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MOLECULAR BIOLOGICAL CHARACTERIZATION OF DEFECTIVE INTERFERING PARTICLES OF VESICULAR STOMATITIS VIRUS

A Thesis Submitted to the

School of Graduate Studies

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In Partial Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

Faculty of Medicine

By

Woo-Young Choi

ABSTRACT

Defective interfering particles (DIPs) of vesicular stomatitis virus (VSV) were generated and/or amplified during serial high multiplicity passage in BHK-21 cells. Two IND-DIPs (IND-ST and IND-LT) were derived from the Indiana (Ind) serotype and two NJ-DIPs (Ogd-DI and Haz-DI) were generated from Ogden and Hazelhurst strains, respectively, of the New Jersey (NJ) serotype of VSV. The biological activity of each DIP was examined by the interference assay. Two IND-DIPs interfered both homotypically and heterotypically. In contrast, two NJ-DIPs interfered homotypically but could not interfere heterotypically. Intracellular RNA synthesis in standard virus infected cells and in cells infected with both standard virus and DIPs revealed that the level of RNA synthesis of the standard virus was markedly reduced if DIP interfered with the production of the standard virus. In contrast, the level of mRNA transcription was not affected if DIPs did not interfere with replication of the standard virus. Furthermore, DIP genomes were replicated only if the DIP interfered with the replication of the standard virus. These data strongly suggest that the first step of DIP-mediated viral interference is at the level of viral RNA synthesis.

In an effort to understand how these DIPs are generated, which genetic elements are involved in viral interference and how the DIPs interfere with the replication of standard virus, the complete sequence analyses of cloned cDNAs from the four different DIPs were carried out by the dideoxy chain termination method. The size of each DIP genome was 2,660 bases (IND-ST), 5,313 bases (IND-LT), 2,984 bases (Ogd-

DI), and 6,110 bases (Haz-DI). The DIP genomes of IND-ST, Ogd-DI, and Haz-DI were derived from the 5' end of the standard virus genome and retained different lengths of inverted complementary termini (54 bp, 18 bp, and 71 bp, respectively), to form panhandle stem structures. In contrast, IND-LT retained the 3' half of the standard virus genome (4,974 bases) and 530 bases of the 5' end of the standard virus genome. This DIP genome represented a single deletion of a part of the L gene and thus contained the same 3' and 5' termini as the standard virus genome. Examination of the DIP genomes revealed that they all retained the 5' end of the standard virus. These findings suggest that the 5' terminus may be essential for control of DIP replication and interference activity.

To understand the mechanism(s) of viral interference mediated by DIPs, a reverse genetic system for VSV was employed. All five viral genes from each of the three different strains of VSV were cloned and placed under the control of the T7 RNA polymerase promoter. Furthermore, cDNAs of four different DIPs were subcloned into a specialized transcription vector containing the T7 RNA polymerase promoter, hepatitis delta virus ribozyme, and the T7 terminator to generate DIP RNAs with the exact 3' and 5' termini identical to those of the authentic DIP genomic RNAs. The T7 RNA polymerase expressed by a recombinant vaccinia virus successfully rescued the cloned DIP genomes, which were amplified by subsequent passages. Furthermore, the rescued recombinant DIPs had the same biological activities as the original DIPs. Future experiments will help to identify cis-acting sequences in the DIP genomes and the roles of individual viral proteins involved in viral interference.

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DEDICATION

This thesis is dedicated to my father, my mother,

my grandmother, my brother, and my sister

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LIST OF ABBREVIATIONS

μCi	microcuries
μg	microgram
μl	microlitre
μmol	micromoles
A	Adenine base in a nucleotide sequence
ATCC	American type culture collection
ATP	adenosine 5'-triphosphate
BHK-21	Baby hamster kidney cell clone 21
Bis	N'N'-Bis-methylene-acrylamide
bp	base pairs
BrdU	5-bromo-2'-deoxyuridine
BRL	Bethesda Research Laboratories
°C	degrees Celsius
C	Cytosine base in a nucleotide sequence
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CPE	cytopathic effect
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate

ds	double-stranded
DEPC	diethylpyrocarbonate
DIP(s)	defective interfering particle(s)
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
Fig.	figure
g	grams
G	Guanine base in a nucleotide sequence or glycoprotein
Haz	Hazelhurst
HDV	hepatitis delta virus
HIFBS	heat inactivated fetal bovine serum
HR	heat resistant
hr(s)	hour(s)
IBI	International Biotechnologies, Inc.
IEC	International Equipment Company
Inc.	Incorporated

IND	Indiana
IPTG	isopropyl β-D-thio-galactoside
kD	kilodaltons
kb	kilobases
kg	kilogram
l	leader
L	large polymerase protein
LT	long truncated DIP
M	matrix protein or moles
MEM	minimal essential medium
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimoles
MOI	multiplicity of infection
MOPS	morpholinopropanesulfonic acid
M(r)	relative molecular mass
mRNA	messenger ribonucleic acid
N	nucleocapsid protein
NEN	New England Nuclear
ng	nanogram

nm	nanometre
NJ	New Jersey
NP-40	Nonidet P-40
Ogd	Ogden
ORF	open reading frame
pTV	transcription vector
P	phosphoprotein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming unit
PMSF	phenylmethylsulfonyl-fluoride
Poly (A)	polyadenosine
PPO	2,5-diphenyl-oxazole
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNase	ribonuclease
RNasin	ribonuclease inhibitor
RNP	ribonucleoprotein complex
rpm	revolutions per minute
RT	reverse transcriptase

sec	seconds
S	Svedberg units
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOB	tryptone, yeast extract, NaCl
SOC	tryptone, yeast extract, NaCl, glucose
ST	short truncated DIP
STD	standard virus
t	trailer
T	Thymine base in a nucleotide sequence
TAE	Tris-acetate, EDTA buffer
TBE	Tris-borate, EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TK$^-$143	thymidine kinase-negative human 143 B cells
Tris	tris (hydroxymethyl) aminomethane
TTBS	TBS plus Tween 20
UV	ultraviolet
vTF7-3	recombinant vaccinia virus containing T7 RNA polymerase gene
VSV	vesicular stomatitis virus
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1: GENERAL INTRODUCTION

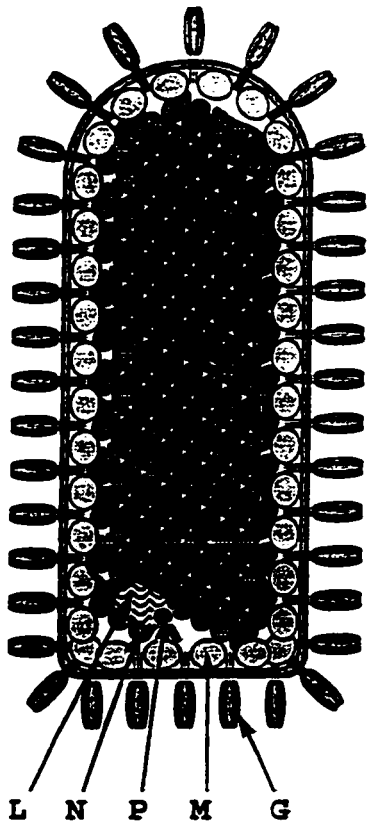
1. VESICULAR STOMATITIS VIRUS

The virion and its components

Vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family, is the prototype of the genus *Vesiculovirus*. Fig. 1 shows a schematic representation of the morphology and structural components of the virion of VSV. The virion is bullet-shaped and consists of a helically wound ribonucleoprotein (RNP) complex surrounded by a membranous envelope layer through which spike projections protrude. The particle size is approximately 180 nm long and 75 nm in diameter. The approximate composition of VSV virions is 74% protein, 20% lipid, 3% carbohydrate, and 3% RNA (Mcsharry and Wagner 1971a,b, Wagner 1975).

There are two main serotypes of VSV which are known as Indiana serotype (VSV_{IND}) and New Jersey serotype (VSV_{NJ}). The VSV_{NJ} serotype consists of two strains; Ogden ($VSV_{NJ\text{-}Ogd}$) and Hazelhurst ($VSV_{NJ\text{-}Haz}$). The characteristics of mRNAs and proteins of the two main serotypes of VSV (VSV_{IND} and VSV_{NJ}) are summarized in Table 1. The genetic information of VSV is contained in an unsegmented single strand of RNA that is complementary to messenger RNA (negative-stranded RNA virus). The RNA has a sedimentation coefficient of 38 to 45S, corresponding to a molecular weight of approximately 4×10^6, and is noninfectious RNA because of the negative polarity (Huang and Wagner 1966). There is one copy of RNA in each virion. As with all negative-stranded viruses, VSV carries a RNA-dependent RNA polymerase

Figure 1. Schematic representation of the morphology and structural components of VSV. The virion is composed of two major structural components: the ribonucleoprotein (RNP) complex, which contains single-stranded RNA tightly bound by the major N (nucleocapsid) protein and the minor proteins L and P, and the membrane associated with two proteins, the integral glycosylated (G) protein and the internal matrix (M) protein, which adheres to the inner membrane surface and binds to the RNP complex. Arrows indicate the individual VSV proteins. The gene order of the VSV genome is shown with symbols representing the expressed proteins. This figure was taken from Fields *et al.* (1996).


L
N
P
M
G

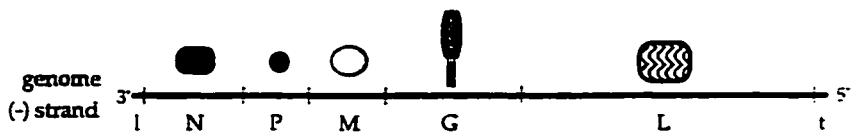

genome
(-) strand
3'
5'
l
N
P
M
G
L
t

TABLE 1

VSV mRNAs and proteins

Genes	mRNA	Proteins			
	No. of nucleotides[a]	No. of amino acids	Predicted M.W. (kD)	Percentage homology[e]	Approx. no. of molecules per virion
	Ind (NJ)	Ind (NJ)	Ind (NJ)		
N	1326 (1329)	422 (422)	47.4 (47.9)	68	1258
P	814 (856)	265 (274)	29.9 (31.4)[c]	32	466
M	831 (758)	229 (229)	26.1 (26.2)	62	1826
G	1665 (1573)	511 (517)[b]	57.4 (58.2)[d]	51	1205
L	6373 (6391)	2109 (2109)	240.7(241.6)	65	50

[a] Without a poly (A) tail
[b] Precursor molecule
[c] Nonphosphorylated
[d] Nonglycosylated
[e] Homology between the amino acid sequence of VSV_{IND} and VSV_{NJ}

that is responsible for both mRNA transcription and genomic RNA replication (Baltimore *et al.* 1970). There are five cistrons in the genomic RNA and the RNA polymerase transcribes five monocistronic messenger RNAs. Each of the messenger RNAs codes for a protein found in the virion. The virion contains five proteins: nucleoprotein (N), phosphoprotein (P, formerly known as NS), matrix protein (M), glycoprotein (G), and large polymerase protein (L) (Kang and Prevec 1969, Wagner *et al.* 1969a, b). Three proteins (N, P, and L) are found to be associated with the virion RNA to form the RNP complex, which is the infectious component of VSV. The major nucleocapsid protein, N protein, is exceedingly insoluble and is very tightly complexed with viral RNA. The RNA is protected from the action of ribonuclease by this protein. The P protein is phosphorylated to varying degrees (Clinton *et al.* 1978, Kingsford and Emerson 1980), the most highly phosphorylated forms of which apparently have the greatest potential for supporting transcription (Banerjee and Barik 1992, Sleat and Banerjee 1993). Recently, cellular casein kinase II and VSV L-protein-associated kinase have been identified as kinases involved in the phosphorylation of the P protein (Beckes and Perrault 1991, Banerjee and Barik 1992, Das *et al.* 1995, Gao and Lenard 1995). The original name, NS, mistakenly indicating nonstructural, is changed now to P protein because of its highly phosphorylated state. The large catalytic polymerase protein is designated L and the L gene represents 60% of the coding potential of the entire genome (Schubert *et al.* 1984). L protein is very labile to heat and sheer forces and must be handled very gently. Both the L and P proteins, as well as the N protein-RNA complex, are essential for transcriptase activity (Emerson and

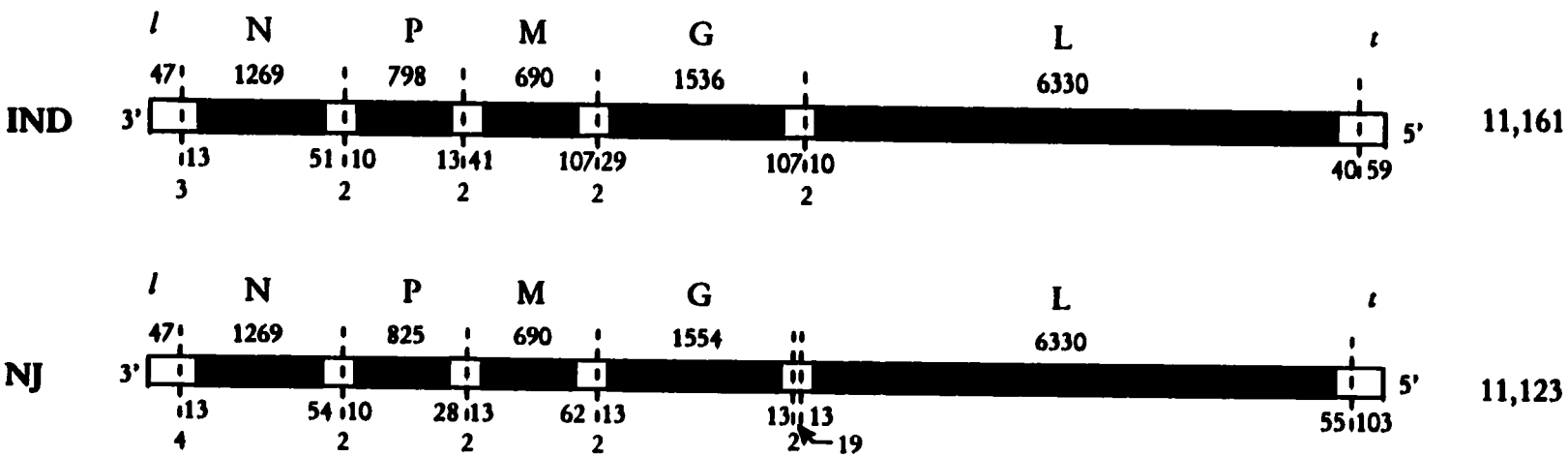
Yu 1975). The VSV membrane contains two proteins: an externally oriented, integral glycoprotein (G) and a internal matrix (M) protein that lies on the inner surface of the virion membrane (Patzer *et al.* 1979, Zakowski and Wagner 1980). The M protein lies within the membrane envelope, perhaps interacting both with the membrane and the nucleocapsid core. A single glycoprotein (G) species spans the membrane and forms the spikes on the surface of the virion. The G protein is the only one of the five virus proteins which contains carbohydrate and is the major antigenic determinant responsible for type specificity and gives rise to neutralizing antibody (Kelley *et al.* 1972, Volk *et al.* 1982).

Structure and organization of the genome

The VSV_{IND} genome consists of a single piece of RNA, 11,161 nucleotides long (Schubert *et al.* 1984) that serves as the template for the synthesis of six separate and nonoverlapping RNAs during transcription (Fig. 2). VSV transcribes an untranslated leader RNA and five capped and polyadenylated mRNAs by a virion-associated RNA-dependent RNA polymerase (Banerjee *et al.* 1985). Early studies demonstrated that the mRNAs were not transcribed in equimolar amounts (Villarreal *et al.* 1976). This unequal transcription was later found to correlate with gene order. Abraham and Banerjee (1976) and Ball and White (1976) reported that the genes are transcribed sequentially, in a polar manner, in the order 3'-N-P-M-G-L-5'. Therefore, attenuation of transcription at each gene junction results in a decrease in the amounts of message synthesized, depending on its distance from the 3'-terminal start site of

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, indicating the first seven nucleotides of the poly (A) site on each mRNA that is encoded by the genome. 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 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. (B) The % nucleotide similarity for each gene between VSV_{IND} and VSV_{NJ} is indicated. (C) The highly conserved intergenic, gene-start and gene-end sequences are shown. Each VSV gene starts with a 3'-terminal gene-start site which contains five conserved nucleotides (UUGUC) and terminates with a conserved poly (A) site containing seven uridine residues which are repeatedly transcribed into poly (A) tails approximately 200 nucleotides long. The poly (A) site is followed by the intergenic region which consists of the conserved sequence, GA, in all but the P-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 gene-start site (Luk *et al.* 1987).

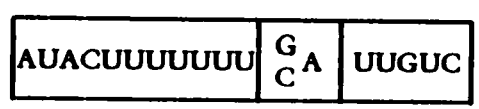
A



B

	% Nucleotide similarity
N	68 %
P	41 %
M	57 %
G	54 %
L	62 %

C



transcription (Iverson and Rose 1981). The N mRNA is transcribed most frequently and the L mRNA least. This is consistent with the idea that the polymerase is needed in catalytic amounts, while the N protein is needed in high amounts for the encapsidation of the genomic minus- and plus-sense RNAs. Therefore, the organization of the genome together with the sequential and attenuated mode of transcription ensures that each gene product is synthesized in the ratios needed during the course of the infection, a characteristic of most, if not all, nonsegmented negative-stranded RNA viruses. During transcription, the polymerase copies 99.4% of the standard genome into complementary RNA. The intergenic regions (3 or 4 bases between the leader N-gene junction and only 2 bases between each two of the other genes) and 59 bases at the 5' terminus are not normally transcribed. Thus, the genome of VSV represents a single, highly efficient transcriptional unit.

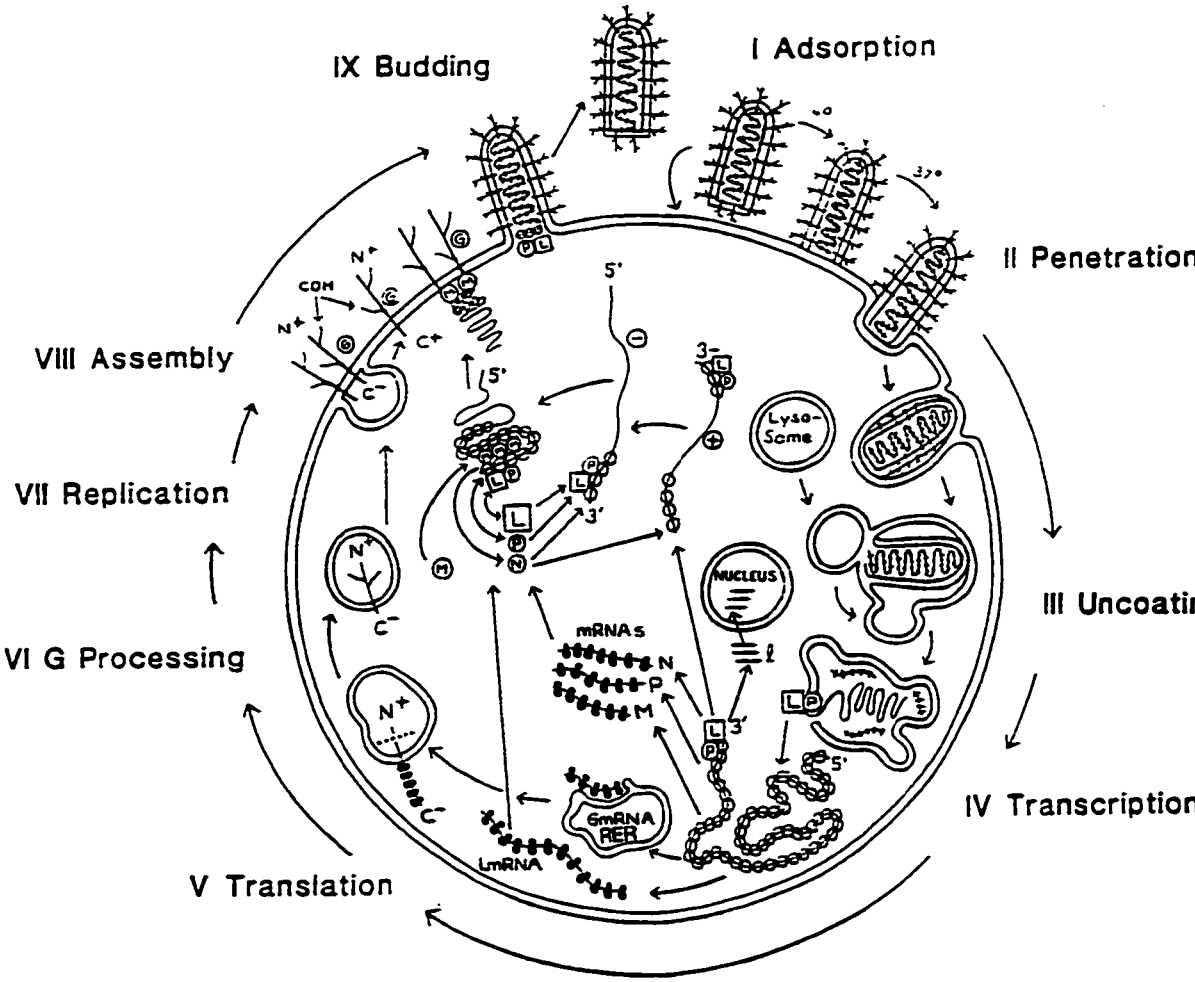
Cycle of infection

The infectious cycle of VSV begins when an infectious virion recognizes a virus receptor on the host cell surface. This is followed by a series of events that terminate in release of progeny virions and death of the cell. Fig. 3 depicts the infection cycle of VSV. The cycle proceeds with adsorption, penetration, uncoating, transcription, translation, replication, assembly, and budding.

(a) Adsorption

The virus attaches to a receptor on the host cell surface by means of a specific viral surface component. VSV adsorption can occur efficiently at 4°C (Wagner *et al.* 1963,

Figure 3. The series 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 that 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). This figure was taken from Wagner (1987).


IX Budding
I Adsorption
II Penetration
III Uncoati
IV Transcription
V Translation
VI G Processing
VII Replication
VIII Assembly
Lyso-
Some
NUCLEUS
mRNAs
N
P
M
GmRNA
RER
LmRNA
COH
5'
3'

Schlegel and Wade 1983) but VSV binding is a pH-dependent event (Matlin *et al.* 1982). 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 of cells comes from the experiments on fusion from within, which occurs when a VSV-infected cell fuses with an uninfected cell by virtue of the G protein inserted into the plasma membrane. Although the host cell receptor has not yet been conclusively identified, VSV does possess high affinity for phosphatidylserine (Schlegel *et al.* 1983).

(b) Penetration and uncoating

Entry of the virus into the cell (penetration) is an energy-dependent event because it requires a physiological temperature. For infection to proceed following penetration, the viral membrane must be removed (uncoating) to permit viral transcription. Since endocytosis appears to be the predominant mode of VSV entry into the cytoplasm, the cytoplasm appears to be the logical site for uncoating. This event requires fusion of viral membrane with the membranes of endocytic vesicles. The intact virus apparently enters the cell by means of a coated pit (Simpson *et al.* 1969), processing to a coated vesicle and then to larger uncoated collecting vacuoles and finally to secondary lysosomes. Fusion of the VSV membrane with the vacuole membrane requires a low pH that can be provided only by lysosomes.

(c) Transcription

The VSV genomic RNA complexed with the N protein serves as the template for transcription. Incoming viral proteins without new protein synthesis can carry out transcription. Only three viral proteins-nucleocapsid (N), large polymerase (L), and

phosphoprotein (P)-are absolutely required for mRNA synthesis, while glycoprotein(G) and matrix (M) do not participate. Although many details of the mechanism of VSV transcription are not yet understood, most of the data point to a single polymerase entry site located at the extreme 3' end of the VSV genome (Emerson 1982). The polymerase initiates at the 3' end of the genome and either makes leader RNA followed by the five mRNAs or makes a full-length, encapsidated, positive-sense copy of the genome. Colono and Banerjee (1977) described a 47-nucleotide leader RNA sequence that, unlike the five mRNAs, is not capped, polyadenylated, or translated, but initiates the transcription process and is made in larger molar amounts than any mRNA. mRNAs of all five VSV genes are capped and polyadenylated and it has been suggested that L protein carries out these processes (Abraham and Banerjee 1976).

(d) Translation

VSV mRNAs are translated immediately after transcription and in fact translation may be coupled with transcription. All five VSV proteins are synthesized throughout the cycle of infection, but the amount of each protein synthesized correlates roughly with the linear sequence of genome cistrons: N, P, M, G, and L proteins (Mudd and Summers 1970, Wagner *et al.* 1970, Hsu *et al.* 1979). The G protein is synthesized from endoplasmic reticulum membrane-associated mRNA by means of a signal sequence, stepwise glycosylation, and migration of vesicles to the cytoplasmic membrane for fusion and insertion of the fully glycosylated G protein (Knipe *et al.* 1977, Rothman *et al.* 1980). The four other VSV proteins are synthesized by cytoplasmic polyribosomes associated with monocistronic mRNAs (Morrison and

Lodish 1975).

(e) Replication

The negative-stranded genomic RNA complexed with N protein serves as template for replication as well. In contrast to transcription, replication, the production of full-length copies of the viral RNA, requires newly synthesized viral proteins (Patton *et al.* 1984). A full-length, all-inclusive positive-stranded copy of the genome, the first step in replication, is produced from the infecting nucleocapsid templates consisting of RNA and N, P, and L proteins only when viral protein synthesis occurs. Unlike mRNAs, the full-length positive strand is neither capped nor polyadelylated. Furthermore, both the full-length positive-strand and the negative-strand genomic RNA are found only in the form of N protein-coated nucleocapsid structures, never as naked RNAs. In this form, they are resistant to ribonucleases, whereas the unencapsidated mRNAs are not (Soria *et al.* 1974). The viral N protein is thought to modulate transcription and replication by its ability to bind to nascent leader RNAs, thus promoting read-through of the termination signals as the full-length RNA is associated into nucleocapsids (Schubert *et al.* 1982). It is becoming increasingly evident that the VSV polymerase serves both messenger RNA transcription and genome replication. The apparent site of entry of the polymerases is the 3' terminus of the VSV genome (Emerson 1982), which codes for the plus-strand leader RNA. Blumberg *et al.* (1981,1983) have proposed that the 5' terminus of the leader RNA serves as the nucleation site for binding of newly synthesized N protein to form the RNP complex, which switches from transcription to replication.

(f) Assembly and budding

The newly synthesized progeny RNA complexed with newly synthesized N, P, and L proteins forms RNP complexes (Rubio *et al.* 1980). Independently synthesized M protein binds to these progeny RNP complex which then migrate to regions of the cytoplasmic membrane that contain the newly inserted but randomly distributed 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 phospholipid. The tightly coiled nucleocapsid is apparently next enveloped in the G protein-converted cytoplasmic membrane, leading to budding and release of fully formed and infectious VSV particles.

2. DEFECTIVE INTERFERING PARTICLES (DIPs)

Biological characteristics of DIPs

Defective interfering particles (DIPs) were first clearly defined by Huang and Baltimore (1970) when they recognized the ubiquity and the similarities of homologous interfering particles among divergent groups of RNA and DNA viruses. To encompass all these interfering viruses, they proposed the general term 'defective interfering particles'. DIPs exhibit the same shape and symmetrical characteristics as parental virus. Some are smaller than parental virus particles because the deleted genome RNA assembles into a shorter nucleocapsid (which determines virus length as it becomes enveloped at the cell surface). They contain virus structural proteins and antigens.

DIPs require homologous parental virus for replication and replicate preferentially

at the expense of helper virus, thereby causing interference. The following characteristics are observed in cells doubly infected with helper virus plus DIPs: (1) primary transcription (from input virus templates) proceeds normally. (2) virion-size RNA replication is strongly inhibited, and, therefore, secondary transcription (from newly replicated viral templates) is also strongly suppressed. (3) The reduced amounts of viral replication, encapsidation, and maturation proteins that result from these reduced levels of viral transcription and translation become devoted largely to replication of DIP RNA genomes and to their maturation into DIPs (Huang and Manders 1972, Perrault and Holland 1972, Khan and Lazzarini 1977). The studies cited above led to the conclusion that this majority class of DIPs are replicative, transcriptionally inert particles that exert their interfering effects by outcompeting standard virus at the level of genome replication.

Wagner *et al.* (1963) first demonstrated that VSV could establish persistent infections in cell culture. DIP effects can lead to two biologically important results: (a) persistent infection of cells that otherwise would have been destroyed by viral cytopathology and (b) complete recovery of cells from virus infection that would have been lethal in the absence of DIPs.

Palma and Huang (1974) demonstrated regular cyclical patterns of VSV_{IND} yields that were inversely correlated with homologous DIP yields when uninfected cells were added regularly to infected cultures. After replication of standard virus to high levels, DIPs soon replicated to high levels (using standard virus as helper). At the maximum level of DIP yields, standard virus replication regularly became depressed until

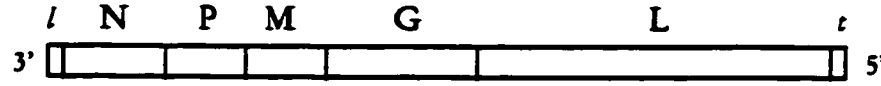
relatively few helper viruses remained to support DIPs replication. Then when DIP levels dropped, virus yields once again cycled up to maximum and the cycle was repeated. Viral and DIP nucleocapsids were undergoing regular cyclical interactions intracellularly and DIP nucleocapsids were cyclically regulating viral synthesis in these persistently infected cells even at times when little or no infectious mature virus was being shed.

Genome structure of DIPs

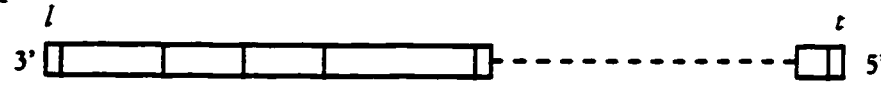
DIPs of VSV and other rhabdoviruses that accumulate during multiplication at high multiplicities of infection (undiluted passage) contain only a portion of the viral genome and are formally equivalent to deletion mutants. Characteristically, these incomplete genomes (in DIPs) interfere with the replication of the complete genome (standard virus). Nevertheless, they are themselves dependent on the polymerase of standard virus for their own multiplication, because DIPs invariably have lost a portion of the L gene and so can not encode a functional polymerase. Since the complete sequence of the L gene (Schubert *et al.* 1984) and partial sequences of several DIPs have been determined, it is possible to map precisely the extent of the deletions in DIP genomes and to characterize the sites of recombination of the deleted molecules (Meier *et al.* 1984). Four types of DIP genomes can be distinguished, these being designated: (a) simple deletion, (b) compound, (c) panhandle, and (d) snapback (Fig. 4) (Kang 1980, Lazzarini *et al.* 1981, Perrault 1981). All four types of genomes have structural features in common; namely, variable portions of the L gene are deleted, the 5'-terminal regions

Figure 4. Structures of representative DIP RNAs. The negative-strand sequences are represented by the open boxes. The black boxes indicate the complementary (positive-strand) sequences. *l* and *t* represent the 3' leader region and 5' trailer region of the standard virus genome, respectively. *t'* is the complement of *t*. The dash line indicates the deleted sequences.

VSV genome

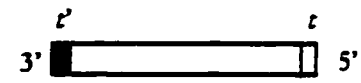


Simple deletion DI

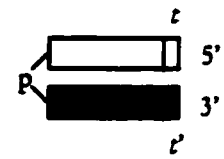


Compound DI

Panhandle DI



Snapback DI



of the deleted genomes are identical in sense and sequence to the 5' terminus of the minus strand of the complete genome, and the 3'-terminal regions are identical to the 5' terminus of either the minus or the plus strand of the complete genome.

(a) 5'-end-derived panhandle or copyback class

This is the simplest structural class of VSV DIP genomes, and they are the most abundant and competitive class (along with the snapback type). As can be seen in Fig. 4, they consist of the 5' end of the standard virus genome and varying portions of the adjacent large (L)-protein gene. The termini of VSV_{IND} and of many VSV_{IND} "panhandle-" or "copyback-" type DIP genomes have been partially sequenced (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'Hara *et al.* 1984, Meier *et al.* 1984). All DIPs of VSV examined so far contain the genomic RNA 5' end (in the negative-strand sense), but this class of DIPs has lost the genomic 3' sequences and replaced them with a complementary copy of the genomic 5' end (i.e., a 3' end identical in sequence to the 3' terminus of the viral antigenomic plus strand). The "panhandle" or "copyback" self-complementary region of these DIP genomes varies from 45 base pairs to about 150 base pairs in length. DIP genomes of this class are therefore mainly negative-sense portions of the L gene and the 5' terminus, plus a small positive-sense (5'-complementary) segment comprising the 3'-stem terminus.

(b) Snapback or hairpin class

As can be seen in Fig. 4, and as the name implies, this type of DIP genome is self-complementary over its entire length. Sequence analysis of this type of DIP genome (Johnson and Lazzarini 1977, Keene *et al.* 1977, 1979, Schubert *et al.* 1979) shows that half of each genome is homologous to the 5' end of the genomic sense of the virus and a portion of the adjacent L gene. The other half is the 3' complement of the 5' half. Schubert and Lazzarini (1981) sequenced the joining region that links the 5' half of the genome of one snapback DIP, DI-011, to its 3' complement and found that this genome is a perfect duplex joined by a single phosphate.

(c) Simple internal-deletion class

The simple internal-deletion type of DIP genome is the least complex DIP genome rearrangement. In the prototype HR-LT (Fig. 4) (Petric and Prevec 1970), the internal deletion excised most of the L-protein gene, leaving both viral termini and the other four genes intact (Perrault and Semler 1979, Epstein *et al.* 1980, Yang and Lazzarini 1983). This is an extremely rare type of VSV DIP genome. It was originally isolated in Prevec's laboratory and found to be unique because it mapped to the 3' half of the genome in annealing studies (Reichmann and Schnitzlein 1979, 1980). It is also unique in being able to transcribe the four nondeleted (N, P, M, G) cistrons (Chow *et al.* 1977, Colonno *et al.* 1977, Johnson and Lazzarini 1977, Johnson *et al.* 1979). Presumably due to its transcribing activity, this DIP has the unique ability to kill cells when infecting alone (Marcus *et al.* 1977) and to interfere heterotypically with the NJ Concan subgroup of VSV, which belongs to the same subgroup as NJ Ogden (but not with the

NJ Hazelhurst subgroup) (Prevec and Kang 1970, Schnitzlein and Reichmann 1976, 1977, Adachi and Lazzarini 1978, Reichmann *et al.* 1978, Khan and Lazzarini 1977).

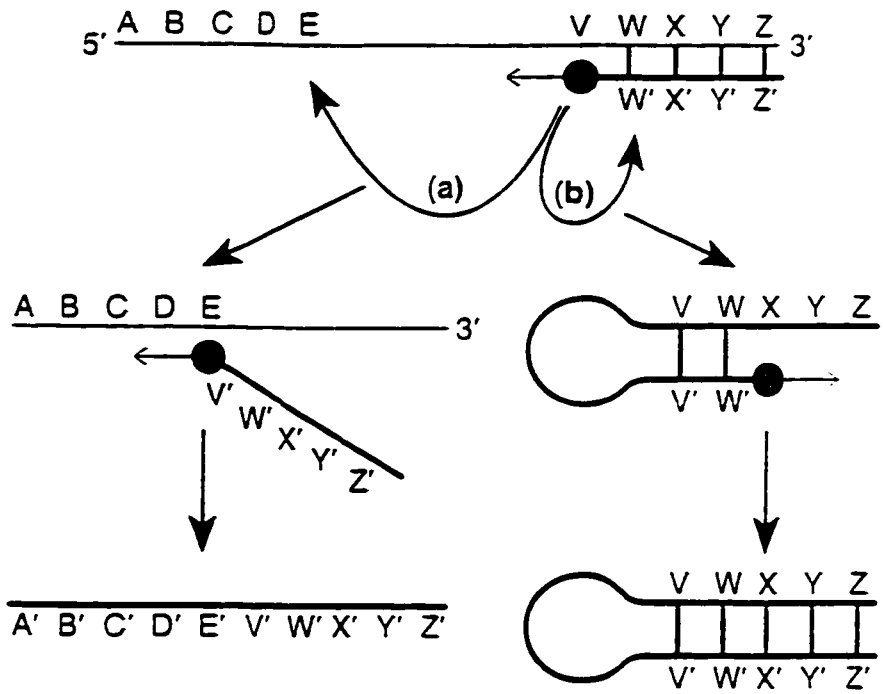
(d) Compound class

This last class of DIP genome structure includes many bizarre, extensively rearranged DIP RNAs (Fig. 4). It differs from the transcribing HR-LT by only containing an added stem sequence (Perrault and Semler 1979, Epstein *et al.* 1980) and this stem addition apparently completely silences the transcription-initiation activity of the viral 3' end. The standard virus genomic 3' end is internal to this added stem by only 72 nucleotides from the DIP 3' end (Keene *et al.* 1981), but this internalization apparently inactivates transcriptional activity. Furthermore, this stem addition renders this DIP genome much more competitive than its transcriptionally active counterpart, HR-LT, which lacks the 3' stem (Perrault and Semler 1979).

Mechanisms of DIPs generation

DIP RNA genomes are most likely generated by "polymerase copy-choice" mechanisms (Leppert *et al.* 1977, Kang 1980, Lazzarini *et al.* 1981, Perrault 1981, Holland 1990, Roux *et al.* 1991). This model postulates that DIP genomes are formed when the viral replicase complex, carrying the nascent viral genome, falls off its template and reattaches at another site. If it rejoins a viral antigenome further downstream (Fig. 5a), the result is a virus genome with an internal deletion. Complex DIP RNA genomes would be generated by multiple polymerase jumps. If the nascent strand folds back on its own 5' end by using intrastrand or interstrand "jumping

Figure 5. "Polymerase copy-choice model" of DIP generation. The polymerase (black circle) detaches from the template strand (thin line), and either (a) rejoins a similar template further downstream, forming a deletion mutant, or (b) folds back on itself, forming a copy-back genome with complementarity between its 5' and 3' ends. The thick line represents the defective genome. [ABCDE] and [VWXYZ] represent the 5' and 3' region of the standard virus genome, respectively. [A'B'C'D'E'] and [V'W'X'Y'Z'] are the complement of [ABCDE] and [VWXYZ], respectively. This figure was taken from Bangham and Kirkwood (1993).



Simple deletion DIP **Panhandle DIP**

Complex type DIP **Snapback DIP**

polymerase" (Fig. 5b), the result is a 'panhandle' or 'snapback' particle. All VSV DIP sequence data published to date support the above model (Yang and Lazzarini 1983, Deand Perrault 1982, Keene *et al.* 1981, O'Hara *et al.* 1984, Nichol *et al.* 1984, Meier *et al.* 1984). The sequence data show that in general there seems to be no sequence specificity either at the sites of polymerase premature termination or at the sites of resumption of synthesis.

Mechanisms of homologous viral interference

The mechanism(s) of DIP-mediated viral interference is not known for rhabdoviruses or for any other RNA virus. However, several models for interference mechanisms have been proposed. Nontranscribing as well as transcribing DIPs will be treated separately here because the interference mechanisms may be quite different.

(a) Nontranscribing DIPs

The early studies of Huang and Manders (1972) and Perrault and Holland (1972) demonstrated that most DIP interference - i.e., by 5' nontranscribing DIPs (panhandle DIP and snapback DIPs) (see Fig. 4) - probably occurs at the level of replication of viral and DIP genomes. These DIPs are unable to transcribe themselves; they do not inhibit primary transcription by the helper virus; they rapidly and strongly suppress helper virus genome replication, while DIP genomes replicate efficiently; and finally, they strongly suppress secondary transcription- an inevitable outcome of the reduction of synthesis of new viral templates. These findings were extended by Palma and Huang (1974) and Stamminger and Lazzarini (1977). Since the reduction of secondary

transcription leads to a relative shortage of viral polymerase and encapsidation proteins, and since the reduced numbered of helper virus templates are devoted mainly to transcription, while DIP nucleocapsids are devoted only to replication, then nearly all the limited viral replication-encapsidation proteins should be shunted to DIP replication at the expense of standard virus replication.

The predominance of panhandle- or copyback-type DIPs and snapback DIPs suggests that the 3'-stem sequence favours replication of both types of DIP RNAs, just as it favours viral genomic RNA replication over complementary RNA replication. Since the natural role of the 5' terminal sequence is to act exclusively as an origin of replication during synthesis of full-length negative strand genomic RNA, its presence at each end of the DIP RNA has been proposed to account, at least in part, for the replicative advantage of this class of DIP and hence for its ability to interfere with the replication of standard virus (Holland 1987). Recently, Wertz *et al.* (1994) analyzed directly both RNA transcription and replication from VSV RNA analogues recovered from cDNA clones. The results showed that the RNA with authentic 5' and 3' ends directed abundant transcription but low replication. In contrast, RNAs with complementary termini derived from either end of the genome replicated well but transcribed poorly or not at all. The results also showed that engineered RNAs containing longer regions of uninterrupted terminal complementarity had a clear replicative advantage over those with shorter regions. Thus, these results indicated that DIP RNAs with complementary termini have the greatest competitive replication advantage.

(b) Transcribing DIP

Transcribing DIPs (simple deletion DIP in Fig. 4) clearly interfere in a different manner than the majority of VSV DIPs. They interfere with other serotypes (Prevec and Kang 1970, Schnitzlein and Reichmann 1976). Bay and Reichmann (1981, 1982) showed that their transcribing ability is required for interference and they interfere with primary transcription of both homotypic and heterotypic helper virus, a phenomenon that does not occur with nontranscribing DIPs. Using chimeric DIPs (HR-LT genomes with thermolabile polymerase), Bay and Reichmann (1981, 1982) observed that at nonpermissive temperature, these unique DIPs could not self-transcribe, and concomitantly they lost the ability to inhibit virus primary transcription and to interfere heterotypically (but they still were replicated). They suggested a model for interference proposing that there are more frequent dissociation events during transcription of the virus nucleocapsids due to their longer lengths. This mechanism would also make these transcribing DIPs unique, since smaller DIP genome size is not a factor in the interference exerted by nontranscribing DIPs. It appears that there are probably at least as many different mechanisms for DIP interference as there are different replication strategies among RNA viruses, but the ability to generate DIP genomes by replicative error is ubiquitous, and each virus eventually selects and amplifies the more competitive of the subgenomic structures generated.

3. REVERSE GENETICS OF NEGATIVE STRANDED RNA VIRUSES

Background

With the development of recombinant DNA technology during the last several years, successful recovery of infectious RNA viruses and functional RNA replicons from cDNA has greatly facilitated molecular genetic analyses of viral proteins and *cis* regulatory elements. This technology allows the use of RNA virus replication machinery to express heterologous sequences.

Until recently, this technology was applicable only to positive-stranded RNA viruses. Specific mutagenesis of cDNA clones has provided important insights into the molecular biology of these viruses. In addition, positive-stranded RNA viral vectors carrying foreign epitopes or expressing heterologous polypeptides have been constructed using polioviruses (Almond and Burke 1990, Percy *et al.* 1992), Sindbis virus (Hahn *et al.* 1992, Levis *et al.* 1987, London *et al.* 1992), Semliki Forest virus (Liljeström and Garoff 1991), or plant viruses (French *et al.* 1986, Takamatsu *et al.* 1987). RNA transcription *in vitro* of linearized full-length cDNA copies of the genomes of these viruses yields viral RNA molecules that can be transfected into appropriate host cells and which initiate productive virus infections. This is possible because the genomic RNA of positive-stranded RNA viruses is used directly as mRNA for translation of the viral proteins responsible for the amplification of input RNA as well as for the packaging of the RNA into infectious virus particles.

In contrast to positive-stranded RNA viruses, the study of negative-stranded RNA viruses has been limited by the ability to perform direct genetic manipulation using

recombinant DNA technology because neither the full-length genomic RNA nor antigenomic RNAs are infectious. The genomic RNA must be encapsidated by the nucleocapsid protein (N) before it can serve as a template for either transcription or replication by the viral polymerase (Garcia-Sastre and Palese 1993). Only the encapsidated RNAs function as templates for replication and transcription of mRNAs (Emerson and Wagner 1972). In contrast to deproteinized genomic or antigenomic RNAs, the RNP complex is infectious after introduction into cells.

Negative-stranded RNA viruses are a large and diverse group of enveloped viruses of both medical and economic significance. There are six families of RNA viruses in which the negative strand is sequestered in the extracellular virion. Viruses of three of these families, the *Rhabdoviridae*, the *Paramyxoviridae*, and the *Filoviridae*, have unitary linear (non-segmented) genomes, whereas viruses of the other three families, *Arenaviridae*, *Bunyaviridae*, and *Orthomyxoviridae*, have multipartite (segmented) genomes comprising, respectively, two, three, and seven or eight subunits. These viruses contain many important human and animal pathogens: parainfluenza virus, mumps virus, measles virus, respiratory syncytial virus (RSV) (*Paramyxoviridae*); vesicular stomatitis virus (VSV), rabies virus (*Rhabdoviridae*); influenza virus types A, B, and C (*Orthomyxoviridae*); lymphocytic choriomenigitits virus (LCMV) (*Arenaviridae*); and several encephalitis and hemorrhagic fever viruses (*Arenaviridae*, *Bunyaviridae*, and *Filoviridae*) (Murphy and Kingsbury 1990). Particular elements essential for their replication and gene expression have been retained throughout negative-stranded RNA virus evolution and illustrate that these viruses have originated

from a common ancestor (Tordo *et al.* 1992).

Reverse genetics was first successfully applied for influenza viruses that belong to the *Orthomyxoviridae* family. New techniques were developed that allowed the rescue of synthetic RNA molecules into influenza A viruses (Luytjes *et al.* 1989) and, subsequently, into other negative-stranded RNA viruses. These techniques are presently being used to study the molecular biology of these viruses. Questions concerning *cis*- and *trans*-acting elements that are involved in transcription and replication of negative-sense RNA viral genomes can be addressed with reverse genetic approaches. Here, the most recent developments on the genetic manipulation of negative-stranded RNA viruses are briefly summarized (Table 2).

Excellent progress has been made in development of reverse genetic systems for both the segmented and the nonsegmented negative strand RNA viruses. Reverse genetics methods have become available for the study of all negative-stranded RNA viruses and genetic engineering of their genomes has become almost as easy as for positive-stranded RNA viruses.

Segmented negative stranded RNA viruses

One approach has utilized synthetic RNA genomes containing a reporter gene in an antisense orientation flanked by the terminal genomic RNA sequences required for packaging and replication. This method was first successful for influenza virus (Luytjes *et al.* 1989). Transcripts that contained authentic terminal sequences from an influenza virus genome segment and an internal chloramphenicol acetyltransferase (CAT)

TABLE 2

Genetic manipulation of negative-stranded RNA viruses

	Viruses	Comments
Segmented viruses	Influenza virus	Transfection of reconstituted ribonucleoprotein and rescue of transfectant virus
	Bunyavirus	Transcription of transfected RNAs by cloned protein cDNAs
Non-segmented viruses	Vesicular stomatitis virus	Rescue of infectious virus from cloned cDNA
	Rabies virus	Rescue of infectious virus from cloned cDNA
	Sendai virus	Rescue of infectious virus from cloned cDNA
	Measles virus	Rescue of infectious virus from cloned cDNA
	Respiratory syncytial virus	Rescue of infectious virus from cloned cDNA
	Parainfluenza virus type 3	Rescue of synthetic analogs of genome RNA by helper virus

reporter gene were encapsidated *in vitro* by purified influenza virus nucleoprotein and the virus polymerase proteins (PA, PB1 and PB2). The construct was replicated, transcribed and translated following transfection of the reconstituted recombinant RNP into influenza virus-infected cells. The helper virus not only provided the proteins needed for further RNA synthesis but, due to the segmented nature of its genome, also allowed packaging of the synthetic genome segment into progeny virus and passage of CAT activity in tissue culture. Thus, reassortant ('transfectant") viruses that possessed specific alterations in different genome segments could be generated (Enami *et al.* 1990). This achievement has rendered influenza virus accessible to experimental investigations of virus protein- and virus-host interactions (Garcia-Sastre and Palese 1993). Castrucci *et al.* (1992) showed that cDNA-derived infectious influenza viruses with altered genetic and biological properties can be produced.

Important advances in influenza virus research have been made which provide insights into the protein requirements for replication, the sequences essential for signalling transcription and replication, and the signals involved in controlling mRNA abundance and polyadenylation. With influenza virus, it has been possible to recover reassortant viruses, termed "transfectant" viruses, that contain altered individual gene segments. The effect of these alterations on virus replication in cells and in animals can be studied. In particular, alterations of the neuraminidase gene have been exploited to examine the effects of altering the structure of this protein on infectivity and pathogenicity of virions. The reverse genetic system of influenza virus still requires live helper virus for providing the viral structural proteins. Thus, a major goal

remaining for the segmented RNA viruses is the development of a system whereby all gene functions can be supplied by cDNA-derived RNAs. This can be utilized for characterizing *trans*-acting factors and their interacting domains.

Recently, Dunn *et al.* (1995) studied bunyavirus transcription using a recombinant RNA template derived from plasmid, which comprises a negative-sense reporter gene (CAT) flanked by the exact 5' and 3' untranslated regions of the Bunyamwera virus S RNA segment. They investigated both the protein and RNA sequence requirements for bunyavirus transcription; only the bunyavirus L and N proteins (not NSs protein) were needed for transcription and 5 nucleotides at the 3'-terminus might be important for recognition of the template by the bunyavirus proteins. The application of reverse genetics for understanding arenavirus gene expression has not yet been reported.

Non-segmented negative stranded RNA viruses

Unlike the segmented negative stranded RNA viruses, the genome of nonsegmentd RNA viruses is transcribed into multiple mRNA species by the virion associated RNA-dependent RNA polymerase. The nucleocapsid also serves as the template for RNA replication, a process in which the RNA polymerase synthesizes full length positive-strand RNA ignoring the stop signals at the intergenic junctions. The positive-strand is incorporated into nucleocapsids, which in turn serve as the templates for the synthesis of progeny nucleocapsids containing negative-strand genomic RNA. Understanding the mechanism of the switch from transcription to replication in this class of viruses has been a major challenge.

Park and colleagues modified the RNP-transfection method developed for influenza virus for use with Sendai virus and first succeeded in demonstrating that a short, artificial RNA construct could be rescued inside a cell into a form that allowed recognition and amplification by the polymerase of a non-segmented virus (Park *et al.* 1991). The synthetic RNA, which was generated *in vitro* by T7 RNA polymerase transcription from a linearized plasmid in order to create precise ends, corresponded to a Sendai virus minigenome in which the entire coding region was replaced with the coding region of the CAT reporter gene. This model genome possessed the virus 3'-terminal sequence including the putative promoter for the polymerase and the signal(s) directing leader RNA transcription/release and initiation of mRNA transcription. The 5' end contained the transcription stop/polyadenylation signal derived from the 5'-terminal cistron (L gene) and encoded the antigenomic promoter for replication. In the form of an RNP, this construct thus represents a functional template for replication of RNPs and also, due to the presence of a leader/reinitiation signal, for transcription of positive-stranded leader RNA and a CAT mRNA. Park *et al.* (1991) demonstrated that after transfection of the *in vitro*-transcribed RNA construct into cells, CAT activity was recovered after subsequent infection of cells with Sendai virus. Control experiments confirmed that CAT expression was Sendai virus-specific. Furthermore, the artificial RNPs were packaged into infectious virus particles, as demonstrated by successful passage of CAT activity by transfer of cell-free supernatants. Thus, it was confirmed that all *cis*-acting sequences required for encapsidation, initiation of replication and transcription of this paramyxovirus reside in the terminal sequences

of the genome.

Rescue of RNAs from cDNA transfected cells by infectious helper virus has also been successful in other paramyxovirus systems such as respiratory syncytial virus (RSV; Collins *et al.* 1991), parainfluenza virus type 3 (PIV-3; Dimock and Collins 1993, De and Banerjee 1993) and measles virus (Sidhu *et al.* 1995). Assays of reporter gene activity expressed from negative-stranded minigenomes similar to the one described for Sendai virus again indicated that they were amplified, transcribed and incorporated into infectious particles. Thus, the investigation of *cis*-acting sequences has been possible for a variety of paramyxoviruses. The experiments revealed that the sequence corresponding to the 3' terminus of the viral genome has to be the end of the transcript, as replication cannot proceed from an internal site (Collins *et al.* 1991, De and Banerjee 1993). In contrast, the 5' end appeared to tolerate additional nucleotides (De and Banerjee 1993). In addition, it was shown that the nucleotides of the 3'-terminal promoter could be replaced by sequences complementary to the 5' end (i.e. the antigenome promoter; Collins *et al.* 1991). Not only promoter sequences can be analyzed by the reporter gene assay (Harty and Palese 1995), but also internal transcription signals using bicistronic model genomes that contain two different reporter genes (Kuo *et al.* 1996). A particular feature of the paramyxovirus polymerase activity, an RNA editing, was also found to be template-directed (Park and Krystal 1992).

Finally, a reverse genetics system has been developed for VSV as well. The replicative cycle of VSV is very similar to that of paramyxoviruses. An efficient system

completely devoid of infectious helper virus was used for VSV. Intracellularly transcribed synthetic RNA corresponding to the genome of a nonexpressing natural VSV copyback-type DIP was rescued by VSV proteins expressed from plasmids (Pattnaik *et al.* 1992). A transcription plasmid, pVSV-DI, was constructed that used the T7 promoter to direct the transcription of a full length cDNA copy of the DIP RNA of VSV. The DIP RNA was synthesized intracellularly by a using recombinant vaccinia virus T7 RNA polymerase expression system, and the precise 3' end of the DIP RNA was generated by autolytic cleavage mediated by a ribozyme placed immediately downstream of the viral cDNA. The three viral nucleocapsid associated proteins, N, P, and L, were synthesized intracellularly from recombinant plasmid DNAs containing the corresponding cDNAs under the control of the T7 promoter. When the DIP RNA was transcribed in the cytoplasm of cells that were simultaneously expressing the N, P, and L proteins, efficient encapsidation and replication of the DIP RNA was achieved. Furthermore, intracellular synthesis of DIP RNA in cells expressing all five VSV proteins from separately transfected cDNA clones resulted in the production of infectious DIPs. The major advantage of this system is that the replication of DIP RNA is supported entirely by cDNA clones and no homologous helper virus is required. Thus, the infectious progeny particles constitute a pure population, all members of which contain RNA that originated from the cDNA clone. The development of this system opens VSV to the analysis of *cis*-acting regulatory sequences as well as the *trans*-acting factors involved in the viral replication cycle.

Stillmann *et al.* (1995) also reported a system using VSV minigenomes where

minigenomic RNAs are expressed from cDNAs and contain either the G gene or both G and M genes flanked by the VSV leader and trailer regions such that all of the sequences thought to be required for VSV replication and the synthesis of viral mRNAs are present. These RNAs can be replicated, transcribed, and packaged into infectious particles when coexpressed with the other VSV proteins.

In a remarkable study, Schnell *et al.* (1994) generated a nonsegmented negative-stranded RNA virus from cloned cDNA for the first time. Schnell *et al.* succeeded in cloning the approximately 12,000 nucleotides of the rabies virus genome between a T7 RNA polymerase promoter and the hepatitis delta virus (HDV) ribozyme sequence. Transcription of the plasmid using T7 RNA polymerase resulted in a full-length RNA with precise 3' and 5' ends (although the 5' end contained three extra Gs derived from the T7 promoter). The plasmid DNA containing the T7 promoter was transfected into cells infected with a recombinant vaccinia virus expressing the T7 polymerase. Three other plasmids (again driven by the recombinant vaccinia virus) were also cotransfected into these cells. The additional plasmids expressed the rabies virus N, P, and L proteins, which had been shown previously (Conzelmann and Schnell 1994) to be sufficient to generate transcriptionally active rabies virus RNPs. The presence of the three viral proteins and of a full-length viral RNA copy led to the formation of cDNA-derived rabies virus in this recombinant-vaccinia-virus-driven system. An important feature of this approach was initiation of the infectious cycle by expressing the antigenomic RNA rather than the genomic RNA in cells expressing the viral N, P, and L proteins. The rationale for starting with the antigenomic RNA was that, unlike the

genomic RNA, it would not be able to hybridize to the N, P, L mRNAs supplied to generate the nucleocapsid and initiate replication. This strategy avoids a potentially deleterious antisense problem an which the mRNAs encoding the N, P, and L proteins would hybridize to the negative-strand genomic RNA.

Both Lawson *et al.* (1995) and Whelan *et al.* (1995) subsequently reported the rescue of the full-length positive strand of VSV and the generation of recombinant VSV from cDNA. Infectious virus has also been obtained from full-length cDNA clones of respiratory syncytial virus (Collins *et al.* 1995), Sendai virus (Garcin *et al.* 1995), and measles virus (Radecke *et al.* 1995); however, parainfluenza virus system has not yet been reported.

4. STATEMENT OF OBJECTIVES

Although VSV has been widely used as a model system to study viral interference mechanisms for many years, the study of VSV has been limited by the inability to perform direct manipulation of the virus by using recombinant DNA technology because the naked genomic RNA is not biologically active or infectious. However, reverse genetic systems for VSV have been developed to allow genetic manipulation of the VSV genome (Pattnaik *et al.* 1992). Therefore, the overall objective of this thesis was to gain a better understanding of the molecular mechanisms of viral interference mediated by DIP to analyze and modify genomes at the molecular level. Specific objectives were to:

1) isolate DIPs from three different strains of VSV(VSV_{IND}/$VSV_{NJ\text{-}Ogd}$/$VSV_{NJ\text{-}Haz}$),
2) characterize DIPs from each strain of VSV to examine their biological characteristics,
3) clone and sequence four different DIP genomes to determine their physical structure, and
3) establish reverse genetic techniques for VSV DIPs.

CHAPTER 2: MATERIALS AND METHODS

1. CELLS AND VIRUSES

Cell cultures

Baby hamster kidney cell clone 21 (BHK-21), obtained from the American Type Culture Collection (ATCC), was used for this study. BHK-21 cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) supplemented with 5–10% heat inactivated fetal bovine serum (HIFBS) (Gibco BRL), 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 2 mM L-glutamine. Infections were routinely carried out in the same medium.

Cells were maintained in 10-cm plastic tissue culture dishes and were serially subcultured by removing cells from dishes with 0.05% trypsin plus 0.53 mM EDTA (Gibco BRL), reseeding in fresh medium at 1/4 confluent saturation density and incubating them at 37°C in a humidified atmosphere of 5% CO_2 for 24 hrs prior to use.

Thymidine kinase-negative human 143 B cells (TK^-143) (Rhim *et al.* 1975), obtained from Dr. Kathryn Wright (University of Ottawa), were also grown as monolayers in DMEM with 5% HIFBS, 100 U/ml of penicillin-streptomycin, 2 mM L-glutamine, and 25 μg/ml of 5-bromodeoxyuridine (BrdU) for the preparation of vaccinia virus.

Preparation and purification of standard viruses

Three strains from two different serotypes of VSV were used in this study: the heat resistant strain of Indiana serotype (VSV_{IND-HR}) and the Ogden strain of New Jersey

serotype ($VSV_{NJ\text{-}Ogd}$) were originally obtained from Dr. L. Prevec, McMaster University; the Hazelhurst strain of New Jersey serotype ($VSV_{NJ\text{-}Haz}$) was originally obtained from Dr. Sue Emerson, National Institutes of Health. Each strain of VSV was purified by three sequential plaque isolations to be free of DIPs before preparing standard virus stocks. The plaque-purified virus was propagated in BHK-21 cells and then infectious virus was measured by plaque assay. To prepare standard virus stocks, BHK-21 cell monolayers were infected with a virus stock at a multiplicity of infection (MOI) of 0.1 plaque-forming unit (PFU) per cell. After viral adsorption for 60 min, the inoculum was removed and fresh medium was added. The infected cells were incubated at 37°C for a further 15–18 hrs, and culture fluids were harvested, clarified by low-speed centrifugation (2,000 rpm, 20 min) in a Sorvall® GLC-2B centrifuge to remove cell debris. Stocks of virus were stored in small volumes at −80°C. Standard virus stocks contained 2×10^9 PFU/ml, 1×10^9 PFU/ml, and 6×10^8 PFU/ml for $VSV_{IND\text{-}HR}$, $VSV_{NJ\text{-}Ogd}$, and $VSV_{NJ\text{-}Haz}$, respectively.

Preparation and purification of DIPs

Four DIPs were used in this study: two DIPs (IND-ST and IND-LT), derived from the Indiana serotype of VSV; two DIPs (Ogd-DI and Haz-DI), derived from the New Jersey serotype of VSV.

Two different DIP seed stocks (IND-ST and IND-LT) of the Indiana serotype of VSV were part of the original DIP stock (Prevec and Kang 1970). These seed stocks were amplified in BHK-21 cells for 15–18 hrs using $VSV_{IND\text{-}HR}$ as the helper.

For the production of new DIPs of the New Jersey serotype of VSV, the Ogden strain and the Hazelhurst strain of the NJ serotype were passed undiluted four times in monolayer cultures of BHK-21 cells as described below. Monolayers of BHK-21 cells (1×10^7 cells in 10-cm plate) were infected at a MOI of 50 of the standard virus. After 1 hr adsorption at 37°C, the inocula were removed and incubation was continued for 15–18 hrs. The culture fluids were harvested, clarified by low-speed centrifugation (2,000 rpm, 20 min), and the supernatant was used to infect a fresh monolayer of BHK-21 cells. Successive undiluted passages were carried out in this fashion. The second high multiplicity passage of virus was used as seed stock since the yield of DIPs was higher when using virus from this passage.

The purification procedure for DIPs from culture supernatant was carried out as described below. Monolayer cultures of BHK-21 cells were infected with appropriate seed stocks of DIPs and matching standard virus at a MOI of 10 per cell. After 15–18 hrs, culture fluids were harvested, and cell debris was removed by centrifugation at 2,000 rpm for 20 min. The supernatants were harvested, and virus particles were recovered by ultracentrifugation at 25,000 rpm for 90 min in a Beckman SW 28 rotor. The virus pellet was resuspended in TNE buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA) and carefully layered on a 5–30% (wt/vol) linear sucrose gradient in TNE buffer prepared in a Beckman SW 41 centrifuge tube. The samples were then centrifuged at 35,000 rpm for 35 min at 4°C. The DIP band was collected and stored in small aliquots at −80°C.

The concentration of DIPs was determined by the methods described by Bradford

(1976) using the Bio-Rad protein microassay kit (Bio-Rad). Bovine serum albumin was used as a standard. The concentrations of DIPs (approximately 50 μg/ml of IND-ST and Ogd-DI, 100 μg/ml of IND-LT and Haz-DI) were adjusted on the basis of the size of each DIP in order to obtain approximately the same number of DIPs in a given volume.

Preparation and purification of recombinant vaccinia virus

Recombinant vaccinia virus, vTF7-3, expressing T7 RNA polymerase (Fuerst *et al.* 1986, 1987, Rosenberg *et al.* 1987) was obtained from Dr. Kenneth Dimock (University of Ottawa). Propagation and purification of the recombinant vaccinia virus vTF7-3 was performed essentially as described by Mackett *et al.* (1985) except that the final step of sucrose gradient centrifugation was not performed. For the preparation of small stocks from a single plaque, recombinant vaccinia virus was selected by plaque assay on TK^-143 cell monolayers in the presence of BrdU (25 μg/ml). After plaque purifications, TK^-143 cells were infected with plaque-purified isolations and incubated in the presence of BrdU until CPE of infection was pronounced. Monolayer cells were scraped from the dish, resuspended in hypotonic solution (10 mM Tris-HCl, pH 9.0), subjected to three rounds of freeze-thawing, and sonicated in a water bath sonicator to release cell-associated virus. Cell debris was pelleted from the lysates by centrifugation at 3,500 rpm for 10 min. The supernatant fluid was titrated by plaque assay on TK^-143 cells.

To prepare large stocks from this small stock, TK^-143 cells were infected at a MOI

of 0.1 at 37°C for 90 min, changed with fresh medium, and incubated for 2 days. Recombinant vaccinia virus stocks contained approximately 1×10^9 PFU/ml.

2. BIOLOGICAL ASSAYS

Plaque assays

Plaque assays were used to determine the titers of all virus stocks (Barrett *et al.* 1989). BHK-21 cells were used for all assays. Infectious virus was titrated by counting plaques on monolayers of cells (35-mm dishes). Serial ten-fold dilutions of virus were prepared and 100 μl of the appropriate dilution was used to infect cells (35-mm dishes). Inocula were adsorbed at 37°C for 1 hr and then an overlay of DMEM containing 0.9% agar Noble (Difco Laboratories/BDH) was added and allowed to harden at room temperature. The total time of incubation was 20–24 hrs at 37°C. The cells were then fixed with ethanol-acetic acid fixer (95% ethanol and 5% acetic acid) for 1 hr at room temperature, the agarose was removed from the dish and plaques were counted without staining. Virus titers were obtained by averaging values from duplicate plates.

Interference assays

DIP-mediated interference activity was measured by a yield reduction assay (YRA) adapted from Barrett *et al.* (1984). Monolayers grown in 35-mm dishes (~80% confluent) were infected with standard virus at a MOI of 3, either alone or with different amounts of DIPs. After 1 hr incubation at 37°C with occasional rocking, the inoculum was removed and 1 ml of the fresh medium was added to each well. At 15–18 hr of

incubation each culture fluid was harvested, centrifuged at 10,000 rpm for 10 min at 4°C, and stored at −80°C until infectivity was determined by plaque assay. Assays were carried out in duplicate.

3. RNA ANALYSES

Labeling of RNA

For the preparation of ^{3}H-labeled VSV RNA, monolayers of BHK-21 cells in 35-mm dishes (~80% confluent) were maintained at 37°C in medium with 2 μg/ml of actinomycin D (Sigma Chemical Co.) for 1 hr prior to infection. Cells were infected with standard virus at a MOI of 3, either alone or with different concentrations of DIPs. For RNA analysis from transfection experiments, cells were infected with the standard virus at a MOI of 3. After a 30 min adsorption period, the inoculum was removed and cells were superinfected with the supernatant from the transfected cells.

After 1 hr of adsorption, fresh medium containing actinomycin D (2 μg/ml) was added. [^{3}H]uridine (20 μCi/ml; DuPont NEN) was added at 4 hr postinfection (p.i.) and the infected monolayers were harvested at 6 hr p.i. The intracellular RNA was isolated from the cells as described below.

Extraction of RNA

Intracellular RNA was extracted from the cells by the method of Pattnaik and Wertz (1990). Labeled cells in 35-mm plates were harvested in 1 ml of phosphate-buffered saline (PBS) containing 10 mM EDTA. After brief centrifugation, cell pellets

were resuspended in 0.4 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.4% Na-deoxycholate, 10 mM EDTA), vortexed well, and incubated on ice for 7 min. Nuclei were removed by centrifugation at 12,000 rpm for 1 min. The clarified supernatant (cytoplasmic extract) was adjusted to 0.1% SDS. RNA was then extracted with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated from the aqueous phase with 2 volumes of ice-cold ethanol in the presence of NaCl (final concentration of 0.1 M) at −20°C for more than 2 hrs. The precipitated RNA was recovered by centrifugation (12,000 rpm, 4°C, 15 min), rinsed with 75% ethanol, and then dissolved in the appropriate volume of diethylpyrocarbonate (DEPC)-treated water.

All solutions that came in contact with the disrupted virus had been treated with DEPC to inactivate traces of ribonuclease (Maniatis *et al.* 1982).

Gel electrophoresis of RNA

The RNA was heated to 65°C for 15 min in electrophoresis sample buffer (50% deionized formamide, 1×MOPS buffer [0.2 M MOPS, 0.05 M Na-acetate, 0.01 M EDTA], 6.5% formaldehyde, 10% glycerol, bromophenol blue), then resolved by electrophoresis on 1% agarose-formaldehyde gels in 1×MOPS/EDTA buffer as previously described (Ausubel 1994), and detected by fluorography (Laskey 1980). Briefly, gels were immersed in fixer (30% methanol and 10% acetic acid) for 60 min with gentle shaking. Gels were washed two times with methanol (30 min each time) and then with 3% PPO (2,5-Diphenyl-Oxazole) (Sigma Chemical Co.) in methanol for at least 3 hrs with gentle agitation. Gels were rehydrated in distilled water for 60 min

with gentle agitation and dried in a gel dryer (Bio-Rad Model 583) at 75°C for 50 min. The dried gels were exposed to X-ray film (Fuji) for the appropriate times.

4. PROTEIN ANALYSES

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resuspended in SDS-PAGE sample buffer (0.05 M Tris-HCl, pH 7.0, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and eluted by heating the sample at 100°C for 5 min. The samples were then analyzed on 10% polyacrylamide gels by using the Bio-Rad Mini-Protean II system (Bio-Rad) and the method of Laemmli (1970). Gels were prepared with a 10% separating gel (10% acrylamide, 0.13% bis, 0.1% SDS, 0.75 M Tris-HCl, pH 8.8) and a 5% stacking gel (5% acrylamide, 0.07% bis, 0.1% SDS, 63 mM Tris-HCl, pH 6.8) and were subjected to eletrophoresis in buffer (192 mM glycine, 25 mM Tris, 0.1% SDS) at 150 volts (constant amperage) until the bromophenol blue reached the bottom of the gel. For fluorography (Chamberlain 1979), gels were submerged in fixer (10% acetic acid, 40% methanol), impregnated with Amplify (Amersham), vacuum dried, and exposed to X-ray film (Fuji) at −80°C.

Analysis of viral proteins

BHK-21 cell monolayers in 10-cm plates (1×10^7 cells) were infected with standard virus at a MOI of 3, either alone or with DIPs (1:100 dilution). After 1 hr incubation at 37°C, the inoculum was removed and the fresh medium was added. The culture

fluids were harvested at 15–18 hr p.i., cleared by centrifugation at 2,500 rpm for 15 min, and then pelleted at 25,000 rpm for 90 min at 4°C in a Beckman SW 28 rotor. Viral pellets were lysed in SDS-PAGE sample buffer, boiled for 5 min, and analyzed by SDS-PAGE in a 10% polyacrylamide gel. Gels were fixed, stained with Coomassie blue and dried in a gel drier (Bio-Rad Model 583).

Preparation of antiserum specific for VSV

Antiserum was prepared against VSV_{IND-HR}, VSV_{NJ-Ogd}, and VSV_{NJ-Haz}. Each strain of VSV was grown in BHK-21 cells and purified by sucrose gradient centrifugation. The virus band in the sucrose gradient was collected and treated with 0.1% NP-40. Disrupted virus (25–30 μg/250 μl of each virus) was first emulsified in complete Freund's adjuvant and inoculated into rabbits (2 kg female New Zealand white rabbit). Rabbits were boosted with disrupted virus samples prepared in incomplete Freund's adjuvant at two week intervals. The antisera were collected 6 weeks after the initial immunization, and all the antisera were heat inactivated at 56°C for 30 min and stored at −80°C. The antisera were tested for the content of specific antibodies by Western blot analysis.

Other antibodies used in this study were a monospecific rabbit antibody raised against an amino-terminal peptide from the L protein, kindly provided by Dr. Manfred Schubert (National Institutes of Health) and a polyclonal antibody raised against purified P protein of VSV_{IND} generously provided by Dr. Amiya Banerjee (The Cleveland Clinical Foundation).

Western blots

Proteins were resolved by 10% SDS-PAGE, and transferred to PVDF membrane (Millipore) using the Bio-Rad Mini-Trans-blot Electrophoresis Cell at 100 volts for 40 min in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) (Burnette 1981). Antigens were detected using the Bio-Rad Immun-Blot assay kit according to the manufacturer's instructions. All washes and incubations were carried out with gentle agitation. Membranes were washed for 10 min in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) and then free protein binding sites on membranes were blocked by incubating for 30 min in TBS containing 3% gelatin. Following two 5 min washes in TBS containing 0.05% Tween 20 (TTBS), membranes were incubated for 1 hr in antibody buffer (TTBS plus 1% gelatin) containing antiserum. Unbound antibody was removed from membranes with two 5 min washes in TTBS. Membranes were incubated for 1 hr with a 1:3,000 dilution of goat anti-rabbit Ig G conjugated with alkaline phosphatase (Bio-Rad) in antibody buffer. Two 5 min washes in TTBS followed by a 5 min wash in TBS were performed. Membranes were developed in carbonate buffer (0.1 m $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8) containing 0.3 mg/ml of NBT (p-nitro blue tetrazolium chloride, Bio-Rad) and 0.15 mg/ml of BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt, Bio-Rad). The colour development was stopped by placing the membranes in distilled water.

Radiolabeling of VSV infected/transfected cells

VSV-infected cells were starved for 1 hr at 15 hr p.i. in methionine-free MEM (Flow Laboratories). Cells were then labeled at 37°C for 2 hrs with methionine-free medium containing L-[^{35}S]methionine (50 μCi/ml; DuPont/NEN). For radiolabeling of transfected cells, the cells were starved in methionine-free MEM for 1 hr at 12 hr posttransfection and then exposed to 50 μCi/ml of L-[^{35}S]methionine for 2 hrs.

Immunoprecipitations

At the end of labeling period, the cells were washed with PBS, scraped into PBS containing 10 mM EDTA and collected by centrifugation at 12,000 rpm for 15 sec. The cell pellet was resuspended in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 0.5% Triton X-100, 1 mM benzamidine HCl, 1 mM PMSF) and stored on ice for 15 min. Cytoplasmic extracts were obtained after pelleting the particulate material from the total cell extracts by centrifugation at 12,000 rpm for 5 min. 1–5 μl of antisera were added, and samples were rotated overnight at 4°C. The antigen-antibody complexes were recovered by incubating with 2.5 mg of protein A-Sepharose CL-4B (Phamacia) for 1 hr. After washing the immune complexes five times with RIPA buffer, immunoprecipitated proteins were analyzed by SDS-PAGE in 10% polyacrylamide gels and detected by fluorography as described above (Chamberlain 1979).

5. MOLECULAR CLONING TECHNIQUES

All procedures and reaction conditions for DNA manipulation and plasmid constructions were carried out according to standard methods (Maniatis *et al.* 1982). All DNA modifying enzymes were purchased from New England Biolabs, Boehringer Mannheim, or Phamacia and used according to conditions suggested by the manufacturers.

Isolation of plasmid DNA

A single bacterial colony was inoculated into TB medium (17 mM KH_2PO_4, 72 mM K_2HPO_4, 1.2% Bacto-Tryptone, 2.4% bacto-yeast extract and 0.4% glycerol) containing 100 μg/ml of ampicillin at 37°C in a shaker-incubator for 12–18 hrs. Small-scale preparations of plasmid DNA were done using the alkaline lysis method (Birnboim and Doly, 1979). Cells from 2 ml of culture medium was pelleted at 12,000 rpm for 30 sec in a microcentrifuge (IEC MicroMaxcentrifuge®) and resuspended in 200 μl of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 4 mg/ml of lysozyme). Following the addition of 300 μl of Solution II (0.2 N NaOH, 1% SDS), the cells were incubated on ice for 3–5 min after inverting the tube several times. 150 μl of 7.5 M ammonium acetate was added and placed on ice for 5 min. After centrifugation at 12,000 rpm for 5 min, 1 volume of isopropanol was added to the supernatant. The DNA was pelleted in a microcentrifuge for 5 min and dissolved in RNase A-treated H_2O at a concentration of 1 μg/ml.

Large-scale preparations of plasmid DNA were also prepared by the alkaline lysis

method (Birnboim and Doly, 1979) and purified by precipitation with 13% (wt/vol) Polyethylene glycol (PEG 8000; Sigma Chemical Co.) in 1.6 M NaCl. The quantity and quality of plasmid DNA was determined on a 1% agarose gel to ensure that the majority of the DNA was supercoiled.

Electrophoresis of DNA

DNA was separated in an agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.0 and 1 mM EDTA) by horizontal gel electrophoresis. Electrophoresis was usually performed in a Wide Mini-Sub™ gel apparatus (Bio-Rad). The gel-loading buffer contained 0.02% bromophenol blue and 5% glycerol in TAE buffer. The location of DNA within the gel was visualized under ultraviolet (UV) illumination by directly adding ethidium bromide (EtBr) (0.5 μg/ml) to the agarose gel. Gels were photographed using polaroid type 667 film (Polaroid).

Purification and recovery of DNA from gels

DNA was purified from agarose gels by using the Geneclean II kit according to the manufacturer's (BIO 101 Inc.) instructions. DNA bands were excised from agarose gels under UV illumination and added to three volumes of NaI stock solution. Following a 5 min incubation at 45°C–50°C to dissolve agarose, Glassmilk® suspension was added and mixtures were rotated for 10–15 min at room temperature. After centrifugation at 12,500 rpm for 5 sec, pellets (Glassmilk/DNA complex) were washed three times with NEW wash solution (NaCl/ethanol/water) and eluted in appropriate volumes of

water at 45°C–50°C for 3–5 min.

Ligation of DNA

Linear plasmid DNAs and DNA fragments for ligation were purified by using the Geneclean II kit as described above prior to ligation. All ligations were carried out at room temperature for 3–5 hrs in 10–20 μl reaction mixtures containing 2 units of T4 DNA ligase (Gibco BRL). An approximately five-fold excess of insert DNA was ligated to 0.05 μg linear plasmid DNA and used to transform *Escherichia coli* strain DH5α, purchased from New England Biolabs.

Preparation of competent *E.coli*

E.coli DH5α was streaked directly from a frozen stock onto the surface of SOB agar plates (2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl containing 250 mM KCl, 20 mM $MgSO_4$, 1.5% bacto-agar) and the plates were incubated at 37°C for 12–16 hrs. One or two well-isolated colonies were cultured in 100 ml of SOB media at 37°C for 2–3 hrs. For efficient transformation, the number of viable cells did not exceed 10^8 cells/ml. Cells were transferred to ice-cold 50 ml polypropylene tubes (Falcon 2070), cooled on ice for 30 min and recovered by centrifugation at 4,500 rpm for 15 min at 4°C. The cell pellets were resuspended in ice-cold 0.1 M $CaCl_2$ by gentle pipetting, recovered by centrifugation again, and resuspended in ice-cold 0.1 M $CaCl_2$ by gentle pipetting. These competent cells were used immediately or stored in small aliquots at −80°C until required. All steps in this procedure were carried out

aseptically.

Transformation of *E.coli*

Transformation of competent cells with plasmid was performed essentially as described by Hanahan (1983) with minor modifications. DNA (1–5 μl) was added to 200 μl of competent *E.coli* on ice for 30 min and heated to 42°C for 90 sec. Following a 1–2 min incubation on ice, 800 μl of SOC medium (SOB medium containing 20 mM glucose) were added and the mixture was agitated at 37°C for 45 min in a rotary shaker. The transformation mixture was spreaded on SOB agar plates containing 100 μg/ml of ampicillin and incubated at 37°C for 15–20 hrs. Ampicillin resistant colonies were picked from SOB plates and in some cases, ampicillin resistant colourless colonies were picked from SOB plates containing 100 mM IPTG (Gibco BRL) and 2% X-Gal (Gibco BRL).

Screening of recombinant bacterial clones

All transformants were screened using the method developed by Sekar (1987). Two ml of TB medium containing 100 μg/ml of ampicillin were incubated with a single colony and cells were grown at 37°C for 12–15 hrs with shaking. 10 μl of the culture medium were removed and mixed with 10 μl of 2×protoplasting buffer (60 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 40% sucrose, 100 μg/ml RNase A, 100 μg/ml lysozyme). A 0.8% agarose gel was prepared in Tris-Borate-SDS buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA and 0.05% SDS). Each of the wells in the gel

were preloaded with 3 μl of the lysis buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA and 2% SDS, 5% sucrose and 0.04% bromophenol blue). 7.5 μl of each sample in protoplasting buffer were then loaded into the wells on the top of the lysis buffer. Electrophoresis was carried out in Tris-Borate-SDS buffer initially at 30 volts for 15 min and then at 120 volts for 1–2 hrs. Gels were stained with EtBr (1 μg/ml in Tris-Borate-SDS buffer) and DNA was visualized with a UV light. The recombinant bacterial clones were selected by the size of the plasmids since the recombinant plasmids would be larger than the vector plasmid. The recombinant plasmid DNAs were prepared as described above, and digested with appropriate enzymes to analyze the size of the insert DNA and the orientation of the insert DNA.

Manual DNA sequencing

All sequence analysis was done by the dideoxynucleotide chain termination method (Sanger *et al.* 1977) using the Sequenase DNA sequencing kit (United States Biochemical Corporation). About 5 μg of plasmid DNA (pBKS clones and pTVDI clones) and 30 ng of primers (forward sequencing primer and reverse sequencing primer for pBKS clones; T7 sequencing primer and SP6 sequencing primer for pTVDI clones) were used for sequencing reactions. After sequencing reactions, samples were denatured at 80°C for 2 min, quickly cooled on ice, and loaded onto sequencing gels. ^{35}S-dATP labeled DNA strands were separated by electrophoresis on 6% polyacrylamide gels (5.7% acrylamide, 0.3% bis, 8 M urea in 1×TBE) using the IBI Model STS-45 Thermoplate Sequencing Apparatus. Electrophoresis of gels was performed at 1,500 volts for 2 –

3 hrs depending on the distance between the template and the primer site. Gels were then transferred to Whatman 3MM paper, dried in a Bio-Rad model 583 gel dryer at 80°C for 40 min, and exposed to X-ray film at −80°C for 1−4 days. Nucleotide sequence data were assembled into contiguous sequences and analyzed using IBI computer programs.

6. cDNA CLONING OF VSV GENES

Preparation of cytoplasmic extracts

Cytoplasmic extracts of BHK-21 cells were prepared essentially as described by Rose *et al.* (1977). Ten 10-cm dishes monolayer cultures of BHK-21 cells which had just reached confluence were washed twice in ice-cold PBS. Cells were trypsinized, collected by low-speed centrifugation at 2,500 rpm for 15 min at 4°C. Pellets were resuspended in 1.5 times the packed cell volumes of homogenizaton buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM magnesium acetate, 7 mM β-mercaptoethanol, 0.1% NP-40), and cells were allowed to swell on ice for 10 min. Cells were disrupted by 20 strokes in a tight-fitting Teflon-glass homogenizer on ice, and the cytoplasmic extracts were freed of nuclei by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was dialysed overnight against dialysis buffer (10 mM HEPES, pH 7.5, 90 mM KCl, 1.5 mM magnesium acetate, 7 mM β-mercaptoethanol) at 4°C, the dialysed material was centrifuged at 12,000 rpm for 10 min at 4°C to remove debris, and samples of the extract were stored at −80°C.

In vitro transcription of VSV mRNA

In vitro transcription was done by the method of Rose *et al.* (1977). The *in vitro* transcription reactions consisted of purified virions from the three different strains of VSV (VSV_{IND-HR}, VSV_{NJ-Ogd}, and VSV_{NJ-Haz}) at a concentration of 200 μg of protein/ml, 1 mM of each of the four rNTPs, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 4 mM DTT, 0.025% Triton X-100, and 1 mM S-adenosylmethionine. 10 μl of cytoplasmic cell extract prepared above (20 mg of protein/ml) containing 50 mM $MgCl_2$ were added to 90 μl of the reaction mixture and preincubated at 30°C for 5 min. mRNA synthesis *in vitro* was carried out at 30°C for 2 hrs and stopped by the addition of 1% SDS and 0.2 M Na-acetate (pH 5.2) at final concentrations. The RNA transcripts (*in vitro* synthesized, poly(A)-containing viral mRNA) were purified by phenol/chloroform/ isoamylalcohol extraction, precipitated with ethanol, and passed through an oligo(dT)-cellulose column. The purified mRNAs were resuspended in DEPC-treated H_2O.

For comparison with *in vitro* transcripts, the genomic RNA was isolated from three different strains of VSV. BHK-21 cells in 10-cm plates were infected with each standard virus (MOI=3). At 15–18 hr p.i., the culture fluids were harvested and virus was purified by centrifugation on 5–30% linear sucrose gradients made in TNE buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). The purified viruses were disrupted in the presence of 1% Nonidet P-40, 0.4% Na-deoxycholate, 10 mM EDTA, and 10 mM Tris-HCl (pH 7.5). Viral genomic RNA was isolated by phenol/chloroform/isoamylalcohol extraction and precipitated with ethanol.

Synthesis of VSV mRNA from cDNA

Synthesis of mRNA from cDNA was done using the SuperScript Choice System for cDNA synthesis (Gibco BRL) according to manufacturer's instructions. For primer annealing, 2.5 μg of mRNA and 250 ng of primer (CD3: 5'-GAGAGTCGACGCGGC CGCTTTTTTTTCATA-3') were incubated at 70°C for 10 min. The conserved poly(A) signal sequences of viral genes of VSV are in boldface, and the *SalI* and *NotI* sites at the 5' end of the oligonucleotide are underlined. Following the addition of reaction mixture containing first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 500 μM of each of the four dNTPs, and 200 units of reverse transcriptase (SuperScript II RT™), the first strand reaction was carried out at 42°C for 1 hr and stopped on ice. Globin mRNA (0.5 kb) was used for a control RNA. The second strand reaction consisted of second strand buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM β-NAD^-), 250 μM of each of the four dNTPs, 1.2 mM DTT, 65 units/ml DNA ligase, 250 units/ml DNA polymerase I, and 13 units/ml RNase H. [α-^{32}P]dCTP (1 μCi/μl) was added for labeling of second strand cDNA synthesis. After the mixture was incubated at 16°C for 2 hr, 10 units of T4 DNA polymerase were added and incubation was continued at 16°C for 5 min. The double-stranded cDNA was extracted with phenol/chloroform/isoamylalcohol and ethanol precipitated.

The resulting blunt ends of the cDNA were converted to termini containing 5' extensions by adding adapters to the cDNA. The adapter was prepared from two complementary oligonucleotides (CD1 and CD2) containing three restriction enzyme

sites, which were annealed and ligated to the blunt-ended double-stranded cDNA.

CD1 5'-GTCGACGCGCCGCG-3'
CD2 3'-CAGCTGCGCGGCGCTTAA-5'
*Sal*I *Not*I *Eco*RI

The blunt-end ligation of the adapters to the cDNA was carried out with 100 units/ml T4 DNA ligase in the presence of 66 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 14 mM DTT, and 200 μg/ml adapters. The reaction mixture was incubated at room temperature overnight, then heated at 70°C for 10 min to inactivate the ligase. The DNA was analyzed by agarose gel electrophoresis and detected by fluorography or by staining with EtBr followed by UV illumination.

Cloning into pBKS vector

The double-stranded cDNAs ligated with synthetic adapters were phosphorylated by T4 polynucleotide kinase and then cloned into pBluescriptKS (Stratagene) vector (pBKS) that had been digested with *Eco*RI and dephosphorylated by using calf intestinal alkaline phosphatase (1 unit/μl) (Boehringer Mannheim). Both orientations of the insert were recovered in plasmids from transformed *E.coli* DH5α cells. The size of the insert was checked by *Not*I digestion and the correct orientation of the insert was selected by appropriate restriction enzyme digestion and sequencing. The resulting constructs from VSV_{IND-HR} were called pBKS-IN, pBKS-IP, pBKS-IM, and pBKS-IG and the resulting plasmids from VSV_{NJ-Ogd} and VSV_{NJ-Haz} were designated pBKS-ON, pBKS-OP, pBKS-OM, pBKS-OG; pBKS-HN, pBKS-HP, pBKS-HM, and pBKS-HG, respectively. pEB2, which contains a cDNA copy of the L gene of VSV_{IND} under the

control of the T7 RNA polymerase promoter in pGem4XB vector, was generously provided by Dr. Manfred Schubert (National Institutes of Health).

7. cDNA CLONING OF VSV DIP GENOME

Preparation of DIP RNA

Monolayers of BHK-21 cells were infected with appropriate DIP stocks and the standard virus at a MOI of 10. For the replication of the two DIPs of VSV_{IND} (Ind-ST and Ind-LT), a heat-resistant strain of Indiana serotype was used as the helper virus. Ogden and Hazelhurst strains of New Jersey serotype were used as the helper viruses for the production of the two New Jersey DIPs (Ogd-DI and Haz-DI), respectively. At 15–18 hr of infection, culture supernatants were harvested, cell debris was removed by centrifugation at 2,500 rpm for 15 min, and virus was pelleted at 25,000 rpm for 1 hr in a Beckman SW 28 rotor. Virus pellets were resuspended in TNE buffer, followed by centrifugation on a 5–30% linear sucrose gradients made in TNE buffer. Samples were centrifuged in a Beckman SW 41 rotor at 35,000 rpm for 35 min. The DIP bands were collected, diluted with TNE buffer, and pelleted at 35,000 rpm in a Beckman SW 41 rotor for 1 hr. DIP pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.4% Na-deoxycholate, 10 mM EDTA), followed by extraction with phenol/chloroform/isoamylalcohol. DIP RNA was precipitated with ethanol in the presence of 5 μg of glycogen as carrier. DIP RNA was then pelleted by centrifugation at 12,000 rpm for 15 min and resuspended in DEPC-treated water.

RT-PCR of VSV DIP genome

For cDNA synthesis of three DIP RNAs (Ind-ST, Ogd-DI, and Haz-DI) by RT-PCR, oligonucleotides representing 17 nucleotides from the 5' terminus of the each genomic RNA (i.e. the 17 nucleotides complementary to the 3' terminus of each genomic RNA) were synthesized as primers for both first strand cDNA synthesis and PCR. The sequence of primer for Ind-ST was 5'-GGGCGGCCGCCGT**ACGAAG ACCACAAAACC**-3' (VSVI3) and the primer sequence for the two NJ-DIPs (Ogd-DI and Haz-DI) was 5'-GGGCGGCCGCCGT**ACGAAGACAAAAAAACCA**-3' (VSVNJ). The same primer was used for the two NJ-DIPs because the first 18 nucleotides from the 5' terminus of NJ_{Ogd} and NJ_{Haz} have the same sequence (Colonno and Banerjee 1978, Liang and Emerson 1988). In the case of Ind-LT the first strand cDNA was synthesized using a primer (VSVLT: 5'-GGGCGGCCGCCGT**ACGAAG ACAAACAAACC**-3') representing 17 nucleotides from the 5' terminus of Indiana antigenomic RNA (i.e. complementary to the 3' terminus of the Indiana genomic RNA) and PCR was performed with a primer (VSVI3) representing 17 nucleotides from the 5' terminus of the Indiana genomic RNA. All primers were flanked with the *Not*I site. The *Not*I site at the 5' end of the oligonucleotide is underlined and the VSV sequences are in bold face.

The reverse transcription reactions were carried out using SuperScript™ II system (Gibco BRL) according to the manufacturer's instructions. The cDNA synthesis reactions consisted of 3 μg of total RNA and 250 ng of primer in a 12 μl reaction volume. After primer annealing at 70°C for 10 min and on ice, 8 μl of reaction

mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM of each of the four dNTPs) was added. The reaction mixture was incubated at 42°C for 60 min and inactivated by heating at 70°C for 15 min. One-tenth of the cDNA in RNase-treated H_2O was then used as template for amplification in PCR.

One-tenth of the cDNA was then used directly for DNA amplification with 5 units of *Taq* DNA polymerase (Gibco BRL) in 100 μl buffer provided by the supplier (Gibco BRL) [containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, and 0.5 μM primer]. The amplification reaction was performed in a thermal cycler (Perkin-Elmer Cetus). The initial template denaturation step was 3 minutes at 94°C and afterwards the cycle profile was for 30 cycles of: denaturation at 94°C for 1 min, primer annealing at 45–50°C for 2 min, and extension at 72°C for 3–6 min. At the end of the cycle, the 72°C extension step was continued for an additional 7 minutes and samples were then cooled at 4°C. The resulting PCR products were extracted with phenol/chloroform/isoamylalcohol and separated on 1% agarose gels, gel purified and dissolved in water.

Cloning of DIP cDNAs

All PCR-amplified products were digested with the restriction endonuclease *Not* I and cloned into the pBKS (Stratagene) vector which had been linearized by cleavage at the *Not*I site and dephosphorylated. The ligation mixture was used to transform *E.coli* DH5α as described above. Ampicillin resistant colourless colonies on the IPTG/X-Gal agar plates were screened by purification of DNA and digestion with the

appropriate enzymes to check the insert size and orientation before sequencing using the Sequenase kit (United States Biochemical). The resulting constructs were called pBKS-ST, pBKS-LT, pBKS-OD, and pBKS-HD, respectively.

Ligase-free subcloning of pBKS-DI into transcription vector (pTV)

All DIP constructs in pBKS vector were subcloned into the special transcription vector (TV), obtained from Dr. Andrew Ball (University of Alabama), a pGEM derivative which contains the T7 promoter to initiate transcription of inserted sequences and the hepatitis delta virus (HDV) ribozyme to cut the 3' end of DIP RNA (Pattnaik *et al.* 1992). To construct plasmid pTV-DI, an overlap PCR amplification strategy was utilized. 5'-1st primers (primer ID5G and HD5G) contained 18 nucleotides complementary to the T7 RNA polymerase promoter sequences of pTV fused with two non-DIP nucleotides (GG) and 17 nucleotides complementary to the 5' end of DIP genome (primer ID5G for Ind-DIP and primer HD5G for NJ-DIP). 3'-1st primers (primer ID3 and HD3) contained 18 nucleotides complementary to the HDV ribozyme antisense-sequences of pTV fused with 17 nucleotides complementary to the 3' end of DIP genome (primer ID3 for Ind-ST and primer HD3 for NJ-DIP). Primer LT3 contained 18 nucleotides complementary to the HDV ribozyme antisense-sequences of pTV fused with 17 nucleotides complementary to the 3' end of Ind-LT. The first PCR was accomplished using primers ID5G and ID3 (IND-ST) or primers ID5G and LT3 (IND-LT) or primers HD5G and HD3 (NJ-DIPs) on plasmid pBKS-DI. These PCR products were then used as template for a second PCR using primer

UNIV5 and UNIV3. Primer UNIV5 contained 18 nucleotides complementary to the T7 RNA polymerase promoter sequences of pTV fused with 32 nucleotides complementary to a portion of pTV next to the T7 promoter (called universal 5' primer). Two non-VSV nucleotides (GG) were contained in the 3' end of primer UNIV5. Primer UNIV3 contained sequences complementary to the first 50 nucleotides of the HDV ribozyme antisense-sequences (called universal 3' primer). The second PCR was done using primers UNIV5 and UNIV3 to amplify the first PCR products. The final PCR product was then used for *in vivo* homologous recombination cloning (Aslanidis and Jong 1990, Shuldiner *et al.* 1991). pTV was passed through the *E.coli* 534 *dam*$^-$*dcm*$^-$ strain to obtain an unmethylated version which could be cut with *Stu*I, digested with *Sma*I and *Stu*I, and mixed with the final PCR product without ligase. This mixture was used to transform *E.coli* DH5α as described above. Ampicillin-resistant colonies were selected and digested with proper enzymes to check the inserts. All PCR-amplified regions were sequenced by using the Sequenase kit (United States Biochemical). The cloned sequences were inserted in both orientations between the bacteriophage T7 promoter and a cDNA copy of the self-cleaving ribozyme from the antigenomic strand of HDV. The resulting plasmids were named pTVDI(+) and pTVDI(−) to reflect the polarity of the T7 transcript they generated: DIP antigenomic or genomic RNA, respectively. All primers (see APPENDIX) used for PCR were synthesized by Gibco BRL or Procyon.

In vitro transcription of pTVDI constructs

Plasmid DNA was incubated at 37°C for 30 min with proteinase K and extracted with phenol/chloroform/isoamylalcohol. The DNA was precipitated in the presence of 2.5 volumes of ethanol and 0.1 volumes of 3 M Na-acetate (pH 5.2). After incubation at −20°C the DNA was pelleted in a microcentrifuge at 4°C, resuspended in an appropriate volume of DEPC-treated H_2O for *in vitro* transcription reactions.

In vitro transcription of pTVDI(−)/(+) was done by using the *In vitro* transcription kit (Promega) according to the manufacturer's instructions. *In vitro* transcription reactions consisted of 5 μg of plasmid DNA in DEPC-treated H_2O, 20 μl of 5×transcription buffer, 10 μl of 100 mM DTT, 100 units of RNasin ribonuclease inhibitor, 2.5 mM of each of the four rNTPs and 40 units of T7 RNA polymerase in a total volume of 100 μl. The mixture was incubated at 37°C for 90 min. RQ1 DNase (RNase-free) was added and the mixture was incubated for 37°C for 15 min. The samples were extracted and precipitated as described above. The RNA was resuspended in an appropriate volume of DEPC-treated H_2O.

8. AMPLIFICATION OF DIP FROM cDNA

Vaccinia virus infection and DNA transfection

Transfection experiments were carried out as described previously (Lawson *et al.* 1995) with minor modifications. BHK-21 cells on 10-cm dishes (~80% confluent) were infected with the vaccinia virus vTF-3 at a MOI of 3. After virus adsorption at 37°C for 60 min, the inoculum was removed, the cells were washed with Opti-MEM (Gibco

BRL) and then transfected with appropriate plasmid DNAs (30 μg of plasmids encoding DIP RNA and 30, 30, 30, 20, and 10 μg of plasmids encoding the G, M, N, P, and L proteins, respectively). For each 10-cm plate, a total of 150 μg of plasmid DNA was added to 0.5 ml of 0.25 M $CaCl_2$. An equal volume of a solution containing 280 mM NaCl, 50 mM HEPES and 1.5 mM Na-phosphate (pH 7.1) was added. The precipitate was allowed to form for 30 min and then added to the culture dish containing 8 ml of Opti-MEM. After 4 hrs transfection, cells were washed twice with DMEM and incubated in 10 ml of DMEM containing 10% HIFBS at 37°C. The culture supernatants were harvested at 48 hr posttransfection for the passage experiments.

In order to examine the expression of cloned VSV genes, BHK-21 cells on 35-mm plates were transfected using Lipofectin™ (Gibco BRL) according to the manufacturer's instructions. Cells were infected with vTF7-3 (MOI=3) for 60 min and washed with Opti-MEM. For each transfection, 2.5 μg of plasmids encoding each protein from three strains of VSV individually or in combination were diluted to 100 μl of Opti-MEM in a polystyrene tube. 10 μl of Lipofectin (1 mg/ml) in 100 μl of Opti-MEM was gently mixed with the DNA solution and the mixture was incubated at room temperature for 15 min. Following addition of 800 μl of Opti-MEM to each tube containing the Lipofectin-DNA complexes, the transfection mixture was added to the washed cells. After a 4 hr incubation at 37°C, cells were washed with DMEM and replaced with fresh DMEM containing 10% HIFBS for a further 12–15 hr incubation at 37°C.

Amplification of rescued DIP

The transfected supernatant (Passage 0) was collected, cleared by centrifugation at 2,500 rpm for 15 min, filtered through 0.2 μm-pore-size (Millipore products) syringe filters (Acrodisc) to remove most of the vaccinia virus, pelleted by centrifugation at 35,000 rpm for 60 min at 4°C in a Beckman SW 41 rotor. The pellet was resuspended in 400 μl of DMEM without serum. To amplify DIPs, another set of BHK-21 cells in 35-mm dishes was infected with standard virus at a MOI of 3. After 30 min adsorption, the inocula were removed and the cells were superinfected with 200 μl of Passage 0 supernatant. After 1 hr incubation, the medium was added and cells were maintained at 37°C for 15–18 hrs. The culture fluids were collected, cleared by centrifugation at 12,000 rpm for 5 min. Subsequent passages were performed by plating the supernatants from those cells onto fresh BHK-21 cells. The rescued DIP stocks were prepared from the culture supernatants (P1) and analyzed by interference assay and by RNA analysis on BHK-21 cells as described previously.

CHAPTER 3: CHARACTERIZATION OF NEW VSV DIPs

1. INTRODUCTION

The first objective of my project was to generate and characterize the biological properties of DIPs of VSV in order to have a better understanding of the homotypic and heterotypic interference phenomena. DIPs are generated by serial undiluted passage of most, if not all, animal viruses (Huang and Baltimore 1977) and have genomes that are deleted forms of the infectious (standard) virus genomes. Since DIPs are defective they require the standard virus to replicate their genomes, and in turn interfere with the production of standard virus. Among the various types of interference that have been described for animal viruses, homologous interference by DIPs may provide a particularly useful tool for studying virus replication and for the understanding of viral disease states (Huang and Baltimore 1970).

Homologous autointerference in VSV was first investigated by Cooper and Bellett (1959) who observed that serial undiluted passage of the virus in cell culture led to lowered yields of the standard virus due to the presence of a transmissible interfering component (now called DIPs). Since then, a number of reports have described possible mechanism(s) of interference by DIP of VSV. Many reports have shown that DIP-mediated interference usually occurs with the same serotype of the standard virus (homotypic interference), however, heterotypic interference (i.e. DIP interferes with the production of a different serotype of standard virus) has also been reported for VSV (Prevec and Kang 1970, Schnitzlein and Reichmann 1976). The mechanism by which

DIPs cause inhibition of standard virus multiplication is still obscure.

VSV provides several advantages in studying the autointerference mechanism. VSV has a very wide host range and grows to high titers and can be prepared in large quantities. Also, VSV DIPs can be easily separated from the standard viruses. Thus, a relatively pure population of DIPs can be obtained. The availability of this high-quality preparation of DIPs in large quantity permitted me to study the nature of DIPs as well as the mechanism of homologous viral interference.

This chapter reports the generation and enrichment of DIPs derived from different strains of VSV through serial undiluted passages and the ability of DIPs of different origins to interfere homotypically and heterotypically. The relationship between their biological activities and RNA synthesis during coinfection with standard virus and DIPs is also presented.

2. RESULTS

Generation of DIPs from VSV

To generate DIPs, standard viruses of New Jersey serotypes of VSV were serially passaged four consecutive times in BHK-21 cells. Initially, cells were infected with a standard virus stock at a MOI of 50 and incubated at 37°C for 15-18 hrs. At each passage the medium was harvested, cleared of cell debris by centrifugation, and frozen at -80°C. The yields of the progeny virus from each of the passages were then determined by plaque assay (data not shown). The virus titer of two different strains of NJ standard viruses ($VSV_{NJ\text{-}O_{gi}}$ or $VSV_{NJ\text{-}Haz}$) gradually declined through the four

undiluted passages. This loss of titer is characteristic of the phenomenon of homologous autointerference as first described by Cooper and Bellett (1959).

To amplify new NJ-DIPs, virus from serial passage 3 of $VSV_{NJ\text{-}Ogd}$ or $VSV_{NJ\text{-}Haz}$ was mixed with the appropriate standard virus (MOI of 20) and each mixture was used to infect BHK-21 cells. For amplifying IND-DIPs, DI stocks (IND-ST and IND-LT), which had been kept in the laboratory of Dr. C.Y. Kang, were individually mixed with Indiana standard virus infected BHK-21 cells at a MOI of 20 and incubated at 37°C for 15-18 hrs. Progeny viruses were harvested and DIPs were then separated from the standard virions using sucrose gradients centrifugation. All working stocks of DIPs exhibited a single size class of DIP on sucrose gradients.

The origin of each DIP is shown in Table 3. IND-ST was originally generated from the wild-type Indiana serotype of VSV and IND-LT was derived from a heat-resistant strain of the Indiana serotype of VSV (Petric and Prevec 1970). Ogd-DI and Haz-DI were newly generated from the New Jersey Ogden and New Jersey Hazelhurst strains of VSV, respectively. The sizes of DIPs as judged from sucrose gradients appeared to be approximately one-half (IND-LT and Haz-DI) and one-fourth (IND-ST and Ogd-DI) that of the standard virions.

Interference activities of DIPs

Purified DIPs were assayed for their ability to interfere with the production of the standard virus. BHK-21 cells were infected with the standard virus alone or with the standard virus plus different amounts of the DIPs. Following incubation at 37°C

TABLE 3

Origins of VSV DIPs

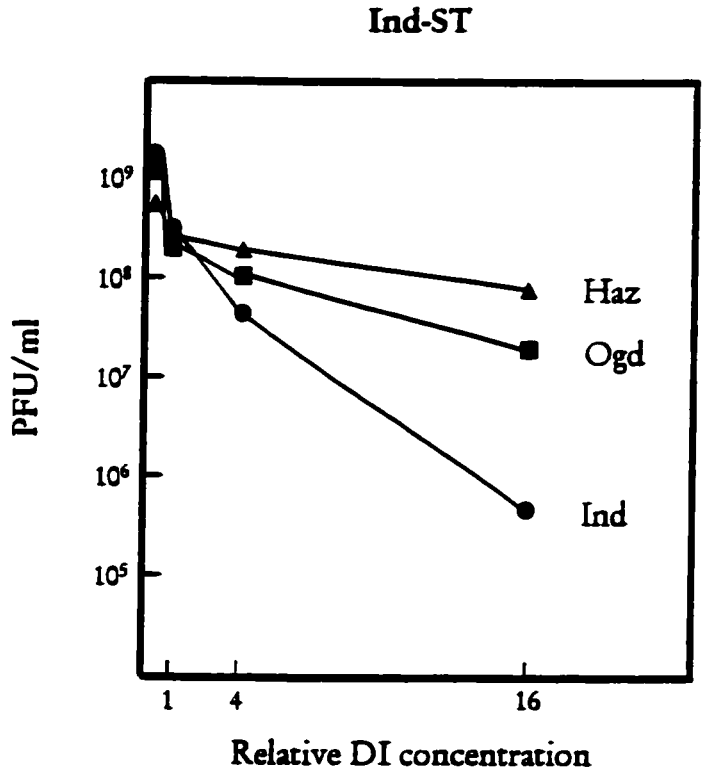
Standard viruses	DIPs
Indiana (Ind)	Ind-ST
Indiana (Ind)-heat resistant (HR)	Ind-LT
New Jersey (NJ) - Ogden (Ogd)	Ogd-DI
New Jersey (NJ) - Hazelhurst (Haz)	Haz-DI

for 15–18 hrs, infectious virus yields were determined by plaque assay on BHK-21 cells (Figs. 6 and 7). As shown in Fig. 6, coinfection of cells with the standard virus and IND-DIPs (IND-ST or IND-LT) resulted in a marked decrease of virus yields compared to infection with the standard virus alone. The virus titers showed a varying amount of reduction in infectious virus yield with increasing concentrations of coinfecting IND-DIPs; strong or weak interference was observed depending upon the standard VSV. Fig. 6A and 6B show that IND-ST and IND-LT interfered efficiently with the standard virus of Indiana serotype (3.4 logs and 4.0 logs reduction in IND-ST and IND-LT, respectively); moderately interfered with the standard virus of NJ-Ogden (2.0 logs and 2.6 logs reduction in IND-ST and IND-LT, respectively) and weakly interfered with the standard virus of NJ-Hazelhurst (0.6 logs and 1.1 logs reduction in IND-ST and IND-LT, respectively). It was noticed that the level of interference by IND-LT was greater than for IND-ST. The degree of interference was dependent on the amount of DIPs added. These results are somewhat different from a previous report (Prevec and Kang 1970) where no heterotypic interference by IND-ST was observed. Several factors that might explain these differences in interference will be discussed below.

The ability of the new NJ-DIPs to interfere with the different standard viruses of VSV was also tested (Fig. 7). When NJ-DIPs were mixed with NJ standard virus and grown in doubly infected cells, a significant reduction in titer of standard virus was observed. As shown in Fig. 7, the yield of NJ-Ogd standard virus was decreased by 3.3 logs and 3.2 logs with Ogd-DI and Haz-DI, respectively. The reduction of NJ-Haz standard virus yield was 2.8 logs with Ogd-DI and 3.2 logs with Haz-DI. The degree

Figure 6. Homotypic and heterotypic interference by IND-DIPs. BHK-21 cells were infected with VSV (MOI=3) alone or plus serial four fold dilutions (1:400, 1:100, and 1:25) of IND-ST (A) or IND-LT (B). Virus was adsorbed for 1 hr at 37°C and medium was added. At 16 hr after infection, the cultures were harvested and the standard virus titer was determined by plaque assay on BHK-21 cells. (●—●) VSV_{IND}; (■—■) VSV_{NJ-Ogd}; (▲—▲) VSV_{NJ-Haz}.

A


Ind-ST
PFU/ml
10⁹
10⁸
10⁷
10⁶
10⁵
Haz
Ogd
Ind
1
4
16
Relative DI concentration

B

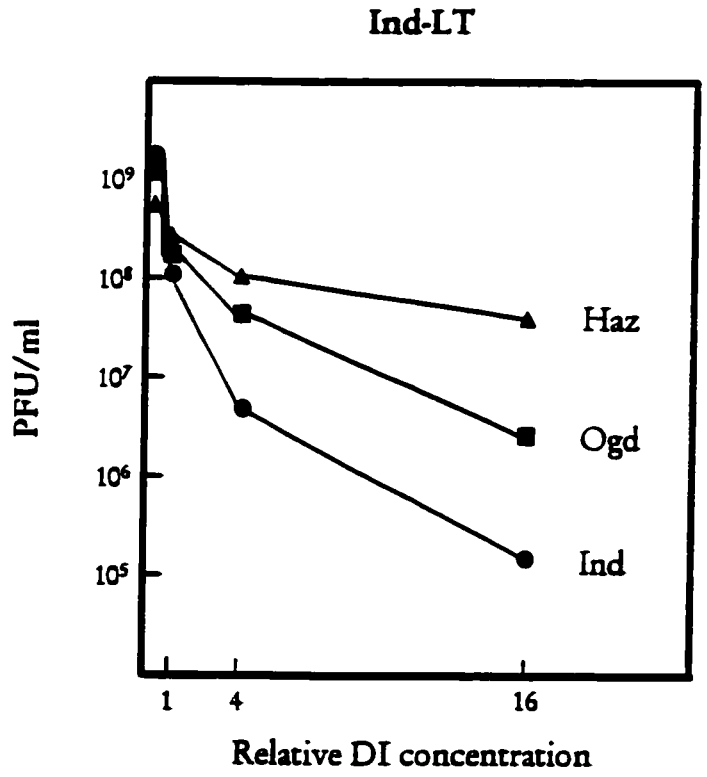
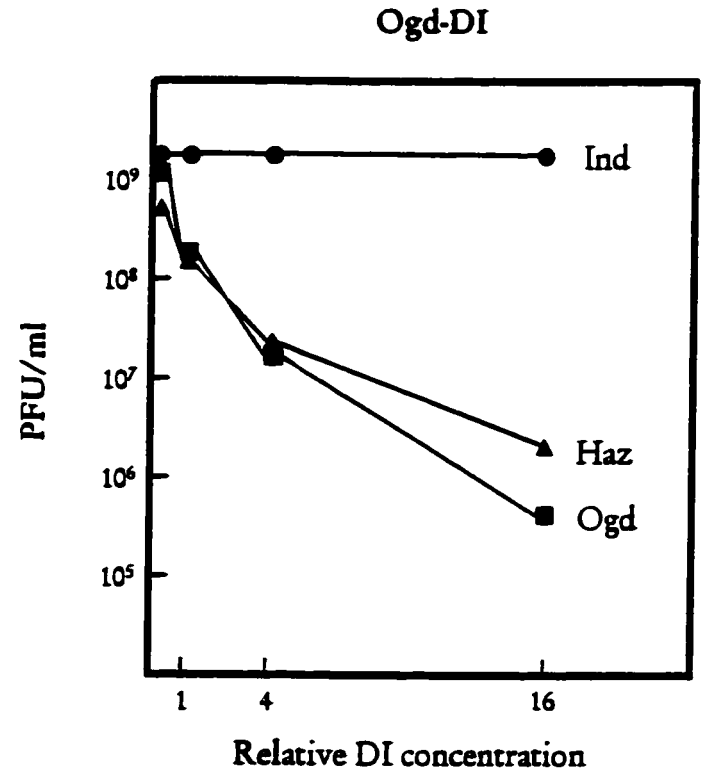
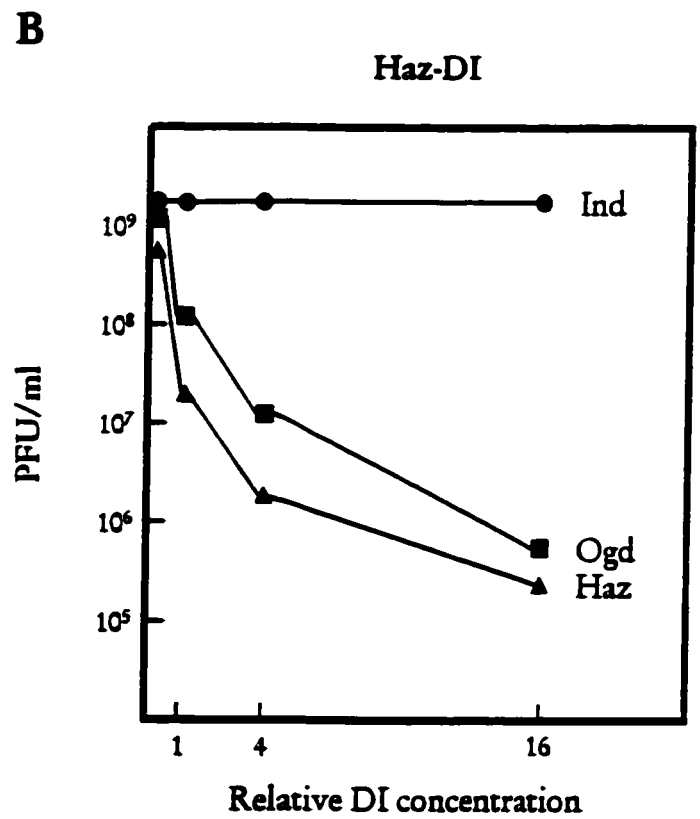

Ind-LT
PFU/ml
10⁹
10⁸
10⁷
10⁶
10⁵
Haz
Ogd
Ind
1
4
16
Relative DI concentration

Figure 7. Homotypic and heterotypic interference by NJ-DIPs. Monolayers of BHK-21 cells were infected with VSV (MOI=3) alone or plus serial four fold dilutions (1:400, 1:100, and 1:25) of Ogd-DI (A) or Haz-DI (B). Virus was adsorbed for 1 hr at 37°C and medium was added. At 16 hr after infection, the cultures were harvested and the standard virus titer was determined by plaque assay on BHK-21 cells. (●—●) VSV_{IND}; (■—■) $VSV_{NJ\text{-}Ogd}$; (▲—▲) $VSV_{NJ\text{-}Haz}$.

A


Ogd-DI
Ind
Haz
Ogd
PFU/ml
10^9
10^8
10^7
10^6
10^5
1
4
16
Relative DI concentration

B


Haz-DI
Ind
Ogd
Haz
PFU/ml
10^9
10^8
10^7
10^6
10^5
1
4
16
Relative DI concentration

of interference was dependent on the concentration of DIPs as seen with IND-DIPs. NJ-DIPs strongly interfered with the production of the homotypic NJ standard viruses. In contrast, the production of the heterotypic IND standard virus was not affected by NJ-DIPs. Neither Ogd-DI nor Haz-DI interfered with the yield of Indiana serotype standard virus. Thus, two NJ-DIPs are capable of interfering the NJ standard virus production but are unable to interfere with IND standard virus.

These data clearly demonstrate that IND-DIPs interfere with all three standard viruses, although with a different degree of interference, while in contrast NJ-DIPs only interfere with homotypic standard virus production. The difference in patterns of interference by IND-DIPs and NJ-DIPs provides an excellent tool to investigate the mechanism(s) of homologous viral interference at the molecular level.

Effects of viral RNA synthesis by DIPs

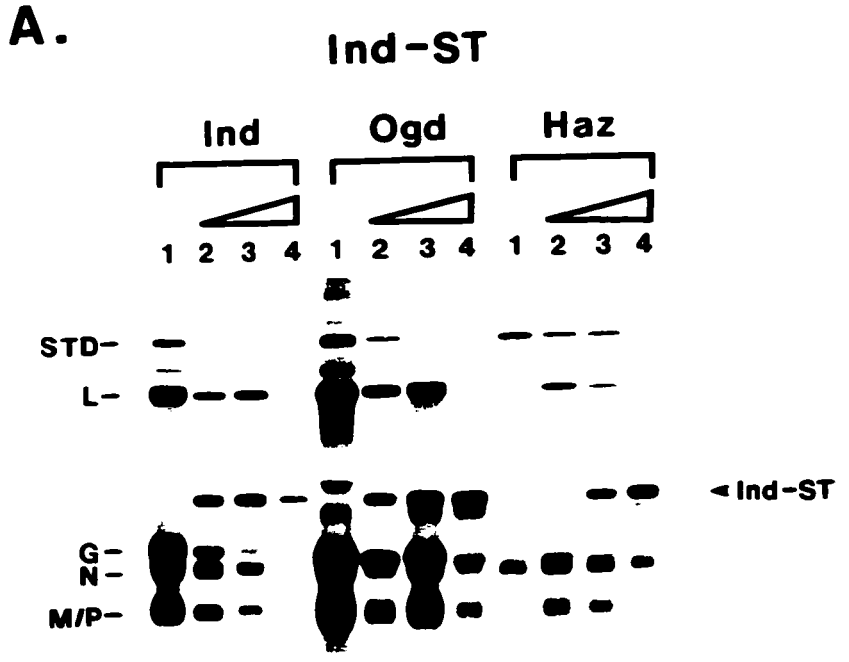
In order to examine the virus-specific intracellular events occurring in cells coinfected with standard virus and DIPs, cells were pretreated with 2 μg/ml of actinomycin D for 1 hr prior to infection and infected either with VSV standard virus alone or coinfected with the standard virus and DIPs. Cells were then labeled with [^{3}H]-uridine for 2 hrs at 4 hr p.i. in the presence of 2 μg/ml of actinomycin D. Addition of actinomycin D did not alter interference and demonstrated that interferon was not involved (Shenk and Strollar 1973). The labeled RNAs were recovered by phenol/chloroform/isoamylalcohol extraction and equal amounts of cytoplasmic RNA were extracted and analyzed by electrophoresis in an agarose-formaldehyde gel (Figs.

8 and 9).

As shown in Figs. 8 and 9, the RNA pattern of IND standard virus was identical to that of NJ standard virus. The standard virus infected cells contained all five VSV mRNAs and the standard virus genomic RNA (each lane 1). Since the sizes of M mRNAs (831 and 758 nucleotides in VSV_{IND} and VSV_{NJ}, respectively) and P mRNAs (814 and 856 nucleotides in VSV_{IND} and VSV_{NJ}, respectively) (Table 1, Fig. 2) are almost the same between the two different serotypes of VSV, it is difficult to distinguish them in the agarose gel.

The pattern of virus-specific RNA synthesis in coinfected cells differs markedly from those in cells infected with the standard virus alone. As increasing amounts of IND-ST were used, the level of mRNA transcription and the genomic RNA replication were decreased significantly depending upon which standard virus was used as helper; a strong inhibition of IND standard virus RNA synthesis, a moderate inhibition of NJ-Ogd standard virus RNA synthesis and a weak inhibition of NJ-Haz standard virus RNA synthesis were observed. This degree of inhibition of virus-specific RNA synthesis correlated with the degree of interference. The interesting point is the replication of DIP genomic RNA during the interference. It seemed that the DIP genomic RNA replication was proportional to the amount of the DIPs used in infection. These results demonstrate that increasing amounts of DIP genomic RNA replication resulted in a strong inhibition of standard virus RNA synthesis. However, an excess amount of IND-ST showed overall reduction of the viral RNA synthesis including the DIP genomes (Fig. 8A, lane 4). These results suggest that a significant

Figure 8. Viral RNA synthesis in cells infected with the standard viruses and IND-DIPs. BHK-21 cells were incubated at 37°C in medium with 2 μg/ml of actinomycin D for 1 hr prior to infection. Standard virus of VSV (MOI=3) was inoculated into the cells singly (each lane 1) or mixedly with 1:400 dilution (each lane 2), 1:100 dilution (each lane 3) and 1:25 dilution (each lane 4) of IND-ST (A) or IND-LT (B). After 1 hr of adsorption, medium containing actinomycin D (2 μg/ml) was added. Cells were exposed to 20 μCi/ml of [^{3}H]uridine between 4 and 6 hr p.i. The labeled RNAs were isolated from the cells and analyzed by electrophoresis in a 1% agarose-formaldehyde gels and detected by fluorography. The positions of the standard genomic RNA (STD) and L, G, N, M and P mRNAs are indicated. Arrowheads represent the position of DIP RNA (A, IND-ST; B, IND-LT). Ind, Indiana standard virus; Ogd, NJ-Ogden standard virus; Haz, NJ-Hazelhurst standard virus.


A.
Ind-ST
Ind
Ogd
Haz
1 2 3 4 1 2 3 4 1 2 3 4
STD-
L-
◄Ind-ST
G-
N-
M/P-

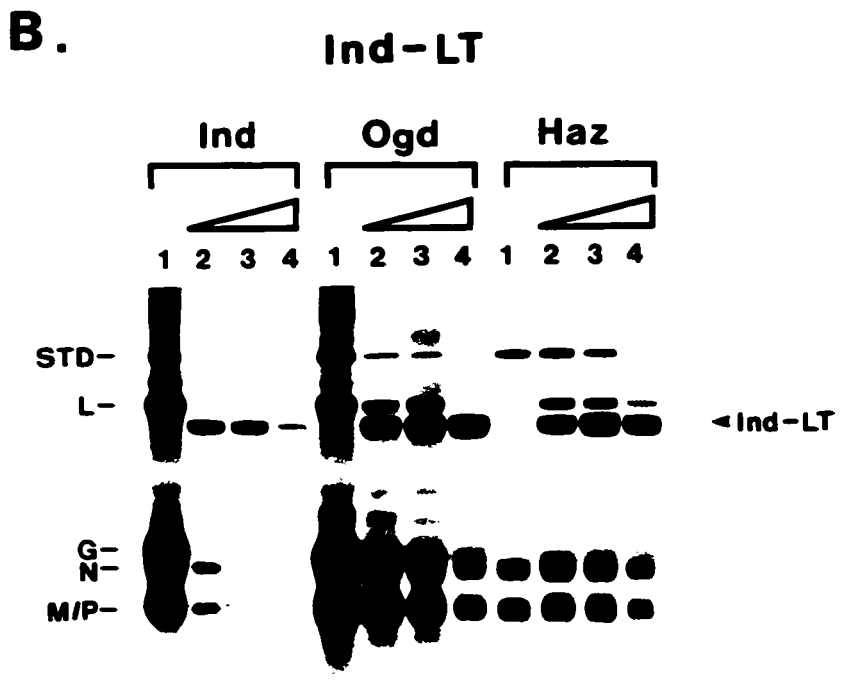
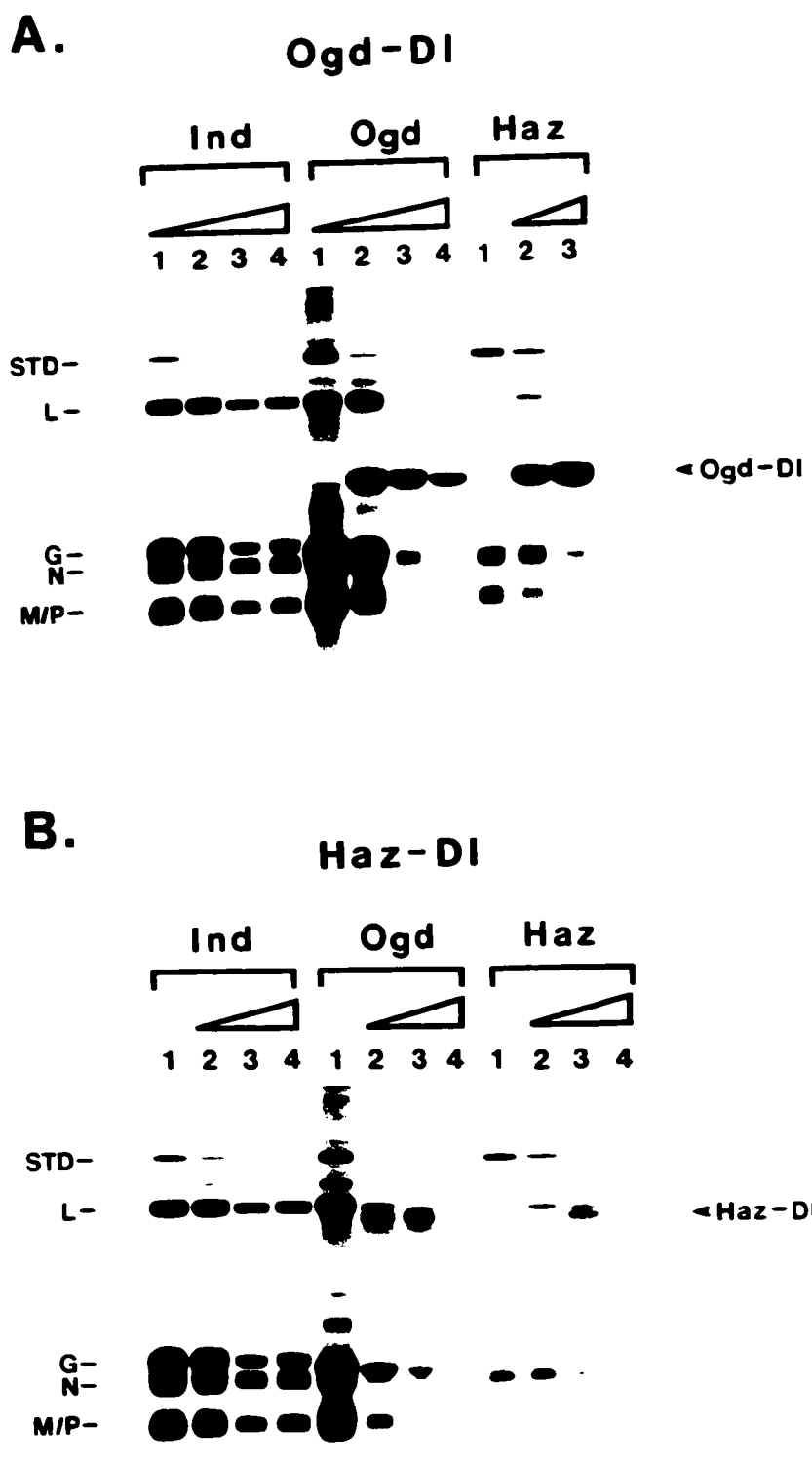

B.
Ind-LT
Ind
Ogd
Haz
1 2 3 4 1 2 3 4 1 2 3 4
STD-
L-
◄Ind-LT
G-
N-
M/P-

Figure 9. Viral RNA synthesis in cells infected with standard viruses and NJ-DIPs. Experiments were carried out as described in the legend to Fig. 8. Each lane 1, standard virus infection; lanes 2-4, standard virus plus 1:400 dilution (each lane 2), 1:100 dilution (each lane 3) and 1:25 dilution (each lane 4) of Ogd-DI (A) or Haz-DI (B) coinfection. The positions of the standard genomic RNA (STD) and L, G, N, M and P mRNAs are indicated. Arrowheads represent the position of DIP RNA (A, Ogd-DI; B, Haz-DI). Ind, Indiana standard virus; Ogd, NJ-Ogden standard virus; Haz, NJ-Hazelhurst standard virus.

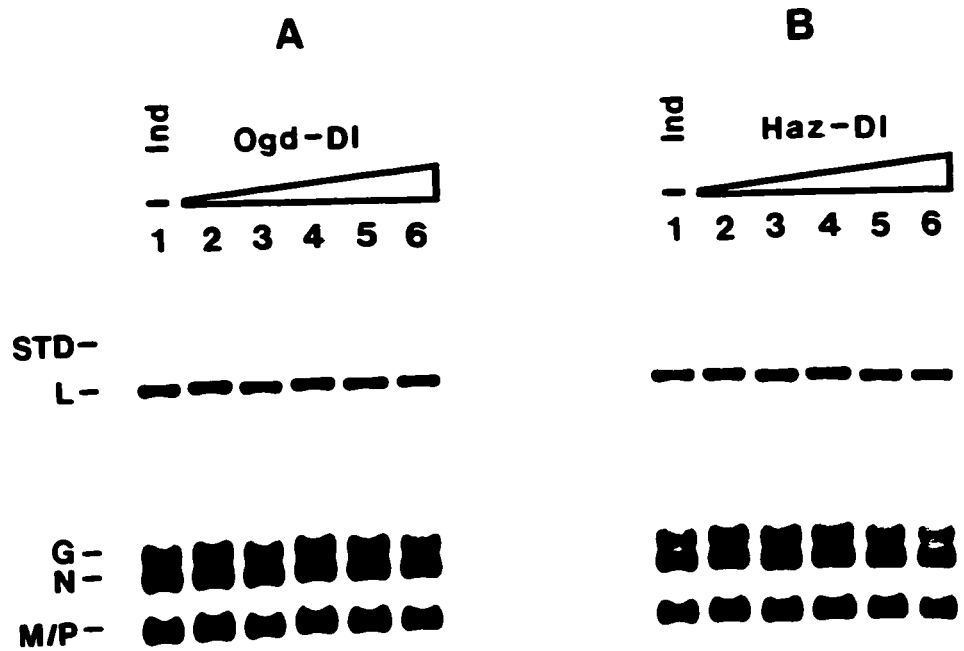

A.
Ogd-DI
Ind
Ogd
Haz
1 2 3 4 1 2 3 4 1 2 3
STD-
L-
◄Ogd-DI
G-
N-
M/P-
B.
Haz-DI
Ind
Ogd
Haz
1 2 3 4 1 2 3 4 1 2 3 4
STD-
L-
◄Haz-DI
G-
N-
M/P-

reduction of viral mRNA transcription causes a decrease in viral protein synthesis for genomic RNA replication. DIPs in cells without standard virus as helper were unable to replicate (data not shown). Thus, the DIP preparations were suitable for subsequent experiments. The overall RNA patterns of IND-LT (Fig. 8B) are essentially the same as IND-ST, with an increase in the replication of DIP genomic RNA.

The ability of NJ-DIPs to inhibit the viral RNA synthesis in cells coinfected with standard virus was also examined (Fig. 9). Similar results were obtained when NJ-DIPs were infected with NJ standard viruses. As increasing amounts of NJ-DIPs were used NJ standard genomic RNA and mRNA synthesis were dramatically decreased. NJ-DIP genomic RNA was replicated in NJ standard virus coinfected cells (Fig. 9A and 9B). A correlation between the amount of DIP genomic RNA replication and inhibition of mRNAs synthesis was also found to exist for NJ-DIPs. It was discovered that neither Ogd-DI nor Haz-DI genomic RNAs were replicated when these DIPs were coinfected with IND standard virus (Fig. 9A and 9B). Furthermore, RNA synthesis of IND standard virus was not affected significantly in the presence of NJ-DIPs. Since NJ-DIPs showed no interference with IND standard virus (Fig. 7), it is concluded that the lack of DIP genomic RNA replication accounted for the lack of interference.

It was observed that standard virus-specific RNA synthesis was noticeably reduced when NJ-DIPs were present. It is possible that amounts of DIPs used may saturate the virus receptors on the cell surface and thus prevent the standard virus from attaching to the cells. To test this possibility, the cells were infected with standard virus first and then DIPs were superinfected. As shown in Fig. 10, the level of RNA synthesis of

Figure 10. Viral RNA synthesis in VSV_{IND}-infected cells after superinfection with NJ-DIPs. BHK-21 cells were treated with 2 μg/ml of actinomycin D for 1 hr prior to infection. Cells were infected with Indiana standard virus of VSV at a MOI=3. After 30 min adsorption, the inoculum was removed. Cells were then mock infected (each lane 1) or superinfected with 1:6400, 1:1600, 1:400, 1:100, and 1:25 dilution (each lane 2, 3, 4, 5, and 6, respectively) of NJ-DIP (A, Ogd-DI; B, Haz-DI) for 45 min. Infected cells were labeled with [^{3}H]uridine as described in Fig. 8. The intracellular RNA was extracted, separated in a 1% agarose-formaldehyde gel and detected by fluorography. The positions of the standard genomic RNA (STD) and L, G, N, M and P mRNAs are indicated.


A
B
Ind
Ogd-DI
1 2 3 4 5 6
Ind
Haz-DI
1 2 3 4 5 6
STD-
L-
G-
N-
M/P-

IND standard virus was not affected in the presence of NJ-DIPs.

Cells infected with mixtures of DIPs and standard viruses under conditions of interference showed replication of DIP genomic RNA and reduced synthesis of standard mRNAs and genomic RNA depending on the input amounts of DIPs (Figs. 8 and 9). At the level of genomic RNA replication, DIP genomic RNAs were preferentially replicated during interference. These results indicate that interference occurs via selective replication of DIP genomic RNA. The most severe interference with the most marked changes in intracellular viral RNA species occurred when cells were producing DIP genomic RNAs. Thus, interference also occurs at the level of viral RNA synthesis. The most likely interpretation of these data is that interference is mediated by the DIP RNAs (Huang and Wagner 1966), but more definitive experiments (e.g. genetic manipulation of DIP genomes) are required to prove this.

3. DISCUSSION

Biological properties of VSV DIPs

DIPs of VSV were generated by serial undiluted passage (Table 3) and homotypic and heterotypic interference of DIPs generated from different strains of VSV were examined (Figs. 6 and 7). DIPs derived from different strains of VSV did not show the same pattern of interference. A pattern of relatedness between standard viruses can be determined based on the ability of DIPs to interfere with different viruses. The results of interference assays showed that IND-DIPs interfered with both the homotypic strain (VSV_{IND}) and heterotypic strains ($VSV_{NJ\text{-}Ogd}$ and $VSV_{NJ\text{-}Haz}$). In

contrast, NJ-DIPs caused interference with only homotypic strains ($VSV_{NJ\text{-}Ogd}$ and $VSV_{NJ\text{-}Haz}$). These interesting data led to further experiments studying viral interference.

The data showing that heterotypic interference by two IND-DIPs (IND-ST and IND-LT) can occur between different serotypes of VSV are somewhat different from a previous report (Prevec and Kang 1970), which showed that IND-LT interfered heterotypically but IND-ST interfered only homotypically. It is difficult to compare these results with those from the earlier report because different cell lines were used, there were differences in DIP heterogeneity and VSV isolates, and the experimental protocols used.

Viral RNA synthesis in DIP-coinfected cells

Virus-specific RNA synthesis in cells infected with either the standard virus alone or with the standard virus plus DIPs was examined (Figs. 8-10). It was found that DIP genomic RNA replicated well if DIPs interfered with the replication of the standard virus. Furthermore, the most dramatic change was a decrease in viral mRNA synthesis and a reduction in standard virus genomic RNA replication. In contrast, when there was no interference, viral RNA synthesis was not affected by DIPs and DIP genomic RNA was not replicated in coinfected cells. These results indicate that standard virus-specific RNA synthesis (both genomic RNA and mRNA) was significantly suppressed during interference at least during the first 6 hrs of infection. The marked reduction of viral mRNA synthesis inevitably leads to a decrease in viral protein synthesis, followed by the inhibition of the replication of standard virus genomic RNA. The

reduction of standard virus genomic RNA replication may well lead to a lack of template for the polymerase complex for both mRNA synthesis and the standard virus genomic RNA replication.

Several earlier reports (Huang and Wagner 1966, Sreevalscan 1970, Huang and Manders 1972, Moyer and Gatchell 1979) have shown similar results by studying only homotypic interference and it was proposed that the interference might occur during viral RNA replication. This interference in RNA synthesis may indicate that the RNA of the DIP is the most important component for interference. The genomic RNA of DIPs might interfere with the replication of the standard virus by competing for the polymerase complex supplied by standard virus as described previously (Palma and Haung 1974, Stamminger and Lazzarini 1977).

The relationship between viral interference and RNA synthesis

The relationship between viral interference and viral RNA synthesis is summarized in Table 4. When DIPs showed interference with the production of standard virus, the mRNA synthesis of standard virus and the replication of the standard genomic RNA were inhibited and DIP RNA was produced in the coinfected cells. If there was no interference, standard virus-specific RNA synthesis was not affected and there was no DIP RNA replication. These results demonstrated that the DIP genome was replicated only when DIP inhibited the RNA synthesis of the standard virus. These results suggest that the DIP-mediated viral interference is at the level of viral RNA synthesis and DIP genomic RNA must be recognized as a template by the either homotypic or

TABLE 4

Effects of interference on RNA synthesis

Characteristics	DIPs	Ind-ST	Ind-LT	Ogd-DI	Haz-DI
Homotypic	Interference	+	+	+	+
	DIP replication	+	+	+	+
	Inhibition of RNA synthesis	+	+	+	+
Heterotypic	Interference	+	+	−	−
	DIP replication	+	+	−	−
	Inhibition of RNA synthesis	+	+	−	−

heterotypic RNA polymerase complexes in order to result in interference. Thus, it can be concluded that the replication of DIP RNA is a prerequisite for interference.

These results imply that a broad size range of RNA genomes can share this property. Perhaps all DIP RNA species have some common denominator, such as a nucleotide base sequence or conformation, which is responsible for the interference phenomenon. In order to test this hypothesis we need to know more about the enzymes responsible for RNA synthesis, the architecture of the RNA synthesizing complexes in the cell and the nucleotide sequence relationship between standard genomic RNA and DIP RNA. Knowledge of the genomic sequences of the standard viruses and DIPs used in this study will help to elucidate the molecular mechanism(s) of interference by DIPs. These studies are presented in the next chapter.

CHAPTER 4: CLONING AND SEQUENCING OF VSV DIPs

1. INTRODUCTION

The genomic structure of DIPs are of particular interest as they retain only a portion of the standard virus genome; they can interfere with standard virus replication and effectively out compete standard virus replicating RNA templates. Thus, their sequences hold the key to what features are important for their efficient replication and/or encapsidation. Also, the array of DIP RNA structures provides information regarding the extent to which virus RNA polymerases are capable of synthesizing 'mistake' or recombinant molecules that may play an important role in RNA virus evolution (Holland *et al.* 1982).

To develop an understanding of how DIPs are generated and how they interfere with the growth of infectious virus, it is necessary to determine the sequence relationships between standard virus and DIP genomes. VSV DIPs are the most extensively characterized. Several VSV IND-DIPs have been examined and have been grouped into a number of classes (Perrault 1981, Lazzarini *et al.* 1981). The vast majority of IND-DIPs studied are derived from the 5' end of the standard virus genome and contain only small portions of the L gene (polymease gene) (i.e.; IND-ST), thus they do not give rise to translatable mRNAs and are completely dependent on the standard helper virus for the proteins necessary for their replication and encapsidation. Sequencing of ends of the IND-DIP RNAs derived from the 5' end of the genome has demonstrated that they have self-complementarity (Keene *et al.* 1978, Perrault and

Leavitt 1977, Perrault *et al.* 1978, Schubert *et al.* 1978, Semler *et al.* 1978). The other rare class of DIPs are 3' derived DIPs. IND-LT (Kang and Prevec 1971) has been grouped to this class and the 3' end and 5' ends of representatives of this class of DIP genomes have been sequenced (Petric and Prevec 1970, Perrault and Semler 1979, Epstein *et al.* 1980). IND-LT has been shown to be transcriptionally active and displays biological properties not shared with the other major classes of VSV DIPs (Perrault and Semler 1979, Keene *et al.* 1981).

In order to further characterize the VSV DIPs at the molecular level, the second objective of my project, molecular cloning and sequencing of DIP genomes, was undertaken and sequences were compared to that of the standard virus. Sequence analysis is one of approaches taken to understand the role of the minimally conserved portion of the standard virus genome in virus replication and interference. In this chapter, I will present complete nucleotide sequences of IND-DIP genomes (IND-ST and IND-LT) and also present the first complete nucleotide sequence of NJ-DIP genomes (Ogd-DI and Haz-DI). A sequence of the DIP genome of VSV, derived from genomic cDNA copies, is described and DIP genome structure and generation models for these VSV DIP genomes will be presented.

2. RESULTS

RT-PCR amplification of DIP RNAs and cloning of DIP genomes

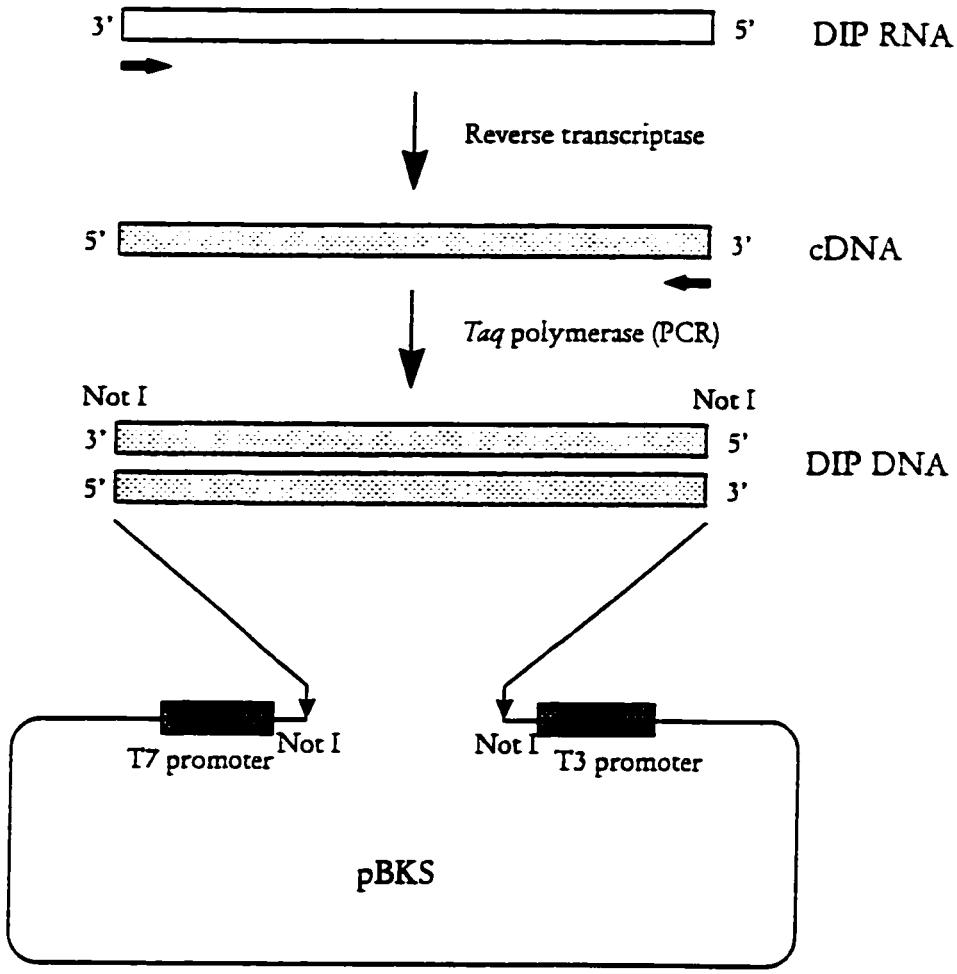
The majority of VSV DIP genomes that have been described are 'panhandle' or 'copyback' DIPs (Fig. 4), which have portions of the L gene 5' end linked to the 3'

inverted complement of the 5' terminus to give small terminal panhandle or stem structures. Because of their unusual sequence organization, one can design PCR amplifications of DIP RNAs by using only a single primer complementary to the 3' end of the standard virus antigenome, which is also complementary to 3' ends of both the DIP "genomes" and "antigenomes". The copyback DIPs are, of course, chimeras in which each chain contains both genomic or (−) sense and antigenomic or (+) sense sequences. This single primer can, at least in theory, amplify copyback DIP RNAs.

To test this hypothesis single primers designed from the published full nucleotide sequence of two VSV serotypes, VSV_{IND} and VSV_{NJ} were used to probe the sequences of the nucleic acids of DIPs (IND-ST, Ogd-DI, and Haz-DI). Based on sequence information (McGeoch 1979, McGeoch and Dolan 1979, Rose 1980, Schubert *et al.* 1980, Gallione *et al.* 1981, Rose and Gallione 1981, Gallione and Rose 1983, Banerjee *et al.* 1984, Schubert *et al.* 1984, Gill and Banerjee 1985, 1986, Rae and Elliott 1986, 1987, Nichol *et al.* 1989, Barik *et al.* 1990), oligonucleotide VSVI3 complementary to the 3' terminus of the DIP genome was used to prime cDNA synthesis using RNA of IND-ST. Similarly, a primer VSVNJ was used to synthesize and amplify cDNA from RNAs of the Ogd-DI or Haz-DI. In the case of IND-LT, which is an internal deletion of the standard virus, the IND-LT RNA contains 3'-and 5'- terminal sequences identical to VSV_{IND} RNA. The cDNA of IND-LT was amplified using primer VSVI3 and another primer (VSVLT) which was complementary to the 3' end of the standard virus genome (see Materials and Methods).

In Fig. 11 the procedure used for cloning VSV DIP genomes is summarized and

Figure 11. The cloning procedure of DIP genomes. The DIP genomic RNAs isolated from DIPs were amplified with a unique primer of negative polarity to the inverted repeats (VSVI3 primer for IND-ST, VSVNJ primer for Ogd-DI or Haz-DI). This primer serves as reverse transcriptase primer as well as PCR primer. For IND-LT two primers (a VSVI3 3' primer and a VSVLT 5' primer) were used for RT-PCR. PCR reactions were performed by using *Taq* polymerase (denaturation; 94°C, 1', hybridization; 45-50°C, 2', extension; 72°C, 3-6', 30 cycles). The PCR products were digested with *Not*I and then cloned into pBKS vector. The open bar indicates (−) sense DIP RNA and the stippled bar represents cDNA of DIP genomes. Black arrows indicate oligonucleotide primers used for cDNA synthesis.

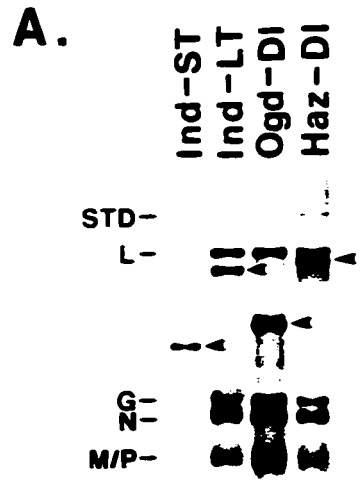

3'
5'
DIP RNA
Reverse transcriptase
5'
3'
cDNA
Taq polymerase (PCR)
Not I
Not I
3'
5'
5'
3'
DIP DNA
T7 promoter
Not I
Not I
T3 promoter
pBKS

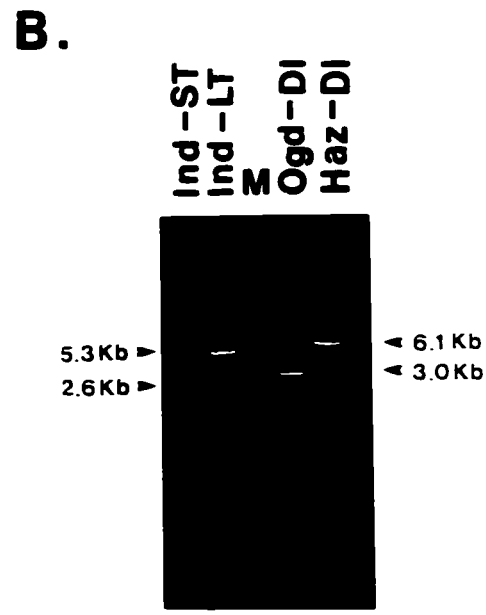
the locations of the oligonucleotide primers used for priming cDNA synthesis are shown. DIP genomic RNAs were isolated from DIPs purified on sucrose gradients. cDNA synthesis was performed using a 3' primer (VSVI3 for IND-ST, VSVLT for IND-LT, VSVNJ for Ogd-DI and Haz-DI) and amplification was carried out using the same primer during the PCR reaction. All primers contained the first 17 nucleotides of the standard virus genome plus a *Not*I site to allow convenient cloning of the PCR product. Single cDNA products of about 2.6, 5.3, 3.0 and 6.1 Kb corresponding to DIP RNAs present in IND-ST, IND-LT, Ogd-DI and Haz-DI respectively were amplified (Fig. 12A and B). The sizes of the PCR products are in good agreement with the size of DIP RNAs relative to marker mRNAs of standard virus in Fig. 12A. Thus, this strategy could synthesize full-length DIP cDNAs. The PCR products were digested with *Not*I and then cloned into pBKS vector that had been digested with *Not*I and treated with phosphatase. The resulting plasmids were called pBKS-ST, pBKS-LT, pBKS-OD and pBKS-HD respectively. The complete nucleotide sequences of each of the PCR products were determined.

Sequencing strategy of DIP cDNA

For sequence analyses of DIP genomes cloned in the pBKS vector, restriction endonuclease mapping of DIP cDNA was performed by digestion of the cDNA with specific restriction enzymes. The cloned fragments yielded the number and size of fragments predicted from the published sequence. However, some restriction patterns from the DIP cDNA's were quite different from those of the published standard

Figure 12. PCR amplification of DIP genomic RNAs. (A) Size determination of DIP RNAs. Cells coinfected with the standard virus and DIP were treated with actinomycin D and labeled with [^{3}H]uridine as described in Fig. 8. Intracellular RNA was isolated, separated in a 1% agarose-formaldehyde gel, and detected by fluorography. The size of the standard virus RNAs and mRNAs were used to determine the size of the DIP RNAs. Arrows indicate the positions of DIP RNAs; IND-ST RNA (Ind-ST lane), IND-LT RNA (Ind-LT lane), Ogd-DI RNA (Ogd-DI lane) and Haz-DI RNA (Haz-DI lane). (B) PCR products of DIP cDNAs. One-tenth of the products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide fluorescence. Approximate sizes of DIP DNAs are indicated by comparison with marker DNA (Lane M; *Bst*EII-digested bacteriophage lambda DNA). Lanes: DNA amplified from IND-ST RNA, IND-LT RNA, Ogd-DI RNA and Haz-DI RNA (Ind-ST lane, Ind-LT lane, Ogd-DI lane and Haz-DI lane respectively).


A.
Ind-ST
Ind-LT
Ogd-DI
Haz-DI
STD-
L-
G-
N-
M/P-


B.
Ind-ST
Ind-LT
M
Ogd-DI
Haz-DI
5.3Kb
2.6Kb
6.1Kb
3.0Kb

viruses.

This initial restriction endonuclease mapping combined with previous sequence information (McGeoch and Dolan 1979, Rowlands 1979, Semler *et al.* 1979, Rose 1980, Schubert *et al.* 1980, Gallione *et al.* 1981, Keene *et al.* 1981, Rose and Gallione 1981, De and Perrault 1982, Yang and Lazzarini 1983) was used to establish the location of restriction endonucleases sites within each pBKS insert (Figs. 13-16). One cDNA clone containing the entire coding sequence of each DIP genome (pBKS-DI) was used for further sequence analyses. Based on the restriction map, pBKS-DI containing the complete cDNA of each DIP genome was digested with specific restriction endonucleases to yield the proper size of fragments required for sequencing. The resulting restriction enzyme fragments (5 fragments for IND-ST in Fig. 13, 10 fragments for IND-LT in Fig. 14, 7 fragments for Ogd-DI in Fig. 15, 12 fragments for Haz-DI in Fig. 16) were then subcloned into the pBKS vector.

DNA sequencing analysis was done by the chain-termination method of Sanger *et al.* (1977). Since the multiple cloning sites of the pBKS vector contain sequences compatible with the primers (forward primer and reverse primer) supplied in the Sequenase Sequencing kit, these two primers served for the sequencing of the insert DNA of the all clones. Several independent sequence determinations were carried out with different template DNA preparations to ensure accuracy. The base sequences can be clearly read for over 300 bases from one end of each clone with little ambiguity. Ambiguities in a sequence could be eliminated by comparing several gels. Nonoverlapping regions of the clones of DIP genome were sequenced by designing

Figure 13. The restriction endonuclease map of IND-ST genome and the sequencing strategy for IND-ST cDNA. The numbers at the top indicate distances from the 3' end of the DIP DNA in nucleotides. The indicated restriction sites were used to generate restriction fragments for further subcloning into pBKS (a 641 bp *Bgl*II–*Sau*3AI fragment, a 556 bp *Sau*3AI–*Bst*XI fragment, a 234 bp *Bst*XI–*Sal*I fragment, a 320 bp *Sal*I–*Pst*I fragment and a 386 bp *Pst*I–*Bgl*II fragment). The 6 cDNA clones were either partially or completely used to determine the nucleotide sequence of the IND-ST genome. The arrows indicate the extent, position and direction of sequencing of clones. Black bar, the cDNA clone of the entire IND-ST genome; open bars, cDNA subclones of IND-ST genome.

IND-ST

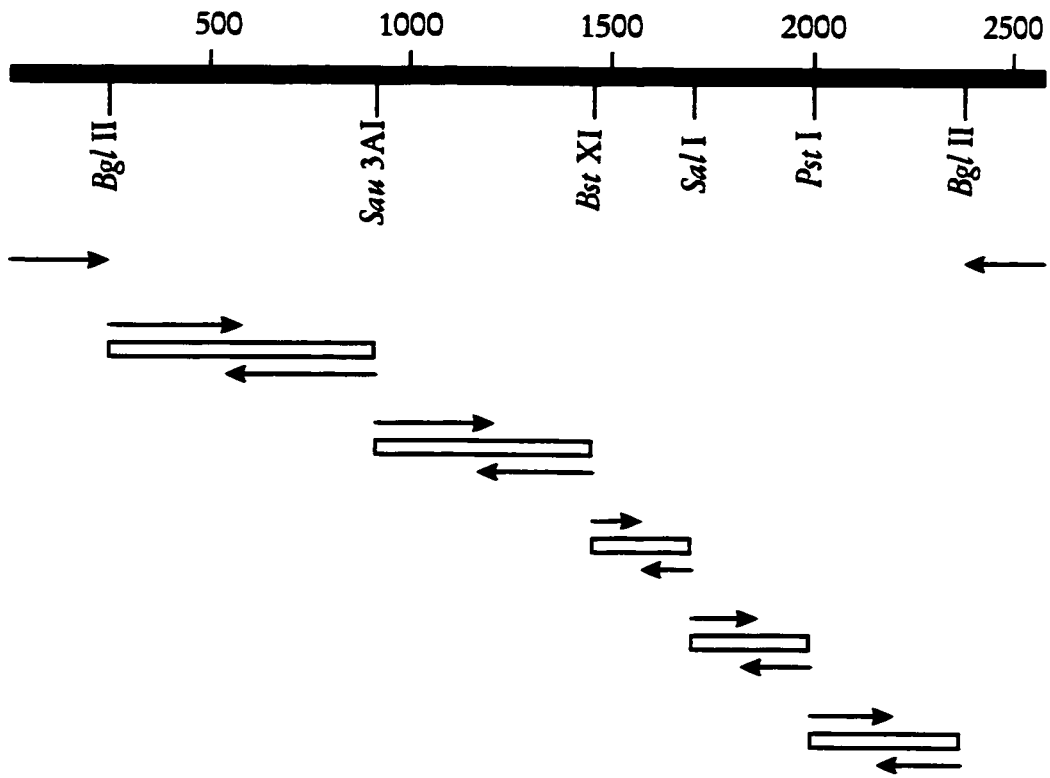


Figure 14. The restriction endonuclease map of IND-LT genome and the sequencing strategy for IND-LT cDNA. The numbers at the top indicate distances from the 3' end of the DIP DNA in nucleotides. The indicated restriction sites were used to generate restriction fragments for further subcloning into pBKS (a 444 bp *Bgl*II–*Pst*I fragment, a 738 bp *Pst*I–*Eco*RV fragment, a 691 bp *Eco*RV–*Eco*RI fragment, a 132 bp *Eco*RI–*Eco*RV fragment, a 553 bp *Eco*RV–*Sac*I fragment, a 297 bp *Bgl*II–*Stu*I fragment, 915 bp & 905 bp *Stu*I–*Stu*I fragments, a 591 bp *Kpn*I–*Stu*I fragment and a 2481 bp *Bgl*II–*Bgl*II fragment). The 11 cDNA clones were either partially or completely used to determine the nucleotide sequence of the IND-LT genome. The arrows indicate the extent, position and direction of sequencing of clones. Black bar, the cDNA clone of the entire IND-LT genome; open bars, cDNA subclones of IND-LT genome.

IND-LT

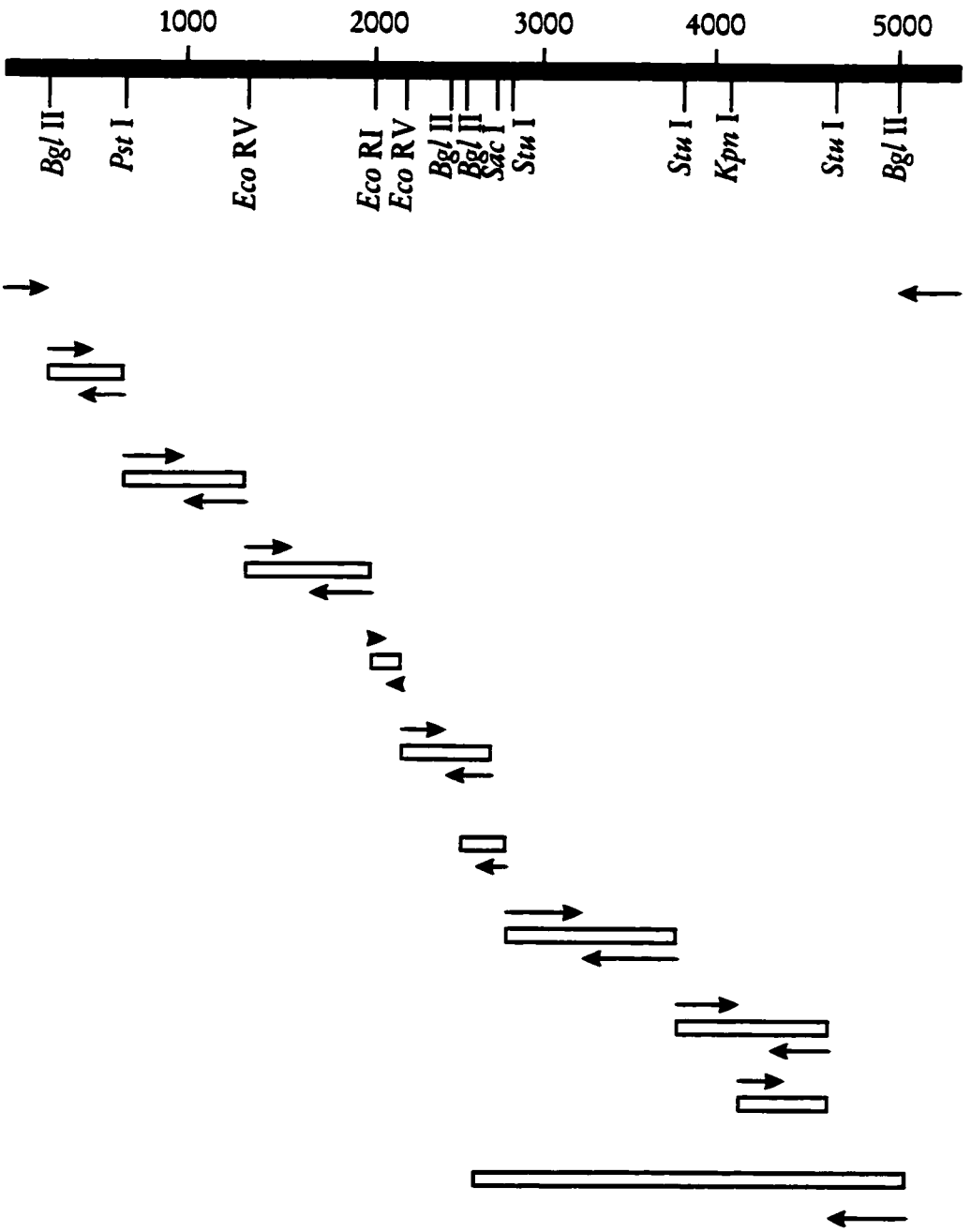

1000
2000
3000
4000
5000
Bgl II
Pst I
Eco RV
Eco RI
Eco RV
Bgl II
Bgl II
Sac I
Stu I
Stu I
Kpn I
Stu I
Bgl II

Figure 15. The restriction endonuclease map of Ogd-DI genome and the sequencing strategy for Ogd-DI cDNA. The numbers at the top indicate distances from the 3' end of the DIP DNA in nucleotides. The indicated restriction sites were used to generate restriction fragments for further subcloning into pBKS (a 438 bp *Not*I–*Xba*I fragment, 270 bp & 847 bp *Xba*I–*Xba*I fragments, a 582 bp *Hinc*II–*Xba*I fragment, a 819 bp & 699 bp *Eco*RV–*Eco*RV fragments and a 567 bp *Pst*I–*Eco*RV fragment). The 8 cDNA clones were either partially or completely used to determine the nucleotide sequence of the Ogd-DI genome. The arrows indicate the extent, position and direction of sequencing of clones. Black bar, the cDNA clone of the entire Ogd-DI genome; open bars, cDNA subclones of Ogd-DI genome.

Ogd-DI

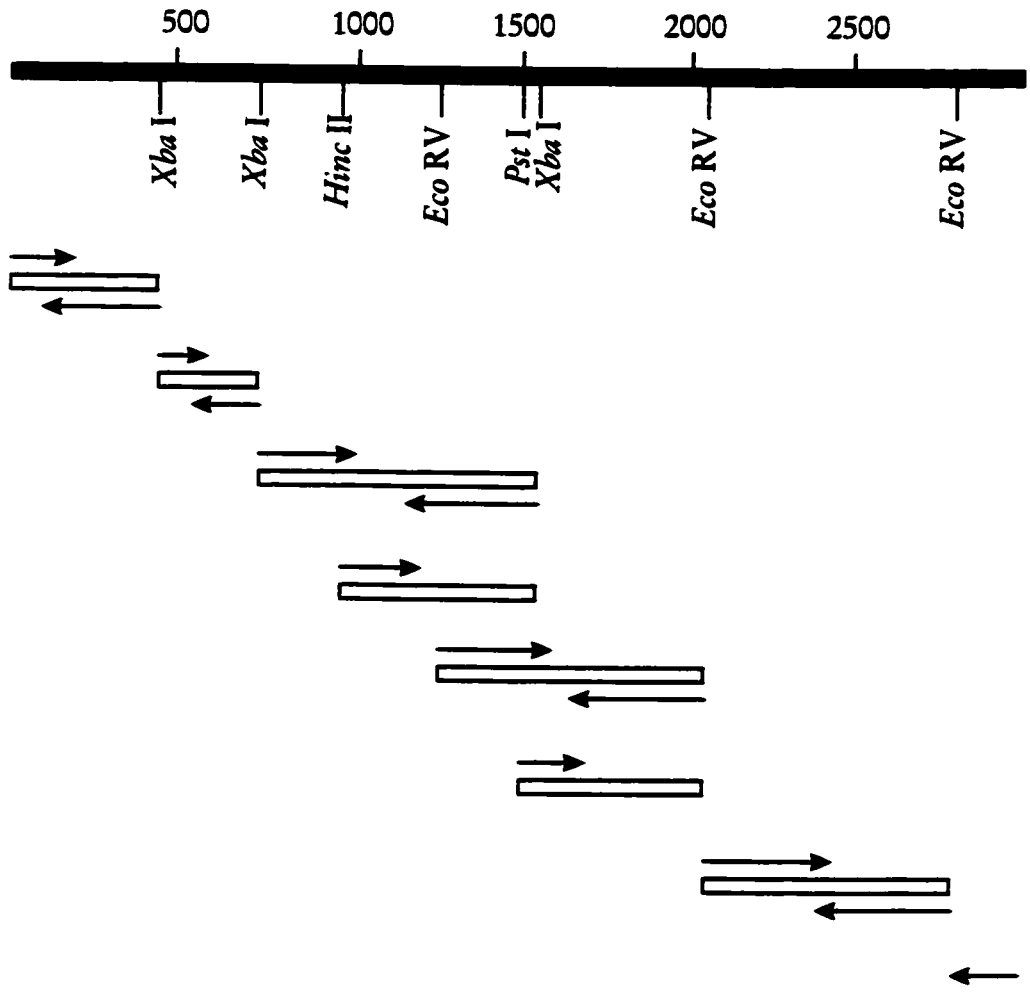

500
1000
1500
2000
2500
Xba I
Xba I
Hinc II
Eco RV
Pst I
Xba I
Eco RV
Eco RV

Figure 16. The restriction endonuclease map of Haz-DI genome and the sequencing strategy for Haz-DI cDNA. The numbers at the top indicate distances from the 3' end of the DIP DNA in nucleotides. The indicated restriction sites were used to generate restriction fragments for further subcloning into pBKS (a 858 bp *Not*I–*Bam*HI fragment, 234 bp & 1522 bp *Acc*I–*Acc*I fragments, 345 bp & 222 bp *Sau*3AI–*Sau*3AI fragments, a 623 bp *Acc*I–*Bam*HI fragment, a 441 bp *Bam*HI–*Sac*I fragment, a 768 bp *Sac*I–*Hinc*II fragment, a 497 bp *Xmn*I–*Eco*RI fragment, a 467 bp *Hinc*II–*Sac*I fragment, a 680 bp *Sac*I–*Sac*I fragment and a 675 bp *Sac*I–*Eco*RV fragment). The 13 cDNA clones were either partially or completely used to determine the nucleotide sequence of the Haz-DI genome. The arrows indicate the extent, position and direction of sequencing of clones. Black bar, the cDNA clone of the entire Haz-DI genome; open bars, cDNA subclones of Haz-DI genome.

Haz-DI

1000
2000
3000
4000
5000
6000
Acc I
Acc I
Bam HI
Sau 3AI
Sau 3AI
Sau 3AI
Acc I
Bam HI
Sac I
Xmn I
Hinc II
Hinc II
Eco RI
Sac I
Sac I
Eco RV

special primers and by comparing to the standard virus genomes.

Sequence analyses of DIP genomes

(a) IND-ST

The complete nucleotide sequence information of IND-ST is shown in Fig. 17. Sequence analyses revealed that the IND-ST genome is 2660 nucleotides in length (i.e. 24% of the standard virus genome) and that 2606 nucleotides at the 5' end of the IND-ST genome are identical to the 5' end of IND-standard virus genome. Sequence analysis of the 3' end of the IND-ST genome showed the 54 terminal bases to be the inverted complement of the 5' terminus, confirming that IND-ST RNA is a copyback type DIP genome possessing a terminal panhandle or stem structure as described previously (Perrault *et al.* 1978, Schubert *et al.* 1979, Rao and Huang 1982, Kolakofsky 1982). Surprisingly, this IND-ST was found to possess the same 54 base inverted complementary termini as found in an earlier report (Nichol *et al.* 1984). However, the size of the DIP genome is different; our IND-ST (2660 nucleotides) is larger than the published IND-ST1 (~2200 nucleotides) (Nichol *et al.* 1984).

Four point mutations (positions 365 [A to G], 483 [A to G], 1736 [T to C] and 2308 [C to T]), two insertions (positions 1265 [T] and 1595 [T]) and one deletion (position 1035 [G]) were detected among different cDNA clones prepared from DIP RNA of the same strain of the standard virus. These mutations may be explained by the high mutability of VSV, the infidelity of reverse transcription during cDNA synthesis, amplification of cDNA during PCR, or combination of these.

Figure 17. Nucleotide sequences of IND-ST genome from the cDNA clones. The sequence of IND-ST was compared with the previously published sequence of the WT IND strain of VSV (Wagner 1987). This cDNA sequence corresponds to (+)-sense polarity. The inverted complementary termini of the DIP genome are underlined. The point mutations (bold letter), insertions (bold underlined) and deletion (*) are indicated.

```
   3'ACGAAGACCA CAAAACCAGA TAAAAAATAA AAACCACAAG AGGGTCTTAA GGATTGCTCA
  61 AATTACCACC ACTGTTGCAA GAGACGGATG GATCACCAGT TGTACAGATC ATTATCATAT
 121 TGCCTGTAAG TCCTGTTTGA GACCCATAGA AGAGATCACC CTGGACTCAA GTATGGACTA
 181 CACGCCCCCA GATGTATCCC ATGTGCTGAA GACATGGAGG AATGGGGAAG GTTCGTGGGG
 241 ACAAGAGATA AAACAGATCT ATCCTTTAGA AGGGAATTGG AAGAATTTAG CACCTGCTGA
 301 GCAATCCTAT CAAGTCGGCA GATGTATAGG TTTTCTATAT GGAGACTTGG CGTATAGAAA
 361 ATCTGCTCAT GCCGAGGACA GTTCTCTATT TCCTCTATCT ATACAAGGTC GTATTAGAGG
 421 TCGAGGTTTC TTAAAAGGGT TGGTAGACGG ATTAATGAGA GCAAGTTGCT GCCAAGTAAT
 481 ACGCCGGAGA AGTCTGGCTG ATTTGAAGAG GCCGGCCAAC GCAGTGTAGG GAGGTTTGAT
 541 TTACTTGATT GATAAATTGA GTGTATCACC TCCATTCCTT TCTCTTACTA GATCAGGACC
 601 TATTAGAGAC GAATTAGAAA CGATTCCCCA CAAGATCCCA ACCTCCTATC CGACAAGCAA
 661 CCGTGATATG GGGGTGATTG TCAGAAATTA CTTCAAATAC CAATGCCGTC TAATTGAAAA
 721 GGGAAAATAC AGATCACATT ATTCACAATT ATGGTTATTC TCAGATGTCT TATCCATAGA
 781 CTTCATTGGA CCATTCTCTA TTTCCACCAC CCTCTTGCAA ATCCTATACA AGCCATTTTT
 841 ATCTGGGAAA GATAAGAATG AGTTGAGAGA GCTGGCAAAT CTTTCTTCAT TGCTAAGATC
 901 AGGAGAGGGG TGGGAAGACA TACATGTGAA ATTCTTCACC AAGGACATAT TATTGTGTCC
 961 AGAGGAAATC AGACATGCTT GCAAGTTCGG GATTGCTAAG GATAATAATA AAGACATGAG
1021 CTATCCCCCT TGGG*AAGGGA ATCCAGAGGG ACAATTACAA CAATCCCTGT TTATTATACG
1081 ACCACCCCTT ACCCAAAGAT GCTAGAGATG CCTCCAAGAA TCCAAAATCC CCTGCTGTCC
1141 GGAATCAGGT TGGGCCAATT ACCAACTGGC GCTCATTATA AAATTCGGAG TATATTACAT
1201 GGAATGGGAA TCCATTACAG GGACTTCTTG AGTTGTGGAG ACGGCTCCGG AGGGATGACT
1261 GCTGTCATTA CTACGAGAAA ATGTGCATAG CAGAGGAATA TTCAATAGTC TGTTAGAATT
1321 ATCAGGGTCA GTCATGCGAG GCGCCTCTCC TGAGCCCCCC AGTGCCCTAG AAACTTTAGG
1381 AGGAGATAAA TCGAGATGTG TAAATGGTGA AACATGTTGG GAATATCCAT CTGACTTATG
1441 TGACCCAAGG ACTTGGGACT ATTTCCTCCG ACTCAAAGCA GGCTTGGGGC TTCAAATTGA
1501 TTTAATTGTA ATGGATATGG AAGTTCGGGA TTCTTCTACT AGCCTGAAAA TTGAGACGAA
1561 TGTTAGAAAT TATGTGCACC GGATTTTGGA TGAGTCAAGG AGTTTTAATC TACAAGACTT
1621 ATGGAACATA TATTTGTGAG AGCGAAAAGA ATGCAGTAAC AATCCTTGGT CCCATGTTCA
1681 AGACGGTCGA CTTAGTTCAA ACAGAATTTA GTAGTTCTCA AACGTCTGAA GTATACATGG
1741 TATGTAAAGG TTTGAAGAAA TTAATCGATG AACCCAATCC CGATTGGTCT TCCATCAATG
1801 AATCCTGGAA AAACCTGTAC GCATTCCAGT CATCAGAACA GGAATTTGCC AGAGCAAAGA
1861 AGGTTAGTAC ATACTTTACC TTGACAGGTA TTCCCTCCCA ATTCATTCCT GATCCTTTTG
1921 TAAACATTGA GACTATGCTA CAAATATTCG GAGTACCCAC GGGTGTGTCT CATGCGGCTG
1981 CCTTAAAATC ATCTGATAGA CCTGCAGATT TATTGACCAT TAGCCTTTTT TATATGGCGA
2041 TTATATCGTA TTATAACATC AATCATATCA GAGTAGGACC GATACCTCCG AACCCCCCAT
2101 CAGATGGAAT TGCACAAAAT GTGGGGATCG CTATAACTGG TATAAGCTTT TGGCTGAGTT
2161 TGATGGAGAA AGACATTCCA CTATATCAAC AGTGTTTAGC AGTTATCCAG CAATCATTCC
2221 CGATTAGGTG GGAGGCTGTT TCAGTAAAAG GAGGATACAA GCAGAAGTGG AGTACTAGAG
2281 GTGATGGGCT CCCAAAAGAT ACCCGAATTT CAGACTCCTT GGCCCCAATC GGGAACTGGA
2341 TCAGATCTCT GGAATTGGTC CGAAACCAAG TTCGTCTAAA TCCATTCAAT GAGATCTTGT
2401 TCAATCAGCT ATGTCGTACA GTGGATAATC ATTTGAAATG GTCAAATTTG CGAAGAAACA
2461 CAGGAATGAT TGAATGGATC AATAGACGAA TTTCAAAAGA AGACCGGTCT ATACTGATGT
2521 TGAAGAGTGA CCTACACGAG GAAAACTCTT GGAGAGATTA AAAAATCATG AGGAGACTCC
2581 AAACTTTAAG TATGAAAAAA ACTTTGATCC TTAAGACCCT CTTGTGGTTT TTATTTTTTA
2641 TCTGGTTTTG TGGTCTTCGT 5'
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(b) IND-LT

Fig. 18 shows the sequence of the IND-LT genome. The genome consists of 5,313nucleotides (i.e. 48% of the standard virus genome). The IND-LT cDNA sequences contain the same 3' and 5' termini as the standard virus genome. IND-LT cDNA retains an exact copy of the 3' half of the standard genome (leader, N, P, M and G genes) and contains a large internal deletion within the L gene. The deletion begins 4974 residues from 3' end of the leader region and extends to 10823 bases of the standard virus genome as previously described (Perrault and Semler 1979, Epstein *et al.* 1980). IND-LT cDNA has a deletion of 5849 bases, which corresponds to 92% of the L gene.

The nucleotide sequence of the IND-LT which was generated from the IND-HR strain of VSV was compared to that published for the IND-WT strain of VSV. A summary of the mutations within the IND-LT and their potential effect on the amino acid sequence is shown in Table 5. Sequence comparison revealed 87 point mutations, three insertions and three deletions. The positions of the mutations within the IND-LT genome are indicated as bold letters. The 87 base substitutions listed result in 49 silent mutations, without a change of the amino acid, and alterations in 28 amino acids. The most interesting mutations in the sequence, however, consisted of single-base deletions (positions 2407 and 2521) and single-base insertions (positions 2409 and 2580) found in the M gene resulting in 17 amino acid changes. These mutations, if expressed, would dramatically affect the primary structure of the M protein. The distribution of these mutations within the IND-LT sequence did not reveal any

Figure 18. Nucleotide sequences of IND-LT genome from the cDNA clones. The sequence of IND-LT was compared with the previously published sequence of the WT IND strain of VSV (Wagner 1987). This cDNA sequence corresponds to (+)-sense polarity. The inverted complementary termini of the DIP genome are underlined. The point mutations (bold letter), insertions (bold underlined) and deletions (*) are indicated. The site of internal deletion in IND-LT begins at position 4974 and extends to position 10823 (arrow marked).

```
   3'ACGAAGACAA ACAAACCATT ATTATCATTA AAAGGCTCAG GAGAAACTTT AACAGTAATC
  61  AAAATGTCTG TTACAGTCAA GAGAATCATT GACAACACAG TCATAGTTCC AAAACTTCCT
 121  GCAAATGAGG ATCCAGTGGA ATACCCGGCA GATTACTTCA GAAAATCAAA GGAGATTCCT
 181  CTTTACATCA ATACTACAAA AAGTTTGTCA GATCTAAGAG GATATGTCTA CCAAGGCCTC
 241  AAATCCGGAA ATGTATCAAT CATACATGTC AACAGCTACT TGTATGGAGC ATTGAAGGAC
 301  ATCCGGGGTA AGTTGGATAA AGATTGGTCA AGTTTCGGAA TAAACATCGG GAAGGCAGGG
 361  GATACAATCG GAATATTTGA CCTTGTATCC TTGAAAGCCC TGGACGGTGT ACTTCCAGAT
 421  GGAGTATCGG ATGCTTCCAG AACCAGCGCA GATGACAAAT GGTTGCCTTT GTATCTACTT
 481  GGCTTATACA GAGTGGGCAG AACACAAATG CCTGAATACA GAAAAAGGCT CATGGATGGG
 541  CTGACAAATC AATGCAAAAT GATCAATGAA CAGTTTGAAC CTCTTGTGCC AGAAGGTCGT
 601  GACATTTTTG ATGTGTGGGG AAATGACAGT AATTACACAA AAATTGTCGC TGCAGTGGAC
 661  ATGTTCTTCC ACATGTTCAA AAAACATGAA TGTGCCTCGT TCAGATACGG AACTATTGTT
 721  TCCAGATTCA AAGATTGTGC TGCATTGGCA ACATTTGGAC ACCTCTGCAA AATAACCGGA
 781  ATGTCTACAG AAGATGTAAC GACCTGGATC TTGAACCGAG AAGTTGCAGA TGAGATGGTC
 841  CAAATGATGC TTCCAGGCCA AGAAATTGAC AAGGCCGATT CATACATGCC TTATTTGATC
 901  GACTTTGGAT TGTCTTCTAA GTCTCCATAT TCTTCCGTCA AAAACCCTGC CTTCCACTTC
 961  TGGGGGCAAT TGACAGCTCT TCTGCTCAGA TCCACCAGAG CAAGGAATGC CCGACAGCCT
1021  GATGACATTG AGTATACATC TCTTACTACA GCAGGTTTGT TGTACGCTTA TGCAGTAGGA
1081  TCCTCTGCTG ACTTGGCACA ACAGTTTTGT GTTGGAGATA GCAAATACAC TCCAGATGAT
1141  AGTACCGGAG GATTGACGAC TAATGCACCG CCACAAGGCA GAGATGTGGT CGAATGGCTC
1201  GGATGGTTTG AAGATCAAAA CAGAAAACCG ACTCCTGATA TGATGCAGTA TGCGAAACGA
1261  GCAGTCATGT CACTGCAAGG CCTAAGAGAG AAGACAATTG GCAAGTATGC TAAGTCAGAA
1321  TTTGACAAAT GACCCTATAA TTCTCAGATC ACCTATTATA TATTATGCTA CATATGAAAA
1381  AAACTAACAG ATATCATGGA TAATCTCACA AAAGTTCGTG AGTATCTCAA GTCCTATTCT
1441  CGTCTAGATC AGGCGGTAGG AGAGATAGAT GAGATCGAAG CACAACGAGC TGAAAAGTCC
1501  AATTATGAGT TGTTCCAAGA GGACGGAGTG GAAGAGCATA CTAGGCCCTC TTATTTTCAG
1561  GCAGCAGATG ATTCTGACAC AGAATCTGAA CCAGAAATTG AAGACAATCA AGGCTTGTAT
1621  GTACCAGATC CGGAAGCTGA GCAAGTTGAA GGCTTTATAC AGGGGCCTTT AGATGACTAT
1681  GCGGATGAGG ACGTGGATGT TGTATTCACT TCGGACTGGA AACAGCCTGA GCTTGAATCC
1741  GACGAGCATG GAAAGACCTT ACGGTTGACA TTGCCAGAGG GTTTAAGTGG AGAGCAGAAA
1801  TCCCAGTGGC TTTTGACGAT TAAAGCAGTC GTTCAAAGTG CCAAACGCTG GAATCTGGCA
1861  GAGTGCACAT TTGAAGCATC GGGAGAAGGG GTCATCATAA AAAAGCGCCA GATAACTCCG
1921  GATGTATATA AGGTCACTCC AGTGATGAAC ACACATCCGT CCCAATCAGA AGCCGTATCA
1981  GATGTTTGGT CTCTCTCAAA GACATCCATG ACTTTCCAAC CCAAGAAAGC AAGTCTTCAG
2041  CCTCTCACCA TATCCTTGGA TGAATTGTTC TCATCTAGAG GAGAATTCAT CTCTGTCGGA
2101  GGTAACGGAC GAATGTCTCA TAAAGAGGCC ATCCTGCTCG GTCTGAGGTA CAAAAAGTTG
2161  TACAATCAGG CGAGAGTCAA ATATTCTCTG TAGACTATGA AAAAAAGTAA CAGATATCAC
2221  AATCTAAGTG TTATCCCAAT CCATTCATCA TGAGTTCCTT AAAGAAGATT CTCGGTCTGA
2281  AGGGGAAAGG TAAGAAATCT AAGAAATTAG GGATCGCACC ACCCCCTTAT GAAGAGGACA
2341  CTAACATGGA GTATGCTCCG AGCGCTCCAA TTGACAAATC CTATTTTGGA GTTGACGAGA
2401  TGGACA*CTCA TGATCCGCAT CAATTAAGAT ATGAGAAATT CTTCTTTACA GTGAAAATGA
2461  CGGTTAGATC TAATCGTCCG TTCAGAACAT ACTCAGATGT GGCAGCCGCT GTATCCCATT
2521  *GGATCACATG TACATCGGAA TGGCAGGGAA ACGTCCCTTC TACAAGATCT TGGCTTTTTT
2581  TGGGTTCTTC TAATCTAAAG GCCACTCCAG CGGTATTGGC AGATCAAGGT CAACCAGAGT
2641  ATCACGCTCA CTGTGAAGGC AGGGCTTATT TGCCACACAG AATGGGGAAG ACCCCTCCCA
2701  TGCTCAATGT ACCAGAGCAC TTCAGAAGAC CATTCAATAT AGGTCTTTAC AAGGGAACGG
2761  TTGAGCTCAC AATGACCATC TACGATGATG AGTCACTGGA AGCAGCTCCT ATGATCTGGG
2821  ATCATTTCAA TTCTTCCAAA TTTTCTGATT TCAGAGAGAA GGCCTTAATG TTTGGCCTGA
2881  TTGTCGAGAA AAAGGCATCT GGAGCTTGGG TCCTGGATTC TGTCAGCCAC TTCAAATGAG
2941  CTAGTCTAGC TTCCAGCTTC TGAACAATCC CCGGTTTACT CAGTCTCTCC TAATTCCAGC
3001  CTTTCGAACA ACTAATATCC TGTCTTTTCT ATCCCTATGA AAAAAACTAA CAGAGATCGA
3061  TCTGTTTCCT TGACACCATG AAGTGCCTTT TGTACTTAGC TTTTTTATTC ATCGGGGTGA
3121  ATTGCAAGTT CACCATAGTT TTTCCATACA ACCAAAAAGG AAACTGGAAA AATGTTCCTT
3181  CCAATTACCA TTATTGCCCG TCAAGCTCAG ATTTAAATTG GCATAATGAC TTAATAGGCA
3241  CAGCCTTACA AGTCAAAATG CCCAAGAGTC ACAAGGCTAT TCAAGCAGAC GGTTGGATGT
3301  GTCATGCTTC CAAATGGGTC ACTACTTGTG ATTTCCGCTG GTACGGACCG AGGTATATAA
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3361  CACATTCCAT CCGATCCTTC ACTCCATCTG TAGAACAATG CAAGGAAAGC ATTGAACAAA
3421  CGAAACAAGG AACTTGGCTG AATCCAGGCT TCCCTCCTCA AAGTTGTGGA TATGCAACTG
3481  TGACGGATGC TGAAGCAGCG ATTGTCCAGG TGACTCCTCA CCATGTGCTT GTTGATGAAT
3541  ACACAGGAGA ATGGGTTGAT TCACAGTTCA TCAACGGAAA ATGCAGCAAT GACATATGCC
3601  CCACTGTCCA TAACTCCACA ACCTGGCATT CCGACTATAA GGTCAAAGGG CTATGTGATT
3661  CTAACCTCAT TTCCATGGAC ATCACCTTCT TCTCAGAGGA CGGAGAGCTA TCATCCCTAG
3721  GAAAGGAGGG CACAGGGTTC AGAAGTAACT ACTTTGCTTA TGAAACTGGA GACAAGGCCT
3781  GCAAAATGCA GTACTGCAAG CATTGGGGAG TCAGACTCCC ATCAGGTGTC TGGTTCGAGA
3841  TGGCTGATAA GGATCTCTTT GCTGCAGCCA GATTCCCTGA ATGCCCAGAA GGGTCAAGTA
3901  TCTCTGCTCC ATCTCAGACC TCAGTGGATG TAAGTCTCAT TCAGGACGTT GAGAGGATCT
3961  TGGATTATTC CCTCTGCCAA GAAACCTGGA GCAAAATCAG AGCGGGTCTT CCCATCTCTC
4021  CAGTGGATCT CAGCTATCTT GCTCCTAAAA ACCCAGGAAC CGGTCCTG*TC TTCACCATAA
4081  TCAATGGTAC CCTAAAATAC TTTGAGACCA GATACATCAG AGTCGATATT GCTGCTCCAA
4141  TCCTCTCAAG AATGGTCGGA ATGATCAGTG GAACTACCAC AGAAAGGGAA CTGTGGGATG
4201  ACTGGGCTCC ATATGAAGAC GTGGAAATTG GACCCAATGG AGTTCTGAGG ACCAGTTCAG
4261  GATATAAGTT TCCTTTATAT ATGATTGGAC ATGGTATGTT GGACTCCGAT CTTCATCTTA
4321  GCTCAAAGGC TCAGGTGTTT GAACATCCTC ACATTCAAGA CGCTGCTTCG CAACTTCCTG
4381  ATGATGAGAC TTTATTTTTT GGTGATACTG GGCTATCCAA AAATCCAATC GAGTTTGTAG
4441  AAGGTTGGTT CAGTAGTTGG AAGAGCTCTA TTGCCTCTTT TTTCTTTATC ATAGGGTTAA
4501  TCATTGGACT ATTCTTGGTT CTCCGAGTTG GTATTTATCT TTGCATTAAA TTAAAGCACA
4561  CCAAGAAAAG ACAGATTTAT ACAGACATAG AGATGAACCG ACTTGGGAAG TAACTCAAAT
4621  CCTGCACAAC AGATTCTTCA TGTTTGAACC AAATCAACTT GTGATATCAT GCTCAAAGAG
4681  GCCTTAATTA TATTTTAATT TTTAATTTTT ATGAAAAAAA CTAACAGCAA TCATGGAAGT
4741  CCACGATTTT GAGACCGACG AGTTCAATGA TTTCAATGAA GATGACTATG CCACAAGAGA
4801  ATTCCTGAAT CCCGATGAGC GCATGACGTA CTTGAATCAT GCTGATTACA ATTTGAATTC
4861  TCCTCTAATT AGTGATGATA TTGACAATTT GATCAGGAAA TTCAATTCTC TTCCGATTCC
4921  CTCGATGTGG GATAGTAAGA ACTGGGATGG AGTTCTTGAG ATGTTAACAT CATGGCCCCA
4981  ATCGGGAACT GGATCAGATC TCTGAAATTG GTCCGAAACC AAGTTCGTCT AAATCCATTC
5041  TTGCGAAAAA ACACAGGAAT GATTGAATGG ATCAATAGAC GAATTTCAAA AGAAGACCGG
5101  AATGAGATCT TGTTCAATCA GCTATGTCGT ACAGTGGATA ATCATTTGAA ATGGTCAAAT
5161  TCTATACTGA TGTTGAAGAG TGACCTACAT GAGGAAAACT CTTGGAGAGA TTAAAAAATC
5221  ATGAGGAGAC TCCAAACTTT AAGTATGAAA AAAACTTTGA TCCTTAAGAC CCTCTTGTGG
5281  TTTTTATTTT TTATCTGGTT TTGTGGTCTT CGT 5'
```

TABLE 5

Number of mutations of Ind-LT

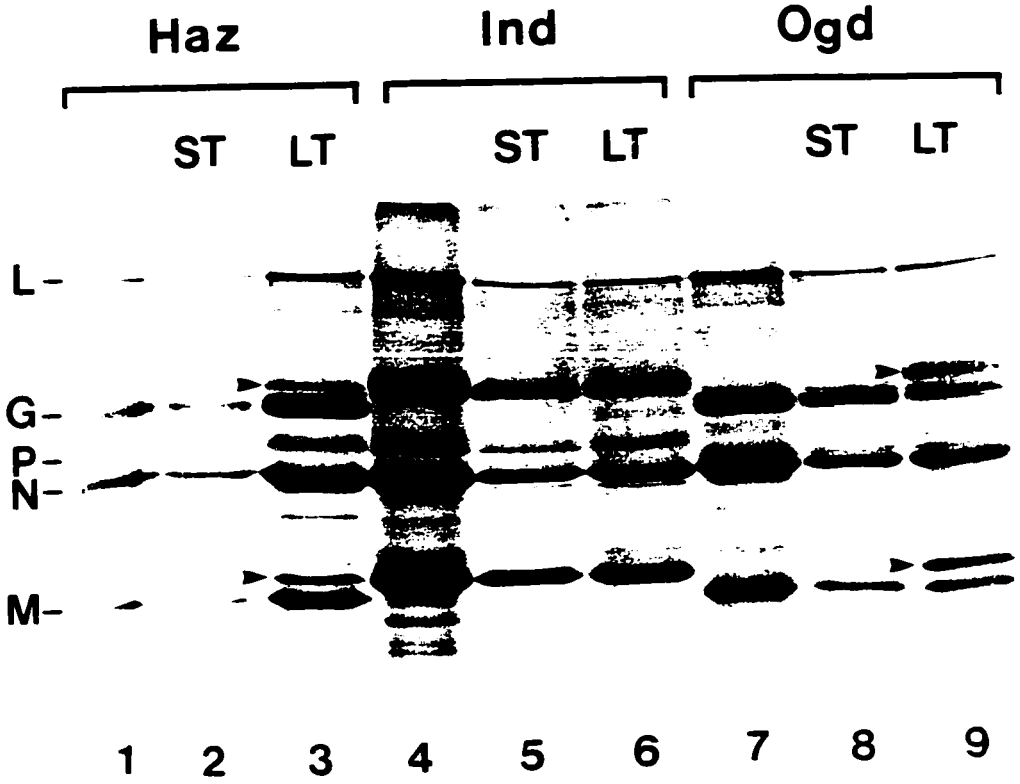
	Transitions	Transversions	Insertions	Deletions	Amino acids changes
N	7	1	–	–	2
P	23	5	–	–	11
M	11	3	2	2	22
G	25	9	1	1	11
ΔL	3	–	–	–	NA[a]

[a] Nonapplicable

significant clustering of mutations in particular regions. At least 2 identical cDNA clones make up the consensus sequence and were compared with the published standard virus sequences.

The IND-LT (5313 nucleotides) was shown to have an organization identical to that of the standard virus. The genome consists of four ORFs. The first ORF (N gene) starts from an AUG at nucleotide 64 and terminates with stop codon UGA at nucleotide 1329. ORF 2 (P gene) is from nucleotide 1396 and terminates with a UAG at nucleotide 2190. ORF 3 (M gene) begins at nucleotide 2249 and terminates with a stop codon UGA at nucleotide 2936. The last ORF (G gene) starts from an AUG at nucleotide 3078 and terminates with a stop codon UAA at nucleotide 4610. The L gene of IND-LT contains only 530 bases out of 6373 bases of the complete L gene. The presence of Indiana serotype-specific proteins generated by IND-LT corresponding to two of the four the predicted ORFs (Fig. 19, lanes 3, 6 and 9) was detected since the sizes of G and M proteins of IND and NJ standard virus were clearly distinguishable by SDS-PAGE. The sizes of G and M proteins encoded by the IND-LT were identical to those of the IND standard virus. Although it is not known what influence the amino acid substitutions listed in Table 2 would have on the performance of the proteins and its multiple functions, these results clearly demonstrate the IND-LT directs protein synthesis. The effects of single-base insertions or deletions could potentially result in the restoration of the reading frame, thus complete proteins would have been synthesized.

Figure 19. SDS-PAGE analysis of virion proteins recovered from coinfections of IND-DIPs with standard IND and NJ viruses. Cells were infected with standard virus alone or with DIPs (1:100 dilution). After 16 hrs of incubation, the viruses were concentrated by ultracentrifugation (25,000 rpm, 90 min). Viral pellets were then resolved by SDS-PAGE. The gel was stained with Coomassie blue and dried in a gel drier. Lane 1, NJ-Haz infected cells; lane 2, NJ-Haz and IND-ST coinfected cells; lane 3, NJ-Haz and IND-LT coinfected cells; lane 4, IND infected cells; lane 5, IND and IND-ST coinfected cells; lane 6, IND and IND-LT coinfected cells; lane 7, NJ-Ogd infected cells; lane 8, NJ-Ogd and IND-ST coinfected cells; lane 9, NJ-Ogd and IND-LT coinfected cells. The positions of the viral proteins (L, G, P, N and M) are indicated. Arrowheads represent proteins synthesized by IND-LT.


Haz
Ind
Ogd
ST
LT
ST
LT
ST
LT
L-
G-
P-
N-
M-
1
2
3
4
5
6
7
8
9

(c) NJ-DIPs

The first complete sequence analyses of NJ-DIP genomes revealed that the Ogd-DI genome contains 2984 nucleotides and the Haz-DI genome contains 6110 nucleotides (Fig. 20 and 21). These two NJ-DIP genomes possessed the 5' end of the L gene. Ogd-DI cDNA started 3535 bases from the beginning of L gene and retained the remainder of the L gene and Haz-DI cDNA had a deletion of 480 bases from the beginning of the L gene but retained the remainder of the L gene. The 3' terminus of the Ogd-DI genome corresponded to the inverted complement of the 5' terminal 18 bases. A full 71 nucleotides of the 3' terminus of Haz-DI genome was the precise complement of the 5' terminus of the Haz-DI genome. Beginning at position 19 (Ogd-DI) or position 72 (Haz-DI) from the 5' end of each DIP genome, however, the sequence was homologous to the 5'-terminus of each standard virus genome (Feldhaus and Lesnaw 1988, Barik *et al.* 1990). Thus, the NJ-DIP genomes contained panhandle stem structures of 18 nucleotides and 71 nucleotides for Ogd-DI and Haz-DI, respectively. This sequence analysis demonstrated that the two NJ-DIP genomes belong to the copyback DIP class, with different lengths of inverted complementary termini. Many sequence differences between the DIPs obtained here and the published sequence of the standard viruses were observed, but have not been shown here because so many mutations were detected. These differences may reflect the fact that these DIPs were derived from different isolates of standard viruses than the viruses for which sequences has been published (Feldhaus and Lesnaw 1988, Barik *et al.* 1990).

All of the DIP sequences obtained were identified in the standard virus genome

Figure 20. Nucleotide sequences of Ogd-DI genome from the cDNA clones. This cDNA sequence corresponds to (+)-sense polarity. The inverted complementary termini of the DIP genome are underlined.

```
   3'ACGAAGACAA AAAAACCAAT AATTAAAAGA GCAACTAGAT TAAGGGACGC CATTTCTTGG
  61 TTCATCCCAC CCGAGTCTCC TCTGTCTACA TGCATTTTGA ATAATATTCA GGCTTTAACA
 121 GGAGAAGATT GGAGCTCTAA ACAACATGGC TTTAAGAGGA CAGGATCGGC ATTACATAGA
 181 TTTTCCACCT CCCGGATGAG CAATGGAGGA TTTGCCTCAC AAAGCCCGGC CACCTTAACT
 241 CGCATGATTG CAACTACAGA CACAATGAGA GACTTTGGTA CAAAAAATTA TGATTTCATG
 301 TTCCAAGCAT CTTTGTTATA TGGACAGATG ACAACAAGTA TTTCAAGATA TGGAACCCCA
 361 GGGTCTTGCA CGGATCATTA CCATATCAGA TGTAAAGGAT GCATTAGAGA GATTGAAGAA
 421 GTAGAACTGA ACACTAGTCT AGAATACAAG ACGCCCGATG TTTCTCACAT ATTGGAAAAA
 481 TGGAGGAACA ATACTGGATC TTGGGGTCAT CAAATCAAAC AATTGAAACC TGCCGAGGGA
 541 AACTGGGAAT CATTGTCTCC TGTAGAGCAA TCATATCAAG TTGCAAGATG TATTGGATTT
 601 CTTTATGGTG AACTGACACA CAAGAAATCG AGACAGGCTG ATGATAGTTC TCTATTCCCC
 661 TTGAGCATCC AACTAAAAGT GAGAGGGAGA GGTTTCTTGC GAGGCCTTCT AGACGGTTTG
 721 ATGAGATCCA GTTGCTGTCA GGTAATTCAT AGACGAAGTG TTTCTACCTT AAAGAGACCT
 781 GCAAATGCAG TTTACGGTGG TCTCATATAC CTCATTGATA AACTAAGTGC CTCAAGTCCC
 841 TTCTTATCAC TTGTAAGAAC CGGACCTATT CGACAAGAAT TAGAACAGGT GCCACACAAG
 901 ATGTCTACAT CGTATCCAAC GAACATTAGG GACTTGGGCT CCATTGTGAG AAATTATTTT
 961 AAATATCAGT GTCGACCAGT TGAGAGAGGA AACTATAAAA CTTATTATAA TCAAATATGG
1021 TTATTTTCTG ATGTTTTGTC CACTGAATTT ATAGGGCCAA TGGCTATATC CAGCTCTCTC
1081 CTTAGACTCC TTTATCGACC TTCTCTGACA AAGAAGGATA GAGAAGAATT GAGGGAGTTA
1141 GCAGCACTAT CATCTAATTT ACGAAGTGGA GAAGATTGGG ATGATTTACA TATCAAATTT
1201 TTCTCAAACG ATCTCCTCTT TTGCTCACAA GAGATAAGAC ATGCTTGCAA ATTTGGGATT
1261 AAAAAAGATA ATGAAGATAT CACTTTTTAC CCGAATTGGG GAACAGAGTA CATTGGGAAC
1321 GTCATTGATA TTCCTGTGTT TTACAGGGCT CAAAATGTCA AAAAGGATAT TAAAGTACCC
1381 CCTCGGATCC AAAATCCCTT GATGTCCGGA CTTCGACTAG GACAGTTACC CACCGGAGCT
1441 CACTACAAGA TGAGAACCAT TATATCCCGT TTTAAAATCC CCTATCATGA TTTTCTAGCT
1501 TGTGGGGATG GGTCGGGAGG AATGACTGCA GCCTTGCTTC GACTTAATAG AGCATCTAGA
1561 GGAATTTTCA ACAGTTTGTT GGATTTATCA GACACGATGT TAAGAGGATC TTCCCCGGAA
1621 CCTCCCAGCG CCCTTGAGAC CCTAGGGGGA GAAAGATCAA GATGTGTAAA CGGAGATAGC
1681 TGCTGGGAAC ATCCTTCCGA TCTTAGTGAT GAGAACACCT GGAAATACTT TCTACATGTT
1741 AAAAGGGGAT GTGGAATGAG TATCAACCTT ATTACTATGG ACATGGAAGT GAAGGACCCT
1801 ACCATGTCAT ACAAAATTGA ATTATTGGTC AGACAATATG TCCCAGTTTT ATTAGAGTCC
1861 GACGGTTGCC TCATTTACAA AACATATGGA ACATATATCG CTACACAAAA AGATAACTCT
1921 TTGACTCTTA TAGGATCACT CTTTCATTCT GTTCAACTTG TCCAAACAGA CTTAAGTTCT
1981 AGTAACACAT CCGAATTGTA TCTAGTCTGT AGAAGATTGA AAGATTACAT CGATACTCCT
2041 TTTGTAGATT GGATAGAATT GTACGATTCT TGGGAGAATC AGTATGCTTT TAAAGATATC
2101 AAAGATGAAT TCCACAGAGC TCGATCCCTA AAGCCAGAGA CAACTCTTGT GGGAATCCCT
2161 CCCCAATTTA TACCAGACCC GGGGGTTAAC CTTGAAACAC TGTTTCAAAT AGCGGGGGTC
2221 CCTACTGGAG TTGCACACGG AATCACTCAT CACATAATAC AGTCTAAAAA CAAATTGATC
2281 TCAAATGCAA TCGGAAGCAT GTGTGTTATC TCTCATTTTG TCATGAACAC AATACGGACA
2341 ACAGACAGTA TGCCTGGACC CCCGTCTGAC GGGGATGTCA ATAAAATGTG TTCTGCACTA
2401 ATTGGAACAT GCTTCTGGCT AAGTTGGATG GAATCAGATC TTAATTTATA TAATACGTGT
2461 CTAAGGTCAA TAACGAAATC CATGCCTGTG AGATGGTTCA GGGCCCTAAA AAATGGAAAA
2521 TGGTTCCAAA AATGGGACTG CAAGGGGGAT GCTATTCCTA AGGATTCCAG ATTGGGAGAT
2581 AGCCTGGCTA ATATAGGAAA CTGGATTAGA GCTTGGGAGT TGATTAGAGA CGGGAATATA
2641 TCTGAGCCTT TTGATGCTAC AGCTGTAGAA GTGTTGACAA CCTCAGTTGA TAAATCTCTG
2701 AGTTGGAAGA GAATCCTAAA AACAACTGGT ATTCCGAGAC TTTTGAACAG TGATATTGAT
2761 GTAATTGACC AATCCATACT AAATGTGCAA ATTGATATCG TAGAGAACCA AGCTTGGCAA
2821 AATTGAATAG ACACTTCTGT CATCCCTAGA AGAAGGGATA AATTAGATAA TATGAAAAAA
2881 ACTGGACTGA AGACAAGCAT CAAGAATCGT ATGATTAATA ATGGTTCCAA GACAATCGAC
2941 CTTCTTTTGG CCTTTTGTAT TAGGAATGGT TTTTTTGTCT TCGT 5'
```

Figure 21. Nucleotide sequences of Haz-DI genome from the cDNA clones. This cDNA sequence corresponds to (+)-sense polarity. The inverted complementary termini of the DIP genome are underlined.

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   3'ACGAAGACAA AAAAACCATT CCTAATACAA AAGGCCAAAA GAAGGTCGAT TGTCTTGGAA
    61 CCATTATTAA TAGCATAGCA TTAGTCGGTC CTCTATGTCA GAAATTTCTT GATTTACACA
   121 AGATCACTCT AATACTAAAT GCAGTTTCTT TAGGAGAGAC TAAGGAACTC CTCACCACCT
   181 TCAAGGGTAA ATACCGCATG AGTTGCGAAA ACATTCCTAT TGCCCGCTTA AGACTTCCCA
   241 GTTTAGGCCC CGTATTTATG TGCAAAGGTT GGACATACAT TCACAAAGAA CGAGTATTAA
   301 TGGATCGAAA CGTTCTCCTC ATGTGCAAGG ATGTCATAAT CGGCCGAATG CAGACATTTT
   361 TGGCCATGAT TGGTAGGGCG GACAATAAAT TTAGTTCCGG ATCAAATATA CACTCTGGCA
   421 AATGTATACC GAATCGGAGA TCGAATCTTG GAGCAGTGTG GCAATAGGGC ATATGATTTG
   481 ATAAAAATGA TTGAACCTAT TTGTAATCTG AAAATGATGG AATTGGCTAG ATTACATCGT
   541 CCTAAAATTC CCAAATTCCC ACATTTTGAG GAGCATGTCA AAGGTTCAGT AAGAGAATTG
   601 ACACAAAAAT CCAACAAAAT ACAAGCATTG TATGATTTGA TTATGTCTAT GAAAGATGTA
   661 GACCTGGTGT TAGTGGTCTA CGGATCATTC CGTCATTGGG GGCATCCATT CATAGGTTAT
   721 TTTGAAGGCT TAGAAAAATT GCATACTCAG GTGAATATGG AAAAGCATAT TGACAAAGAG
   781 TACCCACAGC AGTTGGCAAG TGATTTAGCT AGACTTGTTT TACATAAACA ATTTAGTGAG
   841 TCAAAAAAAT GGTTTGTGGA TCCTTCGAAG ATGTCTCCCA AACATCCTTT TTATGAACAT
   901 GTCGTTAATA AGACATGGCC CACCGCAGCA AAAATCCAAG ATTTTGGAGA CAATTGGCAC
   961 AAACTTCCCC TCACTCAATG TTTCGAAATA CCGGATTTAA TAGATCCTTC AGTGATTTAT
  1021 TCGGACAAAA GTCACTCCAT GAACAAAAAA GAAGTGATAC AACATGTGCG GACAAAGCCG
  1081 AATATTCCAA TTCCAAGTAA AAAAGTACTG CAAACAATGC TCACTAATAG AGCAACGAAC
  1141 TGGAAGGCTT TTTTAAAAAA TATTGATGAG AATGGCCTGG ATGATGATGA CCTTATAATT
  1201 GGACTGAAGG GGAAAGAAAG GGAATTGAAA ATAGCTGGGA GATTCTTTTC ATTAATGTCA
  1261 TGGAGACTAA GGGAGTATTT TGTTATCACA GAATATTTAA TCAAAACGTA CTATGTGCCC
  1321 TTATTCAAGG GACTGACCAT GGCAGATGAT CTAACATCTG TGATCAAGAA GATGATGGAC
  1381 AGTTCATCAG GTCAGGGGCT TGATGATTAC TCATCTGTTT GTCTGGCTAA CCATATTGAC
  1441 TATGAGAAGT GGAACAACCA TCAGAGGAAA GAGTCGAATG GCCCAATCTT CCGAGTTATG
  1501 GGACAATTTC TAGGTTATCC ATCACTCATA GAAAGAACTC ATGAGTTCTT TGAGAAGAGC
  1561 TTAATTTATT ACAACGGACG TCCGATCTCA TGACTATTAG AAACGGTACA TTATGTAATT
  1621 CCACCAAAAA CCGAGTTTGC TGGAACGGGC AGAAAGGAGG ATTAGAAGGA CTGAGACAGA
  1681 AGGGATGGAG TATCGTGAAT CTCTTGGTGA TTCAAAGAGA AGCAAAGATT CGAAACACAG
  1741 CAGTGAAAGT CTTAGCACAG GGGGATAATC AGGTTATATG CACACAATAC AAGACAAAGA
  1801 AAACTAGATC AGAATTAGAA TTGAGGGCAG TCTTACATCA AATGGCAGGA AATAACAATA
  1861 AAATTATGGA GGAGATTAAG AGAGGGACTG AAAAATTGGG CTTGATTATA AATGATGATG
  1921 AAACCATGCA ATCAGCAGAC TATTTGAACT ATGGGAAAAT TCCAATATTC CGCGGGGTTA
  1981 TCCGAGGATT GGAAACAAAA AGATGGTCGC GAGTGACATG TGTTACAAAT GATCAAATTC
  2041 CGACATGTGC CAATTTGATG TCTTCAGTCT CTACCAACGC ATTAACTGTT GCTCATTTTG
  2101 CAGAAAACCC CATAAACGCA ATGATTCAAT ATAATTATTT TGGGACCTTT GCTCGATTAT
  2161 TGCTCTTTAT GCATGACCCT GCAATAAGAC ATTCTCTGTA TACAGTCCAG GAAAAGATAC
  2221 CTGGTTTGCA CACCAGAACA TTCAAATATG CTGTGTTATA TCTAGATCCT TCAATCGGAG
  2281 GGGTGTGTGG TATGGCGTTG TCTCGATTCT TAATCAGAGC ATTTCCAGAT CCAGTAACAG
  2341 AGAGCCTTTC ATTCTGGAAA TTTATTTATG AACATGCCTC TGAGCCTCAT CTTAAAAAGA
  2401 TGGCTGTAAT GTTTGGGGAC CCTCCAATTG CCAAATTCAG AATAGAGCAT ATCAATAAGT
  2461 TATTAGAGGA CCCCACCTCT TTAAACATCT CAATGGGGAT GAGCCCTGCC AATCTACTAA
  2521 AGAGTGAAGT GAAAAACTGT CTGATAGAGT CGAGATCATC TATCAAGAAT GAAATCATCA
  2581 AAGACGCTAC TATCTATATG CATCAAGAGG AAGAGAAGCT TAGAGGATTC TTATGGTCAA
  2641 TCAAACCACT CTTTCCCCGT TTCTTAAGCG AGTTTAAGGC TGGGACATTT TTGGGGGTAT
  2701 CCGAAGGCTT AATAAATTTA TTCCAAAATT CGCGCACAAT CAGAAATTCT TTTAAAAGGA
  2761 GATATCACAA AGATCTCGAT GAATTGATAA TCAAGAGTGA GATATCTTCT TTAAGTCATC
  2821 TTGGATCCAT GCATTATAGA TTAGGGGATA ATCGTATATG GTCCTGTTCA GCATCTAGAG
  2881 CTGATGTATT AAGATACAAA TCTTGGACAA GAAGGGTAGT GGGAACTACA GTGCCTCACC
  2941 CACTGGAGAT GCATGGATCA CCCTCAAAGA AGGAAAAACC TTGTCAACTA TGTAATTCAT
  3001 CTGGTCTTAC TTATATTTCT GTGCATTGTC CAAAGGGAAT CACAGATGTA TTCAATAGGA
  3061 GAGGACCATT GCCGGCTTAC TTGGGGTCTA ATACATCTGA GTCAACTTCC ATCTTACAAC
  3121 CATGGGAAAA AGAGAGTAAA ATACCAATAA TTAAGAGGGC AACCAGATTG AGGGATGCTA
  3181 TATCTTGGTT TGTACCACCA GAATCTCCTC TATCTACGTG TATATTGAAT AACATTAGGG
  3241 CTTTAACAGG AGAGGATTGG AGCTCCAAAC AACATGGCTT TAAGAGAACA GGATCAGCAT
  3301 TACACAGGTT CTCAACTTCG AGAATGAGCA ATGGAGGGTT CGCTTCACAA AGCCCTGCTA
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3361  CTTTAACACG TATGATTGCG ACCACAGATA CAATGAGAGA TTTTGGTACA AAAAATTATG
3421  ACTTTATGTT CCAGGCATCT TTATTGTATG GACAGATGAC AACAAGTATA TCTAGGTACG
3481  GATCTCCAGG TTCCTGTACA GACCATTATC ATGTCAGATG TAAGGATGCA TTCGAGAGAT
3541  CGAAGAAGTC GAATTAAACA CTAGCTTGGA ATACAGGACA CCCGATGTTT ATCATGTACT
3601  GGAAAAGTGG AGGAACAACA CCGGATCTTG GGGTCATCAA ATCAAACAAT TGAAGCCGGC
3661  AGAAGGTAAC TGGGAATCAT TATCACCGGT CGAACAGTCA TATCAAGTTG CGAGATGCAT
3721  TGGATTTCTT TATGGGGAAT TGACACACAA AAAATCAAGA CAGGCAGATG ATAGTTCTTT
3781  ATTCCCGTTA AGCATCCAAT TGAAAGTGAG AGGGAGGGGC TTCCTACGAG GGTTACTGGA
3841  TGGTTTGATG AGATCAAGTT GCTGTCAAGT AATCCATCGC AGAAGTGTTT CCACTTTAAA
3901  GAGACCTGCT AATGCTGTTT ATGGTGGTTT GATATACCTC ATTGATAAAC TAAGTGCATC
3961  AAGTCCCTTC CTATCACTTG TAAGAACCGG TCCTATTCGA CAAGAGTTAG AGCAAGTGCC
4021  ACATAAAATG TCGACATCAT ATCCAACCAA TATCAGGGAT TTAGGTTCTA TTGTGAGAAA
4081  TTATTTCAAA TATCAGTGTC GACCTGTTGA GAGGGGAAAC TACAAGACTT ATTACAATCA
4141  AATATGGCTC TTTTCTGATG TGTTATCGAC TGAATTTATA GGGCCCATGG CTATATCTAG
4201  CTCACTCCTC AAACTCCTTT ATAAACCTTC TCTCACAAAG AAAGACAAAG AGGAATTAAG
4261  AGAGCTTGCA GCACTGTCAT CTAACTTAAG GAGCGGTGAG GATTGGGATG ATTTACATAT
4321  CAAATTTTTC TCAAATGATC TGCTATTCTG CCCACAAGAG ATAAGACATG CCTGCAAATT
4381  TGGAATTCAA AAAGAGAATG AGGGTATCGC TTTGTACCCA AATTGGGGAA TAGAGTATAT
4441  TGGAAATGTG ACTGATATTC CTGTTTTCTA TAGGGCTCAA AGTGTCCAAA AAGATATCAG
4501  AATCCCTCCT CGAATACAAA ATCCCTTGAT GTCCGGACTT CGACTAGCAC AGTTACCCAC
4561  CGGAGCTCAC TACAAGATGA GAACCATTAT ATCCCGTTTT AAAATCCCCT ATCATGATTT
4621  TCTAGCTTGT GGGGATGGGT CGGGAGGAAT GACTGCAGCC TTGCTTCGAC TTAATAGAGC
4681  ATCTAGAGGA ATTTTCAACA GTTTGTTGGA TTTATCAGAC ACGATGTTAA GAGGATCTTC
4741  CCCGGAACCT CCCAGCGCCC TTGAGACCCT AGGGGGAGAA AGATCAAGAT GTGTAAACGG
4801  AGATAGCTGC TGGGAACATC CTTCCGATCT TAGTGATGAG AACACCTGGA AATACTTTCT
4861  ACATGTTAAA AAGGGATGTG GAATGAGTAT CAACCTTATT ACTATGGACA TGGAAGTGAA
4921  GGACCCTACC ATGTCATACA AAATTGAATT ATTGGTCAGA CAATATGTCC CAGTTTTATT
4981  AGAGTCCGAC GGTTGCCTCA TTTACAAAAC ATATGGACAT ATATCGCTAC ACAAAAAGAT
5041  AACTCTTTGA CTCTTATAGG ATCACTCTTT CATTCTGTTC AACTTGTCCA AACAGACTTA
5101  AGTTCTAGTA ACACATCCGA ATTGTATCTA GTCTGTAGAA GATTGAAAGA TTACATCGAT
5161  ACTCCTTTTG TAGATTGGAT AGAATTGTAC GATTATTGGG AGAATCAGTA TGCTTTTAAA
5221  GATATCAAAG ATGAATTCCA CAGAGCTCGA TCCCTAAAGC CAGAGACAAC TCTTGTGGGA
5281  ATCCCTCCCC AATTTATACC AGACCCGGGG GTTAACCTTG AAACACTGTT TCAAATAGCG
5341  GGGGTCCCTA CTGGAGTTGC ACACGGAATC ACTCATCACA TAATACAGTC TAAAAACAAA
5401  TTGATCTCAA ATGCAATCGG AAGCATGTGT GTTATCTCTC ATTTTGTCAT GAACACAATA
5461  CGGACAACAG ACAGTATGCC TGGACCCCCG TCTGACGGGG ATGTCAATAA AATGTGTTCT
5521  GCACTAATTG GAACATGCTT CTGGCTAAGT TGGATGGAAT CAGATCTTAA TTTATATAAA
5581  ACGTGTCTAA GATCAATAAC GAAATCCATG CCTGTGAGAT GGTTCAGGGC CCTAAAAAAT
5641  GGAAAATGGT TCCAAAAATG GGACTGCAAG GGGGATGCTA TTCCTAAGGA TTCCAGATTG
5701  GGAGATAGCC TGGCTAATAT AGGAAACTGG ATTAGAGCTT GGGAGTTGAT TAGAGACGGG
5761  AATATATCTG AGCCTTTTGA TGCTACAGCT GTAGAAGTGT TGACAACCTC AGTTGATAAA
5821  TCTCTGAGTT GGAAGAGAAT CCTAAAAACA ACTGGTATTC CGAGACTTTT GAACAGTGAT
5881  ATTGATGTAA TTGACCAATC CATACTAAAT GTGCAAATTG ATATCGTAGA GAACCAAGCT
5941  TGGCAAAATT GAATAGACAC TTCTGTCATC CCTAGAAGAA GGGATAAATT AGATAATATG
6001  AAAAAAACTG GACTGAAGAC AAGCATCAAG AATCGTATGA TTAATAATGG TTCCAAGACA
6061  ATCGACCTTC TTTTGGCCTT TTGTATTAGG AATGGTTTTT TTGTCTTCGT 5'
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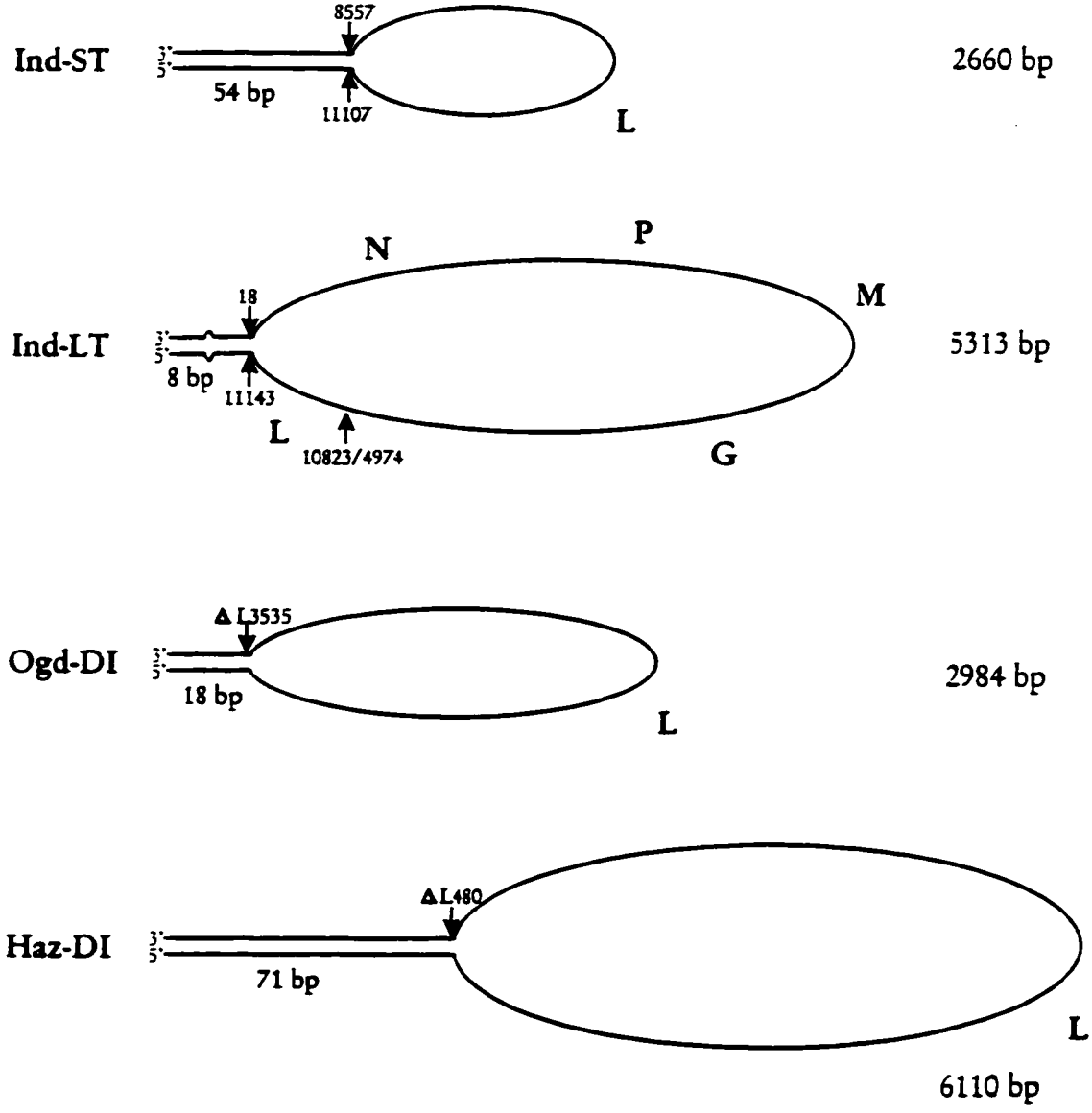
and no host or nonviral sequences were found. The conservation of 5' end sequences in DIP genomes of both serotypes indicates that these sequences are important in RNA polymerase recognition. Analysis of the 3' terminal regions of DIP genomes of both serotypes was also performed to determine sequences downstream of the deletion points where the polymerase resumes synthesis after the premature termination event. The flanking sequences were not similar to the analyzed DIP cDNA. The implications of these findings regarding mechanisms of DIP generation will be discussed.

3. DISCUSSION

Physical mapping of the DIP genomes

The genome structure and terminal sequences of IND-DIPs and novel NJ-DIPs of VSV were determined. Fig. 22 illustrates the deduced physical maps. Sequence similarities and genome organization confirmed that all DIP genomes are derived from their standard viruses. Nucleotide sequence analysis of cloned cDNA from four different DIPs revealed that three DIP genomes (IND-ST, Ogd-DI and Haz-DI) were generated from the 5' end of the standard virus genome and were of the copy back class. IND-LT, which retained the 3' half of the standard genome, was shown to be a simple deletion DIP (position 4974-10823 nucleotides of L gene). The IND-ST cDNA contains 2660 nucleotides and possesses 54 base inverted complementary termini. The IND-ST genome is identical to the 5' end of the IND-standard virus genome except for several mutations. The IND-LT genome comprises 5313 nucleotides with partial inverted complementary termini within the first 18 bases of the 3' and 5'

Figure 22. Genome structure deduced from the sequences of cDNA clones. The cDNAs of the DIP genomes correspond to (+)-sense polarity. The viral genes are shown in the corresponding DIP genome; N, P, M, G and L genes. Δ indicates deleted portions from the beginning of the L gene.


Ind-ST
8557
54 bp
11107
L
2660 bp
N
P
M
Ind-LT
18
8 bp
11143
L
10823/4974
G
5313 bp
Δ L3535
Ogd-DI
18 bp
L
2984 bp
Δ L480
Haz-DI
71 bp
L
6110 bp

termini (i.e. the first 8 bases, position 10 and the last 6 bases have inverted complementarity) and is capable of encoding the four viral proteins N, P, M and G. cDNAs spanning the entire genome of the NJ-DIP genomes were cloned and sequenced for the first time. The Ogd-DI and Haz-DI genomes comprise 2984 and 6110 nucleotides, respectively, with different inverted complementary termini (18 bases for Ogd-DI and 71 bases for Haz-DI). The sequences on the L gene side of the deletion of DIP genomes are 3535 nucleotides (Ogd-DI) and 480 nucleotides (Haz-DI) downstream of the beginning of each L gene.

All VSV DIP cDNAs that have been examined here contain the genomic 5' terminus. The same results were reported by Lazzarini *et al.* (1981). The retention of the 5' end in the DIP genomes is not surprising as these regions are likely to be involved in RNA polymerase binding and initiation of replication. Whether the conserved termini represent replicase binding sites or whether they are important for a three-dimensional configuration essential for replication or encapsidation, or both, cannot be determined at this time.

Generation model of DIPs

It is currently thought that DIP genomic RNAs of negative-stranded RNA viruses originate by a copy-choice mechanism in which the viral replicating enzyme prematurely terminates RNA synthesis, abandons the template, but resumes chain elongation somewhere else on the same template or on another homologous template (Meier *et al.* 1984). In this process, specific sequences that may act as termination and

reinitiation signals for RNA synthesis may be involved. The data presented here support this copy-choice mechanism so that the internal deletion (IND-LT) was generated in the first step of replication, in which the polymerase jumps at position 4974 and resumes at position 10823 from the template of the genome. In the copyback DIP (IND-ST, Ogd-DI and Haz-DI), following premature termination of negative-stranded RNA synthesis, the polymerase, with the nascent RNA strand (–) attached, resumes synthesis at a site 18, 54, or 71 nucleotides (Ogd-DI, IND-ST, Haz-DI respectively) from the 5' end of the nascent RNA strand to form 18, 54, 71 base pair stem structures. These findings are in good agreement with the general 'leaping or jumping replicase complex' model for DIP generation (Leppert *et al.* 1977, Kang 1980, Lazzarini *et al.* 1981, Perrault 1981, Holland 1990, Roux *et al.* 1991).

However, a search for base sequences that might participate in copy-choice events did not reveal any common pattern in the sequences at deletion boundaries, or resemblance with any of the known regulatory signals for transcription and replication. It is not clear as yet which nucleotide sequences are essential for transcription termination. For VSV it appears that no specific terminator or promoter sequence is involved in copy-choice DIP generation. Similarly, the extreme variability in the stem lengths of a large number of VSV copyback DIP RNAs suggests that defined sequences are not involved in termination and initiation of DIP RNA synthesis as described previously (O'Hara *et al.* 1984). This would suggest that these processes are relatively independent of sequence specificity.

A copy-choice mechanism was shown to operate in the generation of VSV

copyback DIP RNAs (IND-ST, Ogd-DI, Haz-DI) and simple deletion IND-LT, the premature termination event falls into a similar pattern, where the enzyme stops RNA synthesis and releases the template upstream of a sequence that resembles part of the transcription initiation signal. Although the entire nucleotide sequence of all four DIP genomes are sequenced, it can not be concluded yet which nucleotide sequences are responsible for generation of DIP.

Sequence analyses and interference

Sequence analysis of the DIP genomes allowed further characterization of the DIP RNAs. Two major types of DIP genomes have been described here for VSV; Copyback DIP genomes (IND-ST, Ogd-DI and Haz-DI) lack the genomic 3' end sequences, but have sequences complementary to the 5' end at the 3' end. These complementary ends have been shown to vary from 18 to 71 nucleotides in length. Since they lack the standard genomic 3' end sequences, which contain the signals for transcription, they are unable to express any mRNA. Thus, these copyback genomes can serve only as templates for replication. Interference might involve competition for polymerase complexes by binding polymerases on the inverted complementary termini efficiently. Recently, Wertz *et al.* (1994) reported that longer regions of terminal complementarity had a clear replicative advantage over those with shorter regions. These "copyback" sequences are thought to be crucial for the interference property of most VSV DIPs. On the other hand, 'internal deletion' DIP genome (IND-LT) conserved the standard genomic 3' and 5' termini, and therefore retained replication

and transcription signals. Since the ability of VSV internal deletion DIP genomes to encode viral proteins has been shown here and by others (Colonno *et al.* 1977, Chow *et al.* 1977, Johnson and Lazzarini 1977, Hsu *et al.* 1985, Re *et al.* 1985), it is assumed that this transcribing DIP has a different interference mechanism from copyback DIPs.

The main structural features that these DIPs share are the homologous bases in the 3' and 5' termini, suggesting that these regions play a more important role in modulating DIP RNA replication and/or encapsidation than other areas of the genome. It is possible that these conserved termini could also be important for DIP interference. Further sequence studies on these and on related DIP genomes will be necessary to resolve these questions.

CHAPTER 5: REVERSE GENETICS OF VSV

1. INTRODUCTION

Many questions remain regarding how DIPs interfere with the standard virus replication. To investigate this question at the molecular level, it would be important to be able to recover the native DIP genomes from cDNA clones under conditions where all viral proteins are also expressed from cloned cDNAs. This approach is known as reverse genetics and provides for molecular genetic analyses of *cis*-acting sequences in the DIP genome and *trans*-acting plasmid-encoded viral proteins for studying the mechanism(s) of interference.

Although VSV has been a good model for studying various aspects of the virus life cycle, one of the limitations in using VSV to study virus replication and assembly has been the inability to introduce specific mutations into the negative-sense genomic RNA of this virus. The difficulty in manipulating the genome of VSV, and for those of other negative-strand viruses, results from the nature of the functional template for replication and transcription. For VSV and other negative-strand viruses, the genomic RNA must be encapsidated by the nucleocapsid protein (N) before it can serve as a template for either transcription or replication by the viral RNA polymerase (Garcia-Sastre and Palese 1993). The difficulty in generating biologically active nucleocapsids of negative-strand RNA viruses has provided a major stumbling block for detailed genetic analysis of this group of viruses.

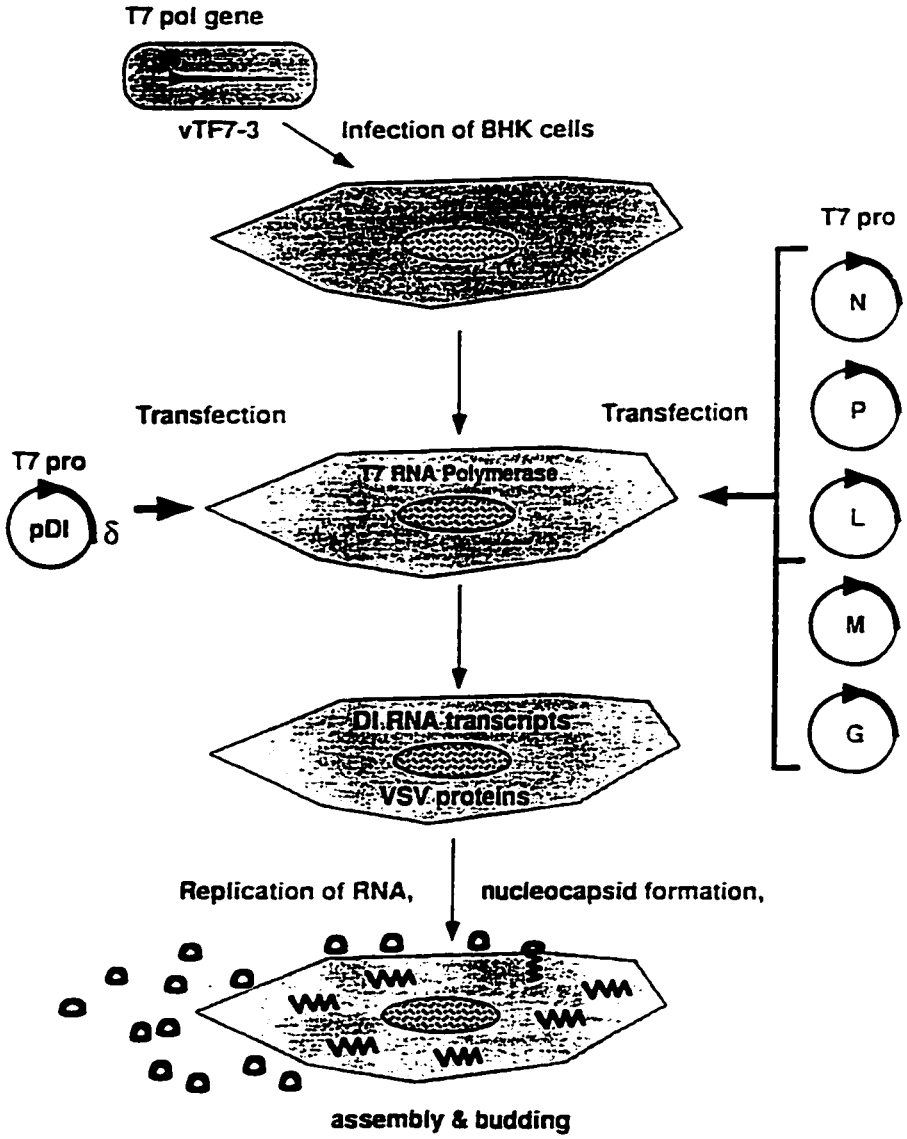
It has become possible to alter negative-strand RNA virus genomes at the cDNA

level and recover the RNAs (Enami *et al.* 1990, Collins *et al.* 1991, Park *et al.* 1991, Pattnaik *et al.* 1992, Calain *et al.* 1992). These accomplishments hold great promise as experimental systems for genetic manipulation of negative-strand virus genomes and there has been considerable progress in developing methods for direct genetic manipulation of the RNAs and proteins involved in the transcription, replication, and packaging of negative-strand RNA viruses. Pattnaik *et al.* (1992) have showed, by using a recombinant vaccinia virus-T7 RNA polymerase expression system, that infectious DIPs of VSV can be recovered from negative-sense transcripts of a plasmid that contains a full-length cDNA derived from the DI-T particle genome when cells express all five VSV proteins from cloned cDNAs (Fig. 23). This replication, assembly, and budding of DIPs from cells expressing all five VSV proteins entirely from plasmids provide a powerful approach for analysis of *cis*-acting signals and detailed structure-function analysis of the VSV gene products in each step of the replicative cycle of the virus.

The final objective of my project was to study the molecular mechanism(s) of the viral interference mediated by DIPs by applying this reverse genetic system. In this chapter, I will examine the ability of VSV proteins to rescue DIPs from cDNAs clones using all five viral genes from the three different strains of VSV. I will also examine the importance of 3' and 5' termini in DIP genomic RNA for template activity by subcloning DIP genomic cDNAs (pBKS-ST, pBKS-OD, and pBKS-HD) into a specialized transcription plasmid (pTV) that expresses either negative-stranded or positive stranded DIP genomic RNA. T7 polymerase transcription would generate

Figure 23. Scheme of infection with a vaccinia virus (vTF7-3) and transfection with plasmids DNAs. This figure was taken from Pattnaik *et al.* (1992).

VSV - VVT7 Expression System



DIP genomic RNAs with the exact 5' ends of the authentic DIP RNAs as well as the exact 3' ends, resulting from the action of the HDV ribozyme. Finally, the successful amplification of VSV DIPs from cloned cDNAs will be described.

2. RESULTS

Cloning of viral genes from two different serotypes of VSV

To obtain full-length cDNA copies of mRNAs representing all five viral genes (N, P, M, G, and L) from the three different strains of VSV (VSV_{IND}, $VSV_{NJ\text{-}Ogd}$, and $VSV_{NJ\text{-}Haz}$), the SuperScript Choice System for cDNA synthesis (Gibco BRL) was used as outlined in Fig. 24. First, mRNAs were transcribed *in vitro* using the endogeneous virion-associated RNA polymerase activity of the standard virus in the presence of rNTP and BHK-21 cell extracts. In previous studies Ball and White (1976) and Rose *et al.* (1977) found that addition of a crude cell extract greatly enhanced the synthesis of full-length VSV mRNA's by the VSV virion transcriptase. The mechanism of action of the cell extract is not known, but host tubulin presumably acts in viral RNA transcription (Moyer *et al.* 1986). Gupta *et al.* (1995) have recently reported cellular casein kinase II was essential for transcription due to its activity for phosphorylation of the P protein. *In vitro* synthesized, poly(A)-containing viral mRNAs were then purified by oligo(dT)-cellulose column chromatography.

The purified mRNAs were analyzed by electrophoresis through a 1.2% agarose-formaldehyde gel (Fig. 25A). After *in vitro* transcription, the products of total VSV mRNA were shown at high yields. The four mRNA transcripts (G, N, P and M

Figure 24. The cloning strategy for viral genes of VSV. The VSV genomic (negative-sense) RNA is represented by the long open bar at the top of the figure. mRNAs were produced from viral genomic RNA by *in vitro* transcription in the presence of rNTP and cell extracts. The mRNAs are represented as small open bars containing a 5'-methylated-guanosine cap (G^m) and a poly A tail ($A_{(n)}$). The black region indicates positive-sense leader RNA. The cDNA (stippled bar) was synthesized by reverse transcriptase with primer CD-3 (arrow marked). The sequence of primer CD-3 is shown in Materials and Methods. The double-stranded DNA (stippled bars) was ligated to *Eco*RI-adapters and then cloned in the *Eco*RI site in the multicloning site of pBKS, which contains the T7 promoter (shaded bar). *l*, leader region; *t*, trailer region; N, nucleocapsid gene; P, phosphoprotein gene; M, matrix protein gene; G, glycoprotein gene; L, large polymerase gene.

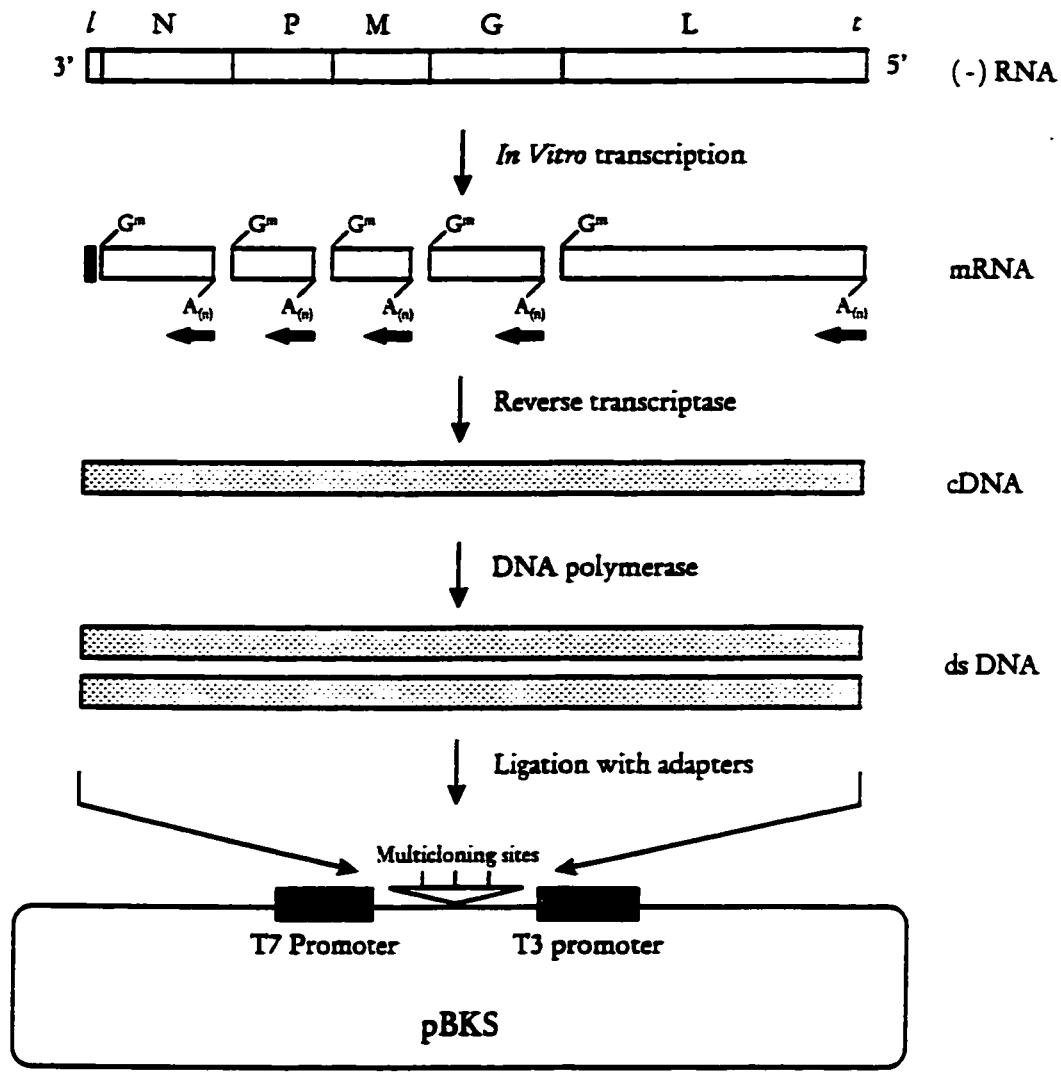
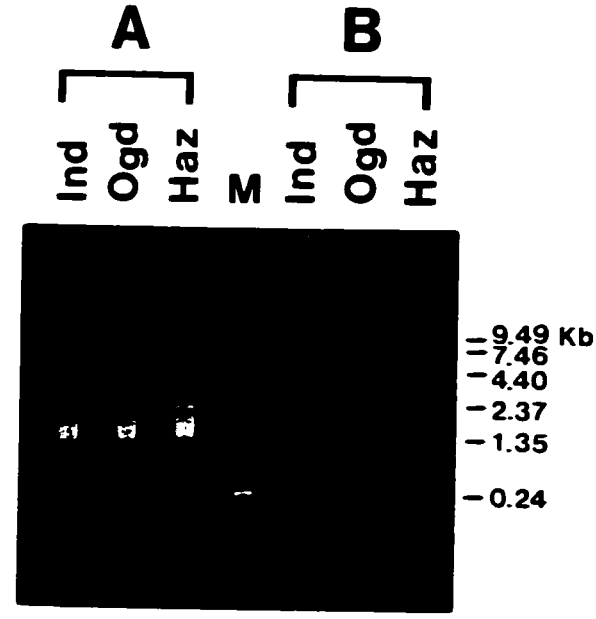
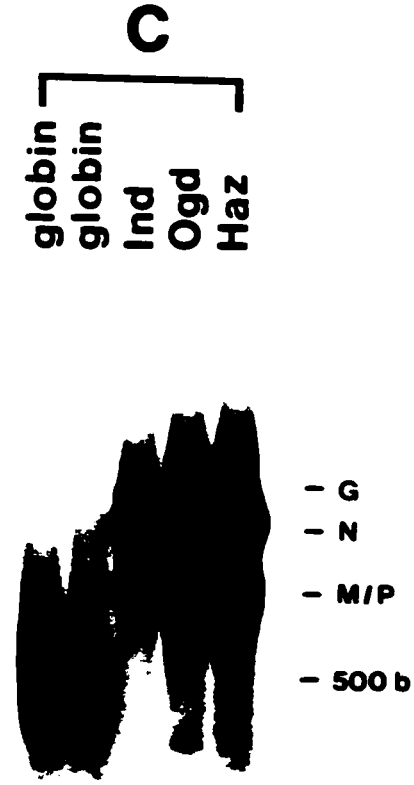

l
N
P
M
G
L
t
3'
5'
(-) RNA
In Vitro transcription
mRNA
Reverse transcriptase
cDNA
DNA polymerase
ds DNA
Ligation with adapters
Multicloning sites
T7 Promoter
T3 promoter
pBKS

Figure 25. *In vitro* mRNA transcription and cDNA products of VSV. (A and B) The mRNA transcripts were analyzed by electrophoresis on a 1.2% agarose-formaldehyde gel and visualized by ethidium bromide fluorescence. (A) Lanes: Ind lane, mRNAs from VSV_{IND} standard virus; Ogd lane, mRNAs from $VSV_{NJ\text{-}Ogd}$ standard virus; Haz lane, mRNAs from $VSV_{NJ\text{-}Haz}$ standard virus. (B) Lanes: Ind lane, the genomic RNA of VSV_{IND} standard virus; Ogd lane, the genomic RNA of $VSV_{NJ\text{-}Ogd}$ standard virus; Haz lane, the genomic RNA of $VSV_{NJ\text{-}Haz}$ standard virus. Lane M shows a series of six synthetic poly(A)-tailed RNAs as RNA molecular size markers. (C) Agarose gel analysis of cDNA synthesis. ^{32}P-labeled first strand cDNAs made from VSV mRNAs were electrophoresed on a 1.2% agarose-formaldehyde gel, and the dried gel was exposed to X-ray film. Lanes: Ind lane, cDNAs synthesized from VSV_{IND} mRNA; Ogd lane, cDNAs synthesized from $VSV_{NJ\text{-}Ogd}$ mRNA; Haz lane, cDNAs synthesized from $VSV_{NJ\text{-}Haz}$ mRNA; globin lane, cDNA from globin mRNA (500 bases) as control marker.


A
B
Ind
Ogd
Haz
M
Ind
Ogd
Haz
9.49 Kb
7.46
4.40
2.37
1.35
0.24


C
globin
globin
Ind
Ogd
Haz
G
N
M/P
500 b

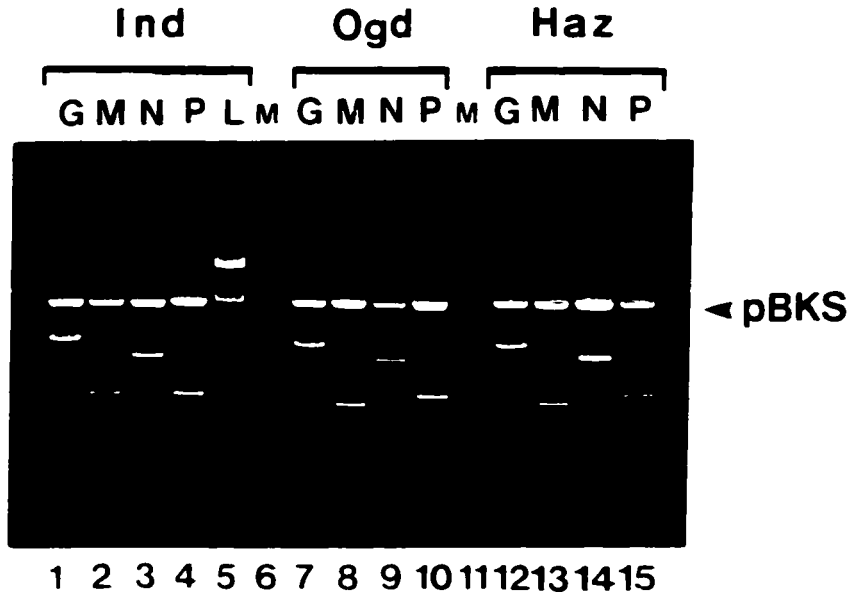
genes) of each strain of VSV were synthesized with the correct sizes; G mRNAs (1,665 bases, 1,573 bases), N mRNAs (1,326 bases, 1,329 bases), P mRNAs (814 bases, 856 bases), and M mRNAs (831 bases, 758 bases) of VSV_{IND} and VSV_{NJ}, respectively, corresponded to the sizes of mRNAs without poly (A) tails. L mRNAs were not detected by *in vitro* transcription and thus attempts to synthesize the full-length large polymerase L protein *in vitro* have not been successful. Before *in vitro* transcription genomic RNAs were isolated from each purified standard virus and compared to the *in vitro* transcripts (Fig. 25B). The size of standard virus genomes is 11,161 bases for VSV_{IND} and 11,123 bases for $VSV_{NJ\text{-}Ogd}$. Although the complete sequence of $VSV_{NJ\text{-}Haz}$ has not been reported, the genome of $VSV_{NJ\text{-}Haz}$ was assumed to be similar in size with that of $VSV_{NJ\text{-}Ogd}$.

The full-length first-strand cDNAs were synthesized by reverse transcriptase (SuperScript II RT™) using purified mRNAs in the presence of dNTP. The primer for first strand cDNA synthesis was designed to anneal to the conserved poly(A) signal of all viral genes of VSV (5'-TTTTTTTCATA-3') and to contain restriction enzyme sites. Second strand cDNA synthesis was catalyzed by *E.coli* DNA polymerase I, RNase H, DNA ligase, and T4 DNA polymerase. To verify whether this procedure was carried out correctly, a small aliquot of each sample was labeled with [α-^{32}P]dCTP and electrophoresed on an agarose-formaldehyde gel after the first strand reaction (Fig. 25C). The globin gene (500 bases) was used as control RNA. It was possible to obtain high yields of full-length ds cDNA for all mRNA species of the three strains of VSV except for the L mRNA. The sizes of the full-length double-stranded cDNA

species copied from the respective mRNA of VSV were of similar size as expected. I therefore proceeded on the assumption that all of the double-stranded cDNA bands represented full-length copies of the mRNAs encoding each viral gene (which were later verified) and carried out the cloning procedure.

Since the products of double-stranded cDNA were blunt-ended cDNAs, adapters containing *Eco*RI 5' extensions were ligated to the double-stranded cDNAs to maximize ligation efficiency. *Eco*RI-adapted double-stranded cDNAs were then inserted into *Eco*RI-digested pBKS vector. *E. coli* DH5α cells were transformed, ampicillin resistant colourless colonies were picked on IPTG/X-Gal agar plates, and plasmid DNAs were prepared. Although this approach did not yield L clones for any of the three strains of VSV, pEB2, which contains L gene of VSV_{IND} under control of the T7 RNA polymerase promoter in pGem4XB vector, was kindly provided by Dr. M. Schubert (1985). To determine the sizes of the inserts, pBKS plasmids with inserts at the *Eco*RI site were screened by digestion with *Not*I; this site was not contained in any of the viral genes (pEB2 was digested with *Xho*I and *Bss*H2) (Fig. 26). The size of the pBKS vector is 2.96 kb, and the inserts corresponded in size to their respective mRNAs. All plasmids containing inserts of the correct size were chosen for sequence analysis. The nucleotide sequence of the insert DNA was determined on both DNA strands for at least 300 nucleotides from the 3' and 5' ends (data not shown). All plasmids contained a T7 promoter immediately preceding the sequence encoding the 5' end of each gene and the nucleotide sequence was determined to show the start sequence at the 5' end of each gene and stop-polyadenylation signals at the 3' end of each gene. This

Figure 26. Selection of transformants with VSV genes by determining the size of inserts. Selection of possible recombinant clones was made by digesting plasmid DNA with *Not*I (IND-L clone was digested with *Xho*I and *Bss*H2) to check the inserts. The digested DNAs were then analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide fluorescence. Lanes: Ind G, M, N, P, L lane, plasmid DNA containing G, M, N, P, L gene of VSV_{IND}, respectively; Ogd G, M, N, P lane, plasmid DNA containing G, M, N, P gene of $VSV_{NJ\text{-}Ogd}$, respectively; Haz G, M, N, P lane, plasmid DNA containing G, M, N, P gene of $VSV_{NJ\text{-}Haz}$, respectively; lane M, *Bst*EII-digested lambda DNA. The arrowhead indicates the size of pBKS vector (2.96 kb).

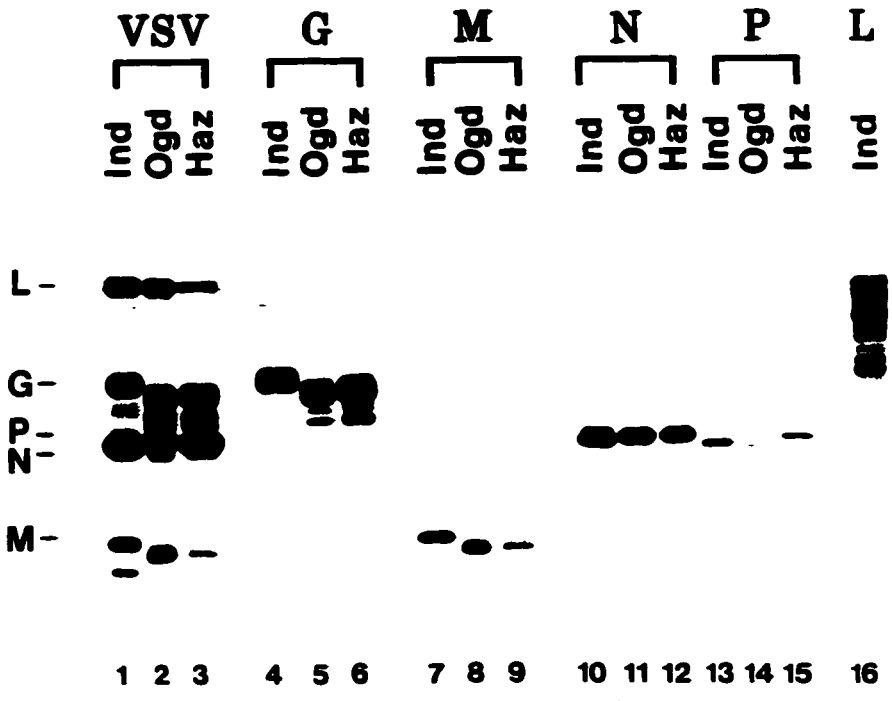

Ind
Ogd
Haz
G M N P L M G M N P M G M N P
pBKS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

confirmed the complete sequence of the mRNA encoding each viral gene for the three strains of VSV.

Expression of VSV proteins using cloned viral genes

To examine the expression of VSV proteins from cDNA clones, plasmids containing the entire coding regions for N, P, M, and G of the three strains of VSV and the IND-L gene, under the control of the promoter for T7 RNA polymerase, were transfected individually into BHK-21 cells which had been infected with vaccinia virus vTF7-3 (Fuerst *et al.* 1986, 1987). Protein expression was examined either by Western blot (immunoblot) analysis (data not shown) or by immunoprecipitations with anti-VSV rabbit serum and anti-IND-P-serum after labeling of the cells with [^{35}S]methionine (Fig. 27). The anti-VSV_{IND} serum did not detect IND-P protein but IND-P protein was recognized by anti-IND-P-serum (Schuster *et al.* 1996). Results showed that transfected cells expressed VSV proteins (lanes 4 through 16) which comigrated with the authentic VSV proteins synthesized in VSV-infected cells (lanes 1 through 3). The levels of protein expression by plasmids approximated those observed in VSV-infected cells. In contrast, the level of P protein expressed by plasmid was somewhat less than that observed in VSV-infected cells (compare lanes 1-3 and 13-15), which might be the result of early termination of T7 transcription, premature termination of translation, or both. Although there were some degraded products from recombinant proteins (lanes 5, 6, and 16), the main protein bands migrated similarly to authentic VSV proteins (lanes 1 to 3). The size of each P protein expressed by plasmid (~ 45 to 48 kD)

Figure 27. Immnoprecipitation of VSV proteins expressed in transfected cells. BHK-21 cells infected with vTF7-3 (MOI=3) and transfected with 2.5 μg of each plasmid, individually, were labeled for 2 hrs with [^{35}S]methionine (lanes 4 to 16). For comparison, cells were infected with VSV standard virus (MOI=3) (lane 1, VSV_{IND}; lane 2, $VSV_{NJ\text{-}Ogd}$; lane 3, $VSV_{NJ\text{-}Haz}$), labeled at 20 hr p.i., and harvested. Cell extracts were subjected to immunoprecipitation with rabbit anti-VSV-serum, and the immunoprecipitates were resolved by SDS-PAGE. Lanes: lane 4, pBKS-IG transfected cells; lane 5, pBKS-OG transfected cells; lane 6, pBKS-HG transfected cells; lane 7, pBKS-IM transfected cells; lane 8, pBKS-OM transfected cells; lane 9, pBKS-HM transfected cells; lane 10, pBKS-IN transfected cells; lane 11, pBKS-ON transfected cells; lane 12, pBKS-HN transfected cells; lane 13, pBKS-IP transfected cells; lane 14, pBKS-OP transfected cells; lane 15, pBKS-HP transfected cells; lane 16, pEB2-IL transfected cells. Lanes 1 and 13, anti-VSV_{IND} serum mixed with anti-IND-P serum (Schuster *et al.* 1996); lanes 4, 7, and 10, anti-VSV_{IND} serum; lanes 2, 5, 8, 11, and 14, anti-$VSV_{NJ\text{-}Ogd}$ serum; lanes 3, 6, 9, 12, and 15, anti-$VSV_{NJ\text{-}Haz}$ serum; lane 16, monospecific anti-IND-L serum (Schubert *et al.* 1985).


VSV
G
M
N
P
L
Ind
Ogd
Haz
Ind
Ogd
Haz
Ind
Ogd
Haz
Ind
Ogd
Haz
Ind
Ogd
Haz
Ind
L-
G-
P-
N-
M-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

was a little smaller than P proteins produced in VSV-infected cells (~ 50 to 55 kD). This difference may be due to the degree of phosphorylation of P protein. The expressed IND-L protein was identical in size to the L protein of the virion (241 kD) with high level expression. Comigration of five proteins expressed by mixedly transfected plasmids and proteins produced by VSV infection indicated that authentic viral proteins could be expressed from cloned cDNA (data not shown).

All five VSV proteins were expressed from cDNA by using the vaccinia virus/T7 RNA polymerase expression system. All plasmids contained the VSV genes under the control of T7 RNA polymerase promoter, functional mRNAs were transcribed from the plasmid templates, and mRNAs were properly translated to produce viral proteins. Proteins produced in this system were indistinguishable from authentic protein produced during VSV infection according to apparent molecular weight and analyses with specific antibodies. These five proteins of VSV_{IND} were subsequently shown to be functional by their ability to replicate and transcribe the RNA of IND-DIP from cDNAs (see below).

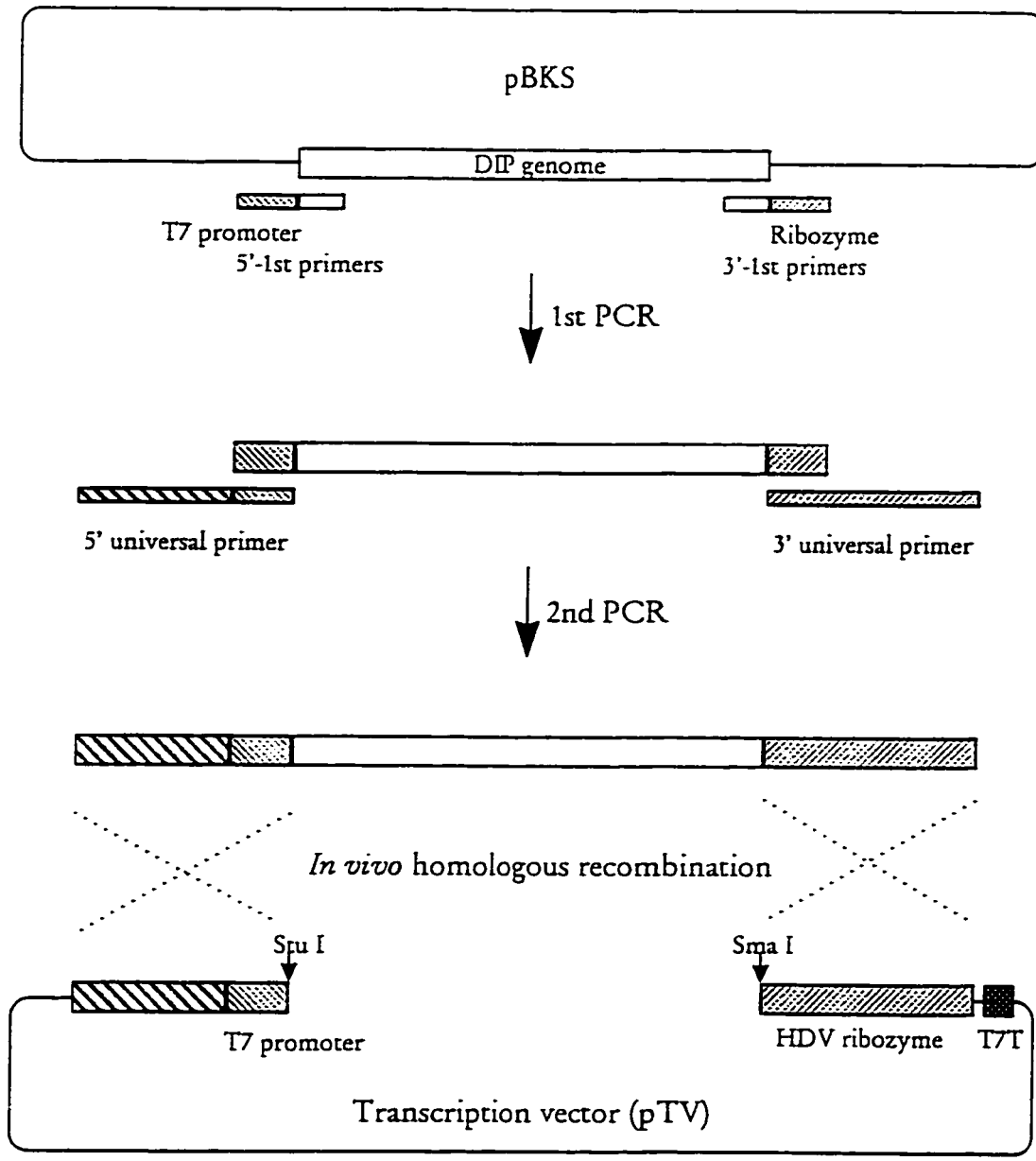
Construction of a transcription vector containing complete cDNA copies of DIP genomes

To produce the authentic 3' and 5' termini of DIP genomes from cDNA clones, subcloning of pBKS-DI sequences into pTV, which contains the T7 promoter, HDV ribozyme and T7 terminator, was performed. The strategy for subcloning DIP genomic sequences by using an overlap PCR amplification and *in vivo* homologous

recombination was as described in Materials and Methods and in Fig. 28. Shuldiner *et al.* (1990) reported that only PCR DNA fragments of less than 2 kb showed efficient *in vivo* homologous recombination. Subcloning of the pBKS-ST insert (2.6 kb) and pBKS-OD insert (3 kb) into pTV was quite efficient. The larger DIP inserts, pBKS-LT (5.3 kb) and pBKS-HD (6.1 kb) were digested with appropriate restriction enzymes prior to PCR reactions. After *in vivo* homologous recombination, internal fragments of pBKS-LT and pBKS-HD were ligated to pTV, which already contained the 5' and 3' ends of each of the DIP genomes. For each DIP genome, plasmids containing the DIP cDNA in opposite orientations were constructed. pTVDI(−) clones express genomic sense DIP RNA and pTVDI(+) clones direct transcription of antigenomic sense DIP RNA. Subcloning of all pBKS-DI into pTV was successful except for pTV-LT(+).

Initially, a T7 RNA polymerase promoter was placed directly upstream of the 5' end sequences so that RNA transcription would begin with the 5' terminal nucleotide of the DIP genome. However, detectable amounts of RNA were not transcribed either *in vitro* or after transfection of the plasmids into cells infected with vTF7-3 (data not shown). To provide an efficient initiation sequence for the T7 RNA polymerase (Pattnaik *et al.* 1992), two additional G residues were inserted between the T7 promoter and the DIP sequences. To generate RNA transcripts ending with the authentic DIP 3'-terminal nucleotide, the 3' DIP sequence was cloned adjacent to the ribozyme sequence from the antigenomic strand of HDV (Ball 1992). Autolytic cleavage should occur at the 5' end of the ribozyme RNA and generate a 3' end of the

Figure 28. Subcloning strategy of pBKS-DI into pTV. The open bars represent DIP DNA. For the first PCR reaction, primers ID5G and ID3 (IND-ST), primers ID5G and LT3 (IND-LT), and primers HD5G and HD3 (NJ-DIPs) were used. A second PCR reaction was performed by using primers UNIV5 and UNIV3 of all DIP genomes. 5'-1st primers: the thin hatched region, 18 nucleotides complementary to T7 promoter sequences of pTV and two non-DIP nucleotides (GG); the open region, 17 nucleotides complementary to 5' end of DIP genome. 3'-1st primers: the thin hatched region, 18 nucleotides complementary to HDV ribozyme antisense-sequences of pTV; the open region, 17 nucleotides complementary to 3' end of DIP genome. 5' universal primer: the thin hatched region, two (non-DIP) G nucleotides and 18 nucleotides complementary to T7 promoter sequences of pTV; the thick hatched region, 32 nucleotides complementary to a portion of pTV next to the T7 promoter. 3' universal primer; the thin hatched region, sequences complementary to the first 50 nucleotides of antisense-HDV ribozyme. The sequence of each primer is shown in Materials and Methods and in the Appendix. The shaded region in the pTV indicates the T7 terminator. The final PCR products were mixed with pTV digested with *Sma*I and *Stu*I. *In vivo* homologous recombination occurred in *E.coli* DH5α.


pBKS
DIP genome
T7 promoter
5'-1st primers
Ribozyme
3'-1st primers
1st PCR
5' universal primer
3' universal primer
2nd PCR
In vivo homologous recombination
Stu I
Sma I
T7 promoter
HDV ribozyme
T7T
Transcription vector (pTV)

upstream RNA corresponding to the authentic 3' terminus of DIP RNA.

To determine the exact 3' and 5' termini of each cloned sequence, the nucleotide sequence at the end of each insert corresponding to the 3' and 5' termini of the DIP cDNA was determined. Fig. 29 depicts sequencing gels showing the T7 promoter region of the pTVDI inserts. All plasmids showed the T7 promoter positioned to transcribe the negative-sense and positive-sense DIP RNA from pTVDI(−) and pTVDI(+), respectively, with two additional nucleotides (GG).

pTVST(−)/(+), pTVOD(−)/(+), and pTVHD(−)/(+) retained the same sequences up to 54 nucleotides, 18 nucleotides, 71 nucleotides, respectively, at each terminus because these DIPs belonged to the copyback class of DIPs. After the terminal complementary sequences, pTVDI(−) showed the 5' sequences of the DIP genome and pTVDI(+) showed the 3' sequences of the DIP genome. The antigenomic ribozyme sequence of hepatitis delta virus was positioned to cleave the 3'-terminus of the transcripts immediately after the last DIP-specific nucleotide for all of the plasmids (data not shown). Sequencing of pTVDI(−)/(+) clones therefore confirmed that they contained correct sequences.

To examine the transcription of RNA from pTVDI(−)/(+) clones by T7 RNA polymerase, *in vitro* transcription was performed (Fig. 30). In each case, RNAs of the appropriate sizes were generated suggesting that cleavage of the primary transcript by the ribozyme occurred. Two major RNA species were transcribed from pTVDI(−), which produced genomic RNA, and from pTVDI(+), which produced antigenomic RNA, although pTVHD(−)/(+) appeared to migrate as one large 6.1 kb band. This

Figure 29. Sequences in the T7 promoter regions of pTV-DI plasmids. The sequences represent DIP cDNAs. Two additional nucleotides (GG) are positioned between the T7 promoter (long arrow) and DIP sequences in all constructs. Two constructs containing the DIP cDNA in opposite orientations are indicated: pTVST(−), pTVOD(−), and pTVHD(−) are predicted to transcribe genomic RNA; pTVST(+), pTVOD(+), and pTVHD(+) are predicted to transcribe antigenomic RNA. Panels: A, sequences from the 5' proximal regions of both pTVST(−) and pTVST(+) are identical up to 54 nucleotides (arrowhead at both sides). From nucleotide 55 pTVST(−) shows 5' end sequences of IND-ST and pTVST(+) shows 3' end sequences of IND-ST; B, sequences from the 5' proximal regions of both pTVOD(−) and pTVOD(+) are identical up to 18 nucleotides (arrowhead at both sides). From nucleotide 19 pTVOD(−) shows 5' end sequences of Ogd-DI and pTVOD(+) shows 3' end sequences of Ogd-DI; C, sequences from the 5' proximal regions of both pTVHD(−) and pTVHD(+) are identical up to 71 nucleotides (arrowhead at both sides). From nucleotide 72 pTVHD(−) shows 5' end sequences of Haz-DI and pTVHD(+) shows 3' end sequences of Haz-DI.

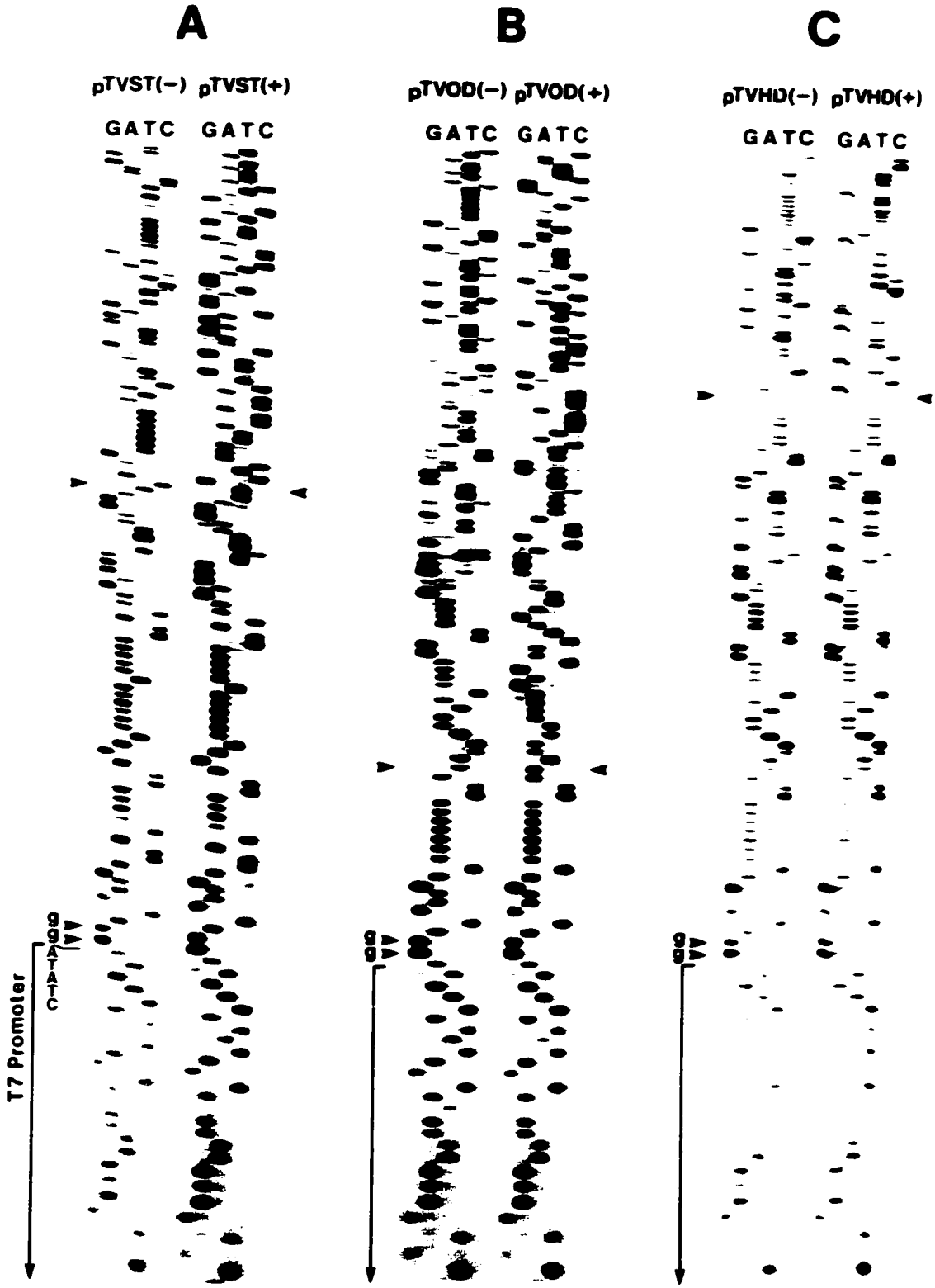
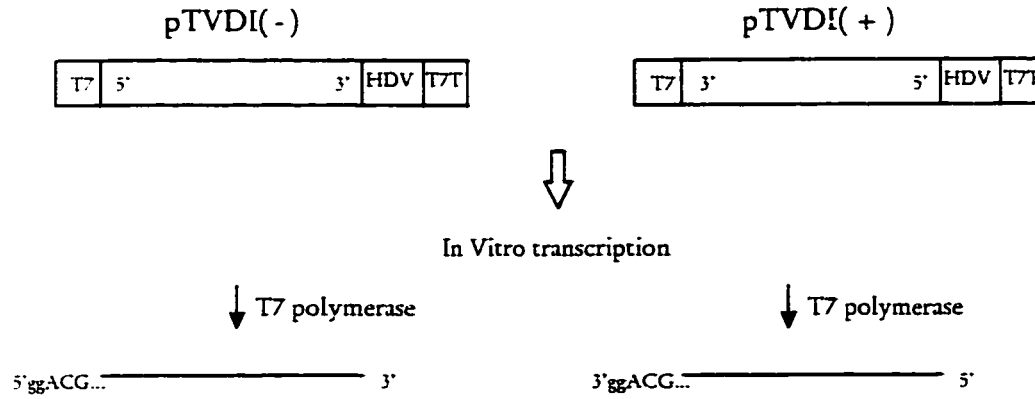
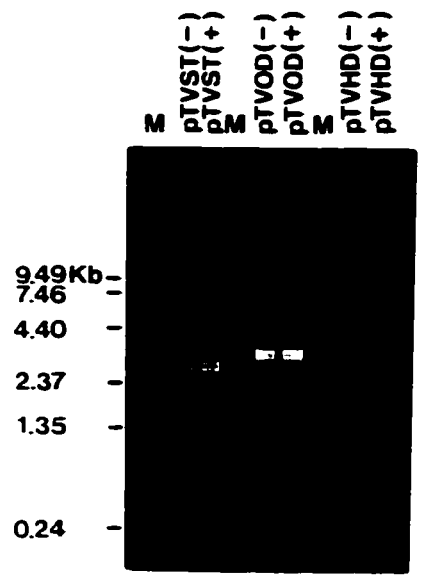

A
pTVST(−) pTVST(+)
GATC GATC
T7 Promoter
B
pTVOD(−) pTVOD(+)
GATC GATC
C
pTVHD(−) pTVHD(+)
GATC GATC

Figure 30. *In vitro* transcription of RNAs from pTVDI(−)/(+) constructs. The open bars represent pTVDI constructs containing the T7 promoter (T7), the HDV ribozyme sequence (HDV) and the T7 terminator (T7T). After *in vitro* transcription, pTVDI(−) is predicted to yield a negative-sense transcript containing two non-DI nucleotides at the 5' end (5'gg) and pTVDI(+) is predicted to yield a positive-sense transcript containing two non-DI nucleotides at the 3' end (3'gg). The transcripts were analyzed by electrophoresis on a 1.2% agarose-formaldehyde gel and visualized by ethidium bromide fluorescence. Lanes: lane pTVST(−), IND-ST genomic RNA transcript; lane pTVST(+), IND-ST antigenomic RNA transcript; lane pTVOD(−), Ogd-DI genomic RNA transcript; lane pTVOD(+), Ogd-DI antigenomic RNA transcript; lane pTVHD(−), Haz-DI genomic RNA transcript; lane pTVHD(+), Haz-DI antigenomic RNA transcript; lane M, a series of six synthetic poly(A)-tailed RNAs as RNA molecular size markers.


pTVDI(-)
T7
5'
3'
HDV
T7T
pTVDI(+)
T7
3'
5'
HDV
T7T
In Vitro transcription
T7 polymerase
T7 polymerase
5'ggACG...
3'
3'ggACG...
5'

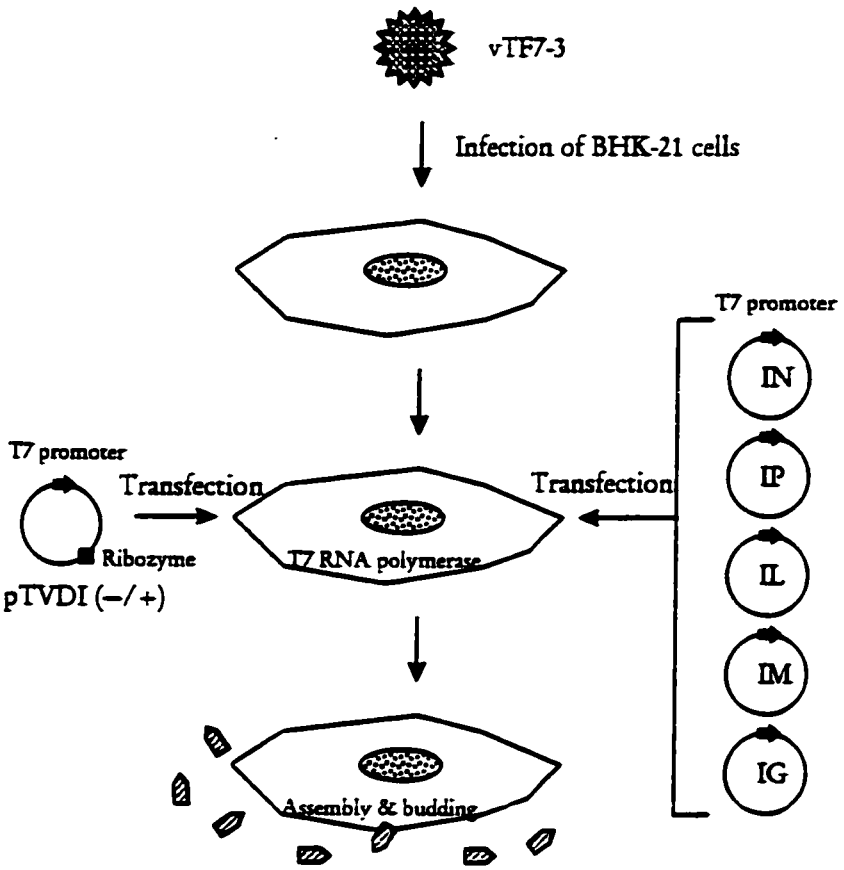

M
pTVST(-)
pTVST(+)
M
pTVOD(-)
pTVOD(+)
M
pTVHD(-)
pTVHD(+)
9.49Kb
7.46
4.40
2.37
1.35
0.24

single band for pTVHD(−)/(+) transcript might be due to low resolution of the agarose gel (1.2%) for large RNAs. When analyzed in a 0.8% agarose gel two bands were resolved for pTVHD(−)/(+) (data not shown). The faster-migrating species is believed to correspond to the full-length DIP genomic RNA [lanes, pTVST(−), pTVOD(−), and pTVHD(−)] or antigenomic RNA [lanes, pTVST(+), pTVOD(+), and pTVHD(+)] transcripts after cleavage by the HDV ribozyme. The species migrating slightly slower are presumably primary transcripts that have not been autocatalytically cleaved by the ribozyme (all lanes except marker lanes). Therefore, *in vitro* transcription of pTVDI(−)/(+) produced correctly sized DIP RNAs of about 2.6 kb, 3 kb, and 6.1 kb for pTVST(−)/(+), pTVOD(−)/(+), and pTVHD(−)/(+), respectively.

Amplification of DIP genomes from cDNA through serial passages

To determine whether DIP genomes transcribed from plasmid DNA could be rescued by the vaccinia virus-T7 RNA polymerase-based expression system, DIP RNA replication was analyzed (Fig. 31). BHK-21 cells in 10-cm plates were infected withvTF7-3. These cells were then transfected either with pTVDI(−), which should produce genomic DIP RNA, or with pTVDI(+), which should produce antigenomic DIP RNA, and with five other plasmids that express the VSV_{IND} N, P, M, G and L proteins. N protein is required to assemble nascent DIP genomic or antigenomic RNA into nucleocapsids. Once formed, these nucleocapsids should serve as templates for synthesis of minus-strand or positive-strand RNA by the L/P polymerase complex,

Figure 31. The protocol of amplification of DIPs from cDNAs and analysis of DIP RNA synthesis. BHK-21 cells (10-cm dishes) were infected with vTF7-3 (shaded star) and transfected with pBKS-IN, pBKS-IP, pEB2-IL, pBKS-IM, pBKS-IG, and either pTVDI(−) or pTVDI(+). The amount of transfected plasmids was with 3:2:1:3:3:3 propositions, respectively. After 48 hr posttransfection, the culture fluids (Passage 0) were harvested, pelleted, and used to inoculate VSV_{IND}-infected cells. Culture fluids were collected at 16 hr p.i. (Passage 1). Culture fluids from Passage 1 were then analyzed for the presence of DIPs by labeling cells with [^{3}H]uridine. Labeled RNAs were analyzed in a 1.2% agarose-formaldehyde gel. Shaded arrow, T7 promoter; shaded rectangle, HDV ribozyme; hatched bullet-shape, DIPs.



48 hrs posttransfection

Collect the culture fluids (P0)

centrifugation ↓ 35,000 rpm, 60 min

Pellet the rescued DI particles

Superinfect VSV_{IND}-infected cells with P0

incubation ↓ 37°C, 16 hrs

Collect the culture fluids (P1)

Infect fresh BHK-21 cells with P1

incubation ↓ 37°C, 4 hrs

Label cells with [^{3}H]uridine

incubation ↓ 37°C, 2 hrs

Harvest cells and isolate RNA

Analyze RNA on agarose-formaldehyde gel

initiating the DIP replication cycle. At 48 hr posttransfection, supernatants from cells transfected with pTVDI(−)/(+) and the protein-expressing plasmids were harvested and clarified. The presence of DIPs in the culture fluids from transfected cells was assayed by the RNA replication assay, as described below. VSV_{IND}-infected cells were incubated with supernatants from transfected cells 30 min postinfection. Cells were exposed to [^{3}H]uridine for 2 hrs at 4 hr postinfection to label newly replicating RNAs. Cytoplasmic extracts were prepared and the RNAs were extracted and analyzed by electrophoresis. If DIPs were released into the supernatant fluids of transfected cells and were able to superinfect the VSV_{IND}-infected cells, replication of DIP RNA should be detected.

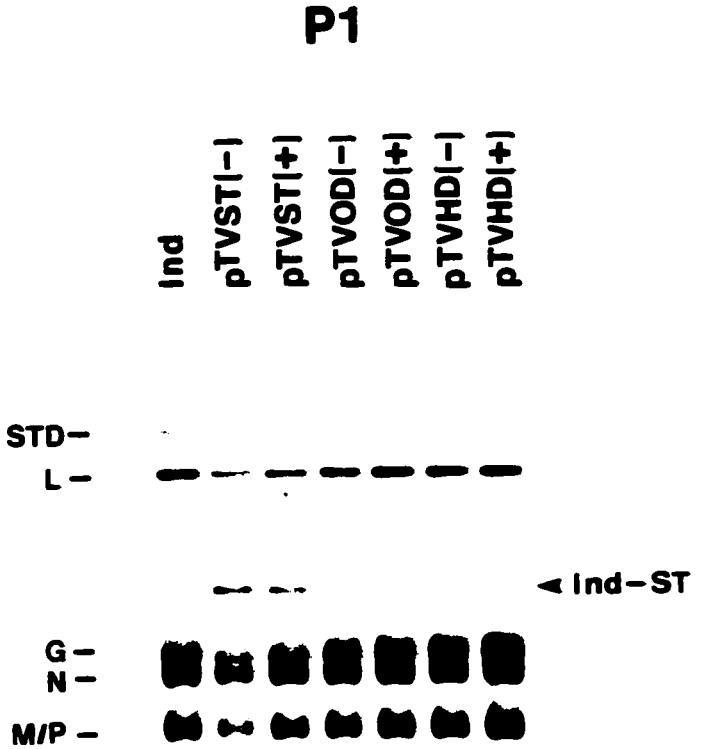
Despite numerous attempts, there was no evidence that DIPs were present in culture fluids of transfected cells (Passage 0). Although Pattnaik *et al.* (1992) reported that plasmid-expressed proteins supported rescue of DIP RNA, the transfection efficiency was very low and insufficient for DIP RNA synthesis in our hands. Since the amounts of DIPs present in the Passage 0 supernatant was very low, the culture fluids from transfected cells were filtered to remove most of the vaccinia virus and concentrated by ultracentrifugation. Subsequent passaging was then attempted to amplify DIPs. The pellets from the supernatants were added to fresh BHK-21 cells (35-mm plates) infected with VSV_{IND} to provide a source of the VSV proteins necessary to support encapsidation and replication. After 15-20 hr the supernatants were collected and clarified (Passage 1). One milliliter of each supernatant fluid from Passage 1 was then added to fresh BHK-21 cells in 10 cm plates. At this passage VSV_{IND} proteins did

not need to be provided to the cells because supernatants from Passage 1 contained adequate amounts of standard virus. The culture fluids were collected at 15-20 hr postinfection (Passage 2). Fresh BHK-21 cells were infected with supernatants from Passage 1 or 2, and labeled with [^{3}H]uridine as described above. The results of this assay for DIPs are shown in Fig. 32.

The results of RNA labeling in the presence of actinomycin D showed clearly that after Passage 1, the RNAs made by both plasmid pTVST(−) and pTVST(+) were copied into full-length DIP RNAs [lanes, pTVST(−), pTVST(+)]. Replication of the negative-sense RNA transcript of pTVST(−) by T7 RNA polymerase was evident from the presence of IND-ST RNA. As for pTVST(+), which served as an indicator for encapsidation and replication, IND-ST were also assembled and released from cells transfected with pTVDI. The amount of each protein expressed in the cells could be optimized by previously developed systems (Pattnaik *et al.* 1991, 1992, Conzelmann and Schnell 1994, Stillman *et al.* 1995). The levels of proteins synthesized in cells transfected with all five plasmids under the conditions used here therefore supported replication, assembly, and budding of DIPs.

In contrast, no RNA synthesis was detected in cells infected with culture fluids from Passage 1 of transfected-pTVOD(−)/(+) and pTVHD(−)/(+) [lanes, pTVOD(−), pTVOD(+), pTVHD(−), and pTVHD(+)]. This result was expected because previous results (Figs. 9 and 10) demonstrated that NJ-DIPs did not interfere with the IND-standard virus and there was no NJ-DIP RNA synthesis in VSV_{IND}-infected cells. This transfection result therefore correlated with the previous interference assay. Only

Figure 32. Assay for the presence of DIPs in the culture fluids following Passage 1. BHK-21 cells were infected with 200 μl of Passage 1 culture fluids and labeled with [^{3}H]uridine as described in Fig. 8. Labeled RNAs were analyzed in a 1.2% agarose-formaldehyde gel. Lanes: lane Ind, culture fluid from VSV_{IND} standard virus-infected cells; lanes pTVST(−), pTVST(+), pTVOD(−), pTVOD(+), pTVHD(−) and pTVHD(+), culture fluid from cells infected with Passage 1 supernatants of cells initially transfected with pTVST(−), pTVST(+), pTVOD(−), pTVOD(+), pTVHD(−) and pTVHD(+), respectively. The arrowhead indicates the position of IND-ST RNA.

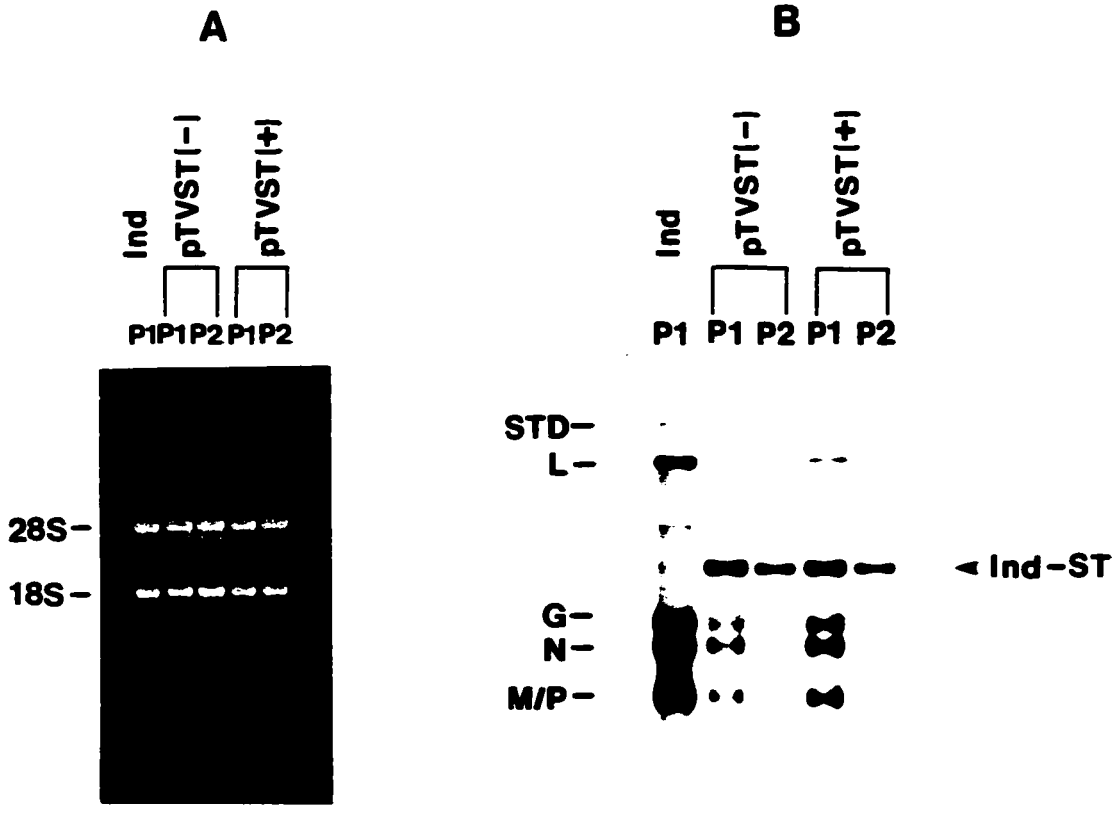

P1
Ind
pTVST(-)
pTVST(+)
pTVOD(-)
pTVOD(+)
pTVHD(-)
pTVHD(+)
STD
L
Ind-ST
G
N
M/P

Indiana standard virus infected cells without any superinfection of culture fluids from Passage 1 also showed no detectable DIP RNA species as control experiment (lane Ind). These results demonstrated that VSV_{IND} proteins expressed from plasmids could support the replication of IND-DIP but not NJ-DIPs.

Following passage, the intracellular RNA of BHK-21 cells was also tested for the presence of IND-ST DIP RNA (Fig. 33). The amounts of extracted RNA were the same in the all samples (Fig. 33A). Analysis of the RNAs also showed that the IND-ST accurately reproduced all of the replicative and transcriptional events (compare lane Ind and other lanes). IND-ST was detected in the culture fluids of cells (Passages 1 and 2), so amplification occurred in cells expressing all five VSV proteins produced by the standard VSV during successive passages. It was expected that the recombinant IND-ST would be propagated by the standard virus, like natural DIPs, since the original IND-ST genome was naturally selected during the genesis of this DIP.

It was found that IND-ST could be rescued, from a clone expressing the genomic or antigenomic RNA in cells expressing all five VSV_{IND} proteins supplied by VSV_{IND} standard virus and passaged and amplified [Fig. 33B, lanes, pTVST(−) and pTVST(+)]. Under these conditions of VSV_{IND}-supported rescue, not only the input negative-sense DIP RNA [pTVST(−)] replicated to form positive-sense RNA, but also the input positive-sense RNA [pTVST(+)] was assembled with the nucleocapsid protein and the newly synthesized polymerase to form an active replication complex to synthesize new negative-sense RNA. Either positive- and negative-sense RNAs are encapsidated but only the nucleocapsids containing the negative-sense RNA are incorporated into budded

Figure 33. Assay for the presence of DIPs in the culture fluids from subsequent passage. BHK-21 cells were infected with 200 μl of each passage culture fluids and labeled with [^{3}H]uridine as described in Fig. 8. Labeled RNAs were analyzed in a 1.2% agarose-formaldehyde gel. (A) The RNAs were visualized by ethidium bromide fluorescence. 28S and 18S rRNAs are indicated. (B) The labeled RNAs were subjected to fluorography. Lanes: lane Ind, culture fluid (Passage 1) from VSV_{IND} virus-infected cells; lane P1[pTVST(−)], culture fluid from cells superinfected with Passage 0 supernatant [initially transfected with pTVST(−)]; lane P2[pTVST(−)], culture fluid from Passage 1-infected cells, lane P1[pTVST(+)], culture fluid from cells superinfected with Passage 0 supernatant [initially transfected with pTVST(+)]; lane P2[pTVST(+)], culture fluid from Passage 1-infected cells. Arrowhead indicates the position of IND-ST RNA.


A
Ind
pTVST(-)
pTVST(+)
P1
P1
P2
P1
P2
28S
18S
B
Ind
pTVST(-)
pTVST(+)
P1
P1
P2
P1
P2
STD
L
Ind-ST
G
N
M/P

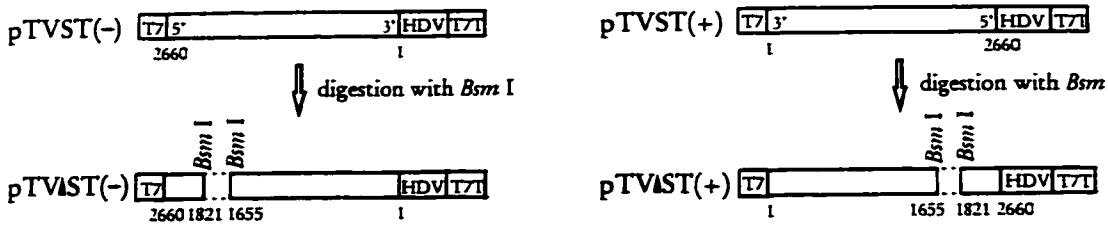
virions. The mechanism by which this selection is exerted is not known at present.

Recovery of genetically altered IND-ST

In order to demonstrate that IND-ST DIP can be recovered from the cDNA clone, a genetic marker was introduced into the cDNA clones of IND-ST genome. As a target, the *Bsm*I sites, which are located at nucleotide 1655 and nucleotide 1821 (pTV has no *Bsm*I site) were selected. The plasmids pTVΔST(−)/(+) were constructed by deleting a part of the pTVST(−)/(+) cDNA spanning the nucleotides 1655-1821 (Fig. 34). Transfection experiments were carried out with pTVΔST(−)/(+) as described above. The "transfectant" DIPs [clones pTVST(−)/(+) and pTVΔST(−)/(+)] were propagated by transfering the supernatants to fresh cells two more times in the presence of the helper standard virus. The propagation of original IND-ST DIPs as well as the truncated IND-ST DIPs was demonstrated by infection with passage 1 supernatants in the presence of helper standard virus (Fig. 34). Both the original IND-ST genomic RNAs [lanes, pTVST(−), pTVST(+)] and the deleted IND-ST genomic RNAs [lanes, pTVΔST(−), pTVΔST(+)] were detected.

To demonstrate the presence of the genetic marker in the "transfectant" IND-ST DIPs and their deleted IND-ST DIPs genome, IND-ST DIP genomic RNAs were purified from the recovered viruses after 2 passages and then carried out a reverse transcription-PCR (RT-PCR) using a unique primer VSVI3 (see Appendix), which is specific for the inverted repeats of IND-ST genome (Fig. 35A). The reverse transcription products were then amplified by PCR using the same primer as RTase

Figure 34. Assay for the presence of deleted IND-ST in the culture fluids following Passage 1. Experiments were carried out as described in the legend of Fig. 32. Lane Ind, culture fluid from VSV_{IND} standard virus-infected cells; lanes pTVST(−), pTVST(+), pTVΔST(−), and pTVΔST(+), culture fluid from cells infected with Passage 1 supernatants of cells initially transfected with pTVST(−), pTVST(+), pTVΔST(−), and pTVΔST(+), respectively. Arrowheads indicate the positions of IND-ST RNA and IND-ΔST RNA.


pTVST(-)
T7
5'
3'
HDV
T7t
2660
1
digestion with Bsm I
Bsm I
Bsm I
pTVΔST(-)
T7
HDV
T7t
2660 1821 1655
1
pTVST(+)
T7
3'
5'
HDV
T7t
1
2660
digestion with Bsm I
Bsm I
Bsm I
pTVΔST(+)
T7
HDV
T7t
1
1655 1821 2660

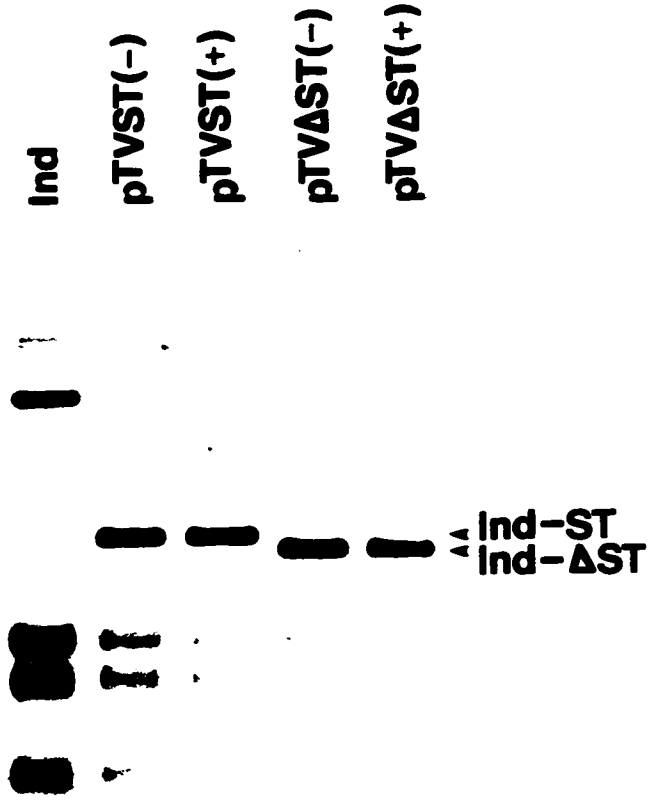
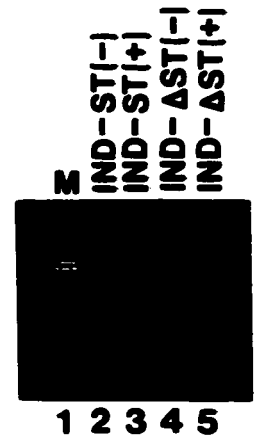
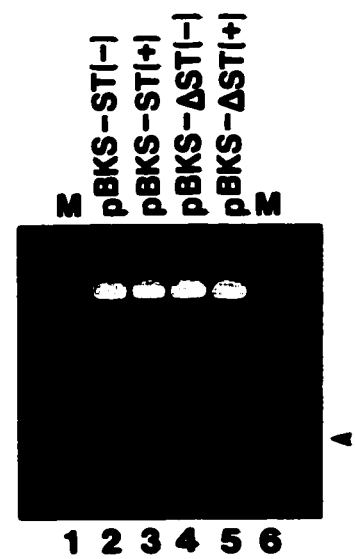

Ind
pTVST(-)
pTVST(+)
pTVΔST(-)
pTVΔST(+)
Ind-ST
Ind-ΔST

Figure 35. Demonstration of the genetic marker in the genome of the transfectant virus IND-ΔST. (A) PCR amplification of IND-ST RNAs and their deleted mutants. Experiments were carried out as described in the legend of Fig. 11. One-tenth of the products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide fluorescence. Approximate sizes of DIP DNAs are indicated by comparison with marker DNA (lane 1, *Bst*EII-digested bacteriophage lambda DNA). Lanes 2, 3, 4, and 5; DNA amplified from IND-ST(−) RNA, IND-ST(+) RNA, IND-ΔST(−) RNA, and IND-ΔST(+) RNA respectively. (B) Identification of a restriction enzyme recognition site in the "transfectant" DIP genome. DNA samples in pBKS were digested with *Bsm*I (pBKS has no *Bsm*I site) and separated on a 1% agarose gel. DNAs were detected by staining with ethidium bromide. Lane 1, *Hae*III-digested ϕX174 DNA, lane 2, 3, 4, and 5; *Bsm*I-digested DNA in pBKS-ST(−), pBKS-ST(+), pBKS-ΔST(−), and pBKS-ΔST(+), respectively, lane 6, *Bst*EII-digested bacteriophage lambda DNA. *Bsm*I fragments are present only in DNA derived from pBKS-ST(−)/(+) [arrow marked].

A


M
IND-ST(-)
IND-ST(+)
IND-ΔST(-)
IND-ΔST(+)
1 2 3 4 5

B


M
pBKS-ST(-)
pBKS-ST(+)
pBKS-ΔST(-)
pBKS-ΔST(+)
M
1 2 3 4 5 6

primer. The PCR amplification of the cDNAs could only take place for the DIP RNAs. DNA fragments of ~2.6 kb [Fig. 35A, lanes, IND-ST(−), IND-ST(+)] and ~2.5 kb [lanes, IND-ΔST(−), IND-ΔST(+)] were obtained from the genomes of "transfectant" IND-ST(−)/(+) and IND-ΔST(−)/(+), respectively. The PCR products were then cloned to pBKS. The presence of the genetic marker in the "transfectant" DIP was verified by digestion of the pBKS-ST(−)/(+) and pBKS-ΔST(−)/(+) with *Bsm*I restriction enzyme (Fig. 35B). After digestion with *Bsm*I, the expected fragment of 166 bp was obtained from the complete IND-ST RNA (Fig. 35B, lanes 2 and 3), whereas the cDNA clone derived from the sequence deleted IND-ST RNA did not produce the 166 bp fragment because *Bsm*I will cut once (lanes 4 and 5). Only pIND-ST(−)/(+)-derived DNAs gave rise to a fragment of 166 bp (arrow marked). Sequencing of DIP DNA of "transfectant" IND-ST and IND-ΔST further confirmed the presence of the expected deletion of 166 bp at the predicted site, while the rest of the determined sequence corresponded to that of the original IND-ST genome (data not shown).

With this method, the presence and absence of the *Bsm*I fragment (166 bp) in the recovered virus RNA was verified and the results for the *Bsm*I fragments were shown in Fig. 35B. The absence of the 166 bp *Bsm*I fragment in the cDNA clone of IND-ΔST clearly demonstrated that the same DIP had been recovered from the cloned DNA.

Characterization of transfectant DIPs

The "transfectant" IND-ST were characterized by the interference assay and RNA synthesis as described in Chapter 3. The culture fluids from Passage 2 were used for

preparation of "transfectant" IND-ST stocks because the supernatants of this passage contained mostly "transfectant" IND-ST and the replication of the standard virus was inhibited markedly (Fig. 33). BHK-21 cells (five 10 cm plates for each "transfectant" DIPs) were infected with one milliliter each of Passage 2 supernatants and incubated at 37°C for 15 to 18 hrs. "Transfectant" DIPs were purified on sucrose gradients. The bandwidth and density of "transfectant" DIPs were the same as those of natural IND-DIP. These results show that the "transfectant" DIPs were propagated by the standard virus, like natural DIPs. The concentrations of two "transfectant" DIPs and natural IND-ST were adjusted to obtain the same amounts of DIPs in each stock. The interference ability of "transfectant" DIPs was investigated by using the standard method. For comparison, the natural IND-ST was also used. Fig. 36 depicts that two "transfectant" IND-ST interfere well with the replication of VSV_{IND} (Panel A) and $VSV_{NJ\text{-}Ogd}$ (Panel B), but poorly with $VSV_{NJ\text{-}Haz}$ (Panel C). This result is consistent with the interference activity of the original IND-ST in that the biological activities of two "transfectant" IND-ST were indistinguishable from the original IND-ST.

Analysis of RNA synthesis also showed that the infection with increasing amounts of the input "transfectant" IND-ST resulted in increasing amounts of inhibition of RNA synthesis of the standard virus as observed with the natural IND-ST (Fig. 37, lanes 1 to 3). The amount of "transfectant" DIP RNA [pTVST(−) and pTVST(+)] replication was almost the same as RNA replication of the natural IND-ST in the presence of Indiana standard virus (compare each lane, 1, 2 and 3 in Fig. 37).

The "transfectant" IND-ST recovered from the plasmid containing the antigenomic

Figure 36. Homotypic and heterotypic interference by transfectant IND-ST. BHK-21 cells were infected with VSV alone (Panel A, VSV_{IND}; Panel B, $VSV_{NJ\text{-}Ogd}$; Panel C, $VSV_{NJ\text{-}Haz}$) at a MOI=3 or plus serial four fold dilutions (1:400, 1:100, and 1:25) of natural IND-ST (IND-ST) or "transfectant" IND-ST [pTVST(−) or pTVST(+)]. Virus was adsorbed for 1 hr at 37°C and medium was added. At 16 hr after infection, the cultures were harvested and assayed for plaque formation on BHK-21 cells. (●—●) natural IND-ST; (▲—▲) "transfectant" IND-ST from pTVST(−); (■—■) "transfectant" IND-ST from pTVST(+); (◆) each standard virus.

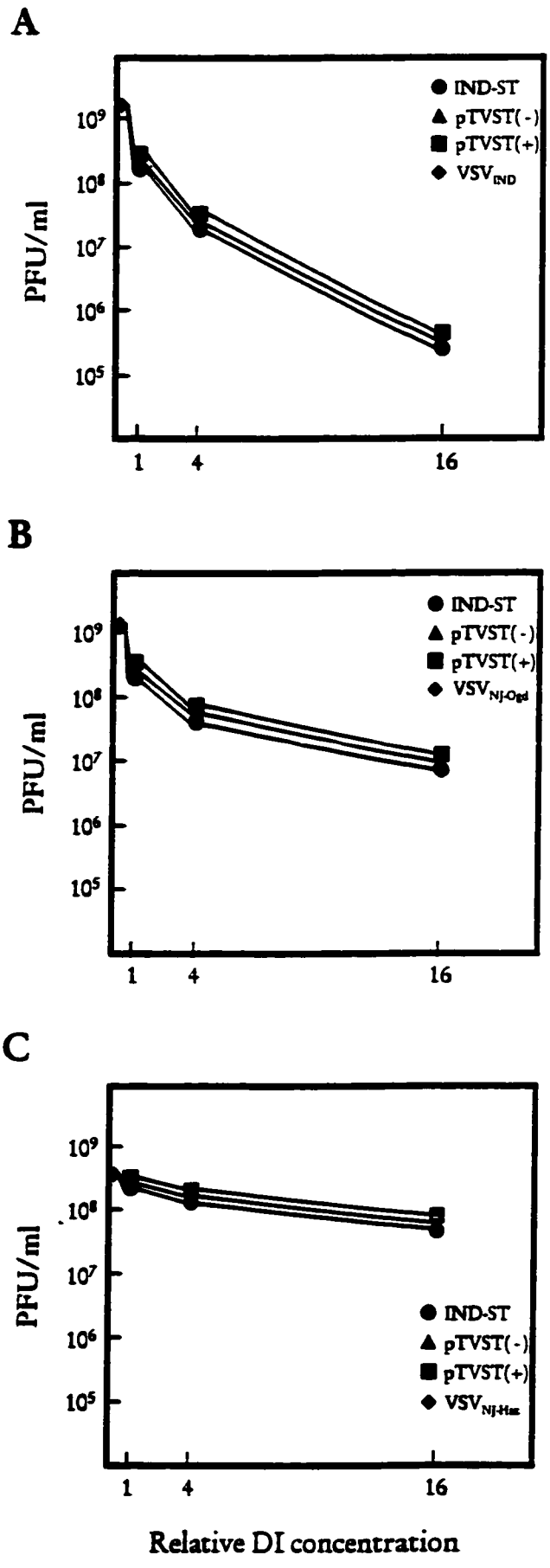
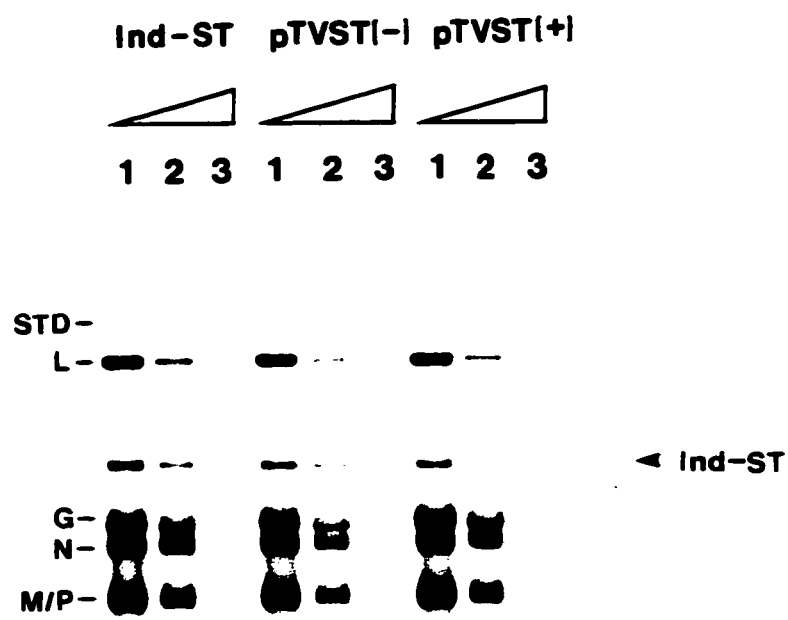

A
IND-ST
pTVST(-)
pTVST(+)
VSV IND
PFU/ml
10^9
10^8
10^7
10^6
10^5
1
4
16
B
IND-ST
pTVST(-)
pTVST(+)
VSV NJ-Ogd
PFU/ml
10^9
10^8
10^7
10^6
10^5
1
4
16
C
IND-ST
pTVST(-)
pTVST(+)
VSV NJ-Hau
PFU/ml
10^9
10^8
10^7
10^6
10^5
1
4
16
Relative DI concentration

Figure 37. Viral RNA synthesis in cells infected with VSV_{IND} standard virus and transfectant IND-ST. Infected cells were treated with 2 μg/ml of actinomycin D for 1 hr and labeled with [^{3}H]uridine as described in Fig. 8. The labeled RNAs recovered were separated in a 1.2% agarose-formaldehyde gel and detected by fluorography. Lanes 1 to 3; standard virus plus 1:400 dilution (each lane 1), 1:100 dilution (each lane 2) and 1:25 dilution (each lane 3) of natural IND-ST (Ind-ST) or "transfectant" IND-ST from pTVST(−) or "transfectant" IND-ST from pTVST(+) coinfection. The positions of the standard genomic RNA (STD) and L, G, N, M and P mRNAs are indicated. Arrowhead represents the position of IND-ST RNA.


Ind-ST
pTVST(-)
pTVST(+)
1 2 3 1 2 3 1 2 3
STD-
L-
Ind-ST
G-
N-
M/P-

template [pTVST(+)] showed the same characteristics as the "transfectant" IND-ST recovered from the plasmid containing the genomic template [pTVST(−)]. These results indicate that DIP RNAs were replicated in the plasmid transfected cells in which all five VSV proteins were expressed. Recovery of DIPs from cDNA was partially verified by showing the same interference ability and the same pattern of RNA synthesis as the natural DIP.

3. DISCUSSION

Establishment of VSV DIP rescue system for studying interference mechanisms

The reverse genetic system provides a useful tool to study the mechanism(s) of viral interference. This system was first introduced for VSV by Pattnaik *et al.* (1992) in which the RNA from DIP of VSV could be recovered from a cloned DIP DNA in the presence of N, P, M, G, and L proteins in *trans*. The transient vaccinia virus/T7 polymerase system was used to express individual VSV proteins from plasmids. The cDNA constructs were designed in a way to allow intracellular transcription of RNAs with termini exactly the same as the authentic sequence. In order to generate a proper 5'-terminus, it was necessary to add two G residues between the T7 promoter and the VSV sequence. These extra nucleotides at the 5' terminus of the RNA transcripts could be trimmed by the viral polymerase (Pattnaik *et al.* 1992). RNAs terminating with proper 3'-terminal sequences were shown to be critical for the replication of VSV DIP RNA (Pattnaik *et al.* 1992). This was achieved by the autolytic activity of the HDV ribozyme (Ball 1992). DIP genomic RNAs transcribed by the T7 RNA polymerase

were recognized by VSV proteins expressed from transfected plasmids. Like all the published systems (Pattnaik *et al.* 1992, Conzelmann and Schnell 1994, Stillman *et al.* 1995), DIPs were recovered from cloned DIP cDNA in the presence of all five VSV proteins expressed from the cloned genes.

The interesting observation made with our system related to the amplification and purification of "transfectant" DIPs. If DIPs recovered from cDNA had been released from transfected cells, these should have been amplified in a manner similar to that of the natural DIPs. This suggests that intracellularly produced RNAs were encapsidated, replicated, and assembled into virions by five VSV proteins supplied also from the transfection of the cDNA clones. Conzelmann and Schnell (1994) have reported that packaging of pSDI transcripts and subsequent expression of β-Gal occurred in only 1 of 1,000 cells correctly transfected with all plasmids and infected with vTF7-3. Since the transfection efficiency was so low and insufficient for RNA synthesis in our experiments, amplification of the transfected RNAs by standard helper VSV was attempted. It was successful and the "transfectant" DIP RNA could be demonstrated by direct biochemical methods.

A reconstituted *in vitro* system has previously been developed to study the requirements for VSV RNA replication (Davis and Wertz 1982, Patton *et al.* 1983, Wertz 1983, De and Banerjee 1984). Although the available *in vitro* replication systems have defined the protein requirements for VSV RNA replication, the ability of these systems to allow analysis of the structure-function relationships of various proteins involved in RNA replication was limited because the input nucleocapsid templates

contained the polymerase. Because of the limitations associated with the existing *in vitro* replication systems, the system described here will provide an opportunity to study the roles of individual viral protein and modifications of DIP genome in viral replication and interference.

The ability to amplify "transfectant" DIPs recovered from cDNA will be particularly useful for genetic analyses and for studies of viral interference mechanism(s) mediated by DIPs since "transfectant" DIPs can be purified in large quantities. Wertz *et al.* (1994) suggested that the possession of complementary termini is a major factor in determining the efficiency of replication and confers a powerful replicative advantage. The two termini of the RNA may well provide the polymerase recognition site and/or nucleating site for the encapsidation process. Now it is possible to genetically engineer "transfectant" DIPs for identification of *cis*-acting sequences involved in interference based on complete sequence analyses of four different DIP genomes. Studies currently in progress are aimed at determining the exact nature of the *cis*-acting signals responsible for viral interference. The system described here also provides an opportunity to study the roles of individual viral protein domains and modifications and different combinations of proteins from other serotypes in viral interference. Thus, the factors and signals for viral interference will be reconstructed entirely from DNA-encoded proteins and RNAs and detailed structure-function analyses of VSV gene products involved in viral interference are now possible.

CHAPTER 6: MECHANISM OF HOMOTYPIC AND HETEROTYPIC VIRAL INTERFERENCE

Although DIPs have been described for a number of animal viruses, the DIPs of VSV have received the most scrutiny with respect to mechanisms of interference (Huang and Wagner 1966, Huang and Baltimore 1970, Prevec and Kang 1970, Holland *et al.* 1976, Schnitzlein and Reichmann 1976, Khan and Lazzarini 1977, Moyer and Gatchell 1979, Perrault 1981, Holland 1987, Von Laer *et al.* 1988). Most previous systems may not be directly relevant to interference by DIPs but do present a number of interesting hypotheses. In this thesis, attempts were made to directly explain the possible viral interference mediated by DIPs. DIPs of VSV were generated and/or amplified by serial undiluted passage of the standard virus. Two IND-DIPs interfered with both Indiana standard virus and New Jersey standard virus. Under these conditions, DIP genomic RNA was synthesized and the RNA synthesis of standard virus was inhibited. In contrast, two NJ-DIPs could not interfere with Indiana standard virus and DIP RNA synthesis was not detected. This suggests that the DIP genomic RNA must be recognized as a template by either the homotypic RNA polymerase complex or the heterotypic RNA polymerase complex in order to demonstrate viral interference.

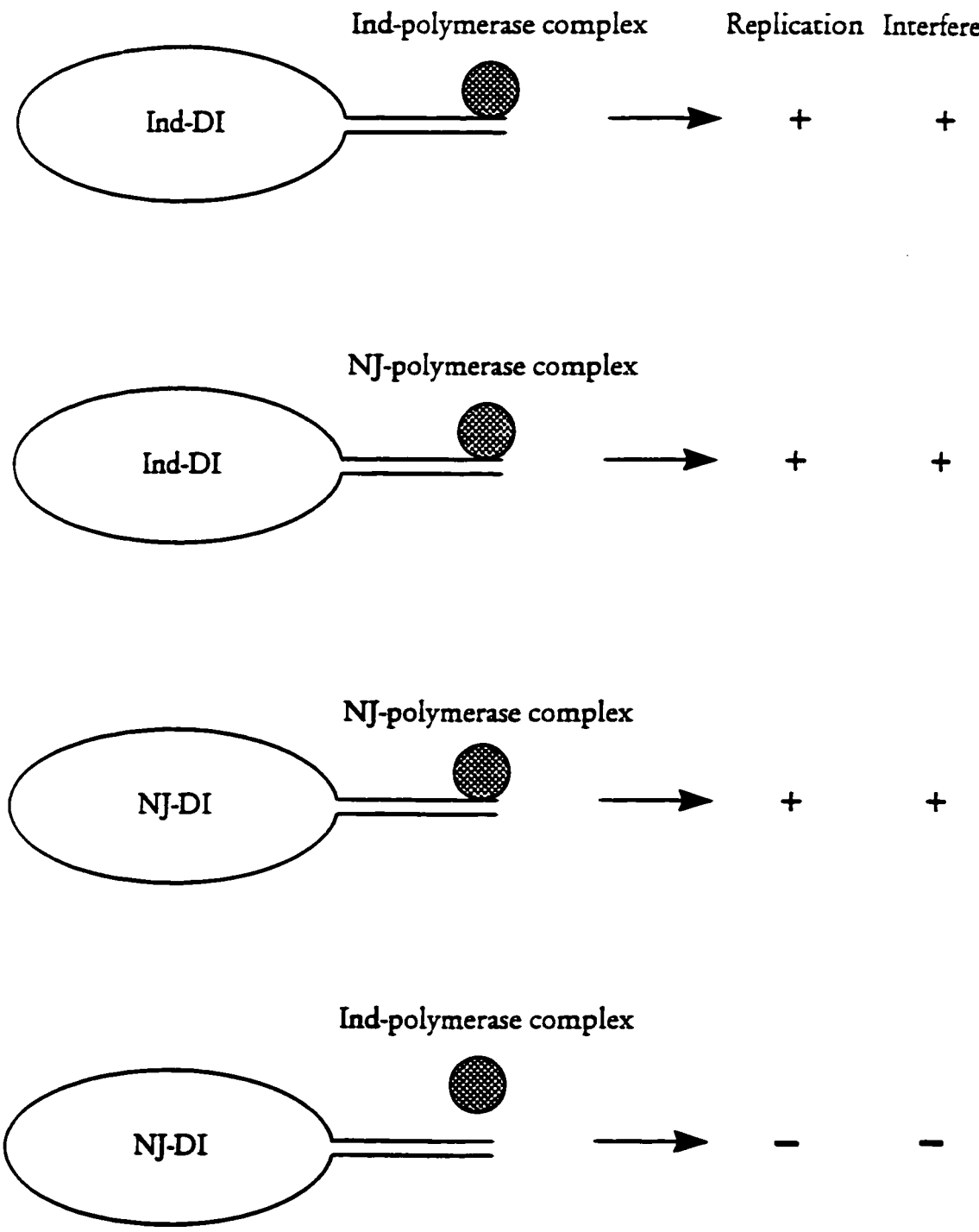
Nucleotide sequence analysis of these DIP genomes revealed that they retained the exact 5' end of the standard virus genome. The data indicate that this region may be important for their replication and interference ability. Since three DIPs (IND-ST,

Ogd-DI, and Haz-DI) are non-transcribing DIPs and contain different lengths of inverted complementary termini and another DIP (IND-LT) is 3' transcribing DIP and contains the same 3' and 5' termini of the standard virus genome, it may be possible that IND-LT has different interference mechanisms from nontranscribing DIPs.

Based on the 5' DIPs, a possible model for homotypic and heterotypic interference is proposed (Fig. 38); If the viral polymerase complex recognizes the termini of the DIP genome, DIP RNA is synthesized by competing with standard virus genomes for the polymerase complex and thus the RNA synthesis of the standard virus is inhibited. The competition by DIP genome for RNA polymerase was originally suggested by Stampfer *et al.* (1969). In contrast, if the viral polymerase complex does not recognize the termini of the DIP genome, there is no DIP RNA synthesis and no inhibition of standard virus RNA synthesis.

The two ends of DIP genomes are likely to be involved in the RNA polymerase recognition site or preferential encapsidation of the DIP genomes over that of the standard virus. Thus, the study of the relationship between the terminal sequence of a DIP genome and the individual proteins consisting of the viral polymerase complex will shed light on the mechanism of interference mediated by DIPs. To analyze this at the molecular level, a system known as reverse genetics in which DIP from cDNA is rescued in cells expressing all five VSV proteins from cloned cDNAs was established. Using this reverse genetics approach, the following studies are now possible; (a) the reconstitution experiment to determine the specificity of homotypic and heterotypic interactions, and which combinations of viral proteins can support DIP genome

Figure 38. Possible model for homotypic and heterotypic viral interference. The stippled circles indicate viral polymerase complex. Replication: +, DIP genomic RNA synthesis by either homotypic polymerase complex or heterotypic polymerase complex; −, no DIP genomic RNA synthesis. Interference: +, DIP interferes with the replication of the standard virus; −, DIP does not interfere with the replication of the standard virus.


Ind-polymerase complex
Replication
Interfere
Ind-DI
+
+
NJ-polymerase complex
Ind-DI
+
+
NJ-polymerase complex
NJ-DI
+
+
Ind-polymerase complex
NJ-DI
-
-

replication/interference, (b) the identification of sequences in the DIP genome that are important for interference by constructing serial deletion DIP cDNA clones or chimeric DIP clones by exchanging the terminal sequences from the two serotpyes of VSV.

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APPENDIX

OLIGONUCLOTIDE SEQUENCES

Primers used for reverse transcription of viral genes

CD3 5'-GAGAGTCGACGCGGCCGCTTTTTTTCATA-3'

Primers used for PCR of DI cloning into pBKS

VSVI3 5'-GGGCGGCCGCCGTACGAAGACCACAAAACC-3'

VSVLT 5'-GGGCGGCCGCCGTACGAAGACAAACAAACC-3'

VSVNJ 5'-GGGCGGCCGCCGTACGAAGACAAAAAAACCA-3'

Primers used for sequencing of viral genes/DI genomes in pBKS

Foward 5'-GTTTTCCCAGTCACGAC-3'

Reverse 5'-GGAAACAGCTATGACCATG-3'

Primers used for sequencing of DI cDNA in pTV

TV 5'-TAATACGACTCACTATA-3'

SP6 5'-GATTTAGGTGACACTATAG-3'

Primers used for PCR of DI subcloning into pTV

ID5G 5'-TTAATACGACTCACTATAGGACGAAGACCACAAAACC-3'

HD5G 5'-TTAATACGACTCACTATAGGACGAAGACAAAAAAACC-3'

ID3 5'-GAGATGCCATGCCGACCCACGAAGACCACAAAACC-3'

HD3 5'-GAGATGCCATGCCGACCCACGAAGACAAAAAAACC-3'

LT3 5'-GAGATGCCATGCCGACCCACGAAGACAAACAAACC-3'

UNIV5 5'-CATTAATGCAGGGGGATCTCGATCCCGCGAAATTAAT ACGACTCACTATAGG-3'

UNIV3 5'-TTCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGAT GCCATGCCGACCC-3'

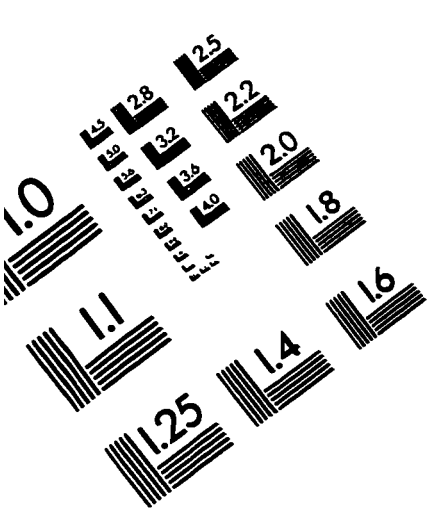
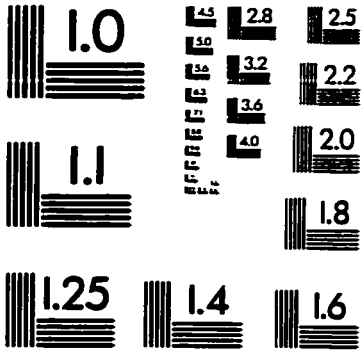
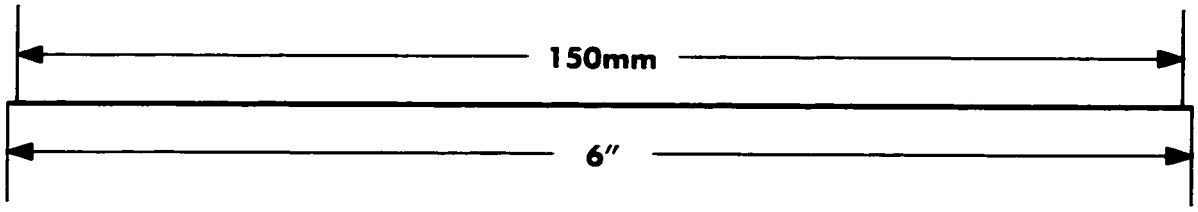
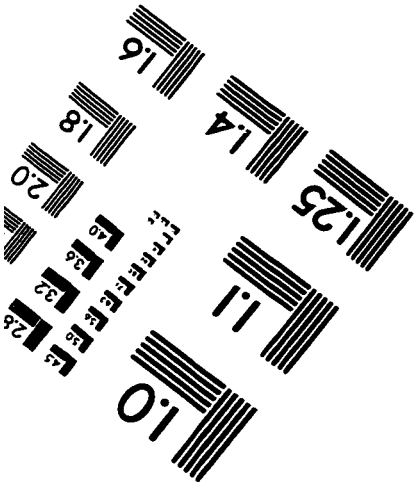


IMAGE EVALUATION TEST TARGET (QA-3)







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