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ANALYSIS OF GENE JUNCTION SEQUENCES OF HUMAN PARAINFLUENZA VIRUS TYPE 3

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A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Transcription of the human parainfluenza virus type 3 (hPIV3) genome occurs by a sequential stop-start mechanism which is directed by short conserved sequence elements found at the boundary of the each of the hPIV3 genes, yielding several monocistronic mRNAs. The general aim of this project was to begin to elucidate the role of these cis acting transcription regulatory sequences. A technique called minigenome rescue, which allows the in vitro manipulation and analysis of cDNA representing minigenome analogs of the hPIV3 genome, was used. Plasmids were constructed containing cDNAs in which the open reading frames (*orf*) of one chloramphenicol acetyltransferase (CAT) or two reporter genes (luciferase and CAT) were flanked by hPIV3 terminal sequences necessary for replication, transcription and encapsidation. In bicistronic cDNAs, natural or mutated hPIV3 gene junction sequences were introduced between the open reading frames of the two reporter genes, with the luciferase *orf* 3' to the CAT *orf* (in the negative-sense minigenome). Plasmids were transfected into vaccinia vTF7-3- infected HEp-2 cells along with other plasmids encoding the proteins necessary for encapsidation and transcription. Transfected cells were then tested for their ability to transcribe the two reporter genes by monitoring enzyme assay (Luc and CAT) and/or Northern blot analysis.

The requirement of the rule of six for efficient rescue of hPIV3 minigenomes was verified using a series of monocistronic cDNAs . Minigenomes whose total length was a

multiple of six nt (6n) were rescued more efficiently than minigenomes that were not 6n nt in length, as assayed by CAT activity. The importance of the positioning or phasing of the CAT mRNA transcriptional start site within the hexamer protected by each NP (Nucleoprotein) molecule in the nucleocapsid was also tested. CAT activity data showed that, in bicistronic minigenomes, the phasing of the transcriptional start site has little effect on the level of expression of the downstream gene.

The abilities of bicistronic minigenomes with wild type or mutant versions of the hPIV3 gene junctions to direct CAT expression were also compared. Deletion or reiteration of the intergenic trinucleotide in the NP/P (junction sequence between the NP and the P gene) junction abolished CAT gene expression, indicating a critical role for the intergenic sequence in efficient transcription termination and downstream gene transcription. As confirmed by both reporter gene assays and Nortern blot analysis, the M/F gene junction, which contains an additional 8 nucleotides in the M gene end sequence, exhibited a high frequency of transcriptional readthrough. Removal of the eight extra nt from the M/F junction reduced the frequency of transcriptional readthrough to the level seen for the other hPIV3 gene junctions. Additional evidence indicated that M or F gene sequences upstream or downstream of the M/F junction do not contribute to transcriptional readthrough at this junction. Thus, the 8 additional nt are necessary and sufficient to direct readthrough transcription at the M/F junction.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
β-Gal	beta galactosidase
bp	base pairs
bPIV3	bovine parainfluenza virus 3
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complememtary DNA
CV-1	African green monkey kidney cell line
DEPC	diethyl pyrocarbonate
DI	defective interfering
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
F	fusion protein
FBS	fetal bovine serum
GE	Gene End Sequence
GS	Gene Start Sequence
HDV	Hepatitis delta virus
HEp-2	human epidermal carcinoma cell lines
hPIV1	human parainfluenza virus type1
hPIV3	human parainfluenza virus type 3
HN	haemagglutinin-neuraminidase protein
IG	Intergenic Sequence
L	large polymerase subunit
LB	Luria- Bertani
LLC-MK2	Rhesus monkey kidney cell line
Luc	luciferase

M	Matrix protein
MV	Measles Virus
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NDV	Newcastle disease virus
NP	nucleoprotein
nt	nucleotide
orf	open reading frame
pfu	plaque forming units
P	polymerase associated phosphoprotein
RNP	ribonucleoprotein
RSV	respiratory syncytial virus
RT	Readthrough transcription
SDS	sodium dodecyl sulphate
SeV	Sendai Virus
SSC	sodium chloride/sodium citrate buffer
SSPE	Sodium chloride/sodium phosphate/ EDTA buffer
STET	sucrose/tris/EDTA/triton-X buffer
SV5	Simian virus 5
SV41	Simian virus 41
TBE	tris/borate/EDTA buffer
TBS	tris buffered saline
TE	tris/EDTA buffer
TLC	thin layer chromatography
VSV	Vesicular stomatitis virus
vTF7-3	Recombinant Vaccinia Virus encoding T7 RNA Polymerase
VWR	Vaccinia virus strain WR

I. INTRODUCTION

A. Overview

Negative strand RNA viruses are a large, diverse group of enveloped viruses of both medical and economic significance. They include members such as influenza viruses, measles virus, mumps virus, respiratory syncytial virus, parainfluenza viruses, rabies virus, Ebola virus and hantaviruses. They are found in hosts from plant and animal kingdoms, and have a wide range of biological properties and genome organization.

The negative sense genomic RNA of these viruses is not translated in vivo (or in vitro) nor is the purified genomic RNA infectious. The genomes of these viruses are complexed with the viral nucleoprotein (NP) as ribonucleoprotein (RNP) complexes which are the actual templates recognized by the viral RNA polymerases. The virion carries its own RNA dependent RNA polymerase, which is responsible for the transcription and replication of the viral genome in the infected cell. Replication involves the synthesis of a replicative intermediate consisting of a complementary copy of the genome, known as the antigenome, which is also encapsidated and used for synthesis of genomic RNA. Viral RNA polymerase also uses the genome as a template to synthesize viral mRNAs. The fact that negative sense RNAs are not infectious and the inability to assemble infectious ribonucleoproteins in vitro from cloned sequences made the generation of negative sense viruses for many years impossible. In addition, the absence of homologous recombination during the replication cycle of negative sense RNA viruses eliminated the possibility of introducing novel sequences or mutations into the genome, as can be done with positive sense RNA viruses. The negative sense RNA viruses were refractory to these techniques until the 1990s when

specific reverse genetics techniques were designed to introduce specific mutations into the genomes of these viruses. The recent development of these techniques allows the in vitro manipulation of cDNA representing analogs of negative sense RNA virus genomes and their rescue into infectious virus particles. Rescue technology permits defined genetic studies of negative sense RNA viruses and will assist the development of engineered vaccine strains. Another application of these techniques is the analysis of events surrounding transcription and replication of the negative sense RNA viruses.

This thesis examines the role of cis acting sequences in the human parainfluenza virus type 3 (hPIV3) genome in the regulation of transcription.

B. *Paramyxoviridae*

The members of the family *Paramyxoviridae* have an affinity for mucoprotein (myxo) receptors on erythrocytes. Structurally, they resemble members of the family *Orthomyxoviridae* (which includes the influenza viruses) and hence their family name (Lamb and Kolakofsky, 1996). Virus particles are pleiomorphic, ranging from 150-300 nm in diameter (Chanock and McIntosh, 1990). Their host range is restricted to warm blooded vertebrates. The family *Paramyxoviridae* is divided into two sub families, *Paramyxovirinae* and *Pneumovirinae*. The *Paramyxovirinae* comprises three genera, *Respiroviruses*, including Sendai Virus (SeV) and human and bovine parainfluenza virus type 3 (hPIV3 and bPIV3); *Morbilliviruses* (e.g., measles and canine distemper viruses); and *Rubulaviruses* (e.g. mumps virus, Newcastle disease virus [NDV] and simian virus 5[SV5]).

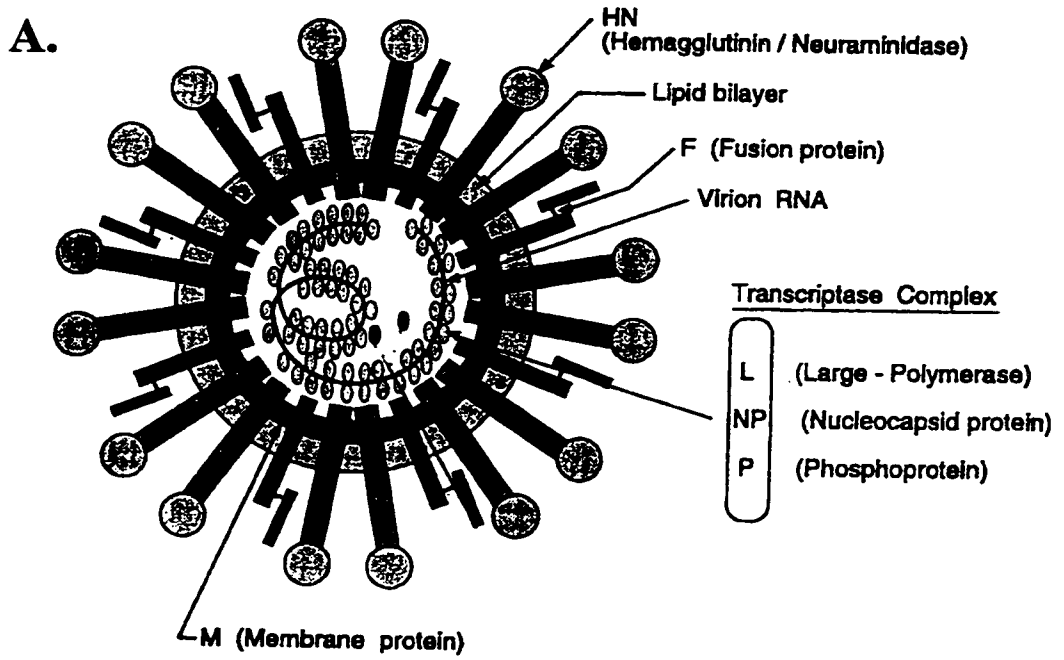
C. Human parainfluenza virus type 3

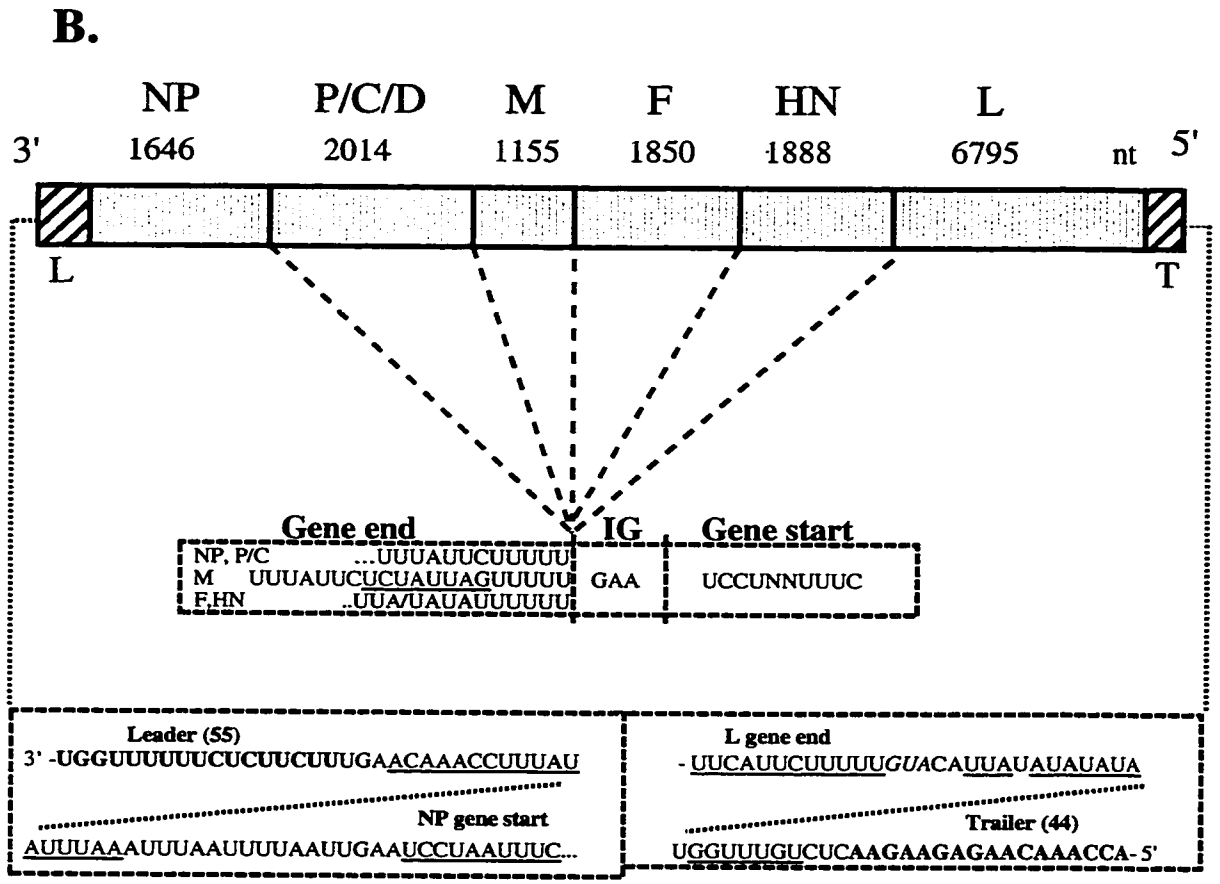
As mentioned above, hPIV3 is a member of the genus *Paramyxovirus*, which also contains parainfluenza types 1, 2 and 4. hPIV3 is the most virulent of the four types (Sanchez *et al.* 1986) and is an important respiratory pathogen of infants, young children and the immunocompromised, second only to RSV in this regard (Wright, 1991). It is involved in lower respiratory tract infections and is a significant cause of croup, bronchiolitis and pneumonia in infants and children less than one year of age (Chanock and McIntosh, 1990). The primary infection rate of hPIV3 is 60-70 % during the first thirty months of life (Bratt and Hightower, 1977). There is currently no approved vaccine or therapy for the prevention or treatment of infections caused by hPIV3.

i)Virion morphology

Virions (Figure 1) are bounded by a lipid bilayer bearing spike-like surface projections composed of the haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. These surface glycoproteins are each anchored in the viral envelope (or in the plasma membrane of the infected cell) by a hydrophobic transmembrane domain, with most of each protein oriented towards the outside and leaving small tails at the inner face of the membrane (Lamb and Kolakofsky, 1996). These tails are thought to play a role in virus assembly through their interaction with the matrix (M) protein (Sanderson *et al.*, 1993), which in turn, interacts with nucleocapsids. The surface glycoproteins confer biological activities to virion preparations. The HN protein mediates adsorption by attachment to sialic acid containing glycoproteins or gangliosides on cells, resulting in virus attachment,

Figure 1 **Schematic of the hPIV3 virion and genome organization** (adapted from Fields and Knipe, 1996). **A)** The hPIV3 virion. Virion components are labelled as follows: nucleocapsid protein, NP; polymerase-associated phosphoprotein, P; matrix protein, M; haemagglutinin-neuraminidase glycoprotein, HN; fusion glycoprotein, F; large polymerase subunit, L.
B) hPIV3 genome organization (3' to 5', negative sense). Genes are shown in the order in which they appear along the genome. The number above each gene refers to the length (in nucleotides) of the transcribed sequences of that gene. In the upper box, the gene end, intergenic (IG) and gene start sequences at the gene junctions are shown. The 8 nt interruption in the M gene end sequence is underlined. In the lower boxes, the sequences of the extracistronic 55 nt leader and 44 nt 5' trailer regions and the adjoining NP gene start or L gene end transcription signals are underlined. Terminal complementarity between the leader and trailer is bold.


A.
HN
(Hemagglutinin / Neuraminidase)
Lipid bilayer
F (Fusion protein)
Virion RNA
Transcriptase Complex
L
NP
P
(Large - Polymerase)
(Nucleocapsid protein)
(Phosphoprotein)
M (Membrane protein)


B.
NP
P/C/D
M
F
HN
L
3'
1646
2014
1155
1850
1888
6795
nt
5'
L
T
Gene end
IG
Gene start
NP, P/C ...UUUAUUCUUUUU
M UUUAUUCUCUAUUAGUUUUU
F,HN ..UUA/UAUAUUUUUU
GAA
UCCUNNUUUC
Leader (55)
3' -UGGUUUUUUCUCUUCUUUGAACAAACCUUUAU
NP gene start
AUUUAAAUUUAAUUUUAAUUGAAUCCUAAUUUC...
L gene end
- UUCAUUCUUUUUGUACAUUAUAUAUAUA
Trailer (44)
UGGUUUGUCUCAAGAAGAGAACAAACCA-5'

agglutination of erythrocytes (hemadsorption and haemagglutination), and the cleavage of sialic acid (neuraminidase). The F protein mediates the lysis of adsorbed erythrocytes (hemolysis), viral entry into cells and cell to cell fusion (Choppin *et al.*, 1980). The nonsegmented, negative sense genome of 15,462 bases is contained within a helically symmetrical nucleocapsid that is enclosed within the lipid bilayer (Kingsbury, 1990). The morphology of the helical nucleocapsids is conferred by the tight association of the genome with ~2600 molecules of the major nucleocapsid protein, NP. The nucleocapsids also contain ~ 300 copies of the nucleocapsid phosphoprotein P and ~50 copies of the major polymerase subunit, L (estimates for Sendai virus, Lamb *et al.*, 1976).

ii) Proteins encoded by hPIV3

The hPIV3 genome encodes at least seven proteins (6 structural and one nonstructural protein), the genes for which are grouped along the genome in the 3' to 5' direction in the following order: RNP associated protein genes, envelope associated protein genes, and the polymerase gene (refer to figure 1).

a) RNP associated protein genes

The first, or most 3' gene codes for the nucleoprotein, NP, which associates tightly with the viral genome and antigenome RNAs creating nuclease resistant RNP structures. NP is the most abundant of the viral proteins synthesized. NP serves several functions in virus replication, including encapsidation of the genome and the antigenome RNAs, association with the P-L polymerase (see below) during transcription and replication, and interaction

with the M protein during virus assembly (Peeples, 1991). The intracellular concentration of unassembled NP is also thought to be the major factor that controls the relative rates of transcription and replication from genome templates (Blumberg *et al.*, 1981).

The second gene encodes P, a phosphoprotein that is a component of the RNA dependent RNA polymerase complex. Together with the L protein P forms the viral polymerase (P-L), and together with unassembled NP forms a complex , which is probably the active form in RNA encapsidation (Horikami *et al.*, 1992). The amino terminal domain of the P protein contains most of the sites at which the protein is phosphorylated and also contains a unique region specifically required for the RNA encapsidation step of genomic replication (Curran *et al.*, 1994).

The P gene gives rise to several other polypeptide products (C, V, D) by means of overlapping reading frames and by RNA editing or pseudotemplated addition of nucleotide (Galinski *et al.*, 1992). The P gene can use multiple start codons and produce proteins from all three orfs. hPIV3 belongs to a group of paramyxoviruses whose P orf spans the entire gene. The P genes of this group of viruses (including SeV, MV, and CDV) contain overlapping P and C protein orfs (Lamb and Paterson, 1991). The C orf is in the N terminal region and positioned in the +1 frame relative to the P orf (Curran *et al.*, 1991). The C proteins are relatively small basic nonstructural proteins (180-204 a.a.) and were first described for Sendai virus (Lamb and Choppin, 1977). C proteins have also been reported to be produced by members of the *Respirovirus* and *Morbillivirus* genera but not by members of the genus *Rubulavirus*. Recently C proteins were shown to be present in small amounts in both virions and in nucleocapsids isolated from cells and virions (Portner *et al.*,

1986; Lamb *et al.*, 1991). The C proteins are not required for mRNA synthesis or for genome replication but were found to inhibit mRNA synthesis in vitro which in turn may play a role in helping the transition from primary transcription to genome replication (Curran et al., 1992, Tapparel, 1997, Durbin *et al.*, 1999). They are also indispensable for in vivo multiplication and pathogenesis (Kurotani *et al.*, 1998). For Sendai virus, the C orf also expresses multiple proteins (C', Y, Y') by ribosomal initiation from multiple start sites (Vidal *et al.*, 1990a).

The pseudotemplated addition of nucleotides to mRNAs is another phenomenon that permits the expression of additional polypeptides from the P gene. This cotranscriptional 'editing' involves the insertion of a precise number of G residues by polymerase stuttering (Vidal *et al.*, 1990b; Kolakofsky *et al.*, 1991, Park and Krystal, 1992). During polyadenylation, a similar function for the viral polymerase has also been described for the addition of A residues. The proposed frame shifting site for hPIV3 (3'-UUAAUUUUUUCCCCC-5') resembles the transcription termination sequence 3'-UUNAUA/UU/CUUUUU-5' (Spriggs and Collins, 1986b; Galinski *et al.*, 1992).

A unique aspect of paramyxovirus cotranscriptional editing is that the number and frequency of G insertions is highly specific. Thus, for Sendai virus and the morbilliviruses, a 1G insertion is the predominant event and for the rubulaviruses, a 2G insertion is the predominant event. For bPIV3 one to six G's are added at equal frequencies, presumably to accommodate two overlapping orfs contained in this gene. The RNA editing step always occurs downstream of the C protein orf. The result of this unusual transcription process is that upon translation, the N terminal half of the P protein sequences can be joined not only

to the C-terminal half of the P protein, but they can also be fused to a very highly conserved cysteine rich domain expressed from another orf, V (Thomas *et al.*, 1988) as the result of a 1G insertion. About half of the mRNAs have the insertion, however, some mRNAs have 2Gs inserted which closes the reading frame resulting in the synthesis of a shorter protein, W, which represents the N terminal half of P alone. The cysteine rich domain of the measles virus V protein can bind zinc (Liston and Briedis,1994), a characteristic feature of transcription regulatory proteins such as the adenovirus E1A protein. The V protein, first identified for SV5 (Peluso *et al,* 1977) is a structural protein associated with the nucleocapsids of rubulaviruses, while it is a nonstructural protein absent in the virions of respiro- and morbilliviruses (Collins *et al.*, 1996). The V and W proteins were found to inhibit genome replication without any effect on mRNA synthesis (Curran *et al.*, 1991).

For bPIV3 and hPIV3, there are also two orfs overlapping the P orf, V and D (Pellet et al, 1991, Galinski *et al.*, 1992). The V protein however, is not believed to be expressed by hPIV3 as the V orf is closed by the presence of several stop codons between the G insertion site and the cysteine rich region (Galinksi *et al.*, 1992).

b)Envelope associated proteins

The third hPIV3 gene, M gene encodes the matrix protein, the most abundant protein in the virion. It is found on the inner surface of the viral envelope and associates with the nucleocapsids via ionic interactions. The M protein is considered to be the central organizer of viral morphogenesis as it can interact with the cytoplasmic tails of the integral membrane glycoproteins, the lipid bilayer, and the nucleocapsids. The self association of M and its

contact with the nucleocapsids may be the driving force in forming a budding virus particle (Peeples, 1991, Sanderson *et al,* 1993a, 1993b).

The products of the F and the HN genes are associated with the envelope and are responsible for virus attachment and virus-cell fusion events (Ebata *et al.*, 1991; Moscona and Peluso, 1992). The fusion F glycoprotein mediates penetration of the host cell by pH-independent fusion of the viral envelope with the plasma membrane. In addition, F glycoproteins on the surface of the infected cell can also mediate fusion with neighboring uninfected cells leading to the formation of syncytia, a characteristic cytopathic effect produced by parainfluenza viruses in cell culture.

For most of the parainfluenza viruses studied to date, syncytium formation is insignificant unless the HN protein is also expressed.(Ebata et al., 1991; Wild *et al.*, 1991; Moscona and Peluso, 1992, Hu *et al.*, 1992; Lamb, 1993). Thus viral penetration requires coordinate attachment and fusion activities. The F protein, which is a type 1 glycoprotein with a C terminal transmembrane domain and cytoplasmic tail is synthesized as an inactive precursor (F_0). During its transport through the endoplasmic reticulum, an N-terminal signal peptide is removed, and the F protein assembles into trimers. F_0 is also cleaved post-translationally by host cell proteolytic enzymes (proteases and carboxypeptidases) thus forming the biologically active protein necessary for infectivity, consisting of the disulfide linked chains F_1 and F_2. The oligomer is then transported through the exocytic pathway to the plasma membrane.

The haemagglutination and neuraminidase functions are encoded by the HN gene. The haemagglutinin specifically functions during attachment, while neuraminidase is

believed to have a role in virus release (Galinski and Wechsler, 1991). The HN protein is a type 2 glycoprotein with N-terminal cytoplasmic domain and a hydrophobic stretch of amino acids near the N terminus that functions as both signal sequence and transmembrane domain. For almost all parainfluenza viruses, the HN protein has fusion promoting activity. HN may be participating through its attachment function, perhaps by positioning the target membrane at a precisely defined optimum distance, or it may have a more direct role which remains to be defined. One model proposes that HN interacts with sialic acid bearing receptors on the surface of a susceptible cell which induces a conformational change in HN (Lamb, 1993). This is turn results in a conformational change in $F_{1,2}$, exposing the fusion peptide (F_0 must have already been cleaved) and completing the "activation" of the F protein. Exposure of the fusion peptide allows it to insert into the target membrane, triggering membrane fusion.

c) The polymerase gene

The L gene, the most 5' and by far the largest hPIV3 gene, encodes the large polymerase unit, L. It is the least abundant of the structural proteins. The P and L proteins form a complex, and both of these components are required for polymerase activity with NP-RNA templates (Hamaguchi *et al.*, 1983). The P-L complex is responsible for catalyzing both transcription and replication (Moscona and Peluso, 1991; Horikami *et al.*, 1992).

iii)Genome organization

The hPIV3 genome is a negative sense ssRNA (Figure 1). The genome is completely sequenced and is 15,462 nucleotides in length (Collins *et al.*, 1996; Cote´ *et al.*, 1987; Dimock *et al.*, 1986; Elango *et al.*, 1986; Galinski *et al.*, 1986a, 1986b, 1987a, 1987b, 1988, 1992; Jambou *et al.*, Prinoski *et al.*, 1987; Spriggs and Collins, 1986a, 1986b, Spriggs *et al.*, 1986, 1987).

Control elements essential for transcription and replication flank the six genes. At the 3' terminus of the hPIV3 genome is the extragenic 55-nucleotide leader region which contains the viral promoter for synthesis of positive sense RNAs. The viral RNA dependent RNA polymerase accesses this promoter for both mRNA transcription and genome replication. At the 5' end of the genome is the 44-nucleotide trailer region. The 3' terminal 38 and 5' -terminal 39 nucleotides share 85 % complementarity (Galinski *et al.*, 1988) likely reflecting a high degree of sequence identity between promoters at the 3' ends of genome and antigenome RNA. The 8 terminal 3' nucleotides of hPIV3, bPIV3, Sendai virus and Newcastle disease virus genomes are identical, also suggesting functional importance (Sakai *et al.*, 1987, Blumberg *et al.*, 1991). Sequence relatedness is less with measles virus and is very low with RSV.

iv) Gene junction sequences

Transcription proceeds along the linear array of genes by a sequential termination reinitiation (stop-start) mechanism during which the polymerase remains template bound. The stop-start mechanism is directed by short conserved sequence elements of approximately

30 nt (Spriggs and Collins, 1986) found at the gene boundaries, which represent transcriptional initiation and termination sequences (Table 1). Manipulation of sequences (Kuo *et al.*,1996b) surrounding the RSV gene start and gene end motifs showed that the transcription signals are contained entirely within these conserved motifs.

These functional sequences share homology with their counterparts in other negative strand RNA viruses (Gupta and Kingsbury, 1984). Based on their location, they are classified into three motifs (sequences as depicted in Table 1):

1) **The gene end motif** (GE) is found at the downstream border of each gene and it is assumed to encode the precise 3' end of the mRNA. It is also assumed to direct transcription termination, as well as polyadenylation by reiterative copying of the short tract of U residues at the downstream end of the signal . The gene end sequence for the M gene of hPIV3 differs from the other hPIV3 gene end sequences and contains an 8 nt insertion. An M-F readthrough transcript is uniquely abundant in hPIV3 infected cells (Spriggs and Collins, 1986; Cote *et al.*, 1987), which suggests that the eight extra nucleotides in hPIV3 infected cells have a role in polymerase readthrough at this junction. In simian virus 41, the apparent lack of a GE motif for the M gene is associated with its transcription only as a readthrough transcript including the downstream F gene (Tsurudome et al., 1991).

2) The hPIV3 genes are separated by the **intergenic trinucleotide** region (GAA) at all junctions. According to the current model for paramyxovirus transcription, the hPIV3 polymerase transcribes the gene until it encounters the gene end sequence. The polymerase complex together with the nascent RNA skips the intergenic trinucleotide sequence and reinitiates transcription by entering the gene start sequence of the next gene immediately

Table 1: Transcription regulatory sequences of hPIV3

Gene	Gene End	Intergenic	Gene Start
NP			UCCUAAUUUCU
NP/P	UAUUUAUUCUUUUU	GAA	UCCUAAUUUCU
P/M	UAUUUAUUCUUUUU	GAA	UCCUAAUUUCU
M/F	UAUUAUUC*UCUAUUAG*U UUUU[1]	GAA	UCCUAAUUUCU
F/HN	UAUUUAUUCUUUUU	GAA	UCCUCAUUUCU
HN/L	UAUUUAUUCUUUUU	GAA	UCCUCGUUUCU
L	UAUUUAUUCUUUUU		

[1]The bold and underlined sequence indicates the eight extra nucleotides in the M/F gene end sequence.

downstream. A GAA sequence is also present in four of the five Sendai virus junctions (Gupta and Kingsbury, 1984) and all but one of the measles virus junctions (Crowley *et al.*, 1988). Other paramyxoviruses, such as RSV and SV5, do not show conservation in the length or sequence of their intergenic sequences (Spriggs and Collins, 1986). A specific role has not been assigned to the intergenic sequence but it has been shown that both an intact gene end sequence and intergenic sequence are required for expression of reporter genes at "normal" levels (Saffran, 1995; Kuo *et al.*, 1996a). Recent evidence for the rhabdovirus VSV also suggests that the intergenic sequence may have a role in transcription termination (Barr *et al.*, 1997; Stillman and Whitt, 1997). Barr *et al,* (1997a) investigated the role of the VSV consensus intergenic dinucleotide sequence 3'-GA-5' in the modulation of transcription. Alteration or removal of the intergenic sequence such that the U tract responsible for synthesis of the upstream mRNA poly (A) tail was effectively positioned adjacent to the consensus downstream gene start signal resulted in almost complete abrogation of downstream mRNA synthesis, thus defining the intergenic sequence as an essential sequence element of the gene junction.

3) The **gene start motif** (GS) is found at the upstream border of each gene and assumed to be involved in mRNA initiation (Spriggs and Collins, 1986). It consists of the sequence 3'-' UCCCNNUUCC-5' which encodes the first 10 nt of mRNA. The Sendai virus GS motif has 80% sequence identity (3'-UCCCANUUUC-5') with that of hPIV3. Other paramyxoviruses have different GS motifs.

Although three distinct functional regions have been identified, transcriptional control undoubtedly involves a coordinated interaction of the three domains and it is possible that these elements are not the only determinants affecting polymerase processivity.

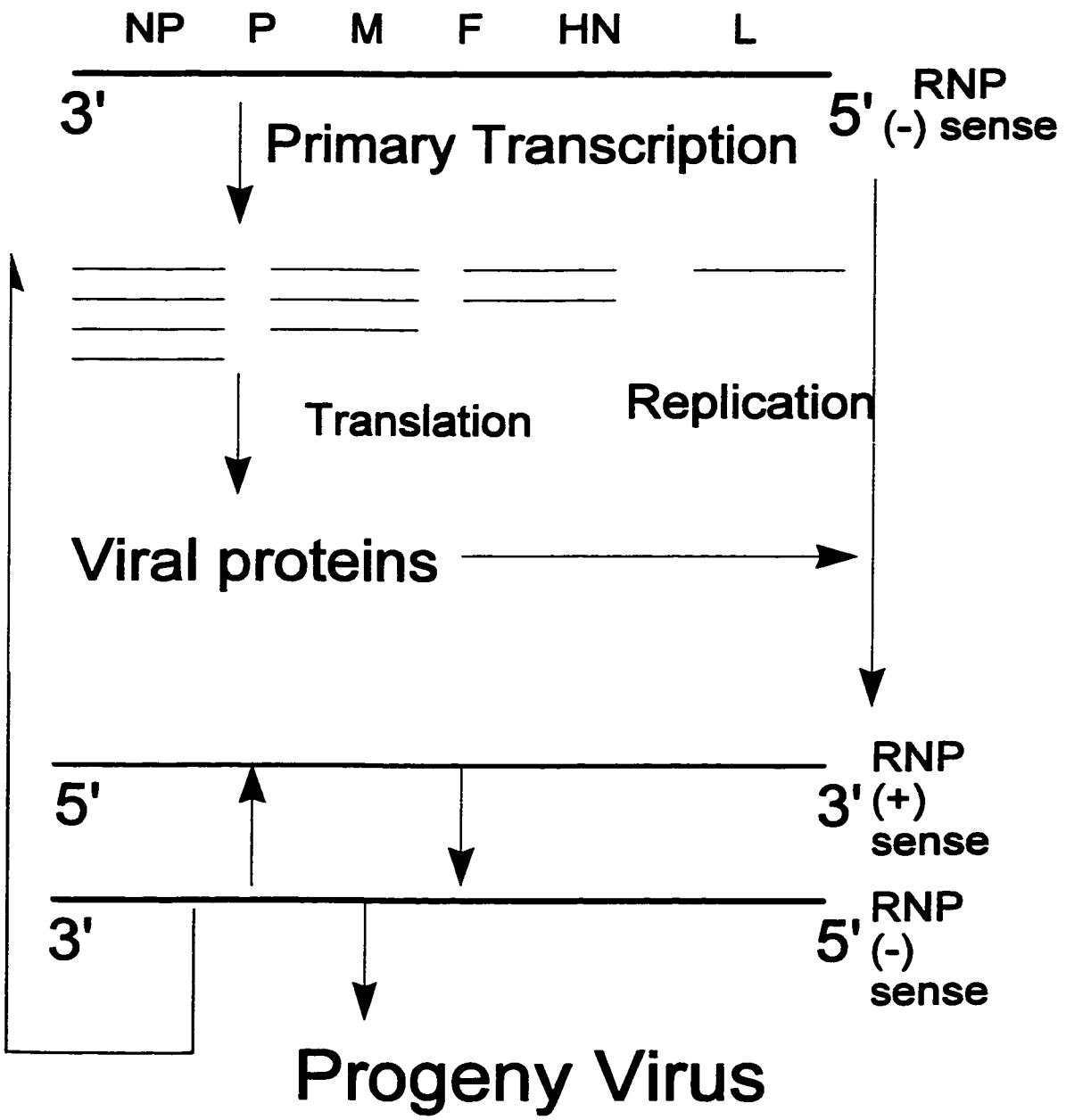
D. Transcription and replication

During viral entry into the cell, virus particles adsorb to the cellular membrane and then fuse with the cellular plasma membrane at the neutral pH found at the cell surface, resulting in the release of the helical nucleocapsids into the cytoplasm. Transcription of the viral genome is the first event after entry of the virus into cells. (Refer to flow chart, Figure 2). This process yields multiple capped and polyadenylated mRNA species. Replication begins under the direction of newly synthesized viral proteins and a full length (+) sense antigenome is made and serves as a template for the synthesis of (-) sense genome RNA. For negative sense RNA viruses, the positive sense RNA molecules that function as intermediates for genome synthesis and those that are used as mRNAs are different. The virus encoded RNA polymerase copies the same template into two different kinds of RNA molecules with different modes, viz. transcription and replication.

i) Transcription

The polymerase acts in processive mode for transcription, in which the internal genome signals define stop and restart sites. Most simply this can be explained by the presence of a "transcriptase" and a "replicase" form of the polymerase complex. The

Figure 2 **Summary of hPIV3 transcription and replication** (adapted from Fields and Knipe, 1996). Primary transcription of the RNP yields mRNAs that are translated into viral proteins, including those necessary for genome replication. Replication results in the synthesis of a full length (+) sense antigenome that serves as a template for synthesis of progeny genomes.


NP
P
M
F
HN
L
3'
5'
RNP
(-) sense
Primary Transcription
Translation
Replication
Viral proteins
5'
3'
RNP
(+)
sense
3'
5'
RNP
(-)
sense
Progeny Virus

transcriptase is able to recognize cis acting stop and start signals and gives rise successively to a short leader RNA (Leppert *et al.*, 1979) and several capped and polyadenylated mRNAs.

a) Models of transcription

While several models have been proposed for nonsegmented negative strand RNA virus transcription the stop-start model is the most widely accepted (Banerjee, 1987) . This model postulates that the same polymerase molecule terminates and reinitiates at each junction. This model also proposes a single entry site for the polymerase at the 3' end of the genome, evidence for which has been put forth for VSV (Emerson, 1982) as well as for several paramyxoviruses (Collins *et al.*, 1980; Glazier *et al.*, 1977; Dickens *et al.*, 1984).

b) Transcription initiation

Paramyxovirus transcription is believed to be initiated at the extreme 3' end of the genome (Collins *et al.*, 1980; Glazier *et al.*, 1977; Dickens *et al.*, 1984). In hPIV3, primary transcription is suggested to be initiated by the synthesis of 3' leader (Galinski and Wechsler, 1991).The leader mRNA of hPIV3 is predicted to be 55 nt nonpolyadenylated RNA. In the case of VSV (Li and Pattnaik, 1999), deletion analysis of the 47 nt leader sequence showed that the first 24 nt contain overlapping signals for both transcription and replication. The sequences downstream from 25 to 47 were found to be necessary for optimal levels of transcription but did not affect replication to a great extent.

c)Polyadenylation

The paramyxovirus mRNA transcripts, which are predominantly monocistronic, are capped and methylated at their 5' ends and polyadenylated at their 3' ends (Barik, 1993). Polyadenylation is thought to occur via transcriptional stuttering at a U stretch in the gene end sequence. It has been observed from studies of Sendai virus DI genomes, that the viral RNA polymerase will not terminate unless it encounters a U downstream of the first three Us of the polyadenylation signal (Hsu *et al.*, 1985). For RSV, mutational analyses have confirmed that sequences immediately 3' to the gene end U tract play a critical role in transcription (Kuo *et al.*, 1997). These sequences may serve to cause polymerase pausing to facilitate reiterative copying of the downstream U tract. In case of hPIV3, the AUUUC sequence 3' to the U stretch may have a similar function.

For the rhabdovirus, VSV, there is evidence (Hwang *et al.*, 1998) that polyadenylation of VSV mRNA dictates efficient transcription termination at the intercistronic gene junctions. Barr *et al.*, (1997b) found that $AUACU_7$ sequence element in the gene end motif is required for the polyadenylation of VSV mRNA. Mutations in the $AUACU_7$ tract that blocked polyadenylation also blocked transcription termination and generated mostly readthrough transcripts.

d) Transcription attenuation

For nonsegmented negative sense RNA viruses, each gene is transcribed into a monocistronic mRNA and the amount of each mRNA is controlled by the distance of the gene from the 3' promoter. At each gene junction, there is a probability that the polymerase

will terminate and fail to reinitiate transcription. The frequency with which the polymerase restarts mRNA synthesis at each junction is high, but not perfect. Presumably, this occurs because of pauses at the junctions. The pause times may be due, at least in part, to processes such as reinitiation and capping or polyadenylation, which are slow relative to transcription. For VSV, pause times increase significantly at each junction (Iverson and Rose, 1981). Thus there is always less mRNA made from downstream genes relative to their upstream neighbors, and there is a gradient of mRNA abundance according to the position of the gene relative to the 3' end of the template. For measles virus, this gradient of mRNA abundance is relatively smooth, indicating that the polymerase has an equal probability of not reinitiating at each junction. Transcription attenuation may be the principal mechanism used by nonsegmented negative sense viruses for regulation of mRNA and protein synthesis.

e) Transcriptional Readthrough

Another transcriptional mechanism which may fine tune the relative amounts of specific mRNAs is transcriptional readthrough, that is inefficient transcriptional termination and polyadenylation. The viral RNA polymerase, in addition to synthesizing monocistronic mRNAs, can ignore the gene end signals for polyadenylation-termination and synthesize a transcriptional readthrough product that contains sequences from upstream and downstream genes as it would during replication. Since only the upstream orf would be normally translated, the downstream orfs on these mRNAs are not expressed. However the polymerase would still be free to reinitiate transcription downstream. In this way the expression of specific gene products in the genome could be selectively down regulated.

In the case of VSV, this is a rare event and only 3 % of the viral transcripts are readthrough RNAs (Master and Samuel, 1984).

f) M/F readthrough transcription

For paramyxoviruses, readthrough transcription at most gene junctions is also low, on the order of ≤10 %, but the M-F junction is an apparent exception to this rule. High frequency readthrough transcription at the M/F junction has been reported for SeV (Gupta and Kingsbury,1985), RSV (Collins and Wertz, 1983), MV (Cattaneo *et al.*, 1987), SV41 (Tsurodome *et al.*, 1991), hPIV1 (Bousse *et al.*, 1997), mumps virus (Takeuchi *et al.*, 1991), and hPIV3 (Spriggs and Collins, 1986). For example hPIV3, SV5 and hPIV1 all display high readthrough transcription (~50%, 40 %, and ~80%, respectively) at the M-F junctions (Spriggs and Collins, 1986; Rassa and Parks, 1998; Bousse *et al.*, 1997). For these paramyxoviruses, readthrough transcription at the M/F junction correlates with sequence differences from the consensus sequence in either the M gene end or the M-F intergenic region.

The hPIV3 M gene end sequence differs from others in containing an extra 8 nt just upstream of the polyadenylation signal. The readthrough transcript could be the result of this sequence somehow hampering the polyadenylation of the upstream M transcript and hence its termination.

For SV5, it has been shown that, ~ 40% of the F mRNA is detected as a bicistronic M-F transcript (Rassa and Parks, 1998). This disproportionate amount of M-F bicistronic RNA also correlates with unique features of the M/F gene junction. The junction between

the M and F genes in SV5 displays several distinguishing characteristics: (1) the nucleotide sequence of the gene end sequence adjacent to the M gene end U tract differs from the consensus sequence; (2) the four U residues which compose the tract for M gene polyadenylation are the shortest of any SV5 gene end U tract; (3) the M-F intergenic region is the longest of the SV5 gene junctions.

In the case of hPIV1, transcriptional readthrough at the M/F junction is particularly high, ~ 80 %. An identical GE sequence AUUCUUUUU, is found (Galinski, 1991) between the M and F, F and HN, HN and L genes (Power *et al.*, 1992) and is unlikely to be responsible for readthrough. The intergenic sequence, GAA is conserved between hPIV1 genes of F and HN and HN and L (Galinski et al , 1991) but the intergenic sequence between M and F of hPIV1 is GCA, suggesting that the intergenic sequence plays a role in terminating hPIV1 transcription.

ii) Replication

Replication of all paramyxoviruses occurs in the host cell cytoplasm, independently of host DNA replication. After translation of the primary transcripts and accumulation of the viral proteins, antigenome synthesis begins. The same polymerase that was engaged in mRNA synthesis copies a similar template but now ignores all the transcriptional signals (and may be editing sites) and synthesizes an exact complementary copy of the genome.

In contrast to transcription, the product of replication is not a free RNA, but an RNP with an encapsidated full length RNA. It has been proposed (Blumberg *et al.*, 1981; Banerjee, 1987; Moyer *et al*, 1991) that the binding of NP protein to nascent leader RNA is

a critical step for the transition from transcription to replication. NP would act as an antiterminator to allow the RNA polymerase to read through the signals for termination, mRNA processing and reinitiation at the intergenic boundaries. This readthrough results in the synthesis of a fully encapsidated antigenomic RNA instead of monocistronic mRNAs. Reinitiation at the junction between the leader and the first protein coding gene would convert the polymerase to the transcription mode, in which elongation of the newly synthesized RNA is independent of the assembly with NP protein.

Encapsidation by the NP protein would be triggered when the abundance of the NP protein reaches a critical level. The encapsidation event therefore plays a crucial role in modulating the balance between transcription and replication (Patton *et al,* 1984). For SV5, a functional promoter was shown to require two discontinuous regions (CRI and CRII) located in the 3' terminal 90 bases of the genome RNA (Murphy *et al.*, 1998). Most importantly, the relative spacing of these two cis-acting regions is a critical factor in determining the level of SV5 RNA replication.

E. Reverse genetics of nonsegmented negative sense RNA viruses.

As mentioned at the beginning of this thesis, genetic manipulation and analysis of negative strand RNA virus biology lagged far behind that of other RNA viruses such as the positive RNA viruses and retroviruses, mainly because the isolated genomes, antigenomes or cDNA copies of negative sense RNA viruses are not infectious. The initiation of an infectious cycle needs the presence of a complete nucleocapsid structure. Only in this form can the RNA function as a template for the virus polymerase. Proteins can be expressed

neither from the genomic RNA, as it is of negative polarity, nor from the antigenome because of the modular organization of the RNA which does not allow translation of all viral proteins by the cellular machinery. Consequently, in vitro reconstitution of functional RNPs from proteins and RNAs also was not possible. Reassortment, a technique frequently exploited for the introduction of mutated genes into segmented viruses such as influenza viruses, does not occur for nonsegmented negative sense RNA viruses (Bergman et al., 1992). These obstacles greatly limited the analyses of viral processes, their regulation, as well as applications such as the development of vaccine candidates.

The rescue of RNAs from representatives of the *Mononegavirales* was first accomplished using small subgenomic RNAs containing only essential sections of the viral genomes. These minirepilicons or minigenomes, based on the influenza model (Luytjes, 1989), carried a CAT coding sequence inserted between viral non-coding termini of SeV (Park *et al.*, 1991), RSV (Collins *et al*, 1991, 1993) or hPIV3 (Dimock and Collins, 1993). Alternatively, naturally arising defective interfering particle (DI) RNAs were rescued (Re, 1991) .

In all these systems, the required proteins mediating encapsidation, transcription and replication were provided either by the homologous viruses or by recombinant vaccinia virus encoding phage T7 RNA polymerase (Fuerst *et al.*, 1986), which was used to drive T7 specific transcription of mRNAs encoding NP, P and L proteins from transfected plasmids, as pioneered for VSV by Pattnaik and Wertz (1990).

Investigations using minireplicons revealed important information about non-coding regulatory regions of the negative-sense viral genomes. The substitution of minigenome by

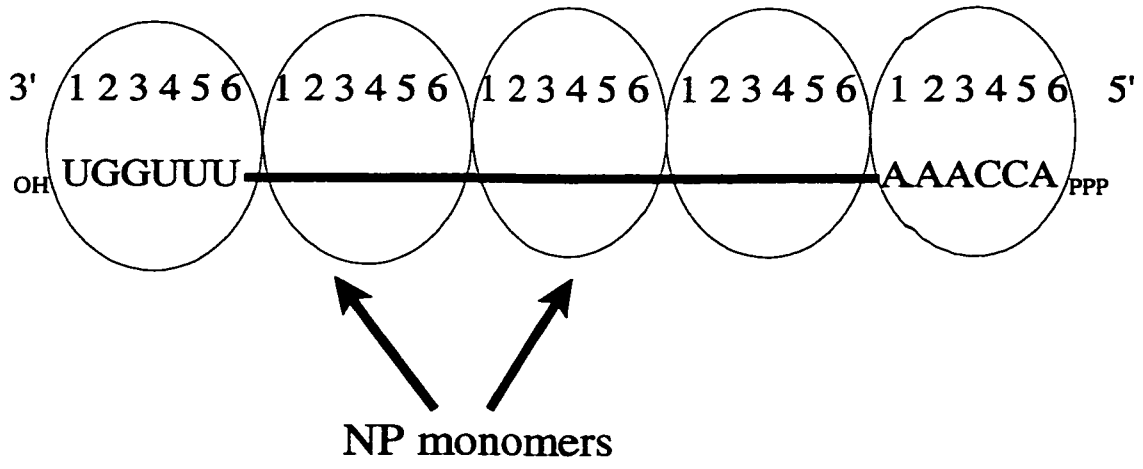
full length viral RNAs and the rescue of infectious virus was first accomplished for the rhabdovirus rabies virus (Schnell *et al.*, 1994). Similar experiments have since been used to rescue VSV (Lawson *et al.*, 1995), MV (Radecke, 1995), RSV (Collins *et al*, 1995) SeV (Garcin *et al*, 1995, Kato *et al*, 1996) and hPIV3 (Durbin *et al*, 1997, Hoffman and Banerjee, 1997).

The genomes of many paramyxoviruses can now be changed at will by site directed mutagenesis, and the resulting phenotypes can be studied and defined in the context of not only viral replication in vitro but also in vivo viral multiplication and pathogenesis.

a) The rule of six

One unexpected outcome of this work was the discovery that for a number of paramyxoviruses, the overall length of a viral RNA or RNA analog must be a multiple of six nucleotides for efficient rescue. This "rule of six" was first recognized when it became possible to perform genome replication starting with cDNA copies of natural Sendai virus DI RNAs (Calain and Roux, 1993). Efficient replication of the DI RNAs required that their total length be a multiple of six nucleotides (Calain and Roux, 1993, 1995; Sidhu *et al.*, 1995). The rule of six is consistent with the prediction that each NP monomer (~58 kd) is associated with precisely six nucleotides (Egelman *et al.*, 1989). Nucleocapsid assembly presumably begins with the first nucleotide at the 5' end of an RNA molecule (Lamb and Kolakofsky, 1996) and continues by assembling six nucleotides at a time until the 3' end is reached (Figure 3). The efficiency of the promoter at the 3' end may depend on its position relative to the NP subunits and this is determined by the total number of nucleotides in the

Figure 3 **The rule of six**: One monomer of NP protein, represented by each ellipse, interacts with exactly six nucleotides of RNA.


3'
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
5'
OH
UGGUUU
AAACCA
PPP
NP monomers

genome. Paramyxovirus genomes contain inverted terminal repeats of 12 nt specific to each genera, hence the 5'- and 3'- terminal 12 nt of the genome and the antigenome of each virus are identical. These dodecamers are definitely important cis acting determinants for genome replication, and they would be precisely covered by the terminal two NP subunits in hexamer length genomes.

The rule of six holds for hPIV3, SeV and MV genome analogs, but does not appear to apply to RSV or to rhabdoviruses such as rabies virus and VSV. It seems to be a feature of viruses that utilize P gene RNA editing, and it has been suggested that this is a method for selecting against antigenomes and genomes that have acquired length changes during replication, and termed genome length correction (Hausmann et al., 1996). When SeV minigenomes containing the P gene editing site A_6G_3, and not of hexamer length, are replicated in transfected cells, minigenomes are selected with insertions (or deletions) in the A_6G_3 purine run, which readjust their length to multiples of six (Hausmann *et al.*, 1996). Pseudotemplated transcription was found to occur at the same purine run used for G insertions during mRNA synthesis when the viral polymerase was copying the genome template during antigenome synthesis (Hausmann *et al.*,1996, Lamb and Kolakofsky, 1996).

b) hPIV3 rescue

As for other paramyxoviruses, the cis acting signals necessary for encapsidation, replication and transcription of hPIV3 genomes or genome analogs are contained in short terminal RNA sequences that can be linked to foreign sequences, such as reporter genes, without loss of function (Dimock and Collins, 1993). Bicistronic minigenomes that contain

two different reporter genes can also be used to analyze internal transcriptional sequences found at gene junction sequences (Saffran, 1995).

In previous work, synthetic minigenomes generated by in vitro transcription with T7 RNA polymerase were rescued using helper hPIV3 following transfection of 293 cells (Saffran, 1995, Dimock and Collins, 1993).

The rescue system (refer to Figures 4 and 5) used throughout the thesis does not require complementation by hPIV3 to supply the NP, P and L proteins necessary for encapsidation and expression of minigenomes. Instead, the cDNAs encoding the minigenome as well as the viral NP, P and L protein coding sequences are transcribed intracellularly by T7 RNA polymerase encoded by recombinant vaccinia virus vTF7-3.

Minigenome cDNAs are cloned between the T7 promoter and ribozyme sequences from the antigenomic strand of hepatitis delta virus (HDV), which is followed by the T7 RNA polymerase transcription termination sequence. Thus, autolytic cleavage of transcripts containing the HDV ribozyme sequence, immediately downstream of the virus sequences, generates RNAs with correct 3' termini. 5' termini are determined by the location of the T7 promoter. Each minigenome contains two extra non viral G residues (Durbin *et al.*, 1997a)at the 5' end which are contributed by the T7 promoter (in order to enhance T7 RNA polymerase transcription), but it is assumed that these would be lost during intracellular hPIV3 polymerase mediated RNA replication (Pattnaik *et al.*, 1992; Pelet *et al.*, 1996).

Figure 4 **The hPIV3 rescue system.** HEp-2 cells are transfected with 4 plasmids encoding hPIV3 minigenome RNA and the hPIV3 NP, P and L proteins needed for RNA encapsidation. Cells are also infected with vaccinia virus vTF7-3 to provide the T7 RNA polymerase required to drive transcription of mRNAs and minigenome RNA. T7p, T7 RNA polymerase promoter; *l*, leader; *t*, trailer, δ, hepatitis δ ribozyme.

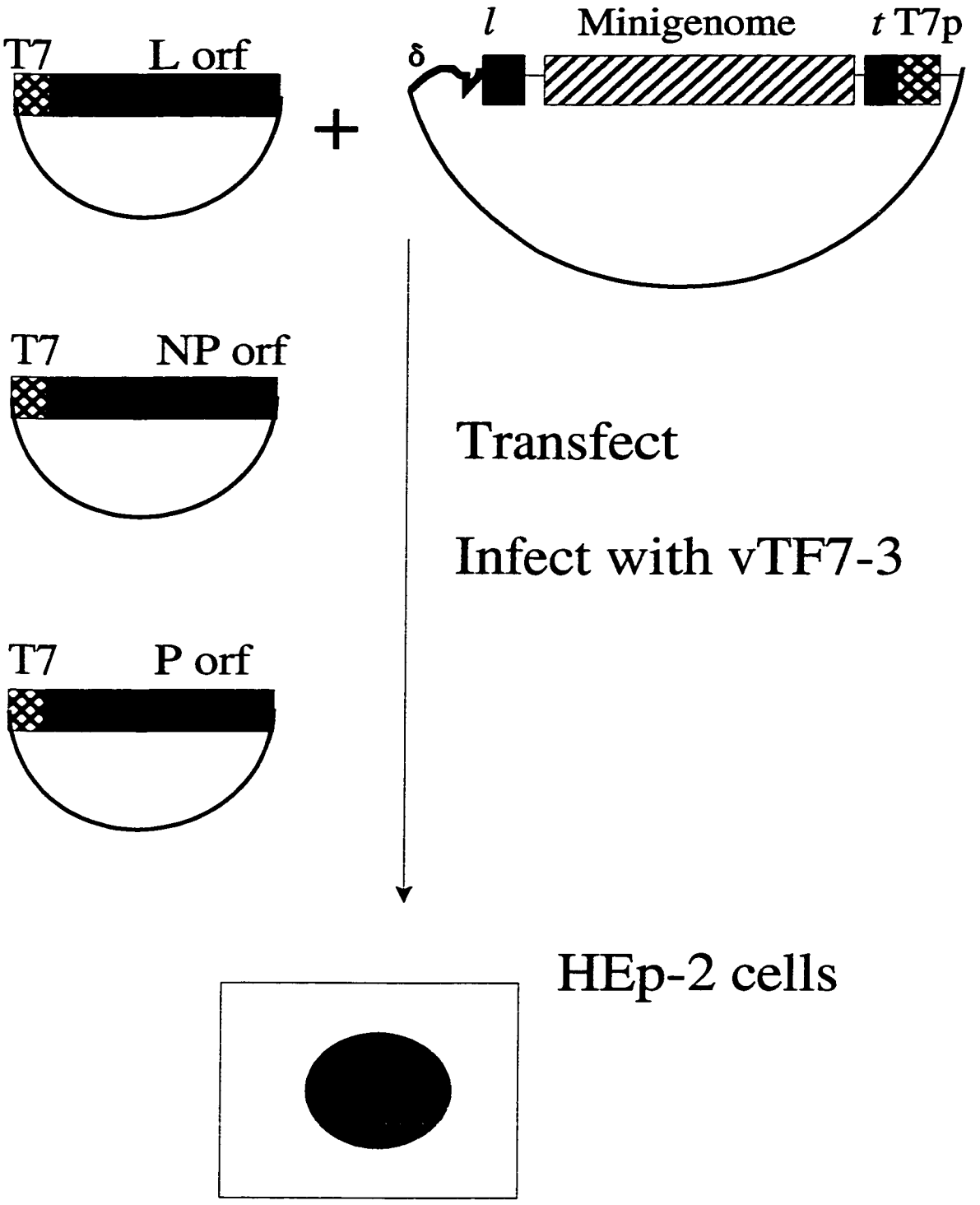
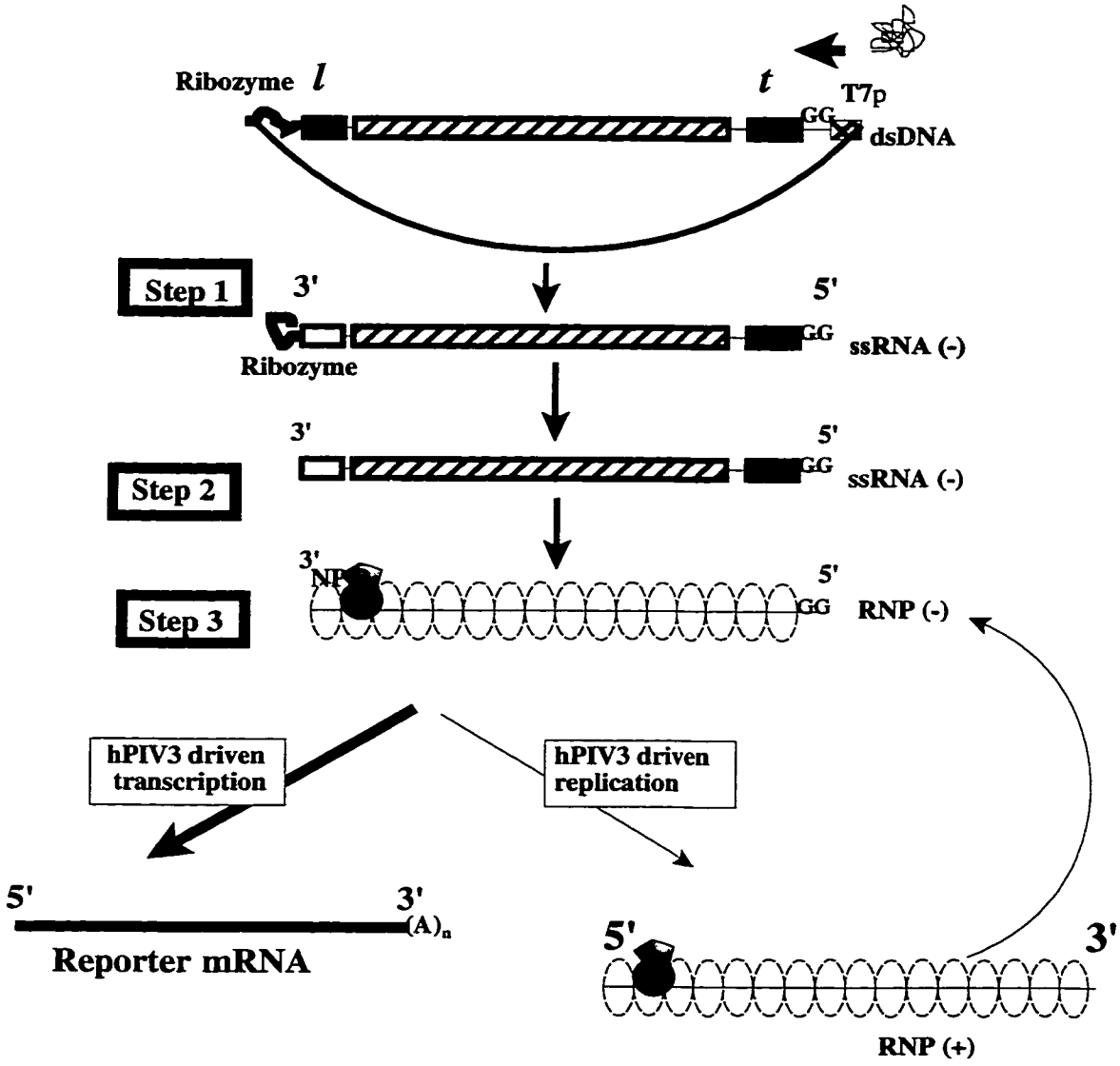

T7
L orf
+
δ
l
Minigenome
t T7p
T7
NP orf
T7
P orf
Transfect
Infect with vTF7-3
HEp-2 cells

Figure 5 **Flow of events during rescue of hPIV3 minigenome analogs.** During the rescue of the minigenome analogs, the following events take place and are shown in the figure in a stepwise manner:-

Step 1: T7 RNA polymerase drives transcription of minigenome cDNA sequences to yield (-) sense minigenome RNA (arrow indicates direction of transcription)

Step 2: Ribozyme cleavage generates minigenome RNA with correct hPIV3 3' end

Step 3 Encapsidation of minigenome RNA by hPIV3 NP, P and L proteins (represented by the ellipses, the circle and the pentagon, respectively) yields templates for transcription (to produce mRNAs for reporter genes) and replication (to produce (+) sense antigenomes, which are encapsidated).


Ribozyme
l
t
T7p
GG
dsDNA
Step 1
3'
5'
GG
ssRNA (-)
Ribozyme
3'
5'
Step 2
GG
ssRNA (-)
3'
NP
5'
Step 3
GG
RNP (-)
hPIV3 driven transcription
hPIV3 driven replication
5'
3'
(A)n
Reporter mRNA
5'
3'
RNP (+)

II. OBJECTIVES

As for other paramyxoviruses, transcription of hPIV3 genome occurs by a sequential stop-start mechanism leading to the synthesis of monocistronic, capped and polyadenylated mRNAs as directed by short conserved sequence elements found at the gene junctions. My initial hypothesis was that all cis acting signals required for transcription termination, polyadenylation and re-initiation reside in these gene junction sequences. Therefore introducing mutations into these sequences was expected to help elucidate the specific function of each sequence. It was also predicted that the eight extra nucleotides in the gene end sequence of the hPIV3 M gene were necessary and sufficient for increased transcriptional readthrough at the M/F junction.

Previous work by Saffran *et al.*, (1995) in our lab utilized hPIV3 minireplicons bearing luciferase and CAT marker genes which were transcribed in vitro from linearized cDNA and complemented by transfection into cells infected with standard hPIV3 as a source of viral proteins needed for encapsidation (Dimock and Collins, 1993). One limitation of this method was that the efficiency of rescue was low, and it was difficult to detect the various RNA species in transfected cells. The vaccinia driven rescue system (Figures 3 and 4) has been reported to give much greater yields of minigenome RNA and enhanced transcription of reporter genes (Calain *et al.*, 1992, Pattnaik *et al.*, 1992). Therefore the first objective of my thesis was:

1) To modify previously constructed cDNAs so that they could be used in the vaccinia driven rescue system.

Once the modified cDNAs had been constructed, the following objectives were established:

2) To confirm that hPIV3 genome analogs obey the rule of six, using monocistronic minigenomes.

3) To construct and test plasmids containing bicistronic cDNAs with luciferase and CAT orfs separated by hPIV3 junctions and flanked by hPIV3 termini.

4) To use these plasmids (modified by site directed mutagenesis) to address the following questions concerning transcription of hPIV3 mRNAs :

a) importance of sequence and spacing of gene start sequences within the nucleoprotein subunit in transcription initiation i.e. the register of the transcriptional start site with respect to the position of the NP monomer

b) to compare the function of the hPIV3 gene junctions

c) to examine the potential antiterminator activity of the 8 extra nucleotides in the M gene end sequence

Initially, reporter gene assays were used to compare the activity/function of the various bicistronic minigenomes. Subsequently, specific minigenomes were analyzed by Northern blotting in order to quantify the various monocistronic and bicistronic RNA species and to distinguish between the potential effects of mutations on polymerase readthrough and termination.

III. MATERIALS AND METHODS

A. Cell culture and virology

1. Cells

The human epidermal carcinoma cell line, HEp-2 was kindly provided by Dr. P.Collins (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The African green monkey kidney cell line, CV-1 and the Rhesus monkey kidney cell line, LLC-MK2 were obtained from the American Type Culture Collection (ATCC). All cell lines were grown in autoclavable minimal essential medium (MEM; Life Technologies) with 10 % (HEp-2) or 5% (LLC-MK2, CV-1) fetal bovine serum (FBS; Life Technologies). Media were supplemented with 2 mM L-glutamine, 0.23% (w/v) sodium bicarbonate and 50 μg/ml gentamycin sulphate (Cell Gro, Mediatech Inc). Cell monolayers were maintained in 100 mm diameter polystyrene dishes (Corning) and grown at 37°C in a 5% CO_2 atmosphere in a Shellab Incubator (Sheldon Manufacturing).

HEp-2 and CV-1 cells were split twice weekly at a ratio of 1:8 while LLC-MK2 cells were split twice weekly at a 1:6 ratio. Passaging was performed on confluent monolayers by first washing once with 5 ml of prewarmed Tris-buffered saline. 0.005% trypsin/ 0.002% EDTA in 2 ml TBS was added and plates were incubated at 37°C for 2-3 minutes. The trypsin was inactivated by the addition of culture medium, and cells were diluted into fresh culture plates.

2. Viruses

A stock of human parainfluenza virus type 3 (hPIV3) strain WASH/47885/57, propagated in HEp-2 cells, was obtained from Dr. P. Collins (NIAID, NIH). Recombinant vaccinia virus vTF7-3 which expresses bacteriophage T7 RNA polymerase (Fuerst et al, 1986) was obtained from ATCC.

3. Preparation of virus stocks

A vTF7-3 stock was prepared as follows. Confluent plates of CV-1 cells were infected at a multiplicity of infection (MOI) of 0.1 plaque forming units (pfu) per cell. Incubation at 37°C for one hour with occasional tilting allowed virus to adsorb to the cells. The inoculum was replaced with 10 ml fresh MEM containing serum, and plates were incubated for 72 hours until the cells had a "lacelike" appearance . The cells were washed, scraped gently into 3 ml TBS, pelleted at 500 rpm for 5 minutes and resuspended in 0.8 ml serum free medium. The cell suspension was subjected to three freeze/thaw cycles and sonicated briefly. Supernatants were pooled, aliquoted and stored at -80°C. Frozen hPIV3 stocks were already available.

4. Plaque assays

Confluent monolayers of HEp-2 cells in 6 well plates (Corning) were used for plaque assays. 10 fold serial dilutions of the virus stock (vTF7-3 or hPIV3) were prepared in 1 ml of serum-free MEM. Cells in duplicate wells were infected for 1 hour at 37°C by adding 0.3 ml of the appropriate dilution of virus. 1.5 ml of agarose/MEM was overlaid and

incubation was continued until visible plaques were seen (3-4 days). 2 ml of 10% (v/v) formol saline was added to each well to fix the cells. After one hour at 37°C, the agarose overlays were washed off, and monolayers were stained for 30 min with 2 ml 0.1% (w/v) crystal violet. Plaques were counted and the virus titres were determined.

B. DNA preparation and analysis

1. Small scale isolation of plasmid DNA (miniprep)

The miniprep DNA isolation procedure was an alkaline lysis method (Sambrook, 1989). Single colonies were used to inoculate 3 ml LB broth containing ampicillin, which was incubated overnight at 37°C. 1.5 ml of the culture was transferred to a microfuge tube, and cells were pelleted by centrifugation at 14,000 rpm for 30 seconds. The supernatant was removed and the cells were completely resuspended in 100 µl ice-cold 50 mM glucose, 25 mM Tris-HCL pH 8.0, 10 mM EDTA, 100 µg/mL DNAse-free RNAse A. Cells were then lysed by adding 200 µl of freshly prepared 1%SDS, 0.2 N NaOH, the tubes were inverted 4-5 times during which the samples quickly became clear and viscous. 150 µl of ice cold 5M Potassium acetate, pH 4.8 was added and the samples were mixed vigourously leading to the loss of viscosity and formation of a large white precipitate. The samples were placed on ice for 5 minutes and then centrifuged at 4°C in a microfuge for 10 minutes. The supernatant was carefully transferred to a fresh microfuge tube. After extraction with 400 µl chloroform, DNA was precipitated by the addition of 1 ml of ethanol at -80 °C for 30 minutes. The precipitated DNA was pelleted at 4°C for 10 minutes and the pellet was air dried and

resuspended in 50 µl sterile double distilled water. 5 µl were examined on an agarose gel as described below.

2. Large scale isolation of plasmid DNA

Large scale DNA preparations were performed using the Wizard ® Plus Midipreps DNA Purification system. DNA was resuspended in sterile dH_2O to approximately 1 µg/µl, according to the column yield. The DNA concentration was determined by spectrophotometry at λ=260 nm.

3. Agarose gel electrophoresis

DNA electrophoresis was performed using 1% agarose gels in 1X Tris-Acetate buffer (TAE; 40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Samples were prepared by adding 1/10 volume of loading buffer (50% glycerol, 0.1 M EDTA, 1% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Electrophoresis was carried out in the Horizon 58 (Gibco/BRL) apparatus at approximately 10 volts/cm. Gels were stained in 1 µg/ml ethidium bromide (EtBr) and destained briefly in H_20. Photography was performed using a Gel Print 2000i (Biophotonics) with a FOTODYNE U.V. light box.

C. Sequence analysis

Automated fluorescent sequencing was performed by the Biotechnology Research Institute of the University of Ottawa (A. Bergeron and C. Dixon).

D. Electroporation

1. Preparation of cells

One l of warm LB broth was inoculated with 1/100 volume of a fresh overnight culture of *E. coli* DH5α F'. Cells were grown with agitation (225 rpm) to an OD_{600} of 0.5-1.0. The flask was chilled on ice for 30 minutes, and cells were centrifuged in a Beckman JA-10 rotor at ~1600 X g for 10 minutes at 4°C. The pellet was resuspended in 1 l ice cold, sterile 15% glycerol and centrifugation was repeated. The pellet was resuspended in 500 ml ice cold 10% glycerol and the centrifugation was repeated. Cells were pelleted by centrifugation in a JA-20 rotor at 1100 X g for 10 minutes at 4°C. Final resuspension was in 3 ml of 15% glycerol. Aliquots of cells were frozen on dry ice and stored at -80°C.

2. Electroporation

1 - 10 ng of DNA in a volume of 1 ul was mixed with 20 µl electrocompetent cells and placed in a sterile cuvette in the electroporation chamber (Life Technologies). Conditions for electroporation were as follows: resistance=4 kΩ, capacitance=330 µF, resistance of medium=low setting. After electroporating, cells were incubated at 37°C for 1 hour in 1 ml SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl supplemented with 10 mM $MgSO_4$ and 20 mM glucose). A 100 µl aliquot of cells was plated on LB agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 50 µg/ml ampicillin (Boehringer Mannheim). Plates were then incubated at 37°C and colonies were picked.

E. Restriction and modification enzymes

Restriction endonucleases and modifying enzymes were purchased from New England Biolabs, Promega Biotech, MBI Fermentas, Life Technologies, Pharmacia Canada, Epicentre Technologies and Ambion. Restriction and modification enzymes were used as recommended by the suppliers.

F. Purification of DNA restriction fragments from agarose gels

DNA fragments to be cloned were purified from 1% agarose gels using the protocol for Amersham Pharmacia Biotech GFX™ PCR and Gel Band Purification kits.

G. Construction of monocistronic and bicistronic cDNAs encoding minigenomes

1. Oligonucleotide insertion

This method was used for the construction of pLC M/F, pLCNP/P 3IG, and the pLC NP/P tc1-6 series (see Appendix). Some of the oligonucleotides used in the construction of mutants by direct cloning were kindly provided by Dr. P. Collins (NIAID, NIH). Oligonucleotides were also synthesized (see Appendix) at the Biotechnology Research Institute (University of Ottawa, A. Bergeron). Complementary oligonucelotides were annealed by heating 1 μg of each DNA pair in 10 μl dH_2O to 95°C for 5 minutes and then allowing the sample to cool slowly. The annealed pairs of oligonucleotides with sticky ends were then ligated into the corresponding restriction site in the parent plasmid to be altered. Reactions typically used 500 ng of the parent plasmid that had been digested with restriction enzyme and 1 μg of the oligonucleotide pair. Ligations were performed in 1 mM ATP, 50

mM Tris -HCL (pH 7.6), 10 mM $MgCl_2$, 1 mM DTT and 5% (w/v) polyethylene glycol-8000, using 2 units of T4 DNA ligase overnight at 16°C. *E.coli* DH5∝ F cells were transformed. The design of the oligonucleotides allowed for screening by gain or loss of restriction sites. Plasmid DNAs were sequenced to verify the mutations.

2. Conventional cloning (ligation of restriction fragments)

The junction constructs pLCNP/P, pLCNP/PΔIG, M/F Δ8, F/HN, HN/L and monocistronic cDNAs pCAT2G0 - pCAT2G5 were constructed by conventional cloning procedures. Restriction fragments from different plasmids, purified following agarose gel electrophoresis, were ligated together to generate the desired products.

3. Site-directed mutagenesis

The Kunkel method of mutagenesis was used (Kunkel et al., 1987) with the aid of the Muta-Gene Phagemid In Vitro Mutagenesis kit (Biorad) to synthesize monocistronic minigenomic cDNA pCAT 2G6, which has six extra nucleotides in the PstI site of the CAT noncoding region of pCAT2G0 (Durbin *et al.*, 1997). 200 pmol of the mutagenic oligonucleotide was phosphorylated in a 30 µl reaction (100 mM Tris, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 0.4 mM ATP) containing 4.5 units T4 polynucleotide kinase (Bio-Rad). The reaction proceeded for 45 minutes at 37°C and was stopped by heating at 65°C for 10 minutes. Phosphorylated oligonucleotides were diluted to 6 pmol/µl with TE. Phosphorylated oligonucleotides and uracil-containing single stranded plasmid (KSIIPIV-CAT, which contains hPIV3 leader and trailer sequences, T7 promoter and CAT coding sequences;

K.Dimock, unpublished) were annealed in a 10:1 ratio in a 10 µl reaction (20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl). A negative control was also prepared that did not contain the oligonucleotide. Samples were heated to 70°C and allowed to cool at ~ 1°C/min to 30°C. Complementary strand synthesis was achieved by the addition of 3 units T4 DNA ligase (Biorad), 0.5 units T7 DNA polymerase and synthesis buffer (final concentration: 0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl pH 7.4, 3.75 mM $MgCl_2$, 0.5 mM DTT). Samples were incubated 5 minutes on ice, 5 minutes at 25°C and 90 minutes at 37°C. An aliquot of this reaction was used to transform *E. coli* DH5∝ F' cells as described below. Transformants were screened for the loss of the PstI restriction site and mutations were confirmed by DNA sequence analysis.

4. Polymerase chain reaction

Hot start PCR was used to amplify parts of the hPIV3 M and F genes. These PCR products were used to replace parts of Luc and CAT orfs upstream and downstream of the M/F gene junction with M and F sequences. pPIV3FL+ (20 ng) was amplified with 10 µM each of oligonucleotides MF+ and MF- in a 100 µL reaction containing 10mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08 % Nonidet P-40, 0. 3 mM $MgCl_2$, 20 nM dNTPs), 0.25U of recombinant Taq DNA polymerase (MBI Fermentas), in a Hybaid Thermal Reactor (94 °C for 45 seconds; 42°C for 1 min; 72°C for 2.5 minutes for 30 cycles)

5. Cloning of PCR products in pCR-TOPO® plasmid

The Topo TA cloning protocol (Invitrogen) was used for cloning PCR products as templates for hPIV3 M and F specific riboprobes. The hPIV3 M and F sequences were amplified from a plasmid containing a cDNA copy of the hPIV3 genome (K. Dimock, unpublished) and the PCR product was ligated into the pCR ®-TOPO vector. 2 µl of this TOPO ligation reaction was used to transform TOP10 One Shot ™ cells.

H. In vitro transcription and riboprobe preparation

In vitro transcription reactions were performed according to protocols recommended by the supplier (Promega) with some modifications. A 100 µl reaction containing transcription buffer (40 mM Tris-HCl, pH 7.9; 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl), 10 mM DTT, 100 units RNAsin (Promega) and 1 mM each dNTP was warmed to 37°C. 2 µg linearized DNA and 60 units T7 RNA polymerase (Ambion or Epicentre) were added and transcription reactions were incubated at 37°C for 3 hours. The RNA synthesis was confirmed by agarose gel electrophoresis of a 3 µl aliquot. Samples were stored at -80°C.

For riboprobe preparation (refer to Table 2 for a list of riboprobes used), a 20 µl in vitro transcription reaction driven by T7, T3 or SP6 RNA polymerase (60 Units) typically contained 1X Transcription buffer, 6.25 µM [α-^{32} P]CTP (400 Ci/mMol, Amersham) along with ATP, GTP and UTP (2.5 mM each). Transcription of longer sequences was performed with an additional 5µM of unlabelled CTP. Reactions were incubated one hour at 37°C. 2 units of RQ1 DNAse (Promega) was added to remove the DNA template and incubation was

Table 2: Riboprobes

Probe	**Plasmid used as template for *in vitro* transcription**	**Position in gene represented by the probe**
CAT probe (negative sense)	pCAT 2G0	213-660 nt of CAT gene
F probe (negative sense)	pIBIF	1730-2214 nt of hPIV3 F gene
M probe (negative sense)	Topo M	712 - 1047 nt of the hPIV3 M gene

continued for 20 minutes at 37°C. Unincorporated nucleotides were removed from the labelled riboprobe by passing the reaction mixture through RNAse free MicroSpin™ G-50 columns (Amersham Pharmacia Biotech)

I. Transfections

Confluent monolayers of HEp-2 cells were split 1:4 into 6 well dishes and incubated at 37°C until they had reached 90 % confluency. The medium was removed and cells (1-1.5 X 10^6 per well) were washed with 1 ml OPTIMEM (Life Technologies) before transfection. The transfection mixture consisted of 0.4 μg each of the test plasmids encoding minigenomes, pTMN (encoding hPIV3 N protein) and pTMP (encoding hPIV3 P protein), and with 0.1 μg of pTML (encoding hPIV3 L protein) in a final volume of 0.1ml of OPTIMEM. To this plasmid mix, 0.1 ml OPTIMEM containing 12 μl of LipofectACE™ (Life Technologies) was added and the mixture was incubated at room temperature for 20 minutes. To this was added 0.8 ml of OPTIMEM containing 2 % fetal bovine serum and vTF7-3 (MOI=10 pfu/cell). Plates were incubated at 37°C for 24 hrs and cells were then harvested for reporter gene assays or RNA analysis.

J. Reporter gene assays

1. Preparation of lysates from transfected cells

Cells from individual wells were harvested and lysed by resuspension in OPTIMEM containing 0.2% Nonidet P-40. Lysates were transferred to 1.5 ml microfuge tubes and

clarified by centrifugation for 1 min at full speed. Supernatants were transferred to fresh microfuge tubes for immediate use or storage at -80°C.

2. CAT assays

The CAT assay (Gorman, 1982) consisted of 130 µl of a CAT mix (250 mM Tris-HCl pH 7.5, 0.5 mM acetyl coenzyme A (Boehringer Mannheim), 0.025 µCi D-threo[dichloroacetyl-1-^{14}C] chloramphenicol (Amersham), and was initiated with 20 µl of a cell lysate or a diluted cell lysate. Reactions were incubated for one hour at 37°C. Extraction of chloramphenicol was achieved by adding 1 ml ethyl acetate. Samples were vortexed, centrifuged 3 minutes at 12,000 rpm at room temperature and 900 µl of the upper organic phase was transferred to a fresh tube. Samples were dried in a Speed-Vac concentrator (Savant). The pellet was resuspended in 10 µl ethyl acetate and samples were spotted on a thin layer chromatography (TLC) silica plate (Baker) and air dried briefly before developing in a 95:5 (v/v) chloroform:methanol environment. The TLC plates were dried and exposed to phosphor screens for at least 4 hours. Visualization of phosphor screen images was done using a phosphorimager (Storm™, Molecular Dynamics) and quantification was done using ImageQuant V. 4.2 software (Molecular Dynamics). CAT activity was expressed as a fraction (%) of the chloramphenicol that had been converted to acetylated species.

3. Luciferase assays

The luciferase assays (Wood and Deluca, 1987) were performed using a commercial Luciferase assay system (Promega). 10 µl of cell extract, prepared as described above for CAT assays, was mixed with 100 µl Luciferase Assay Reagent that had been equilibrated to room temperature. Each sample put in scintillation vials was placed immediately into the scintillation counter (LKB Wallac, 1214 RACKBETA liquid scintillation counter), and luminescence was measured over a one minute period.

4. β-Galactosidase assays

β-Galactosidase assays were used to compare and normalize reporter gene assays for transfection efficiency. 0.1 µg of pSC11 plasmid (Chakarbarti *et al.*, 1985), in which the β-galactosidase gene is driven by the vaccinia p11 promoter, was included in transfection mixes. 25 µl of cell lysate was mixed with 50 µl 2X CPRG reagent (16 mM chlorophenol red-β-D-galactopyranoside, 120 mM Na2HPO$_4$ 7H$_2$O, 80 mM NaH$_2$PO$_4$. H$_2$O, 20 mM Kcl, 2mM MgSO$_4$.7H$_2$O, 10 mM β-mercaptoethanol) in 96 well microtitre plates. The reaction was monitored by measuring the absorbance at 590 nm in a Elisa plate reader (Spectracount™, Packard Instruments) over time intervals of 5 minutes. The net change in absorbance per minute over a total time interval of 45 minutes was assumed to be directly proportional to the β-Galactosidase activity of the sample and to reflect the relative transfection efficiency of each sample.

5. Protein assays

Protein assays were performed using the Bio-Rad DC Protein Assay dye method according to the microassay procedure. A 5 µl aliquot of a 1:10 dilution of each cell lysate was pipetted into wells of a 96 well plate. 25 µl of Reagent A' were added to each well followed by 200 µl of reagent B and the samples were mixed briefly. Absorbance was measured at 750 nm. The values were compared against a standard curve prepared with known concentrations (0.2 mg/ml to 1.5 mg/ml) of bovine serum albumin (BSA).

K. RNA analysis

1. RNA isolation

Total RNA was prepared by homogenising cells from single wells of 6-well plates in 1 ml Trizol Reagent (Life Technologies, Chomczynski and Sacchi, 1987). Homogenized samples were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. RNA was then extracted by adding 200 µl chloroform followed by vigourous shaking and incubation at room temperature for 10 minutes. The samples were centrifuged at 12,000Xg for 15 minutes at 4°C to yield a lower, red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase, which contains the RNA was then transferred to a fresh tube and the RNA was precipitated by mixing with 500 µl isopropanol. Following centrifugation, the pellet was washed with 1 ml 75% ethanol and then redissolved in 20 µl DEPC treated dH_2O. RNA quality was assessed by agarose gel electrophoresis.

Poly A+ RNA was isolated (Aviv and Leder, 1972) using Oligo (dT)- cellulose columns (Molecular Research Center, Inc.). 30-100 µg of total RNA was dissolved in 1 ml DEPC-dH_2O in an Eppendorf tube, heated for 5 minutes at 70°C, quickly cooled on ice, and mixed with an equal volume of 2X Binding Buffer (1.0 M NaCl, 100 mM sodium citrate, 0.2 % SDS). This RNA solution was then passed over an oligo (dT)- cellulose column equilibrated in 1X Binding Buffer. The eluate was collected, heated and passed again over the column. The column was then washed with 5 ml of binding buffer followed by washing with 5 ml of low salt buffer (50 mM sodium citrate, 0.1 % SDS, 0.1 M NaCl) to remove residual ribosomal RNA that may have bound to the column. The bound poly A+ RNA was recovered by eluting the column with 0.6 ml of elution buffer (1 mM sodium citrate, 0.1 % SDS). The eluted RNA was then precipitated by adding 6 µl of polyacryl carrier, NaCl to 0.3 M and 1.5 volumes of isopropanol. Following centrifugation, the pellet was washed with 75 % ethanol, dissolved in 10 µl DEPC-dH_2O and analyzed by Northern blotting and hybridisation.

2. Northern analysis

RNAs were denatured with glyoxal and electrophoresed on 1.2 % agarose gels, prepared according to Sambrook et al (1989). Electrophoresis was carried out until the bromophenol blue had migrated three-quarters of the length of the gel.

Transfer of RNA to positively charged nylon membranes (0.2 µm pore size, Boehringer Mannheim) was accomplished by rapid downward capillary transfer (Sambrook et al., 1989) in 10X SSPE (1.8 M NaCl, 0.1 M sodium phosphate, pH. 7.7, 10 mM EDTA)

using the Turboblotter assembly (Schleicher and Schuell). Membranes were soaked for 10 minutes in 10x SSPE before overnight transfer. After the transfer, membranes were rinsed in 5x SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0) and RNA was fixed by an ultraviolet crosslinking step (Noonberg et al., 1994) for 5 minutes.

Hybridization was performed according to the protocol of Church and Gilbert (1984) with ^{32}P labeled riboprobes. Prehybridisation and hybridisation were performed in a hybridisation oven (Bellco). Prehybridisation of membranes was done at 65 °C for 2 hours in Church buffer (0.25 M Na_2HPO4, pH 7.2, 1 mM EDTA, 10 % SDS, 1 % casein (Engler-Blum et al, 1993) . Hybridisation was done in a similar buffer containing 1X 10^6 cpm/mL of ^{32}P- labelled riboprobes for 12-20 hours at 65 °C. After hybridization, blots were washed 1 hour in 2X SSPE, 0.1% SDS at 68° C followed by a wash in 0.1X SSPE, 0.1% SDS at 68 °C for 40 minutes and a final rinse in 0.1X SSPE. Membranes were air dried and exposed to phosphor screens. The data were analyzed by phosphor imaging (Molecular Dynamics) and the levels of monocistronic and bicistronic transcripts were determined. Some blots were also exposed to Cronex X-ray film (DuPont) and radioactive bands were cut from the blots and counted for radioactivity in the 1214 RACKBETA liquid scintillation counter. The percent readthrough (% RT) was calculated as the fraction of the total mRNA that was detected as a bicistronic transcript.

The % CAT readthrough transcription =

$$\frac{\text{amount of readthrough transcript} \quad X \quad 100}{\text{amount of readthrough transcript + amount of monocistonic transcript}}$$

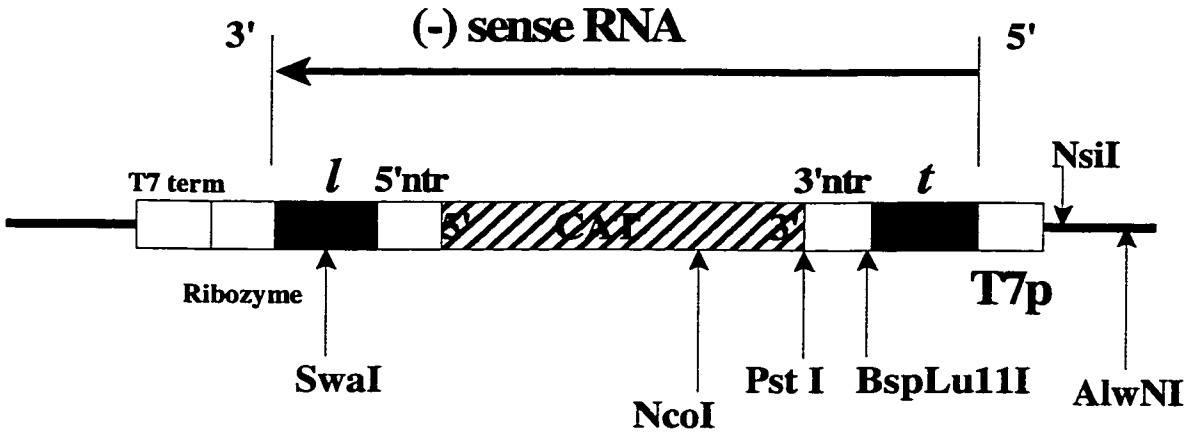
IV. RESULTS

The main objective of this work was to begin the analysis of transcriptional regulatory sequences of hPIV3 using a minigenome rescue system. The initial hypothesis was that all cis acting sequences required for transcription termination and reinitiation reside in hPIV3 gene junctions sequences. To test this hypothesis, plasmids encoding monocistronic and bicistronic minigenomes with wild type and mutated gene junction sequences were designed to be tested in the minigenome rescue system.

A. Constructs

Plasmids encoding hPIV3 minigenome analogs in which viral genes were replaced by the luciferase and/or CAT reporter genes, and which satisfy all of the requirements for rescue in a vTF7-3 driven system (Durbin *et al.*, 1997), were constructed by modifying previous plasmids (Saffran, 1995). These requirements included the addition of a hepatitis delta virus (HDV) ribozyme sequence at the 3' end of the hPIV3 genomic sequences (Perrotta and Been, 1991) and a GG dinucleotide between the 5' end of the hPIV3 sequences and the T7 promoter. All of the minigenomes or minireplicons described below were designed to yield (-) sense RNA when transfected into cells infected with vTF7-3 and contain the following sequences in the 3'→5' direction: 55 nt hPIV3 leader, 10 nt hPIV3 NP gene start, 46 nt non-translated NP sequence, reporter gene sequences and hPIV3 gene junctions, 58 nt non-translated L sequence, 13 nt L gene end sequence, and 44 nt hPIV3 trailer sequence (Refer to Figure 6). The 226 nt hPIV3 terminal sequences have been shown to be

Figure 6 **Schematic of cDNA encoding the monocistronic minigenome analog pCAT2G0.** pCAT2G0 contains the CAT orf which is flanked by hPIV3 5' nontranslated sequences (ntr) of NP gene, 3' ntr of the L gene, leader (*l*) and trailer (*t*). The positions of the T7 terminator (T7 term), HDV ribozyme (Rib) and T7 promoter (T7p) sequences are also indicated. The positions of various restriction endonucleases are also indicated. The direction and the length of the RNA transcribed intracellularly by T7 RNA polymerase is depicted by the dashed horizontal line.

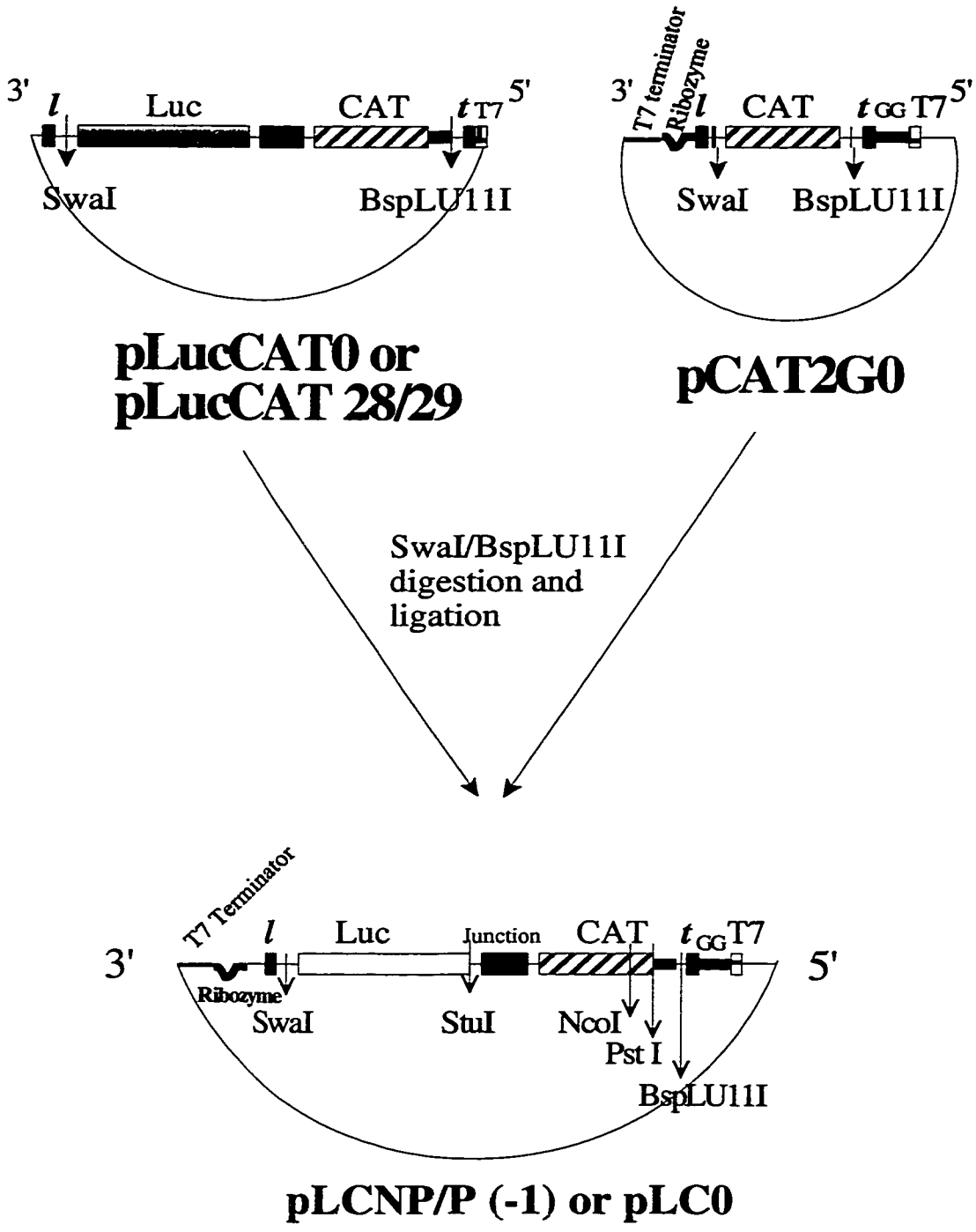

3'
(-) sense RNA
5'
T7 term
l
5'ntr
3'ntr
t
NsiI
5'
CAT
3'
Ribozyme
T7p
SwaI
NcoI
Pst I
BspLu11I
AlwNI

both necessary and sufficient for transcription and replication of minigenomes (Dimock and Collins, 1993). All modifications were confirmed by sequence analysis.

i) Monocistronic constructs

The first monocistronic plasmid constructed was pCAT2G0 (Figure 6), which encodes a minigenome that is 856 nt in length and was derived from pCAT2G (Durbin *et al*, 1997) as follows. pCAT2G encodes a negative sense minigenome consisting of the 3'-terminal 111 nt and 5'- terminal 115 nt hPIV3 sequences fused to a negative sense copy of the CAT orf, and flanked by: (1) the HDV ribozyme sequence and T7 RNA polymerase terminator sequence on the upstream border of the minigenome and (2) the T7 promoter sequence with a GG dinucleotide on the downstream border of the minigenome. There are two BspLU11I sites in pCAT2G, one located at the junction between the L gene end sequence and the trailer, and a second located in the vector. pCAT2G was partially digested with BspLU11I, the 5' BspLU11I overhangs were filled in using the Klenow fragment of DNA polymerase and plasmids were recircularized by ligation. Transformants (pCAT2G0) were screened for plasmids in which the BspLU11I site in the vector had been destroyed. Digestion of pCAT2G0 (Figure 7) with BspLU11I and SwaI , therefore, releases a large restriction fragment that includes the HDV ribozyme, the T7 terminator, the vector, the T7 promoter with 2G's between the promoter and the 5' end of the hPIV3 genome, and the trailer. This BspLU11I-SwaI fragment of pCAT2G0 was used to provide the ribozyme, the T7 terminator and the T7 promoter for many of the bicistronic plasmids described below.

Figure 7 **Construction of pLCNP/P(-1) and pLC0.** Restriction sites described in the text are shown. The SwaI/BspLU11I fragment of pCAT2G0 was used to add the HDV ribozyme and the T7 promoter sequences plus 2 extra G's to the 3' and 5' ends of the pLucCAT0 or pLucCAT28/29 (see Saffran, 1995 and the text) yielding pLC0 and pLCNP/P (-1) respectively.


3'
l
Luc
CAT
t T7
5'
SwaI
BspLU11I
pLucCAT0 or
pLucCAT 28/29
T7 terminator
Ribozyme
3'
l
CAT
t GG T7
5'
SwaI
BspLU11I
pCAT2G0
SwaI/BspLU11I
digestion and
ligation
T7 Terminator
3'
l
Luc
Junction
CAT
t GG T7
5'
Ribozyme
SwaI
StuI
NcoI
Pst I
BspLU11I
pLCNP/P (-1) or pLC0

pCAT2G0 also served as the parental plasmid for a series of constructs designed to verify that hPIV3 minigenomes obey the rule of six. The NcoI/NsiI fragment of pCAT2GO was replaced with the corresponding fragments from a series of previously constructed plasmids (K.Dimock, unpublished) that contain 1-6 additional nucleotides at the PstI site located at the boundary between the CAT noncoding sequences and the L gene noncoding sequences (Figure 6, Table 3). These modifications yielded plasmids pCAT2G1-pCAT2G6 which encode minigenomes that are (6n+1) to (6n+6) nt in length. The NcoI/NsiI or NcoI/AlwNI fragments of these plasmids were also used to adjust the overall length of bicistronic minigenomes to 6n nt.

ii) Bicistronic constructs

Two previously constructed plasmids, pLuc/CAT0 and pLuc/CAT28/29 (Saffran, 1995) were the starting points for the construction of plasmids containing bicistronic minigenomes (Figure 7). The BspLU11I-SwaI fragments of these two plasmids were replaced by the BspLU11I-SwaI of pCAT2G0, yielding plasmids pLC0 and pLCNP/P (-1). pLC0 encodes a bicistronic minigenome that is a multiple of 6 nt (2658 nt) in length, in which the luciferase orf is located 3' to the CAT orf and separated from the CAT orf by nonviral sequences. pLC0, therefore serves as a negative control to verify the function of hPIV3 junction sequences. In pLCNP/P(-1), the luciferase and CAT cistrons are separated by the hPIV3 NP/P gene junction, but the minigenome is 6n-1 (2663 nt) in length. A third plasmid, pLCNP/P (Figure 8) was derived from pLCNP/P (-1) with the corresponding fragment from pCAT2G1, resulting in the addition of a single nucleotide at the PstI site.

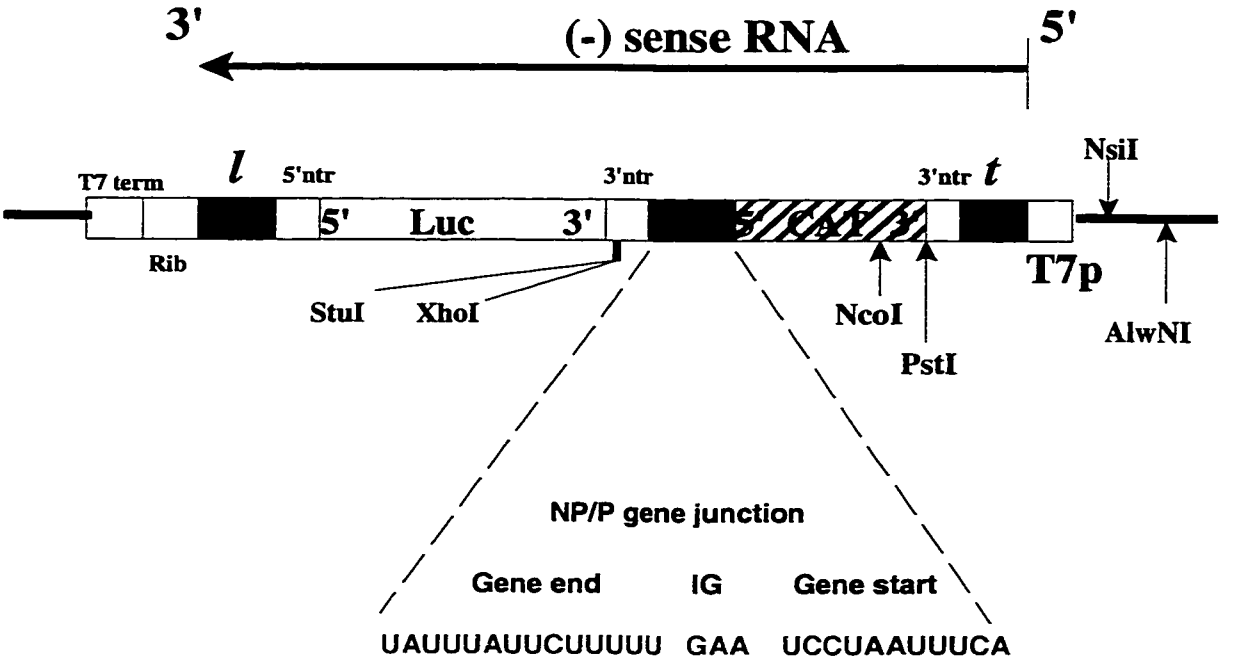
Table 3: Features of plasmids pCAT2G0-pCAT2G6

Construct	Plasmid [1] used to alter PstI site (NcoI/NsiI fragment)	Sequence inserted at the PstI site (underlined nucleotides indicate change in sequence)	Register of the resulting minigenome[2]
pCAT2G0	-	CTGCAG	0
pCAT2G1	5306	CTGCA<u>A</u>G	+1
pCAT2G2	5307	CTGCA<u>AA</u>G	+2
pCAT2G3	5308	CTGCA<u>AAA</u>G	+3
pCAT2G4	5309	CTGCA<u>AAAA</u>G	+4
pCAT2G5	5305	CTGCA_	-1 (+5)
pCAT2G6	PST+6	CTGCA<u>AAAAAA</u>G	+6

[1] The NcoI/NsiI fragment of pCAT2G0 was replaced by the corresponding NcoI/NsiI fragment of the indicated plasmid. Plasmids 5305-5309 and Pst+6 were previously constructed from oligonucleotides made by Dr. K.Dimock.

[2] A register of 0 indicates that the minigenome is predicted to be 6n nt in length.

Figure 8 **Schematic of cDNA encoding the bicistronic minigenome analog pLC NP/P.** Reporter genes Luc and CAT are separated by the hPIV3 NP/P gene junction. The restriction sites used for adjusting the position of the downstream (CAT) mRNA transcriptional initiation site (XhoI/StuI) and the lengths of various constructs (PstI) are indicated. All other regions are as described in the legend to Figure 7 and in the text.


3'
(-) sense RNA
5'
T7 term
l
5'ntr
3'ntr
3'ntr
t
NsiI
5'
Luc
3'
Rib
StuI
XhoI
NcoI
PstI
T7p
AlwNI
NP/P gene junction
Gene end
IG
Gene start
UAUUUAUUCUUUUU
GAA
UCCUAAUUUCA

pLCNP/P was used as the reference plasmid for many of the experiments detailed below.

The Tc series of constructs was designed to assess the importance of the position of the CAT transcriptional initiation site relative to the NP monomer that would cover the site. Self complementary oligonucleotides 7-13 nt in length, and containing either BamHI or AvaII restriction sites to facilitate screening (Table 4) were inserted into the StuI site in noncoding sequences of the luciferase gene in pLCNP/P(-1), to produce plasmids pLCNP/P Tc1-6 (Figure 9). This generated plasmids in which the position of the transcriptional initiation site was shifted from position 1, within the NP monomer covering the transcription start site , to positions 2 - 6. The overall length of these minigenomes were then adjusted to 6n using the appropriate NcoI/BspLU11I fragment from pCAT2G0-5 (Table 4).

Plasmids encoding minigenomes with authentic M/F, F/HN and HN/L junctions (the P/M junction is identical to the NP/P junction) or mutant hPIV3 gene junctions were also engineered (Tables 5 and 6). Some of these plasmids were created (Table 6, Figure 8) by substituting the XhoI/AlwNI fragments from one of several previously constructed plasmids (Saffran, 1995) for the corresponding fragment of one of the pLCNP/P tc plasmids. Alternatively, annealed pairs of complementary oligonucleotides were inserted between the BamHI and SacII sites which flank the nonviral junction in pLC0. All of these constructions were designed to maintain the CAT mRNA transcriptional start site at the position 1 relative to the NP monomer and the overall length of the constructs at 6n, using the appropriate NcoI/NsiI or NcoI/AlwNI fragment from pCAT2G1 or pCAT2G2.

It was necessary to rule out the possibility that M sequences upstream of the M/F junction and/or F sequences downstream of the M/F junction contributed to transciptional

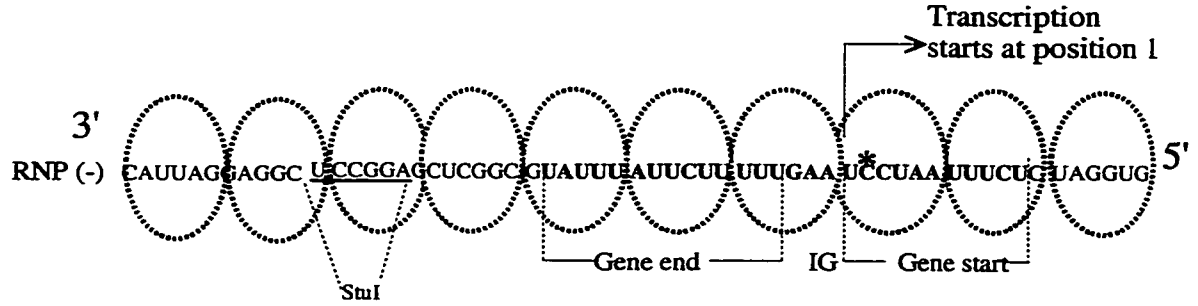
Table 4: Features of plasmids pLCNP/P tc1- 6.

Construct	Oligonucleotides inserted at the StuI site	Number of nt inserted at the StuI site	Number of nt inserted at the PstI site [1]	Number of nt in minigenome (register)
pLCNP/P (-1)	-	-	-1	2663 (-1)
pLCNP/P tc1	**CAC** GGATCC **GTG**[2]	+12	+1	2676 (0)
tc2	**ACAC** GGACC **GTGT**[3]	+13	0	2676 (0)
tc3	**C** GGATCC **G**[2]	+8	-1	2670 (0)
tc4	**AC** GGACC **GT**[3]	+9	+4	2676 (0)
tc5	**AC** GGATCC **GT**[2]	+10	+3	2676 (0)
tc6	**CAC** GGTCC **GTG**[3]	+11	+2	2676 (0)

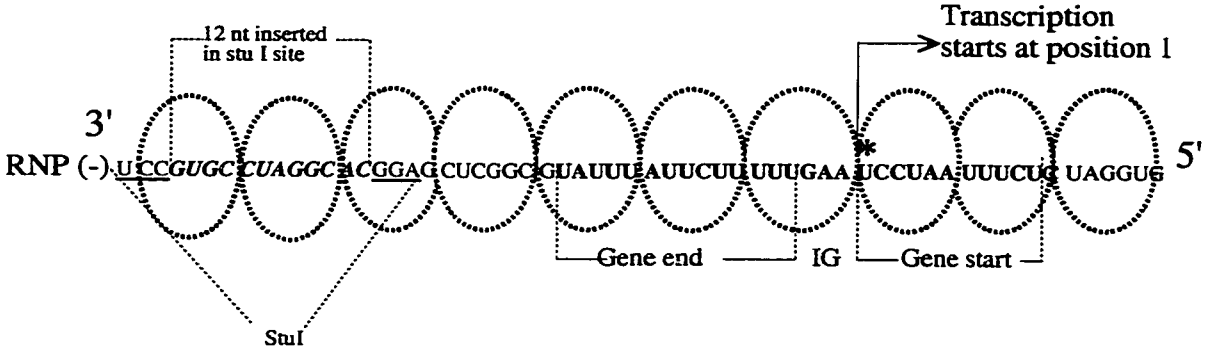
[1] PstI site eliminated with insertion of NcoI/BspLU11I fragment from pCAT2G0-5
[2] New BamHI site introduced
[3] New AvaII site introduced

Figure 9 **Shifting the CAT transcriptional start site.** **(A)** The sequence surrounding the transcriptional start site for the downstream CAT mRNA in a bicistronic minigenome is depicted in the 3' to 5' orientation. Ellipses represent NP monomers. The NP/P gene junction sequences are in bold text and the position of the transcriptional start site (position 1) is indicated by an asterisk. Nucleotides representing the StuI site used for adjusting the position of the CAT mRNA transcriptional initiation site are underlined and nucleotides inserted in the StuI site are bold and italicized. **(B)** Insertion of 12 nucleotides maintains the position of the transcriptional start site relative to the NP monomer. **(C)** Insertion of 13 nucleotides shifts the transcriptional start site to position 2 relative to the NP monomer.

A.


Transcription
starts at position 1
3'
RNP (-) CAUUAGGAGGC UCCGGAGCUCGGCGUAUUUAUUCUUUUGAAUCCUAAUUUCUCUAGGUG 5'
StuI
Gene end
IG
Gene start

B.


12 nt inserted
in stu I site
Transcription
starts at position 1
3'
RNP (-) UCCGUGCCUAGGCACGGAGCUCGGCGUAUUUAUUCUUUUGAAUCCUAAUUUCUCUAGGUG 5'
StuI
Gene end
IG
Gene start

C.

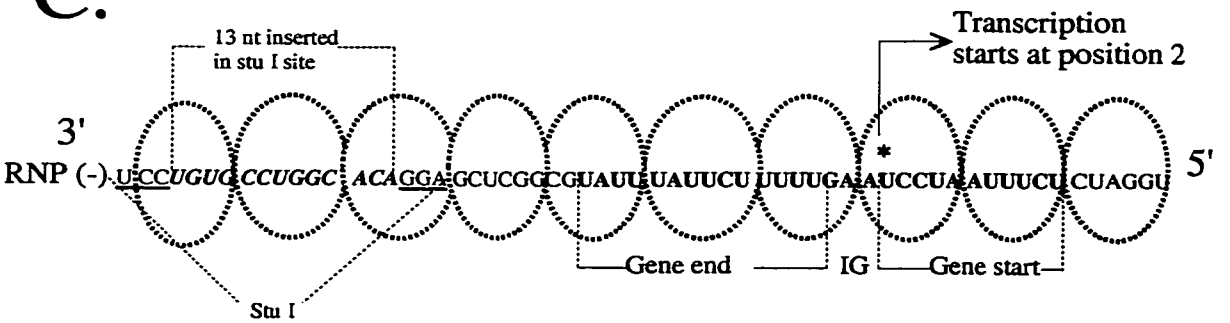

13 nt inserted
in stu I site
Transcription
starts at position 2
3'
RNP (-) UCCUGUGCCUGGCACAGGAGCUCGGCGUAUUUAUUCUUUUGAAUCCUAAUUUCUCUAGGU 5'
Stu I
Gene end
IG
Gene start

Table 5: Bicistronic minigenomes containing different hPIV3 junctions[1]

Construct	Features
pLC0	Nonviral sequences between Luc and CAT orfs
pLCNP/P(-1)	Authentic hPIV3 NP/P junction, out of register
pLCNP/P	Authentic hPIV3 NP/P junction
pLC NP/P ΔIG	hPIV3 NP/P junction lacking the intergenic trinucleotide motifs between GE and GS
pLCNP/P 3IG	hPIV3 NP/P junction containing three intergenic trinucleotide motifs between GE and GS
pLCM/F	Authentic M/F junction
pLCM/F Δ8	Eight nucleotides deleted from M gene end sequence
pLCF/HN	Authentic F/HN junction sequence
pLCHN/L	Authentic HN/L junction sequence

[1] All minigenomes are 6n nt in length except pLCNP/P(-1), which is 6n-1 nt in length.

Table 6: Features of bicistronic constructs containing different hPIV3 junctions

Construct	Change in gene junction[1]	Change in Luc 3' NTR (StuI site)[2]	Change in CAT 3' NTR (Pst I site)[3]	Number of nt in minigenome (register)
pLC0	-	_	_	**2658 (0)**
pLCNP/P	_	_	+1 (pCAT2G1)	2664 (0)
pLCNP/P (-1)	_	_	_	2663 (-1)
NP/P 3IG	+6 (pLCNP/P 3IG)	+12 (pLCNP/P Tc1- XhoI/Nsi I fragment	+1 (pCAT2G1-NcoI/NsiI)	2682 (0)
NP/PΔIG	-3 (34/35)	+8 (pLCNP/P tc3)	+2 (pCAT2G2)	2670 (0)
M/F	+8 (pLCM/F)	+9 (pLCNP/P tc4-XhoI/NsiI)	+2 (pCAT2G2-NcoI-NsiI)	2682 (0)
M/F Δ8	_ (38/39)	+12 (pLCNP/P tc1)	+1 (pCAT2G1)	2676 (0)
F/HN	_ (30/31)	+12 (pLCNP/P tc1)	+1 (pCAT2G1)	2676 (0)
HN/L	_ (32/33)	+12 (pLCNP/P tc1)	+1 (pCAT2G1)	2676 (0)

[1] The XhoI/ NcoI fragment of the plasmid specified in parenthesis was used to insert the desired junction sequence between the Luc and CAT orfs. The change in the number of nucleotides as compared to the NP/P P junction in pLCNP/P(-1) is indicated.

[2] The transcription start site for the CAT orf was adjusted to the +1 position with the XhoI/AlwNI fragment of the plasmid specified in parenthesis, except for pLCM/F and pLCNP/P 3IG where an XhoI /NsiI fragment was used. The number of nucleotides added at the StuI site is indicated.

[3] The register of the construct was adjusted to 6n nt (register of 0) with the NcoI/AlwNI fragment of the plasmid specified in parenthesis, except for pLCM/F and pLCNP/P3IG where an NcoI/NsiI fragment was used. The number of nucleotides added at the PstI site is indicated.

readthrough at this junction during viral replication. Another bicistronic minigenome was designed that contained not only the M/F junction but also 445 nt of M gene sequence upstream and 444 nt of F gene sequences downstream of the hPIV3 M/F junction, disrupting the luc and CAT coding sequences in pLC NP/PTc5. A 943 bp EcoRV and NcoI restriction fragment from pLC NP/PTc5 was excised and replaced with a 892 bp PCR fragment generated with the MF+ and MF- primers. The new construct was designated pL-m/f-C (Figure 10).

B) Reporter gene expression studies

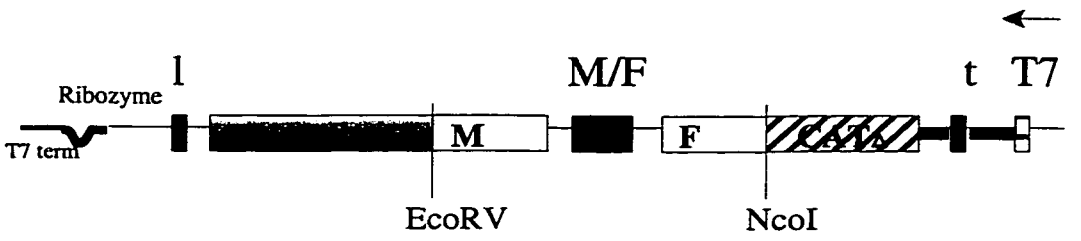
Monocistronic and bicistronic minigenomes were tested for their ability to drive the transcription of reporter genes. HEp-2 cells were infected with recombinant vaccinia virus vTF7-3 and transfected with plasmids pTMNP, pTMP and pTML (encoding hPIV3 NP, P and L proteins) and pSC11. Cells were harvested and assayed for CAT, luciferase and β-galactosidase activities 24 hours post transfection. Protein assays were also carried out on the lysates to normalize for cell number.

i) Rescue of hPIV3 minigenomes obeys the rule of six

Rescue of some paramyxovirus genome analogs demands that the genome analogs be a multiple of 6 nt in length. The rule of six states that each NP monomer is associated with precisely 6 nucleotides. The first series of experiments, therefore, were designed to determine if the rule of six also applied to the rescue of hPIV3 minigenome analogs. Preliminary work has shown that the minigenome encoded by pCAT2G was rescued

Figure 10 **Schematic of pL-m/f-C.** pL-m/f-C was derived from pLCNP/P by substituting hPIV3 M and F sequences for an EcoRV/NcoI fragment containing luciferase and CAT sequences immediately flanking the M/F gene junction. All other regions are as described in the legend to Figure 7 and in the text.

pL-m/f-C



efficiently (Durbin *et al.*, 1997; K.Dimock, J. Rouselle, unpublished) and that little or no CAT activity was observed for negative controls (i.e. if any one of the pCAT2G, pTMN, pTMP or pTML were left out of the transfection mix, or if wild type vaccinia virus (VWR) was used rather than vTF7-3). Rescue efficiency was measured in terms of relative CAT activity. pCAT2G0 was treated as a reference plasmid and its activity was set to 100% for comparision with other constructs.

As shown in Figure 11, when the pCAT2G series of plasmids were tested in the complete rescue system, the minigenomes encoded by pCAT2G0 and pCAT2G6, which are 6n nt in length, were rescued 50-200 times more efficiently than the minireplicons that are not multiples of 6 nt. This clearly demonstrates that efficient rescue of hPIV3 minigenomes, as for several other paramyxoviruses, requires that the minigenomes be a multiple of six nucleotides. In addition, the data indicate that insertion of as as many as six nt at the PstI site in the L gene non coding region of pCAT2G0 has only a small, if any, effect on rescue. This site, therefore appeared to be suitable for adjusting the length of bicistronic minigenomes.

ii) Rescue of bicistronic minigenomes

It was also important to confirm that the bicistronic Luc-CAT minigenomes could be rescued and that junction sequences between Luc and CAT orfs functioned as expected. The ability of pLCNP/P and pLC0 to direct CAT expression in transfected cells was compared. It can be seen (Figure 12) that CAT activity in lysates of cells transfected with pLCNP/P, which has an NP/P junction, was ~120 times greater than in cells transfected with pLC0, which expressed background levels of CAT activity. This experiment confirms that

Figure 11 **Minigenomes which obey the rule of six are efficiently rescued.** vTF7-3 infected HEp-2 cells were transfected with plasmids pTMNP, pTMP and pTML (encoding the hPIV3 NP, P and L proteins, respectively) and with a plasmid encoding a monocistronic hPIV3 minigenome, pCAT2G0-pCAT2G6, as indicated. CAT assays were carried out 24 hr after transfection (n=5). CAT activity in pCAT2G0 transfected cells was set at 100 % and used for comparision with the other constructs.

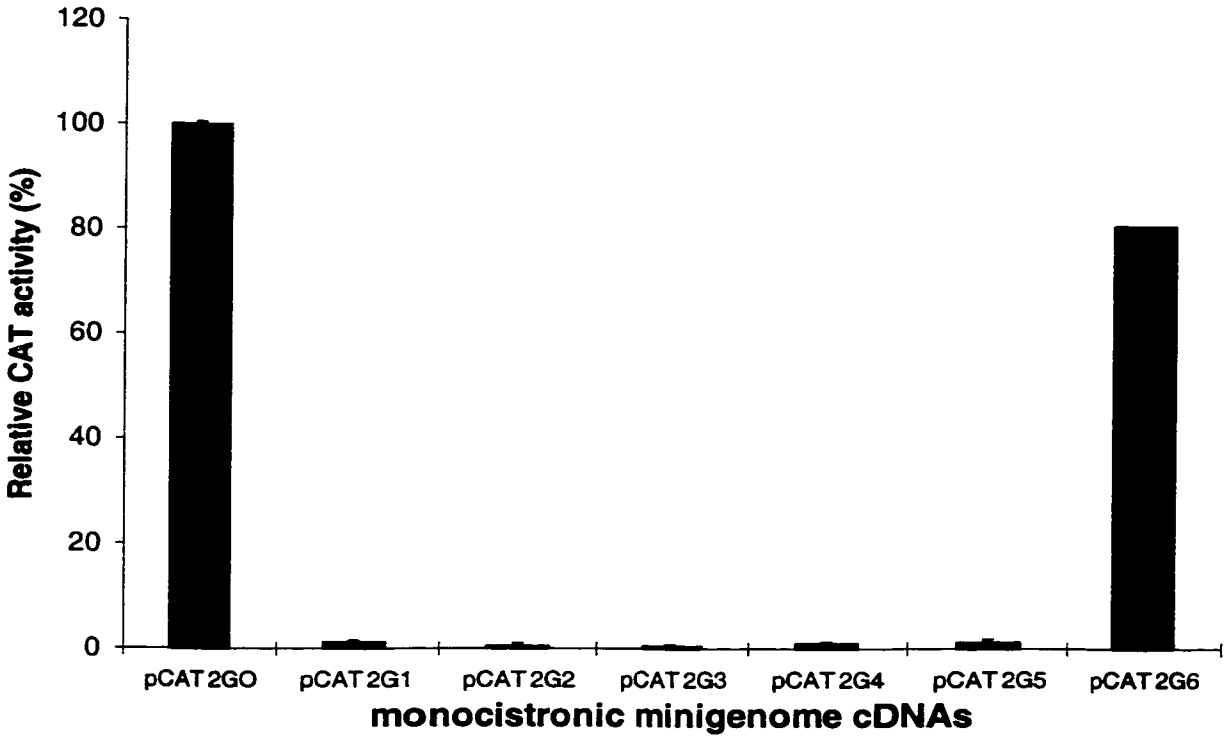
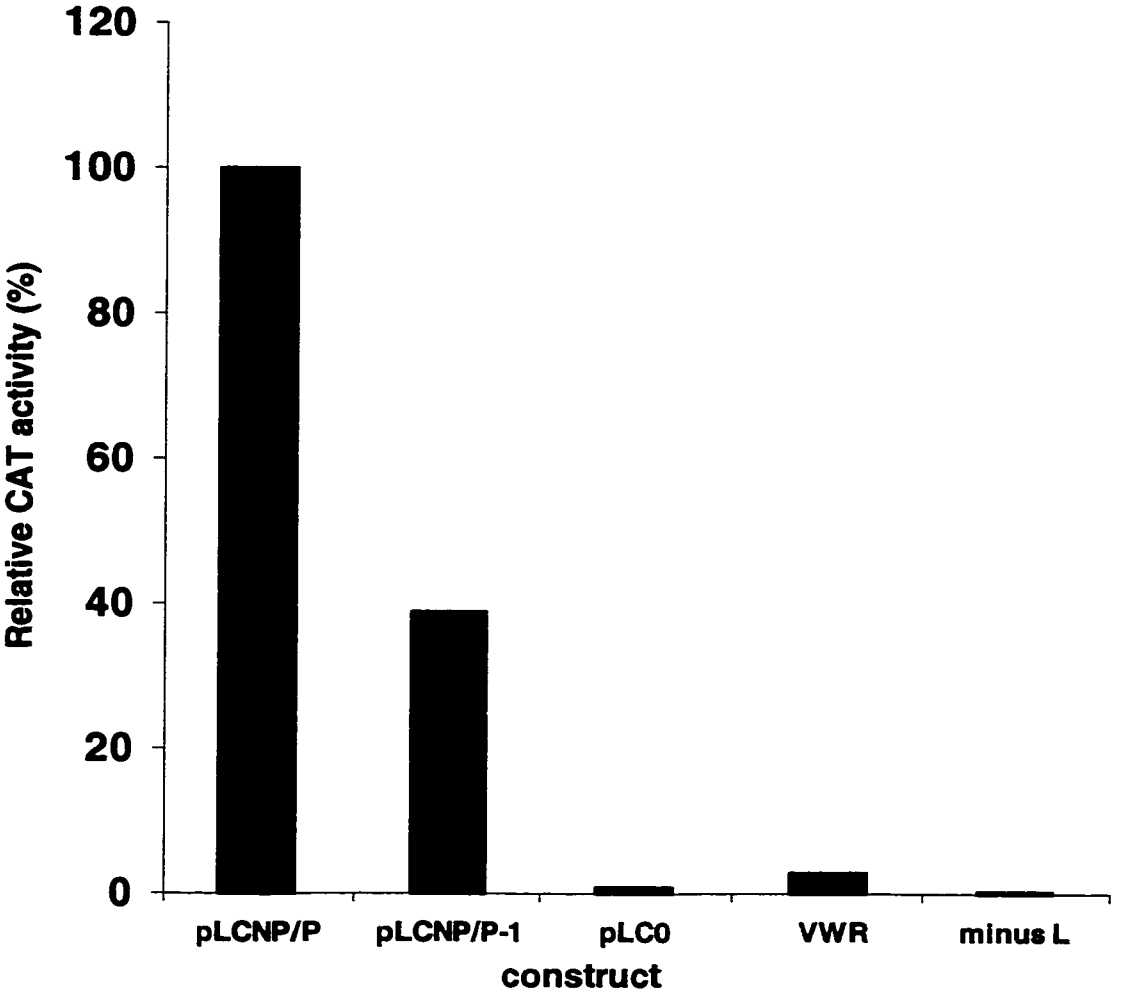

120
100
80
60
40
20
0
Relative CAT activity (%)
pCAT2GO
pCAT2G1
pCAT2G2
pCAT2G3
pCAT2G4
pCAT2G5
pCAT2G6
monocistronic minigenome cDNAs

Figure 12 **Rescue of bicistronic minigenomes.** vTF7-3 infected HEp-2 cells were transfected with plasmids pTMNP, pTMP and pTML and with a plasmid encoding a bicistronic hPIV3 minigenome, as indicated. CAT assays were carried out 24 hr after transfection (n=3). CAT activity in pLCNP/P transfected cells was set at 100 % and used for comparision with other plasmids. There were 2 negative controls for this experiment: cells were transfected with pLCNP/P and with the plasmids encoding the hPIV3 proteins, but were infected with wild type vaccinia virus (VWR); cells were transfected with pLCNP/P, pTMNP and pTMP, but not with pTML (minus L). The CAT activity data were not normalized in this experiment.


120
100
80
60
40
20
0
Relative CAT activity (%)
pLCNP/P
pLCNP/P-1
pLC0
VWR
minus L
construct

the hPIV3 NP/P junction sequence is required for transcription of the downstream CAT sequences and expression of CAT.

Rescue of the pLCNP/P minigenome was also found to be 2.5 fold greater than rescue of the minigenome encoded by pLCNP/P(-1). This suggests that the rule of six is important but that the effect was smaller as compared to what was observed with the monocistronic minigenomes. It should be noted that rescue of bicistronic minigenomes was approximately 3 - 4 fold less efficient than rescue of monocistronic minigenomes as determined by CAT assay. There were two negative controls for this experiment. In the VWR control, cells were transfected with pLCNP/P and with the plasmids encoding the hPIV3 proteins, but were infected with wild type vaccinia virus (VWR). In the minus L control, cells were infected with VTF7-3 and transfected with pLCNP/P, pTMNP and pTMP, but not with pTML.

iii) Shifting the transcription start site to six possible positions relative to the NP subunit has no major effect on downstream CAT gene expression.

One objective of this research was to confirm the role of the eight extra nt in the M gene end sequence which are predicted to function as an antiterminator leading to the synthesis of increased amounts of M-F readthrough transcripts. This could be investigated by deleting the 8 nt and observing the effect of the deletion on transcription of the downstream CAT gene. One side effect of this deletion would be to alter the position of the transcription initiation site of the CAT gene to the +3 position with respect to the NP monomeric subunit at this position in the RNP. Therefore it was important to test whether repositioning the CAT transcriptional start site had any major effects on CAT expression.

It should also be recognized that the position of the transcriptional start site for all of the hPIV3 genes is either +1 or +2.

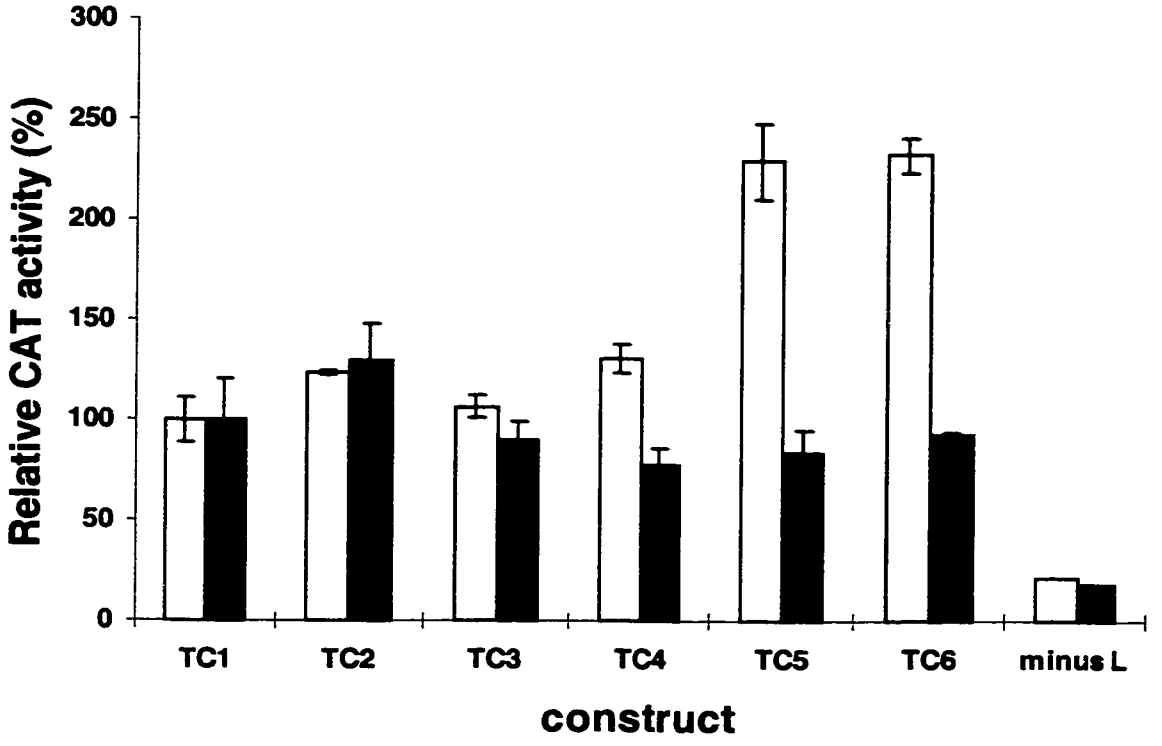
The relative CAT activities of mutants in which the CAT transcriptional start site was shifted to the 6 possible positions within the NP monomeric subunit were compared (Figure 13) using pLCNP/P tc1 as a reference plasmid where the CAT transcription start site is maintained at +1 relative to the NP monomer. The data suggest that the position of the transcriptional start site for the downstream CAT gene has no large effect on transcription of CAT mRNA, as estimated by CAT activity.

The statistical interpretation of the data within each experiment suggests that there is no significant difference ($p>0.05$) between the transcriptional start site mutants. Nevertheless, it was decided that the transcriptional start site for all subsequent constructs be adjusted to position1 relative to the NP monomeric subunit.

iv) The intergenic trinucleotide sequence is required for expression of the downstream gene.

It has been proposed that nontranscribed intergenic sequences are necessary for efficient termination of transcription of upstream genes and that this is an important requirement for reinitiation of downstream gene transcription (Saffran, 1995, Rassa and Parks, 1999, Barr *et al.*, 1997, Stillman and Whitt, 1997, 1998). To investigate the importance of the intergenic hPIV3 trinucleotide sequence, the effect of deletion or reiteration of the sequence on transcription of the downstream CAT gene in bicistronic minigenomes was assessed. Two bicistronic minigenomes, pLCNP/PΔIG and pLCNP/P 3IG

Figure 13 **Effect of shifting the CAT transcriptional start site on the rescue of bicistronic minigenomes.** vTF7-3 infected HEp-2 cells were transfected with plasmids pTMNP, pTMP and pTML and with a plasmid encoding a bicistronic minigenome, pLCNP/P tcC1-tcC6, in which the CAT mRNA transcriptional start site was shifted relative to the NP monomer, as indicated. CAT assays were carried out 24 hr after transfection. White and black bars represent values for two different experiments (n=3) done in triplicate and normalized for transfection efficiency and for protein. CAT activity in pLCNP/P tc1 transfected cells was set at to 100% for comparison with other constructs. In the minus L control, cells were transfected with pLCNP/P tc1, pTMNP and pTMP, but not with pTML (minus L). Lines above the bars represent standard deviation from the mean.


300
250
200
150
100
50
0
Relative CAT activity (%)
TC1
TC2
TC3
TC4
TC5
TC6
minus L
construct

were constructed in which either the IG sequence was completely deleted or two extra IG sequences were inserted between the NP and P cistrons.

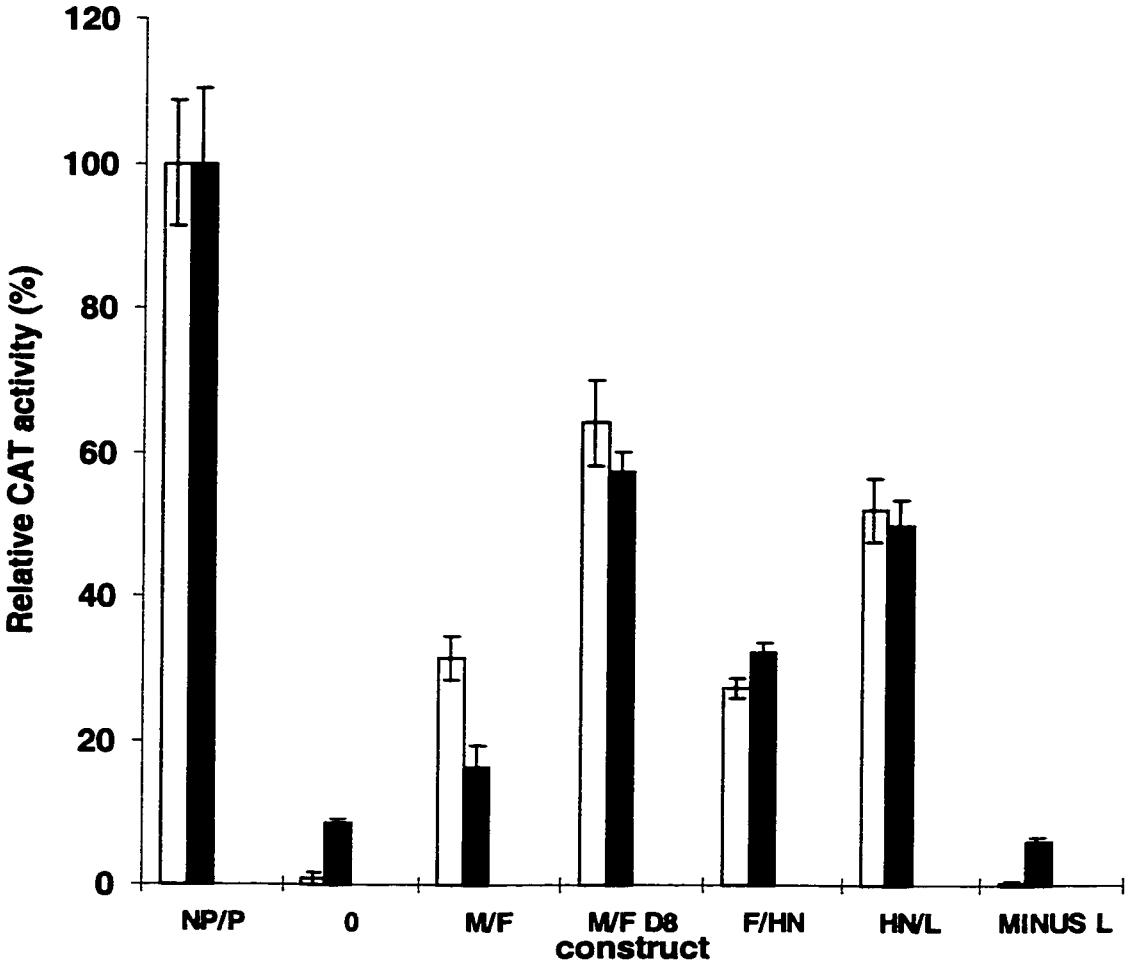
For both of these constructs, little or no CAT activity was observed (data not shown). This indicates that an intact IG sequence is required for transcription of the downstream gene and also suggests that proper spacing between the gene end and gene start sequences is critical.

v) hPIV3 junctions

Two important objectives of this research were: (1) to compare the abilities of the different hPIV3 gene junctions to direct transcription of the downstream mRNA and (2) to determine if the 8 extra nucleotides in the M gene end sequence were necessary and sufficient to cause a high frequency of transcriptional readthrough at the M/F junction. Examination of the various gene junctions in the hPIV3 genome identifies nucleotide differences in the F/HN and HN/L gene start motifs, in addition to the 8 extra nucleotides in the M gene end sequence (Table 1). To determine if nucleotide differences in the various hPIV3 gene junctions affect the ability of these sequences to direct downstream (CAT) transcription, constructs encoding bicistronic minigenomes containing the various junctions were compared for their ability to direct CAT expression. In addition, a construct in which the M/F junction was modified by deletion of the 8 extra nucleotides in the M gene end sequence was tested. As shown in Figure 14, differences in CAT expression were observed.

CAT activity was lowest for the construct containing the M/F junction, which would be expected if the eight extra nucleotides function as an antiterminator, leading to

Figure 14 **Rescue of bicistronic minigenomes containing hPIV3 gene junctions.** vTF7-3 infected HEp-2 cells were transfected with plasmids pTMNP, pTMP and pTML and with a plasmid encoding a bicistronic minigenome containing an hPIV3 gene junction, as indicated. CAT assays were carried out 24 hr after transfection. White and black bars represent values for two different experiments done in triplicate and normalized for transfection efficiency and for protein. CAT activity in pLCNP/P transfected cells was set at to 100% for comparison with other constructs. In the minus L control, cells were transfected with pLCNP/P, pTMNP and pTMP, but not with pTML. Lines above the bars represent standard deviation from the mean.


120
100
80
60
40
20
0
Relative CAT activity (%)
NP/P
0
M/F
M/F D8
F/HN
HN/L
MINUS L
construct

transcriptional readthrough at this junction. However, lower levels of CAT expression could also result from termination without reinitiation, rather than transcriptional readthrough. Deletion of the 8 nucleotides in the M gene end sequence restored much of the ability of the minigenome to direct CAT expression. For minigenomes containing F/HN and HN/L junctions, CAT activity was also lower than observed for minigenomes containing the NP/P junction. These differences could also be attributed to sequence differences.

C) Northern blot analysis of minigenomes

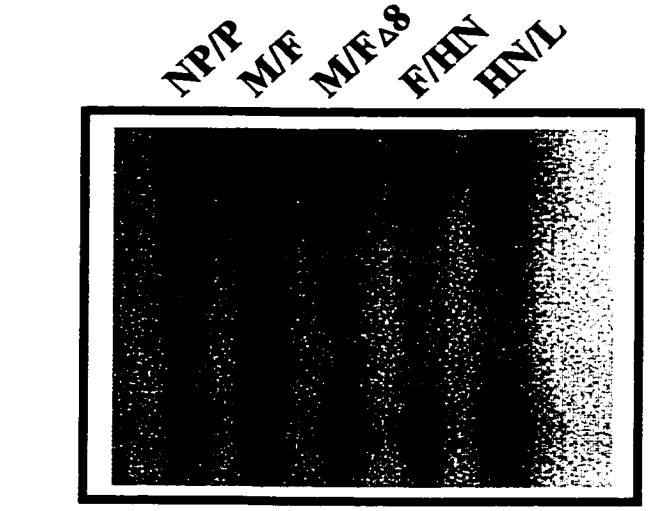
i) Analysis of hPIV3 junctions

The differences seen in CAT expression when constructs containing various gene junctions were compared can be interpreted in several ways. For instance, decreased CAT activity could be the result of polymerase readthrough, improper polyadenylation and termination of the upstream transcript, or a failure of the viral polymerase to reinitiate at the downstream gene. To distinguish between these possibilities, RNA from transfected HEp-2 cells were analyzed by Northern blotting. This approach should permit the identification of the various species of RNAs transcribed from the minigenome by the viral polymerase and help to distinguish between transcriptional readthrough and improper termination.

RNA extracted from HEp-2 cells transfected with plasmids encoding minigenomes with the different hPIV3 gene junctions was probed with a (-) sense [^{32}P] -labeled CAT riboprobe and the % transcriptional readthrough at each junction was determined (refer to the formula given in the Material and Methods). Preliminary results with total RNA (Figure 15A) supported the data obtained from CAT assays. The amount of readthrough was

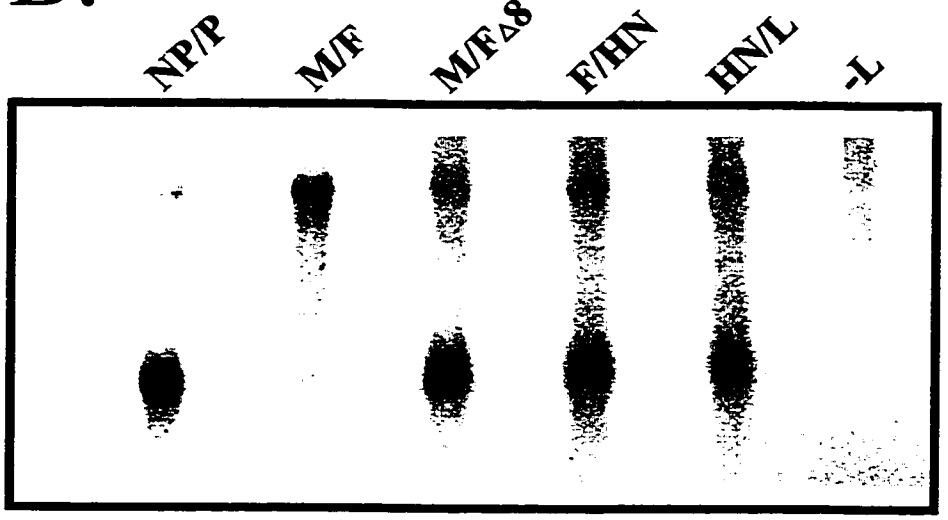
Figure 15 **Northern blot analysis of minigenomes.** vTF7-3 infected HEp-2 cells were transfected with plasmids pTMNP, pTMP and pTML and with a plasmid encoding a bicistronic minigenome containing one of the natural or modified hPIV3 gene junctions, as indicated. Total RNA **(A)** and poly A+ RNA **(B)** from transfected cells were analysed by northern blotting using a ^{32}P-labelled (-) sense CAT riboprobe. The positions of bicistronic Luc-CAT readthrough transcripts and monocistronic CAT mRNA are indicated. The readthrough transcription frequencies (% RT) are indicated below the corresponding lanes for each blot. In the minus L sample, cells were transfected with pLCNP/P, pTMNP and pTMP, but not with pTML.

A.



% RT 37 91 55 57 49

B.



% RT 10 88 12 16 23 ND

estimated to be greater for the M/F junction than for any of the other junctions. However, bicistronic Luc-CAT transcripts will include both polyadenylated transcriptional readthrough products as well as antigenomes. Monocistronic luciferase transcripts were not clearly visible in total RNA blots probed with a (-) sense luciferase probe (not shown). This was thought to be due to large amounts of 18S rRNA that would co-electrophorese with the luciferase transcripts in agarose gels.

These problems were addressed by isolating polyadenylated RNA from transfected HEp-2 cells for northern blot analysis. When polyadenylated RNAs were analyzed by northern blot (Figure 15B) readthrough transcription was lowest for NP/P (10 %) and highest levels for the M/F junction (88 %). Deletion of the 8 extra nucleotides in the M gene end sequence (M/F Δ8) restored the frequency of transcriptional readthrough to the low levels (12 %) observed for the NP/P junction, as expected. These results indicate that the 8 extra nucleotides in the M gene end sequence were both necessary and sufficient for increased transcriptional readthrough. Transcriptional readthrough at the F/HN and HN/L junctions was similar though slightly higher than at the NP/P junction, at 16 and 23 % respectively.

Monocistronic luciferase transcripts could not be detected consistently in blots of polyadenylated RNA despite the use of three different luciferase-specific riboprobes. Possible reasons for this difficulty will be presented in the Discussion.

ii) hPIV3 M and F gene sequences surrounding the M/F gene junction do not contribute in transcriptional readthrough at this junction.

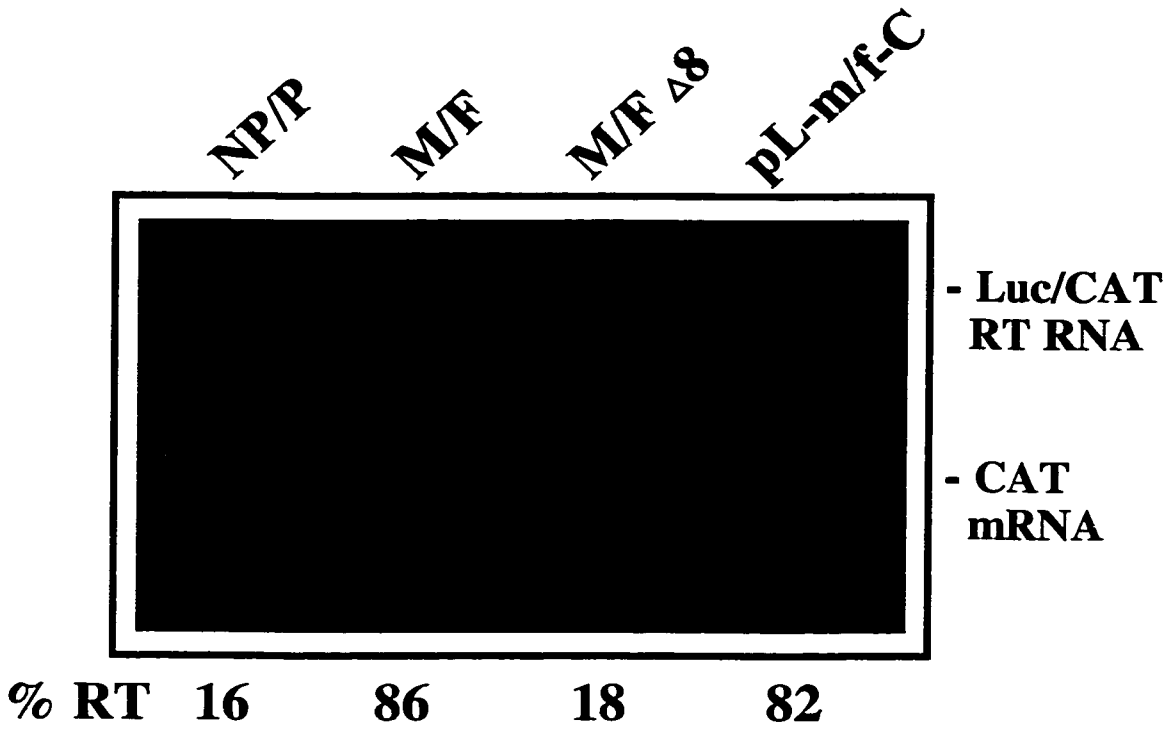
It was necessary to rule out the possibility that M sequences upstream of the M/F

gene junction and/or F sequences downstream of the M/F junction contributed to transcriptional readthrough at this junction during virus replication. Therefore a plasmid was constructed in which 430 bp of M and 450 bp of F gene sequences upstream and downstream of the hPIV3 M/F junction were inserted in place of luc and CAT sequences in pLCM/F (pL-m/f-C). Analysis of polyadenylated RNA from pL-m/f-C transfected cells (Figure 16) by northern blot probed with (-) sense F riboprobe indicated that the frequency of readthrough at the M/F junction (82%) was similar to what was observed for pLC M/F (86%). This result supports the idea that the 8 extra nucleotides in the M/F junction are sufficient to direct transcriptional readthrough.

iii) The frequency of M/F transcriptional readthrough in transfected and hPIV3 infected cells is comparable.

The level of M-F readthrough transcription in hPIV3 infected HEp-2 cells was estimated to be between 60% and 75% by northern blot analysis using a (-) sense F specific riboprobe (nt 60-252 of the F gene, not shown). These results confirm previous reports that 50-80% of F transcripts are found in a bicistronic M-F mRNA (Spriggs and Collins, 1986). Therefore the level of M-F readthrough transcription in this system is very similar to what has been determined for a *bona fide* hPIV3 infection.

Figure 16 **Northern blot analysis of pL-m/f-C.** vTF7-3 infected HEp-2 cells were transfected with plasmids pTMNP, pTMP and pTML and with a plasmid encoding a bicistronic minigenome containing one of the natural or modified hPIV3 gene junctions, as indicated. Poly A+ RNA isolated from transfected cells was analysed by northern blotting with a ^{32}P-labelled negative sense CAT riboprobe. The positions of bicistronic Luc-CAT readthrough transcripts and monocistronic CAT mRNA are indicated. The readthrough transcription frequencies (% RT) are indicated below the corresponding lanes.


NP/P
M/F
M/F Δ8
pL-m/f-C
- Luc/CAT RT RNA
- CAT mRNA
% RT 16 86 18 82

V. DISCUSSION

Paramyxovirus transcription is unique in several ways. Transcription of the viral genome is the first event after the entry of the viral nucleocapsid into the host cell cytoplasm. Of the various models suggested for paramyxovirus transcription, the stop-start model is the most accepted model (Banerjee, 1987). It proposes a single 3' terminal polymerase entry site and a polar gradient of transcription. The viral RNA dependent RNA polymerase acts in a nonprocessive mode for transcription in which the internal genome signals define stop and restart sites. The "transcriptase" is able to recognize cis acting stop and start signals and gives rise successively to a short leader RNA and several capped and polyadenylated mRNAs. During transcription, the polymerase terminates at the end of the leader region and reinitiates at the beginning of the first gene. Reinitiation appears to be a critical event as it defines a polymerase activity that transcribes the template to make mRNAs, as opposed to the one that replicates the genome. During transcription the polymerase transcribes one gene, skips the intergenic trinucleotide sequence and then continues transcription. Thus the entire genome is completely transcribed into monocistronic mRNAs except for the intergenic and trailer sequences. The switchover from transcription to replication is regulated at the level of chain initiation by the concurrent assembly of nascent RNA with NP to form RNP. The relative amounts of the mRNAs or genomes relies upon the concentration of unassembled NP which determines the frequency of genome replication (Vidal and Kolakofsky, 1989).

The main objective of this project was to begin the analysis of transcriptional regulatory sequences of one such negative RNA virus, hPIV3, using a reverse genetics or

rescue technique. This approach allows for the in vitro manipulation of cDNA representing minigenome analogs of negative sense RNA virus genomes and the rescue of the RNA into functional nucleocapsids. Until the last decade or so, negative sense RNA viruses had been refractory to the introduction and recovery of engineered changes to their genomic sequences. The development of techniques for the rescue of negative sense RNA genomes and genome analogs has led to major advances in our understanding the molecular biology of these viruses. These methods permit defined genetic studies of negative sense RNA viral genomes, such as the introduction of mutations into cDNA copies of hPIV3 sequences, and the rescue of altered hPIV3 minigenomes. The minigenomes can then be assessed for their ability to direct transcription of reporter genes.

My starting hypothesis was that all the cis acting sequences necessary for encapsidation, replication and transcription of hPIV3 genomes or genome analogs are contained in short terminal RNA sequences that can be linked to foreign sequences, such as reporter genes, without loss of function (Durbin *et al.*, 1995; Dimock and Collins, 1993). In preliminary experiments, monocistronic and bicistronic cDNAs from previous work in the lab of K. Dimock (Saffran, 1995) and elsewhere (Durbin *et al.*, 1995), in which minigenome cDNAs were cloned between the T7 RNA polymerase promoter and ribozyme sequences from the antigenomic strand of hepatitis δ virus, were modified. The modified plasmids were used to establish basic parameters of a vaccinia virus-driven hPIV3 rescue system, such as requirements for: (1) T7 RNA polymerase to launch rescue, (2) all three of the hPIV3 NP, P and L proteins, and (3) hPIV3 junction sequences for expression of the downstream (CAT)

reporter gene. Once the rescue system was established additional questions about hPIV3 transcription were addressed.

i) hPIV3 and the rule of six

One of the aims of this project was to confirm that hPIV3, like several other nonsegmented, negative strand RNA (Calain and Roux, 1992,1993, 1995; De and Banerjee, 1993; Kalin *et al.*, 1994; Park *et al.*, 1991; Sidhu *et al.*, 1995 ; Saffran, 1995; Durbin *et al,* 1997) viruses obeys the rule of six (Calain and Roux, 1992), which states that the overall length of a viral RNA genome analog must be a multiple of six nucleotides for efficient rescue. A series of monocistronic constructs that encoded minigenomes (6n+1) to (6n+6) nt in length clearly showed that minigenomes 6n and 6n+6 nt in length were rescued 50-200 times more efficiently than minigenomes that were not 6n nt in length, verifying that the rule of six holds true for hPIV3.

Calain and Roux (1993) suggested that the rule of six has a significant effect on replication. This requirement is probably related to the fact that each NP monomer has been predicted to be associated with precisely six nucleotides (Egelman *et al.*, 1989). Nucleocapsid assembly presumably begins at the 5' end of newly replicated RNA and continues by assembling six nucleotides at a time until the 3' end is reached (Lamb and Kolakofsky, 1996). The rule of six is thought to reflect critical interactions between the viral polymerase and the 3' terminal promoter sequences, with initiation of RNA replication being most efficient when the last six bases of the promoter are completely bound by an NP monomer (Calain and Roux, 1993; Pelet *et al.*, 1996). Paramyxovirus genomes contain

inverted terminal repeats of 12 nt. These dodecamers are definitely important cis acting determinants for genome replication, and they would be precisely covered by the terminal two NP subunits in hexamer length genomes. A single nucleotide more, or less, anywhere in the genome would result in the 3' end of the template not being precisely covered by NP monomers.

ii) Hexamer nucleotide phasing of transcription start sites in hPIV3

The rule of six also implies that the viral polymerase interacts with the template bases for RNA synthesis in the context of the NP subunits. The viral polymerase may view the genome (and the regulatory elements for replication and transcription) not just as a linear sequence but as one in which the nucleotides are grouped as hexamers within an NP subunit. The 15,462 nt long hPIV3 genome can be represented as a linear array of 2,577 hexamers and genome replication would begin opposite the first base of the first subunit, at nt 1. The position (phase) of transcriptional control sequences within hexamers may also be important for both mRNA initiation and polyadenylation/termination. For example, when the hexamer phasing of the initiating A residue of the various mRNAs was determined for members of each genus of the family Paramyxovirus, they were found to be strongly conserved, suggesting that the position of the transcriptional start site may be important in the initiation of transcription. For members of the genus Paramyxovirus,these positions are 2, 1, 1, 1, 1 and 2 for the six genes (See Table 7). Pelet *et al.* (1996) first proposed that the relative positioning of a particular base within the hexamer of nucleotides bound by the NP molecules may be important in initiation of transcription. The transcriptional start site of the NP mRNA is strictly conserved at nt 56 among all the members of the subfamily

Table 7: **Subunit phasing at paramyxovirus gene junctions (adapted from Kolakofsky *et al.*, 1998)**

Virus (genome length in nt)	Protein	mRNA start position (nt)	6n+x (Phase)
bPIV3 (15,480)	N	56	2
	N/P	1705	1
	P/M	3703	1
	M/F	4855	1
	F/HN	6751	1
	HN/L	8642	2
	L	15,440	
hPIV3 (15,462)	N	56	2
	N/P	1705	1
	P/M	3721	1
	M/F	4879	1
	F/HN	6733	1
	HN/L	8624	2
	L	15,422	
SeV (15,384)	N	56	2
	N/P	1741	1
	P/M	3637	1
	M/F	4813	1
	F/HN	6637	1
	HN/L	8528	2
	L	15,331	

Paramyxovirinae, opposite the 2nd base of the 10th NP subunit (sequence TAGGGT60). Insertions of 6 nt at nt 47 or 67, which shifted the NP mRNA start site to the same relative position within the next subunit had little effect on transcription (Pelet *et al.*, 1996). The conserved NP mRNA start site can also be moved within the same subunit without much effect on transcription (Pelet *et al.*, 1996).

The next objective of this work, therefore, was to assess the importance of the position of hPIV3 gene start sequences within nucleocapsid subunits (i.e. the phasing or register of the transcriptional start site with respect to an NP subunit) in directing transcription of the downstream (CAT) mRNA. The CAT transcriptional start site in pLCNP/P was repositioned to each of the 6 possible positions within the NP monomer. The results of these experiments, as estimated by CAT activity, indicated that shifting the position of the transcriptional start site of the downstream gene within an NP subunit has no large effect on transcription of the mRNA. Nevertheless, it was decided that the transcriptional start site for all the subsequent minigenomes be adjusted to position 1 relative to the NP subunit. These data are consistent with experimental evidence obtained by others indicating that the requirement for exact positioning of transcription start sites within NP subunits is not stringent. Schneider *et al* (1997) designed a recombinant measles virus in which 3 nt were inserted in the P gene mRNA editing site, and this was compensated for by the deletion of 3 nt at the last (HN/L) junction. Even though the phase of M, F and HN mRNA initiation sites were displaced by three positions, the virus replicated normally in cell culture. In another study with SV5 (He and Lamb, 1999), infectious viruses with shifted transcription start sites were recovered.. Transcription initiation sites for the SV5 P, F and

HN genes were changed from the normal position 1 to position 5, 6, and 5 respectively; for the SV5 SH gene the initiation site was moved from position 2 to position 3 while for the M and L genes was maintained at the same position as in the wild type virus (position 6).

iii) Analysis of hPIV3 gene junctions

The junctional elements are believed to contain the cis acting sequences necessary and sufficient for termination and reinitiation of transcription by the hPIV3 RNA polymerase. The function of the various gene junctions may be to modulate polymerase activity such that during transcription, the different mRNAs are transcribed in quantities that are optimal for virus replication and assembly. Precisely how these signals at the gene junctions alter the behaviour of RNA polymerase during transcription is unknown. It has not been possible to define experimentally exactly which of the different sequence motifs or what combination of sequence motifs are involved in transcription initiation, termination, and related events which occur at the gene junctions or to determine if additional nonconserved flanking sequences are important in these functions. The extreme conservation of the gene junction sequences would suggest that viruses with specific sequences have a selective advantage. It may be that even slight alterations in the signalling ability of a gene junction and the subsequent change in the relative abundance of upstream and downstream mRNAs may render a virus less competitive during the infectious cycle.

To investigate the importance of the intergenic hPIV3 trinucleotide sequence, bicistronic minigenomes, in which the intergenic sequence was deleted, or present in three copies, were generated. For both of these constructs, little or no CAT activity was observed, supporting the idea that the intergenic triplet is essential, and that proper spacing between

gene end and gene start sequences is critical for transcription of the downstream gene. Previous work in the lab (Saffran, 1995) was consistent with these observations, and suggested that spacing was more important than sequence in determining the efficiency of downstream mRNA synthesis. The polymerase may have a limited flexibility in terms of the position at which it can effectively initiate downstream mRNA synthesis once it encounters a transcription termination/polyadenylation signal (Barr *et al.*, 1997). The polymerase may have terminated abruptly at the upstream gene end sequence without reinititating transcription, or it may have read through the downstream gene start signal producing a bicistronic transcript. Which of these interpretations is correct remains to be determined.

For the rhabdovirus VSV, the consensus intergenic sequence, 3'- GA- 5', was investigated by Barr *et al* (1997a), who analyzed the transcription efficiencies of bicistronic minigenomic analogs with altered or deleted intergenic sequences. Positioning of the U tract responsible for polyadenylation of the upstream mRNA immediately adjacent to the consensus downstream gene start signal almost completely abrogated downstream mRNA synthesis, thus defining the intergenic sequence as an essential sequence element of the gene junction. Many of the genome analogs with modified intergenic sequences directed the synthesis of an abundant readthrough transcript with correspondingly diminished levels of the downstream mRNA. Barr *et al.*, (1997), and Hwang *et al.*(1998) have suggested a model of sequential transcription, in which the primary function of the intergenic sequence is to provide a separating sequence between the U tract of the gene end sequence and the gene start sequence, permitting upstream mRNA termination to occur, which is a prerequisite for initiation of downstream mRNA transcription. They suggest that the intergenic sequence

acts in concert with the polyadenylation/stop signal. Stillman and Whitt (1997) on the other hand have suggested that the intergenic sequence is a separate cis acting sequence and not part of the polyadenylation or the downstream mRNA start signal. The polymerase may have to recognize this sequence before stuttering at the polyadenylation site or perhaps this sequence is important for efficient termination and release of the polyadenylated transcript. It was seen (Stillman and Whitt, 1997) that loss of the polyadenylation function led to antitermination of the upstream transcript, leading to readthrough transcription. In addition, naturally occurring readthrough transcripts do not contain intervening poly (A) sequences, supporting the idea that when poly (A) sequences are not added to the 3' end of the upstream transcript, the behavior of viral RNA polymerase at the gene junction sequence changes drastically. Instead of terminating transcription of the upstream mRNA and reinitiating the downstream mRNA, the polymerase simply reads through the gene junction.

The relationship between polyadenylation and transcription termination (Hwang *et al.*, 1998) in higher eukaryotes has been studied (Connelly and Manley, 1988). It has been shown that a bipartite signal consisting of a functional polyadenylation element as the upstream signal, plus various downstream elements (DSE), are required for efficient termination of RNA polymerase II (pol II) transcription. Furthermore, the strength of the polyadenylation signal has been shown to correlate with termination efficiency.

Another of the experimental objectives was to compare the abilities of the different hPIV3 junctions to direct transcription of the downstream mRNA. As measured by CAT activity in transfected cell lysates, termination and reinitiation was most efficient for the NP/P junction while synthesis of the downstream CAT mRNA was lowest for the M/F

junction. A bicistronic cDNA containing an M/F junction from which the 8 extra nt were deleted was also tested. CAT expression directed by this minigenome was restored to near normal (NP/P) levels. These results are consistent with the hypothesis that the 8 extra nt in the M gene end sequence function as an antiterminator sequence leading to elevated transcriptional readthrough. However, another possible interpretation of these results is that the 8 extra nucleotides inhibit reinitiation of transcription and CAT mRNA synthesis. RNA analyses (discussed below) were necessary to distinguish between these two possibilities. Minigenomes with F/HN and HN/L junctions containing minigenome also led to lower CAT expression as compared to minigenomes containing the NP/P junction. While these results could also be attributed to sequence variations among these junctions, in view of the subsequent RNA analyses, they were probably due to differences in transfection efficiency or other variables in the experiments.

iv) Northern blot analysis of minigenomes

Northern blot analysis (Figure 15) of the poly A+ RNA isolated from cells transfected with the hPIV3 junction constructs demonstrated that the readthrough transcription frequency at all the hPIV3 junctions ranged from 13-23% except for minigenomes containing the M/F junction, which exhibited very high (88 %) readthrough transcription frequency. The mutant M/F junction lacking the 8 extra nt also showed a lower level of readthrough transcription, comparable to the other hPIV3 junctions. Another important finding from the northern blot analysis of minigenomes was that M sequences upstream and F sequences downstream of the M/F junction (in the pL-m/f-C minigenome)

did not significantly affect the extent of readthrough transcription. Together, these results clearly indicate that the eight nt in the M gene end sequence are both necessary and sufficient for increased transcriptional readthrough at the M/F junction.

One major concern with these experiments was that the monocistronic luciferase mRNA transcript could not be detected consistently in total RNA or polyA+ RNA preparations, despite the use of two different negative sense luciferase specific riboprobes and even though luciferase activity was readily detected in cell lysates. Detection and quantification of luciferase mRNA would have confirmed conclusions about the extent of termination and readthrough transcription at the various gene junctions. Initially it was thought, for total RNA, that this difficulty was due to the fact that the luciferase mRNA would migrate to the same position as 18S rRNA and that the large amounts of 18S rRNA would inhibit the binding of the luciferase mRNA to the membrane during blotting. However, the same problem was also observed with polyA+RNA. One possible explanation for these observations is that the luciferase mRNA is being rapidly degraded in vaccinia virus-infected and transfected cells. In eukaryotic cells, mRNA decay/degradation occurs rapidly following deadenylation (Drummond *et al.*, 1985). Once polyA tails are shortened to a critical length (~25-60 A residues), decapping of mRNA and ultimately, 5' to 3' exonucleolytic decay results (Muhlrad *et al*, 1994, 1995, Drummond *et al.*, 1985). Inadequate polyadenylation would also leave the mRNA sensitive to nucleolytic degradation. It is possible that the polyA tails of luciferase mRNA are not long enough to prevent rapid turnover however, whether the polyA tails of luciferase mRNA are any shorter, on average, than those of CAT mRNA remains to be determined. There is no obvious reason why this

would be the case. Specific sequences in untranslated regions (UTR) of eukaryotic mRNA sequences can also promote rapid mRNA decay, such as the AU rich elements (ARE) in the 3' UTR of several unstable mammalian mRNAs (Wilson and Triesman, 1988). The 3' nontranslated region of the luciferase mRNA is predicted to include some luciferase nontranslated (58 nt) sequences as well as HPIV3 gene end sequences, but no ARE elements. It also remains possible that an alternative probe would allow for the detection of luciferase mRNA.

v) Transcriptional termination, polyadenylation and readthrough in the Mononegavirales Mutational analysis of paramyxovirus and VSV genome analogs consistently points to sequences located immediately 3' to the U tract within the gene end signal as important in directing polyadenylation and transcription termination (Lamb and Kolakofsky, 1996; Barr *et al.*, 1997b; Kuo *et al.*, 1996, Hwang *et al.*, 1998; Rassa and Parks , 1998, 1999). These sequences are highly conserved in paramyxovirus genomes (Collins *et al.*, 1996) naturally occurring changes in these regions are associated with increased readthrough transcription (Hsu *et al.*, 1985; Spriggs and Collins, 1986; Tsurodome *et al.*, 1991). Hwang *et al.* (1998) noted that the $AUACU_7$ sequence element in the VSV gene end motif had a role in polyadenylation and transcription termination. Mutations in this sequence blocked transcription termination and generated mostly readthrough transcripts, with the C residue immediately adjacent to the U tract being the most critical for termination (Barr *et al.*, 1997b). It is interesting that for many other members of the superfamily *Mononegavirales,* a C residue is found immediately upstream of a U tract (Spriggs and

Collins, 1986, Gupta and Kingsbury, 1984). For RSV, it has been proposed that sequences immediately 3' to the U tract serve to cause polymerase pausing to facilitate reiterative copying i.e. polyadenylation (Kuo *et al.*, 1997). This mechanism by which viral gene end sequence directs polyadenylation and termination (Barr *et al.*, 1998) could be compared to some bacterial rho independent transcription terminators that are contained within the nascent RNA chain as it emerges from the polymerase and not in the template (Carafa *et al.*, 1990).

For SV5, a 3'-AAAUUC-5' motif located 3' to the U tract is a major factor directing efficient polyadenylation termination by the SV5 polymerase. Mutational analysis of the M-F intergenic sequence also suggested that defects in termination and reinitiation of transcription were highly dependent on the length of the U tract in the gene end sequence. SV5 also exhibits naturally occurring elevated readthrough transcription across the M/F junction, and 40% of SV5 F transcripts are bicistronic (Rassa and Parks, 1998, 1999). The SV5 M/F junction differs in several features: (1) the nucleotide sequence of the gene end sequence adjacent to the M gene end U tract differs from the consensus sequence; (2) the four U residues which compose the tract for M gene polyadenylation are the shortest of any SV5 gene end U tract; (3) the M-F intergenic region is the longest of the SV5 gene junctions.

For hPIV3, the AUUC sequence 3' to the U tract presumably has a similar, and critical function in transcriptional regulation and the 8 nucleotide insertion interrupts the proposed polyadenylation termination signal 3'-AUUCUUUUU-5' between the M and F genes. The viral RNA polymerase may be unable to pause efficiently at this site leading to impaired polyadenylation and termination, and a high frequency of readthrough transcription.

It remains a possibility that the 8 nt sequence only functions as an antiterminator in the context of the M gene end sequence (which is similar to the NP and P gene end sequences) and the F gene start sequence. It will be interesting to observe the effect of this 8 nt sequence on readthrough transcription if it is inserted in other hPIV3 junctions at the same position.

vi) Significance of M/F readthrough transcription in the paramyxovirus life cycle

Clearly, elevated synthesis of polyadenylated bicistronic M-F RNA is a common property of a number of paramyxoviruses, but the role of this disproportionate readthrough transcription in the viral life cycle remains to be determined. Because translation of a eukaryotic mRNA is usually limited to the 5' proximal open reading frame (Kozak, 1991) and proteins encoded in more distal open reading frames are typically not expressed, M-F readthrough transcription may represent a mechanism to downregulate F protein synthesis. The 5' NTRs of the F mRNAs of both hPIV3 (193 nt; Spriggs et al., 1986, Côté et al, 1987), hPIV1 (274 nt; Merson et al., 1988) and measles virus (582 nt; Richardson *et al.*, 1986). are significantly longer than the corresponding sequences of other paramyxovirus F mRNAs (53 nt for Sendai virus, 18 nt for hPIV2, 29 nt for SV5). A role for these long NTRs has not been identified. It may be speculated that these long 5' NTRs also contribute to diminished F mRNA translation.

In hPIV1- and hPIV3-infected cells, F protein synthesis may be regulated, therefore, at both transcriptional and translational levels, and the amounts of F protein are noticeably lower than the amounts of HN in these cells (Spriggs and Collins, 1986, Bousse *et al.*, 1997). Why would these viruses evolve a mechanism to downregulate F expression? It has been

shown that overexpression of hPIV1 F following transfection (as compared to what is found in hPIV1-infected cells) was found to downregulate HN expression posttranscriptionally (Bousse *et al.*, 1997, Tanaka *et al.*, 1996). In addition, fusion of cells transfected with plasmids encoding hPIV3 F and HN is inhibited when the mass ratio of the F plasmid to the HN plasmid exceeds 1:10 (Ebata, 1996, K. Dimock, unpublished). These data suggest that the amount of F protein (or the ratios of F and HN) must be tightly controlled in hPIV1- and hPIV3-infected cells to avoid inhibition of HN expression and/or fusion. Excess F might disrupt the stoichiometry of F-HN complex formation and, as a result, its own function, or that of HN. The optimal ratio of F to HN for fusion by these viruses is unknown although studies using reconstituted lipid vesicles showed that maximal fusion occurred when the ratio of Sendai virus F to HN was 2 (Nakanishi *et al.*, 1982) suggesting that efficient fusion required many F molecules. Excessive fusion, and disruption of cellular integrity and functions may be detrimental to virus replication, whereas insufficient fusion is likely to inhibit virus spread. Kato et al (1999) have also suggested that limiting F protein synthesis may be advantageous for the virus to persist in nature.

Another consequence of readthrough transcription is that more polymerase molecules have access to downstream genes. Thus another role for M-F readthrough transcription may be to ensure that appropriate amounts of the HN and L gene products are synthesized. It has been shown that the SV5 HN protein has a very short half life on the cell surface (Leser *et al.*, 1996) and M-F readthrough may function to counteract HN turnover by increasing polymerase access to the HN gene. The intracellular concentration of viral polymerase proteins is important for a productive infection by negative strand viruses (Meier *et al.*, 1987)

and M-F readthrough may also serve to balance the ratio of the upstream P and downstream L gene products.

vii) Future prospects

A recent breakthrough in the study of nonsegmented negative strand RNA viruses is the establishment of rescue systems to recover infectious virus entirely from cDNA. Mutations can now be introduced into viral genomes at will and the resulting phenotypes can be studied. This technology is being used to answer outstanding questions regarding the roles of specific viral sequences in replication and their contribution to pathogenicity. The ability to produce infectious virus from cDNA will allow more defined analyses of the genes and regulatory sequences of the negative strand viruses. Future work on this project should concentrate on the rescue of full length hPIV3 containing altered gene junctions. The patterns of mRNA produced by viable hPIV3 mutants can be compared to what is observed using minigenomes, and the effects that mutations have on the overall fitness of the altered virus can be examined. With reverse genetics techniques, it is also possible to study the effect of mutations in specific viral proteins or protein domains and their importance in virus replication and pathogenicity. For example, learning more about specific protein domains involved in protein-protein interactions critical for viral entry, may identify potential targets for antiviral therapy.

Viral attenuation through specific mutations has obvious practical significance in vaccine development. Such attenuating mutations could include eliminating genes whose products are dispensable for replication in cell culture, rearranging gene order, or modifying

regulatory elements (Jin *et al.*, 1996; Kato *et al.*, 1997; Kurotani *et al.*, 1998; Roberts *et al.*, 1998; Wertz *et al.*, 1998). There are no vaccines currently available for hPIV3. Characterization of mutations associated with attenuation of existing hPIV3 vaccine candidates permits the construction and rescue of recombinant vaccine viruses with improved characteristics of attenuation and stability (potential inhibitors of transcription, replication e.g using antisense to inhibit hPIV3 infection). Rearrangement of the genes within the viral genome may also result in attenuation by altering the expression levels of the different viral proteins, and eliminating the expression of accessory viral genes and/or nonessential protein domains might also be used for attenuating purposes.

In addition to their use as vaccines, these viruses may also have a role to play in somatic gene therapy as nonintegrative vectors. Appropriately attenuated vectors based on parainfluenza viruses, which exhibit a marked tropism for respiratory epithelia, might be designed to express proteins for the treatment of other respiratory diseases or for therapy of conditions such as cystic fibrosis or cancer.

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APPENDIX

Oligonucleotide Sequences

Primers used for sequencing

3061 5'-TTTACGATGCCATTGGGA-3' CAT + sense

3062 5'-CATGTCGGCAGAATGCTT-3' CAT - sense

Oligonucleotide pairs used for cloning hPIV3 junctional sequences

1) pLC NP/P

4528 5'-ATAAATAAGAAAAACTTAGGATTAAAGA-3'
4529 5'-GATCTCTTTAATCCTAAGTTTTTCTTATTTATGC-3'

2) pLC F/HN

4530 5'-ATAATTATAAAAAACTTAGGAGTAAAGA-3'
4531 5'-GATCTCTTTACTCCTAAGTTTTTTATAATTATGC-3'

3) pLC HN/L

4532 5'-ATAAATATAAAAAACTTAGGAGCAAAGA-3'
4533 5'-GATCTCTTTGCTCCTAAGTTTTTTATATTTATGC-3'

4) pLC NP/P ΔIG

4534 5'-ATAAATAAGAAAAAAGGATTAAAGA-3'
4535 5'-GATCTCTTTAATCCTTTTTTCTTATTTATGC-3'

5) pLC M/F Δ8

4538 5'-ATAAATAAGAAAAACTTAGGACAAAAGA-3'
4539 5'-GATCTCTTTTGTCCTAAGTTTTTCTTATTTATGC-3'

6) pLC NP/P 3IG

4614 5'-ATAAATAAGAAAAACTTCTTCTTAGGATTAAAGA-3'
4615 5'-GATCTCTTTAATCCTAAGAAGAAGAAGTTTTTCTTATTTATGC-3'

7) pLC M/F

P13SIGM 5'-ATAAATAAGAGATAATCAAAAACTTAGGACAAAAGA-3'
P13SIGMC 5'-GATCTCTTTTGTCCTAAGTTTTTGATTATCTCTTATTTATGC-3'

Oligonucleotides used for shifting transcription start site of NP/P gene start sequence from 1-6 positions

1) pLCNP/P tc1 oligonucleotide
B6 5'-CACGGATCCGTG-3'

2) pLCNP/P tc 2
A7 5'-ACACGG(TA)CCGTGT-3'

3) pLCNP/P tc3
B2 5'-CGGATCCG-3'

4) pLCNP/P tc4
A3 5'-ACGG(TA)CCGT-3'

5) pLCNP/P tc5
B4 5'-ACGGATCCGT-3'

6) pLCNP/P tc6
A5 5'-CACGG(TA)CCGTG-3'

Oligonucleotides used for adjusting the length of minigenome (by insertion at the Pst I site in the L noncoding sequence)

1) -1 nt (+5) in the Pst site
5305 5'-CAGGGCGGGGCGTAACTGCAAATACAAATACAATA-3'

2) +1 nt in the Pst I site

5306 5'-CAGGGCGGGGCGTAACTGCAAGAATACAAATACAATA-3'

3) +2 nt

5307 5'-CAGGGCGGGGCGTAACTGCAAAGAATACAAATACAATA-3'

4) +3 nt

5308 5'-CAGGGCGGGGCGTAACTGCAAAAGAATACAAATACAATA-3'

5) +4 nt

5309 5'-CAGGGCGGGGCGTAACTGCAAAAAGAATACAAATACAATA-3'

6) +6 nt

PST+6 5'-CAGGGCGGGGCGTAACTGCAAAAAAAGAATACAAATACA ATA-3'

Primers used for PCR amplification of M/F junction

MF+ 5'-ACACACGATATCAGAAGGGTGAAAAATCACTG-3'

MF- 5'-CACACACCATGGTCATATAAAGGAATGATCAG-3'

Primer used for amplication of M sequence for use as template for riboprobe synthesis

M probe 5'-TATCTCTTATTTGCTTTGGC-3'

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1996-present Master of Science, Biochemistry, Microbiology and Immunology, University of Ottawa. In progress; expected summer 2000
Thesis Title: Analysis of gene junction sequences of human parainfluenza virus type 3
Thesis Supervisor: Dr. K. Dimock

1992-1994 Masters of Science, Microbiology, Department of Microbiology, University of Pune , India
Thesis Title: Optimisation of growth medium for the production of cuticle degrading enzymes by the wild strain of the entomophagous fungus *Metarhizium anisopliae.*
Thesis Supervisors: Dr. Mukund Deshpande (National Chemical Laboratory, Pune, India)

1989-1992 Bachelor of Science, Microbiology, Department of Microbiology, University of Pune, Pune, India
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Work Experience

1998-2000 Graduate Student (M.Sc.), University of Ottawa
Thesis title: Analysis of gene junction sequences of human parainfluenza virus type 3

1997 Laboratory Technician (Part time), University of Ottawa,
Project Tasks: Testing and comparision of the efficacy of Molecular Biology Kits manufactured by leading companies like QIAGEN and Bio101
Supervisor: Dr. I. Altosaar

1994-1995	Laboratory Technician, Plant Tissue Culture Division National Chemical Laboratory, Pune, India. Project Tasks: Morphogenesis, somatic embryogenesis and in vitro flowering studies in chick pea (*Cicer arietinum*) Supervisor: Dr. K. Krishnamurthy
Fall 1994	Laboratory Technician, Polchem Hygiene Laboratories Pune, India. Project tasks: Conducting hygiene audits in poultry farms and quality control studies of the disinfectants. Supervisor: Mr. M. Limaye
Summer 1996	Volunteered at the Department of Biology, Carleton University Project tasks: Conducting transformation on sphaeroplasts of *Neurospora crassa* and analysis of the morphological and functional mutants. Supervisor: Dr. J. Vierula

Laboratory skills

Cell Culture:	Culture of mammalian cell lines, preservation of cell lines
Molecular Biology:	PCR, extraction and purification of nucleic acids; cloning, site directed mutagenesis, RT-PCR, electrophoresis, plasmis mediated transcfection of mammalian cells, Northern blotting, preparation of radiolabeled riboprobes,
Biochemical methods:	Reporter enzyme assays, Protein assays
Virology:	Cultivation, quantification, purification of animal viruses (hPIV3, recombinant vaccinia virus)
Others:	Plant Tissue culture techniques, Microprojectile Bomdardment, *Agrobacterium tumefaciens* mediated plant transformation, Fungal transformations etc.

Additional Skills

Languages: Good oral and written skills in English
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Oral and Poster Presentations

Poster presented by P. Jogalekar at the 1998 Meeting of the Canadian Society for Microbiologists (CSM), June 14, 1998, in University of Guelph, Guelph, Ontario.
Title: Analysis of Transcription regulatory sequences in human Parainfluenz a virus Type 3 (P. Jogalekar*, J. Rouselle, H.A. Saffran and K.Dimock)

Poster presented by P. Jogalekar at the CSM 1999 Meeting of the Canadian Society for Microbiologists (CSM), June 15, 1998, in University ofMontreal, Montral, Quebec.
Title: Analysis of Transcriptional Readthrough at the M/F Gene Junction of Human Parainfluenza Virus Type 3 (P. Jogalekar*, H.Saffran, K. Dimock)

Talk presented by P. Jogalekar at the Annual Meeting of the American Society of Virology (ASV), July 12, 1999, in Amherst, Massachussets.
Title: Transcriptional readthrough at the Human Parainfluenza virus 3 M/F Gene junction is determined by additional nucleotides in the M gene end sequence. (P.Jogalekar*, H. Saffran, A.P. Durbin and K.Dimock)