NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

DEFECTIVE INTERFERING PARTICLES OF HUMAN PARAINFLUENZA VIRUS 3 AND ESTABLISHMENT OF PERSISTENT INFECTIONS

A Thesis Submitted to the

School of Graduate Studies

University of Ottawa

In partial fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

Faculty of Medicine

By

Donald G. Murphy

ISBN 0-315-62307-1

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Defective interfering particles of human parainfluenza virus 3 (HPIV3) were generated and/or amplified by serial undiluted passage of the standard virus. Analysis of the progeny virus titer at each cell passage revealed a cyclic pattern of virus production. Viruses produced from serial passages 8 and 9 interfered with replication of the standard virus. When cells were mixedly infected with standard virus and virus from either serial passages 5 or 8, three subgenomic RNA species, in addition to the standard virus genomic RNA, were detected in the progeny virions. Northern blot analysis revealed that all three subgenomic RNAs contained sequences from the 5'-end of the standard virus genome. The data indicates that 5' defective interfering (DI) particles were generated and/or amplified during serial undiluted passage of HPIV3.

Two independent HPIV3 persistent infections of LLC-MK_2 cells were established. One persistently infected culture was established by propagating the few cells which survived from an acute standard virus infection. The other persistently infected culture was readily established with virus from serial undiluted passage 8. This suggests that the DI particles present in serial passage 8 virus protected the cells from standard virus destruction. The two persistently infected cultures were propagated for nearly three years and the virus released from both cell lines at all times examined was noncytocidal. Subgenomic RNAs (putative DI particle genomes) were detected in virions released from both persistently infected cultures at various cell passages. No apparent changes in the size of the viral proteins were observed throughout the persistent infections. The DI particle-size genomes and the noncytocidal

mutant virus are both likely to be involved in the maintenance of the persistent state.

The degree of genetic change undergone by the viral genome during long-term persistence was examined. Nucleotide sequence analysis of the M gene and flanking regions of virus recovered after 147 cell passages (29 months of persistence) revealed that the viral genome did not undergo extensive genetic change. In the M protein coding region, only one conservative amino acid change was observed indicating that M protein mutation is not likely to be involved in the maintenance of the persistent infections. In contrast, the genomic 3'-termini of viruses recovered after 146 cell passages was found to be highly mutated. The mutations observed were either U to C or A to G changes. Such exclusive substitution of one type of nucleotide for another is unlikely to be due to intrinsic polymerase errors but to an extrinsic biased hypermutational activity. An RNA unwinding/modifying activity that could give rise to the biased hypermutations is discussed.

ACKNOWLEDGEMENTS

I would like to thank Dr. C. Yong Kang for his support and guidance and for providing me with an excellent scientific environment to conduct my research. After 2 years as a Master's candidate, I am grateful that he encouraged me to pursue Ph.D. studies.

Special thanks go to Dr. Ken Dimock for his never-ending help and advice, ideas and numerous discussions. I thank his laboratory for providing me with the cDNA clones used in my research project and many other collaborative contributions.

I am glad to have shared the laboratory with Ken Garson and Marie-José Côté. I thank them for all their help, advice and comradery. I am grateful to Doug Storey and Erling Rud who in the early years assisted me with my work. I am also grateful for the help and advice brought by Mark Parrington during my studies.

I thank Lynda Lachance for her work during one summer which contributed to some of the data in this thesis. I would also like to thank Bernadette Mah for her sequencing analysis of some of my clones.

I am grateful to Dr. Mark Galinski for providing me with a full-length clone of the L gene sequence of human parainfluenza virus 3.

Finally, I would like to thank the secretarial staff and support staff of the department who have always been helpful and kind with me.

A predoctoral scholarship from the "Fonds pour la Formation de Chercheurs et l'Aide à la Recherche" was received for 10 semesters of this study. I also acknowledge the financial support of the University of Ottawa.

DEDICATION

This thesis is dedicated to Guylaine

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PAGE

LIST OF ABBREVIATIONS

μCi	microcuries
μg	microgram
μmol	micromoles
A	Adenine base in a nucleotide sequence
AMV	avian myeloblastosis virus
Asn	Asparagine
ATCC	American type culture collection
BHCl	benzamidine-hydrochloride
Bis	N,N'-Bis-methylene-acrylamide
BPB	bromophenol blue
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
BSC-1	African green monkey kidney cells
°C	degrees Celsius
C	Cytosine base in a nucleotide sequence
CA	California
cDNA	complementary DNA
Ci	Curies
cm	centimetre
CPE	cytopathic effect
CT	Connecticut
CV-1	African green monkey kidney cells
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate

ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxyribonucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DI	defective interfering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid disodium salt
ELISA	enzyme-linked immunosorbant assay
EtBr	ethidium bromide
F	fusion protein
Fig.	Figure
FL	Florida
g	gravity or grams
G	Guanine base in a nucleotide sequence
GAR-AP	goat anti-rabbit alkaline phosphatase
h	hour
H	hemagglutinin
HA1	hemadsorption 1
HA2	hemadsorption 2
HeLa	human epitheloid carcinoma cells
HEp-2	human epidermoid carcinoma cells

HIFBS	heat-inactivated fetal bovine serum
HN	hemagglutinin-neuraminidase
HPIV1	human parainfluenza virus 1
HPIV2	human parainfluenza virus 2
HPIV3	human parainfluenza virus 3
HPIV4	human parainfluenza virus 4
I	Inosine
IBI	International Biotechnologies, Inc.
IEC	International Equipment Company
IFN	interferon
Inc.	Incorporated
KB	human epidermoid carcinoma cells
L	large polymerase or mouse cells
LLC-MK_2	rhesus monkey kidney cells
LTD	Limited
M	matrix protein or molar
MD	Maryland
MDBK	Madin-Darby bovine kidney cells
MDCK	Madin-Darby canine kidney cells
MEM	minimal essential medium
MIBE	measles inclusion body encephalitis
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimoles
MOI	multiplicity of infection

mRNA	messenger ribonucleic acid
NEN	New England Nuclear
nm	nanometre
N	variable nucleotide or amino acid
NDV	Newcastle disease virus
NENS	Na-acetate, EDTA, NaCl, and SDS
ng	nanogram
NP	nucleocapsid protein
NS	phosphoprotein
NY	New York
OH	Ohio
P	phosphoprotein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picogram
PMSF	phenylmethylsulfonyl-fluoride
PPLO	pleurapneumonia-like organism
RBC	red blood cell
RF	replicative form
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNP	ribonucleoprotein core
RSV	respiratory syncytial virus
sec	seconds
S	Svedberg units

Sdi^-	virus no longer sensitive to DI particle interference
SH	small hydrophobic protein
SV5	simian virus 5
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	standard saline citrate
STD	standard
SSPE	subacute sclerosing panencephalitis
T	Thymine base in a nucleotide sequence
TBE	Tris-borate, EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
ts	temperature sensitive
TTBS	TBS plus tween 20
UV	ultraviolet
U	Uracil base in a nucleotide sequence
Vero	African green monkey kidney cells
VSV	vesicular stomatitis virus
YT	yeast extract, tryptone, and NaCl

CHAPTER 1: GENERAL INTRODUCTION

1. HUMAN PARAINFLUENZA VIRUS 3: A MEMBER OF THE *PARAMYXOVIRIDAE*

Classification

The main feature which distinguishes the *paramyxoviridae* from all of the other virus families is the size and shape of the nucleocapsids (Matthews, 1982). The members of the *paramyxoviridae* are classified into three genera: *paramyxovirus*, *morbillivirus* and *pneumovirus*. This classification is based on biological and morphological characteristics. Members of the *paramyxovirus* genus possess both hemagglutinin and neuraminidase activities while the *morbilliviruses* possess only hemagglutinin activity. *Pneumoviruses* contain neither of these activities and have narrower nucleocapsids than the other two genera. The most studied members of each genus and the hosts they infect are listed in Table 1.

Pathogenesis and epidemiology

The four human parainfluenza viruses (types 1, 2, 3 and 4) are major respiratory pathogens of infants and children (Chanock *et al.*, 1963) and are second only to respiratory syncytial virus (RSV) in this respect. Human parainfluenza virus 1 (HPIV1) (HA2) and HPIV3 (HA1) were first isolated by Chanock *et al.* (1958). HPIV2 (croup-associated virus) was first recognized by the induction of cytopathic effects in tissue culture cells (Chanock, 1956). HPIV4 was first isolated and characterized by Johnson *et al.* (1960). The name parainfluenza virus was selected since these agents

TABLE 1

PARAMYXOVIRIDAE GENERA AND BEST STUDIED SPECIES

Genus	Species	Host
Paramyxovirus	Sendai virus (murine parainfluenza virus type 1)	Mouse
	Human parainfluenza viruses types 1-4	Human
	Simian virus 5 (canine parainfluenza virus type 2)	Dog
	Mumps virus	Human
	Newcastle disease virus	Chicken
Morbillivirus	Measles virus	Human
	Canine distemper virus	Dog
	Rinderpest virus	Cattle
Pneumovirus	Respiratory syncytial virus	Human

are biologically and antigenically different from the influenza viruses (Andrewes *et al.*, 1959; Cook *et al.*, 1959). Diseases induced by parainfluenza viruses can be inapparent upper respiratory tract or life-threatening lower respiratory tract. All four parainfluenza viruses can cause acute respiratory tract diseases in humans of all ages (Chanock and McIntosh, 1990).

Of all four parainfluenza viruses, HPIV3 is usually responsible for lower respiratory tract disease in infants under 6 months of age. Thus, HPIV3 infection may occur despite the presence of maternally derived neutralizing antibodies. Severe HPIV3 lower respiratory tract infection leads to pneumonia, bronchitis or bronchiolitis. HPIV3 is second only to RSV as a cause of pneumonia and bronchiolitis in infants under 6 months of age (Chanock and McIntosh, 1990). Reinfection of children with HPIV3 is believed to be common (Glezen *et al.*, 1984). However, the development of lower respiratory tract illness with HPIV3 usually occurs with primary infection (Glezen *et al.*, 1984; Chanock and Parrot, 1965). By 2 years of age 60% of children are infected with HPIV3 and this increases to 80% by 4 years of age (Chanock and McIntosh, 1990).

HPIV1 is the leading cause of laryngotracheabronchitis (croup); infection usually involves the larynx and upper trachea. HPIV2 and HPIV3 are also associated with severe croup. Severe illness as a result of infection with HPIV2 is less frequent than with HPIV1 (Chanock and McIntosh, 1990). Primary infections with HPIV1 and HPIV2 usually do not occur in the first 6 months of life, but later (Chanock and Parrot, 1965). In contrast to infection with HPIV3, infants appear to be protected by the maternally derived antibodies to HPIV1 and HPIV2. After 6 months of age there is an increase in infection with HPIV1 and HPIV2, correlating

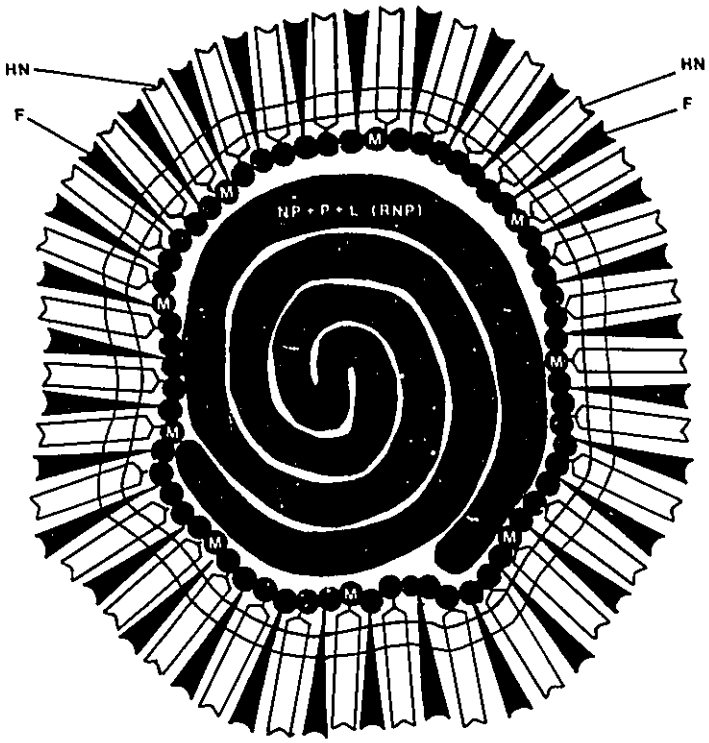
with an increase in croup and other lower respiratory tract diseases. By 5 years of age over 75% of children have been infected with HPIV1 and by this age a majority of children have also been infected with HPIV2 (Chanock and McIntosh, 1990).

Symptoms with HPIV4 infection are believed to be less severe than with the other types and are mainly upper respiratory tract. However, pathogenesis with type 4 virus is likely to be greater than is apparent since the recovery of this agent in tissue culture cells is more difficult than with the other types (Chancola *et al.*, 1964).

Virion morphology

Paramyxoviruses are composed of an internal ribonucleoprotein core (nucleocapsid) that is surrounded by a lipoprotein envelope which is derived from the host cell (Fig. 1). Both roughly spherical and filamentous forms have been observed (Norrby *et al.*, 1970). The average diameter of the spherical forms vary from 150 to 250 nm, but larger forms are quite common. For example, the diameter of HPIV3 has been observed to range from 200 to 800 nm (Waterson *et al.*, 1961). This pleomorphism suggests that paramyxovirus budding is not tightly controlled. This is supported by the apparent packaging of 28S and 18S rRNAs in addition to the viral genome (Kolakofsky and Bruschi, 1975). The viral membrane bears the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins which are visualized as spikes by negative staining. The nucleocapsid contains the single-strand RNA genome. Paramyxovirus Sendai virions have been shown to encapsidate almost equivalent amounts of genomic (negative-strand) and antigenomic (positive-strand) RNA nucleocapsids (Mottet and Roux, 1989). The nucleoproteins associated with the genomic RNA provide the

Figure 1. Schematic representation of a HPIV3 particle. Associated with the genomic RNA are the NP, P and L proteins which constitute the ribonucleoprotein core (dark spiral line). The F and HN glycoproteins protrude through the lipid bilayer (outer ring). The M protein is located on the inner surface of the lipid bilayer interacting with the cytoplasmic domain of the viral envelope glycoproteins.


HN
F
HN
F
M
NP + P + L (RNP)

nucleocapsids' helical morphology (Waterson *et al.*, 1961; Kingsbury, 1990).

Genome organization and virus proteins

The HPIV3 genome and proteins have been well characterized (Guskey and Bergtrom, 1981; Storey *et al.*, 1984, 1987; Jambou *et al.*, 1985, 1986; Ray *et al.*, 1985; Sānchez and Banerjee, 1985; Wechsler *et al.*, 1985a,b; Dimock *et al.*, 1986; Elango *et al.*, 1986; Galinski *et al.*, 1986a,b, 1987a,b, 1988; Luk *et al.*, 1986, 1987; Sānchez *et al.*, 1986; Spriggs and Collins, 1986a,b; Spriggs *et al.*, 1986, 1987; Côté *et al.*, 1987; Prinoski *et al.*, 1987). The HPIV3 genome is a nonsegmented negative-strand RNA molecule of 15,461 nucleotides in length (Galinski *et al.*, 1988). The genome is transcribed into six unique mRNAs which encode the six known structural proteins [nucleocapsid protein (NP), nucleocapsid phosphoprotein (P), matrix protein (M), F protein, HN and large polymerase (L)] and at least one nonstructural C protein (Fig. 2; Table 2). The M protein can also be translated from an abundantly synthesized M/F dicistronic transcript (D.G. Storey, 1986, Ph.D. Thesis, University of Ottawa, Ottawa, Ontario). The HPIV3 gene order is 3'-NP-P+C-M-F-HN-L-5' (Spriggs and Collins, 1986a). This gene order is similar to that of Sendai virus (Dowling *et al.*, 1983; Shioda *et al.*, 1986) and Newcastle disease virus (NDV) (Chambers *et al.*, 1986; Wilde *et al.*, 1986; Toyoda *et al.*, 1987). However, genomes of simian virus 5 (SV5) and mumps virus have an additional gene located between the F and HN genes which encodes a small hydrophobic (SH) protein (Hiebert *et al.*, 1985; Elango *et al.*, 1988).

The leader region (Leppert *et al.*, 1979) at the 3'-terminus of the HPIV3 genome is 52 nucleotides in length whereas the 5'-terminus has a 44-nucleotide trailer region. The first 17 nucleotides at the 3'- and

Figure 2. Genetic map of HPIV3 genome and intergenic, gene-start and gene-end sequences. The exactly conserved gene-end sequences are underlined. The gene-end sequence of the F gene has an A residue at the third position. The fifth and sixth variable nucleotides in the gene-start sequences are AA for NP, P+C and M and GU, CA, and CG for F, HN, and L genes, respectively.

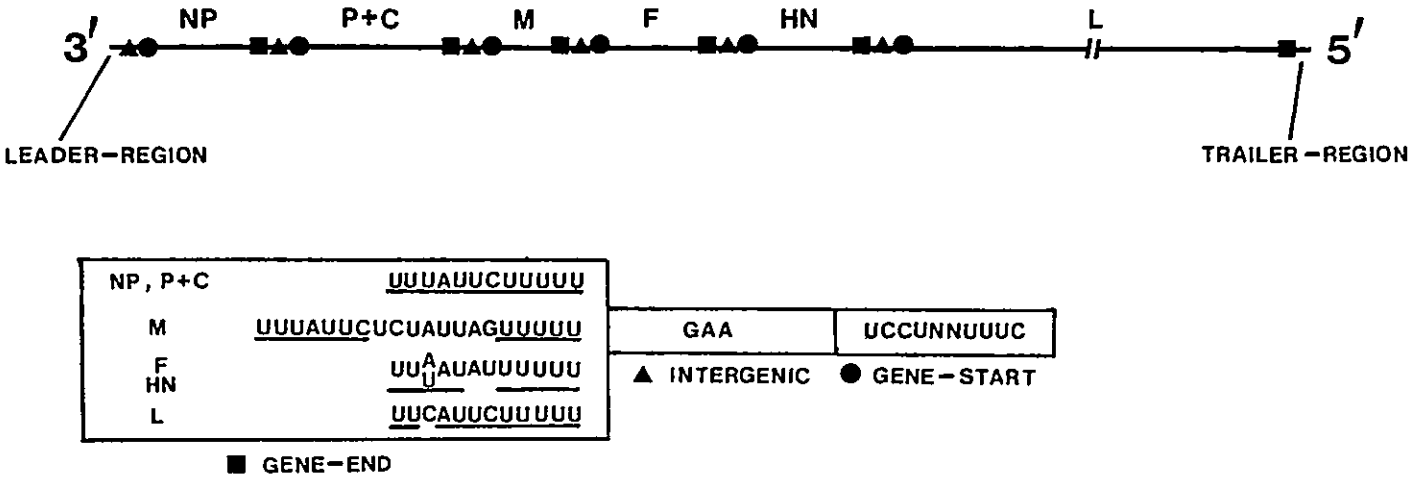

3′
NP
P+C
M
F
HN
L
5′
LEADER–REGION
TRAILER–REGION
NP, P+C
UUUAUUCUUUUU
M
UUUAUUCUCUAUUAGUUUUU
F
HN
UUAUAUUUUUU
L
UUCAUUCUUUUU
GAA
UCCUNNUUUC
▲ INTERGENIC
● GENE–START
■ GENE–END

TABLE 2

HPIV3 TRANSCRIPTION AND TRANSLATION PRODUCTS

	mRNA	Proteins			
				Molecular weight (in thousands)	
genes	No. of nucleotides excluding poly A		No. of amino acids	Predicted	Apparent[a]
NP	1641		515	57.8	68
P+C	2009	P	602	67.5	87
		C	199	23.3	-
M	1150		353	39.5	38
F	1845	F_0	539	60.0	65
		F_1	430	47.6	51
		F_2	91	12.4	12
HN	1888		572	64.3	72
L	6755		2233	255.8	>200

[a] By SDS-PAGE

5'-ends of the HPIV3 genome are inversely complementary. In addition, 33 of the first 39 nucleotides at the ends of the genome are inversely complementary. Thus, the 3'- and 5'-ends could base pair to form a panhandle structure (Dimock *et al.*, 1986; Galinski *et al.*, 1988).

The 3'-end of the HPIV3 genes have a 10-nucleotide transcriptional start sequence that is well conserved (Fig. 2). As well, the 5'-end of the HPIV3 genes have a 12-nucleotide transcriptional-end sequence that is also relatively well conserved (Fig. 2). This gene-end sequence for the M gene differs from the others due to an insertion of 8 nucleotides upstream of the 5 uridine residues. This insertion could be responsible for the abundantly synthesized M/F readthrough transcript. The HPIV3 genes are separated by the trinucleotide sequence GAA (Spriggs and Collins, 1986a; Chanock and McIntosh, 1990).

Members of the *paramyxoviridae* employ different mechanisms to express the P protein and one or more nonstructural proteins. The HPIV3 and Sendai virus P+C genes utilize two open reading frames (ORFs) to express the P protein and the C protein (Galinski *et al.*, 1986b; Luk *et al.*, 1986; Spriggs and Collins, 1986b). In addition, the Sendai virus C ORF expresses four proteins (C', C, Y_1 and Y_2) by ribosomal initiation at multiple start sites (Dillon and Gupta, 1989). The Sendai virus P ORF codes for another small protein, designated X, which is carboxyl-coterminal with P (Curran and Kolakofsky, 1987). Interestingly, a sixth protein, the V protein, is translated from a second mRNA due to the insertion of a single-G residue at nucleotide position 1053 (Vidal *et al.*, 1990). The measles virus P gene also encodes a V protein from a second mRNA as a result of a precise one-G insertion (Cattaneo *et al.*, 1989a). The P proteins of HPIV3 and Sendai virus (79-83 KDa) are larger than

larger than those of mumps virus, SV5 and NDV (44-56 KDa). SV5, mumps virus and NDV do not contain a C ORF in the P gene (McGinnes *et al.*, 1988; Takeuchi *et al.*, 1988; Thomas *et al.*, 1988; Elango *et al.*, 1989). Nonstructural proteins observed in infected cells with the latter 3 viruses are related to the corresponding P proteins (Herler and Compans, 1982; Paterson *et al.*, 1984; McGinnes *et al.*, 1988; Thomas *et al.*, 1988). The SV5 P gene contains a stop codon in the middle and is used to translate a V protein. The P protein is translated from a mRNA containing a precise insert of two-Gs upstream from the internal stop codon (Thomas *et al.*, 1988).

The two HPIV3 surface glycoproteins, HN and F, are required for virus infectivity. The HN protein mediates virus attachment by binding to sialic acid-containing cellular receptors. The neuraminidase portion of the molecule is believed to be responsible for cleavage of sialic acid residues on virus particles to prevent self-aggregation during virus release (Scheid *et al.*, 1972). The F protein mediates the fusion of the virion envelope with the host cell membrane allowing for virus penetration (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). In order to be active, the precursor F protein (F_0) must be cleaved by a host trypsin-like enzyme. This cleavage yields the two disulfide linked subunits, F_2 (amino acids 19-109) and F_1 (amino acids 110-539). The first 18 amino-terminal amino acids are thought to represent the signal peptide that is cleaved (Spriggs *et al.*, 1986; Côté *et al.*, 1987). HN and F are anchored in the membrane by their amino and carboxy termini, respectively. The HN amino-termini hydrophobic sequence is believed to function as a signal peptide which is not cleaved from the mature protein (Elango *et al.*, 1986; Storey *et al.*, 1987). HN and F contain Asn-N-linked sugars (Kohama *et al.*, 1978;

Yoshima *et al.*, 1981) and are likely to be synthesized and transported to the plasma membrane using the general scheme established for such proteins (Hubbard and Ivatt, 1981; Sabatini *et al.*, 1982).

The NP, P and L proteins are associated with the genomic RNA to constitute the ribonucleoprotein core (Storey *et al.*, 1984; De *et al.*, 1990). The NP protein is the most abundant viral protein in virions and is tightly bound to the viral RNA. The NP protein confers stability and flexibility to the ribonucleoprotein core. In contrast, the L protein is the least abundant viral protein in virions. The P protein, a highly phosphorylated protein, is less abundant than the NP protein, but is more abundant than the L protein. The L protein in conjunction with the P protein are believed to carry out the enzymatic processes of transcription and replication (De *et al.*, 1990).

The nonglycosylated M protein is located on the inner surface of the lipid bilayer. The M protein is thought to participate in virus assembly at the cell surface by linking the nucleocapsid with the envelope viral glycoproteins (Buechi and Bächi, 1982).

Replication strategy

The basic schemes for replication of paramyxoviruses and rhabdoviruses are similar except for virus entry and release from the host cell. In both cases, the polymerase associated with the incoming nucleocapsid is thought to enter the template at the 3'-end, and for paramyxoviruses, to sequentially synthesize the plus-strand leader RNA and the NP, P+C, M, F, HN and L mRNAs (primary transcription) by stopping and restarting at each gene junction. The consensus sequences at each gene boundary are believed to mediate initiation and termination of

transcription (Gupta and Kingsbury, 1984). During synthesis the mRNAs are capped and polyadenylated whereas the leader RNA is unmodified. Translation of these transcripts is required to initiate genome replication and continued protein synthesis is required for further replication (Robinson, 1971; Perlman and Huang, 1973; Wertz and Levine, 1973).

The switch from transcription to replication is believed to be controlled by the intracellular level of unassembled NP protein. Polymerases whose nascent leader RNA assemble NP protein would be able to synthesize a full-length encapsidated antigenome. Polymerases whose nascent leader do not assemble NP, before the leader/NP junction, would terminate and restart at the beginning of NP to synthesize all the mRNAs (Glazier *et al.*, 1977; Blumberg *et al*, 1981; Vidal and Kolakofsky, 1989). Full-length antigenomes would serve as template for amplification of the negative-strand genome. A leader RNA synthesized from the 3'-end of the antigenome has been identified in VSV-infected cells. This minus-strand leader RNA would function in a similar manner to the plus-strand leader RNA, preventing synthesis of the negative-strand genome in absence of viral (NP) protein synthesis (Leppert *et al.*, 1979). Transcription of the amplified genome (secondary transcription) allows for translation of all proteins and further synthesis of antigenome and genome nucleocapsids.

Paramyxoviruses replicate in the cytoplasm and assemble at the cell surface. The M protein assembles at the cytoplasmic surface of the nascent envelope likely due to its interaction with the cytoplasmic tail of the envelope glycoproteins. The M protein is believed to stabilize the nucleocapsids at the membrane. Viral budding then takes place (Buechi and Bächi, 1982; McCreedy and Lyles, 1989).

2. DEFECTIVE INTERFERING PARTICLES

Introduction

Defective interfering (DI) particles were first reported for influenza virus by von Magnus (1954). They are generated during the replication of many, and possibly all, animal and plant viruses (Perrault, 1981; Ismail and Milner, 1988; Burgyan *et al.*, 1989). DI particles of the negative-strand RNA viruses, in particular VSV and Sendai virus, have been the most extensively studied. DI particles are subgenomic deletion mutants which cannot replicate by themselves and which interfere specifically with the multiplication of the standard (STD) helper virus that they require for their own generation and replication. DI particles are antigenically identical to the STD virus since they use the structural proteins synthesized by the STD virus (Kang and Prevec, 1969; Huang, 1973).

The study of DI particles is of interest because (i) they contribute to our understanding of virus replication (Meier *et al.*, 1984); (ii) they are thought to play a role in modulating virus infections and recovery from infections (Huang, 1988); (iii) they have been shown to be involved in the establishment of persistent infections *in vitro* and *in vivo* (Barrett and Dimmock, 1986); (iv) understanding their mechanism of interference may help us to combat viral diseases of animals and plants (Fultz *et al.*, 1982a; Cave *et al.*, 1985).

DI particle production

DI particles are generated and amplified by serial undiluted passage of viruses at high multiplicity of infection (MOI) in cell cultures or embryonated eggs. DI RNAs are generated by an aberrant replicative

event and, therefore, their generation does not depend on MOI (Kingsbury and Portner, 1970). High MOI is used to only insure that every cell which is infected with one or more DI particles will also be infected with one or more STD particles so that the former can be replicated and consequently amplified (Holland, 1990).

Establishment of persistent infections in cell cultures is another method to generate DI particles. DI particles have been detected in numerous persistently infected cell cultures and have been implicated as a possible determinant in the maintenance of persistent infections (Holland *et al.*, 1980). Persistently infected cells provide an excellent environment for aberrant replicative events. A subgenomic RNA with a replicative advantage over the STD genome could easily be amplified in this environment.

Biological characteristics

Paramyxovirus DI particles are generally smaller than the STD virus particle. However, the size variability (pleomorphism) of the STD virus population makes it very difficult to achieve a clean separation between DI and STD virions on sucrose gradients (Kingsbury *et al.*, 1970; Roman and Simon, 1976). In contrast, the bullet-shape rhabdovirus particle is proportional to the length of the RNA genome and DI particles can easily be separated by size from STD particles on sucrose gradients (Holland *et al.*, 1976b; Kang and Allen, 1978).

The host cell can also influence the frequency of DI particle generation and amplification (Kang, 1980). Kang *et al.* (1978) showed that different host cells generate different size classes of VSV DI particles at different serial virus passages. Kingsbury and Portner (1970) reported that

four of six Sendai virus plaque purified preparations failed to generate subgenomic RNAs after 6 passages in chick embryo lung cells while all 6 populations yielded subgenomic RNAs when similarly passaged in embryonated eggs. Holland *et al.* (1976a) observed that their HeLa cell line did not generate or replicate VSV DI particles after either serial undiluted passage or direct addition of DI particles. Thus, each virus-cell system must be examined for the generation of DI particles. Nonetheless, cell lines yielding high titer virus tend to generate and amplify DI particles more efficiently than low yielding cell lines (Holland, 1990).

Serial undiluted passage of viruses in cell culture usually leads to cyclic production of plaque-forming virus (Palma and Huang, 1974; Roman and Simon, 1976; Rima *et al.*, 1977). This cyclic production is believed to be due to the interdependence that exists between STD and DI virus particles. Palma and Huang (1974) observed an overlapping cyclic production of both STD and DI virus particles of VSV during serial passages of infected cells. They showed that a decrease in STD virus yield was promptly followed by an increase in DI particles and vice versa. This interference between STD and DI virus yields has been implicated as a possible mechanism in the maintenance of persistent infections (Kawai *et al.*, 1975).

DI particles can modulate the normally lethal cytopathic effects of viruses to allow survival of the infected cells. In cell culture, DI particle modulation of infection with viruses that are normally cytocidal often leads to the establishment of persistent infections (Holland and Villarreal, 1974; Roux and Holland, 1979). DI particles can also modulate infections *in vivo*. The initial experiments by Doyle and Holland (1973) showed that VSV DI particles could delay the time of death of adult mice from an

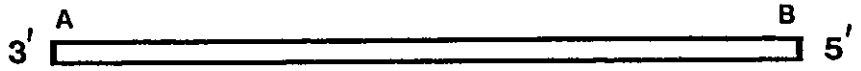
otherwise STD virus lethal infection. Such modulation of virus infection *in vivo* has since then been demonstrated in other virus systems (Barrett and Dimmock, 1986; Huang, 1988). DI particles can also facilitate the establishment of persistent infections *in vivo*. Hamsters coinfected with STD and DI VSV particles can become persistently infected avoiding the otherwise STD virus lethal infection (Fultz *et al.*, 1982b). However, interference with replication may not be the sole mechanism by which DI particles exert their effect *in vivo*, since viruses which interfere well *in vitro* do not always do so *in vivo* (Barrett and Dimmock, 1984).

Structural classes of DI particle genomes of negative-strand RNA viruses

DI particle genomes are efficiently replicated by the RNA polymerase of the STD virus and therefore must share with the latter the essential sequences that are required for replication initiation and encapsidation of the viral genome. Nucleic acid hybridization and sequencing analysis of VSV, Sendai virus and influenza virus DI RNAs have demonstrated that there are four basic classes of DI particle genomes (Fig. 3). (i) The panhandle or copyback type in which the genomic 5'-termini and adjacent sequences are retained, but the 3'-termini and adjacent sequences are lost and replaced by the complement of the 5'-termini. The region of complementarity (stem or panhandle) in most cases ranges from 46 to 200 nucleotides (Kang, 1980; Kolakofsky, 1982). This is the most common type of genome observed for VSV DI particles. (ii) The snapback or hairpin type has retained the genomic 5'-termini and adjacent sequences which constitute half of the molecule. The other half of the molecule is its complement. (iii) The internal deletion type has retained both the genomic 5'- and 3'-termini, but is deleted internally. Sendai virus has been found

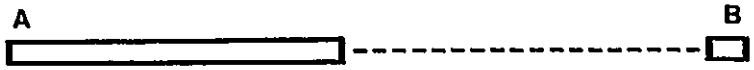
Figure 3. Major structural classes of DI particle genomes of negative-strand RNA viruses. The negative-strand sequences are represented by the open boxes. The black boxes indicate the complementary (positive-strand) sequences. A and B represent the 3'- and 5'-end of the nondefective genome, respectively. B' is the compliment of B. For the internal deletion type, the dash line indicates the deleted sequences.

STD Genome

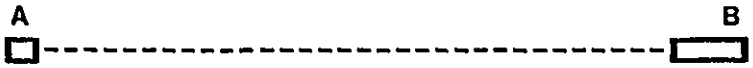


Internal deletion DI

simple deletion

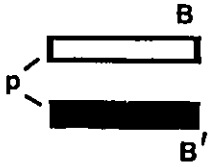


fusion



Panhandle (copyback) DI

Snapback (hairpin) DI



to frequently generate this type of DI genome. Some Sendai virus DI genomes have extensive internal deletions and for this reason have been called "fusion" type DI RNAs (Amesse *et al.*, 1982). (iv) The mosaic or complex type includes all DI particles which have a complex or mosaic internal sequence rearrangement with termini structures of any of the above. Such DI RNAs have been observed for influenza virus (Nayak *et al.*, 1985).

Generation mechanisms of DI particle genomes

DI particle RNA genomes are most likely generated by "polymerase copy-choice" mechanisms (Lazzarini *et al.*, 1981; Perrault, 1981). This model postulates that during replication the polymerase falls off the template carrying its nascent daughter strand and moves to another location on the same or different template reinitiating elongation of the nascent daughter strand. This model now seems even more plausible as Helfman and Perrault (1989) showed that the VSV polymerase can redistribute and copy exogenously added templates.

The generation of panhandle or snapback types structures can be explained by this model. During the copying of the antigenome the polymerase falls off carrying the nascent genomic strand and reinitiates synthesis using the nascent genomic strand as template. Internally deleted DI RNAs could be generated during replication of either the negative- or positive-strand genome. Reinitiation would occur on the same or different template, but not on the nascent daughter strand. Mosaic or complex DI RNA genomes would be generated by multiple polymerase jumps.

Mechanism of interference

DI particles apparently interfere with STD virus production by competing for viral specific gene products that are required for replication (Giachetti and Holland, 1989). It has been suggested that the 3'-terminus of the antigenome (or copyback DI 3'-terminus) is a better promotor for RNA replication than the genomic 3'-terminus. This is supported by the finding that Sendai virus DI RNAs possessing a copyback 3'-terminus have a marked replicative advantage over the internally deleted type, irrespective of the size of the copyback RNA molecule (Re and Kingsbury, 1986). Thus, DI RNAs of the copyback type may be replicated at the expense of the STD genome due to their strong affinity for the replicase proteins.

Re and Kingsbury (1986) have also shown that transcriptionally active DI RNAs of the internal deletion type were outgrown by transcriptionally inactive DI RNAs. This suggests that transcriptionally inactive genomes have a replicative advantage over both DI and STD virus genomes that engage in transcription.

However, these findings do not account for the transcriptionally active internally deleted DI RNAs that do survive and interfere with the replication of the STD genome (Re *et al.*, 1983a). There may be an internal attenuating feature that is present in the STD virus genome that is missing or disrupted by the deletion in the DI genome. Alternatively, these DI RNAs may be equivalent to the STD RNA genome in regards to RNA synthesis and the only competitive advantage would be their small size (Nayak *et al.*, 1985).

3. PERSISTENT INFECTIONS OF CULTURED CELLS WITH RNA VIRUSES

Modes of establishment

Numerous persistent infections of cultured cells have been studied to elucidate the mechanisms by which a virus may initiate and maintain persistence in a host. From these studies, two principal factors have been implicated in the mediation of persistent infections: DI particles and temperature-sensitive (*ts*) viruses (Holland *et al.*, 1980; Youngner and Preble, 1980; Whitaker-Dowling and Youngner, 1987). Another factor which has been implicated, but less often than DI particles and *ts* viruses, is interferon (IFN).

Persistent infections of BHK21 and mouse L cells with STD VSV can only be established in the presence of a thousand-fold excess of DI particles (Holland and Villarreal, 1974; Youngner *et al.*, 1976). However, Youngner *et al.* (1976) were able to establish a persistent infection of L cells with a *ts* mutant recovered 7 days after initiation of persistence in absence of DI particles. Furthermore, a VSV clone recovered after more than 5.5 years of persistence in BHK21 cells, a now highly mutated virus, can also establish a persistent infection of BHK21 cells in absence of DI particles at low MOI (Rowlands *et al.*, 1980). IFN has also been used to establish persistent infections of L cells with VSV. Nishiyama (1977) and Ramseur and Friedman (1977, 1978) pretreated L cells with IFN to minimize the cytolytic effects of STD VSV. Under this condition persistent infections were readily established. In addition, a virus clone recovered after the 40th subculture of VSV-L cells readily established persistent infections without pretreatment of cells with IFN (Nishiyama *et al.*, 1978). In contrast, rabies virus persistent infections of BHK21 cells

can be established with STD virus without addition of DI particles or pretreatment with IFN (Kawai *et al.*, 1975; Wild and Bijlenga, 1981). In all of the above cases, except those involving IFN, the persistently infected cultures were obtained by cultivating the cells which survived the infection.

Paramyxovirus persistent infections of cultured cells with normally cytocidal viruses have been obtained by a variety of methods, including cultivation of cells surviving a STD virus infection, coinfection with DI particles, infection with *ts* viruses and infection at a MOI where the cells were resistant. Persistent viral-cell systems established by cultivation of the cells surviving a STD virus infection include HPIV3-KB (Daniel and Chany, 1962), Sendai-BHK21 (Nagata *et al.*, 1972; Roux and Holland, 1979), Sendai-HeLa (Yoshida *et al.*, 1983), mumps-BHK21 (Truant and Hallum, 1977), NDV-MDBK (Lawton *et al.*, 1986), measles-HeLa (Rustigian, 1962), measles-HEp-2 (Hayes and Zweernik, 1978) and Rinderpest-Vero (Kobune *et al.*, 1981).

Roux and Holland (1979) have shown that coinfection of BHK21 cells with STD virus and DI particles of Sendai virus regularly led to the survival of infected cells and to the establishment of a persistent infection. Rima and coworkers (1977) have shown that measles virus passaged 3 to 5 times from an undiluted inoculum rapidly established a persistent infection of Vero cells. However, less than 5% of the cells survived a STD measles virus infection and these cells could not be subcultured. In addition, persistent infections of Vero cells with canine distemper virus could be established by undiluted passage of virus (ter Meulen and Martin, 1976). These findings indicate that DI particles aid, and in particular circumstances may be necessary, for the establishment

of paramyxovirus persistent infections.

ts viruses have also been shown to play a role in the establishment of persistent infections. However, not all *ts* viruses are able to establish persistent infections. Kanda and Shibuta (1981) were able to readily establish a Sendai-Vero persistent infection with one of eleven *ts* mutants at 37°C (nonpermissive temperature 39°C). This mutant replicated well in the Vero cells, but the infected cells showed no CPE. The fluid of this culture did not contain DI particles. Infection with some of the other *ts* mutants, like the STD virus, required long-term subcultivation of cells for the establishment of persistence. Infection with the remaining *ts* mutants resulted in an abortive infection. Pringle *et al.* (1978) were also able to establish a persistent infection of BSC-1 cells with a *ts* mutant of RSV at the nonpermissive temperature. This persistently infected culture was established by cultivation of the surviving cells. Infection with other *ts* mutants resulted in either an abortive infection or complete cell lysis due to the appearance of STD virus by reversion. Persistent infections have also been readily established by *ts* viruses selected during persistent infections. In contrast to the STD virus, *ts* viruses of Sendai virus and NDV recovered from persistently infected LLC-MK_2 and MDBK cells, respectively, have extremely low cytopathogenicity and can readily establish persistent infections in the normal parental cells (Yoshida *et al.*, 1982; Lawton *et al.*, 1986).

Some viruses are highly cytolytic at a particular MOI while at a different MOI induce little CPE. For example, infection of CV-1 cells with HPIV3 at a low MOI (0.01 to 1) causes a lytic infection with extensive CPE. In contrast, Wechsler and coworkers (1987) observed that at an MOI of 10 most of the cells survive the infection and a persistent

infection can be rapidly established. On the other hand, a persistent influenza C virus infection was readily established in MDCK cells at a low MOI (Goshima and Maeno, 1989). At a high MOI most of the cells died and cultivation of surviving cells was unsuccessful. In addition, some viruses do not give rise to CPE in certain cell lines, independent of the MOI of infection, and for this reason can easily establish a persistent infection. This is the case for mumps virus in human conjunctiva cells (Walker and Hinze, 1962), Vero cells (McCarthy *et al.*, 1981) and HEp-2 cells (Andzhaparidze *et al.*, 1982), bovine parainfluenza virus 3 in human conjunctiva cells (Cole and Hetrick, 1965) and NDV in L cells (Preble and Youngner, 1973).

Factors implicated in the maintenance of persistent infections

Three factors have been implicated in the maintenance of persistent infections initiated with cytolytic viruses: DI particles, virus mutants and IFN. The best studied persistent virus-cell system, which happens to be maintained by DI particles, is the VSV persistent infection of BHK21 cells. Holland and Villarreal (1974) showed that cloned virus (DI-free) recovered from BHK21 cells persistently infected with VSV could not on its own establish a persistent infection of normal BHK21 cells. However, in the presence of a sucrose gradient purified DI particle isolated from the persistently infected cells, the same DI-free virus readily established a persistent infection. Thus, the VSV-BHK21 system is one that is maintained by DI particles. Another well studied persistently infected virus-cell system is that of Sendai-BHK21. Roux and Holland (1980) showed that in these cells DI RNAs were constantly synthesized in 10- to 340-fold greater molar amounts than was the STD-size RNA. They

suggested that the high levels of DI RNAs inside these cells was not fortuitous, but likely required for the maintenance of the persistent state. Kawai *et al.* (1975) demonstrated cyclic patterns of STD and DI virus particle production in a rabies-BHK system. These DI particles effectively suppressed the cytopathic effect of the STD parental virus in mixed infections. This cyclical pattern of virus production resembles the cyclic rising and falling of VSV and DI particle production which occurs when diluted VSV-infected cells are passaged onto fresh uninfected cells (Palma and Huang, 1974). Thus, DI particles also appear to play an important role in the maintenance of this rabies-BHK persistent infection.

ts viruses are often selected during the course of persistent infections and for this reason have been implicated in maintenance of numerous persistent virus-cell systems. In a VSV persistent infection of L cells initiated with DI particles, *ts* viruses were rapidly selected (Youngner *et al.*, 1976). By 10 days after initiation of persistence the frequency of *ts* clones in the virus population was 17.8% and by 63 days 100% of the clones isolated were *ts*. The presence of DI particles or IFN could not be demonstrated in the VSV-L culture fluids. This same group of workers had earlier reported the spontaneous selection of *ts* viruses in a NDV persistent infection of L cells (Preble and Youngner, 1972). They demonstrated that *ts*+ revertants isolated from the persistent infection as well as *ts* mutants induced by nitrous acid treatment of the STD virus were able to establish and maintain persistent infections in L cells. Virus reisolated 8 passages later from these persistent infections was *ts*. Thus, coselection of the *ts* phenotype appeared to be important for maintenance of the persistent infections (Preble and Youngner, 1973). The selective survival of *ts* mutants in a Sendai-BHK21 persistent infection has been

well documented (Nagata *et al.*, 1972; Kimura *et al.*, 1975; Nishiyama *et al.*, 1976). It was later demonstrated that this persistently infected culture also synthesized DI particles (Yoshida *et al.*, 1982). To clarify the role played by the *ts* mutant, a persistent infection was established in LLC-MK_2 cells with a *ts* virus isolated from the Sendai-BHK21 system that was free of DI particles. The virus recovered from this Sendai-LLC-MK_2 system was *ts* and DI particles were not detected (Yoshida *et al.*, 1982). This group of workers also reported the release of *ts* virus, but not of DI particles, from a Sendai-HeLa system. They concluded *ts* mutants play an important role in the maintenance of persistent infections with Sendai virus (Yoshida *et al.*, 1983).

The production of IFN has also been involved in the maintenance of persistent infections. One factor that distinguishes IFN-mediated systems is that the cultures are resistant to both homologous and heterologous viruses. Ito *et al.* (1985) found that a mumps-L system was resistant to both homologous and heterologous virus (VSV or Sindbis virus) challenge. IFN was detected in amounts that varied and each increase in IFN titer was followed by a decrease in virus yield. IFN antiserum enhanced production of infectious virus and caused rapid cell destruction. An influenza C virus persistent infection of MDCK cells produced low levels of IFN and was resistant to superinfection by homologous virus and VSV (Goshima and Maeno, 1989). The cultures were cured by cultivation in the presence of influenza C antiserum and as a result loss their IFN-producing activity and became susceptible to homologous virus (VSV) infection. IFN has been shown to play a role in the maintenance of several VSV-L systems since treatment with IFN antibody led to cell destruction (Ramseur and Friedman, 1977, 1978; Youngner *et al.*, 1978;

Nishiyama, 1977). The involvement of IFN was also reflected in the ability of these cells to resist heterologous virus challenge (Ramseur and Friedman, 1977, 1978; Nishiyama, 1977; Youngner *et al.*, 1978).

Biological properties of virus released from persistent infections

Establishment of persistent infections with viruses that are normally cytocidal leads to the selection of viruses with altered biological properties. Viruses recovered from persistently infected cells have one or more of the following properties: decreased virulence, small plaque phenotype, temperature sensitivity, lack of infectivity, interference with parental virus, and in cases where the persistence was initiated by DI particles, resistance to the DI particle that was used to initiate the persistent infection. Holland *et al.* (1979) have shown that the VSV genome undergoes and accumulates mutations during persistence. They proposed that multiple virus mutations occur and are selected during persistence giving rise to the small plaque or *ts* phenotypes as well as to the other characteristics of viruses recovered from persistently infected cultures.

In the persistent infection of BHK21 cells with VSV, mutant viruses (Sdi^-) arise which are no longer sensitive to interference by DI particles present in the infection (Horodyski and Holland, 1980). Giachetti and Holland (1988) have shown that the resistance of the Sdi^- mutants to interference by wildtype DI particles lies in the polymerase complex of Sdi^- mutants. In an *in vitro* complementation assay the polymerase complex of an Sdi^- mutant was unable to replicate a wildtype DI particle probably due to mutations in the L-NS polymerase genes.

In L cells persistently infected with VSV there is a dominance of *ts* phenotypes (Youngner *et al.*, 1976). One mutant isolated (VSV-Pi) displays reduced transcription of viral mRNA, but produces increased amount of viral genome RNA and proteins compared to STD VSV. VSV-Pi is also defective in its ability to shut off host protein synthesis (Frey and Youngner, 1982, 1984). In mixed infection with STD VSV and VSV-Pi the pattern of gene expression was identical to that observed with VSV-Pi alone. Furthermore, VSV-Pi interfered with the ability of STD VSV to shut off host protein synthesis (Jordan and Youngner, 1987). The L protein of VSV-Pi was clearly implicated as the gene product responsible for transcriptional interference using an *in vitro* transcription assay (Jordan *et al.*, 1989). The VSV persistent infections of BHK21 and L cells show that the viral polymerase proteins play an important role in the evolution of virus mutants.

Characteristics of persistently infected cells

A characteristic of many persistently infected cultures, in which DI particles have been implicated in establishment, is the development of severe cytopathology or crisis. Such is the case for VSV persistent infections of both BHK21 (Holland and Villarreal, 1974) and L (Youngner *et al.*, 1976) cells as well as the Sendai-BHK21 system (Roux and Holland, 1979; Yoshida *et al.*, 1982). In each of these three cases the cells initially underwent several crisis periods which became milder and eventually disappeared as the cells were subcultured. Persistent infections established by cultivation of surviving cells, in which DI particles do not appear to be involved, may (Rustigian, 1966; Youngner *et al.*, 1976) or may not (Pringle *et al.*, 1978) exhibit such crisis periods while persistent infections

which are readily established (in absence of DI particles) do not go through such crisis periods. Several differences exist between DI particle or *ts* virus-mediated persistence versus IFN-mediated persistence. The percentage of cells infected in persistent infections mediated by DI particles or *ts* viruses usually ranges from 70 to 100% while in IFN-mediated persistent infections this is much lower, ranging from <10 to 30% (Ramseur and Friedman, 1977; Nishiyama, 1977; Ito *et al.*, 1985; Goshima and Maeno, 1989). As previously stated (page 25), IFN-mediated persistently infected cultures are resistant to both homologous and heterologous virus challenge while persistent infections not maintained by IFN are resistant to homologous virus infection, but sensitive to heterologous virus challenge. Finally, most IFN-mediated persistent infections can be cured with antiviral antibody or IFN antiserum while those mediated by DI particles or *ts* viruses tend to be unaffected by such treatment.

Most persistently infected cultures become morphologically indistinguishable from uninfected cells after a limited number of passages. In general, persistently infected cells grow slightly slower or to a similar rate as the uninfected cells. In addition, persistently infected cells usually release significantly less virus than acutely infected cells and the amount of virus released tends to vary with cell passage.

Altered virus structural proteins associated with persistent infections

Viruses with biological modifications are selected during persistent infections. These biological modifications are frequently accompanied by alterations in the virus structural polypeptides. A role for altered M protein in the maintenance, and also possibly in the establishment, of

paramyxovirus persistent infections has been documented. Wechsler *et al.* (1987) reported that two of five CV-1 cell lines persistently infected with HPIV3 have an M protein of altered electrophoretic mobility. In one of the persistently infected cell line this alteration was observed within 36 h of the initial infection. This rapid change in M protein suggests a role for M protein in the establishment of persistence. M protein with an altered electrophoretic mobility has also been observed in a HeLa cell line (K11) persistently infected with measles virus (Rozenblatt *et al.*, 1979). In BHK21 cells persistently infected with Sendai virus, the M protein was found to be greatly reduced or absent. The M protein was shown to be synthesized at a normal rate, but unstable relative to the other viral proteins (Roux and Waldvogel, 1982). In the K11 cell line a reduced steady-state amount of M protein has also been observed. The reduced amount of M protein was found to result from degradation of the protein (Young *et al.*, 1985). The instability of the M protein could explain the defective viral maturation observed in these persistently infected cell lines. Alterations in proteins other than M have also been reported. Some of these include the NP, P and M1 proteins of a measles-HEp-2 (Boriskin *et al.*, 1986), Sendai-MDBK (Lawton *et al.*, 1986) and rabies-BHK21 (Wild and Bijlenga, 1981) persistent infection, respectively.

The role of M protein in persistent infections has been highlighted by studies of subacute sclerosing panencephalitis (SSPE). SSPE is a rare, slowly progressive central nervous system disease that results from persistent measles virus infection. SSPE develops 5 to 10 years after acute measles virus infection in 1 per 300,000 cases (Norrby and Oxman, 1990). Sera from SSPE patients, in general, have high antibody titers to all measles virus proteins, except the M protein (Hall *et al.*, 1979;

Wechsler *et al.*, 1979b). Brain tissue from SSPE patients often do not contain detectable measles virus M protein (Hall and Choppin, 1979; Lin and Thormar, 1980). Defects have been demonstrated in M gene transcription (Cattaneo *et al.*, 1987) and translation (Carter *et al.*, 1983; Baczko *et al.*, 1986; Cattaneo *et al.*, 1986; Enami *et al.*, 1989) and in M protein stability (Sheppard *et al.*, 1986; Cattaneo *et al.*, 1988a; Ayata *et al.*, 1989). However, it has now been shown that the M protein is expressed in some SSPE brains (Norrby *et al.*, 1985; Baczko *et al.*, 1986; Sheppard *et al.*, 1986) and that the expression of the other two envelope-associated proteins, F and H, is altered (Norrby *et al.*, 1985; Baczko *et al.*, 1986; Liebert *et al.*, 1986; Cattaneo *et al.*, 1989b). Recently, humoral response to the M protein was demonstrated in the serum and cerebrospinal fluid of all the patients with SSPE tested by ELISA (Dhib-Jalbut *et al.*, 1988). Restricted expression of any of the three viral envelope-associated proteins may play a role in measles persistence by preventing the assembly of virus particles.

Evolution of RNA viruses in cultured cells

The rate of base substitutions for viral RNA genomes is estimated to be about 10^{-3} to 10^{-5} per base per replication. More specifically, the nucleotide substitution frequency for both the VSV and poliovirus polymerase is in the order of 10^{-3} to 10^{-4} (Steinhauer *et al.*, 1989a; Ward *et al.*, 1988). The mutation rate during the replication of Rous sarcoma virus and a spleen necrosis virus-based vector was found to be 1.4×10^{-4} and 2×10^{-5}, respectively, per nucleotide per replication cycle (Dougherty and Temin, 1988; Leider *et al.*, 1988). A major factor involved in the rapid rate of evolution of viral RNA genomes is the lack of a

proofreading enzyme system associated with the RNA polymerase. The mutation rate for DNA genomes is estimated to be between 10^{-8} to 10^{-11} (Drake, 1969). The proofreading and repair mechanisms of DNA polymerases is believed to account for this low rate of misincorporation.

Animal RNA viruses in laboratory cell cultures can undergo rapid genome evolution or maintain genome stability depending on the condition of passage. A STD VSV population after 523 low multiplicity passages in BHK21 cells displays a consensus T_1 fingerprint map indistinguishable from that of the original virus (Steinhauer *et al.*, 1989b). However, nearly every individual clone isolated from passage 523 differs from the consensus sequence. Thus, under these conditions VSV exhibits a quasispecies population distribution (collection of differing, related genomes). On the other hand, VSV passaged at high input multiplicities (undiluted) evolves rapidly and continuously (Spindler *et al.*, 1982). The consensus fingerprint of the total population of VSV after 254 undiluted passages differs from the original virus (Steinhauer *et al.*, 1989b). Oligonucleotide map analysis of individual clones suggests that the virus population is not characterized by a single consensus sequence but by several (perhaps many) rather fit master sequences (subpopulations). Variants existing in these subpopulations would compete among themselves and with the other master sequence populations present in the total population.

The evolution of the VSV genome during long-term persistent infection of BHK21 cells has been examined (Holland *et al.*, 1979). Oligonucleotide map analysis of cloned virus after 14, 21 and 42 months of persistence revealed that the viruses had undergone three, eight and eleven map changes, respectively. Since 85 to 90% of all mutations are

not apparent, it was estimated that roughly 50 to 100 mutations had occurred by 42 months of persistence. After 60 months of persistence the oligonucleotide map studies suggested that the entire genome had undergone 100 to 200 base changes (Rowlands *et al.*, 1980). Clearly, the VSV genome undergoes massive mutation during persistence. VSV is the only negative-strand RNA virus in which evolution of the genome in cultured cells has been studied. It would be of interest to determine whether the genome of other negative-strand RNA viruses also evolve at such a rapid rate.

Virus evolution during undiluted passages or persistent infections of VSV is believed to be driven by DI particles (Holland *et al.*, 1982; Horodyski *et al.*, 1983; DePolo *et al.*, 1987). Oligonucleotide map analysis of a variety of DI particles shed by the persistently infected culture at different times revealed that new DI particles are constantly arising and outcompeting the DI particles present in the cells (Holland *et al.*, 1979). DI particles force the evolution of the STD virus into forms (Sdi^- mutants) which are resistant to interference by the present DI particle. The appearance of new DI particle types now capable of interfering with the mutant virus is believed to provide the evolutionary pressure for selection of new Sdi^- mutants (DePolo *et al.*, 1987). Thus, there is continuous coevolution of virus and DI particles. Giachetti and Holland (1988) have demonstrated that mutation in the polymerase genes is implicated in DI particle resistance of Sdi^- mutants. Semler and Holland (1979) observed that the 5'-end sequence of a Sdi^- mutant from a 5 year old persistently infected culture had 7 mutations in the first 46 nucleotides. The model suggests that as Sdi^- mutants escape from DI particles, there is selection for mutant viral termini that interact more

efficiently with the evolving replicase complex (Giachetti and Holland, 1988).

4. STATEMENT OF OBJECTIVES

1) To generate and/or amplify DI particles of HPIV3 and to characterize their genetic content.
2) To determine whether serial undiluted passage of HPIV3 aids in the establishment of persistent infections of cultured cells.
3) To identify the factors implicated in the maintenance of HPIV3 persistent infections of cultured cells.
4) To investigate the genetic changes undergone by HPIV3 RNA after long-term persistent infections of cultured cells.

CHAPTER 2: MATERIALS AND METHODS

1. CELLS, VIRUSES AND VIRUS PRODUCTION

Cells

LLC-MK_2 (rhesus monkey kidney) and CV-1 (African green monkey kidney) cells were originally obtained from Flow Laboratories (Rockville, MD) and from the American Type Culture Collection (ATCC) (Rockville, MD), respectively.. HEp-2 (human epidermoid carcinoma) cells were kindly provided by Dr. Syed A. Sattar, University of Ottawa, Ottawa, Ontario. Cells were grown as monolayers in 100 mm-diameter polystyrene dishes (Nunc/GIBCO/BRL Life Technologies Inc., Burlington, Ontario). All media reagents and trypsin were purchased from GIBCO. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (HIFBS), 2 mM L-glutamine with or without anti-PPLO agent (1X) and penicillin-streptomycin (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate). Cells were incubated at 37°C in a humidified Shel-lab incubator (John's Scientific Inc., Toronto, Ontario) supplemented with 5% CO_2.

Cell lines were maintained by dispersing confluent monolayers with 1.25% trypsin in phosphate-buffered saline (PBS) (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$). To remove the trypsin, cells were pelleted at 200 x g for 5 min in a Sorvall GLC-2B clinical centrifuge (HL-4 rotor). Cells were resuspended in medium and plated.

Cells were viewed by phase contrast microscopy using a Leitz Diavert inverted light microscope (Wild Leitz Canada LTD, Wellowdale, Ontario).

Cells were photographed with an Olympus OM-2 camera and photomicrographic adapters (Olympus Optical Company, Tokyo, Japan).

Viruses

HPIV3, ATCC strain C-243, was originally obtained from D. A. McLeod, Laboratory Centre for Disease Control, Ottawa, Ontario. A heat resistant strain of VSV (VSV_{IND-HR}) was originally obtained from Dr. L. Prevec, McMaster University, Hamilton, Ontario.

Virus infection and production

STD HPIV3 infections of LLC-MK_2 cells were performed at a multiplicity of infection (MOI) of 1 to 3, unless otherwise mentioned. The virus was allowed to adsorb for 1 h (37°C, 5% $C0_2$), before fresh medium was added. Cells were further incubated for 24-48 h.

STD HPIV3 stocks were produced in a similar manner. After a 24 or 48 h infection period, the medium was harvested, clarified at 2250 x g and 4°C in a 253 rotor (IEC DPR-6000 centrifuge) and frozen in 1 ml aliquots at -80°C.

2. BIOLOGICAL ASSAYS

Plaque assays

HPIV3 was titrated on monolayer cultures of LLC-MK_2 cells in 60 mm-diameter polystyrene dishes (Nunc). Serial ten-fold dilutions of virus were prepared and 0.1 ml of the appropriate dilution was used to infect cells. After a 1 h incubation period at 37°C (5% CO_2), the cells were overlayed with 4 ml of DMEM and supplements containing 0.8% agar

Noble (Difco Laboratories/BDH Inc., Toronto, Ontario). Cells were incubated for 4 days at 37°C (5% CO_2). 2 ml of a 1:8000 dilution of neutral red (Fisher Scientific, Nepean, Ontario) in PBS was added and the cells were further incubated for 24 h. White plaques were counted. All plaque assays were performed in duplicate and the average score was recorded.

VSV_{IND-HR} was also titrated on LLC-MK_2 cells using the above protocol. In this case plaques were scored 24 h after infection and were easily detected without staining.

Interference assays

LLC-MK_2 cells were infected with a mixture of STD virus and the virus preparations to be assayed. The virus was allowed to adsorb for 1 h at 37°C (5% CO_2) and then fresh medium was added. For the interference assay involving the virus recovered from the persistently infected cells, the culture fluid was replaced with fresh medium at 24 h postinfection. All infections were allowed to proceed for 48 h. The medium was harvested and the virus titer was determined by plaque assay.

Hemadsorption

Monolayer cultures were washed twice with warm (37°C) PBS. 2 ml of a 0.5% human O^+ red blood cell (RBC) suspension in PBS was added to the cultures. After incubation at 4°C for 20 min, the nonadsorbed RBCs were removed and monolayers were subsequently washed four times with cold (4°C) PBS. The cells were viewed and photographed through a phase contrast microscope.

3. PERSISTENT INFECTIONS

Establishment of persistent infections

To establish the two persistently infected cultures, LLC-MK_2 cells were infected at a MOI of 5 with STD HPIV3 and at a MOI of 2.5 with HPIV3 that had undergone 8 serial undiluted passages. After 5 days, most of the cells in the STD virus-infected culture had succumbed to the infection. However, the cells that survived were cultured and by 10 days (15 days postinfection) a confluent monolayer was obtained. These cells were passaged and a persistently infected culture was established. This culture was designated PI3/MK_2-P0. In contrast, the serial passage 8 virus-infected monolayer remained intact and the majority of the cells survived the infection. These cells were passaged and a second persistently infected culture was established. This culture was designated PI3/MK_2-P8.

Maintenance of persistent infections

The persistently infected cells were grown in 100 mm-diameter dishes using DMEM and supplements. In times of severe crisis the HIFBS concentration of the medium was raised to 10%. The persistently infected cultures were passaged 1:4 every 6 days, on average. Both cell lines were maintained for 162 passages or 1000 days. Cells were frozen in 70% DMEM, 15% HIFBS and 15% dimethyl sulfoxide (DMSO) (Fisher Scientific) at a concentration of 3×10^6 cells/ml every 8th cell passage, on average. 1 ml aliquots were placed in Nunc cryotubes (1.8 ml volume) and incubated at -20°C for 1 h and at -80°C overnight. Cells were then immersed in liquid nitrogen.

4. ISOLATION AND ELECTROPHORESIS OF RNA

Radiolabeling of HPIV3-infected cells

HPIV3 infected cells were labeled with 5 or 10 μCi/ml of [5,6-^{3}H]uridine (Amersham Canada LTD, Oakville, Ontario) 24 h prior to extraction of RNA. In some experiments, cells were treated with 1 μg/ml of Actinomycin C_1 (Boehringer Mannheim Canada, Dorval, Quebec) 1 h prior to radiolabeling.

Isolation of HPIV3 genomic RNA

The media removed from the culture plates was clarified at 2250 x g for 10 min at 4°C in a 253 rotor. The virus was pelleted at 112,000 x g for 2 h at 4°C in a Sw28 rotor (Beckman ultracentrifuge L8-55M). The virus was resuspended into 100μl of 50 mM Na-acetate, pH 5.2, 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 100 mM NaCl and 0.5% sodium dodecyl sulfate (SDS) (NENS) containing 400 μg/ml of proteinase K (Boehringer Mannheim Canada) for every 35 ml of media. The suspension was transferred to a 1.5 ml microcentrifuge tube which was incubated at 37°C for 30 min. The mixture was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). In some instances, 5 μg of wheat germ tRNA was added to the samples. Three volumes of ethanol were added to precipitate the RNA. The tubes were placed at -80°C.

Isolation of cellular RNA

Monolayers were washed with 2 ml of cold PBS. The PBS was removed and 2 ml of cold PBS was re-added. The cells were scraped with

a rubber policeman and pelleted at 250 x g for 5 min at 4°C in a 253 rotor. The pellet was resuspended in 100 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl and 500 mM sucrose at a concentration of 1.2 x 10^7 cells/ml. Polyvinyl sulfate at 25 μg/ml and spermine-HCl at 30 μg/ml were added. The suspension was made 1% with respect to triton X-100 and Na-deoxycholate. The pellet was dispersed by vortexing and cells were disrupted by six strokes of the pestle of a dounce homogenizer. The nuclei were pelleted at 12,100 x g for 5 min at 4°C in a JA-20 rotor (Beckman centrifuge J2-21M). To the supernatant was added an equal volume of 2XNENS containing 400 μg/ml of proteinase K. After 30 min at 37°C the supernatant was extracted twice with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. To precipitate the RNA 2.5 volumes of ethanol was added. The tubes were placed at -20°C.

Electrophoresis of RNA

RNA was separated in a 1% agarose gel (13.3 x 22.0 cm) prepared in 10 mM phosphate buffer, pH 7.0 (6.1 mM Na_2HPO_4, 3.9 mM NaH_2PO_4). Electrophoresis was at 100 volts for 4 h with constant recirculation of the buffer. The RNA was denatured in the presence of 0.9 M (6%) glyoxal (BDH), 50% DMSO and 10 mM phosphate buffer, pH 7.0 at 60°C for 15 min. After electrophoresis the RNA was either visualized by fluorography or transferred onto a hybridization membrane.

For fluorography, gels were immersed in fixer (30% methanol, 10% acetic acid) for 1 h with gentle shaking. Gels were washed twice (30 min each time) in methanol and then incubated for 16 h in methanol containing 3% 2,5-diphenyloxazole (Fisher Scientific) with gentle agitation. Gels were rehydrated in distilled water for 40 min with gentle agitation.

Gels were dried using the Bio-Rad Model 224 Gel Slab Dryer for 1 h with heat and exposed to Cronex 4L X-ray film (Picker International Canada Inc., Ottawa, Ontario).

5. NUCLEIC ACID HYBRIDIZATION

Northern blots

Following electrophoresis, the RNA was transferred onto GeneScreen Plus (NEN Research Products, Mississauga, Ontario) by capillary action with 1.5 M NaCl and 0.15 M Na-citrate (10 x SSC) for 40 h (Thomas, 1983). To reverse the action of the glyoxal the membrane was immersed into 50 mM NaOH for 15 sec and then neutralized in 1 x SSC and 0.2 M Tris-HCl, pH 7.5 for at least 1 min.

Southern blots

After electrophoresis, the DNA was denatured by immersing the gels into 0.4 M NaOH and 0.8 M NaCl for 30 min and then neutralized in 0.5 M Tris-HCl, pH 7.6 and 1.5 M NaCl for at least 30 min. The DNA was transferred onto GeneScreen Plus (NEN) by capillary action with 10 x SSC for 20 h (Southern, 1975).

Nick translation

DNA was nick translated by a modified procedure of Rigby *et al.* (1977). 1.0 μg of DNA was incubated in the presence of 50 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 1 mM dithiothrietol (DTT), 50 μg/ml bovine serum albumin (BSA), 30 μmol of each dGTP, dCTP, and dTTP, 100 μCi of [α-^{32}P]dATP (800 Ci/mmol, Amersham), 440 pg deoxyribonuclease 1 and

10 units of DNA polymerase 1 of *E. coli* (Pharmacia, Baie d'Urfé, Quebec) at 14°C for 105 min. The sample was spun through a column of sephadex G-50 to separate the incorporated from unincorporated dNTPs. DNA was denatured with 0.5 volumes of 1 M NaOH for 15 min and neutralized with 0.5 volumes of 1 M HCl and 1 volume of 1 M Tris-HCl, pH 7.0.

Hybridization

Membranes were prehybridized in 6 x SSC, 5 x Denhardt's solution (0.1% ficoll, 0.1% polyvinylpyrrolidone and 0.1% BSA), 0.1% SDS and 100 μg/ml denatured salmon testes DNA. Blots were then incubated in prehybridization buffer containing the denatured probe at 65°C for a minimum of 16 h. Three 15 min washes in 2 x SSC and 0.1% SDS and three other 15 min washes in 0.2 x SSC and 0.1% SDS were performed. A final wash was carried out in 3 mM Tris, pH 8.8. All washes were at room temperature. Membranes were air dried and exposed to X-ray film at -80°C.

6. PROTEIN ANALYSIS

Preparation of HPIV3 virion structural proteins

Cells to be labeled with [^{35}S]methionine were fluid changed with 7 ml of methionine-free MEM (Flow Laboratories Inc., Mississauga, Ontario) supplemented with 1% HIFBS, 1% L-glutamine and 5% the normal methionine concentration. Cells to be labeled with [^{3}H]glucosamine were fluid changed with 7 ml of DMEM and normal supplements. Cells were labeled for 24 h with either 15 μCi/ml of L-[^{35}S]methionine (1066 Ci/mmol, Amersham) or 10 μCi/ml of D-[6-^{3}H]glucosamine

hydrochloride (30 Ci/mmol, Amersham). The medium removed from the cells was clarified at 2250 x g for 10 min at 4°C in a 253 rotor. The virus was pelleted at 112,600 x g for 2 h at 4°C in a Sw28 rotor. The virus was resuspended into 50 μl of 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 1 mM phenylmethylsulfonyl-fluoride (PMSF) and 1 mM benzamidine-hydrochloride (BHCl). Samples were frozen at -80°C.

Radiolabeling of HPIV3-infected cells

Cells were washed once with warm PBS and starved for 1 h in the presence of 2 ml methionine-free MEM. Cells were labeled for 2 h with 50 μCi/ml L-[^{35}S]methionine.

Preparation of cell lysates

All solutions were chilled on ice prior to use. Cells were washed once with 2 ml of PBS. Cells were scraped with a rubber policeman into 2 ml of PBS and centrifuged at 250 x g for 2 min at 4°C in a 253 rotor. Cells were resuspended in 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM PMSF and 1 mM BHCl (RIPA) at a concentration of 1.2 x 10^7 cells/ml and transferred to a microcentrifuge tube. An equal volume of RIPA containing 0.2% SDS, 2% Triton X-100 and 2% sodium deoxycholate (RIPA plus detergents) was added to each tube. The tubes were immediately inverted ten times and nuclei and membranes were quickly spun down in a microcentrifuge for 2 min. Cell lysates were frozen at -80°C.

Immunoprecipitations

Cell lysates (up to 300 μl) were thawed on ice and made to 470 μl with cold RIPA plus detergents. Cell lysates were rotated for 16 h at 4°C

in the presence of 30 μl rabbit anti-HPIV3 serum (kindly provided by D. A. McLeod) and 15 mg (100 mg/ml) protein A sepharose CL-4B beads (Pharmacia). Beads were washed four times with 500 μl of cold RIPA plus detergents and resuspended in 50 μl of 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue (BPB) and 10% β-mercaptoethanol (SDS-PAGE sample buffer).

Polyacrylamide gel electrophoresis of proteins

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) on slab gels using a discontinuous buffer system. Resolving gels were composed of 10% acrylamide, 0.13% N'N'-Bis-methylene-acrylamide (Bis), 0.1% SDS and 0.75 M Tris-HCl, pH 8.8. Stacking gels were composed of 5% acrylamide, 0.07% Bis, 0.1% SDS and 63 mM Tris-HCl, pH 6.8. Electrophoresis buffer consisted of 200 mM glycine, 25 mM Tris and 0.1% SDS. Prior to electrophoresis, virion protein samples were mixed with an equal volume of SDS-PAGE sample buffer and all protein samples were heated at 95°C for 5 min. Proteins to be transferred onto a membrane for immunological detection were electrophoresed using the Bio-Rad Mini Protean II Electrophoresis Cell (Bio-Rad Laboratories, Rockville Centre, NY) at 190 volts for 1 h whereas all other proteins were resolved using the Bio-Rad Protean II Slab Electrophoresis Cell at 65 volts for 15 h.

For fluorography gels were submerged into fixer (10% acetic acid, 30% methanol) for 45 min with gentle shaking. The fixing solution was removed and the gel was soaked in Amplify (Amersham) for 25 min. Gels were dried using the Bio-Rad Model 224 Gel Slab Dryer for 105 min with heat and the gels were exposed to X-ray film at -80°C.

Immunoblot assays

Proteins were transferred to nitrocellulose filter (Bio-Rad) using the Bio-Rad Mini Trans-blot Electrophoresis Cell at 90 volts for 90 min in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3 (Burnette, 1981). Antigens were detected using the Bio-Rad Immun-Blot Assay Kit Goat Anti-Rabbit Alkaline Phosphatase (GAR-AP) Conjugate. All washes and incubations were carried out with gentle agitation. The membranes were washed in 75 ml of 20 mM Tris-HCl, pH 7.5 and 500 mM NaCl (TBS) for 10 min. The remaining protein binding sites on the nitrocellulose membrane were blocked by immersing the membrane in TBS containing 3% gelatin for 1 h. Following two 5 min washes in 75 ml of TBS containing 0.05% tween 20 (TTBS), the membrane was incubated overnight in 50 ml of antibody buffer (TTBS plus 1% gelatin) containing 50 μl of anti-HPIV3 rabbit serum. To remove unbound antibody two 5 min washes in TTBS were performed. The membrane was incubated in 100 ml of antibody buffer containing a 1:3000 dilution of GAR-AP for 1 h. Two 5 min washes in TTBS followed by a 5 min wash in TBS were performed. The membrane was developed in 100 ml carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8) containing 30 mg of p-nitro blue tetrazolium chloride and 15 mg of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt for 12-15 min. The colour development was stopped by placing the membrane in distilled water.

7. PLASMID PREPARATION AND ELECTROPHORESIS OF DNA

Transformation of *E.coli* with plasmid DNA

pGEM3-L was introduced into competent *E. coli* strain HB101 by the

method of Golub (1988). pGEM3-L was kindly provided by Dr. Mark Galinski, Cleveland Clinic Foundation, OH while competent HB101 were provided by Dr. Ken Garson, University of Ottawa, Ottawa, Ontario. Briefly, 1 μl of plasmid DNA (20 ng/μl) in 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA (TE) was mixed with 3 μl of competent cells in a microcentrifuge tube on ice. Following a 1 min incubation at 4°C, 100 μl of 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) was added and the mixture was immediately spread on 2YT agar plates (2YT medium plus 1.5% agar) containing 100 μg/ml ampicillin. Plates were incubated at 37°C for 24 h.

Isolation of plasmid DNA

Plasmid DNA was isolated using the method developed by Clewell and Helinski (1969) which was later modified by Goebel (1970). 5 ml of 2YT medium containing 25 μg/ml of ampicillin was incubated with a single colony and cells were grown at 37°C for 8 h with shaking. 2.5 ml was removed and used to inoculate 500 ml of Luria Broth (1% tryptone, 0.5% yeast extract, 1% NaCl) plus 0.1% glucose which was incubated at 37°C overnight with shaking. Cells were pelleted at 11,300 x g for 15 min at 4°C in a JA-10 rotor (Beckman centrifuge J2-21M) and resuspended into 20 ml of TE buffer. Cells were pelleted again at 7,700 x g for 15 min at 4°C in a JA-20 rotor and resuspended into 6 ml of 25% sucrose and 50 mM Tris-HCl, pH 8.0. Following the addition of 1.2 ml of lysozyme (10 mg/ml) and 2.4 ml of 0.25 M EDTA, the cells were incubated on ice for 15 min. To the mixture was added 9.6 ml of Triton Lysate Solution (0.1% Triton X-100, 62.5 mM EDTA, 50 mM Tris-HCl, pH 8.0) and the cells were further placed on ice for 5 min. Membranes were spun down at

39,200 x g for 1 h at 4°C in a JA-20 rotor. For each ml of lysate 1.1 g of CsCl was added. Ethidium bromide (EtBr) was added to a final concentration of 185 μg/ml. Plasmids were centrifuged to equilibrium at 290,000 x g for 17 h at 20°C in a VTi 65 rotor (Beckman ultracentrifuge L8-55M). The plasmid band was collected and the solution was extracted four times with an equal volume of n-butanol saturated with H_2O to remove the EtBr. Three volumes of TE buffer, 0.4 volumes of 3 M Na-acetate, pH 5.2 and 4.5 volumes of cold isopropanol were added. After incubation at -80°C for 30 min the DNA was pelleted at 12,100 x g for 30 min at 4°C in a JA-20 rotor. The DNA was resuspended into 200 μl of H_2O and transferred into a microcentrifuge tube. The DNA was precipitated again with 2.5 volumes of cold ethanol and 0.1 volumes of 3 M Na-acetate, pH 5.2, on ice. The plasmid DNA was pelleted in a microcentrifuge at 4°C for 15 min and dissolved in H_2O at a concentration of 1 μg/ml. The DNA was quantitated using a DMS 200 UV-visible spectrophotometer (Varian Canada Inc., Georgetown, Ontario).

Electrophoresis of DNA

DNA was separated by horizontal gel electrophoresis using 0.8%-1.5% agarose and Tris-borate buffer (TBE) (90 mM Tris, 90 mM Boric acid, 2.5 mM EDTA). Electrophoresis was usually performed with a HE 33 Minnie the Gel-Cycle (Hoefer Scientific Instruments, San Francisco, CA) electrophoresis apparatus. Samples were mixed with 2.5 volumes of 5X loading buffer (25% glycerol, 5X TBE, 0.06% BPB) and electrophoresed at 100 volts. The DNA was visualized under ultraviolet (UV) illumination after staining with EtBr (1 μg/ml in TBE). Gels were photographed using Polaroid Type 667 film (Henry's, Toronto, Ontario).

8. CLONING OF HPIV3 GENOMIC RNA

Reverse transcription

Oligonucleotides synthesized on an Applied Biosystems (Mississauga, Ontario) Model 380B DNA synthesizer were used to prime cDNA synthesis using virion genomic RNA as template and avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences Inc., St. Petersburg, FL). The cDNA reactions consisted of the RNA in 100 mM Tris-HCl, pH 8.3, 130 mM KCl, 10 mM $MgCl_2$ and 2.5 mM DTT, 1 μg of primer, 0.75 mM of each of the four dNTPs and 25 units of RNasin (Promega/BIO/CAN Scientific Inc., Mississauga, Ontario) in a total volume of 38 μl. The mixture was heated at 65°C for 2 min and placed at 40°C for 5 min. 2 μl of reverse transcriptase (17.6 units/μl) was then added and the mixture was further incubated at 40°C for 105 min. The samples were extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. The DNA was precipitated in the presence of 2.5 volumes of ethanol and 0.5 volumes of 8 M NH_4-acetate. After incubation at -20°C the DNA was pelleted in a microcentrifuge at 4°C, resuspended in an appropriate volume of H_2O, and transferred into a 0.5 ml microcentrifuge tube.

Polymerase chain reaction

The cDNA was amplified by the polymerase chain reaction (PCR) process using the Perkin-Elmer Cetus (Norwalk, CT) GeneAmp DNA Amplification Reagent Kit and DNA thermal cycler. The reaction mixtures consisted of cDNA in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin, 100 ng of each of the two primers, 0.5 mM of

each of the four dNTPs and 5 units of Taq DNA polymerase (5 units/μl) in a total volume of 100 μl. The synthetic oligonucleotide primers used to amplify the cDNA were constructed with convenient restriction sites at their 5'-ends. Samples were overlayed with 100 μl of mineral oil. The DNA was amplified using the Step-Cycle file. The initial template denaturation step was 3 min at 94°C. Afterwards the cycle profile was 1 min at 94°C (denaturation), 2 min at 45-55°C (annealing) and 3 min at 72°C (extension). To amplify the M gene and flanking regions of HPIV3 an automatic extension time of 10 sec was added to the 72°C extension step. Thirty cycles were performed. At the end of the 30th cycle, the 72°C extension step was prolonged for an additional 7 min. Samples were then cooled to 4°C. The bottom aqueous phase was removed and extracted once with chloroform/isoamyl alcohol. One-tenth of the sample was analyzed by agarose gel electrophoresis.

Purification of DNA from gels

DNA was purified from gels using a modification of the "freeze-squeeze" method published by Tautz and Renz (1983). Gels were stained with EtBr and DNA visualized under long-wave UV light. Bands of interest were cut out with a scalpel. The agarose slices were placed in 0.5 ml microcentrifuge tubes which had been punctured through the cap and bottom with a 21 gauge needle. The tubes were placed in 1.5 ml decapped microcentrifuge tubes and centrifuged for 1 min. To the agarose in the 1.5 ml tubes was added 100 μl of 0.3 M Na-acetate, pH 5.2. The agarose was transferred with a 1 ml needleless syringe to 0.5 ml tubes which had been punctured through the cap and bottom and plugged with silanized glass wool. The tubes were frozen at -80°C for 30 min. The

tubes were then inserted in 1.5 ml tubes and immediately centrifuged for 4 min. This centrifugation was repeated twice with fresh 1.5 ml tubes for 4 min and 3 min, respectively. The three eluted volumes were pooled and extracted 3 times with H_2O saturated n-butanol. The DNA was then precipitated with 2.5 volumes of ethanol and stored at -20°C.

Restriction digests and ligation in M13

The amplified cDNAs purified from agarose gels and M13 replicative form (RF) DNA (Pharmacia) were digested with 5-10 units of *Sst*1 (Pharmacia) and *Hin*dIII (BRL) in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 50 mM NaCl at 37°C for 2 h. At the end of the incubation period, the samples were extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. 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 and stored at -20°C.

For the ligation reactions, restricted fragments and digested M13 RF DNA were incubated in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 5% polyethylene glycol (PEG-8000) and 3.25 units of T4 DNA ligase (Pharmacia) at 16°C for 7 h in a final volume of 20 μl.

9. M13 PHAGE

Transformation of competent cells with replicative form of M13 DNA

Replicative form of M13 DNA (Messing, 1983) was used to transform either Epicurian coli XL1-Blue cells (Stratagene, La Jolla, Ca) or *E. coli* DH5αF' (BRL) following the manufacturer's protocol. Briefly, 100 μl of competent cells were thawed on ice and transferred to pre-chilled 15 ml

Falcon 2059 polypropylene tubes (Fisher Scientific). To the XL-1 blue cells, 1.7 μl of a fresh 1:10 dilution of β-mercaptoethanol was added. After 10 min on ice, 2 ng of M13 DNA was added and the mixture was further incubated on ice for 30 min. The cells were heat shocked at 42°C for 45 sec. The cells were incubated on ice for at least 2 min before 0.9 ml of SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) pre-heated at 42°C was added. To 200 μl of freshly growing *E. coli* strain JM101 (OD_{550}=1) was added 0.75 ml of the transformed cell mixture, 3 ml of B top agar (1% tryptone, 0.8% NaCl, 0.6% agar), 75 μl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BRL) and 15 μl of 100 mM isopropylthiogalactoside (BRL). The contents were poured onto B agar plates (1% tryptone, 0.8% NaCl, 1.2% agar) and incubated at 37°C overnight.

Growth and analysis of M13 single-stranded DNA

M13 plaques were grown with vigorous shaking in 2 ml of 2YT medium at 37°C for 7 h. The medium was transferred to two 1.5 ml microcentrifuge tubes and centrifuged for 5 min. To 20 μl of supernatant was added 1 μl of 10% SDS and 5 μl of 5X loading buffer. Samples were electrophoresed in a 0.8% agarose gel (13.3 x 22.0 cm) buffered with TBE at 50 volts for 15 h. The remaining supernatant was stored at 4°C (phage stock).

Preparation of phage template for sequencing

50 μl of an overnight culture of *E. coli* strain JM101 grown in 2 ml of 2YT medium at 37°C with shaking was used to inoculate 12 ml of 2YT medium. After a 1 h incubation at 37°C with shaking, 50-100 μl of phage

stock was added and the culture was further incubated at 37°C with shaking for 7 h. The bacteria were pelleted at 12,100 x g for 10 min in a JA-20 rotor. The supernatant was made 2% with PEG-8000 and 250 mM Na-acetate, pH 7.0. Following an overnight incubation at 4°C the phage was pelleted at 12,100 x g for 15 min in a JA-20 rotor. The phage was dissolved in 0.4 ml TE buffer, transferred to 1.5 ml microcentrifuge tubes, extracted once with phenol alone, twice with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. After a 15 min incubation on ice in the presence of 0.5 volumes of 8 M NH_4-acetate and 2 volumes of ethanol, the DNA was pelleted for 15 min in a microcentrifuge. The DNA was washed once with 70% ethanol dried down for 5 min in a Speed Vac Concentrator (Savant Instruments/Emerston Instruments Inc., Richmond Hill, Ontario) and resuspended in H_20.

10. DNA SEQUENCE ANALYSIS

Sequencing of M13 single-stranded DNA

Dideoxynucleotide sequence analysis (Sanger *et al.*, 1977) was performed using the TaqTrack sequencing system (Promega/BIO/CAN Scientific) with [α-^{35}S]dATP essentially as described by the manufacturer. Briefly, 1.5-2.0 μg of M13 single-stranded DNA was annealed with 8 ng of primer (pUC/M13 24-mer forward primer or specific oligonucleotide primers) in 50 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$ with 7.5 μM dGTP, dTTP and dCTP at 70°C for 3 min. The final volume was 25 μl. The primer was extended and labeled by incubation at 37°C for 2 min in the presence of 1.5 μl of [α-^{35}S]dATP (>600 Ci/mmol, Amersham) and 1.5 μl of Taq DNA polymerase (5 units/μl). 6 μl of the labeled, annealed

primer-template mixture was immediately added to 4 tubes containing the appropriate A, C, G, and T deoxy/dideoxy (d/dd)NTP mix. In all 4 reactions the concentration of the three nonspecific dNTPs was 250 μM while the concentration of the specific dNTP was 25 μM. The concentration of the ddNTP in the specific reactions was 350 μM for ddATP, 160 μM for ddCTP, 50 μM for ddGTP and 600 μM for ddTTP. The reaction mixtures were incubated at 70°C for 7 min. The sequencing reactions were terminated by the addition of 4 μl of stop solution (10 mM NaOH, 95% formamide, 0.05% BPB, 0.05% xylene cyanol). The reactions were heated at 90°C for 5 min immediately before loading on a sequencing gel.

Sequencing gels

Sequencing reactions were resolved in either 6% polyacrylamide (5.7% acrylamide-0.3% bis-acrylamide)-7 M urea gels or 8% polyacrylamide (7.6% acrylamide-0.4% bis-acrylamide)-7 M urea gels in TBE buffer. Electrophoresis was performed with the IBI (New Haven, CT) Model STS-45 Thermoplate Sequencing Apparatus at a constant power of 55 watts. After electrophoresis, gels were lifted from dimethyldichlorosilane (BDH) treated glass plates onto a Whatmann 3MM paper and dried in a Bio-Rad Model 583 Gel Drier. Gels were exposed to X-ray film at -80°C.

CHAPTER 3: GENERATION AND CHARACTERIZATION OF DI PARTICLES OF HPIV3

1. INTRODUCTION

The initial objective of my project was to generate and characterize the genetic content of DI particles of HPIV3 in order to gain a better understanding of HPIV3 replication. DI particles of nonsegmented negative-strand RNA viruses contain a portion of the STD virus genome and interfere with the replication of the parental virus. Interference with STD virus replication can modulate the severity and duration of virus infections and/or lead to the establishment of persistent infections. The sequences remaining in the genomes of DI particles are responsible for their biological properties and essential for their amplification.

Successive undiluted passage of virus stocks has been used successfully to generate DI particles. The pleomorphism of paramyxovirus particles makes it very difficult to separate DI virions from STD virions on the basis of size. Therefore, paramyxovirus DI particles have been identified by sucrose gradient sedimentation or agarose gel electrophoresis of RNA genomes (Kolakofsky, 1976; Amesse *et al.*, 1982). DI particles of nonsegmented negative-strand RNA viruses have been reported for VSV (Cooper and Bellet, 1959), rabies virus (Kawai *et al.*, 1975), Sendai virus (Kingsbury *et al.*, 1970), measles virus (Hall *et al.*, 1974) and NDV (Roman and Simon, 1976).

This chapter reports the generation and/or amplification, through serial undiluted passage, of DI particles of HPIV3. The size and genetic content of the DI particle RNAs is presented and compared to that of Sendai virus and VSV.

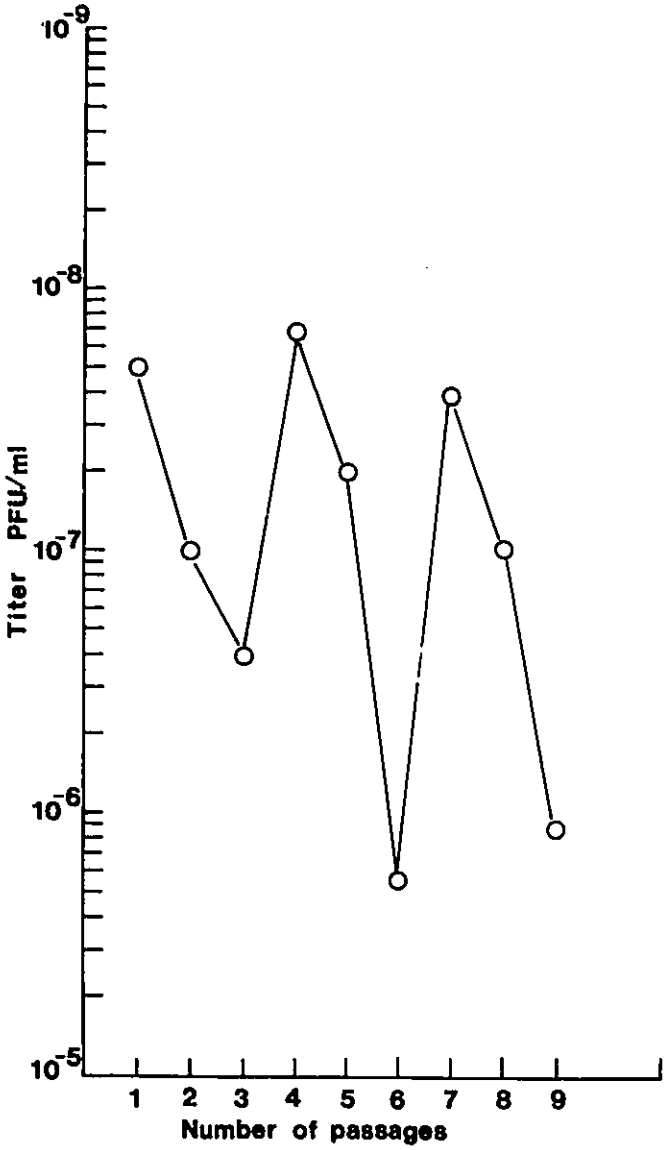
2. RESULTS

Generation of DI particles from HPIV3

To generate and/or amplify DI particles from HPIV3, the STD virus was serially passaged at 48 h intervals nine consecutive times in LLC-MK_2 cells. Initially, cells were infected with a STD virus inoculum of 250 µl corresponding to a MOI of 10. The STD virus originated from a plaque purified clone that had been passaged twice at a relatively low MOI (1-3) in LLC-MK_2 cells. The subsequent infections were performed with 250 µl of culture medium obtained from the previous infection. At each passage the medium was harvested, cleared of cell debris by centrifugation, and frozen at -80°C. The virus titer (plaque-forming units) of the progeny virus from each of the passages was then determined (Fig. 4). In the first three passages, a gradual decline in titer was observed. This was then followed by a burst in virus production at the fourth passage. In the next two successive passages, the titer gradually declined again only to be followed by a second burst in virus production at passage seven. Similarly, the titer gradually declined in the last two successive passages. This cyclic production of plaque-forming virus is characteristic of DI particle production (Palma and Huang, 1974).

Since paramyxovirus particles are pleomorphic, it has been very difficult (Kingsbury *et al.*, 1970), or even impossible (Roman and Simon, 1976), to separate DI virions from STD virions by size in sucrose gradients. Therefore, to detect the presence of DI particles the RNA present both in virions and virus-infected cells was analyzed at each of the nine passages by agarose gel electrophoresis. RNA species migrating faster than the STD virus genome would be indicative of the presence of

Figure 4. Yield of plaque-forming HPIV3 upon serial undiluted passage. The culture fluid from each of the nine passages was clarified and frozen at -80°C until assayed for plaque-forming virus in LLC-MK_2 cells.


10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Titer PFU/ml
1 2 3 4 5 6 7 8 9
Number of passages

DI particles. For this analysis cells were treated with actinomycin C_1 1 h prior to addition of the radiolabel. Only the STD-size (50S) genome was detectable in virions of the first six passages (Fig. 5). However, in the seventh, eighth and ninth passages one subgenomic RNA-size species (DI-3) was observed. Interestingly, DI-3 was detected at passage 5 in virus-infected cells (Fig. 6). Also, a second subgenomic RNA-size species (DI-1) was observed migrating just below the 50S genome and was present as early as passage 2 (Fig. 6).

To investigate whether the virus present in the late passages contained interference activity, LLC-MK_2 cells were infected with a mixture of the STD virus and different concentrations of virus from passages 8 or 9. After 48 h, the culture fluid was assayed for plaque-forming virus. Table 3 shows that as the amount of virus from passages 8 or 9 was reduced, the virus titer increased. Culture fluid from passage 9 had less interfering activity than that of passage 8. These results indicate that virus from passages 8 and 9 interfered with replication of the STD HPIV3.

The subgenomic RNAs in the serially passaged virus were present only at low levels. To amplify the DI particles, viruses from serial passages 5 and 8 were individually mixed with the STD virus (MOI of 50) and each mixture was used to infect LLC-MK_2 cells. The RNA species obtained from both virions and virus-infected cells are shown in Fig. 7. The levels of DI-1 and DI-3 were significantly increased with regards to the level of the 50S genome. Indeed, DI-3 was by far the most abundant RNA species in virions of the STD virus plus serial passage 8 virus mixed infection. DI-1 RNA was present in approximately the same amount as the STD virus in both virions and virus-infected cells. A faint band migrating at

Figure 5. Virion RNA profile of virus particles recovered at each serial undiluted passage. Confluent monolayer cultures of LLC-MK_2 cells were treated 15 h after infection with 1 μg/ml of actinomycin C_1. [^{3}H]uridine was added 1 h after the actinomycin C_1 treatment. At 40 h postinfection the virus particles were concentrated by ultracentrifugation and the RNAs were extracted, denatured with glyoxal and separated in a 1% agarose gel. Lanes: 1 to 9, serial undiluted passages 1 to 9, respectively; 10, ^{3}H-labeled 28S and 18S rRNAs.

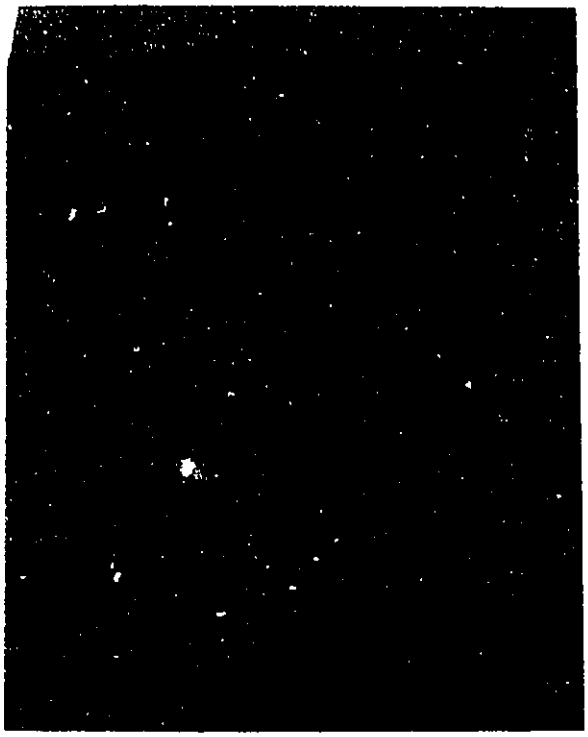


Figure 6. Intracellular RNA profiles of infected LLC-MK_2 cells at each serial undiluted passage. Infected cells were treated with actinomycin C_1 and labeled with [^{3}H]uridine as described in Fig. 5. The intracellular RNA was extracted, denatured with glyoxal and separated in a 1% agarose gel. Lanes: 1 to 9, serial undiluted passages 1 to 9, respectively; 10, ^{3}H-labeled 28S and 18S rRNAs.

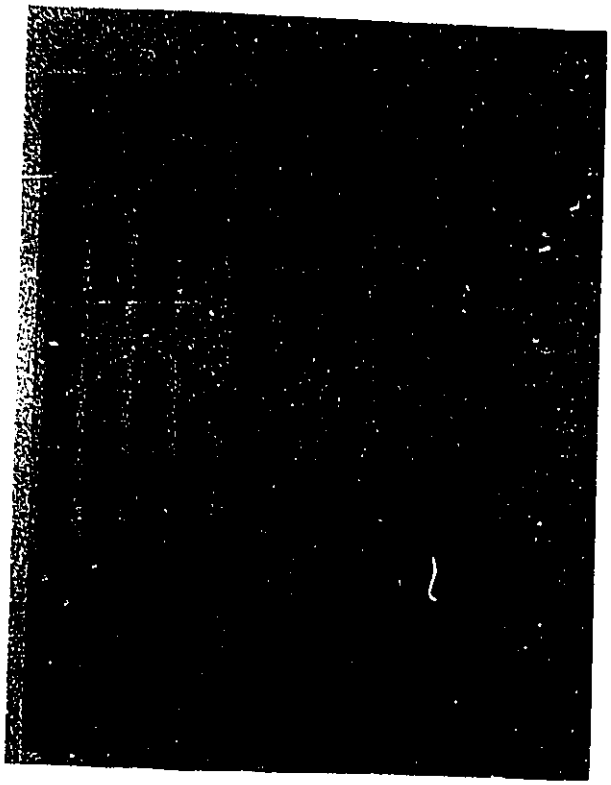


TABLE 3

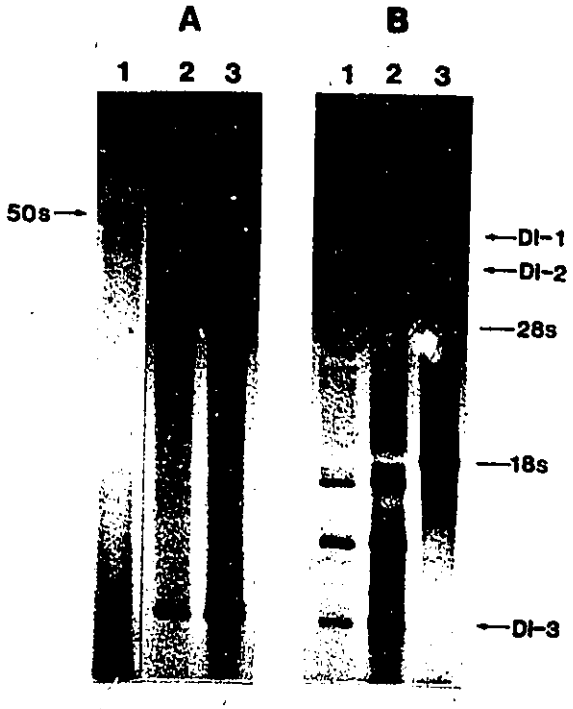
ABILITY OF LATE SERIAL PASSAGED HPIV3 TO REDUCE YIELD OF INFECTIOUS VIRUS[a]

Inoculum[b]	Yield (PFU/ml)	Reduction (%)
STD (0.1 ml)	1.1×10^8	-
STD (0.1 ml) + SP8 (0.1 ml)	3.6×10^7	67
STD (0.1 ml) + SP8 (0.05 ml)	6.3×10^7	43
STD (0.1 ml) + SP8 (0.025 ml)	6.8×10^7	38
STD (0.1 ml) + SP9 (0.1 ml)	6.4×10^7	42
STD (0.1 ml) + SP9 (0.05 ml)	9.2×10^7	16
STD (0.1 ml) + SP9 (0.025 ml)	1.1×10^8	0

[a] STD virus (1.5×10^8); SP8, serial passage 8 virus (1.0×10^7); SP9, serial passage 9 virus (8.7×10^5).

[b] All inoculum volumes were 0.2 ml.

Figure 7. Virion RNA and intracellular RNA profiles of LLC-MK_2 cells coinfected with the STD and serial passage virus. Infected cells were treated with actinomycin C_1 and labeled with [^{3}H]uridine as described in Fig. 5. The RNA was denatured with glyoxal and separated in a 1% agarose gel. (A) Lanes: 1, virion RNA from passage 1 virus; 2, STD virus plus serial passage 5 virus coinfection; 3, STD virus plus serial passage 8 virus coinfection. (B) Lanes: 1, intracellular RNA from STD virus plus serial passage 5 virus coinfection; 2, STD virus plus serial passage 8 virus coinfection; 3, ^{3}H-labeled 28S and 18S rRNAs.


A
B
1 2 3
1 2 3
50s
DI-1
DI-2
28s
18s
DI-3

the same position as subgenomic RNA DI-2 identified in the hybridization analysis (Fig. 9) could also be discerned.

Genetic mapping of HPIV3 DI particles

To determine the genetic contents of DI-1, DI-2 and DI-3 subgenomic RNA species, total cytoplasmic RNA from cells coinfected with the STD virus plus passage 8 virus was hybridized with cloned DNA sequences specific for each of the six HPIV3 structural protein genes (Dimock *et al.*, 1987). Figure 8 shows the map positions of each of the virus-specific inserts. The cDNAs represent at least 80% of the NP (pPI47) gene, 50% of the P/C (pPI28) gene, 95% of the M (pPI3) gene, 80% of the F (pPI14) gene, 50% of the HN (pPI10) gene, and 33% of the L (mpPIg22) gene.

The result of the Northern blot hybridizations obtained from STD virus-infected cells alone is shown in Fig. 9A. All of the clones hybridized to the STD virus genomic RNA, thus confirming viral specificity. More significantly, all the clones hybridized to their corresponding mRNAs. The L mRNA (Fig. 9A, lane 6b) was identified on the basis of its large size, now known to be 6795 nucleotides long (Galinski *et al.*, 1988). Only the HN and L clones hybridized to a single species of HPIV3 mRNA. All of the other clones hybridized to larger transcripts as well as to mRNAs. These are most likely polycistronic transcripts obtained by readthrough transcription as observed for other paramyxoviruses (Collins and Wertz, 1983; Paterson *et al.*, 1984; Wilde and Morrison, 1984; Gupta and Kingsbury, 1985). Clones representing the M and F mRNAs both hybridized to an abundant M-F dicistronic transcript (Spriggs and Collins, 1986a). The P/C clone hybridized to a second transcript which is possibly a P/C-M dicistronic transcript. This

Figure 8. Genetic map of the sequences covered by the cDNA clones.

HPIV3 GENOME

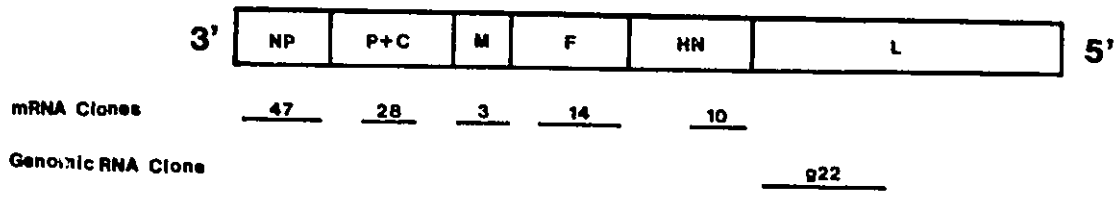
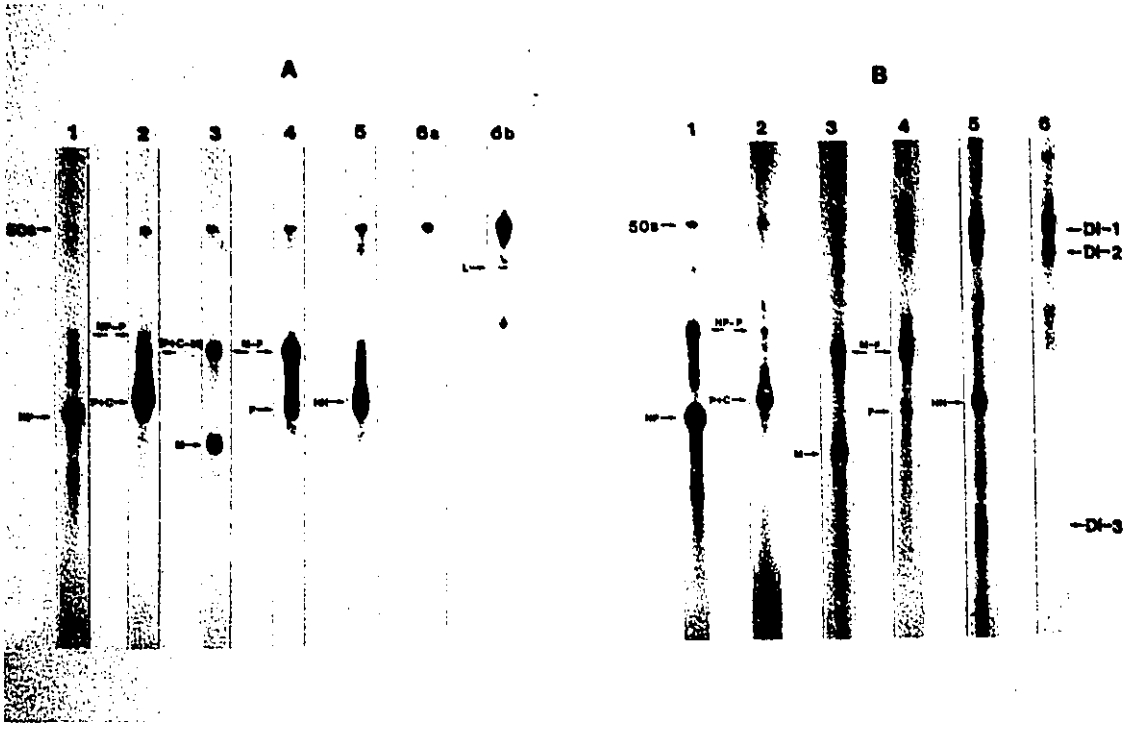


Figure 9. Hybridization of ^{32}P-labeled HPIV3 cDNAs to Northern blots of total cytoplasmic RNA. Total cytoplasmic RNA was isolated from HPIV3-infected LLC-MK$_2$ cells and electrophoresed in a 1% agarose gel. The intracellular RNA was transferred onto GeneScreen Plus and the blots were cut into 4 mm-wide longitudinal strips. The individual strips were hybridized to nick-translated pPI47 (NP) (lane 1), pPI28 (P+C) (lane 2), pPI3 (M) (lane 3), pPI14 (F) (lane 4), pPI10 (HN) (lane 5), and mpPIg22 (L) (lane 6) cDNAs, respectively. (A) Total cytoplasmic RNA isolated from STD virus-infected cells. Lane 6a is a 3 h exposure, while lane 6b is an 8 h exposure. (B) Total cytoplasmic RNA isolated from STD virus plus serial passage 8 virus-coinfected cells. The locations of RNAs DI-1, DI-2, and DI-3 (determined by direct labeling) are indicated.


A
1
2
3
4
5
6a
6b
50s
NP-2
PHC
M
M-2
T
HH
B
1
2
3
4
5
6
50s
NP-2
PHC
M
M-2
T
HH
DI-1
DI-2
DI-3

polycistronic transcript runs approximately in the same position as the M-F dicistronic transcript. Thus, a band unique to the P/C-M dicistronic transcript was not observed following hybridization with the M clone. Both the NP and P/C clones hybridized weakly to a possible NP-P/C dicistronic transcript.

The Northern blot analysis of the intracellular RNA from the STD virus plus passage 8 virus coinfection is shown in Fig. 9B. DI-1 RNA hybridized to the F, HN and L probes, while DI-2 RNA hybridized only to the HN and L probes. Therefore, DI RNAs 1 and 2 contain genetic information from the 5' half of the HPIV3 genome. None of the probes hybridized to DI-3 RNA. However, DI-3 may well be derived from the 5'-end of the HPIV3 genome since the L-specific probe does not cover the 5'-terminal sequences of the HPIV3 genome (Fig. 8). To verify this hypothesis, plasmid pGEM3-L, which contains a full length copy of the L gene, was hybridized to total cytoplasmic RNA of passage 8 virus-infected cells (Fig. 10). DI-3 hybridized to pGEM3-L indicating that DI-3 is also derived from the 5'-end of the HPIV3 genome.

The molecular weights of the DI RNAs were estimated using the HPIV3 genomic RNA and mRNAs as size markers (Table 4). DI-1, DI-2 and DI-3 RNAs correspond roughly to 63, 51 and 5% of the size of the STD virus genome, respectively.

Cloning of HPIV3 DI-3 RNA

A common feature of all DI RNAs of nonsegmented negative-strand RNA viruses is that they have conserved the 5'-terminus of the STD virus genome. The 3'-terminus of these DI RNAs corresponds either to the 3'-terminus of the STD virus genome (internal deletion type) or is

Figure 10. Hybridization of ^{32}P-labeled pGEM3-L to Northern blots of total cytoplasmic RNA. Infected cells were treated with actinomycin C_1 and labeled with [^{3}H]uridine as described in Fig. 5. Total cytoplasmic RNA was isolated, denatured with glyoxal and separated in a 1% agarose gel. RNA was either detected by fluorography (A) or transferred to GeneScreen Plus and hybridized with nick-translated pGEM3-L (B). In both (A) and (B): lane 1, serial passage 2 RNA; lane 2, serial passage 8 RNA.

TABLE 4

PREDICTED MOLECULAR WEIGHTS OF HPIV3 DI RNAs[a]

DI RNA	Estimated molecular weight[b]	% of total genome
DI-1	3.15×10^6	62.8
DI-2	2.50×10^6	49.9
DI-3	2.40×10^5	4.8

[a] As determined by glyoxal denaturation, electrophoresis in a 1% agarose gel and Northern blot hybridization (Fig. 9). The molecular weight markers were HPIV3 genomic RNA (15,461 nucleotides) and mRNAs (Table 2). The average length of a poly A tail was taken as 300 nucleotides (Sanchez and Banerjee, 1985).

[b] The average molecular weight of a ribonucleotide was taken as 324.25.

complementary to the 5'-terminus (copyback type). Now, the first 17 nucleotides of the 3'-terminus of the STD HPIV3 genome are inversely complementary to those of the 5'-terminus. Therefore, the first 17 nucleotides of the 3'-end of HPIV3 DI RNAs should be those of the STD virus genome regardless of whether the DI RNAs are of the internal deletion or copyback types.

DI-3 was estimated to be almost 5% of the STD virus genome length (Table 4) or approximately 775 nucleotides. From the hybridization data and what is known about the primary structure of DI RNAs, it is fair to assume that DI-3 RNA is a simple RNA molecule likely derived from the exact 5'-end of the HPIV3 genome. In an attempt to clone this subgenomic RNA, serial passage 8 virus RNA was reversed transcribed using a synthetic oligonucleotide primer (DM14; 5'-CTCGAGCTCACCAAACAAGAGAAG-3') complementary to the exact 3'-end of the HPIV3 genome (underlined sequence) and AMV reverse transcriptase. DM14 should now be complementary to the 3'-end of the newly synthesized DNA assuming that the cDNA synthesis went to completion using DI-3 RNA as template. Thus, DI-3 cDNA should be amplifiable using DM14 alone by the PCR process. However, after 30 cycles of amplification at various annealing temperatures no specific products were obtained (data not shown).

3. DISCUSSION

Serial undiluted passage and generation of HPIV3 DI particles

Serial undiluted passage was used to generate and/or amplify DI particles of HPIV3. Nine serial passages were performed and a cyclic

pattern of virus production was observed. Subgenomic RNAs were detected in both virions and virus-infected cells. In virions, a subgenomic RNA (DI-3) species was observed only in the late passages. Upon coinfection with the STD virus the subgenomic RNAs were substantially amplified. Viruses from passages 8 and 9 interfered with the STD virus replication. From these findings, it can be concluded that DI particles were amplified during serial undiluted passage of HPIV3. It is not known whether the DI RNAs were generated as a result of the undiluted passages. These DI particle genomes could have been present in the STD virus stock and amplified as a result of the serial undiluted passages.

Serial undiluted passage of HPIV3 in LLC-MK_2 cells resulted in only low levels of subgenomic RNA. Also, LLC-MK_2 cells persistently infected with HPIV3 produce relatively low levels of subgenomic RNAs (chapter 4). In contrast, serial undiluted passage of Sendai virus in chick embryo lung cells (Amesse *et al.*, 1982) or chinese hamster ovary cells (Kolakofsky, 1976) produces virus containing only minor amounts of 50S genomic RNA and major amounts of subgenomic RNAs. In addition, BHK21 cells persistently infected with Sendai virus yield high levels of subgenomic RNAs (Roux and Holland, 1980). Thus, it appears that HPIV3 may not support high levels of DI particle replication as does Sendai virus. The fact that coinfection with a high level of the STD virus significantly amplified the DI RNAs suggests that substantial amounts of HPIV3 polymerase proteins may be required to support high levels of subgenomic RNA replication. The host cell also plays a role in DI particle generation (Kingsbury and Portner, 1970; Kang and Allen, 1978; Kang *et al.*, 1978). It is possible that LLC-MK_2 cells do not allow the generation of high levels of DI particles. However, HPIV3 grows to high titers in LLC-MK_2 cells

and cell lines producing high yields of virus tend to generate and amplify DI particles more efficiently than cell lines producing low levels of virus (Holland, 1990).

An interesting finding is that DI-1 RNA could not be detected in virions recovered from the undiluted passages (Fig. 5), but could distinctively be detected after coinfection with high levels of the STD virus (Fig. 7). This conclusively shows that although not detectable, DI particles may be present in virus populations.

The small size of DI-3 RNA (about 775 nucleotides) does not appear to affect its incorporation into virus particles. In the serial passage 8 and STD virus coinfection, DI-3 RNA, in comparison to the 50S genome, was present in higher amounts in virions than in the infected cells (Fig. 7). Re and Kingsbury (1988) have hypothesized that the efficiency of Sendai virus DI particle nucleocapsid envelopment decreases progressively when the RNA is smaller than 1600 bases. My results do not support this hypothesis. It is possible that DI-3 RNA is packaged along with larger RNA molecules as has been observed with the multiple packaging of Sendai virus RNAs (Hosaka *et al.*, 1966; Kingsbury *et al.*, 1970). If this is the case, then numerous DI-3 RNA molecules would have to be packaged in a single virus particle since DI-3 RNA is by far the most abundant RNA species in the virions.

Virus from serial passages 8 and 9 interfered, albeit weakly, with the STD virus replication in coinfections. This is not unexpected since the viruses released from passages 8 and 9 contained only low levels of DI RNAs. This interference appears to be genuine since as the inoculum from passages 8 and 9 was reduced, the virus titer increased. Interference is unlikely to have been due to a high titer virus inoculum as a result of

mixture of the two virus populations since the infectious virus titers of passages 8 and 9 were too low to have a marked effect on the MOI of infection.

Genetic structures of HPIV3 DI particles

As mentioned above a common feature of all Sendai virus and VSV DI particle RNAs analyzed to date is that they have conserved the 5'-terminus of the STD genome. The majority of Sendai virus DI RNA species have extensive internal deletions with conserved terminal sequences (Amesse *et al.*, 1982; Re *et al.*, 1983a). These fusion type DI RNA species have the 3'-proximal sequences of the NP gene fused to the 5'-proximal sequences of the L gene. Another kind of DI RNA that has been described for both Sendai virus and VSV is the copyback or panhandle type (Leppert *et al.*, 1977; Lazzarini *et al.*, 1981; Re *et al.*, 1983a,b). These RNAs have retained the 5'-end of the STD genome but have a 3'-terminus that is the inverse complement of the genomic 5'-terminus. Complex copyback DI RNAs which contain the 3'-terminal sequences of the STD genome, internal to the 3' copyback sequence, have been identified, but rarely. Such is the case for VSV DI RNA LT2 (Keene *et al.*, 1981) and also possibly for Sendai virus DI RNAs 11a and Ha (Re *et al.*, 1983b). On the basis of the hybridization data, it appears that DI-1, DI-2 and DI-3 RNAs have retained the 5'-terminal sequences of the STD genome. However, the HPIV3 DI RNAs do not appear to have retained the 3'-terminus of the STD genome. However, they could possess 3' sequences of a length insufficient to be detected by hybridization. If DI-1 and DI-2 RNAs have retained the 3'-end of the STD genome then the internal deletion would not be as extensive as observed for the fusion

type DI RNAs of Sendai virus (Re *et al.*, 1983a).

DI-3 RNA is unlikely to be of the internal deletion or copyback type, since it could not be reverse transcribed and subsequently amplified by the PCR process using a synthetic oligonucleotide primer that would be compatible with the 3'-end of both types of DI RNAs. This is assuming that the inability to amplify DI-3 RNA was due to the 3'-end sequence and not to an aberrant 5'-end sequence. However, the possibility of mutations at either the 5'- or 3'-ends that would prevent annealing of the oligonucleotide primer cannot be ruled out. If indeed DI-3 RNA does not possess the 3'-end of the STD virus genome or a copyback sequence at its 3'-end then it would be interesting to determine the exact nature of the 3'-end sequence.

All subgenomic RNA molecules analyzed to date from several paramyxoviruses and VSV have part of the L gene deleted. If this is the case for DI-1 and DI-2 RNAs then the genomes would have multiple (at least 2) deletions. On the other hand, if these DI RNAs retain the full L coding sequence then they could potentially synthesize an L protein. The size of these two subgenomic RNAs and the hybridization data suggests that a deletion in L, if present, would be very small.

DI particles have been reported to aid in the establishment of viral persistent infections in cultured cells. To verify this hypothesis an attempt was made to establish HPIV3 persistent infections of LLC-MK_2 cells with both STD virus and virus from serial undiluted passage 8. The establishment and characterization of HPIV3 persistent infections of LLC-MK_2 cells form the basis of the next chapter.

CHAPTER 4: ESTABLISHMENT AND CHARACTERIZATION OF HPIV3 PERSISTENT INFECTIONS OF LLC-MK_2 CELLS

1. INTRODUCTION

In the past decade there has been increased interest in understanding paramyxovirus persistency due to their involvement, or suspected involvement, in several chronic diseases of man. Persistent measles virus infection is responsible for the slowly progressive human neurological disease SSPE. Measles virus and RSV have been implicated in Paget's disease; a chronic bone disorder (Singer and Mills, 1983; Baslé *et al.*, 1986). Furthermore, a significant proportion of human bone marrows have been found to contain antigens to the paramyxoviruses SV5, HPIV1 and HPIV3 (Goswami *et al.*, 1984). The mechanisms underlying these persistent infections are not well understood and much work remains to be done in this field of research.

Several investigators have demonstrated that HPIV3 can persist in humans (Gross *et al.*, 1973; Muchmore *et al.*, 1981). In these investigations persistent HPIV3 shedding was observed in healthy young adults and in older adult patients with chronic pulmonary disease. Persistent infections of cultured cells with HPIV3 have been documented previously (Daniel and Chany, 1962; Hodes *et al.*, 1979; Wechsler *et al.*, 1987). However, none of these studies implicate DI particles in the establishment or maintenance of the persistent infections.

The second objective of my research project was to determine whether DI particles aid in the establishment of HPIV3 persistent infections. DI particles have been shown to be necessary for the establishment of STD VSV persistent infections and to significantly aid in

the establishment of Sendai virus persistent infections (Holland and Villarreal, 1974; Roux and Holland, 1979). The third objective of my project was to determine the factors implicated in the maintenance of the persistent infections. Three principal factors have been implicated in the maintenance of persistent infections: DI particles, *ts* viruses and IFN.

This chapter deals with the role of DI particles in the establishment of HPIV3 persistent infections of cultured cells. A detail characterization of the viral RNA and proteins synthesized in the persistently infected cells and present in virions is presented. The possible factors involved in the maintenance of the persistent infections are discussed.

2. RESULTS

Establishment of persistent infections

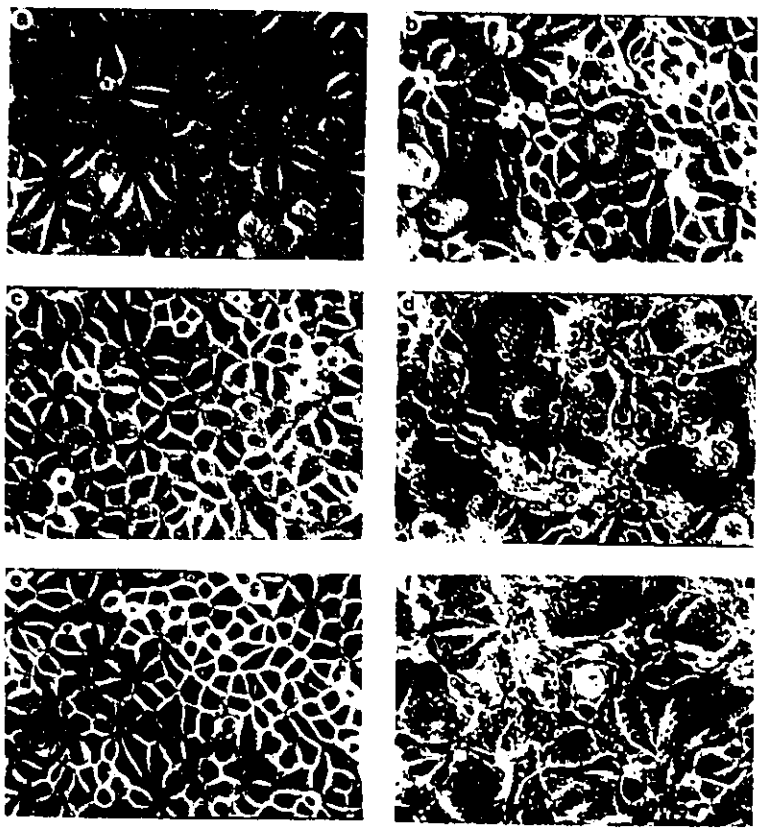
Two independent HPIV3 persistent infections were established in LLC-MK_2 cells. To establish the persistent infections, LLC-MK_2 cells were infected at a MOI of 5 with the STD HPIV3 or at a MOI of 2.5 with HPIV3 that had undergone eight serial undiluted passages (described in chapter 3). In both cases, rounding up of the cells and cell fusion (cytopathic effect) were evident at 48 h postinfection. However, by 5 days postinfection most of the cells that were infected with the STD virus had detached from the surface of the dish and only a few scattered cells remained. On the other hand, the cells that were infected with virus from serial passage 8 had not detached from the plate and the monolayer remained intact. The latter cells were passaged and a persistently infected culture was readily established. This cell culture has been designated PI3/MK_2-P8. The few cells which survived the infection with the STD

virus were propagated by constant feeding. After 10 days (15 days postinfection), a fragile "in crisis" monolayer was obtained. This cell culture has been designated PI3/MK_2-P0. These two cell lines were maintained in culture for 1000 days (33 months) and were passaged 162 times (appendix A).

Biological properties of the persistently infected cells

Early in persistence, the two persistently infected cell cultures were marked by three periods of extensive cytopathology (crisis periods). In the PI3/MK_2-P0 culture the crisis periods occurred at passages 0 to 3 (crisis of 33 days), 11 to 14 (20 days) and 80 to 85 (35 days). In the PI3/MK_2-P8 culture the crisis periods occurred at passages 5 to 8 (33 days), 17 to 28 (72 days) and 52 to 57 (31 days). The crisis periods developed by progressive cell fusion. Severe crisis was characterized by massive cell fusion, presence of vacuolated cells and death of some of the cells. During crisis periods the cells grew very slowly and were loosely attached to the surface of the dish. Between crisis periods and after the last crisis the cells stabilized and were similar to LLC-MK_2 cells except that they were attached more loosely to the surface of the dish, contained some vacuoles and formed more polykaryocytes. Figure 11 illustrates the appearance of LLC-MK_2, STD HPIV3-infected LLC-MK_2, PI3/MK_2-P0 and PI3/MK_2-P8 cells by phase contrast microscopy. The persistently infected cells are shown both in noncrisis and crisis states. Note that the incidence of polykaryocyte formation in crisis periods (Figs. 11D and F) is significantly greater than that seen in a lytic infection (Fig. 11B).

Figure 11. Cytopathic effects induced by acute and persistent HPIV3 infections. (a) Uninfected LLC-MK_2 cells; (b) STD HPIV3-infected LLC-MK_2 cells; (c) PI3/MK_2-P0 cells (passage 92); (d) PI3/MK_2-P0 cells in crisis (passage 80); (e) PI3/MK_2-P8 cells (passage 92); (f) PI3/MK_2-P8 cells in crisis (passage 52). STD virus acutely infected cells were observed for CPE 48 h following infection at a MOI of 3.


a
b
c
d
e
f

The persistently infected cells released less virus (5- to 100-fold as determined by [^{35}S]methionine-labeling of virus particles; Fig. 19) than STD HPIV3 infected cells; the amount of virus released varied with cell passage. The PI3/MK$_2$-P8 culture routinely yielded more virus than the PI3/MK$_2$-P0 culture. Cells from the PI3/MK$_2$-P0 culture were more loosely attached to the surface of the dish than cells of the PI3/MK$_2$-P8 culture.

The number of cells in the persistently infected cultures expressing virus antigens was estimated by hemadsorption. To mediate hemadsorption, the HN protein must be functional and expressed at the surface of infected cells. Figure 12 shows that at least 75% of the cells in both persistently infected cultures were found to express a functional HN at their surface. A similar percentage of cells was found to contain viral antigens by an indirect immunofluorescence assay using HPIV3 rabbit antisera (data not shown).

Involvement of IFN

In several persistently infected cell systems, IFN has been involved in the maintenance of the persistent infections (Meinkoth and Kennedy, 1980; Ramseur and Friedman, 1977). In these systems the cells have been shown to be resistant to superinfection by both homologous and heterologous viruses. In order to determine if IFN played a significant role in the maintenance of the persistent infections, both persistently infected cultures were superinfected with homologous (HPIV3) and heterologous (VSV) viruses. Both the PI3/MK$_2$-P0 and PI3/MK$_2$-P8 cultures were resistant to superinfection with the STD HPIV3 and yielded only small amounts of plaque-forming virus (Table 5). However, they both supported growth of VSV to levels as high as uninfected LLC-MK$_2$ cells.

Figure 12. Hemadsorption assay. (a) uninfected LLC-MK_2 cells, (b) HPIV3-infected LLC-MK_2 cells, (c) PI3/MK_2-P0 cells (passage 124), and (d) PI3/MK_2-P8 cells (passage 124). HPIV3-infected cells were tested for hemadsorption 24 h following infection at a MOI of 1.

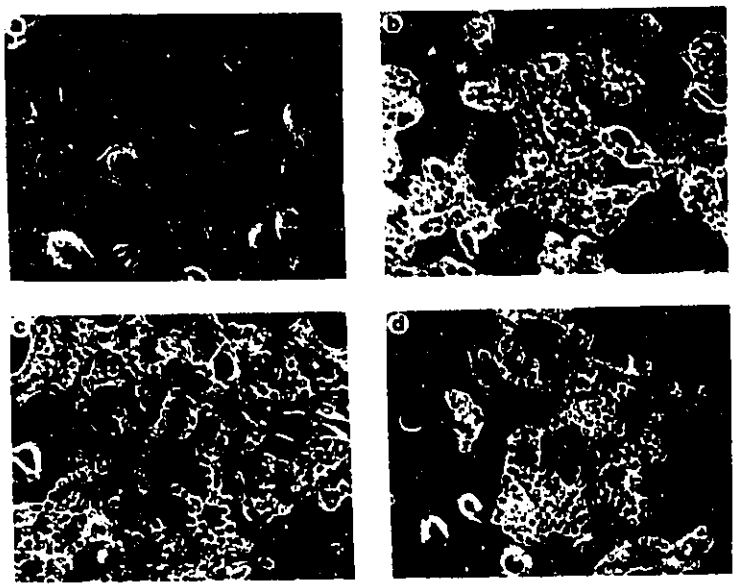

a
b
c
d

TABLE 5

SUSCEPTIBILITY OF HPIV3 PERSISTENTLY INFECTED CELLS TO SUPERINFECTION WITH HPIV3 AND VSV[a]

Cells	HPIV3 (PFU/ml)	VSV (PFU/ml)
LLC-MK_2	3.1×10^8	1.7×10^9
PI3/MK_2-P0		
passage 42	6.0×10^4	2.0×10^9
passage 101	3.7×10^4	2.6×10^9
PI3/MK_2-P8		
passage 38	9.4×10^4	3.1×10^9
passage 101	5.3×10^4	2.1×10^9

[a] LLC-MK_2, PI3/MK_2-P0 and PI3/MK_2-P8 cells were infected with both HPIV3 and VSV at a m.o.i. of 1. After a 1 h adsorption period, cells were washed 3 times with PBS before addition of culture fluid. The culture fluids were harvested at 24 h postinfection and titrated by plaque assay in LLC-MK_2 cells.

This suggests that IFN is not involved in the maintenance of these persistent infections.

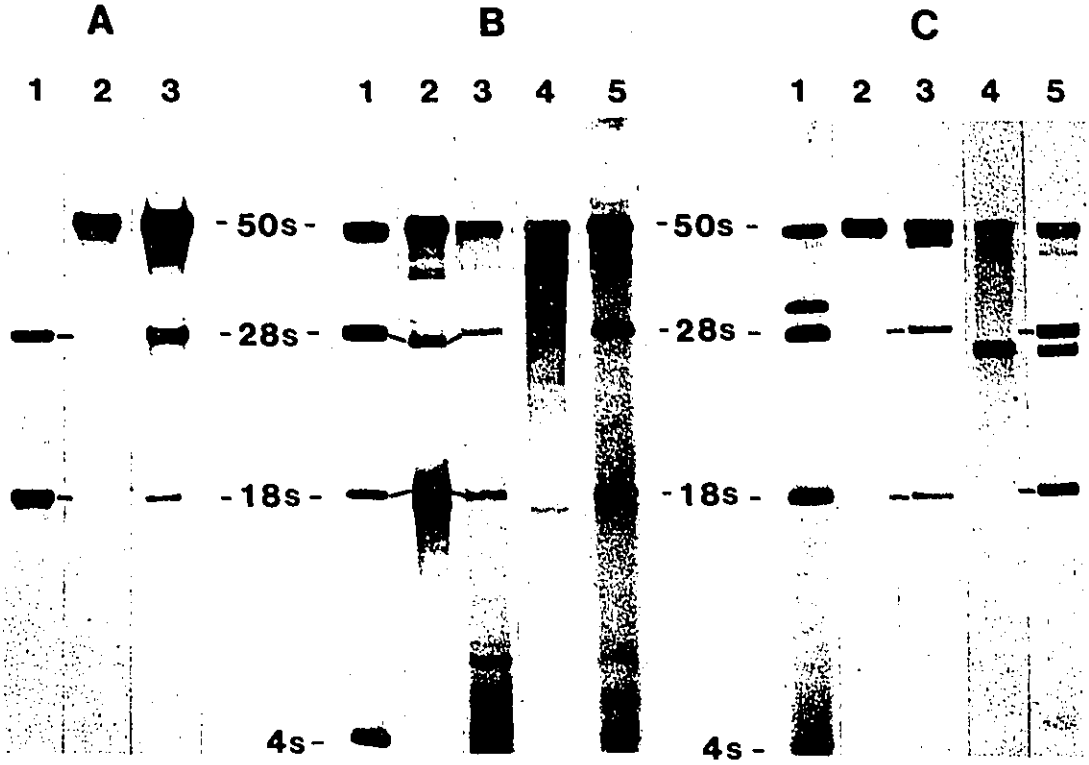
RNA profiles of virus released from the persistently infected cells

DI particles have been shown to be directly involved in the maintenance of persistent infections (Holland and Villarreal, 1974; Kawai *et al.*, 1975). To investigate this possibility in my virus-cell system, the RNA present in virions released by the two persistently infected cultures was analyzed at different times following establishment of persistence. The presence of subgenomic RNAs would be suggestive of DI particle production. In the PI3/MK_2-P8 culture one major species of subgenomic RNA (excluding the 28S and 18S rRNAs) was observed at the cell passages analyzed and the size of the subgenomic RNA species changed with passage of the cells (Fig. 13C). The ratio of subgenomic to 50S RNA molecules varied in these cell passages. Although subgenomic RNAs were present, their levels never markedly exceeded that of 50S RNA.

The virion RNA profile of the PI3/MK_2-P0 culture at similar passages is shown in Fig. 13B. In this cell line a more heterogeneous population of subgenomic RNAs was observed. One subgenomic RNA species of about 17S appeared, disappeared and reappeared at cell passages 89, 100 and 132, respectively. As in the PI3/MK_2-P8 culture the subgenomic RNA levels never greatly exceeded that of 50S RNA. The presence of subgenomic RNAs suggests that both cell lines are producing DI particles.

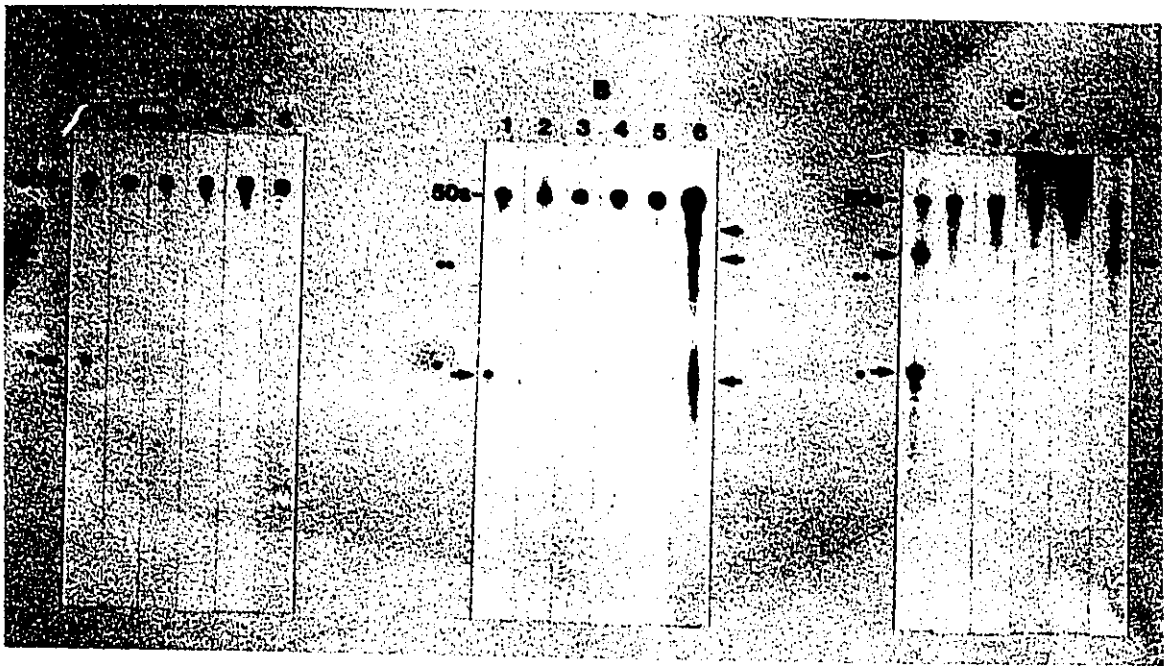
The RNA present in virions was also analyzed by Northern blot hybridization (Figs. 14 and 27). Virion RNAs derived from the STD virus-infected cells, PI3/MK_2-P0 cells (passage 93) and PI3/MK_2-P8 cells (passage 37) were hybridized with cDNA probes (Fig. 8, chapter 1)

Figure 13. RNA profile of virus particles recovered from persistently infected LLC-MK_2 cells at various times following their establishment. Cells were labeled for 24 h with [^{3}H]uridine. Virus particles were concentrated by ultracentrifugation and RNAs were extracted, denatured with glyoxal and separated in a 1% agarose gel. (A) Lane 1, ^{3}H-labeled 28S and 18S ribosomal RNAs; lanes 2 and 3, STD HPIV3 genomic (50S) RNA. Lane 3 is a 2 .times longer exposure of lane 2 revealing the presence of rRNAs. (B) PI3/MK_2-P0 RNA. Lanes 1 to 5, virus particles recovered at cell passages 46, 89, 100, 132 and 145, respectively. (C) PI3/MK_2-P8 RNA. Lanes 1 to 5, virus particles recovered at cell passages 44, 89, 100, 132 and 145, respectively. Cells at passages 132 of (A) and (B) were treated with 1μg/ml of actinomycin C_1 1 h prior to radiolabeling. In certain lanes slight differences exist in the migration of the RNA because some samples were electrophoresed at different times.


A
B
C
1 2 3
1 2 3 4 5
1 2 3 4 5
-50s-
-28s-
-18s-
4s-
-50s-
-28s-
-18s-
4s-

specific for each of the six HPIV3 structural protein genes (Fig. 14). As expected all of the probes hybridized well with the STD-size 50S genome (Fig. 14A). Interestingly, the NP probe hybridized weakly to an additional RNA of about 18S (Fig. 14A, lane 1). Primer extension analysis of the 5'-end of the NP mRNA suggests that there is an RNA species in HPIV3-infected cells that contains NP sequences as well as the leader sequence (M.-J. Côté, 1989, Ph.D. Thesis, University of Ottawa, Ottawa, Ontario). Since the genomic ends of negative-strand RNA viruses are implicated in packaging, it is possible that the additional band detected by the NP mRNA corresponds to such a leader/NP RNA species that would be packaged in virions. Alternatively, this subgenomic RNA could represent a DI particle genome that is derived from the 3'-end of the 50S RNA. A similar hybridization signal was obtained with the NP probe and virion RNA from the PI3/MK_2-P0 culture (passage 93) (Fig. 14B). However, the L probe also hybridized to a similarly migrating subgenomic RNA species. One or both of these signals could correspond to the subgenomic RNA observed in PI3/MK_2-P0 virions (passage 89) migrating just below the 18S rRNA (Fig. 13B, lane 2). The L probe hybridized to two additional slower migrating subgenomic RNA species. In virions of the PI3/MK_2-P8 culture (passage 37) two subgenomic RNAs were detected (Fig. 14C). One RNA species hybridized with the NP and L probes whereas the other RNA species hybridized only with the NP probe. The latter RNA species is a major RNA packaged by the virions and is less likely to correspond to the putative leader/NP RNA species. In addition, this subgenomic RNA species appears to migrate slightly slower than the putative leader/NP RNA species. Note that a subgenomic RNA species migrating to a similar position as the largest of these two subgenomic RNAs was observed at

Figure 14. Analysis of genomic RNAs of virions recovered from the persistently infected cells by Northern blot hybridization. Virus particles were concentrated by ultracentrifugation and RNAs were extracted, denatured with glyoxal, electrophoresed in 1% agarose gels, and transferred onto GeneScreen Plus (NEN). The blots of (A) STD HPIV3 RNA, (B) PI3/MK_2-P0 RNA (passage 93), and (C) PI3/MK_2-P8 RNA (passage 37) were cut into 4 mm-wide longitudinal strips and hybridized with individually nick-translated plasmids: pPI47 (NP) (lane 1); pPI28 (P+C) (lane 2); pPI3 (M) (lane 3); pPI14 (F) (lane 4); pPI10 (HN) (lane 5); mpPIg22 (L) (lane 6) (Dimock et al., 1987). The arrows indicate putative DI RNA species. The dots on the right side of lane 1 in each blot indicate the positions of the 28S (two dots) and 18S (one dot) rRNAs.

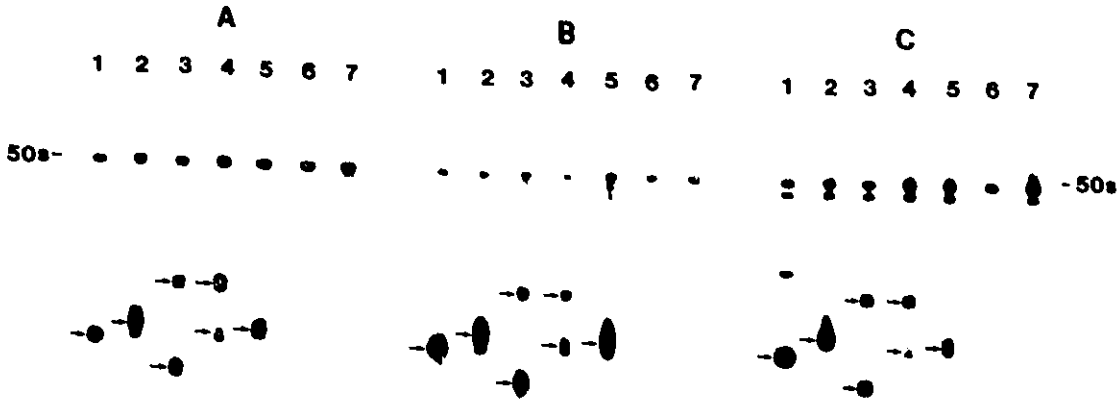

B
1 2 3 4 5 6
50
C

cell passage 44 in PI3/MK_2-P8 virions (Fig. 13C, lane 1). In PI3/MK_2-P8 virions of cell passage 145, three subgenomic RNA species were detected (Fig. 27, chapter 5). Two of these subgenomic RNAs (b and c) hybridized distinctively only with the NP probe. The other subgenomic RNA (a) hybridized with all of the probes except the L probe. The sizes of RNAs b and c suggest that they contain either repeats of NP sequences or additional non-HPIV3-specific sequences.

Viral-specific RNAs in persistently infected cells

Northern blot analysis was further used to determine whether the transcriptional pattern of viral-specific mRNAs had been altered during the course of persistence. Figure 15 shows that the persistently infected cells from both cultures had similar mRNA profiles to that observed in STD HPIV3-infected cells. This suggests that transcription proceeds in a regular fashion at these cell passages. The hybridization data also show that the subgenomic RNA slightly smaller than the 50S genome in the PI3/MK_2-P8 culture at cell passage 104 (Fig. 15C) hybridized with all probes except probe mpPIg22 (Fig. 15C, lane 6). This indicates that the 3'-end of the L gene (negative-strand) has been deleted in this subgenomic RNA since clone mpPIg22 represents only the first 2000 nucleotides of the L gene while clone pGEM3-L (lane 7) represents the entire L gene. Note that a subgenomic RNA with a similar electrophoretic mobility was observed in virions of cell passage 100 (Fig. 13C, lane 3). The NP probe also detected a subgenomic RNA species of about 26S. A similarly migrating subgenomic RNA was observed at cell passages 132 and 145 (Fig. 13C, lanes 4 and 5, respectively). This subgenomic RNA at cell passage 145 was also found to solely hybridize with the NP probe (Fig.

Figure 15. Northern blot analysis of viral RNA species synthesized in persistently infected cells. Total cytoplasmic RNA was isolated from actinomycin C_1-treated cells (1 μg/ml), denatured with glyoxal, separated in a 1% agarose gel and transferred onto GeneScreen Plus (NEN). The blots were cut into 4 mm-wide longitudinal strips and hybridized with individually nick-translated plasmids: pPI47 (NP) (lane 1); pPI28 (P/C) (lane 2); pPI3 (M) (lane 3); pPI14 (F) (lane 4); pPI10 (HN) (lane 5); mpPIg22 (L) (lane 6); pGEM3-L (L) (lane 7). (A) STD HPIV3 infected LLC-MK_2 cells (~15 μg/lane), (B) PI3/MK_2-P0 cells (passage 101; ~20 μg/lane) and (C) PI3/MK_2-P8 cells (passage 104; ~10 μg/lane). Arrows indicate mRNA species.


A
B
C
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
50s
50s

27, chapter 5). These subgenomic RNAs may represent the genomes of DI particles.

Intracellular viral-specific and virion structural proteins

During establishment and maintenance of the persistent state virus genomes undergo a variety of mutations followed by selection of viruses with altered biological activities. These biological modifications are frequently accompanied by alterations in the structural polypeptides. Since the virus recovered from both persistently infected cultures is noncytocidal (discussed below), it was of interest to determine whether any of the viral polypeptides had been altered. For this purpose the intracellular viral proteins were analyzed by both immunoprecipitation and Western blotting. Figure 16 shows the intracellular proteins analyzed by immunoprecipitation. The viral proteins from both persistently infected cultures of cell passage 103 had similar electrophoretic mobilities to those synthesized in STD HPIV3-infected cells. Due to the short intracellular half-life of the M protein, the M protein was detectable only after longer exposure times (data not shown). The amount of L protein, recovered from the immunoprecipitates, in the persistently infected cells was greatly reduced compared to the other viral proteins.

These findings were confirmed by Western blot analysis of proteins synthesized at cell passage 105 (Fig. 17B). In this case the L protein in STD virions was not detected by the antisera. This is probably reflected by the fact that the amount of cell lysate used in the blot analysis was significantly less than in the immunoprecipitation.

The proteins of virions released by both persistently infected cultures (cell passage 109) also migrated to similar positions as those of STD virus

Figure 16. Polyacrylamide gel analysis of viral proteins recovered from HPIV3 acutely and persistently infected LLC-MK_2 cells. Cells were labeled for 2 h with [^{35}S]methionine. Cell extracts of either 100 μl, 200 μl or 300 μl were subjected to immunoprecipitation with rabbit anti-HPIV3 serum and the immunoprecipitates were resolved by SDS-PAGE. Lane 1, STD virion proteins; lane 2, uninfected cells (100 μl); lane 3, STD HPIV3 infected cells (100 μl); lane 4, PI3/MK_2-P0 cells (passage 103, 300 μl); lane 5, PI3/MK_2-P8 cells (passage 103, 200 μl). Lanes 4 and 5 were exposed 4X longer than lanes 2 and 3.

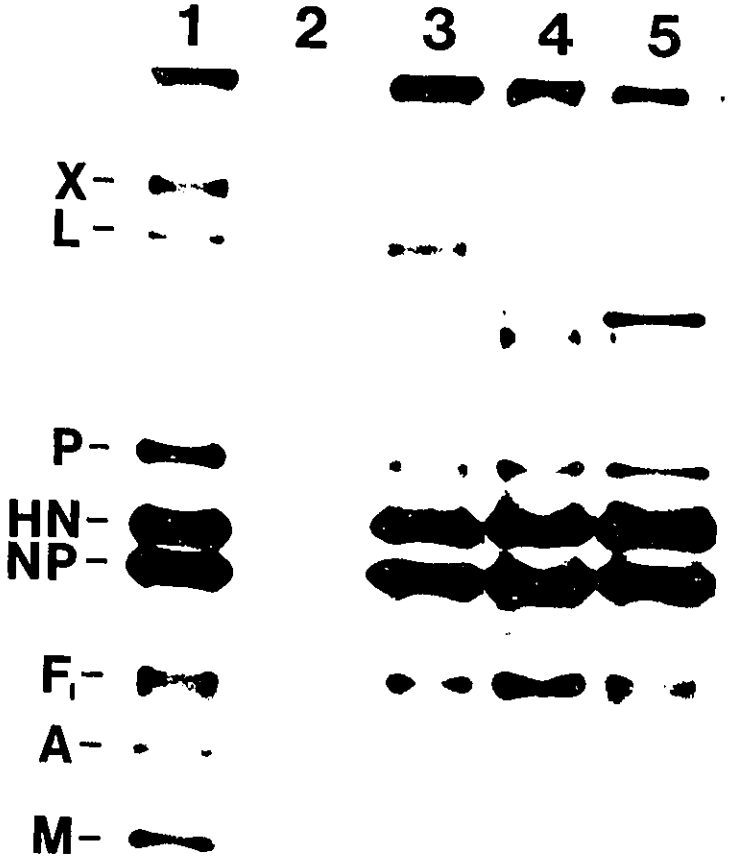
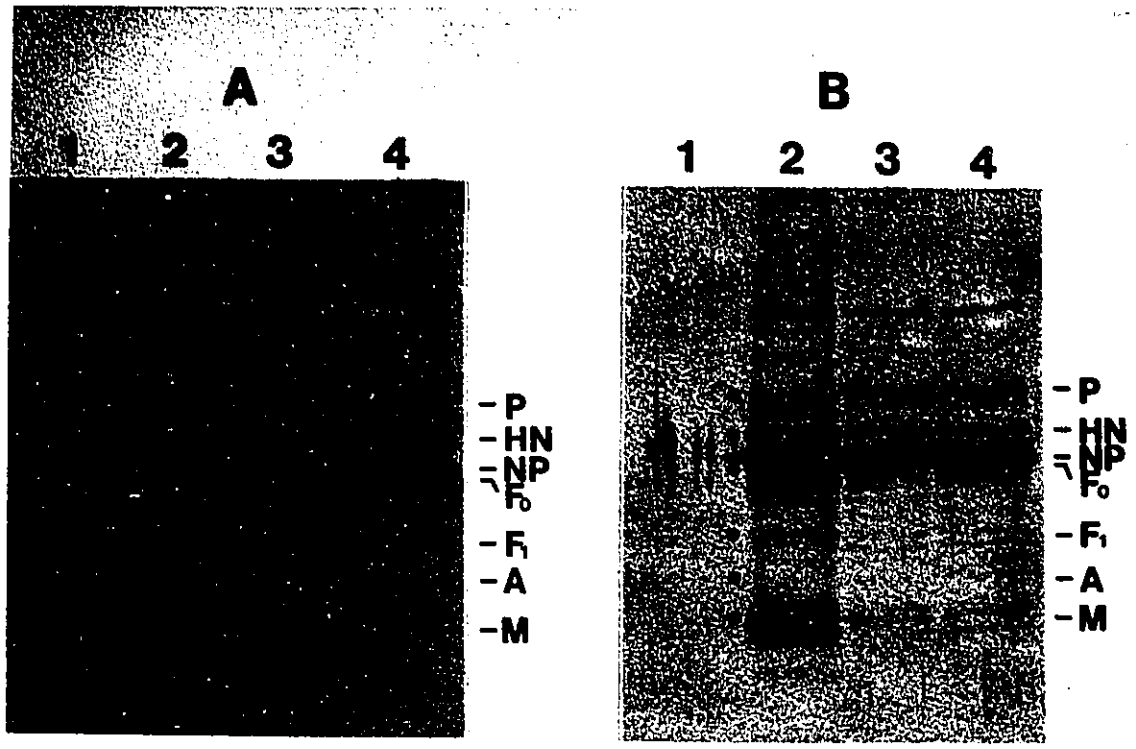

1
2
3
4
5
X-
L-
P-
HN-
NP-
F1-
A-
M-

Figure 17. Western blot analysis of virion-associated and intracellular proteins recovered from HPIV3 acutely and persistently infected cells. Extracellular virions (A) and total cell extracts (B) were separated by SDS-PAGE and electroblotted onto nitrocellulose for immunological detection. In both (A) and (B): lane 1, uninfected LLC-MK_2 cells; lane 2, STD HPIV3-infected LLC-MK_2 cells; lane 3, PI3/MK_2-P0 cells (passage 105); lane 4, PI3/MK_2-P8 cells (passage 105).


A
1
2
3
4
P
HN
NP
F0
F1
A
M
B
1
2
3
4
P
HN
NP
F0
F1
A
M

particles (Fig. 18A). This finding was also confirmed by Western blot analysis of virion proteins of cell passage 105 (Fig. 17A).

To determine whether alterations in the viral proteins occurred in later passages, the virion proteins were analyzed at cell passages 131, 144 and 157. For all three cell passages, the virion structural protein pattern was similar to that of the STD virus particles (Fig. 19). Therefore, no apparent differences were observed in the molecular size of the virion structural polypeptides at all cell passages analyzed.

To further analyze the HN and F glycoproteins of virions, the proteins were labeled with [^{3}H]glucosamine (Fig. 18B). Virions from the PI3/MK$_2$-P8 culture at cell passage 108 contained little of the uncleaved inactive-precursor fusion protein (F_0) compared to both STD virions and virions from the PI3/MK$_2$-P0 culture at cell passage 108. Analysis of [^{3}H]glucosamine-labeled virion proteins of both persistently infected cell lines at passage 115 revealed a similar glycoprotein profile (data not shown).

In all of the virion protein preparations a protein of high molecular weight (designated X) was consistently observed. Protein X was detected in the culture fluid of uninfected LLC-MK$_2$ cells labeled either with [^{35}S]methionine (Fig. 19, lane 9) or [^{3}H]glucosamine (Fig. 18B, lane 4). Since no other major proteins were detected from the culture fluid of uninfected cells, this protein is unlikely to have originated from mycoplasma or from other contaminants in the cell culture. Thus, this protein is likely to be a product of LLC-MK$_2$ cells which is pelletable under the centrifugation conditions.

Figure 18. SDS-PAGE analysis of virions recovered from the persistently infected cells. (A) Cell cultures were treated with 1 μg/ml of actinomycin C_1 for 24 h and proteins were then labeled with [^{35}S]methionine for 24 h. The persistently infected cultures were of passage 109. (B) Virion proteins were labeled with [^{3}H]glucosamine hydrochloride. The persistently infected cultures were of passage 108. The labeled proteins were isolated and analyzed in 10% polyacrylamide gels containing SDS as previously described (Storey et al., 1984). Lanes: 1 (A and B), STD HPIV3; 2 (A and B), PI3/MK_2-P0; 3 (A and B), PI3/MK_2-P8; 4 (B only), culture fluid from uninfected LLC-MK_2 cells.

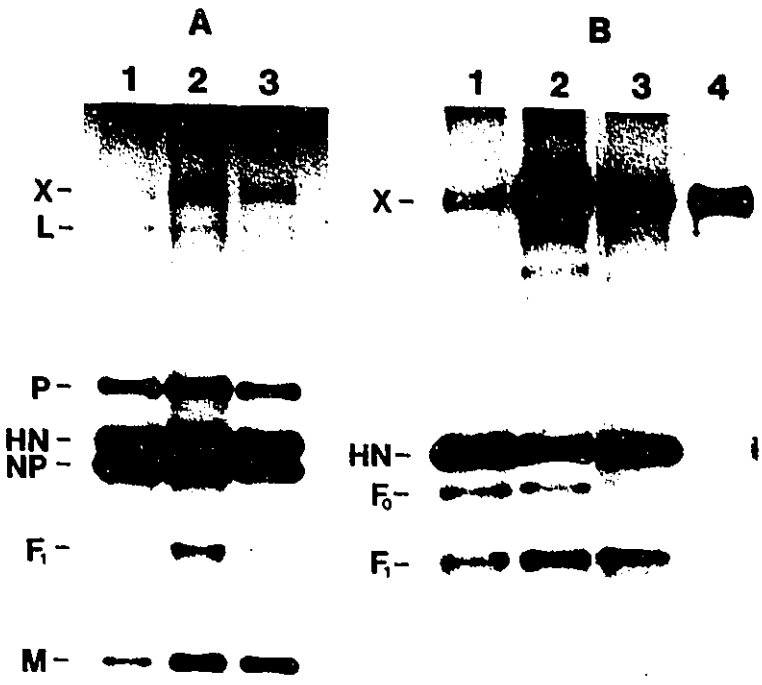
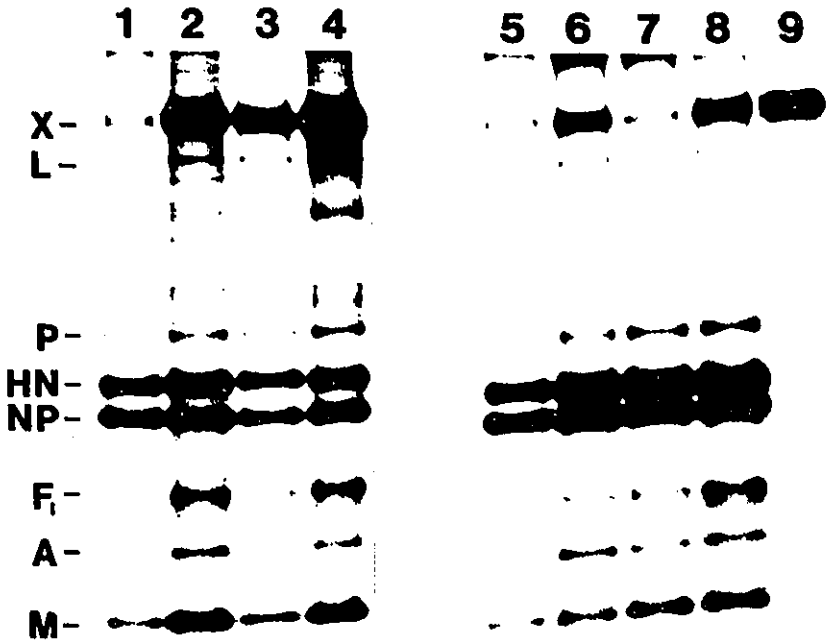

A
1
2
3
X-
L-
P-
HN-
NP-
F1-
M-
B
1
2
3
4
X-
HN-
F0-
F1-

Figure 19. Protein analysis of virus particles recovered from the persistently infected cells. Cells were labeled for 24 h with [^{35}S]methionine. Virus particles were concentrated by ultracentrifugation, resuspended in 50 μl sample buffer, and analyzed by SDS-PAGE. Lanes 1 (0.25 μl) and 5 (0.25 μl), STD HPIV3; lanes 2 (11 μl), 3 (22 μl) and 4 (10 μl), PI3/MK$_2$-P0 virions recovered at cell passages 131, 144 and 157, respectively; lanes 6 (2 μl), 7 (2 μl) and 8 (10 μl), PI3/MK$_2$-P8 virions recovered at cell passages 131, 144, and 157, respectively; lane 9 (5 μl), culture fluid from uninfected LLC-MK$_2$ cells.

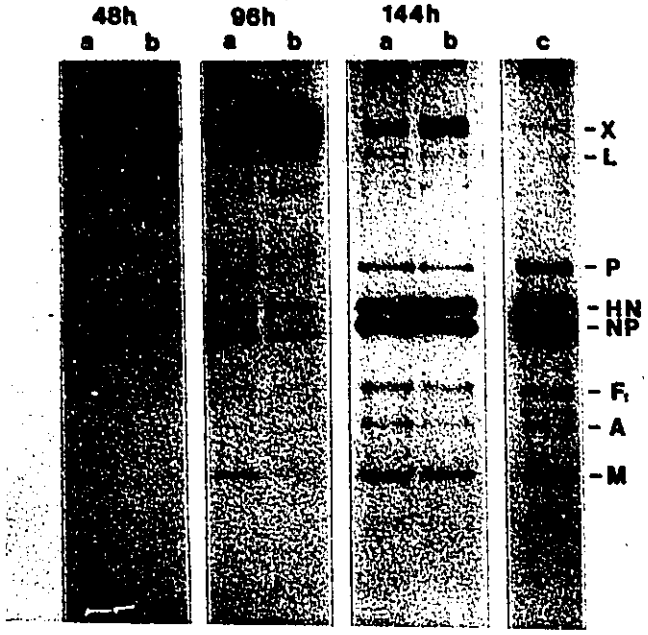

1
2
3
4
5
6
7
8
9
X-
L-
P-
HN-
NP-
F1-
A-
M-

Biological properties of the virus released from the persistently infected cells

The virus released from the two persistently infected cultures was noncytolytic for LLC-MK_2 cells. Culture fluids assayed for plaque-forming virus in LLC-MK_2 cells throughout the course of the persistent infections were always negative (appendix B). Similarly, the PI3/MK_2-P0 and PI3/MK_2-P8 viruses did not form plaques in CV-1 and HEp-2 cells; two cell lines permissive for STD HPIV3 plaque formation. Plaque assays performed at 32°C or by doubling the incubation time were also negative. It was therefore of interest to determine if the virus released from the persistently infected cultures was infectious. To answer this, LLC-MK_2 cells were infected with culture medium recovered from both persistently infected cell lines at cell passage 111. Culture fluids from the infected LLC-MK_2 cells were harvested at 48, 96 and 144 h postinfection. Analysis of the virion proteins by SDS-PAGE (Fig. 20) showed that by 48 h virus production was minimal, since no viral-specific proteins were detected. However, by 96 h low levels of virus production were detected and by 144 h virus production was approaching levels obtained with that of the STD virus-infected cells. These results indicate that the virus released from both persistently infected cultures was infectious.

In an attempt to determine if the nonplaque-forming property could be reversed, virus from both cell lines at cell passages 45, 100, 111 and 131 were passaged once in LLC-MK_2 cells and the progeny virus was plaque assayed in LLC-MK_2 cells. In this manner, plaques were obtained with PI3/MK_2-P0 virus of cell passages 100 and 131 only. When propagated the plaqued-purified viruses grew to titers equivalent to that of the STD virus.

Figure 20. Infectivity of virus released from the persistently infected cells. LLC-MK_2 cells were infected with 1 ml of culture fluid recovered from each persistently infected cell line (passage 111) and analyzed for the production of extracellular virus at the indicated times. Viral proteins were labeled with [^{35}S]methionine 24 h prior to harvest and resolved by SDS-PAGE. Lane a, PI3/MK_2-P0 virus-infected LLC-MK_2 cells; lane b, PI3/MK_2-P8 virus-infected LLC-MK_2 cells; lane c, STD virus-infected LLC-MK_2 cells. Lanes a and b of the 48 h and 96 h harvests were loaded with pelleted material from four-fifths of a plate. Lanes a and b of the 144 h harvest were loaded with pelleted material from one-sixth of a plate. Lane c represents pelleted material from one-twenty-fifth of a plate. LLC-MK_2 cells were infected with STD HPIV3 at a MOI of 3 and incubated for 48 h.


48h
a
b
96h
a
b
144h
a
b
c
X
L
P
HN
NP
F1
A
M

The ability of virus released from the persistently infected cultures at different cell passages to interfere with STD HPIV3 production was examined (Table 6). Virus from the PI3/MK_2-P8 culture strongly interfered with STD virus replication at all three cell passages tested (45, 100 and 131). This interference was most striking with virus of cell passage 131. Note that a subgenomic RNA species was detected in all three passages and that the ratio of subgenomic/50S RNA molecules appears to be highest at cell passage 132 (Fig. 13). This strong interference could be mediated, at least in part, by the subgenomic RNAs which may well represent DI particle genomes. In contrast, virus from the PI3/MK_2-P0 culture of cell passages 100 and 131 did not strongly interfere with STD virus production. However, the interference activity with virus of cell passage 47 was much stronger. Virions of cell passage 46 possessed a strong 50S genome band with no detectable subgenomic RNA species (Fig. 13B, lane 1). To rule out the possibility of the presence of subgenomic RNAs comigrating with the 28S and 18S rRNAs, virion genomic RNA from actinomycin C_1 treated cells was analyzed. Only 50S genomic RNA was detected. No subgenomic RNA species were observed (data not shown). From this data, the possibility that the noncytocidal virus containing a 50S RNA genome has interference activity cannot be excluded. Finally, the PI3/MK_2-P0 and PI3/MK_2-P8 viruses in all mixed infections protected the cells from STD virus destruction.

Involvement of *ts* viruses

Since there have been several reports (Preble and Youngner, 1973; Youngner *et al.*, 1976) describing the selection of *ts* viruses during persistence, experiments were carried out to determine if such a selection

TABLE 6

INTERFERENCE OF VIRUS RELEASED FROM HPIV3 PERSISTENTLY INFECTED CELLS WITH STD VIRUS REPLICATION[a]

	Cell passages		
	45 (PI3/MK_2-P0) 47 (PI3/MK_2-P8)	100	131
Virus	titer (PFU/ml)	titer (PFU/ml)	titer (PFU/ml)
HPIV3 alone	4.4×10^8 (0)[b]	1.0×10^9 (0)	8.7×10^8 (0)
HPIV3 + PI3/MK_2-P0	1.7×10^6 (2.4)	2.0×10^8 (0.7)	1.3×10^8 (0.8)
HPIV3 + PI3/MK_2-P8	1.8×10^6 (2.4)	1.9×10^6 (2.7)	4.9×10^4 (4.2)

[a] LLC-MK_2 cells were infected with a mixture of STD HPIV3 (0.01 ml, MOI = 1) and PI3/MK_2-P0 (1 ml) or PI3/MK_2-P8 (1 ml) culture fluids. The culture fluids from the mixed infections were harvested at 48 h postinfection and titrated by plaque assay.

[b] Log reduction

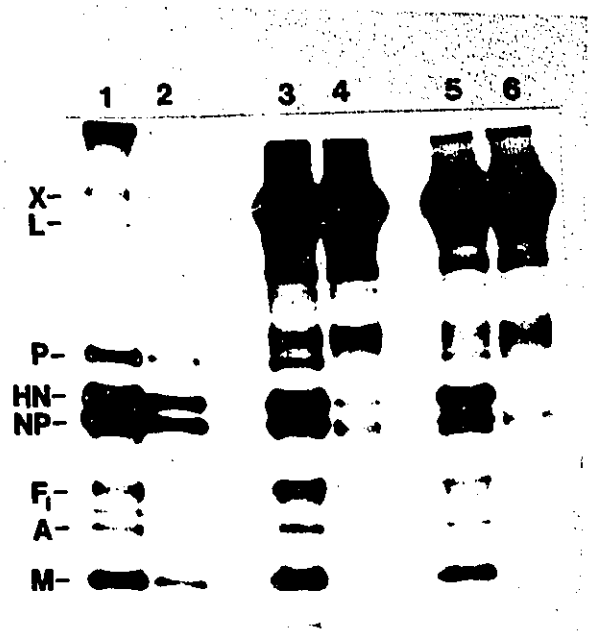
had occurred in the two persistently infected cell lines. The persistently infected cultures, as well as STD virus-infected cells, were incubated at 37°C or 40°C in the presence of [^{35}S]methionine. After a 48 h incubation period, the culture fluids were harvested and virion proteins analyzed by SDS-PAGE (Fig. 21). Virus production from both the STD virus-infected cells and the two persistently infected cell lines was reduced when the cultures were incubated at 40°C. However, this reduction appeared to be somewhat greater for the persistently infected cells. Therefore, the viruses released from the persistently infected cultures are nonplaque-forming mutants which appear to be slightly *ts*.

3. DISCUSSION

Role of DI particles in the establishment of HPIV3 persistent infections

Paramyxovirus infections of cultured cells usually result in death of the infected cells. Persistent infections have been established from such acute virus infections by cultivation of the few surviving cells (described in chapter 1). However, if the virus is subjected to serial undiluted passage, which favors DI particle enrichment, persistent infections are more easily established. In this project a similar phenomenon was observed with HPIV3. A HPIV3 persistent infection of LLC-MK$_2$ cells was readily established with virus that had undergone eight serial undiluted passages. The serial undiluted passage virus was shown to contain low levels of DI particles (chapter 3). In contrast, infection with STD HPIV3 resulted in almost complete destruction of the infected cells. These findings are consistent with the idea that the DI particles present in the serial passage 8 virus inhibited the STD virus replication of a magnitude

Figure 21. SDS-PAGE analysis of virions produced at 37°C and 40°C. Persistently infected cultures, normally maintained at 37°C, were incubated for 48 h at 40°C. LLC-MK_2 cell cultures were infected at a MOI of 3 with STD HPIV3. After a 1 h absorption period at 37°C, the cell cultures were incubated for 48 h at 37°C and 40°C. Cell cultures were labeled with [^{35}S]methionine 24 h prior to harvest. (A) STD HPIV3; (B) PI3/MK_2-P0 (passage 46); (C) PI3/MK_2-P8 (passage 44). For each type of infection, both lanes (37°C and 40°C) were loaded with an equal amount of pelleted material.


1
2
3
4
5
6
X-
L-
P-
HN-
NP-
F1-
A-
M-

sufficient to restrict the cytolytic activity of the STD virus. My findings are similar to those of Roux and Holland (1979) with Sendai virus. Infection of BHK21 cells with STD virus or a mixture of STD virus plus DI particles of Sendai virus resulted in cytopathic effects similar to those I have observed.

There are three possibilities that could explain why some cells survived the STD virus infection. First, DI particles could have been involved. Since it is likely that the STD virus stock was not free of DI particles, infection with STD virus may have resulted in some cells being coinfected with a STD virus particle and a DI particle. Secondly, a subpopulation of the cells may have been resistant to HPIV3-mediated cell killing. Finally, some of the cells could have been infected with a noncytocidal mutant of HPIV3 present in the STD virus stock as a subpopulation.

Persistent infections with HPIV3 in cultured cells have been described previously. Daniel and Chany (1962) established a HPIV3 (strain EA 102) persistent infection of KB cells by propagating the cells which survived the infection. Hodes *et al.* (1979) observed that Vero cells inoculated with undiluted passage HPIV3 (Mills strain) at a MOI of 10 did not develop CPE and readily became persistently infected. Finally, Wechsler and coworkers (1987) reported that HPIV3 (ATCC strain C-243) infection of CV-1 cells at a relatively high MOI (10 PFU/ml) resulted in the immediate establishment of a persistent infection. At a low MOI (0.01 to 1 PFU/ml) most of the cells were destroyed within 5 days. The results of Daniel and Chany (1962) and Hodes *et al.* (1979) are compatible with my findings. However, Hodes *et al.* (1979) did not implicate DI particles in the establishment of the persistent infection, but to a soluble inhibitor of

syncytium formation. This hypothesis was later withdrawn (Hodes, 1982). Wechsler and coworkers (1987) were unable to detect DI particles in their virus stock. However, the finding that a persistent infection could only be established at relatively high MOI suggests the involvement of DI particles.

Synthesis of subgenomic RNAs during HPIV3 persistence

Although HPIV3 persistent infections of cultured cells have been previously documented, DI particles were not detected during the course of the persistent infections. In the two LLC-MK_2 cell lines persistently infected with HPIV3 subgenomic RNAs (putative DI particle genomes) were detected in virions. In the PI3/MK_2-P8 culture the subgenomic RNA population consisted of one major species which varied in size with passage of the cells. The ratio of subgenomic to 50S RNA molecules also varied at each cell passage indicating a possible cycle of production. The cyclic production of STD and DI virus particles has been implicated as the mechanism of maintenance in a rabies virus persistent infection of BHK cells (Kawai *et al.*, 1975). In the PI3/MK_2-P0 culture the subgenomic RNAs existed as a more heterogeneous population and the ratio of subgenomic to 50S RNA molecules also varied with cell passage.

During the course of these persistent infections the subgenomic RNA levels in virions never greatly exceeded that of 50S RNA. This differs from the Sendai virus persistent infections of BHK21 cells reported by Roux and Holland (1980) where subgenomic RNAs were at all times synthesized in 10- to 340-fold greater molar amounts than 50S RNA. This difference could be explained in several ways. First, BHK21 cells may support synthesis of subgenomic RNAs better than LLC-MK_2 cells.

Secondly, the subgenomic RNA in their Sendai-BHK21 cultures appear to be the factor responsible for the maintenance of the persistent state while in the HPIV3 system it is not clear whether subgenomic RNAs are solely responsible for the persistence. Finally, as previously mentioned in chapter 3, HPIV3 may not be able to support replication of high levels of subgenomic RNAs.

Cyclic appearance of cytopathology was observed in the initial stages of persistence. Although the virion RNA was not analyzed in times of crisis, RNA profiles were obtained between crisis periods. For both cultures, very low levels of subgenomic RNAs were observed in times of minimal cytopathology (PI3/MK_2-P0, passage 46; PI3/MK_2-P8, passage 89).

The hybridization data obtained during the course of the persistent infections revealed the generation of various types of subgenomic RNAs. Nucleotide sequence analysis is required before one can say for sure that a particular sequence has been retained or lost. However, hybridization analysis as performed in my experiments provides a good representation of the sequences that have been retained or deleted. DI particle genomes of the internal deletion class were observed during the course of the persistent infections. The extent of the internal gene deletions varied. For example, one subgenomic RNA species retained only NP and L gene coding sequences; the P/C, M, F, and HN gene sequences being deleted (Fig. 14C). This DI particle-size genome can be classified as a fusion type DI RNA. A second internally deleted subgenomic RNA had only the sequences mapping at the 3'-end of the L gene (negative-strand) deleted (Fig. 15C). Another subgenomic RNA hybridized with all probes except the full-length L probe (Fig. 27B, subgenomic RNA (a)). However, this subgenomic RNA is likely to have retained the STD-size genomic

5'-termini since all subgenomic RNAs of nonsegmented negative-strand RNA viruses analyzed to date have retained the 5'-termini of the genome from which they were generated. The latter two subgenomic RNAs appear to be similar in genetic content to the well-characterized DI-LT' of VSV (Perrault and Semler, 1979; Epstein *et al.*, 1980). The DI-LT genome contains genetic information from the 3'-end of the STD VSV genome and has L gene sequences deleted. DI-LT is transcriptionally active yielding the positive-strand leader RNA and four functional mRNAs (Colonno *et al.*, 1977; Johnson and Lazzarini, 1977). DI RNA genomes containing genetic information from each of the viral genes have been reported, but rarely (Kang *et al.*, 1985). Some subgenomic RNAs were found to hybridize to L gene sequences only (Fig. 14B). Such subgenomic RNAs could be of the panhandle or snapback types. Alternatively, they could be of the internal deletion type with 3'-end sequences of a length insufficient to be detected by hybridization. Finally, the remaining subgenomic RNAs were found to hybridize to NP gene sequences only. Interestingly, the size of some of these subgenomic RNAs cannot be accounted for by only one copy of NP gene sequences (Fig. 15C and 27B). The data suggests that these subgenomic RNAs contain repeats of NP gene sequences. One subgenomic RNA hybridizing solely to the NP probe was of the NP gene size (Fig. 14C). The structure of this subgenomic RNA is also interesting since it appears not to have retained the 5'-end of the 50S genome. If it contains 5'-end sequences, these are of a length insufficient to be detected by hybridization.

Virus released from the PI3/MK_2-P0 culture early in persistence (cell passage 47) strongly interfered with the STD virus production. These virus particles contained only minor amounts of subgenomic RNAs

indicating that the interference observed was mediated by virus with the 50S RNA. Yoshida *et al.* (1982) have shown that 50S RNA recovered from a Sendai-LLC-MK_2 persistent infection could strongly interfere with STD Sendai virus replication in a mixed infection. Thus, it is not possible to attribute interference activity with virus recovered from persistent infections to the presence of subgenomic RNAs without physically separating subgenomic RNA-containing particles from STD-size genome-containing particles.

Synthesis of viral proteins during HPIV3 persistence

During long-term persistent infections it is not unexpected to find viral proteins with altered electrophoretic mobilities. In several paramyxovirus persistent infections the M protein has been found to be altered (Rozenblatt *et al.*, 1979; Wechsler *et al.*, 1987). However, all viral proteins synthesized in my two persistently infected cell lines continuously displayed similar electrophoretic mobilities to those synthesized in the STD virus-infected cells. Wild *et al.* (1981) also observed no apparent changes in the molecular size of viral proteins synthesized in a measles virus persistent infection. There is evidence suggesting that virus genomes undergo extensive genetic change during long-term persistent infections (Holland *et al.*, 1979). The genetic change undergone by the virus in my two persistently infected cultures has not resulted in proteins with altered electrophoretic mobilities.

In the persistently infected cultures of cell passage 103 it was observed by immunoprecipitation that the L protein was reduced in amount relative to the other viral proteins. However, in the PI3/MK_2-P8 (passage 104) and PI3/MK_2-P0 (passage 101) cultures the viral mRNA

patterns were normal indicating the presence of a functional L protein.

The virions released from the persistently infected cultures appeared to contain slightly less NP protein relative to the HN protein (and possibly to the other viral proteins) in comparison to STD virus particles. This was especially the case for virions of the PI3/MK_2-P8 culture (Figs. 19 and 21). Indeed, virions from the PI3/MK_2-P8 culture at cell passage 44 contained a marked decrease in NP protein (Fig. 21, lane 5). The significance of this decrease in NP protein is not clear. It is possible that mutations in the NP gene sequence as those observed in the HPIV3 RNA recovered from cell passage 146 are involved (see chapter 5).

Virions from the PI3/MK_2-P8 culture at cell passage 108 and 115 were observed to contain little of the uncleaved inactive precursor fusion protein (F_0) compared to both STD virions and virions from the PI3/MK_2-P0 culture. However, this decrease did not appear to be correlated with an increase in F_1. The data suggests that F protein synthesis is reduced and that the F protein synthesized is successfully processed to the active form (F_{12}). The five CV-1 cell lines persistently infected with HPIV3 were also found to produce virions containing a lower fraction of uncleaved F_0 compared to the STD virus (Wechsler *et al.*, 1987). However, these virions contained a higher fraction of cleaved F_1 compared to STD virions. This high level of active protein may explain the ability of these cells to fuse rapidly with uninfected cells. Such fusion activity was not observed when uninfected LLC-MK_2 cells were added to PI3/MK_2-P0 or PI3/MK_2-P8 cells.

The virus released from both persistently infected cell lines is noncytocidal. Analysis of the virion proteins did not reveal any abnormalities that could account for this phenomenon. UV inactivation

studies have implicated the VSV leader RNA as the molecule responsible for the shut-off of host macromolecular synthesis (Weck *et al.*, 1979; McGowan and Wagner, 1981; Dunigan and Lucas-Lenard, 1983). However, it was shown that the dose of UV-irradiation required to inactivate the VSV leader RNA also altered the L and NS proteins of VSV (Whitaker-Dowling and Youngner, 1988). Thus, any of these products could be responsible for the inhibition of host cell macromolecular synthesis and concomitant cell death.

Mechanism(s) of maintenance of the persistent infections

The mechanism of maintenance of HPIV3 persistent infections is not understood at the present time. The subgenomic RNAs and/or noncytocidal viruses must be considered as responsible for the maintenance of the persistent infections. The subgenomic RNAs are present in low amounts and are not amplified to levels significantly exceeding that of the 50S genome. It is possible that a low level of subgenomic RNA (putative DI particle genome) is sufficient to prevent cell death and maintenance of HPIV3 persistence. In chapter 3 it was shown that low levels of subgenomic RNAs in serial undiluted passage 8 virus prevented cell death and readily led to establishment of the persistent state. Furthermore, the development of severe cytopathology or crisis periods is characteristic of persistent infections maintained by DI particles (Holland and Villarreal, 1974; Roux and Holland, 1979; Yoshida *et al.*, 1982).

In the initial stages of the persistent infections a noncytocidal mutant virus was selected. It is possible that the selection of this noncytocidal mutant, showing slight temperature-sensitivity, was necessary for maintenance of the persistent infections. The rapid selection of *ts*

mutants in NDV and VSV persistent infections of L cells led the authors to conclude that the *ts* phenotype was required for the maintenance of the persistent infections (Preble and Youngner, 1972; Youngner *et al.*, 1976). It is also known that most viruses recovered from persistent infections, in which DI particles were implicated in the establishment, are able to readily establish a persistent infection in the absence of DI particles. It is not known whether the noncytocidal virus recovered from the PI3/MK_2-P0 and PI3/MK_2-P8 cultures is able to readily establish a persistent infection without the subgenomic RNA-containing particles. Such an experiment would be a difficult task since the virus released from the persistently infected cultures is nonplaque-forming.

Tuffereau and Roux (1988) proposed a model for DI particle mediated interference during persistent infections. The model proposes that DI particles or other virus mutants may allow cell survival by interfering with the stable formation inside cells of a viral structure composed of M/HN/nucleocapsids. Interference with the stable formation of such a viral structure would lead to a faster turnover of membrane-associated proteins and as a consequence restrict viral budding. This model differs from the model where DI particle mediated interference is believed to occur via competition of the viral products that are required for viral genome replication. It is not known whether there is a faster turnover of the membrane-associated proteins in my persistently infected cell lines. Interestingly, viral RNA synthesis in PI3/MK_2-P8 cells at passage 104 was found to be higher than in STD virus-infected cells (Fig. 15 legend). However, the persistently infected cells have consistently yielded less virus than STD virus-infected cells. These results suggest that either the

efficiency of translation of the viral mRNAs and/or virion maturation is altered and controls the progeny virus yield.

CHAPTER 5: CLONING AND NUCLEOTIDE SEQUENCE ANALYSIS OF HPIV3 RELEASED FROM PERSISTENTLY INFECTED LLC-MK$_2$ CELLS

1. INTRODUCTION

It is known that the genomes of RNA viruses undergo genetic change during persistence. The fact that viruses evolve during persistent infections makes it impossible to attribute viral persistence to a single factor such as DI particles or to a single genetic lesion (such as one rendering a virus *ts*). DI particles and an evolving genome, alone or in combination, at any one time, may play a role in the maintenance of the persistent infection. DI particle genomes are already known to be present in my HPIV3 persistently infected cultures, but the degree of genetic change undergone by the virus during persistence is unknown. Thus, the fourth objective of my project was to determine the degree of genetic change undergone by the HPIV3 genome after long-term persistence. For this purpose, synthetic oligonucleotide primers complementary to the genomic RNA were constructed. However, when primers, annealed to virion genomic RNA, were extended with reverse transcriptase in the presence of deoxynucleotides and dideoxynucleotides no products were obtained. It is thought that the amount of RNA available from virus particles was insufficient for primer extension analysis. Therefore, an alternate strategy had to be employed. To overcome the low levels of template RNA available, PCR was chosen to amplify first-strand cDNA that had been synthesized from virion genomic RNA. The amplified products were cloned in M13 vectors and analyzed by the dideoxy chain-termination sequencing technique (Sanger *et al.*, 1977). Specific areas of

the HPIV3 genome which could possibly reveal details on the maintenance of the persistent infections were chosen for this analysis.

2. RESULTS

Cloning of M gene and flanking regions

No apparent changes in the molecular size of the viral proteins synthesized in the persistently infected cells were observed. However, the M protein in a number of paramyxovirus persistent infections has often been found to be altered (Rozenblatt *et al.*, 1979; Wechsler *et al.*, 1979a; Roux and Waldvogel, 1982; Young *et al.*, 1985; Wechsler *et al.*, 1987). For this reason, the M protein has been implicated as a possible determinant in the maintenance of paramyxovirus persistent infections. Therefore, for both of my persistently infected cultures the nucleotide sequences of the M protein gene and flanking regions of virus recovered after 147 cell passages (29 months of persistence) and the STD virus were determined.

For this purpose, a synthetic oligonucleotide, DM7 (5'-CTCGAGCTCGAAGAAGTATCTGAATTG-3'), representing the sequence complementary to the 5'-end of the P/C gene (nucleotides 1835-1852 of the P/C gene, Spriggs and Collins, 1986b) was used to prime cDNA synthesis using virion genomic RNA as template. DM7 contained a *Sst*I restriction site at its 5'-end (underlined sequence) plus an additional 3 nucleotides serving to protect the restriction site. DM7 and a second synthetic oligonucleotide, DM8 (5'-CTTAAGCTTTACAACTACATGCTGTAG-3'), corresponding to the 3'-end of the F gene (nucleotides 266-283 of the F gene, Côté *et al.*, 1987), were used to amplify M gene sequences by the PCR process. DM8 was constructed with a *Hin*dIII restriction site at its

5'-end. After 30 cycles of amplification one-tenth of the product was analyzed by agarose gel electrophoresis (Fig. 22A). A DNA band of the expected size (1642 nucleotides) was obtained for all three amplified products. A second DNA band, co-migrating with the 872 nucleotide marker, was also amplified. This latter band was heavier in the PI3/MK_2-P0 DNA (Fig. 22A, lane 3). In this case the annealing temperature of the amplification step was 50°C. For the STD and PI3/MK_2-P8 DNA the annealing temperature was 55°C. The 872 nucleotide-size band was lighter for the latter two amplified DNA species suggesting it arose as a result of primer binding to a similar stretch of nucleotides.

To verify that the 1642 nucleotide-size band contained M gene-specific sequences, the gel was subjected to Southern blot analysis. Figure 22B shows that this DNA hybridized well with the M gene specific probe, pPI3.

Nucleotide sequence analysis of M gene and flanking regions

The expected-size DNA fragments were inserted into the *Sst*I and *Hin*dIII sites of M13mp18 RF DNA which was used to transform Epicurian coli XL1-Blue competent cells (Stratagene). Two clones were selected for each of the three M gene species and sequenced by the dideoxynucleotide chain-termination technique. Figure 23 shows the nucleotide sequence analysis where differences were obtained. The changes identified in the amplified DNA with regards to the published sequences are outlined in Fig. 24. The PI3/MK_2-P0 clones had one and two substitutions, respectively, while both PI3/MK_2-P8 clones had three substitutions. In the PI3/MK_2-P0 and PI3/MK_2-P8 sequences only one amino acid change in the M coding region was selected after 29 months of persistence. The two

Figure 22. Agarose gel electrophoresis and Southern blot analysis of products amplified with primers DM7 and DM8. (A) One-tenth of the amplified products were resolved on a 1% agarose gel and visualized by ethidium bromide fluorescence. (B) The DNA in the gel was denatured and transferred onto GeneScreen Plus (NEN). The DNA was hybridized with plasmid pPI3(M). Lanes: 1, *Bst*EII-digested bacteriophage lambda DNA; 2, STD HPIV3 amplified DNA; 3, PI3/MK_2-P0 (passage 147) amplified DNA; 4, PI3/MK_2-P8 (passage 147) amplified DNA; 5, *Hae*III-digested bacteriophage ϕX174 DNA.

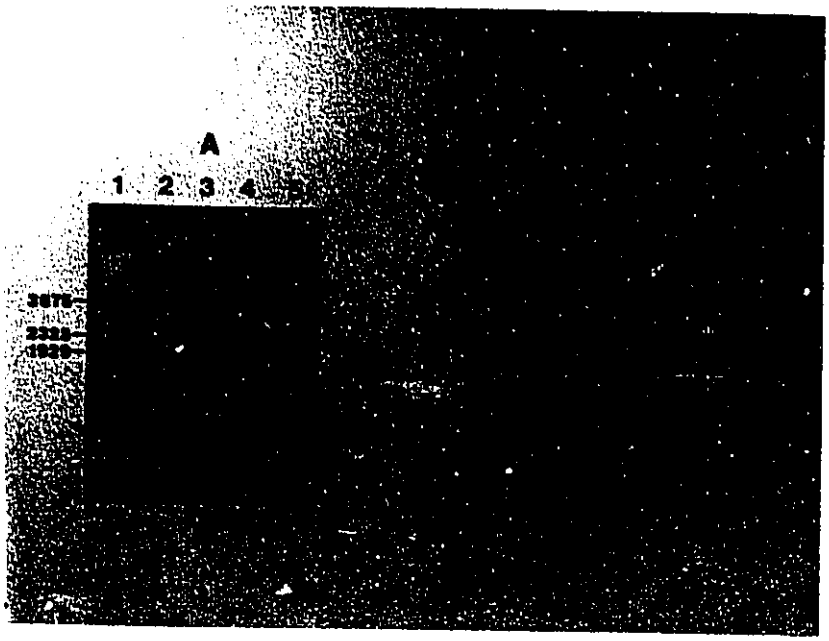
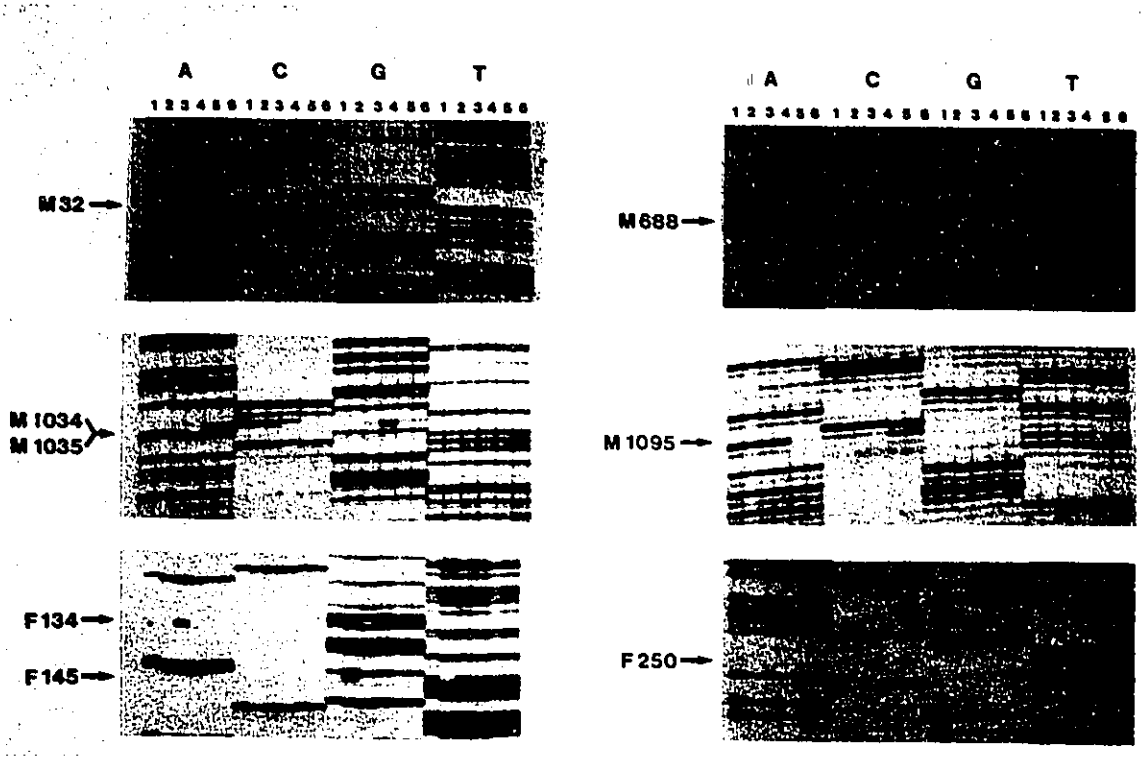

A
1 2 3 4 5

Figure 23. Nucleotide sequence analysis of M gene and flanking regions. Only the regions showing differences with the published sequences are shown. Lanes: 1, HPIV3(2); 2, HPIV3(4); 3, PI3/MK_2-P0(1); 4, PI3/MK_2-P0(5); 5, PI3/MK_2-P8(1); 6, PI3/MK_2-P8(2).


A
C
G
T
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
M32
M688
M1034
M1035
M1095
F134
F145
F250

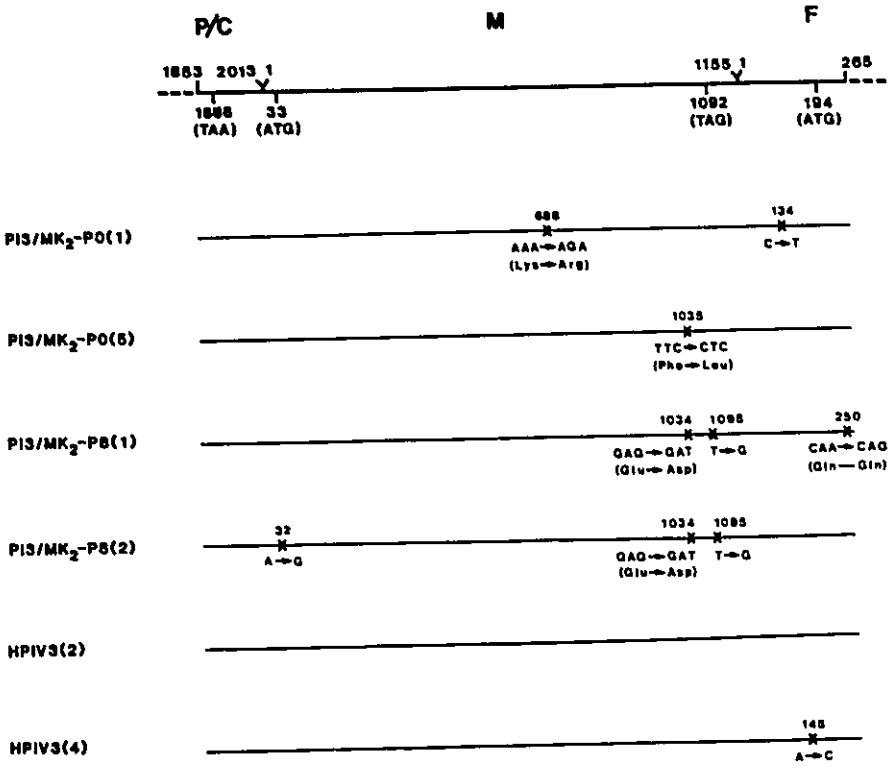
clones analyzed from culture PI3/MK_2-P8 possessed the same amino acid change, glutamic acid to aspartic acid, due to the substitution of G for T at position 1034. The amino acid substitutions in the PI3/MK_2-P0 M sequences were located at a different position in each clone. Clone (1) had an A for G substitution at position 688 which changes lysine to arginine. Clone (5) had a T for C substitution at position 1035 which changes phenylalanine to leucine. In all cases the amino acid substitutions were conservative. One nucleotide difference was observed in one of the two STD virus clones examined.

Comparison of my P/C gene sequence with that of Spriggs and Collins (1986b) revealed two major differences. Their sequence reports a stretch of eight A residues beginning at nucleotide 1898 while only seven were detected in all of my clones. This makes my amplified sequence 1641 nucleotides in length instead of the expected 1642 nucleotides. Secondly, my sequence indicates the presence of an A residue at position 1884 which changes the carboxy-terminal amino acid from cysteine to tyrosine.

Cloning of the genomic 3'-termini

The genomic 3'-end of negative-strand RNA viruses serves as the initiation site for the viral replicase and transcriptase. Therefore, alterations at this site could affect the processes of replication and transcription. Furthermore, UV-irradiation experiments with VSV have suggested that the VSV leader, a 47-nucleotide RNA encoded by the 3'-end of the virus genome, is responsible for the shut off of host macromolecular synthesis (Weck *et al.* 1979; McGowan and Wagner, 1981; Dunigan and Lucas-Lenard, 1983) and thus cell killing. It was therefore of interest to determine whether the sequences in this critical region were

Figure 24. Nucleotide sequence substitutions in the M gene and flanking regions of virus recovered after 147 cell passages. The nucleotide substitutions are presented in the mRNA strand sense, 5' to 3'. In the coding region the positions of the nucleotide substitutions are shown in the triplet sequence of the codon and the resulting amino acid change, where applicable, is given below. The numbering of nucleotides is according to Spriggs and Collins (1986b) for the P/C gene, Prinoski *et al.* (1987) for the M gene, and Côté *et al.* (1987) for the F gene. The locations of the translation initiation and termination codons are shown on the genetic map.

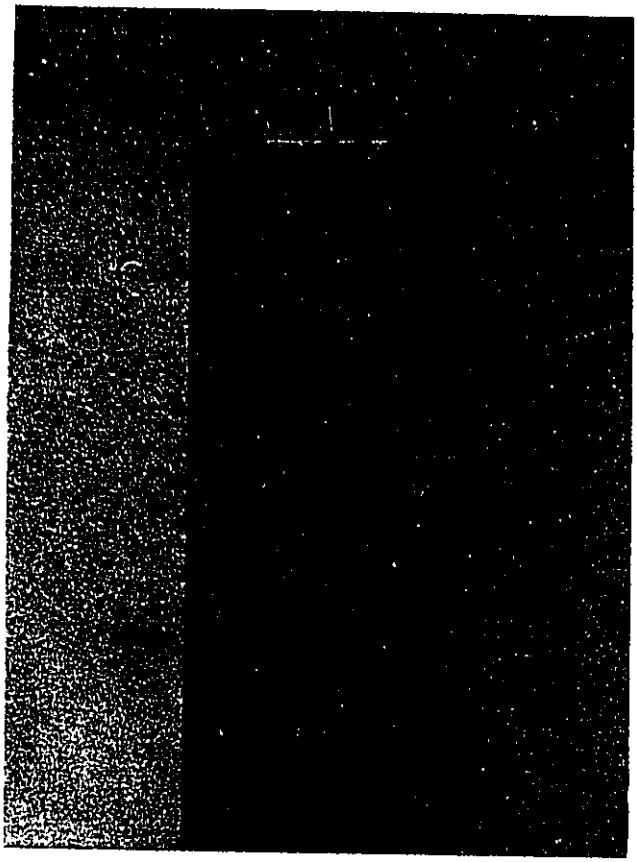

P/C
M
F
1883
2013
1
1155
1
265
1886
(TAA)
33
(ATG)
1092
(TAG)
194
(ATG)
PI3/MK2-P0(1)
686
AAA→AGA
(Lys→Arg)
134
C→T
PI3/MK2-P0(5)
1035
TTC→CTC
(Phe→Leu)
PI3/MK2-P8(1)
1034
GAG→GAT
(Glu→Asp)
1095
T→G
250
CAA→CAG
(Gln→Gln)
PI3/MK2-P8(2)
32
A→G
1034
GAG→GAT
(Glu→Asp)
1095
T→G
HPIV3(2)
HPIV3(4)
145
A→C

altered. For this purpose the 3'-termini of virus recovered after 146 cell passages and STD virus were cloned, amplified by PCR and sequenced. A synthetic oligonucleotide, DM14 (5'-CTCGAGCTCACCAAACAAGAGAAG-3'), representing the sequence complementary to the exact 3'-end of the HPIV3 genome (Dimock *et al.*, 1986) was used to prime cDNA synthesis using virion genomic RNA as template. DM14 contained a *Sst*I site at its 5'-end (underlined sequence). DM15 (5'-CTTAAGCTTTGCTAGATTGATGCC-3') corresponds to the 3'-end of the NP gene (nucleotides 316-330 of the NP gene, Galinski *et al.*, 1986a) and was constructed with a *Hin*dIII site at its 5'-end. DM14 and DM15 were used to amplify the 3'-end of the HPIV3 genomes by the PCR process. After 30 cycles of amplification at an annealing temperature of 45°C, one-tenth of the product was analyzed by agarose gel electrophoresis (Fig. 25). A DNA fragment of the expected size, 333 nucleotides, was obtained.

Nucleotide sequence analysis of the genomic 3'-termini

The DNA fragments were inserted into the *Sst*I and *Hin*dIII sites of M13mp19 RF DNA which were used to transform *E. coli* DH5αF' competent cells (BRL). Four PI3/MK_2-P0 and PI3/MK_2-P8 clones and two STD virus clones were selected and sequenced by the dideoxynucleotide chain-termination method (Fig. 26). Numerous mutations were observed in the PI3/MK_2-P0 and PI3/MK_2-P8 DNA. Three of the PI3/MK_2-P8 clones (4,5, and 6) contained exactly the same base-substitutions and differed at 43 positions from the 3'-end sequence of the HPIV3 genome (Dimock *et al.* 1986; Galinski *et al.*, 1986a). Interestingly, all of these were U to C transitions. Thus, 35.8% (43 of 120) of the U residues in the 303 nucleotide-long product were mutated to C residues (Table 7). The fourth

Figure 25. Agarose gel electrophoresis of amplified genomic 3'-ends. One-tenth of the products amplified with primers DM14 and DM15 were separated in a 1.5% agarose gel and visualized by ethidium bromide fluorescence. Lanes: 1, STD HPIV3 DNA; 2, PI3/MK_2-P0 (passage 146) DNA; 3, PI3/MK_2-P8 (passage 146) DNA; 4, *Hae*III-digested bacteriophage ϕX174 DNA.



clone (PI3/MK_2-P8(8)) contained all the substitutions present in the three other clones plus four A to G transitions. All of the PI3/MK_2-P8 clones contained an U to C (A to G positive-sense) substitution at nucleotide 111 which converts the translational-start codon from AUG to GUG (positive-sense). The closest in frame AUG codon occurs 33 amino acids downstream due to an U to C (A to G positive-sense) transition at position 211. A NP protein was observed to be present in PI3/MK_2-P8 virions of cell passage 144 and to migrate at a similar position as the NP protein present in STD virus particles (Fig. 19B, lane, 7). However, the virions contained less NP protein than HN protein; a condition which is reversed in STD virus particles. The virion RNA profile at cell passage 145 revealed the presence of an abundant subgenomic RNA migrating slightly below the 28S rRNA (Fig. 27A). To determine the genetic composition of this subgenomic RNA, virion RNA from cell passage 146 was subjected to Northern blot analysis (Fig. 27B). This subgenomic RNA hybridized strictly to the NP probe. Furthermore, two slower migrating and less abundant subgenomic RNAs also hybridized to the NP probe. Therefore, the PI3/MK_2-P8 clones could be derived from the 3'-end of the 50S genome or of the subgenomic RNAs.

An interesting finding was that some of the PI3/MK_2-P0 clones possessed numerous A to G transitions in addition to U to C transitions. Also, none of the four clones contained exactly the same nucleotide substitutions. Clone PI3/MK_2-P0(5) had 13 of the 120 U residues changed to C residues (10.8%) and 26 of the 83 A residues changed to G residues (Table 7). Clone PI3/MK_2-P0(10) had the same 13 U to C transitions and 23 A to G transitions (27.7%). Thirteen of the A to G transitions were in the same position as those of clone PI3/MK_2-P0(5). One of the clones,

Figure 26. The 3'-terminal nucleotide sequences of the genome of virus particles released from the persistently infected cells. The prototype sequence represents the published sequences of Dimock *et al.* (1986) and Galinski *et al.* (1986a). The triplet complementary to the NP translational-start codon (dark inverted triangles), the NP gene transcriptional-start sequence (overlined), the leader/NP gene trinucleotide intergenic sequence (dark circles), and the 15 nucleotides corresponding to the two primer sequences (underlined) are indicated.

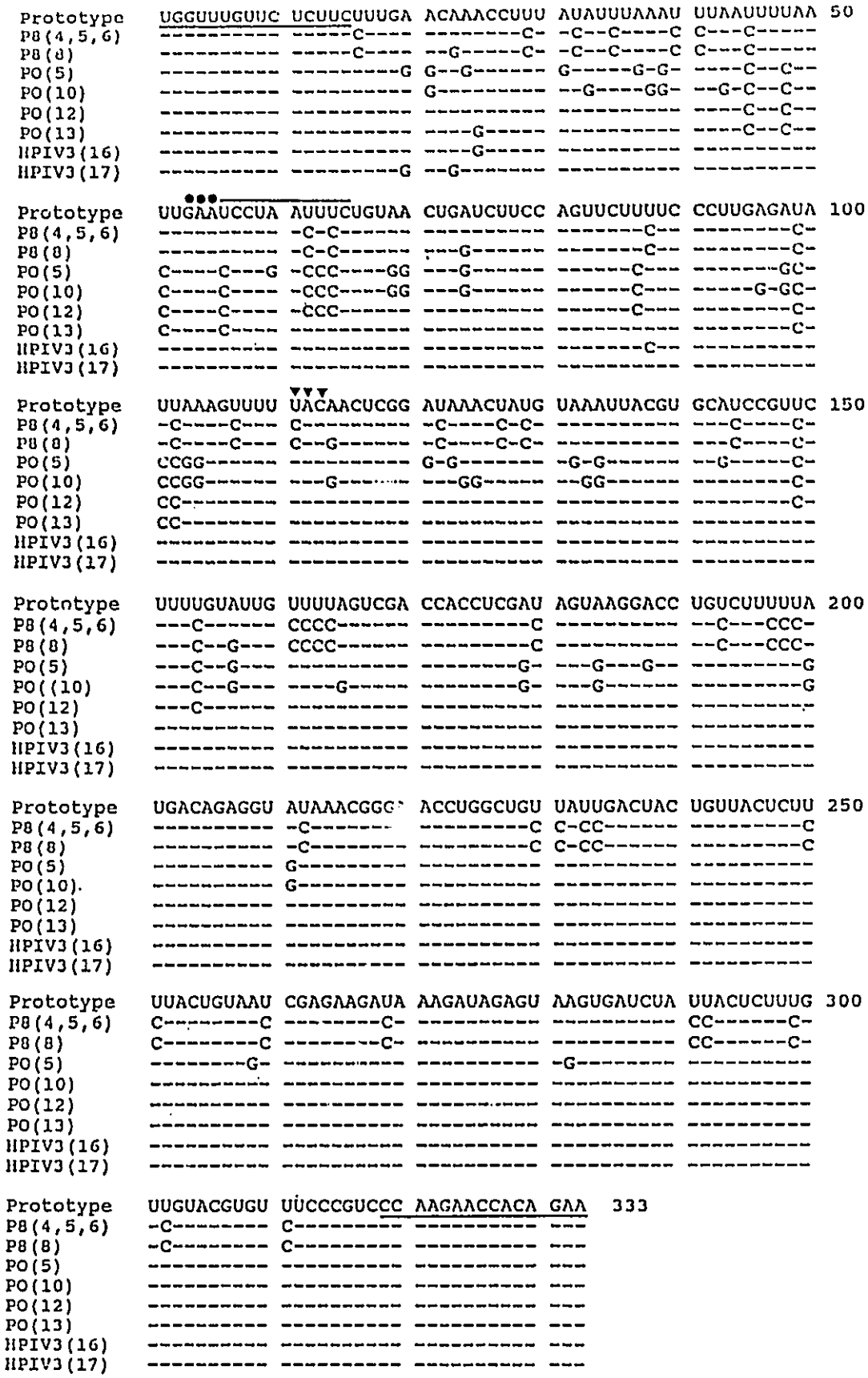


TABLE 7

NUMBER OF U TO C AND A TO G TRANSITIONS

	U to C	A to G
PI3/MK_2-P8(4,5,6)	43 (35.8%)[a]	0 (0.0%)[a]
PI3/MK_2-P8(8)	43 (35.8%)	4 (4.8%)
PI3/MK_2-P0(5)	13 (10.8%)	26 (31.3%)
PI3/MK_2-P0(10)	13 (10.8%)	23 (27.7%)
PI3/MK_2-P0(12)	13 (10.8%)	0 (0.0%)
PI3/MK_2-P0(13)	7 (5.8%)	1 (1.2%)
HPIV3(16)	1 (0.8%)	1 (1.2%)
HPIV3(17)	0 (0.0%)	2 (2.4%)

[a] Percentage of U (120 = 100%) and A (83 = 100%) residues changed to C and G residues, respectively.

Figure 27. RNA profile of virus particles released from the persistently infected cells and Northern blot analysis of PI3/MK_2-P8 virion RNA. Cells were labeled for 24 h with [^{3}H]uridine. Virus particles were concentrated by ultracentrifugation and RNAs were extracted, denatured with glyoxal and separated in a 1% agarose gel. (A) RNA profile of STD virus particles (lane 1), PI3/MK_2-P0 (passage 145) particles (lane 2), and PI3/MK_2-P8 (passage 145) particles (lane 3). Arrows indicate subgenomic RNA species. (B) Northern blot analysis of PI3/MK_2-P8 virion RNA of cell passage 146. Following electrophoresis in a 1% agarose gel, the RNA was transferred onto GeneScreen Plus (NEN). The blot was cut into 4 mm-wide longitudinal strips and hybridized with individually nick-translated plasmids: pPI47(NP)(lane 1); pPI28(P/C) (lane 2); pPI3(M) (lane 3); pPI14(F) (lane 4); pPI10(HN) (lane 5); pGEM3-L (lane 6). Arrows indicate hybridization to subgenomic RNA species.

PI3/MK_2-P0(12), possessed the same 13 U to C transitions as found in the above two clones, but no A to G transitions. Clone PI3/MK_2-P0(13) had 7 U to C transitions (5.8%) and one A to G transition (1.2%). The 7 U to C transitions are a subset of the 13 U to C transitions observed in the other three PI3/MK_2-P0 clones.

In all four PI3/MK_2-P0 clones the translational start codon was not altered. Since subgenomic RNAs are present at cell passage 145 (Fig. 13B, lane 5), it is not possible to state with certainty that the four PI3/MK_2-P0 clones were derived from the 3'-end of the 50S genome. Note that clone 13 would have the potential to code for a functional NP protein since it has no nucleotide substitution past the translational start codon.

In PI3/MK_2-P0 clones 5, 10 and 12 only 2 of the 13 U to C transitions were located in the protein-coding region and in clone 13 all the 7 U to C transitions were in the non-coding region. In addition, most of the A to G transitions were confined to the first 200 nucleotides. In this cell line the mutation frequency was observed to decrease as the distance from the 3'-end increased. In the PI3/MK_2-P8 cell line the U to C transitions were scattered throughout the amplified sequence. However, it is not known if these U to C transitions were maintained throughout the entire NP gene.

Surprisingly, the STD HPIV3 cloned products also possessed U to C and A to G transitions. Clone HPIV3(16) had single A to G and U to C transitions while clone HPIV3(17) had two A to G transitions. Similarly positioned A to G and U to C transitions were observed in the PI3/MK_2-P0 and PI3/MK_2-P8 clones. These results suggest that such biased hypermutational activity operates during both acute and persistent infections.

3. DISCUSSION

Mutation of M gene and role of M protein in HPIV3 persistence

The only other negative-strand RNA virus in which the evolution of the genome in cultured cells has been studied is VSV. Holland *et al.* (1979) used T1 ribonuclease oligonucleotide mapping to estimate the number of mutations undergone by the VSV genome during persistence. The oligonucleotide map of cloned virus recovered after 21 and 42 months of persistence revealed 8 and 11 map changes, respectively. In this type of analysis only 10 to 15% of the oligonucleotides migrate to unique regions. Thus, 85 to 90% of the mutations will not be apparent. Therefore, it is estimated that the virus recovered after 21 and 42 months of persistence will have undergone roughly 50 to 80 and 70 to 110 mutations, respectively. These numbers indicate that significant genome change is occurring during VSV persistence.

From the sequence analysis of the M gene and flanking regions, the HPIV3 genome is estimated to have undergone 10 to 30 mutations over 29 months of persistence. (This estimate does not take into account the biased hypermutations observed at the 3'-end of the genomes. These mutations are likely a result of an extrinsic activity rather than intrinsic polymerase errors). This level of mutation is somewhat less than that observed for VSV. It should be noted that in the case of VSV a selection was made for plaque-forming virus. This would eliminate any more drastically mutated nonplaque-forming virus even if these were the most abundant species. The use of oligonucleotide primers during the reverse transcription step and amplification process is the only selection exerted in my analysis. In this case only virus genomes with highly mutated

primer-binding sites would be selected against. The evolutionary rate of virus genomes is likely to depend on the nature of the virus-host system. The nature of the viral polymerase complex, the number of replication cycles and the pressure exerted by the host cell are all factors contributing to the evolution of the virus genome. Another factor which is believed to drive virus evolution is DI particles. DI particles, however, are more likely to exert their effects on the termini of the STD-size virus genome rather than in the coding regions.

The sequence data also revealed that the M protein was not significantly altered after 29 months of persistence. The amino acid substitutions in the two clones analyzed from the PI3/MK_2-P0 cell line were conservative and located at different positions. These amino acids substitutions were also not observed in the clones from the PI3/MK_2-P8 culture. The same amino acid change, glutamic acid to aspartic acid, was observed in both clones of the PI3/MK_2-P8 cell line. Therefore, this mutation does not change the overall charge of the protein. For these reasons, the observed amino acids substitutions are likely not to be responsible for the maintenance of the persistent state. However, I cannot rule out the possibility that any of these mutations do not affect the function of the M protein.

Biased hypermutation in HPIV3 RNA derived from persistently infected cells

The 3'-end of HPIV3 RNA recovered from both persistently infected cultures was found to be highly mutated. Interestingly, the only types of nucleotide changes observed were U to C and A to G transitions. Such exclusive mutation of one nucleotide for another, known as a biased

hypermutation, is unlikely to be due to the infidelity of the viral polymerase, but to the double-stranded RNA unwinding/modifying activity reported by Bass and Weintraub (1988). This RNA unwinding/modifying activity converts A residues to inosine (I) residues in both strands of the duplex. The unwinding activity has been observed in a number of mammalian tissue culture cells and in Xenopus embryos (Bass and Weintraub, 1988; Wagner *et al.*, 1989).

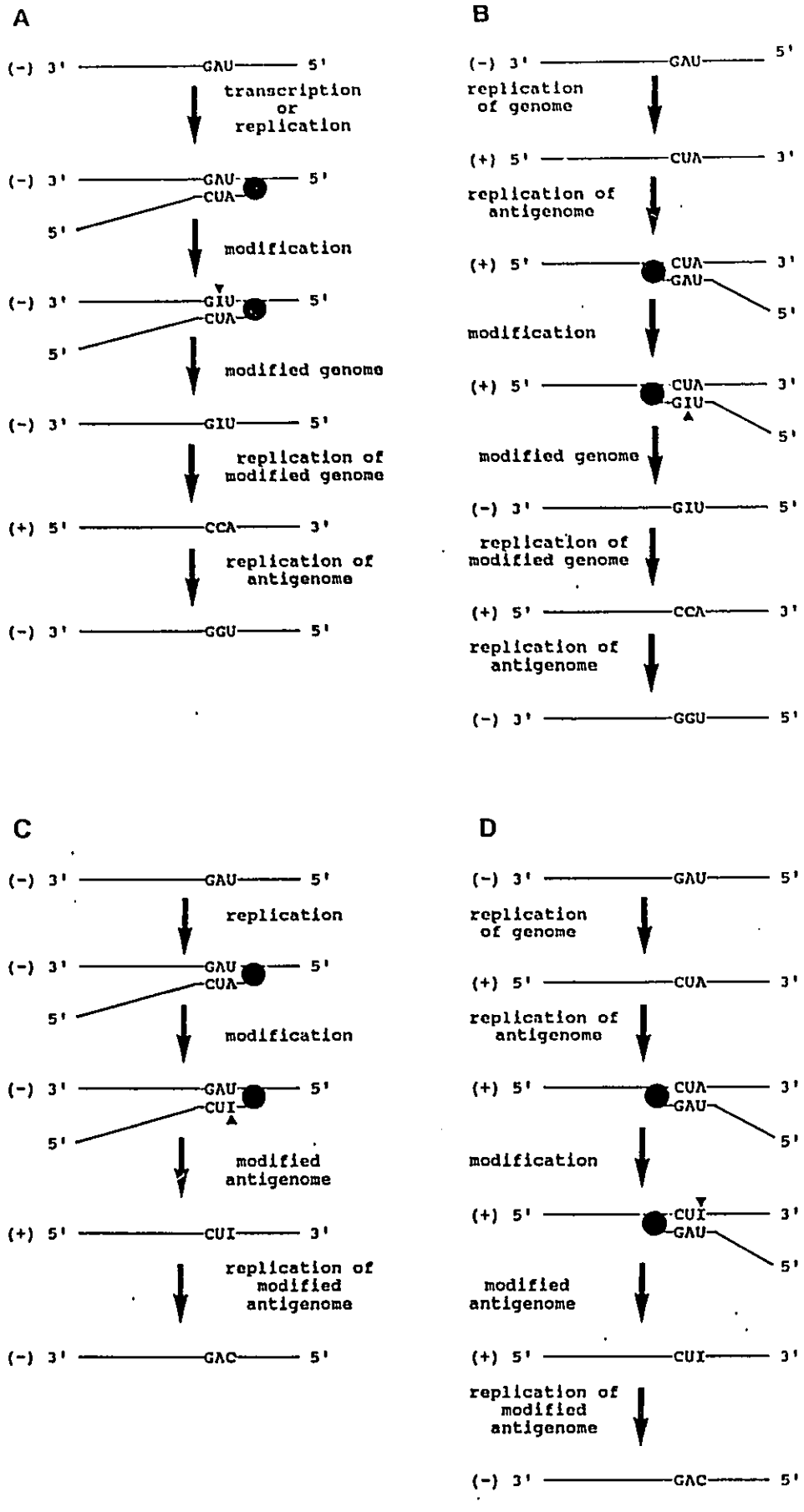
Biased hypermutations have been observed affecting the M gene in a measles inclusion body encephalitis (MIBE) case and SSPE virus strains (YM-V, YM-I) and the H gene in SSPE cell line IP-3-Ca. In the MIBE case 132 of 266 A residues (genome-sense) were mutated to G residues (Cattaneo *et al.*, 1988b). In the YM-V SSPE strain 36 A to G substitutions were observed. When this strain was propagated in cultured cells (now called strain YM-I) 38 additional A to G changes were found. Interestingly, 35 of the 38 changes affected A residues that were mutated in the MIBE case (Wong *et al.*, 1989). This suggests that certain residues may be preferentially mutated. The authors concluded that the 38 additional changes might have been generated during passage of the virus in cultured cells, but that they could not rule out the possibility that a preexisting variant was selected *in vitro*. In a segment of the H gene in cell line IP-3-Ca, 20 U to C (genome-sense) substitutions which resulted in 16 amino acid changes were observed. These mutations were localized in a region spanning 333 nucleotides which includes a total of 97 U residues (Cattaneo *et al.*, 1989b). Similar localized biased mutations were observed in a VSV DI particle genome isolated after 120 undiluted passages (O'Hara *et al.*, 1984). In a stretch of 18 nucleotides near the 5'-end of the DI particle genome 11 of the 14 A residues were mutated to G

residues.

The RNA unwinding/modifying activity is found only on double-stranded RNA. The only time the RNA of negative-strand viruses could find itself in a double-helical structure is either during replication or transcription. During transcription or replication of the genome (negative-strand) or replication of the antigenome (positive-strand) newly synthesized nucleotides may find themselves base-paired with the template. If so, A to I mutations could be introduced in the genome during either replication and transcription of the genome or replication of the antigenome (Figs. 28A and B). In the next round of replication the I residues, now present on the genome, would be converted to C residues on the antigenome. I residues have a preference for base-pairing with C residues (Bass and Weintraub, 1988). During replication of the antigenome, G residues would be incorporated in the newly synthesized genome giving rise to A to G substitutions. In a similar manner, A to I mutations could be introduced in the antigenome during either synthesis of antigenome from genome or synthesis of progeny genome from antigenome (Figs. 28C and D). The I containing antigenome when used as template for the production of progeny genome would give rise to U to C substitutions (Bass *et al.*, 1989; Cattaneo *et al.*, 1989b; Lamb and Dreyfuss, 1989).

Both A to G and U to C hypermutations on the same strand have not yet been reported. In HPIV3 RNA recovered from my persistently infected LLC-MK_2 cells both types of mutations were observed in the genome. If these mutations are a result of the unwinding/modifying activity of Bass and Weintraub (1988) then both the genome and the corresponding antigenome must have been mutated. Clone PI3/MK_2-P8(8) had the same 43 U to C transitions as the other three clones from this cell line plus

Figure 28. RNA unwinding/modification activity. During replication or transcription a short stretch of newly synthesized nucleotides may find themselves based-paired with the template. A to I mutations could be introduced in the genome during either replication or transcription of the genome (A) or replication of the antigenome (B). In a similar manner, A to I mutations could be introduced in the antigenome during replication of the genome (C) or antigenome (D). The dark circle represents the polymerase complex. The triangles indicate the mutations.


A
(-) 3' GAU 5'
transcription or replication
(-) 3' GAU 5'
CUA
5'
modification
(-) 3' GIU 5'
CUA
5'
modified genome
(-) 3' GIU 5'
replication of modified genome
(+) 5' CCA 3'
replication of antigenome
(-) 3' GGU 5'
B
(-) 3' GAU 5'
replication of genome
(+) 5' CUA 3'
replication of antigenome
(+) 5' CUA 3'
GAU
5'
modification
(+) 5' CUA 3'
GIU
5'
modified genome
(-) 3' GIU 5'
replication of modified genome
(+) 5' CCA 3'
replication of antigenome
(-) 3' GGU 5'
C
(-) 3' GAU 5'
replication
(-) 3' GAU 5'
CUA
5'
modification
(-) 3' GAU 5'
CUI
5'
modified antigenome
(+) 5' CUI 3'
replication of modified antigenome
(-) 3' GAC 5'
D
(-) 3' GAU 5'
replication of genome
(+) 5' CUA 3'
replication of antigenome
(+) 5' CUA 3'
GAU
5'
modification
(+) 5' CUI 3'
GAU
5'
modified antigenome
(+) 5' CUI 3'
replication of modified antigenome
(-) 3' GAC 5'

four additional A to G transitions. Clones PI3/MK$_2$-P0(5) and (10) had the same 13 U to C changes as clone PI3/MK$_2$-P0(12) plus numerous additional A to G changes. This suggests that the A to I mutations were first introduced in the antigenome. This would give rise to the U to C transitions observed in the genome. During replication of the mutated antigenome or newly generated genomes containing the U to C substitutions none or various A to I substitutions would be introduced in the genome template. After a round of replication this would give rise to certain genomes having only U to C changes and to others having U to C and A to G changes.

Similar U to C and A to G substitutions were observed in the two STD HPIV3 clones. This suggests that the RNA unwinding and modification also occurs during acute infections. Genomes with numerous nucleotide changes in lytic infections would be selected against. Genomes with few base substitutions would also tend to be selected against, but such genomes could survive as variant genomes. Evidence for this is observed in a substrain of the Edmonston strain of measles virus adapted for growth in suspension in HeLa cells. This substrain differs in the P gene from another Edmonston sequence by 6 A to G (genome-sense) changes in a stretch of 600 nucleotides (Cattaneo *et al.*, 1989b).

It is not known whether the PI3/MK$_2$-P8 and PI3/MK$_2$-P0 clones correspond to the 3'-end of the 50S genome or to the subgenomic RNAs. Assuming that the clones were derived from the 3'-end of the 50S genome, then in cell line PI3/MK$_2$-P8 this would suggest the existence of 50S genomes with conserved NP translational start codons. These would be in a minority with regards to 50S genomes containing mutated start codons. This could explain the apparently lower levels of NP protein in

passage 144 virions (Fig. 19). Such a situation could also explain the very low levels of NP protein in virions of cell passage 44 (Fig. 21). In cell line PI3/MK_2-P0 the NP protein also appears to be underrepresented in comparison to the HN protein, but not as significantly as in PI3/MK_2-P8 virions. The clones obtained from this cell line do not have altered translational start codons. Clone 13 would have the potential to code for a functional NP protein. The other clones would give rise to NP proteins with altered amino acids. These clones could direct the synthesis of nonfunctional NP proteins. If so, this could explain the apparently reduced levels of NP protein in PI3/MK_2-P0 virions.

The leader/NP gene trinucleotide intergenic sequence GAA was not modified in any of the clones. However, the NP transcriptional gene start signal in the clones from both persistently infected cell lines was modified, especially in the PI3/MK_2-P0 clones. If these clones correspond to the 3'-end of the 50S genome than we would expect the transcription of the NP gene to be reduced. Reduction in NP gene transcripts would be another factor that could contribute to the maintenance of the persistent state.

It cannot be excluded that these biased hypermutational events might be responsible for maintenance of the persistent infections. However, until the origin of the mutated sequences is determined this remains only speculative.

CHAPTER 6: CONCLUSIONS

DI particles of HPIV3 were generated and/or amplified by serial undiluted passage of the STD virus. This supports the general belief that all viruses generate DI particles. The amplified DI particle genomes were found to contain sequences from the 5'-end of the STD viral genome. This suggests that they have retained the exact 5'-end of the STD virus genome and correlates with the finding that all subgenomic RNAs of nonsegmented negative-strand RNA viruses analyzed to date have retained the STD viral genomic 5'-termini. The smallest subgenomic RNA, which was about 775 nucleotides in length, could not be amplified by PCR following reverse transcription of its genome. This suggests that this subgenomic RNA has either an altered 3'- or 5'-end. A future experiment would be to determine the exact nature of the ends of this interesting and extremely small subgenomic RNA.

DI particles were found to aid in the establishment of HPIV3 persistent infections of LLC-MK_2 cells since, in contrast with the STD virus, a persistent infection could be immediately established. The virus released from the persistently infected cultures early in persistence, and thereafter, was noncytocidal indicating that a mutant virus was selected during establishment of the persistent infections. Subgenomic RNAs (putative DI particle genomes) were produced during the course of the persistent infections; a phenomenon that until now had not been observed with HPIV3 persistent infections of cultured cells. IFN did not appear to play a role in HPIV3 persistent infections of LLC-MK_2 cells. It is concluded that the persistent infections are likely to be maintained by both the noncytocidal mutant virus and the subgenomic RNAs.

Some of the subgenomic RNAs were found to hybridize exclusively to NP gene sequences. However, the size of some of these subgenomic RNAs indicates that they must contain more than one copy of NP gene sequences. A future experiment would be to determine the genetic composition of these subgenomic RNAs. This may reveal preferential polymerase binding sites.

Nucleotide sequence analysis of the M gene and flanking regions of virus recovered after 147 cell passages (29 months of persistence) revealed few nucleotide substitutions. The M protein coding region of virus released from both cell lines possessed only one conservative amino acid substitution. In two clones analyzed from cell line PI3/MK_2-P0 the amino acid substitution was located at a different position. Furthermore, these two amino acid substitutions were different than the one observed in the clones from the PI3/MK_2-P8 cell line. Since, the amino acid substitutions observed in the M protein were inconsistent and conservative they are not expected to be a major determinant in the maintenance of the persistent infections.

The extensive U to C and A to G substitutions observed in the 3'-terminal genomic sequence of virus recovered at cell passage 146 are likely due to an extrinsic mutational activity rather than to intrinsic polymerase errors. This extrinsic mutational activity is postulated to be the unwinding/modifying activity which converts A residues to I residues of RNA present in a double-helical form. To determine the genome origin of the biased hypermutated sequences, the subgenomic RNAs would have to be separated from the STD-size genome prior to cloning. This could be achieved by isolating the respective RNA bands following electrophoresis in agarose gels or by isolating intracellular nucleocapsids on sucrose

gradients. The fact that the same types of nucleotide substitutions were observed in the STD virus genomic 3'-termini suggests that this activity also operates during acute virus infections of cultured cells. This activity is likely to play an important role in the evolution of RNA viruses.

LIST OF REFERENCES

Amesse, L.S., Pridgen, C.L., and Kingsbury, D.W. (1982) Sendai virus DI RNA species with conserved virus genome termini and extensive internal deletions. *Virology* 118, 17-27.

Andrewes, C.H., Bang, F.B., Chanock, R.M., and Zhdanov, V.M. (1959) Para-influenza viruses 1, 2 and 3: Suggested names for recently described myxoviruses. *Virology* 8, 129-130.

Andzhaparidze, O.G., Boriskin, Y.S., Bogomolova, N.N., and Drynov, I.D. (1982) Mumps virus-persistently infected cell cultures release defective interfering particles. *J. Gen. Virol.* 63, 499-503.

Ayata, M., Hirano, A., and Wong, T.C. (1989) Structural defect linked to nonrandom mutations in the matrix gene of Biken strain subacute sclerosing panencephalitis virus defined by cDNA cloning and expression of chimeric genes. *J. Virol.* 63, 1162-1173.

Baczko, K., Liebert, U.G., Billeter, M., Cattaneo, R., Budka, H., and ter Meulen, V. (1986) Expression of defective measles virus genes in brain tissues of patients with subacute sclerosing panencephalitis. *J. Virol.* 59, 472-478.

Barrett, A.D.T., and Dimmock, N.J. (1984) Modulation of Semliki Forest virus-induced infection of mice by defective interfering virus. *J. Infect. Dis.* 150, 98-104.

Barrett, A.D.T., and Dimmock, N.J. (1986) Defective interfering viruses and infections of animals. *Curr. Top. Microbiol. Immunol.* 128, 55-84.

Baslé, M.F., Fournier, J.G., Rozenblatt, S., Rebel, A., and Bouteille, M. (1986) Measles virus RNA detected in Paget's disease bone tissue by *in situ* hybridization. *J. Gen. Virol.* 67, 907-913.

Bass, B.L., and Weintraub, H. (1988) An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* 55, 1089-1098.

Bass, B.L., Weintraub, H., Cattaneo, R., Billeter, M.A. (1989) Biased hypermutation of viral RNA genomes could be due to unwinding/modification of double-stranded RNA. *Cell* 56, 331.

Blumberg, B.M., Leppert, M., and Kolakofsky, D. (1981) Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. *Cell* 23, 337-845.

Boriskin, Y.S., Bogomolova, N.N., Koptyaeva, I.B., Giraudon, P., and Wild, T.F. (1986) Measles virus persistent infection: Modification of the virus nucleocapsid protein. *J. Gen. Virol.* 67, 1979-1985.

Buechi, M., and Bächi, T. (1982) Microscopy of internal structures of Sendai virus associated with the cytoplasmic surface of host membranes. *Virology* **120**, 349-359.

Burgyan, J., Grieco, F., and Russo, M. (1989) A defective interfering RNA molecule in cymbidium ringspot virus infections. *J. Gen. Virol.* **70**, 235-239.

Burnette, W.N. (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195-203.

Carter, M.J., Willcocks, M.M., and ter Meulen, V. (1983) Defective translation of measles virus matrix protein in a subacute sclerosing panencephalitis cell line. *Nature (London)* **305**, 153-155.

Cattaneo, R., Kaelin, K., Baczko, K., and Billeter, M.A. (1989a) Measles virus editing provides an additional cysteine-rich protein. *Cell* **56**, 759-764.

Cattaneo, R., Rebmann, G., Schmid, A., Baczko, K., ter Meulen, V., and Billeter, M.A. (1987) Altered transcription of a defective measles virus genome derived from a diseased human brain. *EMBO* **6**, 681-688.

Cattaneo, R., Schmid, A., Billeter, M.A., Sheppard, R.D., and Udem, S.A. (1988a) Multiple viral mutations rather than host factors cause defective measles virus gene expression in a subacute sclerosing panencephalitis cell line. *J. Virol.* **62**, 1388-1397.

Cattaneo, R., Schmid, A., Eschle, D., Baczko, K., ter Meulen, V., and Billeter, M.A. (1988b) Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. *Cell* **55**, 255-265.

Cattaneo, R., Schmid, A., Rebmann, G., Baczko, K., ter Meulen, V., Bellini, W.J., Rozenblatt, S., and Billeter, M.A. (1986) Accumulated measles virus mutations in a case of subacute sclerosing panencephalitis: Interrupted matrix protein reading frame and transcription alteration. *Virology* **154**, 97-107.

Cattaneo, R., Schmid, A., Spielhofer, P., Kaelin, K., Baczko, K., ter Meulen, V., Pardowitz, J., Flanagan, S., Rima, B.K., Udem, S.A., and Billeter, M.A. (1989b) Mutated and hypermutated genes of persistent measles viruses which caused lethal human brain diseases. *Virology* **173**, 415-425.

Cave, D.R., Hendrickson, F.M., and Huang, A.S. (1985) Defective interfering virus particles modulate virulence. *J. Virol.* **55**, 366-373.

Chambers, P., Millar, N.S., Bingham, R.W., and Emmerson, P.T. (1986) Molecular cloning of complementary DNA to Newcastle disease virus, and nucleotide sequence analysis of the junction between the genes encoding the haemagglutinin-neuraminidase and the large protein. *J. Gen. Virol.* **67**, 475-486.

Chancola, J., Vargosko, A.J., Kim, H.W., Parrott, R.H., Christmas, E., Jeffries, B., and Chanock, R.M. (1964) Antigenic variation among newly isolated strains of parainfluenza type 4 virus. *Am. J. Hyg.* 79, 357-364.

Chanock, R.M. (1956) Association of a new type of cytopathic myxovirus with infantile croup. *J. Exp. Med.* 104, 555-576.

Chanock, R.M., and McIntosh, K. (1990) Parainfluenza viruses. In *Virology*, pp. 963-988. Edited by B.N. Fields, D.M. Knipe, R.M. Chanock, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman. New York: Raven Press, Ltd.

Chanock, R.M., and Parrott, R.H. (1965) Acute respiratory disease in infancy and childhood: present understanding and prospects for prevention. *Pediatrics* 36, 21-39.

Chanock, R.M., Parrott, R.H., Cook, K., Andrews, B.E., Bell, J.A., Reichelderfer, T., Kapikian, A.Z., Mastrota, F.M., and Huebner, R.J. (1958) Newly recognized myxoviruses from children with respiratory disease. *N. Engl. J. Med.* 258, 207-213.

Chanock, R.M., Parrott, R.H., Johnson, K.M., Kapikian, A.Z., and Bell, J.A. (1963) Myxoviruses: Parainfluenza. *Am. Rev. Respir. Dis.* 88 supp., 152-166.

Clewell, D., and Helinski, D.R. (1969) Supercoiled circular DNA-protein complex in *Escherichia coli*: Purification and induced conversion to an open circular DNA form. *Proc. Natl. Acad. Sci. USA* 62, 1159-1166.

Cole, F.E., Jr., and Hetrick, F.M. (1965) Persistent infection of human conjunctiva cell cultures by myxovirus parainfluenza 3. *Can. J. Microbiol.* 11, 513-521.

Collins, P.L., and Wertz, G.W. (1983) cDNA cloning and transcriptional mapping of nine polyadenylated RNAs encoded by the genome of human respiratory syncytial virus. *Proc. Natl. Acad. Sci. USA* 80, 3208-3212.

Colonno, R.J., Lazzarini, R.A., Keene, J.D., and Banerjee, A.K. (1977) *In vitro* synthesis of messenger RNA by a defective interfering particle of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* 74, 1884-1888.

Cook, M.K., Andrews, B.E., Fox, H.H., Turner, H.C., James, W.D., and Chanock, R.M. (1959) Antigenic relationships among the "newer" myxoviruses (parainfluenza). *Am. J. Hyg.* 69, 250-264.

Cooper, P.D., and Bellett, J.D. (1959) A transmissible interfering component of vesicular stomatitis virus preparations. *J. Gen. Microbiol.* 21, 485-497.

Côté, M.-J., Storey, D.G., Kang, C.Y., and Dimock, K. (1987) Nucleotide sequence of the coding and flanking regions of the human parainfluenza virus type 3 fusion glycoprotein gene. *J. Gen. Virol.* 68, 1003-1010.

Curran, J.A., and Kolakofsky, D. (1987) Identification of an additional Sendai virus non-structural protein encoded by the P/C mRNA. *J. Gen. Virol.* **68**, 2515-2519.

Daniel, P., and Chany, C. (1962) Etude d'une lignee de cellules KB infectees chroniquement par le virus para-influenzae type 3. I. Aspects de la production virale. *Can. J. Microbiol.* **11**, 513-521.

De, B.P., Galinski, M.S., and Banerjee, A.K. (1990) Characterization of an *in vitro* system for the synthesis of mRNA from human parainfluenza virus 3. *J. Virol.* **64**, 1135-1142.

DePolo, N.J., Giachetti, C. and Holland, J.J. (1987) Continuing coevolution of virus and defective interfering particles of viral genome sequences during undiluted passages: Virus mutants exhibiting nearly complete resistance to formerly dominant defective interfering particles. *J. Virol.* **61**, 454-464.

Dhib-Jalbut, S., McFarland, H.F., Mingioli, E.S., Sever, J.L., McFarlin, D.E. (1988) Humoral and cellular immune response to matrix protein of measles virus in subacute sclerosing panencephalitis. *J. Virol.* **62**, 2483-2489.

Dillon, P.J., and Gupta, K.C. (1989) Expression of five proteins from the Sendai virus P/C mRNA in infected cells. *J. Virol.* **63**, 974-977.

Dimock, K., Rud, E.W., and Kang, C.Y. (1986) 3'-Terminal sequence of human parainfluenza virus 3 genomic RNA. *Nucleic Acids Res.* **14**, 4694.

Dimock, K., Storey, D.G., Côté, M.-J., and Kang, C.Y. (1987) Cloning, coding assignments and mapping of human parainfluenza virus 3 genes. In *Biology of Negative Strand Viruses*, pp. 213-220. Edited by D. Kolakofsky and B.W.J. Mahy. New York: Elsevier Biomedical Press.

Dougherty, J.P., and Temin, H.M. (1988) Determination of the rate of base-pair substitution and insertion mutations in retrovirus replication. *J. Virol.* **62**, 2817-2822.

Dowling, P.C., Giorgi, C., Roux, L., Dethlefsen, L.A., Galantowicz, M.E., Blumberg, B.M., and Kolakofsky, D. (1983) Molecular cloning of the 3'-proximal third of Sendai virus genome. *Proc. Natl. Acad. Sci. USA* **80**, 5213-5216.

Doyle, M., and Holland, J.J. (1973) Prophylaxis and immunization in mice by use of virus-free defective T particles to protect against intracerebral infection by vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* **70**, 2105-2108.

Drake, J.W. (1969) Comparative rates of spontaneous mutation. *Nature (London)* **221**, 1132.

Dunigan, D., and Lucas-Lenard, J. (1983) Two transcriptional products of the vesicular stomatitis virus genome may control L cell protein synthesis. *J. Virol.* **45**, 618-626.

Elango, N., Coligan, J.E., Jambou, R.C., and Venkatesan, S. (1986) Human parainfluenza type 3 virus hemagglutinin-neuraminidase glycoprotein: nucleotide sequence of mRNA and limited amino acid sequence of the purified protein. *J. Virol.* 57, 481-489.

Elango, N., Kövamees, J., and Norrby, E. (1989) Sequence analysis of the mumps virus mRNA encoding the P protein. *Virology* 169, 62-67.

Elango, N., Varsanyi, T.M., Kövamees, J., and Norrby, E. (1988) Molecular cloning and characterization of six genes, determination of gene order and intergenic sequences and leader sequence of mumps virus. *J. Gen. Virol.* 69, 2893-2900.

Enami, M., Sato, T.A., and Sugiura, A. (1989) Matrix protein of cell-associated subacute sclerosing panencephalitis viruses. *J. Gen. Virol.* 70, 2191-2196.

Epstein, D.A., Herman, R.C., Chien, I., and Lazzarini, R.A. (1980) Defective interfering particle generated by internal deletion of the vesicular stomatitis virus genome. *J. Virol.* 33, 818-829.

Frey, T.K., and Youngner, J.S. (1982) Novel phenotype of RNA synthesis expressed by vesicular stomatitis virus isolated from persistent infection. *J. Virol.* 44, 167-174.

Frey, T.K., and Youngner, J.S. (1984) Further studies on the RNA synthesis phenotype selected during persistent infection with vesicular stomatitis virus. *Virology* 136, 211-220.

Fultz, P.N., Shadduck, J.A., Kang, C.-Y., and Streilen, J.W. (1982a) Mediators of protection against lethal systemic vesicular stomatitis virus infection in hamsters: Defective interfering particles, polyinosinate-polycytidilate, and interferon. *Infect. Immun.* 37, 679-686.

Fultz, P.N., Shadduck, J.A., Kang, C.-Y., and Streilen, J.W. (1982b) Vesicular stomatitis virus can establish persistent infection in Syrian hamsters. *J. Gen. Virol.* 63, 493-497.

Galinski, M.S., Mink, M.A., Lambert, D.M., Wechsler, S.L., and Pons, M.W. (1986a) Molecular cloning and sequence analysis of the human parainfluenza 3 virus RNA encoding the nucleocapsid protein. *Virology* 149, 139-151.

Galinski, M.S., Mink, M.A., Lambert, D.M., Wechsler, S.L., and Pons, M.W. (1986b) Molecular cloning and sequence analysis of the human parainfluenza 3 virus mRNA encoding the P and C proteins. *Virology* 155, 46-60.

Galinski, M.S., Mink, M.A., Lambert, D.M., Wechsler, S.L., and Pons, M.W. (1987a) Molecular cloning and sequence analysis of the human parainfluenza 3 virus gene encoding the matrix protein. *Virology* 157, 24-30.

Galinski, M.S., Mink, M.A., and Pons, M.W. (1987b) Molecular cloning and sequence analysis of the human parainfluenza 3 virus genes encoding the surface glycoproteins, F and HN. *Virus Res.* **8**, 205-215.

Galinski, M.S., Mink, M.A., and Pons, M.W. (1988) Molecular cloning and sequence analysis of the human parainfluenza 3 virus gene encoding the L protein. *Virology* **165**, 499-510.

Giachetti, C., and Holland, J.J. (1988) Altered replicase specificity is responsible for resistance to defective interfering particle interference of an Sdi$^-$ mutant of vesicular stomatitis virus. *J. Virol.* **62**, 3614-3621.

Giachetti, C., and Holland, J.J. (1989) Vesicular stomatitis virus and its defective interfering particles exhibit *in vitro* transcriptional and replicative competition for purified L-NS polymerase molecules. *Virology* **170**, 264-267.

Glazier, K., Raghow, R., and Kingsbury, D.W. (1977) Regulation of Sendai virus transcription: Evidence for a single promotor *in vivo*. *J. Virol.* **21**, 863-871.

Glezen, W.P., Frank, A.L., Taber, L.H., and Kasel, J.A. (1984) Parainfluenza virus type 3: seasonality and risk of infection and reinfection in young children. *J. Infect. Dis.* **150**, 851-857.

Goebel, W. (1970) Studies on extrachromosomal DNA elements: Replication of the colicinogenic factor Col E_1 in two temperature sensitive mutants of *Escherichia coli* defective in DNA replication. *Eur. J. Biochem.* **15**, 311-320.

Golub, E.I. (1988) "One minute" transformation of competent *E. coli* by plasmid DNA. *Nucleic Acids Res.* **16**, 1641.

Goshima, F.Y., and Maeno, K. (1989) Persistent infection of MDCK cells by influenza C virus: Initiation and characterization. *J. Gen. Virol.* **70**, 3481-3485.

Goswami, K.K.A., Cameron, R., Russell, W.C., Lange, L.S., and Mitchell, D.N. (1984) Evidence for the persistence of paramyxoviruses in human bone marrows. *J. Gen. Virol.* **65**, 1881-1888.

Gross, P.A., Green, R.H., and McCrea Curnen, M.G. (1973) Persistent infection with parainfluenza type 3 virus in man. *Am. Rev. Respir. Dis.* **108**, 894-898.

Gupta, K.C., and Kingsbury, D.W. (1984) Complete sequences of the intergenic and mRNA start signals in the Sendai virus genome: Homologies with the genome of vesicular stomatitis virus. *Nucleic Acids Res.* **12**, 3829-3841.

Gupta, K.C., and Kingsbury, D.W. (1985) Polytranscripts of Sendai virus do not contain intervening polyadenylate sequences. *Virology* **141**, 102-109.

Guskey, L.E., and Bergtrom, G. (1981) High yield growth and purification of human parainfluenza type 3 virus and initial analysis of viral structural proteins. *J. Gen. Virol.* **54**, 115-123.

Hall, W.W., and Choppin, P.W. (1979) Evidence for lack of synthesis of the M polypeptide of measles virus in brain cells in subacute sclerosing panencephalitis. *Virology* **99**, 443-447.

Hall, W.W., Lamb, R.A., and Choppin, P.W. (1979) Measles and subacute sclerosing panencephalitis virus proteins: Lack of antibodies to the M protein in patients with subacute sclerosing panencephalitis. *Proc. Natl. Acad. Sci. USA* **76**, 2047-2051.

Hall, W.W., Martin, S.J., and Gould, E. (1974) Defective interfering particles produced during the replication of measles virus. *Med. Microbiol. Immunol.* **160**, 155-164.

Hayes, E.C., and Zweernik, H.J. (1978) Measles virus persistent infections: Biological and biochemical studies. In *Persistent viruses*, pp. 759-765. Edited by J.G. Stevens, G.J. Todaro and C.F. Fox. New York: Academic Press, Inc.

Helfman, W.B., and Perrault, J. (1989) Redistributive properties of the vesicular stomatitis virus polymerase. *Virology* **171**, 319-330.

Herler, G., and Compans, R.W. (1982) Synthesis of mumps virus polypeptides in infected Vero cells. *Virology* **119**, 430-438.

Hiebert, S.W., Paterson, R.G., and Lamb, R.A. (1985) Identification and predicted sequence of a previously unrecognized small hydrophobic protein, SH, of the paramyxovirus simian virus 5. *J. Virol.* **55**, 744-751.

Hodes, D.S. (1982) Selection of temperature-sensitive mutants in persistent infection by parainfluenza virus type 3. *J. Gen. Virol.* **60**, 401-404.

Hodes, D.S., Weldy, P.L., and Doundoulakis, J.H. (1979) Establishment of persistent infection by parainfluenza virus type 3: role of a syncytium inhibitor. *J. Gen. Virol.* **44**, 515-523.

Holland, J.J. (1990) Defective viral genomes. In *Virology*, pp. 151-165. Edited by B.N. Fields, D.M. Knipe, R.M. Chanock, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman. New York: Raven Press, Ltd.

Holland, J.J., Grabau, E.A., Jones, C.L., and Semler, B.L. (1979) Evolution of multiple genome mutations during long-term persistent infection by vesicular stomatitis virus. *Cell* **16**, 495-504.

Holland, J.J., Kennedy, S.I.T., Semler, B.L., Jones, C.L., Roux, L., and Grabau, E.A. (1980) Defective interfering RNA viruses and the host cell response. In *Comprehensive Virology*, vol. 16, pp. 137-192. Edited by H. Fraenkel-Conrat and R.R. Wagner. New York: Plenum Press.

Holland, J.J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S., and VandePol, S. (1982) Rapid evolution of RNA genomes. *Science* 215, 1577-1585.

Holland, J.J., and Villarreal, L.P. (1974) Persistent noncytocidal vesicular stomatitis virus infections mediated by defective T particles that suppress virion transcriptase. *Proc. Natl. Acad. Sci. USA* 71, 2956-2960.

Holland, J.J., Villarreal, L.P., and Breindl, M. (1976a) Factors involved in the generation of rhabdovirus defective T particles. *J. Virol.* 17, 805-815.

Holland, J.J., Villarreal, L.P., Welsh, R.M., Oldstone, M.B.A., Kohne, D., Lazzarini, R., and Scolnick, E. (1976b) Long-term persistent vesicular stomatitis virus and rabies virus infection of cells *in vitro*. *J. Gen. Virol.* 33, 193-211.

Homma, M., and Ohuchi, M. (1973) Trypsin action on the growth of Sendai virus in tissue culture cells. 3. Structural difference of Sendai viruses grown in eggs and tissue culture cells. *J. Virol.* 12, 1457-1465.

Horodyski, F.M., and Holland, J.J. (1980) Viruses isolated from cells persistently infected with vesicular stomatitis virus show altered interactions with defective interfering particles. *J. Virol.* 36, 627-631.

Horodyski, F.M., Nichol, S.T., Spindler, K.R., and Holland, J.J. (1983) Properties of DI particle resistant mutants of vesicular stomatitis virus isolated from persistent infections and from undiluted passages. *Cell* 33, 801-810.

Hosaka, Y., Kitano, H., and Ikeguchi, S. (1966) Studies on the pleomorphism of HVJ virions. *Virology* 29, 205-221.

Huang, A.S. (1973) Defective interfering viruses. *Annu. Rev. Microbiol.* 27, 101-117.

Huang, A.S. (1988) Modulation of viral disease processes by defective interfering particles. In *RNA genetics*, vol. 3, pp. 195-208. Edited by E. Domingo, J.J. Holland and P. Ahlquist. Boca Raton: CRC Press, Inc.

Hubbard, S.C., and Ivatt, R.J. (1981) Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 50, 555-583.

Ismail, I.D., and Milner, J.J. (1988) Isolation of defective interfering particles of sonchus yellow net virus from chronically infected plants. *J. Gen. Virol.* 69, 999-1006.

Ito, Y., Tsurudome, M., and Hishiyama, M. (1985) Persistence of mumps virus in mouse L 929 cells. *Microbiol. Immunol.* 29, 847-857.

Jambou, R.C., Elango, N., and Venkatesan., S. (1985) Proteins associated with human parainfluenza virus type 3. *J. Virol.* 56, 298-302.

Jambou, R.C., Elango, N., Venkatesan, S., and Collins, P.L. (1986) Complete sequence of the major nucleocapsid protein gene of human parainfluenza type 3 virus: comparison with other negative strand viruses. *J. Gen. Virol.* 67, 2543-2548.

Johnson, K.M., Chanock, R.M., Cook, M.K., and Huebner, R.J. (1960) Studies of a new human hemadsorption virus. I. Isolation, properties and characterization. *Am. J. Hyg.* 71, 81-92.

Johnson, L.D., and Lazzarini, R.A. (1977) Replication of viral RNA by a defective interfering vesicular stomatitis virus particle in the absence of helper virus. *Proc. Natl. Acad. Sci. USA* 74, 4387-4391.

Jordan, J.A., Whitaker-Dowling, P., and Youngner, J.S. (1989) The L protein of a VSV mutant isolated from a persistent infection is responsible for viral interference and dominance over the wild-type virus. *Virology* 169, 137-141.

Jordan, J.A., and Youngner, J.S. (1987) Dominance of temperature sensitive phenotypes. II. Vesicular stomatitis virus mutants from a persistent infection interfere with shut-off of host protein synthesis by wild-type virus. *Virology* 158, 407-413.

Kanda, T., and Shibuta, H. (1981) A temperature-sensitive mutant of Sendai virus which establishes persistent infection in vero cells without cell crisis. *Virology* 108, 318-324.

Kang, C.Y. (1980) Interference induced by defective interfering particles. In *Rhabdoviruses*, vol. 2, pp. 201-219. Edited by D.H.L. Bishop. Boca Raton: CRC Press, Inc.

Kang, C.Y., and Allen, R. (1978) Host function-dependent induction of defective interfering particles of vesicular stomatitis virus. *J. Virol.* 25, 202-206.

Kang, C.Y., Glimp, T., Clewley, J.P., and Bishop, D.H.L. (1978) Studies on the generation of vesicular stomatitis virus (Indiana serotype) defective interfering particles. *Virology* 84, 142-152.

Kang, C.Y., and Prevec, L. (1969) Proteins of vesicular stomatitis virus. I. Polyacrylamide gel analysis of viral antigens. *J. Virol.* 3, 404-413.

Kang, C.Y., Schubert, M., and Lazzarini, R.A. (1985) Frequent generation of new 3'-defective interfering particles of vesicular stomatitis virus. *Virology* 143, 630-635.

Kawai, A., Matsumoto, S., and Tanabe, K. (1975) Characterization of rabies viruses recovered from persistently infected BHK cells. *Virology* 67, 520-533.

Keene, J.D., Chien, I.M., and Lazzarini, R.A. (1981) Vesicular stomatitis virus defective interfering particle containing a muted internal leader RNA gene. *Proc. Natl. Acad. Sci. USA* 78, 2090-2094.

Kimura, Y., Ito, Y., Shimokata, K., Nishiyama, Y., Nagata, L., and Kitoh, J. (1975) Temperature-sensitive virus derived from BHK cells persistently infected with HVJ (Sendai virus). *J. Virol.* 15, 55-63.

Kingsbury, D.W. (1990) Paramyxoviridae and their replication. In *Virology*, pp. 945-962. Edited by B.N. Fields, D.M. Knipe, R.M. Chanock, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman. New York: Raven Press, Ltd.

Kingsbury, D.W., and Portner, A. (1970) On the genesis of incomplete Sendai virions. *Virology* 42, 872-879.

Kingsbury, D.W., Portner, A., and Darlington, R.W. (1970) Properties of incomplete Sendai virions and subgenomic viral RNAs. *Virology* 42, 857-871.

Kobune, F., Yamanouchi, K., Nagahima, K., and Shishido, A. (1981) Establishment of a persistently infected cell line with rinderpest virus. *Arch. Virol.* 68, 271-277.

Kohama, T., Shimizu, K., and Ishida, N. (1978) Carbohydrate composition of the envelope glycoproteins of Sendai virus. *Virology* 90, 226-234.

Kolakofsky, D. (1976) Isolation and characterization of Sendai virus DI-RNAs. *Cell* 8, 547-555.

Kolakofsky, D. (1982) Isolation of vesicular stomatitis virus defective interfering genomes with different amounts of 5'-terminal complementarity. *J. Virol.* 41, 566-574.

Kolakofsky, D., and Bruschi, A. (1975) Antigenomes in Sendai virions and Sendai virus-infected cells. *Virology* 66, 185-191.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680-685.

Lamb, R.A., and Dreyfuss, G. (1989) Unwinding with a vengeance. *Nature (London)* 337, 19-20.

Lawton, P., Karimi, Z., Mancinelli, L., and Seto, J.T. (1986) Persistent infections with Sendai virus and Newcastle disease viruses. *Arch. Virol.* 89, 225-233.

Lazzarini, R.A., Keene, J.D., and Schubert, M. (1981) Origins of defective interfering particles of the negative-strand RNA viruses. *Cell* 26, 145-154.

Leider, J.M., Palese, P., and Smith, F.I. (1988) Determination of the mutation rate of a retrovirus. *J. Virol.* 62, 3084-3091.

Leppert, M., Kort, L., and Kolakofsky, D. (1977) Further characterization of Sendai virus DI-RNAs: A model for their generation. *Cell* 12, 539-552.

Leppert, M., Rittenhouse, L., Perrault, J., Summers, D.F., and Kolakofsky, D. (1979) Plus and minus strand leader RNAs in negative strand virus-infected cells. *Cell* **18**, 735-747.

Liebert, U.G., Backzo, K., Budka, H., and ter Meulen, V. (1986) Restricted expression of measles virus proteins in brains from cases of subacute sclerosing panencephalitis. *J. Gen. Virol.* **67**, 2435-2444.

Lin, F.H., and Thormar, H. (1980) Absence of M protein in a cell-associated subacute sclerosing panencephalitis virus. *Nature (London)* **285**, 490-492.

Luk, D., Masters,. P.S., Sānchez, Ā., and Banerjee, A.K. (1987) Complete nucleotide sequence of the matrix protein mRNA and three intergenic junctions of human parainfluenza virus type 3. *Virology* **156**, 189-192.

Luk, D., Sānchez, Ā., and Banerjee, A.K. (1986) Messenger RNA encoding the phosphoprotein (P) gene of human parainfluenza virus 3 is bicistronic. *Virology* **153**, 318-325.

Matthews, R.E.F. (1982) Classification and nomenclature of viruses. *Intervirol.* **17**, 1-199.

McCarthy, M., Wolinsky, J.S., and Lazzarini, R.A. (1981) A persistent infection of Vero cells by egg-adapted mumps virus. *Virology* **114**, 343-356.

McCreedy, B.J., Jr., and Lyles, D.S. (1989) Distribution of M protein and nucleocapsid protein of vesicular stomatitis virus in infected cell plasma membranes. *Virus Res.* **14**, 189-206.

McGinnes, L., McQuain, C., and Morrison, T. (1988) The P protein and the nonstructural 38K and 29K proteins of Newcastle disease virus are derived from the same open reading frame. *Virology* **164**, 256-264.

McGowan, J.J., and Wagner, R.R. (1981) Inhibition of cellular DNA synthesis by vesicular stomatitis virus. *J. Virol.* **38**, 356-367.

Meier, E., Harmison, G.G., Keene, J.D., and Schubert, M. (1984) Sites of copy choice replication involved in generation of vesicular stomatitis virus defective-interfering particle RNAs. *J. Virol.* **51**, 515-521.

Meinkoth, J., and Kennedy, S.I.T. (1980) Semliki Forest virus persistence in mouse L929 cells. *Virology* **100**, 141-155.

Messing, J. (1983) New M13 vectors for cloning. *Methods Enzymol.* **101**, 20-78.

Mottet, G., and Roux, L. (1989) Budding efficiency of Sendai virus nucleocapsids: Influence of size and ends of the RNA. *Virus Res.* **14**, 175-188.

Muchmore, H.G., Parkinson, A.J., Humphries, J.E., Scott, E.N., McIntosh, D.A., Scott, L.V., Cooney, M.K., and Miles, J.A.R. (1981) Persistent parainfluenza virus shedding during isolation at the South Pole. *Nature (London)* **289**, 187-189.

Nagata, I., Kimura, Y., Ito, Y., and Tanaka, T. (1972) Temperature-sensitive phenomenon of viral maturation observed in BHK cells persistently infected with HVJ. *Virology* **49**, 453-461.

Nayak, D.P., Chambers, T.M., and Akkina, R.K. (1985) Defective-interfering (DI) RNAs of influenza viruses: Origin, structure, expression, and interference. *Curr. Top. Microbiol. Immunol.* **114**, 103-151.

Nishiyama, Y. (1977) Studies of L cells persistently infected with VSV: Factors involved in the regulation of persistent infection. *J. Gen. Virol.* **35**, 265-279.

Nishiyama, Y., Ito, Y., Shimokata, K. (1978) Properties of the viruses selected during persistent infection of L cells with VSV. *J. Gen. Virol.* **40**, 481-484.

Nishiyama, Y., Ito, Y., Shimokata, K., Kimura, Y., and Nagata, I. (1976) Relationship between establishment of persistent infection of hemagglutinating virus of Japan and the properties of the virus. *J. Gen. Virol.* **32**, 73-83.

Norrby, E., Kristensson, K., Brzosko, W.J., and Kapsenberg, J.G. (1985) Measles virus matrix protein detected by immune fluorescence with momoclonal antibodies in the brain of patients with subacute sclerosing panencephalitis. *J. Virol.* **56**, 337-340.

Norrby, E., Marusyk, H., and Örvell, C. (1970) Morphogenesis of respiratory syncytial virus in a green monkey kidney cell line (Vero). *J. Virol.* **6**, 237-242.

Norrby, E., and Oxman, M.N. (1990) Measles virus. In *Virology*, pp. 1013-1044. Edited by B.N. Fields, D.M. Knipe, R.M. Chanock, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman. New York: Raven Press, Ltd.

O'Hara, P.J., Nichol, S.T., Horodyski, F.M., and Holland, J.J. (1984) Vesicular stomatitis virus defective interfering particles can contain extensive genomic sequence rearrangements and base substitutions. *Cell* **36**, 915-924.

Palma, E.L., and Huang, A. (1974) Cyclic production of vesicular stomatitis virus caused by defective interfering particles. *J. Infect. Dis.* **129**, 402-410.

Paterson, R.G., Harris, T.J.R., and Lamb, R.A. (1984) Analysis of gene assignment of mRNAs of a paramyxovirus, simian virus 5. *Virology*, **138**, 310-323.

Perlman, S.M., and Huang, A.S. (1973) RNA synthesis of vesicular stomatitis virus. V. Interactions between transcription and replication. *J. Virol.* **12**, 1395-1400.

Perrault, J. (1981) Origin and replication of defective interfering particles. *Curr. Top. Microbiol. Immunol.* **93**, 151-207.

Perrault, J., and Semler, B.L. (1979) Internal genome deletions in two distinct classes of defective interfering particles of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* **76**, 6191-6195.

Preble, O.T., and Youngner, J.S. (1972) Temperature-sensitive mutants isolated from L cells persistently infected with Newcastle disease virus. *J. Virol.* **9**, 200-206.

Preble, O.T., and Youngner, J.S. (1973) Selection of temperature-sensitive mutants during persistent infection: role in maintenance of persistent Newcastle disease virus infections of L cells. *J. Virol.* **12**, 481-491.

Pringle, C.R., Shirodaria, P.V., Cash, P., Chiswell, D.J., and Malloy, P. (1978) Initiation and maintenance of persistent infection by respiratory syncytial virus. *J. Virol.* **28**, 199-211.

Prinoski, K., Cote, M.-J., Kang, C.Y., and Dimock, K. (1987) Nucleotide sequence of the human parainfluenza virus 3 matrix protein gene. *Nucleic Acids Res.* **15**, 3182.

Ramseur, J.M., and Friedman, R.M. (1977) Prolonged infection of interferon-treated cells by vesicular stomatitis virus: Possible role of temperature-sensitive mutants and interferon. *J. Gen. Virol.* **37**, 523-533.

Ramseur, J.M., and Friedman, R.M. (1978) Prolonged infection of L cells by vesicular stomatitis virus: Defective-interfering forms and temperature-sensitive mutants as factors in the infection. *Virology* **85**, 253-261.

Ray, R., Brown, V.E., and Compans, R.W. (1985) Glycoproteins oh human parainfluenza virus type 3: Characterization and evaluation as a subunit vaccine. *J. Infect. Dis.* **152**, 1219-1230.

Re, G.G., Gupta, K.C., and Kingsbury, D.W. (1983a) Genomic and copy back 3' termini in Sendai virus defective interfering RNA species. *J. Virol.* **45**, 659-664.

Re, G.G., Gupta, K.C., and Kingsbury, D.W. (1983b) Sequence of the 5' end of the Sendai virus genome and its variable representation in complementary form at the 3' ends of copy-back defective interfering RNA species: Identification of the L gene terminus. *Virology* **130**, 390-396.

Re, G.G., and Kingsbury, D.W. (1986) Nucleotide sequences that affect replicative and transcriptional efficiencies of Sendai virus deletion mutants. *J. Virol.* **58**, 578-582.

Re, G.G., and Kingsbury, D.W. (1988) Paradoxical effects of Sendai virus DI RNA size on survival: Inefficient envelopment of small nucleocapsids. *Virology* **165**, 331-337.

Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**, 237-251.

Rima, B. K., Davidson, W. B., and Martin, S. J. (1977) The role of defective interfering particles in persistent infection of Vero cells by measles virus. *J. Gen. Virol.* **35**, 89-97.

Robinson, W.S. (1971) Sendai virus RNA synthesis and nucleocapsid formation in the presence of cyclohexamide. *Virology* **44**, 494-502.

Roman, J.M., and Simon, E.H. (1976) Defective Interfering particles in monolayer-propagated Newcastle disease virus. *Virology* **69**, 298-303.

Roux, L., and Holland, J.J. (1979) Role of defective interfering particles of Sendai virus in persistent infections. *Virology* **93**, 91-103.

Roux, L., and Holland, J.J. (1980) Viral genome synthesis in BHK 21 cells persistently infected with Sendai virus. *Virology* **100**, 53-64.

Roux, L., and Waldvogel, F. A. (1982) Instability of the viral M protein in BHK-21 cells persistently infected with Sendai virus. *Cell* **28**, 293-302.

Rowlands, D., Grabau, E., Spindler, K., Jones, C., Semler, B., and Holland, J. (1980) Virus protein changes and RNA termini alterations evolving during persistent infection. *Cell* **19**, 871-880.

Rozenblatt, S., Gorecki, M., Shure, H., and Prives, C.L. (1979) Characterization of measles virus-specific proteins synthesized *in vivo* and *in vitro* from acutely and persistently infected cells. *J. Gen. Virol.* **29**, 1099-1106.

Rustigian, R. (1962) A carrier state in HeLa cells with measles virus (Edmonston strain) apparently associated with noninfectious virus. *Virology* **16**, 101-104.

Rustigian, R. (1966) Persistent infection of cells in culture by measles virus. I. Development and characterization of HeLa sublines persistently infected with complete virus. *J. Bacteriol.* **92**, 1792-1804.

Sabatini, D.D., Kreibich, G., Morimoto, T., and Milton, A. (1982) Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* **92**, 1-22.

Sānchez, Ā., and Banerjee, A.K. (1985) Studies on human parainfluenza virus 3: Characterization of the structural proteins and *in vitro* synthesized proteins coded by mRNAs isolated from infected cells. *Virology* **143**, 45-54.

Sānchez, Ā., Banerjee, A.K., Furuichi, Y., and Richardson, M.A. (1986) Conserved structures among the nucleocapsid proteins of the paramyxoviridae: Complete nucleotide sequence of human parainfluenza virus type 3 NP mRNA. *Virology* **152**, 171-180.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Scheid, A., Caliguiri, L.A., Compans, R.W., and Choppin, P.W. (1972) Isolation of paramyxovirus glycoproteins. Association of both hemagglutinating and neuraminidase activities with the larger SV5 glycoprotein. *Virology* **50**, 640-652

Scheid, A., and Choppin, P.W. (1974) Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology* **57**, 475-490.

Semler, B.L., and Holland, J.J. (1979) Persistent vesicular stomatitis virus infection mediates base substitutions in viral RNA termini. *J. Virol.* **32**, 420-428.

Sheppard, R.D., Raine, C.S., Bornstein, M.B., and Udem, S.A. (1986) Rapid degradation restricts measles virus matrix protein expression in a subacute sclerosing panencephalitis cell line. *Proc. Natl. Acad. Sci. USA* **83**, 7913-7917.

Shioda, T., Iwasaki, K., and Shibuta, H. (1986) Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L proteins. *Nucleic Acids Res.* **14**, 1545-1563.

Singer, F.R., and Mills, B.G. (1983) Evidence for a viral etiology of Paget's disease of bone. *Clin. Orthop.* **178**, 245-251.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-513.

Spindler, K.R., Horodyski, F.M., and Holland, J.J. (1982) High multiplicities of infection favor rapid and random evolution of vesicular stomatitis virus. *Virology* **119**, 96-108.

Spriggs, M.K., and Collins, P.L. (1986a) Human parainfluenza virus type 3: Messenger RNAs, polypeptide coding assignments, intergenic sequences, and genetic map. *J. Virol.* **59**, 646-654.

Spriggs, M. K., and Collins, P. L. (1986b) Sequence analysis of the P and C protein genes of human parainfluenza virus type 3: patterns of amino acid sequence homology among paramyxovirus proteins. *J. Gen. Virol.* **67**, 2705-2719.

Spriggs, M.K., Johnson, P.R., and Collins, P.L. (1987) Sequence analysis of the matrix protein gene of human parainfluenza virus type 3: Patterns of amino acid sequence homology among paramyxovirus proteins. *J. Gen. Virol.* **68**, 1491-1497.

Spriggs, M.K., Olmstead, R.A., Venkatesan, S., Coligan, J.E., and Collins, P.L. (1986). Fusion glycoprotein of human parainfluenza virus type 3: Nucleotide sequence of the gene, direct identification of the cleavage activation site, and comparison with other paramyxoviruses. *Virology* **152**, 241-251.

Steinhauer, D.A., de la Torre, J.C., and Holland, J.J. (1989a) High nucleotide substitution error frequencies in clonal pools of vesicular stomatitis virus. *J. Virol.* **63**, 2063-2071.

Steinhauer, D.A., de la Torre, J.C., Meier, E., and Holland, J.J. (1989b) Extreme heterogeneity in populations of vesicular stomatitis virus. *J. Virol.* **63**, 2072-2080.

Storey, D.G., Côté, M.-J., Dimock, K., and Kang, C.Y. (1987) Nucleotide sequence of the coding and flanking regions of the human parainfluenza virus 3 hemagglutinin-neuraminidase gene: comparison with other paramyxoviruses. *Intervirol.* **27**, 69-80.

Storey, D.G., Dimock, K., and Kang, C.Y. (1984) Structural characterization of virion proteins and genomic RNA of human parainfluenza virus 3. *J Virol.* **52**, 761-766.

Takeuchi, K., Hishiyama, M., Yamada, A., and Sugiura, A. (1988) Molecular cloning and sequence analysis of the mumps virus gene encoding the P protein: Mumps virus P gene is monocistronic. *J. Gen. Virol.* **69**, 2043-2049.

Tautz, D., and Renz, M. (1983) An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Anal. Biochem.* **132**, 14-19.

ter Meulen, V., and Martin, S.J. (1976) Genesis and maintenance of a persistent infection by canine distemper virus. *J. Gen. Virol.* **32**, 431-440.

Thomas, P. (1983) Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. In *Methods in Enzymology*, vol. 100, pp. 255-266. Edited by R. Wu, L. Grossman and K. Moldave. New York: Academic Press, Inc.

Thomas, S.M., Lamb, R.A., and Paterson, R.G. (1988) Two mRNAs that differ by two non-templated nucleotides encode the amino co-terminal proteins P and V of the paramyxovirus SV5. *Cell* **54**, 891-902.

Toyoda, T., Hamaguchi, M., and Nagai, Y. (1987) Detection of polycistronic transcripts in Newcastle disease virus infected cells and identification of their sequence content. *Arch. Virol.* **95**, 97-110.

Truant, A. L., and Hallum, J. V. (1977) A persistent infection of baby hamster kidney-21 cells with mumps virus and the role of temperature sensitive variants. *J. Med. Virol.* 1, 49-67.

Tuffereau, C., and Roux, L. (1988) Direct adverse effects of Sendai virus DI particles on virus budding and on M protein fate and stability. *Virology* 162, 417-426.

Vidal, S., Curran, J., and Kolakofsky, D. (1990) Editing of the Sendai virus P/C mRNA by G insertion occurs during mRNA synthesis via a virus-encoded activity. *J. Virol.* 64, 239-246.

Vidal, S., and Kolakofsky, D. (1989) Modified model for the switch from Sendai virus transcription to replication. *J. Virol.* 63, 1951-1958.

von Magnus, P. (1954) Incomplete form of influenza virus. *Adv. Virus Res.* 2, 59-79.

Wagner, R.W., Smith, J.E., Cooperman, B.S., and Nishikura, K. (1989) A double-stranded RNA unwinding activity introduces structural alterations by means of adenosine to inosine conversions in mammalian cells and Xenopus eggs. *Proc. Natl. Acad. Sci. USA* 86, 2647-2651.

Walker, D.L., and Hinze, H.C. (1962) A carrier state of mumps virus in human conjunctiva cells. I. General characteristics. *J. Exp. Med.* 116, 739-750.

Ward, C.D., Stokes, M.A.M., and Flanegan, J.B. (1988) Direct measurement of the poliovirus RNA polymerase error frequencies at selected single base sites in viral RNA. *J. Virol.* 62, 558-562.

Waterson, A.P., Jensen, K.E., Tyrrell, D.A.J., and Horne, R.W. (1961) The structure of parainfluenza 3 virus. *Virology* 14, 374-378.

Wechsler, S.L., Lambert, D.M., Galinski, M.S., Heineke, B.E., and Pons, M.W. (1985b) Human parainfluenza virus 3: Purification and characterization of subviral components, viral proteins and viral RNA. *Virus Res.* 3, 339-351.

Wechsler, S.L., Lambert, D.M., Galinski, M.S., Mink, M.A., Rochovansky, O., and Pons, M.W. (1987) Immediate persistent infection by human parainfluenza virus 3: unique fusion properties of the persistently infected cells. *J. Gen. Virol* 68, 1737-1748.

Wechsler, S.L., Lambert, D.M., Galinski, M.S., and Pons, M.W. (1985a) Intracellular synthesis of human parainfluenza type 3 virus-specified polypeptides. *J. Virol.* 54, 661-664.

Wechsler, S.L., Rustigian, R., Stallcup, K.C., Byers, K.B., Winston, S.H., and Fields, B.N. (1979a) Measles virus-specified polypeptide synthesis in two persistently infected HeLa cell lines. *J. Virol.* 31, 677-684.

Wechsler, S.L., Weiner, H.L., and Fields, B.N. (1979b) Immune response in subacute sclerosing panencephalitis: Reduced antibody response to the matrix protein of measles virus. *J. Immunol.* **123**, 884-889.

Week, P.K., Carroll, A.R., Shattuck, D.M., and Wagner, R.R. (1979) Use of U.V. irradiation to identify the genetic information of vesicular stomatitis virus responsible for shutting off cellular RNA synthesis. *J. Virol.* **30**, 746-753.

Wertz, G.W., and Levine, M. (1973) RNA synthesis by vesicular stomatitis virus and a small plaque mutant: Effects of cyclohexamide. *J. Virol.* **12**, 253-264.

Whitaker-Dowling, P., and Youngner, J.S. (1987) Viral interference-dominance of mutant viruses over wild-type virus in mixed infections. *Microbiol. Rev.* **51**, 179-191.

Whitaker-Dowling, P., and Youngner, J.S. (1988) Alteration of vesicular stomatitis virus L and NS proteins by uv irradiation: Implications for the mechanism of host cell shut-off. *Virology* **164**, 171-175.

Wild, T.F., Bernard, A., and Greenland, T. (1981) Measles virus: evolution of a persistent infection in BGM cells. *Arch. Virol.* **67**, 297-308.

Wild, T.F., and Bijlenga, G. (1981) A rabies virus persistent infection in BHK21 cells. *J. Gen. Virol.* **57**, 169-177.

Wilde, A., McQuain, C., and Morrison, T. (1986) Identification of the sequence content of four polycistronic transcripts synthesized in Newcastle disease virus infected cells. *Virus Res.* **5**, 77-95.

Wilde, A., and Morrison, T. (1984) Structural and functional characterization of Newcastle disease virus polycistronic RNA species. *J. Virol.* **51**, 71-76.

Wong, T.C., Ayata, M., Hirano, A., Yoshikawa, Y., Tsuruoka, H., and Yamanouchi, K. (1989) Generalized and localized biased hypermutation affecting the matrix gene of a measles virus strain that causes subacute sclerosing panencephalitis. *J. Virol.* **63**, 5464-5468.

Yoshida, T., Hamaguchi, M., Naruse, H., and Nagai, Y. (1982) Persistent infection by a temperature-sensitive mutant isolated from a Sendai virus (HVJ) carrier culture: its initiation and maintenance without aid of defective interfering particles. *Virology* **120**, 329-339.

Yoshida, T., Hamaguchi, M., Naruse, H., Nishikawa, K., and Nagai, Y. (1983) Characterization of the virus isolated from HeLa cells persistently infected with Sendai virus (HVJ). *Microbiol. Immunol.* **27**, 207-211.

Yoshima, H., Nakanishi, M., Okada, Y., and Kobata, A. (1981) Carbohydrate structures of HVJ (Sendai virus) glycoproteins. *J. Biol. Chem.* **256**, 5355-5361.

Young, K.K.Y., Heinecke, B.E., and Wechsler, S.L. (1985) M protein instability and lack of protein processing associated with nonproductive persistent infection of HeLa cells by measles virus. *Virology* 143, 536-545.

Youngner, J.S., Dubovi, E.J., Quagliana, D.O., Kelly, M., and Preble, O.T. (1976) Role of temperature-sensitive mutants in persistent infections initiated with vesicular stomatitis virus. *J. Virol.* 19, 90-101.

Youngner, J.S., and Preble, O.T. (1980) Viral persistence: Evolution of viral populations. In *Comprehensive Virology*, vol. 16, pp. 73-135. Edited by H. Fraenkel-Conrat and R.R. Wagner. New York: Plenum Press.

Youngner, J.S., Preble, O.T., and Jones, E.V. (1978) Persistent infection of L cells with vesicular stomatitis virus: Evolution of virus populations. *J. Virol.* 28, 6-13.

APPENDIX A

PASSAGE HISTORY OF PI3/MK_2-P0 AND PI3/MK_2-P8 CELLS

Date	Cell lines		Passage number	Days postinfection
	PI3/MK_2-P0	PI3/MK_2-P8		
12-11-85	+	+	0	0
20-11-85		+	1	8
26-11-85		+	2	14
27-11-85	+		1	15
30-11-85		+	3	18
06-12-85	+		2	24
12-12-85		+	4	30
19-12-85	+		3	37
		+	5	37
06-01-86	+		4	54
		+	6	54
09-01-86	+		5	57
13-01-86	+		6	61
		+	7	61
17-01-86	+		7	65
20-01-86	+		8	68
22-01-86		+	8	70
23-01-86	+		9	71
27-01-86		+	9	75
29-01-86	+		10	77
06-02-86	+	+	11	85

11-02-86		+	12	90
12-02-86	+		12	91
14-02-86		+	13	93
20-02-86	+		13	99
		+	14	99
26-02-86	+		14	105
		+	15	105
05-03-86	+		15	112
		+	16	112
10-03-86	+		16	117
11-03-86		+	17	118
17-03-86	+		17	124
21-03-86	+		18	128
		+	18	128
25-03-86	+		19	132
26-03-86		+	19	133
31-03-86		+	20	138
01-04-86	+		20	139
10-04-86		+	21	148
11-04-86	+		21	149
16-04-86	+	+	22	154
22-04-86	+		23	160
24-04-86		+	23	162
25-04-86	+		24	163
29-04-86	+		25	167
02-05-86	+		26	170
		+	24	170

07-05-86	+		27	175
		+	25	175
13-05-86	+		28	181
13-05-86		+	26	181
20-05-86	+		29	188
		+	27	188
23-05-86	+		30	191
27-05-86	+		31	195
		+	28	195
02-06-86	+		32	201
		+	29	201
04-06-86	+		33	203
		+	30	203
10-06-86	+		34	209
		+	31	209
13-06-86	+		35	212
		+	32	212
19-06-86	+		36	218
		+	33	218
26-06-86	+		37	225
		+	34	225
03-07-86	+		38	232
		+	35	232
08-07-86	+		39	237
08-07-86		+	36	237
10-07-86	+		40	239
		+	37	239

18-07-86	+		41	247
		+	38	247
24-07-86	+		42	253
		+	39	253
31-07-86	+		43	260
		+	40	260
07-08-86	+		44	267
		+	41	267
14-08-86	+		45	274
		+	42	274
19-08-86	+		46	279
		+	43	279
21-08-86	+		47	281
		+	44	281
25-08-86	+		48	285
		+	45	285
02-09-86	+		49	293
		+	46	293
06-09-86	+		50	297
		+	47	297
11-09-86	+		51	302
		+	48	302
15-09-86	+		52	306
		+	49	306
18-09-86	+		53	309
		+	50	309

24-09-86	+		54	315
		+	51	315
29-09-86	+		55	320
		+	52	320
03-10-86	+		56	325
		+	53	325
09-10-86	+		57	331
		+	54	331
14-10-86	+		58	336
		+	55	336
20-10-86	+		59	342
		+	56	342
29-10-86	+		60	351
		+	57	351
01-11-86		+	58	354
04-11-86	+		61	357
		+	59	357
O7-11-86		+	60	360
10-11-86	+		62	363
		+	61	363
14-11-86	+		63	367
		+	62	367
19-11-86	+		64	372
		+	63	372
24-11-86	+		65	377
		+	64	377
28-11-86	+		66	381

28-11-86		+	65	381
01-12-86	+		67	384
		+	66	384
05-12-86	+		68	388
05-12-86		+	67	388
08-12-86		+	68	391
11-12-86	+	+	69	394
15-12-86	+	+	70	398
19-12-86	+	+	71	402
22-12-86	+	+	72	405
02-01-87	+	+	73	416
05-01-87	+	+	74	419
09-01-87	+	+	75	423
15-01-87	+	+	76	429
21-01-87	+	+	77	435
27-01-87	+	+	78	441
02-02-87	+	+	79	447
06-02-87	+	+	80	451
13-02-87	+	+	81	458
19-02-87	+	+	82	464
27-02-87	+	+	83	472
06-03-87	+	+	84	479
13-03-87	+	+	85	486
20-03-87	+	+	86	493
23-03-87	+	+	87	496
30-03-87	+	+	88	503
02-04-87	+	+	89	506

08-04-87	+	+	90	512
14-04-87	+	+	91	518
22-04-87	+	+	92	526
27-04-87	+	+	93	531
04-05-87	+	+	94	538
11-05-87	+	+	95	545
21-05-87	+	+	96	555
27-05-87	+	+	97	561
02-06-87	+	+	98	567
08-06-87	+	+	99	573
13-06-87	+	+	100	578
17-06-87	+	+	101	582
23-06-87	+	+	102	588
29-06-87	+	+	103	594
06-07-87	+	+	104	601
13-07-87	+	+	105	608
20-07-87	+	+	106	615
24-07-87	+	+	107	619
28-07-87	+	+	108	623
31-07-87	+	+	109	626
07-08-87	+	+	110	633
17-08-87	+	+	111	643
24-08-87	+	+	112	650
31-08-87	+	+	113	657
04-09-87	+	+	114	661
11-09-87	+	+	115	668
15-09-87	+	+	116	672

21-09-87	+	+	117	678
25-09-87	+	+	118	682
02-10-87	+	+	119	689
09-10-87	+	+	120	696
16-10-87	+	+	121	703
21-10-87	+	+	122	708
26-10-87	+	+	123	713
29-10-87	+	+	124	716
02-11-87	+	+	125	720
09-11-87	+	+	126	727
16-11-87	+	+	127	734
23-11-87	+	+	128	741
30-11-87	+	+	129	748
07-12-87	+	+	130	755
14-12-87	+	+	131	762
21-12-87	+	+	132	769
28-12-87	+	+	133	776
04-01-88	+	+	134	783
11-01-88	+	+	135	790
18-01-88	+	+	136	797
25-01-88	+	+	137	804
01-02-88	+	+	138	811
08-02-88	+	+	139	818
15-02-88	+	+	140	825
22-02-88	+	+	141	832
01-03-88	+	+	142	840
08-03-88	+	+	143	847

14-03-88	+	+	144	853
21-03-88	+	+	145	860
28-03-88	+	+	146	867
04-04-88	+	+	147	874
11-04-88	+	+	148	881
18-04-88	+	+	149	888
25-04-88	+	+	150	895
02-05-88	+	+	151	902
09-05-88	+	+	152	909
16-05-88	+	+	153	916
25-05-88	+	+	154	925
01-06-88	+	+	155	932
09-06-88	+	+	156	940
20-06-88	+	+	157	951
29-06-88	+	+	158	960
07-07-88	+	+	159	968
19-07-88	+	+	160	980
25-07-88	+	+	161	986
02-08-88	+	+	162	994
08-08-88	+	+	-	1000

APPENDIX B

ASSAY OF SUPERNATANT FROM PI3/MK_2-P0 AND PI3/MK_2-P8 CULTURES FOR PLAQUE FORMATION IN LLC-MK_2 CELLS

Cell line	Passage number
PI3/MK_2-P0	1, 12, 26, 38, 47, 99, 100, 116, 118
PI3/MK_2-P8	4, 13, 24, 42, 46, 99, 100, 116, 118

APPENDIX C

OLIGONUCLEOTIDE SEQUENCES

Primers used for reverse transcription and PCR

DM7	5'-CTC GAG CTC GAA GAA GTA TCT GAA TTG-3'
DM8	5'-CTT AAG CTT TAC AAC TAC ATG CTG TAG-3'
DM14	5'-CTC GAG CTC ACC AAA CAA GAG AAG-3'
DM15	5'-CTT AAG CTT AAG ACA CCA AGA ACC-3'

Primers used for sequencing M gene and flanking regions

DM9	5'-CTT TCT TGT TCC AGC GAG TGT GGC-3'
DM10	5'-CCA TTA AGG GAT AAC AAA TCT CCC-3'
DM11	5'-CCC TGT TTT GAT GTG TAC CTG CAG-3'
DM12	5'-CGC TTT GAC TGT TCT TCT CAC TTC-3'
DM13	5'-GAG TGG TAA TGG TTC TAT ATG ACC-3'

PUBLICATIONS AND ABSTRACTS

PUBLICATIONS

Murphy, D.G., Dimock, K., and Kang, C.Y. (1987) Defective interfering particles of human parainfluenza virus 3. *Virology* **158**, 439-443.

Murphy, D.G., Dimock, K., and Kang, C.Y. (1989) Requirements for the establishment and maintenance of human parainfluenza virus 3 persistent infections. In *Genetics and Pathogenicity of Negative Strand Viruses*, pp. 348-355. Edited by D. Kolakofsky and B.W.J. Mahy. New York, Amsterdam & Oxford: Elsevier Biomedical Press.

Murphy, D.G., Dimock, K., and Kang, C.Y. (1990) Viral RNA and protein synthesis in two LLC-MK_2 cell lines persistently infected with human parainfluenza virus 3. *Virus Res.* **16**, 1-16.

Murphy, D.G., Dimock, K., and Kang, C.Y. Numerous U to C and A to G transitions in human parainfluenza virus 3 RNA recovered from persistently infected cells. Manuscript submitted.

ABSTRACTS

Murphy, D.G., Dimock, K., and Kang, C.Y. (1986) Defective interfering particles of human parainfluenza virus 3. 5th Annual Meeting of the American Society of Virology.

Murphy, D.G., Dimock, K., and Kang, C.Y. (1987) "Les particules interferentes defectueuses du virus humain parainfluenza 3". 55th Congress of the "Association Canadienne-Francaise pour l'Avancement des Sciences". p. 224.

Murphy, D.G., Dimock, K., and Kang, C.Y. (1987) Defective interfering particles of human parainfluenza virus 3. 7th International Congress of Virology. R25.14, p. 188.

Murphy, D.G., Dimock, K., and Kang, C.Y. (1988) Characterization of human parainfluenza virus 3 recovered from persistently infected cells. 7th Annual Meeting of the American Society of Virology.

Murphy, D.G., Dimock, K., and Kang, C.Y. (1988) Characterization of human parainfluenza virus 3 recovered from persistently infected cells. Genetics and Pathogenicity of Negative Strand Viruses. *Virus Res.* supp. 2, p. 49.

Murphy, D.G., Dimock, K., and Kang, C.Y. (1989) Human parainfluenza virus 3 persistent infections in LLC-MK_2 cells: characterization of viral RNA and proteins. 39th Annual Meeting of the Canadian Society of Microbiologists. V 13, p. 84.