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DEFECTIVE INTERFERING PARTICLES AND VIRAL INTERFERENCE: A MODEL FOR THE MECHANISM OF HETEROTYPIC INTERFERENCE.

A Thesis Submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
Department of Microbiology and Immunology
School of Medicine

By

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ABSTRACT.

Defective interfering (DI) particles contain deleted viral genomes and interfere with standard virus replication. The majority of vesicular stomatitis virus (VSV) DI particles originate from the 5'-end of the parental virus genome and contain limited intrastrand complementarity. The terminal sequences of DI particle genomes may be crucial for autointerference, however, the molecular mechanism of DI particle mediated autointerference remains unknown.

Among the VSV DI particles characterized to date, DI-LT is unique in two specific ways. First, its genome consists of the 3'-half of the VSV_{IND} genome and it is able to transcribe the positive-strand leader RNA as well as the N, NS, M, and G mRNAs. Second, it is not only able to interfere with the replication of VSV_{IND} (Homotypic interference), but also with VSV_{NJ} (Heterotypic interference). The heterotypic interference activity of DI-LT is limited to the Concan subtype [VSV_{NJ}(Ogden)] of VSV_{NJ}. In order to better understand the mechanism of heterotypic interference mediated by DI-LT, we isolated two DI particles from VSV_{NJ}(Ogden), NJ-PG2 and NJ-121. According to hybridization data these DI particles contained both the positive- and negative-strand leader RNA template regions (like another DI particle, DI-LT2) and they are able to transcribe both leader RNAs and larger RNA transcripts in vitro (unlike DI-LT2). These DI particles were unable to heterotypically interfere with VSV_{IND}. Hence, it is not just the ability of DI-LT to transcribe its genome which allows it to interfere heterotypically. All attempts at cloning the NJ-121 genome (including

PCR amplification in the presence of leader RNA specific oligonucleotide primers) have resulted in the isolation of a NJ-121 clone containing the positive-strand leader RNA template located beside 101 bases of the G protein gene and 1678 bases of the L gene. This indicated that none of the RNA molecules in the NJ-121 RNA preparation contained both leader RNA template regions.

In order to better understand why DI-LT heterotypically interferes only with the Concan subtype of VSV_{NJ}, we sequenced the 5'-end of the VSV_{NJ}(Ogden) genome and compared it to the sequence derived from VSV_{NJ}(Hazelhurst). Since the promoter site of the replicative intermediate (full length positive-strand RNA) is stronger than the promoter site in the negative-strand genomic RNA, it is postulated that differences in the NS protein-binding site of the replicative intermediate may affect the outcome of replication much more than changes in the NS protein-binding site of the negative-strand genomic RNA. The differences within the last 47 nucleotides at the 5'-end VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) (equivalent to the first 47 nucleotides at the 3'-end of the replicative intermediate) were restricted to the NS protein-binding site. It is postulated that the reason why DI-LT interferes with the replication of VSV_{NJ}(Ogden) but not with VSVNJ(Hazelhurst) is due to differences in the affinities of the NS protein-binding sites for their respective NS proteins. A genome with a low affinity interaction with the RNA polymerase would result in lower replicative efficiency when competing with a genome of higher RNA polymerase binding affinity. These results are correlated to differences in the specific requirements of each template (N

protein-RNA complex) for their homotypic NS and L proteins, when reconstituted with heterotypic proteins in *in vitro* transcription assays.

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Lastly I would like to thank my parents, family and wife for providing a loving and supportive living environment and enduring my graduate study years.

This thesis is dedicated to my friend and wife,

Marie Claude Turcotte

and to my parents.

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LIST OF ABBREVIATIONS.

A	Adenine (used as part of a sequence).
Amersham	Amersham Corp., Arlington Heights, Il.
AMV	Avian myeloblastosis virus.
ATP	Adenosine 5'-triphosphate.
B	B broth.
Beckman	Beckman Instruments Inc., Palo Alto, CA.
Bio-Rad	Bio-Rad Laboratories, Richmond, CA.
Bluogal	Halogenated indolyl-β-D-galactoside.
bp	Base pairs.
BRL	Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD.
BSA	Bovine serum albumin.
C	Cytidine (used as part of a sequence).
cDNA	Complementary DNA.
CPM	Counts per minute.
CTP	Cytidine 5'-triphosphate.
dATP	Deoxyribosyladenosine 5'-triphosphate.
dCTP	Deoxyribosylcytosine 5'-triphosphate.
DEAE	Diethylaminoethyl.
dGTP	Deoxyribosylguanine 5'-triphosphate.
DI	Defective Interfering (Particle).
DIFCO	DIFCO Laboratories, Detroit, MI.
DMEM	Dulbecco's modified Eagle's medium.
DMSO	Dimethyl sulphoxide.

DNA	Deoxyribonucleic acid.
dNTP	Deoxyribonucleoside triphosphates.
DTT	Dithiothreitol.
DuPont	E.I. DuPont De Nemours and Co. (Inc.), Wilmington, DE.
E. coli	Escherichia coli.
EDTA	Ethylenediamine tetraacetic acid.
FMC	FMC BioProducts, Rockland, ME.
G	Guanosine (used as part of a sequence).
G Protein	Glycoprotein.
GIBCO	GIBCO Laboratories, Life Technologies, Inc., Grand Island, NY.
GTP	Guanosine 5'-triphosphate.
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.
HR	Heat resistant.
hr	Hour.
IBI	International Biotechnologies Inc., New Haven, CT.
IND	Indiana.
IPTG	Isopropylthio-β-D-galactoside.
i Region	Intergenic region.
K	Kilo (10^3).
Km	Optimal concentration.
L	Polymerase large protein.
l	Leader.

La	Lupus erythematosus protein.
LB	Luria-Burtani medium.
Life Sciences	Life Sciences Inc., St. Petersburg, FL.
M	Matrix protein.
mA	Milliampere.
MEM	Eagle's minimal medium.
min	Minute.
MOI	Multiplicity of Infection.
mRNA	Messenger RNA.
N	Nucleocapsid protein.
βNAD	β-Nicotinamide Adenine Dinucleotide.
New England Biolabs	New England Biolabs Inc., Beverly MA.
NEN	New England Nuclear Research Products, Boston, MA.
NJ	New Jersey.
NP40	Nonidet P-40.
N-RNA	N protein bound RNA.
NS	Phosphoprotein.
P	Phosphoprotein.
PCR	Polymerase chain reaction.
PEG	Polyethylene glycol.
PEI	Polyehtyleneimine.
PFU	Plaque-forming-unit.
Pharmacia	Pharmacia Biotechnology International, Uppsala, Sweden.
PK	Polynucleotide kinase.

Pol 1	Escherichia coli DNA polymerase 1.
Poly(A)	Polyadenosine.
PPO	2,5-Diphenyloxazole.
Promega Biotech	Promega Corp., Madison, WI.
PS	Phopsphatidylserine.
PVP	Polyvinylpyrrolidone.
RNA	Ribonucleic acid.
RNasin	Ribonuclease inhibitor.
RNP	Ribonucleoprotein complex.
RPM	Rotations (or revolutions) per minute.
RT	Reverse transcriptase.
Schleicher + Schuell	Schleicher + Schuell Inc., Keene, NH.
SDS	Sodium dodecylsulphate.
Sigma	Sigma Chemical Co., St. Louis. MO.
SSC	Standard saline citrate.
STD	Standard.
T	Deoxyribosylthymine.
TAQ	Thermus aquaticus.
Tm	Melting temperature.
t Region	Trailer region.
tRNA	Transfer RNA.
TTP	Deoxyribosylthymine 5'-triphosphate.
U	Uridine.
USB	United States Biochemical Corp., Cleveland, OH.
UTP	Uridine 5'-triphosphate.
U.V.	Ultraviolet.

VS	Vesicular Stomatitis.
VSV	Vesicular Stomatitis Virus.

1. INTRODUCTION

Defective interfering virus (DI) particles play a major controlling role in the replication of virus. They are constantly generated at low levels by infectious virus and only amplify to interfering levels when the parent helper virus is abundant (Holland, 1987). This phenomenon, known as autointerference, is achieved by rearrangements and deletions of the standard virus genome in such a way that the resulting incomplete form of the virus can be preferentially replicated. It is generally understood that RNA viruses, except Retroviruses, lack the ability to integrate their RNA into the host genome in the form of double stranded proviral DNA. RNA viruses must therefore rely on alternative mechanisms of attenuation, such as DI particles, to achieve long term virus-host cell persistence. This aspect and the fact that DI particles are generated by defective replicative complexes that rearrange the virus genome makes their study very intriguing. Vesicular Stomatitis Virus (VSV) has been particularly useful for the study of many aspects of the generation of and interference mediated by DI particles. VSV is a unique model for the study of DI particles because the standard virus can be easily separated from DI particles by velocity-gradient centrifugation and it grows to high titer in a wide variety of host cells. Amongst the wide variety of VSV DI particles, DI-LT (originating from virus from the Indiana serotype of VSV, VSV_{IND}) has the special ability of not only interfering with VSV_{IND} (Homotypic interference), but also with VSV_{NJ} (Heterotypic interference). The following chapter will expose the biology, genome, genome products and

DI particles of VSV in order to better understand the molecular aspects of DI particle mediated heterotypic interference.

1.1 Biology of Vesicular Stomatitis Virus (VSV).

1.1.1 Historical Review.

VSV was first reported as a disease of horses and mules in South America in the late nineteenth century (Theiler,1901). The disease symptoms resemble those of foot and mouth disease. Affected animals show elevated temperature with loss of appetite and abnormal salivation. Vesicular lesions occur around the mouth, tongue, hoofs and udders. Unlike foot and mouth disease, VSV disease is seldom fatal (Howartson, 1970).

VSV is classified as a member of the family Rhabdoviridae (Matthews, 1982). VSV is the prototype member of the Vesiculovirus genus and is divided into two serotypes, Indiana (IND) and New Jersey (NJ) (Cartwright and Brown, 1972; Frazier and Shope, 1979). This division is based upon the serological relationships between the viral specific proteins. The major cross-reactive protein is the nucleocapsid (N) protein, whereas type specificity is associated with the glycoprotein (G). The Indiana serotype has been further subdivided into four distinct but antigenically related subtypes: Indiana, Argentina, Brazil, and Cocal (Federer, et al., 1967; and Cartwright and Brown, 1972). The New Jersey serotype has been subdivided into two closely related subtypes, Concan and Hazelhurst (Reichmann, et al., 1978). The subdivisions were based upon reciprocal differences in antibody neutralization of virion infectivity, nucleotide base sequence

homology, oligonucleotide maps of virion RNA, and interference by DI particles. In 1925 cattle arriving in Richmond, Indiana, from Kansas City showed symptoms of the disease which was later transmitted to horses. The infectious agent was isolated from the latter and preserved by animal transfer. This strain is now known as the Indiana serotype. In the following year, an outbreak occurred in New Jersey. Virus isolated from infected animals was found to be serologically distinct from VSV_{IND}. This strain is now known to be the New Jersey serotype.

VSV_{IND} has been extensively studied at the molecular level; however, VSV_{NJ} is the more important cause of disease, being responsible for periodic and unpredictable major epizootics in cattle, horses, and swine throughout the Americas (Hanson, 1952; Hanson,1986; and Mason, 1986). Historically, VSV_{NJ} epizootics in the United States have appeared at approximately ten year intervals. Virus infections first appear in early summer and then disappear with the first frost in early winter. Extensive enzootic virus activity also exists in regions of Mexico, Central America, and South America (Mason, 1986). In these areas, virus activity persists, with regular appearance of the disease in cattle populations. In addition, annual identification of VSV_{NJ} seropositive wildlife, particularly feral swine, in the southeastern Unite States, has indicated that limited enzootic virus activity also exists in this region (Fletcher et al., 1985; Hanson and Karstad, 1959; Stallnecht, et al., 1985; and Stallnecht, et al., 1986).

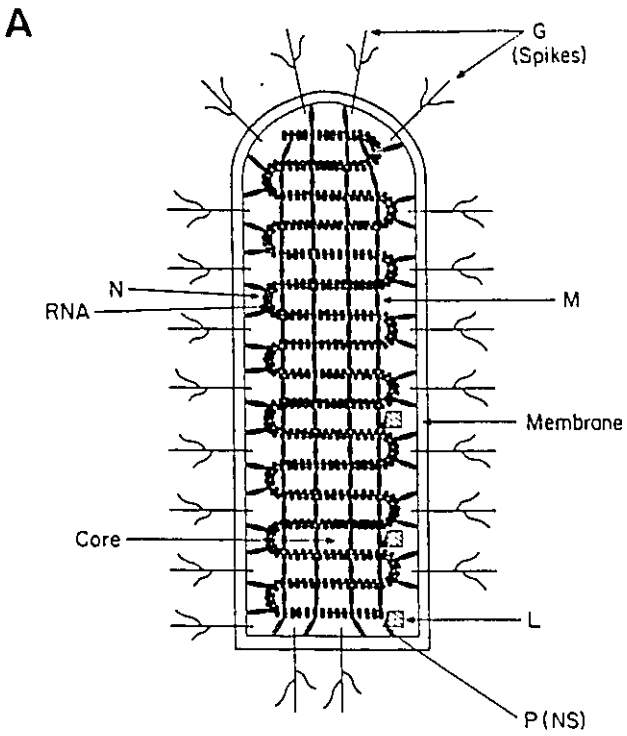
The natural virus reservoir, the mechanism of maintenance of virus in enzootic regions, and the mode of virus transmission are still

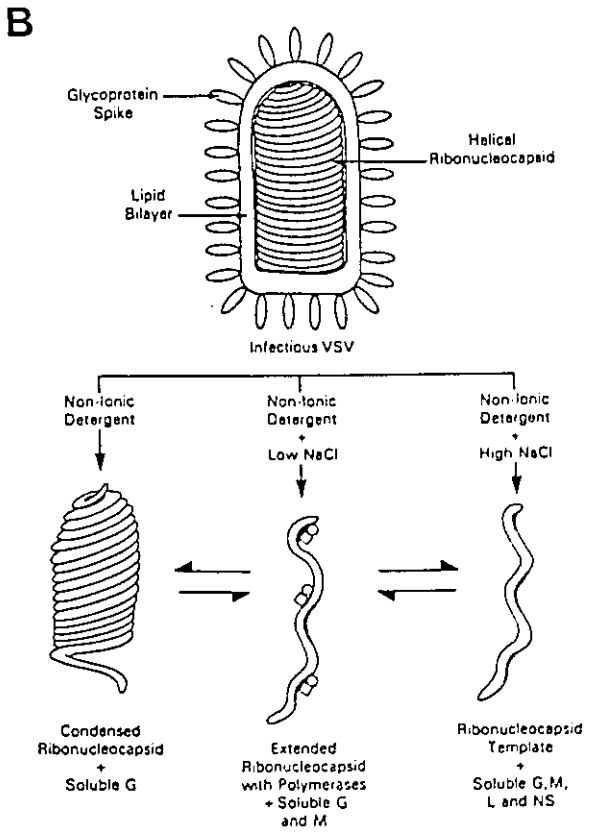
unidentified. Possible insect involvement in the transmission and maintenance of the disease has been suggested based on: the typical seasonal nature of VSV_{NJ} epizootics (Hanson, 1952), numerous VSV isolates from insects (Francy, 1986; Kramer, et al., 1986; Shekokov and Peralta, 1967; and Sudia, et al., 1967), demonstration of experimental transmission of VSV_{IND} by horseflies, mosquitos, and sandflies (Ferris, et al., 1955; and Tesh, et al., 1972), and transovarial transmission of VSV_{IND} in sandflies (Tesh, et al., 1972). VSV in man is a severe influenza-like disease that is rarely fatal and is almost invariably caused by laboratory infection or by transmission from infected animal carcasses (Wagner, 1987).

1.1.2 Virion and its Components.

The first morphological studies on VSV were made by Chow et al. (1954) and Bradish et al. (1956). Figure 1a shows a schematic representation of the morphology and structural components of the virion of VSV. The term "Rhabdovirus" was adopted to group together viruses having a bullet or bacilliform morphology by the International Committee on Nomenclature (Wildy, 1971). The infectious (standard) virion is bullet-shaped, rounded at one end and flat at the other, hence the term B particles was coined to describe them. They measure about 180 nm in length and 65 nm in width. Orenstein et al.(1976) contend that the bullet shape is an artifact of fixation and staining and that the infectious particles are bacilliform with two rounded ends, although they agree that the internal nucleocapsid is itself bullet-shaped. It is very common to note the presence of smaller,

Figure 1. Schematic representation of the morphology and structural components of VSV. (A) The virion is composed of two major structural features: the ribonucleocapsid core, composed of the genomic RNA tightly bound by the N (Nucleocapsid) protein and the two RNA polymerase complex components, the L and NS proteins; and the envelope composed of a cell derived lipid bilayer membrane associated with the integral membrane glycoprotein (G) and the peripheral matrix (M) protein (Wagner, 1987). (B) The controlled disruption of the virion by non-ionic detergent (Triton X-100, or Nonidet P-40) and NaCl. The G protein is released by the detergent alone, leaving a M protein bound ribonucleocapsid in its helical conformation. As the NaCl concentration is progressively increased, the M protein is released with a loss of the helical conformation and finally the L and NS proteins are released (Emerson and Schubert, 1987a). These proteins may be added back to the ribonucleocapsid template to reconstitute polymerase activity (adding L and NS proteins) or to shut off polymerase activity (adding the M protein).


A
G
(Spikes)
N
RNA
M
Membrane
Core
L
P (NS)


B
Glycoprotein
Spike
Helical
Ribonucleocapsid
Lipid
Bilayer
Infectious VSV
Non-Ionic
Detergent
Non-Ionic
Detergent
+
Low NaCl
Non-Ionic
Detergent
+
High NaCl
Condensed
Ribonucleocapsid
+
Soluble G
Extended
Ribonucleocapsid
with Polymerases
+ Soluble G
and M
Ribonucleocapsid
Template
+
Soluble G, M,
L and NS

truncated (T) or defective interfering (DI) particles in uncloned preparations of VSV. These particles are the same width as B particles, however, their length varies from 50 to 80 nm, depending upon the amount of viral RNA that they have retained during their generation (Huang and Baltimore, 1977). It has been demonstrated that the DI particles can be separated from infectious B particles by sucrose velocity-gradient sedimentation (Huang, et al., 1966b; Crick, et al., 1966; and Hackett, et al., 1967). DI particles sediment much slower during the centrifugation due to their smaller size.

Electron micrographs of nonionic detergent disrupted virions shows that the virion contains a long helical nucleocapsid coiled in a regular manner to give the virion its characteristic shape. Figure 1b indicates that the coiled nucleocapsid retains its shape in the absence of salt but is uncoiled in the presence of increasing salt concentrations (Newcomb and Brown, 1981). These and other studies have led to the hypothesis that the secondary structure of the VSV nucleocapsid is due to electrostatically bound matrix (M) protein, which dissociates from the nucleocapsid in the presence of a hypertonic solution (Emerson and Wagner, 1972; Newcomb and Brown, 1981; and Newcomb, et al., 1982).

The approximate composition of the VSV virion is 74% protein, 20 % lipid, 3% carbohydrate and 3% RNA (Wagner, 1975). The condensed helical nucleocapsid is enclosed within a lipoprotein bilayer which is composed of 50% lipid and 50% protein (McSharry and Wagner, 1971; and Patzer, et al.,1978). The lipids are derived from the infected host cell, but are selected in different proportions than those of the

plasma membrane. The principal lipid differences are a larger proportion of cholesterol and, among the phospholipids, much less phosphatidylcholine compared to sphingomyelin and a larger amount of amino phospholipids. This altered lipid composition contributes significantly to the greater rigidity of the VSV membrane compared to that of the host-cell membrane from which it was derived (Barenholz, et al., 1976).

The VSV membrane contains two proteins: an externally oriented, integral glycoprotein (G) and a peripheral matrix (M) protein which lines the inner surface of the virion membrane (Patzer et al., 1979; and Zakowski and Wagner, 1980). The G protein: is N-glycosylated at two asparagine residues, is the major antigenic determinant responsible for type specificity, and gives rise to neutralizing antibody (Kelley, et al., 1972; and Volk, et al., 1982). The G protein of VSV_{IND} has 1-2 molecules of the fatty acid palmitate esterified at a cysteine residue. The function of the fatty acid is unknown but it is postulated that it may have a role in the transport of the G protein to the cell surface (Schlesinger and Malfer, 1982). The fatty acid acylation of the G protein seems to be a non-essential event (Gallione and Rose, 1983). The M protein seems to serve as an attaching component that binds the nucleocapsid to the cell plasma membrane where the G protein is inserted. The M protein is quite basic (pI~9.1) and inhibits transcription by binding to the nucleocapsid (Carrol and Wagner, 1979; and Wilson and Lenard, 1981). The M protein also binds to acidic phospholipid headgroups by which means it appears to attach the nucleocapsid-M protein complex to phosphatidylserine residues lining

the inner surface of virion membrane (Zakowski, et al., 1981; and Wiener, et al., 1983). The characteristics of these proteins and those of the nucleocapsid are summarized in Table 1.

The genetic information of VSV is contained within an unsegmented single strand of RNA that cannot serve as a messenger RNA and hence is called a negative stranded RNA. The single stranded RNA in infectious VSV_{IND} virions has a molecular weight of 3.68×10^6 and contains 11,162 nucleotides (Schubert, et al., 1984). The genome organization will be discussed in section 1.1.3. The genomic RNA is itself not infectious (Huang and Wagner, 1966a).

Associated with the genomic RNA are three proteins which together form the nucleocapsid, which is infectious (Szilagyi and Uryvayev, 1973; and Emerson and Yu, 1975) at a level of 10^{-5} to 10^{-6} that of the virion. Tightly associated with the RNA is the nucleocapsid (N) protein, present in the virion at a level of 1258 molecules (Thomas, et al.,1985). There is an estimated one molecule of N protein for every nine nucleotides of RNA. The RNA within VSV nucleocapsids is completely resistant to digestion with various ribonucleases even when the nucleocapsids are previously treated under high salt conditions (Emerson and Wagner, 1973).

The infectious nucleocapsid also contains two minor proteins: the large (L) protein and the NS protein, originally, but mistakenly called a nonstructural protein. These proteins are present at levels of 50 and 466 molecules per nucleocapsid, respectively (Thomas, et al., 1985). Together with the nucleocapsid template (N-RNA) they form the endogenous RNA-dependant RNA polymerase. The L protein is believed to

Table 1. Properties of VSV mRNAs and Proteins. This table was constructed using data accumulated from several sources. The values within parenthesis are the corresponding values for VSV_{NJ}. The mRNA length, predicted amino acids, predicted molecular weight, and percentage amino acid homology values for each protein were taken from the following sources: N (Gallione, et al., 1981; Banerjee, et al., 1984), NS (Gallione, et al., 1981; Gill and Banerjee, 1985), M (Rose and Gallione, 1981; Gill and Banerjee, 1986), G (Rose and Gallione, 1981; Gallione and Rose, 1983) and L (Schubert, et al., 1984; Feldhaus and Lesnaw, 1988). The number of molecules per virion was calculated by Thomas et al. (1985) for VSV_{IND} virions. The protein characteristics and functions were summarized in Emerson and Schubert (1987).

Protein	mRNA Length*	Predicted Amino Acids	Predicted Molecular Weight	Percentage Homology*****	Molecules Per Virion	characteristics	Function
N	1326 (1329)	422 (422)	47,355 (47,898)	68	1258	Self-aggregates	Encapsidates genomic RNAs. Anti-terminator of replication.
NS(P)	814 (856)	265 (274)	29,878 (31,406)***	32	466	Heterogeneously phosphorylated.	Promotes L binding to nucleocapsid. Polymerase component. Prevents N self-aggregation?
M	831 (758)	229 (229)	26,064 (26,229)	62	1826	Basic. Interacts with Lipids, G and N.	Condenses nucleocapsid. Inhibits transcription. Binds to acidic phospholipid headgroups to attach M-bound nucleocapsid to Phophatidylserine. Clusters G in plasma membrane?
G	1665 (1573)	511 (517)**	57,416 (58,229)****	51	1205	Transmembrane glycoprotein.	Cell attachment. Endosome fusion. Budding.
L	6373 (6391)	2109 (2109)	241,012 (241,581)	65	50	Genetically and physically labile.	Catalytic unit of polymerase. Recognition of signal Sequences. Capping, Methylation, and Polyadenylation.

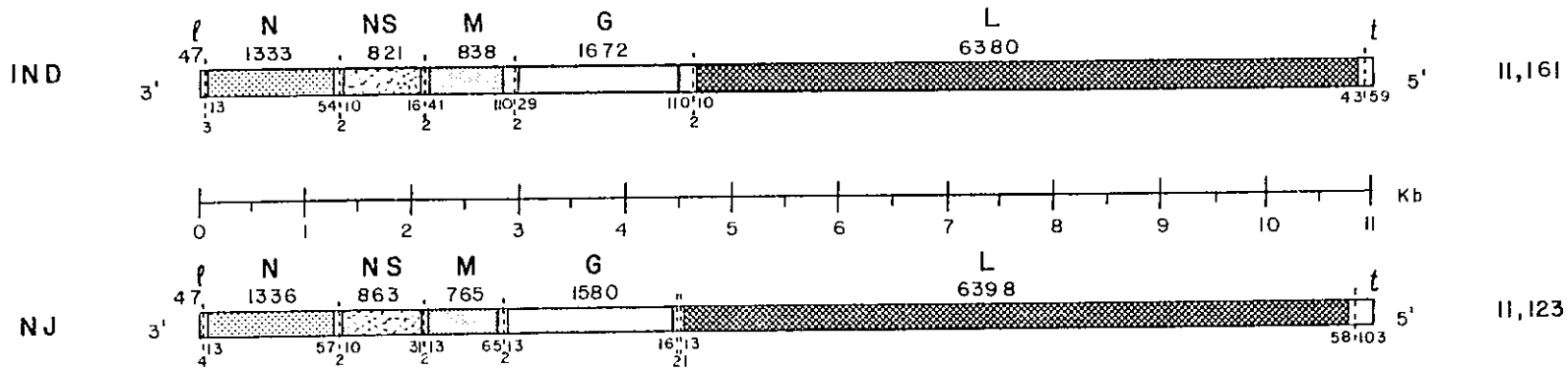
* Without a pol(A) tail.
** Precursor molecule.
*** Nonphosphorylated.
**** Nonglycosylated.
***** Homology between the amino acid sequence of VSV_{IND} and VSV_{NJ}.

be the catalytic subunit of the RNA polymerase. The NS protein is highly phosphorylated and appears to exist in at least two forms depending upon the degree of phosphorylation (Clinton, et al., 1978; and Kingsford and Emerson, 1980). Phosphorylation of the NS protein is thought to be carried out by host-cell protein kinases (Imblum and Wagner, 1974; and Clinton, et al., 1982; Bell, et al., 1984) and possibly by the L protein (Sanchez, et al., 1985). These kinases determine the distribution of phosphorylation amongst threonine and serine residues of the protein (Clinton and Huang, 1984; Clinton and Finley-Whelan, 1984; and Bell, and Prevec, 1985). The NS protein name will soon be changed to P protein due to its highly phosphorylated state (Wagner, 1987). In this thesis, the old name will be used as it is the name used in most of the literature. The L and NS proteins can be dissociated from the nucleocapsid in an high-ionic-strength environment, which results in the loss of infectivity and RNA transcriptase activity. Transcription and infectivity can be restored upon reconstituting the L and NS proteins with denuded nucleocapsids in a low-ionic-strength environment (Emerson and Wagner, 1972, 1973). The N protein-RNA complex in association with the L and NS proteins are essential for transcriptase activity (Emerson and Yu, 1975).

1.1.3 Genome Organization.

VSV encodes five known gene products (Figure 2). The gene order (3'-N-NS-M-G-L-5') is highly conserved amongst Rhabdoviruses. The transcriptional mapping of the VSV genes by UV irradiation revealed that transcription of the viral genes is carried out sequentially. It

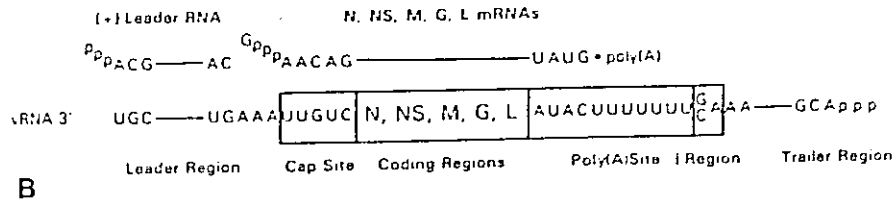
Figure 2. **Maps of the VSV_{IND} and VSV_{NJ} genomes.** (A) The genomes are represented in a 3'- 5' orientation from left to right. The numbers above the bars indicate the nucleotide lengths of the leader (l) RNA and the mRNAs, including the first seven nucleotides of the poly(A) site on each mRNA that is encoded by the genome [note, the mRNAs usually have an additional 200 A-residues on their 3'-ends (Rose, et al., 1977)]. The shaded portions of the bars represent the protein-coding portions of the genomes, and the open bars represent the non-coding portions. The numbers immediately below the bars indicate the lengths of the 5'and 3' untranslated portions of each mRNA (including the stop codon). The dotted lines with numbers indicate the positions and lengths of the intergenic regions. The nontranscribed "trailer" (t) region of the genome is indicated at the extreme 5'-end of the genome. The % nucleotide similarity for each gene, between the two serotypes is indicated below and left of the bars. (B) The highly conserved VSV transcriptional sequences. Each VSV gene starts with a 3'-terminal cap site which contains five conserved nucleotides and terminates with a conserved poly(A) site containing seven uridine residues which are repeatedly transcribed into approximately 200 nucleotide long poly(A) tails. The poly(A) site is followed by the intercistronic (i) region which consists of the conserved sequence, GA, in all but the NS-M junction in VSV_{IND}, which contains, CA. The VSV_{NJ} G-L junction contains a 19-base insertion between the nontranscribed dinucleotide, and the cap site (Luk, et al., 1987). The 3'-terminal and 5'-terminal extracistronic leader and "trailer" regions are indicated. The leader region is transcribed into a 47 nucleotide positive-strand leader RNA. There are 3 adenine residues between the leader transcription termination site and the cap site of the N mRNA in VSV_{IND} and 4 adenine residues in VSV_{NJ} (McGeoch, et al., 1980; Rowlands, et al., 1979). The trailer region is not transcribed and is variable in length.



% Nucleotide similarity

N	68 %
NS	41 %
M	57 %
G	54%
L	62 %

A



B

was found that the UV-target sizes for the transcription of each gene were independent of the size of each gene, but dependent upon the position of the gene in the genome. The gene order of VSV_{IND} was determined by this method (Abraham and Banerjee, 1976; Ball and White, 1976). RNA duplex mapping, using genomic RNA and isolated RNAs, confirmed these results (Herman, et al., 1978). The gene order was ultimately proven by the results of genomic RNA and mRNA cloning and sequencing (Rose and Schubert, 1987). Figure 2a shows the gene order for VSV_{IND} and VSV_{NJ}.

The genomic size and gene order of Rhabdoviruses are highly conserved. The genes are transcribed sequentially, starting with the 3' end of the genome. Attenuation of transcription at each gene junction results in a decrease in the amount of mRNA sythesized, as the distance from the 3'-terminal start site of transcription increases (Iverson and Rose, 1981). The N protein, needed in the largest amount to completely encapsidate the genomic and antigenomic RNAs, is always encoded by the first gene, while the catalytic polymerase L protein is always encoded by the last gene. Therefore, the organization of the genome together with the sequential attenuation of transcription ensures that each gene product is synthesized in the ratios needed during the course of infection and makes VSV a highly efficient transcriptional unit.

From the 11,161 nucleotides of the VSV IND genome, 11,091 nucleotides (99.4%) are transcribed and a total of 10,608 nucleotides (95%) encode the five structural proteins (Emerson and Schubert, 1987a). The extracistronic regions consist of the 50 nucleotide 3'

terminal leader (l) region, which is transcribed into a 47 nucleotide plus-strand leader RNA (Colonno and Banerjee, 1976), and the 59 nucleotide untranscribed 5'-terminal trailer (t) region (Schubert and Lazzarini, 1981). The untranscribed short intercistronic (i) regions consist of only two nucleotides G/CA (Figure 2b), in all but the intercistonic region between the G and L genes in VSV_{NJ}(Ogden), where there is an additional 19 nucleotides (Luk, et al., 1987). The intercistronic regions are flanked directly upstream by the conserved polyadenylation signal, AUACUUUUUU, of the previous gene and directly downstream by the message start sequence (cap site), UUGUC, of the next gene. The 3'-terminus of the genomic RNA contains the origin of replication. A complementary sequence is present at the genomic 5'-end which allows the replication of the anti-genome. During replication all of the signals for transcription termination and reinitiation are ignored to allow the replication of the entire genome. Recently, there has been the discovery of an additional protein, the 7K protein, which has been detected from the in vitro translation of NS mRNA (Herman, 1986). It is translated from the same reading frame as the NS protein and contains the same carboxyl terminal amino acids as the NS protein. The start codon for the 7K protein is situated closer to the 3'- rather than the 5'-end of the mRNA. The function of the 7K protein is unknown. The VSV genome is therefore very compact and very efficiently expressed and replicated in an autoregulatory fashion.

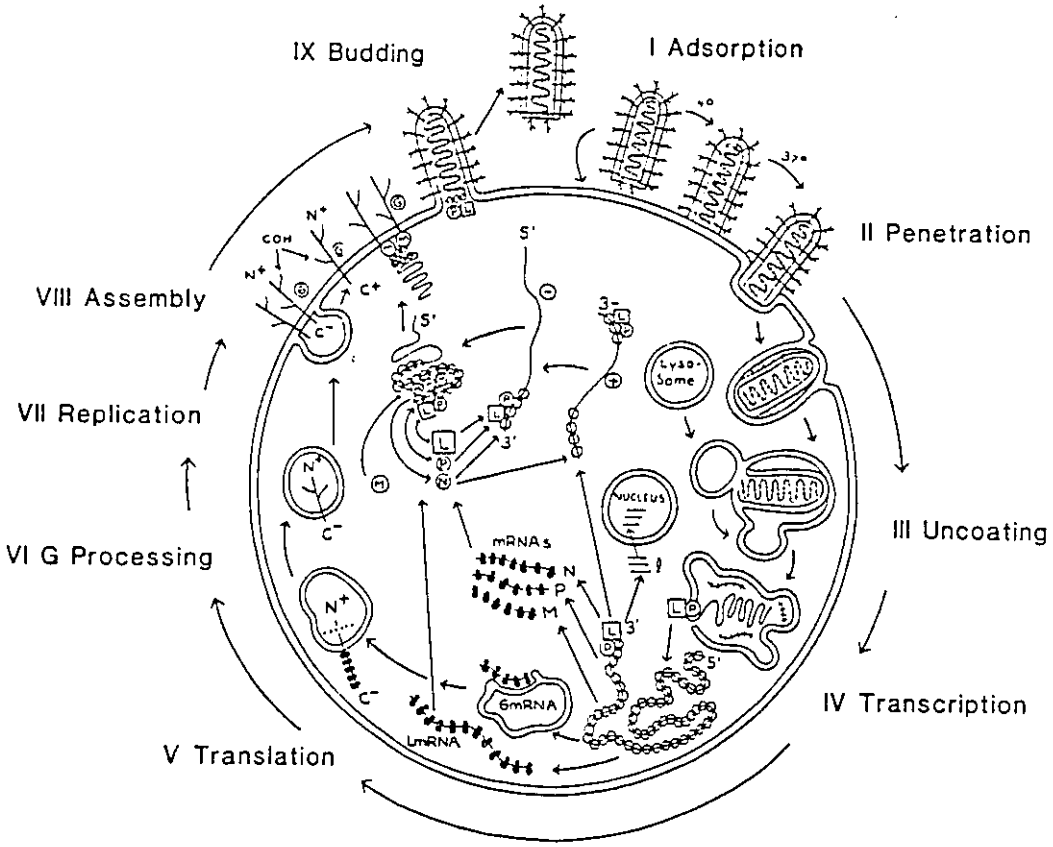
1.1.4 Cycle of Infection.

Figure 3 and the following summary of the sequence of events in the cycle of infection of a susceptible cell with VSV were taken largely from the review of Wagner (1987). Under standard conditions, VSV infection of a monolayer of cells begins with a latent period of about two hours, during which time there are no progeny virus particles detected. Following the latent period is an exponential phase of multiplication and release of progeny to a peak of approximately 1,000 infectious virions per cell at 6-8 hr postinfection (Wagner, 1975). Maximal virus production depends upon: cells (BHK_{21} cells yield 5-10 times as much virus as mouse L cells); cell media (optimal growth conditions for cells); temperature of incubation (virus replicates faster at 37°C, but yields more virus at 31°C); input multiplicity of infection (input multiplicity of 5-10 plaque-forming-units (PFU)/cell ensures infectivity of more than 99% of the cells); serotype of virus (VSV_{IND} grows faster and yields more progeny than VSV_{NJ}) and lastly on the presence of DI particles (DI particles, by their definition will reduce the yield of progeny infectious virions). The important aspects of each component of the infective cycle will be discussed below.

1.1.4.a Adsorption.

VSV adsorption is quite inefficient and difficult to quantitate partially due to the ratio of physical particles to infectious viral particles which is rarely less than 5:1. The attachment to target cells is mediated by the viral glycoprotein (G) (Wagner, 1975). Strong evidence for the interaction of VSV G protein with the surface membrane

Figure 3. The sequence of events in the cycle of infection of a susceptible cell with VSV. The infection begins with a virion adsorbing to the cell surface, penetrating into the cytoplasm and being uncoated after lysosome fusion. The uncoated nucleocapsid then begins to transcribe mRNAs, which are translated into proteins which accumulate in the cytoplasm and lead to the switch from transcription to replication and production of daughter nucleocapsids. The daughter nucleocapsids are then either bound by matrix (M) protein and assembled into virions by budding through the plasma membrane in regions of G protein accumulation, or they are transcribed (secondary transcription) to produce more mRNAs (Wagner, 1987). The sequence of events associated with the infection cycle of VSV are discussed in detail in the text. This figure was taken from Wagner (1987).


IX Budding
I Adsorption
II Penetration
III Uncoating
IV Transcription
V Translation
VI G Processing
VII Replication
VIII Assembly
mRNAs
N
P
M
6mRNA
LmRNA
Lyso-Some
NUCLEUS

of cells comes from the experiments on fusion from within, by which one VSV-infected cell can fuse with an uninfected cell by virtue of the G protein inserted into the plasma membrane, particularly when the virion maturation is blocked in cells infected with temperature-sensitive M protein mutants (Storey and Kang, 1985).

The host cell receptor has not yet been conclusively identified, but because VSV infects cells from very divergent species the receptor must be a common component of almost all cell plasma membranes. Exposure of L cells to neuraminidase or trypsin does not affect adsorption of VSV, a finding that eliminates sialoglycoproteins, sialoglycolipids, or other easily accessible surface proteins as major receptors for VSV adsorption (Schloemer and Wagner, 1975). Similar studies with Vero monkey cells indicated that trypsinization had no affect on the attachment of VSV to cells at 4°C (Schlegel, et al., 1982). On the basis of dose-response curves and Scatchard plots of binding affinities, studied under conditions of excess and nonexcess input, it was revealed that there were two distinct interactions between Vero cells and VSV, indicating separate saturable and nonsaturable sites for attachment (Schlegel, et al., 1982). Prior exposure to trypsin did not diminish nonsaturable binding. Saturable high affinity binding of VSV to Vero cells is inhibited by phosphatidylserine so this membrane lipid may constitute all or part of the VSV receptor (Schlegel, et al., 1983).

1.1.4.b Penetration and Uncoating.

Penetration of host cells closely follows adsorption by a series of events that are not quite clear. Soon after penetration the membrane envelope is removed by an uncoating event. These two events are not readily dissociable and can be considered as one event. Whereas the adsorption to cells in an energy independent event, penetration and uncoating is energy dependent. Unlike paramyxoviruses, VSV does not induce membrane fusion at neutral pH and viral entry into cells is achieved via endocytosis (Matlin, et al., 1982). It has been postulated that VSV and α_2-microglobulin share the same receptor for endocytosis by coated pits (Dickson, et al., 1981).

Electron micrographs demonstrate that VSV accumulates on the cell surface in clathrin coated pits. The virus is engulfed within a coated vesicle, then proceeds to large uncoated collection vacuoles and finally to secondary lysosomes (Lenard and Miller, 1982; White, et al., 1983) which maintain an acidic pH. At this pH the VSV glycoprotein exhibits membrane fusion capacity (Florkiewicz and Rose, 1984). It is believed that the acidic environment of the lysosomes induce glycoprotein mediated fusion of the virion envelope with the lysosomal membrane such that the ribonucleocapsid (RNP) complex is uncoated and is expelled into the cytoplasm. Much of the evidence for the role of the lysosome comes from the use of antiviral lysosomotropic amines which cause a rapid rise in the pH of the lysosomal contents.

1.1.4.c Transcription.

Primary transcription is the first viral metabolic event after cells are penetrated by VSV and their nucleocapsids are released into the cytoplasm. Much of what is understood about VSV transcription is derived from *in vitro* studies. Baltimore *et al*. (1970) first described the virion-associated RNA-dependent RNA polymerase and worked out the basic conditions for VSV transcription *in vitro*. It was later shown that detergent-disrupted purified virions (Bishop, 1971; Moyer and Banerjee, 1975), purified RNP from detergent-disrupted virions (Abraham and Banerjee, 1976; Szilagyi and Uryvayev, 1973) or RNP isolated from infected cells (Galet and Prevec, 1973; Toneguzzo and Ghosh, 1976) are all capable of synthesizing RNA *in vitro* in the presence of four ribonucleoside triphosphates. For optimal transcription, the requirements for adenosine triphosphate (ATP) is ten times higher (K_m = 0.5mM) than for the other three ribonucleoside triphosphates (K_m = 0.05mM) (Testa and Banerjee, 1979). The high requirement for ATP is probably due to its role in initiation of RNA synthesis, polyadenylation and the phosphorylation of the NS protein (Emerson, 1987). The enzymology of the VSV transcriptase was described by Emerson and Wagner (1972, 1973). The N protein-RNA complex, the NS and L proteins are required to function as RNA template and RNA polymerase complexes, respectively.

The N protein is absolutely required for transcription, since neither deproteinized genomic RNA nor various synthetic ribonucleotide polymers will serve as a template for the viral transcriptase (Emerson and Wagner, 1972). One function of the N protein is probably to keep

the genomic RNA in an extended form (by preventing any base pairing) suitable for transcription. The N protein also protects the genomic RNA from nuclease digestion. Therefore, the N protein alone or in combination with the polymerase complex must interact with every nucleotide in the genome. Footprinting studies have shown that the methylation patterns of deproteinized and N-encapsidated genomic RNA are identical (Keene, et al., 1981c). Therefore, the N protein does not interact with the bases directly, but most likely with the phosphate backbone of the RNA.

The L and NS proteins from homologous or heterologous virions are both required for the full function of the transcriptase complex (De and Banerjee, 1984, 1985; Emerson and Yu, 1975; Mellon and Emerson, 1978; Naito and Ishihama, 1976). An important parameter of the in vitro transcription system is the requirement for NaCl. Using detergent-disrupted virions and lowering the optimal salt concentration (0.1M to 0.02M) results in the cessation of RNA synthesis in vitro. In contrast, RNA synthesis by purified RNP free of G and M proteins was found to be insensitive to salt concentration reduction (De, et al., 1982; Pinney and Emerson, 1982a). It was later found that the M protein stopped RNA synthesis by interacting directly with the RNP complex and it was postulated that the M protein may play a role in the regulation of transcription within the cell.

The observation that VSV was able to catalyze in vitro transcription with highly purified RNP components lead to the assumption that host cell factors were not necessary in this process. However, stimulation of RNA synthesis in vitro by the addition of host

cell extracts has been reported (Ball and White, 1978; Rose, et al., 1977). Recently, β-tubulin has been implicated as a positive transcription factor for VSV since in vitro mRNA synthesis is completely abolished by the addition of anti-β-tubulin antibodies (Moyer, et al., 1986). In another set of experiments it was determined that β-tubulin could functionally replace the highly charged amino half of the NS protein in in vitro transcription assays (Chattopadhyay and Banerjee, 1987). In addition, the L protein has been shown to be associated with β-tubulin when the latter is immunoprecipitated from infected cells (Chattopadhyay and Banerjee, 1987).

Reconstitution of transcription experiments using purified N protein-RNA templates, L and NS proteins, demonstrated that the NS protein by itself could bind to saturation on the template, while L protein was unable to bind to template unless NS protein was also present (Mellon and Emerson, 1978). Thus it appears that one function of the NS protein is to promote L interaction with the template. Footprinting studies suggest that the NS protein may be the protein that recognizes the promoter (polymerase binding site at the 3'-end of the genomic and antigenomic RNAs).

The 3'-end of the genome is extremely important in both transcription and replication. The importance of this region is emphasized by the sequence conservation within the first 18 bases of the Indiana, New Jersey, Cocal, Piry, and Chandipura negative-strand genomes (Giorgi, et al., 1983; Keene, et al., 1979). Using dimethyl sulfate methylation patterns to study protein interaction with the genome RNA, it was found that the NS protein changed the methylation

pattern of bases 16-30 at the 3'-end of the standard genome (Keene, et al., 1981c) and bases 17-37 at the 3'-end of DI-particle genomes (Isaac and Keene, 1982). Since only intimate association of proteins with RNA will change their pattern of methylation it was concluded that the NS protein binds tightly to these regions of the genome. It has been postulated that since the NS protein is required for L protein binding, the NS protein probably functions by recognizing the above sequences and thereby directing the L protein to the neighboring initiation sequences at the 3'-end of the genome.

It is now commonly accepted that the leader RNA and the messenger RNAs are sequentially transcribed following their gene order (see section 1.1.3). Sequential transcription of the genes as well as the gene order were established by ultraviolet (UV) light inactivation kinetics: transcription of genes proximal to the 3'-end of the genome are more UV resistant than the transcription of genes further downstream (Abraham and Banerjee, 1976; and Ball and White, 1976). The UV target size of a transcribed gene depends on its position in the genome and is the sum of the target sizes of the preceding genes.

All models of transcription must account for the polarity of the transcripts, as well as considering why the mRNAs are capped, methylated and polyadenylated, while the leader RNA is neither capped, polyadenylated, nor methylated. The first model consisted of precursor cleavage followed by posttranscriptional modification (Colonno and Banerjee, 1976). Evidence for this model consist of the observation of large polycistronic mRNAs containing polyadenylic acid [poly(A)] sequences between genes (Herman, et al., 1978). Leader RNA sequences

have been discovered linked to the 5'-end of N mRNA and trailer regions linked to the 3'-end of L mRNA (Chinchar, et al., 1982; Schubert and Lazzarini, 1981). The second model is the stop-start model (Banerjee, et al., 1977). According to this model, the polymerase initiates and terminates RNA chain synthesis at each gene junction, but all 3'-proximal sequences must be transcribed before the transcription of 5'-adjacent genes can be initiated. Indirect evidence for a stop-start mechanism is provided by defective interfering (DI) particles. The majority of DI particles transcribe only the negative-strand leader RNA. If processing of RNA were involved in generating leader RNAs, some RNA transcribed from the remainder of the DI genome should be found. However, the DI particles effectively terminate transcription at the end of the negative-strand leader RNA template and no other RNAs are synthesized (Emerson, et al., 1977). A variation of the stop-start model states that each gene has its own promoter or polymerase entry site, but although initiation occurs simultaneously at each gene, internal transcriptases are unable to completely traverse an internal gene until the adjacent 3'-sequences are copied (Testa, et al., 1980). Direct evidence that the polymerase can initiate at the beginning of internal genes comes from the identification of a set of small oligonucleotides corresponding to the first 11-14 bases of the N gene, synthesized in molar excess over leader RNA when produced under conditions of matrix (M) protein inhibition of transcription (Chanda and Banerjee, 1981; Naeve and Summers, 1981; Pinney and Emerson, 1982a,b). Evidence for both variations have been obtained by partial in vitro transcription reactions. However, reconstitution studies

yield different results. Partial transcription on reconstituted nucleocapsid complexes synthesized only leader RNA specific transcripts (Emerson, 1982). If the reconstituted complexes were allowed to transcribe in the presence of all four ribonucleoside triphosphates for sufficient time to begin synthesizing messenger RNAs and then two ribonucleoside triphosphates were removed, both leader and mRNA specific oligonucleotides were transcribed. These results would argue that there is a single polymerase entry site, located at the 3'-end of the genome, and that the polymerase molecules can reach internal genes only by transcribing 3'-proximal sequences. Until further experiments, under in vivo conditions, prove one or the other of these variations we cannot firmly state the exact mechanism of transcription.

1.1.4.d Translation.

Translation of each mRNA of VSV is coupled to transcription. All five mRNAs are synthesized throughout the cycle of infection, but the amount of each protein synthesized is roughly in decreasing order from N, through NS, M, G and the L protein (Kang and Prevec, 1969, 1970,1971; Wagner, et al., 1970; Mudd and Summers, 1970; Hsu, et al., 1979). The G protein is synthesized from mRNA on endoplasmic reticulum membrane-associated polyribosomes, inserted into the endoplasmic membrane by means of a signal sequence, anchored in the membrane by a carboxyl-terminal hybrophobic region, and glycosylated in golgi vesicles which migrate to the cytoplasmic membrane where they fuse, inserting the fully glycosylated G protein (Knipe, et al., 1977; Rothman, et al., 1980). The other four proteins are synthesized from

monocistronic messenger RNAs on cytoplasmic polyribosomes (Morrison and Lodish, 1975).

1.1.4.e Replication.

Unlike transcription, replication of VSV requires coupled translation (Huang and Manders, 1972; Pearlman and Huang, 1973; Wertz and Levine, 1973; Davis and Wertz, 1982) and probably one or more host factors (Hill, et al., 1981; Patton, et al., 1983). It is generally accepted that the same polymerase functions for replication as well as transcription. All full length positive-strand and negative-strand RNA molecules and some leader RNAs are encapsidated with N protein (Blumberg, et al., 1981). The viral N protein is thought to modulate the switch from transcription to replication by its ability to bind to nascent leader RNA, thus promoting read-through of the termination signals as the full length nascent RNA is assembled into nucleocapsids (Blumberg, et al., 1981; Schubert, et al., 1982). It has been proposed that the 5' terminus of the leader RNA serves as the nucleation site for binding of newly synthesized N protein to form the ribonucleocapsid (Blumberg, et al., 1981, 1983). The first 18-20 nucleotides of both the positive- and negative-strand leader RNAs are thought to contain the putative nucleation site. Of the five strains of VSV examined, there is a remarkable homology in the first 18 nucleotides with the consensus sequence 3'-UGCUUNUNNUNNUUUGGU-5', where N represents a G, U, or C, but never an A (Giorgi, et al., 1983). The nucleocapsid assembly signal is thought to be a 5-times-repeated A residue in every third position at the 5'-terminus of the leader RNA (Blumberg, et al., 1983;

Giorgi, et al., 1983). The critical role of the N protein in VSV replication and nucleocapsid assembly is emphasized by the studies of Arnheiter et al. (1985), who showed that a monoclonal antibody that reacts only with cytoplasmic unbound N protein, and not with nucleocapsid N protein, selectively inhibits genome replication and not messenger RNA transcription. Another monoclonal antibody to the N protein bound to nucleocapsids, immunoprecipitated N-NS complexes, inhibited transcription and protected cells from VSV. These studies strongly indicate that two conformational forms of N protein regulate replication and transcription. The conformational importance of the N protein is emphasized by the work of Perrault et al. (1980, 1981, 1983, 1984). They found a novel VSV mutant, polR, which efficiently read through the leader RNA termination site under standard in vitro transcription reaction conditions. The mutation occurs within the N protein where it has been shown to have different post-translational modifications. Based on these results, this group proposed that the preexisting assembled RNP template structure (and not assembly of nascent leaders) determine whether read through of leader termination can occur. The switch from transcription to replication is controlled by a modification of the N protein on the RNP template. The direct involvement of the N protein in the replicative process has gained further support by the results of in vitro replication systems in which encapsidation of the nascent RNA chains by the N protein was directly demonstrated. These systems consisted of: infected cell lysates (Batt-Humphries, et al., 1979; Hill, et al., 1981; Ghosh and Ghosh, 1982), lysolecithin permeabilized VSV infected cells (Condra and Lazzarini,

1980) and cell free lysate (Peluso and Moyer, 1983); and an mRNA-dependent rabbit reticulocyte lysate to which was added VSV mRNA and nucleocapsids (Davis and Wertz, 1982; Patton, et al., 1983, 1984; and Wertz, 1983).

It has been observed that the N protein self-aggregates (Blumberg, et al., 1983; Sprague, et al, 1983; and Peluso and Moyer, 1984). In in vitro replication systems, aggregation of N protein is a function of increased concentration and the aggregated form is less able to support replication (Peluso and Moyer, 1984; Howard, et al., 1987). However, at concentrations where the N protein would normally self-aggregate, the NS protein prevented N protein aggregation and supported replication (Howard, et al., 1987). In addition, complexes between N protein and NS protein have been shown to exist both in vivo and in vitro and to be active in replication (Bell, et al., 1984; Peluso and Moyer, 1984; Davis, et al., 1986; and Masters and Banerjee, 1988a). Recently, in vitro (Howard, et al., 1987) and in vivo (Peluso and Moyer, 1984, 1988; Peluso, 1988) experiments have shown that the NS/N protein ratio was critical in determining the optimum levels of replication. Based on these results, a model has been proposed in which the NS protein regulates replication by controlling the availability of N protein. The N protein alone can support replication if present in low concentration. At higher concentration, the N protein aggregates and becomes incompetent in replication unless the NS protein is present to prevent it from aggregating and maintains it in a replication-competent form. Critical to the complex formation is the N/NS protein concentration ratio. When the molar amount of NS protein

exceeds that of N protein, replication is inhibited. Thus, the NS protein may be the factor that controls the balance between VSV RNA replication and transcription, and it may do so by controlling the availability of N protein to support replication. Only the N protein of the N-NS protein complex appears to be bound to RNA during encapsidation, with the NS protein being released (Peluso and Moyer, 1988). In addition, the NS protein seems to be responsible for conferring upon the N protein the RNA sequence binding specificity that occurs during VSV genome replication (Masters and Banerjee, 1988b).

The La protein of systemic lupus erythematosus has been found complexed with both the positive- and negative-strand leader RNAs (Kurilla and Keene, 1983; Wilusz, et al., 1983) and has been proposed as ultimately controlling the switch from transcription to replication. Wilusz and Keene (1984) have proposed that formation of a La-protein-leader RNA complex may serve to ensure adequate levels of viral protein accumulation before the switch to replication takes place.

1.1.4.f Assembly and Budding.

Once viral progeny RNA has been fully replicated and has simultaneously associated with the N, NS and L proteins, it forms newly assembled ribonucleoprotein (RNP) cores (Rubio, et al., 1980). Independently synthesized M protein apparently has marked affinity for associating with the cell membranes (Morrison and McQuain, 1978) as well as with progeny nucleocapsids (Wilson and Lenard, 1981). The interaction of the M protein with the nucleocapsid seems to stop viral transcription (Carroll and Wagner, 1979; Clinton, et al., 1978; De, et

al., 1982; Pinney and Emerson, 1982a,b; Wilson and Lenard, 1981). As the M protein binds to the progeny nucleocapsid the complex condenses into the tightly coiled nucleocapsid structure of the mature virion (Newcomb and Brown, 1981; Newcomb, et al., 1982). Simultaneous with the assembly of the nucleocapsid, the VSV G protein is being synthesized on membrane-associated polyribosomes and processed and glycosylated in the endoplasmic reticulum-Golgi apparatus. Coated vesicles then migrate to the plasma membrane for fusion and insertion of the G protein (Rothman, et al., 1980). It is believed that the nucleocapsid-M protein complex migrates to regions of the cytoplasmic membrane that contain the newly inserted VSV G protein (Wagner, et al., 1971). The nucleocapsid-M protein complex appears to bind to the cytoplasmic surface of the plasma membrane at a region rich in inserted G protein and phosphoserine phopholipids. There is evidence that the positively charged M protein (Carroll and Wagner, 1979) binds to negatively charged headgroups of phophoserine phospholipid (Zakowski, et al., 1981). The tightly coiled nucleocapsid is apparently next enveloped in G-protein-dense cytoplasmic membranes, leading to budding and release of infectious VS virions. The budding process is not totally specific for the VSV G protein. Budding VSV nucleocapsids can become enveloped by virion glycoproteins of other, unrelated viruses, such as retroviruses, to form pseudotypes (Love and Weiss, 1974; Kang and Lambright, 1977; Weiss, et al., 1979; Weiss and Bennett, 1980; Zavada and Zavodska, 1973/74) and specific cell surface antigens (Hecht and Summers, 1976). It has even been demonstrated that an interaction between a precise number of G, M and N proteins in infected cells may

not be an essential prerequisite for VSV maturation and the VSV G protein may not be essential for the formation of a budding virion (Lodish and Porter, 1980a,b). However, the formation of nucleocapsid-M protein complexes seems to promote lateral diffusion of G protein to regions in the cytoplasm destined for nucleocapsid envelopment and budding of VS virions (Jacobs and Penhoet, 1982).

1.1.5 Cellular Response to Infection.

VSV causes rapid changes in the metabolism of host cells leading to death. The cytopathology of VSV is very rapid but does vary according to cell and virus strains and types (Bablanian, 1975). There are two responses to virus infection: the first is the rapid shut down of RNA, DNA and host cell protein synthesis (Wagner, et al., 1984) accompanied by cell rounding by one hour postinfection (the positive-strand leader RNA has been implicated as the viral component responsible for the host cell shutoff of macromolecular synthesis (Grinnell and Wagner, 1984; Remenick, et al., 1988)); the second response, cell death, seems to follow progeny virus release from infected cells. As this thesis will not examine the cellular response to virus infection this vast topic will not be discussed in further detail.

1.2 Defective Interfering (DI) Particles.

Defective interfering (DI) Particles of VSV have been extensively studied due to: their importance in understanding viral interference, their modes of generation, their utility as models for understanding viral replication, their implication in the development of persistent

viral infections, and their relative ease of production and purification. Accordingly, there have been many reviews written on many aspects of DI particle production, structure, and implications in the development of persistent viral infections. Due to space limitation I am forced to condense much of the literature here. Further information may be obtained in the following reviews (Huang and Baltimore, 1977; Reichmann and Schnitzlein, 1979, 1980; Holland, et al., 1980; Faulkner and Lazzarini, 1980; Johnson and Lazzarini, 1980; Kang, 1980; Perrault, 1981; Lazzarini, et al., 1981; Blumberg and Kolakofsky, 1983; Barrett and Dimmock, 1986; Huang, 1987; Schlesinger, 1987; and Holland, 1987).

DI particles were first clearly defined by Huang and Baltimore (1970) when they realized that DI particles from both DNA and RNA viruses could be grouped together by the following common characteristics: they are generated by replication errors resulting in the generation of genomic RNAs containing only part of the standard virus genome, they use the structural proteins synthesized by standard virus and therefore are antigenically indistinguishable from standard virions, they cannot replicate independently of standard virus and are thus defective, they replicate preferentially at the expense of helper standard virus, thereby causing interference, during co-infection the absolute amount of DI particles and the amount relative to standard virus is enhanced; and lastly, they require a replication competent nucleic acid in order to interfere.

DI particle interference was first described by Henle and Henle (1943) as lowered infectivity of influenza viruses passed undiluted in

embryonated chicken eggs. This phenomenon was further investigated by von Magnus (1951, 1954), who demonstrated that serial undiluted passages of influenza virus *in ovo* led to strong interference after several passages and that this interference was due to replicating defective virus particles that he called "incomplete virus particles." Cooper and Bellett (1959) and Bellett and Cooper (1959) were the first to systematically analyze this phenomenon with VSV in cell culture. They proved that the interfering virus consisted of sedimentable particles that accumulated after serial undiluted passages in cell culture. These particles were shown to be transmissible and to sediment more slowly than helper virus. Hackett (1964) obtained electron micrographs showing that DI particles are shorter than standard infectious virions. Crick *et al.* (1966), Huang *et al.* (1966), and Hackett *et al.* (1967) demonstrated that discrete bands of DI particles of VSV could be separated from infectious standard virus by velocity sedimentation in sucrose gradients, which led to the first definitive biological and biochemical characterization of purified DI particles of VSV (Huang and Wagner, 1966a,b). Rhabdovirus DI particles are unique among animal viruses in that their length is a reflection of the length of their RNA. This characteristic makes them easy to purify and has led to widespread studies into their biological and biochemical characteristics.

1.2.1 Biological Characteristics of DI Particles.

DI particles of most viruses have the identical shape and symmetry as their nondefective counterparts. DI particles of VSV are unique in

that they are usually much smaller than standard virus particles. They are smaller because their deleted genomes assemble into shorter nucleocapsids which become enveloped into shorter virus particles at the cell surface. The nucleocapsid has a helical symmetry and is tightly coiled into rod shaped virions. The smallest DI particles are about 10% of the viral genome length, and the largest are about 50-60% (Holland, 1987). It is possible that DI particles containing less than 10% of the standard virion are produced and replicated and able to interfere, but they probably cannot mature due to size constraints in the enveloping process.

The most common VSV DI particles are those derived from the 5'-half of the genome, retaining part of the L gene (Reichmann and Schnitzlein, 1979). These DI particles direct neither transcription nor translation of mRNA both *in vivo* and *in vitro* (Huang and Manders, 1972; Perrault and Holland, 1972). In cells doubly infected with helper virus and DI particles, the following events are observed: primary transcription from standard virus occurs normally, virion-sized RNA replication is strongly inhibited, therefore secondary transcription is reduced, and the reduced levels of viral replication, encapsidation, and structural proteins become devoted to replication of DI RNA genomes and to their maturation into DI particles (Huang and Manders, 1972; Perrault and Holland, 1972; Khan and Lazzarini, 1977). Therefore, something in the structure of the DI particle genomic RNA must endow DI particles with their replicative advantage. The exact reason for their replicative advantage is unknown, but the current theories will be discussed in section 1.2.5.

The ability of DI particles to interfere with virus replication or to replicate seems to be profoundly influenced by the host-cell type (Huang and Baltimore, 1970; Perrault and Holland, 1972; Holland, et al., 1976; Kang, et al., 1981; Youngner, et al., 1981). The molecular basis for the difference in the response of "low-interference" versus "high-interference" cell lines is unknown. In the low-interference cell lines DI particles may be replicated but unable to interfere (Perrault and Holland, 1972) or simply unable to replicate (Holland, et al., 1976). Therefore, host-cell factors involved in replication/encapsidation or other viral processes must be involved, but their nature and site of action is unknown. Kang, et al. (1981) concluded from studies of DI particle replication in mouse-human hybrid cells that human chromosome 16 encodes a factor or factors able to suppress de novo generation of VSV DI particles, but not the amplification of DI particles in a virus inoculum.

The amount of interference exerted by a given DI particle in any cell type can vary enormously. Sekellick and Marcus (1980) showed that single GMK-Vero cells, unable to respond to interferon, normally able to yield over 5,000 plaque-forming-units (PFU) generally yielded 0 PFU if simultaneously infected with virus plus a single DI particle. This all-or-none effect of DI particles is probably operative with most situations involving DI particles and homotypic wild type standard virus. It is not operative in "low-interference" cell lines, nor is it when DI particles are coinfected into cells with heterotypic standard virus. When standard VSV_{NJ} was coinfected with most VSV_{IND} DI particles, the heterotypic DI particles replicated, but did not

interfere significantly (Schnitzlein and Reichmann, 1977a,b; Khan and Lazzarini, 1977; Adachi and Lazzarini, 1978). However, pseudotype DI particles, produced when heterotypic DI particles are replicated with standard virus, do interfere strongly (Schnitzlein and Reichmann, 1977b). If homotypic DI particles are added to viral infections 2.5 hours after infection with the standard virus, interference is greatly reduced (Stampfer, et al., 1969). Standard virus seem to be able to evolve resistance to homotypic DI particles over time, leading to the generation of a new set of DI particles which are able to interfere with the evolved virus (Horodyski and Holland, 1980, 1981, 1983).

Defective interfering particles can allow cells to survive otherwise lethal virus infections in two ways. First, DI particles can lead to the establishment of persistently infected cells (Holland and Villarreal, 1974) but small-plaque mutant virus, ts mutants, and mutations that affect transcription, replication, host-cell shutoff, maturation, surface proteins, and other factors may also be involved in the establishment and maintenance of the persistent state (Holland, 1987). Second, cells may completely recover from virus infection that would otherwise kill cells in the absence of DI particles (Huang and Baltimore, 1970).

In vivo, DI particles can lead to the protection of animals. DI particles were shown to completely protect adult mice from otherwise lethal infections with VSV (Doyle and Holland, 1973). However, at higher doses of standard virus, DI particles provided less protection. The time to death was prolonged and the mice presented an unusual pathology not observed in wild-type infections (Rabinowitz, et al.,

1977). Although adult mice are protected by DI particles, they do not replicate DI particles well during high multiplicity intracerebral passages (Holland and Villarreal, 1975), whereas newborn mice generated and replicated VSV DI particles very efficiently. It has been suggested that dosage, route of inoculation, and timing may be critical in the replication and interference of DI particles in adult mice (Cave, et al., 1984). In hamsters, as few as 100 PFU of VSV was lethal when injected intraperitoneally, but when coinfected with DI particles most hamsters survived (Fultz, et al., 1981, 1982a,b, 1984). Interestingly, most DI-particle-protected hamsters appear to become persistently infected with VSV. DI particles can facilitate the production of interferon. In fact, DI particles that contain totally self-complementary or "snapback" RNA are excellent inducers of interferon (Marcus and Sekellick, 1977). This feature may be important for the protective function of DI particles in vivo.

Defective interfering particles and helper virus exhibit cyclical interactions. It has been observed that VSV_{IND} helper virus yields were inversely correlated to homologous DI particle production (Palma and Huang, 1974) in a cyclic manner when uninfected cells were added regularly to infected cultures. After virus cyclic replication to high levels, DI particles soon replicated to high levels. At the maximum level of DI particle yield, the helper virus level became depressed until relatively few helper virus particles remained to support DI particle replication. Then the DI particle levels dropped allowing the helper virus levels to rise once more and the cycle to repeat itself.

Before discussing the genome structure of VSV DI particles, it should be emphasized that DI particles are very prevalent and are an important consideration to virologists studying viruses *in vitro*. Basically, a high titer virus stock is never "free" of DI particles. As soon as viruses are grown in cell culture to the titers required for molecular studies, they will have a certain quantity of DI particle contamination. Holland *et al.* (1976) estimated that the rate of DI particle generation is about 10^{-7} to 10^{-8} per infectious virus replication in BHK_{21} cells, since expansion of clones much beyond 10^8 PFU regularly gives rise to DI particle contamination. The best method that has been developed to eliminate or greatly reduce the numbers of DI particles present in stocks of VSV (Stampfer, *et al.*, 1971) consists of cloning virus over a series of plaque purifications. As long as the plaque-derived virus is not amplified in cells, it will remain free of DI particles (Holland, *et al.*, 1976). To verify the presence of even a low level of DI particle contamination in a virus stock, the stock is passed undiluted. The most competitive DI particle will predominate as a major DI particle band in sucrose gradients. If the stock virus is contaminated with DI particles, independently passed virus on identical cells will result in the amplification of identical DI particles. However, if the stock virus is free of DI particles, there is no seed inoculum of DI particles to be amplified. Since DI particles of different size and genome type seem to be generated randomly, it has been proposed that the DI particles amplified in independent passes upon identical cells are randomly different because the amplified DI particles represent DI particles generated during the first undiluted

passage (Holland, et al., 1976). Because DI particle generation is random and very frequent, amplification of DI particles to the levels necessary for molecular studies leads to the coamplification of other newly generated DI particles. These new DI particles usually represent a small percentage of the total yield, but can become dominant if they are better competitors and allowed to coamplify over several cell passes.

1.2.2 Genome Organization of DI particles.

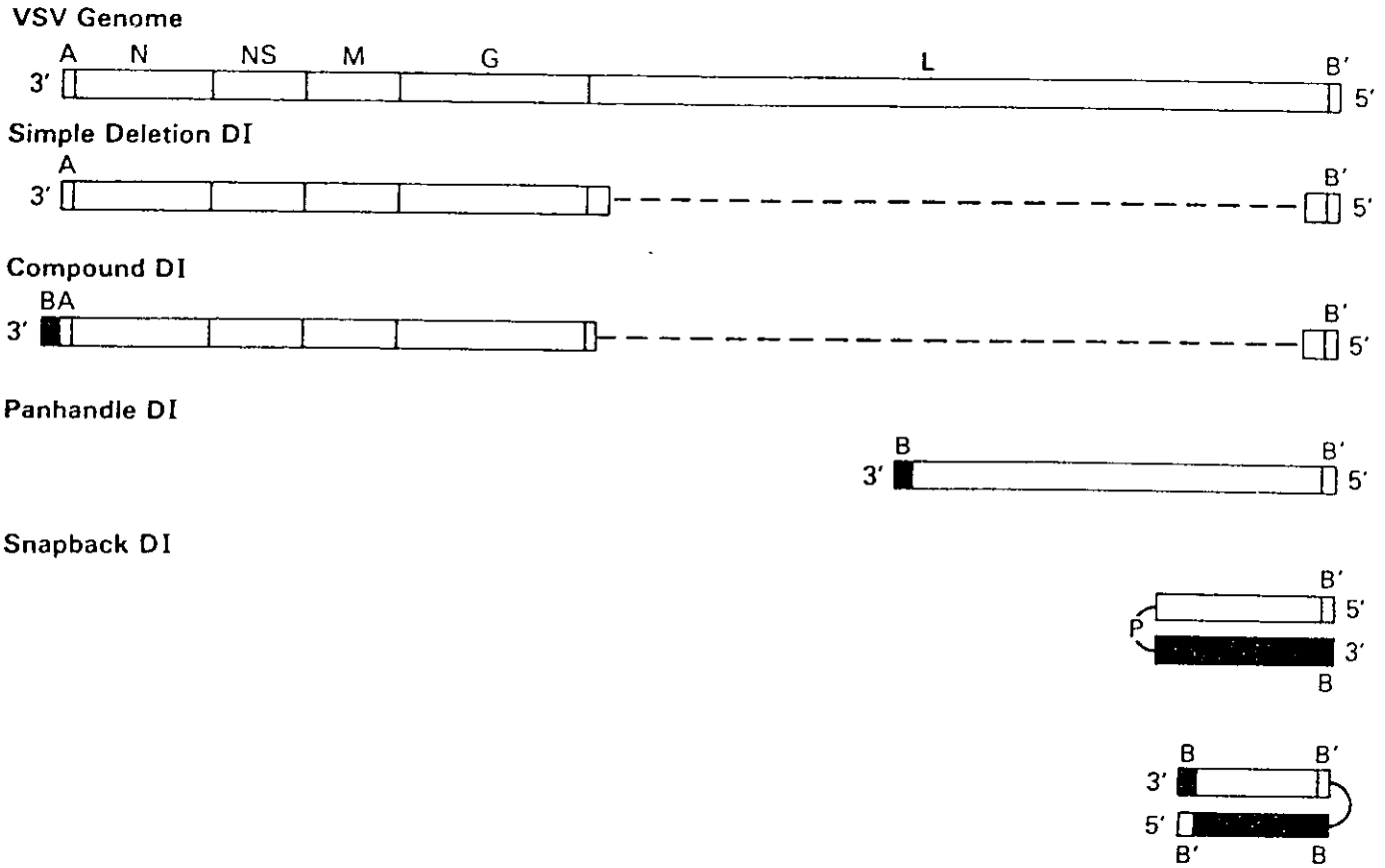
DI particles are viral particles that contain only a portion of the genetic information of the parental virus. The most common VSV DI particles are those derived from the 5'-half of the genome, retaining part of the L gene (Reichmann and Schnitzlein, 1979). The fact that DI particle genomes are efficiently replicated, by enzymes encoded by the helper virus, indicates that the genomes of the DI particle and helper virus share the minimum essential characteristics for replication. They must have competent initiation sites at their 3'-termini, the complement of an initiation site at their 5'-termini and whatever sequence or structure that is required for encapsidation into nucleocapsids. These common features of DI particle genomes have given insight into the requirements for encapsidation, replication and interference. Most VSV DI particles contain terminal sequence complementarity from as few as 45 nucleotides to complete complementarity (Meier, et al., 1984). The first evidence of this structural feature was obtained from electron microscopic observation of circular DI RNAs with small panhandles or stems (Perrault, 1976;

Perrault and Leavitt, 1977). In addition the stems could be isolated by digestion of the DI particle genomic RNA with single strand-specific ribonucleases. By examining several DI particle duplexes the following generalizations were made: the 5'-end sequence of the standard genome and the DI RNA are identical for the length of the duplex, the 3'-end sequence of the DI RNAs is not present within the negative-strand standard virus genome, the 3'-end sequence of DI RNAs corresponds to the 3'-end of the positive-strand replicative intermediate RNA (Perrault, et al., 1978). These generalizations were confirmed by directly sequencing: RNA transcripts from panhandle DI particles, which represent the complement of the 3'-end of DI RNAs (Semler, et al., 1978; Schubert, et al., 1978), RNA transcripts representing the complement of the 3'-end of standard virus genomes (Colonno and Banerjee, 1978), the 3'-end of standard virus genomic RNAs and DI particle genomic RNAs (Keene, et al., 1978, 1979, 1981a,b; Giorgi, et al., 1983), cDNA copied from the 3'-end of standard virus genomes (McGeoch and Dolan, 1979; Rowlands, 1979), the 5'-end of DI RNAs (Schubert, et al., 1979, 1980) and the 5'-end of standard virus genomic RNAs (Semler, et al., 1979). DI particles have four general genomic organizations: the stem or panhandle class, the snapback or hairpin class, the simple internal deletion class and the mosaic rearrangement or compound class (Holland, 1987). Figure 4 illustrates the structure of these four classes of DI particles.

1.2.2.a Stem or Panhandle Class.

This class of DI particles seems to be the most common among VSV DI particles and hence is probably the most competitive type of DI particle. Their genomes (Figure 4) consist of the 5'-end of the standard virus and varying portions of the adjacent large (L)-protein gene. Their 3'-ends consist of a complementary copy of the virion 5'-end (which makes them identical to the 3'-end of the viral replicative intermediate). The length of self-complementarity varies from 45 to 200 base pairs (Kolakofsky, 1982). The terminal sequences of many VSV_{IND} panhandle DI particles have been determined and compared to standard virus sequence confirming the above generalization about the panhandle DI sequence structure (Keene, et al., 1978; Schubert, et al., 1978, 1979, 1980; Semler, et al., 1978, 1979; McGeoch and Dolan, 1979; Rowlands, 1979; Yang and Lazzarini, 1983; Nichol, et al., 1984; O'Hare, et al., 1984a,b; Meier, et al., 1984). Therefore, genomes of this class of DI particles consist mainly of negative-stranded RNA, originating from the L gene, and a small segment of positive-strand RNA, originating from the 3'-end of the replicative intermediate, at their 3'-termini. None of these DI particles contain transcription initiation sites beyond the negative-strand leader RNA termination site (B region in figure 4), hence they are known as nontranscribing DI particles. Since these DI particles and their replicative intermediates both contain the 3'-end of the standard virus replicative intermediate at their 3'-termini, these DI particles are recognized as replicative intermediates. This characteristic may be the basis for

Figure 4. Structures of representative defective interfering (DI) particle RNAs. Regions of the standard virus genome which are conserved are indicated by open boxes. Complementary positive-strand RNA is denoted by black boxes. [A] represents the positive-strand leader RNA template (transcribed by the standard virus) and [B] represents the negative-strand leader RNA template (transcribed by the positive-strand replicative intermediate template). Most DI RNAs contain [B] at their 3'-terminus and like the standard virus, all DI RNAs contain [B'], the complement of [B], at their 5'-terminus. Snapback DI RNAs consist of completely complementary RNAs which are covalently linked. Removal of the nucleocapsid proteins from these DI particles allows immediate annealing into a "hairpin" structure. These structures and the mechanism of their generation is discussed in the text.


VSV Genome
A
N
NS
M
G
L
B′
3′
5′
Simple Deletion DI
A
B′
3′
5′
Compound DI
BA
B′
3′
5′
Panhandle DI
B
B′
3′
5′
Snapback DI
B′
5′
P
3′
B
B
B′
3′
5′
B′
B

their replicative advantage when interfering with standard virus replication, as will be discussed in section 1.2.5.a.

1.2.2.b Snapback or Hairpin Class.

As its name implies this class of DI particles has self-complementarity over its entire length. This type of DI structure was discovered because deproteinized genomic RNA "snaps back" into a stable duplex molecule resembling a hairpin (Lazzarini, et al., 1975; Perrault, 1976; Perrault and Leavitt, 1977). Sequence analysis of this type of DI particle (Johnson and Lazzarini, 1977; Keene, et al., 1977, 1979; Schubert, et al., 1979) shows that one half of each DI genome is homologous to the 5'-end of the virus negative-strand RNA and the other half is its complement. The VSV_{IND} DI particle, DI-011, was sequenced through the region where the 5'-half of the molecule joined the complementary sequence and was found to contain a single phosphate joint (Schubert and Lazzarini, 1981). The negative-strand sequence was later compared to that of the L gene and it was determined that DI-011 contained an exact copy of the last 1,167 nucleotides from the 5'-end of the VSV_{IND} genome (Meier, et al., 1984). Therefore, in this type of DI particle the genome and antigenome are identical in sequence and hence this type of RNA genome is neither positive- nor negative-stranded (DI-011 is represented as the first illustration in Figure 4, Snapback DI genomes). A slightly more complex type of snapback DI genome was encountered when DI-ST2 was sequenced (Nichol, et al., 1984). This DI particle apparently arose when a panhandle DI particle (DI-ST1) had a complementary copy of itself added onto its genome

(Figure 4, second snapback DI genome illustrated). This type of DI particle is a member of the mosaic or compound DI class to be further discussed in section 1.2.2.d.

1.2.2.c Simple Internal Deletion Class.

The simple internal deletion class of DI particles is the least complex type of DI genome rearrangement (Figure 4). Though this type of DI particle is very common in other types of viruses, among VSV DI particles it is very rare and is therefore thought to be a poor competitor (Perrault and Semler, 1979). The most studied simple internal deletion DI particle of VSV is DI-LT. This DI particle was generated from a heat resistant strain of VSV_{IND} (Petric and Prevec, 1970). Its genome has retained both the 3'-end and 5'-end of the standard virus genome and the entire coding regions of the N, NS, M and possibly the G proteins, but has deleted most of the L protein gene (Reichmann and Schnitzlein, 1979, 1980; Perrault and Semler, 1979; Epstein, et al., 1980; Yang and Lazzarini, 1983; Herman, 1984). This DI particle is unique in that it is able to transcribe the N, NS, M, and G mRNAs (or a truncated form of the G mRNA) (Chow, et al., 1977; Colonno, et al., 1977; Johnson and Lazzarini, 1977; Johnson, et al., 1979; Herman, 1983, 1984; Herman and Lazzarini, 1981). Its transcriptional activity has been postulated to control the unique ability of DI-LT to kill cells (Marcus, et al., 1977) and to interfere heterotypically with the Concan subtype of VSV_{NJ}, but not with the Hazelhurst subtype of VSV_{NJ} (Prevec and Kang, 1970; Schnitzlein and

Reichmann, 1976, 1977a,b; Adachi and Lazzarini, 1978; Reichmann, et al., 1978).

1.2.2.d Mosaic Rearrangement or Compound Class.

The last class of DI particles groups together all of the bizarre rearrangements that cannot be placed in the first three classes. The DI particles that predominate following long term undiluted passing of virus and persistent virus infections are of the mosaic type (O'Hara, et al., 1984a). These mosaic DI particles all have stem structures at their 3'-ends but immediately internal to this sequence they have a variety of rearrangements of the standard virus genome. Figure 4 shows two mosaic or compound type DI particles: a snapback DI particle, DI-ST2, and DI-LT2, a DI particle that was isolated from the same virus pool as DI-LT (or as it will now be denoted, DI-LT1) and may have been generated from DI-LT1 (Perrault and Semler, 1979; Epstein, et al., 1980). The major difference between DI-LT1 and DI-LT2 is the addition of a 70 base long stem structure (plus an additional noncoded A) to the 3'-end of DI-LT1 to generate DI-LT2 (Perrault and Semler, 1979; Epstein, et al., 1980; Keene, et al., 1981a,b). This stem addition apparently silences the transcriptional ability of the viral 3'-end, and confers upon DI-LT2 a greater competitiveness in comparison to DI-LT1 (Perrault and Semler, 1979). The second, DI-ST2, was isolated from the same virus pool from which was isolated the panhandle DI particle, DI-ST1 (having a 54 base pair stem). DI-ST2 is twice the length of DI-ST1 and its genome contains the same sequence as DI-ST1 with a positive-strand copy of DI-ST1 linked onto the equivalent of the DI-ST1

5'-end (Nichols, et al., 1984). This DI particle and others isolated from persistent infections show that the VSV genome can be extensively rearranged during the generation of DI particles. VSV DI particles have not been found to contain any cellular RNA sequences.

1.2.3 Ribonucleic Acid Synthesis by DI Particles.

During in vitro transcription, the only product synthesized by almost all VSV DI particles is a short negative-strand (or DI leader) leader RNA of between 46-48 nucleotides in length (Reichmann, et al., 1974; Emerson, et al., 1977; Semler, et al., 1978; Schubert, et al., 1978). The leader RNA is transcribed off the exact 3'-end of DI particle genomes. Due to the lack of transcription initiation sites, in most DI particles, beyond the negative-strand leader RNA transcription stop site, the remainder of the genome is not transcribed.

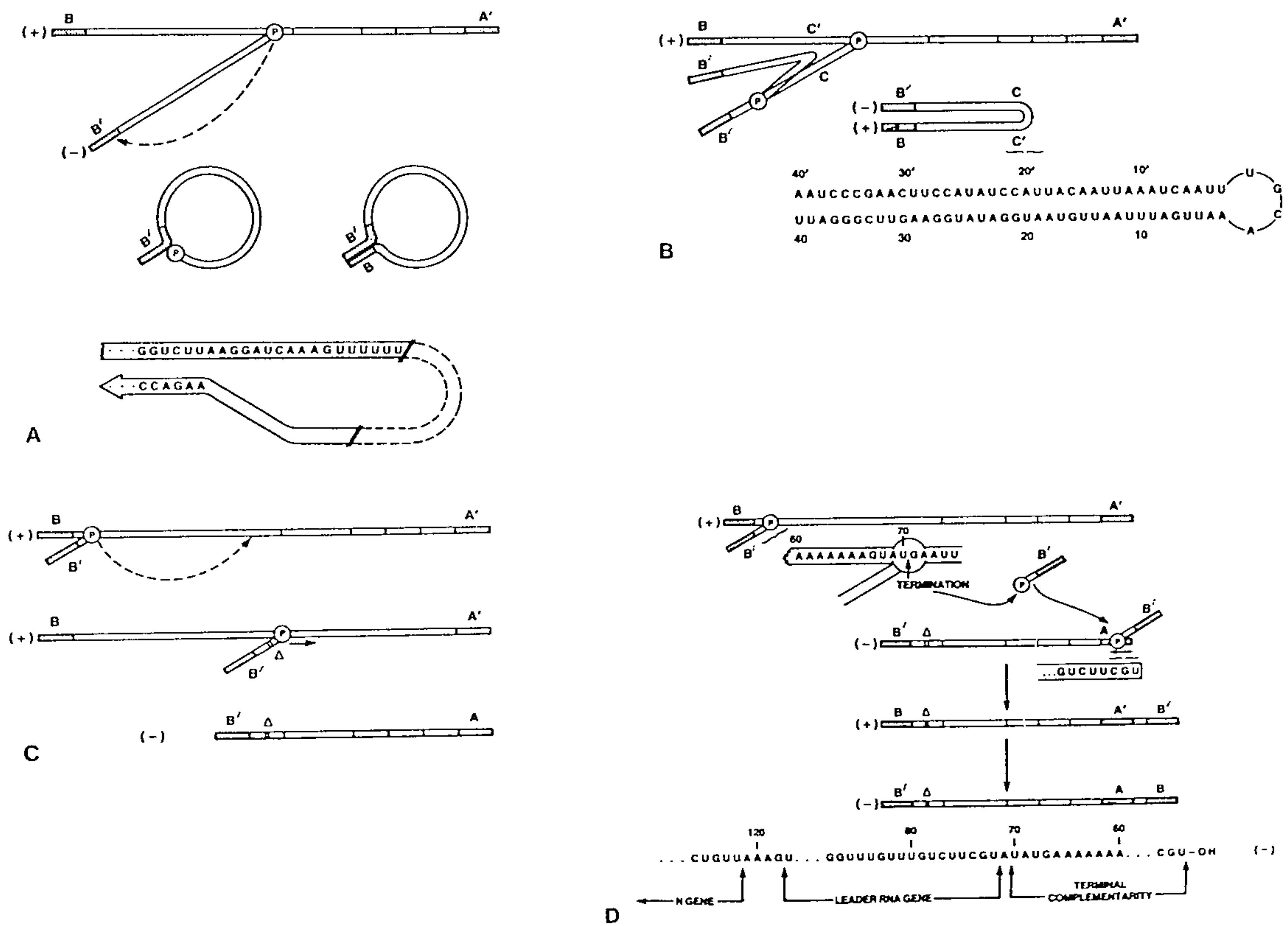
DI-LT1, the simple deletion DI particle described in section 1.2.2.c, is the only well studied DI particle which is able to transcribe in vitro (Colonno, et al., 1977) and in vivo (Chow, et al., 1977; Johnson and Lazzarini, 1977; Herman and Lazzarini, 1981), the positive-strand leader RNA and the N, NS, M, and G mRNAs. In regards to the control of transcription, it is interesting that the compound DI particle, DI-LT2, which only contains an additional 70 base stem structure at the 3'-end of DI-LT1, transcribes solely the 46-nucleotide-long negative-strand leader RNA in vitro (Perrault and Semler, 1979; Keene, et al., 1981a,b). Therefore, the negative-strand leader RNA transcription stop seems to prevent downstream initiation of

transcription. However, DI particles derived from the VSV_{IND} Pol-R1 mutant which contain a mutant N protein that suppresses polymerase termination, are able to synthesize large transcripts, in vitro (Perrault, et al., 1980, 1983). Recently, Liang and Emerson (1988) isolated a VSV_{NJ}(Hazelhurst) DI particle which was able to initiate transcription beyond the negative-strand leader RNA transcription stop site, therefore, transcription inhibition beyond the negative-strand leader RNA transcription termination site is not without exception.

1.2.4 Mechanisms of Generation of DI Particles.

DI particle generation is not a result of mere RNA strand breakage nor is it a result of premature replication termination. This is evident by the conservation of the terminal sequences in the genomes of DI particles. Therefore, a model was proposed (Leppert, et al., 1977; Huang, 1977; Kang, 1980) to explain the generation of DI particles involving viral replicase "template-switching" or "copy-back", in which the polymerase leaves its template (positive-strand), carrying the 3'-end of the nascent chain with it, resumes nascent chain elongation after binding to the nascent chain ribonucleocapsid, and copies the nascent chain back to its 5'-end. This replicase-error (copy-choice) model explains the generation of stem or panhandle class DI particles and has been generalized to explain all four types of DI particles (Lazzarini, et al., 1981; Perrault, 1981), by using interstrand or intrastrand "replicase leaps", or both (Figure 5). All VSV DI particle sequence data published to date support the above model (Yang and Lazzarini, 1983; De and Perrault, 1982; Schubert, et al., 1984; Keene,

Figure 5. Proposed models to explain the origins of the four different classes of DI particle RNAs. [B] Represents the negative-strand leader RNA template (located at the 3'-end of the positive-strand replicative intermediate RNA). [B'] Represents the complement of [B] and is located at the 5'-end of negative-stranded RNA. [A] Represents the positive-strand leader RNA template (located at the 3'-end of the negative-strand standard virus RNA). [A'] Represents the complement of [A] and is located at the 5'-end of positive-strand replicative intermediate RNA. [P] Represents the polymerase complex. (A) Model for the origin of the stem or panhandle class of DI particles. These DI particles are derived from the 5'-end of the standard virus RNA and possess complementary termini. Following premature termination of the negative-strand RNA synthesis, the polymerase, with the nascent strand of RNA attached, resumes synthesis at a site within the nascent strand of RNA and forms the complementary termini. (B) Model for the origin of the snapback or "Hairpin" class of DI particles. This class of DI particles may originate when one polymerase overtakes another and synthesizes across a replication fork. The product RNA forms a completely complementary RNA duplex upon deproteinization. DI-011 is one well studied example of this type of DI particle. The sequence where the DI-011 molecule starts to copy-back onto itself is shown. (C) Model for the origin of the simple internal deletion class of DI particles. In this case, following premature termination of negative-strand RNA synthesis, the polymerase resumes synthesis further downstream (towards the 5'-end) on the same positive-strand RNA template. DI-LT1 is an example of this type of DI particle. (D) Model for the origin of the mosaic rearrangement or compound class of DI particles. DI-LT2 is one of the many types of compound DI particles. This DI particle probably originated following premature termination of negative-strand RNA synthesis at nucleotide 70 and resumption of RNA synthesis at the 3'-end of a simple deletion DI particle (like DI-LT1) to form a positive-strand replicative intermediate molecule. The replicative intermediate molecule is then replicated to form DI-LT2 with the 3'-end sequence shown in the figure.


A
B
C
D
···GGUCUUAAGGAUCAAAGUUUUUU
··CCAGAA
AAUCCCGAACUUCCAUAUCCAUUACAAUUAAAUCAAUU
UUAGGGCUUGAAGGUAUAGGUAAUGUUAAUUUAGUUAA
AAAAAAAQUAUGAAUU
TERMINATION
...QUCUUCGU
. . . CUGUUAAAGU. . . GGUUUGUUUGUCUUCGUAUAUGAAAAAAA. . . CGU-OH
N GENE
LEADER RNA GENE
TERMINAL COMPLEMENTARITY

et al., 1981a,b; O'Hara, et al., 1984a; Nichol, et al., 1984; Meier, et al., 1984). The sequence data shows some weak, imprecise sequence recognition may be operative in a few examples, but in general there seems to be no sequence specificity either at the sites of replicase premature termination nor at the sites of resumption of synthesis. It is interesting that even though the generalized replicase leap mechanism seems to be operative in the generation of DI particles, recombination in negative-strand standard virus has not been observed (Holland, et al., 1982).

1.2.5 Mechanism of Interference Mediated by DI Particles.

The exact mechanism of DI-particle-mediated interference with viral replication is still unknown. However, due to the accumulated mass of data regarding the structure and competitive advantage of DI particles, several theories have been proposed. Because transcribing DI particles have the ability to interfere heterotypically and are poor competitors when competing with nontranscribing DI particles in coinfection experiments, their mechanism of interference is clearly different from that of the nontranscribing DI particles.

1.2.5.a Nontranscribing DI Particles.

Initially, it was suggested that DI particles had a replicative advantage due to their smaller size (Huang and Baltimore, 1970). However, it was soon realized that smaller DI particles are not invariable selected during replication and size alone did not control the replicative advantage of DI particles (Perrault and Holland, 1972a;

Holland, et al., 1976). It was subsequently observed that VSV DI RNAs not only preferentially replicate but are: incapable of transcription on their own, do not inhibit primary transcription by input parental virus, rapidly and strongly suppress viral genome replication, while efficiently replicating themselves, and they strongly suppress secondary transcription (Stampfer, et al., 1969; Perrault and Holland, 1972b: Huang and Manders, 1972). It was concluded that DI particle-mediated interference acted probably at the level of genome replication. Since the reduction of secondary transcription leads to a relative shortage of viral proteins, and since the reduced number of virus templates devoted mainly to transcription, while DI nucleocapsids are devoted mainly to replication, then it was logically concluded that DI RNAs successfully compete for the VSV replicase by virtue of a higher affinity for the enzyme. This concept was supported by quantitative studies of the RNA species in standard and autointerfered infections (Palma, et al., 1974; Stamminger and Lazzarini, 1977). Two models were proposed to explain these observations. The first proposes that the origin of replication at the 3'-end of the VSV negative-strand genome is recognized by a different replicase than that recognizing the 3'-end of the positive-strand replicative intermediate. Since most DI particles contain terminal sequence complementarity the synthesis of both the positive- and negative-strands of DI RNAs would be accomplished by the same replicase (the one recognizing the 3'-end of the standard virus replicative intermediate). Furthermore, an additional kinetic advantage is gained if the synthesis of the positive-strand replicative intermediate is rate-limiting during

standard virus replication (Perrault, et al., 1978). The second model differs in that it postulates that only one replicase is involved in replication. This enzyme having a "low affinity" for the standard virus negative-strand 3'-end and a "high affinity" for the positive-strand replicative intermediate 3'-end (Huang, et al., 1978). Ultimately, in both models, the replicative intermediate of the standard virus is competing with both strands of the DI RNA for available viral proteins.

The importance of stem structures in governing the replicative advantage of DI particles is strongly supported by the observation that the transcribing DI particle, DI-LT1, lacking a stem structure, replicates very poorly in the presence of stem-containing DI particles during single cycles of infection (Perrault and Semler, 1979). The mere presence of the stem structures seems to prevent the DI particle genome from being a template for transcription of mRNA, and allows only replication to occur, as exemplified by DI-LT2 (as described in section 1.2.3). Aside from preventing transcription of mRNAs, VSV DI stem sequences also serve as templates for the synthesis of the short negative-strand leader RNA. This RNA transcript is found in very low levels in standard virus infected cells, but is synthesized in a 50-fold molar excess in interfered infections (Rao and Huang, 1979, 1980; Leppert, et al., 1979; Leppert and Kolakofsky, 1980). It has been proposed that the availability of VSV N protein may control the switch from transcription to replication by binding to the positive-strand leader RNA (Leppert, et al., 1979) promoting the read-though synthesis of positive-strand replicative intermediate RNA. The same process may

occur with negative-strand leader RNAs, promoting the synthesis of negative-strand genomic RNA. As described in section 1.1.5.e, the encapsidation recognition site may reside within a five-times-repeated A residue at every third position from the 5'-end of both the positive- and negative-strand leader RNAs (Blumberg, et al., 1983). Therefore, the advantage the positive-strand replicative intermediate RNA has in its ability to be replicated may also reside in the ability of the negative-strand leader RNA to be encapsidated (Perrault, et al., 1984). DI-mediated interference may occur due to sequestering of the intracellular N protein pool by both strands of the DI particle replicative complex.

1.2.5.b Transcribing DI Particles.

Transcribing DI particles clearly differ in their mechanism of interference. The best studied VSV transcribing DI particle is DI-LT1 which can heterotypically interfere with VSV_{NJ}(Ogden), a member of the Concan subtype (Prevec and Kang, 1970; Schnitzlein and Reichmann, 1976), but not with VSV_{NJ}(Hazelhurst), a member of the Hazelhurst subtype, while being replicated by both standard viruses (Schnitzlein and Reichmann, 1977b, Khan and Lazzarini, 1977; Adachi and Lazzarini, 1978). The addition of a stem to the 3'-end of this DI particle (generating DI-LT2) arrests both the transcription and heterotypic interfering activities (Perrault and Semler, 1979; Epstein, et al., 1980; Keene, et al., 1981a,b) of DI-LT1. The UV-target size of DI-LT1, with regards to interference, is about 42% of the DI-LT1 genome size (Bay and Reichmann, 1979), whereas other DI particles have target sizes

equivalent to their genome sizes. When DI-LT1 was replicated in the presence of thermolabile polymerases, at nonpermissive temperature, DI-LT1 was shown to loose both its ability to heterotypically interfere and to interfere with primary transcription (Bay and Reichmann, 1981, 1982), although not their ability to be replicated by standard virus. It was therefore concluded that the mechanism of homotypic interference for transcribing DI particles was merely based upon the smaller size of the DI particle in comparison to standard virus. However, the mechanism of heterotypic interference has not been elucidated.

1.3 Statement of Research Objectives.

In order to better understand the heterotypic interference phenomenon, two research objectives were established. As it has been observed that the transcribing VSV_{IND} DI particle, DI-LT1, could interfere with VSV_{NJ}(Ogden), the first objective was to determine whether transcribing DI particles could be generated from VSV_{NJ}(Ogden) and if so, could these DI particles heterotypically interfere with VSV_{IND}. If the ability to transcribe was the only criteria for a DI particle to interfere heterotypically, the newly generated DI particles should interfere with VSV_{IND}. The second objective was to compare the genomes of VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst), in an attempt to understand what controls the ability of the VSV_{IND} DI-LT1 to heterotypically interfere with VSV_{NJ}(Ogden) but not with VSV_{NJ}(Hazelhurst). The VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) 3'- and 5'-terminal genomic RNA sequences were examined, due to the importance of these regions in controlling transcription and replication.

2. Materials and Methods.

2.1 Cells and Virus.

Baby hamster kidney cell clone 21 (BHK_{21}) and mouse L_2 cells were obtained from the American Type Culture Collection. Cultures of rat cells transformed with the B77 strain of avian sarcoma virus (R(B77)) were originally obtained from Dr. Howard Temin, McArdle Laboratory, University of Wisconsin. Cells were grown as monolayers in Dulbecco's modified minimum essential medium (DMEM)(GIBCO), supplemented with 5%-7.5% fetal bovine serum (GIBCO) and 2mM glutamine (GIBCO).

VSV_{IND-HR} and VSV_{NJ}(Ogden) were originally obtained from Dr. L. Prevec, McMaster University and VSV_{NJ}(Hazelhurst) was obtained from Dr. Sue Emerson, University of Virginia.

2.2 Growth of Virus.

2.2.1 Standard Virus Growth and Purification.

All three standard viruses were plaque purified prior to any experiments where the quality of the virus required that they be free of DI particles. Plaque purifications were performed as follows: a monolayer of BHK_{21} cells was infected with serial ten fold dilutions of the virus, the virus inocula were adsorbed to the cells for one hour, an agar overlay (0.9% DIFCO agar in 1 X DMEM) was placed over the cells, allowed to set for one half hour, and incubated at 37°C in 5% CO_2 for 24 hours. The plaques were easily observed without staining. Plaques were harvested using a pasteur pipette to extract a plug of agar from over the plaques, the agar plug was placed in one mL of DMEM (without fetal bovine serum) and vortexed, 0.1 mL of ten fold dilutions

of this stock was used to infect new plates of cells and plaques from these plates used to infect a further set of plates. By doing three consecutive plaque purifications a pool of virus "free" of DI particles was obtained.

A heat resistant strain of VSV_{NJ}(Ogden) was generated by heating the plaque purified virus at 45°C for 30 mins, diluting the virus and plaque purifying on R(B77) cells at 40.5°C. Plaques arising from this heat treatment were selected and further plaque purifications were performed at 40.5°C. Three successive plaque purifications were performed to generate a "heat resistant" strain (Kang, et al., 1984). Since VSV has been assigned to Biosafety level C in the guidelines of the Medical Research Council of Canada, the growth and purification of virus was done in the biohazard type C containment facility at the University of Ottawa,

2.2.2 DI Particle Production and Purification.

The following DI particles were produced and purified for use in this work: IND-011 (Lazzarini, et al., 1975), IND-ER1, IND-ER2, NJ-JM2, NJ-121, NJ-121a, NJ-121b, NJ-PG2, and NJ-PG1 (Rud, et al., 1986). The IND denotes that the DI particles are derived from the Indiana serotype of VSV and the NJ denotes the New Jersey serotype of VSV.

IND-011 was generated from the Mudd-Summers strain of the Indiana serotype. We amplified this DI particle by taking DI particles, that had been purified on 5-30% sucrose gradients made in modified TNE (10 mM Tris-HCl, pH 7.6, 1 M NaCl, 1 mM Na_2EDTA), and coinfecting them with VSV_{IND-HR} wild type virus on BHK_{21} cells. The tissue culture media was

harvested after 16-20 hours and centrifuged in a SW27 rotor(Beckman) at 25,000 RPM for two hours at 4°C. The virus pellet was resuspended in modified TNE and centrifuged through a 5-30% sucrose gradient (made in modified TNE) at 35,000 RPM for 35 min at 4°C in a SW41 rotor (Beckman). The visible DI particle band was harvested and stored at -20°C in the gradient buffer containing 50% glycerol. The ratio of DI particles to standard (STD) virus, which would maximize the production of DI particle, was optimised by adding dilutions of the DI particles to a fixed quantity of standard virus and observing the recovery of DI particles on sucrose gradients. IND-ER1 and IND-ER2 were generated from VSV_{IND-HR} plaque purified stock virus after three consecutive high multiplicity of infection (MOI) passages in BHK_{21} cells at 37°C. These DI particles were used as controls in the hybridization experiments.

NJ-JM2 was produced by taking plaque purified VSV_{NJ}(Ogden) and infecting BHK_{21} cells at high MOI over four consecutive passes at 37°C. The DI particles were harvested and amplified as described above for IND-011, except that VSV_{NJ}(Ogden) STD virus was used to amplify NJ-JM2.

NJ-121 was obtained by infecting R(B77) cells with the heat resistant strain of VSV_{NJ}(Ogden) over five consecutive high MOI passes at 40.5°C (Kang, et al., 1984). The DI particles were purified in sucrose gradients and the visible DI particle band pelleted by centrifugation in a SW50.1 rotor (Beckman) at 45,000 RPM for one hour at 4°C. The pelleted DI particles were resuspended in 1 X RSB (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$) and stored at -70°C. NJ-121 was amplified identically to the methods used for NJ-JM2, except on R(B77) cells. NJ-121a and NJ-121b were produced at undetectable levels

in early amplifications of NJ-121 (as observed in sucrose gradients), but as the number of amplifications increased so did their concentration until they became the dominant DI particles. NJ-PG2 and NJ-PG1 were produced by taking the second high MOI passage preparation of heat resistant VSV_{NJ}(Ogden) used for the preparation of NJ-121 and infecting R(B77) cells at high MOI for three additional passages at 40.5°C. NJ-PG2 and NJ-PG1 were generated two years after NJ-121. NJ-PG2 and NJ-PG1 were amplified and stored using the same methods as for NJ-121.

2.2.3 Interference Assays.

The gradient purified DI particles were mixed with known quantities of standard virus and adsorbed onto either BHK_{21}, R(B77), or L_2 cells. The infections were allowed to proceed for 16 hours and the culture media harvested. The culture media were diluted in DMEM (without fetal bovine serum) and the titer of each virus was determined by plaque assay on BHK_{21} monolayers. The DI/STD ratio that reduced the titer of the homotypic (STD virus of same serotype as parent of DI particle) virus by one hundred fold on BHK_{21} monolayers was used in heterotypic (STD virus from a serotype other than that of the parent virus) interference assays. The ratio determined on BHK_{21} cells was also used for the R(B77) and L_2 cells. An additional assay was performed with a ratio of DI/STD that would reduce the yield of homotypic STD virus by one hundred fold on L_2 cells. In order to get a rough estimate of the relative quantity of DI particles required to reduce the titer of homologous standard virus by one hundred fold, the aliquot of DI

particles used to reduce the titer by one hundred fold was assayed by protein concentration determination (protein assay kit, Bio-Rad). The ratio of protein concentration to DI particle genome size was compared for all of the DI particles.

2.2.4 Radiochemical Labelling.

Virion RNA was labelled with either 5 μCi/mL of [^{3}H]uridine (43 Ci/mmol, NEN) or 200 μCi/mL of [^{32}P]orthophosphate (9120 Ci/mmol, NEN) after treating the cells with 1 μg/mL actinomycin D for two hours prior to infection. In order to get maximal incorporation of [^{32}P]orthophosphate into the virion RNA the cells were starved in a phosphate-free minimum essential media (MEM, GIBCO), for 20 hours prior to infection. Radiochemicals were added to the growth media one hour post-infection. In virion RNA preparations, infections were allowed to proceed for 16 hours, whereas in messenger RNA preparations, infections were only allowed to proceed for five hours. Messenger RNA preparations were only labelled with [^{3}H]uridine.

2.3 RNA Extraction and Purification.

2.3.1 Genomic RNA Extraction and Purification.

Labelled virus was recovered from tissue culture media by centrifugation in a SW27 rotor (Beckman) at 25,000 RPM, for two hours, at 4^{o}C. The pelleted virus was disrupted with 0.5% SDS and digested with 200 μg/mL proteinase K at 37^{o}C for 30 min (Storey et al., 1984). Virion RNA was isolated by phenol-m-cresol-8-hydroxyquinoline-chloroform extraction and precipitation with ethanol (Hefti and Bishop,

1975). Specific species of RNA were individually isolated by gel electrophoresis as will be discussed in section 2.4.

2.3.2 Messenger RNA Extraction and Purification.

Messenger RNA (mRNA) was extracted from BHK_{21} cells five hours post-infection with either VSV_{NJ}(Ogden) or VSV_{IND-HR}. The cells were washed twice with phosphate buffered saline (PBS) then once in 1 X TNE (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM Na_2EDTA). The cells were lifted from the plates by leaving the cells in contact with 1 X TNE at 4°C for 15 min. The cells were pelleted for 15 min at 3,000 X g at room temperature and resuspended in 1 X TNE containing 1 % NP40 for 15 min at 4°C. The cytosol fraction was isolated by centrifugation in a JA-20 rotor (Beckman) at 7,000 X g for 30 min at 4°C. An equal volume of 2 X NENS (100 mM sodium acetate, pH 5.2, 200 mM NaCl, 20 mM Na_2EDTA, 1% SDS) was added to the cytosol at room temperature and the mixture digested with 500 µg/mL proteinase K for 30 min at 37°C. The suspension was then phenol extracted, as described for the virion RNA, and ethanol precipitated. Alternatively, the mRNA was isolated from the cells by dounce homogenization in the presence of 1 X TMNS (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 25 mM NaCl, 250 mM sucrose) containing 25µg/mL polyvinylsulfate, 30µg/mL spermine, 1% triton X-100 (v/v), and 1% sodium deoxycholate (v/v). The cytosol fraction was then recovered and mRNA isolated as above.

In order to isolate the mRNA free of non-polyadenylated cellular RNAs and contaminating cellular DNA, the cellular RNA preparations were further purified by oligo(dT)-cellulose chromatography (Aviv and Leder,

1972; Edmonds, et al., 1971; and Maniatis, et al., 1982). The polyadenylated RNA was then ethanol precipitated and quantitated by multiplying the absorbance value at 260 nm by 40 μg/mL. The purity of the RNA was assessed by the ratio of the absorbance units at 260nm and 280nm, which should be close to 2.0. The absorbances were measured in H_2O, using a Beckman Model DU-8B spectrophometer.

2.4 Gel Electrophoresis of RNA and Purification.

2.4.1 Glyoxal Denaturation and Gel Electrophoresis.

Virion and mRNA were denatured in the presence of 1 M deionized glyoxal (prepared as described by Williams and Mason, 1985), 50% DMSO in 10 mM sodium phosphate buffer, pH 7.0 at 65°C for 20 min. The gel consisted of either 1% (for virion RNA) or 1.5% (for mRNA) agarose (BRL) prepared and electrophoresed in 10 mM sodium phosphate, pH 7.0. The electrophoresis was carried out at 100 volts for four hours (McMaster and Carmichael, 1977). Gels that were used for size determination were fixed in 10% glacial acetic acid and 30% methanol, dehydrated in 100% methanol, impregnated with 3% PPO (2,5-diphenyloxazole) in methanol, soaked in water for 30 min to precipitate the PPO, dried under vacuum, and exposed to X-ray film (Cronex-4,Du Pont) at -70°C (Bonner and Laskey, 1974).

Gels that were to be used for hybridization purposes were soaked in 25 mM sodium phosphate, pH 6.5 for 30 min and electroblotted onto Genescreen (NEN) at 25 volts, in a Biorad Electroblot apparatus containing 25 mM sodium phosphate, pH 6.5, for four hours. The blots were dried in a vacuum oven at 80°C for at least two hours.

Gels containing ^{32}P-labelled RNA were exposed wet directly to x-ray film to determine the location of individual RNA bands. These bands were cut out and electroblotted (as above) onto DEAE cellulose membrane (Schliecher & Schuell, NA-45). The RNA was eluted from these membranes in formamide high salt buffer (55% deionized Formamide, 1.8 M sodium acetate, pH 7.4, 2 mM Na_2EDTA, 0.2% SDS) at 65°C. The RNA in this form can be directly added to the hybridization solutions to be used for probing blots.

2.4.2 Methylmercuric hydroxide Denaturation and Gel Electrophoresis.

RNAs for sequence determination were isolated from 1% low melting point agarose (Seaplaque, FMC) gels containing 10 mM methylmercuric hydroxide (Bailey and Davidson, 1976), made up in 1 X EM buffer (50 mM boric acid, 10 mM sodium sulfate, 1 mM Na_2EDTA). These gels were used because the denaturation by methylymercuric hydroxide is reversible with β-mercaptoethanol, whereas, RNA denatured with glyoxal requires elevated temperature and pH conditions above 8 to reverse denaturation (conditions not conducive to intact RNA recovery). These gels were electrophoresed in 1 X EM buffer at 50 volts, for 16 hours. To recover the RNA the gels were either exposed directly to x-ray film, or if the RNA was not radioactive, stained with 1 μg/mL ethidium bromide in 0.5 M ammonia acetate and observed under short wave UV light. The gel bands were diluted in five volumes of 1 X E buffer (20 mM Tris-HCl, pH 8, 10 mM Na_2EDTA, 0.5% SDS, 20 mM β-mercaptoetanol) and heated at 65°C. The diluted gel remained liquid at room temperature. This solution was phenol extracted and ethanol precipitated.

2.4.3 Polyacrylamide Gel Electrophoresis in the Presence of 8 M Urea.

The polyacrylamide stock solution was 40% (2% bis-acrylamide, 38% acrylamide), deionized, degassed and stored at 4°C in the dark. This stock solution was diluted down to the required percentage to prepare gels.

Leader RNA were isolated from *in vitro* transcription reactions by gel electrophoresis on 12% polyacrylamide gels containing 8 M urea in 1 X TBE (2 mM Na_2EDTA in 89 mM Tris-borate buffer, pH 8.3)(Peacock and Dingman, 1967). These gels were 33 cm X 20 cm X 1.5 mm. The leader RNAs were located after exposure to x-ray film and cut out of the gels. The leader RNAs were eluted from the gel slices in 10 mM Tris-HCl, pH 7.4, 0.4 M NaCl, at 37°C and ethanol precipitated. The leader RNAs were then used as probes. The ribonuclease T_1 digestion products of the leader RNAs were observed on 20% polyacrylamide gels containing 8 M urea. These two gels were run at 200 volts (12% gel) and 400 volts (20% gel) for 16 hours.

RNA and DNA sequencing reactions were electrophoresed on 20%, 8% and 6% polyacrylamide gels containing 8 M urea. The 20% gels were 33 cm X 20 cm X 0.4 mm. The 8% and 6% gels were 85 cm X 20 cm X 0.4 mm using the BRL gel electrophoresis system (model S1, BRL) and then changed to 38 cm X 42 cm X 0.4 mm gels of the IBI thermoplate sequencing apparatus (IBI, model STS 45) at a later date. These gels were run at 30 mA constant current.

2.5 Hybridization Experiments to Determine the Genome Content of the DI Particles.

2.5.1 Hybridization of Genomic RNA to Messenger RNA.

To determine if the DI particles contained nucleotide sequences complementary to 3' mRNA, ^{32}P-labelled genomic RNA was hybridized to mRNA blots. Messenger RNA blots were prepared , as described in section 2.4.1, and cut into 4 mm strips. These strips were prehybridized in 50% deionized formamide (v/v), 0.04% polyvinyl-pyrrolidone (PVP)(w/v), 0.04% bovine serum albumin (BSA)(w/v), 0.04% ficoll, 5 X SSC (0.75 M NaCl, 0.075M sodium acetate), 1% SDS, and 0.5 mg/mL salmon sperm DNA. The strips were prehybridized for 18 hours at 45^{o}C. The prehybridization solution was replaced with a hybridization solution containing the same components as the prehybridization solution but only half the amount of PVP, BSA, Ficoll, and denatured salmon sperm DNA. The genomic RNA probes were isolated as described in section 2.4.1 and added directly into the hybridization solution. The hybridization was carried out at 42^{o}C over 48 hours. The strips were then removed from the hybridization solution and washed by the following series of washes: once in 3 X SSC (0.45 M NaCl, 0.045 M sodium acetate) at room temperature for 30 min, once in 3 X SSC at 37^{o}C for 30 min, twice in 2 X SSC (0.3 M NaCl, 0.03 M sodium acetate) containing 0.5% SDS at 42^{o}C for 30 min, once in 0.1 X SSC (0.015 M NaCl, 0.0015 M sodium acetate) at room temperature for 30 min, and once in 0.1 X SSC at 42^{o}C for 30 min. The strips were left to dry at room temperature and exposed to x-ray film at -70^{o}C.

2.5.2 Hybridization of Cloned Messenger RNA to Genomic RNA.

To ensure that the mRNA hybridization results (section 2.5.1) were specific to the DI genomic RNA and not due to contamination from STD virion RNA, nick translated plasmids containing cloned mRNAs were hybridized to genomic RNA blots. The cDNA derived clones representing VSV_{NJ}(Ogden) mRNAs were obtained from the following sources: the N mRNA clones, pN53 and pN77 (Banerjee, et al., 1984), from Dr. A.K. Banerjee (The Cleveland Clinic Foundation, Dept. of Molecular Biology, Cleveland, OH); the NS mRNA clone pNS22 (Gill and Banerjee, 1985) and the M mRNA clone pM24 (Gill and Banerjee, 1986) were graciously provided by Dr. D.S. Gill, before publication (Roche Institute of Molecular Biology, Nutley, NJ); and the G mRNA clone pNJG6 (Gallione and Rose, 1983) was provided by Dr. J.K. Rose (Yale University School of Medicine, Dept. of Cell Biology, New Haven, Ct). All of the cloned mRNAs were placed into the PstI site of pBR322. The L mRNA was not cloned at the time that this work was completed.

The mRNA clones were nick translated (Maniatis, et al., 1975; and Rigby, et al., 1977) using DNase I, E. coli DNA polymerase I, [α-^{32}P]dATP (>800 Ci/mmole, Amersham) and 1-2 μg of each plasmid (including pBR322). The unincorporated nucleotides were removed by chromatography on Sephadex G50 columns. The incorporated peak (1-5 X 10^8 cpm/μg) was added directly to the hybridization solution (identical to section 2.5.1). These nick translated plasmids were hybridized to genomic RNA blots, prepared as described in section 2.4.1. The hybridization and washing conditions were exactly as described in section 2.5.1.

2.5.3 Hybridization of Genomic RNA to Restriction Endonuclease Fragments from cDNA Clones of VSVNJ(Ogden) mRNAs.

To determine which regions of each mRNA cistron were conserved within the DI genomic RNAs, we hybridized ^{32}P-labelled genomic RNA to restriction endonuclease fragments from cDNA clones of VSV_{NJ}(Ogden) mRNA . The mRNA clones described in section 2.5.2 were individually cut with Pst I and the following restriction endonuclease combinations: Hinf I + BamH I (pN53); Hinf I + Xho I (pN77); and Hinf I (pNS22, pM24, pNJG6). The fragments varied from 9-424 base pairs in length. Figure 9 shows the position of the restriction fragments for each of the mRNA derived clones. The restriction digested plasmids were electrophoresed on 4% Nusieve (FMC) agarose gels (1 X TBE). The electrophoresed gels were electroblotted onto Genescreen plus (NEN) membranes in 1/4 X TAE (10 mM Tris-acetate, pH 7.4, 0.5 mM Na_2EDTA) and dried at room temperature. This method was tested with end-labelled restriction endonuclease fragments to assure that the smallest fragments would bind to the membrane. These results will not be shown, but indicated that this method bound even the 9 base pair fragments and that >95% of the DNA was transferred to the membranes.

The blotted restriction endonuclease digested mRNA derived clones (unlabelled) were cut into 8 mm strips and hybridized with ^{32}P-labelled genomic RNA, using a protocol identical to that described in section 2.5.1. The washed blots were let dry at room temperature and exposed to x-ray film at -70^{o}C.

2.5.4 Hybridization of Strand-Specific RNA Transcripts to Genomic RNA.

To determine whether the DI particle genomic RNAs contained only negative stranded RNA, or a mixture of positive- and negative- strand RNA, in vitro RNA transcripts were hybridized with genomic RNA blots. Since it had been previous determined that most of the DI particle genomic RNAs contained a portion of the G protein gene, the G mRNA derived clone pNJG6 was digested with Pst I (producing a 593 base pair fragment corresponding to the 3'-end of the G protein gene and a 980 base pair fragment corresponding to the 5'-end of the G protein gene), ligated (procedure for ligation to be discussed in section 2.8.1) into Pst I digested pGEM-4B (a bacterial plasmid containing the promoter sites for both the T7 RNA polymerase and the SP6 RNA polymerase positioned to transcribe opposite strands of inserted DNA placed within a multiple cloning site (Promega Biotech)) and used to transform Escherichia coli (E. coli) JM101 transformation competent cells (Frozen storage protocol 3 (Hanahan, 1985)). Following colour development in the presence of Bluo-gal (Halogenated Indolyl-β-D-Galactoside, BRL) and IPTG (Isopropylthio-β-D-Galactoside, BRL), colorless colonies were screened for the presence of the G protein gene Pst I fragments. The orientation of these fragments, with regards to which end was located adjacent to either the T7 or SP6 RNA polymerase promoters, was determined by digestion with Hinc II, for the 593 base pair fragment, and Hind III, for the 980 base pair fragment. By this procedure it was determined that the construct pGEM-NJG6-593, would transcribe a positive strand 593 base RNA transcript with SP6 RNA polymerase. The construct pGEM-NJG6-980, however would transcribe a negative strand 980

base RNA transcript with SP6 RNA polymerase. The orientation was verified by double stranded DNA sequencing using sequencing primers complementary to either the SP6 or T7 RNA polymerase promoters (the protocol will be discussed in section 2.8.4.b) The constructs were linearized with either Xba I for T7 RNA polymerase transcription reactions or with Hind III for SP6 RNA polymerase transcription reactions.

The transcription reaction mixtures contained: 40mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM each of ATP, GTP, and UTP, 12 μM CTP, 1 unit/μL RNasin, 0.5 μg linearized plasmid DNA, 50 μCi [α-^{32}P] CTP (>400 Ci/mmole, Amersham), and 10 units of either SP6 RNA polymerase or T7 RNA polymerase. The reactions were incubated at 37^{o}C for one hour, in a final volume of 20 μL. The RNA transcription reaction components were provided in the Riboprobe Gemini transcription system kit (Promega Biotech). The DNA template was digested with one unit of RQ1 DNase (Promega Biotech) for 15 min at 37^{o}C upon completion of the reaction. The RNA was recovered after phenol extraction and ethanol precipitation. These RNA transcripts were used to probe virion genomic RNA blots.

The virion RNA blots were prehybridized and hybridized by the method of Melton et al. (1984). The genomic RNA blots (prepared as in section 2.4.1) were cut into 4 mm wide strips, prehybridized (50% deionized formamide, 5 mM $NaPO_4$, pH 6.5, 5 X SSC, 0.1% SDS, 1 mM Na_2EDTA, 0.05% BSA, 0.05% PVP, 0.05% ficoll and 500 μg/mL denatured salmon sperm DNA) at 60^{o}C for 16 hours, hybridized in a new aliquot of the prehybridization solution containing the strand-specific RNA

transcripts at 60°C for 24 hours, the blots were then washed five times in 0.1 X SSC, 0.1% SDS at 65°C for 20 min, dried at room temperature and exposed to x-ray film at -70°C.

2.6 In Vitro Transcription Assays.

2.6.1 Reaction Conditions and Transcript Identification.

To determine what each DI particle was able to transcribe in vitro, the DI particles were first purified by sucrose velocity gradient centrifugation (as described in section 2.2.2). The DI particles were disrupted in 0.125% NP40 and added to a transcription reaction mixture to give the following final reaction concentrations: 0.05% NP40, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5mM $MgCl_2$, 5 mM DTT (dithiothreitol), 1 mM ATP, 0.1 mM CTP, 0.1 mM UTP, 0.05mM GTP, 50 μCi/reaction [α-32P]GTP (>760 Ci/mmole, Amersham). The enzyme reactions were incubated at 30°C for three hours and terminated by the addition of EDTA and SDS to final concentrations of 10 mM and 1%, respectively. The products of transcription were separated from the unincorporated nucleotide triphosphates by chromatography through Sephadex G50 (Superfine, Pharmacia) columns in the presence of 1 X TNE, 0.1% SDS. The first peak was phenol extracted and ethanol precipitated.

The RNA transcripts were resolved on either a 12% polyacrylamide gel containing 8 M urea (see section 2.4.3), to resolve leader RNA sized transcripts, or a 1.5% agarose gel after glyoxal denaturation (see section 2.4.1), to resolve mRNA sized transcripts. The 12% polyacrylamide gels were exposed to x-ray film at -70°C, whereas the 1.5% agarose gels were fixed and dried (without fluorography) and

exposed to x-ray film at -70°C. It has been previously determined that RNase T1 digestion is able to differentiate positive and negative strand leader RNAs. Therefore, the RNA transcripts were digested with RNase T1 (1000 units/mL, Boehringer Mannheim) for 45 min at 37°C and electrophoresed on 20% polyacrylamide gels containing 8 M urea (see section 2.4.3). These gels were exposed directly to x-ray film at-70°C.

2.6.2 Hybridization of Leader RNAs to Genomic RNAs.

To determine whether the DI particles contained the RNA templates for the positive strand leader RNA, the negative strand leader RNA or both, northern blotted viral RNA was hybridized with leader RNA transcripts, isolated from 12% polyacrylamide gels (as described in section 2.4.3). The RNA transcripts originated from VSV_{IND-HR}, VSV_{NJ}(Ogden), IND-011, NJ-JM2, NJ-121, NJ-PG1, and NJ-PG2. The hybridization and washing conditions were exactly as described in section 2.5.1.. The melting temperature, T_m, a measure of the thermal stability of the hybrids was calculated by the following formula:

$$T_m = 81.5 + 16.6(\log M) + 0.41(\%G + C) - 0.72(\% \text{ formamide}) - 1.4(\% \text{ mismatch}).$$

where M is the molarity of the monovalent cation and (% G + C) is the percentage guanine and cytosine residues in the nucleotide probe. The % mismatch represents the percentage that the probe differs from the target RNA sequence (Howley, et al., 1979; and Hyman, et al., 1973). The hybridization conditions here give a M value of 0.75 and the New Jersey positive strand leader RNA has a % G + C value of 33% (Colonno

and Banerjee, 1978a,b,c), therefore the T_m value is 57°C. As the Indiana positive strand leader RNA has a 25% mismatch with the New Jersey positive strand leader RNA template, the T_m value for a hybrid between these two RNAs would only be 22°C. These values indicate that under the conditions of hybridization used here non-specific hybridization between the two serotypes would be unlikely.

2.7 RNA Sequencing.

2.7.1 Determination of the 3' Terminal Nucleotide.

In order to determine the 3'-nucleotide sequence of the DI particle genomes, RNA was isolated from the NJ-PG1/2 DI particle preparation (as described in section 2.3.1). This RNA was 3'-end labelled (England and Uhlenbeck, 1978) with T_4 RNA ligase in a reaction mixture containing: 5 μg RNA, 50 mM Hepes, pH 7.6 (KOH), 10 mM $MgCl_2$, 50 μM ATP, 3 mM DTT, 50 μCi cytidine 3',5'[5'-^{32}P]bisphosphate (>3,000 Ci/mmol, Amersham), 1 unit/μL T_4 RNA ligase (Pharmacia). The reaction was incubated at 4°C for 16 hours, and the RNA recovered by phenol extraction and ethanol precipitation. This RNA was denatured with 10 mM methylmercuric hydroxide, electrophoresed on 1% agarose gels and recovered as described in section 2.4.2.

To determine the 3'-terminal nuclectide from these RNAs, the individual RNAs were digested with RNase T_2 and the individual ribonucleotides separated by chromatography on thin-layer polyethyleneimine (PEI) plates (Volckaert and Fiers, 1977).

2.7.2 Chemical Modification and Cleavage Method.

To determine the 3'-terminal sequence the individually isolated 3'-labelled RNAs were chemically modified (Peattie, 1979) with the following nucleotide specific agents: dimethyl sulfate (G reaction), diethyl pyrocarbonate (A > G reaction), anyhrous hydrazine containing 3 M NaCl (C > U reaction), and 50% hydrazine (U reaction). The partially modified RNAs were then cleaved with 1 M aniline-acetate, pH 4.5. The RNA cleavage products were electrophoresed on 20% and 6% polyacrylamide gels containing 8 M urea (see section 2.4.3). This method enabled the determination of ~100 nucleotides from the 3'-end of the labelled RNA.

2.7.3 Dideoxynucleotide Sequencing Genomic RNAs.

In order to be able to sequence further within the genomic RNAs, oligonucleotides were made, complementary to specific regions within either the STD or DI particle genomes, and used in primer extension reactions. This procedure was used to confirm the VSV_{NJ}(Ogden) 5'-terminal RNA sequence, to determine the length and sequence of the stem structure in NJ-PG1, to sequence the complete L mRNA sequence of VSV_{NJ}(Ogden) and to determine where the RNA polymerase jumped off the L sequence during the generation of NJ-PG1. The oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer, using the phosphoramidite method (Caruthers, et al., 1987). The oligonucleotide primers (200 ng) were labelled with 50 pmol [γ-^{32}P] ATP (250 μCi of 5,000 Ci/mmol, Amersham) by polynucleotide kinase (1 unit/μL, Pharmacia) in 1 X PK buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 5 mM DTT, 0.1 mM spermidine, 0.1 mM Na_2EDTA) at 37^{o}C for 30 min.

The 5'-end labelled primers were purified on 12% polyacrylamide gels containing 8 M urea (as described in section 2.4.3, for leader RNA purification). The gels were electrophoresed at 30 mA.

The primer extension sequencing reactions began by taking 5μg of RNA, adding 2 X 10^6 CPM of labelled primer, heat denaturing at 95^{o}C, and quick chilling in ice water. This mixture was split into five equal portions and added to reactions containing 100mM Tris-HCl, pH 8.3, 130 mM KCl, 10 mM $MgCl_2$, 25 mM DTT, 80 μg/mL Actinomycin D, 100 μM each dNTP, 2 units/μL AMV reverse transcriptase (Pharmacia). Different dideoxynucleosides triphosphates were added to each of four of the five reactions with a dideoxynucleoside triphosphate/deoxynucleoside triphosphate ratio of 0.5. The fifth reaction contained no dideoxynucleosides triphophates. The reactions were incubated at 42^{o}C for 45 min. The reactions were then ethanol precipitated and resuspended in formamide dye (90% formamide, 1 X TBE, 0.05% xylene cyanol, 0.05% bromophenol blue, and 0.05% Orange G). The reaction products were electrophoresed in 20% and 8% polyacrylamide gels containing 8 M urea (as described in section 2.4.3) and exposed to X-ray film at -70^{o}C.

2.8 Cloning of Genomic RNA Sequences.

2.8.1 Construction of cDNA Banks.

In order to determine the location and distance between the positive- and negative-strand leader RNA templates in the DI particle, NJ-121, we produced two cDNA banks from the NJ-121 RNA preparation. The first was produced using the oligonucleotide, CYK-1 (5'-ACG AAG ACA

AAA AAA CCA TT-3'), which was complementary to the first 20 nucleotides at the 3'-end of VSV_{NJ}(Ogden) standard virion RNA (Rowlands, 1979). If the positive-strand leader RNA template was at the 3'-end of NJ-121, then clones containing DNA copies of both leader RNA templates (assuming that the distance between the two was not too large) or clones containing DNA copies of the negative-strand leader RNA template not adjacent to the end of the cDNA should have been produced. The second cDNA bank was produced using the oligonucleotide, ER-1 (5'-TGA CAA AAG GCC AAA AGA AGG-3'), which was complementary to nucleotides 25-45 from the 3'-end of the negative-strand leader RNA template (as determined by RNA sequence analysis). The first 20 nucleotides were not used because the negative-strand and positive-strand leader RNA template sequences only differ at nucleotide 19 in these sequences. If the negative-strand leader RNA template was located at the 3'-end of NJ-121, then clones containing both leader RNA templates or containing the positive-strand leader RNA template not adjacent to the end of the cDNA clones should have been produced.

The method of cDNA synthesis was a modification of the Gubler and Hoffman (1983) technique as described by Watson and Jackson (1985). Essentially this technique uses two separate reactions for the first and second strand synthesis. The first strand synthesis consisted of: 6 μg RNA, 200 ng primer, 1 X RT buffer (50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 50 mM KCl), 1 mM each dTTP, dCTP, dATP, dGTP, 10 μCi [α-^{32}P]dATP (>3,000 Ci/mmol, Amersham), 10 mM DTT, 1 unit/μL RNasin (Pharmacia), and 1 unit/μL AMV reverse transcriptase (Life Sciences). The reaction was incubated at 42^{o}C for two hours and terminated by phenol extraction

and ethanol precipitation. The second strand synthesis consisted of 95% of the first strand synthesis product, 1 X Pol 1 buffer (4 mM $MgCl_2$, 15 mM β-mercaptoethanol, 65 mM KCl), 100 mM HEPES, pH 7.6, 0.15 mM each dTTP, dCTP, dATP, dGTP; 100 μCi [α-^{32}P]dATP (>3,000 Ci/mmol, Amersham), 0.15 mM β-NAD (Sigma), 0.3 units/μL DNA Pol 1 (New England Biolabs), 0.02 units/μL RNase H (BRL), and 0.02 units E. coli DNA ligase (New England Biolabs). The second strand synthesis was incubated at 16°C for one hour, then at 23°C for an additional hour. The reaction was terminated by phenol extraction and ethanol precipitation.

T_4 DNA polymerase (3'-exonuclease) was used to generate blunt ends on the double stranded cDNA. The double stranded DNA was placed into a reaction containing: 1 X T_4 DNA polymerase Buffer (33 mM Tris-acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.1 mg/mL BSA (nuclease free), 0.5 mM DTT), 0.16 mM each of dTTP, dCTP, dATP, and dGTP, and 0.16 units/μL T_4 DNA polymerase. This reaction was incubated at 37°C for 30 min. The reaction was terminated by phenol extraction and ethanol precipitation.

The cDNA preparations were then either blunt end ligated directly into the Sma I site of pUC19 (Yanishe-Perron et al., 1985)(library made with CYK-1) or they were blunt end ligated with phosphorylated Pst I linkers (Pharmacia)(library made with ER-1). The blunt end ligation reactions consisted of 1 X T_4 DNA ligase buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5% (w/v) PEG 8,000, 1 mM ATP, 1 mM DTT), 0.5 unit/μL T_4 DNA ligase (Pharmacia), the cDNA and either 5 μg Pst I phosphorylated linkers, or 250 ng pUC19 (Sma I cut and

dephosphorylated). The ligations were incubated at 22°C, for four hours. The cDNA, linked with Pst I linkers, was digested with Pst I and size selected on Sepharose 4B (Pharmacia) columns. The cDNA averaging over 1 Kb pairs in length was ligated into the Pst I site of pUC19, by the same protocol used above for blunt end ligation. The plasmids were used to transform competent E. coli JM101 (Hanahan, 1985; frozen storage protocol 3). Ampicillin resistant, colorless colonies were picked from SOB agar plates (1.5% agar, 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM Nacl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) containing 50 μg/mL ampicillin and coated with 50 μL 2% Bluo-Gal (Halogenated Indolyl-β-D-Galactoside, BRL) and 10μL 100mM IPTG (Isopropylthio-β-D-Galactoside, BRL). The colorless colonies were replica plated on B agar plates (2% agar, 1% Bacto tryptone, 0.8% NaCl, 0.001% vitamin B-1) containing 50 μg/mL ampicillin. The bacterial colonies were grown directly on Colony/Plaque screen membranes (NEN) for all but one of the replica plates. These membrane bound colonies were denatured in 0.5 N NaOH, neutralized in 1M Tris-HCl, pH 7.5, and dried. Before being used in hybridization reactions the membranes were washed three times with 3 X SSC, 0.1% SDS at 65°C for a total of 16 hours.

2.8.2 Screening Clone Banks for Leader RNA specific clones.

The membrane bound clones prepared in section 2.8.1 were screened for leader RNA specific colonies. The membranes were prehybridized for five hours at 65°C with 40 mL of 1% SDS, 1M NaCl and 10% dextran sulfate. The probes used were the 47 nucleotide long leader RNA transcripts produced by in vitro transcription (prepared as described

in section 2.6.2) of VSV_{NJ}(Ogden) standard virions (positive-strand leader RNA) and the DI particle, NJ-PG1 (negative-strand leader RNA). The probes were added, separately, to the prehybridization buffer with 500 μg denatured salmon sperm DNA and incubated at 65°C for 24 hours. The hybridized membranes were washed twice with 2 X SSC at 22°C for 10 min, twice with 2 X SSC containing 1% SDS at 65°C for 30 min, once with 0.1 X SSC at 22°C for 30 min and once with 0.1 X SSC at 65°C for 30 min.

The clones which hybridized with the leader RNAs were rehybridized to assure that the hybridization was not an artifact. The clones remaining positive were replated on LB agar plates (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl, 0.2% glucose, and 2% Bacto agar) containing 50μg/mL ampicillin. The plasmid DNA was extracted from these clones, restriction endonuclease digested and grouped according to common restriction site patterns.

2.8.3 Preparation of Plasmid DNA.

The primary screening of clones for common restriction endonuclease cleavage patterns was performed on plasmid DNA prepared by the alkaline lysis method (Birnboim and Doly, 1979). The bacterial colonies were grown overnight at 37°C in LB medium containing 50μg/mL ampicillin. The RNA was digested with 100μg/mL pancreatic RNase at 37°C for 30 min. Clones which contained both leader RNA template regions and other clones of interest were then prepared by a large scale alkaline lysis plasmid preparation (Birnboim and Doly, 1979). The bacterial cultures were grown in LB medium containing 50 μg/mL ampicillin until they

reached an optical density (600nm) of ~0.4. The plasmids in these cells were amplified in the presence of chloramphenicol (170 μg/mL). The DNA prepared by this method was purified on equilibrium CsCl-EtBr gradients after centrifugation in a VTi 65 (Beckman) rotor at 325,000 X g for 18 hours at 20°C. The closed circular forms of plasmids were extracted with 1-butanol and ethanol precipitated (Maniatis, et al., 1982).

2.8.4 DNA Sequencing.

2.8.4.a Chemical Modification and Cleavage Method.

The clones produced using CYK-1 (positive strand leader RNA template specific) were digested with EcoR I and Pst I (situated on either side of the Sma I site in pUC19) and 3'-end labelled by the fill-in reaction: 100μCi[α-^{32}P]dATP (>800 Ci/mmol, Amersham), 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 2 mM each dNTP (-dATP), 5 units DNA Pol 1 (Klenow fragment). After 15 min the reaction was chased with 2 mM dATP for an additional 10 min. This reaction will label only the EcoR I end of the plasmid. The end-labelled fragments were isolated on 1.5% agarose gels (1 X TBE) and eluted using a IBI (Model UEA) electroeluter in low salt buffer (20 mM Tris-HCl, pH 8.0, 5 mM NaCl, 0.2 mM Na_2EDTA) at 100 volts for 30-60 min, depending upon the size of the fragment. The fragments were ethanol precipitated and washed with 70% ethanol.

The end-labelled DNA was then chemically modified by the method of Maxam and Gilbert (1980). The modification reactions contained 50% hydrazine (C+T specific, Fisher), or 50% hydrazine in the presence of 33% saturated NaCl (C specific), or 63% formic acid (G+A specific,

BDH), or 0.5% dimethylsulfate (Aldrich) in 50 mM sodium cacodylate, pH 8.0, 10mM $MgCl_2$, 1 mM Na_2EDTA (G specific). The modification reactions were stopped with four volumes of C+T stop (0.3 M sodium acetate, 0.1 mM Na_2EDTA, 25 μg/mL tRNA) for the C, C+T, and A+G reactions. The G reaction was stopped with 1/4 volume G stop (1.5 M sodium acetate, pH 7.0, 1.0 M β-mercaptoethanol, 100μg/mL tRNA). The stopped reactions were ethanol precipitated twice, cleaved with 10% piperidine (Aldrich) at 90^{o}C for 15 min, diluted with two volumes H_2O and lyophilized four times (resuspending in 25μL H_2O each time). The cleaved DNA was resuspended in 10 μL Formamide-dye (80% deionized formamide, 1 X TBE, 0.1 % xylene cyanol, 0.1% bromophenol blue, 0.1% orange G) heated to 95^{o}C for five min and loaded onto 20%, and 6% polyacrylamide gels containing 8M urea (as described in section 2.4.3).

2.8.4.b Sequence Analysis of Alkali Denatured Double Stranded DNA.

The GemSeq K/RT system (Promega) was used for rapid sequencing of the inserted DNA in either pUC 19 or pGEM4B. The supercoiled plasmid DNA (1-2μg) was denatured with alkali (0.2 M NaOH, 0.2 mM Na_2EDTA) at 22^{o}C, neutralized (0.36 M sodium acetate, pH 5.0) and ethanol precipitated. The DNA was resuspended in 1 X Klenow buffer (10 mM Tris-HCl, pH 7.5, 20 mM NaCl) with 30 ng of oligonucleotide primer. The primers used were: the M13 forward primer (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'), and the M13 reverse primer (5'-CAG GAA ACA GCT ATG ACC ATG-3') for pUC 19; the T7 promoter primer (5'-TAA TAC GAC TCA CTA TAG GG-3'), and the SP6 promoter primer (5'-ATT TAG GTG ACA CTA TAG-3') for

pGEM4B. The primer-DNA mixture (10 μL) was incubated for two hours at 37°C. The mixture was then added to 40 μCi [α-^{32}P]dATP (4 μl,>400 Ci/mmol, Amersham) and 5 units of Klenow (1 μL) and split into four tubes containing T, C, A, and G Klenow nucleotide mixes (3 μL each of a dideoxy/deoxynucleotide mixture provided in the kit). These reactions were incubated together for 15 min, chased (1 μL chase solution) for an additional 15 min and stopped (5 μL stop solution) as described in the kit. The sequencing reaction was heated at 70°C for three min and loaded onto 20%, 8% and 6% polyacrylamide gels containing 8 M urea (as described in section 2.4.3).

2.9 Polymerase Chain Reaction.

2.9.1 Determination of Sequence Between Two Leader RNA Templates in NJ-121.

A new technique has been developed to amplify specific regions of DNA from large genomes (Saiki, et al., 1985) using the thermostable DNA polymerase of Thermus aquaticus (Saiki, et al., 1988). This technique consists of adding two oligonucleotides, each complementary to the opposite strand of DNA, positioned in such a manner that the action of the DNA polymerase copies the DNA back and forth between the two oligonucleotides. The amplification occurs over several (25-30) rounds of: heat denaturation to separate the double stranded DNA (94°C, 1.5 min), hybridization of the primers to the single stranded DNA (50°C, 2.5 min),and polymerization at the optimal temperature of the enzyme (72°C, 4 min). This technique was modified to amplify specific regions of cDNA made from the NJ-121 RNA preparation (as described in section

2.8.1). The cDNA produced with the ER-1 primer was used here, because this cDNA produced clones containing the positive-strand leader RNA template positioned beside part of the G mRNA template. The cDNA was amplified using the following oligonucleotide primers: ER-1 (which is complementary to the negative-strand leader RNA template), or ER-8 (5'-TTC GAA TTC TGA CAA AAG GCC AAA AGA AGG-3') which has TTC GAA TTC, the EcoR1 recognition site, added to the 5'-end of ER-1, and in different trials either ER-3 (5'-TTT AAT TAA AAG TTC CCT CT-3)', identical to ten bases on either side of the junction between the G mRNA and the positive-strand leader RNA template, ER-4 (5'-CCA AAT TGT AAT GG-3'), identical in sequence to bases 35 to 16 from the positive-strand leader RNA template, or ER-9 (5'-TTC GAA TTC CCT CTA GGC CAA ATA ATT G-3'), containing TTC GAA TTC, the EcoR1 recognition site, added to the 5'-end of nucleotides 43 to 25 from the positive-strand leader RNA. If the two leader RNA templates within NJ-121 were positioned in a copy-back configuration (like DI-LI2), and if the distance between the two templates was not too large (only 25 nucleotides apart in case of DI-LI2), this technique should have amplified a length of DNA equivalent to the distance between the two leader RNAs. The reaction consisted of 1 X Taq Pol Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (w/v)), 0.5 mM dNTP, 1 μg ER-1 primer, 1 μg ER-3 primer(or ER-4 primer), 0.1 μg cDNA, and 2.5 units Taq polymerase in a 100 μL reaction. The reaction mixture was overlayed with 100 μL of mineral oil to minimize evaporation. Upon completion of the amplification, the mixture was chloroform (24:1 chloroform/isoamyl alcohol) extracted to get rid of the mineral oil overlay, phenol

extracted, and chloroform extracted once more. The DNA was recovered by ethanol precipitation. As a control 121-16-13, a clone in pUC 19 containing a 495 bp fragment from the 5'-end of VSV_{NJ}(Ogden) standard virus ligated onto a 328 bp fragment from the 3'-end of the standard virus, in a copy-back configuration.

The amplified products were restriction endonuclease digested to confirm that they contained the restriction pattern from the 5'-end of the genome. The restriction endonucleases Hind III, Hinc II and EcoR1 were convenient for this purpose.

2.9.2 Cloning of Amplified DNA and Sequence Identity.

It was determined that the amplified product had two Hind III sites. The sequence derived from the 5'-end of the VSV_{NJ}(Ogden) standard virion RNA indicated that there was two Hind III sites separated by a distance eqivalent to the distance separating the two Hind III sites in the amplified product from NJ-121 cDNA. If the amplified product was derived from the complement of the 5'-end of the standard virus genome (ie., from a stem structure on the 3'-end of a DI particle) the sequence should be identical. To prove that these products came from the complement of the 5'-end of the standard virion, the amplified Hind III fragment was ligated into the Hind III site of pGEM4B (as described in section 2.8.1), colorless colonies were screened for clones containing the Hind III fragment with two internal Hinc II sites. The sequence within these clones was determined by primer extension using alkaline denatured DNA as a template and the T7 and SP6 polymerase promoter primers (as described in section 2.8.4.b).

This sequence was compared to the sequence at the 5'-end of the VSVNJ(Ogden) standard virion RNA.

To determine the sequence between the Hind III sites and the ends of the amplified products, we end-labelled the Hind III fragments of the amplified product (as described in section 2.8.4.a), isolated them individually on 10% polyacrylamide gels (1 X TBE) and strand separated them on 8% polyacrylamide gels (8% acrylamide, 0.24% bis-acrylamide) (Maniatis, et al., 1982). The strand separated fragments were chemically modified and sequenced (as described in section 2.8.4.a).

The amplified products from the reaction using ER-8 and ER-9 as primers were digested with EcoR1 (the amplified product contained an internal EcoR1 site) and ligated into the EcoR1 site of pGEM4B (as described in section 2.8.1). Colorless colonies were selected, and clones containing either the 230 base pair fragment (derived from the 5'-end of the genome) or the 725 base pair fragment (containing the sequence from the internal EcoR1 site to the end of the amplified product opposite the negative-strand leader RNA template) were isolated. The clones containing the 725 base pair fragment were chosen to determine the joining sequence between the positive-strand leader RNA and the copy-back sequence. Four of these clones were prepared in large quantities, cesium chloride gradient purified and sequenced by primer extension using alkali denatured supercoiled DNA as a template and the T7 and SP6 polymerase promoter primers (as described in section 2.8.4.b). The sequence was confirmed on 4 separate clones using the Genesis 2000 automated DNA sequencer. The Genesis 2000 sequencing protocol consisted of a primer extension using alkali denatured double

stranded DNA (section 2.8.4.b) in the presence of the T7 and SP6 polymerase promoter primers, T7 DNA polymerase (Sequenase, USB Corp.) and fluorescent labeled dideoxynucleoside triphosphates, as developed by DuPont (Wilmington, Delaware). The Genesis 2000 was on loan from DuPont, Wilmington, Delaware.

2.10 Computer Analysis of Nucleotide Sequences.

The nucleotide sequences were analyzed on an IBM Personal System 2/ model 30 computer. The software used for the analysis was that of PC/Gene (version 4.05, 1986, Dept. of Medical Biochemistry, University of Geneva, Switzerland, distributed by Intelligenics, Inc., Mountain View, CA) provided under the license of Dr. D. Hickey (University of Ottawa, Dept. of Biology).

3. Results.

3.1 Generation of DI Particles from VSV_{NJ}(Ogden).

One of the goals of this study was to generate DI particles from VSV_{NJ}(Ogden), resembling DI-LT1 in size (50% standard genome size) and conserving the 3'-termini physically and transcriptionally intact (positive-strand leader RNA and N,NS,M, and G mRNA template regions). All of the VSV_{NJ} DI particles characterized in the literature, until this study, retained only portions of the L protein gene sequence (Schnitzlein, and Reichmann, 1977).

DI-LT (presumably containing both DI-LT1 and DI-LT2) was generated from a heat resistant strain of VSV_{IND} (Petric and Prevec, 1970) through a series of high MOI passes after repeated heat treatment at 43°C. A further series of less well defined 3'-DI particles were also generated by plaque purifying the heat resistant strain of VSV_{IND}, several times at 40.5°C and repeatedly passing at high MOI (Kang, et al., 1984, 1985). The same strategy was used here to generate 3'-DI particles from VSV_{NJ}(Ogden).

Two series of heat treated DI particle preparations were generated, NJ-121 and NJ-PG1/2. These preparations had one large DI particle, sedimenting close to the standard virus on sucrose gradients (denoted NJ-121 and NJ-PG2), and in the case of NJ-PG1/2, a smaller DI particle sedimenting much slower (denoted NJ-PG1). Upon further passing the NJ-121 DI particle preparation two smaller DI particles were generated, NJ-121a and NJ-121b. As a control, VSV_{NJ}(Ogden) was passed at high MOI on R(B77) cells (the same cell line used to generate NJ-121 and NJ-

PG1/2) without heat treatment. This treatment generated the NJ-JM2 DI particle preparation.

To accurately determine the size of the DI particles within these preparations, virion associated RNA was denatured with glyoxal and electrophoresed on 1% agarose gels (Figure 6). The sizes of each DI particle genomic RNA and their calculated molecular weights are summarized in Table 2. The two largest DI particles, NJ-121 and NJ-PG2 are just slightly larger than the VSV_{NJ}(Ogden) L mRNA (6580 nucleotides, including a 200-nucleotide-long poly(A) tail). These two DI particles were therefore large enough to contain the full 3'-half of the standard virus genome and retain the 5'-terminal sequence required for replication. Therefore two possibilities were examined: first, did these DI particles contain the template regions for the N, NS, M and G mRNAs; and second, did they contain the template region for the positive-strand leader RNA in a transcriptionally active form?

3.2 Genetic Content of VSV_{NJ}(Ogden) DI Particles.

To determine if these DI particles contained the template regions for the N, NS, M and G mRNAs, the following experiments were carried out: first, VSV_{NJ}(Ogden) mRNA blots were probed with ^{32}P-labelled DI particle genomic RNA; second, DI particle genomic RNA blots were probed with nick-translated clones of VSV_{NJ}(Ogden) mRNAs; third, VSV_{NJ}(Ogden) mRNA clones were digested with restriction endonucleases, the fragments separated on agarose gels and probed with ^{32}P-labelled DI particle genomic RNAs; fourth, DI particle genomic RNA blots were probed with strand-specific RNA transcripts from a clone of VSV_{NJ}(Ogden) G mRNA.

Figure 6. Agarose gel electrophoresis of ^{3}H-labeled viral RNAs. ^{3}H-labeled RNAs were phenol extracted from pelleted DI particle preparations, denatured by treatment with glyoxal (McMaster and Carmichael, 1977) and electrophoresed on a 1% agarose gel. ^{3}H-labeled 18 S (~2200 nucleotides) and 28 S (~5100 nucleotides) rRNA (rRNA lane); standard VSV_{IND} RNA preparation containing standard virion RNA (11,162 nucleotides) (Schubert, et al., 1984), and two DI particles, IND-ER1 and IND-ER2 (IND-STD lane); IND-011 RNA preparation containing standard virion and IND-011 RNAs (2334 nucleotides) (Meier, et al., 1984), (IND-011 lane); VSV_{NJ} (Ogden) standard virus RNA preparation containing the virion RNA (11,123 nucleotide), (NJ-STD lane); NJ-JM2 RNA preparation containing the standard virion and NJ-JM2 RNAs (NJ-JM2 lane); NJ-121 RNA preparation containing the standard virion, NJ-121, NJ-121a and NJ-121b RNAs (NJ-121 lane); NJ-PG1/2 RNA preparation containing the standard virion, NJ-PG2 and NJ-PG1 RNAs (NJ-PG1/2 lane); VSV_{NJ} (Ogden) mRNA preparation containing the standard virion RNA (11,123 nucleotides), the L mRNA (6398 nucleotides) (Feldhaus and Lesnaw, 1988), the G mRNA (1573 nucleotides) (Gallione and Rose, 1983), the N mRNA (1329 nucleotides) (Banerjee, et al., 1984), the NS mRNA (856 nucleotides) (Gill and Banerjee, 1985), and the M mRNA (758 nucleotides) (Gill and Banerjee, 1986) (NJ mRNA lane). In addition to the genomic RNA templated sequences, the mRNAs are known to contain an additional, approximately, 200-nucleotide-long poly(A) tail at their 3'-termini (Rose, et al., 1977). The size of the standard virion RNAs, the VSV_{NJ} (Ogden) mRNAs and the rRNAs were used to determine the size of the DI particle RNAs. The resulting sizes are tabulated in table 2.

rRNA
IND-STD
IND-011
NJ-STD
NJ-JM2
NJ-121
NJ-PG1/2
NJ mRNA
28s-
18s-
ER1
ER2
011
STD
STD
STD
STD
121
121a
121b
PG2
PG1
JM2
-40s
-L
-G
-N
-NS
-M

Table 2. Size of DI particle RNA genomes. a. As determined by glyoxal denaturation and electrophoresis on a 1% agarose gel. The molecular weight markers were eukaryotic rRNA and VSV_{NJ}(Ogden) mRNA (Figure 6). b. Length of total genome was taken as 11,162 nucleotides, the length of the VSV_{IND} standard virion genome (Schubert, et al., 1984). c. Average molecular weight of a ribonucleotide being taken as 342.5 (Galinski, et al., 1982). d. IND-011 was previously published as being 20.9% of the VSV_{IND} genome size (Meier, et al., 1984).

DI Particle	Serotype of Origin.	Estimated No. of Nucleotides. (a,b)	Estimated Molecular Weight. (c)	% of Total Genome.
NJ-121	New Jersey	6750	2.3×10^6	61
NJ-121a	New Jersey	5600	1.9×10^6	50
NJ-121b	New Jersey	4250	1.5×10^6	38
NJ-PG2	New Jersey	6750	2.3×10^6	61
NJ-PG1	New Jersey	3100	1.1×10^6	28
NJ-JM2	New Jersey	2800	9.7×10^5	25
IND-ER1	Indiana	4450	1.5×10^6	40
IND-ER2	Indiana	2700	9.2×10^5	24
IND-011	Indiana	2500	8.6×10^5	22(d)

Messenger RNA (^{3}H-labelled) blots were probed with the genomic RNA of each DI particle (Figure 7). The probes were produced by growing the DI particles in the presence of ^{32}P-orthophosphate, extracting the genomic RNA, purifying the RNA on agarose gels after glyoxal denaturation and cutting out the isolated DI particle genomic RNA bands. None of the VSV_{IND} genomic RNAs hybridized with the VSV_{NJ}(Ogden) mRNA. NJ-JM2 hybridized mainly with the L mRNA and very weakly to the G mRNA. However, the DI particles from the NJ-121 and NJ-PG1/2 DI particle preparations hybridized with all of the mRNAs. Interestingly, NJ-121 and NJ-PG2 although identical in size, did not hybridize to the mRNAs in an identical manner. NJ-PG2 hybridized to the mRNAs in a manner similar to VSV_{NJ}(Ogden) standard virus, whereas, NJ-121 hybridized preferentially to the L and G mRNAs. Also the smaller DI particles, NJ-121a, NJ-121b and NJ-PG1 seemed to hybridize to the mRNAs in a similar pattern as their larger counterparts, however with lesser intensity. It should be noted that the DI particle genomic RNAs hybridized preferentially to the L mRNA.

To be certain that the mRNA hybridization patterns were due to DI particle genomic content and not due to standard virus breakdown products, cDNA clones derived from VSV_{NJ}(Ogden) N, NS, M and G mRNA were nick-translated and used to probe DI particle genomic RNA blots (Figure 8). The VSV_{NJ}(Ogden) N mRNA clones hybridized weakly to the VSV_{IND} standard virus genomic RNA (the N mRNAs share 68% sequence homology), but none of the other mRNA clones hybridized to the VSV_{IND} standard virus genome. VSV_{NJ}(Ogden) standard virus RNA hybridized well with all of the probes (except pBR322). All of the DI particle genomic

Figure 7. Hybridization of ^{32}P-labeled DI particle genomic RNAs to Northern blotted VSV_{NJ}(Ogden) mRNAs. VSV_{NJ}(Ogden) mRNAs were isolated from standard virus infected cells, denatured with glyoxal and electrophoresed on a 1.5% agarose gel. The mRNAs were electroblotted onto Genescreen (NEN), baked, and cut into 4-mm-wide strips (along the axis of migration). The strips were hybridized with in vivo ^{32}P-labeled DI particle genomic RNAs. The DI particles genomic RNA probes were prepared as described in section 2.4.1. The position of each mRNA was determined by using an Enhanced (NEN) strip of ^{3}H-labeled mRNAs (^{3}H-NJ mRNA lane). The DI particle genomic RNAs used as probes are indicated above the according lanes. IND signifies that the origin of the standard (STD) or DI particle was the VSV_{IND-HR} strain. NJ signifies that the origin of the standard or DI particle was the VSV_{NJ}(Ogden) strain.

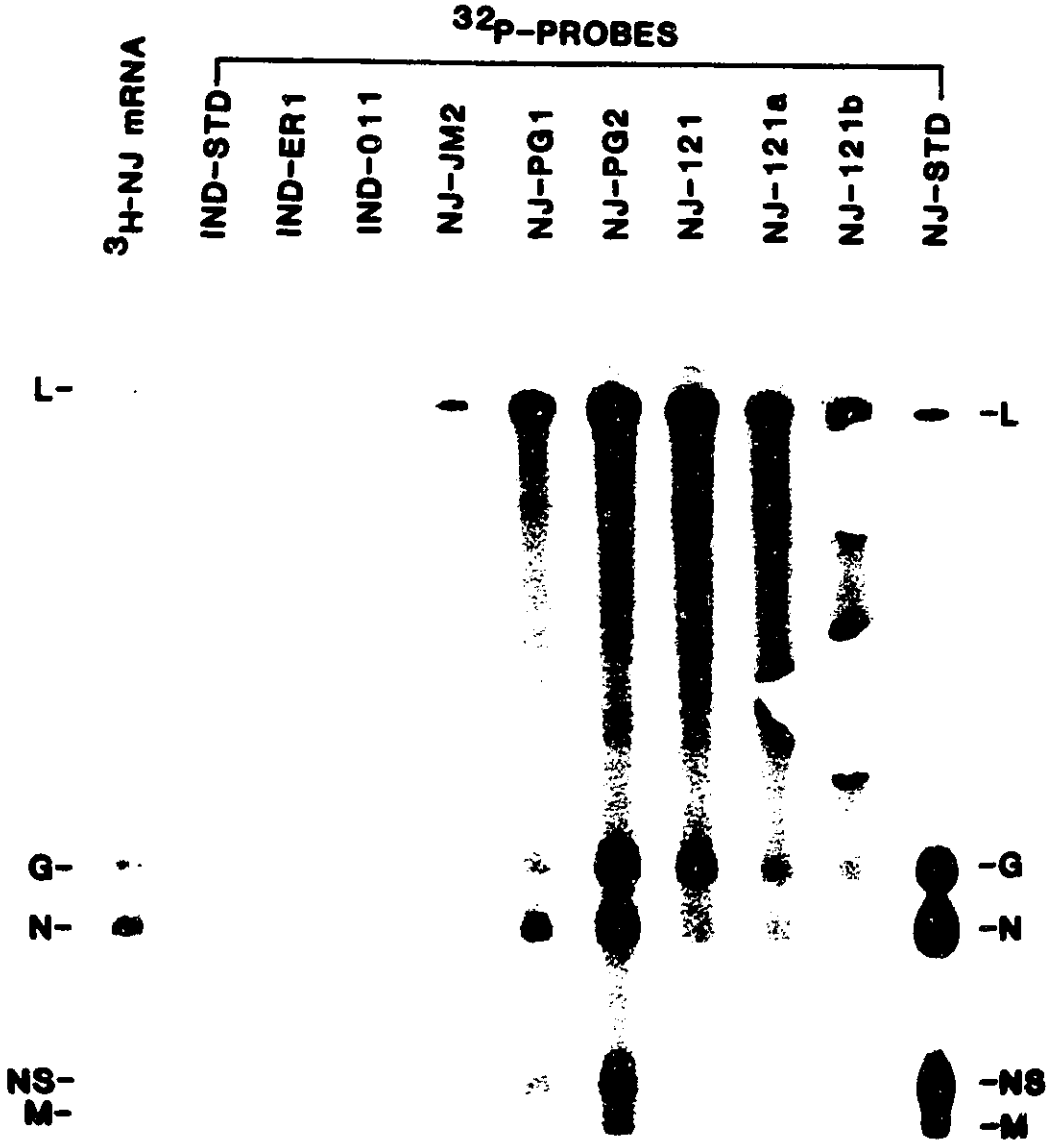
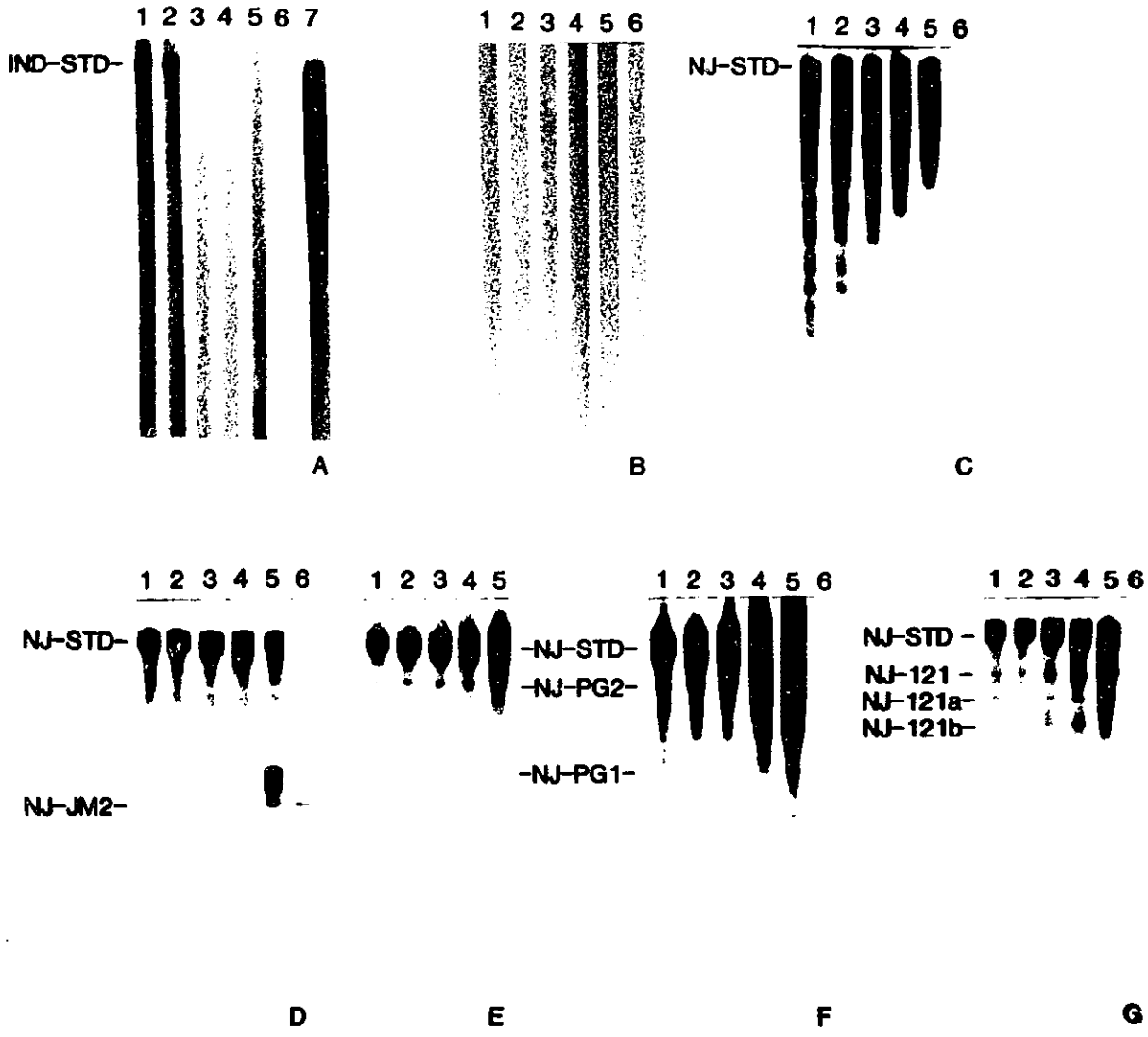

^{32}P-PROBES
^{3}H-NJ mRNA
IND-STD
IND-ER1
IND-011
NJ-JM2
NJ-PG1
NJ-PG2
NJ-121
NJ-121a
NJ-121b
NJ-STD
L-
G-
N-
NS-
M-
-L
-G
-N
-NS
-M

Figure 8. Hybridization of nick translated cDNA clones, derived from VSV_{NJ}(Ogden) mRNAs, to Northern blotted DI particle genomic RNAs. The genomic RNAs from the standard and DI particle genomic RNA preparations (identical RNA preparation to that shown in Figure 6) were denatured with glyoxal, electrophoresed in 1% agarose gels, and electroblotted onto Genescreen (NEN). The blots were cut into 4-mm-wide strips, prehybridized, and hybridized with nick translated cloned VSV_{NJ}(Ogden) mRNAs. Panels A through G represent the genomic RNA blots: (A) VSV_{IND-HR} RNA; (B) IND-011 RNA; (C) VSV_{NJ}(Ogden) RNA; (D) NJ-JM2 RNA; (E) and (F) NJ-PG1/2 RNA; and (G) NJ-121 RNA. Lanes 1 through 6 represent the nick translated cloned mRNAs used to probe the genomic RNA blots: VSV_{NJ}(Ogden) N mRNA clone, pN53 [representing the 5'-end of the N mRNA (Banerjee, et al., 1984)] (lane 1); VSV_{NJ}(Ogden) N mRNA clone, pN77 [representing the 3'-end of the N mRNA (Banerjee, et al., 1984)] (lane 2); VSV_{NJ}(Ogden) NS mRNA clone, pNS22 (Gill and Banerjee, 1985) (lane 3); VSV_{NJ}(Ogden) M mRNA clone, pM24 (Gill and Banerjee, 1986) (lane 4); VSV_{NJ}(Ogden) G mRNA clone, pNJG6 (Gallione and Rose, 1983) (lane 5); and pBR322 (lane 6). Lane 7 in panel A is identical to panel F, lane 5, however, exposed for an identical time as the strips in panel A. Panels E and F are short and long exposures of the same strips to enable the identification of NJ-PG2 and NJ-PG1. Panel C has been overexposed to show the gradient of subgenomic RNA molecules in the VSV_{NJ}(Ogden) standard virus preparation. Upon overexposure of panels D-G a similar pattern is observed.


1 2 3 4 5 6 7
IND-STD-
A
1 2 3 4 5 6
B
1 2 3 4 5 6
NJ-STD-
C
1 2 3 4 5 6
NJ-STD-
NJ-JM2-
D
1 2 3 4 5
-NJ-STD-
-NJ-PG2-
E
1 2 3 4 5 6
-NJ-PG1-
F
1 2 3 4 5 6
NJ-STD -
NJ-121 -
NJ-121a-
NJ-121b-
G

RNAs hybridized with much less intensity than the standard virus, even though the DI particle RNA concentration was much higher (same RNA preparation as that used in Figure 6), indicating that none of the DI particle genomic RNAs encoded a full length copy of any of the mRNAs. NJ-JM2 genomic RNA was hybridized with only the G mRNA probe. The NJ-121 and NJ-PG1/2 DI particle RNA preparations hybridized weakly with all of the mRNA probes. The hybridization with the G mRNA probe seems to be the strongest. Note also that every mRNA probe hybridized to subgenomic RNAs in the VSV_{NJ}(Ogden) standard virus RNA preparation. These RNAs were at very low concentration (Figure 6) in this RNA preparation, indicating that the intensity of hybridization in this set of experiments was due to the degree of sequence conservation and not due the concentration of each RNA. It is interesting to note that in the VSV_{NJ}(Ogden) standard virus RNA preparation, as the RNA sizes increased, they initially contain just N mRNA sequences, then N and NS sequences, then N, NS and M sequences, and finally N, NS, M and G mRNA sequences. These genomic RNAs are unlikely to be due to breakdown and do not seem to amplify well as indicated by their low concentration. These RNAs may represent incompletely replicated forms of the standard virus which may have been packaged into virion or released into the culture media at low levels when cells lysed late in infection.

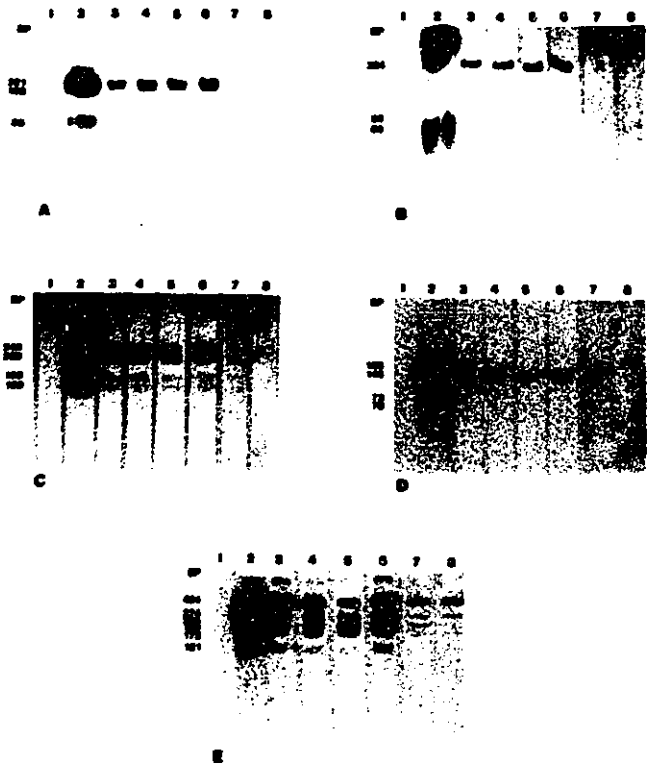
In order to determine which regions of each gene were retained within the DI particle RNAs the cDNA clones derived from the N, NS, M and G mRNAs were digested with restriction endonucleases, the fragments separated on agarose gels, blotted onto Genescreen and probed with ^{32}P-labelled DI particle genomic RNAs (purified on agarose gels after

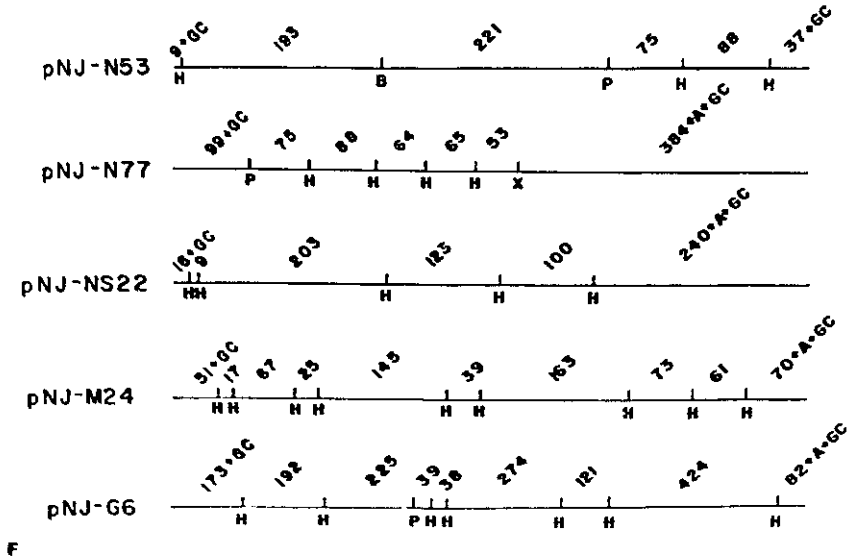
glyoxal denaturation). The resulting hybridization (Figure 9) suggested that the DI particles generated by heat treatment (Figure 9, lanes 2-7) retained small segments of sequence from each gene region. NJ-JM2 (Figure 9, lane 8) does not seem to hybridize to the smaller fragments (originating from the 3'-end of the G mRNA). Therefore the DI particles produced from heat treated VSV_{NJ}(Ogden) appeared to be generated by a series of frequent jumps throughout each gene. Alternatively, the hybridization to all the restriction fragments could have been due to a low level contamination with the small genomic RNAs observed in Figure 3 (panel C).

3.3 Polarity of DI Particle Genomic RNAs.

It was possible that the DI particles contained rearranged genomes arranged in a snapback configuration (like DI-ST2) or that both positive- and negative-strand DI particle genomes were being packaged. Therefore the DI particle genomic RNAs were hybridized with a strand-specific probe. This would indicate whether the DI particle genomes contained both positive- and negative-strand RNA or just negative-strand RNA. Since all of the DI particle genomic RNAs hybridized to the 424 base pair fragment of the G mRNA clone (Figure 9, panel E) and because this fragment originates from the 5'-end of the G protein gene, the 5'-end (980 base pair Pst 1 fragment) of the G protein gene was ligated into pGEM-4B (Pst 1 site). This vector contains the polymerase recognition sites for the T7 and SP6 RNA polymerases. Therefore, upon linearization of the plasmid downstream of the inserted fragment, strand-specific RNA molecules can be transcribed by the appropriate RNA

Figure 9. Hybridization of ^{32}P-labeled DI particle genomic RNAs to restriction endonuclease digested cDNA clones, derived from VSV_{NJ}(Ogden) mRNAs. The cDNA derived from VSV_{NJ}(Ogden) mRNAs was inserted into the Pst I site of pBR322. All of the clones were digested with Pst I and the following restriction endonucleases: Hinf I + BamH I (pNJ-N53); Hinf I + Xho I (pNJ-N77); and Hinf I (pNJ-NS22, pNJ-M24 and pNJ-G6). This produced restriction endonuclease fragments from 9-424 base pairs in length [see the map of the locations and sizes of each restriction endonuclease fragment (F)] The digested clones were electrophoresed on 4% Nusieve (FMC) agarose gels and electroblotted onto Genescreen (NEN). Fragments as small as 9 bp in length were transferred onto Genescreen with >95% efficiency (as determined by transferring end-labeled fragments). Panels A through E represent the restriction endonuclease digested blots of the VSVNJ(Ogden) mRNA clones: pNJ-N53 (A); pNJ-N77 (B); pNJ-NS22 (C); pNJ-M24 (D); and pNJ-G6 (E). Lanes 1 through 8 represent the genomic RNA probes: VSV_{IND-HR} standard virion RNA (lane 1); VSV_{NJ}(Ogden) standard virion RNA (lane 2); NJ-121 RNA (lane 3); NJ-121a RNA (lane 4); NJ-121b RNA (lane 5); NJ-PG2 RNA (lane 6); NJ-PG1 RNA (lane 7); and NJ-JM2 RNA (lane 8). (F) shows the maps of the restriction endonuclease fragments within the VSV_{NJ}(Ogden) mRNA clones: pNJ-N53 [originally called pN53 (Banerjee, et al., 1984)]; pNJ-N77 [originally called pN77 (Banerjee, et al., 1984)]; pNJ-NS22 [originally called pNS22 (Gill and Banerjee, 1985)]; pNJ-M24 [originally called pM24 (Gill and Banerjee, 1986)]; pNJ-G6 [originally called pNJG6 (Gallione and Rose, 1983)]. The restriction endonuclease sites are denoted by the following abbreviations: Hinf I (H); BamH I (B); Pst I (P); and Xho I (X).





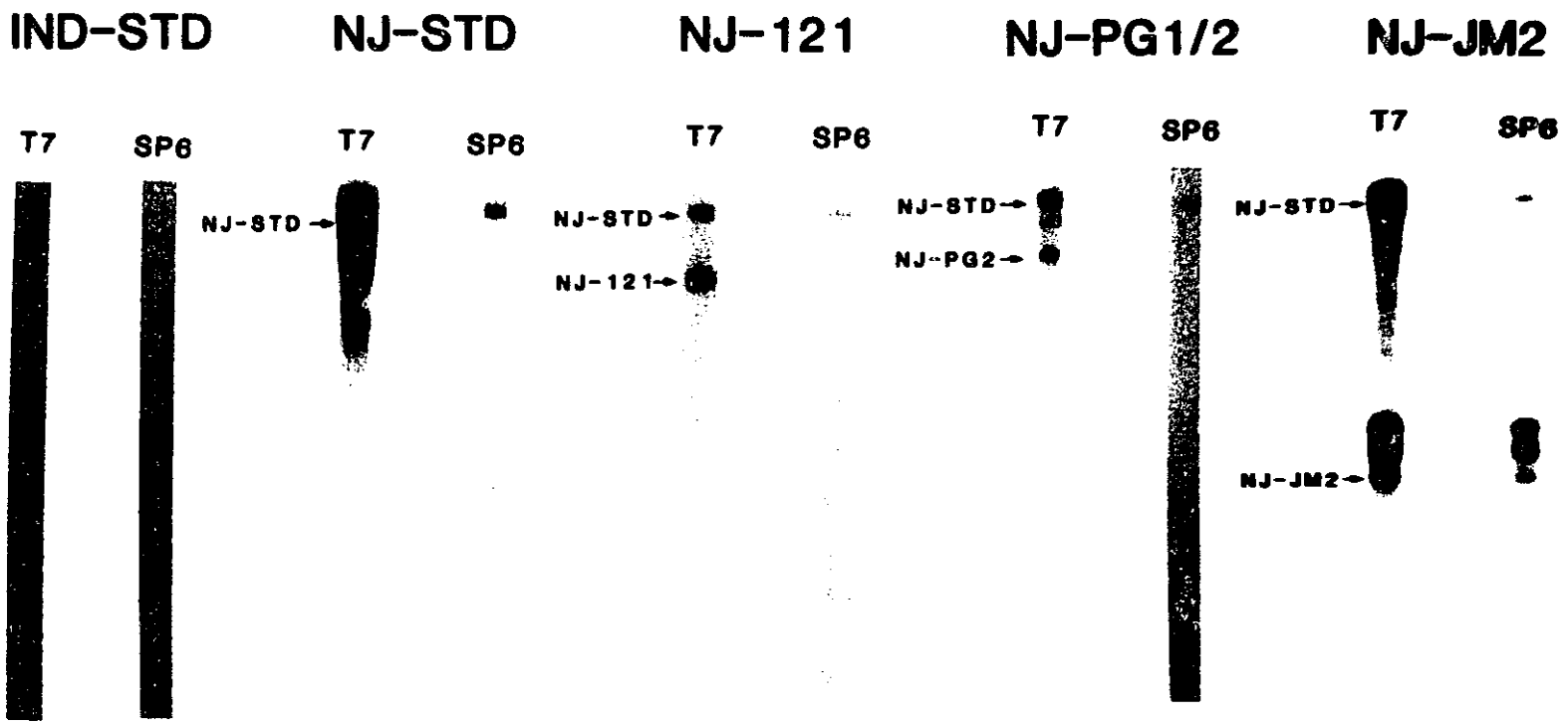
F

polymerases (the orientation and sequence of the inserted fragment was confirmed by sequence analysis, data not shown). After the transcription reaction was completed the DNA was digested with RNase-free DNase and the RNA transcripts used to probe the DI particle genomic RNA (Figure 10). The T7 RNA polymerase products specifically hybridized with negative-stranded RNA and the SP6 RNA polymerase products specifically hybridized with positive-strand RNA. According to these results NJ-JM2 contained both positive- and negative-strand RNA, while the other DI particles contained mostly negative-strand RNA. Notice that the standard virus contained a small amount of positive-strand RNA (detected by the SP6 RNA-polymerase products). Another observation of interest is that the smaller DI particles, NJ-121a, NJ-121b, and NJ-PG1 did not hybridize with either of the probes. These results conflict with the results in Figure 9 (panel E), indicating that the hybridizations in Figure 9 may have been due to the conservation of a very small amount of the G protein gene, or to contamination of the DI particle genomic RNA derived probes with the subgenomic RNAs observed to contaminated the standard virion RNA at very low levels (Figure 9, panel C).

3.4 Transcriptional Capabilities of VSV_{NJ}(Ogden) DI Particles.

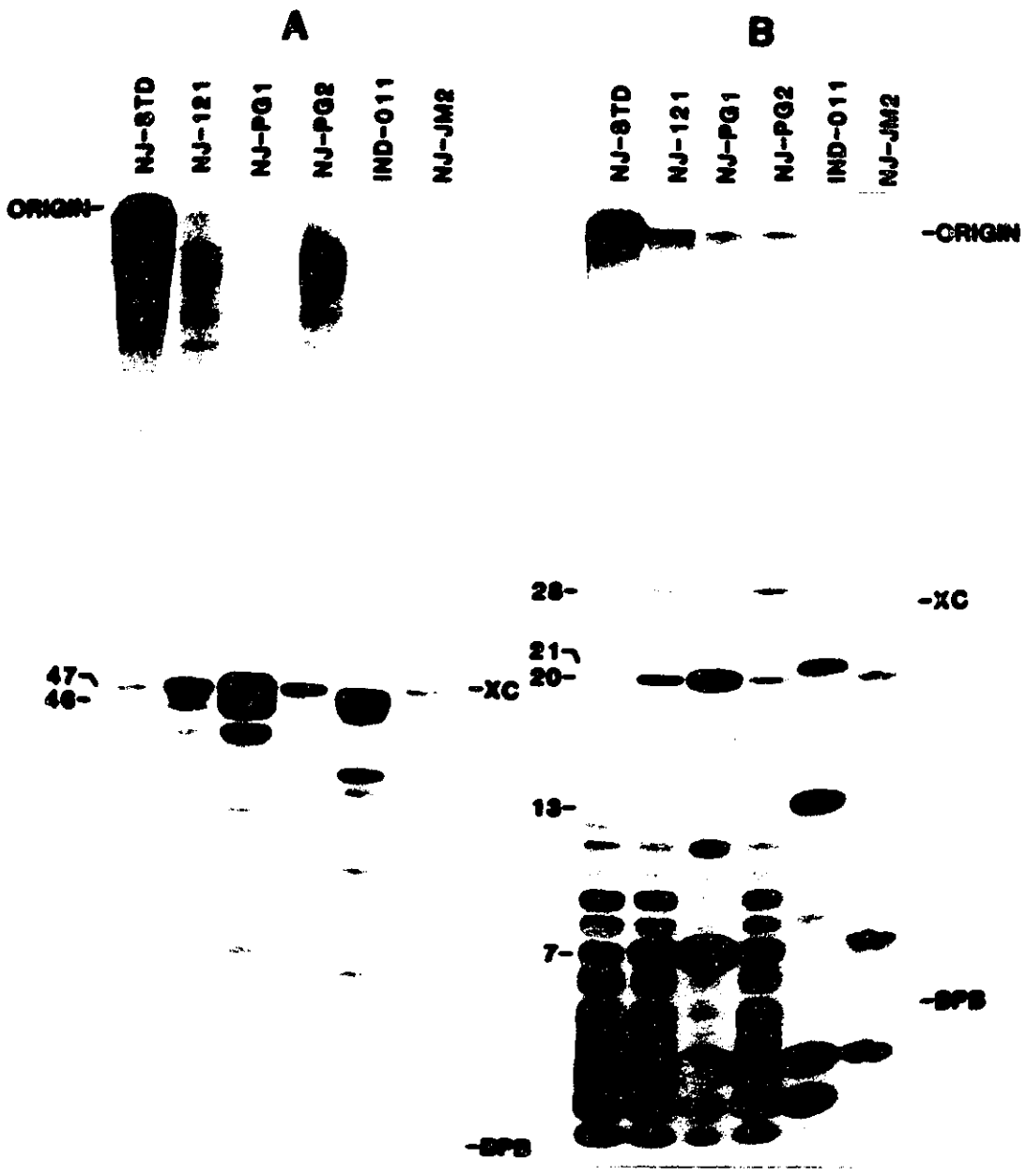
The two largest DI particles, NJ-121 and NJ-PG2, along with the DI particles NJ-PG1, NJ-JM2, IND-011 and VSV_{NJ}(Ogden) standard virus were amplified and purified on sucrose velocity gradients. To ascertain the transcriptional capabilities of these DI particles they were disrupted with detergent (Nonidet P-40) and allowed to transcribe *in vitro* (as

Figure 10. Hybridization of strand-specific RNA transcripts to Genomic RNA blots. Unhybridized strips of the genomic Northern blots, prepared for the experiments in Figure 8, were probed with strand-specific RNA probes produced from the 980 bp Pst I fragment (left side of the pNJ-G6 map in Figure 9) placed into the Pst I site of pGEM-4B. The fragment was oriented so that SP6 RNA polymerase transcribed a negative-strand transcript (detecting positive-strand G mRNA) and T7 RNA polymerase transcribed a positive-strand transcript (detecting the negative-strand G mRNA template). The identification of each Northern blot is indicated as follows: VSV_{IND} standard virus RNA (IND-STD); VSV_{NJ}(Ogden) standard virus RNA (NJ-STD); NJ-121 RNA (NJ-121); NJ-PG1/2 RNA (NJ-PG1/2); NJ-JM2 RNA (NJ-JM2). The blots were probed with either the T7 RNA polymerase transcripts (T7), or the SP6 RNA polymerase transcripts (SP6).


IND-STD
NJ-STD
NJ-121
NJ-PG1/2
NJ-JM2
T7
SP6
T7
SP6
NJ-STD
T7
SP6
NJ-STD
NJ-121
T7
SP6
NJ-STD
NJ-PG2
T7
SP6
NJ-STD
NJ-JM2

described in the Materials and Methods section) in the presence of [α-^{32}P]GTP. The transcripts were analyzed by polyacrylamide gel electrophoresis (Figure 11a,b). All four VSV_{NJ}(Ogden) DI particles (NJ-121, NJ-PG2, NJ-PG1 and NJ-JM2) transcribed a leader RNA identical in size (Figure 11a) to that of the 47-nucleotide-long positive-strand VSV_{NJ}(Ogden) leader RNA (Colonno and Banerjee, 1978). In contrast, the VSV_{IND} DI particle, IND-011, transcribed a 46-nucleotide-long negative-strand leader RNA (Schubert, et al., 1978). In order to distinguish the positive- and negative-strand leader RNAs of VSV_{NJ}(Ogden), RNase T_1 digestion was carried out (Figure 11b). The positive-strand leader RNAs of VSV_{IND} and VSV_{NJ}(Ogden) yield a 28-nucleotide-long product (Colonno and Banerjee, 1978a,b,c), whereas the VSV_{IND} negative-strand leader RNA generated 21- and 13-nucleotide-long digestion products (Schubert, et al., 1978). 28-nucleotide-long products were obtained from the leader RNAs transcribed by VSV_{NJ}(Ogden) standard virus and the DI particles, NJ-121 and NJ-PG2. The RNase T_1 digestion products from NJ-PG1 and NJ-JM2 were 20- and 7-nucleotides-long, indicating that the sequence of the negative-strand leader from VSV_{NJ}(Ogden) differed from the negative-strand leader RNA sequence in VSV_{IND}. The transcripts from NJ-121 and NJ-PG2 also produced the 20- and 7-nucleotide-long RNase T_1 digestion products. As expected, the 21- and 13-nucleotide-long RNase T_1 digestion product were produced by the IND-011 leader RNA transcript (Schubert, et al., 1978). These results indicated that NJ-121 and NJ-PG2 were able to transcribe both the positive- and negative-strand leader RNAs, as well as larger transcripts (Figure 11a). The larger transcripts were denatured with glyoxal and electrophoresed

Figure 11. VSV_{NJ}(Ogden) standard virion and DI particle in vitro RNA transcription products. (A) Polyacrylamide gel electrophoresis of in vitro RNA transcripts from sucrose gradient purified standard virion and DI particle preparations. The transcripts were first analyzed by electrophoresis on a 12% polyacrylamide gel (8 M urea) and autoradiographed at -70°C. (B) The transcripts were also digested with RNase T1 (1 unit/μL) and electrophoresed on 20 % polyacrylamide gels (8 M urea) and autoradiographed at -70°C. The sizes of the transcripts are indicated on the left side of each panel and the migration of xylene cyanol (XC) and bromophenol blue (BPB) on the right side. VSV_{NJ}(Ogden) standard virus transcripts (NJ-STD lane); NJ-121 RNA transcripts (NJ-121 lane); NJ-PG1 RNA transcripts (NJ-PG1 lane); NJ-PG2 RNA transcripts (NJ-PG2 lane); DI-011 RNA transcripts (IND-011 lane); and NJ-JM2 RNA transcripts (NJ-JM2 lane).


A
B
NJ-STD
NJ-121
NJ-PG1
NJ-PG2
IND-011
NJ-JM2
ORIGIN
ORIGIN
47
46
XC
28
21
20
19
7
XC
DPB
DPB

through an agarose gel (Figure 12). The larger transcripts of NJ-121 and NJ-PG2 were not the same size as those produced by VSV_{NJ}(Ogden) standard virus. Hence, the larger transcripts are probably not due to standard virus contamination. The quantity of large transcripts as compared to leader RNA transcribed by NJ-121 and NJ-PG2 was not the same as that transcribed by the standard virus (Figure 11a,b). It appeared that NJ-121 and NJ-PG2 transcribed the leader RNAs in a greater molar excess.

To prove that NJ-121 and NJ-PG2 were able to transcribe both leader RNAs, it was necessary to prove that their genomes contained the template regions for both leader RNAs. Genomic RNA was denatured with glyoxal and electrophoresed on agarose gels. The RNA was then electroblotted onto Genescreen (DuPont), cut into longitudinal strips, and probed with leader RNA transcripts. The leader RNA transcripts were purified in 12 % polyacrylamide gels (Figure 11a). The VSV_{NJ}(Ogden) positive-strand leader RNA produced by the VSV_{NJ}(Ogden) standard virus hybridized with NJ-STD (Figure 13, lanes 2), NJ-121 (Figure 13, panel E, lane 2) and NJ-PG2 (Figure 13, panel F, lane 2). The negative-strand leader RNAs produced by NJ-PG1 and NJ-JM2 hybridized with NJ-JM2 (Figure 13, panel D, lanes 4 and 7), NJ-PG1 (Figure 13, panel E, lanes 4 and 7), NJ-121a,b (Figure 13, panel F,lanes 4 and 7), as well as with NJ-PG2 (Figure 13, panel E, lanes 4 and 7) and with NJ-121 (Figure 13, panel F, lanes 4 and 7). The leader RNA transcripts from NJ-121 and NJ-PG2 (Figure 13, lanes 5 and 6) hybridized with both the VSV_{NJ}(Ogden) standard virus RNA and DI particle RNAs, again indicating that both leader RNAs are being

Figure 12. Large RNA transcripts produced by in vitro transcription of VSV_{NJ}(Ogden) standard virion and DI particles. The RNA transcripts produced by in vitro transcription of VSV_{NJ}(Ogden) standard virion and DI particles, were denatured with glyoxal and electrophoresed on a 1.5% agarose gel. Standard VSV_{NJ}(Ogden) transcripts (NJ-STD); NJ-121 RNA transcripts (NJ-121); NJ-PG1 RNA transcripts (NJ-PG1); NJ-PG2 RNA transcripts (NJ-PG2); and DI-011 RNA transcripts (IND-011). The position of the leader RNA transcript is indicated by a 47mer (its size). BPB denotes the migration of bromophenol blue in this gel system.

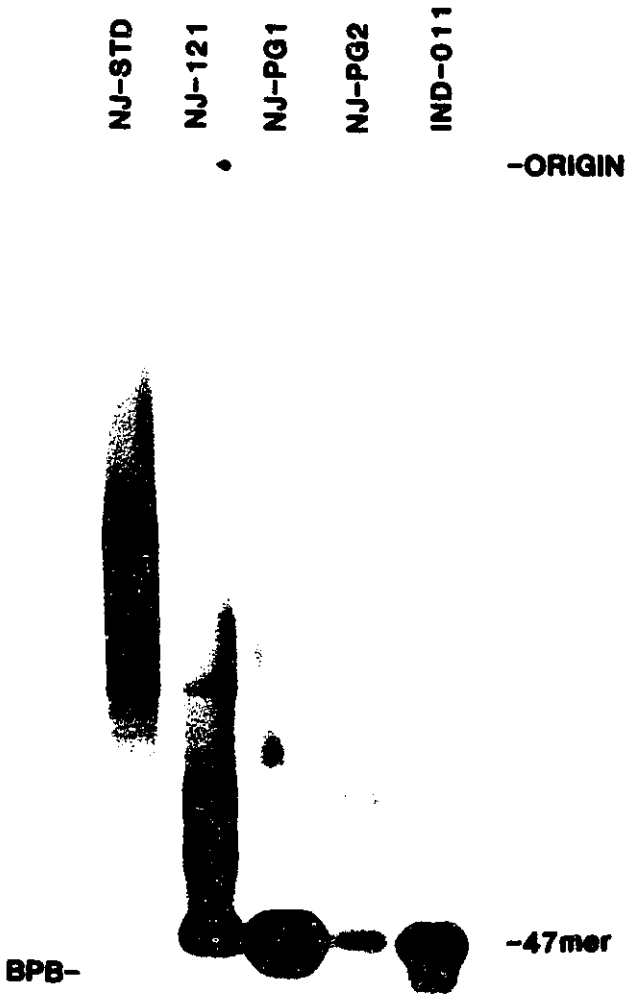
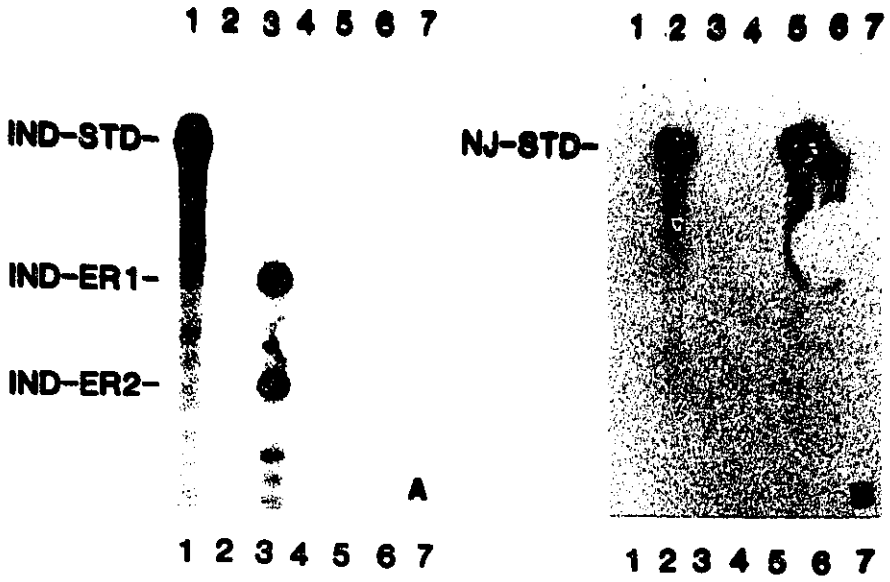
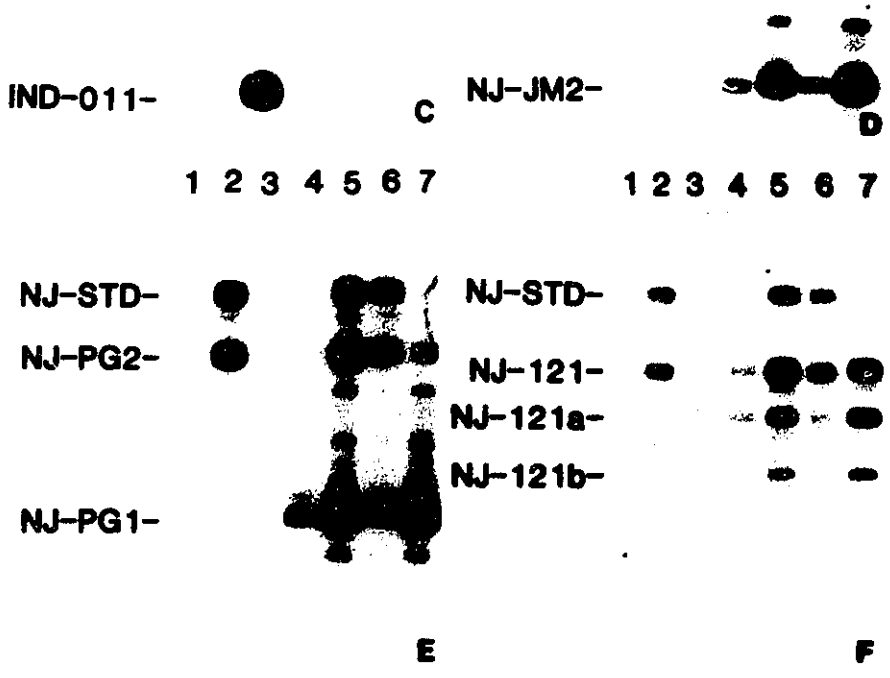

NJ-STD
NJ-121
NJ-PG1
NJ-PG2
IND-011
-ORIGIN
-47mer
BPB-

Figure 13. Hybridization of ^{32}P-labeled leader RNAs to Northern blotted DI particle genomic RNAs. The genomic RNAs from standard virion and DI particle preparations (total RNAs from pelleted virus, not sucrose gradient purified) were electrophoresed and blotted as described in Figure 8 (same RNA blots). The blots were prehybridized and then probed with polyacrylamide purified [α-^{32}P]GTP labeled leader RNAs (only the 47-nucleotide-long products were excised from a 12 % polyacrylamide gel, as in Figure 11a). Panels A through F represent the genomic RNA blots: VSV_{IND-HR} RNA preparation (A); VSV_{NJ}(Ogden) RNA preparation (B); IND-011 RNA preparation (C); NJ-JM2 RNA preparation (D); NJ-PG1/2 RNA preparation (E); and NJ-121 RNA preparation (F). Lanes 1 through 7 represent the leader RNA probes used to probe the genomic RNA blots: VSV_{IND-HR} leader RNA (lane 1); VSV_{NJ}(Ogden) leader RNA preparation (lane 2); IND-011 leader RNA preparation (lane 3); NJ-JM2 leader RNA preparation (lane 4); NJ-121 leader RNA preparation (lane 5); NJ-PG2 leader RNA preparation (lane 6); NJ-PG1 leader RNA preparation (lane 7). It should be noted that the same leader RNA probe preparation was used to probe all six RNA preparations.



IND-STD-

NJ-STD-



transcribed by these DI particles. These results are summarized in Table 3. The leader RNA transcripts from the VSV_{IND} standard virus (Figure 13, lane 1) and IND-011 (Figure 13, lane 3) were added as controls. The positive-strand leader RNA from VSV_{IND} standard virus hybridized with VSV_{IND} standard virus (Figure 13, panel A, lane 1). The negative-strand leader RNA from IND-011 hybridized to VSV_{IND} DI particle RNA (Figure 13, panel A and C, lane 3). The results of these hybridizations (Figure 13) indicate that the template regions for both the positive- and negative-strand leader RNAs were contained within the genomic RNAs of NJ-121 and NJ-PG2 and that both leader RNAs were transcribed.

To summarize the results to this point, two large DI particle preparations were independently generated from VSV_{NJ}(Ogden), NJ-121 and NJ-PG2, which were able to transcribe the 3'-end of the VSV_{NJ}(Ogden) genome. This characteristic qualifies these DI particles as transcribing DI particles and led to a study of their ability to interfere heterotypically. These two DI particles are unique in that they both appeared to contain the template regions for not only the negative-strand leader RNA but also the positive-strand leader RNA (like the VSV_{IND} DI particle DI-LI2) and in addition both leader RNAs seemed to be transcribed (unlike DI-LI2). Therefore, an investigation to determine the location of the leader RNA templates within the NJ-121 genome was undertaken in order to understand why NJ-121 was able to transcribe both leader RNAs while DI-LI2 was unable to transcribe the positive-strand leader RNA.

Table 3. Summary of DI particle hybridization with leader RNA probes. The positive signal (+) were scored after overexposure of the blots shown in Figure 13.

Leader RNA probes	Genomic RNA blots										
	IND-STD	IND-ER1	IND-ER2	NJ-STD	IND-011	NJ-JM2	NJ-121	NJ-121a	NJ-121b	NJ-PG2	NJ-PG1
IND-STD	+	−	−	−	−	−	−	−	−	−	−
NJ-STD	−	−	−	+	−	−	+	−	−	+	−
IND-011	−	+	+	−	+	−	−	−	−	−	−
NJ-JM2	−	−	−	−	−	+	+	+	+	+	+
NJ-121	−	−	−	+	−	+	+	+	+	+	+
NJ-PG2	−	−	−	+	−	+	+	+	+	+	+
NJ-PG1	−	−	−	−	−	+	+	+	+	+	+

3.5 Interference Activities of VSV_{NJ}(Ogden) DI Particles.

The ability of NJ-121, NJ-PG2, NJ-PG1, NJ-JM2 and IND-011 to interfere homotypically (with their parental standard virus) and heterotypically (with standard virus from the other serotype) was examined. Different amounts of sucrose gradient purified DI particles were added to homotypic standard virus to determine a ratio of DI particles to standard virus which would reduce the standard virus yield by 100 fold on BHK_{21} cells. The amount of each DI particle required to reduce the standard virus yield by 100 fold was quantitated by protein concentration determination and found to be equal for all of the DI particles, when corrected for the size of the DI particles. This would indicate that any competitive advantage a particular DI particle might have when competing with other DI particles does not influence the level of homotypic interference with standard virus. The same amount of each DI particle was used to test for heterotypic interference with VSV_{IND} standard virus on BHK_{21} cells (Table 4). These homotypic and heterotypic interference assays were also repeated (using identical amounts of each DI particle and standard virus) on R(B77) and L_2 cells. The level of homotypic interference changed for each cell line, but in none of the cell lines was heterotypic interference (100 fold reduction in standard virus yield) observed.

Due to the low level of homotypic interference observed on L_2 cells, we repeated the assay with an increased amount of each DI particle (twice that required to reduce the homotypic virus titer by 100 fold on BHK_{21} cells). Only three of the DI particles, NJ-121, NJ-PG1, and IND-011, were retested. The ability of these DI particles to

Table 4. Interference by DI particles in BHK_{21}, R(B77), and L_2 cells.

	Titer PFU/ml (% control)					
	BHK 21 Cells		R(B77) Cells		L_2 Cells	
	VSV_{NJ}	VSV_{IND}	VSV_{NJ}	VSV_{IND}	VSV_{NJ}	VSV_{IND}
Standard virus	8.1×10^8 (100%)	1.8×10^9 (100%)	7.8×10^8 (100%)	1.9×10^9 (100%)	1.7×10^7 (100%)	6.3×10^8 (100%)
Standard virus + NJ-121	1.5×10^7 (1.9%)	1.3×10^9 (72%)	1.3×10^7 (1.6%)	1.4×10^9 (73.7%)	3.5×10^6 (21%)	4.0×10^8 (64%)
Standard virus + NJ-PG2	1.3×10^8 (16%)	1.3×10^9 (72%)	1.1×10^8 (14%)	2.8×10^9 (147%)	8.0×10^6 (47%)	4.8×10^8 (76%)
Standard virus + NJ-PG1	1.4×10^7 (1.7%)	1.5×10^9 (83%)	1.1×10^7 (1.4%)	2.0×10^9 (105%)	2.3×10^6 (14%)	4.5×10^8 (71%)
Standard virus + NJ-JM2	1.7×10^7 (2.1%)	1.7×10^9 (94%)	7.8×10^6 (1.0%)	1.5×10^9 (79%)	5.8×10^6 (34%)	5.6×10^8 (89%)
Standard virus + IND-011	2.8×10^8 (34%)	6.3×10^6 (0.35%)	1.7×10^8 (22%)	3.1×10^6 (0.16%)	1.7×10^7 (100%)	1.0×10^8 (16%)

interfere with VSV_{NJ}(Hazelhurst), a member of the Hazelhurst subtype of VSV_{NJ}, was also tested. The VSV_{NJ}(Ogden) DI particles interfered equally well with both VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) standard virus replication on L_2 cells (Table 5). These results indicate that even though NJ-121 and NJ-PG2 were able to transcribe the 3'-end of the VSV_{NJ}(Ogden) standard virus genome, they are unable to interfere heterotypically with VSV_{IND}. This suggested that it was not merely the ability to transcribe beyond the leader RNA sequence which controls the ability of DI-LT1 to interfere heterotypically with VSV_{NJ}(Ogden), but the proteins, which are produced by the transcribed mRNAs, which control this activity. NJ-121 and NJ-PG2 probably do not encode and transcribe the mRNAs for the protein(s) required for heterotypic interference.

3.6 5'-Terminal Genomic RNA Sequence of VSV_{NJ}(Ogden).

In order to understand why DI-LT interferes with VSV_{NJ}(Ogden) but not with VSV_{NJ}(Hazelhurst) the 5'-terminal sequences were determined for VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst). The 5'-terminal sequence analysis was also required in order to determine the distance between the positive- and negative-strand leader RNA templates within the genome of NJ-121.

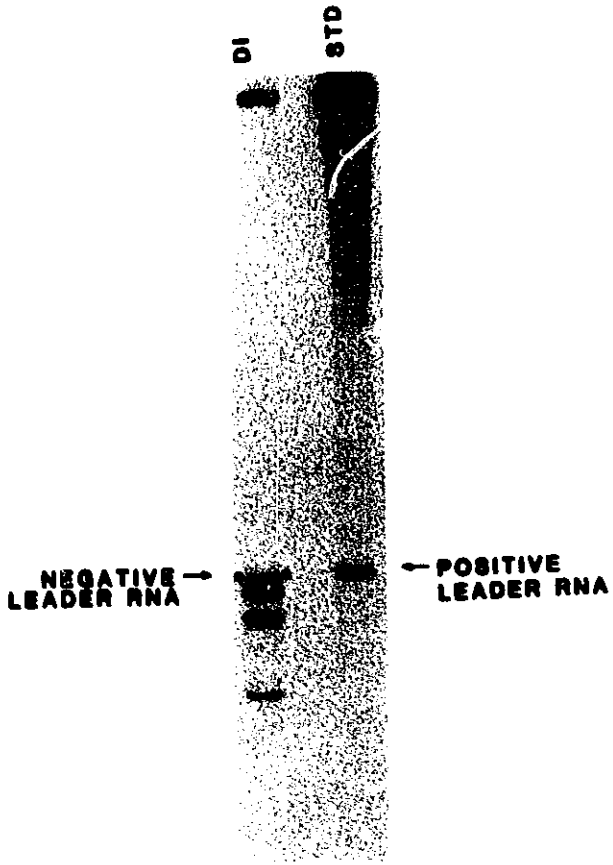
The 5'-end of VSV_{NJ}(Ogden) was cloned using a primer, (CYK-1) 5'-ACG AAG ACA AAA CCA TT-3', complementary to the 3'-terminal 20 nucleotides of the VSV_{NJ}(Ogden) standard virus negative-strand RNA (Rowlands, 1979). This primer should prime cDNA synthesis from both standard virus RNA and NJ-121 RNA. The RNA used as a template for cDNA

Table 5. Interference by DI particles with VSV_{NJ}(Ogden), VSV_{NJ}(Hazelhurst), and VSV_{IND-HR} in L_2 cells.

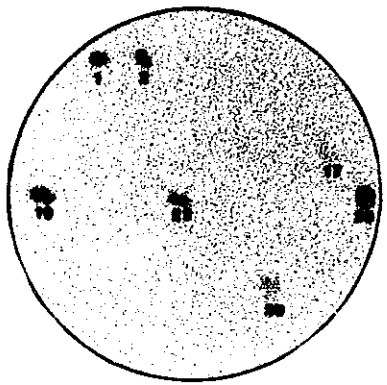
	Titer PFU/ml (% control)		
	VSV_{NJ} (Ogden)	VSV_{NJ} (Hazelhurst)	$VSV_{IND\text{-}HR}$
Standard virus	3.8×10^8 (100%)	2.4×10^8 (100%)	9.5×10^8 (100%)
Standard virus + NJ-121	3.5×10^6 (0.9%)	3.4×10^6 (1.4%)	9.1×10^8 (96%)
Standard virus + NJ-PG1	2.1×10^6 (0.6%)	3.6×10^6 (1.5%)	9.1×10^8 (96%)
Standard virus + IND-011	2.3×10^8 (60%)	1.3×10^8 (54%)	4.0×10^6 (0.4%)

synthesis was from the NJ-121 RNA preparation (Figure 6). The double stranded DNA was blunt-end ligated into the Sma 1 site of pUC19 and the recombinant plasmids transformed into the JM101 strain of E. coli. Three thousand colorless colonies were screened with either the positive-strand leader RNA to detect the 3'-end clones, or the negative-strand leader RNA to detect the 5'-end clones. The probes were derived from in vitro transcripts derived from VSV_{NJ}(Ogden) standard virus (positive-strand leader RNA) and NJ-PG1 (negative-strand leader RNA), purified on polyacrylamide gels (Figure 14a). Note, using this procedure only one clone was detected containing both the positive- and negative-strand leader RNA templates (Figure 14, panels B and C, number 39). This clone was later found to be an artefact of cloning (the 3'-end and 5'-end of the standard virus were ligated together). These results indicated that the genome of NJ-121 did not contain the template for the positive-strand leader RNA located at the 3'-end of the genome with the template for the negative-strand leader RNA located at a close internal location (within 1 Kbase). The clones which hybridized with the positive-strand leader RNA were found to originate from the 3'-end of the standard virus (as confirmed by sequence analysis and restriction endonuclease digestion patterns). Several clones were hybridized with the negative-strand leader RNA. These would be expected to originate from the 5'-end of either VSV_{NJ}(Ogden) standard virion RNA or NJ-121 RNA. The largest cloned insert contained a 1330 base pair fragment. The clones were sequenced by both dideoxynucleotide sequencing alkaline denatured supercoiled DNA (using the M13 forward and reverse primers) and by the Maxam and

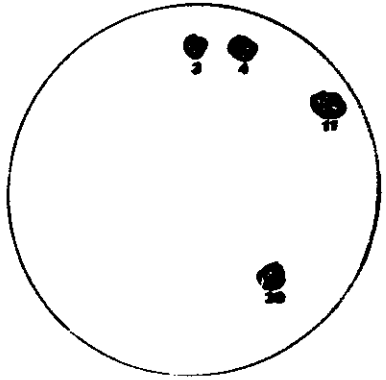
Figure 14. Identification of cDNA clones containing the 5'-end of VSV_{NJ}(Ogden). A oligonucleotide primer, CYK-1, complementary to the first 20 nucleotides of the 3'-end of VSV_{NJ}(Ogden) negative-strand RNA, was used to make cDNA using the NJ-121 RNA preparation as template (Gubler and Hoffman, 1983). The cDNA was blunt end ligated into the Sma I site of pUC 19 (Yanishe-Perron, et al., 1985) and transformed into JM101 (Hanahan, 1985). Insert containing colonies were detected by the use of IPTG (BRL) and Bluogal (BRL). Colorless colonies were transferred to five new LB plates, four on top of Colony/Plaque Screen (NEN) membranes, and grown overnight. The membranes were prewashed three times with 3X SSC, 1% SDS at 65°C for 16 hr, prehybridized and hybridized as described by the manufacturer (NEN). The probes used to detect the VSV_{NJ}(Ogden) 5'-end and 3'-end clones were the negative-strand and positive-strand leader RNAs, respectively (shown in panel A). The negative-strand and positive-strand leader RNAs were synthesized by in vitro transcription, in the presence of [α-^{32}P]GTP, of sucrose gradient-purified NJ-PG1 DI particles and VSV_{NJ}(Ogden) standard virus, respectively, and purified on 12% polyacrylamide gels. The leader RNA transcripts were localized by autoradiography, cut out of the gel, and eluted in 2.5 M ammonium acetate. (B) Membrane hybridized with the positive-strand leader RNA probe. (C) A duplicate membrane hybridized with the negative-strand leader RNA probe. The membranes were washed in the following order: 2X SSC, 25°C, 1 hr; 2X SSC, 1% SDS, 65°C, 1 hr; 0.1X SSC, 25°C, 0.5 Hr; and 0.1X SSC, 60°C, 0.5 hr.



A



B



C

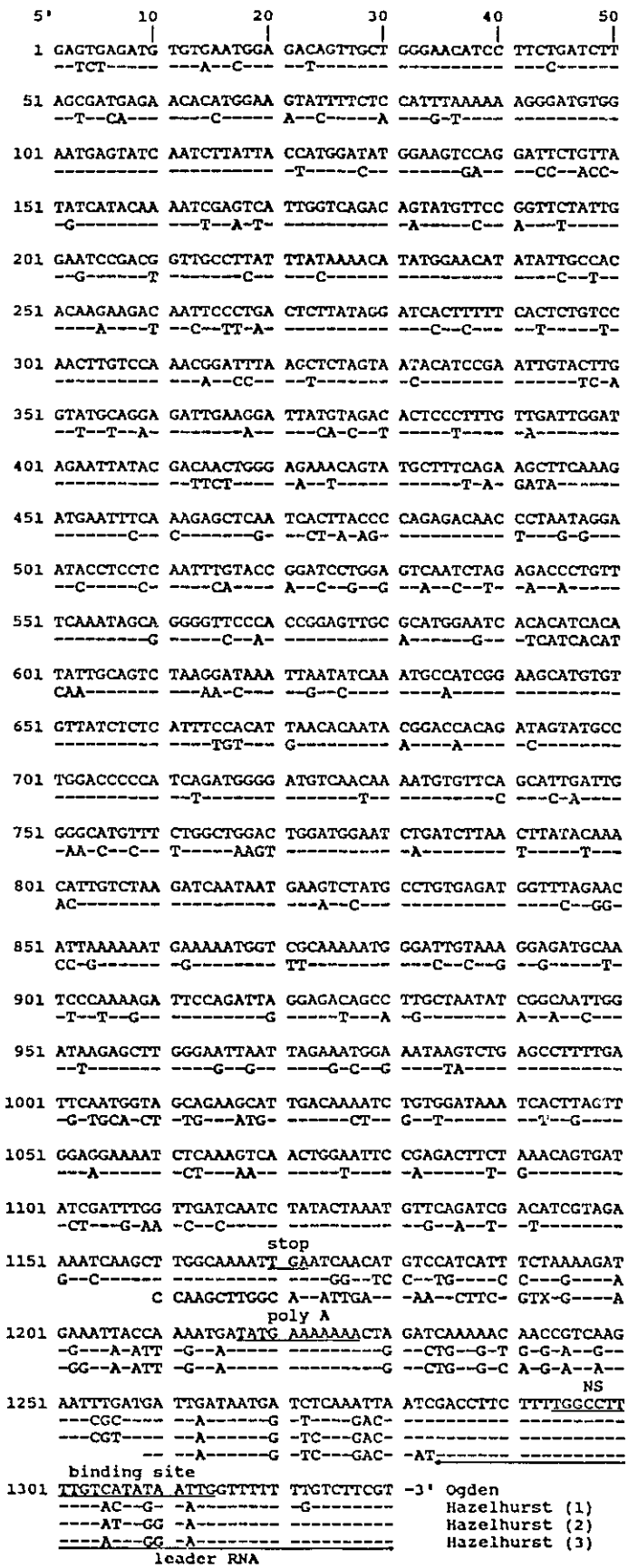
Gilbert sequencing method using end-labelled restriction endonuclease fragments (as described in the Materials and Methods section). The sequence was compared to that derived from three separate VSV_{NJ}(Hazelhurst) sequences (Feldhaus and Lesnaw, 1988; Keene, et al., 1981b; and Liang and Emerson, 1988). The complementary DNA sequence of the 5'-terminal 1330 nucleotides from VSV_{NJ}(Ogden) and the nucleotide differences in the same region of VSV_{NJ}(Hazelhurst) are presented in Figure 15. Dideoxynucleotide sequencing of VSV_{NJ}(Ogden) standard virus genomic RNA confirmed that the observed differences were representative of the entire population of standard virus genomic RNA.

Within the 5'-terminal 1330 nucleotides, the sequence of VSV_{NJ}(Ogden) differs from VSV_{NJ}(Hazelhurst)(Feldhaus and Lesnaw, 1988) at 269 bases (20.3% divergence). Within the leader RNA, bases 1284-1330, 5 bases out of 47 are different (10.6% divergence). The other two reported sequences of VSV_{NJ}(Hazelhurst)(Keene, et al., 1981b; and Liang and Emerson, 1988) also differ from the VSV_{NJ}(Hazelhurst) negative-strand leader RNA sequence at 5 positions. The changes are concentrated within the putative NS binding region of the RNA (Isaac and Keene, 1982). The remainder of the negative-strand leader RNA is unchanged. The restriction of changes within the negative-strand leader RNA, between VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst), to the putative NS binding region may influence the ability of DI-LT to interfere with VSV_{NJ}(Ogden) but not with VSV_{NJ}(Hazelhurst). This intriguing possibility will be further examined in the discussion section.

Within the spacer (S) region, bases 1228-1283, 19 bases of 56 are different (33.9% divergence). This region is of unknown function and

Figure 15. The complementary DNA sequence of the 5'-terminal 1330 nucleotides from VSV_{NJ}(Ogden). This sequence was derived from both dideoxynucleotide sequencing of VSV_{NJ}(Ogden) genomic RNA and DNA sequencing of cDNA clones which hybridized with the negative-strand leader RNA. The nucleotide T at base 1330 is the complementary DNA sequence of the last base of the 5'-terminus. This sequence can be also be interpreted as the sequence (in a DNA form) of the first 1330 nucleotides of the 3'-end of the replicative intermediate RNA (where nucleotide T at base 1330 represents the U of the 3'-terminus). The translation stop codon (nucleotides 1170-1172) and the polyadenylation site (nucleotides 1217-1227) of the L mRNA are indicated, along with the leader RNA (nucleotides 1284-1330) and the putative NS binding site (nucleotides 1294-1314). Hazelhurst (1), (2), and (3) are the sequences from the same regions of VSV_{NJ}(Hazelhurst) as published by Feldhaus and Lesnaw (1987), Keene et al. (1981b), and Liang and Emerson (1988), respectively. Only the nucleotides changes that differ from the sequence of VSV_{NJ}(Ogden) are indicated. Note that Hazelhurst (2) contains a deletion of nucleotides 1188-1193 (ATCCCT) from the same region in Hazelhurst (1). The sequence of Hazelhurst (2) was derived from the 3'-end of a DI particle, whereas the sequence of Hazelhurst (1) was derived by dideoxynucleotide sequencing the standard virion genomic RNA. If this deletion is taken into account, the sequence of Hazelhurst (1) and Hazelhurst (2) from nucleotides 1154-1187 would line up with VSV_{NJ}(Ogden) as follows:

```
        1160       1170       1180
5'- TCAAGCT TGGCAAAATT GAATCAACAT GTCCATC -3'  VSVNJ(Ogden)
    C------ ---------- ----GG--TC C--TG--      VSVNJ(Hazelhurst)(1)
    C------ ---------- ----G----C T--TG-X      VSVNJ(Hazelhurst)(2)
```


5' 10 20 30 40 50
GAGTGAGATG TGTGAATGGA GACAGTTGCT GGGAACATCC TTCTGATCTT
AGCGATGAGA ACACATGGAA GTATTTTCTC CATTTAAAAA AGGGATGTGG
AATGAGTATC AATCTTATTA CCATGGATAT GGAAGTCCAG GATTCTGTTA
TATCATACAA AATCGAGTCA TTGGTCAGAC AGTATGTTCC GGTTCTATTG
GAATCCGACG GTTGCCTTAT TTATAAAACA TATGGAACAT ATATTGCCAC
ACAAGAAGAC AATTCCCTGA CTCTTATAGG ATCACTTTTT CACTCTGTCC
AACTTGTCCA AACGGATTTA AGCTCTAGTA ATACATCCGA ATTGTACTTG
GTATGCAGGA GATTGAAGGA TTATGTAGAC ACTCCCTTTG TTGATTGGAT
AGAATTATAC GACAACTGGG AGAAACAGTA TGCTTTCAGA AGCTTCAAAG
ATGAATTTCA AAGAGCTCAA TCACTTACCC CAGAGACAAC CCTAATAGGA
ATACCTCCTC AATTTGTACC GGATCCTGGA GTCAATCTAG AGACCCTGTT
TCAAATAGCA GGGGTTCCCA CCGGAGTTGC GCATGGAATC ACACATCACA
TATTGCAGTC TAAGGATAAA TTAATATCAA ATGCCATCGG AAGCATGTGT
GTTATCTCTC ATTTCCACAT TAACACAATA CGGACCACAG ATAGTATGCC
TGGACCCCCA TCAGATGGGG ATGTCAACAA AATGTGTTCA GCATTGATTG
GGGCATGTTT CTGGCTGGAC TGGATGGAAT CTGATCTTAA CTTATACAAA
CATTGTCTAA GATCAATAAT GAAGTCTATG CCTGTGAGAT GGTTTAGAAC
ATTAAAAAAT GAAAAATGGT CGCAAAAATG GGATTGTAAA GGAGATGCAA
TCCCAAAAGA TTCCAGATTA GGAGACAGCC TTGCTAATAT CGGCAATTGG
ATAAGAGCTT GGGAATTAAT TAGAAATGGA AATAAGTCTG AGCCTTTTGA
TTCAATGGTA GCAGAAGCAT TGACAAAATC TGTGGATAAA TCACTTAGTT
GGAGGAAAAT CTCAAAGTCA ACTGGAATTC CGAGACTTCT AAACAGTGAT
ATCGATTTGG TTGATCAATC TATACTAAAT GTTCAGATCG ACATCGTAGA
stop
AAATCAAGCT TGGCAAAATT GAATCAACAT GTCCATCATT TCTAAAAGAT
poly A
GAAATTACCA AAATGATATG AAAAAAACTA GATCAAAAAC AACCGTCAAG
TTGTCATATA ATTGGTTTTT TTGTCTTCGT -3' Ogden
Hazelhurst (1)
Hazelhurst (2)
Hazelhurst (3)
binding site
leader RNA

is located between the polyadenylation signal of the L gene and the 47th nucleotide of the negative-strand leader RNA. The polyadenylation signal sequence, bases 1217-1227, is fully conserved. The L gene non-coding region, situated between the end of the L gene stop codon, base 1173, and the polyadenylation signal sequence, has diverged the most with 18 of its 44 bases being different (40.9% divergence). The L protein coding region, bases 1-1172, has 227 differences of its 1172 bases (19.4% divergence). The nucleotide changes within the coding region of the L gene are responsible for 50 amino acid changes in the last 389 amino acids of the carboxyl terminus of the L protein (12.9% divergence). The amino acid sequence for the last (carboxyl) 389 amino acids of the VSV_{NJ}(Ogden) L protein are presented in Figure 16. The amino acid differences within the same region of the VSV_{NJ}(Hazelhurst) L protein are also presented.

3.7 Cloning the 3'-Terminal Region of NJ-121.

In an attempt to understand why NJ-121 was able to transcribe both the positive- and negative-strand leader RNAs we tried to clone the 3'-terminal region of this DI particle in order to determine the location of the leader RNA template regions. As indicated in section 3.6, the use of a primer complementary to the positive-strand leader RNA template did not produce any clones containing both the positive- and negative-strand leader RNA templates. Therefore, within the genome of NJ-121 either there is a large distance between the template regions for the positive-and negative-strand leader RNAs (reducing the probability of obtaining a single clone with both leader RNA template

Figure 16. The carboxyl-terminal 389 amino acids of the VSV_{NJ}(Ogden) L protein. The nucleotide sequence from the last 1330 nucleotides of the VSV_{NJ}(Ogden) genome (Figure 15) was translated into the 389 amino acids shown here (first line). Amino acid 389 (Asn) is the carboxyl-terminal amino acid of the L protein (assuming the L protein is not post-translationally cleaved). The sequence of the identical region of the VSV_{NJ}(Hazelhurst) L protein (second line) is presented here with only the amino acid differences indicated. The VSV_{NJ}(Hazelhurst) L protein sequence was taken from Feldhaus and Lesnaw (1988).

```
                      5                  10                  15
                      |                  |                   |
 1  Val Arg Cys Val Asn Gly Asp Ser Cys Trp Glu His Pro Ser Asp
    Ser  -   -   -   -   -   -   -   -   -   -   -   -   -   -

16  Leu Ser Asp Glu Asn Thr Trp Lys Tyr Phe Leu His Leu Lys Lys
     -   -   -  Lys  -   -   -   -   -   -   -   -  Val  -   -

31  Gly Cys Gly Met Ser Ile Asn Leu Ile Thr Met Asp Met Glu Val
     -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

46  Gln Asp Ser Val Ile Ser Tyr Lys Ile Glu Ser Leu Val Arg Gln
    Lys  -  Pro Thr Met  -   -   -   -   -   -  Leu  -   -   -   -

61  Tyr Val Pro Val Leu Leu Glu Ser Asp Gly Cys Leu Ile Tyr Lys
     -   -   -   -   -   -   -   -   -   -  Cys  -   -   -   -   -

76  Thr Tyr Gly Thr Tyr Ile Ala Thr Gln Glu Asp Asn Ser Leu Thr
     -   -   -   -   -   -   -   -   -   -  Lys  -   -   -   -   -

91  Leu Ile Gly Ser Leu Phe His Ser Val Gln Leu Val Gln Thr Asp
     -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

106  Leu Ser Ser Ser Asn Thr Ser Glu Leu Tyr Leu Val Cys Arg Arg
      -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

121  Leu Lys Asp Tyr Val Asp Thr Pro Phe Val Asp Trp Ile Glu Leu
      -   -   -   -  Ile  -   -   -   -   -   -   -   -   -   -

136  Tyr Asp Asn Trp Glu Lys Gln Tyr Ala Phe Arg Ser Phe Lys Asp
      -   -  Ser  -   -  Asn  -   -   -   -  Lys Asp Ile  -   -

151  Glu Phe Gln Arg Ala Gln Ser Leu Thr Pro Glu Thr Thr Leu Ile
      -   -  His  -   -  Arg  -   -  Lys  -   -   -   -   -  Val

166  Gly Ile Pro Pro Gln Phe Val Pro Asp Pro Gly Val Asn Leu Glu
      -   -   -   -   -   -  Ile  -   -   -   -   -   -   -   -

181  Thr Leu Phe Gln Ile Ala Gly Val Pro Thr Gly Val Ala His Gly
      -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

196  Ile Thr His His Ile Leu Gln Ser Lys Asp Lys Leu Ile Ser Asn
     Val Ile Ile Thr Ser Met  -   -   -  Asn  -   -   -   -   -

211  Ala Ile Gly Ser Met Cys Val Ile Ser His Phe His Ile Asn Thr
      -   -   -   -   -   -   -   -   -   -   -  Val Met  -   -

226  Ile Arg Thr Thr Asp Ser Met Pro Gly Pro Pro Ser Asp Gly Asp
      -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

241  Val Asn Lys Met Cys Ser Ala Leu Ile Gly Ala Cys Phe Trp Leu
      -   -   -   -   -   -   -   -   -   -  Thr  -   -   -   -

256  Asp Trp Met Glu Ser Asp Leu Asn Leu Tyr Lys His Cys Leu Arg
     Ser  -   -   -   -   -   -   -   -   -   -  Thr  -   -   -

271  Ser Ile Met Lys Ser Met Pro Val Arg Trp Phe Arg Thr Leu Lys
      -   -   -   -   -   -   -   -   -   -   -   -  Ala  -   -

286  Asn Glu Lys Trp Ser Gln Lys Trp Asp Cys Lys Gly Asp Ala Ile
      -  Gly  -   -  Phe  -   -   -   -   -   -   -   -   -   -

301  Pro Lys Asp Ser Arg Leu Gly Asp Ser Leu Ala Asn Ile Gly Asn
      -   -   -   -   -   -   -   -   -  Met  -   -   -   -   -

316  Trp Ile Arg Ala Trp Glu Leu Ile Arg Asn Gly Asn Lys Ser Glu
      -   -   -   -   -   -   -   -   -   -   -   -  Ile  -   -

331  Pro Phe Asp Ser Met Val Ala Glu Ala Leu Thr Lys Ser Val Asp
      -   -   -  Ala Ala Ala Val  -  Met  -   -  Thr  -   -   -

346  Lys Ser Leu Ser Trp Arg Lys Ile Ser Lys Ser Thr Gly Ile Pro
      -   -   -   -   -  Lys  -   -  Leu  -  Thr  -   -   -   -

361  Arg Leu Leu Asn Ser Asp Ile Asp Leu Val Asp Gln Ser Ile Leu
      -   -   -   -   -   -  Thr  -  Val Ile  -   -   -   -   -

376  Asn Val Gln Ile Asp Ile Val Glu Asn Gln Ala Trp Gln Asn  *
      -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
```

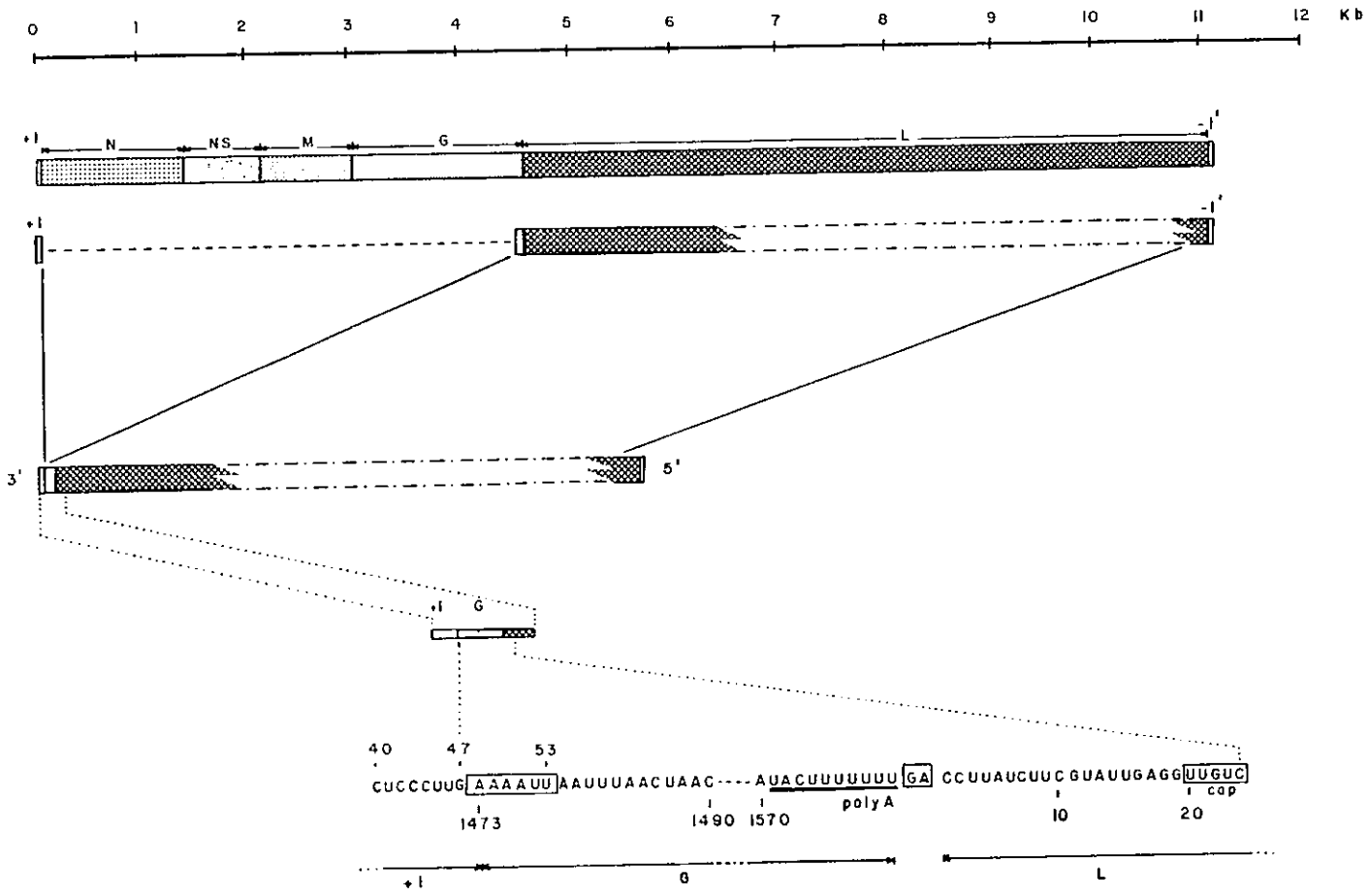
regions), or the positive-strand leader RNA template is positioned internally with the negative-strand leader RNA template located at the 3' terminus (like the genome of IND-LT2). Therefore a primer, ER-1 (5'-TGA CAA AAG GCC AAA AGA AGG-3'), which is complementary to nucleotides 25-45 from the 3'-end of the negative-strand leader RNA template, was produced. This primer should synthesize cDNA from all DI particles containing stem structures at their 3'-ends and all replicative intermediate (positive-strand) RNA molecules (both DI particles and standard virus). The double stranded cDNA produced with ER-1 was blunt-end ligated to Pst 1 linkers, restriction endonuclease digested with Pst 1, size selected by chromatography on sepharose 4B and the cDNA fraction of greater than 1 Kilobase pairs in length was ligated into the Pst 1 site of pUC19. Colorless colonies were probed with positive-strand leader RNA. This approach should have identified clones derived from not only NJ-121 negative-strand RNA (assuming that the negative-strand leader RNA template was located at the 3'end of NJ-121 and the positive-strand leader RNA template was located close to the 3'-end of NJ-121), and replicative intermediate RNA molecules from both NJ-121 and the standard virus. Twenty positive colonies were detected from 3,000 colonies probed. Nineteen of these corresponded to the 3'-end of the standard virus as determined by sequence analysis and Pst 1 restriction digests (one end corresponded to the Pst 1 site within the N protein cistron and the other to the 3'-end of the genome). The other clone contained the positive-strand leader RNA template, the last 101 nucleotides from the G protein cistron, the UUUUUU polyadenylation signal, the intergenic GA, the additional

nontranscribed 19 nucleotides of the G-L gene junction (Luk, et al., 1987) and 1678 nucleotides from the 3'-end of the L protein gene (Figure 17). Located at the junction of the leader RNA and the G protein gene in this clone are six bases, AAAAUU, which correspond to bases 48-53 (the six bases immediately following the positive-strand leader RNA template) from the 3'-end of the genome and bases 1473-1478 of the G cistron. This clone is believed to originate from NJ-121 because of the presence of the consensus sequence at the junction between the leader RNA template and the G protein gene and because this region of the G mRNA hybridized to NJ-121 genomic RNA (Figure 10). The 121 clone was produced from cDNA originating from either the genomic RNA (if NJ-121 resembles DI-LT2) or the replicative intermediate RNA molecule of NJ-121 (if NJ-121 resembles DI-LT2 or only contains the positive-strand leader RNA template at its 3'-end). This clone contained no additional sequence located before the positive-strand leader RNA template.

3.8 Amplification of the Sequence Between the Two Leader RNA Templates in NJ-121.

Due to our inability to find single clones containing both leader RNA templates, the PCR (Polymerase Chain Reaction) method of specifically amplifying DNA sequences (Saiki, et al., 1985) was used to try to determine the distance between the two leader RNA templates within NJ-121 (assuming NJ-121 resembled DI-LT2 in structure). The cDNA produced using ER-1 as a primer was used as a DNA template because this cDNA was the source of the clone representing the NJ-121 positive

Figure 17. Map of NJ-121 according to the results of cDNA cloning NJ-121 genomic RNA. The clone derived by cDNA cloning NJ-121 genomic RNA using the primer ER-1, contained the positive-strand leader RNA template joined to the last 101 nucleotide of the G protein gene, the 7-Us of the pol(A) signal, the intergenic GA, and 1678 nucleotides of the L protein gene. The map at the top of the figure is the VSV_{NJ}(Ogden) standard virus genome. The second map indicates the regions of the standard virus conserved within the genome of NJ-121 (the dotted line indicates the deleted region and the dotted box, the uncloned region of NJ-121). The third map indicates the location of each region within the NJ-121 genome. The junction sequence (AAAUU) between the negative-strand leader RNA template and the G protein gene (shown at the bottom of the Figure) is common to both regions within the standard virion RNA.


0 1 2 3 4 5 6 7 8 9 10 11 12 Kb
+1
N
NS
M
G
L
-1'
+1
-1'
3'
5'
+1 G
40 47 53
CUCCCUUG AAAAUU AAUUUAACUAAC---- AUACUUUUUUU GA CCUUAUCUUC GUAUUGAGG UUGUC
1473
1490 1570
polyA
10
20
cap
+1
G
L

-strand leader RNA template joined to 101 bases of the G protein cistron (section 3.7). This cDNA was amplified in the presence two primers, one primer from each of two sets of primers. The first set consisted of either ER-1 or ER-8 (5'-TTC GAA TTC-3', the EcoR1 recognition site, added to the 5'-end of ER-1). These primers were complementary to the negative-strand leader RNA template. The second set of primers consisted of ER-3 (identical in sequence to ten bases on either side of the junction between the positive-strand leader RNA template and the G protein cistron in the clone from section 3.7), ER-4 (identical in sequence to bases 35-16 of the positive-strand leader RNA template) or ER-9 (5'-TTC GAA TTC-3', the EcoR1 recognition site added to the idetical sequence of bases 43-25 from the positive-strand leader RNA template). These primers were identical in sequence to the positive-strand leader RNA templates. If NJ-121 had two leader RNA templates positioned in a copy-back configuration (ie. the template of the negative-strand leader RNA positioned at the 3'-end of the genome with the template of the positive-strand leader RNA positioned internally, as is the case for DI-LI2) and the distance between the two leader RNA templates was not too large (3 Kilobases seems to be the upper limit of efficient amplification in the PCR technique), this technique should amplify a length of DNA equivalent to the distance between the two leader RNA templates. As a control we amplified a clone, 121-16-13, which consisted of a 495 bp fragment from the 5'-end of the standard virus genome ligated to a 328 bp fragment from the 3'-end of the standard virus genome in a copy-back configuration. The control amplification produced a 500 bp product and the NJ-121 cDNA

amplification produced a 950 bp product (Figure 18). These products were digested with Hind III and Hinc II to produce restriction patterns corresponding to those derived form the 5'-end of the genome. Figure 19 summarizes the restriction endonuclease digestion results. These results indicated that the NJ-121 genome had a 950 bp copy-back stem structure (the complement of the 5'terminal sequence) at its 3'-end. To verify that the sequence was identical to the sequence from the 5'-end of the genome, we first tried to blunt-end ligate the NJ-121 amplified product into the Sma I site of pUC19 without success. Therefore, to confirm that the internal sequence of the amplified product corresponded to the same region of the 5'-end of the genome, the 716 bp Hind III fragment (Figure 19) was ligated into the Hind III site of pGEM-4B for sequence analysis. The resulting sequence corresponded to the sequence from the 5'-end of the genome. This result strongly supported our working hypothesis that the 3' end of NJ-121 contained the negative-strand leader RNA template (the complementary sequence to the 5' end of the standard virion genome) positioned before the template for the positive-strand leader RNA. To determine the terminal sequences of the NJ-121 amplified product and support these results, we end-labelled the Hind III fragments and sequenced the 150 bp and the 110 bp fragments by the Maxam and Gilbert method. The 150 bp product corresponded to the 5'-terminal sequence of the standard virion genome, whereas the 100bp product corresponded to the sequence 891 to 962 nucleotides from the 5'-end of the standard virion genome. Beyond nucleotide 962 the sequence diverges from the standard virus 5'-end sequence. Unfortunately, this divergent sequence

Figure 18. Amplified products from a PCR reaction using leader RNA specific oligonucleotide primers and NJ-121 specific cDNA. The polymerase chain reaction (PCR) was used to amplify the sequence between the two leader RNA template regions in molecules having a stem structure located before the 3'-end of standard virion RNA (like DI-LI2). This was accomplished using two sets of oligonucleotide primers, the first complementary to the negative-strand leader RNA template, and the second identical to the positive-strand leader RNA template. As a control reaction, a clone obtained during our cloning work, 121-16-13 was used as a DNA source. This clone contained a 495 bp copy of the 5'-end of the standard virus genome ligated onto a 328 bp copy of the 3'-end of the standard virus genome in a copy-back configuration (confirmed by sequencing). The use of leader RNA specific primers amplified a ~500bp product, as predicted. Using NJ-121 specific cDNA a ~950 bp product was produced. The gel shown here contains the amplified products produced using ER-1 and ER-4 as oligonucleotide primers.

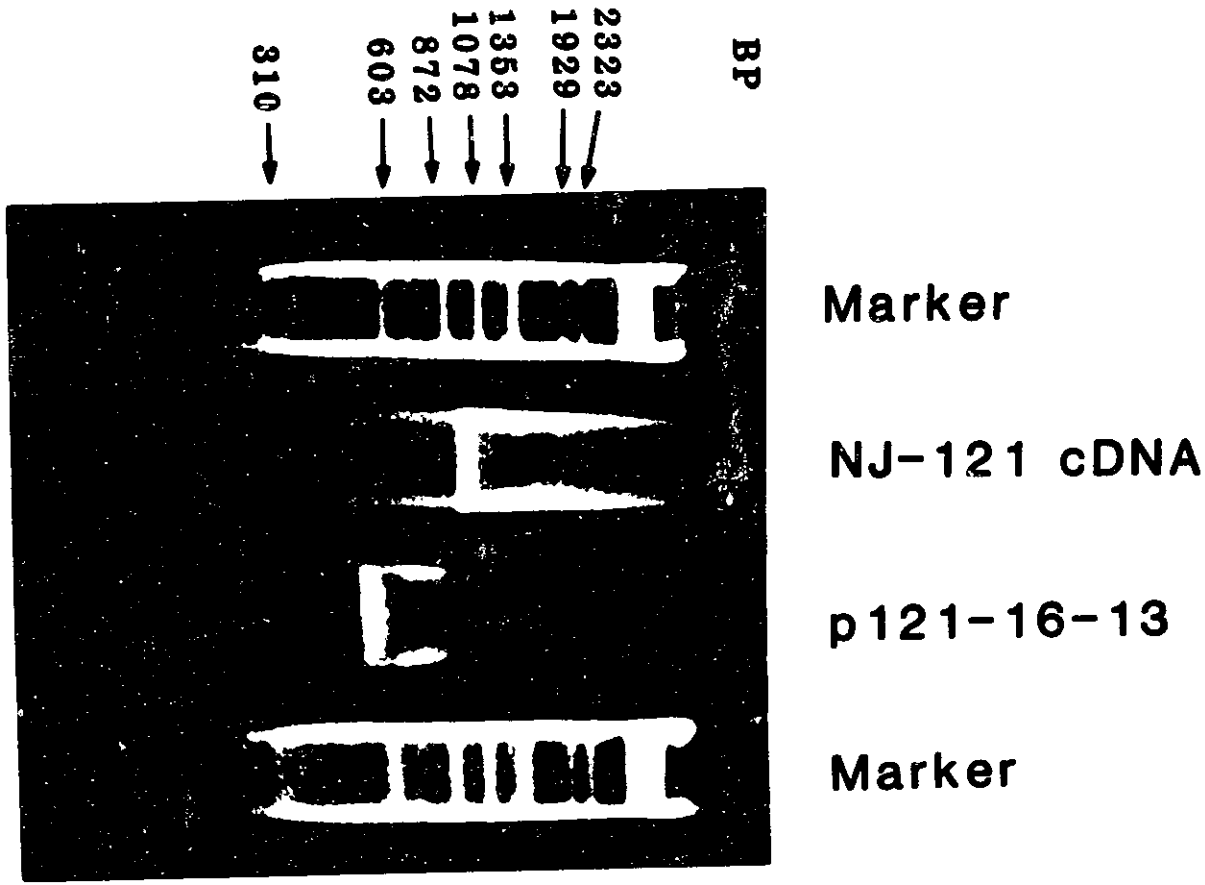
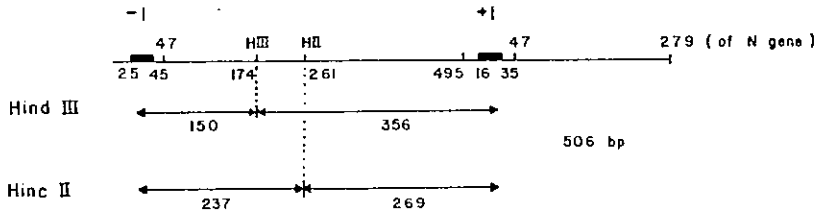

310
603
872
1078
1353
1929
2323
BP
Marker
NJ-121 cDNA
p121-16-13
Marker

Figure 19. Restriction endonuclease maps of both PCR amplified products. The PCR products were restriction endonuclease digested with either Hind III or Hinc II to produce the indicated restriction endonuclease fragments (seen in 4% Nusieve (FMC) agarose gels). The position of the restriction endonuclease cleavage sites was determined by sequence analysis. The internal Hind III fragment from the NJ-121 PCR product was subcloned into the Hind III site of pGEM-4B and sequenced. This sequence corresponded to the sequence from 174-890 base pairs from the 5'-end of the standard virus genome. The PCR products using ER-8, instead of ER-1, and ER-3 or ER-9, instead of ER-4 gave identical results. Maxam and Gilbert sequencing of the 150 bp Hind III fragment confirmed bases 25-174, being located on one end of the amplified product. The ~100 bp product confirmed the sequence 890-962. The sequence beyond this point diverged from the sequence of the 5'-end of the standard virus genome, however it was not legible. To read the sequence beyond base 962, the PCR product made using the primers ER-8 and ER-9 was digested with EcoR I and ligated into the EcoR I site of pGEM-4B. There is an EcoR I site located at position 255 from the 5'-end of the genome. Clones containing the larger restriction endonuclease fragment (725 bp) were sequenced indicating that the sequence beyond position 962 corresponded to the sequence of the primer ER-8 (or ER-1). The black boxes indicate the location of the primer binding sites. The primers would bind to complementary strands of DNA and amplify the regions between them. The +1 primer binding site turned out not to be where it is indicated for the 121 cDNA (see Figure 20).

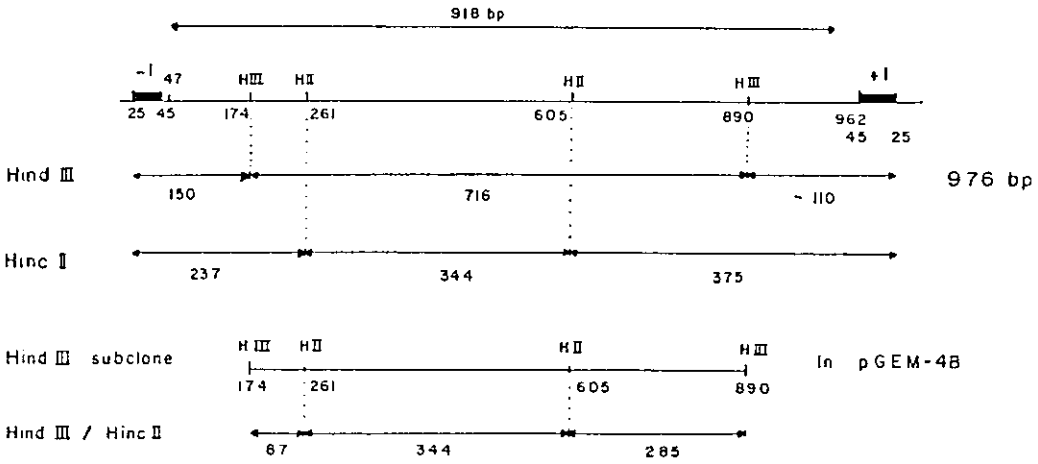
AMPLIFIED PRODUCT

pUC-NJ-16-13 (121-16-13)



AMPLIFIED PRODUCT

121 cDNA

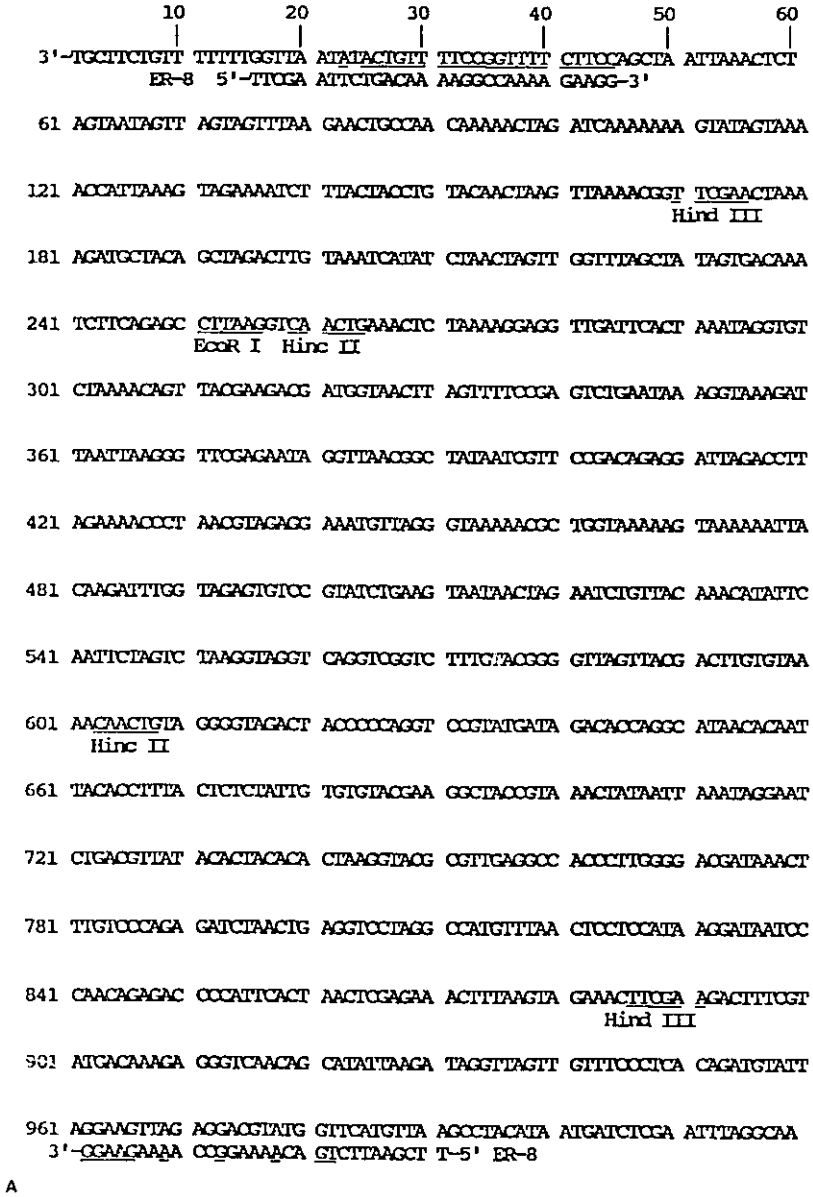


ER4 5'- CCA AAT AAT TGT AAT AAT GG -3'
35 15

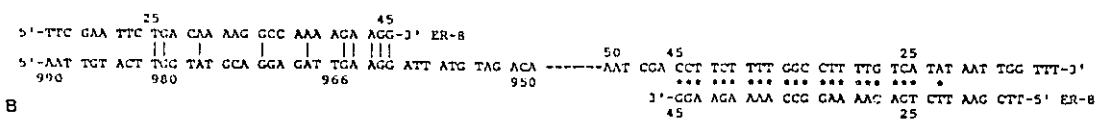
ER1 5'- TGA CAA AAG GCC AAA AGA AGG -3'
25 45

was not legible. Since the divergent sequence should correspond to the junction sequence between the copy-back and the positive-strand leader RNA template in the the putative NJ-121 genome, it was important to positively identify this sequence. We therefore took the product of amplification produced with ER-8 and ER-9 as primers (containing EcoR1 sites at their 5'-termini), EcoR1 digested these products and ligated them into the EcoR1 site of pGEM-4B. The amplified product had an internal EcoR1 site at position 255. Clones containing 725 bp and 230 bp inserts were obtained from the amplified products. We selected 12 colorless colonies and 9 of these had the 725 bp fragment. Four of these clones were sequenced both by dideoxynucleotide sequencing alkaline-denatured DNA using SP6 and T7 sequencing primers. The sequence beyond base 962 corresponded to the sequence of the ER-8 primer (Figure 20a). This indicated that the amplification product from the NJ-121 cDNA was produced by the ER-1 (or ER-8) binding to the cDNA from the 5'-end of the standard virion genome and not from the 3'-end of NJ-121 negative-strand RNA. During the amplification process a single mispriming event at positions 962-966 (from the 5'-end of the genome) has led to the amplification of the 950 bp product (Figure 20b). Bases 962-966 correspond to the 3'-terminal 5 bases in both ER-1 and ER-8 (3'-GGAAG-5'). Computer searching (PC-Gene) has indicated that these 5 bases only occur at this position within the 5'-terminal 1330 nucleotides. From these results it is evident that only one primer was required to specifically amplify this product. Mispriming and amplification have previously been reported (Mullis and Faloona, 1987), however, usually the mispriming event occurs from within a

Figure 20. Sequence analysis of the PCR product derived from NJ-121 cDNA. (A) According to the sequence data obtained from the NJ-121 PCR product, the PCR reaction was initiated by ER-8 (or ER-1) annealing to the DNA strand identical to nucleotides 24-45 from the 3'-end of the replicative intermediate RNA. The same primer then misprimed by annealing to DNA identical to nucleotides 966-962 from the 5'-end of the negative-strand genomic RNA (identical to the sequence produced by the first priming event). The underlined regions above the ER-8 sequence (below nucleotides 16-45 from the 3' end of the replicative intermediate RNA sequence), denote complementary bases, and below the ER-8 sequence (located below nucleotides 962-991 from the 3' end of the replicative intermediate RNA sequence) denote identical bases (complementary to the opposite strand). (B) The NJ-121 PCR product was primed as indicated. * and |, Represent nucleotides complementary and identical to the indicated nucleotides from the 3'-end of the replicative intermediate RNA, respectively.


10 20 30 40 50 60
3'-TGCTTCTGTT TTTTTGGTTA ATATACTGTT TTCCGGTTTT CTTCCAGCTA ATTAAACTCT
ER-8 5'-TTCGA ATTCTGACAA AAGGCCAAAA GAAGG-3'
61 AGTAATAGTT AGTAGTTTAA GAACTGCCAA CAAAAACTAG ATCAAAAAAA GTATAGTAAA
121 ACCATTAAAG TAGAAAATCT TTACTACCTG TACAACTAAG TTAAAACGGT TCGAACTAAA
Hind III
181 AGATGCTACA GCTAGACTTG TAAATCATAT CTAACTAGTT GGTTTAGCTA TAGTGACAAA
241 TCTTCAGAGC CTTAAGGTCA ACTGAAACTC TAAAAGGAGG TTGATTCACT AAATAGGTGT
EcoR I Hinc II
301 CTAAAACAGT TACGAAGACG ATGGTAACTT AGTTTTCCGA GTCTGAATAA AGGTAAAGAT
361 TAATTAAGGG TTCGAGAATA GGTTAACGGC TATAATCGTT CCGACAGAGG ATTAGACCTT
421 AGAAAACCCT AACGTAGAGG AAATGTTAGG GTAAAAACGC TGGTAAAAAG TAAAAAATTA
481 CAAGATTTGG TAGAGTGTCC GTATCTGAAG TAATAACTAG AATCTGTTAC AAACATATTC
541 AATTCTAGTC TAAGGTAGGT CAGGTCGGTC TTTGTACGGG GTTAGTTACG ACTTGTGTAA
601 AACAACTGTA GGGGTAGACT ACCCCCAGGT CCGTATGATA GACACCAGGC ATAACACAAT
Hinc II
661 TACACCTTTA CTCTCTATTG TGTGTACGAA GGCTACCGTA AACTATAATT AAATAGGAAT
721 CTGACGTTAT ACACTACACA CTAAGGTACG CGTTGAGGCC ACCCTTGGGG ACGATAAACT
781 TTGTCCCAGA GATCTAACTG AGGTCCTAGG CCATGTTTAA CTCCTCCATA AGGATAATCC
841 CAACAGAGAC CCCATTCACT AACTCGAGAA ACTTTAAGTA GAAACTTCGA AGACTTTCGT
Hind III
901 ATGACAAAGA GGGTCAACAG CATATTAAGA TAGGTTAGTT GTTTCCCTCA CAGATGTATT
961 AGGAAGTTAG AGGACGTATG GTTCATGTTA AGCCTACATA ATGATCTCGA ATTTAGGCAA
3'-CGAACAAAA CCGGAAAACA GTCTTAAGCT T-5' ER-8

A


25 45
5'-TTC GAA TTC TGA CAA AAG GCC AAA AGA AGG-3' ER-8
5'-AAT TGT ACT TAG TAT GCA GGA GAT TGA AGG ATT ATG TAG ACA -------AAT CGA CCT TGT TTT GGC CTT TTG TCA TAT AAT TGG TTT-3'
990 980 966 950 50 45 25
*** *** *** *** *** *** *** *
3'-CGA AGA AAA CCG GAA AAC AGT CTT AAG CTT-5' ER-8
45 25

B

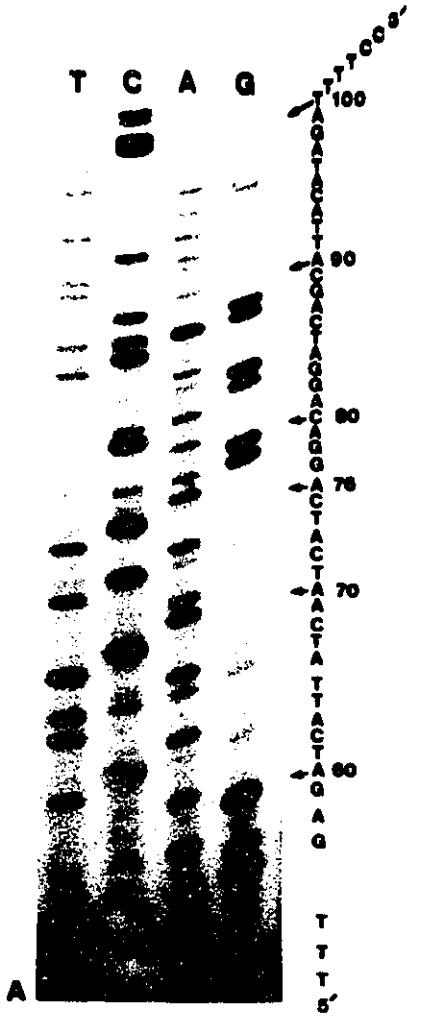
larger amplified product. The observation made here is the first case of a mispriming event which has amplified a DNA sequence using only one primer. It should be noted however, that this primer was homologous to one end of the template DNA. In the control amplification, the 500 bp product was amplified using both the positive- and negative-strand leader RNA specific primers. Since the leader specific primers only amplified the misprimed product (the result of a very low probability event) using the NJ-121 cDNA as a template, it can be concluded that the cDNA from the genome of NJ-121 does not contain the positive- and negative-strand leader RNA templates positioned in a copy-back configuration. The results of cloning using primers specific for the positive-strand leader RNA (Section 3.6), the negative-strand leader RNA (Section 3.7), and PCR reactions using primers complementary to the negative-strand leader RNA and identical to the positive-strand leader RNA (Section 3.8) indicate that the genomic RNA of the NJ-121 does not appear to be a single molecule of RNA containing the template region for both leader RNAs. NJ-121 therefore most probably consists of two molecules, one containing the positive-strand leader RNA template, 101 bases of the G protein gene and the L protein gene, and the other molecule containing a stem structure (negative-strand leader RNA template) and the L mRNA cistron (see Discussion and Figure 22). The only evidence that the molecule containing the negative-strand leader template also contains the L mRNA cistron is the results of hybridizing the genomic RNA to mRNA (Figure 7).

3.9 Sequence of the 3'-end of NJ-PG1.

To confirm that the 5'-terminal sequence of VSV_{NJ}(Ogden) truly corresponds to the complement of the 3'-end of VSV_{NJ}(Ogden) DI particles, the 3'-end of the VSV_{NJ}(Ogden) DI particle, NJ-PG1 was sequenced. The 3'-terminal nucleotide was found to be a U by 3'-end labeling the genomic RNA of NJ-PG1, digesting the labeled RNA with RNase T_2 and separating the ribonucleotides by chromatography on PEI plates. The next 80 nucleotides were sequenced by taking the labeled RNA, chemically modifying the bases and cleaving the modified bases with aniline (Peattie, 1979). The sequence up to base 76 corresponded perfectly to the complement of the 5'-terminal 76 nucleotides of the VSV_{NJ}(Ogden) negative-strand RNA.

In order to verify this sequence and to determine more of the sequence beyond nucleotide 80, NJ-PG1 genomic RNA was used as a template for dideoxynucleotide sequencing using ER-1 (complementary to bases 25-45 from the 3'-end of the negative-strand leader RNA template) as a primer. This primer is complementary to nucleotides 25-45 from the 3'-end of NJ-PG1. The sequence up to nucleotide 80 was confirmed (Figure 21a). The sequence beyond base 76 corresponds to base 2827 (3696 bases from the G-L intercistronic sequence) from the 5'-end of the negative-strand virus genome and continues in the direction of the 5'-end of the genome. Hence, NJ-PG1 is a panhandle DI particle with a 76 base pair stem. In order to determine the identity of the sequence joined onto the 76th nucleotide of NJ-PG1, the L gene (-6522 nucleotides long) had to be sequenced. The first 1678 bp of the L gene were sequenced from the NJ-121 clone (the first 557 identical to that

Figure 21. Complementary DNA sequence of the 3'-end of NJ-PG1 genomic RNA. (A) The sequence derived from dideoxynucleotide sequencing NJ-PG1 RNA in the presence of 5'-end labeled ER-1 (complementary to nucleotides 25-45 from the 3'-end of VSV_{NJ}(Ogden) replicative intermediate RNA molecules). (B) The complementary sequence of NJ-PG1 is identical to the 5'-end of the VSV_{NJ}(Ogden) negative-strand RNA for the first 76 nucleotides (capitalized letters, top left). Beyond this nucleotide, the sequence is identical to nucleotides 2827-2799 from the 3'-end of the VSV_{NJ}(Ogden) positive-strand replicative intermediate RNA (capitalized letters, bottom right). Therefore, it is evident that the NJ-PG1 genome was produced by the RNA polymerase copying from the 3'-end of the replicative intermediate molecule for 2827 nucleotides, leaving the template with the daughter RNA still attached and copying back on the daughter RNA from nucleotide 76. The sequence around the site of premature termination and resumption of synthesis does not indicate common signal sequences (unlike NJ-121).



Nucleotide Sequence From the 5'-End of the VSV_{NJ}(Ogden) Negative-Strand RNA.

Nucleotide Sequence From the 3'-End of the VSV_{NJ}(Ogden) Positive-Strand Replicative Intermediate RNA.

B

published by Luk, et al., 1987). Bases 1679 to 3355 were determined by dideoxynucleotide sequencing VSV_{NJ}(Ogden) standard virion RNA (this sequence was provided by Dr. A.K. Banerjee, Cleveland, Ohio). Dideoxynucleotide sequencing VSV_{NJ}(Ogden) genomic RNA was also used to determine bases 3356 to 3900. The sequence from 3696 to 3900 (corresponding to nucleotides 2827-2622 form the 3'-end of the positive-strand replicative intermediate RNA) was identical to that derived from NJ-PG1. The L gene sequence between bases 3900 and 5192 was at the time of writing this thesis still under investigation (in collaboration with Dr. A. K. Banerjee, Cleveland, Ohio). Only the L protein gene sequence around the point where the NJ-PG1 sequence diverges (the end of the copy-back sequence) from the L protein cistron sequence will be presented here (Figure 21b). The sequence between 5193 and 6522 corresponds to the last 1330 nucleotides at the 5'-end of the genome (presented in Figure 15). The numbering of bases beyond 3900 is based on the sequence of the L protein gene of VSV_{NJ}(Hazelhurst). This numbering system is believed to be accurate because only nucleotide substitutions have been observed within the region of the VSV_{NJ}(Ogden) L gene already sequenced (nucleotides 1-3900 and 5193-6522). In support of the numbering system is the fact that the size of the L protein coding region between VSV_{IND} and VSV_{NJ}(Hazelhurst) is identical (Feldhaus and Lesnaw, 1988) and the sequence avialable for the L gene of VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) is highly conserved (>90% homologous).

Dideoxynucleotide sequencing NJ-JM2 RNA using ER-1 as a primer enabled 110 nucleotides from the 3'-end of NJ-JM2 to be determined.

This sequence was identical to the complement of the 5'-end of the VSV_{NJ}(Ogden) genome. Despite several attempts and varying RNA concentration, the sequence beyond nucleotide 110 could not be determined (the entire sequencing reaction up to base 110 was very weak in comparison to the equivalent reaction using an identical amount of NJ-PG1 RNA). As already mentioned, NJ-JM2 is probably a snap-back DI particle (containing both positive- and negative-stranded RNA). The hairpin loop formed by this genomic RNA probably inhibited the primer extension reaction.

4. Discussion.

Defective Interfering particles only contain a portion of the standard virus genome and are dependent on standard helper virus to replicate. The helper virus provides, in trans, the missing proteins not encoded by the DI particle. Coinfection of cells with DI particles and standard virus of the same serotype leads to inhibition of standard virus replication, a phenomenon known as autointerference or homotypic interference. Most DI particles originating from VSV retain a portion of the 5'-half of the genome (the L protein gene) and none of the 3'-half of the genome. However, there are two well characterized DI particles, DI-LT1 and DI-LT2, which retain most of the 3'-half of the standard virus genome (the positive-strand leader RNA template and the N, NS, M, and G protein genes). DI-LT1 is unique amongst VSV DI particles in two basic ways. It is able to transcribe the positive-strand leader RNA and the N, NS, M, and G mRNAs and it is able to interfere not only with the replication of VSV_{IND} (Homotypic interference), but also with the replication of VSV_{NJ}(Ogden) (Heterotypic interference). The research objectives of this thesis were the examination of two unanswered questions about the heterotypic interference phenomenon. First, can all DI particles retaining a functionally intact VSV 3'-end heterotypically interfere? More precisely, can VSV_{NJ}(Ogden) DI particles retaining a functionally intact positive-strand leader RNA template interfere with VSV_{IND}? Secondly, why was DI-LT mediated heterotypic interference restricted to VSV_{NJ}(Ogden), a member of the Concan subtype of VSV_{NJ}, while VSV_{NJ}(Hazelhurst), a member of the Hazelhurst subtype of VSV_{NJ}, was

unaffected by DI-LT? The 3'- and 5'-terminal RNA sequence differences between VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) were examined in an attempt to understand why DI-LT interferes with the replication of VSV_{NJ}(Ogden) and not with the replication of VSV_{NJ}(Hazelhurst).

4.1 Can DI Particles Transcribing the Positive-Strand Leader RNA of VSVNJ(Ogden) Interfere Heterotypically?

Two DI particle preparations were generated (NJ-121 and NJ-PG1/2) which had DI particles large enough to contain the 3'-half of the genome (Figure 6, Table 2). The method for generating these VSV_{NJ}(Ogden) DI particles was identical to that used for the generation of DI-LT (Petric and Prevec, 1970) and several other 3'-DI particles (Kang, et al., 1985) from VSV_{IND}. The genomic RNA from these DI particles hybridized to all of the mRNAs from the 3'-half of the VSV_{NJ}(Ogden) genome (Figure 7), as well as the L mRNA. The intensity of hybridization to each mRNA did not mimic the pattern produced by the standard virus indicating that the DI particles did not retain a full copy of each gene. Two possible explanations could produce such results: the hybridization may be genuinely due to the genomic RNA content of the DI particles, or due to a combination of the DI particle genomic RNAs and low level contamination with standard virion RNA breakdown products (or the minor RNA bands observed in Figure 8, panel c). Supporting the hypothesis that the hybridization was due to the nature of the DI particles were the results in Figures 7-9. It should be noted that the results in Figure 8 (panel c) were observed upon overexposure and that each of the panels in Figure 8 showed this

gradient of subgenomic RNAs upon overexposure. Therefore, the results in Figures 7-9 could be paartially due to these subgenomic RNAs. Supporting the contamination hypothesis were the results in Figure 10. Figure 10 illustrated that NJ-121, NJ-PG2, and NJ-JM2 retained at least part of the G protein gene, while NJ-121a, NJ-121b, and NJ-PG1 retained very little if any of the G protein gene. The NJ-PG1 sequence (Figure 21) did not contain any G protein gene sequences, further supporting the contamination hypothesis.

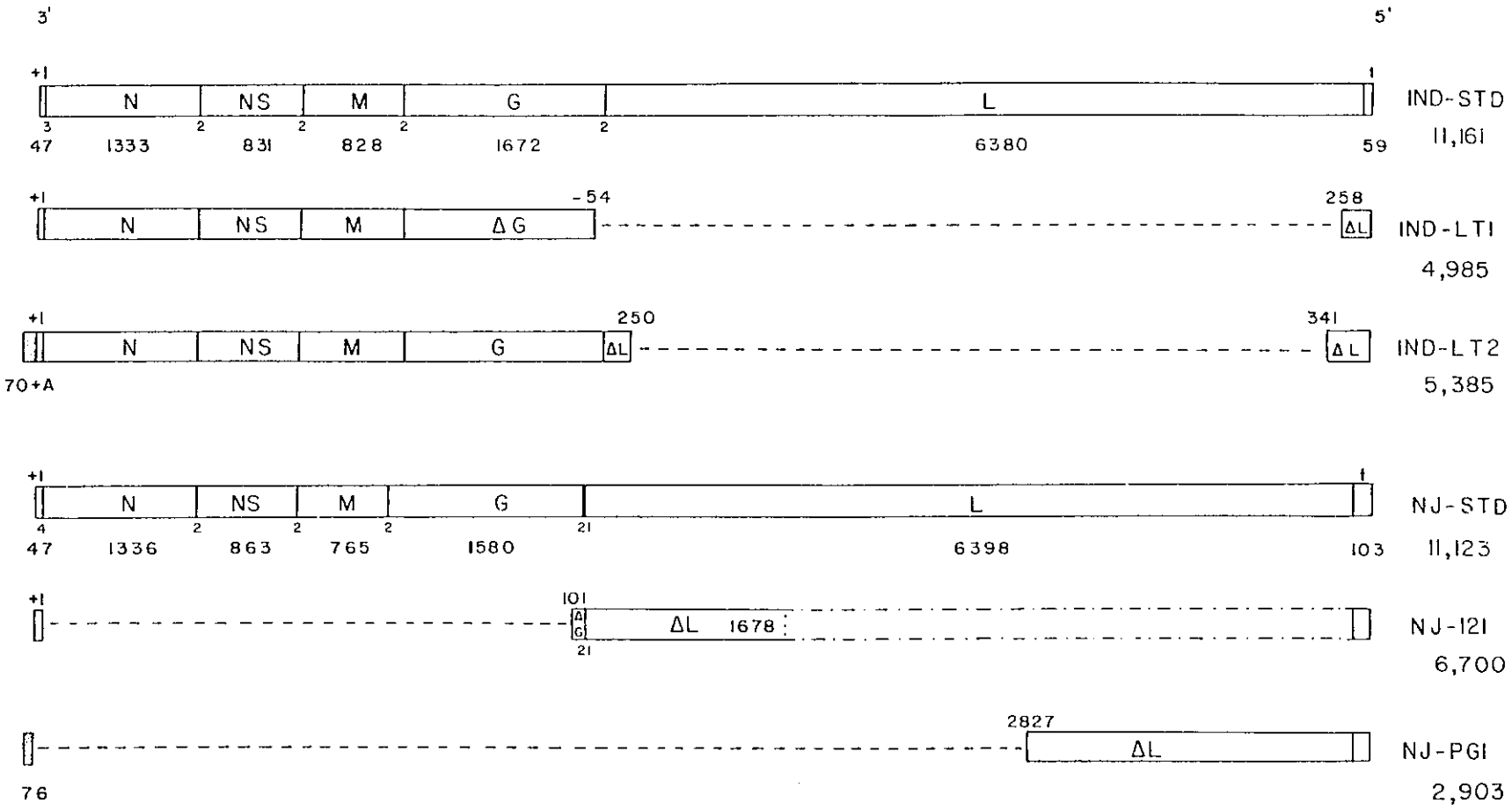
As it first appeared that NJ-121 and NJ-PG2, both larger than DI-LT, seemed to contain a portion of the 3'-half of the VSV_{NJ}(Ogden) standard virus genome (Figures 6-9), the transcription ability of several of these DI particles was examined. NJ-121 and NJ-PG2 transcribed the positive-strand leader RNA (like DI-LT), the negative-strand leader RNA (Figure 11) and larger RNA transcripts not identical to those produced by the standard virus (Figure 12). NJ-JM2 and NJ-PG1 were only able to transcribe the negative-strand leader RNA (like most DI particles).

To prove that both leader RNAs were transcribed by NJ-121 and NJ-PG2, it was necessary to prove that the template regions for both the positive- and negative-strand leader RNAs were contained within the genomes of these two DI particles. The results presented in Figure 13 and tabulated in Table 3, suggested that the genomes of NJ-121 and NJ-PG2 contained the template regions for both leader RNAs and that both leader RNAs were being transcribed by these DI particles. The RNA blots probed in Figure 13 were prepared with the same RNA as that used in Figure 6. By correlating the size and intensity of the RNA bands in

the NJ-121 and NJ-PG1/2 RNA preparations (Figure 6) to the intensity of hybridization by each probe (Figure 13, panels E and F) it was observed that the templates for both leader RNAs in NJ-121 and NJ-PG2 were in approximately equal concentration. According to these results two DI particle preparations, NJ-121 and NJ-PG1/2, were generated containing the DI particles, NJ-121 and NJ-PG2. These two DI particles appeared to encode the template regions for both the positive- and negative-strand leader RNAs (like DI-LT2) and transcribe both of these leader RNAs (unlike DI-LT2). These results may be explained by one of three possibilities: first, NJ-121 and NJ-PG2 may have a genome arrangement like DI-LT2 and somehow are able to transcribe beyond the strong transcriptional termination site of the negative-strand leader RNA template; secondly, NJ-121 and NJ-PG2 may package into mature virions both the genomic (negative-strand) RNA and replicative intermediate (positive-strand) RNA molecules; or thirdly, there are actually two DI particles of virtually identical size and in almost identical concentration in these RNA preparations, one transcribing the positive-strand leader RNA and the other transcribing the negative-strand leader RNA.

After several different approaches were used to clone NJ-121, it was concluded that within the NJ-121 RNA preparation, the molecules able to transcribe the positive-strand leader RNA contained the template region for the positive-strand leader RNA at their 3'-termini, followed by 101 nucleotides from the 5'-end of the G protein gene and the L protein gene (Figure 17 and 22). The size of NJ-121 (Figure 6 and Table 2) corresponds well with the sum of these three regions

Figure 22. Genomic RNA maps of VSV Transcribing DI particles. IND-LT1 and NJ-121 both transcribe the positive-strand leader RNA. IND-LT2 and NJ-PG1 only transcribe the negative-strand leader RNA. The maps of IND-LT1 (also called DI-LT1) and IND-LT2 (also called DI-LT2) are based on the results of Herman (1984) and Yang and Lazzarini (1983). The maps of NJ-121 and NJ-PG1 are drawn according to the results obtained in this thesis. The size of NJ-121 is actually 6,677 nucleotides long if the full L protein gene is retained. +1, Represents the positive-strand leader RNA template and t, represents the trailer region. A triangle placed before a gene name signifies that only part of the gene is retained in the DI particle. The numbers below each gene in the standard virus maps signifies the length of each gene, including the 7 Us of the Poly(A) signal. The shaded boxes signify positive-strand sequences (the copy-back sequence) at the 3'-ends of IND-LT2 and NJ-PG1.


3'
5'
+1
1
N
NS
M
G
L
3
2
2
2
2
47
1333
831
828
1672
6380
59
IND-STD
11,161
+1
-54
258
N
NS
M
ΔG
ΔL
IND-LT1
4,985
+1
250
341
N
NS
M
G
ΔL
ΔL
70+A
IND-LT2
5,385
+1
1
N
NS
M
G
L
4
2
2
2
21
47
1336
863
765
1580
6398
103
NJ-STD
11,123
+1
101
ΔG
ΔL
1678
21
NJ-121
6,700
2827
ΔL
76
NJ-PGI
2,903

(~6700 nucleotides). It was also concluded that no RNA molecules within the NJ-121 RNA preparation contained the positive-strand leader RNA template preceded by a stem structure (containing the negative-strand leader RNA template). Therefore, NJ-121 RNA either consisted of the molecule described above and presented in Figure 22 (NJ-121) and its replicative intermediate packaged in equal concentration, or two different DI particles, one containing the positive-strand leader RNA template (the molecule described above, Figure 22) and the other containing the negative-strand leader RNA template in addition to the L protein gene. The results of probing genomic RNAs with strand-specific probes (Figure 10) indicated that the NJ-JM2 contained both positive- and negative-stranded RNA, but neither NJ-121 nor NJ-PG2 contained positive-stranded RNA. Therefore, both the NJ-121 and NJ-PG2 DI particle preparations most likely each contain two different DI particles of identical size and concentration. One of the DI particles contains the positive-strand leader RNA template at its 3'-end and the second contains the negative-strand leader RNA template at its 3'-end. Since the probability of generating two DI particles of identical size and identical competitive ability (equal concentration) is very low, there must be some selective pressure for packaging such genomic RNAs.

Unfortunately, neither direct RNA sequencing nor dideoxynucleotide sequence analysis of either NJ-121 nor NJ-PG2 resulted in sequence data that could not be explained by contamination of the RNAs with both standard virus and other DI particles. The only satisfactory method of purifying NJ-121 and NJ-PG2 RNA was after glyoxal denaturation and gel electrophoresis, a treatment which modified the G and A residues and

made sequencing practically impossible. However, the NJ-PG2 RNA chemical cleavage pattern was similar to the pattern generated from the 3' end of the standard virion RNA with lower intensity bands from the 3' end of NJ-PG1 genomic RNA (results not shown).

Both the transcribing (NJ-121 and NJ-PG2) and nontranscribing (NJ-PG1 and NJ-JM2) DI particles from VSV_{NJ}(Ogden) were tested for their ability to interfere with the replication of VSV_{IND}. None of the DI particles (except IND-011) were able to heterotypically interfere with the replication of VSV_{IND}, however they all homotypically interfered with the replication of both VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst)(Table 4 and 5). Therefore, it was not merely the ability of DI-LT to transcribe the positive-strand leader RNA of VSV_{IND} that controlled its heterotypic interference ability, but the fact that it is able to transcribe the N, NS, M, and G mRNAs. It was suspected that the NS protein, being part of the polymerase complex, may allow DI-LT to replicate by competing with VSV_{NJ}(Ogden) standard virus for the VSV_{NJ}(Ogden) L protein and thereby interfering with the replication of VSV_{NJ}(Ogden).

In order for a DI particle to interfere with the replication of a standard virus the DI particle must have some replicative advantage. In order for a DI particle to replicate the polymerase complex provided by the standard virus must be compatible with the DI particle genome. However, when most DI particles are replicated by heterotypic standard viruses, the DI particles are replicated but the standard virus is not inhibited. Therefore, an interaction of the heterotypic polymerase complex with the DI particle genome must occur, albeit in a less

efficient manner than when replicated in the presence of a homotypic polymerase complex. The transcribing DI particle, DI-LT, is able to interfere with the replication of VSV_{NJ}(Ogden), indicating that the genome of DI-LT must interact efficiently with the heterotypic polymerase complex. Supporting this hypothesis are the results of in vitro reconstitution of transcription between N protein-bound-RNA templates and heterotypic viral L and NS proteins (De and Banerjee, 1984). The VSV_{NJ}(Ogden) N protein-bound-RNA template had a strict requirement for its own L protein, but was able to transcribe in the presence of either the VSV_{NJ}(Ogden) or the VSV_{IND} NS proteins. In contrast, the VSV_{IND} N protein-bound-RNA template had a strict requirement for its own NS protein, but could transcribe in the presence of either the VSV_{IND} or VSV_{NJ}(Ogden) L proteins. Due to the ability of VSV_{IND} to transcribe in the presence of the VSV_{NJ}(Ogden) L protein, DI-LT which encodes its own NS protein should remain transcriptionally active when coinfected with VSV_{NJ}(Ogden) standard helper virus. Indeed DI-LT is able to transcribe, replicate and heterotypically interfere when coinfected with VSV_{NJ}(Ogden) helper virus (Chow, et al., 1977), although the heterotypic helper virus function is not very efficient (Prevec and Kang, 1970).

It appears that it is not solely the ability of DI-LT to transcribe which confers it with the ability to interfere with VSV_{NJ}(Ogden), but the fact that it transcribes the NS mRNA. When the NS mRNA is translated DI-LT daughter particles can actively transcribe (secondary transcription) when complemented with the VSV_{NJ}(Ogden) L protein. According to this hypothesis, VSV_{NJ}(Ogden) DI particles would have to

encode and transcribe the VSV_{NJ}(Ogden) L protein to be active transcriptionally when coinfected with the VSV_{IND} standard virus. It is unlikely that the DI particles generated in this study would be able to transcribe the L protein. NJ-121 may contain the entire L protein gene, however it contains 101 nucleotides from the G protein gene between the positive-strand leader RNA transcription termination site and the L mRNA transcription initiation site, which may prevent reinitiation at the internal L mRNA transcription initiation site. The six nucleotides (AAAAUU) at the junction between the end of the positive-strand leader RNA template and the G mRNA sequence (Figure 17), contains the AAAA originating from the intergenic sequence located between the positive-strand leader RNA template and N mRNA transcription initiation site and the UU, the first two nucleotides of the VSV mRNA cap site (Figure 2). This sequence may be enough to allow *in vitro* transcription to continue through this region (Figure 11, 12), but it is unlikely that *in vivo* transcription would recognize only the UU of the UUGUC as the mRNA cap site. The *in vivo* sequence specificity for transcription initiation at UUGUC and nonspecificity of *in vitro* transcription initiation has recently been documented for VSV_{NJ}(Ogden) (Luk, et al., 1987). Since there are no UUGUC cap site sequences within the last 101 nucleotides of the G mRNA, the only UUGUC available after the positive-strand leader RNA termination site is within the L protein gene, 130 nucleotides away.

Another parameter which may affect the ability of a DI particle to interfere heterotypically may be the affinity of the N protein-bound-RNA template for the L and NS proteins. If the affinity of the L and

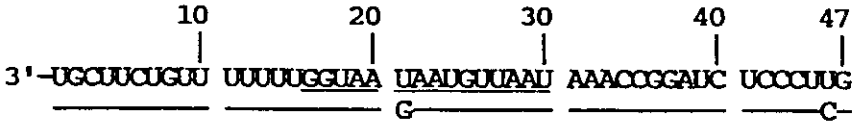
NS proteins for the VSV_{NJ}(Ogden) template is lower than for the VSV_{IND} template, then DI-LT would be replicated preferentially when coinfected with VSV_{NJ}(Ogden). Therefore, VSV_{NJ}(Ogden) DI particles able to transcribe their own L proteins may still compete poorly with VSV_{IND} standard virus for available VSV_{IND} NS proteins and therefore may not be able to interfere with VSV_{IND}. The affinity of the L and NS proteins for the VSV_{NJ}(Hazelhurst) template may be equivalent to the same interactions in VSV_{IND}, hence explaining why DI-LT cannot interfere with VSV_{NJ}(Hazelhurst).

4.2 In What Way do the Genomes of VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) Differ Which May Explain Their Different Response to Coinfection With DI-LT?

DI-LT is able to interfere with the replication of VSV_{NJ}(Ogden), but not with the replication of VSV_{NJ}(Hazelhurst). Since the promoter sites for transcription and replication are located at the 3'-ends of the genomic and antigenomic replicative intermediate RNAs, differences in these sites may affect the replicative advantage of the virus. Within the last 47 nucleotides (equivalent to the negative-strand leader RNA) the two subtypes differ only within the putative NS-binding site (Figure 15, 23). The five nucleotide changes within the putative NS-binding region are localized between nucleotides 19-26 from the 5'-terminus. Due to the importance of the NS binding region as a promoter of transcription and replication, the differences observed in the NS-binding region are further evidence for the evolutionary division of the Concan and Hazelhurst subtypes of VSV_{NJ}. Upon comparing the 3'-end

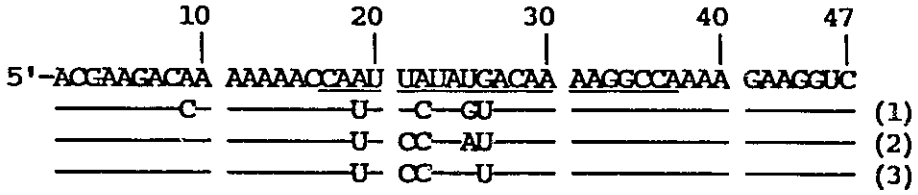
Figure 23. The sequence of the first and last 47 nucleotides of the VSV_{NJ} negative-strand genomic RNA. The sequence of the VSV_{NJ}(Ogden) 3'-terminal and 5'-terminal (representing the sequence complementary to the 3' terminus of DI particle RNAs) 47 nucleotides are shown in their entirety. The nucleotide differences in the VSV_{NJ}(Missouri) and VSV_{NJ}(Hazelhurst) sequences are indicated below the VSV_{NJ}(Ogden) RNA sequence. VSV_{NJ}(Missouri) is a member of the Hazelhurst subtype. The sequences of (1), (2), and (3) were taken from Feldhaus and Lesnaw (1988), Keene *et al.* (1981b), and Liang and Emerson (1988), respectively. The nucleotide sequence of VSV_{NJ}(Missouri) was taken from McGeoch *et al.* (1980). The underlined sequences in the VSV_{NJ}(Ogden) sequences shows the position of the putative NS-binding sites.

3'-End of VSV_{NJ}(Ogden)



3'-End of VSV_{NJ}(Missouri)

5'-End of VSV_{NJ}(Ogden)



5'-End of VSV_{NJ}(Hazelhurst)

sequence between VSV_{NJ}(Ogden) (Rowlands, 1979; Nichol and Holland, 1987) and VSV_{NJ}(Missouri), a member of the Hazelhurst subtype (McGeoch, et al., 1980), there are only two nucleotide differences observed (nucleotides 21 and 46). Therefore, the NS-binding site of the replicative intermediate (the complementary sequence of the 5'-terminal 47 nucleotides) has diverged much more between these two subtypes than the NS-binding site of the genomic RNA, which has remained relatively unchanged. It is interesting to note that during prolonged persistent infections mediated by DI particles, mutants resistant to DI particles are selected (Horodyski and Holland, 1980, 1981; Horodyski, et al., 1983; O'Hare, et al., 1984; DePolo, et al., 1987). These mutant viruses were found to accumulate stepwise base substitutions in their 5'-terminal but not their 3'-terminal genomic RNA sequences. The changes were not restricted to the NS-binding region. Some of these changes may facilitate interactions between the terminal RNA sequence and the polymerase proteins. Recently, it was found that the polymerase proteins (L-NS complex) from these persistant standard virus were unable to replicate DI particles in vitro (Giachetti, et al., 1988), however, when normal standard virus polymerase proteins (L-NS complex) were added the DI particles were replicated. Therefore, mutations in the L-NS polymerase complex confers resistance to DI particle interference, probably due to diminished affinity of the mutant polymerases for wild type DI particle nucleocapsid templates, whereas affinity for mutant virus templates is maintained. These experiments suggest that the polymerase complex must coevolve with viral termini. Therefore, changes in the NS-binding region of the

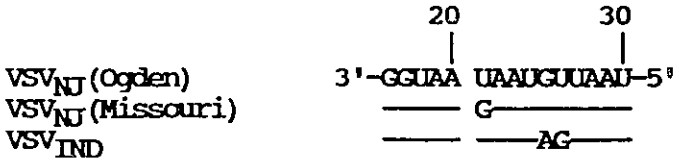
replicative intermediate RNA template may reflect different affinities of the polymerase proteins (L-NS complex) for these sites. Different polymerase binding affinities would correlate well with the ability of VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) to replicate in the presence of DI-LT.

In infected cells, the concentration of negative-strand genomic RNA is much higher than positive-strand antigenomic full length RNA. Accordingly, the polymerase binding site on the antigenomic RNA (replicative intermediate) must be much stronger than the polymerase binding site on the negative-strand RNA (genomic RNA). Likewise, the competitive advantage of DI particles over standard virus in homotypic interference is assumed to be due to the polymerase binding site of the replicative intermediate being on the 3'-ends of both the DI particle genomes and antigenomes. The polymerase binding site and the affinity of the NS protein for this site may control the replicative advantage of a particular virus. A poor polymerase binding site may lead to a slower rate of replication when competing with a stronger polymerase binding site. Therefore, differences in the polymerase binding site at the 3'-end of replicative intermediate RNA would affect replicative efficiencies more than changes at the 3'-end of the negative-strand genomic RNA. Similarly, DI particles containing stronger polymerase binding sites of their genomic and antigenomic RNAs, would have a selective advantage over both standard virus and DI particles with poorer polymerase binding sites. The observation of a greater accumulation of nucleotide differences within the NS-binding region of the replicative intermediate RNA, than within the NS-binding region of

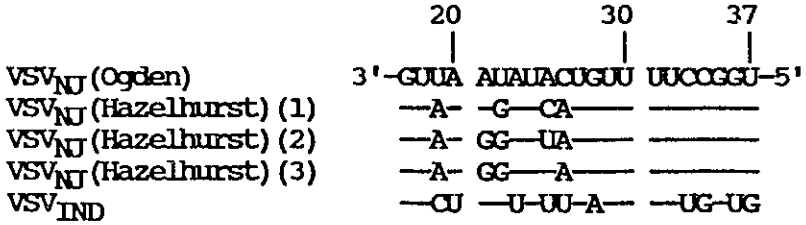
the negative-strand genomic RNA supports this hypothesis (Figure 24). An indirect indication that VSV_{NJ}(Ogden) may have a lower affinity for its own NS protein than VSV_{IND} comes from the results of heterotypic reconstitution of transcription (De and Banerjee, 1984). The VSV_{NJ}(Ogden) template will transcribe in the presence of either the VSV_{IND} or VSV_{NJ} NS proteins. However, the VSV_{IND} template has an absolute specificity for its own NS protein. The nonspecificity of the VSV_{NJ}(Ogden) template may reflect a low NS-binding affinity. Hence, the NS protein would bind to the VSV_{NJ}(Ogden) template less frequently than the VSV_{IND} NS protein would bind to the VSV_{IND} RNA template and the VSV_{NJ}(Ogden) template would therefore be less able to use the available L protein when coinfected with DI-LT. According to this hypothesis since DI-LT does not interfere with the replication of VSV_{NJ}(Hazelhurst) (Reichmann, et al., 1978), the affinity of the VSV_{NJ}(Hazelhurst) NS protein for its NS-binding site must be at least as strong as that of VSV_{IND}. Heterotypic reconstitution of transcription between VSV_{NJ}(Hazelhurst) and VSV_{IND} has not been studied and should indicate the specific transcriptional requirements of VSV_{NJ}(Hazelhurst). It is predicted that the VSV_{IND} NS protein would interact with the VSV_{NJ}(Hazelhurst) L protein, due to the ability of DI-LT to be replicated in the presence of VSV_{NJ}(Hazelhurst) (Adachi and Lazzarini, 1978). This interaction is further supported by the similarity of the NS-L protein binding sites on the NS proteins of VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) (Rae and Elliott, 1986; Emerson and Schubert, 1987b) and the ability of the VSV_{NJ}(Ogden) L protein to interact with the VSV_{IND} NS protein-RNA-N protein complex (De and

Figure 24. The NS-binding site sequence differences in VSV. The sequence of VSV_{NJ}(Ogden), VSV_{IND}, and VSV_{NJ}(Missouri) were taken from Nichol and Holland (1987). The sequence of VSV_{NJ}(Hazelhurst) (1), (2), and (3) were taken from Feldhaus and Lesnaw (1988), Keene, et al. (1981b), and Liang and Emerson (1988), repectively.

NS-Binding Site at the 3'-End of the Negative-Strand RNA.



NS-Binding Site at the 3'-End of the Positive-Strand Replicative Intermediate.



Banerjee, 1984). The NS-L protein binding site on the L protein has not yet been elucidated.

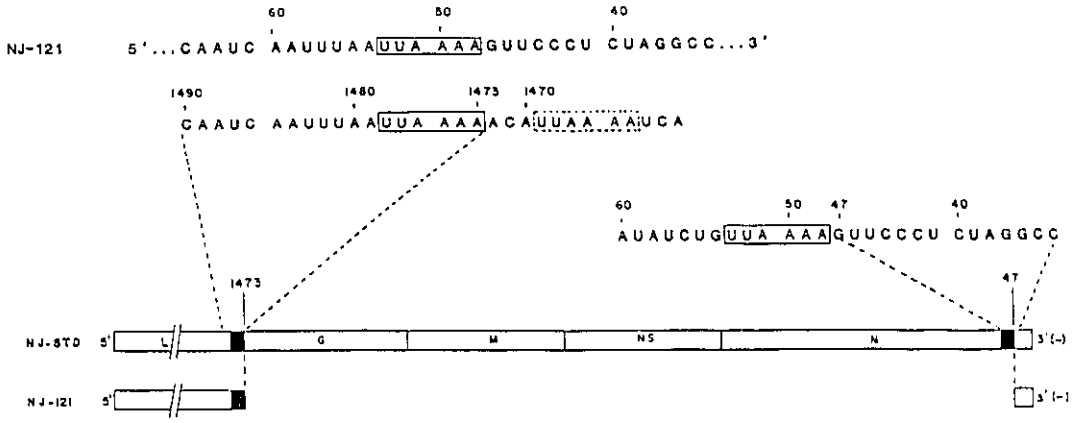
In conclusion, viral heterotypic interference is likely controlled by the specific requirements and affinities of each virion RNA template for the polymerase complex and not merely by the ability of a DI particle to transcribe. Therefore, even if a DI particle were able to transcribe the mRNA for the homotypic proteins that it requires to transcribe efficiently in the presence of heterotypic polymerase components, it may still be unable to interfere with the replication of a heterotypic virus due to differences in RNA template affinities for the polymerase proteins. This correlates well with the hypothesis that DI particles interfere homotypically because they have stronger polymerase binding sites at the 3'-ends of their genomic RNAs than standard virus (Huang, et al., 1978) giving them a replicative advantage during coinfection with standard virus.

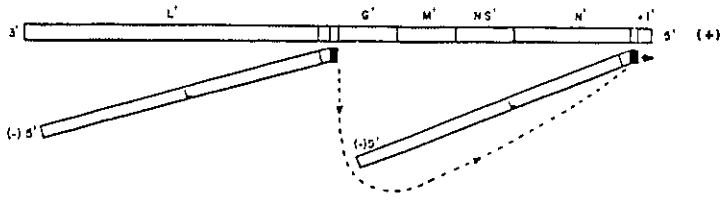
4.3 Sites of Copy Choice Replication Involved in the Generation of VSV_{IJ}(Ogden) DI Particle RNAs.

The copy choice model for the generation of defective interfering (DI) particles of vesicular stomatitis virus suggests that during replication the polymerase prematurely terminates, moves with the nascent daughter strand to another site on the same or a different template molecule, and resumes elongation of the nascent chain. The sites where premature termination and resumption of replication occur have been examined for several VSV_{IND} DI particles (Meier, et al., 1984). The DI particles examined were the simple deletion particle,

DI-LT, the snapback DI particle, DI-011, and the panhandle DI particles, DI-T, DI-T(L), and DI-611. The recombination sites were identified by comparing the nucleotide sequences of the relevant regions of the DI particle RNAs to those of the VSV_{IND} L gene. Sequence homology was not detected between these sites, ruling out the existence of a general terminator or promoter sequence involved in copy choice replication. In several cases, however, premature termination or resumption of RNA replication may be favored by specific signal sequences. The sequences immediately before the start of the deletion in DI-LT contain two hexanucleotides, AUCUGA and GAUUGG, located ten nucleotides apart and five nucleotides from the start of the deletion. These same nucleotides are located thirteen nucleotides apart and seven nucleotides from the end of the deletion. Upon examination of the sequence around the deletion in NJ-121 (the DI particle RNA containing the positive-strand leader RNA template), we find the hexanucleotide, UUAAAA, which is located immediately before the start of the deletion and immediately before the end of the deletion (Figure 25a). Therefore, both the simple internal deletion DI particles of VSV, NJ-121 and DI-LT1 have been generated by the polymerase leaving the template with the daughter RNA at a particular sequence and recognizing a similar sequence just prior to the resumption of replication at the end of the deletion (Figure 25b). Another common characteristic of both DI-LT1 and NJ-121 (and possibly NJ-PG2) is that these DI particles do not compete well with DI particles containing stem structures (Perrault and Semler, 1979). Upon further passing of the NJ-121 and NJ-PG2 DI particle preparations (Figure 6), the recovery of NJ-121 and

Figure 25. Proposed model to explain the origin of the transcribing DI particle, NJ-121. (A) The sequence of the NJ-121 genomic RNA from nucleotides 34-65 is shown in the top line. The boxed-in sequence (UUAAAA) is common to nucleotides 48-53 from the 3'-end of the standard virus genome and nucleotides 1473-1478 from the 3'-end of the G protein gene. The same sequence is repeated at nucleotides 1469-1464 (dotted box) from the 3'-end of the G protein gene. (B) During replication the polymerase likely copied the replicative intermediate RNA (shaded bar) until it reached nucleotide 1473 from the 3'-end of the G protein gene, prematurely terminated replication in this AU rich region, and recognized the same six nucleotides (AAUUUU) (clear box) and resumed replication from nucleotide 47 from the 5'-end of the replicative intermediate RNA. The black boxes represent the UUAAAA sequence. The sequence of the nontranscribing DI particle of identical size to NJ-121 is not known. However, it is postulated that this DI particle genome may consist of most of the L protein gene with a stem structure at its 3'-end.


NJ-121
5'...CAAUC AAUUUAAUUA AAAGUUCCCU CUAGGCC...3'
60
50
40
1490
1480
1473
1470
CAAUC AAUUUAAUUA AAACACUUAA AAUCA
60
50
47
40
AUAUCUGUUA AAAGUUCCCU CUAGGCC
1473
47
NJ-STD
5'
L
G
M
NS
N
3'(-)
NJ-121
5'
3'(-)


L'
G'
M'
NS'
N'
+1'
3'
5'
(+)
(-)5'
(-)3'

NJ-PG2 is greatly reduced in comparison to the recovery of NJ-121a, NJ-121b, and NJ-PG1, respectively, as determined by RNA gel electrophoresis and relative recovery from sucrose velocity gradients (results not shown). This characteristic has made the analysis of both NJ-121 and NJ-PG2 very difficult and consequently very frustrating.

There was no special secondary sequence structure around the termination site of DI-011, the VSV_{IND} snapback DI particle, therefore, it is presumed that snapback DI particles are generated by replication across a replicative fork (Schubert and Lazzarini, 1981, Meier, et al., 1984). NJ-JM2 is thought to be a compound snapback DI particle (like DI-ST2) because both positive- and negative-strand RNA are contained within its genome and it contains sequences from both the G and L genes. Since the size of NJ-JM2 is not large enough to contain the full L gene and part of the G gene, it is presumed that NJ-JM2 contains at least one deletion within the L gene. The generation of such a DI particle might proceed by replication across the replication fork of a replicating internally deleted DI particle (Figure 26). Further sequence analysis of NJ-JM2 will have to be completed to confirm the actual deletion site.

Within the panhandle DI particles examined, DI-T, DI-T(L), and DI-611, the only constant sequence feature is the conservation of the negative-strand leader RNA template (Meier, et al., 1984). NJ-PG1 also has no specific sequence signals common to the sequence around the start and end of the deletion (Figure 21, 27). The panhandle DI particles are thought to be generated by premature termination of replication on the replicative intermediate and the polymerase copying

Figure 26. Proposed model to explain the origin of the snapback DI particle, NJ-JM2. The first event in the generation of NJ-JM2 is the formation of a simple deletion within the L gene (top three lines). This precursor DI particle is then replicated and a snapback DI particle is generated when a polymerase copies through a replicative fork (bottom three lines). A, represents the 3'-end of the standard virus negative-strand genome. B, represents the 3'-end of the standard virus positive-strand replicative intermediate. ('), signifies the complementary sequence. The bottom diagram illustrates the genome organization of NJ-JM2 (B is found at the 3'-end and B' at the 5'-end).

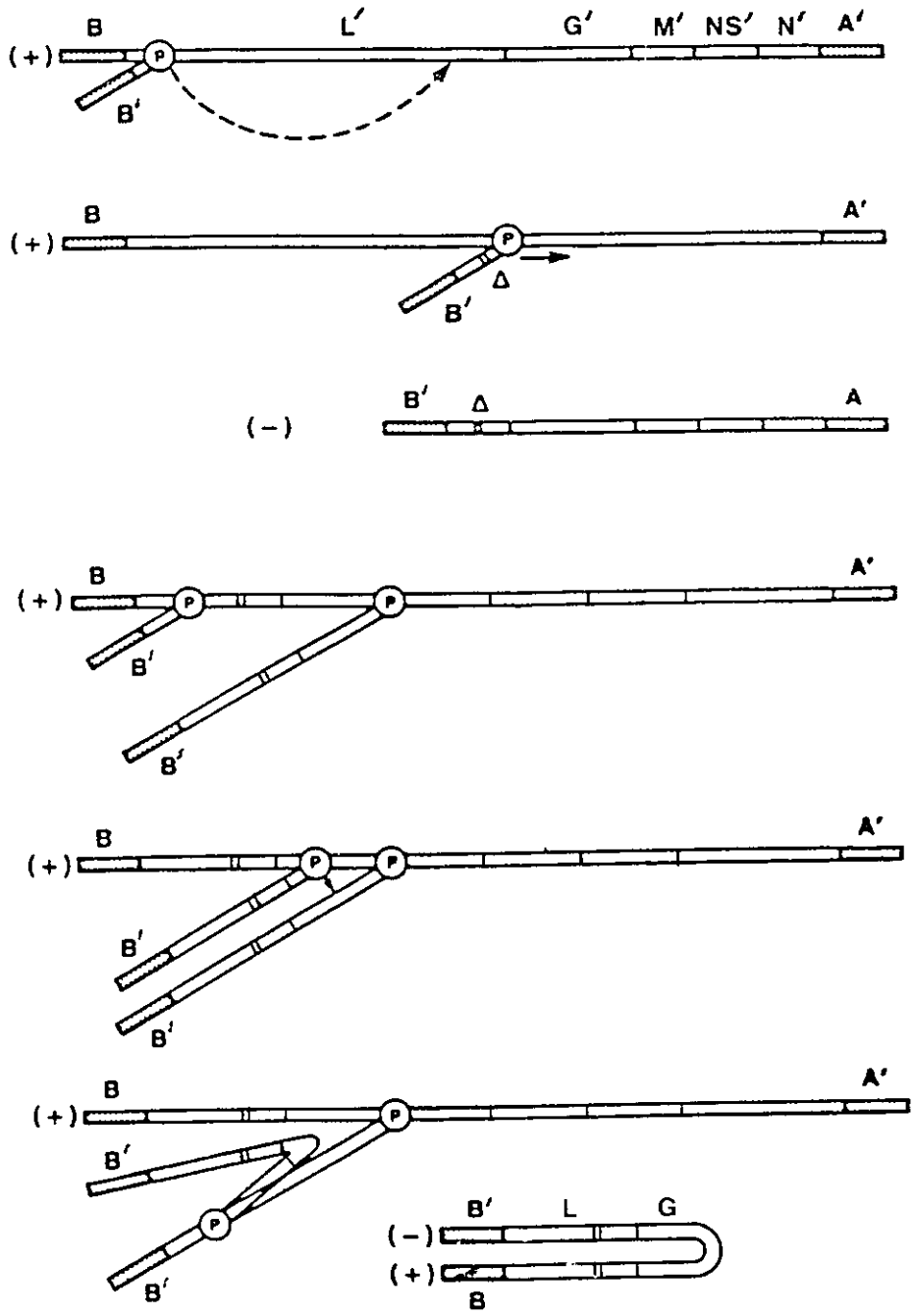
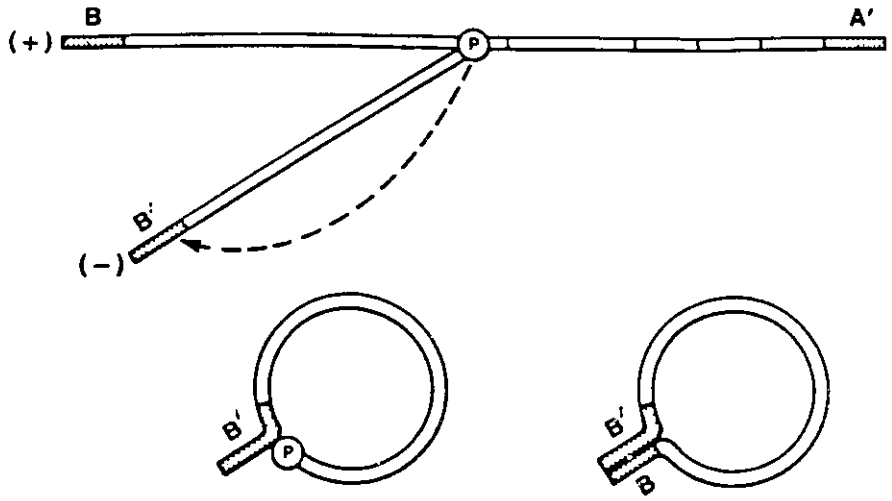
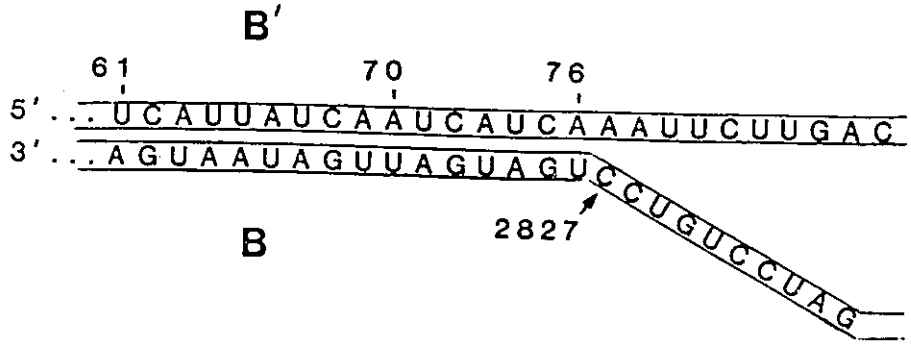

B
L′
G′
M′
NS′
N′
A′
(+)
P
B′
B
A′
(+)
P
Δ
B′
(−)
B′
Δ
A
B
A′
(+)
P
P
B′
B′
B
A′
(+)
P
P
B′
B′
B
A′
(+)
P
B′
P
B′
B′
L
G
(−)
(+)
B

Figure 27. Proposed model for the origin of the panhandle DI particle, NJ-PG1. Following premature termination of negative-strand RNA synthesis, the polymerase, with the nascent RNA strand attached, resumes synthesis at a site 76 nucleotides from the 5'-end of the nascent RNA strand to form a 76 base pair stem structure. The sequences around the resumption site are shown at the bottom.


B
A'
(+)
P
B'
(-)
B'
P
B'
B


B'
61
70
76
5'. . UCAUUAUCAAUCAUCAAAUUCUUGAC
3'. . AGUAAUAGUUAGUAGUCCUGUCCUAG
2827
B

back along the 5'-end of the daughter RNA (negative-strand RNA) to generate their 3'-ends (Lazzarini, et al., 1981; Perrault, 1981). The smallest stem structures found are the size of the negative-strand leader RNA (DI-T, DI-T(L), DI-611), attesting to the importance of this region for the replication and competitive advantage of these DI particles. It is postulated that the DI particle RNA of identical size to the transcribing NJ-121, containing the negative-strand leader RNA template, could have been formed by a polymerase complex falling off the replicative intermediate RNA, after most of the L protein had been copied, with the daughter RNA still attached, and copying back on the daughter RNA to generate a stem structure at its 3'-terminus.

In conclusion the VSV_{NJ}(Ogden) DI particles seem to have been generated by the same type of copy choice mechanisms as that used to generate VSV_{IND} DI particles (Lazzarini, et al., 1981; Perrault, 1981). However, no simple deletion DI particles resembling DI-LT1 (retaining the N, NS, M and G genes) nor compound DI particles resembling DI-LT2 were generated.

5. Conclusion.

The main goal of this thesis was to better understand the mechanism of heterotypic interference observed between the VSV_{IND} DI-LT and VSV_{NJ}(Ogden). DI-LT contains the transcriptionally active template regions for the positive-strand leader RNA and the N, NS, M and G protein mRNAs. To determine if DI particles generated from VSV_{NJ}(Ogden), resembling DI-LT in structure and transcriptional ability, would interfere with VSV_{IND}, DI particles that first appeared to contain partial sequences from throughout the VSV_{NJ}(Ogden) genome were generated and analyzed. After genome analysis was complete it was concluded that NJ-121 and NJ-PG2 did not retain most of the 3' half of the VSV_{NJ}(Ogden) genome. The method of generating these DI particles was identical to that used to generate DI-LT (Prevec and Kang, 1970) and several other DI particles containing sequences from the 3'-half of the VSV_{IND} genome (Kang, et al., 1985). The two largest DI particles, NJ-121 and NJ-PG2, also appeared to contain the transcriptionally active template regions for both the positive- and negative-strand leader RNAs. After several attempts at cloning the sequence between the template regions for the positive- and negative-strand leader RNAs, it was concluded that NJ-121 was probably two DI particles of identical size and in virtually identical concentration. One of the DI particles contained the positive-strand leader RNA template, part of the G protein gene and most of the L protein gene and the other containing the negative-strand leader RNA template and the L protein gene. Neither NJ-121 nor NJ-PG2 were able to heterotypically interfere with the replication of VSV_{IND}. It was concluded that it was not merely the

ability of DI-LT to transcribe the positive-strand leader RNA, but its ability to transcribe the mRNAs for the proteins encoded by DI-LT that enabled it to heterotypically interfere .

Another characteristic of DI-LT is that its heterotypic interference activity is limited to the Concan subtype of VSV_{NJ}. To determine what differed between VSV_{NJ}(Ogden), a member of the Concan subtype, and VSV_{NJ}(Hazelhurst), a member of the Hazelhurst subtype (resistant to interference by DI-LT), which may control the ability of DI-LT to interfere with one subtype and not the other, we compared the nucleotide sequence of the template regions for the positive- and negative-strand leader RNAs. These regions were chosen due to their importance in controlling transcription and replication. The nucleotide differences between VSV_{NJ}(Ogden) and VSV_{NJ}(Hazelhurst) were concentrated within the NS-binding site of the negative-strand leader RNA template. As this region is the putative polymerase binding site of the replicative intermediate RNA, and it is postulated that differences in this region would greatly affect the outcome of replication. Differences in the NS-binding sites may reflect differences in the ability of the NS protein to interact with these sites. A RNA template with a low affinity binding site would not be able to compete with a RNA template containing a higher affinity binding site. This may also explain why DI particles have a replicative advantage over homotypic standard virus in coinfections. Results of heterotypic reconstitution of transcription (De and Banerjee, 1984) indicated that the VSV_{NJ}(Ogden) RNA template is able to interact with either the NS proteins of VSV_{NJ}(Ogden) or VSV_{IND}, while

the VSV_{IND} RNA template would only interact with its own NS protein. These results indicate that the VSV_{NJ}(Ogden) RNA template probably has a lower affinity for the NS protein. DI-LT does not heterotypically interfere with VSV_{NJ}(Hazelhurst) indicating that the polymerase binding site on the VSV_{NJ}(Hazelhurst) RNA template may be as strong as the same site on the VSV_{IND} RNA template. Therefore, it is concluded that the ability of a DI particle to interfere heterotypically depends on the specific replicational requirements of both the DI particle and the heterotypic standard virus. In order for a DI particle to heterotypically interfere it must encode and transcribe the homotypic proteins (L or NS proteins) that it requires to replicate as a stronger competitor when coinfected with heterotypic standard virus. This hypothesis can be tested by taking the NS protein of VSV_{IND}, expressing it in cells, so that 100% of the cells express the NS protein, and coinfecting these cells with a VSV_{IND} DI particle, which normally does not express the NS protein, and VSV_{NJ}(Ogden) standard virus. If our hypothesis is correct, the DI particle should heterotypically interfere with VSV_{NJ}(Ogden). A similar experiment using VSV_{NJ}(Hazelhurst) standard virus should not result in heterotypic interference.

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Zavada, J., and E. Zavodska (1973/74). Complementation and phenotypic stabilization of vesicular stomatitis virus temperature sensitive and thermolabile mutants by avian myeloblastosis virus. Intervirol. 2, 25-32.

7. Curriculum Vitae.

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DATE OF BIRTH: October 7, 1957.

CITIZENSHIP: Canadian.

EDUCATION:

PhD. (Microbiology and Immunology) (University of Ottawa, Ottawa, Ontario, Canada).	1988.
Masters of Science (Virology) (University of Quebec, Institute Armand-Frappier, Laval, Quebec, Canada).	1982.
Bachelor of Science (Biology) (University of Ottawa, Ottawa, Ontario, Canada).	1980.
High School Grade 13 Diploma (Canterbury High School, Ottawa, Ontario, Canada).	1976.

RESEARCH EXPERIENCE:

1980-1982 Evaluation of the efficacy of different combinations of chemical insecticides and insect viruses (Baculoviruses and Cypoviruses) as a control method for the white cutworm, Euxoa scandens (Riley). This work was done under the supervision of Dr. Serge Belloncik (University of Quebec, Institute Aramand-Frappier, Laval, Quebec, Canada).

1982-1983 Cloning and sequencing the S segment of a Canadian isolate of the California encephalitis virus, Snowshoe Hare strain (Bunyaviridae) and oligonucleotide fingerprint analysis of all three RNA segments of several canadian SSH virus isolates. The goal of this work was to determine if genetic reassortment of the three segments of RNA with the Lacrosse virus had been responsible for increasing the virulence of the Canadian SSH isolates thought to be responsible for causing several cases of California encephalitis in Quebec. This work was done under the supervision of Dr. David H.L. Bishop (University of Alabama in Birmingham, Birmingham, Alabama, U.S.A.). This work was done in collaboration with Dr. Serge Belloncik (University of Quebec, Institute Armand-Frappier, Laval,

Quebec, Canada), who provided the SSH virus isolates.

1983-1988 A molecular examination of defective interfering particle-mediated viral heterotypic interference. Several DI particles from VSV_{NJ}(Ogden) were examined for their ability to transcribe <u>in vitro</u> and to interfere with the replication of VSV_{IND}. Two DI particles were isolated that were able to transcribe the 3'-end of the VSV_{NJ}(Ogden) genome. The genomic RNA of these two DI particles, and other DI particles generated with these DI particles were characterized using current molecular biological techniques. One of the transcribing DI particles, NJ-121 was cloned and sequenced. In an attempt to understand why the VSV_{IND} DI-LT was able to heterotypically interfere with VSV_{NJ}(Ogden) and not with VSV_{NJ}(Hazelhurst) the 5'-half of VSV_{NJ}(Ogden) (the L gene and the 5'-terminal 103 nucleotide trailer region) was sequenced and compared to that of VSV_{NJ}(Hazelhurst). It was found that the sequence of the NS-binding region of these two subtypes varied greatly at the 5'-end (but not the 3'-end) of the standard virion genomes. The implications of the variability of this region in regards to heterotypic interference were discussed.

<u>Publications</u>:

Rud, E.W., and C.Y. Kang (1988). The 5' terminal sequence of VSV_{NJ}(Ogden). Is the interaction of the NS protein with the NS binding site responsible for heterotypic interference activity? Virology 164, 551-555.

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