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STUDIES ON RABIES VIRUS POLYMORPHISM

A Thesis Submitted to the
School of Graduate Studies and Research

In Partial Fulfillment of the Requirements
for the Degree of
Masters of Science
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

by

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ABSTRACT

Rabies viruses have very simple genomes made up of single-stranded, negative sense, non-segmented ribonucleic acid (RNA). Most, if not all RNA virus populations may exist as complex mixtures of genetic and phenotypic variants often referred to as quasispecies populations. A quantitative relative fitness assay has previously been used to demonstrate loss of fitness in RNA virus populations due to Muller's Ratchet and to show gains of fitness by natural selection during virus passages. Due to their mutation rates, rapid replication, large population sizes and controlled (variable or constant) host cells, RNA viruses are useful for examining evolutionary processes. This study uses rabies virus as a model to examine virus evolution and virus population biology. The fate of two closely related rabies virus variants (the Western Skunk and Eastern Artic Fox viruses), cloned using end-point dilution techniques, passaged by themselves and in competition to each other in mouse neuroblastoma (MNA) cells was investigated. Competition between the WSK and EAF viruses resulted in the eventual displacement of one of the populations by the other, in agreement with the Competitive Exclusion Principle of population genetics. The sequence of a portion of the P-gene (a central highly variable region) and parts of the non-coding G-L intergenic region, both prior to and after serial passaging of the WSK and EAF viruses, was determined in order to characterize the clonal variation within the populations. A relatively high level of non-synonymous substitutions occurred in the EAF input virus for the P-gene, demonstrating that a variety of mutant forms of viral RNA exists. However for both the WSK and EAF viruses, the consensus sequence (for both parts of the genome analyzed) remained stable over time (after passaging) indicating overall evolutionary stasis. *In vivo* competition studies showed the presence of both

viruses within the same section of the brain, however, segregated into distinct neurons. This may be the result of transmission bottlenecks on the passage from one neuron to the next.

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LIST OF ABBREVIATIONS

AbAm	Antibiotic/Antimycotic
ADRI	Animal Disease Research Institute
AIDS	Acquired Immune Deficiency Syndrome
BHK	Baby Hamster Kidney
°C	Degree Celsius
cDNA	Complementary Deoxyribonucleic Acid
cm^2	Centimeter Squared
CNS	Central Nervous System
CPE	Cytopathic Effect
CVS	Challenge Virus Standard
DEPC	Diethylpyrocarbonate
DI	Defective Interfering
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EAF	Eastern Arctic Fox
EDTA	Ethylenediaminetetraacetic Acid
ERA	
FAT	Fluorescent Antibody Test
FBS	Fetal Bovine Serum
FITC	Fluorescein-5-isothiocyanate
G	Glycoprotein
HEPES	N-(2-hydroxyethyl) piperazine-N'-2 (ethane sulfonic acid)
L	Polymerase
LB	Luria Bertani
M	Matrix protein
mabs	Monoclonal Antibodies
MEM	Minimum Essential Meduim
mg	Microgram
ml	Milliliter
MNA	Mouse Neuroblastoma Cells
MOI	Multiplicity Of Infection
mRNA	messenger Ribonucleoprotein
N	Nucleoprotein
NCAM	Neural cell adhesion molecule

ORF	Open Reading Frame
P	Phospoprotein
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PV strain	Pastuer Virus strain
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
rpm	revolutions per minute
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
Rx	Restriction
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
$TCID_{50/ml}$	Tissue Culture Infectious Dose 50%
TPB	Tryptose Phosphate Broth
TTBS	Tris-Tween Buffer Solution
μl	Microliter
UV	Ultraviolet
v/v	volume per volume
VSV	Vesicular Stomatitis Virus
w/v	weight per volume
WHO	World Health Organization
WSK	Western Skunk Virus

Literature Survey

Introduction

Viruses with an RNA genome (of which the rabies virus is one) or viruses that use RNA as a replicative intermediate are the most abundant group of subcellular parasites (Domingo et. al., 1996). They cause severe diseases such as rabies, hepatitis, yellow fever and viral encephalitis and infect a wide array of animals, plants and bacteria. RNA viruses cause many emergent viral diseases, the most notorious recent example being the AIDS pandemic (Domingo et. al., 1996). Genetic variability has been observed for all RNA viruses examined, and their potential for rapid evolution is still increasingly recognized as the basis of their ubiquity and adaptability (Kilbourne, 1991; Holland et. al, 1992; Domingo et. al., 1994).

The molecular mechanisms underlying RNA virus variation are mutation, homologous and non-homologous recombination, and genetic reassortment in viruses with a segmented genome such as the influenza viruses and reoviruses (Domingo et. al., 1996). Genetic diversification is a complex process involving at least two stages:

1. Mutant generation during viral replication in infected cells, and
2. Propagation of the mutants in the cells where they are generated, then spread first throughout the organism and then expansion by infection to new individual hosts (Domingo et. al., 1996).

The two stages are driven by different mechanisms (Domingo et. al., 1996), mutant generation being essentially governed by the stochastic process of mutagenesis, whereas the fate of each individual variant depends on its fitness (or ability to produce infectious progeny) in the ensemble of mutants and also on random sampling events in a complex network of influences.

It is often reported that RNA virus populations exhibit a heterogeneous population structure within single individuals, often referred to as 'quasispecies', due to the limited replication fidelity caused by an absence of proof reading/repair and post replicative error correction of the RNA replicases (Holland et. al., 1992; Drake, 1993; Domingo and Holland, 1994, 1997). In simple terms, the quasispecies refer to an equilibrium process of mutation and natural selection, which generates a population of variable genomes (Holmes and Moya, 2002). These genetic variants are organized around one or a set of genotypes of highest fitness known as master sequences (Eigen and Schuster, 1977; Eigen, 1987; Nowack, 1992; Eigen, 1996a; Eigen, 1996b). This quasispecies model of mixed RNA virus populations implies a significant adaptive potential, because it allows the rapid selection of the mutant(s) with the highest fitness in any new environmental condition (Morimoto et. al., 1998). However, in the rabies virus little is known about the relationship between this potential diversity available for selection and evolution in nature and actual colonization of new ecological niches such as new animal vector species (Kissi et. al., 1999).

Rabies has been recognized since antiquity and it continues to be a worldwide problem. The situation of rabies in the world is constantly evolving and differs greatly from one continent to another. The main reservoirs of the rabies virus in North America are wildlife species. In 1996, reports on the surveillance of rabies in the United States showed an increase in the total number of cases, especially in raccoons, skunks, foxes and coyotes. In 2000, 7,369 cases of rabies were reported in the United States with raccoons accounting for about 40% of reported cases. Here in Canada, over the past two years there has been an increase in raccoon rabies due to an incursion of the raccoon

strain. In Africa though, there is still a high proportion of humans contracting rabies due primarily to canine rabies. Around 100-200 rabid patients are reported annually (WHO reports) which reflect only a partial figure of the rabies situation in Africa. Worldwide, there are an estimated 60,000 human deaths each year from dog transmitted rabies, with the Asian continent accounting for the majority of human cases reported and 10,000,000 post-exposure vaccination regimens administered as a consequence of this problem (Trimarchi, 2000).

Rabies is a viral infection of the central nervous system (CNS) of mammals caused by a neurotropic virus (Trimarchi, 2000). This disease, which is maintained in host populations of canines, other terrestrial carnivores such as the fox and bats, is transmitted through infectious saliva, transferred by bites of clinically rabid animals to other susceptible hosts (Trimarchi, 2000). The disease is characterized by a long incubation period, followed by acute, progressive encephalitis culminating in the death of nearly every infected animal (Trimarchi, 2000).

Rabies viruses have very simple genomes with no known regulatory elements that must accommodate numerous adaptations to the host species and fulfill a complex infection cycle *in vivo* in order to survive in their populations. For example, rabies virus infects both neuronal and non-neuronal cells, but by far the predominant target of rabies virus infection *in vivo* is the neuron (Murphy et. al., 1973). However, it is not clear whether virus replication in non-neuronal tissue, with the exception of the salivary glands, actually represents an integral part of the pathogenesis of rabies nor is it known whether a single virus population or different subpopulations of a particular strain are responsible for the infection of neuronal and non-neuronal cells (Morimoto et. al., 1998).

Hence, the main objective of this study is to address problems of virus evolution and virus population biology. What happens when one virus variant (mutant) has to compete with other variants *in vitro* and *in vivo*? Therefore the fate of two known rabies virus variants was investigated when passaged by themselves and in competition to each other *in vitro*. The fact that rabies viruses are transmitted in an infected animal from cell to cell in neuronal networks lets one assume that the dynamics of the competition *in vivo* are quite different from what happens in a cell culture flask. The existence of rabies virus strains as quasispecies may have profound implications for the epidemiology of rabies (Morimoto et. al., 1998) and may also contribute to the pathogenesis of the disease. For example, the spill over of a rabies virus strain (Morimoto et. al., 1998) from one species to another may be facilitated by pre-existing subpopulations that have a selective advantage in a new host.

Rabies Virus

History

Rabies has been one of the most widely and consistently feared zoonotic diseases since the earliest recorded human history (Trimarchi, 2000). The transmission of rabies to humans by the bite of mad dogs was included in the Eschunna code of ancient Mesopotamia in the 23rd century B.C. The fact that a wide range of animal species were susceptible to the disease was realized at an early date. In the *Historia Animalium* Aristotle, in the 4th century B.C., stated clearly that the bite of a dog mad with furious rabies would transmit the disease to animals of all other species, with fatal results although for unexplained reasons he had reservations about the susceptibility of man (Aristotle, *Historia Animalium*). The disease was first shown to be of viral etiology by

Pasteur in the 1880s who described the involvement of the CNS and salivary glands in the disease and achieved attenuation of the virus by animal passage and developed the theoretical and practical basis of immunizing injections. In 1885 he reported the landmark first human post-exposure vaccination of Joseph Meister, and by 1896 he reported on the treatment of 350 exposed persons, of whom only one developed rabies.

Primary Taxonomic Considerations

Rabies is the prototype virus of the genus *Lyssavirus*, family *Rhabdoviridae*, order *Mononegavirales* (Wunner et. al., 1995). Previously known simply as the rabies and rabies-related virus group (Shope et. al., 1970), the genus *Lyssavirus* is presently composed of genotype 1, classical rabies virus and six other genotypes of viruses that are closely related antigenically and genetically and that cause a clinical disease indistinguishable from rabies. Genotype 1 includes the majority of field viruses of global distribution in terrestrial mammals and in insectivorous and hematophagous bats of the Western hemisphere, as well as the laboratory and vaccine strains (WHO, 1994). The distribution of the non-rabies lyssaviruses (genotypes 2-6) is restricted to the Old World. They have a narrower geographic distribution than rabies and, although they occasionally infect humans and domestic animals, they seem to infect preferentially certain specific host species (Tordo, Charlton and Wandeler, 1998). They include genotype 2, Lagos bat virus, isolated from African bats, genotype 3, Mokola virus, isolated from African terrestrial mammals; genotype 4, Duvenhage virus, isolated from African bats; genotypes 5 and 6, European bat lyssaviruses 1 and 2 respectively, isolated from European bats (Kissi et. al., 1995); and genotype 7, Australian bat lyssavirus, isolated from Australian flying foxes (Hooper et. al., 1997). The non-rabies lyssaviruses are of particular interest

since classical rabies vaccines may fail to protect animals against exposure to some of them (Koprowski et. al., 1985; Lafon et. al., 1987) especially the more divergent Lagos bat and Mokola viruses. Lyssaviruses are serologically distinct from other rhabdoviruses (Shope and Tesh, 1987).

Structure of the Virus

The rabies virion forms a rigid bullet-shaped particle measuring approximately 180nm in length and 75nm in width (Hummeler et. al., 1967; Vernon et. al., 1972). Although the diameter of the particle is relatively constant, the length is more variable and extends from 130nm to 300nm with 180nm being the mean. Such a variation in length is due both to differences in the various virus strains studied and to the probable presence in cell cultures of defective interfering (DI) particles physically and antigenically indistinguishable from full length particles, but significantly shorter (Clark et. al., 1980; Wunner and Clark, 1980). These DI particles, which have been better studied in Vesicular Stomatitis Virus (VSV) (Lazzarini et. al., 1981; Blumberg and Kolakofsky, 1983) usually possess a truncated genome. They are therefore defective in transcription and replication activity and require the presence of homologous infectious particles to assure their multiplication (Tordo and Poch, 1988).

The virion particle is composed of two structural units: a central cylinder and a lipoprotein membrane. The central cylinder is composed of a tightly coiled ribonucleoprotein (RNP) complex with helical symmetry. This is contained within the lipoprotein membrane that is provided by the cell membrane during budding and through which protrudes an array of knobbed glycoprotein spikes 10nm in length (Figure 1).

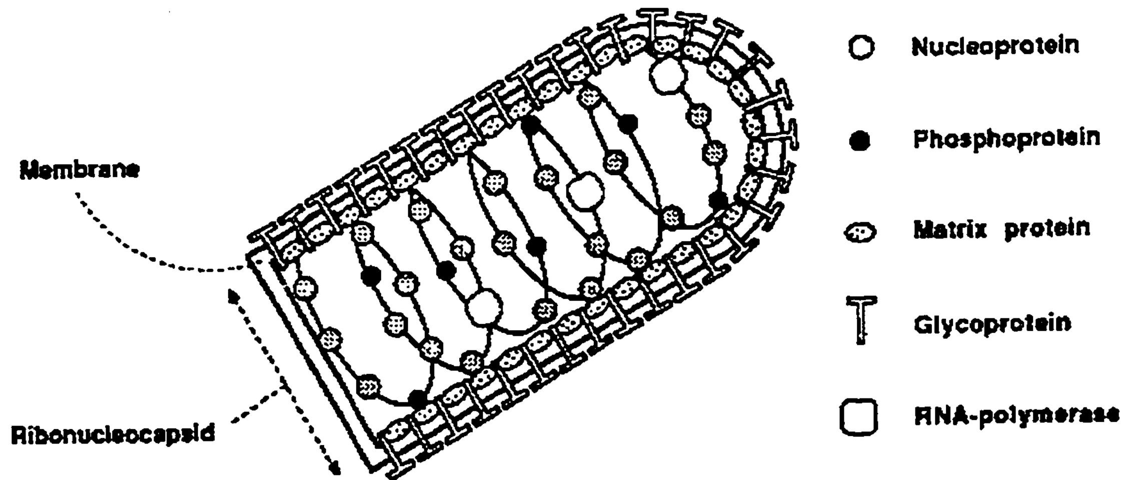


Figure 1: **Schematic view of the rabies virus.** The five viral proteins are also shown.
Source: Tordo, N. and O. Poch. 1988. Structure of rabies virus. In *Rabies*. Edited by J. B. Campbell and K. M. Charlton. Kluwer Academic Publishers, Boston. pp 25-45.

The virus is relatively stable at pH 5 through 10 and is somewhat resistant to air-drying and freeze-thaw cycles (Trimarchi, 2000). It is however labile to pasteurization temperatures, UV light and lipid solvents, ethanol, iodine disinfectants and quaternary ammonium compounds (Kaplan, 1996).

Genome

The rabies virus genome consists of a single-stranded, non-segmented RNA molecule of negative sense polarity of approximately 12,000 nucleotides in length with a molecular mass of approximately 4.6 X 10^6 kDa. The viral RNA is transcribed into five polyadenylated, monocistronic mRNA species that are translated into the five viral proteins: the nucleoprotein (N), phospoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) (Figure 2). The negative polarity of the rabies genome prevents direct translation into viral proteins requiring an autonomous transcription step facilitated by the viral RNA polymerase (Tordo, 1996).

The nucleoprotein is the most highly conserved of all rabies proteins (Rupprecht et. al., 1991) and encapsidates the RNA genome. N interacts with the phosphoprotein and polymerase to form the RNP complex. The RNP functions in the transcription and replication of the virion. Accumulations of the RNP constitute the intracytoplasmic inclusions in infected cells, which have diagnostic importance because they can be detected by direct observation with histological methods and by antigen detection methods employing N protein-specific antibodies (Trimarchi, 2000). The glycoprotein, the sole surface antigen of the viral particle, is the only viral component to elicit the production of neutralizing antibodies (Flamand et. al., 1993) and is therefore an important component of all rabies vaccines. The G protein also plays a role in the stimulation of T

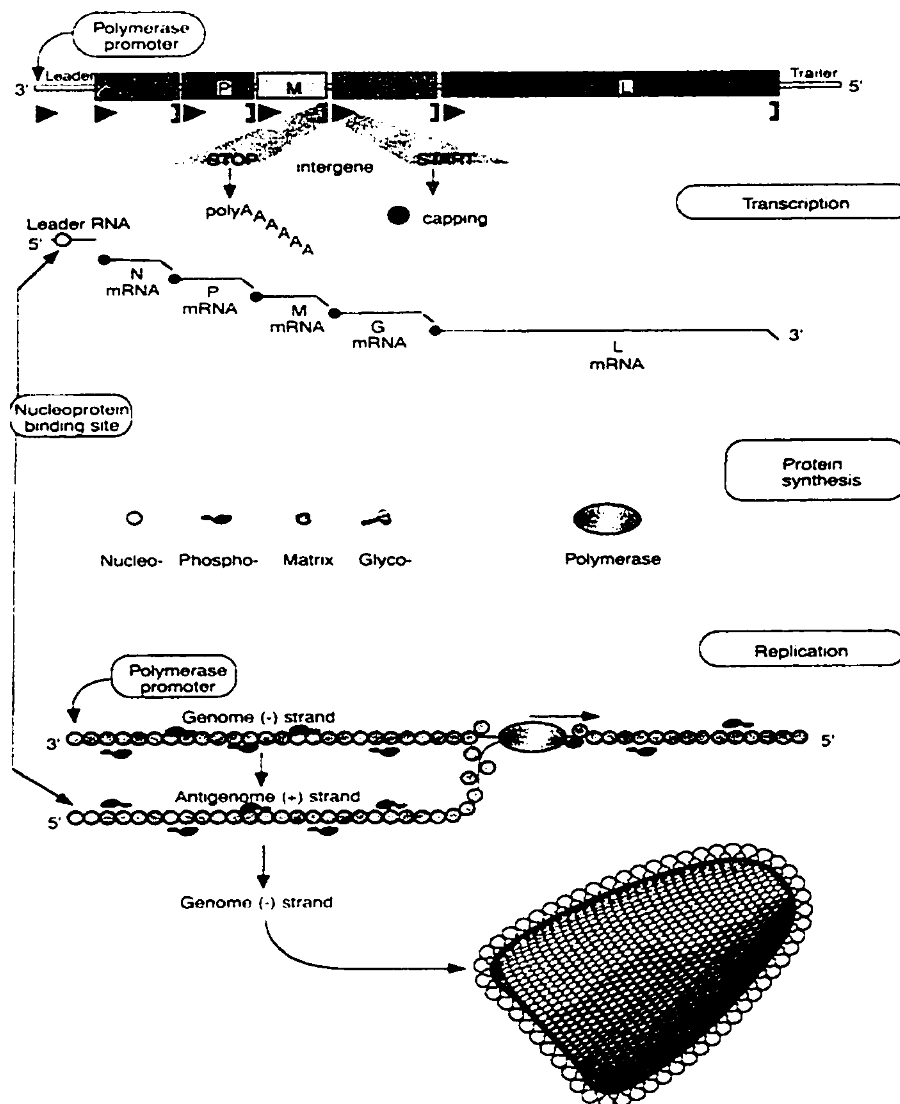


Figure 2: **Transcription, translation and replication of the rabies virus**
Source: Tordo, N., K. Charlton and A. Wandeler. 1998. Rhabdoviruses: Rabies. In *Topley & Wilson's Microbiology and Microbial Infections*. Edited by L. Collier. 9th edition, volume 1, Virology. Arnold Press, London. pp 665-692.

cells (Wiktor et. al., 1985) and functions in receptor binding, mediating the attachment of the virus to the host cells (Perrin et. al., 1982). It is also involved in fusiogenic activity in the endosomes to release the RNP into the cytoplasm where synthesis of the virus takes place. The L protein is the RNA-dependent RNA polymerase that possesses most of the required enzymatic activities: RNA synthesis (De and Banerjee, 1984); mRNA capping and methylation (Horikami and Moyer, 1982); and mRNA polyadenylation (Hunt et. al., 1984; Hunt and Hutchinson, 1993). The phosphoprotein and the matrix protein have not been studied as extensively as the nucleoprotein and glycoprotein. Studies of these proteins have been hampered by the limited availability of monoclonal antibodies directed against them (Nadin-Davis et. al., 1997), but the phosphoprotein is believed to constitute a part of the viral replicase activity by interacting with the L gene product (Wunner, 1991) and specific binding of the P gene product to the nucleoprotein has been documented (Chenik et. al., 1994; Fu et. al., 1994). New studies have shown that in addition to being considered a co-factor for the RNA polymerase function, the P protein binds to LC8 dynein light chain (Jacob et. al., 2000; Raux et. al., 2000), a component of both cytoplasmic dynein and myosin V, which are involved in a wide range of intracellular motile events. The significance of this interaction is yet to be fully determined, but the studies speculate that dynein may be involved in the axonal transport of rabies virus along the microtubules of neurons (Jacob et. al., 2000; Raux et. al., 2000). The matrix protein is located inside the envelope of the viral particle where it likely interacts with the cytoplasmic domain of the glycoprotein (Wunner, 1991). Evidence indicating an inhibitory effect of the matrix protein on virion-associated transcription was reported by Ito et. al. (1996).

Pathogenesis and Pathology

All warm-blooded mammals are susceptible to rabies virus infection but maintenance of specific rabies virus strains occurs in particular reservoir hosts only, for example, dogs, foxes, skunks and bats. Rabies viruses have evolved a pathogenesis, both within the individual animal (cell to cell viral life cycle) and through the process of animal to animal transmission that facilitates the maintenance of a particular variant in a certain reservoir species population (Trimarchi, 2000).

Rabies virus does not generally cause host cell destruction. It is synthesized in the cytoplasm of infected cells and is released by budding through cell membranes (Murphy, 1986). When an animal becomes infected following exposure from the bite of an infected animal, the virus may invade peripheral nerves or nerve endings directly or may first be "amplified" by invasion of striated muscle cells prior to infection of the nerve endings (Charlton, 1988). Substantial stimulation of the immune system does not occur during the early events of the viral replication. The viral G protein mediates binding of the rabies virus to the host cell (Trimarchi, 2000). Several studies indicate that the nicotinic acetylcholine receptor is a receptor for the rabies virus (Baer and Lentz, 1991), but there is also evidence that the membrane oligosaccharides and lipoprotein components of neurons may also serve as rabies virus receptors (Tsiang, 1988). Two additional receptors for the rabies virus have been proposed over the last few years. The first is the neural cell adhesion molecule (NCAM) (Thoulouze et. al., 1998), also called CD56, D2CAM, Leu19 or NKH-1, a cell adhesion glycoprotein of the immunoglobulin (Ig) superfamily which has been described for neurons, astrocytes, myoblasts, myotubes, activated T cells and

NK cells (Goridis and Brunet, 1992; Lanier et. al., 1989). The second, P75NTR, is a low affinity nerve-growth factor receptor (Tuffereau et. al., 1998).

Virus replication in the CNS occurs mainly in neurons, with extensive distribution in the brain and spinal cord (Timarchi, 2000). Recognizable clinical signs of rabies generally do not appear until several replication cycles have occurred in the brain (Kaplan, 1985). Centrifugal spread occurs simultaneously via anterograde axoplasmic flow from the CNS to peripheral nerves and to some non-nervous tissues, including, most importantly, the salivary glands (Trimarchi, 2000). This accounts for the appearance of rabies virus in some tissues and fluids up to a few days before the recognized onset of rabies symptoms (Charlton, 1988). Although infectious dose of virus in the saliva of vectors is paramount for the maintenance of the virus in host populations, virus presence in saliva may be sporadic during and just prior to the clinical period (Constantine, 1967, Fekadu et. al., 1982).

Maintenance of the virus in the reservoir population by direct host-to-host transmission is dependent on simultaneous infection of the brain and salivary glands: it is the impact on behavior resulting from infection of the limbic system that induces biting behavior, and infection of the salivary gland tissue results in infectious doses of virus in the saliva to serve as an infectious inoculum for bite transmission (Trimarchi, 2000). This pathogenic pattern has permitted the entrenchment of the virus in host populations and the continued risk to humans of exposure (Murphy, 1986).

Disease

The incubation period (which varies from 2-12 weeks, but could be longer) is followed by an acute, undelayed progression of encephalitis culminating in the death of

nearly every infected animal. The range in length of illness in most species is 1-10 days. Most of the clinical signs of rabies are expressions of neurological dysfunction (Charlton, 1994) which include a range of symptoms such as disorientation, hallucinations, nuchal rigidity, aerophobia, pharyngeal spasms, hydrophobia, hypersalivation, dysphagia, focal or generalized seizures, cardiac and respiratory arrhythmias, hypertension, and paralysis, leading to coma and death (Matyas et. al., 1999). On the criteria of the predominance of paralysis or of excitability and biting, the clinical syndrome has been classified either as dumb (paralytic) or furious (encephalitic) (Tordo et. al., 1998). This distinction is quite useful, however it is appropriate to consider rabies primarily as paralytic, the encephalitic form being due to hyper-responsiveness and aggressive behavior preceding and then superimposed on the paralytic stage.

Rabies Virus Populations in Individual Hosts and in Host Populations

Rabies virus is widely distributed and can infect a wide range of mammalian species but in developed countries it persists predominantly in wildlife species, notably foxes, skunks, raccoons and bats (Wandeler, 1987). In general, within a discrete geographical region a single terrestrial mammalian species is the predominant host for the virus; for example in Europe the red fox is the principal vector (Steck and Wandeler, 1980) whilst in the American Midwest and Canadian Prairie Provinces rabies is predominantly spread by the striped skunk (Charlton et. al., 1988).

In order for rabies to survive it is essential that an infected animal transmit the virus during a period of virus excretion to enough other susceptible individuals. For this to occur lyssavirus strains must be adapted to the physiological traits and population biology of their hosts (Bacon, 1985). They must have a host-specific pathogenicity and

pathogenesis (length of incubation period, duration and extent of clinical illness) (Wandeler et. al., 1994). During host passage from the site of entry through the central nervous system to the salivary glands, viruses may also experience a number of population bottlenecks and subsequent clonal growth under different selective constraints, during which they must maintain their genetic integrity and overall adaptation to their host's biology (Wandeler et. al., 1994).

Quasispecies and Viral Polymorphism

As mentioned earlier a hallmark of RNA genomes is the error-prone nature of their replication and transcription. Mutation rates during RNA genome replication and transcription are generally in the range of 10^{-4} to 10^{-5} substitutions per nucleotide copied (Holland et. al., 1992; Drake, 1993; Mansky and Temin, 1995). In contrast, error rates during replication of DNA of eukaryotic and prokaryotic cells are in the range of 10^{-8} to 10^{-11} misincorporations per base pair per replication round (Drake, 1991; Beckman and Loeb, 1993). Hence, populations of RNA viruses must be genetically heterogeneous, as shown by a number of studies, for example Morimoto et. al. (1998) and reviewed by Domingo and Holland (1994). Infected organisms, and even different parts of the same organ, often contain distinguishable sets of mutants (Delassus et. al., 1992). Direct reverse transcription and polymerase chain reaction (PCR) amplification of viral RNA combined with rapid nucleotide sequencing has allowed examination of genomic sequences of viruses replicating *in vivo* without the perturbations in population composition brought about by adaptation of viruses to cell culture (Meyerhans et. al., 1989).

The dynamic distributions of mutant swarms that constitute viral populations are referred to as quasispecies, a term coined by Eigen and Schuster (Eigen and Schuster, 1979) to describe early replicons on earth. According to its original theoretical formulation, quasispecies is an organized (rated) spectrum of mutants dominated by a master sequence that may coincide with the average or consensus sequence of the population (Domingo et. al., 1996). In real viral populations, perturbations in the environment introduce an additional element of complexity, and both master sequences and any given mutant distribution often have a fleeting existence (Domingo et. al., 1996). A critical element of quasispecies theory is that the frequency of any individual virus in the quasispecies is a function of both its own replication rate and the probability that it will arise by the erroneous replication of other members of the population (Eigen and Schuster, 1979). Due to its emphasis on mutant generation and on the competitive rating among variant genomes, the concept of quasispecies has become an adequate descriptor of RNA viruses at the population level and has facilitated links between population biology and virology (Eigen and Biebricher, 1988; Holland et. al., 1992). This quasispecies structure/polymorphism in rabies virus has been documented by a number of investigators (Benmansour et. al., 1992; Morimoto et. al., 1998; Kissi et. al., 1999). In a study carried out by Morimoto et. al. (1998) it was shown that passage of a mouse-adapted rabies virus strain CVS-24 in BHK cells resulted in the rapid selection of a dominant variant, that differed genotypically and phenotypically from the dominant variant present in mouse brain or neuroblastoma-cell passaged CVS-24, indicating that this particular rabies virus strain consisted of variants with different biological properties and that changes in the host environment rapidly resulted in shifts in the dominant

variant. This was due to the substitutions noticed in the viral glycoprotein (G) sequence which accumulated in the cell culture thereby changing the tropism for nervous tissues and hence virulence (this may be directly co-related to other rabies virus variants). Although some strains of rabies virus are maintained in a single host species and rarely spill over to other species, others such as the silver-haired bat rabies virus strain appear to be more promiscuous (Rupprecht et. al., 1995 and Morimoto et. al., 1996). This property may be a consequence of the latter containing variants that alone or in co-operation may have the capacity to rapidly adapt to a new host. This has direct implications in rabies control programs, since important to any control program is a clear understanding of the disease epidemiology, including knowledge of the precise nature of the viral agent and how this changes temporally, geographically and according to host species (Nadin-Davis et. al., 1993).

The quasispecies structure has numerous implications for the biology of RNA viruses (Domingo et. al., 1996):

1. The first implication is relevant to viral pathogenesis. Mutant swarms constitute huge reservoirs of variants with potentially useful phenotypes in the face of an environmental change (among others, tissue-specificity as shown by Morimoto et. al., 1998, antigenically altered or drug-resistant variants) (Domingo and Holland, 1992).
2. A second implication of relevance to interpret viral evolution is that despite high mutation rates and variations in the fine composition of mutant spectra, the average or consensus sequence may remain invariant for many generations (Domingo et. al., 1978; Steinhauer et. al., 1989). Such conditions are referred to

as population equilibrium, and they explain the evolutionary stasis of RNA viruses in spite of continuous viral replication (Domingo et. al., 1996). For example, one virus may show stasis in one biological context and rapid evolution in another, as with avian influenza A viruses that undergo rapid genetic change upon colonizing human hosts (Webster et. al., 1992).

3. The third implication of the quasispecies structure is that it predicts important effects of genetic bottlenecks and viral fitness (Domingo et. al., 1996). Any genome sampled at random from a mutant spectrum is likely to harbor deleterious mutations relative to the consensus, thus generating progeny with decreased fitness (Domingo et. al., 1978; Eigen and Schuster, 1979), due to the extreme heterogeneity of viral populations. Only when a quasispecies is poorly adapted to a new environment will newly arising mutants have a high probability of being more fit than the ensemble.

Fitness and Population Bottlenecks

Fitness is one of the central concepts of evolutionary biology (Darwin). Population biologists usually define fitness of a particular genotype as its relative contribution to the next generation (Wilson and Bossert, 1971), hence the number of offsprings generated by one particle. Viral fitness, or its ability to replicate infectious progeny can vary a million-fold within short time intervals (Domingo et. al., 1996). Paradoxically, functional and structural studies suggest extreme limitations to virus variation (Domingo et. al., 1996).

Changes in the size of a population have been shown to have important effects on genetic variation (Domingo et. al., 1996) and on the survival potential of biological species. As already mentioned, RNA viruses have high mutation rates, which can have a

positive effect on the virus. However, high mutation rates can also have a negative effect on the population of these viruses. The observation of fitness decrease of RNA viruses on serial bottleneck events agrees with an important concept in population genetics known as "Muller's Ratchet" (Muller, 1964). Muller (Muller, 1964) had predicted that when an asexual population is small and the mutation rate is high, the population will decline in fitness due to the loss of the fittest mutations in a "kind of irreversible ratchet mechanism". Fitness losses have indeed been observed in clones of RNA viruses propagated through genetic bottlenecks (Chao, 1990; Chao et. al., 1992; Duarte et. al., 1992; Clarke et. al., 1993). Unfortunately, it is not known whether severe genetic bottlenecks in rabies virus occur anywhere from the site of entry to the place of accessing the nervous system during its passage through the nervous system to the salivary glands and through the transmission to another susceptible animal (Wandeler et. al., 1994).

It has however been observed by a number of investigators including Novella et. al. (1995) that repeated transmission of large RNA virus populations from host to host in a constant environment can lead to a significant increase in the fitness of the virus population. Model studies with Vesicular Stomatitis Virus (VSV), a rhabdovirus related to the rabies virus, have indicated up to a million-fold variation in fitness depending on the passage history of the virus (Duarte et. al., 1994; Novella et. al., 1995a; Novella et. al., 1995; Elena et. al., 1996). The transmission population size needed to maintain fitness of VSV was very dependent on the initial fitness, that is, its previous evolutionary history (Novella et. al., 1995; Elena et. al., 1996).

It has also been shown that genetic bottlenecks are not the only way by which a viral quasispecies is driven to low fitness values. Any drastic change in the selective

environment can do so immediately (Domingo et. al., 1996). In a study carried out by Novella et. al. (1995) it was shown that adaptation of the wild-type VSV to persist in sandfly cells decreased VSV replicative fitness in mammalian cells, with the persistent virus showing a two million fold greater fitness in sandfly cells than in BHK-21 cells or in mouse brain. This effect can have profound implications in the development of vaccines using attenuated viruses.

Adaptation

Adaptation occurs through natural selection (Elena et. al., 2000), which can act whenever fitness differences exist among genotypes. Four conditions must be met for natural selection to operate (Elena et. al., 2000); reproduction, a genetic basis for phenotypic traits, phenotypic variation among individuals and phenotype-associated fitness differences. Viruses with RNA as their genetic material adhere to the above conditions (Elena et. al., 2000). The genomes of these viruses have higher replication rates than that of their cellular host. Due to the fact that the replication in RNA viruses is quite imperfect, (Elena et. al., 2000) mutation arises at a high rate, and most of the changes in the amino acid sequence have fitness effects. Adaptation includes concepts such as the Competitive Exclusion Principle and the Red Queen Hypothesis.

Competitive Exclusion Principle and Red Queen Hypothesis

Competition among genotypes for a limited resource implies that the fittest genotype will displace all others, therefore increasing the average fitness of the population (Elena et. al., 2000). This has been demonstrated in RNA viruses, for example in VSV clones of approximately equal relative fitness (Clarke et. al., 1994). This was in agreement with the Competitive Exclusion Principle of population genetics that states

that in the absence of niche differentiation one competing species will always out compete the other (Gause, 1971).

In competition between neutral VSV clones, both the winners and the losers gained fitness relative to a reference VSV clone (Clarke et. al., 1994) in support of the Red Queen Hypothesis (van Valen, 1973). According to this hypothesis each species is competing in a zero sum game for the same fixed resources against others and "no species can ever win and new adversaries grinningly replace the losers" (van Valen, 1973). Among competing viral quasispecies populations (or variants) in an equilibrium situation, only infrequently arising, vastly superior mutants are likely to upset the equilibrium and exclude all others (Domingo et. al., 1996).

Rabies viruses form two types of association with their host species (Holmes et. al., 2002). In the first association, the virus establishes a stable infection cycle within a particular mammalian species, with transmission occurring through infected saliva in bite wounds (Holmes et. al., 2002). Whether the virus always causes disease in these situations is unclear, although fatal rabies is common in infected dogs, foxes and raccoons the same does not always appear to be true of bats (Bear, 1991; Ronsholt et. al., 1998). Adaptation of a particular virus strain to its principal host is indicated by the high frequency and magnitude of its excretion, on the one hand, and by the host's high susceptibility to it on the other (Wandeler, et. al., 1994), factors which facilitate transmission from an infected to a susceptible individual after a biting incident.

The second form of virus-host interaction occurs when the virus jumps species boundaries to infect new hosts (Holmes et. al., 2002). Such cross-species transmission, usually results in sporadic cases of disease without further transmission (Holmes et. al.,

2002), the most obvious example of these "spill-over" infections being human rabies, which generally leads to a fatal outcome if symptoms arise, but where no subsequent transmission takes place. Occasionally, however rabies viruses are able to establish productive infections in new host species (Tordo et. al., 1993; Nadin-Davis et. al., 1994; Smith et. al., 1995). An important example of such a successful host switch involved the transfer of the virus from dogs to the red fox (*Vulpes vulpes*) in Northeast Europe during the 1930s (Bourhy et. al., 1999). After the initial cross-species transmission event, rabies virus was able to spread rapidly westward and southward through European red fox populations in the subsequent 60 years (Anderson et. al., 1981; Bourhy et. al., 1999). Adaptations to different hosts have also been documented to some extent by *in vivo* experiments on susceptibility and by observation on virus excretion in experimentally infected animals in field specimens submitted for diagnosis (Blancou, 1988; Blancou et. al., 1991; Charlton et. al., 1991; Winkler and Jenkins, 1991; Aubert, 1992; Wachendorfer and Frost, 1992).

The rabies virus has already shown that it can adapt quickly to a new host. As cross-species transmission has been shown to initiate rabies epidemics occasionally (Holmes et. al., 2002) it is important to determine why some host transfers are successful and others are not. Is it due to the quasispecies nature of the virus or some other ecological or genetic characteristic? Already mentioned is the study by Morimoto et. al. (1996) which showed an adaptive process by the virus and which was also highlighted in a study by Kissi et. al. (1999), who observed substantial genetic variation in the G gene of the rabies virus from viruses passaged through different host species, with greatly

elevated rates of non-synonymous over synonymous substitutions per site, indicative of positive selection (Holmes et. al., 2002).

Objectives

This study hopes to address issues of virus evolution and virus population biology, by determining the fate of two known rabies virus variants when they are passaged by themselves and in competition to each other. It is expected that the results of this study would have implications for the epidemiology of rabies. The fact that not much is known about virus population dynamics in the rabies virus as it moves from the site of entry to their shedding in to the saliva (Wandeler, personal communication), nor is the interaction of polymorphism and host adaptation understood, makes this an interesting topic of study. The main objectives of this study are:

1. **To study competition *in vitro* between two closely related rabies variants.** The Competitive Exclusion Principle of population genetics states that in the absence of niche differentiation one competing species (variant) will always out compete the other (Gause, 1971). By studying competition *in vitro* between two closely related rabies variants within the same host (Mouse Neuroblastoma Cells), this principle will be studied. The colonization of a host by two rabies variants and the propagation of the infection by both variants may happen in nature, but has not been shown.
2. **To study competition between the two closely related variants *in vivo*.** It is imperative for the survival of rabies that an infected animal transmits the virus during a period of virus excretion to enough other susceptible individuals. Since rabies is transmitted through the saliva by the bite of an infected animal, the presence of the virus will be examined in the salivary glands of the infected animals. This has direct implications in the fitness of the virus. Though viral fitness is usually defined as its ability to replicate

infectious progeny, it can also be defined as its ability to be transmitted from host to host.

3. **To characterize the clonal variation (that is quasispecies nature) within populations of each rabies virus variant using molecular techniques.** The degree of variation exhibited by the two rabies virus variants, at the P-gene locus and the G-L intergenic region both prior to and after 12 *in vitro* passages in mouse neuroblastoma cells will be examined.

Materials and Methods

Cell Culture

Mouse Neuroblastoma (MNA) Cells

The MNA cell line was the cell line of choice used in this study for the propagation of virus. The cells were initially obtained from BioWhittaker (Walkersville, MD) product number 07-305R. They were routinely grown in 75cm^2 plastic flasks (Falcon BD Sciences) using Eagles minimum essential medium (MEM) (Life Technologies, Burlington, Ontario) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL), 5% tryptose phosphate broth (TPB) (Sigma Chemical Company, St. Louis, MO) and 1% antibiotic/ antimycotic (AbAm) solution (100units/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B) (Sigma Chemical Company) at 37°C and 5% CO_2.

For passaging of cell cultures, the monolayers were washed twice with trypsin-EDTA (Gibco-BRL). Each monolayer received approximately 2.5ml of trypsin-EDTA with flasks being rotated to spread the solution evenly. The trypsin was then decanted immediately and the flasks placed at 37°C in a 5% CO_2 incubator for 2-4 minutes with 1ml of trypsin. Once cells had been trypsinized fresh growth medium was then added according to split ratio and cell suspension divided into new 75cm^2 plastic flasks (Falcon BD Sciences). The split ratio was dependent on how soon the formation of complete monolayers was needed and the number of new 75cm^2 flasks used. 20ml of the growth medium was then added to the new flasks. Flasks were then incubated at 37°C for 3-4 days until the formation of complete monolayers.

Viruses

The two rabies virus variants used in this study, the Western skunk (WSK) virus (92L1169) and the Eastern Artic fox (EAF) virus (98N4235) were received in the form of virus positive supernatant (which had been passaged in MNA cells and were now at the 4th passage) through the courtesy of Dr. A. Wandeler and Jan Armstrong, Animal Disease Research Institute (ADRI), Nepean, Ontario, Canada. The virus variants were originally isolated from specimens submitted for routine rabies diagnosis positive for rabies. Virus supernatant when received was thawed, aliquoted into nunc vials (Nalge Nunc International, Denmark) of approximately 1ml in each and stored at –80°C until needed for cloning by limited dilution purification. The titer of the WSK virus when received was $10^{5.9}$ $TCID_{50/ml}$ and that of the EAF virus was $10^{5.7}$ $TCID_{50/ml}$.

Virus Titration

The virus dose capable of infecting 50% of a number of the tissue culture wells in a 96 well plate is called the tissue culture infectious dose 50 ($TCID_{50}$), virus titer. To determine virus titers, cell suspensions of MNA cells in 96-well plates (Nalge Nunc International) seeded at 2 X 10^5 cells/ml, 100µl/ well, were infected with 100µl of the virus supernatant that had been serially diiuted tenfold (10^{-1}, 10^{-2}, 10^{-3} to 10^{-8}) in MEM supplemented with 10% FBS, 5% TPB and 1% AbAm. 96 well plates were then placed in a 37°C, 5% CO_2 incubator for 5 days. After incubation the cells were fixed in 75% acetone and subjected to fluorescent staining test/technique (fluorescent antibody test-FAT) developed by Goldwasser and Kissling (Goldwasser et. al., 1959) and subsequently modified by Dean and Abelseth (Dean and Abelseth, 1973) and by Kissling (Kissling, 1975) with a rabies polyclonal antibody fluorescein-5-isothiocyanate (FITC) conjugate

provided by the Rabies Diagnostic Lab at ADRI. Rabies antigen/intracytoplasmic inclusions formed were viewed using a Leica UV fluorescent microscope. Virus titers were determined using the Spearman-Karber (Finney, 1978) method tables. All titrations were carried out in triplicate.

Cloning of Virus by Limiting Dilution Purification Technique

The WSK virus and the EAF virus after having been passaged (separately) were subjected to two consecutive limiting dilution purifications. This approach was used because the field strains of rabies virus do not form plaques. Titrated stocks of each viral variant were diluted to contain 10 $TCID_{50/ml}$. Monolayers of MNA cells seeded at 2 X 10^5 cell/ml, 100µl per well in a 96 well plate were infected with 100µl of the MNA passaged virus supernatant at 1:100 and 1:1000 dilutions for the WSK and 1:10,000 and 1:100,000 dilutions for the EAF and incubated for 1 hour at 37°C. The virus inoculum was then removed and the cultures were replenished with 100µl of maintenance medium (MEM supplemented with 2% FBS, 5% TPB and 1% AbAm) and incubated at 37°C for 5 days. Tissue culture supernatants, identified according to origin, were then harvested by transferring with a multi-channel pipette (Bioht 1200) to a new 96 well plate and frozen until needed. The cells were then fixed with 75% acetone and subjected to FAT with diagnostic conjugate as previously explained. All wells were examined and selected supernatants from wells exhibiting a single focus of infection were then passaged individually in MNA cells to produce sufficient material for storage and characterization. All such viral 'clones' were then subjected to a second round of limited dilution purification.

Preparation of Virus Stocks and Propagation

Preparation of virus stocks and propagation of each clone of the WSK virus variant and the EAF virus variant after the second limit dilution purification was first carried out in 24 well plates. 1ml of suspended MNA cells (2 X 10^5 cells/ml) were inoculated in suspension with approximately 100µl of chosen virus clone supernatants and incubated at 37°C for 4 days. The presence of virus and rate of infection in each of the 24 wells were monitored by seeding 10µl of the cell/virus suspension from each well, using a single bioht pipettor, into the well of a 96-mini well terezaki plate (Robbins Scientific Corp., Sunnyvale, CA). These seeded terezaki plates which are called monitors, were incubated (for the amount of time the 24 well plate (or culture flask being used) was incubated), fixed with 75% acetone, stained with diagnostic conjugate and viewed under UV as previously described. These monitors indicated the level of virus antigen/ infection present in the cell monolayer before passaging. After the 96-hour incubation when the cells had formed a complete monolayer, all cells were trypsinized and transferred to ambitubes with the addition of MEM supplemented with 10% FBS, 5% TPB, 1% AbAm and 1% HEPES buffer (Sigma) to propagate the virus clones. Ambitubes were first used to grow up clones because virus was in small quantities. Monitors were once again made to monitor infection within the ambitubes. Ambitubes were incubated at 37°C for 3 days and then all cells were trypsinized and transferred to 25cm^2 plastic tissue culture flasks (Falcon). Infection was monitored and those flasks that showed positive infection for rabies were passaged into new 25cm^2 flasks at a concentration of 2 X 10^5 cells/ml and incubated for 3 days at 37°C. To grow up sufficient quantities of the virus clones, cells in the 25cm^2 flasks were trypsinized and passaged into new 75cm^2 flasks at a concentration

of 2 X 10^5 cells/ml, incubated at 37°C for 3 days with virus infection levels monitored. Supernatant was eventually harvested (after propagation of virus clones) and stored at –80°C until needed for either the virus passaging experiment or RNA isolation.

Verification of Clones

To verify by antigenic characterization that the virus clones which had been propagated are actually clones of the specific variants (the WSK virus and the EAF virus) to be studied, 96 mini-well terezaki plate monitors made during propagation of the clones were stained by indirect immunofluoresence using the following monoclonal antibodies (mabs); 5DF12 (anti-N), 24FF11 (anti-N), 26AF11 (anti-N), 38FG5 (anti-N) (generated at the University of Berne, Switzerland and provided through the courtesy of Dr. A. Wandeler), M868 (anti-N), M960 (anti-P), M964 (anti-P) and M993 (anti-N) (generated at the ADRI Monoclonal Antibody Unit) (some of these mabs will be further described below). 12µl of each mab was dispensed in to each well and the plate incubated at 37°C for 45 min. Plates were rinsed three times and washed three times, 10 minutes per wash with Tris-Tween Buffer Solution (TTBS) (50mM Tris, 200mM NaCl, 0.5% v/v Tween-20) and then incubated with FITC-conjugated goat anti-mouse immunoglobulin (ICN/Cappel) at a dilution of 1:900 for 45 minutes at 37°C. Plates were again rinsed three times and washed once for 10 minutes with TTBS, counter-stained with 0.5% w/v Evan's Blue (Sigma) and examined for fluorescence, the intensity of which was graded from one plus (+) positive to three plus (+++) positive.

Maintenance of Rabies Virus Infected MNA Cells over 12 Cell Passages

Using the cloned viruses (passage 25 of the WSK virus and passage 15 of the EAF virus) obtained from the end point dilution technique, three separate MNA cell culture

suspensions were infected with the WSK virus, the EAF virus or a combination of the WSK/EAF viruses at a multiplicity of infection (MOI) of 0.2 for a passaging experiment. MNA cells were seeded at a concentration of 2 X 10^5 cells/ml in a 75cm^2 tissue culture flask with MEM supplemented with 10% FBS, 5% TPB and 1% AbAm. Various infected cells were incubated at 37°C for 4 days and passaged at a concentration of 2 X 10^5 cells/ml into new 75cm^2 flasks for twelve passages. At every passage supernatant was harvested and stored at –80°C until needed for titration. For supernatants harvested from cells inoculated with both viruses, duplicate titrations were performed. One plate was stained with M960 biotin (described below) and Strepavidin-PE (1:500) specific for the EAF strain and the second plate stained with 24FF11 FITC (described below) to detect the WSK strain. Plates were analyzed for fluorescence using the Leica fluorescent microscope. Monitors were also made at each passage and these were fixed and stained as previously explained to detect infected cells. For cells infected with both viruses, two monitors were made for staining with respective conjugated mabs for the detection of infected cells.

Monoclonal Antibodies (Mabs)

A collection of five mabs was purified from ascites fluid. M868 (anti-N), M960 (anti-P), M964 (anti-P) and M993 (anti-N) hybridomas were generated at the ADRI Monoclonal Antibody Unit while 24FF11 (anti-N) was generated at the rabies laboratory at the University of Bern, Switzerland.

Monoclonal Antibody Purification

Ascites fluid was clarified by filtration in Spin-X® centrifuge tube filters (Corning Costar Corp.) for approximately 60 minutes (in 20 minute increments) at 14,000 rpm at

4°C (Eppendorf Centrifuge 5417R). Purification of mabs from the filtrate was carried out by Protein G affinity chromatoghraphy using the MAb Trap G II Kit (Pharmacia Biotech), which is a fast and effective kit for the purification of monoclonal and polyclonal immunoglobulin G (Ig G) from ascites fluid, serum and cell culture supernatants. The column was first equilibrated with binding buffer (diluted 1:10) (provided in the kit) before applying the filtrate with a syringe. After washing the column with 7ml of binding buffer, mabs were eluted using 5ml of elution buffer (diluted 1:10) (provided in the kit), collecting the purified IgG fractions in several polyethylene tubes (Falcon). To identify the fraction(s) containing the mab, aliquots of these preparations were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by gel staining with Coomassie Brilliant Blue (Bio-Rad) and gel drying using a Bio-Rad Gel Dryer (Model 583). The protein concentration of pooled aliquots containing the mab was determined using a microtitre plate method of the Bio-Rad Protein Assay Kit based on the method of Bradford (Bradford, 1976). Protein dye reagent (Bio-Rad) diluted 1:5 in Milli Q water was added to serial dilutions of recovered mab followed by measurement at 595nm with a microplate reader (Multiskan® MCC1340, Labsystems Inc., Finland). The relative protein concentration was calculated by comparing to a bovine gamma globulin standard (Bio-Rad) curve.

Concentrating and Desalting Eluted Monoclonal Antibodies

Eluted mabs were concentrated and desalted using Centriprep-30 (Centriprep Concentrators) with a membrane MW cut-off of 30,000 daltons, hence substances above 30,000 daltons are retained and substances below 30,000 daltons pass through the membrane and are discarded. IgG with a MW of 150,000 daltons will be retained. The

filtrate collector assembly was removed from the Centriprep and the protein sample, adjusted using phosphate buffer saline (PBS) to a final volume of 15ml, was poured into the sample container to the sample fill line. The filtrate collector was carefully inserted back into the sample container with a gentle downward force, sealed and centrifuged for 30 min at 2400rpm (1220g) and at 4°C (#216 swinging bucket rotor in Centra-8R centrifuge, IEC, Needham Heights, MA). After centrifugation the filtrate was discarded, additional PBS was added once again to 15ml and the centriprep unit was centrifuged again. PBS was added three times to the protein sample and centrifuged a total of six times until volume of entire sample had been reduced to approximately 1ml. After the final spin a micropipette was used to remove the retentate (concentrated, desalted mab), which was transferred to a sterile 2ml polypropylene screw cap vial. Sample protein concentration was determined using the Bio-Rad Protein Assay as previously described and the mabs were stored at –20°C until needed for labeling with either fluorescein-5-isothiocyanate (FITC) or biotin succinimide ester (biotin). Immediately prior to labeling, mab concentration was adjusted with 0.1M carbonate buffer to 1mg/ml.

Labeling Monoclonal Antibodies with FITC

FITC, which had been allowed to reach room temperature to prevent condensation upon opening, was weighed out on an analytical scale (Mettler H10T) and made to 1mg/ml in dimethyl sulfoxide (DMSO). 100μl of FITC was added to 1000μl mab solution (1mg/ml) and placed in an amber bottle to protect the reaction from light. This conjugation reaction was incubated at 37°C in a water bath for 2 hours and then a Centriprep-30 unit was used to remove unreacted dye and concentrate/desalt the mab as described above. Once the filtrate was colorless (no more apple green color, indicating all

the unreacted dye had been removed) mabs were then transferred to amber tubes and the absorbance of each FITC conjugate measured at 280nm and at 495nm using a spectrophotometer (UV1201 UV-VIS, Shimadzu). The F: P ratio of the FITC labeled mab was calculated by dividing the absorbance of the mab at 495nm by the absorbance of the mab at 280nm. Ideally the absorbance ratio should fall between 0.5-1.0.

$$\text{F: P ratio} = A_{495}/A_{280}$$

M868 gave the F: P ratio of 0.93; M960, 1.20; M964, 0.75; M993, 0.60 and M24FF11, 1.15.

Labeling Monoclonal Antibodies with Biotin

To label mabs with biotin the protocol outlined in Monoclonal Antibodies Principles and Practice 2nd Edition (Goding, J.W.) was followed. Biotin, which had been allowed to warm up to room temperature, was weighed out on an analytical scale and dissolved to 1mg/ml in DMSO. 150µl of ester solution was added to 1000µl of antibody solution (1mg/ml), mixed and left to incubate at room temperature for 2 hours. Conjugated mabs were dialyzed for 48 hours in 10L of PBS at 4°C and then transferred to clear bottles.

Both FITC labeled mabs and biotin labeled mabs were brought to 50% glycerol (by mixing equal volumes of glycerol and conjugated mab) and then stored at –20°C until needed for direct immunofluorescence.

Testing Labeled Monoclonal Antibodies

Conjugated mabs were then tested to determine the optimal dilution for use. 1:50 dilutions of the labeled mabs were used to generate two-fold serial dilutions in TTBS (1:50, 1:100, 1:200 to 1:25,600). 12µl of each dilution were dispensed into two 96 mini-

well terezaki plates containing MNA cells infected separately with ERA rabies virus, the WSK rabies virus and the EAF rabies virus. The ERA rabies virus shows the same characteristic staining pattern as that of the WSK rabies virus to the conjugated mabs. Seeded terezaki plates, which had been stored at –80°C, were received from Jan Armstrong at ADRI. Plates were then incubated at 37°C for 45 minutes. Plates receiving FITC labeled mabs were rinsed three times with TTBS and washed one time for 10 minutes in the dark, then counter-stained with 0.5% w/v Evan's blue for about 30-60 seconds and examined for fluorescence of infected cells using the Leica UV fluorescent microscope. Plates on which biotin-labeled mabs had been dispensed were rinsed three times and then washed three times, 10 minutes per wash, with TTBS. Wells were then stained with 10µl Strepavidin phycoerythrin at a dilution of 1:500 (1mg/ml) (Molecular Probes, Eugene, OR) and incubated for 45 min at 37°C. Plates were again rinsed three times and washed once for 10 min with TTBS, counter-stained with 0.5% w/v Evans blue and examined for fluorescence using the Leica UV fluorescent microscope.

Molecular Techniques

RNA Isolation

Total RNA was isolated from virus supernatant for the WSK and EAF virus using TRIzol reagent (Gibco BRL). 750µl of TRIzol LS (Gibco BRL) was added to 250µl of virus supernatant in a microfuge tube and mixed by vortexing (Vortex, VWR Canlab). A water sample was processed in parallel as a negative control. Since small amounts of RNA are normally obtained when isolating RNA from supernatant, 5µl of molecular biology grade glycogen solution (1mg/ml) (Gibco BRL) was added to act as a carrier for

RNA precipitation. After mixing 200µl of molecular biology grade chloroform was added. Sample tubes were capped securely and shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2-3 minutes. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C (Eppendorf Microcentrifuge, Brinkmann Instruments, Westbury, NY). Following centrifugation the top aqueous layer was removed, and transferred to a new microfuge tube. RNA was precipitated from the aqueous phase by addition of 500µl of molecular biology grade isopropyl alcohol, followed by inverting the sample several times and incubation at room temperature for 10 min. Samples were then centrifuged for 10 min at 12,000 rpm and 4°C. The precipitate forms a gel like pellet on the bottom or side of the microfuge tube. Supernatant was decanted and RNA pellet washed with 1ml of 75% ethanol, re-centrifuged briefly and liquid decanted. Pellet was then dried in a speedvac (Savant Oligoprep OP120) for 15-30 min and RNA dissolved in 20µl of RNase free water (prepared by treatment with diethylpyrocarbonate (DEPC)) according to the directions in the Sambrook et. al. manual. RNA was stored at –80°C until needed for RT-PCR.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was employed to amplify non-contiguous regions of the viral RNA genome, i.e. the phosphoprotein (P) gene and the G-L intergenic region for both viral variants. All primers used in this study were provided through the courtesy of Dr. Susan Nadin-Davis, ADRI and are fully described in Table I. RT reactions were performed in a volume of 20µl at 37°C for 1½ –2 hours with Moloney murine leukemia virus reverse transcriptase (M-MLV, 200U/µl) (Gibco-BRL) as described (Nadin-Davis, 1998). Primer 887 and 179 were used initially for reverse transcription of the P-gene and G-L intergenic

Table I. **Oligonucleotides used for RT-PCR and Gene Sequencing of the WSK and EAF Strains of Rabies Virus**: Base positions are indicated with reference to the PV strain nucleotide sequence (Tordo et. al., 1986). Degenerate base positions are bracketed.

Oligonucleotides used for RT-PCR and Gene Sequencing of the WSK and EAF Strains of Rabies Virus

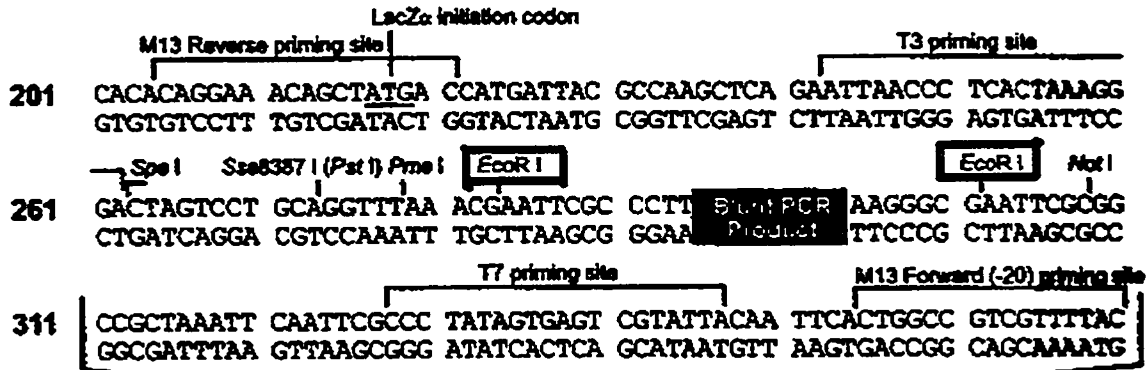
Primer	Nucleotide sequence (5'-3')	Sense	PV strain co-ordinates
887=Nseq8	CTACTTCTCCGGGGAAACCAGAAG	P(+)	1249-1272
888=RabPrev	GG(AG)AGCCA(TC)AGGTC(AG)TCGTCAT	P(-)	2575-2596
653=Pseq10	GAGATGGCAGAGGA(AG)ACTGTAGATCT	P(+)	1568-1593
905=PseqR	CCTTAACTATGTC(AG)TCAAG(AG)TTCA	P(-)	2208-2231
388=M1seq2rev	CATCAAGGTTCATTTTTAACTGCT	P(-)	2196-2219
361=Oligo6SX2	CTCTAGAGCTCGTTCAGCCTCTAACTCGATT	G(-)	5459-5478
179=Gseq3	TGTTGAGGTTCACCTTCCCGATGT	G(+)	4619-6040
191=Gseq5	TCTAGCAGTTTCGGTGACCAACGG	G(+)	5088-5111

region respectively. A water sample was processed in parallel as a negative control, while RNA extracted from rabies-infected brain material was processed in parallel as a positive control. A portion of the RT product (cDNA) was subjected to PCR amplification using primers 888 and 361 for the P-gene and G-L intergenic region respectively. All PCRs were performed with the Expand™ system, as directed by the manufacturer (Roche Molecular Biochemicals). The Expand™ enzyme is a thermostable DNA polymerase mix that yields high copy fidelity. The Gene Amp PCR system 9600 (Perkin Elmer Celus, Emeryville, CA) was used for thermal cycling. Amplification of the P-gene was carried out by first denaturing at 93°C for 2 minutes then 10 cycles of denaturation at 93°C for 10 seconds, annealing at 48°C for 30 seconds and polymerization/extension at 68°C for 4 minutes. An additional 20 cycles of amplification was performed at 93°C for 10 seconds, 48°C for 30 seconds, 68°C for 4 minutes (+ 20 seconds auto-extension per cycle), held at 68°C for 5 minutes before finally holding at 4°C. Since the WSK virus yielded low amounts of the P amplicon a nested PCR was performed on both the WSK and EAF PCR products using internal Primers 653 and 905. The cycle employed was 93°C for 2 minutes, then 10 cycles of 93°C for 10 seconds, 55°C for 30 seconds and 68°C for 4 minutes. An additional 20 cycles was then performed at 93°C for 10 seconds, 55°C for 30 seconds, 68°C for 4 minutes (+20 seconds auto-extension per cycle), held at 68°C for 5 minutes before holding at 4°C. The expected amplicon size was 664bp. Amplification of the G-L region was carried out by first denaturing at 93°C for 2 minutes, then 10 cycles of 93°C for 10 seconds, 55°C for 30 seconds and 68°C for 4 minutes, followed by 20 cycles of 93°C for 10 seconds, 55°C for 30 seconds, 68°C for 4 minutes (+20 seconds auto-extension per cycle). After a final hold at 68°C for 5 minutes the reactions were

cooled and held at 4°C. The expected amplicon was 860bp in size. Analysis of PCR products was carried out by DNA gel electrophoresis through 1% agarose with the use of 100bp DNA size marker. Bands were visualized by ethidium bromide staining under UV light. PCR products were stored at –20°C until needed for molecular cloning and DNA sequencing.

Molecular Cloning, Amplification of Plasmid and Plasmid Purification

Since the Expand™ enzyme/system generates blunt-ended PCR products all amplicons were cloned using the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (Invitrogen, Carlsband, CA). In a microfuge tube between 0.5-4µl of the PCR product (depending on yield as observed by DNA gel electrophoresis) was added to 1µl of salt solution, 1µl of TOPO® vector (plasmid, Figure 3) and sterile water was added to a total volume of 6µl. The reaction was mixed gently and incubated at room temperature for 30 minutes. 2µl of the reaction was then added to a vial of commercially prepared TOP 10 One Shot® Chemically Competent *E coli* cells (Invitrogen) and mixed gently by tapping the vial against the hand. This transformation mix was then incubated on ice for 30 minutes and was mixed gently every 10 minutes. Cells were then heat-shocked at 42°C in a water bath (Baxter Scientific, Multiblock heater) for 30 seconds and were then immediately transferred to ice. 250µl of room temperature SOC medium was then added aseptically, tubes capped tightly and shaken horizontally (200rpm) (Labline Orbit environ shaker, Labline Institute Inc., Melrose Park, IL) at 37°C for 1hour. 10µl, 50µl and 100µl of the transformations were then spread unto pre-warmed (at 37°C) LB agar plates with Ampicillin (100µg/ml) and Kanamycin (25µg/ml). An unseeded LB agar plate and one onto which SOC medium was spread acted as negative controls. Plates were then



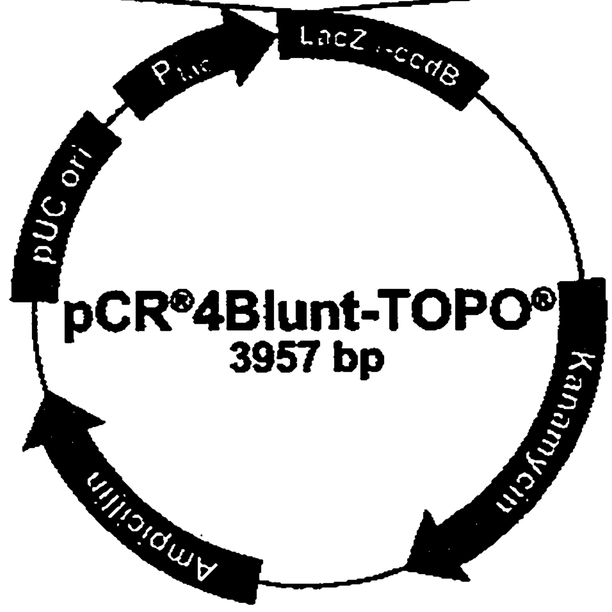


Figure 3: **Map of pCR®4Blunt-TOPO® (plasmid)**
Source: Invitrogen Zero Blunt® TOPO® PCR Cloning Kit for Sequencing Instruction Manual.

incubated overnight at 37°C. For plasmid propagation, transformed *E. coli* colonies picked at random were re-grown on fresh LB agar Ampicillin + Kanamycin plates for reference and were simultaneously cultured overnight in 3ml of LB liquid broth containing ampicillin and kanamycin in 6ml sterile polystyrene culture tubes (Falcon). Plates were incubated at 37°C overnight and then stored at 4°C for several weeks. Tubes were incubated at 37°C overnight with agitation in an orbital shaker. The following day cells were harvested from the liquid cultures and plasmid purification was carried out using the Wizard™ PCR preps DNA purification system (Promega, Madison, WI) using the Vac-Man laboratory vacuum manifold (Promega) with a vacuum source. Plasmid DNA was then stored in microfuge tubes at –20°C until needed for restriction analysis.

Restriction Enzyme Analysis

Since Eco R1 sites flank either side of the polylinker region into which the PCR fragments were inserted (Figure 3), plasmids were analyzed for the presence of the insert by restriction endonuclease digestion using Eco R1 (4U/reaction) (Gibco BRL). For each restriction (Rx) endonuclease digestion a master mix containing 1µl of 10x Rx buffer (Gibco BRL), 1µl of BSA molecular biology grade (1mg/ml) (Gibco BRL), 4U of Eco R1 and sterile water to a total volume of 7µl was prepared. 7µl of the master mix and 3µl of plasmid DNA (total volume of 10µl) were mixed and the reaction was incubated in a dri-block heater (Baxter Scientific, Multi Block heater) at 37°C for 2 hours. The reaction was terminated by the addition of 1µl of 10x DNA loading buffer and heating at 65°C for 5 minutes. Tubes were centrifuged briefly to collect any condensate and samples analyzed by DNA agarose gel electrophoresis using the 1Kbp DNA ladder and 100bp

DNA ladder for size determination. Plasmid DNA/clones were kept at –20°C until needed for DNA sequencing.

DNA Sequencing

All sequencing was performed manually. Sequencing of the P-gene and G-L intergenic region PCR products and recombinant plasmid DNA was performed with ^{32}P-labeled primers using the Promega fmol DNA sequencing kit as suggested by the manufacturer. Primers 388 and 191 (see Table I) were used to sequencing the P-gene and G-L intergenic region respectively. Thermal cycling of sequencing reactions was performed in a Model 480 cycler (Perkin Elmer, Foster City, CA) after a 2 minute denaturation of 95°C. Sequencing of the P-gene product was carried out for 30 cycles of 95°C for 45 seconds, 50°C for 30 seconds and 70°C for 30 seconds before being held at 4°C. Sequencing of the G-L intergenic region products was carried out for 30 cycles of 95°C for 45 seconds, 60°C for 30 seconds and 70°C for 30 seconds then held at 4°C. The sequencing reactions were stopped by the addition of 3μl of fmol Sequencing Stop Solution (Promega). Reactions were then heated at 80°C in a water bath (Baxter Scientific, Multiblock heater) for 3 minutes, chilled on ice immediately and then loaded (2-3μl of each reaction) unto 6% polyacrylamide denaturing gels using a SA-88 sequencing gel apparatus (BRL-Life Technologies). Gels were run for 2½-3 hours for short gels and 7-8 hours for long gels. The unused portion of the sequencing reaction was stored at -20°C. After electrophoresis, the gel was transferred to Whatman 3MM chromatography paper and dried using the Bio-Rad Gel Dryer (Model 583) at 80°C for 40-50 minutes. Autoradiography was carried out by exposing the gel to KODAK Scientific Imaging film (Eastman KODAK Company, Rochester, N.Y.) for 24-48 hours,

depending on the 'freshness' or extent of decay of the radioisotope (^{32}P), at -80°C to minimize the signal to noise ratio. After exposure of the gel to the film, the film was developed and nucleotide sequences were read.

Sequence Analysis

Nucleotide sequences were read manually and the data entered into DNASIS. Protein sequences were predicted from these data using DNASIS whilst sequences were aligned using CLUSTALX (Jeanmougin et. al., 1998).

Animal Inoculation

Twelve albino guinea pigs of approximately 4-5 weeks old obtained from the Animal Care Colony at ADRI were inoculated with the WSK virus and the EAF virus according to Table II. The ADRI Animal Care Committee approved the experimental design. The titer of both viruses was adjusted to $10^{4.80}$ $TCID_{50/ml}$, diluted in MEM containing 2% FBS and 5% TPB. No antibiotic was added to the MEM since guinea pigs, which have a unique bacterial flora in their intestines, could be adversely affected by inclusion of antibiotics. Guinea pigs were anesthetized with isofluorane through the inhalation system and received the virus suspensions as shown in Table II using tuberculin syringes. Remaining virus suspensions (that had been inoculated into the guinea pigs) were titrated (see section 'Virus Titration') to re-confirm the titer of viruses inoculated into the guinea pigs. Animals were checked daily and once clinical signs of rabies were noticed, the guinea pigs were euthanized with a mixture of oxygen and carbon dioxide and the animals necropsied. Brain, spinal cord, muscle and salivary gland

Table II. **Site of Inoculation, Amount and Virus given to each of the Guinea Pigs:** 12 albino guinea pigs of approximately 4-5 weeks old were inoculated with either the WSK or EAF viruses individually or in co-infection. The titer of both viruses was adjusted to $10^{4.80}$ $TCID_{50/ml}$.
i.m= intra-muscular
i.c= intra-cerebral

Site of Inoculation, Amount and Virus given to each of the Guinea Pigs

Guinea Pig	Site of inoculation	Amount given (ml)/$TCID_{50}$ per animal	Virus given
1&2	i.m	0.1	Western
3&4	i.c	0.03	Western
5&6	i.m	0.1	Eastern
7&8	i.c	0.03	Eastern
9&10	i.m	0.1	Mixture
11&12	i.c	0.03	Mixture

tissues were removed and stored at –80°C until needed for cryostat sectioning and titrations.

Cryostat Sectioning

Cryostat sections of the brain (left hemisphere, right hemisphere, medulla and cerebellum), salivary glands, spinal cord, right muscle and left muscle of the hind legs were prepared using Cryostat #855 (Reichert-Jung, Cambridge Instruments GmbH, Nubloch, Germany) from frozen tissues that had been embedded into rectangular wooden blocks with OCT tissue compound. Sections of 12 microns in width were taken at –10°C, mounted onto clear glass slides (Surgipath Medical Industries, Winnipeg, Manitoba) and exposed to UV light for two minutes before being stored in a slide box at –80°C until needed for analysis by direct immunofluorescence using labeled mabs and for FAT.

Staining Cryostat Sections

Cryostat sections were fixed for 20 minutes in undiluted cold acetone in a Copeland jar at room temperature. Sections were then air dried for 3-5 minutes. A circle was then drawn with a wax pencil and slides mounted onto a slide holder in a petri dish for staining. 100μl of diagnostic conjugate at a dilution of 1:400 or labeled mabs (24FF11 FITC (1:100), 24FF11 biotin (1:200), M960 FITC (1:50 or 1:100), M960 biotin (1:50) or a combination of both mabs) was then added to the sections using an automatic dispenser (Bioht) making sure that the conjugate or labeled mabs do not run out of the circle. The petri dish was then incubated at 37°C for 45 minutes. Conjugate was then drained off the slides, which were washed twice in PBS, leaving slides in PBS for 2-3 minutes during the second wash. For slides to be stained with more than one monoclonal antibody, the slides were once again air-dried for 2-3 minutes before application of the second mab,

incubation at 37°C for 45 minutes and washing twice in PBS. Lastly, for use with biotin labeled mabs, Strepavidin PE (1mg/ml) at a dilution of 1:500 was applied, incubated at 37°C for 45 minutes and washed twice once again in PBS. Once all the slides had been washed in PBS, they were gently blotted with a paper towel, placed on a slide tray and left to dry in the dark for about 20 minutes. Mounting media (glycerol+ Sorensen's buffer) was then placed on sections and cover slips were applied. Sections were then examined for fluorescence of infected cells using a Leica fluorescent microscope and pictures taken using a KODAK camera and software.

Titrating Virus in Brains and Salivary Glands

Rabies virus titers were determined, as described above, for the brains (pooled portions of various tissues of the brain consisting of the cerebellum, left hemisphere, right hemisphere and the medulla oblongata) and salivary glands of guinea pigs that developed rabies (the brains and salivary glands of guinea pigs infected with both viruses were titrated twice). A 10% suspension in MEM supplemented with 10% FBS, 5% TPB and 1% AbAm of the guinea pig brain was made and serially diluted ten fold in MEM supplemented with 10% FBS, 5% TPB and 1% AbAm. For the salivary glands a 20% suspension was made before being serially diluted tenfold. MNA cells were seeded at 2 X 10^5 cells/ml (100μl/well) in a 96 well plate and inoculated with serial dilutions of the brain and salivary gland suspensions. Plates were incubated at 37°C for 5 days and monolayers of cells fixed and stained for FAT with diagnostic conjugate as previously explained. For those guinea pigs that were infected with both viruses labeled mabs were also employed.

Experiments and Results

The main objective of this study was:

To address aspects of virus evolution and virus population biology, by determining the fate of two known rabies virus strains when they are passaged by themselves and in competition with each other. There are two aspects of this objective which are of interest:

(i) Does one strain compete out the other?

(ii) Does each strain change?

The experimental design is summarized in Table III.

Competition *in vitro*

To study competition *in vitro* between two closely related rabies viruses, the Western Skunk Virus (a member of the cosmopolitan lineage) and the Eastern Arctic Fox Virus (a member of the Arctic lineage), the MNA cell line was used as the cell line of choice for viral propagation and assaying for virus infectivity (Iwasaki and Clark, 1977; Clark, 1980; Umoh and Blenden, 1983; Tsiang, 1985; Rudd and Trimarchi, 1987).

1.1 Obtaining a Homogenous Population of the Virus Variants

RNA populations exhibit a heterogeneous population structure (Holland et. al., 1992; Drake, 1993; Domingo and Holland, 1994, 1997) within single individuals, often referred to as 'quasispecies'. This heterogeneous population structure has been demonstrated for rabies virus (Benmansour et. al., 1992; Morimoto et. al., 1998; Kissi et. al., 1999) and is assumed to be a common feature of all rabies virus strains. It was therefore important to start the experimentation with a homogenous population structure. Hence, the WSK and EAF viruses were cloned separately by subjection to two consecutive limit dilution purifications to generate two final clones, $92L1169P_{22}B_6$ sub

Table III. **Experimental Design**: Experiments were designed, performed and divided based on the three sub-objectives of the study.

Experimental Design

Objectives	Experiment	Analysis
1. Competition *in vitro*	- Cloning of viruses by limited dilution purification - Mab purification and conjugation to flourochromes -12 passages in MNA cells WSK, EAF WSK/EAF	-Titrate supernatant for each passage for WSK, EAF and WSK/EAF (done twice) -Stain and read 96 well plates to determine titer at every passage -Estimate percentage of infected cells
2. Competition *in vivo*	-Guinea pig inoculation i.c, i.m. -Cryostat sectioning of different tissues	-Titrate brain and salivary glands -Demonstrate virus in tissue sections by immunofluorescence
3. Clonal variation *in vitro*	-12 passages in MNA cells WSK, EAF	-RNA isolation and RT-PCR -Molecular cloning -Restriction endonuclease digestions -Sequencing 84 clones for input virus -Sequencing 84 clones for output virus

clone D_6, with a titer of $10^{5.83}$ $TCID_{50/ml}$ for the WSK strain and $98N4235P_{15}$ D_5 sub clone B_3 with a titer of $10^{7.00}$ $TCID_{50/ml}$ for the EAF strain. The designations B_6, D_6, D_5 and B_3 used to identify the clones, refer in each case to the well from which the virus supernatant was initially harvested from a 24 well plate.

1.2 Characterization of Reactivity Patterns of the Virus Variants to the Various Purified Monoclonal Antibodies

The purpose of this experiment was to demonstrate that original viral stocks exhibit expected reactivities to the selected mabs used in the study. Indeed the precise strains selected for this study (the WSK strain and the EAF strain) were chosen based on their antigenic discrimination which had already been well established by Dr. Wandeler and staff. This analysis confirms these prior findings. A short panel of three Anti-N mabs (M868, M993 and 24FF11) and two Anti-P mabs (M960 and M964) (that had been previously tested) was available at the Center of Expertise for Rabies (ADRI). Mabs were purified using Protein G affinity columns and conjugated to the fluorochrome FITC or biotin succimide ester. Optimization of the purified, labeled mabs involved dilution of the mabs two-fold to determine the staining properties and the optimal dilution for use to detect virus antigen with minimal background staining which will clearly distinguish between the two strains. Table IV shows the characteristic pattern of binding of these purified mabs to the two virus strains. Whilst mabs M868 and M993 bound both viral strains under optimal conditions mab 24FF11 and M960 clearly distinguish between the two strains with 24FF11 (dilution of 1:400) specific for the WSK and M960 (dilution of 1:200) specific for the EAF virus. These two mabs were therefore used as the mabs of

Table IV. Characteristic Reactivity Patterns of the Virus Variants to the Various Purified Monoclonal Antibodies: Purified, conjugated monoclonal antibodies were used in direct immunofluorescence against WSK virus infected MNA cells and EAF virus infected MNA cells. Binding and staining properties of the mabs were observed using the Leica fluorescent UV microscope.

+++= three plus positive, specific (grading the intensity of fluorescence)

- = negative, non-specific (no fluorescence)

Characteristic Reactivity Patterns of the Virus Variants to the Various Purified Monoclonal Antibodies

Monoclonal Antibody	Mab Properties	Western Skunk Virus	Eastern Arctic Fox Virus
24FF11	Anti-N	+++	-
M868	Anti-N	+++	+++
M964	Anti-P	-	+++
M960	Anti-P	-	+++
M993	Anti-N	+++	+++

choice for variant discrimination throughout the study though M964 was also specific for the EAF virus.

1.3 Verification of Clones

To verify that the virus clones (92L1169P_{22} B_6 sub clone D_6 for the WSK virus and 98N4235P_{15} D_5 sub clone B_3 for the EAF virus) which had been identified and propagated are actually clones of the specific variants and exhibit mab reactivities typical of strains as demonstrated in section 1.2, clones were subjected to indirect immunofluorescence with the following mabs: 5DF12 (Anti-N, which binds to most rabies virus strains, hence acting as a positive control), 26AF11, 38FG5, M993 (additional positive controls which are all Anti-N mabs that bind both variants), 24FF11 (which is specific for the WSK strain), M960 and M964 (which are specific for the EAF strain). Unlabelled mabs were detected by subsequent incubation with FITC-conjugated goat anti-mouse immunoglobulin as the secondary antibody. The staining pattern of these mabs against the WSK clone and the EAF clone, shown in Table V confirm previous studies carried out at the Center of Expertise for Rabies (ADRI), when tested against un-cloned WSK and EAF viruses (results not shown). 24FF11 is specific to the WSK while M960 and M964 are specific to the EAF virus. M964 gives a very diffuse, very intense staining pattern. These results show that mabs specific to each variant are not cross-reactive between the variant clones. Similar levels of staining between the specific mabs were also observed. This confirms the antigenic homogeneity of the virus clones 92L1169P_{22} B_6 sub clone D_6 and 98N4235P_{15} D_5 sub clone B_3.

Table V. The Staining Pattern of 92L1169P_{22} B_6 sub clone D_6 (WSK) and 98N4235P_{15} D_5 sub clone B_3 (EAF) when Tested with Various Mabs: All mabs were subjected to indirect immunofluorescence using FITC conjugated goat anti-mouse immunoglobulin as the secondary antibody and viewed using the Leica fluorescent UV microscope. Once again the intensity of fluorescence is being graded.

The Staining Pattern of $92L1169P_{22}$ B_6 sub clone D_6 (WSK) and $98N4235P_{15}$ D_5 sub clone B_3 (EAF) when Tested with Various Mabs.

Monoclonal Antibody	Mab Properties	$92L1169P_{22}$ B_6 sub clone D_6	$98N4235P_{15}$ D_5 sub clone B_3
5DF12	Anti-N	+++	+++
24FF11	Anti-N	+++	-
26AF11	Anti-N	+++	+++
38FG5	Anti-N	+++	+++
M960	Anti-P	-	+++
M964	Anti-P	-	+++
M993	Anti-N	+++	+++

1.4 Maintenance of Rabies Virus Infected MNA Cells

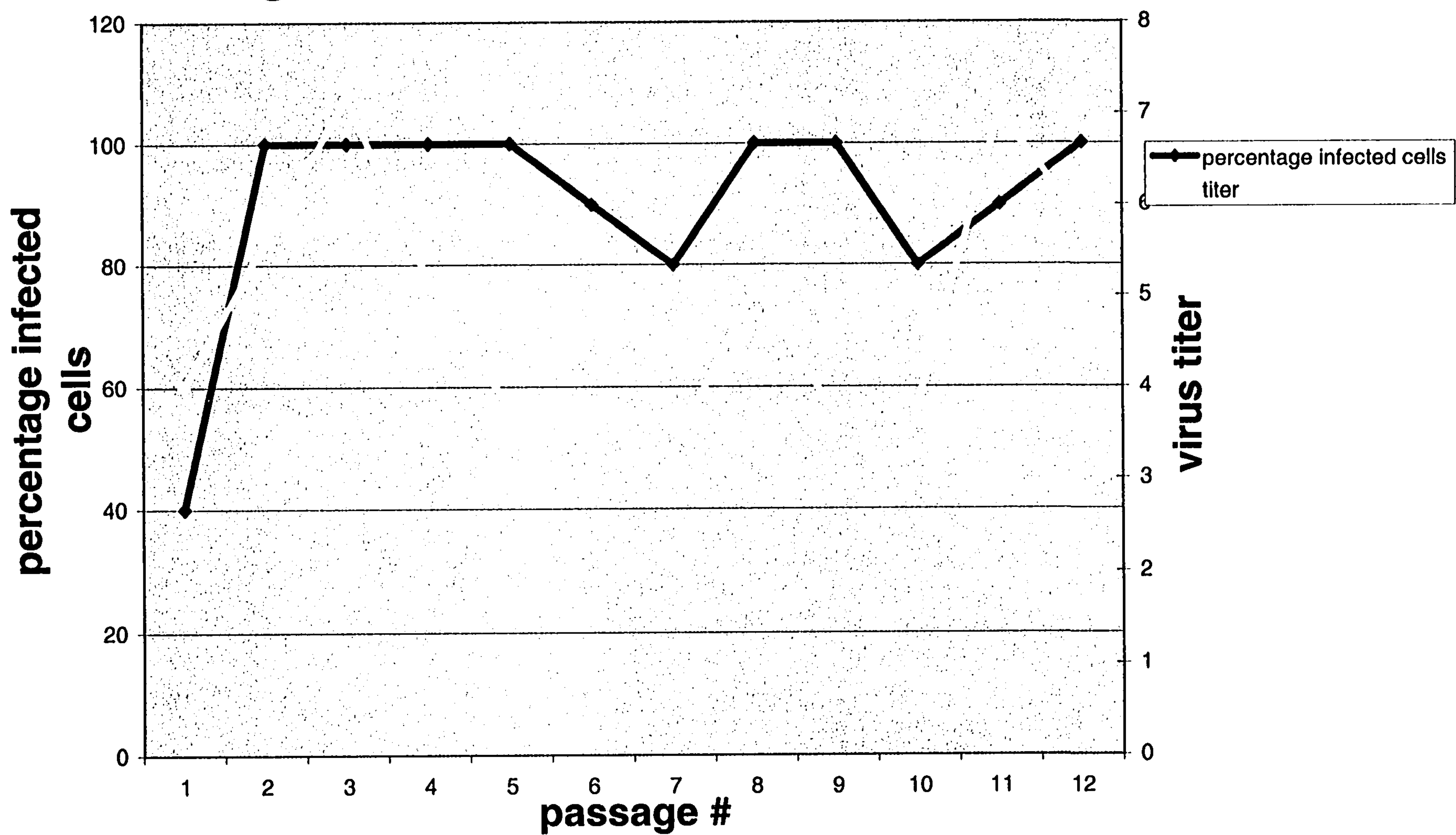
The objective of the passaging experiment was two-fold. The first objective was to study the competition between the two virus variants as they were passaged within the same cell line together and secondly to study the heterogeneity of each virus population when passaged separately using molecular biology techniques as the tools for studying polymorphism.

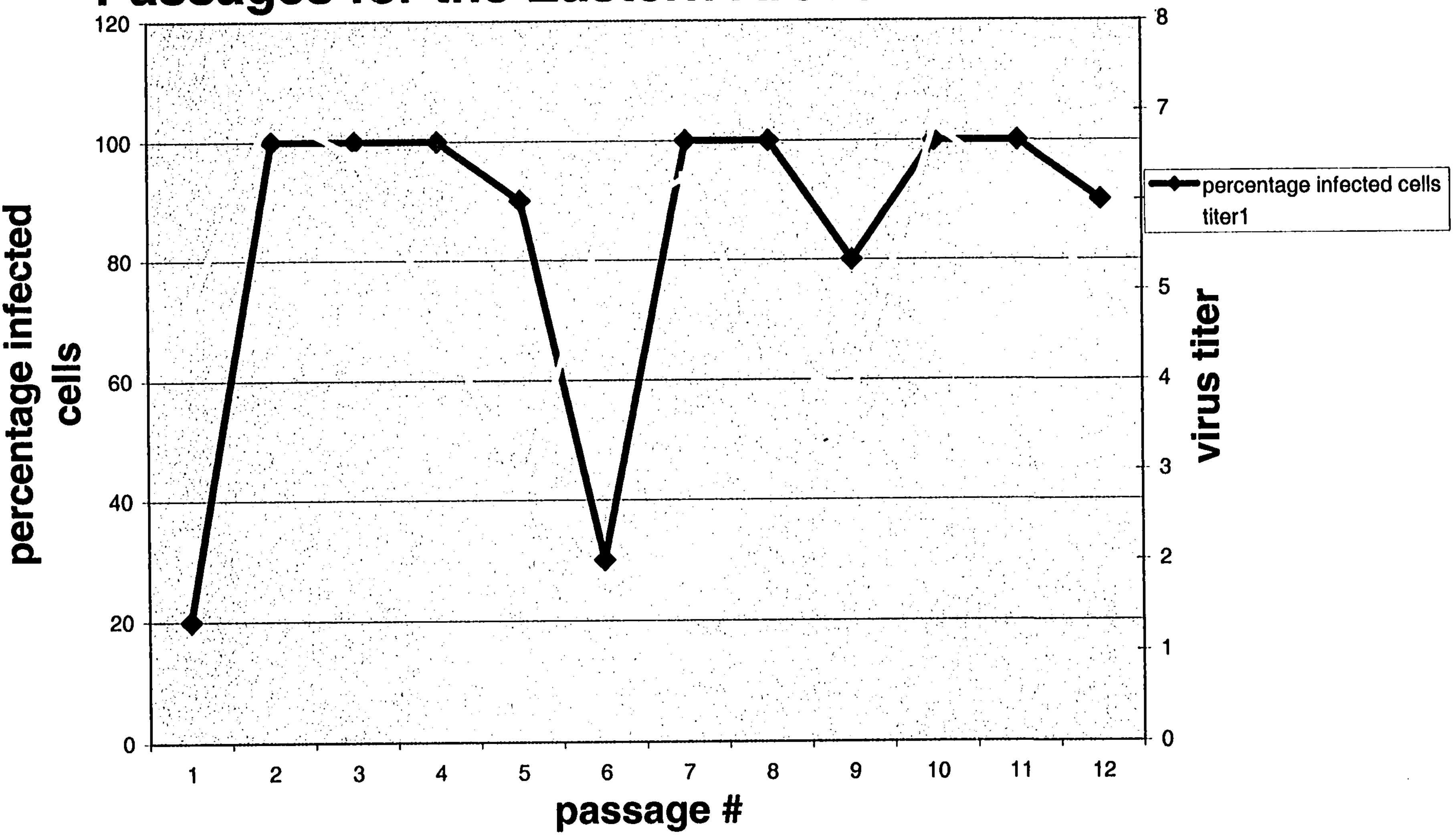
MNA cells were infected with the WSK clone, the EAF clone and a combination of the WSK/EAF variant clones in suspension at a MOI of 0.2 and passaged 12 times. A MOI of 0.2 was used because preliminary experiments showed that for the EAF virus, approximately 50% of the cell monolayer is infected when cell monolayer is approaching 100% before passaging. This will reduce the occurrence of defective interfering particles. The WSK virus on the other hand was much slower at replicating in the cell monolayer and required passaging at least once for virus antigen to be detected at high levels. Before MNA cells were infected, it was important to verify the titer of the variant clones that were stored at -80°C until needed for passaging. The titer of the EAF clone (98N4235P_{15} D_5 sub clone B_3) when re-titrated was found to be $10^{7.33}$ $TCID_{50/ml}$, which correspond to its previous titer ($10^{7.00}$ $TCID_{50/ml}$) before storage, whereas the titer of the WSK clone (92L1169P_{22} B_6 sub clone D_6) fell by more than a log from $10^{5.83}$ $TCID_{50/ml}$ to $10^{4.42}$ $TCID_{50/ml}$ indicating that the WSK virus may not be as stable during freezing and thawing as the EAF. The titer of the WSK clone was titrated a second time to confirm this result, obtaining a titer similar to the previous result ($10^{4.25}$ $TCID_{50/ml}$). To infect cells at a MOI of 0.2, 30.2ml of the WSK virus would be needed at a titer of $10^{4.42}$ $TCID_{50/ml}$. As this amount of virus was not available, it was necessary to passage the WSK clone three times

from P_{22} to P_{25} to increase its titer; passaging on MNA cells at approximately 60-70% monolayer, and not at a 100% monolayer, was done to prevent the accumulation of defective interfering particles and interferon. The titer of the final preparation of the WSK clone (92L1169P_{25} B_6 sub clone D_6) increased to $10^{5.58}$ $TCID_{50/ml}$ and at this titer 2ml of virus was needed to infect MNA cells at a MOI of 0.2.

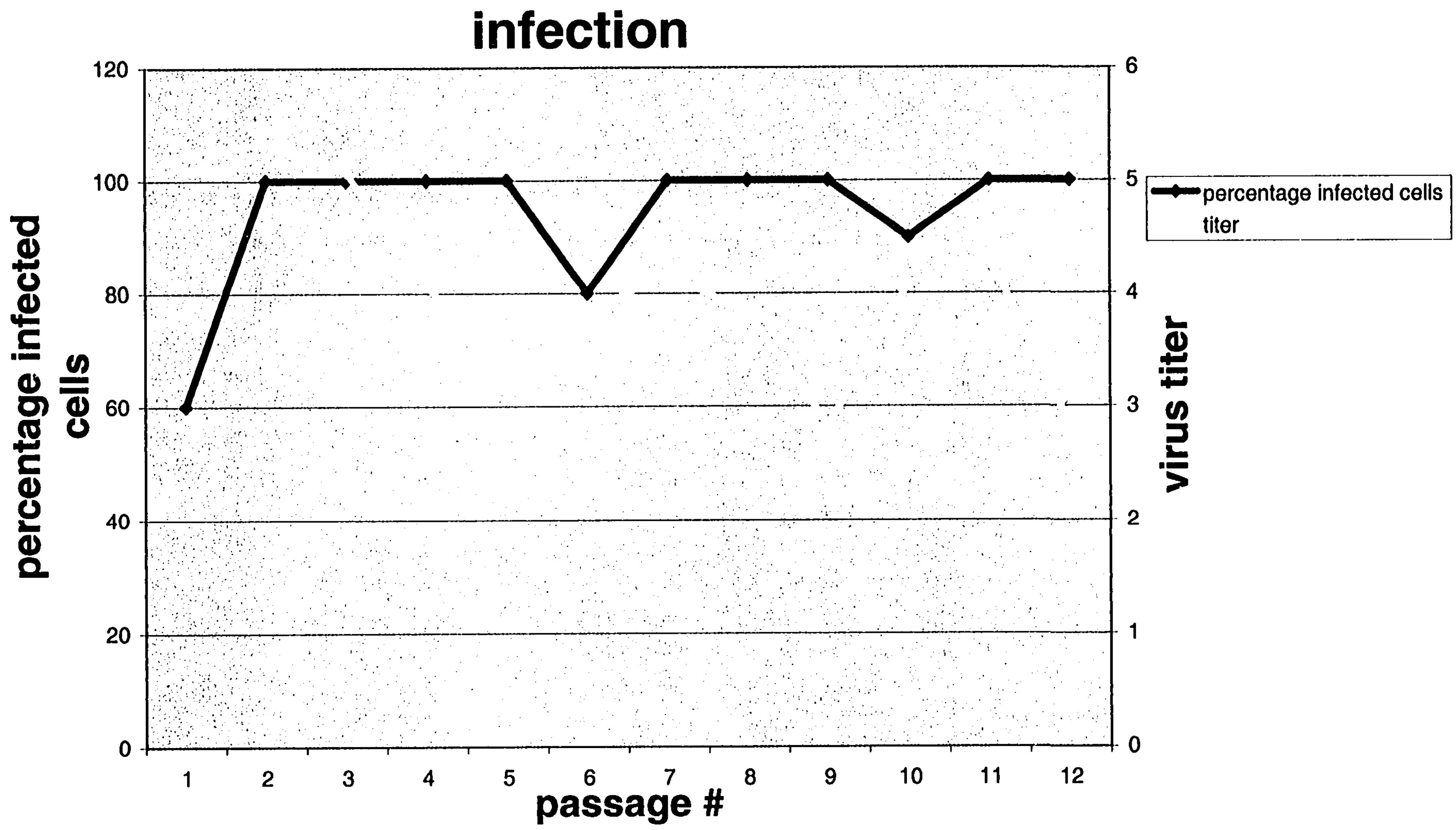
To study competition *in vitro* between the WSK (WSK clone 92L1169P_{25} sub clone D_6) and EAF (EAF clone 98N4235P_{15} D_5 sub clone B_3) it was important to passage these viruses individually in MNA cells to make sure that the viruses can be propagated over a 12 passage period and that the infection can be maintained. At every passage, supernatant was harvested and the titer of the viruses determined. Supernatant containing both viruses was titrated twice and stained with the respective mabs (24FF11 FITC at a dilution of 1:400 to detect the WSK and M960 Biotin at a dilution of 1:200 with Strepavidin PE at 1:500 to detect the EAF). The percentage of infected cells was quantitatively estimated as an average over six wells in the virus monitor. Each well was divided into four quadrants equaling 25% and the presence of virus antigen in the infected cells in each quadrant was estimated quantitatively. Figures 4A-4D show the cell infection levels and virus titers over 12 passages for the WSK virus and EAF virus passaged both separately and as a co-infection at a MOI of 0.2. This experiment confirmed that the infection can be maintained over a twelve-passage period for both the WSK virus and the EAF virus. It was observed that the cell infection levels and virus titers fluctuated in a cyclical pattern, and suggested that virus propagation can be maintained indefinitely. It was also observed that as the percentage of infected cells increased or decreased, the virus titers also increased or decreased, although lagging

Figure 4A-4B. **Cell Infection Levels and Virus Titers Over 12 Passages for A, the WSK Virus and B, the EAF Virus:** MNA cells at a concentration of 2 X 10^5 cells/ml were infected with either the WSK virus or the EAF virus separately at a MOI of 0.2 and passaged 12 times. At every passage supernatant was harvested and the titer of the virus determined. Monitors were also made at every passage to determine quantitatively the percentage of infected cells taken as an average over six wells. Cells were subjected to FAT using diagnostic conjugate.

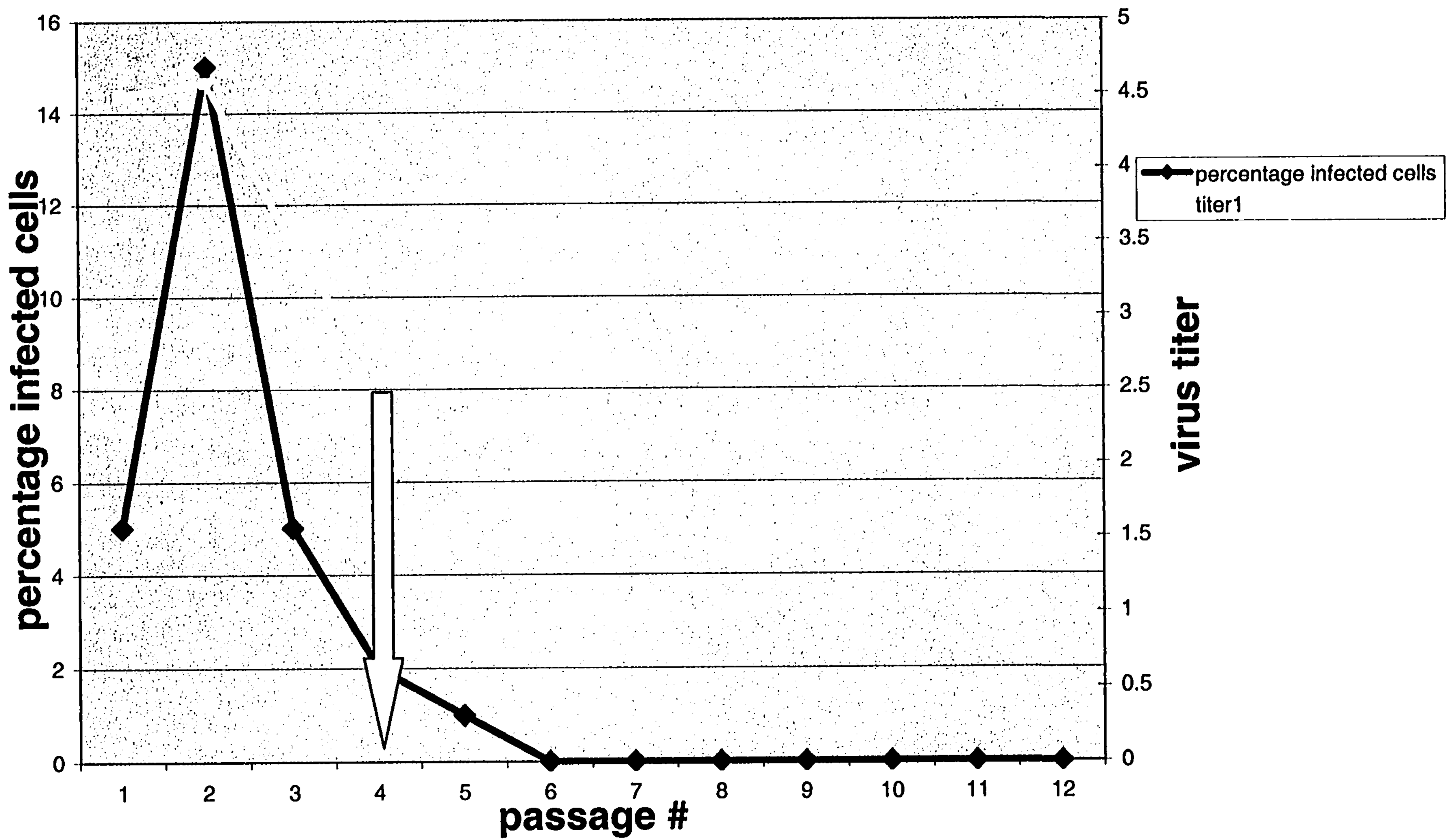

Cell Infection Levels and Virus Titers Over 12 Passages for the Western Skunk Virus
percentage infected cells
virus titer
passage #
percentage infected cells
titer
120
100
80
60
40
20
0
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12


Cell Infection Levels and Virus Titers Over 12 Passages for the Eastern Arctic Fox Virus
percentage infected cells
virus titer
passage #
percentage infected cells
titer1
0
20
40
60
80
100
120
0
1
2
3
4
5
7
8
1
2
3
4
5
6
7
8
9
10
11
12

Figures 4C-4D. **Cell Infection Levels and Virus Titers Over 12 Passages for C, the WSK virus in co-infection and D, the EAF virus in co-infection:** MNA cells at a concentration of 2 X 10^5 cells/ml were infected with the WSK and EAF viruses in co-infection at a MOI of 0.2 and passaged 12 times. At every passage supernatant containing both viruses was titrated twice and stained with their respective mabs. Monitors were also subjected to direct immunofluorescence with the respective labeled mabs. In figure 4D the arrow indicates the loss of the EAF virus after the 4th passage.


Cell Infection Levels and Virus Titers Over 12 Passages for the Western Skunk Virus in Co-infection
percentage infected cells
virus titer
passage #
0
20
40
60
80
100
120
0
1
2
3
4
5
6
1
2
3
4
5
6
7
8
9
10
11
12
percentage infected cells
titer

Cell Infection Levels and Virus Titers Over 12 Passages for the Eastern Arctic Fox Virus in Co-infection



behind the cell infection levels. When the WSK and the EAF viruses were passaged as a co-infection, it was observed that there was a loss of the EAF virus by the fourth passage (Figure 4D).

Passaging the un-cloned WSK virus ($92L1169P_4$) and the EAF virus ($98N4235P_4$) individually in MNA cells without a known MOI was previously carried out. MNA cells were inoculated with 100µl of the viruses individually in T_{25} tissue culture flasks and split at a fixed ratio of 1:6 between passages every 3-4 days, once cell monolayer has reached approximately 100 %. Cell infection levels and virus titers fluctuated in a cyclical pattern for both the WSK and EAF viruses as observed in Figures 4a and 4b (results not shown) showing the possibility of continuous growth of viruses in cell culture prior to viral cloning.

Competition *in vivo*

To study competition between the WSK and the EAF viruses *in vivo*, twelve albino guinea pigs of approximately 4-5 weeks old were inoculated with the WSK ($92L1169P_{20}$ B_6 sub clone D_6), the EAF ($98N4235P_{13}$ D5 sub clone B_3) and a combination of the WSK/EAF according to Table II (See materials and methods). Inoculating intra-muscularly should simulate what happens in naturally infected animals. Though intra-cerebral inoculation is not usually a natural occurrence (since animals are not often bitten in the head by a rabid animal), it is important to study the brain infection. The titer of both viruses was adjusted to $10^{4.80}$ $TCID_{50/ml}$ in MEM containing 2% FBS and 5% TPB, but no antibiotic. As mentioned in the materials and methods guinea pigs which have a unique bacterial flora in their intestines could be adversely affected by inclusion of

antibiotics in the media. A titer of $10^{4.80}$ $TCID_{50/ml}$ was used because $92L1169P_{20}$ B_6 sub clone D_6 was already at this titer, while $98N4235P_{13}$ D_5 sub clone B_3 which had a titer of $10^{6.42}$ $TCID_{50/ml}$ could be readily diluted to $10^{4.80}$ $TCID_{50/ml}$. This ensured that an equal amount of both viruses was inoculated into the guinea pigs, preventing one variant clone from having an advantage over the other variant clone. Animals were maintained under pathogen free conditions, checked daily and once they showed signs of rabies were euthanized, with the brain and salivary glands processed for analysis by direct immunofluoresence with the labeled mabs and for FAT with diagnostic conjugate.

2.1 Detecting the Presence of Virus Antigen in the Brains and Salivary Glands

The brains (pooled portions of various tissues of the brain consisting of the cerebellum, left hemisphere, right hemisphere and the medulla oblongata) and salivary glands of the guinea pigs that developed rabies (five guinea pigs) were titrated unto tissue culture to check for the presence of virus antigen. If one of the factors determining the fitness of a virus is its ability to be transmitted from host to host, and the rabies virus is transmitted through the saliva by the bite of an infected animal, it is necessary to check for the presence of virus antigen in the salivary glands by virus isolation. From the results in Table VI no virus antigen was detected when the salivary glands of the guinea pigs that developed rabies were titrated, but the brains all show the presence of virus antigen. In guinea pigs that were infected with both the WSK strain and the EAF strain, the brains and salivary glands were titrated twice and stained directly with the respective labeled mabs. Guinea pig #278N showed only the presence of the WSK when the brain was titrated, while only the EAF was detected when the brain of guinea pig #275N was titrated. The remaining seven guinea pigs that did not develop rabies were euthanized and

Table VI. **Detection of Virus Antigen in Brains and Salivary Glands of Guinea Pigs Infected with Rabies:** Brains and salivary glands of guinea pigs that succumbed to the rabies virus infection were titrated to check for the presence of virus antigen and subjected to FAT using diagnostic conjugate at a dilution of 1:300. Guinea pig brains infected with both the WSK and EAF viruses were titrated twice and stained with their respective labeled mabs.

Detection of Virus Antigen in Brains and Salivary Glands of Guinea Pigs Infected with Rabies

Guinea pig #	Virus Given	Site of inoculation	Virus titer in brain $TCID_{50/ml}$	Virus titer in salivary glands $TCID_{50/ml}$
274N	WSK	i.c.	$10^{7.75}$	No virus antigen detected
277N	WSK	i.c.	$10^{6.75}$	No virus antigen detected
271N	EAF	i.c.	$10^{7.17}$	No virus antigen detected
278N	WSK/EAF	i.c.	$10^{6.33}$ WSK only	No virus antigen detected
275N	WSK/EAF	i.c.	$10^{4.50}$ EAF only	No virus antigen detected

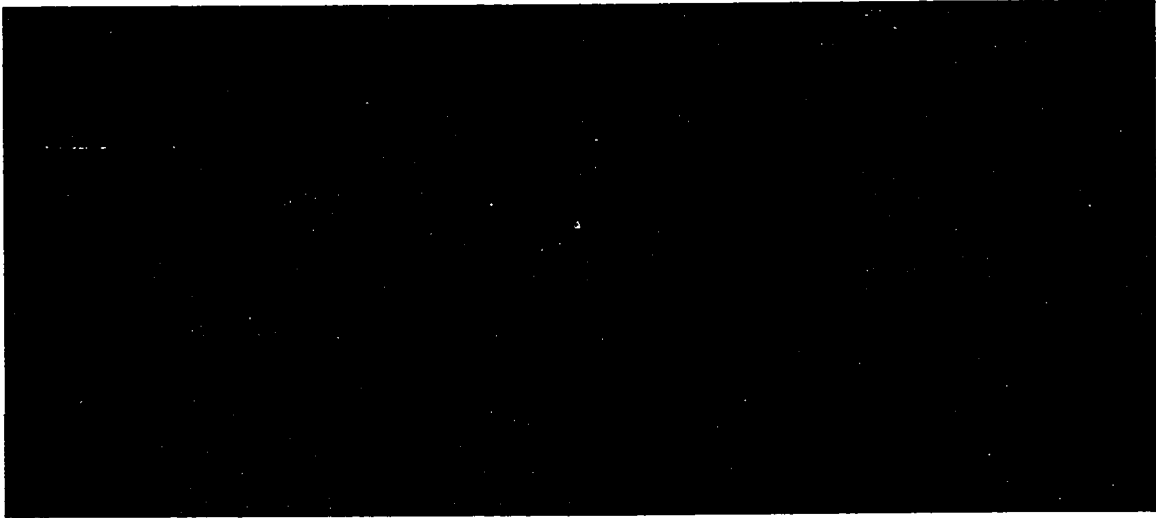
blood from each animal (approximately 25ml) was collected into a vacutainer containing gel and clot activator. Approximately 5ml of serum was collected from each sample and heat inactivated. The presence of antibodies against the rabies virus was investigated in a serum neutralization assay (results not shown). No antibodies against the rabies virus were detected in any of the seven guinea pigs.

2.2 Studying Virus Occurrence within the Guinea Pig Brains- Cryostat Sectioning and Staining

Cryostat sections (10 sections per sample) of the brain (cerebellum) of each guinea pig that developed a rabies infection were made. Brain sections (3 sections per sample) of guinea pigs that were inoculated with one virus were stained with diagnostic conjugate at a dilution of 1:600 to detect for the presence of virus antigen. Figures 5A-5C shows a widespread infection appearing as accumulations of viral RNP in infected cells, characterized by apple green granulations thereby demonstrating the presence and replication of virus in the brains of all three guinea pigs. Figure 6A shows the presence of both the WSK and the EAF present in the brain of guinea pig #278N, which had been infected with both viruses, indicating that both viruses can infect the brain of an animal simultaneously, though titration of the brain of this guinea pig indicated the presence of only the WSK virus. The infection appears localized and though both viruses are present they do not appear to infect the same cells. Figure 6B shows a different section of the brain of guinea pig #278N indicating the presence of only the WSK virus. The WSK virus appears to be dominant over the EAF virus in guinea pig #278N. When guinea pig #275N brain was stained, only the EAF virus was detected (Figure 6C), corresponding to the results obtained when the brain was titrated.

Figure 5A-5C. **Pictures Showing the Presence of the Rabies Virus in Guinea Pigs 271N (A), 277N (B) and 274N (C):** Cryostat sections (12 microns in width) of the brain were taken at -10°C and mounted onto clear glass slides. Sections were exposed to UV light for 2 minutes, fixed in cold acetone and subjected to FAT using diagnostic conjugate at a dilution of 1:600. Sections were examined for fluorescence of infected tissue using the Leica fluorescent microscope. Pictures were taken with the SPOT camera and analyzed using the Image Pro Software.

271N 277N



274N

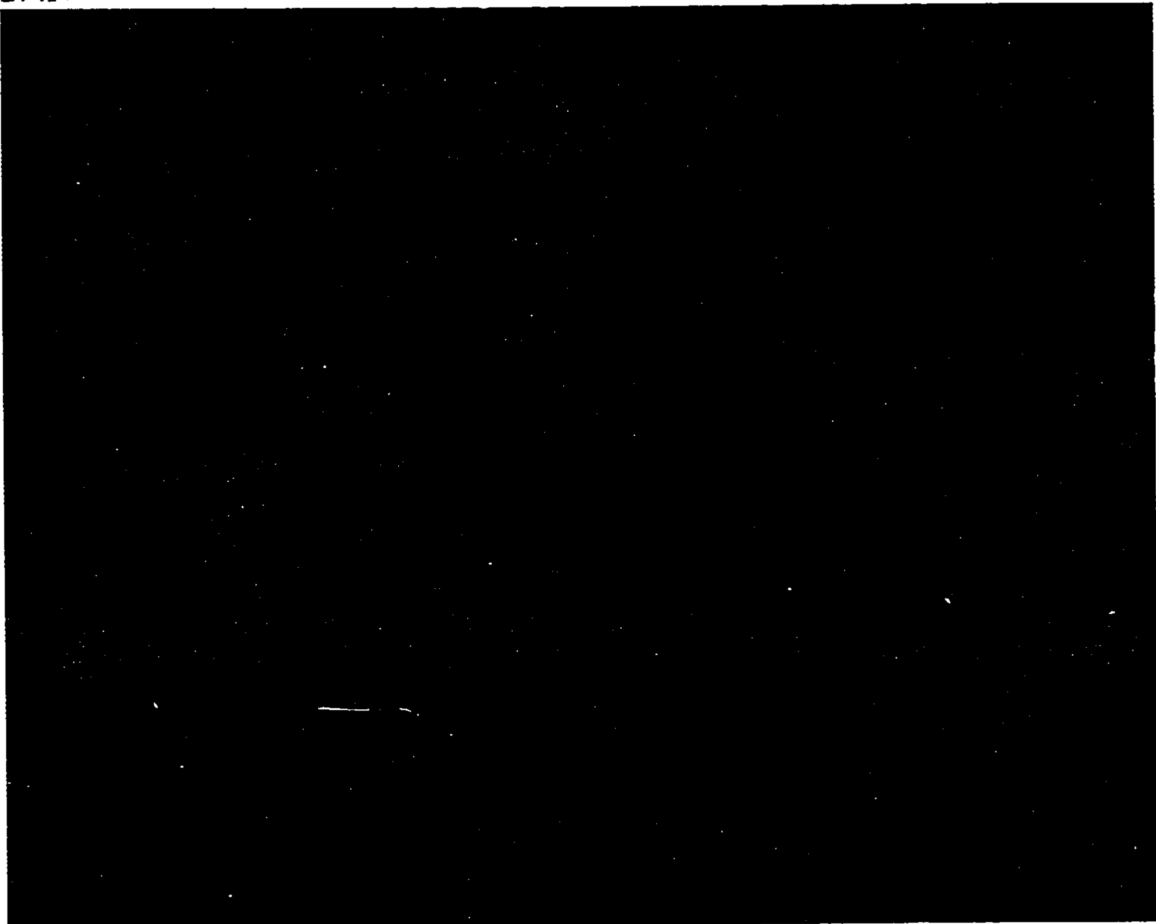
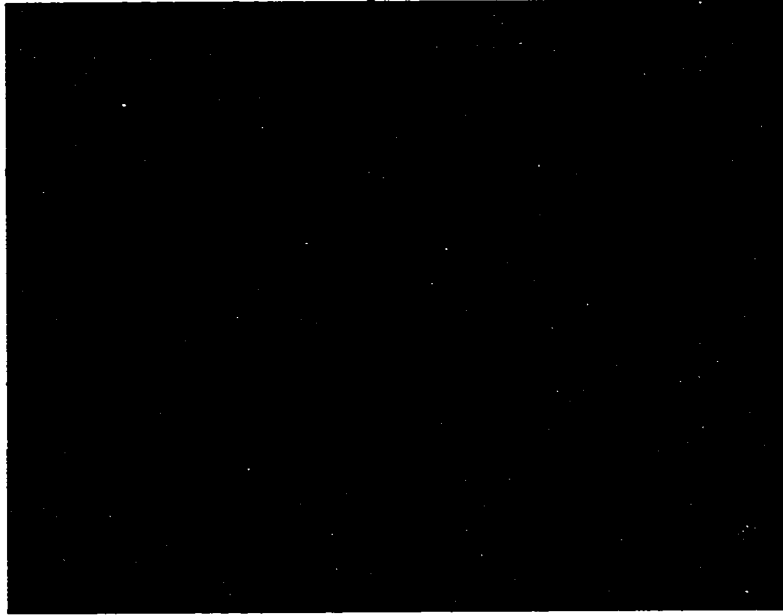


Figure 6A-6B. **Cryostat Sectioning and Staining in Guinea Pig 278N**: Guinea Pig 278N was infected with both the WSK and EAF viruses. Fig 6A shows the presence of both viruses. Fig 6B shows the presence of the WSK virus in that section.

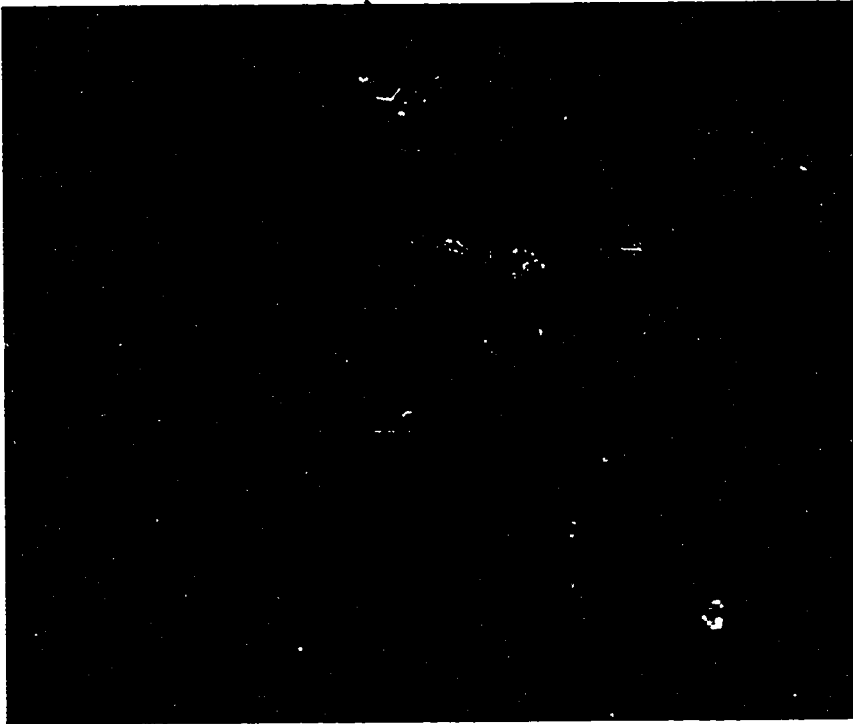
278N: 24FF11 Biotin + Strepavidin PE and 960 FITC



278N: 24FF11 FITC and 960 Biotin + Strepavidin PE

Figure 6C. Cryostat Sectioning and Staining of Guinea Pig 275N: Only the EAF virus was detected.

275N: 24FF11 Biotin + Strepavidin PE and 960 FITC



Clonal Variation *in vitro*

As a first approach to examine the possibility that within a given rabies virus strain, there are virus subpopulations present, the cloned virus preparations of the WSK and EAF which were prepared so as to exhibit minimal heterogeneity were passaged 12 times in MNA cells, and the effect of the passages determined using molecular techniques. It was important to start out with a homogenous population to study how much variation accumulates in these viruses during passaging. The initial virus supernatant before passaging (used to infect MNA cells at a MOI of 0.2) was designated the input virus (92L1169P_{25} B_6 sub clone D_6 for the WSK virus and 98N4235P_{15} D_5 sub clone B_3 for the EAF virus), while virus supernatant after the 12th passage is designated the output virus (92L1169P_{37} B_6 sub clone D_6 for the WSK virus and 98N4235P_{27} D_5 sub clone B_3 for the EAF virus).

3.1 Heterogeneity of the Virus Population

The existence of subpopulations within individual strains of the rabies virus has profound implications for the pathogenesis of the virus. To analyze this heterogeneity, two regions of the rabies virus genome, the P-gene (which contains a central highly unconserved region) and the G-L intergenic region (which does not code for any protein, and which therefore might be expected to exhibit relatively high levels of variation), were analyzed before and after 12 passages of the virus variants.

3.1.1 RNA Isolation and RT-PCR

RNA was first extracted from the supernatants of the input and output viruses for the WSK and the EAF strains using TRIzol reagent. The two areas of interest in the viral RNA genome (the P-gene and the G-L intergenic region) were amplified by RT-PCR

using the Expand System which employs a high fidelity DNA polymerase cocktail (Expand™ enzyme) to generate blunt ended PCR products. Verification of the generation of amplicon and estimation of PCR product concentration was achieved by DNA gel electrophoresis of PCR product aliquots (results not shown). Since the WSK virus yielded low amounts of the P amplicon a nested PCR was performed on both the WSK and EAF PCR products, resulting in an amplicon of approximately 664bp in size. The reason a nested PCR was also carried out on the EAF P-gene PCR product even though P-gene amplification for the EAF was strong after first round PCR, was to allow molecular cloning of the same size fragments for both virus types. More importantly the two viruses were subjected to the same degree of amplification so that any difference in the level of mutation that is identified between the two strains cannot be attributed to the PCR itself. Amplification of the G-L region resulted in a principal product of approximately 1400bp and lower yields of an amplicon of the expected size of 860bp. Sequence analysis of molecular clones of the larger product (see later) indicated that the 1400bp product corresponded to PV strain co-ordinates 4619-6040 due to unanticipated priming within the L open reading frame (ORF) at the latter co-ordinates due to a significant sequence match at this position with primer 179 (data not shown). The 1400bp product was thus appropriate for use in G-L sequence analysis. The G-L amplicon generated actually includes a significant portion of the G-gene, but the sequence window studied is within the intergenic region only. Table I (see Materials and Methods) shows the base positions of all the primers used in this study (during RT-PCR and nucleotide sequencing) in respect to the WSK and EAF P-gene and G-L intergenic region. These

base positions correspond to the base positions of a fully characterized reference strain, the PV strain of the rabies virus (Tordo et. al., 1986).

3.1.2 Molecular Cloning and Restriction (Rx) Endonuclease Digestions

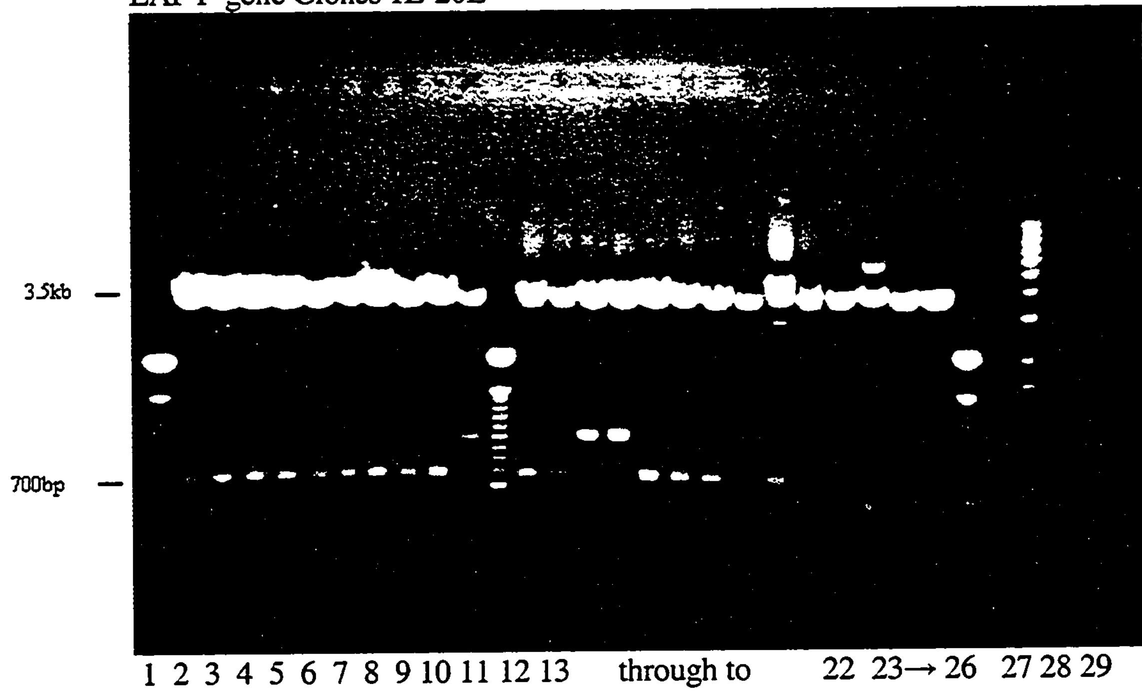
PCR products of the P-gene and G-L intergenic region for the input and output viruses (WSK input, EAF input, WSK output, EAF output) were cloned into the TOPO® vector (Figure 3 in Materials and Methods) and the plasmid transformed into a vial of TOP10 One Shot® Chemically Competent *E. coli* cells. For each viral strain and each region of interest, the objective was to pick 20 colonies for both input and output virus; hence a total of 80 molecular clones were obtained for each genomic region (160 molecular clones in total). The greater the number of clones sequenced the greater the chance of identifying variation (any significant difference in the nucleotide sequence). However, 20 clones/analysis was the maximum number that could be processed reasonably given manpower and time constraints. Once colonies had been picked, *E. coli* cells were then cultured for plasmid purification using a Wizard Miniprep Kit.

To check that the clones that were picked for sequence analysis contained the PCR product inserts, Rx endonuclease digestions of the purified plasmids were performed. Since EcoR1 sites flank either side of the polylinker region into which the PCR fragments were inserted (Figure 3), cleavage of the plasmid DNA by EcoR1 to generate a fragment corresponding to the amplicon was a convenient means of assessment. These digestions were then analyzed by DNA gel electrophoresis. Figure 7 shows the DNA gel for the EAF P-gene clones 1EP-20EP respectively for the input virus. For most clones examined, the plasmid DNA was cleaved into two products: a product of approximately 700bp corresponding to the size expected for the inserted PCR product (664bp) and flanking

Figure 7. **DNA Gel Electrophoresis Showing the Restriction Endonuclease Digestion for the EAF virus clones:** Purified plasmids were subjected to Eco R1 digestion at 37°C for 2 hours and then analyzed on a 1% agarose gel. A fragment of approximately 3.5kb and a fragment of approximately 700bp were obtained. The control plasmid loaded unto lane 28 appears only faintly and has an approximate size of 3.5kb. When gel was viewed at a higher illumination the control plasmid fragment appears stronger (result not shown).

Lanes
1= 100bp marker
2-11= Clones 1EP-10EP
12= 100bp marker
13-22= Clones 11EP-20EP
23= 1WP
24= 3WP
25= 5WP
26= 12WP
27= 100bp marker
28= Control plasmid
29= 1kb marker

EAF P-gene Clones 1E-20E



sequences derived from the cloning junction and a plasmid fragment of approximately 3.5kb in size, as expected. It is noticeable that most of the clones picked contained the insert for the EAF virus. DNA gel electrophoresis for the WSK P-gene, clones 1WP-20WP for the input virus produced the same Rx endonuclease pattern (results not shown). In some cases the clone did not give the expected Rx endonuclease pattern; either the insertion sequence was the incorrect size (Figure 7, lanes 11, 15 and 16) or there was no apparent insert (Figure 7, lanes 24-26). Overall a cloning efficiency of 85% was estimated. The cloning efficiency values indicate the proportion of transformed colonies containing the correct plasmid construct. Therefore, additional colonies had to be investigated until 20 clones all containing the amplicon were identified. Hence, an additional 3 clones were needed. 10 more colonies were then analyzed for the presence of the insert (results not shown), of which five clones produced the expected Rx pattern and 5 did not, thereby yielding a total cloning efficiency of 73.3%,. When the WSK P-gene clones (1WP-20WP) for the input virus were analyzed, the percentage of clones containing the correct plasmid construct was 85%. An additional 10 colonies were then analyzed (1BWP-10BWP, input virus) with a final resulting cloning efficiency of 86.7%.

Cloning efficiencies for the EAF and the WSK P-gene output viruses (results not shown) were 80% - 90%. A total of 30 colonies were analyzed for the EAF P-gene output virus and 25 colonies for the WSK P-gene output virus.

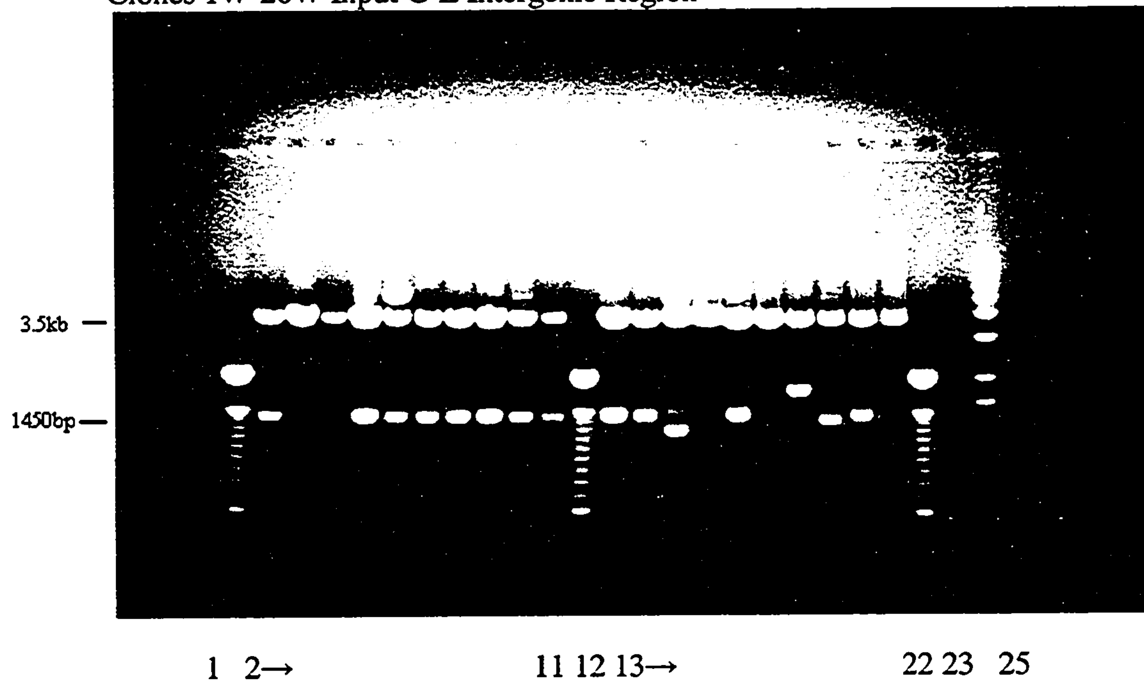
Figures 8A and 8B show the restriction enzyme analysis for the plasmid clones of the WSK virus and EAF virus G-L intergenic region respectively. A fragment of approximately 1400bp was expected, as well as a plasmid fragment of approximately 3.5kb in size. As with the P-gene clones, some of the clones analyzed for the G-L

Figure 8A-8B. **DNA Gel Electrophoresis Showing the Restriction Endonuclease Digestions for A, the WSK virus clones; and B, the EAF virus clones for the G-L amplicon:** Purified plasmids were subjected to Eco R1 digestion at 37°C for 2 hours and then analyzed on a 1% agarose gel. A fragment of approximately 3.5kb and a fragment of approximately 1450bp were obtained for the WSK virus clones. The EAF virus produced a different Rx pattern resulting in fragments of approximately 3.5kb, 900bp and 550bp.

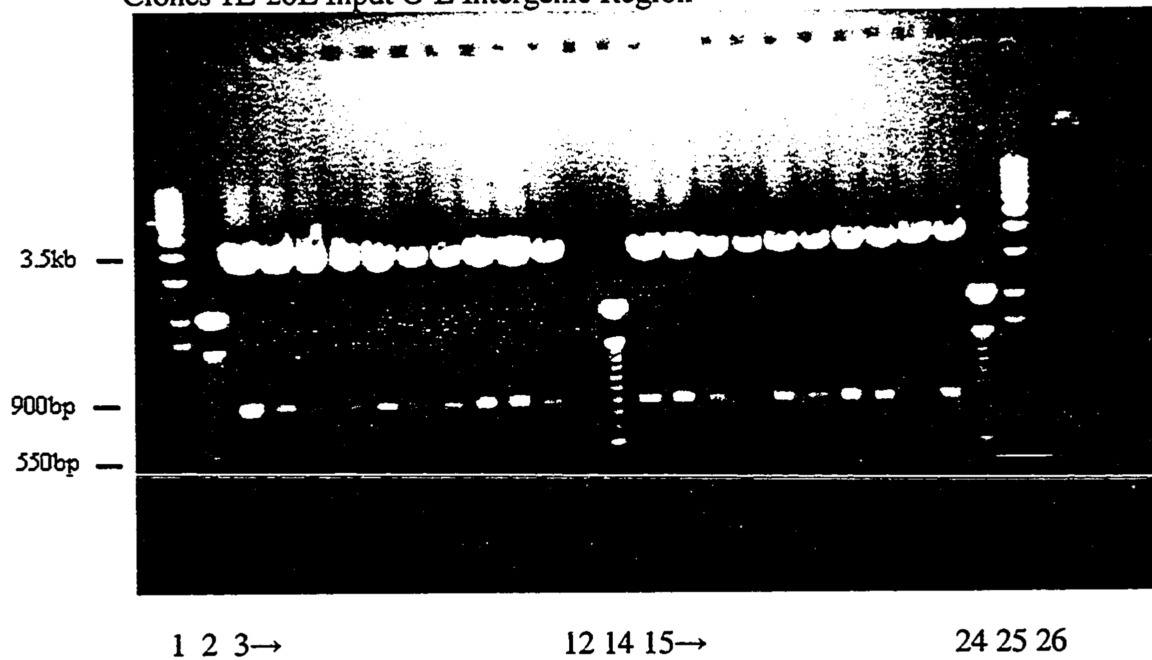
8A. Lanes
1= 100bp marker
2-11= Clones 1WGL-10WGL
12= 100bp marker
13-22= Clones 11WGL-20WGL
23= 100bp marker
25= 1kb marker

8B. Lanes
1= 1kb marker
2= 100bp marker
3-12= Clones 1EGL-10EGL
14= 100bp marker
15-24= Clones 11EGL-20EGL
25= 100bp marker
26= 1kb marker

Clones 1W-20W Input G-L Intergenic Region



Clones 1E-20E Input G-L Intergenic Region



amplicon insert did not produce the expected Rx pattern. Figure 8A shows that the cloning efficiency for the WSK G-L intergenic region input virus (1WGL-20WGL) was 60%; that is only 12 clones produced the expected Rx pattern. Clones 2WGL, 3WGL, 13WGL, 14WGL, 16WGL, 17WGL, 18WGL and 20WGL corresponding to lanes 3, 4, 15, 16, 18, 19, 20 and 22 did not produce the expected RX pattern. Hence, an additional 10 colonies were analyzed for the presence of the insert (results not shown) yielding 7 additional clones for sequencing to produce a total cloning efficiency of 63.3%. Figure 8B shows the restriction endonuclease pattern for the EAF virus G-L intergenic region which differs from that of the WSK G-L intergenic region. Three bands, the first band being the linearized plasmid of approximately 3.5kb in size and two smaller fragments of approximately 900bp and 550bp are obtained instead of two. The combined size of these two smaller fragments is 1450bp consistent with the size of the clonal amplicon and indicating the presence of an EcoR1 site within the G-L amplicon for the EAF. The percentage of colonies producing the expected Rx endonuclease pattern for the EAF G-L intergenic region input virus (1EGL-20EGL) was 95%; hence only one colony analyzed did not produce the expected Rx pattern (Figure 8B). An additional 5 colonies were then analyzed producing a total cloning efficiency of 96%.

Cloning efficiencies for the WSK and the EAF G-L intergenic region output viruses (results not shown) were 52.5% and 80% respectively. A total of 40 colonies were analyzed for the WSK G-L intergenic region output virus and 30 colonies for the EAF G-L intergenic region output virus.

3.1.3 Nucleotide Sequence Analysis

DNA sequencing of the initial PCR products prior to cloning (consensus sequence) and all molecular clones selected on the basis of Rx endonuclease analysis was performed manually as described (see Materials and Methods). Electronic copies of all data were generated using the DNAsis program to allow comparison between clones (Figures 9A-9B, 10A-10B, 11 and 12). For both the P-gene (nucleotides 1,864-2,183 with respect to the PV strain) and the G-L intergenic region (nucleotides 5,126-5,445 with respect to the PV strain) a 320 nucleotide sequence window was compared. The residues of the P protein (amino acid 118-223) predicted from the nucleotide sequence are indicated in Figures 13 and 14 for the WSK and EAF viruses (consensus sequence) respectively. Analysis of the molecular clones indicated three highly divergent clones for the WSK strain P gene input virus which were later identified to be of the raccoon strain, and were thus contaminants that were not included in the final analysis. The procedure used to investigate the source of this contamination of the P- gene PCR with the raccoon strain sequence, which was shown to be a PCR carry over problem rather than a contamination of the original virus stock will be explained in detail in the appendix. A summary of the results are given below:

WSK input P-gene

- DNA sequence comparison showed all 20 clones to be the same as the consensus sequence.

WSK output P-gene

- DNA sequence comparison showed all 20 clones to be the same as the consensus sequence.

Figures 9A-9B. Nucleotide Sequence Analysis of the EAF virus clones for the Input and Output Viruses P-gene Respectively.

Viral genomic RNA was isolated from the input and output viruses and subjected to RT-PCR. PCR products were cloned using the TOPO® kit, sequenced and read manually. Electronic copies of all data were generated using the DNAsis program for comparison and aligned using CLUSTALX. Nucleotide sequence analysis for the WSK virus clones for the P-gene and G-L intergenic region are not shown since all clones were the same as the consensus sequence. Dots indicate conserved sequences between the clones.

EAF -P- gene Input Virus

```
                          10         20         30         40         50
EAF-P        1 ATGGTCACAA ACCGTGGAGG AAATCATATC TTATGTCACA GTCAACTTTC    50
CL1EP        1 .......... .......... .......... .......... ..........    50
CL2EP        1 .......... .......... .......... .......... ..........    50
CL3EP        1 .......... .......... .......... .......... ..........    50
CL4EP        1 .......... .......... .......... .......... ..........    50
CL5EP        1 .......... .......... .......... .......... ..........    50
CL6EP        1 .......... .......... .......... .......... ..........    50
CL7EP        1 .......... .......... .......... .......... ..........    50
CL8EP        1 .......... .......... .......... .......... ..........    50
CL9EP        1 .......... .......... .......... .......... ..........    50
CL11EP       1 .......... .......... .......... .......... ..........    50
CL12EP       1 .......... .......... .......... .......... ..........    50
CL15EP       1 .......... .......... .......... .......... ..........    50
CL16EP       1 .......... .......... .......... .......... ..........    50
CL17EP       1 .......... .......... .......... .......... ..........    50
CL18EP       1 .......... .......... .......... .......... ..........    50
CL19EP       1 .......... .......... .......... .......... ..........    50
CL20EP       1 .......... .......... .......... .......... ..........    50
CL6BEP       1 .......... .......... .......... .......... ..........    50
CL7BEP       1 .......... .......... .......... .......... ..........    50
CL8BEP       1 .......... .......... .......... .......... ..........    50
                          60         70         80         90        100
EAF-P       51 CTAACCCCCC AGGGAAGTCC TCGGAAGATA AGTCAACCCA GACAACTGGC   100
CL1EP       51 .......... .......... .......... .......... ..........   100
CL2EP       51 .......... .......... .......... .......... ..........   100
CL3EP       51 .......... .......... .......... .......... ..........   100
CL4EP       51 .......... .......... .......... .......... ..........   100
CL5EP       51 .......... .......... .......... .......... ..........   100
CL6EP       51 .......... .......... .......... .......... ..........   100
CL7EP       51 .......... .......... .......... .......... .........T   100
CL8EP       51 .......... .......... .......... .......... ..........   100
CL9EP       51 .......... .......... .......... .......... ..........   100
CL11EP      51 .......... .......... .......... .......... ..........   100
CL12EP      51 .......... .......... .......... .......... ..........   100
CL15EP      51 .......... .......... .......... .......... ..........   100
CL16EP      51 .......... .......... .......... .......... ..........   100
CL17EP      51 .......... .......... .......... .......... ..........   100
CL18EP      51 .......... .......... .......... .......... ..........   100
CL19EP      51 .......... .......... .......... .......... ..........   100
CL20EP      51 .......... .......... .......... .......... ..........   100
CL6BEP      51 .......... .......... .......... .......... ..........   100
CL7BEP      51 .......... .......... .......... .......... ..........   100
CL8BEP      51 .......... .......... .......... .......... ..........   100
                         110        120        130        140        150
EAF-P      101 CGAGAACTCA AGAAGGAGAC AACATCCGTT TCTTCCCAGA GAGACAGTCA   150
CL1EP      101 .......... .......... .......... .......... ..........   150
CL2EP      101 .......... .......... .......... .......... ..........   150
CL3EP      101 .......... .......... .......... .......... ..........   150
CL4EP      101 .......... .......... .......... .......... ..........   150
CL5EP      101 .......... .......... .......... .......... ..........   150
CL6EP      101 .......... .......... .......... .......... ..........   150
```

EAF Pgene Input virus

```
CL7EP     101 .......... .......... .......... .......... ..........   150
CL8EP     101 .......... .......... .......... .......... ..........   150
CL9EP     101 .......... .......... .......... .......... ..........   150
CL11EP    101 .......... .......... .......... .......... ..........   150
CL12EP    101 .......... .......... .......... .......... ..........   150
CL15EP    101 .......... .......... .......... .......... ..........   150
CL16EP    101 .......... .......... .......... .......... ..........   150
CL17EP    101 .......... .......... .......... .......... ..........   150
CL18EP    101 .......... .......... .......... .......... ..........   150
CL19EP    101 .......... .......... .......... .......... ..........   150
CL20EP    101 .......... .......... .......... .......... ..........   150
CL6BEP    101 .......... .......... .......... .......... ..........   150
CL7BEP    101 .......... .......... .......... .......... ..........   150
CL8BEP    101 .......... .......... .......... .......... ..........   150
                     160        170        180        190        200
EAF-P     151 ATCCTCGAAA GCCAGGATGG TGGCTCAAGC CGCCTCCGGT CCCCCAGCCC   200
CL1EP     151 .......... .......... .......... .......... ..........   200
CL2EP     151 .......... .......... .......... .......... ..........   200
CL3EP     151 .......... .......... .......... .......... ..........   200
CL4EP     151 .......... .......... .......... .......... ..........   200
CL5EP     151 .......... .......... .......... .......... ..........   200
CL6EP     151 .......... .......... .......... .......... ..........   200
CL7EP     151 .......... .......... .......... .......... ..........   200
CL8EP     151 .......... .......... .......... .......... ..........   200
CL9EP     151 .......... .......... .......... .......... ..........   200
CL11EP    151 .......... .......... .......... .......... ..........   200
CL12EP    151 .......... .......... .......... .......... ..........   200
CL15EP    151 .......... .......... .......... .......... ..........   200
CL16EP    151 .......... .......... .......... .......... ..........   200
CL17EP    151 .......... .......... .......... .......... ..........   200
CL18EP    151 .......... .......... .......... .......... ..........   200
CL19EP    151 .......... .......... .......... .......... ..........   200
CL20EP    151 .......... .......... .......... .......... ..........   200
CL6BEP    151 .......... .......... .......... .......... ..........   200
CL7BEP    151 .......... .......... .......... .......... ..........   200
CL8BEP    151 .......... .......... .......... .......... ..........   200
                     210        220        230        240        250
EAF-P     201 TCGAGTGGTC TGCTACCAAC GAGGAGGATG ATCTGTCAGT AGAAGCTGAG   250
CL1EP     201 .......... .......... .......... .......... ..........   250
CL2EP     201 .......... .......... .......... .......... ..........   250
CL3EP     201 .......... .......... .......... .......... ..........   250
CL4EP     201 .......... .......... .......... .......... ..........   250
CL5EP     201 .......... .......... .......... .......... ..........   250
CL6EP     201 .......... .......... .......... .......... ..........   250
CL7EP     201 .......... .......... .......... .......... ..........   250
CL8EP     201 .......... .......... .......... .......... ..........   250
CL9EP     201 .......... .......... .......... .......... ..........   250
CL11EP    201 .......... .......... .......... .......... ..........   250
CL12EP    201 .......... .......... .......... .......... ..........   250
CL15EP    201 .......... .......... .......... .......... ..........   250
CL16EP    201 .......... .......... .......... .......... ..........   250
CL17EP    201 .......... .......... .......... .......... ..........   250
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EAF P gene Input virus

```
CL18EP      201 .......... .......... .......... .......... ..........       250
CL19EP      201 .......... .......... .......... .......... ..........       250
CL20EP      201 .......... .......... .......... .......... ..........       250
CL6BEP      201 .......... .......... .......... .......... ..........       250
CL7BEP      201 .......... .......... .......... .......... ..........       250
CL8BEP      201 .......... .......... .......... .......... ..........       250
                       260        270        280        290        300
EAF-P       251 ATCGCTCACC AGGTTGCCGA AAGCTTTTCC AAGAAGTACA AGTTTCCCTC       300
CL1EP       251 .......... .......... .......... .......... ..........       300
CL2EP       251 .......... .......... .......... .......... ..........       300
CL3EP       251 .......... .......... .......... .......... ..........       300
CL4EP       251 .......... .......... .......... .......... ..........       300
CL5EP       251 .....C.... .......... .......... .......... ..........       300
CL6EP       251 G......... .......... .......... .......... ........C.       300
CL7EP       251 .......... .......... .......... .......... ..........       300
CL8EP       251 G......... .......... .......... .......... ........C.       300
CL9EP       251 .......... .......... .......... .......... ..........       300
CL11EP      251 .......... .......... .......... .......... ..........       300
CL12EP      251 .......... .......... .......... .......... ..........       300
CL15EP      251 .......... .......... .......... .......... ..........       300
CL16EP      251 .......... .......... .......... .......... ..........       300
CL17EP      251 .......... .......... .......... .......... ..........       300
CL18EP      251 .......... .......... .......... .......... ..........       300
CL19EP      251 .......... .......... .......... .......... ..........       300
CL20EP      251 .......... .......... .......... .......... ..........       300
CL6BEP      251 .......... .......... .......... .......... ..........       300
CL7BEP      251 .......... .......... .......... .......... ..........       300
CL8BEP      251 .......... .......... .......... .......... ..........       300
                       310        320        330        340        350
EAF-P       301 TCGATCATCT GGGATATTTC .......... .......... ..........       350
CL1EP       301 .......... .......... .......... .......... ..........       350
CL2EP       301 .......... .......... .......... .......... ..........       350
CL3EP       301 ........T. .......... .......... .......... ..........       350
CL4EP       301 .......... .......... .......... .......... ..........       350
CL5EP       301 .......... .......... .......... .......... ..........       350
CL6EP       301 .......... .......... .......... .......... ..........       350
CL7EP       301 .......... .......... .......... .......... ..........       350
CL8EP       301 .......... .......... .......... .......... ..........       350
CL9EP       301 .......... .......... .......... .......... ..........       350
CL11EP      301 .......... .......... .......... .......... ..........       350
CL12EP      301 .......... .......... .......... .......... ..........       350
CL15EP      301 .......... .......... .......... .......... ..........       350
CL16EP      301 .......... .......... .......... .......... ..........       350
CL17EP      301 .......... .......... .......... .......... ..........       350
CL18EP      301 .......... .......... .......... .......... ..........       350
CL19EP      301 .......... .......... .......... .......... ..........       350
CL20EP      301 .......... .......... .......... .......... ..........       350
CL6BEP      301 .......... .......... .......... .......... ..........       350
CL7BEP      301 .......... .......... .......... .......... ..........       350
CL8BEP      301 .......... .......... .......... .......... ..........       350
```

EAF - P-gene Output virus.

```
                 10         20         30         40         50
EAFOP     1 ATGGTCACAA ACCGTGGAGG AAATCATATC TTATGTCACA GTCAACTTTC   50
1EOP      1 .......... .......... .......... .......... ..........   50
2EOP      1 .......... .......... .......... .......... ..........   50
4EOP      1 .......... .......... .......... .......... ..........   50
6EOP      1 .......... .......... .......... .......... ..........   50
7EOP      1 .......... .......... .......... .......... ..........   50
8EOP      1 .......... .......... .......... .......... ..........   50
9EOP      1 .......... .......... .......... .......... ..........   50
10EOP     1 .......... .......... .......... .......... ..........   50
11EOP     1 .......... .......... .......... .......... ..........   50
13EOP     1 .......... .......... .......... .......... ..........   50
15EOP     1 .......... .......... .......... .......... ..........   50
16EOP     1 .......... .......... .......... .......... ..........   50
17EOP     1 .......... .......... .......... .......... ..........   50
18EOP     1 .......... .......... .......... .......... ..........   50
19EOP     1 .......... .......... .......... .......... ..........   50
20EOP     1 .......... .......... .......... .......... ..........   50
1BEOP     1 .......... .......... .......... .......... ..........   50
4BEOP     1 .......... .......... .......... .......... ..........   50
5BEOP     1 .......... .......... .......... .......... ..........   50
6BEOP     1 .......... .......... .......... .......... ..........   50
                 60         70         80         90        100
EAFOP    51 CTAACCCCCC AGGGAAGTCC TCGGAAGATA AGTCAACCCA GACAACTGGC  100
1EOP     51 .......... .......... .......... .......... ..........  100
2EOP     51 .......... .......... .......... .......... ..........  100
4EOP     51 .......... .......... .......... .......... ..........  100
6EOP     51 .......... .......... .......... .......... ..........  100
7EOP     51 .......... .......... .......... .......... ..........  100
8EOP     51 .......... .......... .......... .......... ..........  100
9EOP     51 .......... .......... .......... .......... ..........  100
10EOP    51 .......... .......... .......... .......... ..........  100
11EOP    51 .......... .......... .......... .......... ..........  100
13EOP    51 .......... .......... .......... .......... ..........  100
15EOP    51 .......... .......... .......... .......... ..........  100
16EOP    51 .......... .......... .......... .......... ..........  100
17EOP    51 .......... .......... .......... .......... ..........  100
18EOP    51 .......... .......... .......... .......... ..........  100
19EOP    51 .......... .......... .......... .......... ..........  100
20EOP    51 .......... .......... .......... .......... ..........  100
1BEOP    51 .......... .......... .......... .......... ..........  100
4BEOP    51 .......... .......... .......... .......... ..........  100
5BEOP    51 .......... .......... .......... .......... ..........  100
6BEOP    51 .......... .......... .......... .......... ..........  100
                110        120        130        140        150
EAFOP   101 CGAGAACTCA AGAAGGAGAC AACATCCGTT TCTTCCCAGA GAGACAGTCA  150
1EOP    101 .......... .......... .......... .......... ..........  150
2EOP    101 .......... .......... .......... .......... ..........  150
4EOP    101 .......... .......... .......... .......... ..........  150
6EOP    101 .......... .......... .......... .......... ..........  150
7EOP    101 .......... .......... .......... .......... ..........  150
8EOP    101 .......... .......... .......... .......... ..........
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9EOP     101 .......... .......... ....C..... .......... ..........  150
10EOP    101 .......... .......... .......... .......... ..........  150
11EOP    101 .......... .......... .......... .......... ..........  150
13EOP    101 .......... .......... .......... .......... ..........  150
15EOP    101 .......... .......... .......... .......... ..........  150
16EOP    101 .......... .......... .......... .......... ..........  150
17EOP    101 .......... .......... .......... .......... ..........  150
18EOP    101 .......... .......... .......... .......... ..........  150
19EOP    101 .......... .......... .......... .......... ..........  150
20EOP    101 .......... .......... .......... .......... ..........  150
1BEOP    101 .......... .......... .......... .......... ..........  150
4BEOP    101 .......... .......... .......... .......... ..........  150
5BEOP    101 .......... .......... .......... .......... ..........  150
6BEOP    101 .......... .......... .......... .......... ..........  150
                    160        170        180        190        200
EAFOP    151 ATCCTCGAAA GCCAGGATGG TGGCTCAAGC CGCCTCCGGT CCCCCAGCCC  200
1EOP     151 .......... .......... .......... .......... ..........  200
2EOP     151 .......... .......... .......... .......... ..........  200
4EOP     151 .......... .......... .......... .......... ..........  200
6EOP     151 .......... .......... .......... .......... ..........  200
7EOP     151 .......... .......... .......... .......... ..........  200
8EOP     151 .......... .......... .......... .......... ..........  200
9EOP     151 .......... .......... .......... .......... ..........  200
10EOP    151 .......... .......... .......... .......... ..........  200
11EOP    151 .......... .......... .......... .......... ..........  200
13EOP    151 .......... .......... .......... .......... ..........  200
15EOP    151 .......... .......... .......... .......... ..........  200
16EOP    151 .......... .......... .......... .......... ..........  200
17EOP    151 .......... .......... .......... .......... ..........  200
18EOP    151 .......... .......... .......... .......... ..........  200
19EOP    151 .......... .......... .......... .......... ..........  200
20EOP    151 .......... .......... .......... .......... ..........  200
1BEOP    151 .......... .......... .......... .......... ..........  200
4BEOP    151 .......... .......... .......... .......... ..........  200
5BEOP    151 .......... .......... .......... .......... ..........  200
6BEOP    151 .......... .......... .......... .......... ..........  200
                    210        220        230        240        250
EAFOP    201 TCGAGTGGTC TGCTACCAAC GAGGAGGATG ATCTGTCAGT AGAAGCTGAG  250
1EOP     201 .......... .......... .......... .......... ..........  250
2EOP     201 .......... .......... .......... .......... ..........  250
4EOP     201 .......... .......... .......... .......... ..........  250
6EOP     201 .......... .......... .......... .......... ..........  250
7EOP     201 .......... .......... .......... .......... ..........  250
8EOP     201 .......... .......... .......... .......... ..........  250
9EOP     201 .......... .......... .......... .......... ..........  250
10EOP    201 .......... .......... .......... .......... ..........  250
11EOP    201 .......... .......... .......... .......... ..........  250
13EOP    201 .......... .......... .......... .......... ..........  250
15EOP    201 .......... .......... .......... .......... ..........  250
16EOP    201 .......... .......... .......... .......... ..........  250
17EOP    201 .......... .......... .......... .......... ..........  250
18EOP    201 .......... .......... .......... .......... ..........  250
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19EOP    201 .......... .......... .......... .......... ..........  250
20EOP    201 .......... .......... .......... .......... ..........  250
1BEOP    201 .......... .......... .......... .......... ..........  250
4BEOP    201 .......... .......... .......... .......... ..........  250
5BEOP    201 .......... .......... .......... .......... ..........  250
6BEOP    201 .......... .......... .......... .......... ..........  250
                    260        270        280        290        300
EAFOP    251 ATCGCTCACC AGGTTGCCGA AAGCTTTTCC AAGAAGTACA AGTTTCCCTC  300
1EOP     251 .......... .......... .......... .......... ..........  300
2EOP     251 .......... .......... .......... .......... ..........  300
4EOP     251 .......... .......... .......... .......... ..........  300
6EOP     251 .......... .......... .......... .......... ..........  300
7EOP     251 .......... .......... .......... .......... ..........  300
8EOP     251 .......... .......... .......... .......... ..........  300
9EOP     251 .......... .......... .......... .......... ..........  300
10EOP    251 .......... .......... .......... .......... ..........  300
11EOP    251 .......... .......... .......... .......... ..........  300
13EOP    251 .......... .......... .......... .......... ..........  300
15EOP    251 .......... .......... .......... .......... ..........  300
16EOP    251 .......... .......... .......... .......... ..........  300
17EOP    251 .......... .......... .......... .......... ..........  300
18EOP    251 .......... .......... .......... .......... ..........  300
19EOP    251 .......... .......... .......... .......... ..........  300
20EOP    251 .......... .......... .......... .......... ..........  300
1BEOP    251 .......... .......... .......... .......... ..........  300
4BEOP    251 .......... .......... .......... .......... ..........  300
5BEOP    251 .......... .......... .......... .......... ..........  300
6BEOP    251 .......... .......... .......... .......... ..........  300
                    310        320        330        340        350
EAFOP    301 TCGATCATCT GGGATATTTC .......... .......... ..........  350
1EOP     301 .......... .......... .......... .......... ..........  350
2EOP     301 .......... .......... .......... .......... ..........  350
4EOP     301 .......... .......... .......... .......... ..........  350
6EOP     301 .......... .......... .......... .......... ..........  350
7EOP     301 .......... .......... .......... .......... ..........  350
8EOP     301 .......... .......... .......... .......... ..........  350
9EOP     301 .......... .......... .......... .......... ..........  350
10EOP    301 .......... .......... .......... .......... ..........  350
11EOP    301 .......... .......... .......... .......... ..........  350
13EOP    301 .......... .......... .......... .......... ..........  350
15EOP    301 .......... .......... .......... .......... ..........  350
16EOP    301 .......... .......... .......... .......... ..........  350
17EOP    301 .......... .......... .......... .......... ..........  350
18EOP    301 .......... .......... .......... .......... ..........  350
19EOP    301 .......... .......... .......... .......... ..........  350
20EOP    301 .......... .......... .......... .......... ..........  350
1BEOP    301 .......... .......... .......... .......... ..........  350
4BEOP    301 .......... .......... .......... .......... ..........  350
5BEOP    301 .......... .......... .......... .......... ..........  350
6BEOP    301 .......... .......... .......... .......... ..........  350
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Figures 10A-10B. **Nucleotide Sequence Analysis of the EAF virus clones for the Input and Output Viruses G-L Intergenic Region Respectively.**

EAF - G-L intergenic region - Input Virus

```
                        10         20         30         40         50
EAFGL        1 AGGGATCGAT ACCAAAGGTT GTGGACAGGT CAGGGGTTAC CTCAGATCAC     50
CL1EGL       1 .......... .......... .......... .......... ..........     50
CL2EGL       1 .......... .......... .......... .......... ..........     50
CL3EGL       1 .......... .......... .......... .......... ..........     50
CL4EGL       1 .......... .......... .......... .......... ..........     50
CL5EGL       1 .......... .......... .......... .......... ..........     50
CL6EGL       1 .......... .......... .......... .......... ..........     50
CL7EGL       1 .......... .......... .......... .......... ..........     50
CL8EGL       1 .......... .......... .......... .......... ..........     50
CL9EGL       1 .......... .......... .......... .......... ..........     50
CL10EGL      1 .......... .......... .......... .......... ..........     50
CL11EGL      1 .......... .......... .......... .......... ..........     50
CL12EGL      1 .......... .......... .......... .......... ..........     50
CL13EGL      1 .......... .......... .......... .......... ..........     50
CL14EGL      1 .......... .......... .......... .......... ..........     50
CL15EGL      1 .......... .......... .......... .......... ..........     50
CL16EGL      1 .......... .......... .......... .......... ..........     50
CL17EGL      1 .......... .......... .......... .......... ..........     50
CL18EGL      1 .......... .......... .......... .......... ..........     50
CL20EGL      1 .......... .......... .......... .......... ..........     50
CL1BEGL      1 .......... .......... .......... .......... ..........     50
                        60         70         80         90        100
EAFGL       51 TTCACGTTTG GGCACGGACA GAGATCATGG TGAGTCCCCT GACAGTAGAC    100
CL1EGL      51 .......... .......... .......... .......... ..........    100
CL2EGL      51 .......... .......... .......... .......... ..........    100
CL3EGL      51 .......... .......... .......... .......... ..........    100
CL4EGL      51 .......... .......... .......... .......... ..........    100
CL5EGL      51 .......... .......... .......... .......... ..........    100
CL6EGL      51 .......... .......... .......... .......... ..........    100
CL7EGL      51 .......... .......... .......... .......... ..........    100
CL8EGL      51 .......... .......... .......... .......... ..........    100
CL9EGL      51 .......... .......... .......... .......... ..........    100
CL10EGL     51 .......... .......... .......... .......... ..........    100
CL11EGL     51 .......... .......... .......... .......... ..........    100
CL12EGL     51 .......... .......... .......... .......... ..........    100
CL13EGL     51 .......... .......... .......... .......... ..........    100
CL14EGL     51 .......... .......... .......... .......... ..........    100
CL15EGL     51 .......... .......... .......... .......... ..........    100
CL16EGL     51 .......... .......... .......... .......... ..........    100
CL17EGL     51 .......... .......... .......... .......... ..........    100
CL18EGL     51 .......... .......... .......... .......... ..........    100
CL20EGL     51 .......... .......... .......... .......... ..........    100
CL1BEGL     51 .......... .......... .......... .......... ..........    100
                       110        120        130        140        150
EAFGL      101 TCAACATGAT TCAACTGAGC AGGGCGATCT GCCCCTCTTG AAGGACATAA    150
CL1EGL     101 .......... .......... .......... .......... ..........    150
CL2EGL     101 .......... .......... .......... .......... ..........    150
CL3EGL     101 .......... .......... .......... .......... ..........    150
CL4EGL     101 .......... .......... .......... .......... ..........    150
CL5EGL     101 .......... .......... .......... .......... ..........    150
CL6EGL     101 .......... .......... .......... .......... ..........    150
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EAF - Cr - L intergenic region - Intact Virus

```
CL7EGL     101 .......... .......... .......... .......... ..........   150
CL8EGL     101 .......... .......... .......... .......... ..........   150
CL9EGL     101 .......... .......... .......... .......... ..........   150
CL10EGL    101 .......... .......... .......... .......... ..........   150
CL11EGL    101 .......... .......... .......... .......... ..........   150
CL12EGL    101 .......... .......... .......... .......... ..........   150
CL13EGL    101 .......... .......... .......... .......... ..........   150
CL14EGL    101 .......... .......... .......... .......... ..........   150
CL15EGL    101 .......... .......... .......... .......... ..........   150
CL16EGL    101 .......... .......... .......... .......... ..........   150
CL17EGL    101 .......... .......... .......... .......... ..........   150
CL18EGL    101 .......... .......... .......... .......... ..........   150
CL20EGL    101 .......... .......... .......... .......... ..........   150
CL1BEGL    101 .......... .......... .......... .......... ..........   150
                      160        170        180        190        200
EAFGL      151 GCAATAGCCC ACAATCATCT TGCATCTCAG TAAAGTGTGC ATAATTATAA   200
CL1EGL     151 .......... .......... .......... .......... ..........   200
CL2EGL     151 .......... .......... .......... .......... ..........   200
CL3EGL     151 .......... .......... .......... .......... ..........   200
CL4EGL     151 .......... .......... .......... .......... ..........   200
CL5EGL     151 .......... .......... .......... .......... ..........   200
CL6EGL     151 .......... .......... .......... .......... ..........   200
CL7EGL     151 .......... .......... .......... .......... ..........   200
CL8EGL     151 .......... .......... .......... .......... ..........   200
CL9EGL     151 .......... .......... .......... .......... ..........   200
CL10EGL    151 .......... .......... .......... .......... ..........   200
CL11EGL    151 .......... .......... .......... .......... ..........   200
CL12EGL    151 .......... .......... .......... .......... ..........   200
CL13EGL    151 .......... .......... .......... .......... ..........   200
CL14EGL    151 .......... .......... .......... .......... ..........   200
CL15EGL    151 .......... .......... .......... .......... ..........   200
CL16EGL    151 .......... .......... .......... .......... ..........   200
CL17EGL    151 .......... .......... .......... .......... ..........   200
CL18EGL    151 .......... .......... .......... .......... ..........   200
CL20EGL    151 .......... .......... .......... .......... ..........   200
CL1BEGL    151 .......... .......... .......... .......... ..........   200
                      210        220        230        240        250
EAFGL      201 AGGGCTGGGT CATCAAAGCT TTTCCGTCGA GAAAAAAACT GTAGGCTGAA   250
CL1EGL     201 .......... .......... .......... .......... ..........   250
CL2EGL     201 .......... .......... .......... .......... ..........   250
CL3EGL     201 .......... .......... .......... .......... ..........   250
CL4EGL     201 .......... .......... .......... .......... ..........   250
CL5EGL     201 .......... .......... .......... .......... ..........   250
CL6EGL     201 .......... .......... .......... .......... ..........   250
CL7EGL     201 .......... .......... .......... .......... ..........   250
CL8EGL     201 .......... .......... .......... .......... ..........   250
CL9EGL     201 .......... .......... .......... .......... ..........   250
CL10EGL    201 .......... .......... .......... .......... ..........   250
CL11EGL    201 .......... .......... .......... .......... ..........   250
CL12EGL    201 .......... .......... .......... .......... ..........   250
CL13EGL    201 .......... .......... .......... .......... ..........   250
CL14EGL    201 .......... .......... .......... .......... ..........   250
```

EAF - G-L intergenic region - Input Virus

```
CL15EGL     201 .......... .......... .......... .......... ..........   250
CL16EGL     201 .......... .......... .......... .......... ..........   250
CL17EGL     201 .......... .......... .......... .......... ..........   250
CL18EGL     201 .......... .......... .......... .......... ..........   250
CL20EGL     201 .......... .......... .......... .......... ..........   250
CL1BEGL     201 .......... .......... .......... .......... ..........   250
                       260        270        280        290        300
EAFGL       251 AGAGCAGCTG GCAACACTTC TCATCTTCGG ACGTAGATCA AAATGCTTGA   300
CL1EGL      251 .......... .......... .......... .......... ..........   300
CL2EGL      251 .......... .......... .......... .......... ..........   300
CL3EGL      251 .......... .......... .......... .......... ..........   300
CL4EGL      251 .......... .......... .......... .......... ..........   300
CL5EGL      251 .......... .......... .......... .......... ..........   300
CL6EGL      251 .......... .......... .......... .......... ..........   300
CL7EGL      251 .......... .......... .......... .......... ..........   300
CL8EGL      251 .......... .......... .......... .......... ..........   300
CL9EGL      251 .......... .......... .......... .......... ..........   300
CL10EGL     251 .......... .......... .......... .......... ..........   300
CL11EGL     251 .......... .......... .......... .......... ..........   300
CL12EGL     251 .......... .......... .......... .......... ..........   300
CL13EGL     251 .......... .......... .......... .......... ..........   300
CL14EGL     251 .......... .......... .......... .......... ..........   300
CL15EGL     251 .......... .......... .......... .......... ..........   300
CL16EGL     251 .......... .......... .......... .......... ..........   300
CL17EGL     251 .......... .......... .......... .......... ..........   300
CL18EGL     251 .......... .......... .......... .......... ..........   300
CL20EGL     251 .......... .......... .......... .......... ..........   300
CL1BEGL     251 .......... .......... .......... .......... ..........   300
                       310        320        330        340        350
EAFGL       301 TCCAGGAGAG GTCTATGATG .......... .......... ..........   350
CL1EGL      301 .......... .......... .......... .......... ..........   350
CL2EGL      301 .......... .......... .......... .......... ..........   350
CL3EGL      301 .......... .......... .......... .......... ..........   350
CL4EGL      301 .......... .......... .......... .......... ..........   350
CL5EGL      301 .......... .......... .......... .......... ..........   350
CL6EGL      301 .......... .......... .......... .......... ..........   350
CL7EGL      301 .......... .......... .......... .......... ..........   350
CL8EGL      301 .......... .......... .......... .......... ..........   350
CL9EGL      301 .......... .......... .......... .......... ..........   350
CL10EGL     301 .......... .......... .......... .......... ..........   350
CL11EGL     301 .......... .......... .......... .......... ..........   350
CL12EGL     301 .......... .......... .......... .......... ..........   350
CL13EGL     301 .......... .......... .......... .......... ..........   350
CL14EGL     301 .......... .......... .......... .......... ..........   350
CL15EGL     301 .......... .......... .......... .......... ..........   350
CL16EGL     301 .......... .......... .......... .......... ..........   350
CL17EGL     301 .......... .......... .......... .......... ..........   350
CL18EGL     301 .......... .......... .......... .......... ..........   350
CL20EGL     301 .......... .......... .......... .......... ..........   350
CL1BEGL     301 .......... .......... .......... .......... ..........   350
```

EAF - G-L intergenic Region Output virus.

```
                         10         20         30         40         50
EAFGLO      1 AGGGATCGAT ACCAAAGGTT GTGGACAGGT CAGGGGTTAC CTCAGATCAC    50
1EGLO       1 .......... .......... .......... .......... ..........    50
2EGLO       1 .......... .......... .......... .......... ..........    50
3EGLO       1 .......... .......... .......... .......... ..........    50
5EGLO       1 .......... .......... .......... .......... ..........    50
7EGLO       1 .......... .......... .......... .......... ..........    50
8EGLO       1 .......... .......... .......... .......... ..........    50
9EGLO       1 .......... .......... .......... .......... ..........    50
10EGLO      1 .......... .......... .......... .......... ..........    50
11EGLO      1 .......... .......... .......... .......... ..........    50
13EGLO      1 .......... .......... .......... .......... ..........    50
14EGLO      1 .......... .......... .......... .......... ..........    50
15EGLO      1 .......... .......... .......... .......... ..........    50
16EGLO      1 .......... .......... .......... .......... ..........    50
17EGLO      1 .......... .......... .......... .......... ..........    50
18EGLO      1 .......... .......... .......... .......... ..........    50
19EGLO      1 .......... .......... .......... .......... ..........    50
2BEGLO      1 .......... .......... .......... .......... ..........    50
3BEGLO      1 .......... .......... .......... .......... ..........    50
4BEGLO      1 .......... .......... .......... .......... ..........    50
6BEGLO      1 .......... .......... .......... .......... ..........    50
                         60         70         80         90        100
EAFGLO     51 TTCACGTTTG GGCACGGACA GAGATCATGG TGAGTCCCCT GACAGTAGAC   100
1EGLO      51 .......... .......... .......... .......... ..........   100
2EGLO      51 .......... .......... .......... .......... ..........   100
3EGLO      51 .......... .......... .......... .......... ..........   100
5EGLO      51 .......... .......... .......... .......... ..........   100
7EGLO      51 .......... .......... .......... .......... ..........   100
8EGLO      51 .......... .......... .......... .......... ..........   100
9EGLO      51 .......... .......... .......... .......... ..........   100
10EGLO     51 .......... .......... .......... .......... ..........   100
11EGLO     51 .......... .......... .......... .......... ..........   100
13EGLO     51 .......... .......... .......... .......... ..........   100
14EGLO     51 .......... .......... .......... .......... ..........   100
15EGLO     51 .......... .......... .......... .......... ..........   100
16EGLO     51 .......... .......... .......... .......... ..........   100
17EGLO     51 .......... .......... .......... .......... ..........   100
18EGLO     51 .......... .......... .......... .......... ..........   100
19EGLO     51 .......... .......... .......... .......... ..........   100
2BEGLO     51 .......... .......... .......... .......... ..........   100
3BEGLO     51 .......... .......... .......... .......... ..........   100
4BEGLO     51 .......... .......... .......... .......... ..........   100
6BEGLO     51 .......... .A........ .......... .......... ..........
                        110        120        130        140        150
EAFGLO    101 TCAACATGAT TCAACTGAGC AGGGCGATCT GCCCCTCTTG AAGGACATAA   150
1EGLO     101 .......... .......... .......... .......... ..........   150
2EGLO     101 .......... .......... .......... .......... ..........   150
3EGLO     101 .......... .......... .......... .......... ..........   150
5EGLO     101 .......... .......... .......... .......... ..........   150
7EGLO     101 .......... .......... .......... .......... ..........   150
8EGLO     101 .......... .......... .......... .......... ..........   150
```

```
9EGLO     101 .......... .......... .......... .......... ..........  150
10EGLO    101 .......... .......... .......... .......... ..........  150
11EGLO    101 .......... .......... .......... .......... ..........  150
13EGLO    101 .......... .......... .......... .......... ..........  150
14EGLO    101 .......... .......... .......... .......... ..........  150
15EGLO    101 .......... .......... .......... .......... ..........  150
16EGLO    101 .......... .......... .......... .......... ..........  150
17EGLO    101 .......... .......... .......... .......... ..........  150
18EGLO    101 .......... .......... .......... .......... ..........  150
19EGLO    101 .......... .......... .......... .......... ..........  150
2BEGLO    101 .......... .......... .......... .......... ..........  150
3BEGLO    101 .......... .......... .......... .......... ..........  150
4BEGLO    101 .......... .......... .......... .......... ..........  150
6BEGLO    101 .......... .......... .......... .......... ..........  150
                     160        170        180        190        200
EAFGLO    151 GCAATAGCCC ACAATCATCT TGCATCTCAG TAAAGTGTGC ATAATTATAA  200
1EGLO     151 .......... .......... .......... .......... ..........  200
2EGLO     151 .......... .......... .......... .......... ..........  200
3EGLO     151 .......... .......... .......... .......... ..........  200
5EGLO     151 .......... .......... .......... .......... ..........  200
7EGLO     151 .......... .......... .......... .......... ..........  200
8EGLO     151 .......... .......... .......... .......... ..........  200
9EGLO     151 .......... .......... .......... .......... ..........  200
10EGLO    151 .......... .......... .......... .......... ..........  200
11EGLO    151 .......... .......... .......... .......... ..........  200
13EGLO    151 .......... .......... .......... .......... ..........  200
14EGLO    151 .......... .......... .......... .......... ..........  200
15EGLO    151 .......... .......... .......... .......... ..........  200
16EGLO    151 .......... .......... .......... .......... ..........  200
17EGLO    151 .......... .......... .......... .......... ..........  200
18EGLO    151 .......... .......... .......... .......... ..........  200
19EGLO    151 .......... .......... .......... .......... ..........  200
2BEGLO    151 .......... .......... .......... .......... ..........  200
3BEGLO    151 .......... .......... .......... .......... ..........  200
4BEGLO    151 .......... .......... .......... .......... ..........  200
6BEGLO    151 .......... .......... .......... .......... ..........  200
                     210        220        230        240        250
EAFGLO    201 AGGGCTGGGT CATCAAAGCT TTTCCGTCGA GAAAAAAACT GTAGGCTGAA  250
1EGLO     201 .......... .......... .......... .......... ..........  250
2EGLO     201 .......... .......... .......... .......... ..........  250
3EGLO     201 .......... .......... .......... .......... ..........  250
5EGLO     201 .......... .......... .......... .......... ..........  250
7EGLO     201 .......... .......... .......... .......... ..........  250
8EGLO     201 .......... .......... .......... .......... ..........  250
9EGLO     201 .......... .......... .......... .......... ..........  250
10EGLO    201 .......... .......... .......... .......... ..........  250
11EGLO    201 .......... .......... .......... .......... ..........  250
13EGLO    201 .......... .......... .......... .......... ..........  250
14EGLO    201 .......... .......... .......... .......... ..........  250
15EGLO    201 .......... .......... .......... .......... ..........  250
16EGLO    201 .......... .......... .......... .......... ..........  250
17EGLO    201 .......... .......... .......... .......... ..........  250
```

```
18EGLO     201 .......... .......... .......... .......... ..........  250
19EGLO     201 .......... .......... .......... .......... ..........  250
2BEGLO     201 .......... .......... .......... .......... ..........  250
3BEGLO     201 .......... .......... .......... .......... ..........  250
4BEGLO     201 .......... .......... .......... .......... ..........  250
6BEGLO     201 .......... .......... .......... .......... ..........  250

                      260        270        280        290        300
EAFGLO     251 AGAGCAGCTG GCAACACTTC TCATCTTCGG ACGTAGATCA AAATGCTTGA  300
1EGLO      251 .......... .......... .......... .......... ..........  300
2EGLO      251 .......... .......... .......... .......... ..........  300
3EGLO      251 .......... .......... .......... .......... ..........  300
5EGLO      251 .......... .......... .......... .......... ..........  300
7EGLO      251 .......... .......... .......... .......... ..........  300
8EGLO      251 .......... .......... .......... .......... ..........  300
9EGLO      251 .......... .......... .......... .......... ..........  300
10EGLO     251 .......... .......... .......... .......... ..........  300
11EGLO     251 .......... .......... ...C...... .......... ..........  300
13EGLO     251 .......... .......... .......... .......... ..........  300
14EGLO     251 .......... .......... .......... .......... ..........  300
15EGLO     251 .......... .......... .......... .......... ..........  300
16EGLO     251 .......... .......... .......... .......... ..........  300
17EGLO     251 .......... .......... .......... .......... ..........  300
18EGLO     251 .......... .......... .......... .......... ..........  300
19EGLO     251 .......... .......... .......... .......... ..........  300
2BEGLO     251 .......... .......... .......... .......... ..........  300
3BEGLO     251 .......... .......... .......... .......... ..........  300
4BEGLO     251 .......... .......... .......... .......... ..........  300
6BEGLO     251 .......... .......... .......... .......... ..........  300

                      310        320        330        340        350
EAFGLO     301 TCCAGGAGAG GTCTATGATG .......... .......... ..........  350
1EGLO      301 .......... .......... .......... .......... ..........  350
2EGLO      301 .......... .......... .......... .......... ..........  350
3EGLO      301 .......... .......... .......... .......... ..........  350
5EGLO      301 .......... .......... .......... .......... ..........  350
7EGLO      301 .......... .......... .......... .......... ..........  350
8EGLO      301 .......... .......... .......... .......... ..........  350
9EGLO      301 .......... .......... .......... .......... ..........  350
10EGLO     301 .......... .......... .......... .......... ..........  350
11EGLO     301 .......... .......... .......... .......... ..........  350
13EGLO     301 .......... .......... .......... .......... ..........  350
14EGLO     301 .......... .......... .......... .......... ..........  350
15EGLO     301 .......... .......... .......... .......... ..........  350
16EGLO     301 .......... .......... .......... .......... ..........  350
17EGLO     301 .......... .......... .......... .......... ..........  350
18EGLO     301 .......... .......... .......... .......... ..........  350
19EGLO     301 .......... .......... .......... .......... ..........  350
2BEGLO     301 .......... .......... .......... .......... ..........  350
3BEGLO     301 .......... .......... .......... .......... ..........  350
4BEGLO     301 .......... .......... .......... .......... ..........  350
6BEGLO     301 .......... .......... .......... .......... ..........  350
```

Figure 11. **Nucleotide Sequence Analysis of the Consensus Sequence for the WSK Input and Output Viruses for the P-gene.**

```
                    10         20         30         40         50
WSKP       1 ATGGTCACAG ACCGTAGAAG AGATTATATC CTATGTCATG GTCAACTTTC    50
WSKPO      1 .......... .......... .......... .......... ..........    50
                    60         70         80         90        100
WSKP      51 CCAACTCTCC AGGAAGGTCT TCAGAGGACA AATCAACCCA AACTACCGGC   100
WSKPO     51 .......... .......... .......... .......... ..........   100
                   110        120        130        140        150
WSKP     101 AGGGTGCCCA AGAAGGAGAC AACATCCACT CCCTCTCAGA GAGAAAGCCA   150
WSKPO    101 .......... .......... .......... .......... ..........   150
                   160        170        180        190        200
WSKP     151 ATCTTCGAAA GCCAGGATGG CGGCTCAAGC TGCCTCTGGC CCTCCAGCCC   200
WSKPO    151 .......... .......... .......... .......... ..........   200
                   210        220        230        240        250
WSKP     201 TTGAATGGTC TGCCACTAAT GAGGAAGATG ATCTATCAGT AGAGGCTGAA   250
WSKPO    201 .......... .......... .......... .......... ..........   250
                   260        270        280        290        300
WSKP     251 ATCGCTCACC AGATTGCGGA GAGTTTCTCC AAAAAGTACA AGTTTCCCTC   300
WSKPO    251 .......... .......... .......... .......... ..........   300
                   310        320        330        340        350
WSKP     301 TCGATCCTCA GGGATATTCT .......... .......... ..........   350
WSKPO    301 .......... .......... .......... .......... ..........   350
```

Figure 12. Nucleotide Sequence Analysis of the Consensus Sequence for the WSK Input and Output Viruses for the G-L Intergenic Region.

```
                    10         20         30         40         50
WSKGL      1 AGGGATCGAT ACCAAAGGTT GTGGACTGGC CAAGGAGTGT GTCTGATGTC    50
WSKGLO     1 .......... .......... .......... .......... ..........    50
                    60         70         80         90        100
WSKGL     51 TCCGTGCTTG GGCATGGACA GAGGTCATAG TGTATCCCAT GATAGCAGAC   100
WSKGLO    51 .......... .......... .......... .......... ..........   100
                   110        120        130        140        150
WSKGL    101 TCAACATGAG TTAATTGAGA AAGGCAATCT GCCTCCCATG AAGGACATAA   150
WSKGLO   101 .......... .......... .......... .......... ..........   150
                   160        170        180        190        200
WSKGL    151 GCAATAGCTC ACAATCATCT TACATCTTAG CAAAGTGTGC ATAATTATAA   200
WSKGLO   151 .......... .......... .......... .......... ..........   200
                   210        220        230        240        250
WSKGL    201 AGGGCTGGGT CATCAAATCT TCTCAGTCGA GAAAAAAACT GTAGATCAAA   250
WSKGLO   201 .......... .......... .......... .......... ..........   250
                   260        270        280        290        300
WSKGL    251 AGAGCAACTG GCAACACTTC TCATCCTGAG ACCTACATCA AGATGCTGGA   300
WSKGLO   251 .......... .......... .......... .......... ..........   300
                   310        320        330        340        350
WSKGL    301 TCCTGGAGAG GTCTATGATG .......... .......... ..........   350
WSKGLO   301 .......... .......... .......... .......... ..........   350
```

Figure 13. Amino Acid Residues of the P Protein for the WSK virus (Consensus Sequence).

```
 File : WSKP
 Range :       2 -     320   Mode : Normal
 Codon Table : Universal

            10          19          28          37          46          55
5'  TGG TCA CAG ACC GTA GAA GAG ATT ATA TCC TAT GTC ATG GTC AAC TTT CCC AAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Trp Ser Gln Thr Val Glu Glu Ile Ile Ser Tyr Val Met Val Asn Phe Pro Asn

            64          73          82          91         100         109
    TCT CCA GGA AGG TCT TCA GAG GAC AAA TCA ACC CAA ACT ACC GGC AGG GTG CCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ser Pro Gly Arg Ser Ser Glu Asp Lys Ser Thr Gln Thr Thr Gly Arg Val Pro

           118         127         136         145         154         163
    AAG AAG GAG ACA ACA TCC ACT CCC TCT CAG AGA GAA AGC CAA TCT TCG AAA GCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Lys Lys Glu Thr Thr Ser Thr Pro Ser Gln Arg Glu Ser Gln Ser Ser Lys Ala

           172         181         190         199         208         217
    AGG ATG GCG GCT CAA GCT GCC TCT GGC CCT CCA GCC CTT GAA TGG TCT GCC ACT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Arg Met Ala Ala Gln Ala Ala Ser Gly Pro Pro Ala Leu Glu Trp Ser Ala Thr

           226         235         244         253         262         271
    AAT GAG GAA GAT GAT CTA TCA GTA GAG GCT GAA ATC GCT CAC CAG ATT GCG GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Asn Glu Glu Asp Asp Leu Ser Val Glu Ala Glu Ile Ala His Gln Ile Ala Glu

           280         289         298         307         316
    AGT TTC TCC AAA AAG TAC AAG TTT CCC TCT CGA TCC TCA GGG ATA TTC T 3'
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
    Ser Phe Ser Lys Lys Tyr Lys Phe Pro Ser Arg Ser Ser Gly Ile Phe
```

Figure 14. **Amino Acid Residues of the P Protein for the EAF virus (Consensus Sequence).**

```
File : EAFOP
Range :         2 -      320    Mode : Normal
Codon Table : Universal

              10          19          28          37          46          55
5'  TGG TCA CAA ACC GTG GAG GAA ATC ATA TCT TAT GTC ACA GTC AAC TTT CCT AAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Trp Ser Gln Thr Val Glu Glu Ile Ile Ser Tyr Val Thr Val Asn Phe Pro Asn

              64          73          82          91         100         109
    CCC CCA GGG AAG TCC TCG GAA GAT AAG TCA ACC CAG ACA ACT GGC CGA GAA CTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Pro Pro Gly Lys Ser Ser Glu Asp Lys Ser Thr Gln Thr Thr Gly Arg Glu Leu

             118         127         136         145         154         163
    AAG AAG GAG ACA ACA TCC GTT TCT TCC CAG AGA GAC AGT CAA TCC TCG AAA GCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Lys Lys Glu Thr Thr Ser Val Ser Ser Gln Arg Asp Ser Gln Ser Ser Lys Ala

             172         181         190         199         208         217
    AGG ATG GTG GCT CAA GCC GCC TCC GGT CCC CCA GCC CTC GAG TGG TCT GCT ACC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Arg Met Val Ala Gln Ala Ala Ser Gly Pro Pro Ala Leu Glu Trp Ser Ala Thr

             226         235         244         253         262         271
    AAC GAG GAG GAT GAT CTG TCA GTA GAA GCT GAG ATC GCT CAC CAG GTT GCC GAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Asn Glu Glu Asp Asp Leu Ser Val Glu Ala Glu Ile Ala His Gln Val Ala Glu

             280         289         298         307         316
    AGC TTT TCC AAG AAG TAC AAG TTT CCC TCT CGA TCA TCT GGG ATA TTT C 3'
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -
    Ser Phe Ser Lys Lys Tyr Lys Phe Pro Ser Arg Ser Ser Gly Ile Phe
```

The Consensus sequence of the WSK input is the same as that of the WSK output.

EAF input P-gene

- 15 clones identical to the consensus sequence.
- Nucleotide substitutions occurred in:
  - Clone 3EP at position 309 (2,172, PV) C to T; Ser to Phe.
  - Clone 5EP at position 256 (2,119, PV) T to C, a.a. stays the same, Ala.
  - Clone 6EP at position 251 (2,114, PV) A to G, Ile to Val

    and at position 299 (2,162, PV) T to C, Ser to Pro.
  - Clone 7EP at position 100 (1,963, PV) C to T, a.a. stays the same, Gly.
  - Clone 8EP at position 251 (2,114, PV) A to G; Ile to Val

    and at position 299 (2,162, PV) T to C, Ser to Pro.

All the nucleotide substitutions that occurred are transitions. Nucleotide substitutions occurring in the protein-coding region can also be characterized by their effects upon the product of translation, the protein. A substitution is said to be synonymous or silent if it does not cause an amino acid change, otherwise, it is nonsynonymous. In clones 3EP, 6EP and 8EP nonsynonymous changes occur, while synonymous substitutions occur in clones 5EP and 7EP.

EAF output P-gene

- 19 clones identical to the consensus sequence.
- Nucleotide substitution occurred in:
  - Clone 9EOP at position 125 (1,988, PV) T to C, Ser to Pro.

The consensus sequence of the EAF input is the same as that of the EAF output.

WSK input G-L intergenic region

- All 20 clones were similar to the consensus sequence

WSK output G-L intergenic region

- All 20 clones confirmed identical to the consensus sequence

The consensus sequence of the WSK input is the same as that of the WSK output.

EAF input G-L intergenic region

- All 20 clones identical to the consensus sequence

EAF output G-L intergenic region

- 18 clones conform to the consensus sequence
- Nucleotide substitutions occurred in:
    - Clone 11EGLO at position 274 (5,399, PV) T to C
    - Clone 6BEGLO at position 62 (5,187, PV) G to A

Nucleotide substitutions that occur are transitions.

The consensus sequence of the EAF input is the same as that of the EAF output.

Discussion

Introduction

Due to the limited replication fidelity of the viral polymerase activity, caused by an absence of proofreading/repair and post-replicative error correction, it is often reported that RNA virus populations (Holland et. al., 1992; Drake, 1993; Domingo and Holland, 1994, 1997) exhibit a heterogeneous population structure within single individuals. This population, often referred to as quasispecies, is characterized by one or several master sequences and a large spectrum of related variants (Kissi et. al., 1999). The master sequence, which will often be more abundant than other variant sequences is the most fit genome sequence, but may or may not be identical to the average or consensus sequence (Domingo et. al., 1996).

It has long been known that mutant swarms (the mutant spectra that populate viral quasispecies) contain potentially useful variants, for example those with increased resistance to antiviral agents, altered interferon inducing capacity, or antibody- escape and cytotoxic T lymphocyte (CTL)-escape mutants, among others (Domingo, 2000). A new feature of quasispecies dynamics which may turn out to be important for viral adaptation has been revealed: the presence of a memory of past evolutionary history, imprinted on minority components of the mutant spectrum (Ruiz-Jarabo et. al., 2000). Due to the existence of a molecular memory, a viral quasispecies may be capable of reacting swiftly to a selective constraint which has already been experienced by the same virus population (Domingo, 2000).

Though rabies viruses have very simple genomes with no known regulatory elements, they must accommodate numerous adaptations to the host species and fulfill a complex infection cycle *in vivo* in order to survive in their host populations. The

quasispecies structure/polymorphism in rabies virus, which has been documented by a number of investigators (Benmansour et. al., 1992; Morimoto et. al., 1998; Kissi et. al., 1999), has been implicated in the epidemiology and pathogenesis of the virus (Morimoto et. al., 1998). Understanding the evolution of rabies virus is important for determining the basis of its genetic and phenotypic flexibility (Kissi et. al., 1999). The optimization of virus quasispecies in new environments and the following modification of fitness have been widely and empirically used for the production of modified live vaccines (Vodopija and Clark, 1991; Lafay et. al., 1994). In wild isolates, previous results indicated a relative genetic stasis and a limited process of genetic radiation around a prototype sequence (Amengual et. al., 1997; Kissi et. al., 1995) together with an adaptive process to different animal species.

To further address some issues of virus evolution and virus population biology (quasispecies population and viral polymorphism, fitness and population bottlenecks, Mullers Ratchet, Competitive Exclusion Principle), the fate of two known rabies virus variants passaged individually and in competition to each other *in vitro* was determined and a comparative analysis of the degree of variation exhibited by the two rabies virus variants at the P – gene locus and the G – L intergenic region, both prior to and after 12 cell passages was undertaken. The behavior of the two closely related variants *in vivo* was also studied.

Clonal Variation *in vitro*

RNA virus quasispecies are highly effective in the exploration of new genomic sequences (Eigen and Biebricher, 1988). This quasispecies model of mixed RNA virus populations implies a significant adaptive potential because it allows the rapid selection

of the mutant(s) with the highest fitness in any new environmental condition (Morimoto et. al., 1998; Drake and Holland, 1999; Domingo et. al., 2000). The determination of the quasispecies structure of RNA viruses (Domingo et. al., 1978, 1980, 1985; de la Torre and Holland, 1990; Benmansour et. al., 1992; Morimoto et. al., 1998; Drake and Holland, 1999; Domingo et. al., 2000) indicates that multiple variants could coexist during propagation of the virus *in vivo* or *in vitro*.

Sequencing which has been used previously in a wide range of rabies virus studies, for example in the studies of phylogenetic and epidemiologic relationships among isolates is used in this investigation to analyze parts of the rabies virus genome to determine how rapidly polymorphism arises from a uniform (cloned) virus population after a population bottleneck. In total, the portion of the P-gene (320 nucleotides) and part of the G-L intergenic region (320 nucleotides) analyzed in this study, represents 5.3% of the genome of the rabies virus which is approximately 12000 bases long (PV strain) (Tordo et. al., 1986). Moreover, the targeted sequences: a central highly variable region of the P-gene (Nadin-Davis et. al., 2000) and the G-L non-coding region, which does not code for any protein and which therefore might be expected to ehibit relatively high levels of variation, are amongst the most variable parts of the genome.

It is noticed from this investigation that DNA sequence comparison between the consensus sequence of the WSK virus input and output viruses for the P-gene and all the clones analyzed show the nucleotides to be the same, indicating that even though the WSK virus had been passaged on cell monolayers 12 times there was no change in its nucleotide sequence in the portion of the genome analyzed, indicating the conserved nature of the virus. Conversely, some rapid changes observed in an earlier study with

rabies virus isolates from Algeria (Benmansour et. al., 1992) could be due to passages with lower population size or population bottlenecks, allowing rapid changes in the extent and nature of genetic polymorphism (Domingo and Holland, 1997). The EAF virus on the other hand does not appear to be as conserved as the WSK virus for the portion of the P-gene analyzed. This study shows that even though the consensus sequences of the EAF virus input and output viruses for the P-gene are the same, there is a relatively high level of non-synonymous substitutions occurring in three input clones and 1 output clone, demonstrating that a variety of mutant forms of viral RNA exists. All nucleotide substitutions that occurred are transitions with clones 6EP and 8EP (Figure 9A) possessing two nucleotide substitutions in the P-gene at the same loci, suggesting a minor population within the quasispecies complex in addition to that of the consensus sequence.

The genetic factors that control viral emergence may involve either host susceptibility to infection or viral infectiousness (Holmes et. al., 2002). Due to the fact that RNA viruses typically can show high levels of genetic variation, as shown in this study in the EAF input virus population, some strains may differ in their ability to replicate in new hosts or may be able to adapt quickly. This process has been documented in the rabies virus *in vitro* by Morimoto et. al. (Morimoto et. al, 1996, 1998) who showed that substitutions in the viral glycoprotein (G) sequence which accumulated in the cell culture changes the tropism for nervous tissues, thereby changing virulence. That this is an adaptive process was highlighted in the study of Kissi et. al. (Kissi et. al., 1999), who observed substantial genetic variation in the G gene from viruses passaged through different host species, with greatly elevated rates of non-synonymous (dN) over synonymous (dS) substitutions per site, indicative of positive selection.

The analysis of the results in this investigation also indicates that the input virus for the EAF P-gene was less homogenous than that of the output virus for the EAF P-gene (unlike the WSK virus which was conserved in both the input and output viruses), even though both viruses were cloned by two consecutive limit dilution purifications to produce a homogenous population exhibiting minimal heterogeneity. To increase the titer of the WSK virus for the passaging experiment, the WSK virus after being cloned was passaged twice in MNA cells prior to the start of the experiment. This passaging could have allowed additional adaptation of the WSK virus to the MNA cells, hence minimizing the observed variation, as compared to the EAF virus. The EAF virus on the other hand was at a sufficiently high titer after being cloned and did not need to be passaged. The relatively high level of nucleotide substitutions observed in the EAF virus clones for the P-gene indicate that the cloned virus was not a completely homogenous population suggesting that what are considered to be homogenous rabies virus strains actually consist of different variants and these variants may have distinctive biological properties. An adaptation of the EAF virus to the MNA cells is indicated by the fact that nucleotide substitutions occurred in only one of the 20 clones analyzed from the output virus, hence there appears to be less variation than for the input virus. The P protein of the rabies virus is an RNA polymerase cofactor which binds to both the L and N viral proteins. Studies carried out by Jacob et. al. and Raux et. al. (Jacob et. al., 2000; Raux et. al., 2000) have also speculated that the P protein may be involved in the axonal transport of rabies virus along the microtubules of neurons through association with the dynein light chain (the role of the central region is unclear, but the LCD8 binding site is

conserved within the central region), therefore P protein variation could have subtle effects on viral transport.

In contrast to the P-gene, the G-L intergenic region exhibits limited variation in the output virus only (Figure 10A-10B); hence, the results from the two loci are not consistent. An explanation for this may be that factors determining or selective pressures for adaptation on the different parts of the genome of the rabies virus (and RNA viruses in general) may vary. That these nucleotide substitutions observed in the two output clones may be due to mis-incorporation caused by polymerase error, is unlikely, due to the fact that the Expand High Fidelity PCR System is composed of a unique enzyme mix containing thermostable Taq DNA polymerase and a proofreading polymerase (Barnes, 1994), which is designed to give PCR products with high yield, high fidelity and high specificity from episomal and genomic DNA (Roche, Expand High Fidelity PCR System Instructions). Due to the inherent 3'-5' exonuclease proofreading activity of the proofreading polymerase, Expand High Fidelity PCR System results in a 3-fold increased fidelity of DNA synthesis (8.5 X 10^{-6} error rate) compared to Taq DNA polymerase (2.3 X 10^{-5} error rate) (Roche, Expand High Fidelity PCR System Instructions).

An implication of the quasispecies nature for the biology of RNA viruses is provided by the stability of the consensus sequence through time. If the consensus sequence is assumed to represent the genome of highest fitness, then the observation that this remains intact despite high mutation rates could mean that the viral population forms a cooperative structure (Holmes and Moya, 2002). This is referred to as population equilibrium (Domingo et. al., 1996) and it explains evolutionary stasis of RNA viruses in spite of continuous viral replication. This condition is observed in this study where it is

shown that the consensus sequences for the output and input viruses for both parts of the genome analyzed for the WSK and EAF viruses remain the same. Similar experiments on VSV also revealed a stable consensus sequence over multiple passages in cell culture (Steinhauer et. al., 1985).

Competition *in vitro*

The determination of the quasispecies structure of RNA viruses (Domingo et. al., 1978, 1980, 1985; de la Torre and Holland, 1990; Benmansour et. al., 1992) indicates that multiple variants could coexist during propagation of the virus *in vitro* or *in vivo*. This heterogeneous population structure, which has been demonstrated for rabies virus by a number of investigators, is assumed to be a common feature of all rabies virus strains. Hence, before studying competition and clonal variation *in vitro* the two rabies viruses (the WSK and the EAF viruses) were cloned so as to exhibit minimal heterogeneity before passaging so that the fate of the two distinct virus variants in an artificially polymorphic ('di-morphic') virus population when passaged could be investigated. The extent of genetic stability is known to depend on the passage conditions of the viruses (Spindler et. al., 1982; Steinhauer and Holland, 1987; Steinhauer et. al., 1989). Hence a MOI of 0.2 was used to infect MNA cells (a stable cell line for rabies virus propagation) and cells were passaged at a constant concentration of 2×10^5 cells/ml between passages, using the same media to maintain a constant environment for the viruses. The times at which cytopathic effects (CPEs) occur in cell cultures depends on the MOI. For VSV (a rhabdovirus very similar to the rabies virus) at high MOIs, CPE occurs within several hours, whereas at low MOIs, CPE is not evident for 24 hrs or longer. Rabies virus on the other hand shows either little (Tuffereau and Martinet-Edelist, 1985) or no CPE (Ermine

and Flamand, 1977). A MOI of 0.2 was used to infect MNA cells because the EAF virus infected approximately 50% of the cell monolayer as the monolayer approaches 100% before passaging. The WSK virus on the other hand, had a substantially slower virus cycle and had lower viral production (when the titers of the two rabies virus strains were compared). At a MOI of 0.2 the occurrence of defective interfering (DI) particles is also reduced. Most rabies virus investigations use a low MOI of between 0.1 – 0.5 (Kissi et. al., 1999; Morimoto et. al., 1998).

Competition between the WSK and EAF viruses resulted in the eventual displacement of one of the populations by the other, consistent with the Competitive Exclusion Principle of population genetics (Gause, 1971), which states that in the absence of niche differentiation one competing species will always out compete the other. Figures 4C and 4D show that while the WSK virus infection was maintained and propagated over 12 passages in co-infection, the EAF virus was no longer detected after the 4th passage. This result is contrary to what was expected since, during virus propagation of both strains individually in MNA cells during the preliminary studies, the EAF virus replicated in the cell monolayer at a faster rate and yielded on average higher titers than the WSK virus. It was therefore expected that the EAF virus would out compete the WSK virus and not the other way around. From Figures 4C and 4D it is noticeable that at the first passage, the percentage of cells infected with the WSK virus and the EAF virus is at 60% and 5% respectively. The percentage of cells infected with the WSK virus rises to approximately 100% for the WSK virus by the 2nd passage, while the EAF virus, which increases to 15% by the 2nd passage, decreases to 5% by the third passage until it is no longer detected in the cell monolayer. The titer of the EAF also decreases along with the

decrease in the percentage of infected cells to a point at which the amount of virus antigen can no longer be detected. It would appear that though the WSK virus was much slower at replicating in the cell monolayer than the EAF virus, it is inferred from these results that once the infection has been established for the WSK virus, it is hardier than the EAF virus and more adaptable to cell culture in the presence of another virus population. This has implications in the behavior of the rabies virus and lyssaviruses in general in host populations.

Studies in competition between VSV clones of approximately equal relative fitness also resulted in the eventual displacement of one of the populations by the other (Clark et. al., 1994). Although competing VSV quasispecies coexisted for many generations, highly advantageous mutations occurred stochastically in variants of one of the two competing quasispecies, leading to a sudden exclusion of the other (Clark et. al., 1994). Ayala (Ayala, 1969, 1971) on the other hand, rejected Competitive Exclusion as a general principle and showed that two species of *Drosophila* could co-exist for many generations while competing for limited resources. This difference of behavior of Drosophila species and VSV quasispecies (and rabies virus strains as shown in this study) may reflect the genetic and phenotypic inflexibility of Drosophila within the time frame of the experiment, as compared to the highly dynamic nature of RNA virus populations and the large number of viral particles involved (Domingo et. al., 1996).

In the competitions between neutral VSV clones, both the winners and the losers gained fitness relative to a reference VSV clone (Clark et. al., 1994), in support of the Red Queen hypothesis (van Valen, 1973). According to this hypothesis, each species is competing in a zero sum game for the same fixed resources against others and no species

can ever win and new adversaries grinningly replace the losers (van Valen, 1973). Among competing viral quasispecies populations (or variants) in an equilibrium situation, only infrequently arising, vastly superior mutants are likely to upset the equilibrium and exclude all others (Domingo et. al., 1996) and until such "competitive exclusion" occurs, most members of a quasispecies are slowly improving their fitness. The fitness of a virus, defined as the overall replication and survival ability, hence the number of offspring generated by one particle of the virus, can be expressed (Clarke et. al., 1994) as the ratio of the progeny produced by the variant(s) under analysis to the progeny of a competing reference wild-type (wt) strain, which is assigned a fitness of 1.0. Though in this investigation, the fitness of the WSK and EAF viruses in co-infection were not measured, the fact that the EAF virus levels dropped off after the 4th passage, hence no replication to produce infectious progeny would indicate a low fitness level.

Before competition between the WSK and EAF viruses *in vitro* could be investigated it was important to passage these viruses individually in MNA cells to make sure they can be propagated over a 12 passage period and that the infection can be maintained. Investigating and studying the behavior of these viruses in cell culture individually was also paramount to the investigation in co-infection. From Figures 4A and 4B it was observed that the cell infection levels and virus titers fluctuated in a cyclical pattern (also observed in Figure 4C) suggesting that virus propagation can be maintained indefinitely. As the percentage of infected cells increased or decreased, the virus titers also increased or decreased, although lagging behind the cell infection levels. This observation might be due to the presence of DI particles in the cell monolayer. DI particles are mutated virus particles with an incomplete genome. These DI particles,

which have been better studied in VSV (Lazzarini et. al., 1981; Blumberg and Kolakofsy, 1983), usually possess a truncated genome and are therefore defective in transcription and replication activity, requiring the presence of homologous infectious particles to assure their multiplication. The DI particles can replicate rapidly since they have shortened genomes and they interfere with normal genomes for encapsulation into the virion structures. It is noticed that at the initial infection of MNA cells with the WSK and EAF viruses individually, the titer and percentage of cells infected with the viruses increases. The titer of the viruses then drops while the % of infected cells remains at 100% for about 3 to 4 passages before a decrease is noticed. Since normal virion particles and DI particles cannot be distinguished by FAT in cell monolayer, it is not possible to determine the amount of DI particles present. However, the decrease in virus titers, which measure the actual amount of infectious particles present, is followed after a lag period by a drop in the percentage of infected cells possibly due to significant production of DI particles. Thus as MNA cells are passaged, the number of DI particles on the cell monolayer could decrease since they are not able to replicate and hence will not be transferred to new cells. Subsequently, in the new passage, complete virion particles would have the opportunity of infecting new MNA cells (since the MNA cells are still actively dividing) thereby eliciting a rise in the virus titer, until the accumulation of DI particles decreases the titer once again. These results have implications in the pathogenesis and infection of viruses, especially in persistent infections, indicating that cells infected with viruses that do not cause cell destruction may eventually eliminate the infection.

Competition *in vivo*

In order for rabies to survive it is essential that the virus is transmitted by an infected animal during a period of virus excretion to enough other susceptible individuals. For this to occur, rabies virus strains must be adapted to the physiological traits and population biology of their hosts (Bacon, 1985). In the investigation of competition *in vivo* between the WSK and EAF viruses, the fate of these two variants in an artificially polymorphic ('di-morphic') virus population when the viruses are replicating *in vivo* and experience the bottlenecks of neuron to neuron transmission are studied. 12 guinea pigs were inoculated with either the WSK or EAF viruses individually or in co-infection, intra-cerebrally or intra-muscularly (see table in Materials and Methods). Already characterized labeled monoclonal antibodies specific to each variant were used to identify each strain in direct immunofluorescence. Guinea pigs were chosen as the *in vivo* host, due to the fact that rabies infection in guinea pigs have not been fully investigated and also because guinea pigs have mid sized brains as compared to mice and will therefore produce better cryostat sections for analysis.

Of the 12 guinea pigs inoculated with the WSK and EAF viruses accordingly, only 5 guinea pigs developed a viral infection. All the guinea pigs that developed rabies were inoculated intra-cerebrally, though one which was inoculated intra-cerebrally with the EAF virus did not develop rabies and had to be euthanized along with those given virus intra-muscularly at the termination of the experiment. An explanation for this infection failure could be the depth at which that particular guinea pig was inoculated. It could have been inoculated just outside the brain cavity and not have penetrated through, hence instead of establishing a direct brain infection the virus had to be propagated as a natural

infection. Another explanation might be that the guinea pig mounted an antibody response. The serum neutralization assay (results not shown) proved that this was not the case.

The titer of both viruses inoculated into the guinea pigs was at $10^{4.80}$ $TCID_{50/ml}$. From this investigation it can be assumed that at this titer (and titers lower than this), the guinea pigs are not susceptible to the rabies virus when inoculated intra-muscularly, showing that animals may be exposed to substantial quantities of virus with no apparent effect (no disease, and no measurable immune response, as shown in this investigation). This has also been shown in studies by Dr. Wandeler at staff and ADRI, in North American raccoons, which showed that to achieve 80% mortality in the raccoons one has to inoculate over $10^{6.00}$ $TCID_{50/ml}$ raccoon strain rabies viruses by the intra-muscular route. Numerous authors have also provided evidence that lyssavirus infection with involvement of the central nervous system is not invariably fatal, however, as stated by Bell (Bell, 1975) and Fekadu (Fekadu, 1991), few studies have clearly defined criteria for differing latency, abortive infection, or recovery (with or without sequelae) by simultaneous measurements of serum and CSF antibody, combined with a sensitive viral detection system (Wandeler et al, 1994).

The result in Table VI shows that no virus antigen was detected in the salivary glands of the guinea pigs that developed rabies, while the brains of these guinea pigs showed the presence of virus antigen. The fitness of a virus, which is determined by its ability to produce progeny, can also be defined as the ability for that virus to be transmitted. Since the rabies virus is transmitted through the saliva by the bite of an infected animal, the results may speculate that these viruses are not at their highest fitness

levels, but the fact that no virus antigen was detected in the salivary glands may also be attributed to a number of factors. Once the symptoms of rabies was noticed the guinea pigs were euthanized (though one of the guinea pigs died before being euthanized). This may not have given the virus the opportunity to transmit itself to the salivary glands. The virus may also have been present in the salivary gland but not have replicated sufficiently to be detected by immunoflourescence. Genetic bottlenecks which are probably frequent during the natural lifecycle of RNA viruses such as in airborne or droplet transmission or in this case in the invasion of new tissues and organs (the salivary gland) by restricted subsets of infectious particles may have taken place. The presence of virus antigen in the brains of the guinea pigs (Figures 5A-5C and 6A-6C) indicates that replication took place. An increase in titer in the brain of the guinea pigs (except guinea pig #275N) might be an indication of an increase in fitness of the virus within the brain. A serum neutralization assay (results not shown) showed all 7 guinea pigs that did not develop rabies to be negative for antibodies against the virus. Since a neutralization assay was not carried out in the guinea pigs that developed rabies, these results cannot be conclusive.

Adaptations to different hosts have been documented to some extent by *in vivo* experiments on susceptibility and by observation on virus excretion in experimentally infected animals and in field specimens submitted for diagnosis (Blancou, 1988; Blancou et. al., 1991; Charlton et. al., 1991; Winkler and Jenkins, 1991; Aubert, 1992; Wachendorfer and Frost, 1992). Adaptations of a particular virus strain to its principle host are indicated by the high frequency and magnitude of its excretion on the one hand and by the host's high susceptibility to it, on the other. These properties allow for transmission from an infective to a susceptible individual in the case of a biting incident

(Wandeler et. al., 1994). It has not been shown if two different rabies virus strains can adapt and propagate within the same host. This investigation suggests that two rabies virus strains can simultaneously infect the same host (guinea pig #278) and can be detected within the brain as shown in Figure 6A, which shows the presence of both the WSK and EAF viruses. However, titration of the brain of this guinea pig detected the presence of the WSK virus only, indicating a dominance of the WSK virus over the EAF virus in this competition. A total displacement of the EAF virus may eventually take place as shown in the *in vitro* studies that abided by the Competitive Exclusion Principle. The same brain cells do not appear to be infected by both viruses. The brain of guinea pig #275N showed only the presence of the EAF virus which corresponded to the results obtained when the brain was titrated, indicating a dominance of the EAF virus over the WSK virus in this guinea pig. What factors played a part in the EAF virus out competing the WSK virus and causing its fitness loss in this case cannot be ascertained for certain. Fitness losses have indeed been observed in clones of RNA viruses propagated through genetic bottleneck passages (Chao, 1990; Chao et. al., 1992; Duarte et. al., 1992; Clarke et. al., 1993), though it is not known at what point the genetic bottlenecks occurred. It may have happened anywhere from the site of entry, to accessing the nervous system, during its passage through the nervous system and eventually to the salivary glands.

Conclusion and Directions for the Future

This investigation addresses some issues of virus evolution and virus population biology by studying the fate of two known rabies virus variants in competition to each other both *in vitro* and *in vivo*. The interest in evolutionary questions in microbiology may have been triggered by technical advances in molecular biology; hence, a comparative analysis of the degree of variation exhibited by the two rabies virus variants at the P-gene locus and the G -L intergenic region, both prior to and after 12 cell passages was undertaken using molecular techniques.

It was observed that competition between the two virus variants resulted in the eventual displacement of one of the populations by the other, which is consistent with the Competitive Exclusion Principle of Population genetics stated by Gause in 1971. Hence, between the variants *in vitro* and *in vivo*, competition and not co-existence between variants is observed. It is expected that if the experiment were repeated, similar results would be obtained. This needs to be studied further, to determine if all rabies virus variants will show consistent results. Virus propagation can also be maintained indefinitely over a period of time as observed in this study, having direct implications in the pathogenesis and infection of viruses, especially in persistent infections.

This study also showed that evolution of polymorphism is not as rapid as one might expect and as shown in some studies. DNA sequence comparison between the consensus sequence of the WSK virus input and output viruses for the P-gene and G-L intergenic region and all the clones analyzed, showed the nucleotides to be the same. The EAF virus on the other hand did not appear as conserved as the WSK virus, showing a relatively high level of non-synonymous substitutions occurring in a number of clones. Whether or not there is any co-operation between the various quasispecies populations *in vitro* or in a

natural infection has not been proven and needs to be studied further. Despite the laboratory evidence for selectively driven host adaptation in rabies virus as shown by a number of investigators, the molecular mechanisms controlling this process are not fully understood. There is also little information about what genetic changes, if any take place at all, that are involved in host transfer in natural infections. An implication of the quasispecies nature for the biology of RNA viruses is provided by the stability of the consensus sequence through time, a condition observed in this study for the output and input viruses for both parts of the genome analyzed for the WSK and EAF viruses. The observation that the consensus sequence remains intact despite high mutation rates indicate that the viral population forms a cooperative structure, referred to as population equilibrium. The selective pressures that act upon the genome of the rabies virus during adaptation are not fully understood and needs to be further analyzed and studied.

The behavior of naturally polymorphic virus populations in their natural hosts from the site of infection via the central nervous system to the site of excretion needs to be studied. It is not known at what point severe genetic bottlenecks occur. The fact that this study shows the brain of a host (guinea pigs) could be infected with the two variants, but in different cells, hence different neurons, indicates genetic bottlenecks are taking place. No virus antigen was detected in the salivary glands of those guinea pigs that developed the rabies infection, but it is likely that only one variant may have eventually succeeded in being transmitted to the salivary glands; the variant that gets there first.

This study also shows that not all exposures to the rabies virus are fatal, as shown by a number of other studies. However, exposure outcome which depends not only on the host in question but also on the origin and amount of the infecting virus isolate is not

fully understood and needs to be analyzed further. Thus, the concept of a 'susceptible host' species requires qualification and clarification.

Studies on the evolution and population biology of rabies virus (and RNA viruses in general) have profound implications for its epidemiology and pathogenesis.

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Appendix

APPENDIX 1

Analysis of the molecular clones indicated three highly divergent clones (with the same nucleotide sequence) for the WSK strain P-gene input virus, which were later identified to be of the raccoon strain and were thus contaminants that were not included in the final analysis. To show that the source of this contamination of the P-gene PCR with the raccoon strain sequence was due to a PCR carry over problem and not a contamination of the original virus stock, a panel of 8 already characterized monoclonal antibodies for raccoon rabies virus detection was employed. The mab reactivies of the original cloned WSK virus stock that was used in the passaging experiment (92L1169 P_{25} B_6 sub clone D_6) was determined in indirect immunofluorescence (on Terezaki plates seeded with MNA cells infected with the WSK virus clone), using FITC- conjugated goat anti-mouse immunoglobulin as the secondary antibody (as previously explained). Table VII shows the expected mab reactivies for the WSK strain and the raccoon strain. Antigenic discrimination had already been well established by Dr. Wandeler and staff. Table VIII shows the results of the mab reactivities of the WSK virus clone (92L1169 P_{25} B_6 sub clone D_6).This confirms that the original virus stock was not contaminated with the raccoon strain and that the contamination occurred as a result of PCR carry over. Figure 15 shows the nucleotide sequence comparison between various raccoon strain samples and one of the contaminants (clone lWP).

APPENDIX 1: Table VII. Characteristic Pattern of the Raccoon Rabies Virus and the Western Skunk Rabies Virus to the Following Monoclonal Antibodies

Monoclonal Antibodies	Raccoon Virus	Western Skunk Virus
5DF12	+++	+++
26AF11	-	+++
M323	+++	-
M951	+++	-
M964	+++	-
M972	+++	-
M1348	+++	-
M1806	+++	-

APPENDIX 1: Table VIII. Characteristic Reactivity Patterns of the WSK Virus Clone (92L1169P_{25} B_6 sub clone D_6) to the Following Monoclonal Antibodies

Monoclonal Antibody	WSK virus clone
5DF12	+++
26AF11	+++
M323	-
M951	-
M964	-
M972	-
M1348	-
M1806	-

Figure 15. Nucleotide Sequence Comparison between Various Raccoon Strain Samples and one of the Contaminants

Fig 12 APPENDIX 1

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                      10         20         30         40         50
CP_R89.RAC      1 CATCGAGGAC AATCAGGCTC ATCTCCAAGG AGAGCCTATA GAAGTAGATA    50
CP_NY2          1 ...A...... .......... .......... .......... ..........    50
CP_NB.RAC       1 .......... .......... .......... .......... ..........    50
CP_ONT2.RAC     1 ...A...... .......... .......... .......... ..........    50
CP_ONT1.RAC     1 ...A...... .......... .......... .......... ..........    50
CL1WP           1 ---------- ---------- ---------- ---------- ----------    50
                      60         70         80         90        100
CP_R89.RAC     51 GTCTGCCCGA TGACATGAGA CGGCTCCACT TGGATAATGA AAAACCGTCT   100
CP_NY2         51 .......... ...T...... .......... .....G.... ..........   100
CP_NB.RAC      51 .......... .......... .......... .K....G... ..........   100
CP_ONT2.RAC    51 .......... .......... .......... .K...G.... ..........   100
CP_ONT1.RAC    51 .......... .......... .......... .K...G.... ..........   100
CL1WP          51 ---------- ---------- ---------- ---------- ----------   100
                     110        120        130        140        150
CP_R89.RAC    101 GGTTTTGACA AGGTGACAGA AGAAGGGGAG AGCAAGTGTC ATGAAGACTT   150
CP_NY2        101 .......... ........A. .......... .......... ..........   150
CP_NB.RAC     101 .......... ........A. .......... .......... ..........   150
CP_ONT2.RAC   101 .......... ........A. .......... .......... ..........   150
CP_ONT1.RAC   101 .......... ........A. .......... .......... ..........   150
CL1WP         101 ---------- ---------- ---------- ---------- ----------   150
                     160        170        180        190        200
CP_R89.RAC    151 TCAGATGGAT GAAGGGGAGG ACCCCAGCCT CTTGTTCCAG TCATACCTGG   200
CP_NY2        151 .......... .......... .......... .......... ..........   200
CP_NB.RAC     151 .......... .......... .......... .........A ..........   200
CP_ONT2.RAC   151 .......... .......... .......... .......... ..........   200
CP_ONT1.RAC   151 .......... .......... .......... .......... ..........   200
CL1WP         151 ---------- ---------- ---------- ---------- ----------   200
                     210        220        230        240        250
CP_R89.RAC    201 ATAATGTCGG AGTTCAAATA GTCAGGCAAA TGAGGTCAGG AGAGAGATTC   250
CP_NY2        201 .......... .......... .......... .......... ..........   250
CP_NB.RAC     201 .......... .......... .......... .......... ..........   250
CP_ONT2.RAC   201 .......... .......... .......... .......... ..........   250
CP_ONT1.RAC   201 .......... .......... .......... .......... ..........   250
CL1WP         201 ---------- ---------- ---------- ---------- ----------   250
                     260        270        280        290        300
CP_R89.RAC    251 CTTAAAATAT GGTCTCAGAC TGTGGAGGAG ATCATATCCT ATGTCATGAT   300
CP_NY2        251 .......... .......... .......... .......... ..........   300
CP_NB.RAC     251 .......... .......... .......... .......... ..........   300
CP_ONT2.RAC   251 .......... .......... .......... .......... ..........   300
CP_ONT1.RAC   251 .......... .......... .......... .......... ..........   300
CL1WP         251 -------[.. .......... .......... .......... ........G.   300
                     310        320        330        340        350
CP_R89.RAC    301 CAATTTCCCA GGCTCTTTGG GGAGGCCTTC TGAAGACAAG GCCACTCAAA   350
CP_NY2        301 .......... .......... .......... .......... ........G.   350
CP_NB.RAC     301 .......... .......... .......... .......... ..........   350
CP_ONT2.RAC   301 .......... .......... .......... .......... ........G.   350
CP_ONT1.RAC   301 .......... .......... .......... .......... ........G.   350
CL1WP         301 .......... .......... .......... .......... ..........   350
                     360        370        380        390        400
CP_R89.RAC    351 CTGCCAATCG GGAACCCAAG AAAGGAGTGG CATCAGTTTC GTCTCAACTC   400
CP_NY2        351 .......... .......... .......... .......... ..........   400
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Fig 12 APPENDIX 1.

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CP_NB.RAC     351 .......... .......... .......... .......... ..........   400
CP_ONT2.RAC   351 .......... .......... .......... .......... ..........   400
CP_ONT1.RAC   351 .......... .......... .......... .......... ..........   400
CL1WP         351 .......... .......... .......... .......... ..........   400
                         410        420        430        440        450
CP_R89.RAC    401 GAAGGTCAAT CATCCAAAGC GAGAGCGGCA GCCCAAACTG CCTCCGGTCC   450
CP_NY2        401 .......... .......... .......... .......... ..........   450
CP_NB.RAC     401 .......... .......... .......... .......... ..........   450
CP_ONT2.RAC   401 .......... .......... .......... .......... ..........   450
CP_ONT1.RAC   401 .......... .......... .......... .......... ..........   450
CL1WP         401 .......... .......... .......... .......... ..........   450
                         460        470        480        490        500
CP_R89.RAC    451 ACCTGCCCTA GAATGGTCTG CCACCAACGA AGAAGACGAT TTGTCTGTGG   500
CP_NY2        451 .......... .......... T......... .......... ..........   500
CP_NB.RAC     451 .......... .......... .......... .......... ..........   500
CP_ONT2.RAC   451 .......... .......... T......... .......... ..........   500
CP_ONT1.RAC   451 .......... .......... T......... .......... ..........   500
CL1WP         451 ........AT .......... .......... .......... ..........   500
                         510        520        530        540        550
CP_R89.RAC    501 AGGCAGAGAT AGCTCATCAA ATTGCCGAGA GCTTCTCCAA GAAGTACAAA   550
CP_NY2        501 .......... .......... .......... .......... ..........   550
CP_NB.RAC     501 .......... .......... .......... .......... ..........   550
CP_ONT2.RAC   501 .......... .......... .......... .......... ..........   550
CP_ONT1.RAC   501 .......... .......... ..C....... .......... ..........   550
CL1WP         501 .......A.. .......... .......... .......... ..........   550
                         560        570        580        590        600
CP_R89.RAC    551 TTCCCCTCTC ---------- --------.. .......... ..........   600
CP_NY2        551 .......... ---------- --------.. .......... ..........   600
CP_NB.RAC     551 .......... ---------- --------.. .......... ..........   600
CP_ONT2.RAC   551 .......... ---------- --------.. .......... ..........   600
CP_ONT1.RAC   551 .......... ---------- --------.. .......... ..........   600
CL1WP         551 .......... GATCATCAGG GATATTCT.. .......... ..........   600
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