Biol. 97, 289-307.

Leamnson, R.N. and Reichmann, M.E. (1974). The RNA of defective vesicular stomatitis virus particles in relation to viral cistrons. J. Mol. Biol. 85, 551-568.

Lees, J.F., Pringle, C.R. and Elliott, R.M. (1986). Nucleotide sequence of the bunyamwera virus mRNA segment: Conservation of structural features in the bunyavirus glycoprotein gene product. Virology 148, 1-14.

Lenard, J. (1978). Virus envelopes and plasma membranes. Ann. Rev. Biophys. Bioeng. 1, 139-165.

Lenard, J. and Compans, R. (1974). The membrane structure of lipid-containing viruses. Biochim. Biophys. Acta 344, 51-94.

Lenard, J., Wilson, T., Mancarella, D., Reidler, J., Keller, P. and Elson, E. (1981). Interaction of Mutant and wt M Protein of VSV with Nucleocapsids and Membranes. In: Replication of Negative Strand Viruses, p865-870. Ed. D.H.L. Bishop and R.W. Compans. Elsevier, North-Holland.

Leppert, M., Kort, L. and Kolakofsky, D. (1977). Further characterisation of Sendai virus Dl-RNAs: A model for their generation. Cell 12, 539-552.

Leppert, M., Rittenhouse, L., Perrault, J., Summers, D.F. and Kolakofsky, D. (1979). Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell 18, 735-747.

Lesnaw, J.A., Dickson, L.R. and Curry, R.H. (1979).

Proposed replicative role of the NS polypeptide of VSV: Structural analysis of an electrophoretic variant. J. Virol. 31, 8-16.

Lesnaw, J.A. and Reichman, M.E. (1975). RNA synthesis by temperature-sensitive mutants of vesicular stomatitis virus, New Jersey serotype. Virology 63, 492-504.

Lingappa, V.R., Katz, F.N., Lodish, H.F. and Blobel, G. (1978). A signal sequence for insertion of a transmembrane glycoprotein. J. Biol. Chem. 253, 8867-8870.

Little, S.P. and Huang, A.S. (1977). Synthesis and distribution of vesicular stomatitis virus specific polypeptides in the absence of progeny production. Virology 81, 37-47.

Little, S.P. and Huang, A.S. (1978). Shedding of the glycoprotein from vesicular stomatitis virus infected cells. J. Virol. 27, 330-339.

Lodish, H.F. and Froshauer, S. (1977). Rates of initiation of protein synthesis by two purified species of VSV mRNA. J. Biol. Chem. 252, 8804-8811.

Lodish, H.F. and Porter, M. (1980a). Specific incorporation of host cell surface proteins into budding VSV particles. Cell 19, 161-169.

Lodish, H.F. and Porter, M. (1980b). Translational control of protein synthesis after infection by vesicular stomatitis virus. J. Virol. 36, 719-733.

Lodish, H.F. and Porter, M. (1981). VSV mRNA and inhibition of translation of cellular mRNA - Is there a P function in VSV? J. Virol. 38, 504-517.

Lodish, H.F. and Rose, J.K. (1977). Relative importance of

7-methylguanosine in ribosome binding and translation of vesicular stomatitis virus messenger RNA in wheatgerm and reticulocyte cell-free systems. J. Biol. Chem. 252, 1181-1188.

Lodish, H.F. and Rothman, J.E. (1979). The assembly of cell membranes. Sci. Am. 240, 38-53.

Lodish, H.F. and Weiss, R.A. (1979). Selective isolation of mutants of vesicular stomatitis virus defective in production of the viral glycoprotein. J. Virol. 30, 177-189.

Lockart, R.Z. (Jr.) (1973). Criteria for Acceptance of a Viral Inhibitor as an Interferon and a General Description of the Biological Properties of Known Interferons. In: Interferon and Interferon Inducers, p11-35. Ed. N.B. Finter. Elsevier, North-Holland.

Love, D.N. and Weiss, R.A. (1974). Pseudotypes of vesicular stomatitis virus determined by exogenous and endogenous avian RNA tumour viruses. Virology 57, 271-278.

McAllister, P.E. and Wagner, R.R. (1976). Differential inhibition of host protein synthesis in L cells infected with RNA$^-$ mutants by VSV. J. Virol. 18, 550-558.

McGowan, J.J., Emerson, S.U. and Wagner, R.R. (1982). The plus strand leader RNA of VSV inhibits DNA-dependant transcription of adenovirus and SV40 genes in a soluble whole-cell extract. Cell 28, 325-333.

McGowan, J.J. and Wagner, R.R. (1981). Inhibition of cellular DNA synthesis of VSV. J. Virol. 38, 356-367.

McGeoch, D.J. (1979). Structure of the gene N: gene NS intercistronic junction in the genome of vesicular stomatitis virus. Cell 17, 673-681.

McGeoch, D.J., Dolan, A. and Pringle, C.R (1980). Comparisons of the nucleotide sequences in the genomes of the New Jersey and Indiana serotypes of vesicular stomatitis virus. J. Virol. 33, 69-77.

Macpherson, I.A. and Stoker, M.G.P. (1962). Polyoma transformation of hamster cell clones an investigation of genetic factors affecting cell competence. Virology 16, 147-151.

McSharry, J. (1980). The Lipid Envelope and Chemical Composition of Rhabdoviruses. In: Rhabdoviruses, Vol. 1, p107-118. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

McSharry, J.J., Compans, R.W. and Choppin, P.W. (1971). Proteins of vesicular stomatitis virus and of phenotypically mixed vesicular stomatitis virus-simian virus 5 virions. J. Virol. 8, 722-729.

McSharry, J.J. and Wagner, R.R. (1971). Lipid composition of purified vesicular stomatitis virus. J. Virol. 7, 59-70.

Maack, C.A. and Penhoet, E.E. (1980). Biochemical characterisation of the tsE1 mutant of VSV (New Jersey). J. Biol. Chem. 255, 9249-9 .

Magee, A.L., Koyama, A.H., Malfer, C., Wen, D. and Schlessinger, M. (1984). Release of fatty acids from virus glycoproteins by hydroxylamine. Biochim. Biophys. Acta 798, 156-166.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Manley, J.L., Fire, A., Cano, A., Sharp, P.A. and Gefter, M.L. (1980). DNA-dependant transcription of adenovirus

genes in a soluble whole cell extract. Proc. Natl. Acad. Sci. (USA) 77, 3855-3859.

Marcus, P.I. (1980). The Interferon System and Rhabdoviruses. In: Rhabdoviruses, Vol. III, p1-12. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Marcus, P.I. and Sekellick, M.J. (1974). Cell killing by viruses. I. Comparison of cell killing, plaque forming and defective interfering particles of vesicular stomatitis virus. Virology 57, 321-338.

Marcus, P.I. and Sekellick, M.J. (1975). Cell killing by viruses. II. Cell killing by VSV: A requirement for virion derived transcription. Virology 63, 176-190.

Marcus, P.I. and Sekellick, M.J. (1977). Defective interfering particles with covalently linked (±) RNA induce interferon. Nature (London) 266, 815-819.

Marcus, P.I. and Sekellick, M.J. (1978). The rate of primary transcription of VSV is inhibited by interferon action. J. Gen. Virol. 38, 391-408.

Marcus, P.I., Sekellick, M.J., Johnson, L. and Lazzarini, R.A. (1977). Cell killing by viruses. V. Transcribing defective interfering particles of VSV function as cell killing particles. Virology 82, 242-246.

Marks, M.D., Kennedy-Morrow, J. and Lesnaw, J.A. (1985). Assignment of the temperature-sensitive lesion in the replication mutant A1 of vesicular stomatitis virus to the N gene. J. Virol. 53, 44-51.

Marnell, L. and Summers, D.F. (1984). Characterisation of the phosphorylated small enzyme subunit, NS, of the VSV RNA polymerase. J. Biol. Chem. 259, 13518-13524.

Marsh, M., Bolzan, E. and Helenius, A. (1983). Penetration

of Semliki forest virus from acid prelysosomal vacuoles. Cell 32, 931-940.

Marvaldi, J., Lucas-Lenard, J., Sekellick, M.J. and Marcus, P.I. (1977). Cell killing by viruses. IV. Cell killing and protein synthesis inhibition by VSV require the same gene functions. Virology 79, 267-280.

Marvaldi, J., Sekellick, M.J., Marcus, P.I. and Lucas-Lenard, J. (1978). Inhibition of mouse L cell protein synthesis by ultraviolet-irradiated VSV requires viral transcription. Virology 84, 127-133.

Masters, P.S. and Samuel, C.E. (1984). Detection of *in vivo* synthesis of polycistronic mRNAs of vesicular stomatitis virus. Virology 134, 277-286.

Matlin, K.S., Reggio, H., Helenius, A. and Simons, K. (1982). Pathway of vesicular stomatitis virus entry leading to infection. J. Mol. Biol. 156, 609-631.

Maxam, A. and Gilbert, W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavage. Meth. Enzymol. 65, 499-560.

Meier, E., Harmison, G.G., Keene, J.D. and Schubert, M. (1984). Sites of copy choice replication involved in generation of vesicular stomatitis virus defective-interfering particles RNAs. J. Virol. 51, 515-521.

Meinkoth, J. and Kennedy, S.I.T. (1980). Semliki forest virus persistence in mouse L929 cells. Virology 100, 141-155.

Mellon, M.G. and Emerson, S.U. (1978). Rebinding of transcriptase components (L and NS proteins) to the nucleocapsid template of VSV. J. Virol. 27, 560-567.

Messing, J., Gronenborn, B., Muller-Hill, B. and Hofscheider, P.H. (1977). Filamentous coliphage M13 as a cloning vehicle: Insertion of a HindII fragment of the lac regulatory region in M13 replicative form in vitro. Proc. Natl. Acad. Sci. (USA) 74, 3642-3646.

Messing, J. and Vieira, J. (1982). A new pair of M13 vectors for selecting either DNA strand of double digest restiction fragment. Gene 19, 269-276.

Minato, N., Bloom, B.R., Jones, C., Holland, J. and Reid, L. (1979). Mechanism of rejection of virus persistently infected tumour cells by athymic nude mice. J. Exptl. Med. 149, 1117-1133.

Moreau, M-C. (1974). Inhibition of a vesicular stomatitis mutant by rifampicin. J. Virol. 14, 517-521.

Morrison, T.G., Stampfer, M., Baltimore, D. and Lodish, H.F. (1974). Translation of vesicular stomatitis messenger RNA by extracts from mammalian and plant cells. J. Virol. 13, 62-72.

Morrongiello, M.P. and Simpson, R.W. (1979). Conditional lethal mutants of vesicular stomatitis virus. IV. RNA species detected in non-permissive cells infected with host-restricted mutants. Virology 93, 506-514.

Moss, B., Gershowitz, A., Wei, C-M. and Boone, R. (1976). Formation of the guanylated and methylated 5'-terminus of vaccinia virus mRNA. Virology 72, 341-351.

Moyer, S.A. and Banerjee, A.K. (1975). Messenger RNA species synthesised in vitro by the virion-associated RNA polymerase of vesicular stomatitis virus. Cell 4, 37-43.

Moyer, S.A., Grubman, M.J., Ehrenfeld, E. and Banerjee, A.K. (1975). Studies on the in vivo and in vitro messenger

RNA species of vesicular stomatitis virus. Virology 67, 463-473.

Moyer, S.A. and Summers, D.F. (1974). Phosphorylation of vesicular stomatitis virus in vivo and in vitro. J. Virol. 13, 455-465.

Mudd, J.A. and Summers, D.F. (1970). Protein synthesis in vesicular stomatitis virus-infected HeLa cells. Virology 42, 328-340.

Muesing, M.A., Smith, D.H., Cabradilla, C.D., Benton, C.V., Lasky, L.A. and Capon, D.J. (1985). Nucleic acid structure and expression of the human AIDS/ lymphadenopathy retrovirus. Nature (London) 313, 450-458.

Mukherjee, P.K. and Simpson, R.W. (1984). Reversible restriction of vesicular stomatitis virus in permissive cells treated with inhibitors of prostaglandin biosynthesis. Virology 135, 345-355.

Murphy, F.A. and Harrison, A.K. (1979). Electron Microscopy of the Rhabdoviruses of Animals. In: Rhabdoviruses, Vol. 1, p65-106. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Muthukrishnan, S., Both, G.W., Furiuchi, Y. and Shatkin, A.J. (1975). 5'-terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature (London) 255, 33-37.

Naeve, C.W. and Summers, D.F. (1980). Electron microscopy of vesicular stomatitis virus replicative ribonucleoproteins. J. Virol. 34, 764-771.

Naeve, C.W. and Summers, D.F. (1981). Initiation of Transcription by Vesicular Stomatitis Virus Occurs at Multiple Sites. In: The Replication of Negative Strand

Viruses. Ed. D.H.L. Bishop and R.W. Compans. Elsevier, North-Holland.

Naito, S. and Ishihama, A. (1976). Function and stucture of RNA polymerase from vesicular stomatitis virus. J. Biol. Chem. 251, 4307-4314.

Najarian, R., Caput, D., Gee, W., Potter, S.J., Renard, A., Merryweather, J., Van Nest, G. and Dina, D. (1985). Primary structure and gene organisation of human hepatitis A virus. Proc. Natl. Acad. Sci. (USA) 82, 2627-2631.

Nakai, T. and Howatson, A.F. (1968). The fine structure of vesicular stomatitis virus. Virology 35, 268-281.

Neuberger, A., Gottschalk, A., Marshall, D. and Spiro, R.G. (1972). Carbohydrate-Peptide Linkages in Glycoproteins and Methods for their Elucidation. In: The Glycoproteins: Their Compositions, Structure and Function, p450-490. Ed. A. Gottschalk.

Newcomb, W.W. and Brown, J.C. (1981). Role of VSV matrix protein in maintaining the viral nucleocapsid in the condensed form found in native virions. J. Virol. 39, 295-299.

Newcomb, W.W., Tobin, G.J., McGowan, J.J. and Brown, J.C. (1982). In vitro reassembly of vesicular stomatitis virus skeletons. J. Virol. 41, 1055-1062.

Nishioka, Y. and Silverstein, S. (1978). Alterations in the protein synthetic apparatus of friend erythroleukaemia cells infected with vesicular stomatitis virus or herpes simplex virus. J. Virol. 25, 422-426.

Nishiyama, Y., Ito, J. and Shimokata, K. (1978). Properties of viruses selected during persistent infection of L

cells with VSV. J. Gen. Virol. 40, 481-486.

Nowakowski, M., Bloom, B.R., Ehrenfeld, E. and Summers, D.F. (1973). Restricted replication of VSV in human lymphoblastoid cells. J. Gen. Virol. 12, 1272-1278.

Nuss, D.L. and Koch, G. (1976). Translation of individual host mRNAs in MPC-11 cells is differentially suppressed after infection by VSV. J. Virol. 19, 572-578.

Nuss, D.L., Opperman, H. and Koch, G. (1975). Selective blockage of initiation of host protein synthesis in RNA virus infected cells. Proc. Natl. Acad. Sci. (USA) 72, 1258-1262.

Obijeski, J.F. and Simpson, R.W. (1974). Conditional lethal mutants of vesicular stomatitis virus. II. Synthesis of virus-specific polypeptides in non-permissive cells infected with RNA-host restricted mutants. Virology 57, 369-375.

Odenwald, W., Arnheiter, H., Dubois-Dalcq, M. and Lazzarini, R.A. (1984). The Nucleocapsid Coiling of VSV at the Inner Surface of Plasmid Membrane Immunolocalisation of the Matrix Protein. In: The Molecular Biology of Negative Strand Viruses. Ed. D.H.L. Bishop and R.W. Compans. Academic Press Inc., New York (In press).

Odenwald, W.F., Arnheiter, H., Dubois-Dalcq, M. and Lazzarini, R.A. (1986). Stereo images of vesicular stomatitis virus assembly. J. Virol. 57, 922-932.

Okayama, H. and Berg, P. (1982). High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. 2, 161-170.

Ongradi, J., Cunningham, C. and Szilagyi, J.F. (1985a). The role of polypeptides L and NS in the transcription process of vesicular stomatitis virus New Jersey using

the temperature-sensitive mutant tsE1. J. Gen. Virol. 66, 1011-1023.

Ongradi, J., Cunningham, C. and Szilagyi, J.F (1985b). Temperature sensitivity of the transcriptase of mutants tsB1 and tsF1 of vesicular stomatitis virus New Jersey is a consequence of mutation affecting polypeptide L. J. Gen. Virol. 66, 1507-1513.

Ongradi, J. and Szilagyi, J.F. (1981). The Role of the L and NS Polypeptides in the Transcription by Vesicular Stomatitis Virus New Jersey Serotype. In: Replication of Negative Strand Viruses, p837-844. Ed. D.H.L. Bishop and R.W. Compans. Elsevier, North-Holland.

Orenstein, J., Johnson, L., Shelton, E. and Lazzarini, R.A. (1976). The shape of vesicular stomatitis virus. Virology 71, 291-301.

Pal, R., Grinnell, B.W., Snyder, R.M. and Wagner, R.R. (1985). Regulation of viral transcription by the matrix protein of vesicular stomatitis virus probed by monoclonal antibodies and temperature-sensitive mutants. J. Virol. 56, 386-394.

Palese, P. and Schulman, J.L. (1976). Differences in RNA patterns of influenza A viruses. J. Virol. 17, 876-884.

Patton, J.T., Davis, N.L. and Wertz, G.W. (1983). Cell free synthesis and assembly of vesicular stomatitis virus nucleocapsids. J. Virol. 45, 155-164.

Patton, J.T., Davis, N.L. and Wertz, G.W. (1984). N protein alone satisfies the requirement for protein synthesis during RNA replication of VSV. J. Virol. 49, 303-309.

Peluso, R.W. and Moyer, S.A. (1984). Vesicular Stomatitis Virus Proteins Required for the In Vitro Replication of

Defective Interfering Particle Genome RNA. In: Non-segmented Negative Strand Viruses (Paramyxoviruses and Rhabdoviruses), p153-160. Ed. D.H.L. Bishop and R.W. Compans. Academic Press Inc., San Francisco.

Perlman, S.M. and Huang, A.S. (1973). RNA synthesis of VSV. V. Interactions between transcription and replication. J. Virol. 12, 1395-1400.

Perrault, J. (1976). Cross-linked double-stranded RNA from a defective vesicular stomatitis virus particle. Virology 70, 360-371.

Perrault, J. (1981). Origin and replication of defective interfering particles. Curr. Top. Microbiol. Immunol. 93, 151-207.

Perrault, J., Clinton, G.M. and McClure, M.A. (1983). RNP template of VSV regulates transcription and replication functions. Cell 35, 175-185.

Perrault, J. and Holland, J.J. (1972). Absence of transcriptase activity or transcription-inhibiting ability in defective interfering particles of vesicular stomatitis virus. Virology 50, 159-170.

Perrault, J. and Leavitt, R.W. (1977). Inverted complementary terminal sequences in single-stranded RNAs and snap-back RNAs from vesicular stomatitis virus defective interfering particles. J. Gen. Virol. 38, 35-50.

Perrault, J. and McLear, P.W. (1984). ATP dependence of VSV transcription initiation and modulation by mutation in the nucleocapsid protein. J. Virol. 51, 635-642.

Perrault, J. and Semler, B.L. (1979). Internal genome deletions in two distinct classes of defective

Pringle, C.R. (1978). The tdCE and hrCE phenotypes: Host range mutants of vesicular stomatitis virus in which polymerase function is affected. Cell 15, 597-603.

Pringle, C.R. (1982). The genetics of vesiculoviruses. Arch. Virol. 72, 1-49.

Pringle, C.R. (1986). Genetics of Rhabdoviruses (in press).

Pringle, C.R., Devine, V., Wilkie, M., Preston, C.M., Dolan, A. and McGeoch, D.J. (1981). Enhanced mutability associated with a temperature-sensitive mutant of vesicular stomatitis virus. J. Virol. 39, 377-383.

Pringle, C.R., Duncan, B.I. and Stevenson, M. (1971). Isolation and characterisation of temperature sensitive mutants of VSV, New Jersey serotype. J. Virol. 8, 836-841.

Pringle, C.R. and Szilagyi, J.F. (1980). Gene Assignment and Complementation Group. In: Rhabdoviruses, Vol. II, p141-161. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Prinz, P. (1973). Relationship of sigma virus to vesicular stomatitis virus. Adv. Virus Res. 18, 143-157.

Privalsky, M.L. and Penhoet, E.E. (1977). Phosphorylated protein component present in influenza virions. J. Virol. 24, 401-405.

Pustell, J. and Kafatos, F.C. (1982a). A convenient and adaptable package of DNA sequence analysis programs for microcomputers. Nucl. Acids Res. 10, 51-60.

Pustell, J. and Kafatos, F.C. (1982b). A high speed, high capacity homology matrix: Zooming through SV40 and polyoma. Nucl. Acids Res. 10, 4765-4782.

Rabson, A.B. and Martin, M.A. (1985). Molecular organisation of the AIDS retrovirus. Cell 40, 477-480.

Racaniello, V.R. and Baltimore, D. (1981). Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. (USA) 78, 4887-4891.

Racevski, J. and Sarkar, N.H. (1978). Synthesis and processing of precursor polypeptides to murine mammary tumour virus structural proteins. J. Virol. 25, 374-383.

Reading, C.L., Penhoet, E.E. and Ballou, C.E. (1978). Carbohydrate structure of vesicular stomatitis virus glycoprotein. J. Biol. Chem. 253, 5600-5612.

Ramseur, J.M. and Friedman, R.H. (1977). Prolonged infection of interferon-treated cells by vesicular stomatitis virus: Possible role of temperature sensitive mutants and interferon. J. Gen. Virol. 37, 523-533.

Ratner, L., Haseltine, W., Patavca, R., Livak, K.L., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.S., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, S.R. (Jr.), Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, M.T., Gallo, R.C. and Wong-Staal, F. (1985). Complete sequence of the AIDS virus, HTLV-III. Nature (London) 313, 277-284.

Reichmann, M.E., Schnitzlein, W.M., Bishop, D.H.L., Lazzarini, R.A., Beatrice, S.T. and Wagner, R.R. (1978). Classification of the New Jersey serotype of vesicular stomatitis virus into two subtypes. J. Virol. 25, 446-449.

Reid, L.M., Jones, C.L. and Holland, J. (1979). Virus carrier state suppresses tumourigenicity of tumour cells in athymic (nude) mice. J. Gen. Virol. 42, 609-614.

Rettenmier, C.W., Dumont, R. and Baltimore, D. (1975).

Screening procedure for complementation dependent mutants of vesicular stomatitis virus. J. Virol. 15, 41-46.

Repik, P., Flamand, A., Clark, H.F., Obijeski, J.F., Roy, P. and Bishop, D.H.L. (1974). Detection of homologous RNA sequences among six rhabdovirus genomes. J. Virol. 13, 250-252.

Rhodes, D.P., Abraham, S.A., Colonno, R.J., Jelnick, W. and Banerjee, A.K. (1977). Characterisation of vesicular stomatitis virus mRNA species synthesised in vitro. J. Virol. 21, 1205-1112.

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L. and Strauss, J.H. (1985). Nucleotide sequence of yellow fever virus: Implications for flavivirus gene expression and evolution. Science 229, 726-733.

Riedel, H., Kondor-Koch, C. and Garoff, H. (1984). Cell surface expression of fusogenic vesicular stomatitis virus G protein from cloned cDNA. EMBO J. 3, 1477-1483.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase 1. J. Mol. Biol. 113, 237-251.

Rinke, J. and Steitz, J.A. (1982). Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La lupus antibodies. Cell 29, 149-159.

Rodriguez-Boulan, E. and Pendergast, M. (1980). Polarised distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. Cell 20, 45-54.

Rodriguez-Boulan, E. and Sabatin, D.D. (1978). Asymetric budding of viruses in epithelial monolayers: A model

system for the study of epithelial polarity. Proc. Natl. Acad. Sci. (USA) 75, 5071-5075.

Romanowski, V., Matsuura, Y. and Bishop, D.H.L. (1985). Complete sequence of the S RNA of lymphocytic choriomeningitis virus (WE strain) compared to that of Pichinde arenavirus. Virus Res. 3, 101-114.

Rose, J.K. (1975). Heterogenous 5'-terminal structures occur on vesicular stomatitis virus mRNAs. J. Biol. Chem. 250, 8098-8104.

Rose, J.K. (1977). Nucleotide sequences of ribosome recognition sites in messenger RNAs of vesicular stomatitis virus. Proc. Natl. Acad. Sci. (USA) 74, 3672-3676.

Rose, J.K. (1978). Complete sequence of the ribosome recognition sites in vesicular stomatitis virus mRNAs: Recognition by the 40S and 80S complexes. Cell 14, 345-353.

Rose, J.K. (1980). Complete intergenic and flanking sequences from the genome of vesicular stomatitis virus. Cell 19, 415-421.

Rose, J.K., Adams, G. and Gallione, C. (1984). The presence of cysteine in the cytoplasmic domain of the vesicular stomatitis virus glycoprotein is required for palmitate addition. Proc. Natl. Acad. Sci. (USA) 81, 2050-2054.

Rose, J.K. and Gallione, C.J. (1981). Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. 39, 519-528.

Rose, J.K. and Iverson, L. (1979). Nucleotide sequence from the 3' ends of vesicular stomatitis virus mRNAs

determined from cloned DNA. J. Virol. 32, 404-411.

Rose, J.K. and Knipe, D. (1975). Nucleotide sequence complexities, molecular weights and poly(A) content of the vesicular stomatitis virus mRNA species. J. Virol. 15, 994-1003.

Rose, J.K. and Lodish, H.F. (1976). Translation in vitro of vesicular stomatitis virus mRNA lacking 5'-terminal 7-methylguanosine. Nature (London) 262, 32-37.

Rose, J.K., Lodish, H.F. and Brock, M.L. (1977). Giant heterogenous polyadenylic acid on vesicular stomatitis virus mRNA synthesised in vitro in the presence of S-adenosylhomocysteine. J. Virol. 21, 683-693.

Rose, J.K., Welch, W.J., Sefton, B.M., Esch, F.S. and Ling, N.C. (1980). Vesicular stomatitis virus glycoprotein is anchored in the viral membrane by a hydrophobic domain near the carboxy terminus. Proc. Natl. Acad. Sci. (USA) 77, 3884-3888.

Rosen, C.A., Ennis, H.L. and Cohen, P.S. (1982). Translational control of vesicular stomatitis virus protein synthesis: Isolation of an mRNA sequestering particle. J. Virol. 44, 932-938.

Rosen, C.A., Siekierka, J., Ennis, H.L. and Cohen, P.S (1984). Inhibition of protein synthesis in vesicular stomatitis virus infected chinese hamster ovary cells: Role of virus mRNA ribonucleoprotein particle. Biochemistry 23, 2407-2411.

Rossmann, M.G., Arnold, E., Erikson, J.W., Frankenberger, E.A., Griffith, J.P., Hecht, H-J., Johnson, J.E, Kamer, G., Luo, M., Mosser, A.G., Rueckert, R.R., Sherry, B. and Vriend, G. (1985). Structure of a human common cold

virus and functional relationship to other picornaviruses. Nature (London) 317, 145-153.

Rothman, J.E. and Fine, R.E. (1980). Coated vesicles transport newly synthesised membrane glycoproteins from endoplasmic reticulum to plasma membrane in 2 successive stages. Proc. Natl. Acad. Sci. (USA) 77, 780-784.

Rothman, J.E. and Lodish, H.F. (1977). Synchronised transmembrane insertion and glycosylation of a nascent protein. Nature (London) 269, 775-780.

Rowlands, D.J. (1979). Sequences of vesicular stomatitis virus RNA in the region coding for leader RNA, N protein mRNA, and their junction. Proc. Natl. Acad. Sci. (USA) 76, 4793-4797.

Rowlands, D.J., Grabau, E., Spindler, K., Jones, C., Semler, B.L. and Holland, J.J. (1980). Virus protein changes and RNA termini alterations evolving during persistent infection. Cell 19, 871-880.

Roy, P. and Bishop, D.H.L. (1971). Nucleoside triphosphate phosphotransferase. A new enzyme activity of oncogenic and non-oncogenic 'budding' viruses. Biochem. Biophys. Acta 235, 191-206.

Roy, P., Repik, P., Hefti, E. and Bishop, D.H.L. (1973). Complementary RNA species isolated from vesicular stomatitis (HR strain) defective virions. J. Virol. 11, 915-925.

Rubio, C., Kolakofsky, C., Hill, V.M. and Summers, D.F. (1980). Replication and assembly of VSV nucleocapsids: Protein association with RNPs and the effects of cycloheximide on replication. Virology 105, 123-135.

Sagata, N., Yasunaga, T., Tsuzuku-Kawamura, J., Ohisha, K.,

Ogawa, Y. and Ikawa, Y. (1985). Complete nucleotide sequence of the genome of bovine leukaemia virus: Its evolutionary relationship to other retroviruses. Proc. Natl. Acad. Sci. (USA) 82, 677-681.

Sanchez, A., De, B.P. and Banerjee, A.K. (1985). In vitro phosphorylation of NS protein by the L protein of vesicular stomatitis virus. J. Gen. Virol. 66, 1025-1036.

Sanchez-Pescador, R., Power, M.D., Barr, P.J., Steiner, K.S., Stampien, M.M., Brown-Shimer, S.L., Gee, W.W., Renard, A., Randolph, A., Levy, J.A., Dina, D. and Lucino, P.A. (1985). Nucleotide sequence and expression of an AIDS-associated retrovirus. Science 227, 484-492.

Sanger, F. and Coulsen, A.R. (1978). The use of thin acrylamide gels for DNA sequencing. FEBS Letters 87, 107-110.

Sanger, F., Coulsen, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980). Cloning in single stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. 143, 161-178.

Sanger, F., Coulsen, A.R., Hong, G.F., Hill, D.F. and Peterson, G.B. (1982). Nucleotide sequence of bacteriophage DNA. J. Mol. Biol. 162, 729-773.

Sanger, F., Nicklan, S. and Coulsen, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. (USA) 74, 5463-5476.

Satake, M., Coligan, J.E., Elango, N., Norby, E. and Ventatesan, S. (1985). Respiratory syncytial virus envelope glycoprotein (G) has a novel structure. Nucl. Acids Res. 13, 7795-7812.

Scheefers, H., Sheefers-Borchel, U., Edwards, J. and Brown, D.T. (1980). Distribution of virus structural proteins and protein-protein interactions in plasma membrane of baby hamster kidney cells infected with Sindbis or VSV. Proc. Natl. Acad. Sci. (USA) 77, 7277-7281.

Schlegel, R., Dickson, R., Willingham, M.C. and Pastan, I. (1982). Amantidine and densylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of $_2$-macroglobulin. Proc. Natl. Acad. Sci. (USA) 79, 2291-2295.

Schlegel, R., Tralka, T., Willingham, M. and Pastan, I. (1983). Inhibition of VSV binding and infectivity by phosphatidylserine: Is phosphatidylserine a VSV binding site? Cell 32, 639-646.

Schlegel, R. and Wade, M. (1985). Biologically active peptides of the vesicular stomatitis virus glycoprotein. J. Virol. 53, 319-323.

Schmidt, M.F. and Schlesinger, M.J. (1980). Relation of fatty acid attachment to the translation and maturation of VSV and Sinbis virus membrane glycoproteins. J. Biol. Chem. 255, 3334-3339.

Schnitzer, T.J., Dickson, C. and Weiss, R.A. (1979). Morphological and biochemical characterisation of viral particles produced by the ts045 mutant of vesicular stomatitis virus at restrictive temperature. J. Virol. 29, 185-195.

Schnitzlein, W.M. and Reichmann, M.E. (1977). Interference and RNA homologies of New Jersey serotype isolates of vesicular stomatitis virus and their defective particles. Virology 77, 490-500.

Schnitzlein, W.M. and Reichmann, M.E (1985). Characterisation of New Jersey vesicular stomatitis virus isolates from horses and black flies during the 1982 outbreak in Colorado. Virology 142, 426-431.

Schnitzlein, W.M., O'Banion, M.K., Poirot, M.K. and Reichmann, M.E. (1983). Effect of intracellular VSV mRNA concentration on the inhibition of host cell protein synthesis. J. Virol. 45, 206-214.

Schnitzer, T.J. and Lodish, H.F. (1979). Non-infectious VSV particles deficient in the viral nucleocapsid. J. Virol. 29, 443-447.

Schubert, M., Harmison, G.G., Meier, E. (1984). Primary structure of the vesicular stomatitis virus polymerase (L) gene: Evidence for a high frequency of mutation. J. Virol. 51, 505-514.

Schubert, M., Keene, J.D., Herman, R.C. and Lazzarini, R.A. (1980). Site on the vesicular stomatitis virus genome specifying polyadenylation and the end of the L gene mRNA. J. Virol. 34, 550-559.

Schubert, M., Keene, J.D. and Lazzarini, R.A. (1979). A specific internal RNA polymerase recognition site of VSV RNA is involved in the generation of DI particles. Cell 18, 749-757.

Schubert, M., Keene, J.D., Lazzarini, R.A. and Emerson, S.U. (1978). The complete sequence of a unique RNA species synthesised by a DI particle of VSV. Cell 15, 103-112.

Schubert, M. and Lazzarini, R.A. (1981). Structure and origin of a snap-back DI particle RNA of vesicular stomatitis virus. J. Virol. 37, 661-672.

Seif, I., Coulon, P., Rollin, P.E. and Flamand, A. (1985).

Rabies virulence: Effect on pathogenicity and sequence characterisation of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. 53, 926-934.

Sekellick, M.J. and Marcus, P.I. (1979). Persistent infection. II. Interferon-inducing temperature sensitive mutants as mediators of cels sparing: Possitble role in persistent infection VSV. Virology 95, 36-47.

Sekellick, M.J. and Marcus, P.I. (1980). Persistent Infections by Rhabdoviruses. In: Rhabdoviruses, Vol. III, p67-97. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Sela, M., Anfurisen, C.B. and Harrington, W.F. (1957). The correlation of ribonuclease activity with specific aspects of tertiary structure. Biochim. Biophys. Acta 26, 506-513.

Semler, B.L. and Holland, J.J. (1979). Persistent vesicular stomatitis virus infection mediates base substitutions in viral RNA termini. J. Virol. 32, 420-428.

Semler, B.L., Perrault, J., Abelson, J. and Holland, J.J. (1978). Sequence of a RNA templated by the 3'-OH RNA terminus of defective interfering particles of vesicular stomatitis virus. Proc. Natl. Acad. Sci. (USA) 75, 4704-4708.

Sen, A., Sherr, C.J. and Todaro, G.J. (1977). Phosphorylation of murine type C viral p12 proteins regulate their extent of binding to homologous viral RNA. Cell 10, 489-496.

Shaw, G.M., Harper, M.E., Hahn, B.M., Epstein, L.G., Gajdusek, D.C., Price, B.W., Navia, B.A., Petito, C.K.,

O'Hara, C.J., Groopman, J.E., Cho, E-S., Oleske, E.M., Wong-Staal, F. and Gallo, R.C. (1985). HTLV-III infection in brains of children and adults with AIDS encaphalopathy. Science 227, 177-182.

Shimotohno, K., Takahashi, Y., Shimizu, N., Gojobori, T., Golde, D.W., Chien, I.S.Y., Mirva, M. and Sugimura, T. (1985). Complete nucleotide sequence of an infectious clone of human T-cell leukaemia virus type 2: An open reading frame for the protease gene. Proc. Natl. Acad. Sci. (USA) 82, 3101-3105.

Simonsen, C.C., Batt-Humphries, S. and Summers, D.F. (1979). RNA synthesis of VSV infected cells: In vivo regulation of replication. J. Virol. 31, 124-132.

Simpson, R.B. (1979). The molecular topography of RNA polymerase promoter interaction. Cell 18, 277-285.

Simpson, R.W. and Hauser, R.F. (1966). Structural components of vesicular stomatitis virus. Virology 29, 654-667.

Simpson, R.W., Hauser, R.F. and Dales, S. (1969). Viropexis of vesicular stomatitis virus by L cells. Virology 37, 285-290.

Simpson, R.W., Obijeski, J.F. and Morrongiello, M.P. (1979). Conditional lethal mutants of vesicular stomatitis virus. III. Host range properties, interfering capacity, and complementation patterns of specific hr mutants. Virology 93, 493-505.

Sinacore, M.S. and Lucas-Lenard, J. (1982). The effect of the vesicular stomatitis virus associated protein kinase on viral mRNA transcription in vitro. Virology 121, 404-413.

Skehel, J.J. and Hay, A.J. (1978). Nucleotide sequences at the 5' termini of influenza virus RNAs and their transcripts. Nucl. Acids Res. 5, 1207-1219.

Skehel, J.J., Stevens, D.J., Daniels, R.S., Douglas, A.R., Kuossow, M., Wilson, I.A. and Wiley, D.C. (1984). A carbohydrate side chain on haemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. (USA) 81, 1779-1893.

Skem, T., Sommergruber, W., Blaas, D., Gruendler, P., Fraundorfer, F., Pieler, C., Fogy, I. and Keuchler, E. (1985). Human rhinovirus 2: Complete nucleotide sequence and proteolytic processing signals in the capsid protein region. Nucl. Acids Res. 13, 2111-2126.

Smith, A.J.H. (1980). DNA Sequence Analysis by Primed Synthesis. In: Methods in Enzymology, Vol. 65, p560-580. Ed. L. Grossman and K. Moldave.

Smith, A.E., Smith, R., Griffin, B. and Fried, M. (1979). Protein kinase activity associated with polyoma virus middle T antigen in vitro. Cell 18, 915-924.

Sokol, F. and Clark, H.F. (1973). Phosphoproteins structural components of rhabdoviruses. Virology 52, 246-263.

Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A. and Wain-Hobson, S. (1985). Nucleotide sequence of the visna lentivirus: Relationship to the AIDS virus. Cell 42, 369-382.

Soria, M., Little, S.P. and Huang, A.S. (1974). Characterisation of vesicular stomatitis virus nucleocapsids. I. Complementary 40s RNA molecules in

nucleocapsids. Virology 61, 270-280.

Spindler, K.R., Horodyski, F.M. and Holland, J.J. (1982). High multiplicities of infection favour rapid and random evolution of vesicular stomatitis virus. Virology 119, 96-108.

Sprague, J., Condra, J.H., Arnheiter, H. and Lazzarini, R.A. (1983). The expression of a recombinant DNA gene coding for the VSV nucleocapsid protein. J. Virol. 45, 773-781.

Stamminger, G.M. and Lazzarini, R.A. (1974). Analysis of the RNA of defective VSV particles. Cell 3, 85-93.

Stamminger, G.M. and Lazzarini, R.A. (1977). RNA synthesis in standard and autointerfered vesicular stomatitis virus infections. Virology 77, 202-211.

Stamminger, G.M. and Lazzarini, R.A. (1979). Analysis of the RNA of the defective VSV particles. Cell 3, 85-93.

Stanners, C.P., Francoeur, A.M. and Lam, T. (1977). Analysis of VSV mutant with attenuated cytopathogenicity: Mutation in viral function, P, for inhibition of protein synthesis. Cell 11, 273-281.

Steele, J.H. (1975). History of Rabies. In: The Natural History of Rabies, Vol. 1, p1-28. Ed. G.M. Baer. Academic Press Inc., New York.

Stevens, J., Todero, G. and Fox, C. (1978). Persistent Viruses. Academic Press Inc., New York.

Stoker, M.C.P. and MacPherson, I.A. (1961). Studies on transformation of hamster cells by polyoma virus in vitro. Virology 14, 359-370.

Storey, D.G. and Yong Kang, C. (1985). Vesicular stomatitis virus infected cells fuse when the intracellular pool of functional M protein is reduced in the presence of G

protein. J. Virol. 53, 374-383.

Subak-Sharpe, J.H. (1967). Doublet patterns and evolution of viruses. Br. Med. Bull. 23, 161-168.

Subak-Sharpe, J.H. (1969). The Doublet Pattern of the Nucleic Acid in Relation to the Origin of the Virus. In: Handbook of Molecular Cytology. Ed. A. Lima-de-Faria. North-Holland, Amsterdam and London.

Sutcliffe, J.G. (1978). pBR322 restriction map derived from the DNA sequence: Accurate DNA size markers up to 4361 nucleotides long. Nucl. Acids Res. 5, 2721-2728.

Sverdlow, E.D., Tsarev, S.A. and Begar, V.A. (1980). Subunits of E.coli RNA polymerase forming 3'-OH binding site at initial stages of transcription process. F.E.B.S. Letters 114, 111-114.

Szilagyi, J.F (1975). Structure and Functional Activity of the RNA Polymerase of Vesicular Stomatitis Virus. In: In vitro Transcription and Translation of Viral Genomes, p129-134. Ed. A.N. Haenni and G. Beard. INSERM, Paris.

Szilagyi, J.F. and Pringle, C.R. (1979). Effect of temperature sensitive mutation on activity of the RNA transcriptase of VSV New Jersey. J. Virol. 30, 692-700.

Szilagyi, J.F., Pringle, C.R. and Macpherson, T.M. (1977). Temperature dependent host range mutation in vesicular stomatitis virus affecting polypeptide L. J. Virol. 22, 381-3 .

Szilagyi, J.F. and Uryvayev, L. (1973). Isolation of an infectious ribonucleoprotein from vesicular stomatitis virus containing an active RNA transcriptase. J. Virol. 11, 279-286.

Taylor, P. (1984). A fact homology program for aligning

biological sequences. Nucl. Acids Res. 12, 447-456.

Temin, T.M. and Mizutani, S. (1970). RNA dependant DNA polymerase in virions of Rous sarcoma virus. Nature 226, 1211-1213.

Teninges, D., Contamine, D. and Brun, G. (1980). Drosophila Sigma Virus. In: Rhabdoviruses, Vol. III, p113-134. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Testa, D. and Banerjee, A.K. (1977). Two methyl transferase activities in purified virions of vesicular stomatitis virus. J. Virol. 24, 786-793.

Thacore, H.R. (1978). Effect of interferon on transcription and translation of VSV in human and simian cell cultures. J. Gen. Virol. 41, 421-426.

Thacore, H.R and Youngner, J.S. (1973a). Rescue of VSV from interferon induced resistance by superinfection with vaccinia virus. I. Rescue in cell cultures of different species. Virology 56, 505-511.

Thacore, H.R and Youngner, J.S. (1973b). Rescue of VSV from interferon induced resistance by superinfection with vaccinia virus. II. Effect of UV-inactivated vaccinia and metabolic inhibitors. Virology 56, 512-522.

Thacore, H.R and Youngner, J.S. (1975). Abortive infection of a rabbit cornea cell line by VSV: Conversion to productive infection by superinfection with vaccinia virus. J. Virology 16, 322-329.

Theiler (1901). Quoted in 'Vesicular Stomatitis and Related Viruses'. Howatson, 1970.

Thomas, P.S. (1980). Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. (USA) 71, 5201-5205.

Thomas, D., Newcomb, W.W., Brown, J.C., Wall, J.S., Hainfield, J.F., Trus, B.L. and Steven, A.C. (1985). Mass and molecular composition of vesicular stomatitis virus: A scanning transmission electron microscope analysis. J. Virol. 54, 598-607.

Thomas, J.R. and Wagner, R.R. (1983). Inhibition of translation in lysates of mouse L cells infected with VSV: Presence of a defective ribosome-associated factor. Biochemistry 22, 1540-1546.

Thornton, G.B., De, B.P. and Banerjee, A.K. (1984). Interaction of L and NS proteins of vesicular stomatitis virus with its template ribonucleoprotein during RNA synthesis in vitro. J. Gen. Virol. 65, 663-668.

Toneguzzo, F. and Ghosh, H.P. (1978). In vitro synthesis of vesicular stomatitis virus membrane glycoprotein and insertion into membranes. Proc. Natl. Acad. Sci. (USA) 75, 715-719.

Unger, J.T. and Reichmann, M.B. (1973). RNA synthesis in temperature sensitive mutants of vesicular stomatitis virus. J. Virol. 12, 570-578.

Vandepol, S.B. and Holland, J.J. (1986). Evolution of vesicular stomatitis virus in athymic nude mice: Mutations associated with natural killer selection. J. Gen. Virol. 67, 441-451.

Vandepol, S.B., Lefrancois, L., Holland, J.J. (1986). Sequences of the major antibody binding epitopes of the Indiana serotype of vesicular stomatitis virus. Virology 148, 312-325.

Villareal, L.P., Breindl, M. and Holland, J.J. (1976). Determination of molar ratios of vesicular stomatitis

virus induced RNA species in BHK-21 cells. Biochemistry 15, 1663-1667.

von Magnus, P. (1954). Incomplete forms of influenza virus. Adv. Virus Res. 2, 59-79.

Wagner, R.R. (1975). Reproduction of Rhabdoviruses. In: Comprehensive Virology, Vol. 4. pl-75. Ed. H. Frankel-Conrat and R.R. Wagner). Plenum Press, New York.

Wagner, R.R. and Huang, A.S. (1966). Inhibition of RNA and interferon synthesis in Krebs-2 cells infected with VSV. Virology 28, 1-7.

Wagner, R.R., Levy, A., Snyder, R., Ratcliff, G. and Hyatt, D. (1963). Biologic properties of two plaque variants of VSV (Indiana serotype). J. Immunol. 91, 112-1 .

Wagner, R.R., Prevec, L., Brown, F., Summers, D.F., Sokol, F. and MacLeod, R. (1972). Classification of rhabdovirus proteins: A proposal. J. Virol. 10, 1228-1230.

Wagner, R.R., Schnaitman, T.C., Snyder, R.M. and Schnaitman, C.A. (1969). Protein composition of the structural components of vesicular stomatitis virus. J. Virol. 3, 611-618.

Wagner, R.R., Snyder, R.M. and Yamazaki, S. (1970). Proteins of vesicular stomatitis virus: Kinetics and cellular sites of synthesis. J. Virol. 5, 548-558.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alizon, M. (1985). Nucleotide sequence of the AIDS virus, LAV. Cell 40, 9-17.

Watanabe, Y., Sakuma, S. and Tanaka, S. (1974). A possible biological function of the protein kinase associated with vaccinia and vesicular stomatitis virus. F.E.B.S. Letter 41, 331-341.

Weck, P.K., Carroll, A.R., Shattuck, D.M. and Wagner, R.R. (1979). Use of UV irradiation to identify the genetic information of VSV responsible for shutting of cellular RNA synthesis. J. Virol. 30, 746-753.

Weck, P.K. and Wagner, R.R. (1978). Inhibition of RNA synthesis in mouse myeloma cells infected with VSV. J. Virol. 25, 770- 780.

Weck, P.K. and Wagner, R.R. (1979a). Transcription of vesicular stomatitis virus is required to shut off cellular RNA synthesis. J. Virol. 30, 410-413.

Weck, P.K. and Wagner, R.R. (1979b). VSV infection reduces the number of active DNA-dependant RNA polymerases in myeloma cells. J. Biol. Chem. 254, 5430-5434.

Weiss, R.A. (1980). Rhabdovirus Pseudotypes. In: Rhabodoviruses III, p51-65. Ed. D.H.L. Bishop. CRC Press Inc., Florida.

Weiss, R.A. and Bennett, P. (1980). Assembly of glycoproteins in mutants of vesicular stomatitis virus studied by phenotypic mixing with retroviruses. Virology 100, 252-274.

Wertz, G.W. and Davis, N.L. (1979). RNase III cleaves VSV genome-length RNAs but fails to cleave viral mRNAs. J. Virol. 30, 108-115.

Wertz, G.W. and Levine, M. (1973). RNA synthesis by vesicular stomatitis virus and a small plaque mutant: Effects of cycloheximide. J. Virol. 12, 253-264.

Wertz, G.W. and Youngner, J.S. (1970). Interferon production and inhibition of host synthesis in cells infected with VSV. J. Virol. 16, 476-484.

Wertz, G.W. and Youngner, J.S. (1972). Inhibition of

protein synthesis in L-cells infected with VSV. J. Virol. 9, 85-89.

White, J., Matlin, K. and Helenius, A. (1981). Cell fusion by Semliki forest, influenza and vesicular stomatitis virus. J. Cell Biol. 89, 674-679.

Whitton, J.L., Hundley, F., O'Donnell, B. and Desselberger, U. (1983). Silver staining of nucleic acids: Applications in virus research and in diagnostic virology. J. Virol. Meth. 7, 185-198.

Wiktor, T.J. and Clark, H.F. (1972). Chronic rabies virus infection of cell cultures. Infect. Immunol. 6, 988-994.

Wild, F., Cathala, F. and Huppert, J. (1975). Vesicular stomatitis virus (measles) pseudotypes: Tool for demonstrating defective measles infections. Intervirology 6, 185-192.

Wiley, D.C., Wilson, I.A. and Skehel, J.J. (1981). Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) 289, 366-373.

Wilson, T. and Lenard, J. (1981). Interaction of wild type and mutant M protein of vesicular stomatitis virus with nucleocapsids in vitro. Biochemistry 20, 1349-1354.

Wilson, I.A., Skehel, J.J. and Wiley, D.C. (1981). Structure of haemagglutinin membrane glycoprotein of influenza virus at 3 resolution. Nature (London) 289, 366-373.

Wilusz, J., Kurilla, M.G. and Keene, J.D. (1983). A host protein (La) binds to a unique species of minus-sense leader RNA during replication of vesicular stomatitis

virus. Proc. Natl. Acad. Sci. (USA) 80, 5827-5831.

Wilusz, J., Youngner, J.S. and Keene, J.D. (1985). Base mutations in the terminal non-coding regions of the genome of VSV isolated from persistent infections of L cells. Virology 140, 249-256.

Winter, G. and Fields, S. (1982). Nucleotide sequence of human influenza A/PR/8/34 segment 2. Nucl. Acids Res. 10, 2135-2143.

Witt, D.J. and Summers, D.F. (1980). Relationship between virion-associated kinase-effected phosphorylation and transcription activity of vesicular stomatitis virus. Virology 70, 34-39.

Witte, O.N. and Baltimore, D. (1977). Mechanism of formation of pseudotypes between vesicular stomatitis virus and murine leukaemia virus. Cell 11, 505-511.

Wu, F-S. and Lucas-Lenard, J. (1980). Inhibition of ribonucleic acid accumulation in mouse L cells infected with VSV requires viral ribonucleic acid transcription. Biochemistry 19, 804-810.

Wunner, W.H. and Pringle, C.R. (1972a). Protein synthesis in BHK-21 cells infected with vesicular stomatitis virus. I. ts mutants of Indiana serotype. Virology 48, 101-111.

Wunner, W.H. and Pringle, C.R. (1972b). Protein synthesis in BHK-21 cells infected with vesicular stomatitis virus. II. ts mutants of the New Jersey serotype. Virology 50, 250-253.

Yamazaki, S. and Wagner, R.R. (1970). Action of interferon: Kinetics and differential effects on viral functions. J. Virol. 6, 421-428.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985).

Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

Yaoi, Y. and Amano, M. (1970). Inhibitory effect of ultraviolet inactivated VSV on initiation of DNA synthesis in cultured chick embryo cells. J. Gen. Virol. 9, 69-75.

Yaoi, Y., Mitsui, H. and Amano, M. (1970). Effect of UV-irradiated VSV on nucleic acid synthesis in chick embryo cells. J. Gen. Virol. 8, 165-172.

Yaun, R., Bickle, T.A., Ebbers, W. and Brack, C. (1975). Multiple steps in DNA recognition by restriction endonuclease from E.coli K. Nature (London) 256, 556-560.

Ye, R., Pal, R., Ogden, J.R., Snyder, R.M. and Wagner, R.R. (1985). Monoclonal antibodies to the matrix protein of vesicular stomatitis virus (New Jersey serotype) and their effects on viral transcription. Virology 143, 657-662.

Youngner, J.S., Dubovi, J., Quagliana, P.O., Kelly, M. and Preble, O.T. (1976). Role of temperature sensitive mutants in persistent infections initiated with vesicular stomatitis virus. J. Virol. 19, 90-101.

Youngner, J.S., Preble, O.T. and Jones, E.V. (1978). Persistent infection of L cells with vesicular stomatitis virus: Evolution of virus populations. J. Gen. Virol. 28, 6-13.

Youngner, J.S. and Quagliana, P.O. (1976). Temperature sensitive mutants of VSV are conditionally defective particles that interfere with and are rescued by wild

type virus. J. Virol. 19, 102-107.

Youngner, J.S. and Preble, O.T. (1980). Viral Persistence: Evolution of Viral Populations. In: Comprehensive Virology, Vol. 16, p73-135. Ed. H. Frenkel-Conrat and R.R. Wagner. Plenum Press, New York.

Zakowski, J.J., Petri, W.A. and Wagner, R.R. (1981). Role of matrix protein in assembling the membrane of vesicular stomatitis virus: Reconstitution of matrix protein with negatively charged phospholipid. Biochemistry 23, 3902-3907.

Zakowski, J.J. and Wagner, R.R. (1980). Localisation of membrane-associated proteins in vesicular stomatitis virus by use of hydrophobic membrane probes and cross-linking reagents. J. Virol. 36, 93-102.

Zavada, J. (1972). Pseudotypes of VSV with the coat of murine leukaemia and of avian myeloblastosis viruses. J. Gen. Virol. 15, 183-191.

Zavada, J., Bubenik, J., Widmaier, R. and Zavadova, Z. (1975). Phenotypically mixed vesicular stomatitis virus particles produced in human tumour cell lines. Cold Spring Harbor Symp. Quant. Biol. 39, 907-915.

Zavada, J., Cerny, L., Zavadova, Z., Bozonova, J. and Alstein, A.D. (1979). A rapid neutraliation test for antibiotics to bovine leukaemia virus with the use of rhabdovirus pseudotypes. J. Natl. Cancer Inst. 62, 95-98.

Zavada, J. and Rosenbergova, M. (1972). Phenotypic mixing of vesicular stomatitis virus with fowl plague virus. Acta Virol. 16, 103-114.

Zavada, J., Russ, G., Zavadova, Z. and Sabo, A. (1983).

Vesicular stomatitis virus phenotyically mixed with retrovirus: An efficient detection method. Acta Virol. 27, 110-115.

Zilberstein, A., Snider, M.D. and Lodish, H.F. (1981). Synthesis and assembly of VSV glycoprotein. Cold Spring Harbor Symp. Quant. Biol. 16, 785-495.

Zillig, W., Zechel, K. and Halbwachs, H. (1970). A new method of large scale preparation of highly purified DNA dependant RNA polymerase from E.coli. Hoppe-Seyler's Z. Physiol. Chem. 351, 221-224.

Zinkernagel, R.M., Althage, A. and Holland, J. (1978). Target antigens for H_2 restricted vesicular stomatitis virus specific cytoxic T-cells. J. Immunol. 121, 744-748.

Zoller, M.J. and Smith, M. (1982). Oligonucleotide-directed mutagenesis using M13-derived vectors: An efficient and general procedure for the production of point mutations in any fragment of DNA. Nucl. Acids Res. 10, 6487-6500.

APPENDIX

Figure 1

Nucleotide sequence and deduced amino acid sequence of the protein encoded by the putative second open reading frame (ORF) in the NS mRNA of

(a) VSV NJ, Missouri strain

(b) VSV NJ, Ogden strain

(c) VSV IND, Mudd-Summers strain

(d) VSV IND, San Juan strain

The tables of codon usage, base composition and amino acid composition are printed below.

Figure 2

Comparison of the proteins encoded by the putative second open reading frame in the mRNAs of

(i) VSV NJ, Missouri strain and VSV NJ, Ogden strain

(ii) VSV IND, San Juan strain and VSV IND, Mudd-Summers strain

(iii) VSV NJ, Missouri strain and VSV IND, San Juan strain

The sequences have been aligned for maximum homology by the HOMOL program of Taylor (1984).

Matched residues are indicated by *

Figure 3

Comparison of the hydropathic profiles of the proteins encoded by the putative second open reading frame in the NS mRNAs of the four strains of VSV are as predicted by the program of Kyte and Doolittle (1982). The degree of hydrophobicity increases with distance above the mid-line; the degree of hydrophilicity increases with distance below the mid-line. Letters below the plots refer to amino acid residues.

1 VSV NJ, Missouri strain

2 VSV IND, San Juan strain

3 VSV IND, Mudd-Summers strain

4 VSV NJ, Ogden strain

Parameters: group length = 9

shift = 1

spacing between plotted points = 10

```
         10          20          30          40          50          60
          *           *           *           *           *           *
CAG AGA TCA TGG ACA CTA TTG ATC GGC TCA AAA CTT ACT TGG CTA CTT ATG ATA ATT TGG
                                                                Met Ile Ile Trp

         70          80          90         100         110         120
          *           *           *           *           *           *
ATT CTG CCT TGC AGG ATG CAA ATG AGT CTG AAG AAA GAA GAG AGG ATA AAT ATC TCC AAG
Ile Leu Pro Cys Arg Met Gln Met Ser Leu Lys Lys Glu Glu Arg Ile Asn Ile Ser Lys

        130         140         150         160         170         180
          *           *           *           *           *           *
ACC TCT TCA TCG AAG ATC AAG GAG ATA AAC CAA CTC CGT CAT ATT ATC AGG AAG AAG AAT
Thr Ser Ser Ser Lys Ile Lys Glu Ile Asn Gln Leu Arg His Ile Ile Arg Lys Lys Asn

        190         200         210         220         230         240
          *           *           *           *           *           *
CGT CAG ATT CAG ATA CTG ACT ATA ATG CTG AAC ATC TTA CGA TGT TGT CGC CGG ATG AAA
Arg Gln Ile Gln Ile Leu Thr Ile Met Leu Asn Ile Leu Arg Cys Cys Arg Arg Met Lys

        250         260         270         280         290         300
          *           *           *           *           *           *
GAA TAG ACA AAT GGG AAG AAG ATT TGC CCG AAT TGG AAA AGA TTG ATG ATG ATA TAC CAG
Glu ---

        310         320         330         340         350         360
          *           *           *           *           *           *
TAA CTT TTT CTG ACT GGA CAC AAC CTG TAA TGA AGG AAA ACG GAG GGG AGA AAT CAC TAT

        370         380         390         400         410         420
          *           *           *           *           *           *
CTC TGT TTC CTC CGG TTG GAT TAA CAA AAG TTC AGA CAG ACC AAT GGA GGA AGA CAA TTG

        430         440         450         460         470         480
          *           *           *           *           *           *
AGG CAG TCT GTG AGA GCT CTA AAT ATT GGA ATT TGT CAG AAT GTC AGA TTA TGA ACT CAG

        490         500         510         520         530         540
          *           *           *           *           *           *
AAG ATC GTC TTA TCC TCA AAG GCC GAA TTA TGA CTC CTG ACT GCA GTT CAT CAA TCA AAT

        550         560         570         580         590         600
          *           *           *           *           *           *
CTC AGA ACT CCA TAC AGA GTT CCG AAT CTC TCT CCT CCT CGC ATT CAC CCG GTC CAG CAC

        610         620         630         640         650         660
          *           *           *           *           *           *
CAA AGT CAC GGA ATC AAC TAG GTC TAT GGG ATT CAA AAT CAA CAG AAG TAC AAC TGA TCT

        670         680         690         700         710         720
          *           *           *           *           *           *
CGA AGA GGG CCG GAG TGA AGG ACA TGA TGG TGA AAT TGA CAG ACT TTT TTG GAA GTG AAG

        730         740         750         760         770         780
          *           *           *           *           *           *
AGG AAT ATT ATT CAG TAT GCC CAG AAG GGG CCC CAG ATT TGA TGG GAG CAA TCA TCA TGG

        790         800         810         820         830         840
          *           *           *           *           *           *
GAC TAA AAC ATA AAA AGC TTT TTA ACC AAG CAA GAA TGA AGT ATC GTA TCT AAT TAA TTC

        850
          *
CGA TGA TCA ATA TG
```

TTT Phe	0	0.0%	TCT Ser	1	1.5%	TAT Tyr	0	0.0%	TGT Cys	2	3.1%
TTC Phe	0	0.0%	TCC Ser	1	1.5%	TAC Tyr	0	0.0%	TGC Cys	1	1.5%
TTA Leu	1	1.5%	TCA Ser	1	1.5%	TAA ---	0	--	TGA ---	0	--
TTG Leu	0	0.0%	TCG Ser	1	1.5%	TAG ---	1	--	TGG Trp	1	1.5%
CTT Leu	0	0.0%	CCT Pro	1	1.5%	CAT His	1	1.5%	CGT Arg	2	3.1%
CTC Leu	1	1.5%	CCC Pro	0	0.0%	CAC His	0	0.0%	CGC Arg	1	1.5%
CTA Leu	0	0.0%	CCA Pro	0	0.0%	CAA Gln	2	3.1%	CGA Arg	1	1.5%
CTG Leu	4	6.2%	CCG Pro	0	0.0%	CAG Gln	2	3.1%	CGG Arg	1	1.5%
ATT Ile	4	6.2%	ACT Thr	1	1.5%	AAT Asn	2	3.1%	AGT Ser	1	1.5%
ATC Ile	4	6.2%	ACC Thr	1	1.5%	AAC Asn	2	3.1%	AGC Ser	0	0.0%
ATA Ile	5	7.7%	ACA Thr	0	0.0%	AAA Lys	2	3.1%	AGA Arg	0	0.0%
ATG Met	5	7.7%	ACG Thr	0	0.0%	AAG Lys	6	9.2%	AGG Arg	3	4.6%
GTT Val	0	0.0%	GCT Ala	0	0.0%	GAT Asp	0	0.0%	GGT Gly	0	0.0%
GTC Val	0	0.0%	GCC Ala	0	0.0%	GAC Asp	0	0.0%	GGC Gly	0	0.0%
GTA Val	0	0.0%	GCA Ala	0	0.0%	GAA Glu	2	3.1%	GGA Gly	0	0.0%
GTG Val	0	0.0%	GCG Ala	0	0.0%	GAG Glu	2	3.1%	GGG Gly	0	0.0%

------ BASE COMPOSITION ------

	T	C	A	G	Y	R	ALL
NUMBER	214	160	293	187	374	480	854
PERCENT	25.1	18.7	34.3	21.9	43.8	56.2	100.0

------ AMINO ACID COMPOSITION ------

RES	NUM	%	RES	NUM	%	RES	NUM	%	RES	NUM	%
Phe	0	0.0	Leu	6	9.2	Ile	13	20.0	Met	5	7.7
Val	0	0.0	Ser	5	7.7	Pro	1	1.5	Thr	2	3.1
Ala	0	0.0	Tyr	0	0.0	His	1	1.5	Gln	4	6.2
Asn	4	6.2	Lys	8	12.3	Asp	0	0.0	Glu	4	6.2
Cys	3	4.6	Trp	1	1.5	Arg	8	12.3	Gly	0	0.0

b

```
         10          20          30          40          50          60
         *           *           *           *           *           *
CAG ATA TCA TGG ACA GTG TTG ATA GGC TCA AGA CTT ACT TAG CCA CTT ATG ATA ATT TGG
                                                          Met Ile Ile Trp

         70          80          90         100         110         120
         *           *           *           *           *           *
ATT CTG CCT TGC AGG ATG CCA ATG AAT CTG AGG AAA GAC GAG AGG ATA AAT ATC TCC AAG
Ile Leu Pro Cys Arg Met Pro Met Asn Leu Arg Lys Asp Glu Arg Ile Asn Ile Ser Lys

        130         140         150         160         170         180
         *           *           *           *           *           *
ACC TCT TCA TCG AAG ATC AAG GAG ATA AAC CAA CTC CGT CAT ATT ATC AGG AAG AAG AAT
Thr Ser Ser Ser Lys Ile Lys Glu Ile Asn Gln Leu Arg His Ile Ile Arg Lys Lys Asn

        190         200         210         220         230         240
         *           *           *           *           *           *
CGT CAG ATT CAG ATA CTG ATT ATA ATG CTG AAC ATC TTA CGA TGC TGT CAC CGG ATG AAA
Arg Gln Ile Gln Ile Leu Ile Ile Met Leu Asn Ile Leu Arg Cys Cys His Arg Met Lys

        250         260         270         280         290         300
         *           *           *           *           *           *
GAA TAG ACA AGT GGG AAG AAG ATT TGC CTG AAT TAG AAA AGA TTG ATG ATG ATA TAC CGG
Glu ---

        310         320         330         340         350         360
         *           *           *           *           *           *
TGA CCT TTT CTG ATT GGA CAC AGC CTG TAA TGA AGG AAA ATG GGG GAG AGA AAT CAT TGT

        370         380         390         400         410         420
         *           *           *           *           *           *
CTC TGT TCC CTC CAG TCG GGT TAA CAA AGA TTC AAA CAG AAC AAT GGA AAA AAA CCA TTG

        430         440         450         460         470         480
         *           *           *           *           *           *
AGG CGG TTT GTG AGA GTT CAA AAT ATT GGA ATT TAT CAG AAT GCC AAA TTC TTA ACT TGG

        490         500         510         520         530         540
         *           *           *           *           *           *
AAG ACA GCC TCA CTC TCA AAG GCC GAT TGA TGA CTC CTG ATT GTA GTT CTT CAG TAA AAT

        550         560         570         580         590         600
         *           *           *           *           *           *
CTC AAA ATT CTG TCC GGA GGT CAG AAC CTC TCT ACT CCT CTC ATT CTC CAG GTC CCC CAC

        610         620         630         640         650         660
         *           *           *           *           *           *
TCA AGG TAT CAG AGT CCA TCA ATT TAT GGG ATT TAA AGT CCA CTG AAG TAC AAT TGA TCT

        670         680         690         700         710         720
         *           *           *           *           *           *
CCA AGA GAG CCG GAG TTA AGG ACA TGA CAG TCA AAT TGA CAG ACT TCT TTG GAA GTG AGG

        730         740         750         760         770         780
         *           *           *           *           *           *
AAG AGT ATT ATT CAG TAT GCC CAG AAG GGG CGC CAG ACT TGA TGG GAG CTA TCA TCA TGG

        790         800         810         820         830         840
         *           *           *           *           *           *
GAC TGA AGT ACA AGA AAC TCT TCA ATC AGG CAA GAA TGA AAT ATC GTC TTT AAT TCC TTT

        850
         *
TCA TGA TCA ATA TG
```

TTT Phe			TCT Ser	1	1.5%	TAT Tyr	0	0.0%	TGT Cys	1	1.5%
TTC Phe	0	0.0%	TCC Ser	1	1.5%	TAC Tyr	0	0.0%	TGC Cys	2	3.1%
TTA Leu	1	1.5%	TCA Ser	1	1.5%	TAA ---	0	--	TGA ---	0	--
TTG Leu	0	0.0%	TCG Ser	1	1.5%	TAG ---	1	--	TGG Trp	1	1.5%
CTT Leu	0	0.0%	CCT Pro	1	1.5%	CAT His	1	1.5%	CGT Arg	2	3.1%
CTC Leu	1	1.5%	CCC Pro	0	0.0%	CAC His	1	1.5%	CGC Arg	0	0.0%
CTA Leu	0	0.0%	CCA Pro	1	1.5%	CAA Gln	1	1.5%	CGA Arg	1	1.5%
CTG Leu	4	6.2%	CCG Pro	0	0.0%	CAG Gln	2	3.1%	CGG Arg	1	1.5%
ATT Ile	5	7.7%	ACT Thr	0	0.0%	AAT Asn	3	4.6%	AGT Ser	0	0.0%
ATC Ile	4	6.2%	ACC Thr	1	1.5%	AAC Asn	2	3.1%	AGC Ser	0	0.0%
ATA Ile	5	7.7%	ACA Thr	0	0.0%	AAA Lys	2	3.1%	AGA Arg	0	0.0%
ATG Met	5	7.7%	ACG Thr	0	0.0%	AAG Lys	5	7.7%	AGG Arg	4	6.2%
GTT Val	0	0.0%	GCT Ala	0	0.0%	GAT Asp	0	0.0%	GGT Gly	0	0.0%
GTC Val	0	0.0%	GCC Ala	0	0.0%	GAC Asp	1	1.5%	GGC Gly	0	0.0%
GTA Val	0	0.0%	GCA Ala	0	0.0%	GAA Glu	1	1.5%	GGA Gly	0	0.0%
GTG Val	0	0.0%	GCG Ala	0	0.0%	GAG Glu	2	3.1%	GGG Gly	0	0.0%

BASE COMPOSITION

	T	C	A	G	Y	R	ALL
NUMBER	226	162	281	185	388	466	854
PERCENT	26.5	19.0	32.9	21.7	45.4	54.6	100.0

AMINO ACID COMPOSITION

RES	NUM	%	RES	NUM	%	RES	NUM	%	RES	NUM	%
Phe	0	0.0	Leu	6	9.2	Ile	14	21.5	Met	5	7.7
Val	0	0.0	Ser	4	6.2	Pro	2	3.1	Thr	1	1.5
Ala	0	0.0	Tyr	0	0.0	His	2	3.1	Gln	3	4.6
Asn	5	7.7	Lys	7	10.8	Asp	1	1.5	Glu	3	4.6
Cys	3	4.6	Trp	1	1.5	Arg	8	12.3	Gly	0	0.0

c

```
         10          20          30          40          50          60
          *           *           *           *           *           *
ATG AAA AAA ACT AAA CAG ATA TCA TGG ATA ATC TCA CAA AAG TTC GTG AGT ATC TCA AGT

         70          80          90         100         110         120
          *           *           *           *           *           *
CCT ACT CTC GTC TAG ATC AGG CGG TAG GAG AGA TAG ATG AGA TCG AAG CAC AAC GAG CTG
                                               Met Arg Ser Lys His Asn Glu Leu

        130         140         150         160         170         180
          *           *           *           *           *           *
AAA AGT CCA ATT ATG AGT TGT TCC AAG AGG ACG GAG TGG AAG AGC ATA CTA GGC CCT CTT
Lys Ser Pro Ile Met Ser Cys Ser Lys Arg Thr Glu Trp Lys Ser Ile Leu Gly Pro Leu

        190         200         210         220         230         240
          *           *           *           *           *           *
ATT TTC AGG CAG CAG ATG ATT CTG ACA CAG AAT CTG AAC CAG AAA TTG AAG ACA ATC AAG
Ile Phe Arg Gln Gln Met Ile Leu Thr Gln Asn Leu Asn Gln Lys Leu Lys Thr Ile Lys

        250         260         270         280         290         300
          *           *           *           *           *           *
GCT TGT ATG TAC CAG ATC CGG AAG CTG AGC AAG TTG AAG GCT TTA TAC AGG GGC CTT TAG
Ala Cys Met Tyr Gln Ile Arg Lys Leu Ser Lys Leu Lys Ala Leu Tyr Arg Gly Leu

        310         320         330         340         350         360
          *           *           *           *           *           *
ATG ACT ATG CAG ATG AGG ACG TGG ATG TTG TAT TCA CTT CGG ACT GGA AAC AGC CTG AGC

        370         380         390         400         410         420
          *           *           *           *           *           *
TTG AAT CCG ACG AGC ATG GAA AGA CCT TAC GGT TGA CAT TGC CAG AGG GTT TAA GTG GAG

        430         440         450         460         470         480
          *           *           *           *           *           *
AGC AGA AAT CCC AGT GGC TTT TGA CGA TTA AAG CAG TCG TTC AAA GTG CCA AAC ACT GGA

        490         500         510         520         530         540
          *           *           *           *           *           *
ATC TGG CAG AGT GCA CAT TTG AAG CAT CGG GAG AAG GGG TCA TCA TAA AAA AGC GCC AGA

        550         560         570         580         590         600
          *           *           *           *           *           *
TAA CTC CGG ATG TAT ATA AGG TCA CTC CAG TGA TGA ACA CAC ATC CGT ACC AAT CAG AAG

        610         620         630         640         650         660
          *           *           *           *           *           *
CCG TAT CAG ATG TTT GGT CTC TCT CAA AGA CAT CCA TGA CTT TCC AAC CCA AGA AAG CAA

        670         680         690         700         710         720
          *           *           *           *           *           *
GTC TTC AGC CTC TCA CCA TAT CCT TGG ATG AAT TGT TCT CAT CTA GAG GAG AAT TCA TCT

        730         740         750         760         770         780
          *           *           *           *           *           *
CTG TCG GAG GTA ACG GAC GAA TGT CTC ATA AAG AGG CCA TCC TGC TCG GTC TGA GGT ACA

        790         800         810         820         830         840
          *           *           *           *           *           *
AAA AGT TGT ACA ATC AGG CGA GGG TCA AAT ATT CTC TGT AGA CTA TGA AAA AAA GTA ACA

        850
          *
GAT ATC AC
```

TTT Phe	0	0.0%	TCT Ser	0	0.0%	TAT Tyr	0	0.0%	TGT Cys	2	3.0%
TTC Phe	1	1.5%	TCC Ser	1	1.5%	TAC Tyr	2	3.0%	TGC Cys	0	0.0%
TTA Leu	1	1.5%	TCA Ser	0	0.0%	TAA ---	0	--	TGA ---	0	--
TTG Leu	2	3.0%	TCG Ser	1	1.5%	TAG ---	0	--	TGG Trp	1	1.5%
CTT Leu	2	3.0%	CCT Pro	1	1.5%	CAT His	0	0.0%	CGT Arg	0	0.0%
CTC Leu	0	0.0%	CCC Pro	0	0.0%	CAC His	1	1.5%	CGC Arg	0	0.0%
CTA Leu	1	1.5%	CCA Pro	1	1.5%	CAA Gln	0	0.0%	CGA Arg	0	0.0%
CTG Leu	4	6.0%	CCG Pro	0	0.0%	CAG Gln	5	7.5%	CGG Arg	1	1.5%
ATT Ile	3	4.5%	ACT Thr	0	0.0%	AAT Asn	1	1.5%	AGT Ser	2	3.0%
ATC Ile	2	3.0%	ACC Thr	0	0.0%	AAC Asn	2	3.0%	AGC Ser	2	3.0%
ATA Ile	1	1.5%	ACA Thr	2	3.0%	AAA Lys	2	3.0%	AGA Arg	1	1.5%
ATG Met	4	6.0%	ACG Thr	1	1.5%	AAG Lys	8	11.9%	AGG Arg	3	4.5%
GTT Val	0	0.0%	GCT Ala	2	3.0%	GAT Asp	0	0.0%	GGT Gly	0	0.0%
GTC Val	0	0.0%	GCC Ala	0	0.0%	GAC Asp	0	0.0%	GGC Gly	2	3.0%
GTA Val	0	0.0%	GCA Ala	0	0.0%	GAA Glu	0	0.0%	GGA Gly	0	0.0%
GTG Val	0	0.0%	GCG Ala	0	0.0%	GAG Glu	2	3.0%	GGG Gly	0	0.0%

------ BASE COMPOSITION ------

	T	C	A	G	Y	R	ALL
NUMBER	199	171	275	203	370	478	848
PERCENT	23.5	20.2	32.4	23.9	43.6	56.4	100.0

------ AMINO ACID COMPOSITION ------

RES	NUM	%	RES	NUM	%	RES	NUM	%	RES	NUM	%
Phe	1	1.5	Leu	10	14.9	Ile	6	9.0	Met	4	6.0
Val	0	0.0	Ser	6	9.0	Pro	2	3.0	Thr	3	4.5
Ala	2	3.0	Tyr	2	3.0	His	1	1.5	Gln	5	7.5
Asn	3	4.5	Lys	10	14.9	Asp	0	0.0	Glu	2	3.0
Cys	2	3.0	Trp	1	1.5	Arg	5	7.5	Gly	2	3.0

```
         10          20          30          40          50          60
          *           *           *           *           *           *
CAG ATA TCA TGG ATA ATC TCA CAA AAG TTC GTG AGT ATC TCA AGT CCT ATT CTC GTC TGG

         70          80          90         100         110         120
          *           *           *           *           *           *
ATC AGG CGG TAG GAG AGA TAG ATG AGA TCG AAG CAC AAC GAG CTG AAA AGT CCA ATT ATG
                          Met Arg Ser Lys His Asn Glu Leu Lys Ser Pro Ile Met

        130         140         150         160         170         180
          *           *           *           *           *           *
AGT TGT TCC AAG AGG ATG GAG TGG AAG AGC ATA CTA AGC CCT CTT ATT TTC AGG CAG CAG
Ser Cys Ser Lys Arg Met Glu Trp Lys Ser Ile Leu Ser Pro Leu Ile Phe Arg Gln Gln

        190         200         210         220         230         240
          *           *           *           *           *           *
ATG ATT CTG ACA CAG AAT CTG AAC CAG AAA TTG AAG ACA ATC AAG GTT TGT ATG CAC CAG
Met Ile Leu Thr Gln Asn Leu Asn Gln Lys Leu Lys Thr Ile Lys Val Cys Met His Gln

        250         260         270         280         290         300
          *           *           *           *           *           *
ATC CAG AAG CTG AGC AAG TTG AAG GCT TTA TAC AGG GGC CTT TAG ATG ACT ATG CAG ATG
Ile Gln Lys Leu Ser Lys Leu Lys Ala Leu Tyr Arg Gly Leu ---

        310         320         330         340         350         360
          *           *           *           *           *           *
AGG AAG TGG ATG TTG TAT TTA CTT CGG ACT GGA AAC AGC CTG AGC TTG AAT CTG ACG AGC

        370         380         390         400         410         420
          *           *           *           *           *           *
ATG GAA AGA CCT TAC GGT TGA CAT CGC CAG AGG GTT TAA GTG GAG AGC AGA AAT CCC AGT

        430         440         450         460         470         480
          *           *           *           *           *           *
GGC TTT CGA CGA TTA AAG CAG TCG TGC AAA GTG CCA AAT ACT GGA ATC TGG CAG AGT GCA

        490         500         510         520         530         540
          *           *           *           *           *           *
CAT TTG AAG CAT CGG GAG AAG GGG TCA TTA TGA AGG AGC GCC AGA TAA CTC CGG ATG TAT

        550         560         570         580         590         600
          *           *           *           *           *           *
ATA AGG TCA CTC CAG TGA TGA ACA CAC ATC CGT CCC AAT CAG AAG CAG TAT CAG ATG TTT

        610         620         630         640         650         660
          *           *           *           *           *           *
GGT CTC TCT CAA AGA CAT CCA TGA CTT TCC AAC CCA AGA AAG CAA GTC TTC AGC CTC TCA

        670         680         690         700         710         720
          *           *           *           *           *           *
CCA TAT CCT TGG ATG AAT TGT TCT CAT CTA GAG GAG AGT TCA TCT CTG TCG GAG GTG ACG

        730         740         750         760         770         780
          *           *           *           *           *           *
GAC GAA TGT CTC ATA AAG AGG CCA TCC TGC TCG GCC TGA GAT ACA AAA AGT TGT ACA ATC

        790         800         810
          *           *           *
AGG CGA GAG TCA AAT ATT CTC TGT AGA CTA TG
```

TTT Phe	0	0.0%	TCT Ser	0	0.0%	TAT Tyr	0	0.0%	TGT Cys	2	3.0%
TTC Phe	1	1.5%	TCC Ser	1	1.5%	TAC Tyr	1	1.5%	TGC Cys	0	0.0%
TTA Leu	1	1.5%	TCA Ser	0	0.0%	TAA ---	0	--	TGA ---	0	--
TTG Leu	2	3.0%	TCG Ser	1	1.5%	TAG ---	1	--	TGG Trp	1	1.5%
CTT Leu	2	3.0%	CCT Pro	1	1.5%	CAT His	0	0.0%	CGT Arg	0	0.0%
CTC Leu	0	0.0%	CCC Pro	0	0.0%	CAC His	2	3.0%	CGC Arg	0	0.0%
CTA Leu	1	1.5%	CCA Pro	1	1.5%	CAA Gln	0	0.0%	CGA Arg	0	0.0%
CTG Leu	4	6.0%	CCG Pro	0	0.0%	CAG Gln	6	9.0%	CGG Arg	0	0.0%
ATT Ile	3	4.5%	ACT Thr	0	0.0%	AAT Asn	1	1.5%	AGT Ser	2	3.0%
ATC Ile	2	3.0%	ACC Thr	0	0.0%	AAC Asn	2	3.0%	AGC Ser	3	4.5%
ATA Ile	1	1.5%	ACA Thr	2	3.0%	AAA Lys	2	3.0%	AGA Arg	1	1.5%
ATG Met	5	7.5%	ACG Thr	0	0.0%	AAG Lys	8	11.9%	AGG Arg	3	4.5%
GTT Val	1	1.5%	GCT Ala	1	1.5%	GAT Asp	0	0.0%	GGT Gly	0	0.0%
GTC Val	0	0.0%	GCC Ala	0	0.0%	GAC Asp	0	0.0%	GGC Gly	1	1.5%
GTA Val	0	0.0%	GCA Ala	0	0.0%	GAA Glu	0	0.0%	GGA Gly	0	0.0%
GTG Val	0	0.0%	GCG Ala	0	0.0%	GAG Glu	2	3.0%	GGG Gly	0	0.0%

------ BASE COMPOSITION ------

	T	C	A	G	Y	R	ALL
NUMBER	196	163	250	203	359	453	812
PERCENT	24.1	20.1	30.8	25.0	44.2	55.8	100.0

------ AMINO ACID COMPOSITION ------

RES	NUM	%	RES	NUM	%	RES	NUM	%	RES	NUM	%
Phe	1	1.5	Leu	10	14.9	Ile	6	9.0	Met	5	7.5
Val	1	1.5	Ser	7	10.4	Pro	2	3.0	Thr	2	3.0
Ala	1	1.5	Tyr	1	1.5	His	2	3.0	Gln	6	9.0
Asn	3	4.5	Lys	10	14.9	Asp	0	0.0	Glu	2	3.0
Cys	2	3.0	Trp	1	1.5	Arg	4	6.0	Gly	1	1.5

First sequence: NJ MIS
Second sequence: NJ OG

```
     1 MIIWILPCRMQMSLKKEERINISKTSSSKIKEINQLRHIIRKKNRQIQILTIMLNILRCCRRMKE
       ********** * * * ********************************** ********* ****
     1 MIIWILPCRMPMNLRKDERINISKTSSSKIKEINQLRHIIRKKNRQIQILIIMLNILRCCHRMKE

MATCHED =    59  OUT OF    65  PERCENT  90
Mutation weight =  5  Gap weights =  3k +  7, k up to 2000
EVOLUTIONARY DISTANCE =      30
```

First sequence: IND SJ
Second sequence: IND M-S

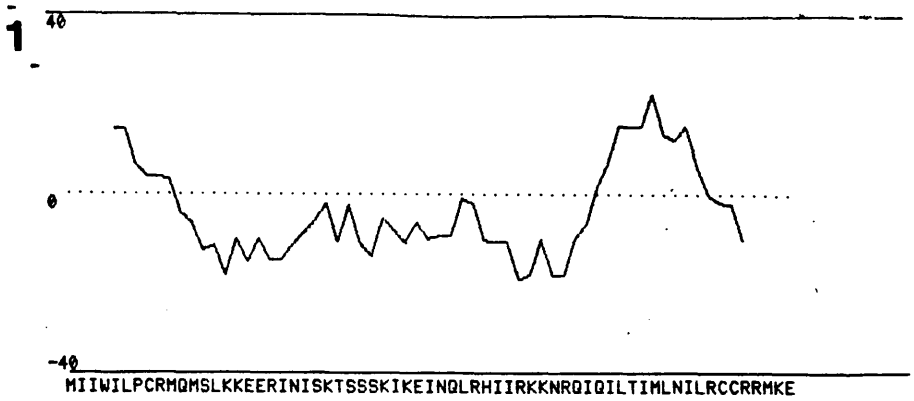
```
     1 MRSKHNELKSPIMSCSKRTEWKSILGPLIFRQQMILTQNLNQKLKTIKACMYQIRKLSKLKALYRGL
       ****************** ****** ********************** ** ** ************
     1 MRSKHNELKSPIMSCSKRMEWKSILSPLIFRQQMILTQNLNQKLKTIKVCMHQIQKLSKLKALYRGL

MATCHED =    62  OUT OF    67  PERCENT  92
Mutation weight =  5  Gap weights =  3k +  7, k up to 2000
EVOLUTIONARY DISTANCE =      25
```

First sequence: NJ MIS
Second sequence: IND SJ

```
     1 M        IIWILPCRMQMSLKKEERINISKTSSSKIKEINQLRHIIRKKNRQIQILTIMLNILRCCRRMKE
       *          * *      *      *           **    *     **  *       *
     1 MRSKHNELKSPIMSCSKRTEWKSILGPLIFRQQMILTQNLNQKLKTIKACMYQIRKLSKLKALYR     GL

MATCHED =    12  OUT OF    72  PERCENT  16
Mutation weight =  5  Gap weights =  3k +  7, k up to 2000
EVOLUTIONARY DISTANCE =     290
```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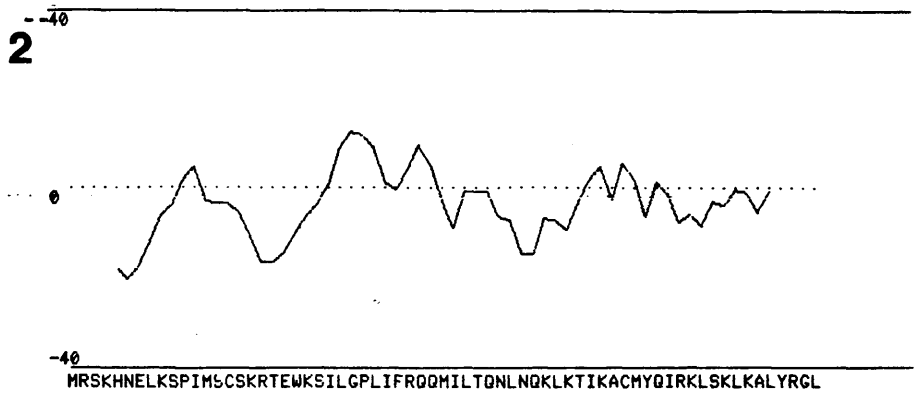
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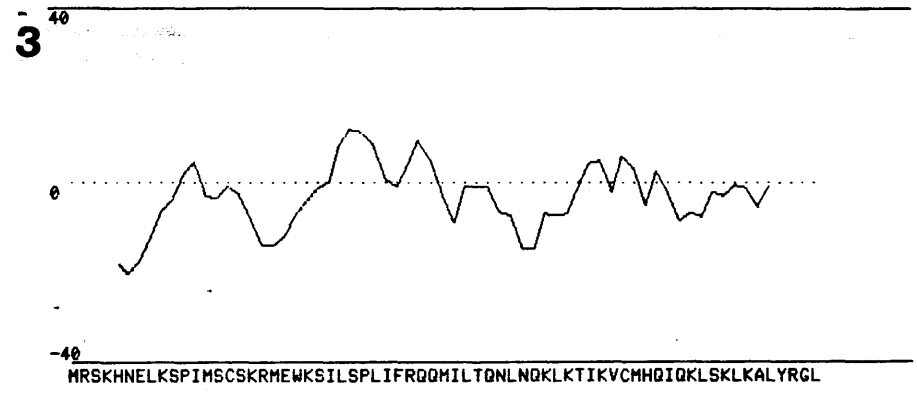
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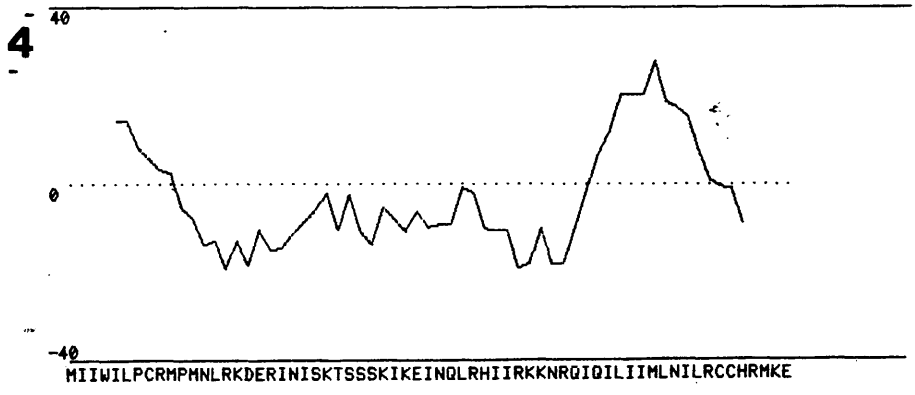
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CHARACTERISATION OF THE MUTATIONS RESPONSIBLE FOR THE ELECTROPHORETIC MOBILITY DIFFERENCES IN THE NS PROTEINS OF VESICULAR STOMATITIS VIRUS - NEW JERSEY COMPLEMENTATION GROUP E MUTANTS

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Running Title: VSV NS protein mutants

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SUMMARY

Temperature sensitive (ts) mutants of vesicular stomatitis virus, New Jersey serotype, classified in complementation group E contain lesions in the NS gene, which manifest as marked electrophoretic mobility differences of the mutant NS proteins in SDS polyacrylamide gels. We have cloned full-length cDNA copies of the mutant NS mRNAs, and have determined their nucleotide sequences. tsE1 and tsE3 have single nucleotide changes, and tsE2 has two nucleotide changes, compared to the wild-type NS gene. Three of the mutations are clustered in a region of 18 nucleotides. All the nucleotide differences result in amino acid substitutions, which in each case change the charge of the amino acid concerned. Analysis of the wild-type and mutant NS protein sequences by the method of Chou and Fasman indicate that single amino acid substitutions can radically alter the predicted secondary structure, and these data are discussed in relation to the observed electrophoretic mobility differences.

INTRODUCTION

The negative strand RNA genome of vesicular stomatitis virus (VSV) encodes five proteins, L, G, N, NS and M. The L, N and NS proteins, together with the genomic RNA, comprise the ribonucleoprotein core of the virus particle, and the M and G proteins form the viral envelope (Wagner, 1975). The NS protein is a component of the RNA polymerase complex (Banerjee et al, 1977) but its precise role(s) in transcription and replication is still uncertain. Complete nucleotide sequences of the NS mRNAs encoded by representative viruses of the two VSV serotypes (Indiana, IND and New Jersey, NJ) have been reported, and predict the molecular weight of the NS protein of IND viruses to be 29,900 (Gallione et al, 1981; Hudson et al, 1986) and that of NJ viruses to be 31,400 (Gill and Banerjee, 1985; Rae and Elliott, 1986). However, the NS proteins migrate on SDS polyacrylamide gels with apparent molecular weights of 40-70,000, depending on the degree of bisacrylamide cross-linking (Bell et al, 1984; Bell and Prevec, 1985; Kingsford and Emerson, 1980; Knipe et al, 1980; Obijeski et al, 1974; Wunner and Pringle, 1972). The aberrant mobility of NS is clearly seen when it migrates more slowly than the N protein, which has a molecular weight of 47,300 (IND serotype; Gallione et al, 1981) or 47,800 (NJ serotype; Banerjee et al, 1984) eg. the studies reported by Lesnaw et al (1979), Maack and Penhoet (1980), Patton et al (1984) and Pringle et al (1981). The NS protein is phosphorylated but the phosphate groups are not responsible for the anomalous mobility - indeed, the more heavily phosphorylated NS molecules migrate faster in SDS polyacrylamide gels (Hsu et al, 1982) while NS that has been dephosphorylated with bacterial alkaline phosphatase migrates more slowly (Hsu and Kingsbury, 1980). The NS protein contains many acidic residues (Gallione et al, 1981; Gill and Banerjee, 1985; Hudson et al, 1986; Rae and Elliott, 1986), and it is

thought that these residues hinder SDS binding which results in the aberrant migration (discussed by Marnell and Summers, 1984).

Temperature sensitive mutants of VSV-NJ were isolated by Pringle et al (1971) following 5-fluorouracil treatment of infected cells. Three mutants, classified in complementation group E, were shown to have lesions in the NS gene (Evans et al, 1979; Lesnaw et al, 1977; Maack and Penhoet, 1980). These mutants form a heterogenous group with respect to their phenotype, accompanied by marked differences in the electrophoretic migration of the NS protein. In order to characterise these mutants further, we have determined the nucleotide sequence of cloned full-length cDNA copies of their NS mRNAs. We report here that a single amino acid substitution in an NS protein can radically alter its electrophoretic mobility in SDS polyacrylamide gels.

MATERIALS AND METHODS

Molecular Cloning and Nucleotide Sequencing

Stocks of VSV-NJ tsE1, tsE2, tsE3, tsE1/R1 and tsE3/R1 were generously provided by J.F. Szilagyi and were used directly to infect cells for RNA preparation without further passage. BHK-C13 cells were infected in the presence of actinomycin D and total cellular RNA prepared by the guanidinium thiocyanate-CsCl method exactly as described previously (Chirgwin et al, 1979; Rae and Elliott, 1986). cDNA synthesis followed the method of Gubler and Hoffman (1983) as modified by Rae and Elliott (1986) and mutant NS gene specific plasmids were identified by in situ colony hybridisation (Grunstein and Hogness, 1976) using a ^{32}P-labelled fragment (the large Pst I fragment, bases 1-523) from the wild-type NS gene plasmid pNJNS1 (Rae and Elliott, 1986). Full-length cDNAs were identified by sizing in agarose gels and designated pNJE1, pNJE2 and pNJE3. Nucleotide sequences were determined by the dideoxy chain terminator method (Sanger et al, 1977) by subcloning restriction enzyme fragments into M13mp18 DNA as detailed previously (Rae and Elliott, 1986).

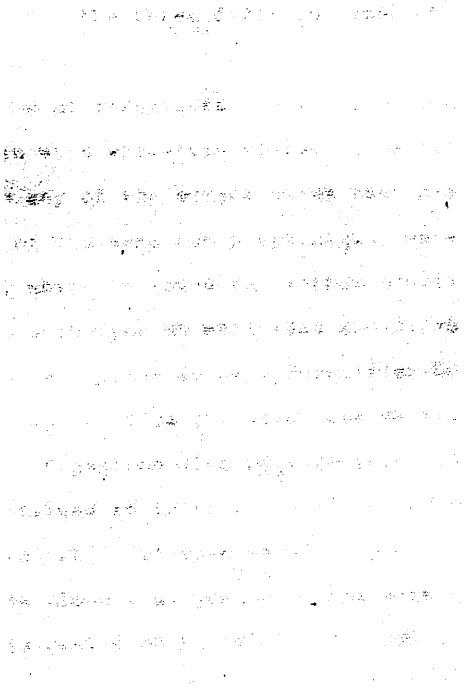
In Vitro Translation and SDS Polyacrylamide Gel Electrophoresis

RNAs were translated in a rabbit reticulocyte lysate (Amersham International Ltd, Bucks, UK) using 10ug total RNA in a 25µl reaction containing 50µCi ^{35}S-methionine. Radiolabelled translation products were separated on SDS polyacrylamide gels (acrylamide:bisacrylamide 75:1; Watret et al, 1985) using the discontinuous buffer system of Laemmli (1970).

Computer Analysis

Nucleotide sequences were stored and manipulated with a DEC PDP11/44 computer using the programs devised by Staden (1982).

ropathy profiles were generated using the SOAP algorithm of Kyte nd Doolittle (1982). Protein secondary structure predictions used the method of Chou and Fasman (1978), and were graphically displayed using the PLOTCHOU program contained in the updated package of the University of Wisconsin Genetics Computer Group (originally described by Devereux et al, 1984). The graphic output was drawn on a Hewlett Packard HP7475A plotter in the Department of Molecular Biology, University of Edinburgh, with the assistance of Dr A. Coulson.



RESULTS

Molecular Cloning and Nucleotide Sequence Determination of the NS mRNAs of tsE1, tsE2 and tsE3

Total cellular RNA was extracted from actinomycin D-treated infected cells (Rae and Elliott, 1986), and an aliquot analysed by *in vitro* translation in a reticulocyte lysate. As seen in Figure 1 the NS proteins of *ts*E1, *ts*E2 and *ts*E3 had different electrophoretic mobilities in an SDS 12% polyacrylamide gel compared to the wild-type NS protein. The *in vitro* products were also analysed on gels containing 10% and 15% polyacrylamide (data not shown), and the apparent molecular weights of the NS proteins were determined relative to the G (62,000), N (47,800) and M (26,000) proteins. The averages of estimations from the three different concentrations of gels are given in Table 1.

No evidence of revertants, as would be indicated by the presence of an NS protein with wild-type migration pattern (Evans *et al*, 1979), was observed in any of the mutant virus mRNA preparations. Therefore, other aliquots of the same RNA preparations were used as templates for cDNA synthesis, which followed the method previously described for cloning cDNA to wild-type NS mRNA (Rae and Elliott, 1986). Mutant NS specific recombinant plasmids were identified by *in situ* hybridisation using a probe prepared from the wild-type NS plasmid, pNJNS1 (Rae and Elliott, 1986). Digestion with *Pst*I revealed that the mutant NS gene inserts all contained an internal *Pst*I restriction enzyme site, characteristic of the wild-type NS gene insert. Apparently full-length cDNA clones, as judged by the size of the insert, were obtained and designated pNJE1 (from *ts*E1) pNJE2 (from *ts*E2) and pNJE3 (from *ts*E3). Nucleotide sequence analysis confirmed that these three mutant NS cDNA clones were complete copies since they all contained the 5' and 3' consensus sequences found in all VSV mRNAs (McGeoch,

; Rose, 1980).

The complete nucleotide sequences of the mutant NS genes (Figure 2) were determined by the dideoxy chain terminator method (Sanger et al, 1979). All the mutant NS mRNAs were the same length (876 bases excluding polyadenylic acid) and encoded the same length of polypeptide (274 amino acids) as the wild-type NS mRNA. tsE1 and tsE3 each had single nucleotide changes while tsE2 had two nucleotide substitutions. Portions of representative sequencing gels showing these changes are displayed in Figure 3. Three of the nucleotide changes occur within an 18 base regions (nucleotide 93-108) while the second mutation in tsE2 is at position 413 (Figure 2). In all cases the nucleotide change resulted in an amino acid substitution with a change in the charge of amino acid concerned. These are summarised in Table 1.

Figure 4 shows a hydropathy profile (Kyte and Doolittle, 1982) of the wild-type NS protein. The region marked 'a', the most hydrophilic domain of the protein, is where three amino acid substitutions are clustered, and 'b' indicates the position of the second mutation in tsE2.

The amino acid sequences of the wild-type and mutant NS proteins were analysed using the secondary structure prediction algorithm of Chou and Fasman (1978), and a graphic output obtained (Figure 5). Although these predictions are based solely on the primary amino acid chain, and do not take into account the effect of post-translation modifications such as phosphorylation, it is obvious that mutations occuring in amino acid residues 28-33 have a marked effect on the predicted secondary structure. The mutation in tsE1 predicts a loss of a region of α-helix and a change in the position of a β-turn. The mutation in tsE3 manifests as a loss of the same α-helical region, although no change in β-turn is predicted. The mutation at amino acid 32 is tsE2 results in an additional region of α-helix

additional β-turns, whereas the mutation at amino acid 114 has a minor effect in establishing a small region of α-helix. No other effects of these mutations were predicted in the remainder of the NS protein.

Nucleotide Sequence Determination of Non-ts Revertants of tsE1 and tsE3

We have also cloned and sequenced full-length cDNA copies of the NS mRNA of non-ts revertants of tsE1 (tsE1/R1; Szilagyi and Pringle, 1979) and tsE3 (tsE3/R1; Evans et al, 1979) [it is worth noting that no revertants have yet been isolated from the double mutant tsE2]. These revertants have a wild-type pattern of RNA synthesis and electrophoretic mobility of the NS protein (Evans et al, 1979). The revertant NS genes were identical to the wild-type sequence (Rae and Elliott, 1986) at the site of mutation, but two additional point mutations were noted: a silent T to C change at position 471 and a T to G change at position 513 which results in a Met to Arg substitution (amino acid 168; Figure 2). Both revertant NS gene sequences were identical. The region encompassing the additional changes in the NS gene (ie. nucleotides 471 and 513) was sequenced in three other independent plasmid isolates containing the tsE1/R1 NS insert, and confirmed the substitutions given above. The amino acid substitution at residue 168 did not affect the predicted secondary structure of the revertant NS protein compared to wild-type (data not shown).

DISCUSSION

The complementation group E mutants of VSV-NJ exhibit a variety of phenotypes and different electrophoretic mobility patterns of the NS protein (Pringle et al, 1970; Evans et al, 1979). By determining the nucleotide sequences of cloned cDNA copies of the mutant NS mRNAs we have precisely mapped the lesions in the NS genes. Our data show that the altered migration of the mutant NS proteins is not due to the synthesis of truncated proteins but rather the effects of amino acid substitutions.

The relative electrophoretic mobility of proteins in polyacrylamide gels containing SDS is a widely used method of estimating their molecular weights (Shapiro et al, 1967). The migration of the protein depends on a high level of SDS binding (1.4g of SDS per 1g of protein) so that an approximately constant negative charge per unit mass is achieved (Creighton, 1983); under these conditions the electrophoretic mobility in gels is proportional to the logarithm of the length of the polypeptide chain. However, the amino acid composition or conformation of the protein may perturb the stoichiometric binding of SDS, resulting in aberrant electrohphoretic migration. De Jong et al (1978) reported that the mobility of α-crystallin A chains was altered by single amino changes, and Carstens et al (1986) reported that a single amino acid substitution in a mutant baculovirus polyhedral protein affected its electrophoretic migration. In addition, the latter workers showed that the single amino acid change grossly affected the predicted secondary structure of the mutant protein. Our data also demonstrate that single amino acid substitutions can radically influence the predicted secondary structure of a protein.

A potential criticism of our approach is that we have sequenced cloned cDNAs rather than obtaining a consensus sequence derived from

oxy sequencing on an RNA template. However, our results are in mplete agreement with the data of D. McGeoch and A. Dolan who btained sequence information for approximately 200 nucleotides at the 5' end of the NS genes of tsE1, tsE2, tsE3, tsE1/R1 and tsE3/R1 by primer extension on genomic RNA (D. McGeoch, personal communication; and data cited in Pringle, 1986). Therefore we feel that the sequences presented here are an accurate reflection of the differences in the wild-type, mutant and revertant NS genes.

We do not think that the presence of additional changes in the NS genes of the revertant viruses is due to a cloning artefact, since the recombinant plasmids were isolated in different cloning experiments which were performed on separate occassions. Furthermore, four individual clones of the tsE1/R1 NS genes contained the additional base substitutions. It also seems unlikely that these changes represent a variant wild-type virus which had somehow contiminated the revertant selection procedures, since these were also carried out at different times (C.R. Pringle, personal communication). The occurrence of other changes in the NS genes of non-ts revertants is implied from the data of Maack and Penhoet (1980) who isolated non-ts revertants of tsE1 with different mobilities of the NS protein compared to both tsE1 and wild-type viruses. We suggest that this could be due to a different amino acid substitution at position 32, or additional amino acid changes elsewhere in the NS protein, any of which could influence the conformation of the protein.

In contrast to the situation in VSV M gene mutants, where point mutations have been located throughout the M protein (Gopalkrishna and Lenard, 1985), three of the four mutations in the NS mutants are clustered around the most hydrophilic region of the NS protein (Figure 4). This region of the NS protein represents an obvious area to explore via in vitro mutagenesis in order to correlate active sites in the protein with the different functions ascribed to NS.

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FIGURE LEGENDS

Figure 1

Electrophoretic mobility differences in the NS proteins of wild-type (wt) and ts mutants E1, E2 and E3 of VSV-NJ. RNA extracted from infected cells was translated in a rabbit reticulocyte lysate, and the translation products analysed on a 12% polyacrylamide gel. M, wild-type VS virion proteins as marker. Viral proteins are identified at the right, and solid arrows denote the position of the NS protein.

Figure 2

Nucleotide and deduced amino acid sequence of the NS mRNA of VSV NJ, showing the changes detected in the NS mRNAs of tsE1, tsE2 and tsE3, as well as the additional changes observed in the non-ts revertants tsE1/R1 and tsE3/R1 (rev).

Figure 3

Portions of representative sequencing gels showing the mutations in the NS genes of the E group mutants

(a) wild-type NS, positions 91-110 are indicated

(b) tsE1, showing G-A change at position 104

(c) tsE2 showing A-G change at position 108

(d) tsE3 showing A-G change at position 93. This gel actually shows the minus strand sequence; the complementary sequence is written as well to compare with (a)

(e) tsE2, showing A-G change at position 353 compared to the wild-type sequence shown in (f)

gure 4

Hydropathy profile of the NS protein of VSV-NJ. 'a' indicates the region (amino acids 28-33) where three mutations are clustered, and 'b' indicates the position of the second mutation (amino acid 115) in tsE2.

Figure 5

Predicted secondary structures of the NS proteins of wild-type and complementation E group mutants VSV-NJ. The generation of these graphic representations is described in Methods. The complete plot of wild-type (wt) NS protein is given, and the relevant portions of the plots of the E group mutants. The arrows indicate differences in the predicted secondary structures due to amino acid substitutions. ⋀⋁ represents α helix, ∿∿ represents β strand, ~~ represents undefined and a change of direction indicates β turn. The open ovals represent hydrophilic regions, and shaded ovals hydrophobic regions.

Table 1 Summary of the Mutations in the NS Genes of Vesicular Stomatitis Virus E Group Mutants

Virus	Apparent Mol Wt of NS Protein ($x10^{-3}$)[a]	Nucleotide Position and Change	Amino Acid Position and Change	Charge Change	Phenotype[b]
wild type	59	—	—	—	Transcriptase +ve RNA synthesis +ve
tsE1	50	104 G→A	32 Glu→Lys	-ve charged polar → +ve charged polar	Transcriptase -ve RNA synthesis -ve
tsE2	52	108 A→G	33 Asp→Gly	-ve charged polar → nonpolar	Transcriptase +ve RNA synthesis +ve
		353 A→G	115 Lys→Glu	+ve charged polar → -ve charged polar	
tsE3	54.5	93 A→G	28 Glu→Gly	-ve charged polar → nonpolar	Transcriptase +ve RNA synthesis -ve

(a) average of estimations from 10%, 12% and 15% polyacrylamide gels
(b) Pringle *et al*, 1971; Szilagyi and Pringle, 1979

REFERENCES

BANERJEE, A.K., ABRAHAM, G. & COLONNO, R. (1977). Vesicular stomatitis virus: Mode of transcription. Journal of General Virology 34, 1-8.

BANERJEE, A.K., RHODES, D.P. & GILL, D.S. (1984). Complete nucleotide sequence of the mRNA coding for the N protein of vesicular stomatitis virus (New Jersey serotype). Virology 137, 432-438.

BELL, J.C., BROWN, E.G., TAKAYESU, D. & PREVEC, L. (1984). Protein kinase activity associated with immunoprecipitates of vesicular stomatitis virus phosphoprotein NS. Virology 132, 229-238.

BELL, J.C. & PREVEC, L. (1985). Phosphorylation sites on phosphoprotein NS of vesicular stomatitis virus. Journal of Virology 54, 697-702.

CARSTENS, E.B., KREBS, A. & GALLERNEAULT, C.E. (1986). Identification of an amino acid essential to the normal assembly of Autographa californica nuclear polyhedrosis virus polyhedra. Journal of Virology 58, 684-688.

CHIRGWIN, J.M., PRZYBLYA, A.E., MACDONALD, R.J. & RUTTER, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.

CHOU, P.Y. & FASMAN, G.D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. Advances in Enzymology 47, 45-147.

CREIGHTON, T.E. (1983). 'Proteins'. W.H. Freeman and Co., New York.

De JONG, W.W., ZWEERS, A. & COHEN, L.H. (1978). Influence of single amino acid substitutions on electrophoretic mobility of sodium dodecyl sulfate-protein complexes. Biochemical and Biophysical Research Communications 82, 532-539.

DEVEREUX, J., HAEBERLI, P. & SMITHIES, O. (1984). A comprehensive

set of sequence analysis programs for the VAX. Nucleic Acids Research 12, 387-395.

EVANS, D., PRINGLE, C.R. & SZILAGYI, J.F. (1979). Temperature-sensitive mutants of complementation group E of vesicular stomatitis virus New Jersey serotype possess altered NS polypeptides. Journal of Virology 31, 325-333.

GALLIONE, C.J., GREENE, J.R., IVERSON, L.E. & ROSE, J.K. (1981). Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus N and NS proteins. Journal of Virology 39, 529-535.

GILL, D.S. & BANERJEE, A.K. (1985). Vesicular stomatitis virus NS proteins: Structural similarity without extensive sequence homology. Journal of Virology 55, 60-66.

GOPALAKRISHNA, Y. & LENARD, J. (1985). Sequence alterations in the temperature-sensitive M-protein mutants (complementation group III) of vesicular stomatitis virus. Journal of Virology 56, 655-659.

GRUNSTEIN, M. & HOGNESS, D.S. (1975). Colony hybridisation: A method for the isolation of cloned DNAs that contain a specific gene. Proceedings of the National Academy of Sciences (USA) 71, 3961-3965.

GUBLER, U. & HOFFMAN, B.J. (1983). A simple and very efficient method for generating cDNA libraries. Gene 25, 263-269.

HSU, C., MORGAN, E.M. & KINGSBURY, D.W. (1982). Site-specific phosphorylation regulates the transcriptase activity of vesicular stomatitis virus NS protein. Journal of Virology 43, 104-112.

HSU, C.-H. & KINGSBURY, D.W. (1982). NS phosphoprotein of vesicular stomatitis virus: subspecies separated by electrophoresis and isoelectric focusing. Journal of Virology 42, 342-345.

HUDSON, L.D., CONDRA, C. & LAZZARINI, R.A. (1986). Cloning and expresasion of a viral phosphoprotein: Structure suggests NS may

function by mimicking an RNA template. Journal of General Virology (in press).

KINGSFORD, L. & EMERSON, S.U. (1980). Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virions and cytoplasm of infected cells. Journal of Virology 33, 1097-1105.

KNIPE, D., ROSE, J.K. & LODISH, H.F. (1975). Translation of individual species of vesicular stomatitis viral mRNA. Journal of Virology 15, 1004-1011.

KYTE, J. & DOOLITTLE, R.F. (1982). A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology 157, 105-132.

LESNAW, J.A., DICKSON, L.R. & CURRY, R.H. (1979). Proposed replicative role of the NS polypeptide of vesicular stomatitis virus: Structural analysis of electrophoretic variant. Journal of Virology 31, 8-16.

MAACK, C.A. & PENHOET, E.E. (1980). Biochemical characterization of the tsE1 mutant of vesicular stomatitis virus (New Jersey). Journal of Biological Chemistry 255, 9249-9254.

MARNELL, L.L. & SUMMERS, D.F. (1984). Characterisation of the phosphorylated small enzyme subunit, NS, of the vesicular stomatitis virus RNA polymerase. Journal of Biological Chemistry 259, 13518-13524.

MCGEOCH, D.J. (1979). Structure of the gene N: Gene NS intercistronic junction in the genome of vesicular stomatitis virus. Cell 17, 673-681.

OBIJESKI, J.F., MARCHENKO, A.T., BISHOP, D.H.L., CANN, B. & MURPHY, F.A. (1974). Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. Journal of General Virology 22, 21-33.

PATTON, J.T., DAVIS, N.L. & WERTZ, G.W. (1984). N protein alone

satisfies the requirement for protein synthesis during RNA replication of vesicular stomatitis virus. Journal of Virology 49, 303-309.

PRINGLE, C.R. (1986). In: 'The Viruses: The Rhabdoviruses'. Series edited by H. Fraenkel-Conrat and R.R. Wagner. Plenum Press, New York and London (in press).

PRINGLE, C.R., DEVINE, V., WILKIE, M., PRESTON, C.M., DOLAN, A. & MCGEOCH, D.J. (1981). Enhanced mutability associated with a temperature-sensitive mutant of vesicular stomatitis virus. Journal of Virology 39, 377-389.

PRINGLE, C.R., DUNCAN, I.B. & STEVENSON, M. (1971). Isolation and characterisation of temperature-sensitive mutants of vesicular stomatitis virus, New Jersey serotype. Journal of Virology 8, 836-841.

RAE, B.P. & ELLIOTT, R.M. (1986). Conservation of potential phosphorylation sites in the NS proteins of the New Jersey and Indiana serotypes of vesicular stomatitis virus. Journal of General Virology (in press).

ROSE, J.K. (1980). Complete intergenic and flanking gene sequences from the genome of vesicular stomatitis virus. Cell 19, 415-421.

SANGER, F., NICKLEN, S. & COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences (USA) 74, 5463-5467.

SHAPIRO, A.L., VINUELA, E. & MAIZEL, J.V. (1967). Molecular weight estimations of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochemical and Biophysical Research Communications 28, 815.

STADEN, R. (1982). Automation of computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acids research 10, 4731-4751.

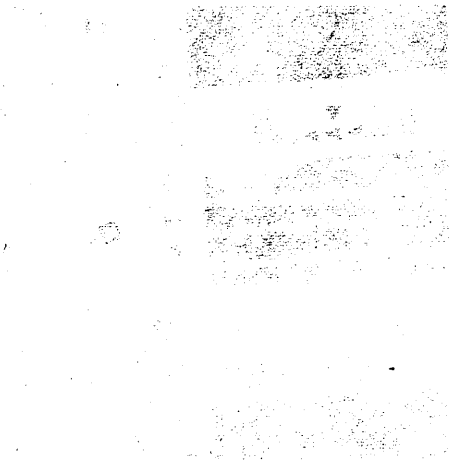
SZILAGYI, J.F. & PRINGLE, C.R. (1979). Effect of

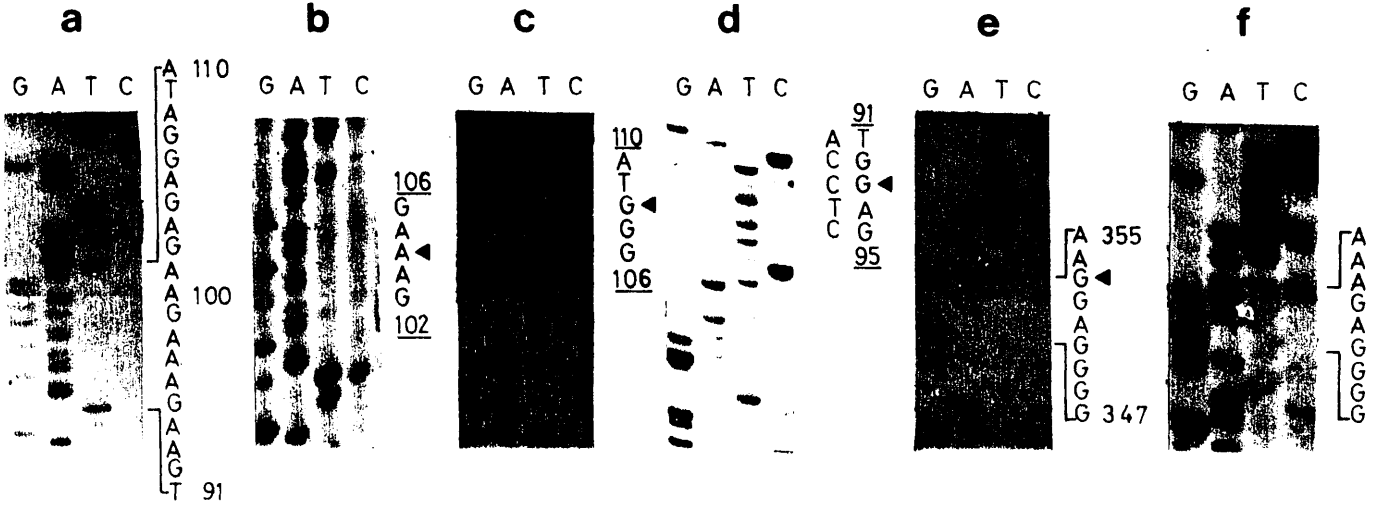
temperature-sensitive mutation on activity of the RNA transcriptase of vesicular stomatitis virus New Jersey. Journal of Virology 30, 692-700.

WAGNER, R.R. (1975). Reproduction of rhabdoviruses. In: 'Comprehensive Virology', pl-93, eds. H. Fraenkel-Conrat and R.R. Wagner. Plenum Press, New York.

WATRET, G.E., PRINGLE, C.R. & ELLIOTT, R.M. (1985). Synthesis of bunyavirus-specific proteins in a continuous cell line (XTC-2) derived from Xenopus laevis. Journal of General Virology 66, 473-482.

WUNNER, W.H. & PRINGLE, C.R. (1972). Comparison of structural polypeptides from vesicular stomatitis virus (Indiana and New Jersey serotypes) and Cocal virus. Journal of General Virology 16, 1-10.

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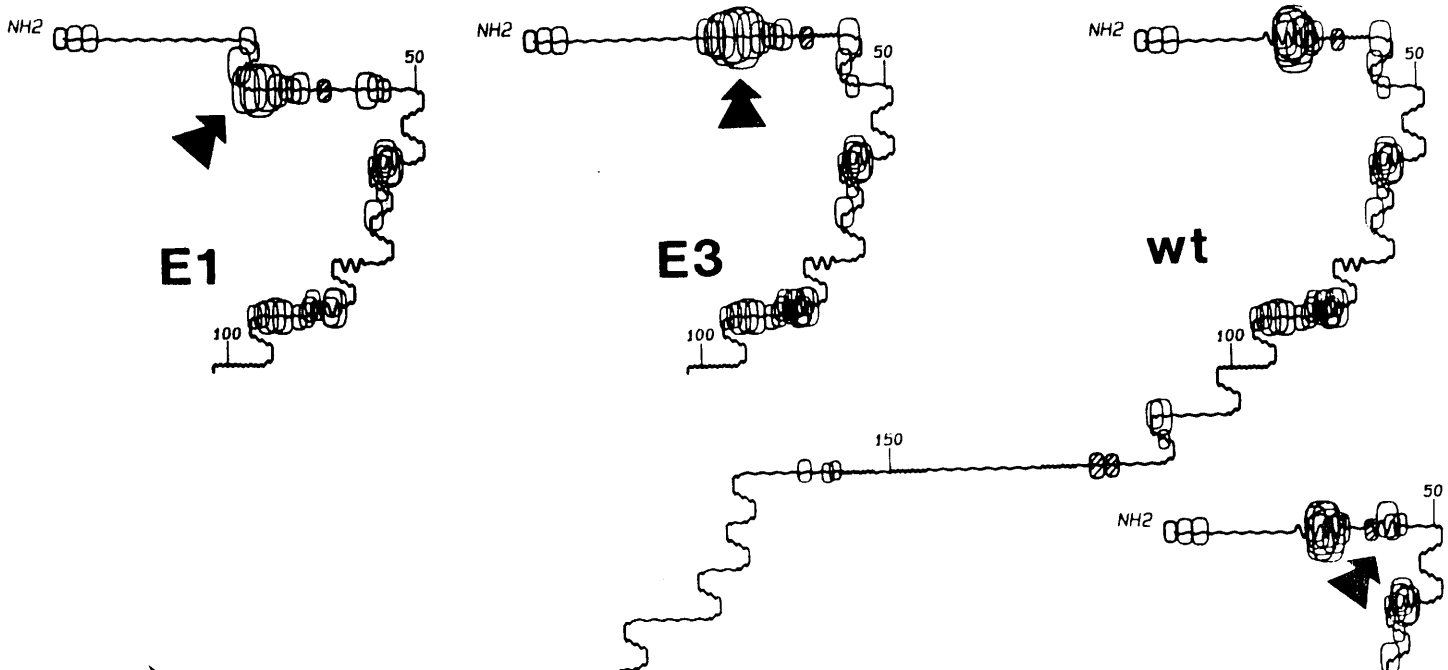
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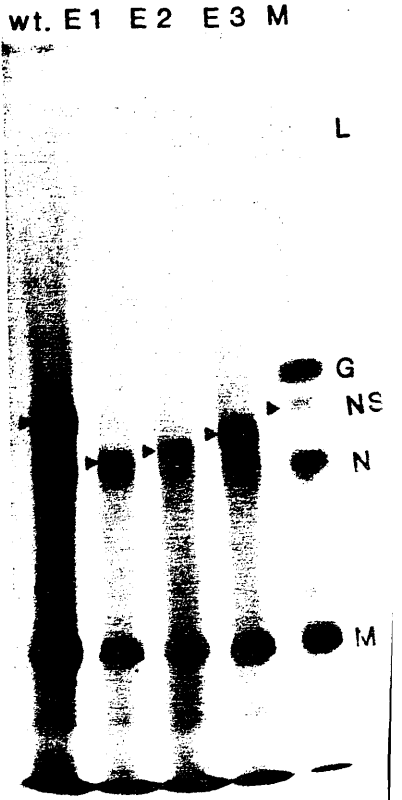
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Conservation of Potential Phosphorylation Sites in the NS Proteins of the New Jersey and Indiana Serotypes of Vesicular Stomatitis Virus

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SUMMARY

A full length cDNA copy of the NS mRNA of the Missouri strain (Hazelhurst subtype, New Jersey serotype) of vesicular stomatitis virus (VSV) has been cloned and sequenced. The mRNA is 856 nucleotides long (excluding polyadenylic acid) and encodes a protein of 274 amino acids (mol. wt. 31 000). Comparison with the NS gene of the Ogden strain (Concan subtype, New Jersey serotype) showed 15% difference at the nucleotide level and 10% difference at the amino acid level; the majority of the changes were located in the 3′ half of the mRNA. Comparison with the NS genes of two strains representing the Indiana serotype showed about 50% nucleotide and 33% amino acid sequence homology between the serotypes. In a four-way comparison of the proteins, two regions of higher homology were noted which may be of functional importance. Eighteen potential phosphorylation sites (Ser or Thr) were conserved between the four proteins; five of these sites correspond to the residues which have been suggested to be constitutively phosphorylated and may be essential for NS activity.

INTRODUCTION

The NS (so-called non-structural) protein of vesicular stomatitis virus (VSV) is multifunctional and has been shown to play a role in mRNA transcription, genome replication and virion maturation (for review, see Pringle, 1986). In the virus particle NS is complexed with the L protein and is closely associated with the negative strand RNA–nucleocapsid (N) protein complex. NS is a phosphoprotein with a molecular weight of about 30000 (Gill & Banerjee, 1985) although in SDS–polyacrylamide gels its migration is anomalously slow probably due to its highly acidic nature (Marnell & Summers, 1984). It is documented that the degree of phosphorylation of NS plays a regulatory role in transcription *in vitro* (Kingsford & Emerson, 1980; Hsu *et al.*, 1982) and the ability to bind nucleocapsids (Clinton *et al.*, 1978), and recently Sánchez *et al.* (1985) reported that the viral L protein specifically phosphorylates NS *in vitro*.

Detergent treatment of VSV particles yields a transcribing ribonucleoprotein complex (TNP), comprising the virion RNA and L, N and NS proteins, which retains essentially all the transcriptase activity of the virion (Bishop & Roy, 1972; Emerson & Wagner, 1972; Szilágyi & Uryvayev, 1973). Fractionation and reconstitution experiments with TNP have been used in an attempt to define the roles of the different proteins in transcription but have generated conflicting results. Ongrádi *et al.* (1985*a*, *b*) have suggested that L alone is the transcriptase while NS exerts a controlling effect, whereas the laboratories of both Emerson and Banerjee have reported that a combination of L and NS is required to synthesize RNA *in vitro* (Emerson & Yu, 1975; Mellon & Emerson, 1978; Kingsford & Emerson, 1980; De & Banerjee, 1984, 1985).

As an alternative approach to studying the role of NS we undertook to clone a cDNA copy of the NS mRNA with the long term view to express the cDNA in a eukaryotic system. For these studies we chose the Missouri strain of the New Jersey serotype of VSV because temperature-sensitive mutants of this strain with lesions in the NS gene had previously been isolated and characterized in our Institute (Pringle *et al.*, 1971; Evans *et al.*, 1979). Here we report the cloning

of a full length cDNA of the Missouri strain NS mRNA, its complete nucleotide sequence and a comparison with the NS genes of three other VSV strains which indicates conservation of certain potential phosphate-accepting amino acids.

METHODS

Virus and cells. The Missouri strain of VSV New Jersey serotype was obtained from J. F. Szilágyi. Virus stocks were prepared and titrated in BHK-21 cells as described by Pringle *et al.* (1971).

Production of infected cell RNA. BHK cells were infected with 10 p.f.u./cell of VSV in the presence of 10 μg/ml actinomycin D. After adsorption for 1 h at 31 °C, the monolayers were washed with medium and incubated for 6 h at 31 °C in the presence of actinomycin D. The cells were then harvested by scraping and washed with phosphate-buffered saline. Total cellular RNA was prepared by the guanidinium isothiocyanate/caesium chloride method of Chirgwin *et al.* (1979).

Synthesis of complementary DNA. Double-stranded cDNA was synthesized according to the method of Gubler & Hoffman (1983), using 50 μg total cellular RNA as template, 1 μg of oligo(dT)$_{12\text{-}18}$ as primer and 40 units of reverse transcriptase in a 40 μl first-strand reaction. Double-stranded cDNA was tailed with dCMP residues, annealed to dGMP-tailed *Pst*I-cut pBR322 DNA (Bethesda Research Laboratories), and recombinant plasmids used to transform competent *Escherichia coli* MC1061 (Pringle *et al.*, 1984).

Identification of NS gene-specific recombinant plasmids. Tetracycline-resistant, ampicillin-sensitive transformed *E. coli* were initially screened with a ^{32}P-labelled, partially alkali-digested vesicular stomatitis virion RNA probe by colony hybridization (Pringle *et al.*, 1984) to identify VSV cDNA-containing clones. NS gene-specific plasmids were identified by a combination of Northern blot hybridization and message selection/*in vitro* translation of infected cell RNA using methods detailed by Pringle *et al.* (1984) and Elliott (1985).

Nucleotide sequence determination and analysis. The NS gene cDNA insert was excised from the pBR322 vector by *Pst*I digestion, and purified by agarose gel electrophoresis. The insert was further digested with *Hpa*II, *Hae*III or *Hin*fI and ligated into bacteriophage M13mp18 DNA. Nucleotide sequence determination followed the dideoxy chain termination method of Sanger *et al.* (1977, 1980). Sequence data were manipulated and analysed with a DEC PDP11 computer as described previously (Lees *et al.*, 1986).

RESULTS AND DISCUSSION

Cloning and identification of NS gene cDNA

Total cellular RNA extracted from actinomycin D-treated, VSV-infected BHK cells was used as the template for cDNA synthesis. With oligo(dT)$_{12\text{-}18}$ as primer and the reaction conditions of Gubler & Hoffman (1983) double-stranded DNA was produced and, following dCMP tailing, the cDNA was annealed to dG-tailed, *Pst*I-cut pBR322 plasmid DNA. Colony hybridization with ^{32}P-labelled VSV genomic RNA was used to identify plasmids containing VSV-specific inserts, and greater than 15% of the colonies screened contained viral sequences. This high proportion presumably reflects the inhibitory effect of the actinomycin D treatment on cellular mRNA synthesis and the efficient production of VSV mRNAs. NS cDNA-specific plasmids were identified by a combination of Northern blotting and message selection/*in vitro* translation. For this, plasmid DNA was prepared from 5 ml overnight cultures (Birnboim & Doly, 1979), and a sample of the DNA was ^{32}P-labelled by nick translation. The radiolabelled DNA was then hybridized to infected cell RNA which had been fractionated by agarose gel electrophoresis and immobilized on a nitrocellulose membrane (Pringle *et al.*, 1984). VSV-infected cell RNAs ^{32}P-labelled *in vivo* were included as markers. An example is shown in Fig. 1 (*a*): it can be seen that the M and NS mRNAs were poorly resolved and therefore hybridization to this region of the blot identified both M and NS cDNA-containing plasmids. To distinguish between these, plasmid DNAs were immobilized on nitrocellulose filters and hybridized to infected cell RNA. After extensive washing the hybridized RNA was eluted, translated in an *in vitro* system and the products were analysed by polyacrylamide gel electrophoresis (Elliott, 1985). The M and NS proteins were clearly resolved by electrophoresis (Fig. 1 *b*), enabling NS cDNA-containing plasmids to be identified. For comparison, the behaviour of an N gene cDNA-containing plasmid in these experiments is also shown in Fig. 1 (*a*) and (*b*). Digestion of the NS-specific plasmids with *Pst*I showed that the inserts contained a characteristic internal *Pst*I site (Fig. 1 *c*). The frequency of NS gene-containing plasmids was estimated to be 0·7% in our library. One plasmid, designated pNJNS1, contained an insert of approximately 900 base pairs in total, and was selected for sequence determination.

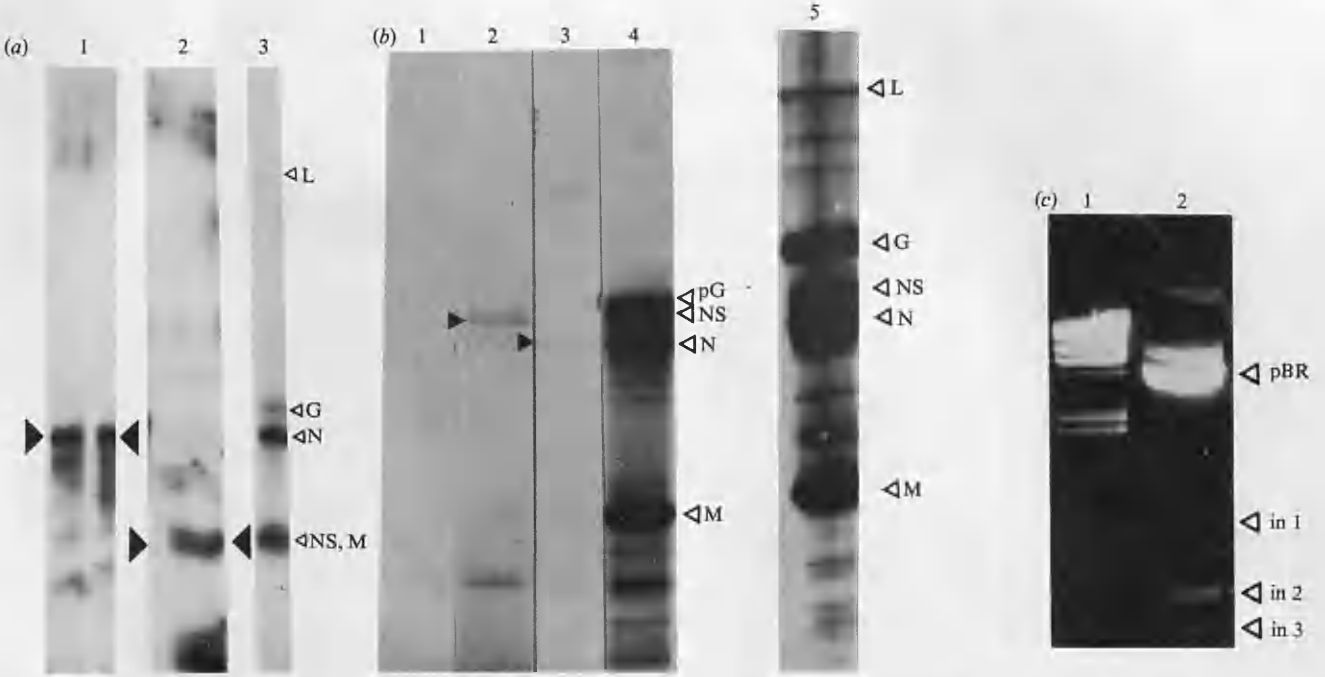

Fig. 1. Characterization of a full length cDNA clone of the NS mRNA of the Missouri strain of VSV. (*a*) Northern blot analysis. Total RNA extracted from VSV-infected cells was fractionated by formaldehyde–agarose gel electrophoresis and transferred to a nitrocellulose membrane (Pringle *et al.*, 1984). The filter was hybridized with ^{32}P-labelled plasmid DNA: (1) pNJN1, reacting with the N mRNA; (2) pNJNS1, reacting with either the NS or M mRNA; (3) a marker track of ^{32}P-labelled VSV mRNAs. (*b*) Message selection/*in vitro* translation. Plasmid DNA was bound to nitrocellulose and hybridized to total infected cell RNAs. The hybridized RNAs were eluted and used to programme a rabbit reticulocyte *in vitro* system. The translation products were analysed by polyacrylamide gel electrophoresis. (1) Selected with pBR322 DNA; (2) selected with pNJNS1 DNA; (3) selected with pNJN1 DNA; (4) translation *in vitro* of infected cell RNA; (5) marker track of [^{35}S]methionine-labelled VSV proteins. VSV proteins are indicated at the right. pG is the *in vitro* equivalent of the VSV G protein. The solid arrowheads indicate the translation products of the selected mRNAs. (*c*) Agarose gel electrophoresis of a partial *Pst*I digest of pNJNS1. (1) *Hin*dIII-digested λ DNA markers; (2) partial *Pst*I digest of pNJNS1 showing the intact insert (in1; approx. 900 bp) and the complete *Pst*I digestion products in2 and in3. pBR is the pBR322 vector DNA.

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            Met Asp Ser Ile Asp Arg Leu Lys Thr Tyr Leu Ala Thr Tyr Asp Asn Leu     17
AACAGAGATC ATG GAC AGT ATT GAT CGG CTC AAA ACT TAC TTG GCT ACT TAT GAT AAT TTG     61
      T              G       A         G           A   C
                     Val

Asp Ser Ala Leu Gln Asp Ala Asn Glu Ser Glu Glu Arg Arg Glu Asp Lys Tyr Leu Gln    37
GAT TCT GCC TTG CAG GAT GCA AAT GAG TCT GAA GAA AGA AGA GAG GAT AAA TAT CTC CAA   121
                     C       A       G         C

Asp Leu Phe Ile Glu Asp Gln Gly Asp Lys Pro Thr Pro Ser Tyr Tyr Gln Glu Glu Glu    57
GAC CTC TTC ATC GAA GAT CAA GGA GAT AAA CCA ACT CCG TCA TAT TAT CAG GAA GAA GAA   181

Ser Ser Asp Ser Asp Thr Asp Tyr Asn Ala Glu His Leu Thr Met Leu Ser Pro Asp Glu    77
TCG TCA GAT TCA GAT ACT GAC TAT AAT GCT GAA CAT CTT ACG ATG TTG TCG CCG GAT GAA   241
                     T                                       A

Arg Ile Asp Lys Trp Glu Glu Asp Leu Pro Glu Leu Glu Lys Ile Asp Asp Asp Ile Pro    97
AGA ATA GAC AAA TGG GAA GAA GAT TTG CCC GAA TTG GAA AAG ATT GAT GAT GAT ATA CCA   301
          G                       T       A                                 G

Val Thr Phe Ser Asp Trp Thr Gln Pro Val Met Lys Glu Asn Gly Gly Glu Lys Ser Leu   117
GTA ACT TTT TCT GAC TGG ACA CAA CCT GTA ATG AAG GAA AAC GGA GGG GAG AAA TCA CTA   361
  G   C           T           G                         T   G   A               G

Ser Leu Phe Pro Pro Val Gly Leu Thr Lys Val Gln Thr Asp Gln Trp Arg Lys Thr Ile   137
TCT CTG TTT CCT CCG GTT GGA TTA ACA AAA GTT CAG ACA GAC CAA TGG AGG AAG ACA ATT   421
          C       A   C   G           G A     A       A         AA   A   C
                                        Ile         Glu         Lys

Glu Ala Val Cys Glu Ser Ser Lys Tyr Trp Asn Leu Ser Glu Cys Gln Ile Met Asn Ser   157
GAG GCA GTC TGT GAG AGC TCT AAA TAT TGG AAT TTG TCA GAA TGT CAG ATT ATG AAC TCA   481
      G   T           T   A                   A           C   A     C T       TG
                                                                    Leu     Leu

Glu Asp Arg Leu Ile Leu Lys Gly Arg Ile Met Thr Pro Asp Cys Ser Ser Ser Ile Lys   177
GAA GAT CGT CTT ATC CTC AAA GGC CGA ATT ATG ACT CCT GAC TGC AGT TCA TCA ATC AAA   541
      C A C   C   T                 T G               T   T       T     G A
        Ser     Thr                 Leu                                 Val

Ser Gln Asn Ser Ile Gln Ser Ser Glu Ser Leu Ser Ser Ser His Ser Pro Gly Pro Ala   197
TCT CAG AAC TCC ATA CAG AGT TCC GAA TCT CTC TCC TCC TCG CAT TCA CCC GGT CCA GCA   601
      A   T   T G C   G   G   A     C       A       T       T   A       C C
                Val Arg Arg         Pro     Tyr                           Pro

Pro Lys Ser Arg Asn Gln Leu Gly Leu Trp Asp Ser Lys Ser Thr Glu Val Gln Leu Ile   217
CCA AAG TCA CGG AAT CAA CTA GGT CTA TGG GAT TCA AAA TCA ACA GAA GTA CAA CTG ATC   661
 TC     GT  TCA GAG TCC A C AA  T             T   G   C   T               T
Leu     Val Ser Glu Ser Ile Asn               Leu

Ser Lys Arg Ala Gly Val Lys Asp Met Met Val Lys Leu Thr Asp Phe Phe Gly Ser Glu   237
TCG AAG AGG GCC GGA GTG AAG GAC ATG ATG GTG AAA TTG ACA GAC TTT TTT GGA AGT GAA   721
  C       A             T               CA  C                   C               G
                                        Thr

Glu Glu Tyr Tyr Ser Val Cys Pro Glu Gly Ala Pro Asp Leu Met Gly Ala Ile Ile Met   257
GAG GAA TAT TAT TCA GTA TGC CCA GAA GGG GCC CCA GAT TTG ATG GGA GCA ATC ATC ATG   781
  A   G                                   G       C               T

Gly Leu Lys His Lys Lys Leu Phe Asn Gln Ala Arg Met Lys Tyr Arg Ile               274
GGA CTA AAA CAT AAA AAG CTT TTT AAC CAA GCA AGA ATG AAG TAT CGT ATC TAA TTAATTC   842
      G   G T C   G   A   C   C   T   G               A         C T       CC  T
              Tyr                                               Leu

CGATGATCAATATG                                                                   856
TC
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of the NS mRNA of the Missouri strain of VSV. The complete sequence of the NS mRNA was obtained by subcloning fragments of the insert of pNJNS1 into M13mp18 DNA and using the methodology of Sanger *et al.* (1977, 1980). The sequence is compared with that of the Ogden strain (Gill & Banerjee, 1985) and differences are indicated below the Missouri strain sequence.

Nucleotide sequence of the NS mRNA of VSV (Missouri strain)

The two insert fragments of pNJNS1 were digested with *Hpa*II, *Hae*III or *Hin*fI, subcloned into bacteriophage M13mp18 and their sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977, 1980). The sequence spanning the internal *Pst*I site was

obtained by digesting intact pNJNS1 with *Hpa*II, isolating the appropriate fragment from a polyacrylamide gel and ligating the purified fragment into M13mp18 DNA.

The complete sequence of the insert from pNJNS1 is shown in Fig. 2, and reveals that pNJNS1 contains a full length cDNA copy of the NS mRNA because (i) at the 5′ end of the insert, following a string of approximately 25 G residues (not shown on figure) is the sequence AACAGAGATC, which corresponds to the consensus sequence 5′-AACAGNNAUC-3′ reported to be at the 5′ end of all VSV mRNAs (McGeoch, 1979; Rose, 1980; Franze-Fernandez & Banerjee, 1978), and (ii) at the 3′ end there is the sequence TATG followed by a polyadenylate stretch of 12 residues [corresponding to the oligo$(dT)_{12\text{-}18}$ primer] before a 'tail' of approximately 25 C residues (not shown in the figure). Rose (1980) and Rhodes & Banerjee (1980) reported that the sequence . . . UAUGAAAA . . . occurs at the mRNA–poly(A) junction in each mRNA. Furthermore, the sequence of approximately 200 bases at the 5′ end of the NS mRNA is identical to the sequence obtained independently by D. J. McGeoch and A. Dolan by primer extension on the viral genomic RNA from within the N gene, across the intergenic region and into the NS gene (D. J. McGeoch, personal communication).

The NS mRNA is 856 nucleotides long excluding the 3′ poly(A) tract, and encodes a protein of 274 amino acids (mol. wt. 31000) beginning at AUG (bases 11 to 13) and terminating at UAA (bases 833 to 835). The next largest AUG-initiated open reading frame in either the (+) [mRNA]- or (−) [genomic RNA]-sense RNA would encode a protein of 65 amino acids, beginning at bases 51 to 53 in the mRNA. The amino acid composition of the NS protein indicates that the protein is relatively rich in aspartate and methionine residues, and relatively deficient in alanine and glycine residues compared to an 'average' protein (Klapper, 1977), i.e. Asp 9·2% compared to 5·5%, Met 4·2% compared to 1·7%, Ala 3·5% compared to 9·0% and Gly 4·2% compared to 7·5%. There are a total of 46 potential phosphorylated residues in the NS protein (33 Ser and 13 Thr). The NS protein has an overall charge of −18·5 at pH 7·0, assuming +1 charge for Lys and Arg, +0·5 for His and −1 charge for Asp and Glu at this pH.

Comparison of VSV NS genes and proteins

Nucleotide sequences of the NS genes of four strains of VSV, representative of the New Jersey (NJ) and Indiana (IND) serotypes, are now available, and a summary of the comparisons of these sequences is given in Table 1.

The mRNAs of the New Jersey serotype strains are both 856 nucleotides in length (excluding polyadenylic acid) compared to 814 nucleotides for the Indiana serotype strains. There is about 50% nucleotide homology between the NS genes of the two serotypes and the distribution of homology is displayed as a computer-generated dot matrix in Fig. 3 (*a*). This shows that there are only short regions of significant homology scattered throughout the gene sequences. For comparison the nucleotide sequences of the G and N genes of the NJ and IND serotypes show 54% and 68% homology respectively (Gallione & Rose, 1983; Banerjee *et al.*, 1984).

The two NJ strains are 85% homologous at the nucleotide level, and as shown in Fig. 2 the majority of the nucleotide differences occur in the 3′ half of the mRNA. A limited comparison of 151 bases at the 5′ end and 208 bases at the 3′ end of the N protein mRNAs of the Missouri and Ogden strains showed a similar degree of homology (D. J. McGeoch, personal communication; Pringle, 1986).

Comparison of the amino acid sequences of the NS proteins showed about 33% homology between the two serotypes, although the dot matrix comparison (Fig. 3*b*) indicated a clear similarity between the two proteins. The conservation of the NS proteins is thus considerably lower than that of the G proteins (51%) or the N proteins (69%) of the two serotypes (Gallione & Rose, 1983; Banerjee *et al.*, 1984). There are 27 amino acid differences between the Missouri and Ogden strains (Fig. 2), notably a stretch of six contiguous mismatched residues at positions 190 to 195. The 10% difference in the amino acid sequences between the NS proteins of the Missouri and Ogden strains [which represent the Hazelhurst and Concan subtypes of the NJ serotype (Reichmann *et al.*, 1978)] manifests itself as a difference in the electrophoretic mobility of the proteins in SDS–polyacrylamide gels (Schnitzlein & Reichmann, 1985). The only other available comparison of VSV gene sequences is from Gopalakrishna & Lenard (1985) who compared the

Table 1. *Comparison of the NS genes and proteins of four strains of VSV*

Serotype	Subtype	Strain	Length of mRNA (no. of nucleotides	Size of protein (no. of amino acids)	Homology (%) Nucleotide MIS	OG	SJ	M–S	Amino Acid MIS	OG	SJ	M–S	Reference
New Jersey	Hazelhurst	Missouri (MIS)	856	274	100	85	50	50	100	90	34	33	This paper
	Concan	Ogden (OG)	856	274		100	51	50		100	34	34	Gill & Banerjee (1985)
Indiana	Indiana	San Juan (SJ)	814	265			100	97			100	95	Gallione *et al.* (1981; with corrections reported in Gill & Banerjee, 1985)
	Cocal	Mudd–Summers (M–S)	814	265				100				100	Hudson *et al.* (1986)

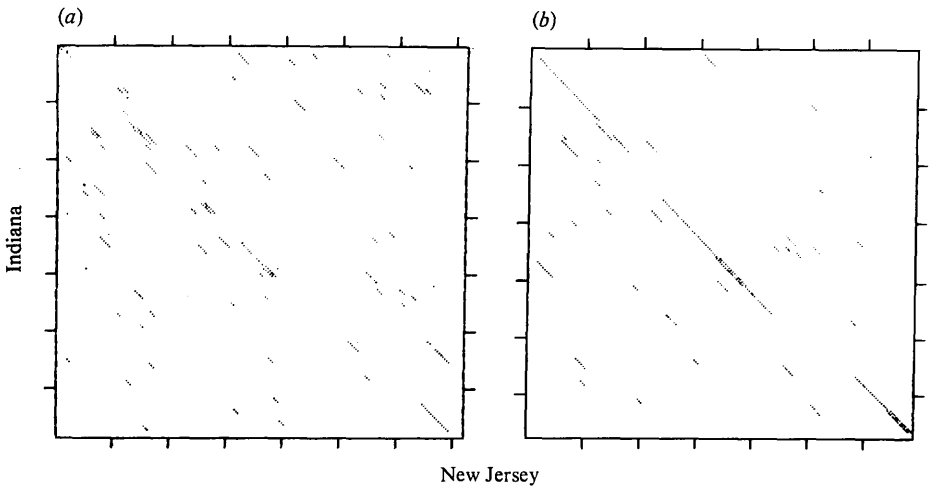

Fig. 3. Computer-generated dot matrix comparisons of (*a*) the nucleotide sequences and (*b*) the amino acid sequences of the NS genes of the Missouri strain (New Jersey serotype) and the San Juan strain (Indiana serotype) of VSV. In (*a*) a window of 12 nucleotides was used and in (*b*) a window of 7 amino acid residues was used. Regions of homology are denoted by a diagonal line, and the level of homology by the thickness of the line (60% homology is the minimum plotted).

M genes of three strains of VSV IND (namely San Juan, Glasgow and Orsay), and reported 1·8% nucleotide and 2·6% amino acid difference between the three strains.

The classification of VSV isolates into the IND and NJ serotypes was based on the finding of little or no reciprocal cross-neutralization of infectivity (Cartwright & Brown, 1972). The IND serotype was further subdivided into four subtypes (Indiana, Argentina, Brazil and Cocal) and the NJ serotype into the Hazelhurst and Concan subtypes on the basis of neutralization, RNA hybridization, oligonucleotide fingerprinting and interference by defective interfering particles (Cartwright & Brown, 1972; Clewley *et al.*, 1977; Crick & Brown, 1973; Federer *et al.*, 1967; Reichmann *et al.*, 1978; Repik *et al.*, 1974). It is apparent from the literature that the various isolates of VSV have been passaged many times in different laboratories (see Clewley *et al.*, 1977; Reichmann *et al.*, 1978) and hence conclusions drawn from sequence comparisons of these laboratory-passaged strains may not be strictly extrapolated to the original isolates. However, the information given above suggests that the IND serotype contains more closely related strains than the NJ serotype. It will be interesting to see whether this trend is maintained when sequence data from other strains become available. However, it is worth noting that Schnitzlein & Reichmann (1985) regard the Hazelhurst subtype as 'an uncommon manifestation of the New Jersey serotype' because of its apparently more limited host range, and this may account for the greater sequence divergence than in the IND serotype.

Fig. 4 is a four-way comparison of the amino acids of the NS proteins which have been aligned using the HOMOL program of Taylor (1984). Three regions of the sequences are of note. First, the regions designated A and C which have relatively high homology (62% and 68%) compared to the overall homology of 33%. The conservation of the amino acid sequences in these regions suggests that they may be of functional significance. Second, the region designated B where there is the most difference between the two NJ strains, and a deletion in the IND sequences was introduced in order to align the proteins. This suggests that this domain is a non-essential or dispensable portion of the protein. It would be of considerable interest to determine whether deletion of this region in the NJ protein affected its function.

Eighteen potential phosphorylation sites (Ser and/or Thr) are conserved between the four NS proteins. This represents 48% of the sites in the Mudd–Summers strain which contains the fewest Ser or Thr residues. None of the aligned potential phosphate acceptors is found in the

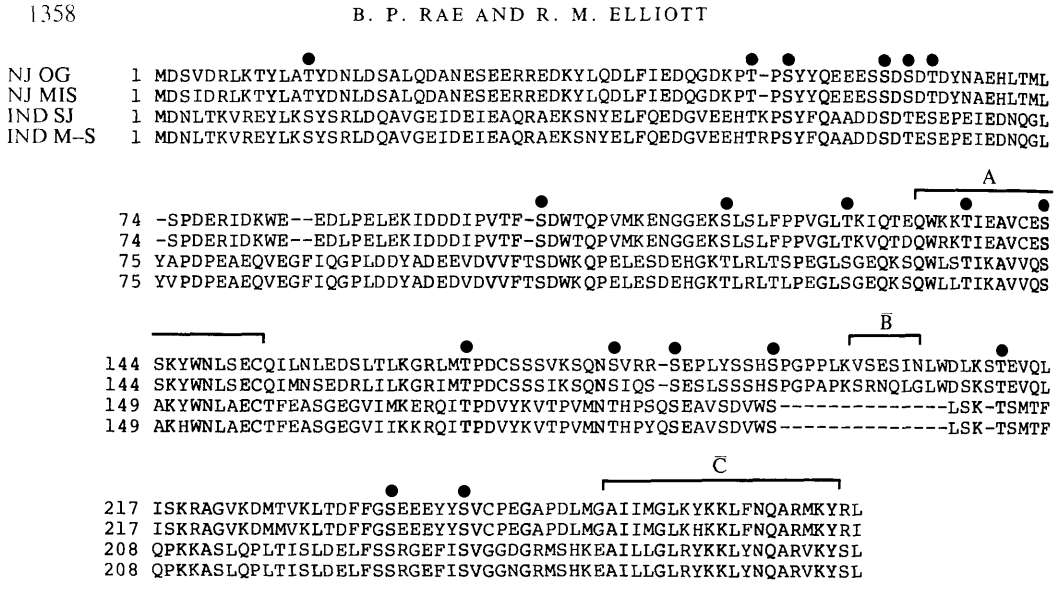

Fig. 4. Alignment of the NS proteins of four strains of VSV using the HOMOL program of Taylor (1984). The shaded regions denote amino acids conserved between the four strains and the filled circles denote conserved potential phosphorylation sites (Ser or Thr). The regions A, B and C are described in the text. OG, Ogden strain, NJ serotype; MIS, Missouri strain, NJ serotype; SJ, San Juan strain, IND serotype; M-S, Mudd-Summers strain, IND serotype.

conserved carboxy terminal domain (region C in Fig. 4). Phosphorylation of the NS protein has been studied in a number of laboratories by analysis of the products generated by enzymic or chemical cleavage of the protein. Hsu *et al.* (1982) reported that NS1 (the less phosphorylated class of NS molecules) was phosphorylated primarily at residues in two chymotryptic peptides, while the more highly phosphorylated molecules contained additional chymotryptic phosphopeptides. Studies by Marnell & Summers (1984) and Bell & Prevec (1985) indicated that the majority of phosphate residues resided in the amino terminal portion of the NS molecule, and Hsu & Kingsbury (1985) localized these residues to a peptide lying between amino acids 35 and 78. As shown in Fig. 4, our comparison reveals five potentially phosphorylated residues in this region which are conserved in the four VSV strains. Hsu & Kingsbury (1985) suggested that the same five sites were constitutively phosphorylated in NS and might be essential for a basal level of NS activity. The availability of a full length cDNA clone for expression in eukaryotic cells and the advent of oligonucleotide mediated site-directed mutagenesis (Zoller & Smith, 1982) will enable us to test this hypothesis experimentally.

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REFERENCES

BANERJEE, A. K., RHODES, D. P. & GILL, D. S. (1984). Complete nucleotide sequence of the mRNA coding for the N protein of vesicular stomatitis virus (New Jersey serotype). *Virology* **137**, 432–438.

BELL, J. C. & PREVEC, L. (1985). Phosphorylation sites on phosphoprotein NS of vesicular stomatitis virus. *Journal of Virology* **54**, 697–702.

BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.

BISHOP, D. H. L. & ROY, P. (1972). Dissociation of vesicular stomatitis virus and the relation of the virion proteins to the viral transcriptase. *Journal of Virology* **10**, 234–243.

CARTWRIGHT, B. & BROWN, F. (1972). Serological relationships between different strains of vesicular stomatitis virus. *Journal of General Virology* **16**, 391–398.

CHIRGWIN, J. M., PRZYBLYA, A. E., MACDONALD, R. J. & RUTTER, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

CLEWLEY, J. P., BISHOP, D. H. L., KANG, C-Y., COFFIN, J., SCHNITZLEIN, W. M., REICHMANN, M. E. & SHOPE, R. E. (1977). Oligonucleotide fingerprints of RNA species obtained from rhabdoviruses belonging to the vesicular stomatitis virus subgroup. *Journal of Virology* **23**, 152–166.

CLINTON, G. M., BURGE, B. W. & HUANG, A. S. (1978). Effects of phosphorylation and pH on the association of NS protein with vesicular stomatitis virus cores. *Journal of Virology* **27**, 340–346.

CRICK, J. & BROWN, F. (1973). Interference as a measure of cross-relationships in the vesicular stomatitis group of rhabdo viruses. *Journal of General Virology* **18**, 79–82.

DE, B. P. & BANERJEE, A. K. (1984). Specific interactions of vesicular stomatitis virus L and NS proteins with heterologous genome ribonucleoprotein template lead to mRNA synthesis *in vitro*. *Journal of Virology* **51**, 628–634.

DE, B. P. & BANERJEE, A. K. (1985). Requirements and functions of vesicular stomatitis virus L and NS proteins in the transcription process *in vitro*. *Biochemical and Biophysical Research Communications* **126**, 40–49.

ELLIOTT, R. M. (1985). Identification of non-structural proteins encoded by viruses of the Bunyamwera serogroup (family Bunyaviridae). *Virology* **143**, 119–126.

EMERSON, S. U. & WAGNER, R. R. (1972). Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis virus B and T virions. *Journal of Virology* **10**, 297–309.

EMERSON, S. U. & YU, Y. H. (1975). Both NS and L proteins are required for *in vitro* RNA synthesis by vesicular stomatitis virus. *Journal of Virology* **15**, 1348–1356.

EVANS, D., PRINGLE, C. R. & SZILÁGYI, J. F. (1979). Temperature-sensitive mutants of complementation group E of vesicular stomatitis New Jersey serotype possess altered NS polypeptides. *Journal of Virology* **31**, 325–333.

FEDERER, K. B., BURROWS, R. & BROOKSBY, J. B. (1967). Vesicular stomatitis virus – the relationship between some strains of the Indiana serotype. *Research in Veterinary Science* **8**, 103–117.

FRANZE-FERNANDEZ, M. T. & BANERJEE, A. K. (1978). *In vitro* RNA transcription by the New Jersey serotype of vesicular stomatitis virus. I. Characterisation of the mRNA species. *Journal of Virology* **26**, 179–187.

GALLIONE, C. J. & ROSE, J. K. (1983). Nucleotide sequence of a cDNA clone encoding the entire glycoprotein gene from the New Jersey serotype of vesicular stomatitis virus. *Journal of Virology* **46**, 162–169.

GALLIONE, C. J., GREENE, J. R., IVERSON, L. E. & ROSE, J. K. (1981). Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus N and NS proteins. *Journal of Virology* **39**, 529–535.

GILL, D. S. & BANERJEE, A. K. (1985). Vesicular stomatitis virus NS proteins: structural similarity without extensive sequence homology. *Journal of Virology* **55**, 60–66.

GOPALAKRISHNA, Y. & LENARD, J. (1985). Sequence alterations in the temperature-sensitive M-protein mutants (complementation group III) of vesicular stomatitis virus. *Journal of Virology* **56**, 655–659.

GUBLER, U. & HOFFMAN, B. J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263–269.

HSU, C. & KINGSBURY, D. W. (1985). Constitutively phosphorylated residues in the NS protein of vesicular stomatitis virus. *Journal of Biological Chemistry* **260**, 8990–8995.

HSU, C., MORGAN, E. M. & KINGSBURY, D. W. (1982). Site specific phosphorylation regulates the transcriptase activity of vesicular stomatitis virus NS protein. *Journal of Virology* **43**, 104–112.

HUDSON, L. D., CONDRA, C. & LAZZARINI, R. A. (1986). Cloning and expression of a viral phosphoprotein: structure suggests vesicular stomatitis virus NS may function by mimicking an RNA template. *Journal of General Virology* **67** (in press).

KINGSFORD, L. & EMERSON, S. U. (1980). Transcriptional activities of different phosphorylated species of NS protein purified for vesicular stomatitis virions and cytoplasm of infected cells. *Journal of Virology* **33**, 1097–1105.

KLAPPER, M. H. (1977). The independent distribution of amino acid near neighbor pairs into polypeptides *Biochemical and Biophysical Research Communications* **78**, 1018–1024.

LEES, J. F., PRINGLE, C. R. & ELLIOTT, R. M. (1986). Nucleotide sequence of the Bunyamwera virus M RNA segment: conservation of structural features in the Bunyavirus glycoprotein gene product. *Virology* **148**, 1–14.

McGEOCH, D. J. (1979). Structure of the gene N:gene NS intercistronic junction in the genome of vesicular stomatitis virus. *Cell* **17**, 673–681.

MARNELL, L. L. & SUMMERS, D. F. (1984). Characterisation of the phosphorylated small enzyme subunit, NS, of the vesicular stomatitis virus RNA polymerase. *Journal of Biological Chemistry* **259**, 13518–13524.

MELLON, M. G. & EMERSON, S. U. (1978). Binding of transcriptase components (L and NS proteins) to the nucleocapsid template of vesicular stomatitis virus. *Journal of Virology* **27**, 560–567.

ONGRÁDI, J., CUNNINGHAM, C. & SZILÁGYI, J. F. (1985*a*). The role of polypeptides L and NS in the transcription process of vesicular stomatitis virus New Jersey using the temperature-sensitive mutant *ts*E1. *Journal of General Virology* **66**, 1011–1023.

ONGRÁDI, J., CUNNINGHAM, C. & SZILÁGYI, J. F. (1985*b*). Temperature sensitivity of the transcriptase of mutants *ts*B1 and *ts*F1 of vesicular stomatitis virus New Jersey is a consequence of mutation affecting polypeptide L. *Journal of General Virology* **66**, 1507–1513.

PRINGLE, C. R. (1986). Rhabdovirus genetics. In *The Viruses: The Rhabdoviruses*. Series edited by H. Fraenkel-Conrat & R. R. Wagner. New York & London: Plenum Press (in press).

PRINGLE, C. R., DUNCAN, I. B. & STEVENSON, M. (1971). Isolation and characterisation of temperature-sensitive mutants of vesicular stomatitis virus, New Jersey serotype. *Journal of Virology* **8**, 836–841.

PRINGLE, C. R., LEES, J. F., CLARK, W. & ELLIOTT, R. M. (1984). Genome sub-unit reassortment among bunyaviruses analysed by dot hybridization using molecularly cloned complementary DNA probes. *Virology* **135**, 244–256.

REICHMANN, M. E., SCHNITZLEIN, W. M., BISHOP, D. H. L., LAZZARINI, R. A., BEATRICE, S. T. & WAGNER, R. R. (1978). Classification of the New Jersey serotype of vesicular stomatitis virus into two subtypes. *Journal of Virology* **25**, 446–449.

REPIK, P., FLAMAND, A., CLARK, H F., OBIJESKI, J. F., ROY, P. & BISHOP, D. H. L. (1974). Detection of homologous RNA sequences among six rhabdovirus genomes. *Journal of Virology* **13**, 250–252.

RHODES, D. P. & BANERJEE, A. K. (1980). Poly(A)-adjacent sequence of the 14·5 S mRNA of vesicular stomatitis virus (New Jersey serotype). *Virology* **105**, 297–300.

ROSE, J. K. (1980). Complete intergenic and flanking gene sequences from the genome of vesicular stomatitis virus. *Cell* **19**, 415–421.

SÁNCHEZ, A., DE, B. P. & BANERJEE, A. K. (1985). *In vitro* phosphorylation of NS protein by the L protein of vesicular stomatitis virus. *Journal of General Virology* **66**, 1025–1036.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 5463–5467.

SANGER, F., COULSON, A. R., BARRELL, B. G., SMITH, A. J. H. & ROE, B. A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *Journal of Molecular Biology* **143**, 161–178.

SCHNITZLEIN, W. M. & REICHMANN, M. E. (1985). Characterisation of New Jersey vesicular stomatitis virus isolates from horses and black flies during the 1982 outbreak in Colorado. *Virology* **142**, 426–431.

SZILÁGYI, J. F. & URYVAYEV, L. (1973). Isolation of an infectious ribonucleoprotein from vesicular stomatitis virus containing an active RNA transcriptase. *Journal of Virology* **11**, 279–286.

TAYLOR, P. (1984). A fast homology program for aligning biological sequences. *Nucleic Acids Research* **12**, 447–456.

ZOLLER, M. J. & SMITH, M. (1982). Oligonucleotide directed mutagenesis using M13 derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucleic Acids Research* **10**, 6487–6500.

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