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DETERMINATION OF THE MECHANISM OF VIRAL INTERFERENCE MANIFESTED BY A MOUSE-ADAPTED STRAIN OF INFLUENZA A VIRUS

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By

Jane E. Bailly, BSc., MSc.

ABSTRACT

Mouse-adapted influenza A virus, FM-MA, interferes with the replication of wild-type strains on coinfection of MDCK cells to the extent that >99% of the infectious progeny virus are FM-MA. The interference phenotype was previously mapped to FM-MA segment 2 which encodes the PB1 polymerase protein with a single amino acid substitution relative to the parent, FM, at position 538. To identify the point at which FM-MA interferes with wild-type A/HK/1/68 (HK), the relative levels of FM-MA and HK transcription and genome replication from PB1, NP and M1 genes were determined in coinfected MDCK cells using RT-PCR. All stages of FM-MA macromolecular synthesis (primary and secondary transcription, genomic RNA, complementary RNA and protein) were enhanced relative to HK, a phenotype which mapped to FM-MA segment 2. The kinetics of viral RNA synthesis in single or mixed infections indicated not only that the presence of FM-MA specifically compromised HK transcription and replication in coinfected cells but also that FM-MA's ability to interfere was due in part to its capacity for increased primary transcription relative to HK. FM-MA genomes were also selectively assembled into progeny virus from cells coinfected with HK and FM-MA, a step which was distinct from the capacity for enhanced RNA synthesis. This suggests that interference of wild-type virus growth by FM-MA in mixed infections resulted from two distinct events: a preferential synthesis of FM-MA-specific macromolecules

which was then augmented by a preferential assembly of FM-MA genomes. The ability of FM-MA to selectively amplify its own genes in cells coinfected with HK did not depend on recognition of an FM-MA-specific promoter by the mutant polymerase. Alternate explanations for this selectivity are proposed.

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Do you not see how necessary a World of Pains and troubles is to school an Intelligence and make it a soul? -John Keats

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A	adenosine
AP	ammonium persulfate
bp	base pair
BSA	Bovine serum albumin
C	cytidine
°C	degrees Celsius
ca	cold-adapted
cm	centimeter
cRNA	complementary ribonucleic acid
CHX	cycloheximide
Ci	Curie
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
g	gram(s)
G	guanosine

HA	influenza virus hemagglutinin protein
hr	hour(s)
IAA	isopropyl alcohol
IPTG	isopropylthio-β-galactoside
kb	kilobase
kDa	kilodalton
M	molar
M1	influenza virus matrix protein
M2	influenza virus ion channel protein
MDCK	Madin Darby canine kidney
MEM	minimum essential medium
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mM	millimolar
mmol	millimole(s)
MOI	multiplicity of infection
mol. wt.	molecular weight
mRNA	messenger ribonucleic acid
μg	microgram
μl	microliter
NA	influenza virus neuraminidase protein

NP	influenza virus nucleoprotein
NP40	Nonidet P40
ng	nanogram(s)
NS1,2	influenza virus nonstructural proteins
nt	nucleotide(s)
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque-forming unit(s)
p.i.	post-infection
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription followed by polymerase chain reaction
SDS	sodium dodecyl sulphate
T	thymidine
TBE	Tris-borate-EDTA buffer
tRNA	transfer ribonucleic acid
U	units

X	times
x	denotes genetic cross
v	volume
V	volts
w	weight
wt	wild-type

1. INTRODUCTION

1.1 OVERVIEW

Influenza is an illness which afflicts humans as well as a broad range of animal populations. The etiologic agent, the influenza virus, was first isolated over 60 years ago (Smith, 1933). Abundant information is now available on its structure, biochemistry, epidemiology and host interactions. Yet current measures to control influenza through prophylaxis and therapy are insufficient and its impact, both in terms of morbidity and mortality and in economic terms, continues to be felt. In the following chapter, I will attempt to describe the measures currently available for control of influenza.

Research aimed at both development and improvement of antiviral strategies clearly must remain a priority. One step along the path to undermining viral processes may be to elucidate existing mechanisms by which one influenza virus inhibits the replication of a coinfecting virus. This phenomenon, termed viral interference, has been observed during the coinfection of cells with 2 strains of influenza virus, as described later in this chapter. If it could be understood how a virus inhibits a coinfecting virus, it may be possible to recreate the inhibitory event in a controlled manner. Unfortunately, there is almost no mechanistic information available on viral interference.

In this thesis I analyze the viral interference manifested by a mouse-adapted variant of influenza A virus, FM-MA. Interference by FM-MA derives from a mutation in PB1, one of the polymerase protein components of the influenza virus

RNA-dependent RNA polymerase complex. This complex is responsible for the transcription and replication of the influenza virus genome, processes which are unique to the virus in their dependence on RNA templates. A precise description of the role of PB1 in interference in this system may thus identify virus-specific targets for therapeutic interventions which do not compromise host functions. Influenza virus genome expression is highly regulated due largely to controls operating at the level of transcription and replication. A study of polymerase function as it relates to the time course of genome expression may therefore also provide a deeper understanding of how these controls are manifested.

1.2 INFLUENZA VIRUS: GENERAL

Influenza viruses are members of the family Orthomyxoviridae which is composed of 3 serotypes, A, B and C. Types A and B share morphological and molecular features absent in C leading to their placement in a distinct genus (Kingsbury, 1990). However their structural differences remain such that genetic recombination between types has not been observed in nature (Kilbourne, 1975; 1980). The 3 serotypes are distinguishable by the antigenicity of their nucleoprotein (NP) and matrix (M1) proteins. Members within a single type demonstrate antigenic cross-reactivity of these structural proteins. The serotypes also distinguish themselves by their epidemiology. Influenza A viruses possess a broad host range which includes humans, birds, horses, pigs, seal, mink and whale (Webster, 1992) and are associated with epidemic and pandemic disease. Types B and C are mainly restricted to humans. The following description of influenza

viruses will be confined to the features of influenza pathophysiology as well as the morphology and life cycle of influenza A virus pertinent to the research performed in the current study.

1.3 THE DISEASE

A unique property of the influenza A variant used in this study is its ability to interfere with wild-type strains in mixed infections. An understanding of the mechanism of action of interference could expose an aspect of the viral life cycle susceptible to intervention. In order to appreciate the importance of this, it is appropriate to begin with a description of influenza, the disease caused by influenza virus, its cost in terms of morbidity and mortality in human and animal populations and the measures undertaken to control it. This description is intended to demonstrate the limitations of the current approaches to control and the importance of exploring novel means to modulate influenza virus infection.

In humans, influenza virus infection is normally restricted to epithelial cells in the upper respiratory tract, producing respiratory and systemic symptoms lasting 1-2 weeks. Immunocytologic studies of infected nasal epithelial cells have identified viral proteins in ciliated, intermediate, basal and goblet cells (Ebisawa, 1969; Liu, 1961; Tateno, 1965) suggesting the occurrence of viral replication. Respiratory pathology is attributable to desquamation of these cells (Carson, 1985; Winther, 1990). Symptoms include sore throat, sneezing, cough and runny nose. Influenza may also produce systemic symptoms including myalgia, chills, headache and fever. These may be effects of the release of toxic products from virus-killed

cells or of viral antigen-antibody complex stimulation of complement (Fenner et al., 1974). Polymorphonuclear phagocytes recruited by the infection can be both mechanically harmful and may liberate endogenous leukocyte pyrogen (IL1) (Sweet and Smith, 1980) which can stimulate prostaglandin E production. The prostaglandin then acts on the hypothalamus to produce fever (Bernheim et al., 1979). Uncomplicated influenza is self-limiting and the majority of these symptoms resolve within one week.

Influenza is occasionally fatal. Fatalities are rare [1 in 10,000 in population under 65 yrs (Couch, 1993)] and usually occur in patients with an existing cardiopulmonary condition, especially increased pulmonary capillary pressure (Small, 1990). Eighty-90% of deaths occur in the elderly population (Anderson, 1991) where such conditions are more common [54 deaths per 10,000 in population >65 yrs (Couch 1993)].

Ferrets provide the best animal model for influenza since the defined, upper respiratory pathology and fever approximate the human disease.

1.4 CONTROL OF INFLUENZA: VACCINES

An evaluation of the economic impact of influenza estimates direct costs of $1-5 billion annually and total costs of up to $15 billion per year (Schoenbaum, 1987). Complete eradication of influenza is unrealistic because of the changing antigenicity of the virus and the decrease in human immunity with time. However prevention through annual vaccination represents an effective control strategy. Ideally, a vaccine is intended to stimulate immunologic responses which correlate

with immunity to infection. In the case of influenza, anti-HA IgG in serum and secretory IgA play a critical role in prevention of infection (Couch et al., 1981; Couch and Kasel, 1983; Couch et al., 1984; Clements et al., 1984).

Commercially available vaccines consist of whole, killed virus or a split-virus (subvirion) vaccine chemically treated for reduced reactogenicity in children under 13 yrs (Bernstein et al., 1982). The composition of the vaccine changes yearly to keep pace with antigenic variation of epidemic strains due to genetic drift. Generally, it is a mixture of 15 ug each of 2 subtypes of influenza A (H3N2 and H1N1) that have cocirculated since 1977 and 1 influenza B. The inactivated vaccine stimulates a high titre of serum antibody (Clements and Murphy, 1986). Field trials have demonstrated it to be 60-90% effective in reducing incidence and preventing fatal complications when antigenically matched with epidemic strains (Meyer et al., 1978; Gross et al., 1987; La Montagne et al., 1978; Quinnan et al., 1983). The efficacy of the vaccine is undermined by underuse: <30% of the target population of 45-50 million receive the vaccine (Douglas, 1980).

The protection against influenza virus infection offered by killed virus vaccine is incomplete (Hoskins et al., 1979; Meyer et al., 1978) especially in young children (Couch, 1993). Secretory IgA, an important determinant of protection against infection and disease, is poorly induced by the killed vaccine (Clements and Murphy, 1986). Intranasal inoculation of a live vaccine offers a more efficient means of stimulating secretory IgA in the respiratory tract. The most promising live vaccines consist of reassortant viruses which have genes encoding internal

proteins contributed by an attenuated cold-adapted (ca) virus, A/Ann Arbor/6/60 (H2N2) and 2 genes encoding the surface proteins NA and HA from the circulating wild-type virus. Field studies have shown these ca reassortants to be safe and immunogenic when introduced intranasally (Lazar et al., 1980; Murphy et al.,1981; Clements et al., 1983; Belshe and Van Voris, 1984) and effective in prevention of natural influenza (Couch et al., 1986). These live virus vaccines have been shown to stimulate nasal IgA more efficiently than inactivated virus vaccines in adults (Clements and Murphy, 1986) and, importantly, produce higher systemic and local antibody titers in children (Murphy et al., 1982; Johnson et., 1986) thereby reducing the incidence of infection (Clover et al., 1991; Piedra and Glezen, 1991). Live influenza vaccines may also promote superior cytotoxic T cell memory than killed virus vaccine (Ennis et al., 1981) which may offer cross protection from challenge by other subtypes. Live ca reassortant vaccines demonstrated to be safe and efficacious await only optimization of immunization schedules and development of a manufacture-distribution system before commercial availability (Couch, 1993).

1.5 CONTROL OF INFLUENZA: ANTIVIRALS

Amantadine HCl treatment is used to complement the inactivated virus vaccine and plays an important role in influenza control. Amantadine is highly effective in preventing influenza A virus infection. It also has therapeutic efficacy; when given in the first 48 hr of the illness, symptoms may by reduced in severity and duration (Van Voris et al., 1981; Youkin et al., 1983; Tominack and Hayden, 1987). Amantadine works by blocking the influenza virus M2 protein which normally

functions as an ion channel, permitting pH regulation critical at several steps in infection. Amantadine compromises M2-facilitated acidification of the virus particle leading to a failure of nucleocapsid uncoating (Bukrinskaya, 1982; Martin and Helenius, 1991a). Resistant strains are easily generated in vitro when viruses are passaged in the presence of amantadine. Amantadine is not metabolized and may lead to renal impairment due to accumulation of the active drug. Side effects include complaints of the gastrointestinal (nausea, loss of appetite), psychomotor (difficulty in concentrating, impaired problem solving) and central nervous (nervousness, light headedness) systems. Rimantadine, an unlicensed analogue of amantadine that is extensively metabolized by the liver, demonstrates a similar efficacy with fewer side effects (Monto and Arden, 1992). However, both drugs are ineffective against influenza B virus which causes 20% of epidemic influenza annually (Douglas, 1990).

Other strategies for control of influenza virus infection are under investigation. These include rationally designed inhibitors of neuraminidase (von Itzstein et al., 1993), DNA vaccination (Fynan et al., 1995), synthetic peptide vaccines (Levi et al., 1995) and inhibition of viral polymerase function using short, capped oligonucleotides (Chung et al., 1994).

1.6 INFLUENZA VIRION STRUCTURE

Influenza virions are pleomorphic particles measuring 80-120 nm. The RNA genome, representing 0.8-1% of the virion mass (Choppin and Compans, 1975) consists of 8 separate, single-stranded RNA segments of negative polarity with

respect to functional mRNA. Each genome segment is separately encapsidated by an arginine-rich phosphoprotein, nucleoprotein (NP), with a periodicity of 1 NP per 20 nucleotides (nt) (Compans and Choppin, 1975) which still leaves the RNA susceptible to RNases (Murti et al., 1980). Each RNA molecule is also associated with 3 polymerase proteins which resolve on 2-dimensional electrophoresis into 2 basic (PB1 and PB2) and 1 acidic (PA) species (Horisberger, 1980). The polymerase proteins, associated as a complex, are found at one end of the virion nucleocapsid (Murti et al., 1988). The unit consisting of RNA molecule, NP and polymerase proteins is termed a ribonucleoprotein (RNP). The isolated RNA genome of the virus is noninfectious. However, RNPs, which are enzymatically active in vitro, are infectious when transfected into cells (Hirst and Pons, 1973). The 5' and 3' termini of each genomic RNA segment associate through base-pairing of partially complementary sequences. In virions and infected cells the RNA segments form circular structures with the duplexed termini constituting a "panhandle" (Hsu et al., 1987). These circular forms undergo supercoiling (Pons et al., 1969) giving the RNP a right-handed, helical configuration (Heggeness et al, 1982). Individual RNPs are approximately 15 nm in diameter and, depending on the length of the segment, 50-130 nm long. The 8 RNPs are surrounded by a shell of matrix (M1) protein which associates with both the viral lipid bilayer via stretches of hydrophobic amino acids (Gregoriades and Frangione, 1981) and the viral RNP (Wakefield and Brownlee, 1989; Ye et al., 1989). NS2, previously believed to be a nonstructual protein, has recently been detected in virions (Yasuda et al., 1993).

While nothing is known about its function in the virus particle, it has been found to associate with M1 (Yasuda et al., 1993). The layer of matrix protein is enclosed by a host-derived lipid membrane (Landsberger et al., 1971) curiously devoid of host membrane-associated proteins (Holland and Kiehn, 1970; Schulze, 1970) but containing viral proteins. These include M2, a nonglycosylated protein present in small amounts and shown recently to function as an ion channel (Pinto et al., 1992). The viral envelope also contains 2 types of integral membrane glycoproteins which protrude from the surface. The majority of these projections are trimers of hemagglutinin (HA) molecules with each monomer consisting of disulfide-linked HA1 and HA2 polypeptide chains. It is the HA which mediates attachment of virions to host cells (Rogers et al.,1983) and which elicits most of the neutralizing antibodies during infection (Wharton et al., 1989). The remaining spikes are composed of neuraminidase (NA) tetramers which assume a characteristic mushroom shape visible under electron microscopy (Blok et al., 1982).

Determination of the crystal structures of HA (Wilson et al., 1983) and NA (Varghese et al., 1983) has provided valuable information on receptor-binding epitopes (Weis et al., 1988) and allowed mapping of antigenic sites. HA and NA vary individually by as much as 80% at the nucleic acid level (Wharton et al., 1989) manifesting in extensive antigenic variation which forms the basis for classifying influenza A viruses into different subtypes. There are 15 serologically distinct HA subtypes and 9 NA groups (Hinshaw et al., 1982; Rohm et al., 1996). Three subtypes of HA and 2 of NA have been isolated from humans while all are found in

the avian population. With this genetic reservoir there exists the potential for introduction of new subtypes through reassortment of human and avian viruses during mixed infection of an intermediate, possibly porcine (Scholtissek et al., 1985), host.

1.7 GENOMIC ORGANIZATION

The influenza virus genome consists of 10 genes which reside on 8 RNA segments ranging in size from 890-2341 nt (reviewed in Lamb, 1989). The genome segments, first demonstrated to be distinct RNA molecules using two-dimensional oligonucleotide fingerprinting (McGeoch et al., 1976), were subsequently resolved by polyacrylamide gel electrophoresis (PAGE) in a matrix containing 6 M urea (McGeoch et al., 1976; Palese, 1977; Scholtissek et al., 1978). Two different influenza A virus strains can simultaneously coinfect a cell and generate reassortant progeny containing a mixture of genes from the two parents. Because RNA segments often demonstrate strain-specific mobilities during partially-denaturing PAGE due to differences in secondary structure, PAGE can be exploited to identify the contributions of parental genes to progeny reassortants.

The ten viral genes encode ten known protein products. The assignment of proteins to individual genome segments was deduced using several approaches. These included hybrid-arrested translation of mRNA using single (complementary) RNA segments (Inglis et al., 1977, 1979; Lamb and Choppin, 1979, 1981) and PAGE analysis of the genome segments of reassortant viruses possessing distinguishable proteins (Palese and Schulman, 1976; Palese, 1977). The three

largest segments, 1-3, encode the polymerase proteins PB2, PB1 and PA, respectively. Segments 4 and 6 code for the surface glycoproteins HA and NA. Segment 5 encodes the nucleocapsid protein, NP. Three and 2 polyadenylated mRNAs are expressed from segments 7 and 8, respectively, by RNA splicing. Segment 7 encodes the matrix (M1) and M2 proteins; a protein product has not been identified for the third mRNA. Segment 8 codes for nonstructural proteins NS1 and NS2.

1.8 INFLUENZA VIRUS LIFE CYCLE

1.8.1 VIRUS ENTRY AND UNCOATING

Influenza virions bind sialic acid-containing receptors of host respiratory epithelial cells (Wiley and Skehel, 1987). Following receptor-mediated endocytosis, they then enter the cell in clathrin-coated and uncoated endosomes (Matlin et al., 1982). The low pH environment of the late endosome activates the hemagglutinin glycoprotein which then mediates membrane fusion between the viral and lysosomal membranes via its HA2 subunit (White et al, 1983; Wharton, 1987). This releases the viral nucleocapsid into the cytoplasm of the cell where the RNPs separate from the M1 protein. The viral RNAs and associated nucleoprotein and polymerase proteins are then quickly delivered to the nucleus [$t_{1/2}$=10 min after penetration into the cytoplasm of Chinese Hamster Ovary (CHO) cells (Martin and Helenius, 1991b)]. This transport does not require cytoskeletal elements (Martin and Helenius, 1991) but likely relies on nuclear localization signals present on NP, PB1, PB2 and PA (Nath and Nayak, 1990; Mukaigawa and Nayak, 1991; Nieto et

al., 1994). Transport of the RNPs through nuclear pores into the nucleus is active (Martin and Helenius, 1991). The matrix protein migrates to the nucleus independently of and more slowly than the RNPs and enters by passive diffusion through nuclear pores [$t_{1/2}$=25 min after penetration into the cytoplasm of CHO cells (Martin and Helenius, 1991)].

1.8.2 VIRAL TRANSCRIPTION

In the nucleus of infected cells vRNPs are both transcribed into mRNA and replicated via a (+) stranded intermediate molecule, complementary RNA (cRNA). (Both transcription and replication reviewed by Krug et al., 1989). The first event of viral RNA synthesis is primary transcription, defined as mRNA synthesis using the inoculum viral RNAs as templates. Influenza mRNA transcription is distinctive in its use of cap 1 (5'm^7GpppNm)-containing RNA fragments derived from cellular polymerase II transcripts as primers (Bouloy et al., 1978; 1979; Krug et al., 1979; Plotch et al., 1979). A viral cap-dependent endonuclease (Plotch et al., 1981) cleaves host transcripts 10-13 nt from their 5' ends, preferably following a purine (Beaton and Krug, 1981; Plotch et al., 1981; Shaw and Lamb, 1984). Transcription begins by incorporation of a G residue onto the free 3' end of the host primer as templated by the penultimate C of the viral RNA template (Plotch et al., 1981). Hydrogen bonding between host RNA fragments and virion RNA is not essential (Krug et al., 1980) and is only believed to occur between the 3' terminal A residue of the host primer and the 3' terminal U of the vRNA (Beaton and Krug, 1981; Lamb et al., 1981; Plotch et al., 1981). Priming activity is, however, dependent on the 5'

methylated cap of the host-donated primer (Bouloy et al., 1980; Plotch et al., 1979). Viral transcripts are elongated by the sequential addition of ribonucleotides, as directed by the viral template, until the polymerase reaches a tract of 5-7 uridine residues 17-22 nt from the 5' end of the vRNA at which point transcription ends and a poly A+ tail of 50-150 residues is added (Hay et al., 1977a,b; Robertson et al., 1981). The available evidence suggests that polyadenylation results from reiterative copying of the uridine stretch at the 5' end of the vRNA. This is presumed to occur due to a "stuttering" of the viral polymerase as it encounters and fails to disrupt the panhandle formed by partially duplexed 5' and 3' vRNA termini (Hsu et al., 1987; Luo et al., 1991).

There is some processing of viral mRNAs in the nucleus. Certain internal adenosine residues are methylated (Narayan et al., 1987) and transcripts encoding M1 and NS1 proteins are spliced to give 2 or 1 smaller mRNAs, respectively (Lamb et al., 1980; Lamb and Lai, 1980; Lamb et al., 1981). Splicing is presumed to be carried out by host enzymes since sequences at the intron/exon junctions of viral transcripts (Lamb and Lai, 1980) resemble eukaryotic consensus donor and acceptor sequences (Mount, 1982). These, along with Borna virus RNA processing (Cubitt et al., 1994), constitute the only documented examples of nonDNA-directed splicing. The timing and extent of splicing of viral transcripts are strictly controlled (Alonso-Caplen and Krug, 1991; Valcarcel et al., 1991) since the products are required at different times in the viral life cycle. This regulation appears to be mediated by one or more viral proteins (Smith and Brown, 1984; Smith and Inglis,

1985).

1.8.3 VIRAL REPLICATION

Viral mRNAs contain non-templated nt at their 5' and 3' ends and lack a copy of the 5' terminal 17-22 vRNA residues and are therefore unsuitable replicative intermediates. The first step in replication involves the production of full-length "antigenomes" representing each of the 8 genome segments. Each of these template molecules contains all of the genetic information of the corresponding parental vRNA in complementary form and is therefore termed a complementary RNA (cRNA). Synthesis of cRNA initiates without a primer, generating a pppA 5' terminus templated by the 3' terminal nt of the vRNA (Hay et al., 1977; Hay et al., 1982). Elongation of cRNA proceeds through the polyadenylation site, allowing transcription of sequences complementary to the 5' vRNA end (Skehel and Hay, 1978). Synthesis of cRNA, like mRNA, occurs in the nucleus. However, cRNAs stay in the nucleus throughout infection while mRNA is exported to the cytoplasm to be translated (Herz et al., 1981; Shapiro et al., 1987). The transition from viral mRNA to cRNA requires de novo protein synthesis suggesting that virus-specific products are involved in unprimed RNA initiation or antitermination (Barrett et al., 1979; Robertson et al., 1981; Ulmanen et al., 1981; Hay et al., 1982). NP has been identified as the putative "switching factor" (Beaton and Krug, 1986; Shapiro and Krug, 1988). One possible model (Beaton and Krug, 1986) draws on the observation that vRNA and cRNA but not mRNA are encapsidated by NP. It proposes that the site of initiation of nucleocapsid assembly is the 5' end of an RNA

molecule. When cRNA is initiated without a primer, the growing chain becomes covered in NP; the process of encapsidating the 3' terminus of the cRNA melts the secondary structure of the vRNA conferred by duplexed termini, permitting readthrough. It follows that when mRNA synthesis initiates with a capped primer the site of encapsidation initiation is sterically blocked, hindering NP binding and antitermination. This is supported by the observation that antitermination was inhibited when a capped primer was used in in vitro transcription assays (Beaton and Krug, 1986). The role proposed for NP in this scenario is not unlike that defined for the vesicular stomatitis virus (VSV) nucleocapsid protein which is required for replication (Hill and Summers, 1982). Definitive identification of the factors directing the influenza virus polymerase decision to synthesize cRNA vs mRNA has not been possible. However, in the model described above, the mode of priming and the abundance of soluble NP are strong determinants. It is possible that another viral protein is required for replication. By analogy to the VSV system this may be a nonstructural protein (Davis et al., 1986). Based on observations that influenza virus NS1 is an RNA binding protein (Yoshida et al., 1981) and that ts mutants with defects in segment 8 (encoding the nonstructural proteins) show reduced vRNA synthesis at the nonpermissive temperature (Wolstenholme et al., 1980) NS1 has been proposed to participate in the switching of transcription to replication (Shapiro and Krug, 1988). A role for NS1 in replication was, however, not substantiated by in vitro RNA transcription assays (Skorko et al., 1991).

In the second step of replication the cRNAs act as templates for the

production of the 8 full-length genomic RNAs (vRNAs) in the nucleus (Shapiro et al., 1987). vRNA synthesis initiates without a primer, generating pppA 5' termini (Hay et al., 1977; Hay et al., 1982). Subsequent RNA elongation absolutely requires the presence of soluble NP (Shapiro and Krug, 1988). Newly-synthesized genomes are transported to the cytoplasm as vRNP (Herz et al., 1981; Shapiro et al., 1987) for assembly into progeny virions. Export of vRNP to the cytosol was reported to require association with M1 in the nucleus (Martin and Helenius, 1991). In support of this finding, a ts mutant encoding a single amino acid substitution in M1 was defective in nuclear-cytoplasmic transport of vRNPs at the nonpermissive temperature (Enami et al., 1993).

1.8.4 VIRAL ASSEMBLY

Influenza virus replicates in the nucleus but buds from the plasma membrane. The formation of progeny influenza virions therefore requires the assembly of viral components located in different parts of the cell. The vRNPs assembled in the nucleoplasm, matrix protein in the nucleus and cytoplasm and viral cytoplasmic glycoproteins HA and NA must colocalize with host plasma membrane at a site which ensures directional budding. Influenza particles assemble, bud and detach from the apical plasma membrane of polarized epithelial cells. This orientation of virion release has a profound impact on the pathogenicity of the virus in determining the direction of viral dissemination. Both HA and NA possess sorting signals; these apical determinants along with components of the cytoskeleton target the viral proteins to the appropriate membrane site (Nayak and

Jabber, 1989). Matrix protein may act as a transport chaperone in associating with vRNPs in the nucleus (Rees and Dimmock, 1981; Ye et al., 1980) and promoting their export to the plasma membrane (Martin and Helenius, 1991). To account for its relative abundance in progeny viruses, M1 in the cytosol may also be recruited to the plasma membrane (Martin and Helenius, 1991). How M1 colocalizes with HA and NA at the plasma membrane is unknown since the presence of both glycoproteins or the cytoplasmic tail of HA are not essential for viral assembly (Nayak, 1994). Morphogenesis also requires assembly of a full complement of 8 individual vRNPs. This may occur by a random selection of multiple vRNPs such that a reasonable number of progeny virions possess at least 1 copy of each genome segment (Hirst, 1962; Compans and Dimmock, 1970). Low infectivity ratios (10-20 particles per PFU) and the observation that "extra" genome segments can be artificially incorporated into virus (Enami et al., 1991) support this model. There are also proponents for a mechanism predicting a nonrandom segregation of genes during morphogenesis. Selective packaging may account for genetic linkage of homologous genes following mixed infection of cells (Lubeck et al., 1979) and observations that the proportions of RNA segments in released virus do not reflect relative quantities of intracellular vRNA (Smith and Hay, 1982; Akkina et al., 1984; Duhaut and McCauley, 1996).

1.9 THE INFLUENZA VIRUS POLYMERASE COMPLEX

Investigation of the molecular nature of the polymerase complex and determination of the precise role of each of its protein components has been

impeded by the difficulty both in isolating enzymatically active polymerase proteins in sufficient quantities and in reconstituting the complex. Polymerase protein functions have been studied using a variety of in vitro transcription systems which derive their enzymatic activity from detergent-disrupted virus (Plotch and Krug, 1978), infected cell nuclear extracts (Beaton and Krug, 1984), thioredoxin-renatured purified polymerase proteins (Szewczyk et al., 1988) or, more recently, recombinant proteins (Kobayashi et al., 1992). These model in vitro systems possess the enzymatic capability to catalyze the various steps of transcription. Dissection of the transcription process in vitro along with in vivo rescue systems, genetic studies exploiting ts mutants and UV cross-linking experiments have contributed much to our understanding of polymerase kinetics, requirement for cellular factors, the movement of the polymerase complex during RNA synthesis. It has also allowed assignment of function to the individual polymerase protein components of the complex. This information is summarized in the following paragraphs.

The core enzyme is a heterocomplex composed of 1 each of PB2, PB1 and PA (Horisberger, 1980; Honda et al., 1990). The preferred primer (Honda et al., 1986) and temperature and divalent cation conditions producing optimum enzyme activity have been investigated (Bishop et al., 1971) as have the inhibitory effects of various drugs including cycloheximide (inhibitor of protein synthesis), actinomycin D (inhibitor of DNA-dependent transcription), alpha-amanitin (inhibitor of RNA polymerase I) (Barrett et al., 1979; Mark et al., 1979) and ribavirin (Scholtissek, 1975). PB2, PB1 and PA can assemble in the absence of vRNA and

NP in infected cells (Detjen et al., 1987; St. Angelo et al., 1987) or colocalize at one end of the vRNA template as a complex (Murti et al., 1988). UV cross-linking studies have documented the initiation of this complex at the 3' end of the vRNA and its movement down the template in tandem with elongating mRNA during transcription (Braam et al., 1993).

Using an in vivo, vaccinia virus vector-driven system the minimum protein requirement for RNP expression was determined to be PB2, PB1, PA and NP (Huang et al., 1990). The functions of 3 of these proteins have been delineated. UV cross-linking studies have established that PB2 associates with caps on host cell primer fragments during the endonuclease reaction (Ulmanen et al., 1981). In support of this, PB2 was seen to bind a photoreactive derivative of m^7GTP (Blaas et al., 1982). This physical association was subsequently shown to reflect a true biological interaction when the binding of polymerase to capped primers and the cap-dependent endonucleolytic event were found to be compromised at elevated temperatures in a ts mutant defective in segment 1 (Ulmanen et al., 1983). PB2 is also homologous to a cap-binding protein involved in eukaryotic protein synthesis initiation (Rychlik et al., 1987). Subsequent to cap binding, PB2 remains associated with the other polymerase proteins in the complex which detaches from the cap and moves 3' along the vRNA template (Braam et al., 1983).

The function of PB1 has also been discerned from UV cross-linking studies. PB1 is the only polymerase protein found in contact with the first G residue incorporated into the mRNA on the terminus of the host primer (Ulmanen et al.,

1981) and it is always positioned at the 3' end of elongating transcripts (Braam et al., 1983). This suggests that PB1 catalyzes the initiation of transcription and subsequent ribonucleotide additions and is therefore the catalytic component of the complex. Recently, PB1 alone was shown to be sufficient for RNA synthesis in vitro (Toyoda et al., 1996). The involvement of PB1 in transcription is supported genetically by a ts mutant defective in PB1 which exhibited impaired mRNA synthesis at the nonpermissive temperature (Krug et al., 1975).

Structure-function analyses have identified certain domains on PB1 likely to be involved in catalysis. PB1 contains 5 motifs, each with 1 strictly invariant amino acid residue, which are common to all RNA-dependent RNA/DNA polymerases. These motifs constitute the "polymerase module" (Kamer and Argos, 1984; Argos et al., 1988; Poch et al., 1989; Muller et al., 1994). Using site-directed mutagenesis, four of the motifs were examined and shown to be critical to polymerase activity (Biswas and Nayak, 1994). Based on sequence comparison to HIV-1, for which three-dimensional structural information is available (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993) it was proposed that three of these form the catalytic core and play a role in nucleoside triphosphate binding (Muller et al., 1994). The other two common motifs may be involved with template or primer positioning relative to the active site (Muller et al., 1994). A 48 amino acid region at the amino terminus of PB1 which mediates association with PA in vivo (Perez and Donis, 1995) and two nuclear localization signals (Nath and Nayak, 1990) have also been identified. A three-way alignment of influenza A, B and C PB1

amino acid sequences has identified blocks of highly conserved sequence for which function has not yet been assigned (Perez and Donis, 1995).

The precise function of PA has not been determined. It has been shown to possess protease activity (Sanz-Ezquerro et al., 1996). Genetic evidence suggests it plays a critical role in replication but not necessarily in mRNA synthesis since ts mutants possessing lesions in PA are deficient in vRNA but not mRNA synthesis at the nonpermissive temperature (Krug et al., 1975; Mahy, 1983). PA, in combination with PB1, PB2 and NP has been shown to be necessary and sufficient for RNA replication in an in vivo recombinant polymerase system (Huang et al., 1990).

1.10 THE vRNA PROMOTER

Progress has been made in determining the cis-acting signals controlling transcription initiation. The termini of each of the genome segments contain conserved noncoding sequence including a 5' 13 nt sequence 5'AGUAGAAACAAGG. There is also a highly conserved sequence at the 3' end with 3'UCGUUUUCGUCC found in segments 4, 5, 6 and 8 and 3'UCGCUUUCGUUCC in segments 1-3 and 7 (Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980; Winter and Fields, 1980). These termini, along with several segment-specific residues, display inverted partial complementarity. The genomic RNAs in virions and infected cells assume a circular configuration with the duplexed RNA termini forming the "panhandle" (Hsu et al., 1987). The viral polymerase has been found associated with the double-stranded region leading to

the suggestion that the panhandle may provide important regulatory signals and that it may, in fact, constitute the promoter (Hsu et al., 1987; Honda et al., 1988). It was subsequently demonstrated by reverse genetics that the 22 5' terminal and 26 3' terminal bases contain the regulatory signals necessary for transcription, replication and packaging of virions (Luytjes et al., 1989).

Various independent in vitro RNA synthesis studies initially suggested that the vRNA promoter resided solely in the 3' end of genomic RNA and that neither the 5' terminus nor the secondary structure of the panhandle were required for transcriptional activity (Parvin et al., 1989; Yamanaka et al., 1991; Seong and Brownlee, 1992a,b; Piccone et al., 1993). Using saturation mutagenesis, the 3' terminal residues 1-4 and 9-11 were determined to be critical for transcription (Honda et al., 1987; Seong and Brownlee, 1992b; Piccone et al., 1993). In support of this a polymerase binding site was identified at position 9-12 (Fodor et al., 1993). The 5' termini of vRNA have subsequently been shown to be an integral part of the vRNA promoter (Hagen et al., 1994; Tiley et al., 1994). These experiments used recombinant RNA polymerase, unlike previous studies which relied on polymerase isolated from packaged RNP that was enzymatically depleted of template and which therefore potentially contained undegraded, endogenous 5' ends. In vitro transcription (Hagen et al., 1994; Fodor et al., 1995) and endonuclease activity (Hagen et al., 1994; Cianci et al., 1995) showed a strict requirement for both 5' and 3' vRNA termini and polymerase was reported to bind preferentially to the 5' end (Tiley et al., 1994). The sequence and structural requirements for polymerase

binding have been determined (Tiley et al., 1994; Fodor et al., 1995). Based on these studies, an "RNA fork" model has been proposed to describe the mechanism of vRNA promoter recognition by the polymerase (Fodor et al., 1995). In this model the individual polymerase proteins form a heterotrimer prior to association with the vRNA segment. The polymerase complex then binds initially to a free, single-stranded 5' end of the vRNA template which has a crucial double-stranded region at positions 10-12 on the 3' terminus and 11-13 on the 5' terminus. The effect of base pair replacements on transcription activity demonstrated that the precise sequence in the duplex region is less important than its ability to base-pair (Fodor et al., 1995). The duplex structure of the vRNA and the affinity of the bound polymerase for the 3' end then brings the free 3' terminus in apposition to the catalytic site of the polymerase. The presence of 5' and 3' termini then activates cap-binding and endonuclease functions of the polymerase. Thus, the "fork" configuration of vRNA functions acts as a scaffold to align the polymerase proteins for transcription initiation. A polymerase binding site on the 5' terminus of vRNA may also influence the choice between mRNA and cRNA synthesis. If the polymerase remained attached to the 5' end, possibly influenced by the presence of a capped primer, steric interference would prevent transcription of the bound sequence and promote termination at the polyadenylation site (Fodor et al., 1994).

1.11 THE cRNA PROMOTER

Promoter requirements for vRNA synthesis have also been investigated (Li and Palese, 1992; Seong and Brownlee, 1992b; Kimura et al., 1993; Tiley et al.,

1994; Pritlove et al., 1995). A panhandle involving base-pairing of specific residues was required for optimum cRNA promoter activity (Tiley, 1994; Pritlove et al., 1995) as was determined for the vRNA promoter however the nt on the 5' and 3' termini found to be essential for polymerase binding to cRNA and vRNA templates were not identical (Tiley et al., 1994). Interestingly, cap-binding activity was also found to be stimulated by presence of templates corresponding to cRNA however, the binding of the polymerase to the 5' and 3' termini of these molecules did not stimulate endonuclease activity (Cianci et al., 1995). This suggests that transcription and replication may involve distinct enzymatic modes directed by polymerase binding. Differential positioning of the polymerase complex upon binding or its subsequent movement on the different templates in addition to the presence of a capped 5' terminus on the nascent RNA chain may provide an explanation for the observation that subgenomic, polyadenylated transcripts are generated in vivo from vRNA but not cRNA templates (Tiley et al., 1994).

1.12 PROMOTER-GENERAL

Nucleotides immediately adjacent to the conserved terminal sequences on vRNA and cRNA were also reported to be important in the synthesis of mRNA and vRNA, respectively (Zheng et al., 1996). These residues are not conserved between segments but may demonstrate high conservation within one segment derived from different strains (Desselberger et al., 1980). The existence of segment-specific sequences controlling RNA synthesis may help to explain the preferential expression of different viral genes during infection.

Experiments testing the ability of the influenza virus polymerase to recognize an internal promoter have produced conflicting data. Li and Palese (1992) showed that synthetic CAT RNA templates with 1, 5 or 13 extra nt at their 3' termini demonstrated reduced promoter activities in vitro (55, 26 or 6%, respectively, relative to a template with authentic termini) but only the first was functional in vivo when transfected as RNP into influenza virus-infected cells. Piccone (1993) observed that a 3' terminal extension of 30 heterogeneous nt to model vRNA templates abolished in vitro and in vivo promoter activity. In contrast, addition of 20-30 nt to the 3' end of a synthetic RNA that was transfected as RNP cores was reported not to affect transcription in vivo (Yamanaka et al., 1991). Synthetic RNA templates containing 3' extensions of 5-2600 nt were transcribed (albeit at levels which decreased with increasing template length) when transfected into cells expressing NP, PA, PB1 and PB2 from pGEM vectors based on differences in polymerase abundance (Mena et al., 1994). Interestingly, promoter activity was not observed by this group when the helper system supplying the polymerase was influenza virus. An explanation for this has been proposed (Mena et al., 1994). In influenza virus-infected cells there is less polymerase than in cells expressing cloned polymerase genes. In helper virus-infected cells, a transfected viral template with a terminal extension may compete unsuccessfully with viral RNA possessing authentic termini for limiting amounts of polymerase and may therefore be incapable of supporting transcription. Polymerase availability and the potential presence of competing wt templates must be considered in further assessment of

the ability of the influenza virus polymerase to recognize an internal promoter.

1.13 CONTROL OF VIRAL GENE EXPRESSION

Influenza virus gene expression is highly regulated. To define the controls operating on the synthesis of viral macromolecules, numerous studies have examined the relationship between the levels of viral mRNAs and their encoded proteins or between the three species of RNA- mRNA, cRNA and vRNA (Hay et al., 1977; Barrett et al., 1979; Mark et al., 1979; Smith and Hay, 1982; Enami et al., 1985; Shapiro et al., 1987). Preferential synthesis of NS1 and NP mRNA early in infection and appearance of glycoprotein mRNAs later were seen to correspond to changes in abundance of their respective proteins (Hay et al., 1977; Shapiro et al., 1987). This led to the proposal that viral gene expression was regulated at the transcriptional rather than translational level (Hay et al., 1977). Shapiro et al. (1987) defined an "early" phase of infection (<2.5 hr p.i.) when preferential synthesis of specific vRNAs, mRNAs and proteins were closely coupled. Since equimolar amounts of template (cRNA) RNAs were measured during this phase, the rate of synthesis of gene-specific levels of vRNA from cRNA appeared to then direct the rate of mRNA transcription. Proteins synthesized during this phase (NS1, NP, PB2, PB1 and PA) were termed "early" proteins. "Late" proteins (M1 and HA) were synthesized after 2.5 hr p.i.. Thus, temporal fluxes in mRNA and protein were said to result from differential replication rather than reflect a transcriptional control (Smith and Hay, 1982; Shapiro et al., 1987).

A more recent study (Hatada et al., 1989) measuring the accumulation of

viral macromolecules documented segment-specific mRNA production during primary transcription, an observation incompatible with earlier studies showing primary transcription to be unregulated (Hay et al., 1977). Hatada et al. (1989) also observed simultaneous replication initiation for all genes, implying "early" and "late" gene expression were not a reflection of replicative control. Interestingly, a post-transcriptional control on late gene expression was also observed since synthesis of M1 and HA was delayed relative to their mRNA production. This control was found to operate at the level of transport out of the nucleus (Vogel et al., 1994). NS1 is now known to play an important role in post-transcriptional regulation of viral gene expression (Alonso-Caplen et al., 1992; Qiu and Krug, 1994). NS1 is a poly A+-binding protein (Qiu and Krug, 1994). According to a model proposed by these authors, when sufficient de novo-synthesized NS1 accumulates, it blocks transport of both cellular and late viral mRNAs. Sequestered cellular mRNAs are then cleaved to generate capped primers required for viral mRNA synthesis and are rapidly degraded (Krug, 1989). At a certain point in infection, post-translational modification (Vogel et al., 1994) inactivates NS1, allowing export of late viral mRNAs to the cytoplasm where they are translated.

A perturbation in the regulation of influenza virus gene expression at any step in infection can compromise virus production. Control may be deranged due to aberrant activity or expression of a viral protein required for RNA synthesis. For example, viruses with mutated NS2 produce deleted PA vRNA and hence defective particles (Odagiri et al., 1994). Regulated gene expression may be compromised

at a subsequent step in infection, such as export of newly synthesized RNP into the cytoplasm. Viruses with mutant M1 were reported to accumulate vRNP in the nucleus, abolishing virion production (Enami et al., 1993; Whittaker et al., 1995). Host factors also play a role in the regulation of viral gene expression. Reduced production of M1 and increased expression of M2 leading to abortive infection are seen in mouse L (Inglis and Brown, 1984) and embryonic brain (Bradshaw et al., 1990) cells due to increased splicing activity in these host cells. Control of viral gene expression can also be perturbed by a coinfecting virus. Because a cell can be simultaneously infected with multiple strains of influenza virus, the possibility exists that expression of one genome may inhibit the multiplication or assembly of the other. Simultaneous infection of cells with two strains of influenza virus normally yields equivalent amounts of the parental strains as well as a minority of reassortant progeny. However, coinfection of cells may result in growth suppression of one or both of the coinfecting strains, a phenomenon termed interference. Interference of wt influenza A virus replication has been reported to occur in cells coinfected with defective interfering (DI) particles, influenza B viruses or a nondefective, attenuated virus.

1.14 VIRAL INTERFERENCE

1.14.1 DEFECTIVE INTERFERENCE

Defective interfering particles comprise a class of viruses which possesses two phenotypic traits: (1) They are unable to replicate autonomously, usually owing to a genome that lacks essential sequence. Although genetically "defective", their

replication is sustained when the required factor(s) is supplied by a coinfecting homologous virus genome. (2) In a cell coinfected with DI and wt virus, the DI genome is preferentially amplified which results in reduced helper virus multiplication. DI particles are thus termed "interfering".

DI particles are observed in association with the growth of most RNA and DNA animal viruses (Holland, 1990). Among influenza viruses, DI particles generated by aberrant replication of viral RNAs are amplified by serial undiluted passage [first reported by von Magnus (1947); reviewed by Nayak, (1980); Nayak and Sivasubramanian (1983); Perrault (1983); Nayak et al., (1985)]. In this high multiplicity environment, cells infected with DI particles are likely to be coinfected with helper virus which permits the replication of the DI RNAs.

The structure of viable DI particle genomes is well characterized. A DI genome contains one or more subgenomic or DI RNAs. DI RNAs are of negative polarity and range in size from 178-859 nt (Nayak, 1980). Reported DI RNA sequences all show internal deletions relative to the corresponding standard genome segment but retain the authentic 5' and 3' genomic termini (Nayak, 1980; Lazzarini et al., 1981; Jennings et al., 1983; Nayak et al., 1985). DI RNAs may be generated by a single deletion or, less commonly, multiple deletions, or may (in a single documented case) possess a mosaic sequence resulting from intergenic recombination (Fields and Winter, 1982). The "copyback" and "snapback" DI RNAs which retain the 5' genomic end but replace the 3' end with a complementary copy of the 5' terminus, found in VSV and Sendai viruses (Kolakofsky, 1976; Perrault,

1981; Yang and Lazzarini, 1983), have not been observed in influenza DI particles. DI RNAs derive mainly from polymerase genes (Jennings et al., 1983; Davis and Nayak, 1979; Nayak et al., 1985), most of these originating from PB2. Subgenomic RNAs derived from HA, NA, NP and NS have been reported however they did not possess an interfering phenotype (Nayak et al., 1985).

There is no in vitro system with which to dissect the molecular events of DI RNA generation however two models have been proposed based on sequence studies. One model suggests that internal deletions may be generated by a "jumping" polymerase where the polymerase complex physically leaves the RNA template during the synthesis of (+) or (-) RNA and reattaches along with the nascent RNA chain further along the same template or on a different template where RNA synthesis resumes (Nayak et al., 1985). No common sequence motifs have been found which could direct polymerase detachment/reattachment (Nayak et al., 1985). The fact that polygenic and copyback DI RNAs are not seen makes it unlikely that the polymerase completely disengages from the template (Nayak et al., 1989). Therefore a second related scheme, the "rolling" polymerase model, proposes that the polymerase complex generates an internal deletion in the nascent RNA by "ignoring" a looped-out region of template and "rolling" over or shifting onto a downstream site brought into juxtaposition by the secondary structure of the RNP without leaving the template (Nayak and Sivasubramanian, 1983; Nayak et al., 1985). The greater length of the polymerase genes may offer more opportunity for secondary structure and could account for the abundance of DI RNAs of

polymerase gene origin.

How DI viruses interfere with standard virus replication is unknown. However certain features of the system have been well-documented (Nayak et al., 1989): (1) Helper virus yield is reduced in cells coamplified with DI particles. (2) The total yield of virus particles and synthesis of viral macromolecules in these cells is decreased. (3). There is a reduction in cytopathic effect caused by standard virus infection. (4). Interference by DI particles is not mediated by interferon.

In an attempt to understand how DI particles interfere with standard virus multiplication, DI viral macromolecular synthesis has been studied. DI RNAs are transcribed into polyA+ mRNA in vitro and in vivo (Chanda et al., 1983; Chambers et al., 1984). DI-specific polypeptides have also been detected in infected cells and in the in vitro translation products of cytoplasmic mRNA from DI-infected cells (Akkina et al., 1984b; Chambers et al., 1984). However, the DI polypeptides are apparently not incorporated into progeny DI particles possibly owing to decreased stability, defects in nuclear import or inability to associate with other proteins (Akkina et al., 1984b). When considering the mechanism of DI interference, DI mRNAs and their putative polypeptides must therefore be considered.

DI RNAs are preferentially transcribed and replicated over standard genome segments in coinfected cells (Chambers et al., 1984). The production of standard polymerase gene transcription (Chambers et al., 1984) and replication of standard polymerase genes (Carter and Mahy, 1982) are specifically reduced. This leads to a decrease in the production of functional polymerase proteins in the cell (Akkina

et al., 1984b) which could in turn reduce overall viral macromolecular synthesis and virus production.

How a DI RNA preferentially inhibits the transcription and replication of its progenitor polymerase gene is not clear. DI-mediated interference was reported to be partially overcome by increasing the dosage of input standard virus (Akkina et al., 1984b). This may be diagnostic of a competition between standard and DI templates for a limited number of polymerase proteins (Akkina et al., 1984b). The nature of the competition and the precise advantage of DI RNAs are not known. Since influenza virus DI RNAs possess the same 5' and 3' termini as their progenitor segments, it is unlikely that they have increased affinity for polymerase. It is also unlikely that they are simply more available for replication due to an impaired capacity for transcription, a mechanism invoked to explain VSV DI-mediated interference (reviewed in Perrault, 1981). Preferential amplification of DI RNAs have therefore been attributed to their smaller size (Akkina et al., 1984b). Because preferential primary transcription of DI RNAs was not observed in vitro (Chanda et al., 1983), the relative in vivo levels of DI and standard RNA may be attributable to selective secondary transcription of DI RNAs (Nayak et al., 1989). Since secondary transcription depends on vRNA synthesis, DI-mediated interference could therefore originate during replication or secondary transcription.

DI RNAs may also influence packaging of genome segments into progeny virus. This is supported by the observation that in cells infected with viruses containing segment 1 DI RNAs, the DI and its progenitor RNA accumulated in the

cell (Duhaut and McCauley, 1996). However the quantity of the "parent" segment 1 in mature virus was selectively reduced suggesting that DI RNA segment 1 was preferentially packaged. Thus, DI-mediated interference with standard virus multiplication may be the result of multiple events including preferential synthesis of DI transcripts and preferential replication of DI RNAs due to a competitive size advantage. DI transcripts may direct production of defective polypeptides which may also play a role by complexing with RNP and possibly blocking transcription and replication (Nayak et al., 1989) [However, not all DI virus preparations contain detectable DI polypeptides and interfering ability did not correlate with level of DI peptides (Chambers et al., 1984)]. There is also a specific reduction in wt polymerase protein which then reduces overall viral macromolecular synthesis. Finally, the advantage conferred by preferential transcription and replication of DI RNAs may be augmented by a specific selection of DI genomes during assembly into virus.

The ability of deletion mutants to decrease the yield of wt virus, suppress intracellular synthesis of viral macromolecules and protect cells in vitro and in vivo from virus-induced cell death define a clear potential application in antiviral therapy (Nayak et al., 1981; Dimmock et al., 1986; Bangham and Kirkwood, 1990; Chambers and Webster, 1991; Morgan and Dimmock, 1992). However, because deletion mutants are replication-defective they can not be clonally purified in the absence of helper virus which severely limits their study or therapeutic applications.

1.14.2 HETEROTYPIC INTERFERENCE

Non-defective interference in mixed infections has also been reported. Heterotypic interference of coinfecting wt strains of influenza type A and B viruses has been observed in cell culture as a reciprocal repression of viral protein synthesis and virus growth, usually with influenza type A showing the most suppression (Mikheeva and Ghendon, 1982; Kaverin et al., 1983; Aoki et al., 1984). The interference was dose-dependent and could be overcome by altering the relative MOIs in the inocula (Mikheeva and Ghendon, 1982; Kaverin et al., 1983) or delaying superinfection of cells with the stronger competitor (Aoki et al., 1984). The mechanism of interference is unknown. Although interference was not attributable to differential binding or penetration (Kaverin et al., 1983), it was observed at the level of primary transcription (Mikheeva and Ghendon, 1982) or at steps subsequent to primary transcription (Kaverin et al., 1983; Aoki et al., 1984) making the construction of an all-inclusive model for heterotypic interference difficult. However, inhibition of one virus by a coinfecting strain early in infection would provide a basis for understanding why reassortment does not occur in heterotypic coinfections (Sugiura, 1975). Differences in influenza type A and B polymerase requirements or specificity may result in the production of nonfunctional heterologous complexes during mixed infection and manifest in mutual viral interference. Influenza A virus polymerase was shown to be capable of transcribing and replicating a reporter gene containing noncoding sequences from an influenza B NS gene albeit at reduced levels compared to a reporter gene flanked by

homologous influenza A noncoding sequences (Muster et al., 1991). A chimeric type A/B influenza virus was then constructed which formed plaques in Madin-Darby bovine kidney cells but which was attenuated relative to wt influenza type A virus (Muster et al., 1991).

The biological relevance of heterotypic interference relates to its potential generation during vaccination with live attenuated influenza type A and B viruses, which for logistical reasons may be administered simultaneously as a divalent vaccine. Interference between pairs of cold-adapted influenza A and B vaccine viruses has been observed in humans (Spencer et al., 1979) and in embryonated eggs and mice (Romanova et al., 1994). Conditions for overcoming interference in the latter system involved adjustment of relative dosages of infecting viruses.

1.14.3 INTERFERENCE BY CONDITIONAL LETHAL OR ATTENUATED VARIANTS

Some virus variants with conditional lethal or attenuated phenotypes also demonstrate interference, inhibiting the growth of wt virus in mixed infections. This phenomenon has been reported for both RNA and DNA viruses. Among RNA viruses, the dominance of conditional defective or attenuated viruses in mixed infection with wt has been documented for rhabdoviruses (Youngner and Quagliana, 1976; Youngner et al., 1986), togaviruses (Keranen, 1977), picornaviruses (Dodds et al., 1985), reoviruses (Chakraborty et al., 1979; Ikegami and Gomatos, 1972) and orthomyxoviruses (Whitaker-Dowling, 1990). The type of interference demonstrated in these systems is termed "interference dominance"

because it involves the functional inactivation of wt genes or proteins by a dominant negative mutant during coinfection and results in the reduction of wt virus yield (reviewed by Whitaker-Dowling, 1987). There are few data available for construction of a mechanism to explain the reduction in wt yield by a mutant virus however several models have been proposed (Whitaker-Dowling, 1987). The "rotten apple" hypothesis asserts that a mutant polypeptide that retains essential binding domains interacts with wt components of a multimeric complex thereby inactivating it. In another scheme, the mutant and wt viruses compete for a limiting factor with the mutant possessing greater binding affinity or a greater abundance of binding sites. Binding of the factor by the mutant may irreversibly sequester it. Another mechanism proposes that mutant virus expression alters the stoichiometry of structural proteins in the cell thereby creating an imbalance which compromises wt virus growth.

Among influenza viruses there are examples of dominant mutant viruses with attenuated phenotypes. The dominance of a small plaque variant in mixed infection with wt virus was found to be due to its receptor-binding protein, HA, which had greater affinity than wt HA for host cells (Noronha-Blob and Schulze, 1976). The temperature-sensitive, cold-adapted strain A/AA/6/60 and reassortants possessing the 6 nonglycoprotein genes of the ca strain (see section 1.4) also demonstrate interference, inhibiting the growth of parental and heterologous subtypes of influenza A viruses in mixed infection of MDCK cells and embyonated eggs (Whitaker-Dowling et al., 1990). Interference is manifested as a reduction in wt

viral yields by up to 3000X and the preferential expression of mutant proteins (Whitaker-Dowling et al., 1990). The various phenotypic characteristics of the cold-adapted virus have been mapped (Snyder et al., 1985). The ca phenotype is specified by segment 3 (PA) while temperature sensitivity maps to segments 1 and 2 (PB2, PB1). Segments 1-3 and 7 (PB2, PB1, PA and M) all contribute to the attenuated phenotype of these viruses. Interference of wt virus growth has been mapped to segment 7 (Whitaker-Dowling et al., 1991). The ca virus affects all stages of wt macromolecular synthesis during coinfection with the principal block occurring during wt vRNA synthesis (Maloy et al., 1994).

1.15 BACKGROUND OF THE CURRENT STUDY

The mouse-adapted strain FM-MA was produced following 12 passages of wt A/FM/1/47 (FM) in mouse lung (Brown, 1990). FM-MA is a non-defective, nonattenuated variant and thus represents a distinct class of interfering virus. FM-MA interferes with H3N2, H1N1 and H2N2 subtypes of wt influenza virus strains in mixed infections of MDCK cells (Brown et al., 1992). Wild-type progeny from such coinfections typically represent less than 1% of the virus yield. Since the parent, FM, does not demonstrate this dominance in mixed infections with other wt strains, it was clear that FM-MA had acquired mutation(s) on mouse passage that conferred the interference phenotype.

Interference has been mapped genetically to segment 2 (PB1) of FM-MA (Brown et al., 1992). Sequencing of this segment identified a single mutation relative to FM resulting in an amino acid change at position 538 (asp->gly) of the

FM-MA PB1 polymerase protein (Smeenk et al., 1996). PB1 is the catalytic component of the heterotrimeric polymerase complex composed of PB1, PB2 and PA (as described in more detail in section 1.9). The FM-MA segment 2 mutation did not reside within the PB1 domains for which function has been assigned including 5 motifs common to all RNA-dependent RNA polymerases, 2 nuclear localization signals, a domain potentially involved in vRNA positioning and an amino terminal domain responsible for binding PA. In addition to its primary role in nucleoside triphosphate binding and catalysis, PB1 serves other functions including protein-protein interactions with PB2, PA and possibly NP as well as RNA template and primer binding thus imposing strict constraints on PB1 structure. The PB1 mutation in FM-MA maps to one of the domains conserved among influenza A, B and C PB1 proteins (Muller et al., 1994) suggesting that it may be affecting structural domains controlling a critical, yet undefined, function.

1.16 OBJECTIVES OF THE CURRENT STUDY

The purpose of this project was to determine the mechanism of interference, due to the PB1 mutation, by FM-MA of wt influenza virus in mixed infections. Since PB1 is active in RNA synthesis, it was clearly important to measure mutant and wt RNA levels during interference in order to detect any suppression of wt transcription or replication which could lead to inhibition of viral growth. Measurement of transcription and genome replication in cells coinfected with FM-MA and a prototype H3N2 strain, A/HK/1/68, required discrimination between the 3 classes of RNA (vRNA, cRNA and mRNA) for each strain and also differentiation between

HK and FM-MA RNA. RT-PCR was employed to target vRNA, cRNA or mRNA produced from 2 early genes (PB1 and NP) and one late gene (M1) using segment-specific primers that were complementary to unique sequences in the 3 classes of molecules and homologous to both strains. Strain-specific restriction digestion of PCR products permitted discrimination of the relative levels of HK and FM-MA RNA as the ratio of virus-specific PCR products. Protein synthesis and exported genomes were also measured for the two viruses to develop a complete profile of macromolecular synthesis during interference.

1.17 RELEVANCE OF CURRENT STUDY

The biological relevance of interference relates to its potential application in antiviral intervention. First, a live attenuated reassortant vaccine containing the FM-MA PB1 gene may constitute an effective therapeutic vaccine. An interfering, avirulent reassortant of FM-MA X HK protected mice against fatal influenza when coinfected intranasally with a virulent strain (Brown et al., 1992). The application of such interfering reassortants as therapeutic vaccines may be quite broad since FM-MA was shown to interfere in vitro with H3N2, H1N1 and H2N2 influenza subtypes (Brown et al., 1992). Second, a detailed understanding of the precise events involved in FM-MA interference with wt growth may make it possible to recreate the inhibitory event in a controlled manner. Finally, the identification of a site in PB1 that can affect polymerase activity also offers new possibilities for the analysis of polymerase function.

MATERIALS AND METHODS

2.1 Virus and Cells

2.1.1 General

The wt A/FM/1/47 (H1N1) and A/HK/1/68 (H3N2) strains were obtained from stocks of World Health Organization (WHO) reference viruses by 2 serial plaque isolations. The mouse-adapted variant of FM, FM-MA, was previously produced by 12 passages in mouse lung followed by plaque to plaque isolation in Madin-Darby canine kidney (MDCK) cells (Brown, 1990). Reassortant viruses that contained combinations of FM-MA and HK genome segments were produced previously (Brown, 1990). Virus stocks were grown for 2 days in the allantoic cavity of 10 day old chicken embryos following infection with 10^3 PFU of influenza virus. Clarified (3000 rpm x 15 min) allantoic fluid was stored at -70°C.

MDCK cells used in virus infections were cultured in Eagle's minimal essential medium (MEM) containing 5% neonatal bovine serum (Biocell), 100 µg/ml streptomycin and 100 U/ml penicillin in the presence of 3-5% CO_2.

2.1.2 Plaque Assay.

To determine the titer of virus stocks, virus was diluted in cold, sterile phosphate-buffered saline (PBS; Appendix I) containing 0.2 % gelatin (w/v). Aliquots (100 µl) were inoculated onto confluent monolayers of PBS-washed MDCK

cells in 6 well dishes and adsorbed for 1 hr at 37°C. Warmed cell overlay (2.5 ml) consisting of **199 Medium** (Appendix I) supplemented with 0.2% bovine serum albumin (BSA), 100 µg/ml DEAE dextran, 0.5% Oxoid agar #28 (w/v), 1 µg/ml trypsin, 100 µg/ml streptomycin, 100 U/ml penicillin, 2.2 mg/ml sodium bicarbonate and 0.2% glucose (w/v) was added to each well and allowed to solidify prior to incubation at 37°C for 2 days. Plaques were fixed with **Carnoy's solution** (Appendix I) for 20 min, air-dried and counted.

2.2 RNA Isolation

2.2.1 Cell Infection

Monolayers of MDCK cells in 100 mm dishes were washed with warmed PBS (Appendix I) prior to adsorption with virus for 1 hr at 37°C. Cells were either singly-infected (MOI=5) or coinfected (MOI=3, each virus) with a virus inoculum (<0.6 ml) mixed prior to adsorption. Infected cells were washed extensively (4 x 10 ml PBS) prior to incubation in 5 ml MEM supplemented with 0.2% bovine serum albumin (BSA), 100 µg/ml streptomycin and 100 U/ml penicillin for various times. If released virus was passaged, the culture medium included 1 µg/ml trypsin. For detection of primary transcription, virus adsorption and cell culture infection were performed in the presence of 100 µg/ml cycloheximide (CHX). Released virions were harvested from the culture supernatant by adsorption to human red blood cells (Cowley, 1984; see 2.2.3)

2.2.2 Infected Cellular RNA Extraction

RNA was extracted from infected cell monolayers using a protocol described

by Chomczynski and Sacchi (1987) with several modifications. Briefly, cell monolayers in 100 mm dishes were washed (3 x 10 ml) with ice-cold PBS and lysed in 1 ml **Denaturing Solution** (Appendix I). Cell lysates were scraped into 15 ml polypropylene tubes, acidified with sodium acetate (NaOAc) (pH 4.0) to 0.2 M and extracted by vigorous shaking with **TE** (Appendix I)-saturated phenol: chloroform: isoamyl alcohol (24:24:1). Following a 15 min incubation on ice, samples were centrifuged at 9500 rpm for 25 min at 4°C. RNA present in the aqueous phase was precipitated with an equal volume of isopropanol at -20°C for 1 hr -> overnight. Pelleted RNA (9500 rpm x 25 min at 4°C) was dissolved in 0.3 ml of **Denaturing Solution** (Appendix I) and reprecipitated in a 1.5 ml Eppendorf tube with an equal volume of isopropanol at -20°C for >1hr. Following centrifugation (13,000 rpm for 15 min at 4°C) RNA pellets were washed with 70% ethanol, air-dried and resuspended in RNase-free dH_2O.

2.2.3. Virion RNA Extraction.

RNA was likewise extracted from virus in culture supernatants following concentration of virions by adsorption to human red blood cells as described (Cowley, 1984). Briefly, cell culture medium (5 ml for a 100 mm dish) was clarified (9000 x 15 min at 4 °C) prior to adsorption with **PBS** (Appendix I)-washed human red blood cells (0.05% v/v) for 1 hr at 4°C. Virus was concentrated (3000 rpm x 5 min at 4°C) and washed twice in ice-cold **PBS** before lysis in 1 ml of **Denaturing Solution** (Appendix I). Acidification of lysates, extraction and purification of RNA was performed as described for total infected cellular RNA (Section 2.2.2).

2.3 Northern Blot Analysis

2.3.1. Electrophoresis, Electroporation and probing of RNA

Total cellular RNA (10-20 µg) was resolved by electrophoresis using gels prepared according to Laemmli (1970) consisting of a separating phase of 4.5% acrylamide (w/v) in 125 mM Tris-HCl (pH 6.8), 4 M urea, 0.4% SDS (w/v), 0.05% (w/v) ammonium persulphate (AP) and 0.05% TEMED (v/v) and a stacking gel containing 3.75% acrylamide in 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% AP and 0.1% TEMED. RNA samples were loaded in an equal volume of 2X **RNA Sample Buffer** (Appendix I) and subjected to electrophoresis at 80-85 V for 16 hr at room temperature. RNA was visualized by staining with ethidium bromide (2.5 µg/ml for 15 min) prior to electroblotting onto nylon membrane (Pall Biodyne B) in 0.5X **TBE** (Appendix I) at 20 V for 16 hr at 4°C using a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). After air-drying, RNA was cross-linked to membranes by UV-irradiation (5 min, Bio-Rad transilluminator). Membranes were prehybridized for 30 min at 42°C in **Modified Denhardt's Solution** (Appendix I) prior to hybridization in **Modified Denhardt's Solution** (1 ml per cm^2 of membrane) containing radioactive probe at 42°C for 2-16 hr. Membranes were washed in 0.1-5X **SSC** (Appendix I) containing 0.1% SDS at 20-42°C prior to autoradiography.

Autoradiographic signals were quantified by densitometry using GPTools software (BioPhotonics Corporation, Mississauga). The linear range of densitometric signals was identified using an X-ray standard with a range of predetermined exposures.

2.3.2 Preparation of Radioactive Probes.

Oligonucleotide probes were end-labelled with γ[^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Pharmacia) as described (Sambrook et al., 1989 pp.10.59-10.61) and purified from unincorporated [^{32}P] using G-50 Sephadex (Pharmacia) spin column chromatography (Maniatis, 1982; p. 466). Approximately 10 ng of labelled oligonucleotide was used per ml of hybridization fluid.

2.4 RNase Protection.

2.4.1 Subcloning of Influenza Virus Genes

cDNAs representing influenza genome segments, previously cloned into pSP64 as described (Brown, 1990), were subcloned behind bacteriophage promoters Sp6 and T7 in pGEM-7Zf(+) vectors. Viral gene insert DNA was isolated by restriction digestion of pSP64 subclones using 2 different enzymes which each generated a unique cut followed by electrophoresis through 0.6% (w/v) low melting temperature agarose (Bio-Rad) in 1X **TBE** (Appendix I), phenol-chloroform extraction of excised viral cDNA bands and DNA precipitation and washing in 70% ethanol. Inserts were ligated into compatible sites of restriction-digested pGEM-7Zf(+) with T4 DNA ligase at 14°C for 16 hr as described in Sambrook et al., (1989, pp. 1.68-1.69) for sticky-end ligation.

2.4.2 Transformation of E. coli by electroporation:

Preparation of electro competent cells

A 10 ml overnight culture of E. coli DH5α, inoculated from a single colony, was amplified in 1 liter of **LB broth** (Appendix I) to an OD_{600} of 0.5-1. Cells were

cooled on ice for 30 min prior to centrifugation at 4000 x g for 15 min at 4°C. Cell pellets were washed twice in 0.5 liter of cold, sterile dH_2O and once in cold 10% glycerol (v/v) prior to final resuspension in 2-3 ml of 10% glycerol, distribution into aliquots and freezing on dry ice. Twenty µl of electro competent DH5α were combined with 1 µl of a 4-fold diluted ligation mixture (dilution with 0.5X **TE**, Appendix I) transferred to a Cell-Porator (Gibco-BRL) Electroporation Chamber and pulsed at 2000 V/cm. The cell suspension was inoculated into 2 ml of **SOC** (Appendix I) and shaken at 37°C for 1 hr prior to plating on LB agar [LB broth supplemented with 1.5% bacto-agar (w/v)] overlaid with 100 µg/ml ampicillin, 20 µg of Xgal and 0.01 mmol isopropylthio-β-D-galactosidase (IPTG).

Preparation of plasmid DNA (small-scale):

Transformant colonies were identified on plates by failed α complementation. Specifically, insert DNA was targetted to a cloning site in the coding region of a portion of the bacterial β-galactosidase gene encoding the first 146 amino acids of the enzyme in pGEM-7Zf(+). When stimulated by IPTG, this fragment of β-galactosidase can associate with the host-encoded carboxy-terminal portion of β-galactosidase creating an active enzyme that catabolizes X-gal to produce a blue product. Subcloning of insert DNA into the cloning site within the β-galactosidase gene segment in the pGEM plasmid interrupts the reading frame of the enzyme and produces an inactive product that cannot associate with the host-encoded portion of β-galactosidase and which therefore does not metabolize the substrate. Colourless bacterial colonies on IPTG/Xgal-containing plates therefore likely

contain plasmid DNA which has an insert. Using a modification of Birnboim and Doily's method (1979), presumed transformant colonies were inoculated singly into 1.5 ml of **LB broth** (Appendix I). Briefly, following incubation overnight at 37°C, cells were pelleted, resuspended in 200 µl of **Lysozyme Solution** (Appendix I), incubated for 5 min at room temperature and lysed with 400 µl of 0.2 M NaOH, 1% SDS (w/v) for 5 min at room temperature. Three hundred µl of 7.5 M ammonium acetate were then added and samples were chilled on ice for 10 min prior to centrifugation at 13,000 rpm for 10 min at 4°C. Plasmid DNA was precipitated from the supernatant by the addition of 500 µl isopropanol and centrifugation for 15 min at 4°C. Plasmid DNA was digested with diagnostic restriction enzymes and subjected to electrophoresis in 1% agarose (w/v) to identify transformants with the desired inserts.

Larger scale plasmid DNA preparation:

Plasmid DNA free of RNA was prepared from 50-100 ml of transformed bacteria cultured overnight in **LB broth** (Appendix I) at 37°C using a commercial protocol and reagents (Qiagen) as per manufacturer's instructions.

2.4.3 In Vitro Transcription and Purification of RNA Probes and RNase Digestion.

The following protocol derives elements from both Hatada et al. (1989) and Ausubel (1989). Single-stranded hybridization probes (0.2-0.9 kb) of high specific activity were prepared for use in RNase protection assays by cleaving RNA-free plasmids to completion with a restriction enzyme generating a 5' overhang and

transcribing with bacteriophage RNA polymerase Sp6 or T7 to produce runoff transcripts of prescribed size. Transcription reaction components DTT (10 mM), 0.2 mM each of ATP, CTP and GTP, 2 U of human placental RNase inhibitor RNAguard (Pharmacia) and **Transcription Buffer** (Appendix I) and 10 µl of $\alpha[^{32}P]$-UTP (>3000 mCi/mmol) were assembled and warmed at 37°C for 2 min before addition of the template DNA to avoid precipitation of the template by spermidine. Approximately 500 ng of template DNA that had been digested, phenol-chloroform extracted, precipitated, washed (70% ethanol) and redissolved in RNase-free dH_2O was used in each transcription reaction along with 10 U of T7 or Sp6 RNA polymerase (Pharmacia) giving a final reaction volume of 20 µl. Reactions were incubated at 37°C for 1 hr followed by treatment with 10 U of RNase-free DNase I (Pharmacia) for 15 min at 37°C to remove the template DNA. Transcripts were extracted with phenol/chloroform/IAA (24:24:1) and precipitated in the presence of 20 µg of carrier yeast tRNA with ammonium acetate (NH_4OAC) (0.5 M final) and 75% ethanol. Pelleted probe RNA, obtained following centrifugation for 15 min at 4°C, was either gel-purified (see below) or reprecipitated twice with NH_4OAC and ethanol to remove unincorporated $\alpha[^{32}P]$ UTP. The final RNA pellet was washed with 75% ethanol/25% 0.1 M NaOAC (pH 5.2), dried and redissolved in 300 µl of **Hybridization Buffer** (Appendix I). For each RNase protection reaction 30 ul of this probe mixture were combined with 10 ug of ethanol precipitated, dried total cellular RNA or 1 µg in vitro-synthesized, unlabelled control transcripts, incubated for 5 min at 85°C prior to transfer to 37°C for 3 hr. Following RNA annealing, 270 µl

of ice-cold **RNase Digestion Buffer** (Appendix I) was added followed by incubation in the presence of RNase. Unprotected RNA was generally digested for 1 hr at 30°C with 40 µg/ml RNase A and 400 U of T1 RNase (Gibco-BRL) however, a range of enzyme treatments were tested (0-40 µg/ml RNase A; 100-700 U of RNase T1). Reactions were transferred to fresh 1.5 ml tubes and stopped by incubation in 0.5% SDS (w/v) and 50 µg Proteinase K for 30 min at 37°C. RNA products were extracted with an equal volume of phenol/chloroform/IAA (24:24:1), precipitated in the presence of 10 µg yeast tRNA with 70% ethanol for 60 min at -80°C and redissolved in 5 µl **Denaturing RNA Loading Buffer** (Appendix I). Samples were resolved by PAGE in 6% polyacrylamide containing 4 M urea (see section 2.3.1).

2.4.4 Gel purification of [^{32}P]-labelled probes:

RNA transcripts precipitated once in NH_4OAC-ethanol (see above) were resuspended in 10 µl of **Denaturing RNA Loading Buffer** (Appendix I), heated at 80°C for 5 min and resolved by electrophoresis through 6-8% PAGE-Urea (see 2.3.1) at 200 V x 5 hr. The wet gel was subjected to autoradiography for 1 min to locate the desired transcript which was then excised and eluted in 400 µl of 2 M NH_4OAC, 1% SDS (w/v) and 25 µg/ml tRNA for 2 hr at 37°C. The probe was precipitated in 70% ethanol and redissolved in 150 µl **Hybridization Buffer** (Appendix I). A negative control consisting of yeast tRNA and radioactive probe was always performed to identify any nonspecific protected products.

2.5 RT-PCR

Total infected cellular RNA (2 µg) or 10% of the yield of virion RNA from a

100 mm plate were mixed with 100 ng of a segment-specific oligonucleotide primer targetted to one of vRNA, cRNA or mRNA (Table 1). The samples were reverse transcribed with dNTP (1 mM each), 10 mM DTT, 0.5 U/µl human placental RNase-inhibiting RNAguard (Pharmacia), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 200 U of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco-BRL) in a final volume of 40 µl for 60 min at 37°C.

Second strand synthesis and PCR amplification were carried out on 2 µl of the first strand synthesis product in the presence of segment-specific forward and reverse primers (Table 2), one or both of which were end-labelled (see section 2.3) with $\gamma[^{32}P]$-ATP using T_4 polynucleotide kinase (Pharmacia) and purified by chromatography through G-50 Sephadex (Pharmacia). The PCR reactions included dNTPs (250 uM each), 75 mM Tris-HCl (pH 8.8), 20 mM $(NH4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$ and 0.1 % NP40 (BDH) in a final volume of 100 µl. The samples were heat denatured prior to addition of 1 U Ultratherm DNA polymerase (Bio/Can) and subjected to 5-35 cycles (generally 20) of 94°C, 30 s; 37-42°C, 30 s; 72°C, 2 min followed by incubation at 72°C for 10 min. Products were digested with either Ava II (segments 2 and 5) or Hind III/BspD I (segment 7) overnight at 37°C as specified by manufacturers to generate strain-specific products. Digested products were resolved by nondenaturing SDS-PAGE. Specifically, electrophoresis samples were loaded in 62.5 mM Tris-HCl (pH 6.8), 10 % glycerol (v/v), 2% SDS (w/v), 10 ug/ml Bromophenol Blue and 5% 2-mercaptoethanol onto gels prepared according to Laemmli (1970). These consisted of a 4.5-7% acrylamide separating

phase containing 375 mM Tris-HCl (pH 8.9), 0.4% SDS (w/v), 0.05% AP (w/v) and 0.05 TEMED (w/v) and a 3% acrylamide stacking gel in 125 mM Tris-HCl (pH 6.8), 0.1% SDS (w/v), 0.1% AP (w/v) and 0.1% TEMED (v/v). Electrophoresis was performed at 150 V for 8 hr in 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 0.1% SDS at room temperature. Gel bands were excised and counted in a scintillation counter following fixing, drying and autoradiography.

2.6 [^{32}P]-Labelling of vRNA

MDCK monolayers were singly-infected (MOI=3) or coinfected (MOI=3, each virus). Following adsorption for 1 hour at 37°C, cells were washed twice with warmed normal saline (50 mM Tris, 150 mM NaCl) prior to incubation for 20 hr in 1-2 ml of phosphate-free Eagle's MEM (ICN) supplemented with 1 mCi/ml [^{32}P]-phosphoric acid (Amersham) and 1 µg/ml trypsin. Released virus was purified from culture supernatant by hemadsorption to human red blood cells (Cowley, 1984 as described in 2.2.3). [^{32}P]-labelled vRNA was extracted (Chomczynski and Sacchi, 1987, with modifications described in 2.2.2) and resolved by SDS-PAGE-urea (see 2.3.1) using acrylamide concentrations of 3-4.5% in the separating phase and 3.75% in the stacking phase (Laemmli, 1970).

2.7 In Vitro RNA Synthesis From Viral Ribonucleoprotein (RNP)

2.7.1 Isolation of RNP

Enzymatically active RNP was isolated from infected MDCK cells in a modification of a procedure described by Albo et al. (1992). Briefly, MDCK cells (typically 2 x 10^8 cells) were infected with virus (MOI=1) for 16 hr in MEM

supplemented with 0.2% BSA. The cells were washed twice in **Solution A** [10 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl] at room temperature prior to lysis in **Solution A** containing 1% NP40 (1 ml lysis solution per 100 mm dish). Samples were diluted to 20 ml in **Solution A** and layered onto 10 ml of **Solution A** containing 15% sucrose prior to centrifugation (22,000 rpm x 18 hr at 22°C). Pelleted RNP was dissolved in **HB Buffer** (Appendix I). In some cases the preparation was then fractionated by centrifugation on a discontinuous 30-50-70% glycerol (v/v) gradient containing 100 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT in a Beckman SW50.1 rotor at 45,000 rpm for 5 hr at 4°C. Fractions were collected from the bottom of the gradient and aliquots were analyzed by nondenaturing SDS-PAGE (as in 2.4) using a 10% acrylamide (w/v) concentration in the separating phase and 3.75% acrylamide (w/v) in the stacking phase. Electrophoresis was performed at 100 V for 3 hr followed by 200 V for 2 hr in 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 0.1% SDS at room temperature.

2.7.2 Silver Staining to Identify Peak RNP-Containing Gradient Fractions.

Proteins in SDS polyacrylamide gels were identified by silver staining essentially as described by Merril (1982) with some modifications to incubation times. Gels were fixed in 40% methanol/10% acetic acid (v/v) for 30 min followed by 10% ethanol/5% acetic acid (v/v) for 30 min. Proteins were oxidized in 0.34 mM potassium dichromate, 0.3 mM nitric acid for 5 min and washed with dH_2O (3 x 5 min) prior to treatment with 1.2 mM silver nitrate for 20 min, washing with dH_2O and colour development in 280 mM sodium carbonate/0.0017% paraformaldehyde (v/v).

2.7.3 RNA Synthesis In Vitro

RNP isolated from MDCK cells was used in in vitro transcription reactions. RNA synthesis was carried out at 30°C for 1 hr using approximately 50-100 µg of protein in 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 50 mM KCl, 10 mm NaCl, 1 mM DTT, 0.1% NP40 and 0.5 mM each of ATP, CTP and GTP, 50 µM $\alpha[^{32}P]$UTP (Amersham; 3000 Ci/mmol), 20 U of human placental RNAguard RNase inhibitor (Pharmacia) and 350 µM ApG as primer in a final reaction volume of 20 µl. Reactions were stopped with the addition of 150 µl 50 mM NaOAc (pH 5.2)/50 mM EDTA, extracted with an equal volume of phenol:chloroform (1:1) and precipitated in 2 volumes of ethanol (1 hr -80°C). RNA was recovered by centrifugation (13,000 rpm x 15 min) and resuspended in 20 µl RNase-free dH_2O. Samples were spotted onto 3M Whatman (2-10 µl) and air-dried. Filters were washed (3 x 200 ml) in ice-cold 10% Trichloroacetic acid (TCA) followed by 2 washes (2 x 200 ml) of 70% ethanol. Air-dried filters were placed in scintillation vials containing 10 ml dH_2O prior to measuring radioactivity. Samples (10 µl) of extracted in vitro transcription reactions were also resolved by partially-denaturing SDS-PAGE (section 2.3.1).

2.8 Detection of Viral Proteins

2.8.1. Pulse-labelling of Proteins

Infected (MOI=3) MDCK monolayers (100 mm dish) were pulse labelled for 1 hr at various times post-infection by the addition of 1-2 ml of methionine/cysteine-free DMEM (ICN) containing 40 uCi/ml $[^{35}S]$met, $[^{35}S]$cys (New England Nuclear). Infected cell monolayers and released virions harvested by hemadsorption (Cowley,

1984) were lysed in 1 ml of 1X **Sample Buffer** (Appendix I) and subjected to SDS-PAGE (see 2.4) through gels prepared with 12.5% acrylamide (w/v) in the separating phase and 3% acrylamide (w/v) in the stacking phase. Gels were either fixed, dried and used for autoradiography or were transferred by electroblotting (see 2.3.1) to Biodyne (Pall) in 48 mM Tris, 39 mM glycine, 20 % methanol and 0.037% SDS (pH 8.3) at 25 V for 16 hr at 4°C.

2.8.2. Western Blot Analysis

Western blot analysis was performed essentially as described (Sambrook et al., 1989, pp. 18.60-18.75) on air-dried membranes. Blocking consisted of a 1 hr incubation at room temperature in 5% nonfat dried milk (w/v) in **PBS** (Appendix I). Primary antibody incubation was performed at 4°C for 2 hr with shaking using a combination of anti-H1N1 and anti-H3N2 polyclonal antibody (1/125 dilution of each). This was followed by extensive washing in PBS (3 x 250 ml, 10 min each) and once in 150 mm NaCl, 50 mM Tris-HCl (pH 7.5) before incubation with protein A conjugated with alkaline phosphatase [1/1000 dilution of 1 mg/ml stock of protein A-alkaline phosphate conjugate (Sigma) in 150 mM NaCl, 50 mM Tris (pH 7.5) containing 2% milk (w/v)] for 1 hr at room temperature. Membranes were washed [4 x 10 min at room temperature in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5)] prior to colour development with 0.0165% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.033% nitroblue tetrazolium (NBT) in 100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl (pH 9.5).

2.9 Statistical Analysis

Where replicate experiments were performed, data were presented as the mean value plus or minus 1 standard deviation. Standard deviation (S) was calculated using the following formula:

$$s^2 = \frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n - 1}$$

RESULTS

The specific goal of this study was to determine how FM-MA interferes with a prototype virus, HK, in mixed infections. FM-MA's interference phenotype was originally observed as a selective (>99%) release of FM-MA infectious progeny virus from MDCK cells coinfected with HK and FM-MA (Brown et al., 1992). Little mechanistic information could be deduced from this observation- only that the inhibition of HK growth by FM-MA occurred at some point during or prior to viral release. In order to identify the critical stage(s) at which FM-MA interferes with HK, it was appropriate to describe the interference system at the macromolecular level by measuring levels of mutant and wild-type RNA and protein in cells coinfected with the two viruses. The potential role of viral assembly in FM-MA's interference phenotype was also examined by detecting levels of exported genomes. The nature of the mutation in FM-MA responsible for interference- an altered PB1 polymerase protein- directed me first to look at transcription and replication in the coinfected cell with two specific goals: A) To determine the relative levels of all 3 classes of FM-MA and HK RNA. B) To detect any interaction of FM-MA and HK leading to a suppression of HK RNA production.

In a cell coinfected with FM-MA and HK, each virus contributes 8 different genome segments and each segment specifies 3 classes of RNA (cRNA, mRNA and vRNA). The technical challenge of a thorough documentation of RNA expression for two coinfecting genomes was two-fold:

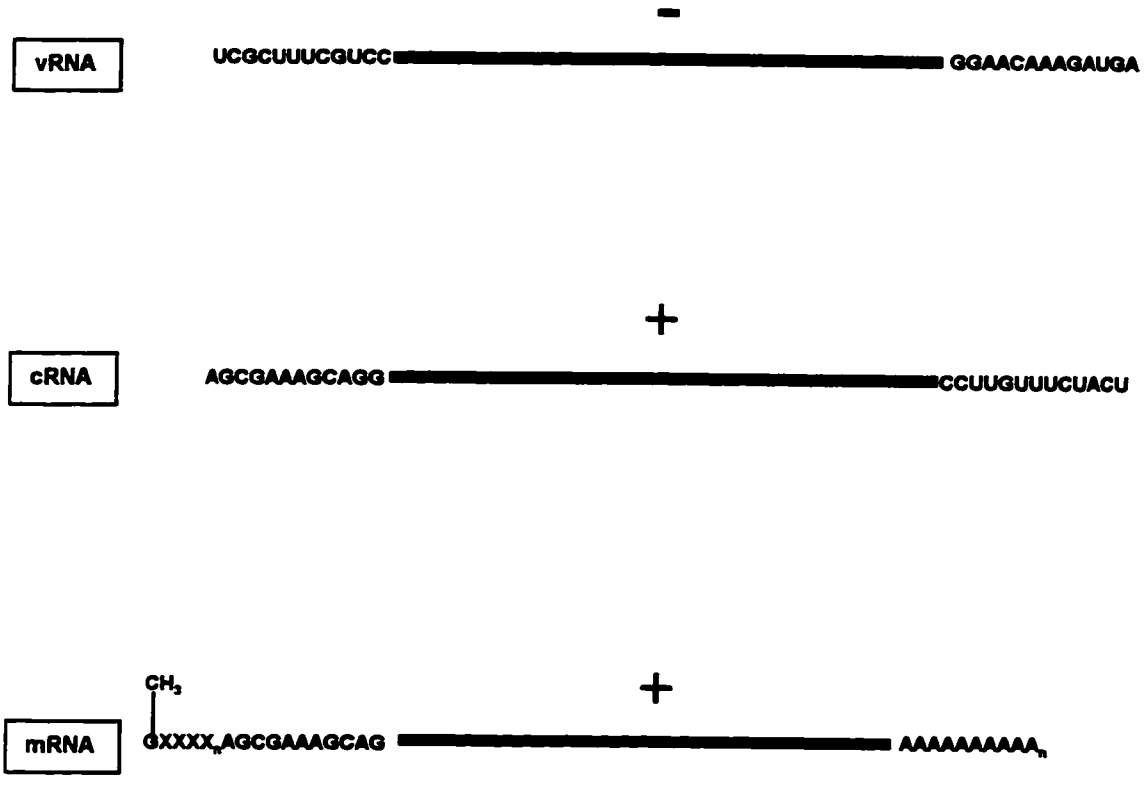
1. Discriminate the 3 RNA classes for one segment of a given viral strain. vRNA is distinguishable by its negative polarity, however cRNA and mRNA are both plus-stranded and resemble each other closely (Fig. 1). Messenger RNA possesses a 5' cap attached to a stretch of approximately a dozen heterologous nt specified by the host and it falls short of the full length of the vRNA segment by terminating at the polyadenylation site 17-22 nt from the 5' end of the vRNA (see section 1.8.2). By contrast, cRNA represents a faithful, full-length copy of the vRNA. Differentiation between cRNA and mRNA expressed from a particular genome segment must therefore target the 5' or 3' end.

2. Differentiate between HK and FM-MA RNA molecules expressed from a given genome segment. Sequence diversity between strains can be exploited to permit this. There exists as little as 5.4% (segment 7) or as much as 41% (segment 4) sequence diversity between HK and FM-MA genes.

3.1 Approach #1 to viral RNA detection and quantification: RNase protection.

An initial attempt to detect and quantify viral RNA depended on RNase protection, a technique proven useful in distinguishing similar RNA molecules. In this approach, a radioactive RNA probe representing a portion of a single FM-MA (or HK) gene was transcribed from plasmid by a bacteriophage polymerase. The probe was hybridized in vitro to total cellular RNA from MDCK cells infected with HK and/or FM-MA. Homologous hybrids were formed between FM-MA probe and FM-MA RNA in the target sample. Heterologous hybrids were generated between FM-MA probe and HK RNA molecules. Single-stranded RNA constituting regions of

Fig. 1. Three RNA classes (vRNA, cRNA and mRNA) produced from each influenza virus genome segment.

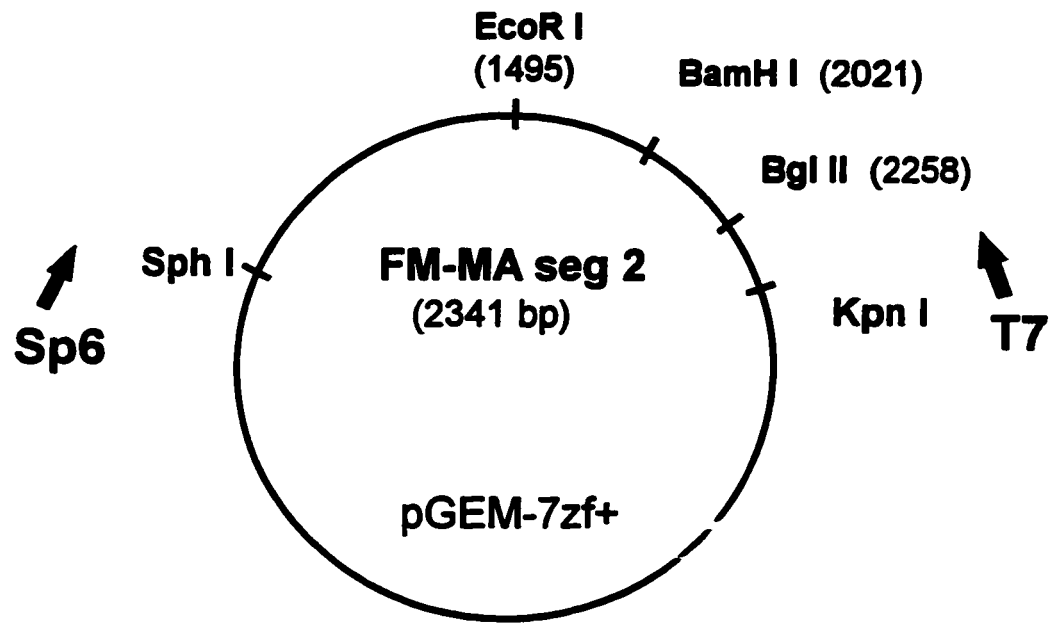

-
vRNA
UCGCUUUCGUCC
GGAACAAAGAUGA
+
cRNA
AGCGAAAGCAGG
CCUUGUUUCUACU
+
CH3
mRNA
GXXXXnAGCGAAAGCAG
AAAAAAAAAn

nonhomology in heterologous hybridizations were digested by single-stranded RNA-specific endonucleases RNase T1 (cleaves 3' to a guanosine) and RNase A (cleaves 3' to a pyrimidine). FM-MA cellular transcripts were represented by undegraded probe whereas probe hybridized to HK RNA was digested. Degraded and undegraded products were distinguished by their differential migration in partially-denaturing PAGE. When an excess of radioactive probe was used, the quantity of protected probe accurately reflected the concentration of target RNA in the sample. Since target RNA sequences diverging from probe sequence by as little as one nucleotide can be degraded, this approach can, in theory, be used to quantify the RNA levels of all 8 FM-MA (or HK) genes by preparing 8 different probes for each strain and targetting the characteristic features of each class as outlined above. The technique also permits differentiation between RNA species of a given strain. Probes of either polarity can be generated by virtue of distinct bacteriophage promoters, Sp6 and T7, flanking the cloned viral sequences. Therefore (+) sense probes can be transcribed to target vRNA. If the (-) polarity transcripts contain sequences representing the 3' end of the gene where cRNA and mRNA sequences diverge, they can be used to differentiate between these species for the probe strain. Because it falls short of the full length of the gene, mRNA will protect a probe sequence that is shorter than cRNA-protected RNA by 15 (segments 2 and 6) or 16 (segments 1,3,4,5,7 and 8) nt.

In vitro-synthesized HK and FM-MA RNA molecules were distinguished using RNase protection.

The potential of RNase protection to discriminate HK and FM-MA target RNA molecules was first tested using a fully in vitro system. A [^{32}P]-labelled, 891 nt probe of (-) polarity was generated by T7 polymerase transcription of an Eco RI-digested FM-MA segment 2 subclone (Fig. 2). Unlabelled target RNAs of 2325 nt were transcribed from Bgl II-digested FM-MA (Fig. 2) or HK segment 2 subclones by Sp6. FM-MA and HK nt sequence diverge by 17% in this transcribed stretch. Hybridization of the FM-MA-specific probe with strain-specific transcripts was therefore expected to yield a protected fragment of 763 nt only in the case of FM-MA target RNAs. Following RNase T1 and RNase A digestion and PAGE analysis (Fig. 3) of the products, homologous hybridizations (FM-MA probe/FM-MA target) were found to produce the expected 763 nt fragment (lanes 2 and 3) while heterologous hybridizations (FM-MA probe/HK target) resulted in probe degradation following RNase digestion (lane 4), as anticipated. Undigested probe was included in the PAGE analysis as a size standard (lane 1). Because yeast tRNA was used as a carrier to facilitate product recovery following RNase treatment, a control assay was performed using yeast tRNA alone as target to identify any nonspecific products (lane 5). Protected probe was not recovered from this sample, indicating that the hybridization signals in test samples represented viral RNAs. This experiment demonstrated the ability of RNase protection to discriminate FM-MA and HK RNAs. Based on the specific activity of the [^{32}P]UTP and the U content in the

Fig. 2. FM-MA segment 2 subclone in pGEM-7Zf+. FM-MA segment 2 DNA representing the entire PB1 gene (2341 bp) was subcloned into Sph I/Kpn I-digested pGEM-7Zf+. The plasmid was linearized in single-enzyme digestions with EcoR I, BamH I or Bgl II. Numbers on plasmid map refer to the nt position in segment 2 sequence relative to position 1 of mRNA. The direction of transcription by Sp6 or T7 polymerases is indicated with an arrow. Sp6 transcription of Bgl II-digested plasmid generated a 2325 nt (+) sense RNA. T7 transcription of EcoR I or BamH I-digested plasmid produced (-) sense RNAs of 891 and 365 nt, respectively, as shown below the plasmid map.


EcoR I
(1495)
BamH I (2021)
Bgl II (2258)
Sph I
FM-MA seg 2
(2341 bp)
Kpn I
Sp6
T7
pGEM-7zf+

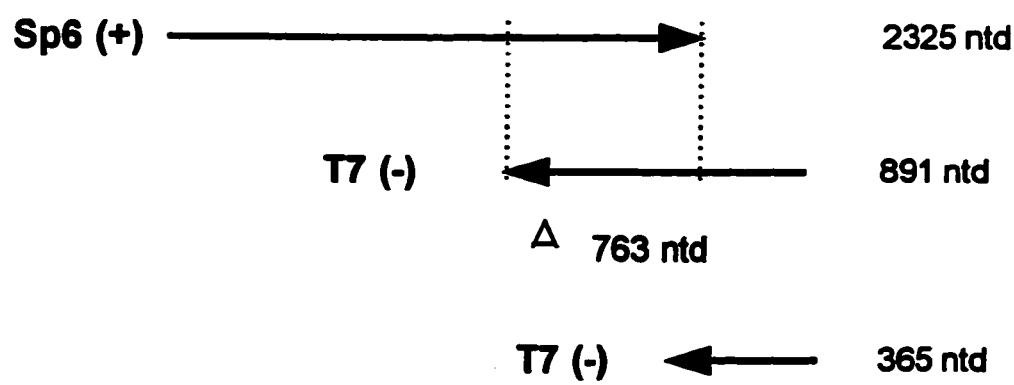
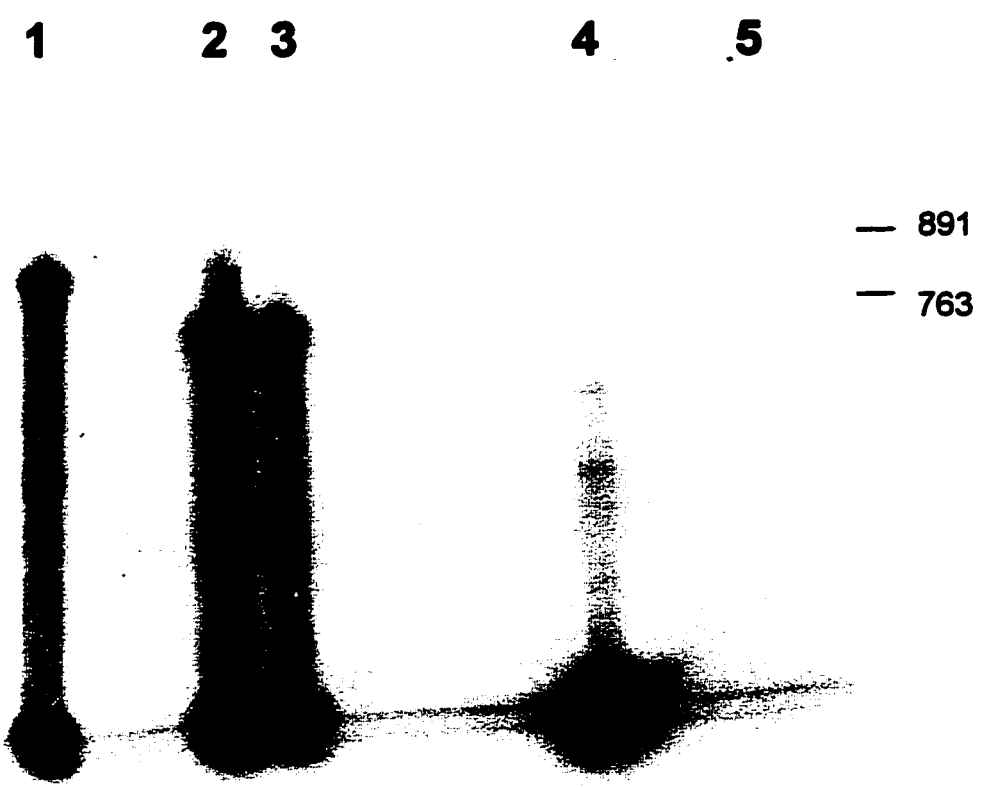

Sp6 (+)
2325 ntd
T7 (-)
891 ntd
Δ 763 ntd
T7 (-)
365 ntd

Fig. 3. PAGE analysis of RNase protection products generated with a FM-MA segment 2 probe and FM-MA segment 2 target transcripts generated in vitro. A 891 nt [^{32}P]-labelled probe of (-) sense was generated by T7 transcription of a FM-MA segment 2 subclone (shown in **Fig. 2**) which had been linearized with EcoR I. The probe was hybridized with unlabelled (+) sense target transcripts of 2325 nt generated by Sp6 transcription of FM-MA (**Fig. 2**) or HK segment 2 subclones linearized with Bgl II. Following digestion with RNases T1 and A, products resulting from hybridizations between FM-MA probe and FM-MA target transcripts (lanes 2 and 3) or FM-MA probe and HK target transcripts (lane 4) or probe in the absence of viral target (lane 5) were resolved by PAGE in 6% acrylamide containing 4M urea and subjected to autoradiography. Undigested probe was included in the PAGE analysis as a size standard (lane 1). Product sizes are indicated (in nt) at right.


1
2
3
4
5
891
763

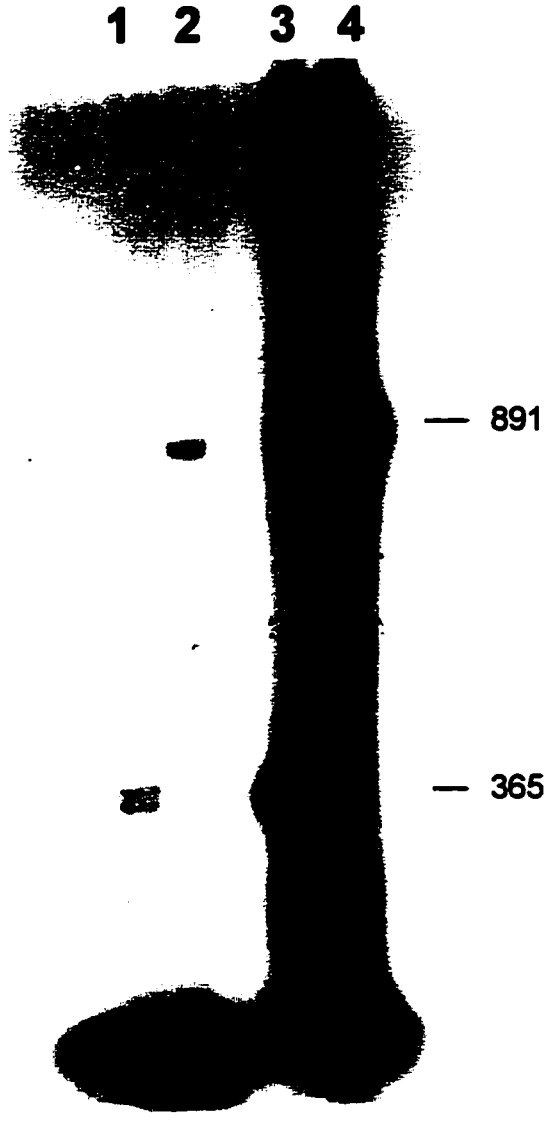
protected product, the number of RNA hybrids and hence the amount of FM-MA RNA in the target sample could be measured. The amount of HK RNA could likewise be quantified using HK-specific probes.

RNA molecules of different classes (cRNA and mRNA) in a context of total infected cellular RNA were distinguished by RNase protection.

The potential of RNase protection to identify virus RNAs generated in vivo in the more complex target sample of infected cellular RNA was assessed next (Fig. 4). Two FM-MA-specific (-) sense probes of 365 nt (lane 3) or 891 nt (lane 4) were first prepared by T7 transcription of segment 2-containing plasmid linearized with BamH I or EcoR I, respectively (Fig. 2). Because these probes were complementary to the 3' end of the gene and included sequence represented in cRNA but not mRNA, annealing of either probe with total RNA from cells infected singly with FM-MA followed by RNase digestion was expected to produce 2 protected fragments differing in length by 15 nt and representing the 2 (+) sense RNA species. Protected products of the 365 nt probe should be 320 and 305 nt; RNase protection with the 891 nt probe should yield products of 846 and 831 nt.

RNase protection was performed using the 2 probes individually in hybridizations with total cellular RNA from MDCK cells infected for 2 hr with FM-MA. Products which resolved on PAGE analysis as 2 closely-migrating bands of expected size were produced in assays using either the 365 nt (Fig. 4 lane 1) or 891 nt (lane 2) probe. In each case, the double signal was presumed to represent cRNA (upper band) and mRNA (lower band) in the target sample. Samples of

Fig. 4. PAGE analysis of the products of RNase protection of total RNA from FM-MA infected cells with FM-MA segment 2 transcripts. [^{32}P]-labelled (-) sense transcripts of 365 and 891 nt were generated by T7 transcription of a FM-MA segment 2 subclone (**Fig. 2**) linearized with either BamH I or EcoR I, respectively. Labelled probes were hybridized individually with total RNA isolated from MDCK cells infected 2 hr with FM-MA (MOI=5). Following digestion with RNases T1 and A, products resulting from hybridization with the 365 nt (lane 1) and 891 nt (lane 2) probes were resolved by PAGE in 6% acrylamide containing 4M urea and subjected to autoradiography. Undigested 365 nt (lane 3) and 891 nt (lane 4) probes were included in the PAGE analysis as size standards. Product sizes (in nt) are indicated at right.


1 2 3 4
891
365

undigested 365 nt (lane 3) and 891 nt (lane 4) probe were included on the gel. It was concluded that RNase protection was useful in distinguishing viral RNAs of different classes present in a total infected cellular RNA sample.

HK and FM-MA RNA were not distinguishable in a context of infected cellular RNA by RNAse protection.

The ability of RNase protection to differentiate HK and FM-MA RNA molecules in a sample of total infected cellular RNA was assessed next. The (-) polarity FM-MA segment 2 probe of 365 nt (Fig. 2) was hybridized with total RNA isolated from cells infected singly for 2 hr with HK or FM-MA. Using previously-established conditions of RNase digestion, this probe was found to protect both HK and FM-MA (+) sense RNA (data not shown). Increasing the concentration of RNase (A or T1) resulted in the degradation of probe involved in either homologous or heterologous hybridizations. Further attempts to optimize hybridization conditions (temperature, formamide and salt) did not allow differential detection of HK versus FM-MA RNA. It was concluded that RNase protection was an unsuitable approach to the measurement of HK and FM-MA RNA during interference since this objective absolutely requires strain-specific detection.

3.2 Approach #2 to viral RNA detection and quantification: Northern blot analysis

FM-MA and HK RNA from infected MDCK cells are differentiated by Northern blot analysis.

The second approach to virus-specific RNA detection was Northern blotting

of total infected cellular RNA followed by probing with strain-specific oligonucleotides. MDCK cells were infected singly with HK, singly with FM-MA or coinfected with the 2 viruses for 1, 2, 4 or 6 hr. Twenty µg of total infected cellular RNA for each sample were resolved by partially-denaturing PAGE. The RNA was transferred to a biodyne membrane and probed with a 18 nt probe 100% or 67% homologous to HK and FM-MA segment 4 vRNA, respectively (Fig. 5). Under appropriate hybridization conditions (42°C, 2 hr), the probe detected HK vRNA (lanes 1, 4, 7 and 10) but not FM-MA vRNA (lanes 2, 5, 8 and 11). Since the probe demonstrated virus-specific hybridization, it was concluded that probe annealing to total RNA from cells infected with both HK and FM-MA (lanes 3, 6, 8 and 12) represented only HK RNA.

Oligonucleotide probes complementary to FM-MA segments 4 or 7 vRNA also showed selective hybridization (Fig. 6) in annealing to total RNA from cells infected singly with FM-MA (lanes 2, 5, 8 and 11) but not HK (lanes 1, 4, 7 and 10). Hybridization signals in coinfected samples (lanes 3, 6, 9 and 12) were therefore presumed to specifically represent FM-MA vRNA. The FM-MA-specific probes used in this experiment targetted regions of nonhomology between strains, demonstrating 67% (segment 4) or 78% (segment 7) complementarity with HK sequence.

Since the goal of these experiments was not only to detect but also to measure levels of HK and FM-MA RNA during interference (coinfection), the hybridization signals in coinfected samples were quantified by densitometry. As each oligonucleotide possesses sequence-dependent hybridization kinetics, signal

Fig. 5. Northern blot analysis of infected cellular RNA showing HK segment 4 vRNA levels. Total RNA was isolated from MDCK cells infected singly (MOI=5) with HK (lanes 1, 4, 7, 10), singly (MOI=5) with FM-MA (lanes 2, 5, 8, 11) or coinfected (MOI=3, each virus) with both viruses (lanes 3, 6, 9, 12) for 1, 2, 4 or 6 hr. RNA samples (20 μg) were resolved by PAGE in 4.5% acrylamide containing 4M urea prior to electroblotting, probing with a [^{32}P]-labelled HK segment 4-specific oligonucleotide and autoradiography. Duration of the infection (in hr) is indicated at the top of the autoradiograph. The location of segment 4-specific and 18S ribosomal RNA bands is indicated at right.

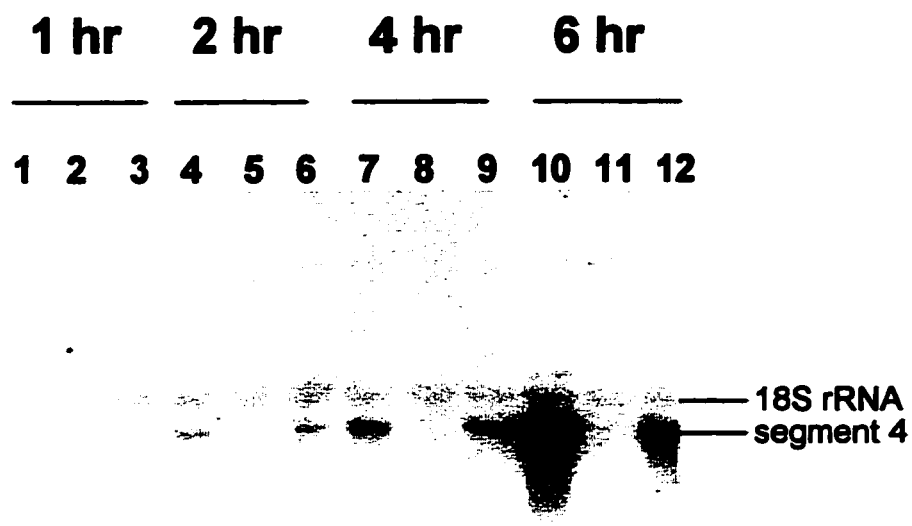
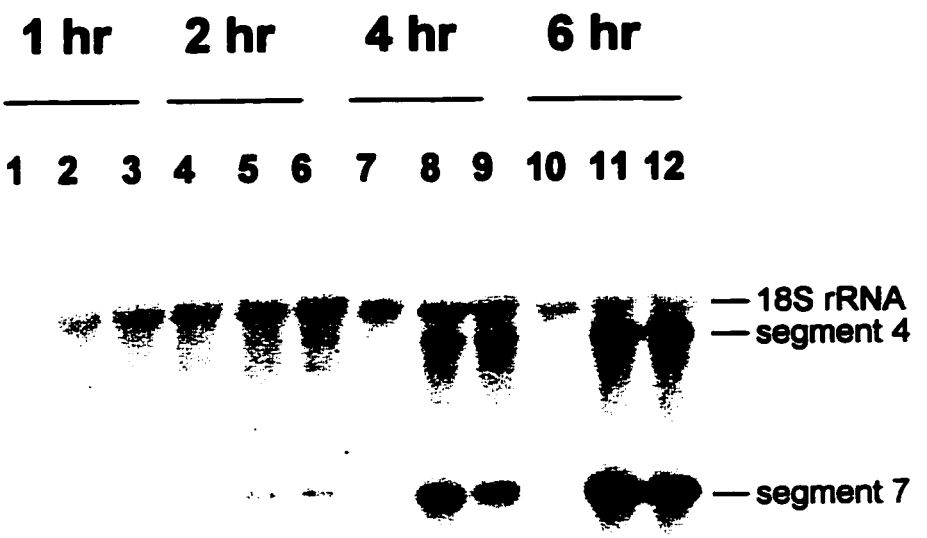

1 hr
2 hr
4 hr
6 hr
1 2 3 4 5 6 7 8 9 10 11 12
18S rRNA
segment 4

Fig. 6. Northern blot analysis of infected cellular RNA showing FM-MA segment 4 and 7 vRNA levels. Total RNA was isolated from MDCK cells infected singly (MOI=5) with HK (lanes 1, 4, 7, 10), singly (MOI=5) with FM-MA (lanes 2, 5, 8, 11) or coinfected (MOI=3 each) with both viruses (3, 6, 9, 12) for 1, 2, 4 or 6 hr. RNA samples (20 μg) were resolved by PAGE in 4.5% acrylamide containing 4M urea prior to electroblotting, probing with [^{32}P]-labelled FM-MA segment 4 or segment 7-specific oligonucleotides and autoradiography. Duration of the infection (in hr) is indicated at the top of the autoradiograph. The location of segment 4 and 7-specific and 18S ribosomal RNA bands is indicated at right.


1 hr
2 hr
4 hr
6 hr
1 2 3 4 5 6 7 8 9 10 11 12
18S rRNA
segment 4
segment 7

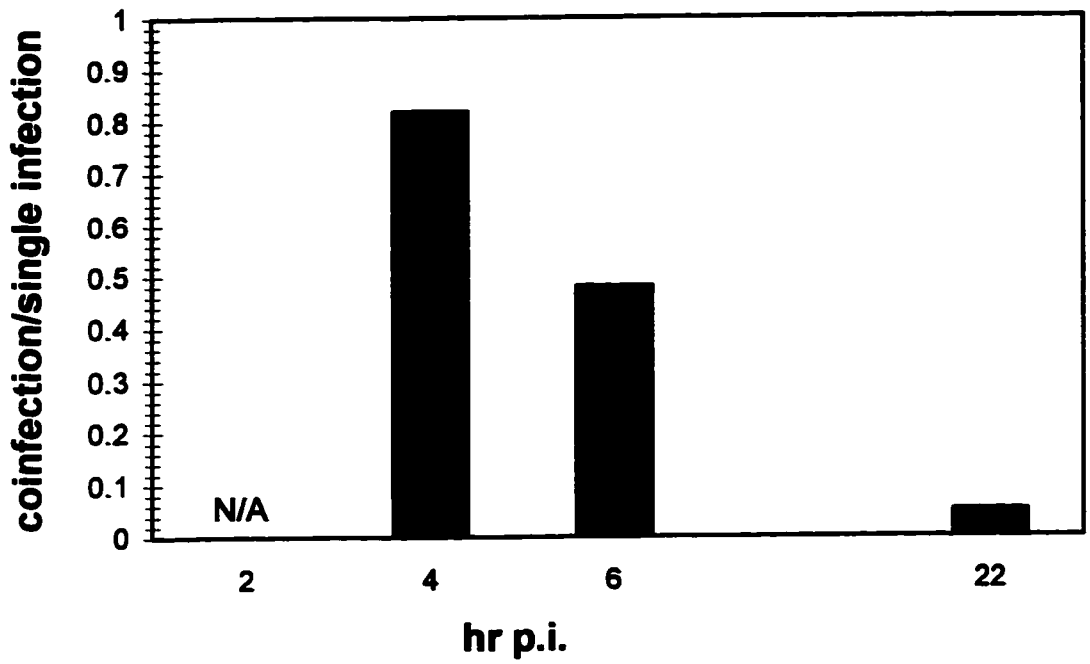
intensities produced by HK and FM-MA-specific probes could not be directly compared. However, signals generated by probings of different RNA samples with the same oligonucleotide (assuming identical conditions of hybridization and autoradiography) could be legitimately compared. The virus-specific hybridization signal in a coinfected sample was therefore quantified by densitometry and expressed relative to the densitometric signal in the corresponding singly-infected sample for the same timepoint. For example, HK vRNA status at 1 hr p.i. in coinfected cells was expressed as a ratio of the densitometric values of the hybridization signals in coinfected vs HK-infected samples. A value of 1 indicated that HK RNA levels were identical in coinfected vs singly-infected cells. A value of less than 1 indicated that HK RNA levels in the coinfected cells were (relatively) repressed. Using this approach, interference of HK RNA expression by FM-MA during mixed infections could be detected as diminished HK RNA levels in the presence of a coinfecting FM-MA as compared to levels in cells infected only with HK.

HK segment 4 vRNA levels in cells coinfected with FM-MA were repressed relative to levels in singly-infected cells at 4 hr p.i. (Fig. 7A). This repression increased over the course of infection. By 22 hr p.i. HK segment 4 vRNA levels in coinfected cells were 5% of the levels in singly-infected cells. This trend was also seen for HK segment 4 (+) sense RNA (data not shown) where, in cells coinfected with FM-MA, HK segment 4 cRNA + mRNA levels were 20% of the level seen in the HK-infected cell by 22 hr p.i..

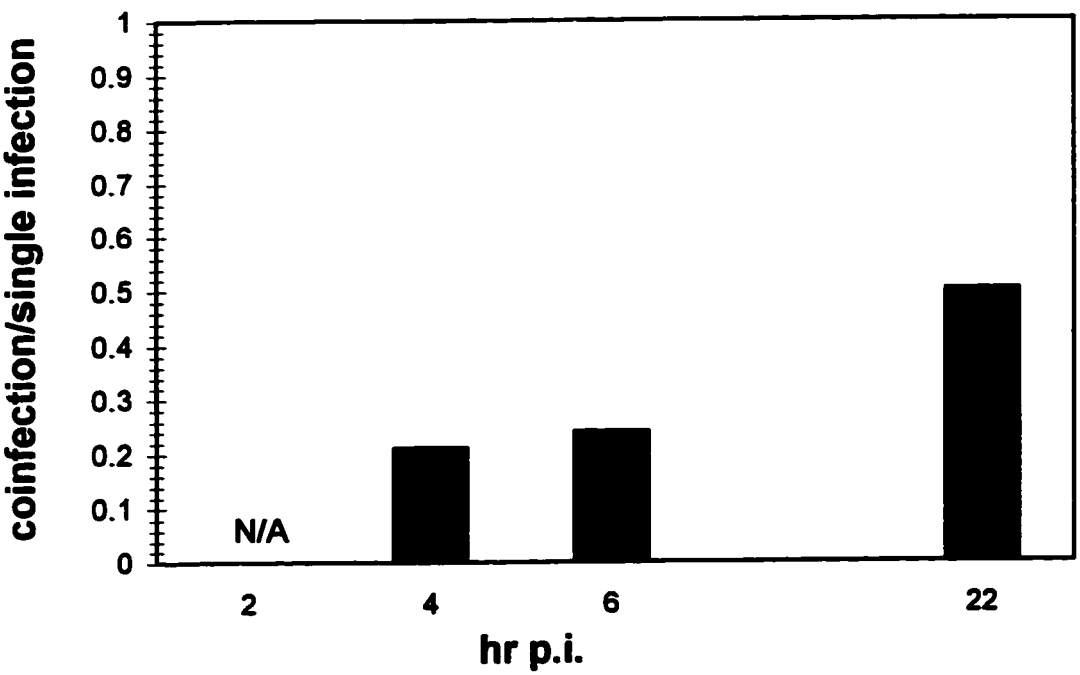
Fig. 7A. Effect of FM-MA on HK segment 4 vRNA levels in coinfected cells. HK vRNA in cells infected singly with HK (MOI=5) or coinfected with HK and FM-MA (MOI=3 each virus) was quantified by densitometric analysis of a Northern blot probed with a HK segment 4 vRNA-specific oligonucleotide. Relative levels of HK segment 4 vRNA in coinfected vs singly-infected cells were expressed as a ratio of the densitometric measurements for each timepoint.

B. Effect of FM on HK segment 4 vRNA in coinfected cells. HK vRNA in cells infected singly with HK or coinfected with HK and FM was quantified by densitometric analysis of a Northern blot probed with a HK segment 4 vRNA-specific oligonucleotide. Relative levels of HK segment 4 vRNA in coinfected vs singly-infected cells were expressed as a ratio of the densitometric measurements for each timepoint. Densitometric signals in RNA samples at 2 hr p.i. were not in the linear range and were therefore not included in the analysis (N/A).

A


coinfection/single infection
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N/A
2
4
6
22
hr p.i.

B


coinfection/single infection
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N/A
2
4
6
22
hr p.i.

HK RNA levels were clearly repressed due to the presence of FM-MA. To determine if this repression was specifically a manifestation of FM-MA's interference phenotype, a similar comparison was made between HK vRNA levels in cells coinfected with the non-interfering parent, FM, and levels in cells infected with HK alone (Fig. 7B). In the presence of FM, HK segment 4 vRNA levels at 4 hr p.i. were 25% of their value in cells infected with HK alone. This suggests that HK RNA levels were adversely affected by the presence of FM. However, unlike the trend seen in mixed infections with HK and FM-MA, in cells coinfected with FM, HK vRNA levels showed less repression at later times in infection. By 22 hr p.i., HK segment 4 vRNA levels in cells coinfected with FM were 60% of their level in cells infected with HK alone. HK (+) sense RNA showed a similar trend in mixed infection with FM (data not shown); HK segment 4 cRNA + mRNA levels in the presence of FM were 30% of their value in cells infected with HK alone at 4 hr p.i.. However, by 22 hr p.i., HK (+) sense RNA was only slightly repressed by the presence of FM, demonstrating 90% of the level seen in singly-infected cells.

It was concluded that HK RNA (both + and - sense) were repressed in cells coinfected with FM-MA relative to levels in singly-infected cells and that this repression increased with time. HK RNA (+ and -) levels were also decreased in cells coinfected with the non-interfering parent, FM, relative to levels in cells infected with HK alone but demonstrated a recovery from repression over the course of infection. These trends were also observed for HK segments 2 and 7 (data not shown).

It should be noted that to make a legitimate comparison of HK RNA levels in coinfected vs singly-infected cells, the samples being compared must contain the same amount of total cellular RNA. In the experiments described above, RNA content in each sample was measured spectrophotometrically. Aliquots of equivalent total RNA subjected to PAGE were stained with ethidium bromide prior to Northern blot analysis for a visual comparison of RNA content in rRNA bands. A more accurate standardization would have been provided by hybridization of each Northern blot with a probe detecting a cellular transcript such as catalase or phosphoglycerate kinase which, when analyzed by densitometry, would allow a numerical comparison of samples and assure equivalent RNA content between samples.

The disadvantages of this approach for the description of RNA events during interference were twofold. First, identification of the precise impact of FM-MA on HK RNA expression during coinfection is difficult since the presence of either FM or FM-MA appeared to inhibit HK RNA accumulation relative to its abundance in singly-infected cells. This is likely due to the relative infectious doses given in singly-infected vs coinfected cell experiments. An MOI of 5 was used in single infections, while cells coinfected with 2 viruses received an MOI of 3 for each virus. This makes a direct comparison of viral RNA levels in coinfected vs singly-infected samples difficult. However, the contrasting trends of increasing HK RNA "suppression" (defined in relation to single-infection data) over time in the presence of FM-MA versus a decrease in HK RNA suppression over the course of coinfection

with the noninterfering parent, FM, could be cited as evidence of FM-MA's interference phenotype. To avoid relying on a comparison of coinfected to singly-infected RNA levels to identify trends, the absolute amount of HK RNA in the coinfected cells could have been determined by comparing autoradiographic signals in coinfected RNA samples with those obtained from predetermined quantities of purified virion RNA. Absolute amounts of HK RNA (+ and -) could have been monitored over the course of coinfection with FM-MA. These values could then be compared directly to both HK RNA levels over the course of coinfection with FM and, importantly, to a profile of FM-MA RNA levels (measured in the same manner) during coinfection with HK.

The second limitation of this Northern analysis approach is that the cRNA and mRNA molecules for a given segment could not be distinguished. mRNA and cRNA differ at their 5' and 3' ends and the resultant difference in their length (50-150 nt) should translate to differential migration in PAGE. Although many electrophoresis conditions were tested (% acrylamide, buffer composition, duration and voltage of electrophoresis), a difference in mobility of sufficient magnitude to allow RNA class-specific quantification was not achieved.

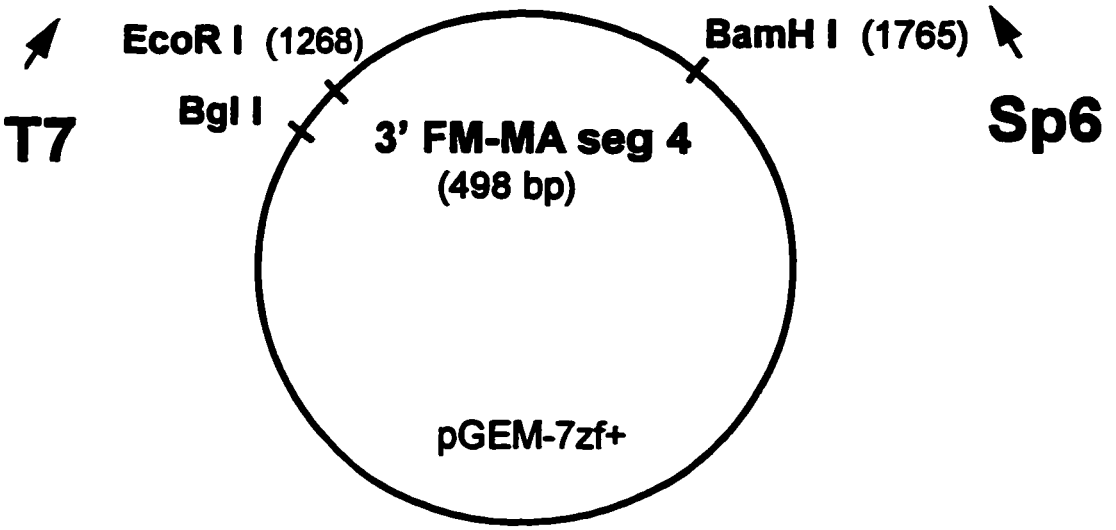
RNase H cleavage of viral RNA to improve resolution of cRNA and mRNA in Northern blot analysis.

In an attempt to better detect the mobility difference between cRNA and mRNA for a particular segment, RNase H cleavage of these molecules was performed. RNase H cleaves regions of double-stranded RNA. Plus-stranded viral

RNA sequences (both cRNA and mRNA) were specifically targetted for cleavage by hybridization of total infected cellular RNA with a (-) sense oligonucleotide probe complementary to sequence in the 3' terminal third of the RNA. Following RNase H digestion, PAGE and Northern blot analysis, 3'-terminal cRNA and mRNA-derived cleavage products (identified by probing) would still differ in size by only 50-150 nt (+/- polyA+ tail). However under appropriate conditions of electrophoresis, this difference should be more easily detected than in intact cRNA and mRNA given the smaller size of the cleaved RNA fragments. cRNA and mRNA-derived cleavage products could be differentiated, quantified by densitometry and presumed to accurately represent cRNA and mRNA levels, assuming that RNase H cleavage is complete.

The possibility of targetting specific RNA sequences for cleavage by oligonucleotide-directed RNase H digestion was first tested using an in vitro system. A 498 bp DNA sequence containing the 3' end of FM-MA segment 4 was subcloned into pGEM-7Zf+ digested with EcoR I and BamH I (Fig. 8). In vitro transcription of Bgl I-digested plasmid by Sp6 in the presence of [^{32}P]UTP produced a (-) sense transcript of 787 nt (Fig. 8) which was resolved by PAGE (Fig. 9, lanes 1-3). Hybridization of this transcript with a (+) sense oligonucleotide (1391+) spanning nt 1391->1419 followed by RNase H cleavage and PAGE yielded products of 324 and 463 nt (lane 5) as predicted. RNase H cleavage of the same transcript targetted by a second (+) sense oligonucleotide (1499+) representing nt 1499->1518 produced the expected cleavage fragments of 345 and 442 nt (lane 6).

Fig. 8. FM-MA segment 4 subclone in pGEM-7Zf+. FM-MA-specific DNA representing the 3' end of segment 4 (nt 1268-1765) was subcloned into EcoR I/ BamH I-digested pGEM-7Zf+. The plasmid was linearized in single enzyme digestions with Bgl I or BamH I. Numbers on the plasmid map refer to the nt position in segment 4 sequence relative to position 1 of mRNA. Direction of transcription by Sp6 or T7 is indicated with an arrow. Sp6 and T7 transcripts generated from (respectively) Bgl I or BamH I digestion of the subclone and the expected product sizes following oligonucleotide targetting and RNase H digestion are shown below the plasmid map.


EcoR I (1268)
BamH I (1765)
T7
Bgl I
3' FM-MA seg 4
(498 bp)
Sp6
pGEM-7zf+

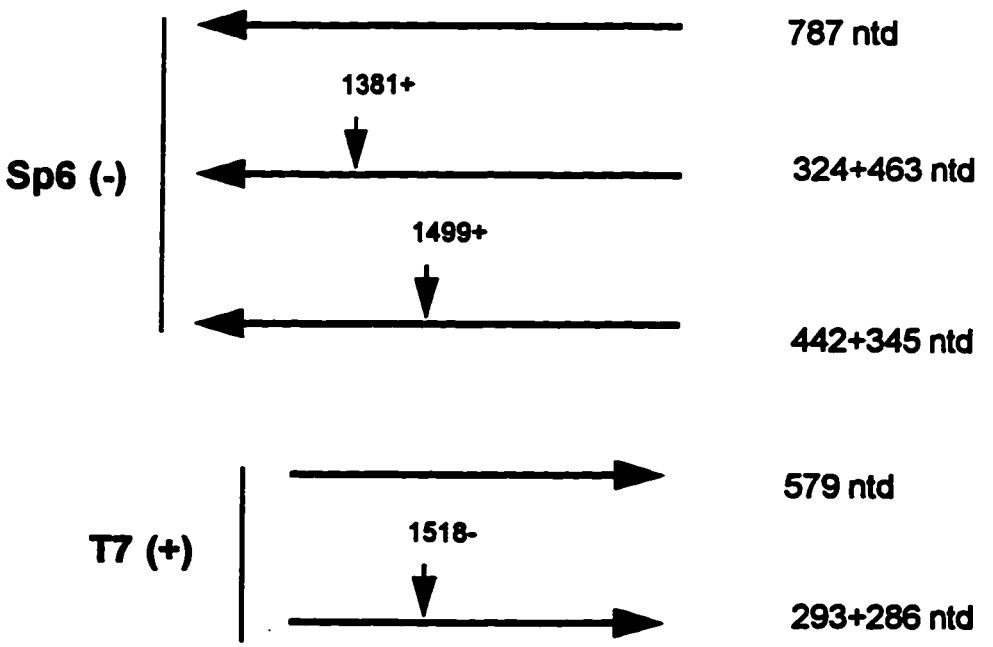
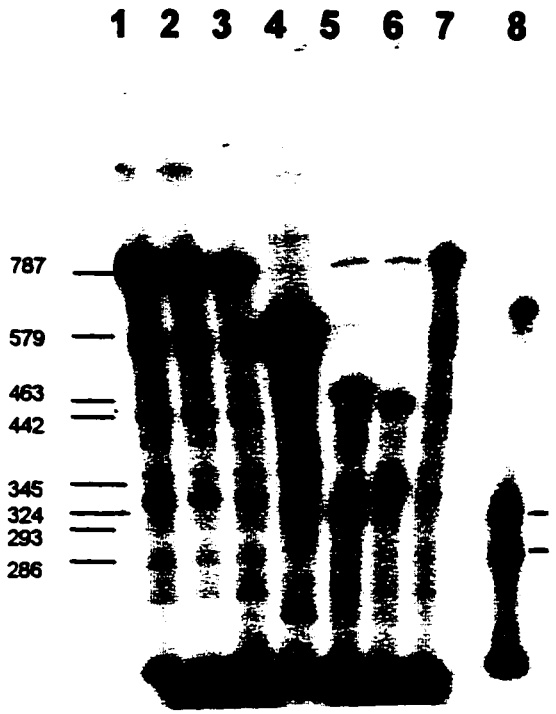

787 ntd
1381+
Sp6 (-)
324+463 ntd
1499+
442+345 ntd
579 ntd
T7 (+)
1518-
293+286 ntd

Fig. 9. Oligonucleotide-directed cleavage of FM-MA segment 4 transcripts by RNase H. A (-) sense transcript of 787 nt (lanes 1-3) was generated by Sp6 transcription in the presence of [^{32}P] of an FM-MA segment 4 subclone (**Fig. 8**) linearized with Bgl I. This transcript was targetted for digestion by 2 (+) sense segment 4-specific oligonucleotides (1381+, lane 5 and 1499+, lane 6) and one (-) sense segment 4-specific oligo (1518-, lane 7). A 579 nt (+) sense RNA (lane 4), generated by T7 transcription of the BamH I-digested segment 4 subclone (**Fig. 8**) was targetted for RNase H digestion by a (-) sense segment 4-specific oligonucleotide (1518-, lane 8). Digested products were resolved by PAGE in 6% acrylamide containing 4 M urea followed by autoradiography. Product sizes (in nt) are indicated at left.


1 2 3 4 5 6 7 8
787
579
463
442
345
324
293
286

When this latter experiment was repeated with an oligonucleotide (1518-) with the opposite polarity (1518->1499), the labelled 787 nt transcript was not cleaved (lane 7).

A [^{32}P]-labelled (+) sense segment 4 transcript of 579 nt was generated in vitro by T7 transcription of BamH I-digested plasmid (Fig. 8; Fig. 9, lane 4). RNase H cleavage directed by 1518- produced 2 fragments of 286 and 293 nt, as expected (Fig. 9, lane 8). It was concluded that RNase H digestion in conjunction with specific oligonucleotides could produce directed cleavage of RNA molecules of either polarity. Although cleavage was seen to be incomplete (Fig. 9 lanes 5, 6 and 8), it was assumed that RNase H activity could be increased or optimized.

The directed cleavage of in vivo-synthesized FM-MA cRNA and mRNA molecules in a context of total cellular RNA was attempted. Total RNA (2 µg) from MDCK cells infected 6 hr with FM-MA was hybridized with a (-) sense FM-MA-specific oligonucleotide probe (1518-) and digested with RNase H using conditions established to give cleavage of in vitro-synthesized FM-MA transcripts. Since the target RNA was unlabelled in this experiment, cleavage was assayed by resolving the products of RNase H digestion by PAGE followed by Northern blot analysis using a labelled oligonucleotide probe complementary to sequence 3' to the cleavage site and present in both mRNA and cRNA (data not shown). Only full-length segment 4 transcripts were detected following RNase H digestion indicating that cleavage had not occurred. Cleavage products were not generated under a range of RNase H (0.1-20 U), oligonucleotide (0.01-10 µg) or target cellular RNA

(0.5-20 μg) concentrations. Because reliable and complete cleavage of target molecules was essential for the measurement of cRNA and mRNA levels, RNase H cleavage was not pursued.

Since viral RNA detection relying on Northern blot analysis would not provide unequivocal information on viral RNA levels or even distinguish RNA class-specific molecules without investing much effort in refining the system, this approach was abandoned.

3.3 Approach #3 to the detection and quantification of viral RNA during interference: Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) followed by strain-specific restriction digestion.

Numerous studies have demonstrated the potential of RT-PCR to detect low abundance RNAs. Accurate quantification of these RNAs has been hampered by the fact that, after an exponential increase of product, the efficiency of amplification declines as a result of the consumption of reaction components and the production of inhibitory pyrophosphates which make it difficult to extrapolate the starting amount of cDNA from the PCR yield. This problem has been addressed by coamplifying an internal standard with the same sequence as the target template but for a small intron, deletion or mutated restriction site (used to distinguish standard from target following PCR). When performed in the same tube, the two amplification processes share all the potential variables: reagent concentrations, cycle conditions and number, primer/template melting conditions and length of amplicon. Because amplification occurs with the same efficiency for both standard

and target DNA, their ratio remains constant irrespective of the cycle number or consumption of reaction components. The ratio of PCR products thus precisely reflects their initial relative concentrations even when there are differences (up to 12X) in initial quantities of RNA (Cottrez et al., 1994). Equal efficiency of standard and target has been demonstrated not only in the exponential phase of amplification but also in the nonexponential phase (Gilliland et al., 1990; Cottrez et al., 1994). Determining the product ratios and knowing the amount of added standard then permits calculation of the quantity of target RNA in the starting material.

The principle that the relative levels of two almost identical RNA templates are maintained in the pool of cDNAs produced during coamplification by RT-PCR was the basis for determining the relative levels of HK and FM-MA RNA in cells coinfected with the two viruses. Since the amount of RNA was unknown for both strains, it was not possible to determine absolute RNA quantities without the use of a third competitive molecule, a standard of known concentration, which would have made strain-specific identification of products extremely difficult. However, by using primers in both RT and PCR steps that were completely conserved between strains, RT-PCR did permit calculation of the relative quantity of HK and FM-MA RNA in a given sample. Strict control of input RNA between samples and PCR cycle number was unnecessary since any factor influencing RT or PCR efficiency affected both strains within one sample equally and did not alter the relative levels of HK and FM-MA cDNAs. Because every sample was internally

controlled at each step of the amplification, strain-specific RNA ratios detected in different samples could be legitimately compared.

RT-PCR followed by strain-specific restriction digestion proved to be a convenient method to detect the 3 RNA classes specified by a given genome segment for both HK and FM-MA in a sample of total RNA from cells coinfected with the 2 strains. Selection for the segment and RNA class to be amplified was made during the RT step of this assay. Primers used in RT (Table 1) were chosen that specifically targetted one of cRNA, mRNA or vRNA for a particular genome segment (Fig. 10A). For example, to amplify vRNA, a segment-specific (+) sense oligonucleotide was used to prime the RT reaction. To amplify cRNA, a segment-specific (-) sense oligonucleotide was used that targetted the unique region of cRNA not present in mRNA. To amplify mRNA, the RT reaction was primed with a (-) sense oligonucleotide that was complementary to the polyA+ tail and which contained several additional nt to specify the desired gene. Each RT reaction contained only one primer and thus directed amplification of a single RNA class. Reverse transcription was followed by PCR amplification using nested primers homologous to sequences present in all 3 RNA classes (Fig. 10B). One or both primers used in PCR (Table 2) were end-labelled with [^{32}P] to facilitate detection and quantification of products. Primers used in RT and PCR were complementary to regions completely conserved between HK and FM-MA so that the RNA of both strains would be amplified equivalently.

Since the goal of the study was a documentation of RNA levels during

Table 1. RNA class-specific oligonucleotides used to prime reverse transcription

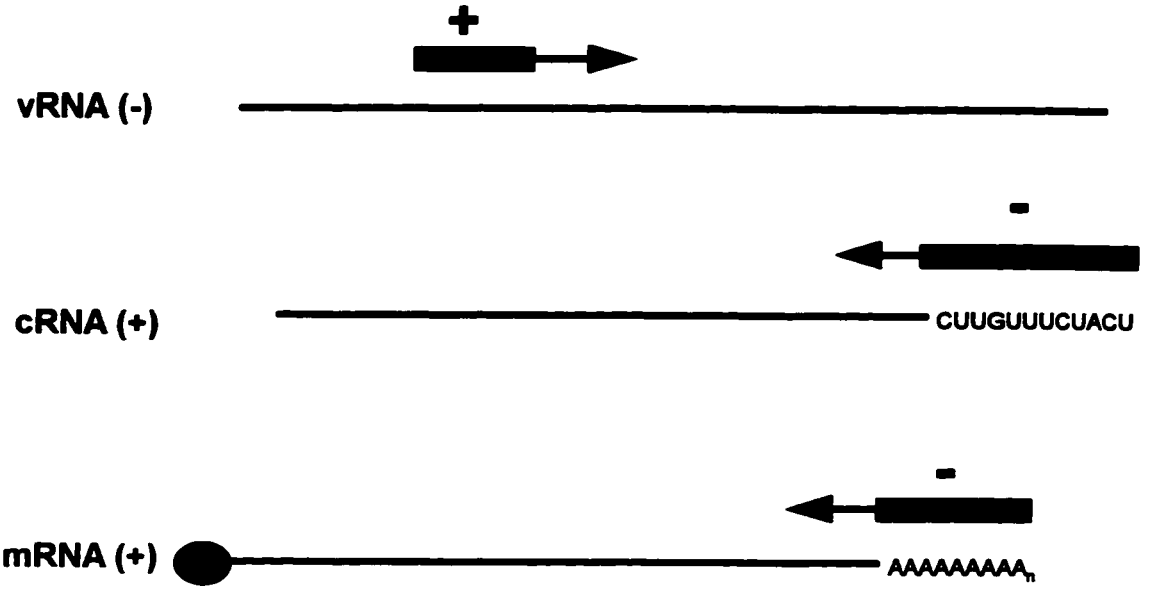
Seg	Specificity	Start ntd	Sense	Sequence
2	vRNA	1759	-	CTGTGGGAGCAAACCCG
	cRNA	2341	+	AGTAGAAACAAGGCATTT
	mRNA	2320	+	$(TTT)_8CA$
5	vRNA	791	-	GAAATGCTGAGATCGAAG
	cRNA	1565	+	AGTAGAAACAAGGGTATT
	mRNA	1540	+	$(TTT)_7CTTTAA$
7	vRNA	16	-	GGTAGATATTGAAAGATG
	cRNA	1027	+	AGTAGAAACAAGGTAGTTTTT
	mRNA	1005	+	$(TTT)_5ACTCCAGCT$

Fig 10. Strategy for RNA class-specific amplification of viral RNAs.

A. Reverse transcription of viral RNA using a primer specific for one of vRNA, cRNA or mRNA.

B. PCR using nested primers, one or both of which were labelled with [^{32}P] (indicated with an asterisk). Primers used in both reverse transcription (**Table1**) and PCR (**Table 2**) were homologous to sequences conserved between HK and FM-MA strains.

A. Reverse Transcription using RNA class-specific primers



B. PCR using nested primers

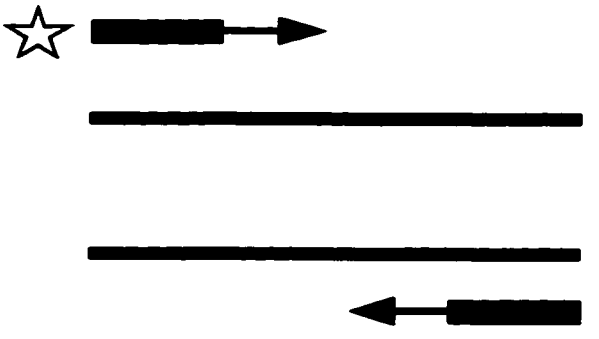


Table 2. Oligonucleotides used to prime PCR

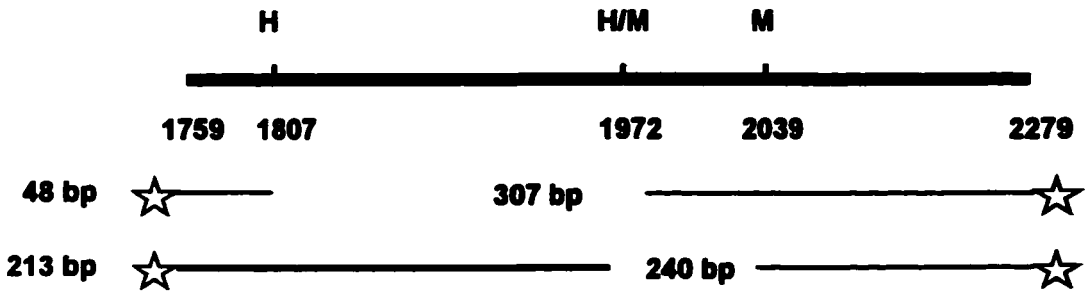
Seg	Forward start ntd	Forward sequence (5'-3')	Reverse start ntd	Reverse sequence (5'-3)	Amplicon (bp)
2	1759	CTGTGGGAGCAAACCCG	2279	TCTTCAATGGTGGAACAGA	520
5	815	TCTTTCTGGCACGGTCTG	1536	TCGTCAGCATCCACAGCA	721
7	16	GGTAGATATTGAAAGATG	976	TCGTCAGCATCCACAGCA	960

interference, the starting material for reverse transcription was total RNA isolated from cells infected with both HK and FM-MA. The resultant RT-PCR products consisted of double-stranded HK and FM-MA cDNAs of equal length. Differentiation between products of the 2 strains was done by digestion of RT-PCR reactions with restriction enzymes which produce strain-specific product profiles. The digested products were subjected to PAGE, autoradiography and scintillation counting of the excised virus-specific bands. Relative levels of HK and FM-MA-specific RNA for a given segment were calculated as the ratio of strain-specific PCR products. This approach therefore satisfied the two technical requirements of documenting RNA expression for two coinfecting virus genomes by allowing:

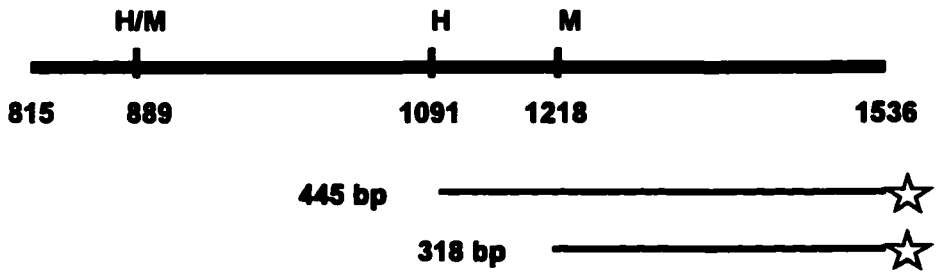
1. Discrimination between the 3 RNA classes for a given genome segment (RT-PCR). 2. Differentiation between HK and FM-MA RNA molecules (strain-specific restriction digestion). RNA levels of 3 genes were monitored by RT-PCR: segment 2 (PB1), segment 5 (NP) and segment 7 (M1). These segments were chosen for study because they provided information on early (PB1, NP) and late (M1) viral genome expression during interference and because they contained sufficient strain-specific sequence homology to permit RT and PCR primer construction and sufficient virus-specific sequence diversity to allow strain-specific restriction digestion of RT-PCR products. The strategy for amplification and restriction digestion of RNA specified by each gene is detailed in Figure 11A (segment 2), 11B (segment 5) and 11C (segment 7).

Fig. 11. Strategy for virus-specific restriction digestion of segment 2 (A), segment 5 (B) or segment 7 (C)-specific RT-PCR products. PCR products were digested singly with Ava II (segments 2 and 5) or double-digested with Hind III and BspD I (segment 7) to generate strain-specific restriction profiles. Restriction digestion sites are identified as HK (H) or FM-MA (M)-specific or common to both viruses (H/M).

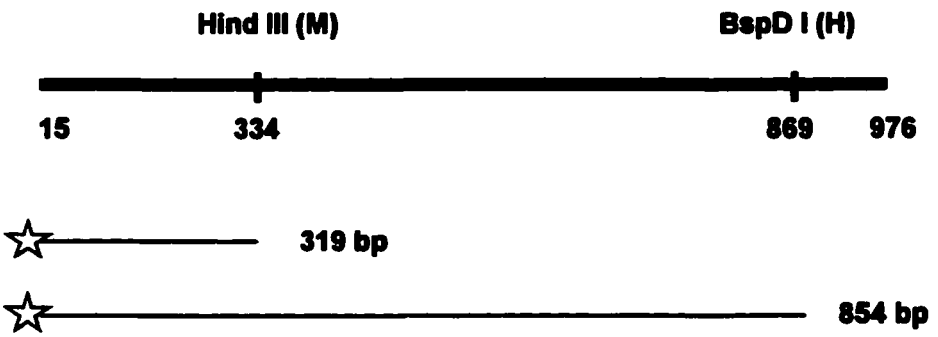
A. Digestion of segment 2 PCR products



B. Digestion of segment 5 PCR products



C. Digestion of segment 7 PCR products



3.3.1. Relative levels of HK and FM-MA-specific products reflected relative levels of input RNAs.

To confirm that the relative levels of HK and FM-MA-specific RT-PCR products accurately reflected the strain-specific input RNA levels, a control experiment was performed. Purified HK and FM-MA vRNA were combined in various ratios and subjected to reverse transcription using a segment 5-specific vRNA primer followed by PCR, Ava II digestion, PAGE and autoradiography (Fig. 12A). Following scintillation counting of the virus-specific Ava II fragments, the relative levels of HK and FM-MA-specific RT-PCR products were found to approximate the ratios predicted by input vRNA levels (Fig. 12B). The RT-PCR product ratios deviated by 10-29% from the expected values.

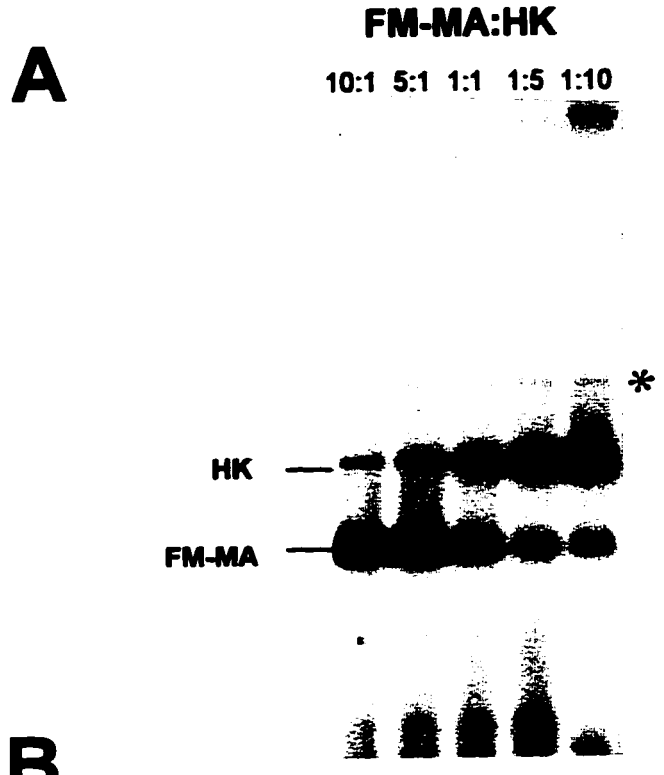
HK and FM-MA DNA strands not used as templates following a denaturation step in PCR can potentially associate to form heterodimeric DNA molecules which are not digested by Ava II. Unless the two strains' cDNAs are present in equal concentrations, the formation of such heterodimers can affect the apparent strain-specific product ratios. Following segment 5-specific RT-PCR, some undigested product was detected and deduced to be heterodimers of HK/FM-MA cDNAs (Fig. 12, see asterisk). Clearly, although the heteroduplex molecules formed a minor component of the total product, their potential impact on the relative quantification of HK and FM-MA RNA must be considered. An effort was therefore made to keep the number of PCR cycles low (20) since the limiting reagent conditions of the plateau phase of PCR amplification could contribute to the pool of unused cDNAs

Fig. 12. Relative levels of FM-MA and HK-specific segment 5 RT-PCR products generated from various input vRNA ratios.

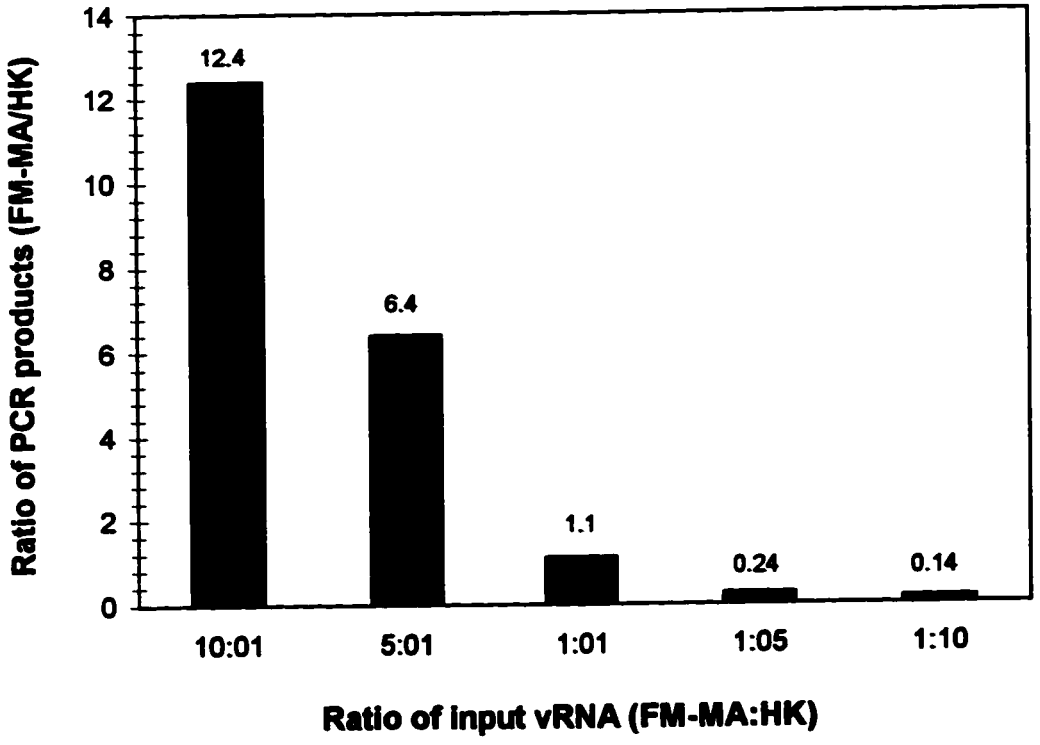
A. Purified FM-MA and HK vRNA combined in various ratios were subjected to segment 5-specific RT-PCR (20 PCR cycles) followed by Ava II digestion, PAGE in 10% acrylamide with a 3.75% stacking gel and autoradiography. Virus-specific restriction digestion products are indicated on left. Heteroduplex product of PCR is indicated with an asterisk.

B. Relative levels of HK and FM-MA-specific PCR products were calculated as the ratio of scintillation counts generated from excised virus-specific Ava II products.

A


FM-MA:HK
10:1 5:1 1:1 1:5 1:10
*
HK
FM-MA

B


Ratio of PCR products (FM-MA/HK)
14
12
10
8
6
4
2
0
12.4
6.4
1.1
0.24
0.14
10:01
5:01
1:01
1:05
1:10
Ratio of input vRNA (FM-MA:HK)

and hence to heteroduplexing.

3.3.2. PCR cycle number did not affect relative levels of HK and FM-MA cDNAs.

To confirm that the relative levels of FM-MA and HK cDNAs produced in RT-PCR do not depend on PCR cycle number, a control experiment was performed. Segment 5-specific RT-PCR was performed on a sample of total RNA isolated from cells infected for 4 hr with HK and FM-MA (MOI=3 each). It should be emphasized that the relative levels of HK and FM-MA-specific cDNAs produced were not expected to be equivalent but rather to reflect the relative RNA levels in the cell during coinfection. Aliquots of the PCR reaction were taken after 5, 10, 15, 20, 25, 30 and 35 cycles and subjected to Ava II digestion, PAGE and autoradiography (Fig. 13A). Following scintillation counting of the virus-specific restriction fragments, the relative levels of FM-MA and HK-specific products were found to be fairly constant (Fig. 13B; FM-MA/HK= 4.6+/-0.63) over a range of 15-35 cycles (HK cDNAs were not detectable after only 5 or 10 cycles). This suggests that HK and FM-MA-specific cDNAs were amplified equivalently during PCR regardless of the amplification phase (exponential or nonexponential).

3.3.3. HK RNA synthesis (vRNA, cRNA and mRNA) was suppressed relative to FM-MA during coinfection.

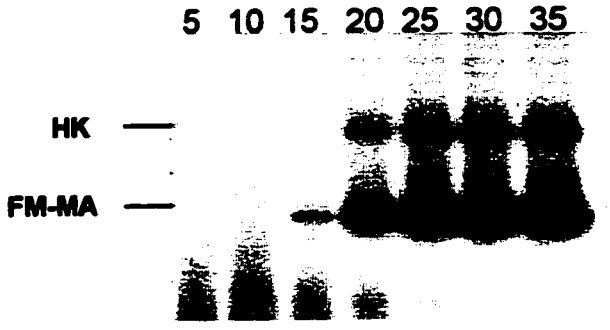
RT-PCR was then used to address the main objective of this study: **documentation of the relative levels of HK and FM-MA RNA during interference**. Specifically, RT-PCR was used to detect and quantify the relative

Fig. 13. Relative levels of FM-MA and HK-specific segment 5 RT-PCR products over a range of PCR cycle numbers.

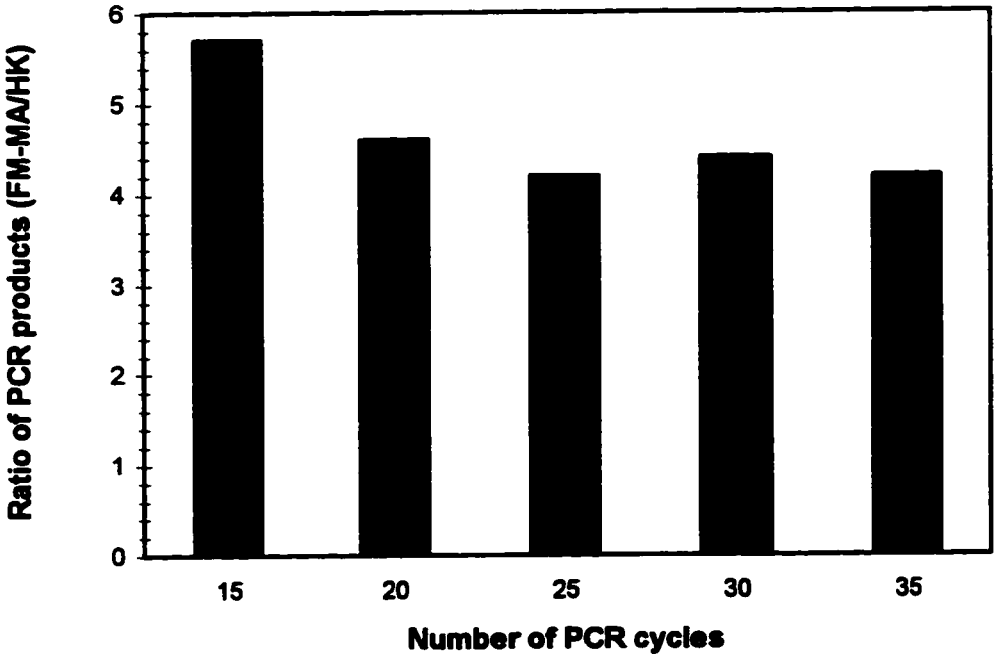
A. Total cellular RNA from MDCK cells coinfected (MOI=3,each virus) with HK and FM-MA (MA) for 6 hr was reverse transcribed using a segment 5 primer specific for vRNA (**Table 1**). A fraction (1/40) of the yield was PCR amplified using segment 5-specific primers (**Table 2**). Aliquots of the PCR reactions were withdrawn after 5, 10, 15, 20, 25, 30 or 35 cycles. Products were digested with Ava II and resolved by electrophoresis through 10% acrylamide with a 3.75% stacking gel followed by autoradiography. Virus-specific restriction digestion products are indicated on the left.

B. Relative levels of HK and FM-MA-specific segment 5 PCR products were calculated as the ratio of scintillation counts generated from excised virus-specific Ava II products.

A


5 10 15 20 25 30 35
HK
FM-MA

B


Ratio of PCR products (FM-MA/HK)
0
1
2
3
4
5
6
15
20
25
30
35
Number of PCR cycles

levels of HK and FM-MA RNA in MDCK cells coinfected with the 2 viruses over a 24 hr period. Total cellular RNA or virion RNA released from cells coinfected with HK and FM-MA (MOI=3 each) for 2, 4, 6, 12, or 24 hr were reverse transcribed using segment 2-specific primers (Table 1). Each RT reaction targetted only one of vRNA, cRNA or mRNA. PCR amplification was performed on a fraction of the RT reaction using nested primers homologous to sequences present in all 3 RNA classes, producing DNA molecules of 520 bp in each case. The segment 2-specific primers used in both RT (Table 1) and PCR (Table 2) were complementary to regions conserved between HK and FM-MA so that the RNA of both strains was amplified equivalently. Differentiation between products of the two strains was done by strain-specific digestion with Ava II followed by PAGE (Fig. 14A), autoradiography and scintillation counting of excised virus-specific bands. Relative levels of HK and FM-MA segment 2-specific RNA were calculated as the ratio of virus-specific Ava II products (Fig. 14B). FM-MA segment 2 RNA levels exceeded those of HK at each timepoint for cRNA, mRNA and intracellular or released vRNA by a factor of 2.4-7.5. HK segment 5 RNA levels showed a larger ratio of FM-MA to HK than segment 2, suggesting that the magnitude of interference is segment-specific (Fig. 15A). FM-MA segment 5 RNA levels exceeded those of HK by a factor of 4-13.6, with a noticeable increase in interference through infection, peaking at 12 hr p.i.. FM-MA dominance over HK segment 5 vRNA levels was more pronounced in released virus than for intracellular molecules.

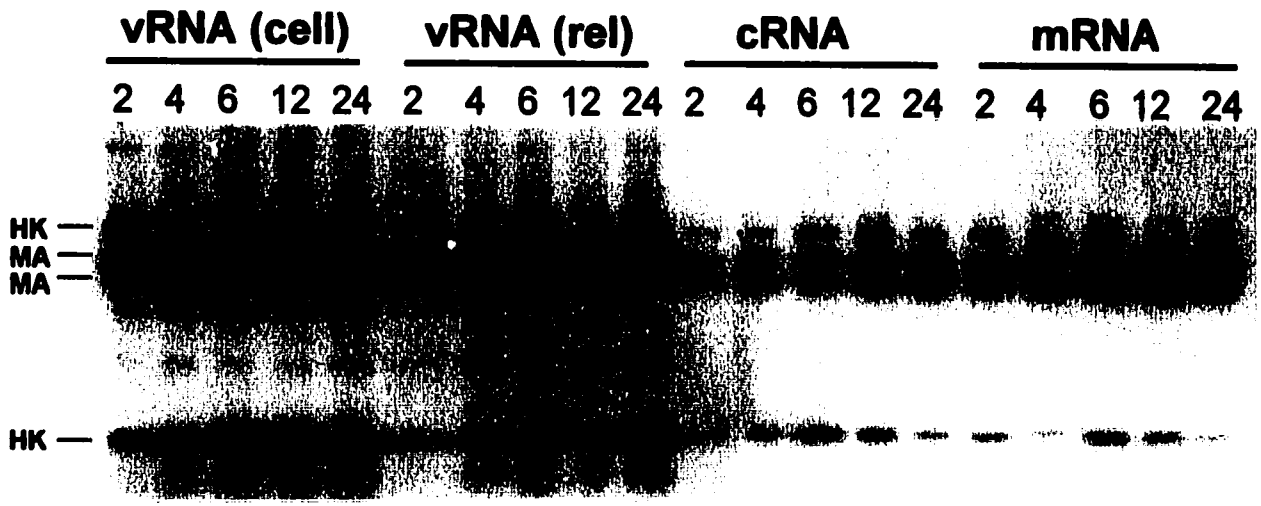
Relative levels of HK and FM-MA segment 7-specific RNA were likewise

Fig. 14. Relative levels of HK and FM-MA segment 2 RNA produced during coinfection.

A. Total cellular RNA or released virion RNA from MDCK cells coinfected (MOI=3 each virus) with HK and FM-MA (MA) for 2, 4, 6, 12 or 24 hr was reverse transcribed using segment 2 primers specific for one of vRNA, cRNA or mRNA (**Table 1**). A fraction (1/40) of the yield was PCR amplified (20 cycles) using [^{32}P]-labelled primers (**Table 2**). Products were digested with Ava II and resolved by electrophoresis through 10% acrylamide with a 3.75% stacking gel followed by autoradiography and quantification by scintillation counting. The RNA species targetted for amplification (cellular vRNA, cRNA and mRNA or released virion RNA [vRNA rel]) are indicated at the top of the autoradiograph. The FM-MA (MA) and HK-specific restriction-digestion products are identified on the left.

B. Relative levels of HK and FM-MA-specific segment 2 RNA were calculated as the ratio of virus-specific PCR products. The mean ratio (plus or minus one standard deviation) of FM-MA/HK RNA for each timepoint was calculated from three replicate coinfection timecourses.

A


vRNA (cell)
vRNA (rel)
cRNA
mRNA
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
HK
MA
MA
HK

B

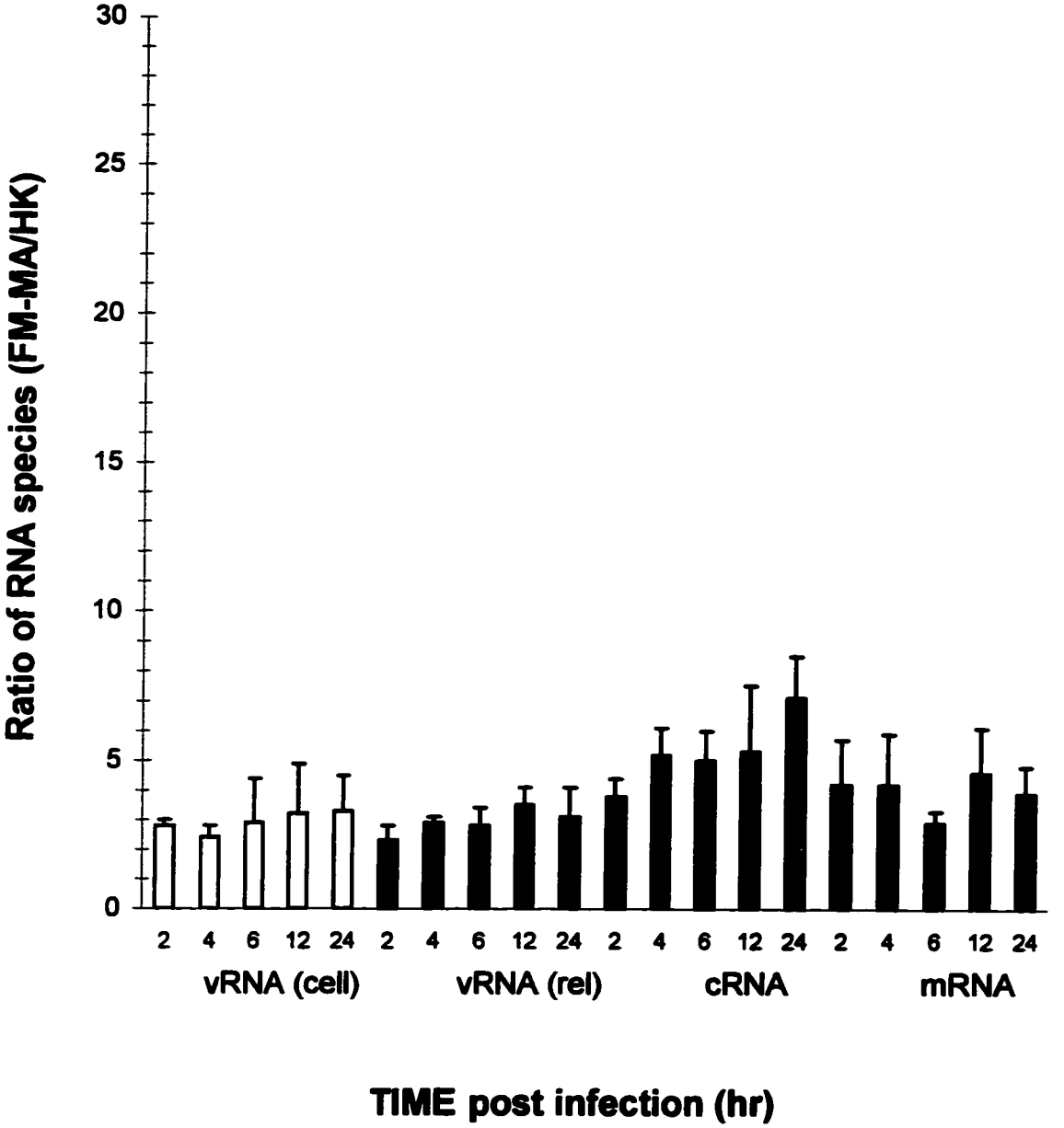
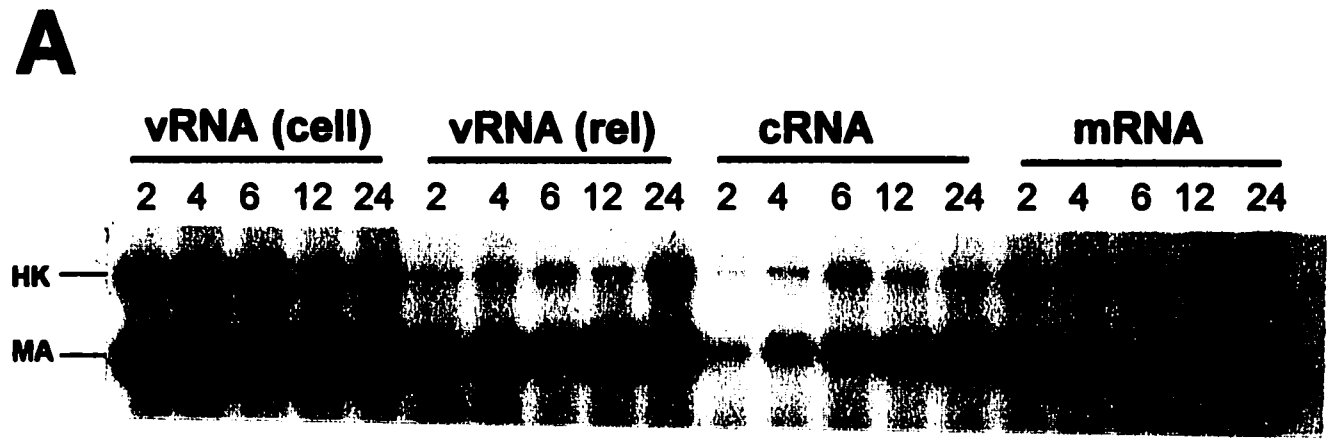

Ratio of RNA species (FM-MA/HK)
30
25
20
15
10
5
0
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
vRNA (cell)
vRNA (rel)
cRNA
mRNA
TIME post infection (hr)

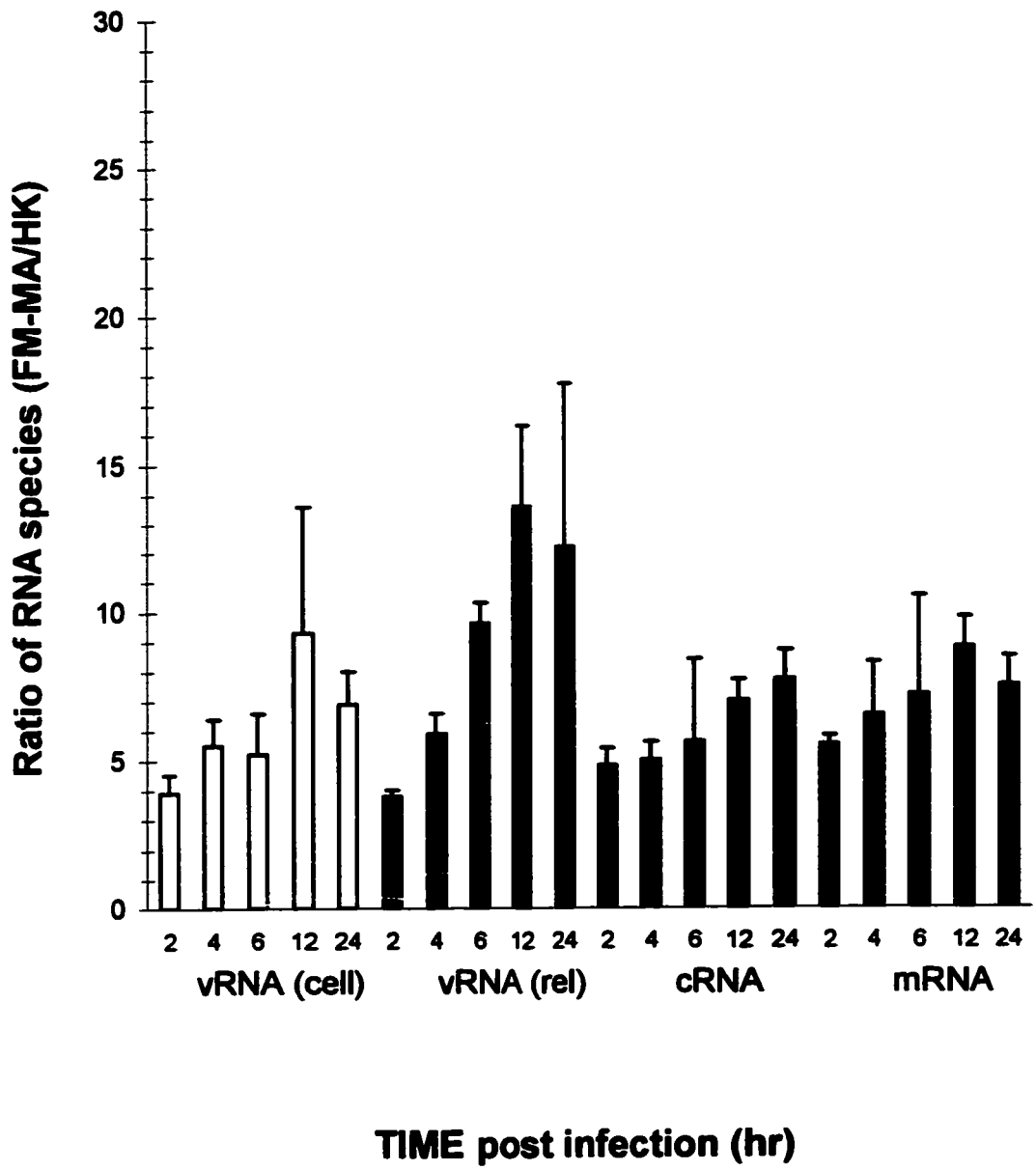
Fig. 15. Relative levels of HK and FM-MA segment 5 RNA produced during coinfection.

A. Total cellular RNA or released virion RNA from MDCK cells coinfected (MOI=3 each virus) with HK and FM-MA (MA) for 2, 4, 6, 12 or 24 hr was reverse transcribed using segment 5 primers specific for one of vRNA, cRNA or mRNA (**Table 1**). A fraction (1/40) of the yield was PCR amplified (20 cycles) using one unlabelled and one [^{32}P]-labelled primer (**Table 2**). Products were digested with Ava II and resolved by electrophoresis through 10% acrylamide with a 3.75% stacking gel followed by autoradiography and quantification by scintillation counting. The RNA species targetted for amplification (cellular vRNA, cRNA and mRNA or released virion RNA [vRNA rel]) are indicated at the top of the autoradiograph. The FM-MA (MA) and HK-specific restriction-digestion products are identified on the left.

B. Relative levels of HK and FM-MA-specific segment 5 RNA were calculated as the ratio of virus-specific PCR products. The mean ratio (plus or minus one standard deviation) of FM-MA/HK RNA for each timepoint was calculated from three replicate coinfection timecourses


A
vRNA (cell)
vRNA (rel)
cRNA
mRNA
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
HK
MA

B


Ratio of RNA species (FM-MA/HK)
30
25
20
15
10
5
0
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
vRNA (cell)
vRNA (rel)
cRNA
mRNA
TIME post infection (hr)

determined (Fig. 16A). Since segment 7 forward and reverse PCR primers lie 5' and 3' to the splice donor and splice acceptor sites, respectively, mRNA amplifications gave both M1 and M2 cDNAs. HK M1 RNA showed more suppression relative to the corresponding segment of FM-MA than did HK PB1 or NP RNA. FM-MA M1 RNA levels exceeded those of HK by a factor of 7.1-24.3, with the most interference seen later in infection (generally, 12 hr p.i.). The level of HK vRNA in released virus at 2 and 4 hr p.i. was too low to establish a ratio with FM-MA (Fig. 16B). However, in the remaining released vRNA samples, FM-MA predominated over HK to a greater extent than was seen intracellularly. Thus, **dominance of FM-MA was observed at the level of transcription and replication for the 3 genes analyzed, with M1 RNA showing the most dominance and PB1 RNA showing the least. In the case of NP and M1 RNA, the FM-MA/HK ratio increased over the course of infection, peaking at 12 hr, and was larger in released virion genomes than in intracellular vRNA.**

3.3.4. FM did not produce more RNA than HK during coinfection.

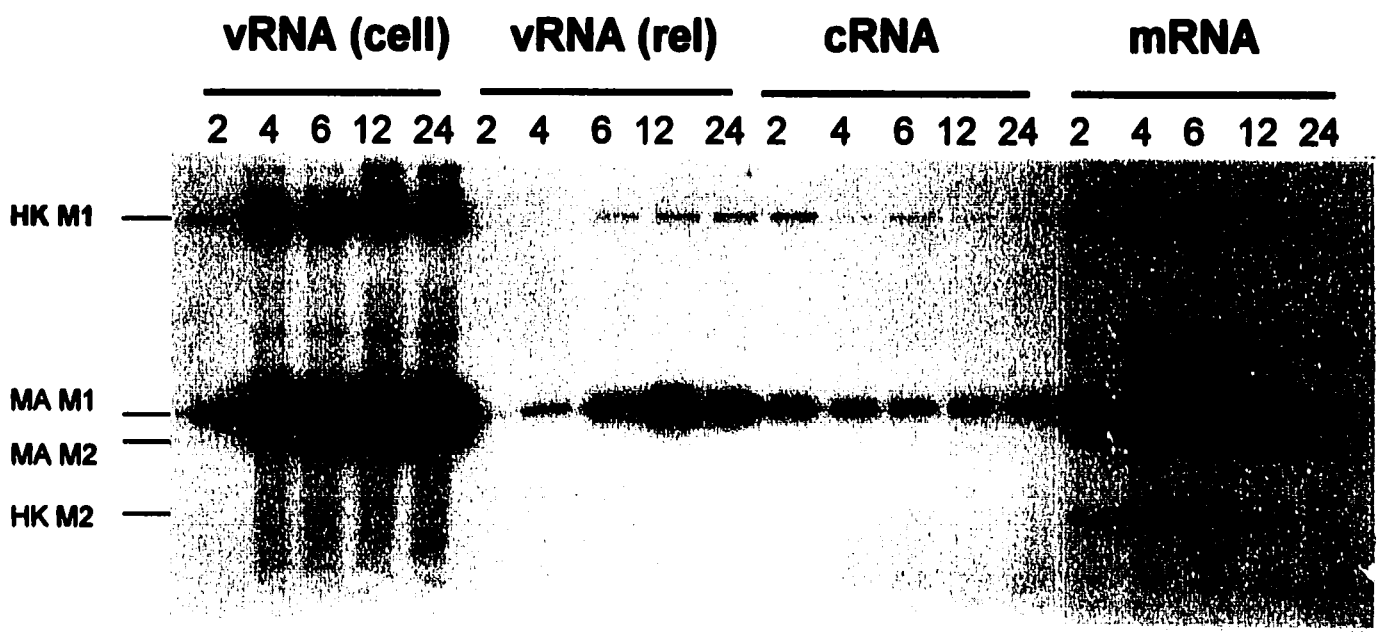
As a control, relative levels of HK and FM (noninterfering parent of FM-MA) RNA were compared. Segment 5 RNA produced during coinfection of MDCK cells with FM and HK was amplified and the virus-specific products were identified and quantified in the manner described in the previous experiments. NP vRNA, cRNA and mRNA levels and released virion RNA levels were approximately equal for the two strains (Fig. 17). This was expected since the parental FM strain does not

Fig. 16. Relative levels of HK and FM-MA segment 7 RNA produced during coinfection.

A. Total cellular RNA or released virion RNA from MDCK cells coinfected (MOI=3 each virus) with HK and FM-MA (MA) for 2, 4, 6, 12 or 24 hr was reverse transcribed using segment 7 primers specific for one of vRNA, cRNA or mRNA (**Table 1**). A fraction (1/40) of the yield was PCR amplified (20 cycles) using a 5' [^{32}P]-labelled and 3' unlabelled primers (**Table 2**). Products were digested with Hind III and BspD I and resolved by electrophoresis through 10% acrylamide with a 3.75% stacking gel followed by autoradiography and quantification by scintillation counting. The RNA species targetted for amplification (cellular vRNA, cRNA and mRNA or released virion RNA [vRNA rel]) are indicated at the top of the autoradiograph. The FM-MA (MA) and HK-specific restriction-digestion products are identified on the left.

B. Relative levels of HK and FM-MA-specific segment 7 RNA were calculated as the ratio of virus-specific PCR products. The mean ratio of FM-MA/HK RNA for each timepoint (plus or minus 1 standard deviation) was calculated from three replicate coinfection timecourses. The HK-specific released virus RNA was not detectable above background at 2 and 4 hr p.i.. $vRNA_{rel}$ data from these timepoints were therefore not analyzed (N/A).

A


vRNA (cell)
vRNA (rel)
cRNA
mRNA
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
HK M1
MA M1
MA M2
HK M2

B

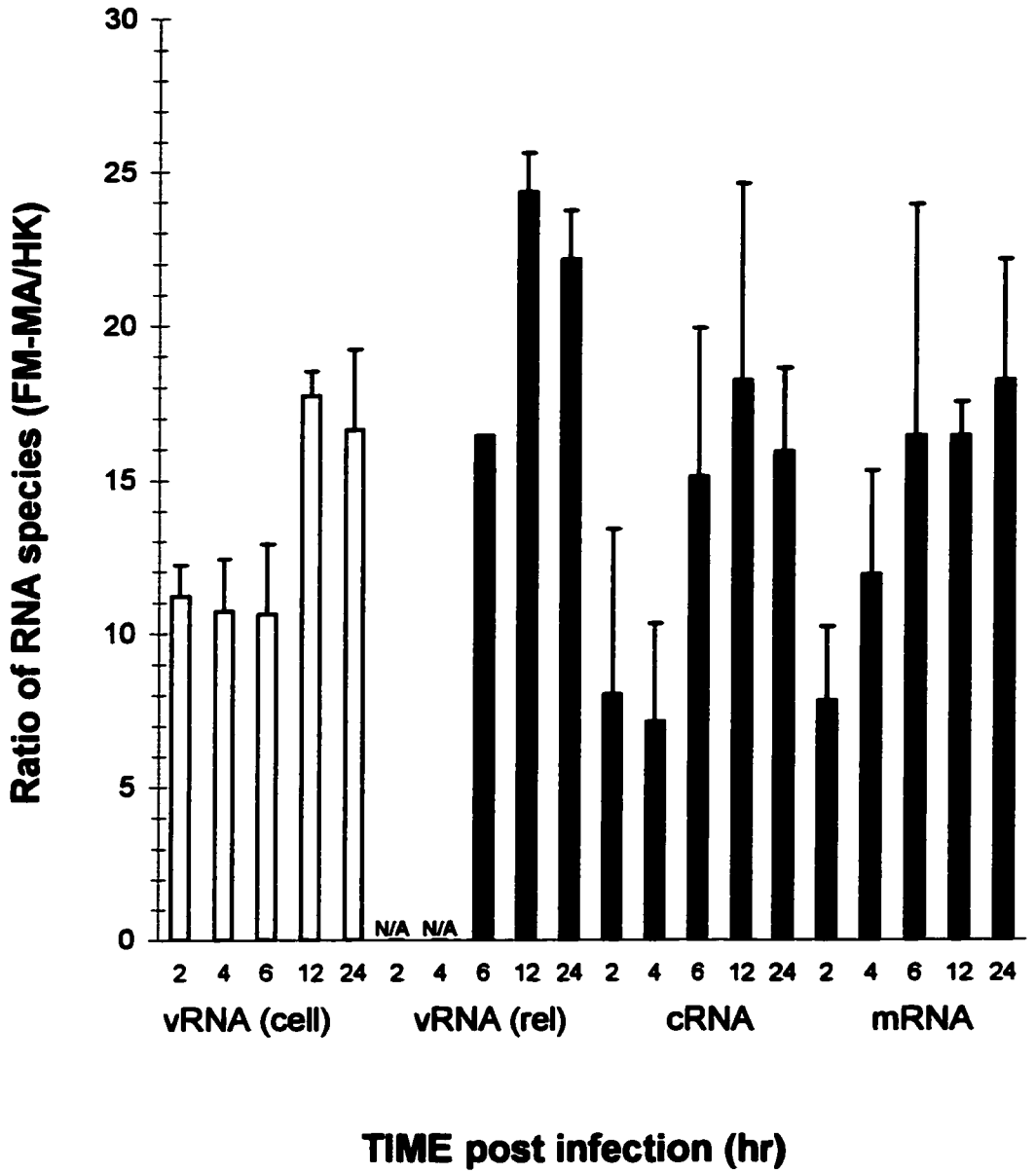
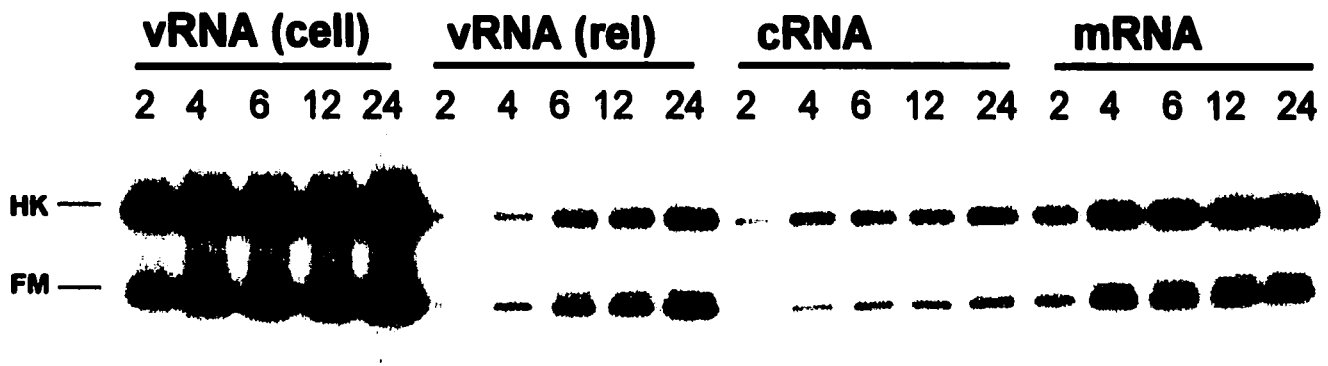

Ratio of RNA species (FM-MA/HK)
30
25
20
15
10
5
0
N/A N/A
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
vRNA (cell)
vRNA (rel)
cRNA
mRNA
TIME post infection (hr)

Fig. 17. Relative levels of segment 5 RNA produced by HK and the noninterfering parent, FM, during coinfection.

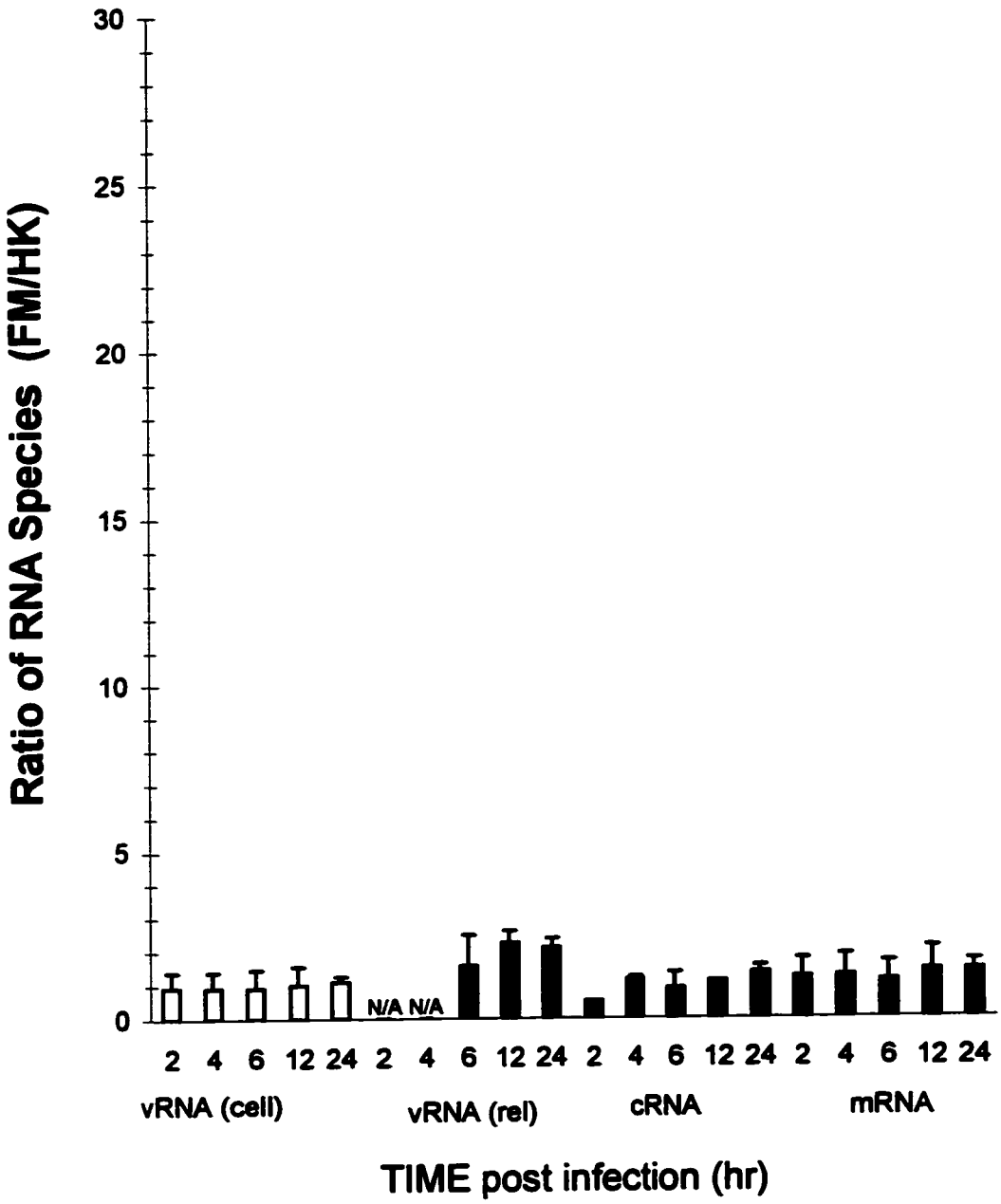
A. HK and FM segment 5-specific RNA species produced during coinfection of MDCK cells (MOI=3 each virus) were amplified by RT-PCR using segment-specific primers (**Tables 1 and 2**) and the products were analyzed as described in **Fig. 15**.

B. Mean relative levels of HK and FM RNA produced at each timepoint for two coinfection timecourses were calculated as the ratio of virus-specific PCR products. The $vRNA_{rel}$ at 2 and 4 hr p.i. were not detectable above background. Data from these timepoints were not analyzed (N/A).

A


vRNA (cell)
vRNA (rel)
cRNA
mRNA
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
HK
FM

B


Ratio of RNA Species (FM/HK)
30
25
20
15
10
5
0
N/A N/A
2 4 6 12 24 2 4 6 12 24 2 4 6 12 24 2 4 6 12 24
vRNA (cell)
vRNA (rel)
cRNA
mRNA
TIME post infection (hr)

interfere with HK virus replication on coinfection (Brown et al., 1992).

3.3.5. Predominance of FM-MA over HK was seen during primary transcription.

RT-PCR was used to determine the relative levels of primary transcription of HK and FM-MA segment 5 mRNA in coinfected cells (MOI=3 each) incubated in the presence of 100 μg/ml cycloheximide (Fig. 18). FM-MA segment 5 RNA accumulation exceeded that of HK at both 2 and 4 hr p.i. (5 fold) whereas relative levels of FM and HK primary transcription seen during coinfection were approximately equivalent at these timepoints.

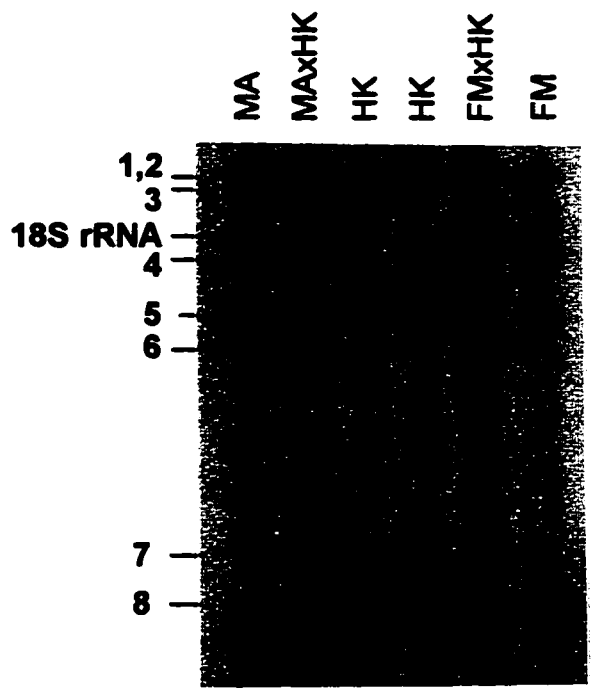
3.3.6. FM-MA released more virion RNA than HK in mixed infections.

Independent confirmation of the dominance of FM-MA but not FM virion RNA release during coinfection with HK was provided by metabolically labelling RNA in MDCK cells coinfected with HK and FM-MA or HK and FM (Fig. 19). [^{32}P]-labelled RNA from released virions was resolved by PAGE. Certain HK and FM-MA genome segments migrate differentially depending on electrophoretic conditions, including segment 4 (HA), segment 6 (NA) and segment 8 (NS1, NS2). HK segment 6 vRNA levels were suppressed relative to FM-MA (Fig. 19, lane 2) but not to FM (lane 5) in the viral yields of mixed infections. The magnitude of FM-MA dominance over HK demonstrated here for segment 6 appeared to be similar to that observed for segments 2 and 5 using RT-PCR (Fig. 14 and 15). Using different electrophoretic conditions, interference with HK segment 4 and 8 was also detected in virus released from cells coinfected with HK and FM-MA but not in mixed infections with

Fig. 18. Relative levels of HK, FM-MA and FM segment 5-specific primary transcripts in mixed infections. MDCK cells were coinfected with either HK and FM-MA (MA) or HK and FM (MOI=3, each virus) in the presence of 100 µg/ml cycloheximide. Total cellular RNA was isolated at 2 or 4 hr post infection, reverse transcribed using a mRNA-specific segment 5 primer (**Table 1**) and amplified by PCR as described in **Fig. 15**. Virus-specific bands resulting from Ava II digestion of PCR products are indicated on the left.

HKxMA2
HKxMA4
HKxFM2
HKXFM4
HK
MA or FM

Fig. 19. SDS-PAGE analysis of [^{32}P]-labelled released virion RNA from single or mixed infections. MDCK cells were either singly infected (MOI=3) with FM , HK or FM-MA (MA) or coinfected (MOI=3, each virus) in the presence of [^{32}P]. Released virions were harvested at 22 hr p.i. by hemadsorption and virion RNA was extracted and resolved by electrophoresis through 4.5% acrylamide with a 3.5% stacking gel. Viruses used in infections are indicated at top of autoradiograph, virus genome segments and position of 18S ribosomal RNA are indicated at left. Segment 8 vRNA molecules migrated into a 10% acrylamide plug.


MA
MAxHK
HK
HK
FMxHK
FM
1,2
3
18S rRNA
4
5
6
7
8

HK and FM (data not shown). The magnitude of interference for these 2 segments was greater than that seen for segment 6.

Since coinfection of cells with HK and FM-MA yielded >100-fold more infectious FM-MA progeny than HK (Table 3, from Brown et al., 1992) it was surprising that intracellular RNA and released genomic RNA levels did not reflect this level of inhibition of HK.

Table 3. Yield of viruses with parental and reassortant combinations of HA and NA from crosses of FM-MA x HK and FM x HK

	Total	H3N2 HK parent	H1N1 FM parent	H1N2 reassortant	H3N1 reassortant	Relative yield (FM vs HK) [fold]
FM-MA x HK	1.3×10^5	7.0×10^2	1.8×10^5	2.2×10^4	5.0×10^2	$10^{2.4}$
FM x HK	5.2×10^5	3.1×10^5	1.3×10^5	9.0×10^4	1.6×10^4	$10^{-0.4}$

From: Brown et al., 1992

This may be explained in part by the observation that the viral yield of cells coinfected with FM-MA and HK included higher levels of H1N2 and H3N1 reassortant viruses than parental H3N2 (HK) (Brown et al., 1992). These nonparental viruses are composed of a mixture of HK and FM-MA genome segments. The RT-PCR assay does not discriminate between HK vRNA from parental versus reassortant virions produced in mixed infections. HK vRNA present in these reassortants will decrease the FM-MA/HK ratio.

Another explanation is that the HK vRNA in released virions detected by RT-PCR may not all represent infectious virus. To address this possibility, the viral yields of cells coinfected with HK and FM-MA were used to reinfect MDCK cells. The relative levels of HK and FM-MA segment 5 vRNA in the original coinfection

and the subsequent passage in MDCK cells were determined by RT-PCR (Fig. 20). The relative level of FM-MA segment 5 vRNA was greater following reinfection of cells than in primary coinfections suggesting that a proportion of the HK vRNA released from mixed infection with FM-MA was derived from noninfectious particles. Passaging with a diluted aliquot of the viral yield from cells coinfected with HK and FM-MA produced a larger ratio of FM-MA/HK vRNA than did a higher MOI infection. This is consistent with the production of noninfectious HK particles during initial coinfection with FM-MA which, when passaged at low MOI, enter the secondary cell but cannot replicate without helper function provided by a coinfecting virus.

3.3.7. FM-MA RNA in progeny virus predominated even when HK infection preceded FM-MA infection.

MDCK cells were infected simultaneously with HK and FM-MA or singly with HK followed by superinfection with FM-MA 1, 2, 4 or 6 hr later. Relative levels of HK and FM-MA segment 5 vRNA for each infection were determined by RT-PCR 12 hr after initial infection by HK (Fig. 21). FM-MA intracellular vRNA predominated marginally when HK infection preceded FM-MA infection by 1 hour. When FM-MA superinfection was delayed 2 or more hours, HK vRNA accumulated to a higher level than FM-MA vRNA in the cell. Interestingly, FM-MA vRNA predominated in the virus yield from these delayed coinfections even when the intracellular pool of HK vRNA exceeded that of FM-MA. This suggests that FM-MA genomes were preferentially assembled into progeny virus.

Fig. 20. Relative levels of HK and FM-MA segment 5 vRNA following passaging.

MDCK cells were coinfected with FM (F) and HK (H) or FM-MA (M) and HK (MOI=3, each virus) for 12 hr in 5 ml of MEM. The virus yield (0.1 or 1 ml of culture supernatant) was used to infect fresh monolayers of MDCK cells for 12 hr. Relative levels of virus-specific segment 5 vRNA levels were determined in the original coinfection (indicated on the histograph with a " I") and passaged (indicated with a "II") cells ($vRNA_{cell}$) and in released virus ($vRNA_{rel}$) from both primary infections and passaged cells by RT-PCR as described in **Fig. 15**. Colour coding of data bars signifies as follows: white-HKXFM intracellular RNA; Light gray-HKXFM released viral RNA; black-HKXFM-MA intracellular RNA; dark gray-HKXFM-MA released viral RNA.

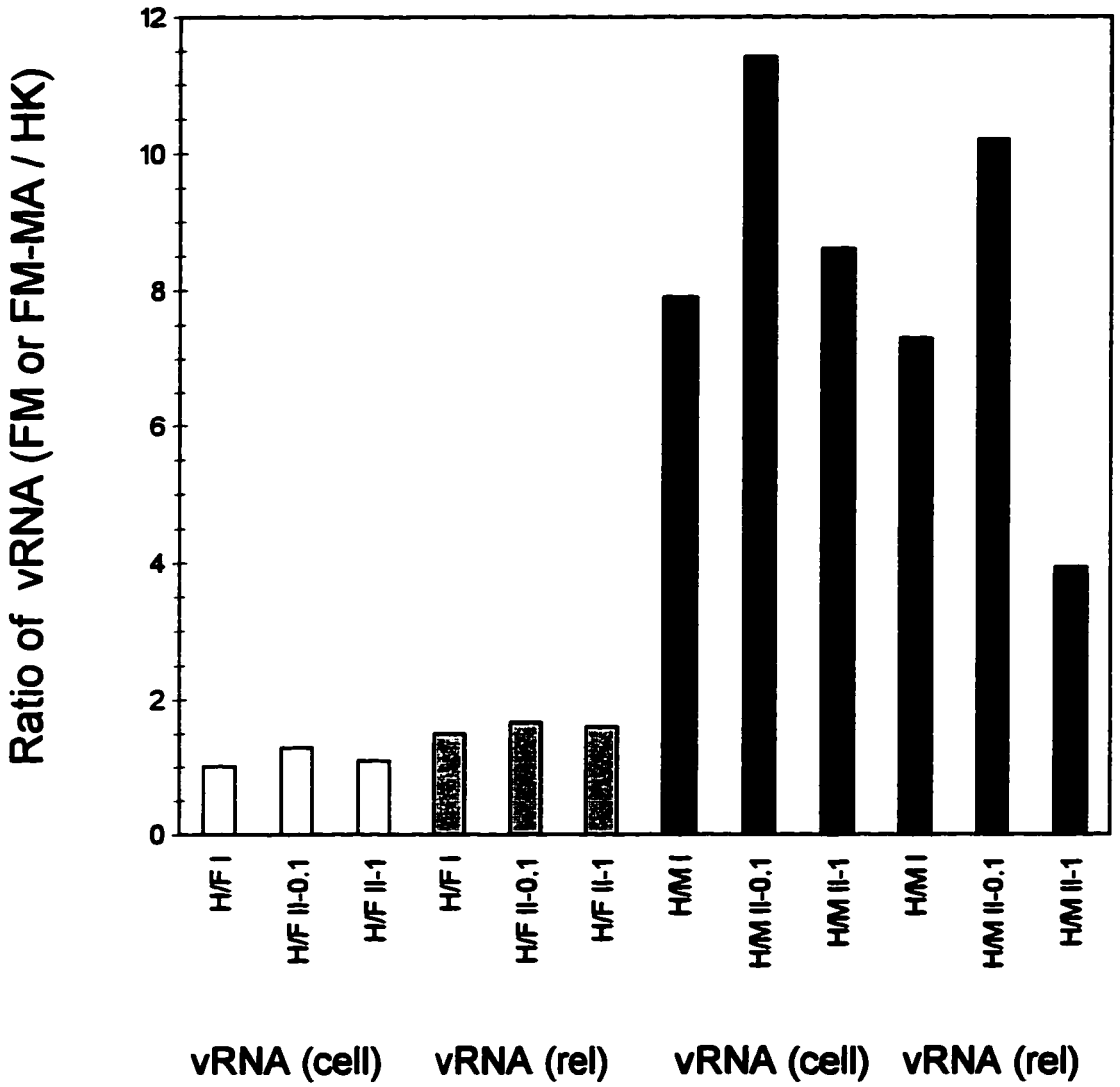
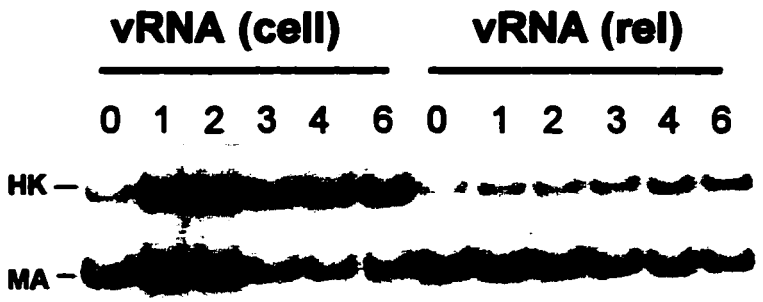

Ratio of vRNA (FM or FM-MA / HK)
12
10
8
6
4
2
0
H/F I
H/F II-0.1
H/F II-1
H/F I
H/F II-0.1
H/F II-1
H/M I
H/M II-0.1
H/M II-1
H/M I
H/M II-0.1
H/M II-1
vRNA (cell)
vRNA (rel)
vRNA (cell)
vRNA (rel)

Fig. 21. Effect of delayed infection with FM-MA following initial infection by HK on relative segment 5-specific vRNA levels in coinfected cells.

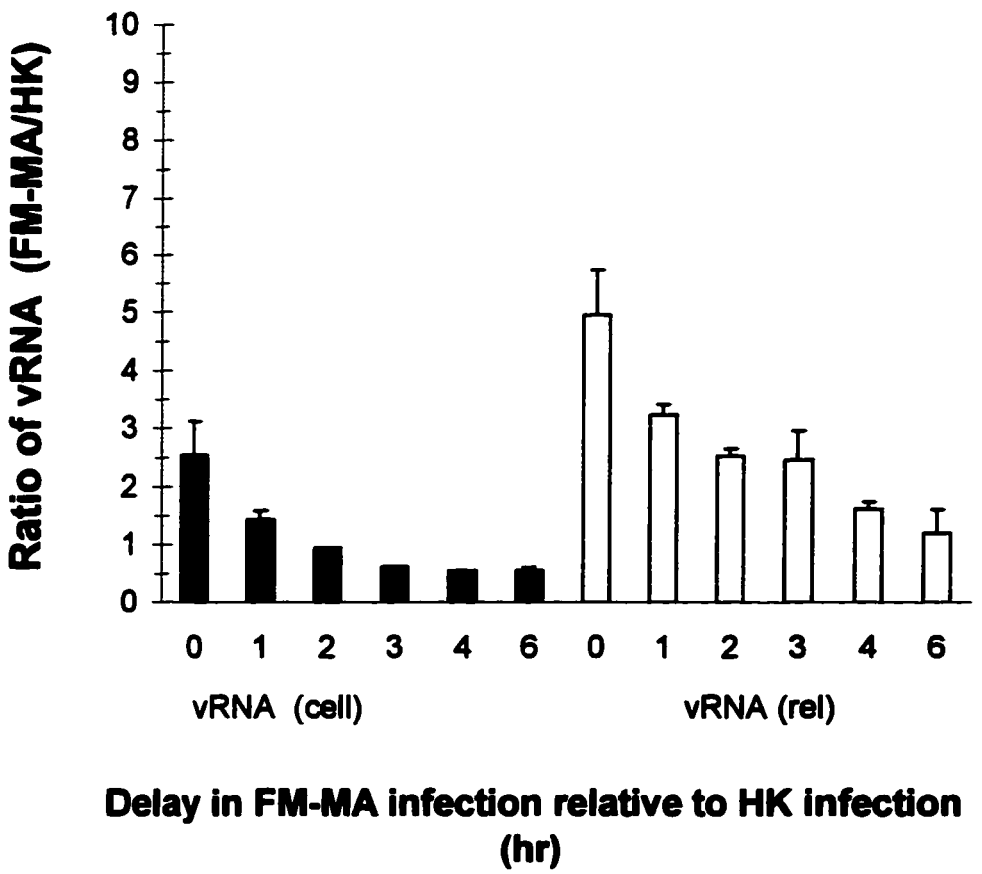
A. MDCK cells were infected simultaneously with HK and FM-MA (MOI=3 each virus) or singly with HK (MOI=3) followed by superinfection with FM-MA (MOI=3) 1, 2, 3, 4 or 6 hr later. Total cellular RNA and released virion RNA were isolated 20 hr after HK infection, reverse transcribed using a vRNA-specific segment 5 primer (**Table 1**) and amplified by PCR as described in **Fig. 15**.

B. Relative levels of HK and FM-MA segment 5 vRNA for each infection were calculated as the ratio of virus-specific restriction digested PCR products. The mean ratio (plus or minus one standard deviation) of FM-MA/HK RNA for each timepoint was calculated from two replicate timecourses.

A


vRNA (cell)
vRNA (rel)
0 1 2 3 4 6 0 1 2 3 4 6
HK
MA

B


Ratio of vRNA (FM-MA/HK)
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 6 0 1 2 3 4 6
vRNA (cell)
vRNA (rel)
Delay in FM-MA infection relative to HK infection (hr)

3.3.8. Effect of increasing the dosage of HK on interference.

To determine if FM-MA dominance over HK RNA levels could be overcome by increasing the dosage of HK, MDCK cells were coinfected simultaneously with FM-MA at a constant MOI of 3 and increasing amounts of HK (MOI of 10, 30 or 40). The relative levels of virus-specific segment 5 intracellular vRNA were determined by RT-PCR at 12 hr p.i. (Fig. 22). Although increasing the dosage of HK increased the level of HK NP vRNA relative to FM-MA, FM-MA vRNA predominated in the cell even when the infecting dose of HK exceeded that of FM-MA by a factor of 13.3. In contrast, the relative levels of FM and HK RNA following coinfection reflected the MOI of each virus.

3.3.9. FM-MA inhibited HK protein synthesis in coinfected cells

To assess the kinetics of interference at the level of total protein accumulation, the proteins of cells coinfected with HK and FM-MA were subjected to SDS-PAGE followed by Western blot analysis (Fig. 23). Since FM-MA and HK matrix proteins differ in their electrophoretic mobilities, the relative amounts of each protein in coinfected cells can be determined. FM-MA matrix protein predominated throughout infection and is the main species in released virions. FM-MA and HK NP proteins also differ in their electrophoretic mobilities however, the relative NP levels in coinfected cells were obscured by the post-translational modification of NP late in infection which produces two forms of NP: uncleaved (mol. wt. 56 kDa) and cleaved (mol. wt. 53 kDA) (Zhirnov and Bukrinskaya, 1981).

Relative rates of protein synthesis were also examined by pulse-labelling

Fig. 22. Effect of increasing dosages of HK on relative segment 5-specific HK, FM-MA (MA) and FM vRNA levels in mixed infections. MDCK cells were simultaneously infected with FM-MA (MOI=3) and HK (MOI=10, 30 or 40) or FM (MOI=3) and HK (MOI=10, 30 or 40). Total cellular RNA was isolated 12 hr p.i., reverse transcribed using a vRNA-specific segment 5 primer (**Table1**) and amplified by PCR as described in **Fig. 15.** PCR products were digested with Ava II giving virus-specific bands following PAGE and autoradiography. Relative virus MOIs for each infection are indicated at the top of the autoradiograph.

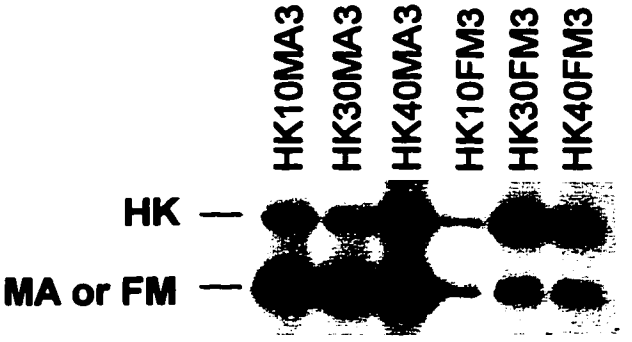
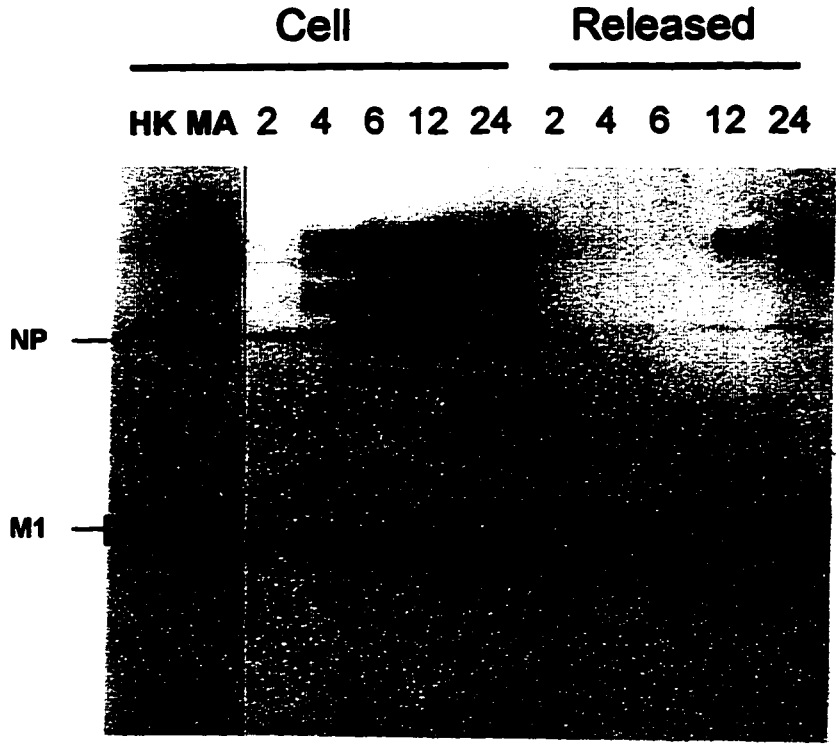

HK10MA3
HK30MA3
HK40MA3
HK10FM3
HK30FM3
HK40FM3
HK
MA or FM

Fig. 23. Western blot analysis of proteins produced in cells coinfected with HK and FM-MA. MDCK cells were coinfected with HK and FM-MA (MOI=3 each). Cell lysates or released virus lysates harvested at 2, 4, 6, 12 or 24 hr p.i. were resolved by SDS-PAGE and electroblotted. Western blot analysis was performed with a combination of anti-FM (1/125) and anti-HK (1/125) polyclonal antibodies followed by protein A-alkaline phosphatase incubation and colour development. Lysates from cells infected singly with HK or FM-MA (MA) for 4 hr were included as viral protein mobility references. Location of viral NP and M1 proteins are indicated on the left.


Cell
Released
HK MA 2 4 6 12 24 2 4 6 12 24
NP
M1

cells coinfected with HK and FM-MA at 0, 2, 3, 4, or 6 hr p.i. with [^{35}S]met,[^{35}S]cys. Infected cells were lysed immediately following labelling and subjected to SDS-PAGE (Fig. 24). At each timepoint, the rate of FM-MA M1 protein synthesis exceeded that of HK. Thus, FM-MA protein predominated in coinfected cells due to an increased rate of synthesis relative to HK rather than increased protein stability.

3.3.10. In single infections, FM-MA polymerase was more efficient than HK polymerase at primary transcription but is less efficient than HK polymerase later in infection

To determine whether dominance of FM-MA RNA in cells coinfected with HK arose from actual suppression of HK RNA synthesis by FM-MA or simply an increased level of FM-MA RNA synthesis relative to an unperturbed level of HK transcription and replication, the kinetics of HK, FM-MA and FM RNA accumulation in singly-infected cells were evaluated. MDCK cells were infected singly with HK, FM-MA or FM. Equivalent numbers of cells infected singly with HK or FM-MA were combined for RNA extraction. cDNA synthesis was performed using either a segment 5-specific primer that targetted vRNA (Fig. 25) or mRNA (Fig. 26) at the RT step. Following PCR, strain-specific restriction digestion, PAGE, autoradiography and scintillation counting were carried out to determine the relative efficiency of HK to FM-MA polymerases at each timepoint in singly infected cells. The results are presented as the ratio of strain-specific Ava II-digested PCR products (Fig. 25B; Fig. 26B). HK and FM polymerase efficiencies were similarly

Fig. 24. Pulse-labelling of proteins produced in cells coinfected with HK and FM-MA. MDCK cells coinfected with HK and FM-MA (MOI=3, each virus) were pulse-labelled for 1 hr following virus adsorption (t=0) or at 2, 3, 4 or 6 hr p.i. in methionine/cysteine-free DMEM containing 40 μCi/ml [^{35}S]methionine, [^{35}S]cysteine. Infected cells were lysed immediately following labelling and subjected to SDS-PAGE in 12.5% acrylamide with a 3.75% stacking gel prior to autoradiography. Cells infected singly with FM-MA (MA) or HK (MOI=3) were pulse labelled for 1 hr at 4 hr p.i.; these lysates were included in the PAGE analysis. Time of pulse-labelling is indicated (in hr p.i.) at top of autoradiograph. Viral proteins are identified on the left.

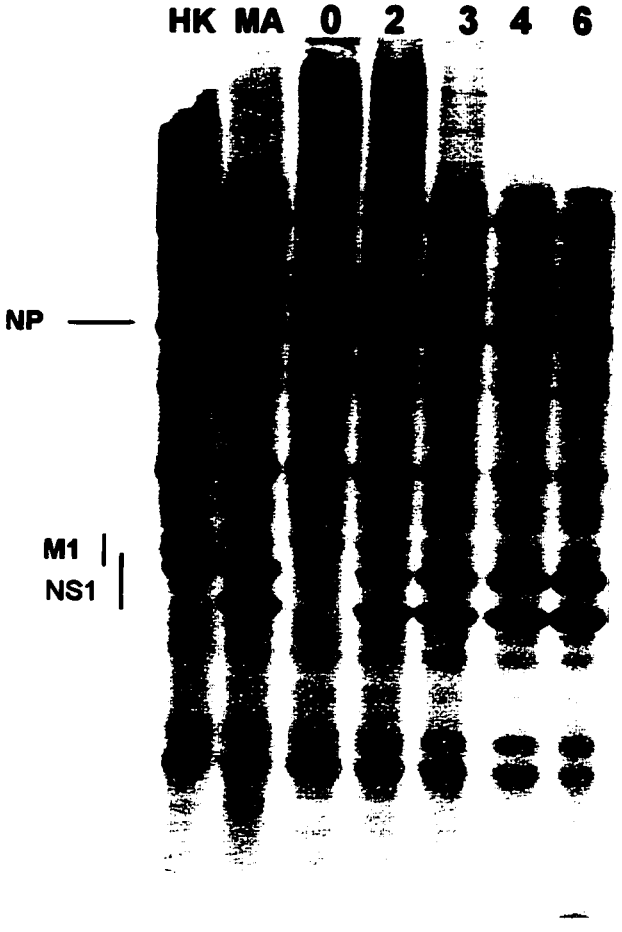
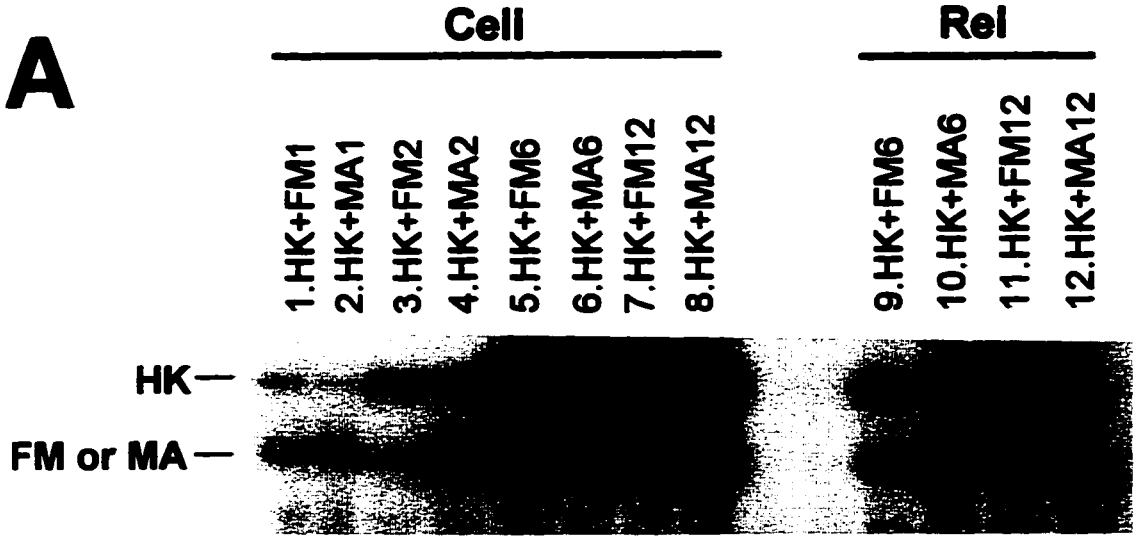

HK
MA
0
2
3
4
6
NP
M1
NS1

FIG. 25. Relative levels of HK, FM-MA and FM vRNA in singly-infected MDCK cells.

A. MDCK cells were infected singly (MOI=3) with HK, FM-MA (MA) or FM for 1, 2, 6 or 12 hr. Total RNA was extracted from pooled samples of singly-infected cells (10^7 cells for each single infection) or equal volumes of culture supernatants from two single infections and subjected to reverse transcription using a segment 5 primer (**Table 1**) specific for vRNA followed by PCR amplification, Ava II digestion, PAGE and autoradiography as described in **Fig. 15**.

B. Relative levels of FM-MA (M) to HK (H) or FM (F) to HK vRNA in each pooled cell or released virion sample were calculated as the ratio of strain-specific restriction-digested [^{32}P]-labelled PCR products.


A
Cell
Rel
1.HK+FM1
2.HK+MA1
3.HK+FM2
4.HK+MA2
5.HK+FM6
6.HK+MA6
7.HK+FM12
8.HK+MA12
9.HK+FM6
10.HK+MA6
11.HK+FM12
12.HK+MA12
HK
FM or MA

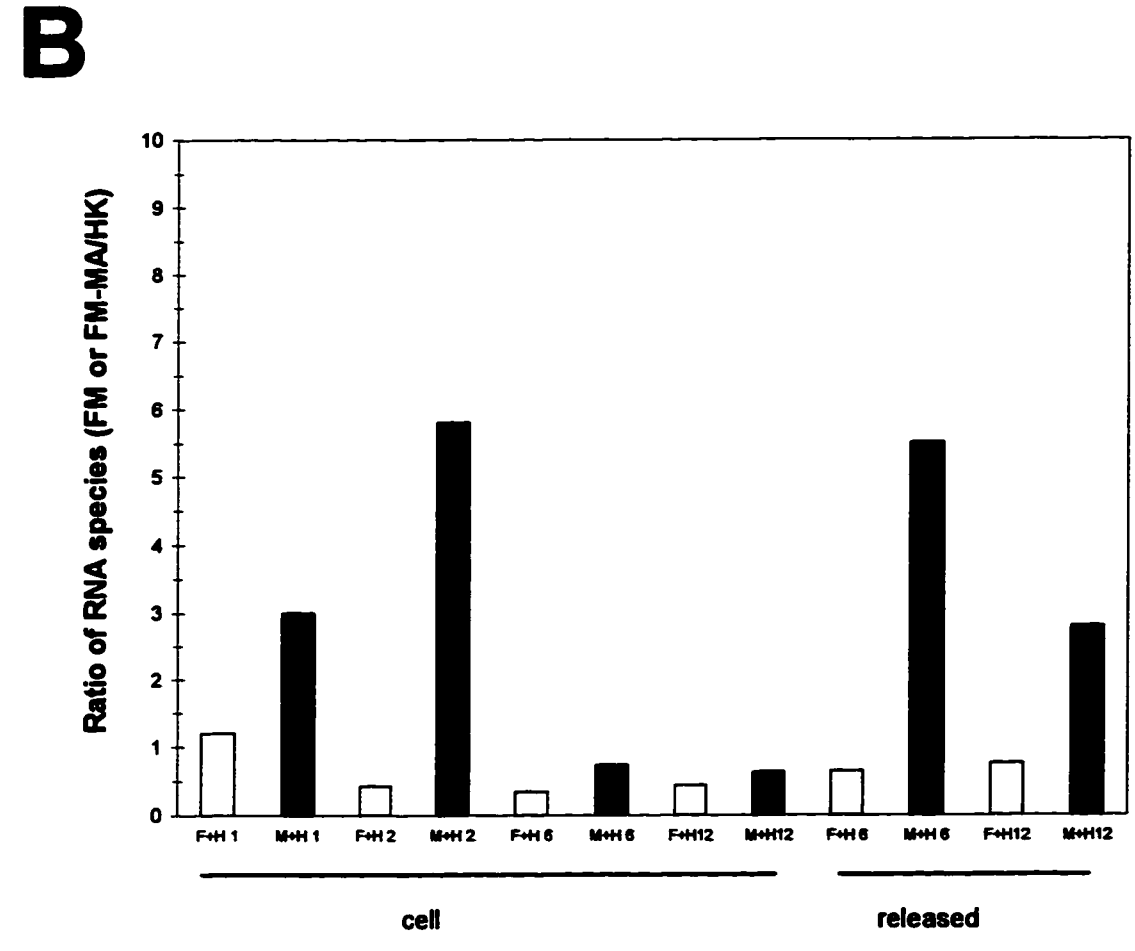
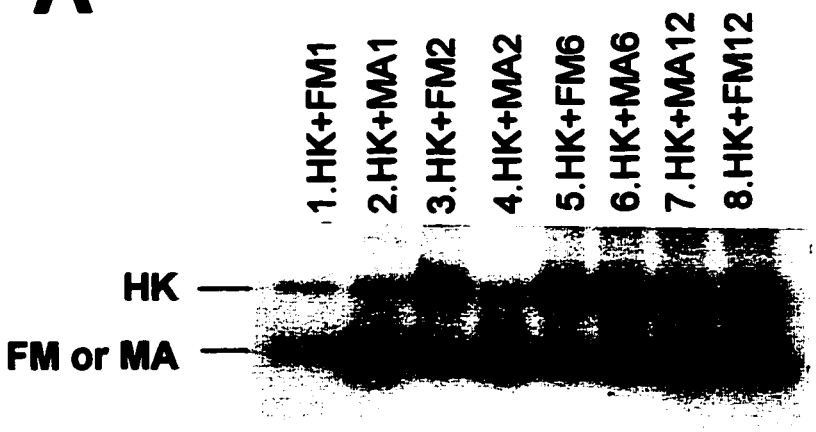

B
Ratio of RNA species (FM or FM-MA/HK)
0
1
2
3
4
5
6
7
8
9
10
F+H 1
M+H 1
F+H 2
M+H 2
F+H 6
M+H 6
F+H12
M+H12
F+H 6
M+H 6
F+H12
M+H12
cell
released

Fig. 26. Relative levels of HK, FM-MA and FM mRNA in singly-infected MDCK cells.

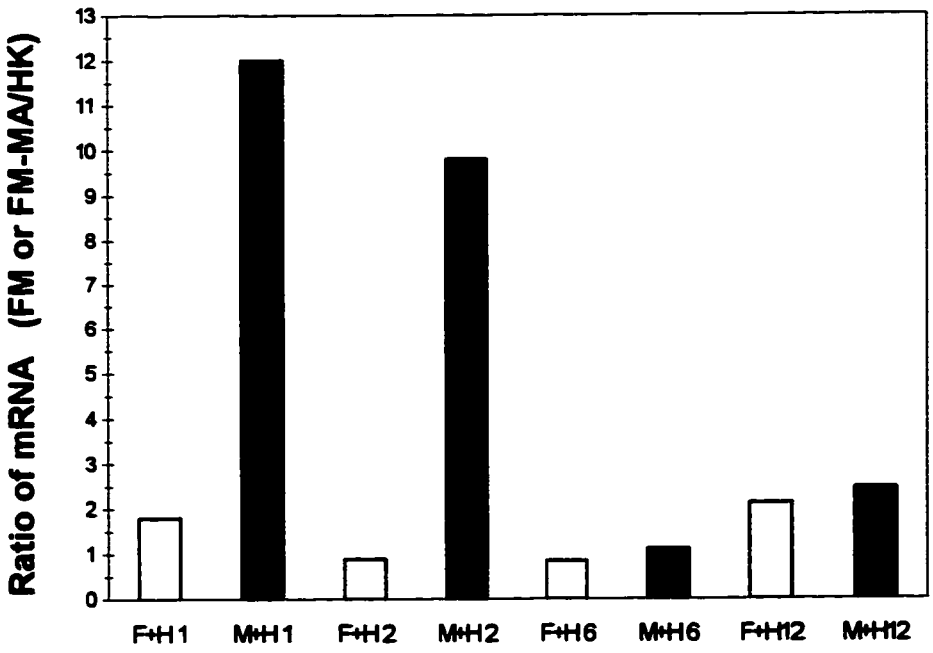
A. MDCK cells were infected singly (MOI=3) with HK, FM-MA (MA) or FM for 1, 2, 6 or 12 hr. Total RNA was extracted from pooled samples of singly-infected cells (10^7 cells for each single infection) or equal volumes of culture supernatants from two single infections and subjected to reverse transcription using a segment 5 primer specific for mRNA (**Table 1**) followed by PCR amplification, Ava II digestion, PAGE and autoradiography as described in **Fig 15**.

B. Relative levels of FM-MA (M) to HK (H) or FM (F) to HK mRNA in each pooled sample were calculated as the ratio of strain-specific restriction-digested [^{32}P]-labelled PCR products.

A


1.HK+FM1
2.HK+MA1
3.HK+FM2
4.HK+MA2
5.HK+FM6
6.HK+MA6
7.HK+MA12
8.HK+FM12
HK
FM or MA

B


Ratio of mRNA (FM or FM-MA/HK)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
F+H1
M+H1
F+H2
M+H2
F+H6
M+H6
F+H12
M+H12

compared.

HK, FM-MA and FM polymerases have different RNA synthesis profiles over the course of a single virus infection. FM-MA produced 12X more NP mRNA (Fig. 26A, lane 2) and 3X more NP vRNA (Fig. 25A, lane 2) than did HK early in infection (1 hr p.i.). FM did not show this relative increase in early transcription or replication efficiency. The superior polymerase activity of FM-MA was not maintained throughout infection since by 6 or 12 hr p.i. HK had generated more vRNA than either FM or FM-MA (Fig. 25A, lanes 5-8). Thus, over the course of a single virus infection, HK is more efficient at generating vRNA molecules than FM-MA. In a coinfection with FM-MA, HK does not demonstrate this efficiency suggesting that the two viruses interact in a coinfected cell in a manner that compromises HK transcription and replication.

The comparison of RNA accumulation kinetics during single infections also showed that, although HK synthesized more intracellular vRNA than did FM-MA by 6 and 12 hour p.i., FM-MA released more vRNA than HK (Fig. 25A, lanes 10 and 12). The preferential assembly of FM-MA vRNA relative to HK in coinfected cells has already been noted (Fig. 21). Because the kinetics of virion production in cells infected singly with HK or FM-MA also favoured FM-MA, the enhanced assembly efficiency of FM-MA appears to be intrinsic to the mutant rather than resulting from a competition with HK during assembly in coinfected cells.

To confirm that the mutant polymerase is inherently more efficient at primary transcription than are HK or FM polymerases, the individual primary transcription

efficiencies of HK, FM-MA and FM polymerases were evaluated in cells infected singly with each virus in the presence of cycloheximide. Equal numbers of cells infected singly with HK or FM-MA were pooled as described above. RNA extracted from these pools was used for segment 5-specific RT-PCR (Fig. 27). FM-MA synthesized more mRNA than did HK by 1 hr (2.3X) and 2 hr (3X) p.i.. HK and FM primary transcription were approximately equal at these timepoints. Thus, the FM-MA polymerase is more active than HK polymerase during primary transcription.

3.3.11. FM-MA's enhanced RNA synthesis phenotype mapped to segment 2.

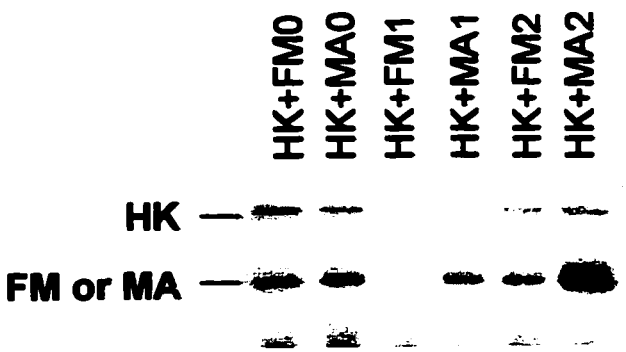
FM-MA contains mutations in segments 1, 2, 4, 6 and 7 relative to the parent, FM (Smeenk and Brown, 1994; Smeenk et al, 1996). Interference was genetically mapped to only one of these, the PB1 gene (segment 2) (Brown et al., 1992). A possible role for segment 8 (NS1,2) was also indicated (Brown et al., 1992) however no changes were found in the FM-MA NS genes relative to FM. To confirm that the enhanced RNA synthesis phenotype of FM-MA described above likewise mapped to the FM-MA PB1 gene, HK x FM-MA reassortants were chosen that each contained a mutant PB1 gene derived from FM-MA but which possessed different combinations of the remaining segments derived from FM-MA and HK (Table 4A). Each of these was tested for its ability to demonstrate enhanced RNA synthesis in a coinfection with HK. The system of RT-PCR followed by strain-specific digestion used in this determination required that the coinfecting viruses possess distinct alleles. Therefore, depending on the composition of the reassortant being assessed either segment 7 (Fig. 28A, lanes 1-4) or segment 5 (lanes 6-12)

Fig. 27. Relative levels of HK, FM-MA and FM primary transcripts in singly-infected MDCK cells.

A. MDCK cells were infected singly (MOI=3) with HK, FM-MA (MA) or FM in the presence of 100 µg/ml of cycloheximide. Total RNA was isolated from pooled infected cell samples following 1 hr adsorption (t=0) or at 1 or 2 hr p.i. and subjected to segment 5-specific RT-PCR followed by strain-specific Ava II digestion, PAGE and autoradiography as previously described (**Fig. 15**).

B. Relative levels of FM-MA (M) to HK (H) or FM (F) to HK primary transcripts in each pooled sample were calculated as the ratio of strain-specific restriction-digested [^{32}P]-labelled PCR products.

A


HK+FM0
HK+MA0
HK+FM1
HK+MA1
HK+FM2
HK+MA2
HK
FM or MA

B

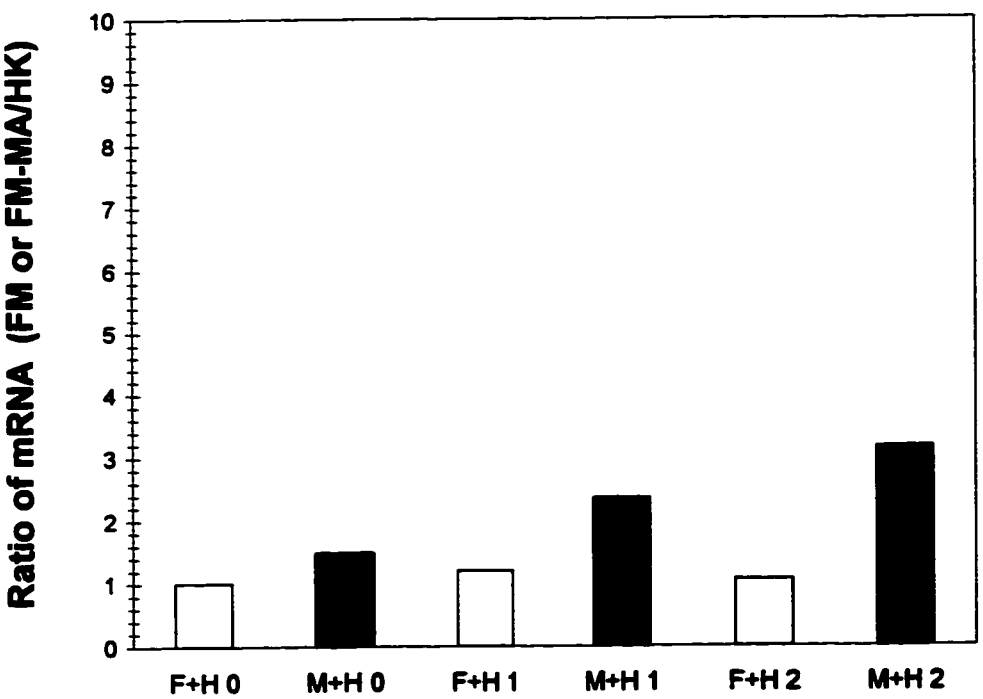

Ratio of mRNA (FM or FM-MA/HK)
10
9
8
7
6
5
4
3
2
1
0
F+H 0
M+H 0
F+H 1
M+H 1
F+H 2
M+H 2

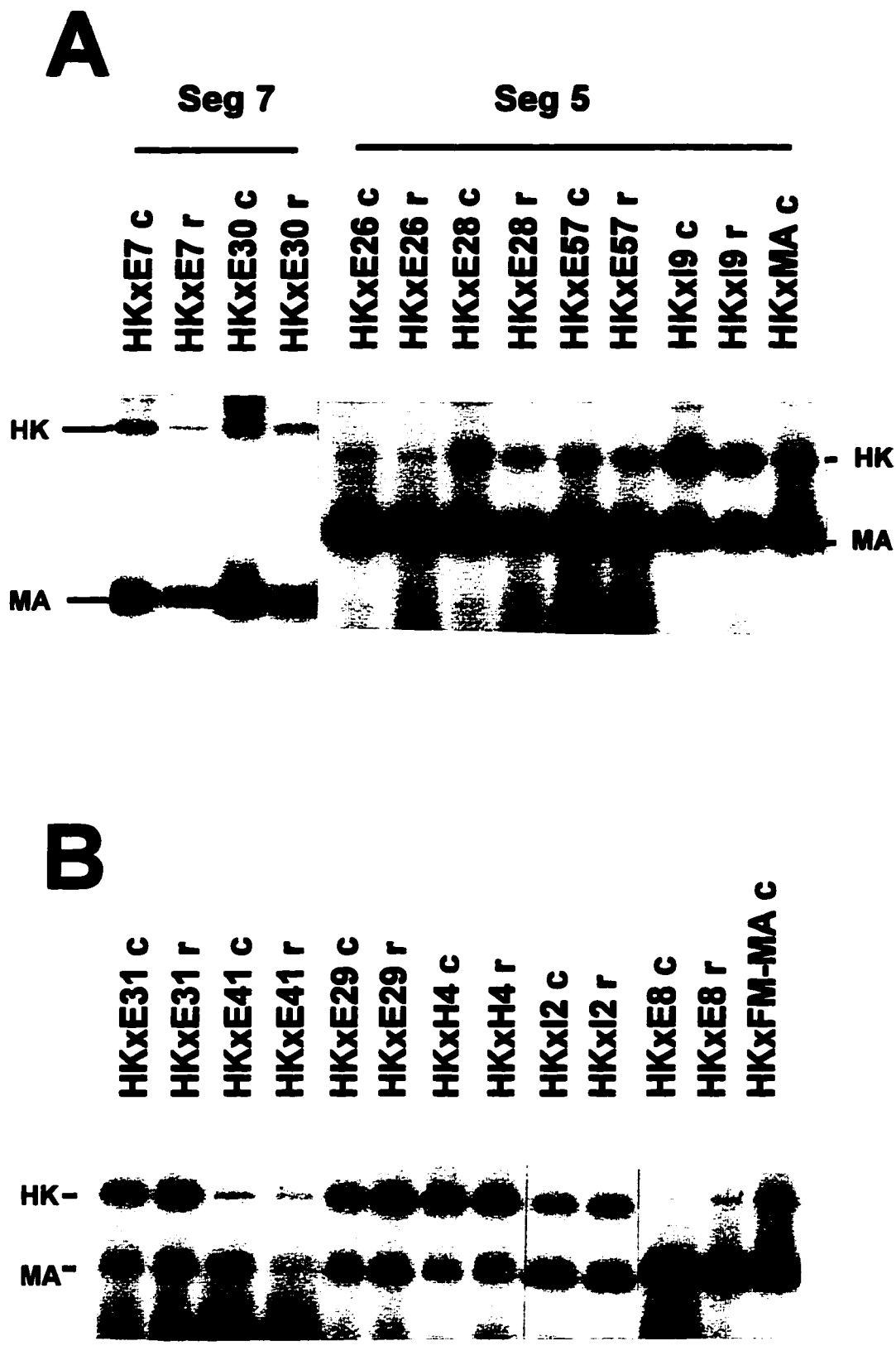
TABLE 4. Origin of RNA segments in HK x FM-MA reassortants

	Segment:	1	2	3	4	5	6	7	8
	FM-MA	M	M	M	M	M	M	M	M
	HK	H	H	H	H	H	H	H	H
A	E7	H	M	M	H	H	M	M	M
	E30	M	M	M	M	H	H	M	M
	E26	H	M	M	M	M	H	M	M
	E28	M	M	H	M	M	H	M	H
	E57	M	M	H	M	M	H	H	M
	I9	H	M	H	M	M	M	H	H
B	H4	M	H	M	M	M	M	M	M
	E31	H	H	M	M	M	H	M	M
	E41	H	H	H	M	M	H	M	H
	I2	M	H	H	M	M	M	M	H
	E29	H	H	H	M	M	H	H	M
	E8	M	H	H	H	M	M	H	M
C	A01	M	M	M	H	M	M	H	M
	E45	H	H	H	M	H	H	M	M
	H11	H	H	H	H	H	H	M	H

Fig. 28. Relative levels of HK and FM-MA vRNA in cells coinfected with HK and HK x FM-MA reassortants.

A. Total cellular RNA (c) or released virion RNA (r) isolated from MDCK cells coinfected with HK and FM-MA (MA) segment 2-containing reassortants (**Table 4A**) was reverse transcribed using segment 7 (lanes 1-4) or segment 5 (lanes 5-13)-specific vRNA primers followed by PCR and strain-specific restriction digestion as described in **Fig. 15** to determine relative levels of HK and FM-MA-specific RNA.

B. Relative levels of HK and FM-MA segment 5 vRNA were likewise determined in cells coinfected with HK and HK segment 2-containing reassortants (**Table 4B**).


A
Seg 7
Seg 5
HKxE7 c
HKxE7 r
HKxE30 c
HKxE30 r
HKxE26 c
HKxE26 r
HKxE28 c
HKxE28 r
HKxE57 c
HKxE57 r
HKxI9 c
HKxI9 r
HKxMA c
HK
MA
HK
MA
B
HKxE31 c
HKxE31 r
HKxE41 c
HKxE41 r
HKxE29 c
HKxE29 r
HKxH4 c
HKxH4 r
HKxI2 c
HKxI2 r
HKxE8 c
HKxE8 r
HKxFM-MA c
HK-
MA-

was monitored to detect relative vRNA levels. Reassortants containing FM-MA PB1 all demonstrated enhanced RNA synthesis with one exception, I9. I9 retained the A-G substitution at nt 1634 relative to FM (see below, Fig. 31) but did not demonstrate the dominance phenotype (lanes 11 and 12). Intra- or extragenic mutation may have occurred during generation of I9 leading to suppression of the mutant phenotype (Brown, 1990).

HK x FM-MA reassortants possessing various FM-MA genes but containing HK PB1 (Table 4B) were likewise tested for their ability to interfere with HK during coinfection (Fig. 28B). With the exception of E8 (lanes 11 and 12) these reassortants did not demonstrate enhanced RNA synthesis relative to HK. Genotyping of E8 by RT-PCR confirmed the absence of the FM-MA segment 2 allele but revealed multiple subgenomic cDNA species of segment 2 (data not shown). E8 stock virus therefore may contain deletion mutants that may be defective interfering particles which could confer a dominant phenotype on coinfection with HK.

3.3.12. The mutant polymerase conferred enhanced RNA synthesis to a HK gene.

FM-MA polymerase selectively conferred enhanced RNA synthesis to its own genome segments in the presence of coinfecting HK virus. The possibility that this selective amplification was due to recognition by FM-MA polymerase of mutant promoter sequences could not be ruled out since amplification and cloning of FM-MA genes for the purpose of sequencing depended on primers homologous to the

5' and 3' termini (13 and 12 nt, respectively) that contain the promoters for viral RNA polymerase. Thus the FM-MA promoters have not been sequenced. To address the possibility that FM-MA-specific promoter usage by the mutant polymerase determined selective amplification of FM-MA genes in coinfected cells, A01, a reassortant encoding a FM-MA polymerase complex, was examined for its ability to confer enhanced RNA synthesis to a HK segment 7 component of this reassortant (Table 4C). Since detection of enhanced or repressed HK segment 7 RNA levels by RT-PCR followed by strain-specific restriction digestion required the use of a corresponding FM-MA allele, reassortants E45 and H11 possessing HK polymerase genes and a FM-MA segment 7 were selected for coinfection with A01 (Table 4C).

In cells coinfected with A01 and E45 or A01 and H11, the mutant polymerase encoded by A01 conferred enhanced RNA synthesis to its HK segment 7 allele (Fig. 29, lanes 1-4). RT-PCR products from a HK x FM-MA coinfection were included as a control for digestion (lane 5). These data show that **selective amplification of FM-MA genes by FM-MA polymerase during coinfection with HK does not depend on recognition of FM-MA-specific promoters by the mutant polymerase.** The ability of A01 to confer enhanced RNA synthesis to FM-MA segment 5 in these coinfections was also observed (data not shown).

A panel of FM x FM-MA reassortants was used to confirm that FM-MA's enhanced RNA synthesis phenotype mapped to FM-MA segment 2 and to assess the possible contribution(s) of other mutant genes to this phenotype. The

Fig. 29. Control of HK segment 7 levels by FM-MA polymerase. Relative levels of HK and FM-MA (MA) segment 7 vRNA in or released (rel) from cells coinfected with HK x FM-MA reassortants (**Table 4C**) were determined as described in **Fig. 16**.

Cell
Rel
A01xE45
A01xH11
A01xE45
A01xH11
HKxMA
HK
MA

reassortants selected for the experiment contained mutant genome segments 1, 2, 4, 6 and 7 in isolation or in combination on a predominantly FM background (Table 5). It should be noted that a single gene reassortant containing FM-MA segment 2 on a parental background was not available for study. Each reassortant was paired with HK in a mixed infection of MDCK cells. Relative levels of HK and FM segment 5 vRNA were determined in infected cellular RNA and released virions 12 hr p.i. by RT-PCR followed by Ava II digestion, PAGE and autoradiography (Fig.30). It was previously demonstrated (Fig. 29) that the mutant polymerase can confer enhanced RNA synthesis to a heterologous (HK) genome segment. Therefore it was assumed that reassortants possessing a mutant PB1 could likewise confer enhanced RNA synthesis to an associated FM segment 5 and that relative segment 5 RNA levels would be a suitable indicator of the RNA dominance phenotype in mixed infections.

The FM-MA segment 2-containing reassortants showed enhanced levels of segment 5 vRNA relative to HK both in the cell and in released virus (Fig. 30A: Z18, Z19, Z9). Reassortants containing a parental segment 2 in association with one or two of the 4 other possible mutant genome segments did not show this dominance in intracellular vRNA (Z16, Z5, Z7). Interestingly, two of these FM segment 2-containing reassortants (Z16, Z5) demonstrated preferential vRNA release relative to HK. Both of these possessed a mutant segment 6 (encoding NA).

Each reassortant used in this experiment was genotyped to confirm the parentage of the PB1 gene. Viral RNA from stocks used for infections was

Table 5. Origin of RNA segments in FM X FM-MA reassortants

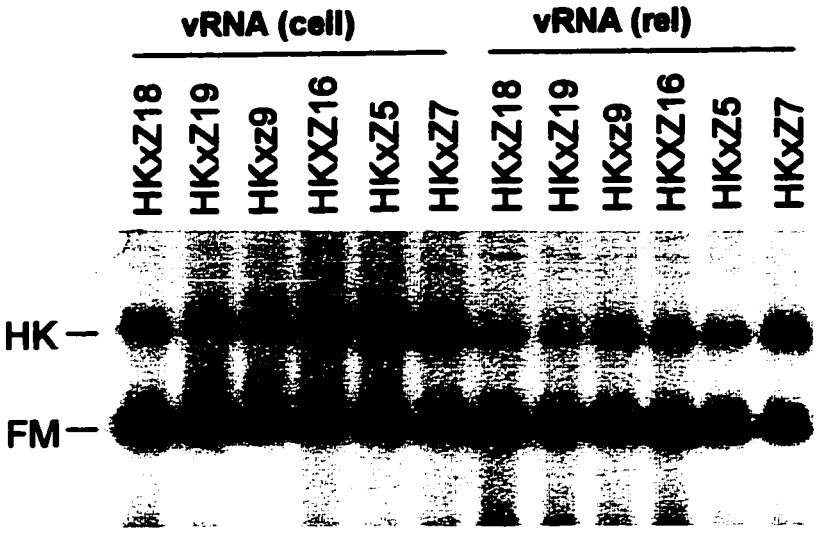
Seg	1	2	3	4	5	6	7	8
FM-MA	M	M	M	M	M	M	M	M
FM	F	F	F	F	F	F	F	F
z5	M	F	F	M	F	M	F	F
z7	M	F	F	F	F	F	M	F
z9	F	M	F	F	F	F	M	F
z16	F	F	F	F	F	M	F	F
z18	M	M	F	F	F	M	F	F
z19	M	M	F	F	F	M	F	F

Fig. 30. Relative levels of HK and FM segment 5 vRNA in cells coinfected with HK and FM x FM-MA reassortants.

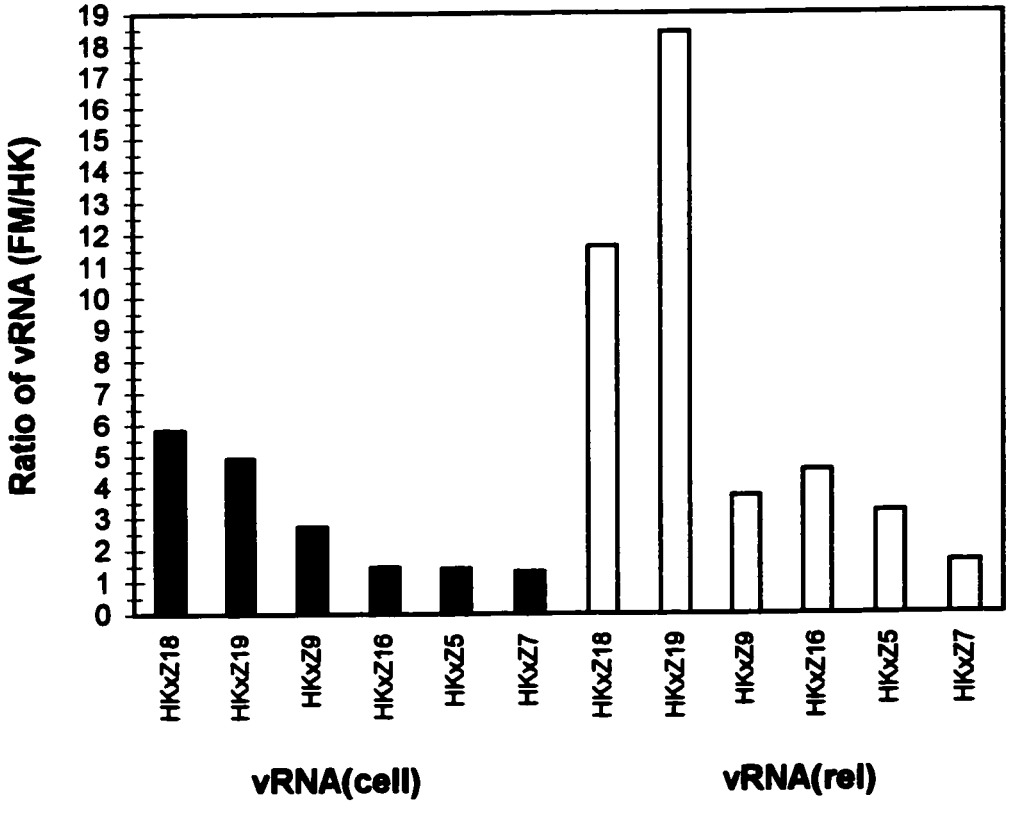
A. Total cellular RNA ($vRNA_{cell}$) or released virion RNA ($vRNA_{rel}$) isolated from MDCK cells coinfected (MOI=3, each virus) for 12 hr with HK and FM x FM-MA reassortants (**Table 5**) was reverse transcribed using a segment 5-specific vRNA primer followed by PCR and strain-specific restriction digestion as described in **Fig. 15** to determine relative levels of HK and FM-specific RNA. Identity of virus-specific Ava II products is indicated at left of autoradiograph.

B. Relative levels of HK and FM segment 5 vRNA were determined by scintillation counting of excised virus-specific Ava II products.

A


vRNA (cell)
vRNA (rel)
HKxZ18
HKxZ19
HKxz9
HKXZ16
HKxZ5
HKxZ7
HKxZ18
HKxZ19
HKxz9
HKXZ16
HKxZ5
HKxZ7
HK
FM

B


Ratio of vRNA (FM/HK)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
HKxZ18
HKxZ19
HKxZ9
HKxZ16
HKxZ5
HKxZ7
HKxZ18
HKxZ19
HKxZ9
HKxZ16
HKxZ5
HKxZ7
vRNA(cell)
vRNA(rel)

extracted and amplified by RT-PCR using primers flanking a strain-specific Dpn II restriction enzyme site present only in FM cDNAs. Diagnostic enzyme digestion of products with Dpn II confirmed the presence of the FM PB1 gene in 3 reassortants, Z16, Z5, Z7 (Fig. 31, lanes 4-6), including 2 (Z16, Z5) which demonstrated preferential vRNA release relative to HK in mixed infections (Fig. 30A, lanes 10 and 11).

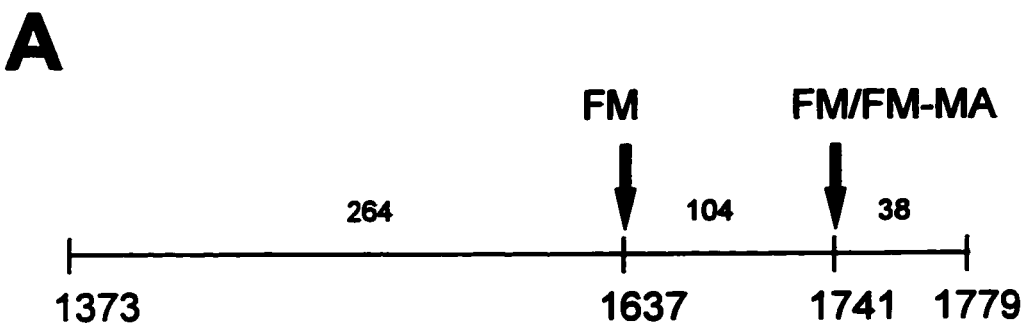
3.3.13. In vitro synthesis of RNA by FM and FM-MA.

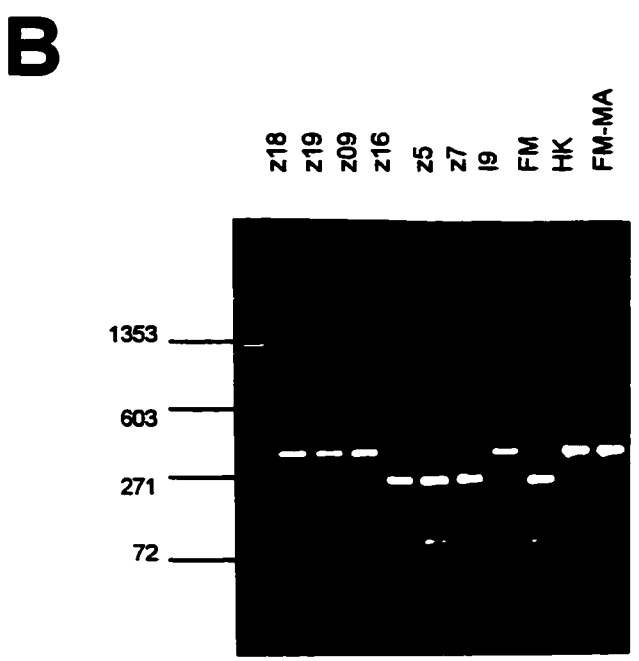
HK and FM-MA were shown to possess different kinetics of RNA synthesis in single virus infections (Figures 25-27). FM-MA out-polymerized HK early in infection (<2 hr p.i.) while HK polymerized more RNA than FM-MA by 6 hr p.i. and sustained its superior polymerase activity over the course of infection. FM did not demonstrate enhanced RNA synthesis relative to HK early in infection. It was of interest to know whether the change in FM-MA PB1 modified polymerase activity relative to FM polymerase leading to potentially critical differences in polymerase kinetics during infection. To better dissect the processes of RNA synthesis, an in vitro system was established using enzymatically active RNP from infected cells. FM and FM-MA polymerase activities were examined in vitro to critically compare the kinetics of RNA synthesis. Manipulation of the essential components of the enzyme assay system (pH, temperature, divalent cation) would then allow establishment of conditions of optimum polymerase activity for both FM and FM-MA. Any differences in polymerase kinetics or optimum conditions may be due to the FM-MA mutations in PB1 or PB2.

Fig. 31. Genotyping of FM x FM-MA reassortants to determine parental origin of segment 2.

A. FM x FM-MA reassortants **(Table 5)** used in previous experiment **(Fig. 30)** were genotyped by RT-PCR. RNA from viral stocks were extracted and subjected to RT-PCR using segment 2-specific primers flanking a Dpn II restriction enzyme site unique to FM.

B. Following digestion with Dpn II, RT-PCR products were subjected to electrophoresis in 2% agarose containing ethidium bromide. Reassortants are identified at the top of gel; sizes shown at left (in nt).

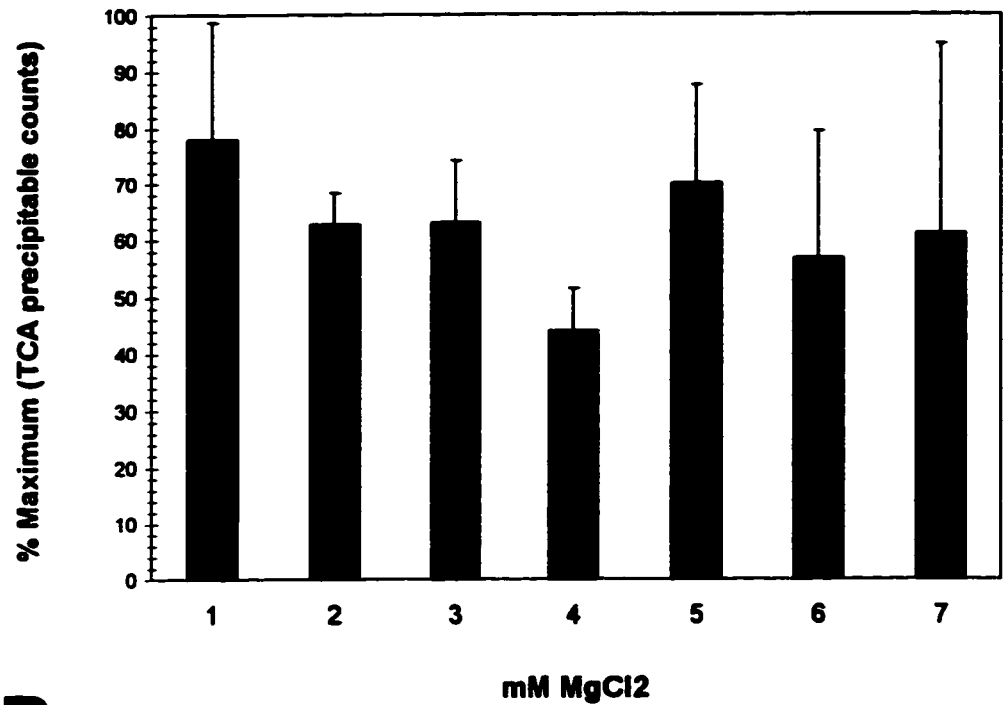

A
FM
FM/FM-MA
264
104
38
1373
1637
1741
1779


B
z18
z19
z09
z16
z5
z7
I9
FM
HK
FM-MA
1353
603
271
72

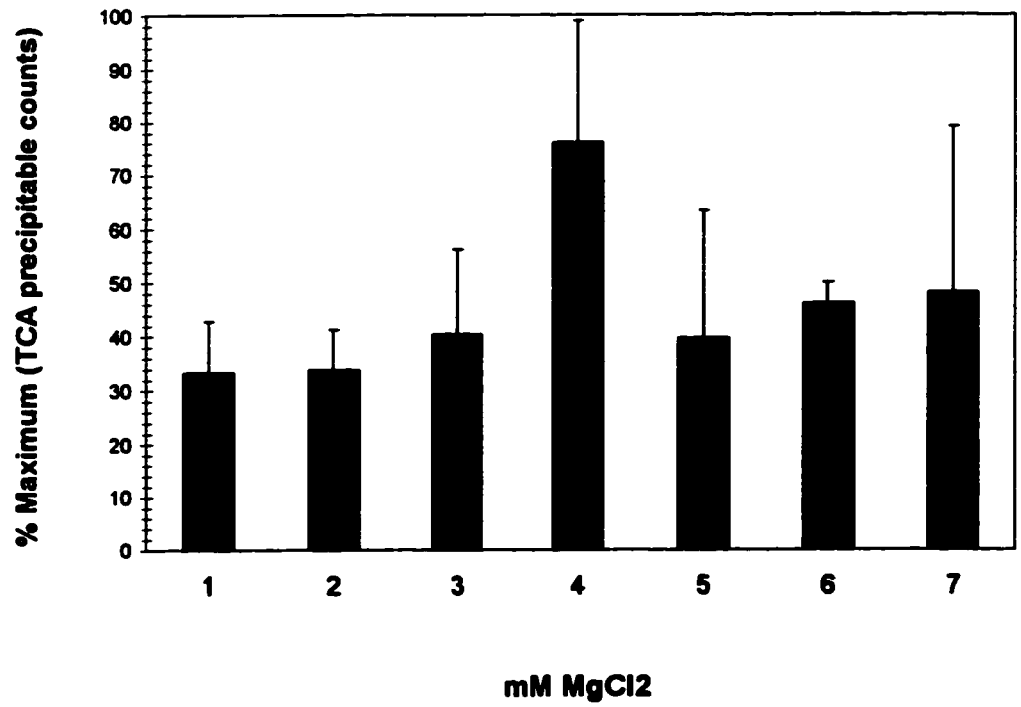
Viral RNP was isolated following NP40-lysis of MDCK cells infected singly with FM or FM-MA. The RNP was partially purified by centrifugation over a 15% glycerol cushion. The RNA polymerase activities of pelleted RNP preparations were determined in vitro in ApG-primed reactions which incorporated [^{32}P]UTP. Following PAGE analysis of the reaction products, segment-sized and subgenomic transcripts were identified (data not shown). Polymerase activity was measured by scintillation counting of trichloroacetic acid precipitates of aliquots of the reaction mixtures. Enzymatic activities of FM and FM-MA polymerases were determined over a range of $MgCl_2$ concentrations (Fig. 32). In order to compare FM and FM-MA polymerase activities, enzymatic activity at each magnesium concentration was expressed as a % of the highest TCA count obtained for either FM or FM-MA polymerase in a given assay. FM-MA polymerase demonstrated a slightly higher overall enzymatic activity over the range of magnesium concentrations tested and an optimum concentration (1 mM) distinct from that of FM polymerase (4 mM).

Fig. 32. In vitro transcription using FM or FM-MA RNP from infected MDCK cells: optimization of $MgCl_2$. RNP (60 µg) from cells infected singly with FM-MA (**A**) or FM (**B**) was used in in vitro transcription reactions in the presence of [^{32}P]UTP and a range of $MgCl_2$ concentrations. Polymerase activity in each reaction was expressed as a % of the maximum TCA-precipitable counts in a given concentration range (one value of 100% was assigned to the highest activity produced by either FM or FM-MA in a given assay) plus or minus one standard deviation. Values represent the averages of 3 replicate concentration ranges.

A


% Maximum (TCA precipitable counts)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
mM MgCl2

B


% Maximum (TCA precipitable counts)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
mM MgCl2

DISCUSSION

Simultaneous infection of cells with 2 strains of influenza virus may result in growth inhibition of one of the viruses, a phenomenon termed viral interference. Interference between influenza viruses is well-documented. Although different interfering viruses have been reported to inhibit the growth of a coinfecting strain at various stages in infection, the precise mechanism(s) by which they do so have not been elaborated. Deconstructing the molecular events leading to viral interference is an important aim. Understanding how the highly regulated processes of viral transcription, replication and assembly are subverted by interfering viruses can be the means of identifying targets susceptible to antiviral intervention.

FM-MA, a mouse-adapted variant of a human influenza A virus, is dominant over homologous and heterologous wt strains in mixed infections. FM-MA was shown to be nondefective, growing to high titer in chicken allantoic cavity and mouse lung (Brown et al., 1992). Interference by FM-MA is defined by the repression of wt virus and the predominance (>99%) of FM-MA in the infectious viral yield of coinfected cells. The goal of this study was to determine the molecular mechanism of interference by FM-MA with a prototype wt virus, HK, during mixed infection of MDCK cells. Specifically, I attempted to identify the stage(s) at which FM-MA interacted with HK in a way that compromised HK growth. FM-MA's interference phenotype was originally mapped to the FM-MA PB1 polymerase

protein that possessed a single amino acid substitution at position 538 relative to the parent, FM (Brown et al., 1992). Since PB1 is the catalytic component of the viral polymerase complex, it was logical to focus the investigation on a measurement of the intracellular RNA levels of FM-MA and HK viruses during coinfection to identify any perturbation of HK RNA synthesis. Protein synthesis in the coinfected cells and released virus were also measured to complete the macromolecular profile. As a control, intracellular RNA and released virus were measured in cells coinfected with HK and the noninterfering parent, FM.

Cells infected with a single strain of influenza A virus contain eight different RNA genome segments. Each genome segment directs the synthesis of three classes of RNA molecules: (+) sense cRNA, mRNA and (-) sense vRNA. Therefore a cell infected with one strain of influenza A virus contains 27 distinct types of RNA molecules: 8 vRNA, 8 cRNA and 11 mRNA, due to splicing of segment 7 (M gene) and 8 (NS gene) transcripts. Many attempts have been made in the past at separate quantification of the three RNA species from the eight genome segments in cells infected with a single viral strain in order to determine the controls operating on influenza virus transcription and replication. These studies employed different quantification systems: **1.** PAGE analysis of double-stranded hybrids formed between purified vRNA and labelled polyA+/- RNA from infected cells (Hay et al., 1977; Barrett et al., 1979). **2.** Filter hybridization of labelled infected cellular RNA (polyA+/-) with immobilized, purified RNA or cDNA probes (Enami et al., 1985; Shapiro et al., 1987). **3.** RNase protection of infected cellular RNA with [^{32}P]-

labelled RNA probes of +/- polarity (Hatada et al., 1989). These varied approaches were laborious and produced incompatible profiles of viral RNA accumulation in infected cells, underlining the technical difficulty of viral RNA quantification.

Determination of the molecular mechanism of interference by FM-MA required individual measurement of the viral RNA species in cells infected with 2 different viral strains, FM-MA and HK. The technical challenges of this study included those inherent in previous investigators' attempts to measure influenza virus transcription and replication: **1. Distinguish the individual RNA genome segments. 2. Differentiate between the 3 RNA classes for a given genome segment.** My use of a mixed infection system introduced an additional technical challenge: **3. Differentiate FM-MA and HK RNA molecules.**

To measure individual levels of the three RNA classes expressed from selected genome segments in cells infected with FM-MA and HK, three approaches were attempted:

1. RNase protection of total RNA from infected cells with segment-specific, virus-specific [^{32}P]-labelled RNA probes. RNase protection was useful in distinguishing in vitro-synthesized HK and FM-MA (+) sense RNA transcripts. It also differentiated between in vivo-synthesized cRNA and mRNA for a given FM-MA segment. However, in my hands, this technique did not permit differentiation of in vivo-synthesized HK and FM-MA RNA molecules. Since virus-specific RNA detection was essential to my objective, this approach was not pursued.

2. Northern blot analysis of total RNA from virus-infected cells. This approach

allowed strain and segment-specific RNA detection using oligonucleotide probes targetting regions of nonhomology between HK and FM-MA. A quantitative comparison of HK and FM-MA RNA levels, however, was difficult since virus-specific RNA detection relied on oligonucleotides possessing distinct hybridization kinetics. Quantitative data on individual viruses provided by densitometric analyses of HK and FM-MA RNA over a 22 hr period could not be directly compared. Also, the resolution of RNA molecules required to allow differentiation between cRNA and mRNA molecules from a given virus genome segment was not achieved. This approach was therefore abandoned.

3. Measurement of the relative levels of HK and FM-MA RNA in coinfected cells using RT-PCR. RT-PCR was the basis for what proved to be the simplest approach to detecting individual viral RNA species. The class of RNA molecules to be amplified was determined in the RT step: each RT reaction included a primer specific for one of cRNA, mRNA or vRNA. Since the starting material for the RT reactions was total RNA from cells infected with both HK and FM-MA, it contained RNA from both strains. RT primers were complementary to sequence completely conserved between HK and FM-MA so that the probability of conversion to cDNA was equal for both viruses' RNA. PCR was then performed on an aliquot of the RT reaction, again using primers directed to regions of complete nucleotide homology between HK and FM-MA so that cDNAs of both strains were amplified equivalently. The products of PCR were double-stranded HK and double-stranded FM-MA cDNAs of equal length. The relative levels of virus-specific products were

determined by digestion of the PCR products with restriction enzymes chosen to give strain-specific restriction profiles. When PCR primers were labelled with [^{32}P], the relative levels of HK and FM-MA PCR products could be calculated as the ratio of scintillation counts for virus-specific restriction digestion products excised from PAGE gels. In a given RT-PCR reaction, HK and FM-MA RNA was amplified equivalently at both RT and PCR steps. Therefore, the ratio of virus-specific PCR products represented the relative virus-specific RNA levels in the starting material. The observation that virus-specific PCR products reflected the input vRNA ratios (Fig. 12) supported this foundational concept. An approach to the complete documentation of HK and FM-MA RNA levels during interference based on RT-PCR fulfilled the following requirements: **1. Genome segment- specific and RNA class-specific detection** (performed at the RT step). **2. Differentiation of virus-specific RNAs** (performed following PCR by strain-specific restriction enzyme digestion of PCR products). It should be noted that this system provided a system of relative rather than absolute quantification.

FM-MA dominated over HK at all levels of macromolecular synthesis.

RT-PCR was used to determine the relative RNA levels of HK and FM-MA for the 3 classes of RNA expressed from each of the 3 genes selected for study: segment 2 (PB1), segment 5 (NP) and segment 7 (M). In MDCK cells coinfected with HK and FM-MA, FM-MA accumulated more intracellular RNA (vRNA, cRNA and mRNA) and released more genomic RNA than did HK over a 24 hr period. By contrast, in cells coinfected with HK and the noninterfering parent, FM,

transcription, replication and virus release were approximately equal for the two strains. The two most noteworthy trends in these data were:

1. Among segments 2, 5 and 7, the magnitude of FM-MA dominance was segment-specific with FM-MA segment 7 showing the most dominance and segment 2 the least.

2. The magnitude of FM-MA dominance was not equal for the different types of RNA measured. Most notably, FM-MA dominance in released virion RNA was greater than in intracellular vRNA.

Both of these observations contributed to the construction of a model for FM-MA interference.

Independent confirmation of a preferential release of FM-MA viral RNA from cells coinfected with HK and FM-MA was provided by metabolically labelling infected cells with [^{32}P]. The segments monitored for interference in this experiment were those possessing strain-specific mobilities, namely segments 4, 6 and 8. Although these did not overlap with the segments monitored by RT-PCR (Segments 2, 5 and 7) this experiment did substantiate three observations made in the RT-PCR study:

1. FM-MA released more virion RNA than HK from cells coinfected with the two viruses.

2. FM-MA dominance in virion RNA released from coinfected cells was segment-specific. Although the released vRNA was not quantified in this experiment, a visual inspection of band intensities showed that the magnitude of FM-MA

dominance in released virion RNA was greater for segments 4 and 8 than for segment 6.

3. HK and the noninterfering parent, FM, released approximately equal (by visual inspection) amounts of virion RNA from coinfected cells.

FM-MA also synthesized and accumulated more protein than HK during a 24 hr coinfection of MDCK cells as shown by pulse-labelling and Western blot analyses, respectively. This suggests that FM-MA's enhanced RNA synthesis relative to HK during mixed infections (described above) established another aspect of its dominance in macromolecular synthesis- dominance in protein synthesis. The possible relationship between virus-specific protein levels and FM-MA's interference phenotype will be examined further in the discussion.

FM-MA dominance over HK involved an inhibition of HK macromolecular synthesis

A predominance of FM-MA RNA and protein in cells coinfected with HK and FM-MA may signify:

1. **A specific repression of HK macromolecular synthesis.**

2. **A superior FM-MA polymerase activity which allows the mutant to synthesize more RNA (and hence protein) relative to an unperturbed complement of HK macromolecules.**

3. **A combination of stimulated FM-MA polymerase activity and HK repression.** The first and third scenarios would involve an interaction between the two viruses in a coinfected cell.

To distinguish between these, viral RNA synthesis was monitored by RT-PCR in cells infected singly with HK or FM-MA. This was done specifically to examine the kinetics of RNA synthesis for each virus on its own. It was shown that, in the absence of a coinfecting virus, HK could synthesize more intracellular vRNA and mRNA molecules than FM-MA by 6 hr p.i. (Fig. 25). This suggests that the HK polymerase is not inherently less active than that of FM-MA over the course of infection. That HK was unable to synthesize more vRNA and mRNA during a coinfection with FM-MA indicates that the 2 viruses must have interacted in the coinfected cell in a way that compromised HK RNA synthesis leading to a repression in HK RNA accumulation.

The specific repression of HK was also demonstrated by comparing HK macromolecular synthesis in cells coinfected with HK and FM-MA relative to levels in cells infected with HK alone (ie. in the presence or absence of FM-MA):

1. Northern blot analysis (Fig. 7) showed that over a 24 hr period, HK segment 4 RNA (+ and -) in cells coinfected with FM-MA (but not FM) underwent increasing repression relative to levels in cells infected singly with HK.

2. Western blot analysis (Fig. 23) showed that in mixed infections with FM-MA, HK M1 protein levels were markedly reduced relative to M1 levels in cells infected singly with HK (4 hr p.i.). By contrast, FM-MA M1 levels were approximately equal in the presence or absence of HK.

Further data support the hypothesis that FM-MA dominance is not attributable to an inherently more active polymerase that allows it to synthesize

more RNA molecules and complete its infection cycle before HK. The effect of increasing the dose of HK relative to a constant level of FM-MA was examined (Fig. 22). Increasing the relative MOI of HK should shorten HK's replication cycle since, in these cells, HK will more quickly accumulate the level of NP required for transition from transcription to replication. However, FM-MA dominance was maintained even when the MOI of HK exceeded that of FM-MA by a factor of 13.3. This indicates that FM-MA interference could not be overcome by increasing the dose of HK and suggests that the interference involves a specific interaction of FM-MA and HK.

It is therefore possible to conclude from these various experiments that one aspect to FM-MA interference with HK is an inhibition of HK macromolecular synthesis. Given that interference was originally reported as a drastic repression of HK in the infectious viral yield of cells coinfected with HK and FM-MA relative to the yield of HK progeny from cells infected singly with HK (Brown et al., 1992), this was not surprising.

FM-MA dominance is due in part to enhanced primary transcription.

Given that FM-MA's interference phenotype maps to a mutation in PB1, the catalytic component of the influenza virus polymerase complex, it was possible that a second aspect of FM-MA dominance over HK in mixed infections was increased FM-MA polymerase activity due to the mutation. An examination of the relative RNA levels in cells infected singly with HK or FM-MA revealed several interesting aspects of each virus' inherent RNA synthesis capabilities in the absence of a

coinfecting strain. First, as noted above, HK was able to synthesize more vRNA than FM-MA by 6 hr p.i. (Fig. 25). However, FM-MA produced more vRNA (3X) and mRNA (12X) than did HK at 1 hr p.i. in these single virus infections (Figs. 25 and 26, respectively). This suggests that, in the absence of a coinfecting virus, FM-MA is inherently more efficient at some early event in RNA synthesis than HK.

To determine if this early efficiency was due to a greater capacity of FM-MA for primary transcription, single virus infections were performed in the presence of cycloheximide. Cycloheximide inhibits protein synthesis required for replication and hence secondary transcription. Incubation of infected cells in the presence of cycloheximide allows one to look at primary transcription in isolation. FM-MA showed a stronger primary transcription than HK, producing 2X more mRNA or 3X more mRNA than HK by 1 or 2 hr p.i., respectively (Fig. 27). This suggests that FM-MA polymerase is inherently more efficient at primary transcription than HK polymerase. The noninterfering parent, FM, did not show this superior primary transcription relative to HK.

It is again important to determine whether, during coinfection, FM-MA interacts with and inhibits HK during primary transcription or simply out polymerizes it. In single virus infections in the presence of cycloheximide, FM-MA produced 3X more primary transcripts than HK by 2 hr p.i. (Fig. 27). During a coinfection of MDCK cells with HK and FM-MA in the presence of cycloheximide, FM-MA predominated to an even greater extent, producing 5x more primary transcripts than HK by 2 hr p.i.. The fact that FM-MA dominance over HK at the level of primary

transcription was greater during coinfection than in a comparison of single virus infections points to a specific interaction of HK and FM-MA during primary transcription in coinfected cells. It is likely that FM-MA's slightly superior primary transcription efficiency (as seen in singly-infected cells) actually compromised HK primary transcription in coinfected cells.

The involvement of an event early in infection in the interference phenotype was confirmed by the observation that FM-MA's dominance in intracellular vRNA levels was decreased substantially or eliminated when coinfection of cells with FM-MA was delayed relative to HK by 1 or 2 hr, respectively (Fig. 21). Other examples of influenza viral interference manifested at the level of primary transcription are available. Coinfection of chicken embryo fibroblasts with Fowl Plague Virus and B/Japan/73 in the presence of cycloheximide yielded transcription of only one partner (Mikheeva and Ghendon, 1982). Heterotypic interference was also observed between A/WSN and B/Kanagawa; when infection of one virus preceded the other by 1-2 hr, primary transcription of the superinfecting virus was selectively inhibited (Aoki et al., 1984). Hypotheses to explain how preferential primary transcription of one partner in a mixed infection could occur or how it then manifested in interference with the growth of the other strain were not put forward by these investigators.

It appears that FM-MA has a slightly enhanced primary transcription activity relative to HK which, when the viruses are together in a mixed infection, causes a repression of HK primary transcription. The data do not allow precise identification

of the basis for FM-MA's enhanced primary transcription ability. FM-MA's apparent superior capacity for primary transcription may actually result from a more rapid uncoating of FM-MA genomes. The mutant polymerase may be more efficient at a step subsequent to uncoating relating more directly to polymerase function- possibly involving initiation or elongation of mRNA molecules. If there is a competition for cellular factors, such as capped primers, during one of these steps and FM-MA is more efficient at recruiting them, HK primary transcription could be compromised. If FM-MA competed more successfully than HK for a limiting factor required for primary transcription, this would explain why HK is repressed at this stage during a mixed infection with FM-MA. The segment-specific magnitude of interference with RNA synthesis (seg 2 < 5 < 7) may be established in the interaction between HK and FM-MA during primary transcription. Since primary transcription of influenza virus genes has been shown to be segment-specific (Hatada et al., 1989), it is possible that the magnitude of interference varies between segments due to different individual frequencies of reinitiation of mRNA transcription from vRNA.

The in vitro polymerase activities of FM and FM-MA RNP from singly-infected MDCK cells were compared in an attempt to detect any quantitative (magnitude of enzyme activity) or qualitative (optimum reaction conditions) differences in transcriptase activity between the mutant and parent polymerases (Fig. 32). The in vitro transcription assays compared FM and FM-MA polymerase activity over a range of $MgCl_2$ concentrations. Enzyme activity was measured as incorporation of

[^{32}P]UTP into TCA-precipitable RNA. FM and FM-MA polymerases demonstrated slightly different magnesium optima, suggesting that mutation in FM-MA genes may contribute to a biochemical difference in the enzyme complex. The overall activity of FM-MA polymerase (calculated as the average value over the $MgCl_2$ range for 3 replicate assays) was slightly greater than that of FM (62% vs 45%, respectively) although the activity of FM-MA (78%) at its optimum magnesium concentration was almost identical to FM's optimized activity (74.7%). The mutations in FM-MA genes thus conferred only slight (if any) enhancement of polymerase activity within the limited range of conditions attempted in these assays.

The interaction between FM-MA and HK during primary transcription lead to an overall repression of HK macromolecular synthesis in coinfected cells.

How does preferential synthesis of FM-MA mRNA during primary transcription then lead to a generalized repression of HK transcription, replication and protein synthesis during coinfection? A segment-specific impairment of HK primary transcription could lead to a dysregulation of HK protein levels. Influenza mRNA and protein levels normally show a strict segment-specific regulation with PB1, PA and PB2 synthesized in low amounts relative to NP and M1 that accumulate to a much higher level (Hatada et al., 1989; Krug et al., 1989). This regulation may maximize replication efficiency given that the polymerase proteins are only required in catalytic quantities whereas NP and M1 protein are required in large amounts in order to encapsidate vRNA. Any event which alters the timing or balance of viral gene expression could be detrimental to viral replication. Although

HK M1 protein synthesis was substantially suppressed relative to FM-MA in mixed infections, the HK-specific quantities of NP were reduced by at most 2 fold (Brown, 1990). Therefore HK protein stoichiometry was altered. Consistent with this, in cells coinfected with FM-MA and HK, interference at the level of mRNA synthesis was not equal for all HK segments (segment 2<5<7). Based on these data, it is therefore likely that the presence of FM-MA altered the balance of intracellular HK proteins.

There is abundant evidence that overexpression of one viral gene can have an inhibitory effect on viral replication. Increasing PA protein expression from cloned cDNA reduced the accumulation of co-expressed influenza proteins (Sanz-Ezquerro et al., 1995). Low levels of recombinant polymerase protein L of vesicular stomatitis virus (VSV) were able to complement L gene ts mutants whereas high levels did not, indicating that overexpression of the polymerase protein may be inhibitory (Meier et al., 1987). It was suggested that cells expressing high levels of recombinant L protein may not be able to efficiently assemble a functional polymerase complex since L protein is present in a large molar excess over the other polymerase protein, NS, and the ribonucleotide template. In our system, assuming that FM-MA proteins cannot function in a helper capacity as efficiently as homologous proteins in HK viral transcription and replication, HK protein dysregulation could compromise the overall efficiency of HK RNA synthesis and viral growth.

To address this latter point (FM-MA proteins may not function efficiently as

helper proteins for HK): there is some precedent for the concept that heterologous protein interactions are less stable than homologous combinations. Mixed infections of cells with two pathogenic avian influenza A strains resulted in the production of pathogenic recombinants only when polymerase and NP proteins were all encoded by one or the other wild-type parent and non-pathogenic recombinants possessed a mixed RNA polymerase complex (Rott et al., 1979). Genome segment complements which include P genes derived from the same parent strain are selected during influenza virus reassortment suggesting that they have a survival advantage (Lubeck et al., 1979). One possible interpretation is that heterologous protein combinations produce protein complexes with reduced activity or altered function and yield nonviable progeny (Lubeck et al., 1979).

Efficient virion assembly contributed to FM-MA dominance over HK in mixed infections

In addition to enhanced primary transcription, FM-MA also demonstrated enhanced virion assembly relative to HK. The evidence for this was as follows:

1. In mixed infections with HK, FM-MA predominated more in the viral yield than intracellularly suggesting a greater capacity to assemble virus.

2. When FM-MA infection was delayed relative to HK, which resulted in higher relative levels of HK RNA synthesis in coinfected cells, FM-MA genomes were still preferentially assembled into released virus. This indicated that despite a greater abundance of available HK vRNA molecules, FM-MA genomes were preferentially released from the cells as virions.

This is supported by experiments conducted on cells infected singly with HK or FM-MA:

3. By 6 or 12 hr p.i., HK (in the absence of FM-MA) accumulated more intracellular vRNA than FM-MA did under the same conditions of infection (Fig. 25). Yet, at these two timepoints, FM-MA released more vRNA from singly-infected cells than HK. This suggests that FM-MA is more efficient than HK at virion assembly and/or release. Furthermore, this efficiency was inherent to FM-MA rather than resulting from a competition with HK during assembly from coinfected cells since it was demonstrated by FM-MA both in the presence and absence of a coinfecting HK. The noninterfering parent, FM, did not display enhanced viral assembly relative to HK in single viral infections (Fig. 25).

Dominance of FM-MA in mixed infections with HK thus involves two distinct events:

1. Enhanced primary transcription by FM-MA leading to enhanced RNA and protein accumulation relative to HK.

2. Enhanced viral assembly relative to HK.

These two phenotypes could be uncoupled: FM-MA lost its enhanced RNA synthesis advantage in a delayed superinfection of HK-infected cells with FM-MA resulting in a predominance of intracellular HK RNA while retaining its dominance in the viral yield due to its enhanced assembly efficiency (Fig. 21). Both phenotypes were manifested by FM-MA but not the parent, FM, indicating that both mapped to one of the 5 genes (segments 1, 2, 4, 6 and 7) mutated relative to the

parent, but not necessarily to the same gene. It was therefore of interest to determine which mutant genes were responsible for FM-MA's phenotypic characteristics. FM-MA's enhanced RNA synthesis phenotype was shown to cosegregate with the FM-MA PB1 (segment 2) gene in mapping studies using HK x FM-MA (Fig. 28) and FM x FM-MA (Fig. 30) reassortants. Since interference was originally mapped genetically to FM-MA segment 2 (PB1) (Brown et al., 1992), this was not unexpected. However, assembly efficiency did not cosegregate with FM-MA segment 2 in FM x FM-MA reassortants: two FM segment 2-containing reassortants showed the preferential assembly phenotype in coinfections with HK (Fig. 30). This suggests that FM-MA segment 2 (PB1) is not required for preferential virus release. These two FM segment 2-containing reassortants both possess a mutated segment 6 (NA). NA catalyzes the cleavage of sialic acid receptors on host cells thereby permitting the release of progeny virus particles from infected cells. It is conceivable that mutation in the FM-MA NA gene (as yet uncharacterized) may confer enhanced elution of FM-MA virus from infected cells. The precise events in virus assembly are still unknown, thus it is not possible to determine how FM-MA NA specifically recognizes and releases its own progeny from a cell coinfected with another strain. A more thorough examination of available reassortants is required to conclusively map this phenotype. However, the fact that a single gene reassortant (Z16) possessing the FM-MA NA gene was capable of enhanced viral assembly in mixed infections with HK is strong evidence that the phenotype maps to the mutant NA gene.

FM-MA's enhanced RNA synthesis phenotype manifested in dominant intracellular RNA accumulation (cRNA, mRNA and vRNA) for multiple segments relative to a coinfecting virus, HK. This phenotype was mapped to FM-MA segment 2 (PB1). An important biological question arises from these data. How does a virus with a mutated polymerase complex selectively amplify its own RNA in the context of a coinfecting virus? Even if the FM-MA polymerase is more efficient than the HK polymerase in some aspect of primary transcription, what directs it to preferentially synthesize its own and not HK RNA during secondary transcription and replication? There are at least three possible models to account for FM-MA-specific RNA synthesis in a mixed infection:

1. **FM-MA genome segments contain a determinant for recognition by the FM-MA polymerase complex.** It is possible the FM-MA polymerase is directed to amplify FM-MA-specific RNA (mRNA, vRNA and cRNA) by a structural feature on the RNA molecules themselves. Since the recognition determinant would have to be present in all eight genome segments, it would most likely occur in the only elements common to all eight segments- the promoters (consisting of the 13 and 12 nt at the 5' and 3' vRNA termini, respectively).

2. **There is a physical segregation of HK and FM-MA infections within the cell.** In this scenario FM-MA-specific replication and secondary transcription could be explained by de novo-synthesized FM-MA polymerase complex entering a compartment of the nucleus populated only by FM-MA RNA molecules. Any advantage accrued by FM-MA during primary transcription and resulting in

increased protein production would then be specifically transferred to FM-MA cRNA, vRNA and mRNA synthesis.

3. The ability of FM-MA to "choose" its own genes for RNA synthesis may be predetermined by the association of the mutant polymerase with FM-MA genome segments in the virion. This association could clearly direct selectivity in FM-MA primary transcription since the polymerase is prebound to FM-MA segments and need not distinguish these from HK genes. Preferential enhanced replication of FM-MA genes by the mutant polymerase is less easily explained but may likewise arise from cRNA synthesis by input FM-MA polymerase if it were still bound to genomic RNA after repeated cycles of transcription. Enhanced vRNA and secondary transcript levels may then result simply from indiscriminate RNA synthesis off a more abundant pool of FM-MA template RNAs by de novo synthesized FM-MA or HK polymerase complexes.

Only the first of these models (FM-MA-specific RNA synthesis due to promoter recognition) was testable using available tools. The FM-MA polymerase encoded by a HK x FM-MA reassortant conferred enhanced RNA synthesis to the heterologous HK segment 7 component of this reassortant. This indicates that the ability of FM-MA polymerase to selectively transcribe and replicate its own genes in the presence of a coinfecting virus does not derive from the recognition of a FM-MA-specific promoter by the mutant polymerase.

There are some observations which may support the second model (FM-MA-specific RNA synthesis in mixed infections with HK due to segregation of infecting

viruses). Cellular RNA synthesis has been shown to occur in a subnuclear structure termed the "nuclear cage" composed of a flexible tangle of DNA fibers, RNA and protein (Jackson and McCready, 1981). This structure is also the site of influenza virus RNA synthesis (Jackson et al., 1982). It has been suggested that attachment of viral RNA to nuclear cage components could provide a structural basis for the tightly coordinated processes of influenza virus RNA transcription and replication from eight separate genome segments (Jackson et al., 1982). It is possible, then, that FM-MA and HK maintain separate infections in the nucleus because they occupy distinct physical spaces defined by such nuclear substructures. Physical segregation of influenza virus genomes within a cell could also explain why mixed infections typically yield a majority (75%) of parental types and a minority of reassortants instead of equal numbers of the 256 possible combinations of the 2 genome sets expected if the 2 viruses were interacting in a random interdispersed manner (Brown, 1990).

In this study I have shown that interference by FM-MA with a coinfecting wt virus, HK, is due to FM-MA's enhanced primary transcription, resulting in preferential RNA and protein accumulation and its enhanced viral assembly. The enhanced RNA synthesis phenotype was mapped to the PB1 polymerase gene of FM-MA. The identification of a novel site on PB1 affecting polymerase activity offers new possibilities for the analysis of polymerase function. It also identifies a potential virus-specific target for therapeutic intervention which does not affect host function. The data presented in this study also disclose other investigatable

antiviral approaches. The possibility that an alteration of viral protein stoichiometry leads to diminished virus growth could be exploited as a general approach to the development of prophylactic or therapeutic measures. Simply overexpressing a heterologous or mutated (Inokuchi and Hirashima, 1987) protein may "immunize" cells against influenza virus multiplication or treat those already infected thus offering an alternative to antiviral therapy.

This research may contribute to another application of viral interference in the development of a live, attenuated virus vaccine. It has been suggested that interference is a desirable quality in a live vaccine (Whitaker-Dowling et al., 1990). This relates to the theoretical potential of an attenuated, interfering virus vaccine to function both in protection against infection and in treatment of acute infections due to its ability to inhibit an epidemic strain. An interfering avirulent reassortant of FM-MA x HK protected mice against fatal influenza when coinfected intranasally with a virulent strain (Brown et al., 1992). The application of such interfering reassortants as therapeutic vaccines may be quite broad since FM-MA was shown to interfere with H3N2, H1N1 and H2N2 influenza subtypes (Brown et al., 1992).

CONCLUSIONS

1. FM-MA demonstrated enhanced primary transcription relative to HK. This may be the event which initiated FM-MA dominance over HK. FM-MA dominance manifested at all stages of macromolecular synthesis (primary

and secondary transcription, genomic RNA, complementary RNA, and protein) in cells coinfected with FM-MA and HK.

2. FM-MA dominance over HK in mixed infections resulted from a combination of enhanced FM-MA RNA synthesis and a repression of HK macromolecular synthesis.

3. FM-MA demonstrated enhanced assembly of viral genomes, a phenotype which was distinct from its capacity for enhanced RNA synthesis.

4. The enhanced RNA synthesis phenotype mapped to FM-MA segment 2 (PB1 gene).

5. FM-MA's capacity for enhanced genome assembly did not map to the PB1 gene.

6. The ability of FM-MA to selectively amplify its own genes did not depend on recognition of an FM-MA-specific promoter.

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APPENDIX I: SOLUTIONS

Carnoy's Fixative

3 parts 100% methanol
1 part acetic acid

Denaturing RNA Loading Buffer

80% (v/v) formamide
1 mM EDTA (pH 8)
0.1% (w/v) Bromophenol Blue
0.1% xylene cyanol (w/v)

Denaturing Solution

4 M guanidinium thiocyanate
25 mM sodium citrate (pH 7)
0.5% sarcosyl (w/v)
0.1 M 2-mercaptoethanol

Fixative for Acrylamide Gels

7% acetic acid
25% methanol
in dH2O

HB BUFFER

10 mM Kcl
5 mM mercaptoethanol
10 mM Tris-HCl (pH 8.5)

Hybridization Buffer

80% formamide (v/v)
50 mM PIPES (pH 6.4)
0.4 M NaCl
1 mM EDTA

Modified Denhardt's Solution

1 volume 20x SSC [3 M NaCl, 0.3 M sodium citrate (pH 7)]
3 volumes 50x Denhardts [1% BSA (w/v); 1% Ficoll (w/v); 1% polyvinylpyrrolidone (w/v), 1% SDS (w/v)])

PBS (Phosphate Buffered Saline)

8.0 mM Na2HPO4
2.5 mM NaH2PO4
145 mM NaCl
pH 7.4 sterilize by autoclaving

RNase Digestion Buffer

50 mM Tris-HCl (pH 7.5)
5 mM EDTA, 0.3 M NaCl

2X Sample Buffer

125 mM Tris-HCl pH 6.8
20% (v/v) glycerol
4% (v/v) sodium dodecylsulphate
10% (v/v) 2-mercaptoethanol
0.1% (w/v) bromophenol blue
in dH2O

SOC

2% bactotryptone (w/v)
0.5% bacto yeast extract (w/v)
10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose

STE Buffer

100 mM Tris-HCl pH 8.0
1 M NaCl
10 mM EDTA
sterilize by autoclaving

Tank Buffer

25 mM Tris
192 mM glycine
0.1% sodium dodecylsulphate

TBE Buffer

89 mM boric acid
89 mM Tris
20 mM EDTA
sterilize by autoclaving

TE Buffer

10 mM Tris-HCl pH 7.4
1 mM EDTA pH 8.0
sterilize by autoclaving

Transcription Buffer

40 mM Tris-HCl (pH 8)
10 mM $MgCl_2$
1 mM spermidine
50 mM NaCl

2YT

16 g Bacto tryptone
10 g yeast extract
5 g NaCl
dH2O to 1 liter
sterilize by autoclaving