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In vitro and *in vivo* genetic stability of the rabies era glycoprotein expression cassette of ADRG1.3, a recombinant adenovirus vaccine against rabies

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IN VITRO AND IN VIVO GENETIC STABILITY OF THE RABIES ERA GLYCOPROTEIN EXPRESSION CASSETTE OF ADRG1.3, A RECOMBINANT ADENOVIRUS VACCINE AGAINST RABIES

Danielle Renée Roberts

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ABSTRACT

Rabies is a fatal zootic disease, yet preventable with the implementation of modern prophylactic measures. In North America, a wide variety of wildlife animals, including raccoons, skunks, and foxes, serve as reservoirs of rabies and thus maintain the rabies zootic. The protection of principal terrestrial wildlife reservoirs using oral vaccination has emerged as the most viable approach to rabies control with the development of several rabies oral vaccine candidates. One such vaccine under development is AdRG1.3, an adenovirus-rabies glycoprotein recombinant created by L. Prevec and F. Graham (McMaster University; Hamilton, Ontario) that can potentially immunize a broader range of animal species than the currently licensed vaccine. In order to be considered for licensing and large-scale application, one prerequisite is to demonstrate the genetic stability of the AdRG1.3 vaccine.

The genetic stability of the rabies glycoprotein (G) expression cassette of AdRG1.3 was therefore examined using both *in vitro* and *in vivo* models. For the *in vitro* study, the AdRG1.3 vaccine was serially passaged 20 times in 293 cell culture and from the twentieth passage a total of 67 AdRG1.3 virus clones were obtained. The G gene expression cassette (including an SV40 polyadenylation signal sequence) along with flanking human adenovirus type 5 sequences were amplified from these clones using PCR to generate an amplified product approximately 2.25 kb long. These products were then purified and subjected to DNA sequence analysis. No changes were observed in the 1870 nucleotide sequence window (containing both the G gene (1572 nt) and the SV40 polyA sequence (132 nt)) of any of the 67 clones.

The use of a double immunostain assay (DIA) with the ability to detect rabies G and HAd5 antigens simultaneously in cell culture was also investigated. Application of the DIA

in conjunction with a panel of anti-G monoclonal antibodies detected the expression of epitopes from all 6 antigenic sites of rabies G by 72 hours post-infection in AdRG1.3-infected cell culture; HAd5 antigens were detectable by 24 hours post-infection. These findings show that the G gene insert is stably expressed in a conformationally appropriate form by the recombinant HAd5 vector.

The genetic stability of the G gene cassette of AdRG1.3 was also evaluated upon *in vivo* passage using five independent series of cotton rats (*Sigmodon hispidus*). From the fifth *in vivo* passage of AdRG1.3, a total of 105 virus clones were obtained from these five independent series of animals. The complete G gene expression cassette was amplified from these clones by PCR and sequenced as for the *in vitro* study; no base changes were observed in the targeted 1870 nucleotide sequence window of any of these clones. Therefore, these results show that the G gene expression cassette of AdRG1.3 remains stable within the adenovirus vector upon passage of the vaccine both in cell culture and in animals.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab/Am	antibiotic/antimycotic
AChR	acetylcholine receptor
ADP	adenovirus death protein
ADRI	Animal Diseases Research Institute
APHIS	Animal and Plant Health Inspection Service
ARG	adenovirus-rabies glycoprotein
BHV	bovine hepatitis virus
BSC	biosafety cabinet
BSL	biosafety level
CAV	canine adenovirus
cELISA	competitive enzyme-linked immunosorbent assay
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations (United States of America)
CNS	central nervous system
CO_2	carbon dioxide
C of E	centre of expertise
CPE	cytopathic effect
CsCl	cesium chloride
C-terminal	carboxy terminal
CVS	Challenge Virus Standard
cyt c	cytochrome c
DEPC	diethyl pyrocarbonate
DIA	double immunostain assay
DIOC	direct instillation into the oral cavity
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EBL-1	European bat lyssavirus 1
EBL-2	European bat lyssavirus 2
EDTA	ethylene diaminetetraacetic acid
env	envelope
ERA	Evelyn Rokitnicki Abelseth
F	fusion glycoprotein
FA	fluorescent antibody
FAT	fluorescent antibody technique
FASTA	FAST All
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate

G	glycoprotein
gag	group-specific antigen
GITC	guanidine isothiocyanate
GMT	geometric mean titre
H_2	hydrogen gas
HAd2	human adenovirus type 2
HAd5 (wt)	human adenovirus type 5 (wild type)
HBV	hepatitis B virus
HCl	hydrochloric acid
HEP	High Egg Passage
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSV	herpes simplex virus
i.c.	intracerebral
i.e.	for example
IF	immunofluorescence
IgG	immunoglobulin G
IL	interleukin
IUPAC	International Union of Pure and Applied Chemistry
mAb	monoclonal antibody
Mar	monoclonal antibody resistant
moi	multiplicity of infection
MEM	minimum essential medium
MLP	major late promoter
mRNA	messenger RNA
NCAM	neural cell adhesion molecule
nt	nucleotide
N-terminal	amino terminal
OLF	Ottawa Laboratory Fallowfield
OMNR	Ontario Ministry of Natural Resources
ORF	open reading frame
ORV	oral rabies vaccination
p	passage
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHYLIP	phylogeny inference package
PIC	point infection control
polyA	polyadenylation

REA	restriction endonuclease analysis
RG	rabies glycoprotein
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SAD	Street Alabama Dufferin
SAG	SAD Avirulent Gif
SIV	simian immunodeficiency virus
SV40	simian virus 40
TC	tissue culture
TE	Tris-EDTA
T_H cell	T helper cell
TVR	trap-vaccinate-release
USA	United States of America
USDA	United States Department of Agriculture
UV	ultraviolet
VBS	Veterinary Biologics Section
VNA	virus neutralizing antibody
VRG	vaccinia-rabies glycoprotein
VSV	vesicular stomatitis virus

Nucleotide abbreviations

A	adenine
C	cytosine
G	guanine
T	thymine

Symbols

®	registered trademark
™	trademark

Amino acid abbreviations

A	Alanine	R	Arginine
N	Asparagine	D	Aspartate
C	Cysteine	Q	Glutamine
E	Glutamic acid	G	Glycine
H	Histidine	I	Isoleucine
L	Leucine	K	Lysine
M	Methionine	F	Phenylalanine
P	Proline	S	Serine
T	Threonine	W	Tryptophan
Y	Tyrosine	V	Valine
B	Asparagine	Z	Glutamine
X	Any amino acid	*	Termination codon

Units of measurement

bp	base pair
°C	degrees celcius
cm	centimetre
cm^2	squared centimetre
FA_{50}	$TCID_{50}$ value determined via. fluorescence assay
g	gravity
kb	kilobase
M	molarity (mol/litre)
ml	milliliters
mM	millimolar
m/v	mass per volume
nm	nanometer
pH	*pondus hydrogenii*
rpm	revolutions per minute
$TCID_{50}$	median tissue culture infectious dose
µg	micrograms
µL	microlitres
µm	micrometer
µM	micromolar
v/v	volume per volume

INTRODUCTION

RABIES VIRUS

Classification

Rabies virus belongs to the *Lyssavirus* genus of the virus family *Rhabdoviridae*. Its linear, non-segmented, single-stranded RNA (ribonucleic acid) genome of negative polarity places it in the same order (*Mononegavirales*) as the *Paramyxoviridae* and *Filoviridae* families. The rhabdoviruses infecting mammals are classified into the *Vesiculovirus, Lyssavirus* and *Ephemerovirus* genera. Vesicular stomatitis virus (VSV) is the prototype virus of the vesiculoviruses, rabies is the prototype virus of the lyssaviruses, and Adelaide River Virus the type virus of the ephemeroviruses. The lyssaviruses, which encompass the rabies and rabies-related viruses, were initially classified serologically into four serotypes, namely: 1) classical rabies, 2) Lagos bat, 3) Mokola, and 4) Duvenhage (Schneider *et al.*, 1973). Subsequently, phylogenetic analyses targeting N gene sequences classified these viruses to four distinct genotypes, in excellent agreement with the serological studies but also identified two groups of lyssaviruses harboured by European bat species that were assigned to genotypes 5 (European bat lyssavirus-1, (EBL-1)) and 6 (EBL-2) (Bourhy *et al*, 1993; Amengual *et al*, 1997). In 1996, a new lyssavirus was isolated from fruit-eating bats (flying foxes, or *Pteropus alecto*) in Australia (Fraser *et al*, 1996), thus representing a seventh genotype, the Australian bat lyssaviruses (Gould *et al*, 1998). Similar phylogenetic outcomes have been achieved by genetic analysis of the glycoprotein gene (Badrane and Tordo, 2001; Badrane *et al*, 2001) and the phosphoprotein gene (Nadin-Davis *et al*, 2002).

Structure

The rabies virus genome is embedded within a ribonucleoprotein (RNP) core and encased in a bullet-shaped capsid approximately 180 to 200 nm in length and 75 to 80 nm in diameter (Hummeler *et al*, 1967; Vernon *et al*, 1972) in its mature state. The lipid envelope

surrounding the capsid is derived from the cell in which the virus grew (Rose and Whitt, 1996). Peplomers, which are projections of viral glycoprotein homotrimers, cover the surface of the virion (Dietzschold *et al*, 1982; Gaudin *et al*, 1992).

Genome

Replication of the compact genome, approximately 11-12 kilobases (kb) in length, is exclusively cytoplasmic using a virus-encoded RNA dependent RNA polymerase, as single-stranded RNA viruses lack the capacity to utilize host cell transcriptional machinery. The 5 major virus genes include: N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), and L (RNA polymerase). Transcription of the genome produces five monocistronic messenger RNA (mRNA) transcripts from the negative sense RNA genome, although the exact mechanism and its regulation remain unclear. Transcription appears to be both sequential and polar; a transcriptional gradient occurs from 3′ to 5′ such that there is much more N message than L message transcribed during infection (Iverson and Rose, 1981; Villareal *et al*, 1976).

Upon phosphorylation, the P proteins undergo trimerization and bind the L protein, which stabilizes the trimer (Gao *et al*, 1996). Together, these proteins form the viral transcriptase-replicase complex (Emerson and Yu, 1975). The L protein, as indicated above, constitutes the RNA polymerase and becomes active when bound to P; this complex transcribes the genomic template, which is encapsulated with the N protein (an essential member of the transcription complex), to produce mRNAs. Replication of the complete RNA genome produces a full-length positive sense intermediate RNA. The structural M protein, the smallest and most abundant viral protein, is involved in the condensation of nucleocapsids during virus assembly, disruption of the cytoskeleton, and inhibition of host cell functions (Rose and Whitt, 2001).

However, the key antigens in the induction of protection are the rabies N and G proteins, making them the most suitable candidates for inclusion in rabies vaccines.

Nucleoprotein

The N protein, which together with the P and L proteins and RNA genome forms the aforementioned RNP complex, is the most highly conserved protein among rabies viruses (Dietzschold *et al*, 1979; Schneider *et al*, 1973). It is also a major target antigen for T-helper (T_H) cells, which cross-react among the lyssaviruses (Celis *et al*, 1988a; Celis *et al*, 1988b; Ertl *et al*, 1989). A recombinant raccoon poxvirus vector expressing N protected mice against lethal rabies infection *in vivo* and inhibited the replication of rabies virus *in vitro* (Lodmell *et al*, 1993), while a recombinant vaccinia vector expressing N was able to protect mice against challenge following intradermal, but not intramuscular, inoculation (Sumner *et al*, 1991). Lodmell *et al.* (1993) suggest the possibility that portions of N are more accessible or surface exposed than previously thought and, if homologous regions exist between N and G, anti-N antibodies may potentially react with G. Immunization with N purified from a recombinant baculovirus expression system showed N to be comparable to RNP in its ability to protect mice from challenge (Fu *et al*, 1991) and in doing so primed animals for production of virus-neutralizing antibodies (VNAs). These findings suggested that including N along with G in recombinant vaccine constructs could increase immunogenicity of the vaccination. However, a more recent publication investigating this idea suggests that beside its adjuvant effect there is doubt to whether including N in a subunit vaccine is advantageous (Drings *et al*, 1999).

Glycoprotein

Rabies glycoprotein, the only surface-exposed viral protein, is responsible for the attachment and binding of virus receptors (Wunner *et al*, 1984) and membrane fusion

(Gaudin *et al*, 1993). Moreover, it is the only rabies antigen responsible for inducing (Wiktor *et al*, 1973) and reacting with (Dietzschold *et al*, 1987) VNAs and thus stimulates protective immunity against rabies (Cox *et al*, 1977). It has also been reported to stimulate cytotoxic T-lymphocytes (MacFarlan *et al*, 1984), generating virus-specific CD8+ and CD4+ T cells upon rabies infection (de Mattos *et al*, 2001). Induction of CD4+ T cells is a critical part of the protective immune response against rabies (Dietzschold and Ertl, 1991) while the role of CD8+ T cells in this immune response remains unclear.

The glycoprotein of the Evelyn Rokitniki Abelseth (ERA) rabies strain has typical type I transmembrane protein structure; it consists of a C (carboxy)-terminal cytoplasmic domain (44 amino acids (aa)) that interacts with internal viral proteins, a hydrophobic transmembrane segment (22 aa) that anchors the protein in the viral envelope, and a glycoslyated N (amino)-terminal ectodomain (439 aa) involved in receptor recognition, fusion, pathogenicity, and antigenicity. The translated protein is 524 aa in length (Anilionis *et al*, 1981), but subsequent cleavage of the ectodomain signal sequence (19 aa) yields a mature glycoprotein 505 aa long.

As mentioned previously, the glycoproteins are organized into homotrimers which form spikes approximately 8.3 nm high and 8.4 nm wide that protrude from the viral envelope (Gaudin *et al*, 1992), giving rise to approximately 445 homotrimers per virion (Flamand *et al*, 1993). They acquire this organization, with the assistance of chaperone proteins and folding enzymes (Doms *et al*, 1993), in the host cell endoplasmic reticulum (Gaudin, 1997; Whitt *et al*, 1991) and proceed to the Golgi complex for glycosylation. Within the ectodomain, three to four putative N-linked glycosylation sites have been observed, depending on the virus (Langevin and Tuffereau, 2002), although not all sites are actually used; residue 37 is inefficiently glycosylated (Shakin-Eschelman *et al*, 1992),

residues 204/247 are glycosylated in some viral strains (Langevin and Tuffereau, 2002), and residue 319 is always glycosylated (Wunner *et al*, 1985b). No O-linked glycosylation has been observed on the rabies glycoprotein.

Studies suggest that multiple receptors exist for rabies virus. The idea that the nicotinic acetylcholine receptor (AChR) serves as a receptor stemmed from observing sequence similarities between a region of the G ectodomain and snake venom neurotoxins (Lentz *et al*, 1982), a hypothesis later confirmed by subsequent studies (Gastka *et al*, 1996; Hanham *et al*, 1993). The murine low-affinity nerve growth receptor $p75^{NTR}$ (Tuffereau *et al*, 1998) and the neural cell adhesion molecule (NCAM) (Thoulouze *et al*, 1998) have been identified as receptors for street rabies virus and challenge virus standard (CVS) respectively. Some gangliosides (Superti *et al*, 1986), as well as phospholipids and glycolipids (Wunner *et al*, 1984), have been implicated to be rabies virus receptor molecules.

Rhabdovirus attachment, fusion, and uptake have been extensively studied using VSV. This virus was found to enter the host cell via receptor-mediated endocytosis, employing clathrin-coated vesicles (Matlin *et al*, 1982) with maximal binding occurring at acidic pH 6.5 to 6.0 (Fredericksen and Whitt, 1998; Matlin *et al*, 1982). Modification of the ectodomain structure of VSV G subsequently blocked or modified fusion activity (Zhang and Ghosh, 1994; Fredericksen and Whitt, 1996). This pH-dependence of virus-binding correlates well with the pH-induced conformational changes in G structure (Fredericksen and Whitt, 1998) necessary for fusion and subsequent release of RNP into the host cell (Matlin *et al*, 1982). Gaudin and colleagues (1991) have likewise shown that the glycoprotein of rabies virus undergoes conformational changes at acidic pH, a state that appears to be reversible upon adjustment to neutral pH conditions. Further work with rabies G has suggested that a

transient state of the protein may be responsible for viral aggregation at low pH and may be involved in the first steps of viral fusion (Gaudin *et al*, 1993).

Antigenicity of the glycoprotein

The development of hybridoma technology brought with it the ability to characterize the antigenicity of proteins using monoclonal antibodies (mAbs). Wiktor and Koprowski (1978) were the first to employ mAbs to detect antigenic variants of rabies virus, which eventually led to limited epitope mapping of rabies virus glycoproteins. Three antigenic sites were mapped (Lafon *et al*, 1983) using 25 mAbs and 90 Mar (monoclonal antibody resistant) mutants of the CVS rabies strain (Wiktor and Koprowski, 1980). Five antigenic sites were similarly mapped to the ERA rabies virus G (Lafon *et al*, 1984); all of these sites were conformational, requiring secondary structure of the G protein for mAb recognition to occur (Wunner *et al*, 1985a). Analysis of Mar mutants aided the continued discovery of antigenic sites. Site I was identified by a single mAb (Wunner *et al*, 1985b) while site II was comprised of discontinuous epitopes (residues 34 to 42 and 198 to 200) (Wunner *et al*, 1985a) linked by disulfide bonds. Site III was continuous, corresponding to amino acids 330 through 340 (Seif *et al*, 1985) and contains a critical antigenic determinant of virus pathogenicity at residue 333 (Dietzschold *et al*, 1983). Sites IV and V have not been definitively described (Tordo, 1996). Site 6, defined by a linear epitope identified from a mAb (6-15C4) produced against the Pitman-Moore rabies strain, was the first antigenic site found that does not depend on the native conformation of the glycoprotein for binding virus neutralizing antibody (Bunschoten *et al*, 1989). The core of this epitope, which spans residues 254 through 275, was characterized by Pepscan analysis to be an octapeptide, with five of the eight residues required for binding of mAb 6-15C4 (van der Heijden *et al*, 1993). Although studies of serum from vaccinated humans and mice indicate that antibodies to the

site 6 epitope constitute a minor population of the rabies virus-specific serum antibodies induced by rabies vaccination (van der Heijden *et al*, 1993), the discovery of a linear epitope suggested the possibility of generating a synthetic peptide vaccine against rabies.

In 1991, Benmansour *et al* suggested a new system of nomenclature where the term "antigenic site" would be reserved for regions of G defined by several mAbs derived from separate fusions. The majority of anti-G mAbs (97%) tested by Lafon *et al* (1984) belonged to discrete antigenic sites II and III; these would be designated as major antigenic sites, while groups of overlapping epitopes would be considered minor antigenic sites and regions defined by a single MAb would be classified as epitopes (Benmansour *et al*, 1991).

Lindsay Elmgren (1999) explored the complex antigenic nature of rabies glycoprotein using two-way competitive binding assays, which allowed for confidence in epitope placement that could not be attained when employing one-way competition. Lyssavirus genotypes 1 through 6 were employed in these assays, from which four major antigenic sites (OLF I, II, III, and IV) and two sub-sites (OLF Ia and IIIa) of the lyssavirus glycoprotein were identified. In Elmgren's studies, antigenic sites were defined as non-overlapping regions of the glycoprotein, with minor sites designated where overlap occurs and given the suffix "a". Virus neutralization tests using anti-G mAbs demonstrated that only OLF sites I and IIIa have neutralizing properties. Sites I, Ia, and IIIa as described by Elmgren (OLF nomenclature) appeared to correspond to sites I, II and III (respectively) as described by Lafon *et al* (1983, 1984)(Wistar nomenclature). OLF site III corresponded to Wistar site V, while OLF site II may correlate with Wistar site IV. OLF site IV, although defined by a single mAb (M1078), had not been described previously and was thus considered by Elmgren to justify designation as a distinct antigenic site.

Role of glycoprotein in pathogenicity

Amino acid position 333 of rabies G has been shown to be a key determinant of pathogenicity of several tissue culture-adapted strains of rabies, such as ERA, CVS, and Flury HEP (High Egg Passage). An arginine → glutamine mutation at position 333 in antigenic site III rendered the rabies virus variant nonpathogenic in immunocompetent adult mice (Dietzschold *et al*, 1983; Seif *et al*, 1985). Later work concluded that virulence is strongly linked to the maintenance of a positively-charged (basic) residue (either arginine or lysine) at position 333 (Tuffereau *et al*, 1989). However, strains like ERA that are adapted to nonneuronal cell lines *in vitro* are already highly attenuated *in vivo* even though the antigenic site III of G is left unchanged (Lawson *et al*, 1989). This suggests that there are also other factors influencing the pathogenicity phenotype of the virus.

Research has shown that the pathogenicity of rabies virus *in vitro* inversely correlates with the extent of apoptosis and expression of G (Morimoto *et al*, 1999), since the downregulation of G expression in infected neurons is associated with decreased apoptosis and increased pathogenicity. The ability of the virus to induce apoptosis is mostly attributable to G (Prehaud *et al*, 2003) which, when overexpressed, enhances apoptosis and the antiviral immune response (Faber *et al*, 2002). The role of apoptosis in pathogenicity was reinforced by Pulmanousahakul *et al* (2001) when they found that a recombinant rabies virus overexpressing the proapoptotic protein cytochrome *c* was also associated with enhanced immune response and attenuated pathogenicity, a finding that could be valuable in vaccine design.

RABIES DISEASE AND EPIDEMIOLOGY

Rabies is an acute viral encephalomyelitis that is enzootic worldwide, with the exception of some island nations and Antarctica. Although Australia is also considered rabies-free it is home to a rabies-related bat lyssavirus (Fraser *et al*, 1996). Rabies exhibits a broad host range in that all mammals are susceptible to infection to some degree, but a hierarchy of species susceptibility appears to exist (WHO, 1973). For example, foxes are very susceptible (Sikes, 1962), raccoons are moderately susceptible, and opossums seem to be especially resistant to rabies infection (Baer *et al*, 1990). Humans serve as a dead-end host for rabies infection and are most often infected through the bite of a rabid animal. Since the virus is trophic for muscle and nervous tissue, it travels by axonal transport and replicates in the central nervous system (CNS) of the host before spreading to other organs via the peripheral nervous system. For example, the virus travels from the wound to the CNS and upon replication proceeds to the salivary glands from which it is then secreted and transmitted to another animal or human through a virus-laden bite. Although the disease can have both "furious" and "dumb/paralytic" clinical manifestations (the first of which is most prevalent), both result in death by paralysis.

Rabies continues to be endemic throughout the world in domestic and wildlife animal reservoirs. In parts of the world where pet control and vaccination programs are limited in their availability, such as Asia and Africa, dogs remain the most important reservoirs of the virus and are the most frequent vector for transmission to humans (de Mattos *et al*, 2001). In more developed countries where these preventative measures are readily available for domesticated animals, rabies is maintained in wildlife species.

However, the existence of a wide variety of wildlife rabies reservoirs in the Americas represents a more complex situation than that of Europe, where vulpine species are the only

major reservoir of rabies (Dietzschold and Schnell, 2002). In North America, the predominant terrestrial species that maintain the rabies zootic are arctic foxes (*Alopex lagopus*) (Blancou *et al*, 1991), red foxes (*Vulpes vulpes*)(Crandell, 1991), grey foxes (*Urocyon cinereoargenteus*), coyotes (*Canis latrans*), striped skunks (*Mephitis mephitis*) (Charlton *et al*, 1988; Charlton *et al*, 1991), and raccoons (*Procyon lotor*). Generally, the Canadian situation has seen the skunk as the main terrestrial reservoir of rabies in the western prairies region, and the fox in Ontario and arctic regions. It is only recently that raccoon rabies emerged in Canada; it was first reported in July 1999 in Ontario (Rosatte *et al*, 2001) and in September 2000 in New Brunswick (Sibbald, 2001) as a result of movement of raccoon rabies north along the eastern seaboard of the United States (Winkler and Jenkins, 1991).

Bat rabies, belonging to genotype 1 (classical rabies), was first recognized in North America in 1953 (Baer and Smith, 1991); it has been described among several insectivorous bat species of the United States of America (USA) and Canada (de Mattos *et al*, 2001) and the hematophagous bat species (primarily *Desmodus rotundus*) throughout Latin America from Mexico to Argentina (Baer, 1991; Childs *et al*, 1994). In the USA it has been suggested by Smith (1988) that distinct virus types co-circulate with the most commonly reported bat species.

The only two diagnosed human rabies cases that have occurred in Canada within the last 15 years have been attributable to bat rabies: a 9-year old boy in Quebec in 2000 (MMWR, 2000) and a 52-year-old man in British Columbia in 2003 (Parker *et al*, 2003). The most commonly diagnosed species in Canada is the big brown bat (*Eptesicus fuscus*); other species include the little brown bat (*Myotis lucifugus*), the silver-haired bat (*Lasionycteris noctivagans*), the hoary bat (*Lasiurus inereus*), and the red bat (*Lasiurus*

borealis). Each of these bat species is associated with one or more distinct virus variants (Nadin-Davis *et al*, 2001; Tordo *et al*, 1997) that are all epidemiologically quite separate from the viruses associated with terrestrial hosts.

Each terrestrial wildlife host of rabies is associated with one or more rabies variants that are geographically limited in range. Several distinct strains (antigenically and genetically characterized) that circulate and persist in a specific host have the potential to spill over into a broad range of mammalian species (Tordo, 1996; Tordo *et al*, 1998), and this spillover to other hosts may, on rare occasions, lead to the emergence of new viral/host associations, provided the virus acquires sufficient fitness to adapt to its new host (Badrane and Tordo, 2001).

When discussing the variants, it is preferable to use the name of the reservoir species as the adjective; for example, dog-to-dog transmission of disease should be defined as canine rabies, whereas skunk-to-dog transmission of disease should be defined as skunk rabies (Aiello and Mays, 1998). It is important to be able to quickly and accurately identify the strain responsible for disease in a given animal and/or area so that the correct control measures can be applied to prevent further spread (Nadin-Davis *et al*, 2003). This is especially critical when cases of wildlife rabies are found in proximity to rural or urban communities, where they present the greatest threat to humans and domestic animals.

ORAL IMMUNIZATION AND WILDLIFE RABIES

The use of selective population reduction to control wildlife rabies has been practiced for centuries but has not been generally regarded as a humane, cost-effective, or ecologically sound method to control rabies infection (Debbie, 1991; Hanlon *et al*, 1999). Culling of animal populations is rather futile, due primarily to the resilience, reproductive potential, and high habitat carrying capacities of the target hosts, especially *Carnivora* species (Wandeler,

2000). The traditional vaccination of domestic animals by injection with killed virus is not feasible for large-scale immunization of wildlife hosts, although a trap-vaccinate-release (TVR) rabies control effort by the Ontario Ministry of Natural Resources (OMNR) has shown success in controlling skunk and fox rabies in urban Toronto (Rosatte *et al*, 1992). However, the protection of principal terrestrial wildlife reservoirs using oral vaccination has emerged as a more viable approach to rabies control with the development of several rabies oral vaccine candidates over the past half-century. The primary goal of oral vaccination is to establish herd immunity, a phenomenon dependent on a variety of factors including vaccine efficacy, specificity, and uptake of vaccine by animals within the target population (Wandeler, 2000). Since any vaccination is not 100% effective, a small percentage of vaccine recipients will respond poorly and therefore not be adequately protected; however, if the majority of the population is immune to the infectious agent, then the probability of a susceptible animal contacting an infected animal is so low that the susceptible individual is not likely to become infected (Kuby, 1997). Therefore, a reduction in rabies infection of wildlife species through effective oral vaccination programs should also in turn reduce the possibility of exposure of domestic animals and humans to the disease.

In the early 1960's, Baer and colleagues at the US Centers for Disease Control and Prevention found that ERA, a live attenuated rabies virus mentioned earlier in this discussion, was able to induce immunity in red foxes upon oral administration. This finding was later published following a WHO conference in Europe (Baer *et al*, 1971) and supported by others (Black and Lawson, 1970; Black and Lawson, 1973). The first field application of ERA, renamed SAD (Street Alabama Dufferin) for use in Europe, took place in 1978 in the Rhone Valley of Switzerland (Steck *et al*, 1982) and led to a significant part of the country being freed from rabies over the next four years (Kappeler and Wandeler, 2000). This was

the first example of pathogen elimination from a non-domestic animal population by means of vaccination rather than by means of selective population reduction (Artois *et al*, 2001). ERA was later released in Canada in 1985 (Johnston *et al*, 1988; MacInnes *et al*, 1988), and the aerial distribution of this vaccine in baits, in combination with parenteral vaccination of inactivated rabies vaccine in urban areas, led to substantial success in the control of fox rabies in southern Ontario (Rosatte *et al*, 1993; Rosatte *et al*, 1997).

However, it was found that attenuated viruses like SAD/ERA were pathogenic for laboratory and wild rodents (Artois *et al*, 1992) as well as target species such as the striped skunk (Rupprecht *et al*, 1990). Due to this residual pathogenicity, use of these strains for domestic animal vaccination was discontinued (Pastoret *et al*, 1996). Avirulent strains resisting neutralization with anti-SAD mAbs were collected as alternate vaccine candidates; these escape mutants included SAG1 (SAD Avirulent Gif) (Flamand *et al*, 1989), which has a single amino acid substitution in position 333 of the glycoprotein, and SAG2, a double avirulent mutant later selected in the interest of enhancing vaccine safety (Schumacher *et al*, 1993; Lafay *et al*, 1994).

Unfortunately, SAD-derived attenuated rabies strains proved to be inefficient in some rabies vectors, such as raccoons in North America (Rupprecht *et al*, 1986). With the advent of molecular biology and its applications for vaccine design came the development of recombinant virus vectors expressing exogenous protein(s). The first recombinant rabies vaccine constructed was VRG (vaccinia-rabies glycoprotein), a vaccinia vector (Copenhagen strain) expressing rabies glycoprotein (ERA strain) (Kieny *et al*, 1984). Preliminary work showed VRG to induce high VNA titres and protection from intracerebral rabies challenge when administered to mice and rabbits by scarification (Kieny *et al*, 1984; Wiktor *et al*, 1984). Soon after, VRG was used to successfully immunize raccoons (Rupprecht *et al*,

1986; Rupprecht *et al*, 1988) and foxes (Tolson *et al*, 1988a; Brochier *et al*, 1991; Pastoret and Brochier, 1996) by oral administration. The vaccine demonstrated some success in controlling fox rabies in Western Europe (Brochier *et al*, 1995); the first field applications of VRG took place in Belgium in 1988 (Brochier *et al*, 1991) and in France in 1989 (Aubert *et al*, 1994). The first release of VRG in North America occurred in the summer of 1990 on Parramore Island, Virginia, US. The efficacy of this vaccination campaign was assessed in raccoons using biomarker analysis, VNA titre determination, and challenge (Rupprecht *et al*, 1993). A significant finding of this study was that there seems to be no minimal VNA titre at which animal survival after rabies virus exposure can be predicted; it seems that an anamnestic VNA response could be a better predictor of protection, which indicates that other vaccination parameters are integral to inducing an adequate immune response (Rupprecht *et al*, 1993).

VRG is used currently to control rabies in coyotes (Fearneyhough *et al*, 1998), grey foxes (Fearneyhough *et al*, 1996), and raccoons (Robbins *et al*, 1998) in the US. When raccoon rabies entered Ontario in 1999, the OMNR attempted to contain the first 3 cases reported using a point infection control (PIC) strategy (Rosatte *et al*, 2001). In areas of reported raccoon rabies cases, a combination of population reduction and TVR was employed, along with oral rabies vaccination (ORV) with baits; although this did not eliminate raccoon rabies in Ontario, it resulted in a lower prevalence than observed during the epizootic in the US and will continue to be used in response to future outbreaks (Rosatte *et al*, 2001).

VRG has been shown to be apathogenic for both target and non-target species (Brochier *et al*, 1989; Artois *et al*, 1990; Artois *et al*, 1992), an improvement over the attenuated rabies vaccines. Also, in comparison to wild rabies virus, VRG has proven to be

more stable in terms of temperature variation and preservation of viral titre over a period of time under both *in vitro* and field conditions (Pastoret *et al*, 1996). Unfortunately, although an initial study using VRG in skunks showed high rates of immunization (Tolson *et al*, 1987) later trials did not observe similar seroconversion (Charlton *et al*, 1992) and thus the search for a broader host range vaccine continues.

To this end, recombinant adenovirus vectors have been investigated as rabies oral vaccine candidates. Many of the features of adenovirus that make them ideal vectors for gene therapy – stability, wide tropism, capacity for incorporating foreign DNA (deoxyribonucleic acid) molecules, ease of manipulation, and high-level protein expression – also make them ideal candidates for oral rabies vaccination.

ADENOVIRUSES

Classification

The taxonomy of the *Adenoviridae* family of viruses has been recently updated to include four accepted genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus* (Benko *et al*, 2000). The *Aviadenovirus* genus is composed solely of bird viruses, while the *Mastadenovirus* genus consists of viruses from a wide variety of mammalian species including, but not limited to, human, simian, bovine, and canine adenoviruses (Shenk, 1996). The more recently established *Atadenovirus* and *Siadenovirus* genera originate from a broader range of hosts. The atadenoviruses were named as a result of the tendency for these viruses to possess high A+T genomic content (Benko and Harrach, 1998; Both, 2002) and they have been found to infect several ruminant, avian, reptilian, and marsupial hosts. The two known siadenoviruses were isolated from avian and amphibian hosts (Davison and Harrach, 2002). A fifth genus being proposed includes the only

confirmed fish adenovirus (Benko *et al*, 2002). The *Mastadenovirus* type strain human adenovirus type 2 (HAd2) was discovered in the adenoid tissue of a military recruit in 1953 (Enders, 1956), and three years later the term "adenoviruses" was coined.

Approximately fifty-one human adenovirus serotypes have been distinguished based on specific neutralizing ability, which is governed by viral hexon and fiber protein epitopes (Norrby *et al*, 1976). The various serotypes are classified into six subgroups (A through F) based on their hemagglutination properties (Rosen, 1960). Adenovirus type 2 and 5, which belong to subgroup C/hemagglutination group III of human adenoviruses, have been extensively used in structural, replication, and recombination studies. These serotypes have been favoured because they are readily available and easily grown in cell culture. Human adenovirus type 5 (HAd5) will be the primary focus of this thesis.

Structure

The adenovirus virion is a non-enveloped, icosahedral particle consisting of a protein shell surrounding a DNA core. Each capsid is approximately 80-110 nm in diameter and is comprised of 252 capsomeres: 240 hexons and 12 pentons (Russell, 1998). For HAd5 and the majority of adenoviruses, each penton base is associated with a single, knobbed fiber protein that mediates cellular binding of the virus. Trimerization of the fiber is crucial for its attachment to either the capsid or the cellular receptor (Chroboczek *et al*, 1995).

Capsids are assembled with progeny viral genomes in the nucleus to form immature, non-infectious virions that must undergo cleavage by the viral protease (encoded by L3) to become mature, infectious virions (Flint *et al*, 2000).

Genome

The adenovirus genome is composed of linear, double-stranded, non-segmented DNA. Davison and colleagues found deficiencies in the interpretation and annotation of

genomic data in primary publications and proceeded to re-annotate the complete HAd5 genome published by Chroboczek *et al.* (1992); the resulting report is considered to be the most accurate third party annotation of the 35,938 bp (base pair) HAd5 virus genome (Davison *et al*, 2003). The genome is characterized by inverted terminal repeats that are 102 bp long in HAd5 (Davison *et al*, 2003) and the denatured single strands of these regions can form "pan-handle" structures important in DNA replication (Cann, 2001). Also important are the terminal proteins covalently linked to the 5′ ends of the genome, which were first identified by their ability to mediate circularization of the viral DNA through protease-sensitive, non-covalent interaction (Robinson *et al*, 1973).

The virus is heavily dependent on the cellular apparatus for transcription, which along with viral DNA replication and virion assembly exclusively takes place in the host cell nucleus. Transcription is divided into two phases ("early" and "late") that are separated by the onset of viral DNA replication. The five early mRNAs (E1A, E1B, E2, E3, and E4) encode early proteins necessary for viral DNA replication. They regulate the expression of other viral genes (including late proteins), encode the viral DNA binding protein, and facilitate evasion of the host immune response (Russell, 1998). Early gene products also modulate host cell functions, including the induction of cell cycle progression and inhibition of apoptosis (Shenk, 1996). Minor "intermediate" transcripts and major "late" transcripts (L1 through L5) are also produced, the latter of which primarily encode proteins for virion structure and assembly (Russell, 1998).

Clinical Manifestations

In addition to being the leading cause of acute respiratory disease in military recruits (Dingle and Langmuir, 1968), adenoviruses can cause pharyngitis and pneumonia, especially in young children. Ocular and enteric infections can also occur, but are not as prevalent as

respiratory illness. Infection can spread between individuals via aerosols, the fecal-oral route, and in the case of ocular infection, through direct inoculation into the conjunctiva. Most adenovirus infections, many of which are asymptomatic (Levinson and Jawetz, 1998), resolve spontaneously and result in long-lasting serotype-specific immunity. Live, non-attenuated vaccines against serotypes 4 and 7 are available, but exclusively for military health purposes.

ADENOVIRUS VECTORS

There are several aspects of adenoviruses that make them useful vectors; they can infect both dividing and non-dividing cells, are trophic for a wide variety of cell types and tissues, and their genomes can accommodate a significant quantity of foreign DNA (approximately 2 kb) without significant effects on stability (Russell, 2000). Bett and colleagues (1993) observed that inserts resulting in a net genome size of ~105% of that of wild type human adenovirus type 5 remained stable, whereas genomes of slightly larger net size rendered the virus unstable at a rate relative to the size of the insert. Virus variants resulting from this instability appeared to undergo rearrangement to generate smaller genomes, contributing to overall genetic instability of the virus population (Bett *et al*, 1993)

In the case of first generation adenovirus vectors, where the E1 or E3 regions are removed, up to 6.5 kb of foreign DNA can be inserted, often under the control of a heterologous promoter (Russell, 2000). As E1 gene products are essential to virus replication, E1-deleted vectors are deemed replication-defective and require a permissive cell line to provide E1 functions in trans to propagate *in vitro*. On the other hand, the E3 gene is considered non-essential since its deletion does not interfere with virus replication (Imler, 1995), making E3-deleted vectors replication-competent. E3 proteins, such as the adenovirus

death protein (ADP) and gp19K, are accessory proteins that serve to subvert host defense mechanisms (Russell, 2000).

Adenovirus vectors have been extensively used to express foreign genes for gene therapy and vaccination applications. E3-substituted adenovirus vectors have been employed primarily to express immunogenic proteins for vaccination, including but not limited to: hepatitis B virus (HBV) surface/core antigens (Morin *et al*, 1987; Ye *et al*, 1991), respiratory syncytial virus (RSV) F/G proteins (Hsu *et al*, 1992), human immunodeficiency virus (HIV) gag/env proteins (Dewar *et al*, 1989; Prevec *et al*, 1991), simian immunodeficiency virus (SIV) gag protein (Flanagan *et al*, 1997), herpes simplex virus (HSV) gB (Johnson *et al*, 1988), and bovine herpes simplex virus (BHV) gD (Papp *et al*, 1997).

Although human adenoviruses have a restricted host range, it appears from the success of immunization in non-permissive and semi-permissive animal species that antigen levels, sufficient to induce detectable humoral responses, are produced in the absence of viral replication (Imler, 1995). For example, good VNA titres against VSV G were obtained using the same immunization protocol in both permissive and non-permissive species with a replication-competent adenovirus type 5 expression vector (Prevec *et al*, 1989).

AdRG1, the first human adenovirus-rabies recombinant virus, was developed by Ludvik Prevec and colleagues at McMaster University (Hamilton, Ontario, Canada) (Prevec *et al*, 1990). The construct includes the rabies glycoprotein gene (ERA strain), flanked by an SV40 (Simian virus 40) immediate early promoter and polyadenylation signal, inserted within an E3 gene deletion in a human adenovirus type 5 virus vector. This vaccine elicited good VNA tires by either the parenteral or oronasal route in dogs and mice, the latter of which were protected from lethal intracerebral rabies challenge (Prevec *et al*, 1990). Oral vaccination of striped skunks and red foxes elicited high rates of seroconversion and survival

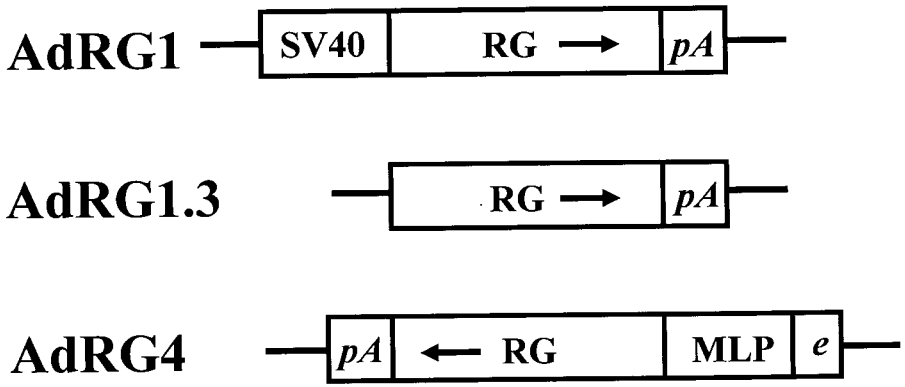
of all challenged animals following immunization was observed (Charlton *et al*, 1992). Vaccine administered directly into the oral cavity (DIOC) was effective over a broad range of doses, which suggests that the high titre of adenovirus produced *in vitro* for insertion into wildlife baits will compensate for expected losses in efficacy in the field (Charlton *et al*, 1992).

Further research on AdRG1 employed the polymerase chain reaction (PCR) and restriction enzyme analysis (REA) to assess the genetic stability of AdRG1 upon the tenth and twentieth passage in 293 cell culture (Lutze-Wallace *et al*, 1995a). The integrity of the heterologous glycoprotein insert was maintained upon *in vitro* passage. Similar analysis of AdRG1 isolated from the feces and saliva of skunks infected with the recombinant virus found two potential mutations in the SV40 region associated with the glycoprotein insert (Lutze-Wallace *et al*, 1995b).

As previously indicated, high doses of vector are required for effective oral immunization under field conditions. Assuming that the magnitude of an animal's immune response correlates with the level of glycoprotein expression generated by the recombinant vector, Yarosh and colleagues (1996) examined ways to facilitate increased gene expression from adenovirus vectors. Two recombinant viruses were generated based on the construction of the AdRG1 vaccine: AdRG1.3 and AdRG4; a schematic comparison of these constructs is detailed in Figure 1. AdRG1.3 retained the SV40 polyadenylation sequence but the SV40 promoter was removed and the translation initiation sequence was modified in an attempt to improve translation efficiency. In this case, expression of the glycoprotein was primarily driven from the HAd5 E3 promoter. In AdRG4 the polyadenylation sequence was also retained and the SV40 promoter was replaced with a different exogenous element, the HAd2 major late promoter (MLP). It is important to note that the direction of transcription of

FIGURE 1: **Schematic of RG and accompanying exogenous elements, inserted into deletions within the E3 region of human Ad5**. The details of AdRGl construction have been previously described (Prevec *et al*, 1990). In AdRGl, the RG (rabies glycoprotein) gene is accompanied by SV40 immediate early promoter and polyA addition signal while in AdRG1.3 the SV40 promoter has been deleted. Both AdRGl and AdRG1.3 have the RG sequences in parallel to the transcription direction of Ad5 E3 and MLP transcripts. In AdRG4, the RG gene is accompanied on the upstream side by a modified HAd2 MLP containing three spliced leaders and an intron sequence as well as upstream promoter-enhancer elements (*e*) from the SV40 promoter, and on the downstream side by the SV40 polyA addition signal. AdRG4 is oriented so that the RG gene is antiparallel to the host HAd5 E3 and MLP transcripts. The exogenous sequences in AdRGl and AdRG4 are placed within an *Xbal* deletion (nucleotides 28592-30470) of HAd5 E3 while AdRG1 .3 contains its insert between two *BglII* sites in the HAd5 E3 region (nucleotides 28133-30818).

Figure used with permission from Dr. L. Prevec (Yarosh *et al*, 1996).


AdRG1
SV40
RG
pA
AdRG1.3
RG
pA
AdRG4
pA
RG
MLP
e

AdRG4 is anti-parallel to that of the AdRG1 and AdRG1.3 constructs.

Of the three vectors tested, AdRG1.3 was the most effective in terms of levels of antibody induced at a specified dose as well as the number of animals responding at lower doses (Yarosh *et al*, 1996). It produced the greatest expression of G *in vitro* and elicited the highest level of VNA in skunks upon oral administration. These findings indicate that the AdRG1.3 vaccine could be a valuable alternative to VRG, and a suitable vaccine for animals not protected by the currently licensed vaccines, namely the striped skunk (Wandeler, 1991). However, before this recombinant vaccine can be licensed for widespread use the safety and stability of AdRG1.3 must be ascertained.

PRE-EXISTING IMMUNITY TO ADENOVIRUS

The majority of human adenovirus serotypes are nonpathogenic in animals, and animal adenoviruses appear to be only pathogenic within their species of origin (Taylor, 1977). Although human and animal adenoviruses have demonstrated a relatively restricted host range and do not, or weakly, replicate in other species (Betts *et al*, 1962; Prevec *et al*, 1989; Oualikene *et al*, 1994; Horwitz, 1996), it is reasonable to question whether pre-existing anti-adenovirus antibodies in the target population will represent a barrier to immunization with a recombinant adenovirus vaccine.

Canine adenovirus type 1 (CAV-1), which has been identified as a natural pathogen in red foxes (Green, 1925) and has been isolated from other target species such as coyotes (Gier *et al*, 1978) and striped skunks (Karstad *et al*, 1975), seems to be the only significant animal adenovirus causing disease in North American wildlife species. It is therefore expected that many of these animals will have developed VNA against CAV. Serological surveys have reported varying levels of CAV exposure in raccoons (6-16%) (Parker *et al*, 1961; Jamison *et al*, 1973; Bolin *et al*, 1982; Sumner *et al*, 1988; Hable *et al*, 1992) and

skunks (32-63%) (Alexander *et al*, 1972), while higher seroprevalance suggests CAV-1 is enzootic in wolves (13-95%) (Choquette and Kuyt, 1974; Stephenson *et al*, 1982; Zarnke and Bullard, 1987), coyotes (33-100%, age-dependent) (Trainer and Knowlton, 1968; Davidson *et al*, 1992; Cypher *et al*, 1988; Gese *et al*, 1997), red foxes (4-100%) (Davidson *et al*, 1992; Truyen *et al*, 1998) and grey foxes (56%) (Davidson *et al*, 1992).

However, it was recently reported that pre-existing immunity to CAV in foxes did not impair the ability of a HAd5 vector expressing rabies G (ERA strain) to elicit an anti-rabies immune response (Vos *et al*, 2001). Moreover, they demonstrated that maternal immunity to rabies does not affect the immune response to rabies G in fox cubs when given the HAd5 recombinant vaccine (Vos *et al*., 2001).

Another aspect to consider is the prevalence of anti-HAd5 antibodies in the target animal species. Serological studies of primates (including humans) provide evidence that an interchange of organisms between humans and animals can occur (Kalter *et al*, 1967). Despite the lack of proof for the existence of human adenoviruses in animals, serological evidence has shown that a small fraction of dogs develop antibody to HAd (Carmichael and Barnes, 1961; Carmichael and Barnes, 1962; Lundgren *et al*, 1969; Winters, 1979). As well, Maxwell (1999) determined, using cELISA (competitive enzyme-linked immunosorbent assay), that 8-25% (depending on the inhibition cutoff) of field raccoons had antibody against an epitope on HAd5. The animal sera were unable to neutralize Ad5, suggesting that although the raccoons were not exposed to the HAd5 serotype they may have been exposed to other human or mammalian adenoviruses that share a common epitope with HAd5 (Maxwell, 1999). However, this may not be problematic as a recent study in mice indicates that oral vaccination with HAd5 vectors expressing rabies G is not impaired by pre-existing immunity to the vaccine carrier (Xiang *et al*, 2003). Similarly, Babiuk and Tikoo (2000)

recently showed that seropositive cattle can be immunized with a bovine adenovirus vector. They propose that the levels of mucosal immunity are not sustained like those of serum antibody levels, thus allowing the infection of mucosal cells of the upper respiratory tract in the presence of high serum antibody levels; upon viral entry into these cells, levels of antigen sufficient to induce an immune response in the host can be produced (Babiuk and Tikoo, 2000).

COTTON RAT MODEL

The cotton rat, reportedly the most abundant rodent in the southern United States as well as parts of Mexico and Latin America (Walker, 1975), has been investigated as a model for biomedical research for over sixty years. There are seven species of cotton rats, the most common being *Sigmodon hispidus*, which also has the largest geographical distribution.

Among the first publications discussing the cotton rat as a disease model was a report by Dr. Charles Armstrong from the National Institutes of Health regarding the rodent's susceptibility to paralytic poliomyelitis (Armstrong, 1939). This led to many significant advances in polio research over the next forty years. Around this time, a project in Great Britain under the code-name "Operation Tyburn" used cotton rats in developing a vaccine against endemic typhus (causative agent *Rickettsia tsutsumagushi*), which decimated British military troops in Asia during World War II (Buckland *et al*, 1945). Although this research was halted at the end of the war, the cotton rat continued to be an important animal model for several respiratory diseases caused by paramyxoviruses. In 1970, susceptibility of the cotton rat to RSV infection was first reported (Prince, 1994) and has since been used to investigate the pathology and immunology of RSV disease (Murphy *et al*, 1990; Piazza *et al*, 1995; Piedra *et al*, 1993;). It has also been employed as a model of parainfluenza virus type 3

(Murphy *et al*, 1981; Porter *et al*, 1991; Hall *et al*, 1991) and measles virus (Wyde *et al*, 1992; Niewiesk, 1999).

In 1984, Pacini and colleagues described the pathogenesis of human adenoviruses in the cotton rat, reporting the animal to be susceptible to at least four human adenovirus serotypes (1,2,5, and 6). To date, cotton rats are the only small animals known to be susceptible to human adenoviruses. It has been observed that in cotton rat infections, the magnitude of viral yield and disease is directly proportional to the viral input dose, allowing for a "made-to-order" severity of disease (Prince *et al*, 1993). Although this finding indicates that HAd5 is not fully adapted to cotton rats (Ginsberg and Prince, 1994), it does add to the utility of the cotton rat as a laboratory model of disease since the severity of disease can be controlled experimentally.

RATIONALE and STATEMENT OF OBJECTIVES

Rabies, most likely the oldest recorded infection of mankind (Fu, 1997), continues to be a significant threat to human and animal health throughout much of the world despite many advances in diagnosis, post-exposure treatment, and control measures (Tordo *et al*, 1997). Compared to the less developed world where the incidence of fatal human rabies cases is high and most often attributable to canine rabies, more developed nations like Europe and North America experience few human rabies fatalities, largely due to effective immunization programs for domestic animals and access to adequate post-exposure prophylaxis for humans. However, in these nations the rabies zootic is maintained in wildlife species. Oral immunization strategies have been successful in controlling rabies infection in some wildlife reservoirs of the disease, such as foxes in Ontario and Europe and coyotes in Texas. Adenoviruses are one of several viral vectors that have been constructed for use as oral rabies vaccines in animals. Of significant interest is AdRG1.3, an adenovirus-rabies glycoprotein recombinant vaccine that has been proven experimentally to induce high levels of rabies virus-neutralizing antibody in skunks, a host species that had previously been refractory to vaccination by the oral route with other vaccine candidates. Under *in vitro* conditions, this recombinant vaccine exhibits detectable expression of rabies glycoprotein in non-permissive cell culture, which confirms the feasibility of vaccinating animals that are generally regarded as non-permissive. These findings make AdRG1.3 a potentially broader host range vaccine than the vaccines currently licensed for control of wildlife rabies.

Besides efficacy, the safety and stability of a vaccine must be established for licensing purposes. To this end, the major objective of this thesis is to assess the genetic stability of the rabies glycoprotein insert of AdRG1.3 under both *in vitro* and *in vivo* conditions. *In vitro* stability studies will be carried out in permissive cell culture while novel

in vivo studies will make use of the cotton rat model of adenovirus infection; clones collected from each of these studies will be analyzed by nucleotide sequencing of the entire glycoprotein insert.

HYPOTHESIS

An adenovirus vector, being a DNA virus, is much less prone to mutation than an RNA virus. In addition, the size of the recombinant virus falls within the range shown previously to be stable. Therefore, it is predicted that the rabies glycoprotein expression cassette of the AdRG1.3 vaccine construct will prove to be genetically stable upon *in vitro* and *in vivo* passage.

MATERIALS AND METHODS

CELL CULTURE

A 293 human embryonic kidney cell line transformed with the left-hand portion of the HAd5 wild type genome (Graham *et al*, 1977) was originally obtained from Dr. L. Prevec (McMaster University; Hamilton, Ontario). The cells were routinely grown in 75 cm^2 vented cap polystyrene Falcon™ tissue culture flasks (Becton Dickinson (BD) Biosciences; Mississauga, ON) using Eagle's minimum essential medium (MEM) (Invitrogen Life Technologies; Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL; Burlington, ON) at 37°C and 5% carbon dioxide (CO_2).

The cell line was routinely passaged once per week at a split ratio of 1:4. The monolayers were rinsed twice with trypsin-EDTA (ethylene diaminetetraacetic acid) (Gibco-BRL), rocking the flasks to ensure even distribution of the solution. The monolayers were then incubated at 37°C and 5% CO_2 with 1 ml of trypsin-EDTA for 3-5 minutes. Upon trypsinization, the cells were resuspended in fresh MEM (10% FBS) and divided into new 75 cm^2 flasks according to the split ratio, which was dependent on how soon the formation of complete monolayers was needed. A 20 ml volume of fresh MEM (10% FBS) was then added to each flask, carefully pipetting to evenly mix the cells and media. The seeded flasks were then incubated at 37°C and 5% CO_2.

VIRUSES

AdRG1.3 (HAV5RG1.3) 2001-01B was originally received from Artemis Technologies, Inc. (Guelph, ON), with original vaccine seed stock produced by Microbix Biosystems, Inc. (Toronto, ON) in a collaborative agreement with OMNR. HAV5wt #127 Lot 15 was produced by the Rabies Centre of Expertise (C of E) at the Animal Diseases Research Institute (ADRI) (Nepean, ON) from virus originally obtained from Dr. L. Prevec (McMaster University; Hamilton, ON).

ANTIBODIES

All anti-rabies glycoprotein monoclonal antibodies used in this study were taken from a collection of anti-lyssavirus monoclonal antibodies supplied to the Rabies C of E by the Monoclonal Antibody Unit, both part of the ADRI, Canadian Food Inspection Agency (CFIA) (Table 1). The Monoclonal Antibody Unit is responsible for maintaining hybridomas and supplying mAb supernatant to the Rabies C of E. The hybridoma cell lines were originally produced at the University of Bern (Switzerland) or at ADRI by, or under the direction of, Dr. A. Wandeler. Hybridoma secreted mAbs, stored at –20°C, were used as cell culture supernatants.

The anti-HAd5 murine mAb 7G1-8-10 was produced by Mrs. J. Armstrong and Dr. A. Wandeler of the Rabies C of E (CFIA). It is directed against the fiber portion of the adenovirus capsid, neutralizes HAd5, and displays nuclear staining in immunofluorescent assays. Anti-adenovirus rabbit serum (1189R) was obtained from a rabbit immunized (at day 0 and day 30) sub scapular with cesium chloride (CsCl) gradient purified human adenovirus type 5/complete Freunds mixture. This was performed by Mrs. J. Armstrong and Dr. A. Wandeler of the Rabies C of E (CFIA). Fluorescein-5-isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) is commercially available from MP Biomedical (Irvine, CA).

PREPARATION OF VIRUS STOCKS

In cell cultures infected with adenovirus, it is characteristic for low amounts of virus particles to be released into cell culture supernatant; therefore, in order to produce high titered viral suspensions, homogenates of virus-infected cells were prepared.
To obtain a high titre viral stock of AdRG1.3, 100 ml of trypsinized 293 cells ($10^{6.1}$ cells/ml) were infected with AdRG1.3 2001-01B at a multiplicity of infection (moi) of 0.1. (for

Table 1: Monoclonal antibodies used for detection of rabies glycoprotein expression

mAb	Antigenic site (OLF)*	Antigenic source	Neutralizing ability	Linear vs. Conformational epitope
10EC9	I	SAD[1] (whole cell)	Yes	Linear
M725	I	ERA[2] (ARG virus)[3]	Yes	Conformational
M818	I	ERA (ARG virus)	Yes	Linear
16EH11	Ia	HEP[4] (whole cell)	No	Conformational
M1089	Ia	FOX[5] (ARG virus)	No	Conformational
M778	II	ERA (ARG virus)	No	Conformational
M1094	II	FOX (ARG virus)	No	Conformational
10ED8	III	SAD (whole cell)	No	Conformational
M1100	III	FOX (ARG virus)	No	Conformational
M724	IIIa	ERA (ERA virus)	Yes	Conformational
16AH8	IIIa	HEP (whole cell)	Yes	Conformational
M785	IIIa	ERA (ARG virus)	Yes	Conformational
M1078	IV	FOX (ARG virus)	No	Conformational

[1] Street Alabama Dufferin rabies vaccine strain

[2] Evelyn Rokiniki Abelseth rabies vaccine strain

[3] Adenovirus Rabies Glycoprotein recombinant

[4] High Egg Passage rabies vaccine strain

[5] Fox rabies isolate from southern Ontario

* Ottawa Laboratory Fallowfield nomenclature

calculation of moi, see Appendix A-3). Virus was absorbed for 1 hour at 37°C, followed by the addition of MEM. At 4 days post infection (dpi), 100% of the cell monolayer was infected. The infected cells were lysed with a Dounce homogenizer (25 strokes with the tightest-fitting pestle) then centrifuged at 1500 rpm (revolutions per minute) (514 g) for 15 minutes (GH-3.8 rotor; Beckman Coulter Allegra® 6R refrigerated centrifuge). The 86 ml of viral supernatant recovered was dispensed into 1 ml aliquots and frozen at -70°C until use. (ADRI Lot#301, HAV5RG1.3 2001-01B, lot 1, p1, 2001-12-14).

This same procedure was used to produce 20 ml of high titre viral stock of HAV5wt (ADRI Lot#304, HAV5wt, lot 15, p4, 2002-02-27).

DETERMINATION OF VIRUS TITRES

Cell suspensions of 293 cells in flat-bottomed polystyrene Nunclon™ 96 well plates (Nalge Nunc International; Rochester, NY) seeded at $4x10^5$ cells/ml, 100 microlitre (μl)/well, were infected with 100 μl of virus that had been serially diluted tenfold (10^{-1},10^{-2}, or 10^{-3} through 10^{-8},10^{-9}, or 10^{-10}) in MEM supplemented with 10% FBS and 1% antibiotic/antimycotic (AbAm)(100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) obtained from the Sigma Chemical Company (St. Louis, MO). The plates were then incubated at 37°C and 5% CO_2.

Titration via. endpoint dilution determination. When virus titre was determined using cytopathic effect (CPE) indicative of adenovirus infection to establish the endpoint dilution, the plates were incubated for 7 to12 days. Using an inverted tissue culture microscope, the wells were checked periodically and scored as either positive or negative for viral CPE.

Titration via indirect immunofluorescence. When virus titre was determined by using indirect immunofluorescence (IF), the plates were incubated for 4 days, at which time the monolayers were fixed with cold acetone (75% v/v). The fixed monolayers were then

incubated for 1 hour at 37°C with 100 µl/well of anti-HAd5 murine mAb 7G1-8-10 diluted 1:10 in fluorescent antibody (FA) buffer. FA buffer was comprised of 50 mM Tris (hydroxymethyl) amino ethane, 200 mM sodium chloride, and 0.5% Tween 20 (polyoxyethylenesorbitan monolaurate) dissolved in tissue culture grade water and adjusted to pH 7.2 to 7.4. Prior to application, the mAb was centrifuged at 1500 rpm (514 g) for 8 minutes (GH-3.8 rotor; Beckman Coulter Allegra® 6R refrigerated centrifuge). The plates were rinsed with FA buffer, then incubated for 1 hour at 37°C with 100 µl/well of goat anti-mouse FITC conjugate antibody diluted 1:1500 in FA buffer. Prior to application, the conjugate antibody was centrifuged at 10,000 rpm (7796 g) for 10 minutes (SS-34 rotor, Sorvall® RC-5B refrigerated centrifuge; Du Pont Canada, Inc.). Plates were rinsed with FA buffer before counterstaining for 1 minute with Evan's Blue dye (Sigma), diluted 1:200 in FA buffer. Plates were examined using fluorescence microscopy (Leica DM IL), and wells were scored as either positive or negative for adenovirus infection.

Calculations. Virus titres were calculated using the Spearman-Karber method (Lorenz and Bogel, 1973)(see Appendix A-1). The $TCID_{50}$ (median tissue culture infectious dose) value of a virus suspension represents the number of virus particles required to infect 50% of cells in cell culture. The FA_{50} value of a virus suspension signifies the $TCID_{50}$ value determined by the fluorescence assay. All titrations were performed in triplicate (unless otherwise indicated) along with a control virus lot of AdRG1.3 adjusted to $10^{8.0}$ $TCID_{50}$/ml. A geometric mean titre (GMT) was then calculated from these titrations (see Appendix A-2).

AdRG1.3 ONE-STEP GROWTH CURVE

25 cm^2 tissue culture flasks (BD Biosciences) were seeded with 5 ml of 293 cells suspended at $4x10^5$ cells/ml in MEM supplemented with 10% FBS. The flasks were incubated at 37°C and 5% CO_2 until monolayers were formed (approximately 3 days). A cell

count was performed on the contents of one T25 flask using a Neubauer hemocytometer. AdRG1.3 virus stock was diluted in MEM to infect fresh 293 monolayers at a moi of 2. Existing media was poured off from the 293 monolayers before 0.5 ml of diluted virus was added. Cultures were incubated at 37°C and 5% CO_2 for 1 hour with periodic rocking to ensure even adsorption of virus onto the monolayer. The inoculum was then poured off and Earle's balanced salt solution (Gibco-Invitrogen) was used to rinse the monolayers of any residual inoculum. 5 ml of maintenance media (MEM supplemented with 2% FBS and 1% Ab/Am) was added to each flask, and the infected monolayers were incubated at 37°C and 5% CO_2 until sampling.

From each time point, two samples were obtained to determine amount of: a) extracellular virus, and b) intracellular virus. Infected cell culture supernatant was collected and frozen at -70°C. The monolayers were then rinsed with Dulbecco's balanced salt solution (Gibco-Invitrogen) prior to the addition of 0.5 ml of fresh MEM, and stored at -70°C. Extracellular virus was titrated from the infected cell supernatant while intracellular virus was titrated from the frozen monolayers after thawing, resuspension, and sonication.

Sonication of infected cells. The model 450 Sonifer sonicator (Branson Ultrasonics Corporation; Danbury, CT) was used to liberate intracellular virus from the samples used to construct the AdRG1.3 growth curve. The AdRG1.3-infected 293 monolayers collected from each time point were removed from storage at –70°C, thawed, shaken, and transferred to 5 ml round-bottom polystyrene tubes (BD Falcon). These thin-walled tubes were used in order to limit temperature rise, and their small diameter raised the height of the liquid to be sonicated; this permitted a greater surface area of the liquid to be exposed to the cooling bath for more effective heat transfer and thereby limited viral and genetic degradation within the samples. Using a microtip, 2 ml of each sample was sonicated by employing four 20-second

pulses (duty cycle set at 15%), with a 20-second pause between each pulse to prevent heating of the samples. A period of 15 minutes was allowed after each sample to allow the settling of any aerosols that may have been generated during sonication. The probe was sterilized for 20 seconds with ethanol (70% v/v) prior to the sonication of each subsequent sample. Sonicated samples were frozen at -70°C until titration.

DETECTION OF RABIES GLYCOPROTEIN (IN VITRO)

Fresh, confluent 293 monolayers were trypsinized and resuspended in MEM (supplemented with 10% FBS and 1% Ab/Am) at a concentration of $4x10^5$ cells/ml. Cells were infected in suspension with AdRG1.3 (ADRI Lot #301) that had been previously diluted 10^{-6} in MEM (10% FBS, 1% Ab/Am), pipetting gently to mix. 96-well Nunc tissue culture plates were seeded with 200 µl of this mixture and incubated at 37°C and 5% CO_2 until fixed with 100% cold methanol at specific time points (9,12, 15, 18, 20, 24, 36, 48, 60, 72, 84, and 96 hours post-infection). Fixed plates were stored at 4°C until evaluation by either indirect IF or the double immunostain assay.

Double Immunostain Assay. The purpose of this assay was to simultaneously detect the expression of rabies G and HAd5 proteins in fixed, infected cell culture monolayers. Fixed monolayers in 96-well tissue culture plates (Nunc) were rehydrated with 100 mM Tris HCl buffer pH 7.6 prior to the blocking step, in which 2 drops per well of heat inactivated normal goat serum (10% v/v) (Kirkegaard and Perry Laboratories (KPL); Gaithersburg, MD) were applied for 45 minutes. [Note that: a) between each step of this assay the monolayers were rinsed three times with 100 mM Tris HCl buffer pH 7.6, then vacuumed, and b) during each reagent application, the plates were rocked to ensure even coverage of the monolayers in the wells.] Polyclonal anti-adenovirus rabbit serum and anti-G murine mAb M818 were diluted into 100 mM Tris HCl buffer pH 7.6 (1/1000 and 1/50 dilutions, respectively); equal

volumes of this mixture were applied to the wells for 1 hour at 37°C. Next, 2 drops per well of biotinylated goat anti-mouse IgG (KPL) were applied for 1 hour at 37°C. 2 drops per well of streptavidin-horseradish peroxidase (HRP) (KPL) were then measured out; into this volume, alkaline phosphatase (AP) labeled anti-rabbit antibody (KPL) was added at a 1/500 dilution. Equal volumes of this mixture were applied to the wells for 45 minutes.

To visualize antigen detection by colorometric means, the DAB (3,3'-diaminobenzidine) reagent set (KPL) and HistoMark® RED test system (KPL) were used as described by the manufacturer. DAB deposited a brownish-gold specific stain in the presence of HRP, which therefore indicated G expression in the monolayer. HistoMark® RED deposited a red specific stain in the presence of AP, which therefore indicated the presence of HAd5 antigen. Stained monolayers were rinsed twice with Tris HCl buffer pH 7.6 and dried before adding glycerol to the wells for visualization under an inverted tissue culture microscope (Olympus CK2).

Indirect Immunofluorescence. Fixed monolayers in 96-well Nunc tissue culture plates were rehydrated with FA buffer prior to incubation with 100 µl/well of murine anti-G mAb for a minimum of 50 minutes at 37°C. A panel of 11 mAbs were employed (Table 1), with 2 wells per plate being stained with each particular mAb. The plates were rinsed 3 times with FA buffer before incubation with 100 µl/well of goat anti-mouse FITC conjugate antibody for a minimum of 40 minutes at 37°C. Prior to application, the conjugate antibody was centrifuged at 10,000 rpm (7796 g) for 10 minutes (SS-34 rotor; Sorvall® RC-5B refrigerated centrifuge). Plates were rinsed with FA buffer before counterstaining for 1 minute with Evan's Blue dye (diluted 1:200 in FA buffer). Plates were examined using fluorescence microscopy (Lietz DM IL) and wells were scored as either positive or negative for rabies glycoprotein.

IN VITRO PASSAGE OF ADRG1.3

Fresh confluent 293 monolayers were trypsinized, adjusted to $4x10^5$ cells/ml, and suspended in MEM (supplemented with 10% FBS and 1% Ab/Am). In a T25 vented tissue culture flask, the cells were infected in suspension with 1 ml of AdRG1.3 (ADRI Lot #301) at a moi of 0.1 and pipetted gently to mix. The infected cells were incubated at 37°C and 5% CO_2 for 4 days, after which virus was harvested using two freeze/thaw cycles followed by Dounce homogenization (25 strokes with the tightest-fitting pestle). The homogenized mixture was centrifuged at 1500 rpm (514 g) for 15 minutes (GH-3.8 rotor; Beckman Coulter Allegra® 6R refrigerated centrifuge), and the clarified cell culture supernatant obtained was frozen at –70°C. 300 µl of the supernatant was titrated by indirect immunofluorescence in order to reduce the time period between serial passages. Each serial passage was performed in the same manner, with the AdRG1.3 inoculum adjusted to infect cells with 1 ml of virus at a consistent moi of 0.1 every passage. Virus titrations were also done using the endpoint dilution method in order to confirm the titre calculated from indirect IF. A total of 20 serial passages of AdRG1.3 in cell culture were performed.

IN VIVO PASSAGE OF ADRG1.3

Animal requirements. A total of 57 cotton rats (*Sigmodon hispidus*), approximately 3 to 4 weeks of age, were obtained from the animal colony at the ADRI (Nepean, ON). Prior to inoculation, animals were housed in groups. Water and feed pellets were freely provided to meet dietary requirements. Shelters of polyvinylchloride tubing were provided as part of the environmental enrichment strategy in place in the animal colony, and the nesting material consisted of wood shavings. With the exception of group housing, the same conditions were used to house the animals post-inoculation. Once infected, the cotton rats were housed separately to prevent cross-contamination between individual animals.

Preparation of inoculum. For the initial passage of AdRG1.3 in cotton rats (p1), the virus was adjusted to $10^{7.00}$ $TCID_{50}$/ml by diluting in Dulbecco's balanced salt solution. Antibiotics were not included in the inoculum preparation in case of toxicity and/or adverse effects on normal flora present in the rodents, while MEM was not used for dilution in the event that foreign bovine proteins would prompt an immunogenic reaction in the cotton rats. The animals were sedated with isoflurane prior to intranasal inoculation and allowed a period of recovery before each subsequent induction.

Processing of animal tissue. Euthanasia was carried out in a chamber by carbon dioxide overdose. Necropsy of the animals took place in a biological safety cabinet (BSL-3) within the animal colony. A separate set of dissection tools (forceps, scissors, scalpel) was used for each animal to prevent cross-contamination. The tissues were harvested sequentially from the least likely to the most likely infected with adenovirus: brain, kidney, liver, spleen, small intestine, and lungs. Only the lung tissues were used in this study; the remaining tissues were collected for other studies not related to this thesis. All tissues were frozen in 1.8 ml screw-cap cryovials (Nunc) except for the lungs. The gross pathology of the lungs was examined and any lesions observed were noted prior to processing of the tissue. The lungs were homogenized with a mortar and pestle using white quartz sand (silicon dioxide, 50-70 mesh)(Sigma-Aldrich) and made into a 10% m/v suspension with Earle's balanced salt solution (Gibco-Invitrogen).

Retrieval of virus. The lung homogenates were spun down in 15 ml conical polypropylene centrifuge tubes (BD Falcon) at 3500 rpm (2800 g) for 10 minutes at 4°C (GH-3.8 rotor; Beckman Coulter Allegra 6 refrigerated centrifuge). The supernatants were dispensed into 1 ml aliquots and frozen at -70°C. Aliquots destined for virus titration and subsequent passage

in cotton rats were filtered using a Millex-GV 0.22 μm syringe-tip filter (Millipore; Billerica, MA).

Virus purification using hydrocarbon. 800 μl of filtered viral supernatant recovered from the lung homogenate suspension from cotton rat A5 was used to evaluate the effect of Arcton 113 (1,1,2 –trichloro -1,2,2-trifluoroethane) (Sigma-Aldrich) on viral infectivity. 400 μl of Arcton 113 was added to each of two 1.5 ml locked-cap centrifuge tubes (Eppendorf; Mississauga, ON) containing 400 μl of virus supernatant (1:1 ratio). The tubes were mixed by inverting several times and placed on ice for a specified period of time: tube 1 (20 minutes) and tube 2 (40 minutes). The tubes were then spun in a refrigerated microfuge (rotor #1378, Baxter Canlab Biofuge A) at 6000 rpm (approximately 4000 g) for 10 minutes. This allowed separation of the mixture into an upper aqueous phase (containing virus) and lower organic phase (containing the fluorocarbon). The aqueous phase was removed and frozen at –70°C for analysis, while the organic phase was decontaminated with Virkon™ (4% m/v)(Antec International; Sudbury, UK) and disposed of using appropriate measures.

ISOLATION OF ADRG1.3 CLONES

Limiting Dilution Technique. In order to obtain discrete virus clones from the viral samples obtained in the study, the limiting dilution technique was used. 293 cells suspended at $4x10^5$ cells/ml were seeded at 100 μl/well in 96-well plates. Previously titrated *in vitro* p20 and *in vivo* p5 AdRG1.3 virus samples were diluted in MEM supplemented with 10% FBS and 1% Ab/Am to achieve approximately 1 plaque per well. The seeded 293 cells were then infected with 100 μl/well of the diluted virus and incubated at 37°C and 5% CO_2. The plates were checked daily for CPE, and wells exhibiting greater than one plaque/foci (or none) were eliminated from the study. Viral infection in wells containing a single plaque

was allowed to progress 10 to 12 days before collection of the virus clones (approximately 150 μl of virus suspension).

***In vitro* amplification of clones.** These suspensions were infected in cell culture to acquire a larger volume of each in order to successfully perform DNA extraction and possibly the double immunostain. 293 cells suspended at $4x10^5$ cells/ml in MEM supplemented with 10% FBS and 1% Ab/Am were aliquotted into 5 ml capped polystyrene tubes (BD Biosciences) at 1 ml/tube. The complete volume of each virus clone obtained from limiting dilution was added to a tube and vortexed, and the contents of each tube was seeded into the corresponding well of a 24-well tissue culture plate (BD Falcon). The plates were then incubated at 37°C and 5% CO_2 for 5 to 6 days, at which time the clones were harvested as infected cell suspensions and frozen at -70°C.

MOLECULAR ANALYSIS

DNA extraction. In a 1.5 ml capped microcentrifuge tube (Eppendorf), 200 μl of the virus clone suspension was added to 800 μL of 6 M (molar) guanidine isothiocyanate (GITC) (Invitrogen). To each tube, 20 μl of a 50% suspension (w/v) (in sterile milli-Q water) of acid-washed glass beads (≤106 μM) (Sigma) was added. The tubes were inverted occasionally over a period of 10 minutes. The glass beads were allowed to settle by gravity, after which the supernatant was removed and beads were washed twice with 1 ml cold ethanol (70% v/v). The beads were dried in a speedvac for 15 minutes, suspended in 30 μl of 0.1x TE (Tris-EDTA) buffer pH 8.0, and incubated at 65°C for 10 minutes to elute DNA from the beads. 5 μl of the elution was set aside for PCR amplification, and both this aliquot and the remaining elution were frozen at -70°C.

PCR amplification of the rabies G expression cassette. PCR was used to amplify the rabies G gene insert and flanking adenovirus sequences in DNA extracted from *in vitro* and

in vivo AdRG1.3 passage samples. All PCR primers used in this study were provided by Dr. Susan Nadin-Davis and are outlined in Table 2. Primer AdRG1.3A targeted 5′ sequences upstream of the adeno-rabies G junction while the ARG-rev1/2 oligonucleotides targeted 3' sequences downstream of the adeno-rabies G junction. When primer AdRG1.3A was used in combination with either ARG-rev1 or ARG-rev2, it allowed amplification of the complete G gene open-reading frame (ORF) and flanking sequences.

All PCRs were performed with the Expand™ High Fidelity$_{PLUS}$ system as directed by the manufacturer (Roche Diagnostics; Laval, QC). This system employs a blend of *Taq* DNA polymerase and a new thermostable proofreading protein that lacks polymerase ability; the synergy between the two components yields high fidelity products suitable for DNA sequence analysis (error rate ~$7.7x10^{-6}$). Sterile diethyl pyrocarbonate (DEPC)-treated water was used as the negative PCR control and unpassaged AdRG1.3 extract was used as the positive PCR control.

For the *in vitro* p20 AdRG1.3 clones, the following PCR program was carried out: initial denaturation at 94°C (2:00); [denature at 94°C (0:15), anneal at 60°C (0:30), extension at 72°C (2:00)] x 10; [denature at 94°C (0:15), anneal at 60°C (0:30), extension at 72°C (2:00), plus (0:10) autoextend] x 25; final extension at 72°C (7:00); hold at 4°C. Primers #781 (AdRG1.3 A) and #1037 (ARG-rev2) were used to obtain an amplicon approximately 2.25 kilobases (kb) in length. For the *in vivo* p5 AdRG1.3 clones, the following PCR program was carried out: initial denaturation at 95°C (2:00); [denature at 94°C (0:15), anneal at 60°C (0:30), extension at 72°C (2:00)] x 10; [denature at 94°C (0:15), anneal at 60°C (0:30), extension at 72°C (2:00), plus (0:10) autoextend] x 30; final extension at 72°C (7:00); hold at 4°C. Primers #1060 (AdRG1.3 A) and #1036 (ARG-rev1) were used to obtain an amplicon approximately 2.15 kb in length.

Table 2: Oligonucleotides used for PCR amplification of rabies G expression cassette of AdRG1.3

Primer	Coordinates and orientation*	Oligonucleotide sequence (5'- 3')	Synthesis number
AdRG1.3A	27840 - 27865 (+)	GCGGACGGCTACGACTGAATGTTAAG	781, 1060
ARG-rev1	30889 - 30911 (-)	GTGCTGCTGAATAAACTGGACAG	1036
ARG-rev2	30992 - 31013 (-)	GATGGACAGGAACAGGAGGAAA	1037

* orientation within the HAd5 genome sequence

(Note: the use of ARG-rev1 instead of ARG-rev2 in the PCR amplification of AdRG1.3 *in vivo* p5 clones was an arbitrary decision. The *in vitro* clones were amplified several months earlier than the *in vivo* clones, and PCR complications arose when employing the PCR protocols of the former to the latter. Although the source of the problem was determined later to be the concentration of PCR buffer used, the first successful amplification of the G gene from the AdRG1.3 *in vivo* p5 clones came from using primers AdRG1.3A and ARG-rev1 so this set was used for the remaining clones. There is only a 102 base pair difference in the PCR products obtained from the two PCR primer sets employed and this region was not targeted for nucleotide sequencing.)

Thermocycling was performed using the GeneAmp® PCR system 9700 (Applied Biosystems; Foster City, CA), and PCR products were verified by DNA gel electrophoresis using 0.8% agarose and a 1kb DNA ladder (Gibco-BRL). Bands were visualized using ethidium bromide staining under UV (ultraviolet) illumination. Photographs of agarose gels (not included in the body of this thesis) were taken using the Kodak 1D Image analysis system (Eastman Kodak Co.; Rochester, NY).

PCR product purification. Each PCR product intended for sequence analysis was purified using the Wizard® PCR Preps DNA purification system (Promega; Madison, WI). This system was used as directed by the manufacturer for use with the Promega Vac-Man® laboratory vacuum manifold. The DNA eluted from the purification resin is considered free of PCR reaction components such as primer-dimers, amplification primers, and deoxynucleotriphosphates (dNTPs); these would interfere with the sequence analysis if not removed. The purified PCR products were then stored at -20°C until sequenced.

DNA sequencing of G expression cassette. All sequencing reactions were performed using the Thermo Sequenase™ Primer Cycle sequencing kits as directed by the manufacturer

(Amersham Biosciences; Baie d'Urfé, QC). Infrared (IR) fluorescence-labeled custom-made primers were obtained from LI-COR Biosciences (Lincoln, NE) as lyophilized nucleotides (1 nanomol preparation), each of which were dissolved in 1 ml Tris-EDTA (TE) buffer and stored at -20°C (Table 3). The primers for sequencing in the forward direction were labeled with IR 700 dye while the primers for sequencing in the reverse direction were labeled with IR 800 dye. Primers ADRG1For and ADRG4Rev were used to obtain a partially overlapping sequence read over the complete cassette targeted. Any remaining sequence ambiguities were resolved using the other sequencing primers available (Table 3).

Sequencing reactions were carried out using the following program: initial denaturation at 95°C (2:00); [92°C (0:30), 50°C (0:30), 72°C (1:00)] x 30; hold at 4°C. Reactions were carried out using the GeneAmp® PCR system 9700 (Applied Biosystems). To aid in visualization and increase the density of the reaction products for loading onto denaturing polyacrylamide sequencing gels, 4 to 6 μl of red IR^2 stop solution (LI-COR) was added before freezing at –20°C.

The LI-COR Global Edition IR^2 automated sequencing system was used to examine the sequencing reaction products. This system included the LI-COR 4200 L DNA Analyser (using 66 cm plates for polyacrylamide gel electrophoresis) and its corresponding DNA analysis software, e-Seq version 2.0. The sequencing gel apparatus was prepared and operated following the procedures described in the LI-COR DNA Sequencing Manual (1999). The reaction products were denatured at 95°C for 3 minutes and immediately placed on ice for loading. Approximately 0.2 to 0.6 μl of each sequencing reaction were loaded onto the gel when using a 64-well sharkstooth comb; 0.5 to 1.0 μl of each sequencing reaction was loaded when using a 48-well sharkstooth comb.

Table 3: Oligonucleotides used for nucleotide sequencing of rabies G expression cassette of AdRG1.3

Primer	Coordinates and orientation*	Oligonucleotide sequence (5'- 3')
ADRG1For	*upstream* -45 to -22 (+)	GAGAGCTTGCCCGTAGCCTGATTC
ADRG2For	468 - 491 (+)	GAAGAGTCTCTACACAATCCGTAC
ADRG3For	987 -1009 (+)	GAGTGTCTGGATGCACTAGAGTC
ADRG4Rev	*downstream* 1895 - 1917 (-)	ACTGGACAGAAATTTGCTAACTG
ADRG5Rev	1240 -1263 (-)	GGATTAAGACATTGCCGTCAGGTC
ADRG6Rev	620 - 643 (-)	GTAGAAGACACCGCTACTCCTGAG

* orientation within the AdRG1.3 sequence window

Manipulation of sequencing data. Following a 12-hour electrophoresis, the sequence image files captured by the e-Seq 2.0 software were used to generate sequence data. Using AlignIR software (LI-COR), the forward and reverse sequences obtained for each clone were combined into one file and assembled to generate a full-length sequence of approximately 1870 nt. From this program a file containing groups of complete sequences in FASTA format was exported, manipulated in Notepad, and loaded into CLUSTAL X software (V.1.8) (Thompson *et al*, 1997) for sequence alignment and comparison. The aligned sequences were run through the DNAPARS algorithm of the PHYLIP V.3.61 phylogeny inference package (Felsenstein, 1993) to generate the sequence format presented in the Results section (Figure 4).

PHOTOGRAPHY

Photograph of fixed, stained, virus-infected cell monolayer (Results section, Figure 6) was taken with a Canon PowerShot S45 digital camera adapted to an inverted tissue culture microscope.

RESULTS

IN VITRO GENETIC STABILITY

One-step growth curve of AdRG1.3

One-step growth curves can be used to determine the essential growth properties of a virus, as characterized by a single cycle of viral infection. Differences in viral growth properties in cell culture, as reflected by the one-step growth curve, can be used to compare the fitness of different viruses in the laboratory setting (Flint *et al*, 2000); for example, to investigate whether the recombinant AdRG1.3 virus displays higher or lower fitness in cell culture as compared with the HAd5 wild type virus. This could have serious implications if wild type virus contamination were to occur during production of the AdRG1.3 vaccine. As well, one-step growth curves have been frequently employed to study mutant viruses to determine what stages of the replication cycle are affected by a particular genetic lesion; therefore, if any AdRG1.3 G gene mutants arise upon *in vitro* or *in vivo* passage, a one-step growth curve could indicate whether this affects the *in vitro* replication of the HAd5 expression vector.

A one-step growth curve of AdRG1.3 in cell culture was established in order to observe the kinetics of the replicating adenovirus recombinant vaccine *in vitro* over a 30-hour period. The key to producing a one-step growth curve is the synchronous infection of cell culture with virus; this was accomplished by infecting 293 cells with a sufficient number of virus particles to ensure that most of the cells are infected rapidly (Flint *et al*, 2000). Therefore, a multiplicity of infection (moi) of 2 was used, which predicts that for every one cell there are two virus particles available to infect it, thus preventing multiple rounds of infection. The growth curve was initiated at two separate times (GC infection #1 and GC infection #2) to enable more convenient sampling for the chosen time points (12, 14, 16, 18,

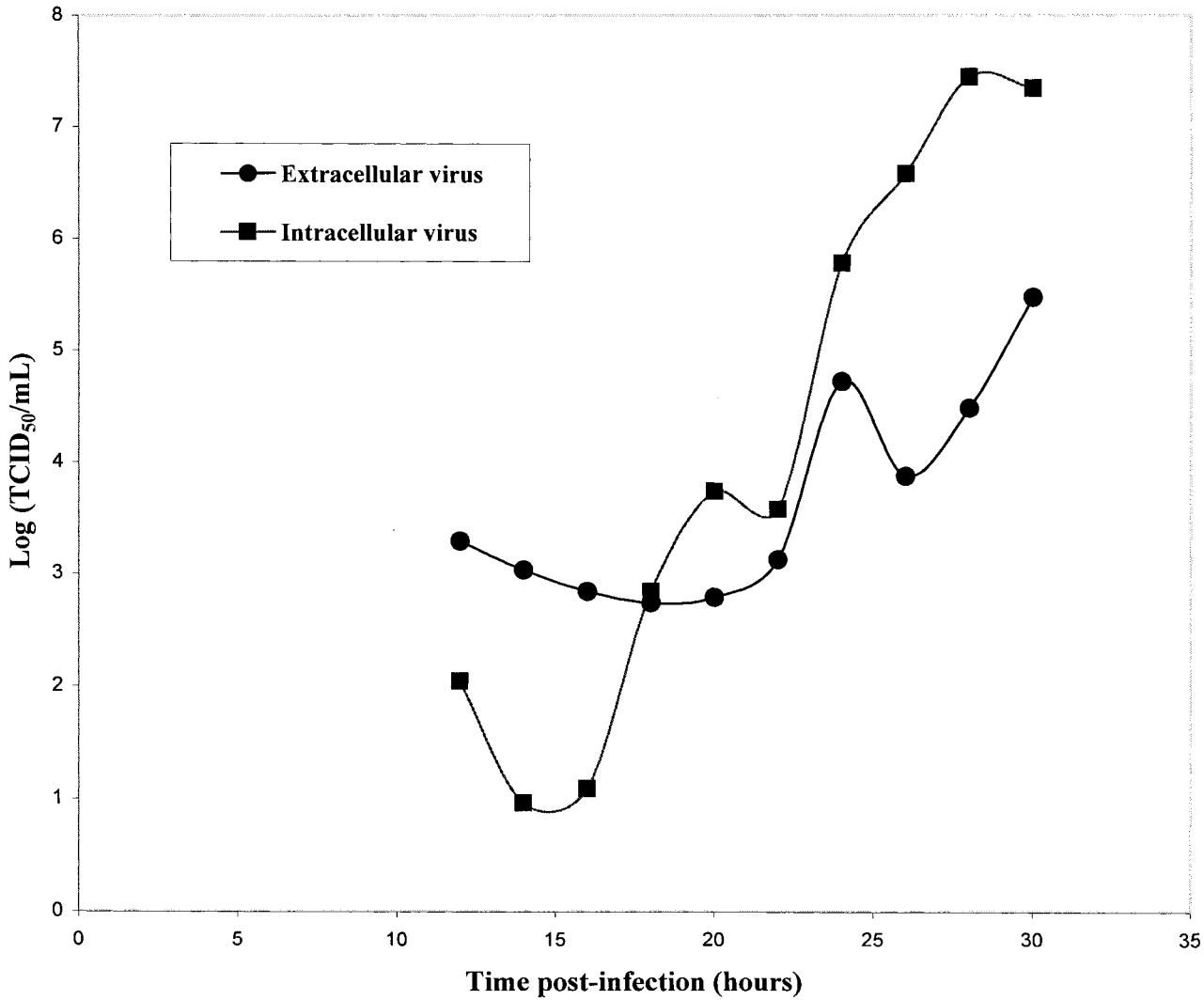
20, 22, 24, 26, 28, and 30 hours post-infection). The same media, cell passage, and virus lot were used for both, as outlined in the Materials and Methods section. These time points were chosen since it has previously been established in the literature that the eclipse period of HAd5 is approximately 11 hours (Flint *et al*, 2000); this represents the time when viral DNA is being uncoated from the capsids but no infectious virus particles have not yet been produced.

Extracellular viral titres were determined from the supernatants collected, whereas intracellular viral titres were determined from a cell-free extract prepared by freeze/thaw of the infected monolayers followed by centrifugation to remove cellular debris. Both were calculated for each time point using the endpoint dilution method; the log values of these titres (Appendix B) were plotted to produce a one-step growth curve of AdRG1.3 (Figure 2). At 12 to 16 hours post infection, intracellular virus titre remained relatively low but readily increased as the adenovirus infection progressed, reaching an intracellular titre of $10^{7.45}$ $TCID_{50}$/mL at 28 hrs p.i. As expected in the growth phase, an increase in intracellular titre was seen before an increase in extracellular titre, as new virus particles were assembled and released from the host cell. However, extracellular virus titre was initially higher than expected; this was most likely attributable to residual inoculum that may have remained after rinsing the monolayers with PBS.

***In vitro* passage of AdRG1.3**

In order to study the genetic stability of the G gene insert of AdRG1.3 under *in vitro* conditions, the vaccine was serially passaged twenty times in 293 cells at a consistent moi of 0.1. From each passage, viral supernatant was harvested by freeze/thaw and Dounce homogenization of infected cells followed by centrifugation. Adenovirus titres were

FIGURE 2: **Geometric mean titres of AdRG1.3 recovered upon synchronous infection of 293 cells in order to establish a one-step virus growth curve.** 293 cells were infected synchronously at a moi of 2. Extracellular virus titres were determined from infected cell culture supernatant. Intracellular virus titres were determined from infected monolayers upon freeze/thawing and sonication to liberate virus particles.


Extracellular virus
Intracellular virus
Log (TCID50/mL)
Time post-infection (hours)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
35

determined from these supernatants by indirect immunofluorescence and used to calculate the dilution of passaged virus required to infect the subsequent passage at a moi of 0.1; these titre values were verified by titration via the endpoint dilution method, which was performed in parallel (Appendix C) but requires a longer incubation period than indirect IF.

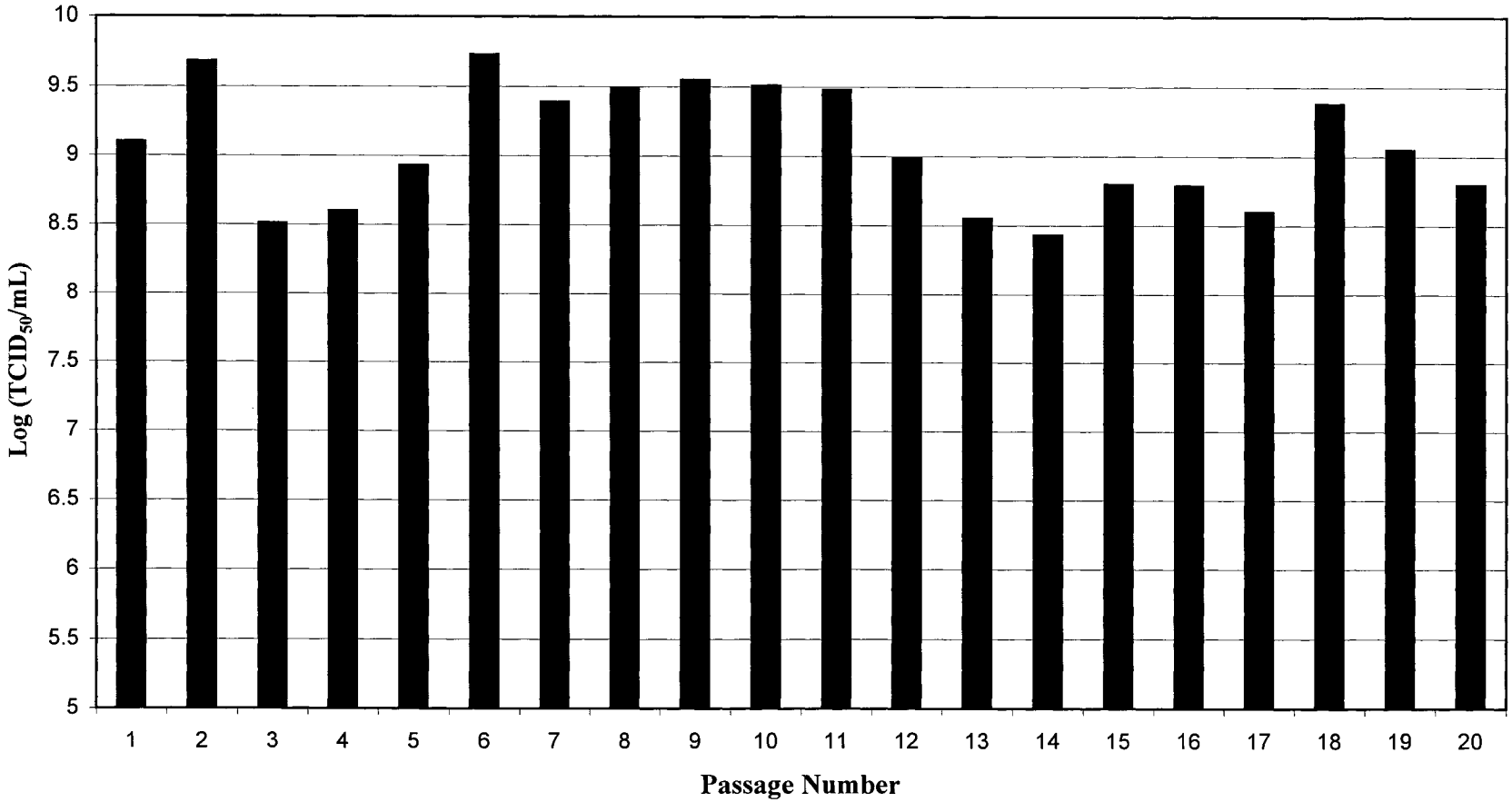
With the exception of passages 6 and 7, there was minimal discrepancy between the titres resulting from these titration methods. As seen in Figure 3, the adenovirus titres recovered from the *in vitro* passage of AdRG1.3 remained within a 1.3 $\log_{10}$ value of each other, from a minimum titre of $10^{8.43}$ $TCID_{50}$/ml to a maximum titre of $10^{9.73}$ $TCID_{50}$/ml.

Molecular analysis of AdRG1.3 after 20 passages *in vitro*

AdRG1.3 clones. 67 virus clones, designated p20-1 through p20-67, were obtained from the twentieth passage of AdRG1.3 *in vitro*. All clones were isolated using the limiting dilution technique and propagated once *in vitro* (293 cell culture) in order to increase the volume of virus necessary for DNA extraction and genetic analysis.

DNA sequence analysis. Following DNA extraction from each AdRG1.3 clone, as well as unpassaged AdRG1.3 (ADRI Lot#301), PCR amplification of the G gene cassette and flanking adenovirus sequences was performed. The resulting PCR products (approximately 2.25 kb for clones, 2.15 kb for unpassaged virus) were purified and sequenced as described in the Materials and Methods section. The targeted sequence window was 1870 nucleotides in length and included the complete G gene coding sequence (1572 nt), downstream SV40 polyadenylation (polyA) sequence (132 nt), and flanking HAd5 sequences. Base 1 of the 1870 nt sequence window corresponds to base 28060 of the HAd5 genome, and base 1870 of the same sequence window corresponds to base 30850 of HAd5 (Genbank assession no.

FIGURE 3: **Geometric mean titres of AdRG1.3 recovered upon serial passage *in vitro*.** AdRG1.3 was serially passaged 20 times in 293 cell culture at a consistent moi of 0.1. Virus was recovered from each passage by freeze/thawing and Dounce homogenization of infected cells. The cell culture supernatant obtained was titrated for human adenovirus by the endpoint dilution method.


10
9.5
9
8.5
8
7.5
7
6.5
6
5.5
5
Log (TCID50/mL)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Passage Number

M73260: Chroboczek *et al*, 1992). The nucleotide sequence obtained from unpassaged AdRG1.3 (AdRG1.3 p0), obtained as seed stock from Artemis, served as the parent sequence to which all AdRG1.3 clone sequences were compared; details of this sequence and its comparison with the first 10 *in vitro* p20 clones are described in Figure 4.

Among the 67 *in vitro* AdR1.3 clones analyzed, no rabies ERA G gene mutants were found; therefore, the G gene expression cassette, including the SV40 polyA signal sequence, appears to be stable in an adenovirus type 5 vector upon *in vitro* passage of the virus.

Comparison to the published ERA G gene sequence. The ERA rabies G gene sequence has been described previously by Anilionis *et al* (1981). When the protein sequence predicted from this published nucleotide sequence (Figure 5 B) was compared with that predicted for the ERA G gene sequence of AdRG1.3 (Figure 5A), the AdRG1.3 glycoprotein exhibited one amino acid substitution at residue 27 (proline in place of leucine). This predicted amino acid replacement could be a consequence of the selection of a specific molecular clone from the ERA virus.

FIGURE 4: **Original unpassaged AdRG1.3 G gene sequence (p0) and its comparison with 10 *in vitro* passaged clones.** PCR amplification using primers #1060 (AdRG1.3A) and #1036 (ARG-rev1) was performed on DNA extracted from unpassaged AdRG1.3 (ADRI Lot #301), producing a product approximately 2.2 kb in length. PCR amplification using primers #1060 (AdRG1.3A) and #1037 (ARG-rev2) was performed on DNA extracted from clones isolated from the twentieth *in vitro* passage of AdRG1.3, producing a product approximately 2.3 kb in length. Purified PCR products were sequenced using various combinations of IR dye-labeled sequencing primers (Table 3) to yield sequence data of 1870 nt in length. Nucleotide sequence alignment and comparison was performed using the CLUSTALX and PHYLIP (V.3.61) software packages.

NOTES:

1) The double-underlined sequence indicates the rabies ERA G gene coding sequence.
2) The single-underlined sequence indicates the SV40 polyA signal sequence.
3) Base 1 of the 1870 nt sequence window corresponds to HAd5 base 28060.
4) Base 1870 of the 1870 nt sequence window corresponds to HAd5 base 30850.
5) The **TCTAGA** sequence is a *XbaI* restriction site.
6) The symbol (▼) indicates the cloning junction between both ends of the G expression cassette and HAd5 sequence.

```
Name                                                Sequences
----                                                ---------
                1                                                                  60
AdRG1.3         TGCTAGTTGA GCGGGACAGG GGACCCTGTG TTCTCACTGT GATTTGCAAC TGTCCTAACC
p20-1           .......... .......... .......... .......... .......... ..........
p20-2           .......... .......... .......... .......... .......... ..........
p20-3           .......... .......... .......... .......... .......... ..........
p20-4           .......... .......... .......... .......... .......... ..........
p20-5           .......... .......... .......... .......... .......... ..........
p20-6           .......... .......... .......... .......... .......... ..........
p20-7           .......... .......... .......... .......... .......... ..........
p20-8           .......... .......... .......... .......... .......... ..........
p20-9           .......... .......... .......... .......... .......... ..........
p20-10          .......... .......... .......... .......... .......... ..........

               61                          ▼                                      120
AdRG1.3         CTGGATTACA TCAAGATCCT CTAGACATGG TTCCTCAGGC TCTCCTGTTT GTACCCCTTC
p20-1           .......... .......... .......... .......... .......... ..........
p20-2           .......... .......... .......... .......... .......... ..........
p20-3           .......... .......... .......... .......... .......... ..........
p20-4           .......... .......... .......... .......... .......... ..........
p20-5           .......... .......... .......... .......... .......... ..........
p20-6           .......... .......... .......... .......... .......... ..........
p20-7           .......... .......... .......... .......... .......... ..........
p20-8           .......... .......... .......... .......... .......... ..........
p20-9           .......... .......... .......... .......... .......... ..........
p20-10          .......... .......... .......... .......... .......... ..........

              121                                                                 180
AdRG1.3         TGGTTTTTCC ATTGTGTTTT GGGAAATTCC CTATTTACAC GATACCAGAC AAGCTTGGTC
p20-1           .......... .......... .......... .......... .......... ..........
p20-2           .......... .......... .......... .......... .......... ..........
p20-3           .......... .......... .......... .......... .......... ..........
p20-4           .......... .......... .......... .......... .......... ..........
p20-5           .......... .......... .......... .......... .......... ..........
p20-6           .......... .......... .......... .......... .......... ..........
p20-7           .......... .......... .......... .......... .......... ..........
p20-8           .......... .......... .......... .......... .......... ..........
p20-9           .......... .......... .......... .......... .......... ..........
p20-10          .......... .......... .......... .......... .......... ..........

              181                                                                 240
AdRG1.3         CCTGGAGCCC GATTGACATA CATCACCTCA GCTGCCCAAA CAATTTGGTA GTGGAGGACG
p20-1           .......... .......... .......... .......... .......... ..........
p20-2           .......... .......... .......... .......... .......... ..........
p20-3           .......... .......... .......... .......... .......... ..........
p20-4           .......... .......... .......... .......... .......... ..........
p20-5           .......... .......... .......... .......... .......... ..........
p20-6           .......... .......... .......... .......... .......... ..........
p20-7           .......... .......... .......... .......... .......... ..........
p20-8           .......... .......... .......... .......... .......... ..........
p20-9           .......... .......... .......... .......... .......... ..........
p20-10          .......... .......... .......... .......... .......... ..........
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           241                                                       300
AdRG1.3      AAGGATGCAC CAACCTGTCA GGGTTCTCCT ACATGGAACT TAAAGTTGGA TACATCTTAG
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           301                                                       360
AdRG1.3      CCATAAAAAT GAACGGGTTC ACTTGCACAG GCGTTGTGAC GGAGGCTGAA ACCTACACTA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           361                                                       420
AdRG1.3      ACTTCGTTGG TTATGTCACA ACCACGTTCA AAAGAAAGCA TTTCCGCCCA ACACCAGATG
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           421                                                       480
AdRG1.3      CATGTAGAGC CGCGTACAAC TGGAAGATGG CCGGTGACCC CAGATATGAA GAGTCTCTAC
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........
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                481                                                       540
AdRG1.3           ACAATCCGTA CCCTGACTAC CGCTGGCTTC GAACTGTAAA AACCACCAAG GAGTCTCTCG
p20-1             .......... .......... .......... .......... .......... ..........
p20-2             .......... .......... .......... .......... .......... ..........
p20-3             .......... .......... .......... .......... .......... ..........
p20-4             .......... .......... .......... .......... .......... ..........
p20-5             .......... .......... .......... .......... .......... ..........
p20-6             .......... .......... .......... .......... .......... ..........
p20-7             .......... .......... .......... .......... .......... ..........
p20-8             .......... .......... .......... .......... .......... ..........
p20-9             .......... .......... .......... .......... .......... ..........
p20-10            .......... .......... .......... .......... .......... ..........

                541                                                       600
AdRG1.3           TTATCATATC TCCAAGTGTG GCAGATTTGG ACCCATATGA CAGATCCCTT CACTCGAGGG
p20-1             .......... .......... .......... .......... .......... ..........
p20-2             .......... .......... .......... .......... .......... ..........
p20-3             .......... .......... .......... .......... .......... ..........
p20-4             .......... .......... .......... .......... .......... ..........
p20-5             .......... .......... .......... .......... .......... ..........
p20-6             .......... .......... .......... .......... .......... ..........
p20-7             .......... .......... .......... .......... .......... ..........
p20-8             .......... .......... .......... .......... .......... ..........
p20-9             .......... .......... .......... .......... .......... ..........
p20-10            .......... .......... .......... .......... .......... ..........

                601                                                       660
AdRG1.3           TCTTCCCTAG CGGGAAGTGC TCAGGAGTAG CGGTGTCTTC TACCTACTGC TCCACTAACC
p20-1             .......... .......... .......... .......... .......... ..........
p20-2             .......... .......... .......... .......... .......... ..........
p20-3             .......... .......... .......... .......... .......... ..........
p20-4             .......... .......... .......... .......... .......... ..........
p20-5             .......... .......... .......... .......... .......... ..........
p20-6             .......... .......... .......... .......... .......... ..........
p20-7             .......... .......... .......... .......... .......... ..........
p20-8             .......... .......... .......... .......... .......... ..........
p20-9             .......... .......... .......... .......... .......... ..........
p20-10            .......... .......... .......... .......... .......... ..........

                661                                                       720
AdRG1.3           ACGATTACAC CATTTGGATG CCCGAGAATC CGAGACTAGG GATGTCTTGT GACATTTTTA
p20-1             .......... .......... .......... .......... .......... ..........
p20-2             .......... .......... .......... .......... .......... ..........
p20-3             .......... .......... .......... .......... .......... ..........
p20-4             .......... .......... .......... .......... .......... ..........
p20-5             .......... .......... .......... .......... .......... ..........
p20-6             .......... .......... .......... .......... .......... ..........
p20-7             .......... .......... .......... .......... .......... ..........
p20-8             .......... .......... .......... .......... .......... ..........
p20-9             .......... .......... .......... .......... .......... ..........
p20-10            .......... .......... .......... .......... .......... ..........
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           721                                                       780
AdRG1.3      CCAATAGTAG AGGGAAGAGA GCATCCAAAG GGAGTGAGAC TTGCGGCTTT GTAGATGAAA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           781                                                       840
AdRG1.3      GAGGCCTATA TAAGTCTTTA AAAGGAGCAT GCAAACTCAA GTTATGTGGA GTTCTAGGAC
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           841                                                       900
AdRG1.3      TTAGACTTAT GGATGGAACA TGGGTCGCGA TGCAAACATC AAATGAAACC AAATGGTGCC
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           901                                                       960
AdRG1.3      CTCCCGATCA GTTGGTGAAC CTGCACGACT TTCGCTCAGA CGAAATTGAG CACCTTGTTG
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........
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           961                                                        1020
AdRG1.3      TAGAGGAGTT GGTCAGGAAG AGAGAGGAGT GTCTGGATGC ACTAGAGTCC ATCATGACAA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           1021                                                       1080
AdRG1.3      CCAAGTCAGT GAGTTTCAGA CGTCTCAGTC ATTTAAGAAA ACTTGTCCCT GGGTTTGGAA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           1081                                                       1140
AdRG1.3      AAGCATATAC CATATTCAAC AAGACCTTGA TGGAAGCCGA TGCTCACTAC AAGTCAGTCA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

           1141                                                       1200
AdRG1.3      GAACTTGGAA TGAGATCCTC CCTTCAAAAG GGTGTTTAAG AGTTGGAGGG AGGTGTCATC
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........
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          1201                                                        1260
AdRG1.3      CTCATGTGAA CGGGGTGTTT TTCAATGGTA TAATATTAGG ACCTGACGGC AATGTCTTAA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1261                                                        1320
AdRG1.3      TCCCAGAGAT GCAATCATCC CTCCTCCAGC AACATATGGA GTTGTTGGAA TCCTCGGTTA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1321                                                        1380
AdRG1.3      TCCCCCTTGT GCACCCCCTG GCAGACCCGT CTACCGTTTT CAAGGACGGT GACGAGGCTG
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1381                                                        1440
AdRG1.3      AGGATTTTGT TGAAGTTCAC CTTCCCGATG TGCACAATCA GGTCTCAGGA GTTGACTTGG
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........
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          1441                                                        1500
AdRG1.3      GTCTCCCGAA CTGGGGGAAG TATGTATTAC TGAGTGCAGG GGCCCTGACT GCCTTGATGT
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1501                                                        1560
AdRG1.3      TGATAATTTT CCTGATGACA TGTTGTAGAA GAGTCAATCG ATCAGAACCT ACGCAACACA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1561                                                        1620
AdRG1.3      ATCTCAGAGG GACAGGGAGG GAGGTGTCAG TCACTCCCCA AAGCGGGAAG ATCATATCTT
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1621                                                        1680
AdRG1.3      CATGGGAATC ACACAAGAGT GGGGGTGAGA CCAGACTGTG AGGACTGGCC CGGGATCGGG
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........
```

```
          1681                                                        1740
AdRG1.3      CTCGAGCAAC TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1741                                                        1800
AdRG1.3      TTTCACAAAT AAAGCATTTT TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAA
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1801                                                        1860
AdRG1.3      TGTATCTTAT CATGTCTGGA TCGTCTAGAG TACCCGGGGA TCTTATTCCC TTTAACTAAT
p20-1        .......... .......... .......... .......... .......... ..........
p20-2        .......... .......... .......... .......... .......... ..........
p20-3        .......... .......... .......... .......... .......... ..........
p20-4        .......... .......... .......... .......... .......... ..........
p20-5        .......... .......... .......... .......... .......... ..........
p20-6        .......... .......... .......... .......... .......... ..........
p20-7        .......... .......... .......... .......... .......... ..........
p20-8        .......... .......... .......... .......... .......... ..........
p20-9        .......... .......... .......... .......... .......... ..........
p20-10       .......... .......... .......... .......... .......... ..........

          1861     1870
AdRG1.3      AAAAAAAAAT
p20-1        ..........
p20-2        ..........
p20-3        ..........
p20-4        ..........
p20-5        ..........
p20-6        ..........
p20-7        ..........
p20-8        ..........
p20-9        ..........
p20-10       ..........
```

FIGURE 5: **Predicted translation product of the G gene open reading frame (ORF) of the (A) AdRG1.3 expression cassette and (B) ERA rabies virus.** These translation products include the 19 aa signal sequence (residues –19 to –1), for a total of 524 aa. Protein sequence for (A) predicted from the nucleotide sequence of AdRG1.3 p0 (unpassaged vaccine). Protein sequence for (B) published by Anilionis *et al*, (1981). Protein sequence alignment and comparison was performed using CLUSTALX, and Protpars of PHYLIP (V.3.61) software packages.

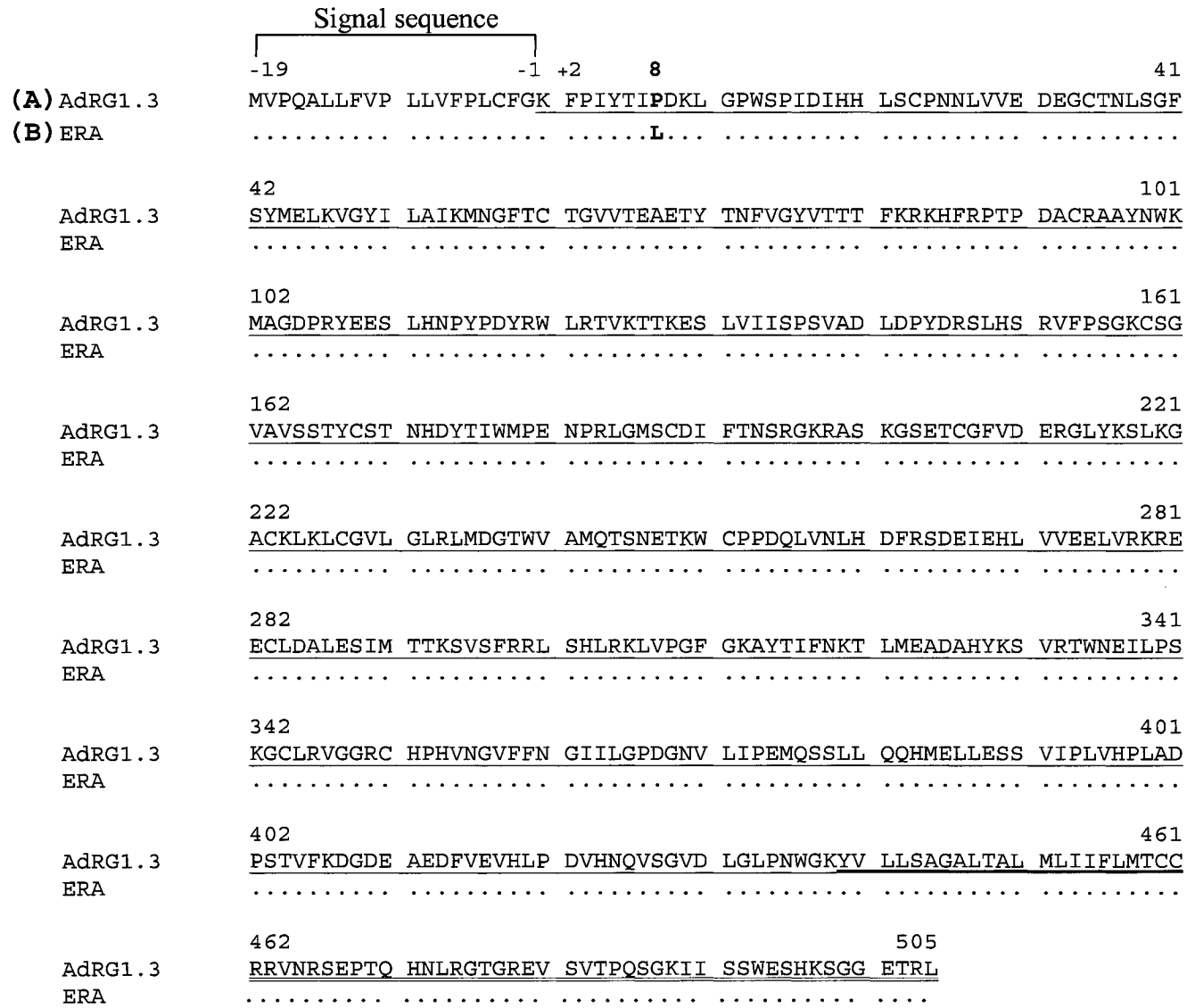
NOTES:

1) At residue **+8,** substitution of **P** (proline) in place of **L** (leucine) (in AdRG1.3 and ERA respectively).
2) The 19 aa signal sequence preceding the N-terminal K (lysine) residue of the glycoprotein.
3) The single-underlined sequence indicates the 439 aa ectodomain.
4) The solid-underlined sequence indicates the 22 aa transmembrane domain.
5) The double-underlined sequence indicates the 44 aa C-terminal domain.

Amino acid designation (single letter code):

A	Alanine	R	Arginine
N	Asparagine	D	Aspartate
C	Cysteine	Q	Glutamine
E	Glutamic acid	G	Glycine
H	Histidine	I	Isoleucine
L	Leucine	K	Lysine
M	Methionine	F	Phenylalanine
P	Proline	S	Serine
T	Threonine	W	Tryptophan
Y	Tyrosine	V	Valine
B	Asparagine	Z	Glutamine
X	Any amino acid	*	Termination codon

(IUPAC-IUB, 1984)


Signal sequence
-19 -1 +2 8 41
(A) AdRG1.3 MVPQALLFVP LLVFPLCFGK FPIYTIPDKL GPWSPIDIHH LSCPNNLVVE DEGCTNLSGF
(B) ERA ....L... ....
42 101
AdRG1.3 SYMELKVGYI LAIKMNGFTC TGVVTEAETY TNFVGYVTTT FKRKHFRPTP DACRAAYNWK
ERA ....
102 161
AdRG1.3 MAGDPRYEES LHNPYPDYRW LRTVKTTKES LVIISPSVAD LDPYDRSLHS RVFPSGKCSG
ERA ....
162 221
AdRG1.3 VAVSSTYCST NHDYTIWMPE NPRLGMSCDI FTNSRGKRAS KGSETCGFVD ERGLYKSLKG
ERA ....
222 281
AdRG1.3 ACKLKLCGVL GLRLMDGTWV AMQTSNETKW CPPDQLVNLH DFRSDEIEHL VVEELVRKRE
ERA ....
282 341
AdRG1.3 ECLDALESIM TTKSVSFRRL SHLRKLVPGF GKAYTIFNKT LMEADAHYKS VRTWNEILPS
ERA ....
342 401
AdRG1.3 KGCLRVGGRC HPHVNGVFFN GIILGPDGNV LIPEMQSSLL QQHMELLESS VIPLVHPLAD
ERA ....
402 461
AdRG1.3 PSTVFKDGDE AEDFVEVHLP DVHNQVSGVD LGLPNWGKYV LLSAGALTAL MLIIFLMTCC
ERA ....
462 505
AdRG1.3 RRVNRSEPTQ HNLRGTGREV SVTPQSGKII SSWESHKSGG ETRL
ERA ....

DETECTION OF RABIES GLYCOPROTEIN *IN VITRO*

The double immunostain assay (DIA) is an immunocytochemical technique developed as a tool to detect virus vector that no longer expresses the inserted transgene by allowing simultaneous detection of rabies glycoprotein and HAd5 antigens in fixed monolayers. The most recent improvements upon the original protocol were made by Mr. Geoff Turner of the Rabies C of E (ADRI). The discrete distribution of the rabies G epitopes (mainly expressed on the host cell surface) and HAd5 antigens (expressed intracellularly, particularly in the host cell nucleus) aids their simultaneous detection in cell culture.

Previous work showed that staining of the glycoprotein without an amplification step resulted in more diffuse staining (unpublished); therefore, staining of the glycoprotein in this assay was carried out using an indirect approach involving a biotin-streptavidin amplification step. After the application of an unlabelled anti-G mAb (murine) (Table 1), a biotinylated anti-isotype secondary antibody (goat anti-mouse IgG) was applied, followed by streptavidin conjugated with a horseradish peroxidase (HRP) enzyme. (Streptavidin binds biotin molecules with very high affinity). 3,3'-diaminobenzidine (DAB), a substrate that yields a brownish-gold stain in the presence of HRP, was then applied in order to visualize the staining of rabies glycoprotein.

Detection of the adenovirus antigen was also done indirectly, but did not require such extensive amplification as performed to detect the glycoprotein. After the application of unlabelled anti-HAd5 polyclonal rabbit serum, alkaline phosphatase (AP)-labelled anti-rabbit antibody was applied. HistoMark® RED (KPL), a substrate that yields a red stain in the presence of AP, was then applied in order to visualize the staining of the HAd5 antigens.

The steps from both antigen detection methods were combined to create the double immunostain assay. (Refer to Materials and Methods for specific details.)

Detection of ERA glycoprotein epitopes *in vitro*. The double immunostain assay was used to observe at what time during *in vitro* AdRG1.3 infection the rabies G epitopes become detectable by immunocytochemistry. 293 cells were infected in suspension with AdRG1.3 and seeded in 96-well plates. At each of a series of time points, from 9 hours to 96 hours post-infection, two plates were fixed with cold methanol (100% v/v). The first plate was stained using the DIA, the second plate by indirect immunofluorescence.

A total of 11 monoclonal antibodies directed against the 6 antigenic sites of G (Table 1) were used in conjunction with the double immunostain. Two wells per fixed plate were dedicated to staining with each of the 11 mAbs, enabling the detection of different G protein epitopes as the adenoviral infection progressed. The expression of all ERA G protein epitopes targeted by the anti-G mAbs was detected by 72 hours post-infection, with the majority of epitopes being detectable by 60 hours (Table 4); an example of this staining is shown in Figure 6. (Note: HAd5 antigens were detectable by 24 hours post-infection using the DIA). In contrast, when using indirect IF the expression of all epitopes targeted by the anti-G mAb panel was detected by 18 hours post-infection, with the majority of epitopes being detectable by 9 hours (Table 5) and the fluorescence signal generally increasing as the *in vitro* infection progressed. The mAb M1100 exhibited the weakest signal over a period of 96 hours, whereas mAb M818 exhibited the strongest signal.

From this work, it was evident that the DIA in combination with an anti-G mAb panel directed toward the six antigenic sites of the rabies glycoprotein could be useful for *in vitro* screening of AdRG1.3 clones that have acquired a mutation (or mutations) in the G gene insert that affects the glycoprotein expression phenotype. Moreover, it can be used to confirm that the G gene insert remains intact and that it's product is being consistently expressed by the recombinant HAd5 vector. Although detection of G is observed earlier

Table 4: Detection of AdRG1.3 glycoprotein gene insert expression in cell culture by the double immunostain assay (DIA)

	Hours post-infection											
mAb	**9**	**12**	**15**	**18**	**20**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
10EC9	X	X	X	X	X	X	X	O	O	O	O	O
M818	X	X	X	X	X	X	X	O	O	O	O	O
M1089	X	X	X	X	X	X	X	X	O	O	O	O
16EH11	X	X	X	X	X	X	O	O	O	O	O	O
M778	X	X	X	X	X	X	X	X	O	O	O	O
M1094	X	X	X	X	X	X	X	O	O	O	O	O
M724	X	X	X	X	X	X	X	O	O	O	O	O
M1100	X	X	X	X	X	X	X	X	X	O	O	O
16AH8	X	X	X	X	X	X	X	O	O	O	O	O
M1078	X	X	X	X	X	X	X	X	O	O	O	O
10ED8	X	X	X	X	X	X	X	X	O	O	O	O

X	signifies that the corresponding G epitope is not detectable by that specific mAb
O	signifies that the corresponding G epitope is detectable by that specific mAb

FIGURE 6: **Staining of an AdRG1.3 viral plaque with the double immunostain assay.** Detection of rabies G (yellow/brown) and adenovirus fiber (red/pink) proteins in AdRG1.3-infected 293 cell culture. Virus-infected monolayers, in 96-well tissue culture plates, were fixed with 100% methanol 4 days post-infection and stained using the double immunostain assay. This figure shows a single AdRG1.3 plaque. (100x magnification, inverted tissue culture microscope; Olympus CK2)

AdRG1.3 plaque (100x magnification)

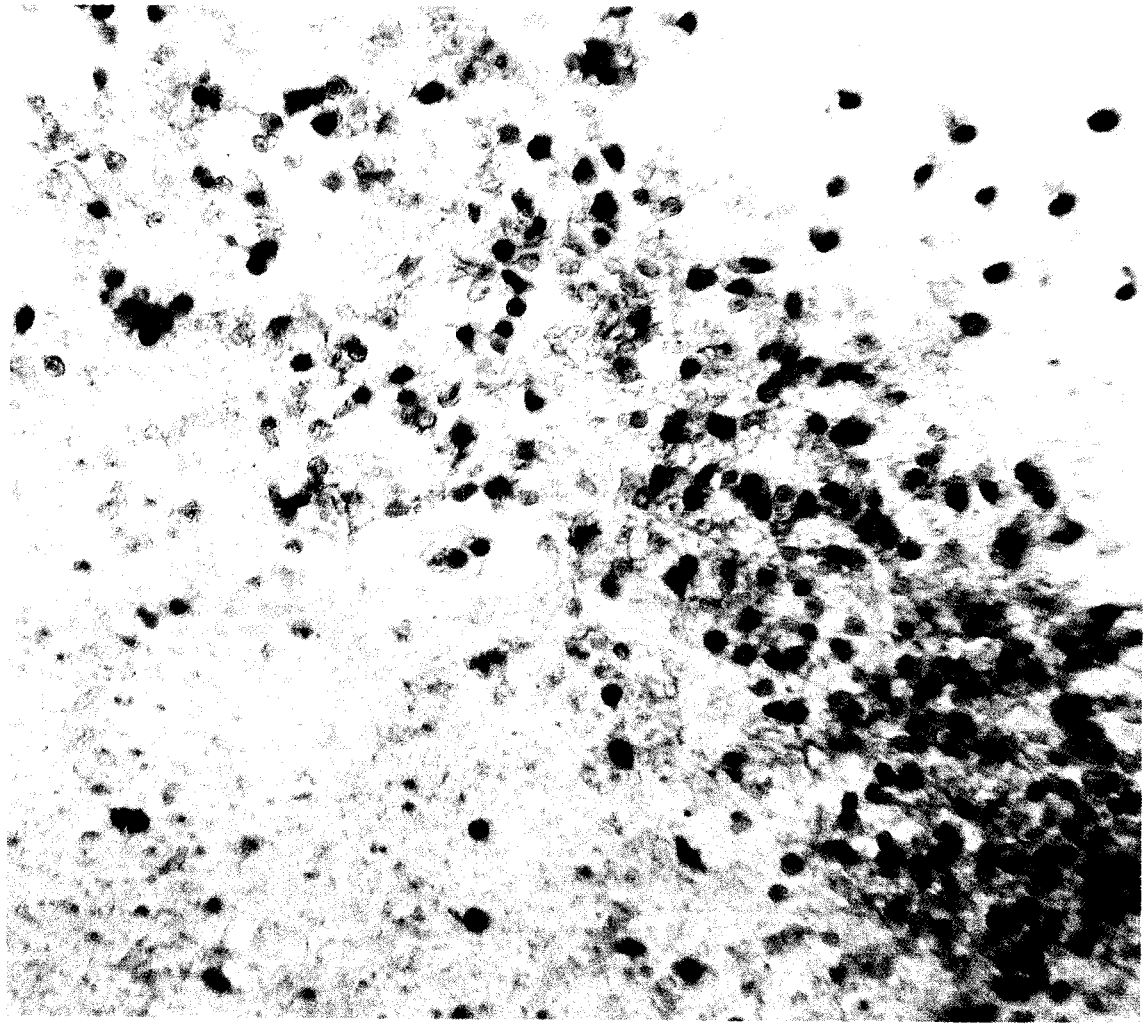


Table 5: Detection of AdRG1.3 glycoprotein gene insert expression in cell culture by indirect immunofluorescence

	Hours post-infection											
mAb	**9**	**12**	**15**	**18**	**20**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
10EC9	●	●	●●	●●●	●●	●●	●●	●●	●●	●●●	●●●	●●●
M818	●●	●●	●●	●●	●●●	●●●	●●	●●	●●	●●●	●●●	●●●
M1089	●●	●	●●	●●●	●●	●●	●●	●●	●●	●●	●●	●●●
16EH11	●●	●	●●	●●	●●	●●	●●	●●	●●	●●●	●●●	●●●
M778	●	X	●●	●●	●●	●●	●●	●●	●●	●●	●●	●●
M1094	●●	●	●	●	●	●●	●●	●●	●	●	●	●●
M724	●●	●	●●	●●	●●	●●	●●	●●	●●	●●●	●●●	●●●
M1100	X	X	X	●	●	●	●	●	●●	●	●	●
16AH8	●	●●●	●	●	●	●	●●	●●	●●	●●	●●	●●
M1078	●	●●●	●●	●●	●●	●●	●●	●●	●●	●●●	●●●	●●●
10ED8	●	●	●●	●●	●	●	●●	●●	●●	●●	●●	●●●

●●●	represents a strong fluorescence signal
●●	represents a medium strength fluorescence signal
●	represents a weak fluorescence signal
X	represents no fluorescence signal detected

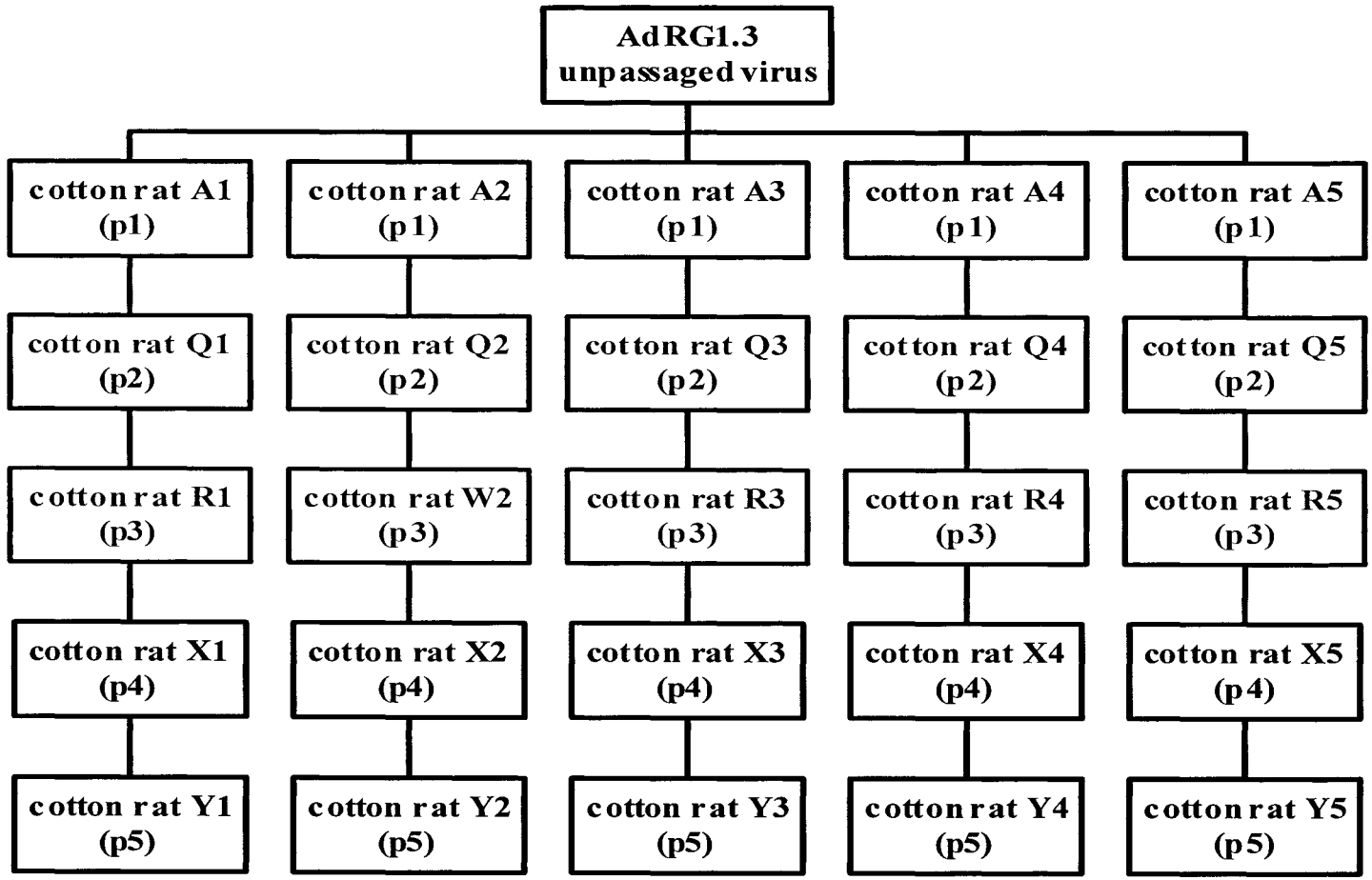
using indirect immunofluorescence, the DIA gives results within 72 hours and offers a technically simpler approach that does not require fluorescence microscopy capabilities.

IN VIVO GENETIC STABILITY

Cotton rat model of adenovirus infection. In order to study the *in vivo* genetic stability of AdRG1.3, the cotton rat model was used. The study was designed so as to follow Veterinary Biologics general licensing considerations (USDA-APHIS, 2000), which suggest a minimum of five serial passages when assessing the genetic stability testing of a live recombinant vaccine in animals. Five independent series of cotton rats were used in order to ensure re-isolation and continued serial passage of the virus.

Experimental design. The overall scheme of the experiment is illustrated in Figure 7. The initial AdRG1.3 inoculum was prepared in phosphate buffered saline (PBS) at $10^{7.00}$ $TCID_{50}$/ml. This titre was chosen based on a previous experiment at ADRI (1991-02-05) that showed that when adenovirus was administered orally to cotton rats at titres of $10^{6.0}$ to $10^{9.0}$ $TCID_{50}$/ml, a titre of $10^{7.0}$ $TCID_{50}$/ml allowed the best recovery of virus from rat tissue with minimal disease in experimental animals (unpublished findings). On day 1 of the first passage, 200 µl of virus suspension was administered intranasally to the animals (series 1, cotton rats A1 though A5) in 4 x 50 µl doses. The cotton rats were observed daily for changes in appetite, thirst, appearance, and activity, any of which could indicate the development of disease. No significant observations were noted throughout the course of the entire *in vivo* passage study. On the fifth day post-inoculation the animals were euthanised and tissues were harvested upon necropsy (see Materials and Methods). The lung tissue from each animal was homogenized and used to prepare a10% m/v suspension in PBS which, after clarification and filtration, was used as the inoculum for subsequent passage of the virus in a new series of animals. Thus virus derived from cotton rat A1 was inoculated into cotton rat

FIGURE 7: **Schematic diagram of *in vivo* AdRG1.3 passage in the cotton rat (*Sigmodon hispidus*) model.** AdRG1.3 was serially passaged five times through five independent series of animals (Series 1 through 5). The AdRG1.3 passage recovered from each animal is denoted by "p1" through "p5".


AdRG1.3
unpassaged virus
cotton rat A1
(p1)
cotton rat A2
(p1)
cotton rat A3
(p1)
cotton rat A4
(p1)
cotton rat A5
(p1)
cotton rat Q1
(p2)
cotton rat Q2
(p2)
cotton rat Q3
(p2)
cotton rat Q4
(p2)
cotton rat Q5
(p2)
cotton rat R1
(p3)
cotton rat W2
(p3)
cotton rat R3
(p3)
cotton rat R4
(p3)
cotton rat R5
(p3)
cotton rat X1
(p4)
cotton rat X2
(p4)
cotton rat X3
(p4)
cotton rat X4
(p4)
cotton rat X5
(p4)
cotton rat Y1
(p5)
cotton rat Y2
(p5)
cotton rat Y3
(p5)
cotton rat Y4
(p5)
cotton rat Y5
(p5)

Q1, from cotton rat A2 into cotton rat Q2, and so on for each series for the remainder of the passages as summarized in Figure 7. Preliminary attempts to detect adenovirus in impression smears of cotton rat lung tissue via indirect immunofluorescence were unsuccessful. Therefore, the 0.22 μm-filtered supernatants collected from lung homogenates were titrated for adenovirus via indirect IF in order to confirm recovery of virus within a short period of time. The endpoint dilution method was performed in parallel to confirm virus titres.

Recovery of virus. For each series of cotton rats, virus titres between 10^4 and 10^5 $TCID_{50}$/ml were recovered after the first and second passages *in vivo* (Table 6). However, following the third passage (for cotton rat series 1, 3, 4, and 5) and the second passage (for cotton rat series 2), little or no virus was recovered. Two attempts to re-passage virus samples recovered from cotton rat Series 1 (A1 to A5) through two new groups of animals were also unsuccessful, with little to no virus recovery beyond three *in vivo* passages of virus; results of these attempts are noted in Appendix D. Therefore, it was necessary to concentrate and/or increase the infectivity of the recovered virus samples in order to continue the *in vivo* passage of AdRG1.3. Although common virus purification methods such as cesium chloride or sucrose density gradient centrifugation are effective in concentrating virus, they often result in a substantial loss of infectious virus titre (Hermens *et al*, 1999). The Veterinary Services Memorandum No. 800.201 (USDA-APHIS, 2000) states that recovered material may be concentrated, but not by *in vitro* propagation; therefore, an alternative method to purify virus using Arcton 113 was investigated.

Treatment of virus with Arcton 113

Fluorocarbons, such as Arcton 113 or Freon 112 in *n*-heptane, have been used to purify several viruses such as vaccinia (Epstein, 1958), foot and mouth disease virus (Brown and Cartwright, 1960), and poliovirus (Manson *et al*, 1957). In the case of these viruses

Table 6: Geometric mean titres[1] recovered upon 5 serial passages of AdRG1.3 in the cotton rat (*Sigmodon hispidus*)

Passage	Cotton rat series 1	Cotton rat series 2	Cotton rat series 3	Cotton rat series 4	Cotton rat series 5
1	$10^{4.25}$ (A1)	$10^{4.25}$ (A2)	$10^{4.48}$ (A3)	$10^{4.05}$ (A4)	$10^{4.42}$ (A5)
2	$10^{4.92}$ (Q1)	$10^{4.08}$ (Q2)[A]	$10^{4.50}$ (Q3)	$10^{4.33}$ (Q4)	$10^{4.99}$ (Q5)
3	$10^{3.08}$ (R1)[A]	$10^{6.17}$ (W2)	$10^{2.83}$ (R3)[A]	$10^{3.66}$ (R4)[A]	$10^{3.49}$ (R5)[A]
4	$10^{6.00}$ (X1)	$10^{4.58}$ (X2)	$10^{6.16}$ (X3)	$10^{6.42}$ (X4)	$10^{6.16}$ (X5)
5	$10^{4.67}$ (Y1)	$10^{2.99}$ (Y2)	$10^{4.57}$ (Y3)	$10^{4.83}$ (Y4)	$10^{4.50}$ (Y5)

[1]determined by endpoint dilution (measured in $TCID_{50}$/ml)

[A] indicates that virus was propagated in cell culture between *in vivo* passages to amplify viral titres for continued passage in cotton rats (see text for details of virus amplification)

Note: designation in parentheses indicates cotton rat from which the passaged virus was recovered

which are naked and thus devoid of lipid, it is likely that fluorocarbons selectively remove host lipid-containing membranes (Brady and Furminger, 1976) and although this purification technique also works successfully with enveloped viruses such as influenza (Hamparian *et al*, 1958), these viruses are more susceptible to inactivation by the fluorocarbons than are the lipid-free viruses (Brady and Furminger, 1976).

Once it was observed that adenovirus recovery from lung tissue consistently declines upon serial passage of AdRG1.3 in the cotton rat, purification of adenovirus recovered from the lungs of cotton rat A5 (0.2 μm-filtered) was attempted using Arcton 113 as described in the Materials and Methods section. The Arcton 113-treated virus was titrated in duplicate (rather than in triplicate) by indirect IF so as to preserve volume of recovered virus for continued passage and to reduce the amount of time required to establish titre.

As seen in Table 7, treatment with Arcton 113 caused a decrease in virus titre rather than an increase in infectivity, possibly due to loss of liberated virus into the organic phase during treatment.

Table 7: Geometric mean titres[1] recovered upon treatment of AdRG1.3-infected cotton rat lung homogenates with Arcton 113

Length of treatment (minutes)	GMT[1]
0	$10^{5.50}$
20	$10^{4.62}$
40	$10^{4.37}$

[1]determined by indirect immunofluorescence (measured in FA_{50}/ml)

***In vitro* propagation of recovered material.** As previously stated, the Veterinary Services Memorandum No. 800.201 (USDA-APHIS, 2000) suggests that material recovered upon vaccine passage *in vivo* cannot be propagated *in vitro*. However in this case, where a limited

volume of virus was recovered and common virus concentration methods were not feasible (or ineffective, as in the case of treatment with hydrocarbon Arcton 113), *in vitro* propagation appeared to be the best approach to increasing recovered virus infectivity. Since the rabies glycoprotein insert of AdRG1.3 was proven to be genetically stable upon *in vitro* passage of the vaccine, the use of *in vitro* propagation of material recovered upon *in vivo* passage was justified.

Therefore, material recovered from cotton rats R1, Q2, R3, R4, and R5 was passaged once in 293 cell culture to increase viral titres to $10^{7.75}$, $10^{7.83}$, $10^{7.58}$, $10^{7.33}$, and$10^{7.41}$ $TCID_{50}$/mL, respectively. The infected cell culture supernatants collected were inoculated (4 x 50 µl doses) into the next group of cotton rats (X1, W2, X3, X4, and X5, respectively) by the intranasal route, with the remainder of the passages carried out as described previously. A progressive decline in recoverable virus was again observed for all 5 series of animals following the *in vitro* viral amplification step (Table 6); however, adequate titres of virus were recovered from the fifth passage of AdRG1.3 *in vivo* to successfully carry out molecular analysis (Figure 8).

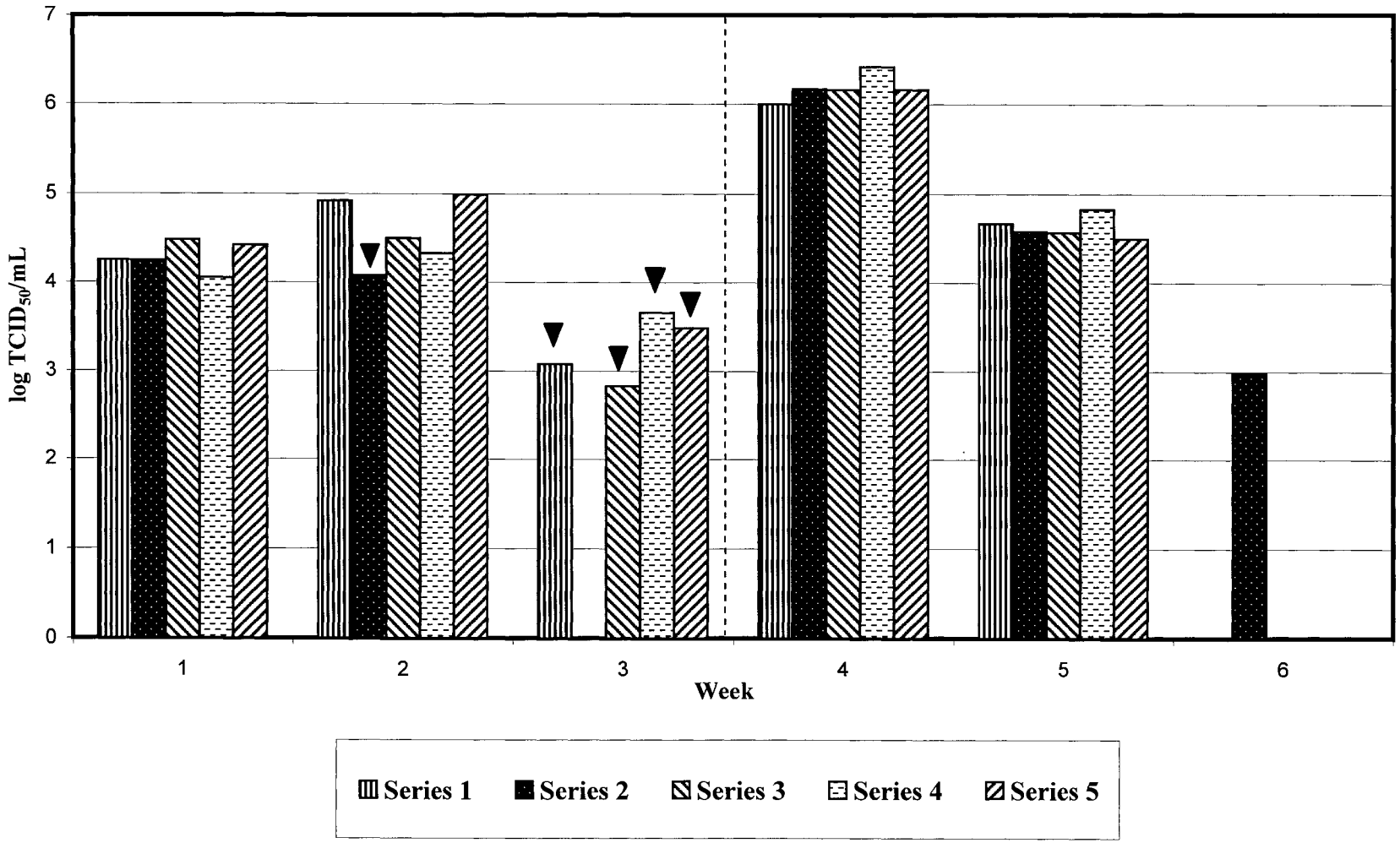
MOLECULAR ANALYSIS

AdRG1.3 clones. 105 virus clones were obtained from the fifth passage of AdRG1.3 *in vivo*, designated according to the animal from which the virus was originally derived: Y1-1 through Y1-29, Y2-1 through Y2-9, Y3-1 through Y3-23, Y4-1 through Y4-23, and Y5-1 through Y5-21. All clones were isolated using the limiting dilution technique and propagated once *in vitro* (293 cell culture) to increase the volume of virus necessary for genetic analysis.

DNA sequence analysis of AdRG1.3 clones. Following DNA extraction, PCR amplification of the G gene and flanking adenovirus sequences from each AdRG1.3 clone

FIGURE 8: **Geometric mean titres of AdRG1.3 recovered upon serial passage *in vivo*.** AdRG1.3 was serially passaged five times through five independent series of cotton rats (*Sigmodon hispidus*). Virus was administered to the animals intranasally and recovered from lung tissue at 5 days post-infection. Recovered virus was filtered (0.2 μm) and used to inoculate the subsequent group of animals. Virus was titrated by the endpoint dilution method (values shown here) to confirm the titres calculated by indirect immunofluorescence.

▼ Inverted arrows indicate virus amplified by passage in cell culture when virus became less/non-recoverable from animals.


7
6
5
4
3
2
1
0
log TCID50/mL
1
2
3
4
5
6
Week
Series 1
Series 2
Series 3
Series 4
Series 5

was carried out. The PCR products obtained (2.15 kb for *in vivo* clones) were purified and sequenced. The length of the resulting sequences to be analyzed was 1870 nucleotides. The nucleotide sequence obtained from unpassaged AdRG1.3 (AdRG1.3 p0) served as the parent sequence to which all AdRG1.3 clone sequences were compared.

Among the 105 *in vivo* AdR1.3 clones analyzed, no rabies ERA G gene mutants were found; therefore, the G gene expression cassette, including the SV40 polyA signal sequence, appears to be stable in an adenovirus type 5 vector upon *in vivo* passage of the virus.

DISCUSSION

AdRG1.3, an adenovirus-rabies glycoprotein recombinant vaccine, is a potential candidate for the oral immunization of terrestrial wildlife hosts against rabies. Among the general licensing considerations described in the Veterinary Services Memorandum No. 800.201 (USDA-APHIS, 2000) (in accordance with the 9 CFR), and the regulations set by the Veterinary Biologics Section of the Canadian Food Inspection Agency, is the assessment of the genetic stability of live vaccines derived from recombinant DNA technology. For a vaccine to be useful, it should not undergo significant mutation upon propagation of working seed to produce the vaccine or within the target species (Lutze-Wallace *et al*, 1995a). The glycoprotein must be efficiently expressed in the host in order to induce an effective anti-rabies immune response; any mutations that may be introduced into the G gene have the potential to adversely affect immunogenicity of the vaccine. Missense mutations, in which a base-pair change causes a change in the amino acid residue encoded, may or may not elicit antigenic changes to the protein. These include silent mutations, which alters the codon but not the amino acid encoded and thus is not expected to change the antigenic nature of the protein. A nonsense mutation, in which a base pair change causes a change from an amino acid-coding codon to a chain-terminating codon, can produce a truncated product which could, depending on where it occurs, radically alter the protein's immunogenicity. As well, mutations in the flanking regulatory elements would not change the structure of the protein itself, but could alter the level of protein expressed from the vector.

To this end, the genetic stability of the ERA rabies glycoprotein cassette of AdRG1.3 was assessed upon *in vitro* and *in vivo* passage of the virus. This not only examined the stability of the G gene upon serial propagation of the vaccine in cell culture but also investigated the possible emergence of mutants upon passage in a live host.

Rabies glycoprotein in rabies virus background

Due to the limited replication fidelity of RNA viruses, they are often referred to as "quasispecies". The quasispecies model of mixed virus populations is characterized by one or more dominant sequences along with a large spectrum of related variants. This population complexity lends itself to greater adaptability in response to changes in the host environment (Novella *et al*, 1995). In a constant environment, a stable dominant virus species within the pool of related viruses has been demonstrated (Domingo, 1992; Eigen *et al*, 1981). However, rapid shifts in the dominant variant have been observed with the rabies CVS strain upon environmental changes (Morimoto *et al*, 1998).

An investigation into the quasispecies structure of the rabies genome showed that at the quasispecies level, the glycoprotein (nucleotides 3909 to 4388) exhibited a mutation frequency of 21.9×10^{-4} mutations per bp – a frequency that increased significantly by 22.1×10^{-4} mutations per bp upon passage in adult mice (Kissi *et al*, 1999). The mechanisms of genomic evolution observed in the G gene region in response to environmental changes (i.e. passage in neuronal and non-neuronal cell culture; passage in heterologous hosts) included 1) the accumulation of limited mutations (including point, deletion, and insertion mutations) with no substitution of the dominant sequence, as well as 2) a less frequent but sudden selective overgrowth of a favoured variant (Kissi *et al*, 1999). The latter has also been observed *in vitro* with VSV (Clarke *et al*, 1994).

Kissi and colleagues (1999) implicate multiple factors influencing the heterogeneity of the rabies virus genome, including the duration of infection, route of transmission, the host immune response, and virus and host protein cooperation. Therefore, it is reasonable to believe that the rabies glycoprotein, when placed in a significantly different virus

background, will be under very different genetic and environmental influences and thus subjected to different selective pressures.

ORAL WILDLIFE VACCINES - SAFETY AND STABILITY

It has been stated that a vaccine should "remain non-pathogenic regardless of the route of infection", "demonstrate genetic stability that precludes reversion to virulence", "exhibit adequate thermostability", and "produce high virus titers *in vitro*" (Dietzschold *et al*, 2004) when being utilized for the oral immunization of wildlife.

Live attenuated rabies virus vaccines

The ERA vaccine, a live attenuated rabies virus released in Canada in 1985 (Johnston *et al*, 1988; MacInnes *et al*, 1988), has been successful in protecting foxes against rabies through oral immunization (Baer *et al*, 1971; Black and Lawson, 1970; Black and Lawson, 1973). Application of ERA (renamed SAD in Europe) led to a significant portion of Switzerland being freed from rabies in the early 1980's (Kappeler and Wandeler, 2000), and the aerial distribution of the ERA in baits, in combination with parenteral vaccination of inactivated rabies vaccine in urban areas, led to substantial success in the control of fox rabies in southern Ontario (Rosatte *et al*, 1993; Rosatte *et al*, 1997). Although ERA is considered a safe, immunogenic vaccine for foxes (Lawson *et al*, 1987; Lawson *et al*, 1989), the vaccine has shown residual pathogenicity in skunks (Lawson *et al*, 1989), another target species, by some routes of inoculation (Tolson *et al*, 1988b). This pathogenicity was also observed in non-target species such as rodents (Lawson *et al*, 1987). A similar attenuated rabies vaccine, SAD_{B19}, demonstrated potential for immunization of raccoons, although not to the same extent as seen in the immunization of foxes (Rupprecht *et al*, 1989). Host factors, including virus-host protein interactions and immune response (as well as attractiveness of the oral bait for species targeted for immunization), may account for

differences observed in the pathogenicity and efficacy of the SAD/ERA rabies vaccines by the oral route in these animals.

In the interest of enhancing safety, derivatives of the SAD vaccines were developed, including escape mutant SAG1 (Flamand *et al*, 1989) that has a single substitution mutation in position 333 of the glycoprotein. This mutation has been shown to render the rabies virus apathogenic for adult mice via the intracerebral route (Coulon *et al*, 1983; Seif *et al*, 1985; Flamand *et al*, 1993). The SAG1 strain was found to be as effective in foxes as the SAD_{Bern} vaccine from which is was derived. However, unlike SAD_{Bern} which is pathogenic via the intracerebral, intramuscular, or oral route, the SAG1 strain is avirulent (Artois *et al*, 1992; Le Blois *et al*, 1990). In a further step to reduce/eliminate reversion of the live attenuated vaccine to the more virulent phenotype, the SAG2 strain was isolated. A double avirulent mutant, SAG2 encodes a Glu (glutamate residue) (GAA) rather than Arg (arginine) (CGU/CGC/CGA/CGG) at residue 333 (Lafay *et al*, 1994). The Glu_{333} G protein is commonly referred to as GA (Faber *et al*, 2002). This new codon differs by two nucleotides from all the arginine-coding triplets; thus, the double avirulent mutant with Glu_{333} is less likely to revert to the virulent Arg_{333} strain than a single avirulent mutant, since two reversion mutations in the G gene open reading frame would need to occur rather than only one.

The reverse genetics approach has allowed researchers to develop safe modified-live rabies viruses (Schnell *et al*, 1994) whose efficacy may be optimized in different wildlife species (Morimoto *et al*, 2001) by incorporating a G protein identical to that of the target virus into a vaccine strain (Dietzschold and Schnell, 2002). Co-expression of immune-enhancing proteins such as cytokine IL-2 (interleukin-2) and pro-apoptotic proteins such as cytochrome c (cyt c) may potentially enhance the antiviral immune response elicited by the vaccine (Dietzschold and Schnell, 2002). Moreover, genetic manipulations that alter the

pathogenicity of rabies virus, without affecting immunogenicity, have been investigated using this approach (Morimoto *et al*, 2000). A recent study examined three recombinant rabies viruses constructed using reverse genetics technology: 1) a rabies virus (RV) vector (SPBNGA) expressing GA, 2) a RV vector expressing a second GA protein (SPBNGA-GA), and 3) a RV vector expressing cyt c (SPBNGA-Cyto c (+)) (Dietzschold *et al*, 2004). The incorporation of the additional genetic elements did not appear to significantly affect the thermostability of the viruses, and none of the three experimental vaccines were pathogenic in mice via intracerebral (i.c.) inoculation. However, after 5 i.c. passages of the constructs in adult mice, an $Asn_{194} \rightarrow Lys_{194}$ mutation occurred in the G gene of all three constructs (except the second G gene of SPBNGA-GA). This mutation was associated with a considerable increase in the pathogenicity of SPBNGA and SPBNGA-Cyto c (+), but not SPBNGA-GA. Although this indicates that the second GA protein of SPBNGA-GA may account for its reduced pathogenicity, further work must be done to determine whether it is solely responsible (Dietzschold *et al*, 2004).

Recombinant rabies vaccines

Recombinant rabies vaccines offer a safer alternative to live attenuated rabies vaccines for the oral immunization of wildlife animals. Recombinant viruses expressing the immunogenic rabies G protein have the ability to induce a protective immune response without causing rabies in the vaccinated host. If possible, "the profile of immunization should be different from that of an active infection" (Van Kampen, 2001). The tropism of the viral vectors often differ from that of rabies virus and therefore could induce an immune response via a different route than that of modified-live rabies vaccines. For example, adenoviruses infect pharyngeal lymphoid tissue upon oral application (Fenner *et al*, 1987). Intratracheal delivery of an adenoviral vector was shown to induce elevated systemic IgG

and mucosal IgA antibodies to the virus and the expressed transgene, indicating both localized and systemic antibody responses (Van Ginkel *et al*, 1995).

Efficient expression and proper conformation of rabies G is crucial for stimulating immunity against rabies; thus, it is crucial that the G expression cassette inserted within recombinant rabies vaccines remain genetically stable. In comparison to the live attenuated rabies vaccines, where rabies G is endogenous to the virus/vector, one must consider the selective pressure placed on the glycoprotein when expressed as a heterologous gene in a different viral vector background.

Vaccinia-rabies glycoprotein recombinant vaccine (VRG)

The first recombinant rabies vaccine constructed was VRG, a vaccinia vector expressing rabies glycoprotein (ERA strain) (Kieny *et al*, 1984). VRG has been shown to be apathogenic for both target and non-target species (Brochier *et al*, 1989; Artois *et al*, 1990; Artois *et al*, 1992) and can be used to successfully immunize raccoons (Rupprecht *et al*, 1986; Rupprecht *et al*, 1988) and foxes (Tolson *et al*, 1988a; Brochier *et al*, 1991; Pastoret and Brochier, 1996) by oral administration. It was first released in North America in 1990 (Rupprecht *et al*, 1993) and is currently used to control rabies in coyotes (Fearneyhough *et al*, 1998), grey foxes (Fearneyhough *et al*, 1996), and raccoons (Robbins *et al*, 1998) in the US. In 1999, the Ontario Ministry of Natural Resources (OMNR) included oral vaccination with VRG as part of its point infection control (PIC) strategy in an attempt to contain raccoon rabies when it spread into Canada (Rosatte *et al*, 2001).

Genetic stability was reported by Desmettre *et al* (1990) after 10 passages of VRG in Vero cells, although the details of genetic analysis are limited. A report published by the WHO regarding the oral immunization of dogs against rabies (WHO, 1998) discusses the genetic stability testing that has been performed on the VRG vaccine. VRG inoculated into

mice by three different routes (i.c., scarification, and oral) was recovered from mouse tissues upon sacrifice. Isolated virus was infected on Vero cells and from 600 positive isolates the rabies G and flanking thymidine kinase gene sequences were amplified by PCR. This G expression cassette did not appear to be deleted in any of these isolates, although REA did indicate some changes in the length of the downstream polyA sequence element. Further to this work, passaging of the viruses in cell culture revealed that 200 of the isolates gave rise to a low proportion of plaques where only 2 of 3 anti-G mAbs would detect G expression *in vitro*. Nucleotide sequence analysis of 14 isolates found that frameshift mutations (caused by G nucleotide insertions) in rabies G resulted in early termination of translation. As well, one isolate produced plaques that were not stained by any of the 3 anti-G mAbs; limited sequence analysis found that a similar frameshift mutation had also occurred, causing premature termination of protein expression. It was concluded that mutations causing the G protein to become non-functional occurred with low frequency in the case of VRG.

However, REA only identifies mutations that occur at restriction sites, and staining with mAbs only detects changes that affect the phenotype of G expression. Although non-functional mutations were observed only at low frequency in the case of VRG, these methods overlook changes that may occur at the genetic level but do not affect the phenotype of G expression.

Adenovirus-rabies glycoprotein recombinant vaccines (ARG)

ARG vaccines express the rabies glycoprotein gene (ERA strain), which is inserted within a gene deletion in a HAd5 vector, usually in the E3 or E1 gene region. One such vaccine, AdRG1, was found to elicit good VNA tires by either the parenteral or oronasal route in dogs and mice, the latter of which were protected from lethal i.c. rabies challenge (Prevec *et al*, 1990). Oral vaccination of striped skunks and red foxes elicited high rates of

seroconversion and survival of all challenged animals following immunization was observed (Charlton *et al*, 1992).

Upon 20 serial passages of AdRG1 in 293 cells, REA of viral stocks obtained from the tenth and twentieth passages showed no gross rearrangements in the viral genome (Lutze-Wallace *et al*, 1995a). PCR and nucleotide sequencing of the G gene expression cassette showed that the consensus sequence remained unchanged after 20 passages. A small number of plaque-purified viruses from the twentieth passage were examined by PCR and REA, which revealed no gross mutations in the expression cassette. In addition, staining of the plaque-purified viruses using FAT (fluorescent antibody technique) showed that the G protein was expressed (Lutze-Wallace *et al*, 1995a).

Further research brought about the creation of the AdRG1.3 vaccine, which is virtually identical to AdRG1 except that the SV40 promoter was removed and the translation initiation sequence was modified in an attempt to improve translation efficiency (Yarosh *et al*, 1996). Compared to AdRG1, AdRG1.3 had higher levels of G expression *in vitro* and elicited the highest level of VNA in skunks upon oral administration. These findings indicated that the AdRG1.3 vaccine could be a valuable alternative to VRG, to protect animals not protected by the currently licensed vaccines, namely the striped skunk (Wandeler, 1991). However, before AdRG1.3 can be licensed for widespread application many conditions must be met, including establishing the genetic stability of the vaccine.

This study sought to improve upon the methods of genetic characterization that were carried out for the AdRG1 vaccine. Rather than directly characterizing viral stocks recovered after *in vitro* passage, viral clones isolated using the limiting dilution method were analyzed; this may allow detection of changes in a small population of the viral stock that would not be identified by the previous methods employed. As well, rather than using only

REA to demonstrate genetic stability, nucleotide sequencing of the entire G gene expression cassette and flanking HAd5 sequences was carried out. As mentioned previously, REA only identifies mutations that occur at restriction sites and staining with mAbs only detects changes that affect the phenotype of G expression; therefore, complete sequencing of the G insert should allow detection of mutations, such as missense mutations or those occurring in the regulatory elements, that would not be identified by the previous methods used.

AdRG1.3 – Predictions regarding genetic stability

To re-iterate the hypothesis of this thesis, the G gene expression cassette of AdRG1.3 is predicted to be genetically stable because the size of the recombinant virus falls within the range shown previously to be stable, and being a DNA virus should be much less prone to mutation than an RNA virus.

It is known that HAd5 genomes can accommodate a significant quantity of foreign DNA (approximately 2 kb) without significant effects on stability (Russell, 2000). It has been observed that inserts resulting in a net genome size of ~105% of that of wild type HAd5 remain stable, whereas genomes of slightly larger net size render the virus unstable at a rate relative to the size of the insert (Bett *et al,* 1993). In the case of AdRG1.3, the G gene expression cassette (~2.4 kb) is inserted within a deletion (~2.7 kb) of the E3 region of HAd5. Since this results in a net genome size slightly less than 100% of that of wild type HAd5, the previous observations would predict that genetic stability would not be affected.

Also, DNA viruses tend to be more genetically stable but less adaptable than RNA viruses; although both undergo spontaneous mutation, the rate is much lower in DNA viruses (10^{-8} to 10^{-11} per replication) than in RNA viruses (10^{-3} to 10^{-6} per replication), thought to be primarily due to differences in the replication enzymes (Condit, 1996). The DNA-dependent DNA polymerase has proofreading function, while RNA-dependent RNA-polymerase and

RNA-dependent DNA-polymerase (reverse transcriptase) do not. Therefore, the adenovirus vector into which the glycoprotein expression cassette is inserted offers a more genetically stable background than rabies virus, at least at the level of transcription.

Stability of G gene expression cassette upon *in vitro* passage of AdRG1.3

The AdRG1.3 recombinant vaccine was serially passaged 20 times in cell culture in order to assess the genetic stability of the glycoprotein gene upon *in vitro* passage. The human embryonic kidney 293 cell line, transformed with sheared human adenovirus type 5 DNA (Graham *et al*, 1977), is suitable for the propagation of adenoviruses and was therefore the cell line of choice for *in vitro* passage of AdRG1.3.

In vitro passage of virus

67 viral clones were isolated by limiting dilution of virus collected from the twentieth passage of AdRG1.3 in 293 cells. The complete G gene expression cassette and flanking adenovirus sequences of each clone were amplified with the Expand High Fidelity Plus PCR system (Roche). This system was used because of its increased fidelity during amplification reaction, so as to limit the introduction of mutations from the amplification process itself; it is reported to have a six-fold increase in fidelity as compared with reactions carried out with *Taq* DNA polymerase alone, due to the incorporation of a new thermostable proofreading protein that lacks polymerase ability. The nucleotide sequence of the G gene, SV40 polyadenylation signal, and adjacent adenovirus DNA (a total of 1870 nt) was determined from the PCR products obtained for each clone and compared to the sequence obtained from the original, unpassaged virus (designated p0). No mutations were found in any of the 67 clones, indicating that the G gene expression cassette remains stable in the adenovirus vector background upon serial passage *in vitro*.

However, it should be acknowledged that one can not rule out the possibility of a low level of mutation beyond the detection limits of this analysis. As mentioned previously, the mutation rate at a specific site for DNA viruses is approximately 10^{-8} to 10^{-11} per replication (Condit, 1996). From this data, one may assume that a minimum of 10^{8} to 10^{11} clones would need to be examined in order to detect a spontaneous mutation at one specific site in the adenovirus genome; therefore, one would not expect to find a spontaneous mutation in the 67 viral clones that were examined in this study. These experiments did however explore the possibility that the G expression cassette mutated to a much higher extent than the adenovirus backbone; within the detection limits of the system this proved not to be the case. One must also consider the possible introduction of errors upon amplification of genomic DNA by PCR. The Expand High Fidelity Plus PCR system (Roche) employed here reports an error rate of 4.8×10^{-6}, as determined by lacI-based PCR Fidelity Assay (Frey and Suppmann, 1995). If a mutation had been found in one of the viral clones examined, one would need to exclude the possibility that the mutation was introduced by the amplification reaction itself in order to conclude that this change was in fact a real mutation. This could be accomplished by repeating PCR amplification of the G gene expression cassette of that clone, as it is highly unlikely that the exact same error would be introduced upon amplification a second time.

Stability of G gene upon *in vivo* passage of AdRG1.3

An *in vitro* system does not necessarily reflect the situation *in vivo*; experiments making use of a cell culture system do not take into account factors in a living system, such as route of vaccine administration or immune response. Thus, it is valuable to assess the genetic stability of the vaccine in an animal model.

Cotton rat model

The cotton rat model of infection, the only small animal found to be suitable for the propagation of human adenoviruses (Pacini *et al*, 1984; Prince *et al*, 1993) was chosen for the *in vivo* passage of AdRG1.3. Human group C adenoviruses, which includes HAd5, can replicate in the lung of cotton rats; in these animals, the magnitude of adenovirus yield and disease is directly proportional to the viral input dose, allowing for a "made-to-order" severity of disease (Prince *et al*, 1993). Thus, cotton rats served as small-animal surrogates for the target species of AdRG1.3 vaccination – namely foxes, raccoons, and skunks.

As with all animal models, there are inherent limitations of the cotton rat model of HAd5 infection. In this study, the cotton rats were inoculated intranasally so as to emulate the route of administration in nature (via the oropharyngeal cavity). However, this does not take into consideration the nature of the viral bait in the field (i.e. bait availability, uptake, and dose), nor the species or health status of the target animal, all of which are complex environmental variables that affect not only the efficacy of the vaccine but also the selective pressure placed on the virus genome during infection. Therefore, the results of the *in vivo* passage of AdRG1.3 can only confirm whether G gene mutants arise in a live animal host under controlled conditions.

Another aspect to consider is the source of virus used during the propagation of AdRG1.3 through a series of cotton rats. In this study, virus was collected from the lungs, filtered, and inoculated directly into the intranasal cavity of the next series of animals in order to have successful continued passage of the virus and to maintain a consistent route of administration. However, this does not reflect the mode of transmission of human adenovirus among the target animal species. Previous work with the AdRG1 vaccine indicates that adenovirus can only be isolated from the oral fluids and feces of vaccinated

foxes, skunks, and raccoons for only a brief period post-vaccination; the short period of excretion of the adenovirus in the feces suggests that there is little or no replication of the virus in the intestines, and thus minimal to no transmission between animals when the vaccine is applied in the field (Charlton *et al*, 1992).

In vivo passage of virus

The AdRG1.3 recombinant virus was passaged 5 times in cotton rats in order to assess the genetic stability of the glycoprotein gene upon *in vivo* passage. Virus was passaged simultaneously through 5 independent series of animals, and from virus recovered from the 5 animals of the fifth passage of AdRG1.3, a total of 105 clones were isolated by the limiting dilution technique. The complete G gene expression cassette and flanking adenovirus sequences of each clone were amplified with PCR, and as with the viral clones isolated from *in vitro* passage of AdRG1.3, the Expand High Fidelity Plus PCR system (Roche) was utilized. The nucleotide sequence of the G gene, SV40 polyadenylation signal, and adjacent adenovirus DNA (a total of 1870 nt) was determined from the PCR products obtained for each clone and compared to the sequence obtained from the original, unpassaged virus (designated p0). No mutations were found in any of the 105 clones, indicating that the G gene expression cassette remains stable in the adenovirus vector background upon serial passage *in vivo*.

This is in contrast to mutations found by Lutze-Wallace and colleagues (1995b) when examining the genetic stability of the AdRG1 vaccine. They examined 111 AdRG1 isolates, recovered from the feces and oral fluids of skunks given the AdRG1 vaccine, using REA and PCR/REA. The G gene expression cassette of 3 isolates exhibiting altered REA patterns, sequenced after amplification by PCR, showed two distinct mutations: 1) a 72 base pair

insertion in the SV40 promoter region, and 2) a 54 base pair deletion within the G coding sequence (Lutze-Wallace *et al*, 1995b).

HAd5 has been clearly shown to replicate in the cotton rat and can produce pathological effects in the lungs, yet the dose-dependence of viral replication mentioned previously indicates that the virus is not fully adapted to the cotton rat (Ginsberg and Prince, 1989; Prince *et al*, 1993). This aspect of infection adds utility to the cotton rat model since the severity of disease can be controlled experimentally. This characteristic dose-dependence was seen upon *in vivo* passage of AdRG1.3; as the titre of adenovirus administered to the animals decreased, so did the output virus titre. Similarly, when virus recovered from the cotton rats' lungs required *in vitro* propagation to continue the *in vivo* passage of AdRG1.3, consequent inoculation of high-titred virus corresponded with increased recovery of virus. Therefore, since the G gene of AdRG1.3 has been observed here to be genetically stable upon passage in animals to which HAd5 is not completely adapted, these findings may provide insight into the genetic stability of the vaccine in other species to which HAd5 is not presumably adapted, including skunk species.

Double immunostain assay (DIA)

As with VRG, the rabies G gene of AdRG1.3 is expressed not on the surface of the adenovirus virions but presumably on the surface of infected cells (WHO, 1992), although the process by which this occurs is poorly understood. Correct conformation of the expressed glycoprotein and its antigenic sites is necessary for inducing immunity against rabies. Therefore, it is valuable to have an assay that confirms that the rabies G gene is being expressed by the adenovirus vector in infected cells, and that its product is in the proper conformation.

The DIA is a double-staining immunocytochemical method that simultaneously detects the expression of rabies glycoprotein and HAd5 antigens in AdRG1.3-infected cell culture. This assay, in conjunction with a panel of 11 mAbs (Table 1) whose specificity encompasses all 6 antigenic sites of rabies G, was able to detect the expression of all targeted G epitopes by 72 hours post-infection. Of these 11 mAbs, 2 recognized linear (sequential) epitopes (mAbs 10EC9 and M818) and 9 recognized conformational (non-sequential) epitopes; this confirms that the G gene product expressed by the adenovirus vector has acquired appropriate 3-dimensional structure.

Monoclonal antibodies display specific reactivity patterns based on the antigenic determinants present. Successful vaccination against rabies is more likely if the mAb reactivity patterns for the adeno-expressed G and ERA G are similar, if not identical. Since the majority of anti-G mAbs recognized conformational epitopes, the single amino acid difference identified between ERA and the AdRG1.3 glycoprotein (Figure 5) does not generate any detectable difference in antigenic profile between the two proteins.

Since the G gene remained stable upon passage of AdRG1.3 in both cell culture and animals, there was no need to screen for AdRG1.3 G gene mutants using the DIA in this case. However, preliminary work (not presented in this thesis) indicates the potential usefulness of this assay in screening vaccine seed for: 1) recombinant virus that has mutated or lost its transgene, and/or 2) HAd5 wild type virus contamination. A study investigating the effect of co-passaging the AdRG1.3 virus with HAd5 wt virus is underway in Dr. Nadin-Davis's laboratory. The two viruses will be co-passaged 10 times in 293 cell culture, with each virus also being passaged independently as a control. Virus will be harvested from the cells at each passage, and the DIA will be used to look at detectable expression of the adenovirus and rabies G epitopes while real-time PCR will be used to quantify the relative

amounts of each virus recovered, as determined from the genetic material present. This work may provide insight into whether there is a difference in fitness of the two viruses. If HAd5 wt were to have a fitness advantage over AdRG1.3 in cell culture, this would have serious implications if wild type contamination of AdRG1.3 vaccine seed were to occur during production; for example, if HAd5 wt were to out-compete the AdRG1.3 recombinant, the resulting vaccine would be ineffective and fail to induce an anti-rabies immune response in the vaccinated animal.

Genetic stability of other adenovirus recombinants

Although recombinant adenovirus vectors have been extensively studied for their use in vaccination and gene therapy applications, the literature regarding their genetic stability is rather limited.

Parks and Graham (1997) defined a lower limit of 27 kb for efficient DNA packaging and stable amplification using high-capacity adenovirus vectors. These vectors, also referred to as helper-dependent or "gutless", have all viral coding sequences deleted to allow the incorporation of large amounts of foreign DNA. Research on these types of vectors has shown that the instability of smaller genomes can result in dimerization (Morsy *et al*, 1998) or concatamerization (Fisher *et al*, 1996; Haecker *et al*, 1996; Kumar-Singh and Farber, 1998) of the vector genomes. Thus, a balance between 27 and 36 kb should be maintained to promote genetic stability of the adenovirus vector genome (Schiedner *et al*, 2002).

Lieber and colleagues (1996) used the cre-*loxP* system (used for site-specific recombination) in order to produce adenoviruses with extended genome deletions so as to minimize the expression of immunogenic/cytotoxic viral proteins. When they studied the transgene expression from these helper-dependent second-generation adenovirus vectors, viral DNA concentrations of the deleted viruses were found to be unstable; however in the

presence of first-generation adenovirus vectors, the deleted vectors seemed to stabilize, possibly because the latter are able to provide the appropriate stabilizing adenovirus proteins (Lieber *et al*, 1996).

Possible contributors to the genetic stability of the G gene insert of AdRG1.3

The G protein is essential to the rabies virus because it is necessary for the attachment and binding of virus receptors (Wunner *et al*, 1984), membrane fusion (Gaudin *et al*, 1993), and for inducing (Wiktor *et al*, 1973) and reacting with (Dietzschold *et al*, 1987) rabies VNA. Although the G protein (90% homology among rabies virus strains)(Coll, 1995) is not as highly conserved as the N protein (98% to 99.6% homology)(Rose and Whitt, 1996), there is selective pressure upon the rabies virus to maintain the G gene with a relatively high degree of sequence conservation so as to maintain the protein's functionality. Since the rabies G protein is not needed by the adenovirus vector to carry out any viral functions, it would be reasonable to assume that there may not be the same pressure to maintain the gene encoding it upon passage of AdRG1.3 in cell culture. However, the stability of the transgene seen upon *in vitro* passage of AdRG1.3 indicates otherwise. This genetic stability could be partially due to the adenovirus region (E3) into which the G gene has been inserted. Although this region of the virus genome is considered non-essential for adenovirus replication because its gene products serve primarily as accessory proteins to subvert host defense mechanisms (Russell, 2000), it seems unlikely "that a region consisting of almost 10% of the viral genome, which (has) probably persisted for millions of years, would be without functions that (are) advantageous to the virus" (Ginsberg and Prince, 1994). Since the transcription of the G gene of AdRG1.3 is presumably under control of the endogenous E3 promoter (Yarosh *et al*, 1996), it may be under similar selective pressure as the E3 gene it

has partially replaced. However, the contribution of the endogenous promoter to the genetic stability of the transgene would need to be investigated further.

The E3 region appears to be historically critical in establishing persistent infections in tonsils and adenoids (Rowe *et al*, 1953); as such, the genes encoded in the E3 region should provide advantageous functions *in vivo*. Since this region has been replaced by the G gene expression cassette in AdRG1.3, it would be reasonable to assume that the pressure to maintain this region might be reduced. However, the findings from the passage of AdRG1.3 *in vivo* indicate that this is not the case. As with the findings from the passage of AdRG1.3 *in vitro*, this may also be partially due to the G gene being under the control of an endogenous adenovirus promoter. As well, even though the rabies glycoprotein is not useful to the adenovirus, it also does not appear to interfere with the infectivity and replication of the host vector; therefore, the benign nature of G expression may allow the genetic region encoding it to be maintained in the HAd5 genome.

In regards to rabies glycoprotein expression, the SV40 polyA addition element, which constitutes much of the downstream region of the G gene expression cassette of AdRG1.3, may contribute to the stability of gene expression from the E3 locus of the HAd5 vector. The SV40 element theoretically serves to direct proper processing of the 3′ end of the mRNA transcribed from the G gene. Although the role, if any, of the SV40 polyA addition sequence remains to be determined, Prevec and colleagues (1990) found that the use of SV40 control elements frequently resulted in high-level expression of the DNA insert. The SV40 polyA element was also found to be genetically stable upon *in vitro* passage; this may, in turn, contribute to the stability of resulting mRNA transcripts and thus glycoprotein expression.

Veterinary vaccine licensing considerations (Canada) – Future work

In Canada, the production, evaluation, distribution, importation and registration of veterinary vaccines and other biologics is regulated by the Veterinary Biologics Section (VBS) of the Canadian Food Inspection Agency. The "New Product Submission Check-List" (http://www.inspection.gc.ca/english/anima/vetbio/info/vb301e.pdf) highlights the documentation and data required when applying to license a vaccine in Canada. In addition to data supporting the genetic stability (passage studies) of a live vaccine, the purity, safety, and identity of the master seed must be assured, as well as the cells in which the vaccine is produced. Data supporting host animal immunogenicity, efficacy, and safety (both in the lab and the field) are required, including three consecutive pre-licensing serials. Expiration of the vaccine (either accelerated or real time results) should be examined, and since AdRG1.3 is a biotechnology-derived product to be distributed among wildlife animal populations, an environmental risk assessment is essential.

Conclusions

At present time, there exist multiple terrestrial wildlife reservoirs of rabies in Canada; namely skunks, foxes, and raccoons. The most viable approach to rabies control is through large-scale oral vaccination programs that target these hosts. The currently licensed vaccine, a vaccinia-rabies glycoprotein recombinant (VRG), has been successful in controlling rabies in foxes and raccoons; however, skunks remain refractory to vaccination with VRG. Therefore, an effective oral vaccine exhibiting a broader host range is needed to protect all major terrestrial rabies reservoirs. The ultimate goal to which this genetic stability study contributes is the eradication of rabies from terrestrial species reservoirs in populated areas of Canada.

AdRG1.3, an adenovirus-rabies glycoprotein recombinant, has been shown to produce high levels of glycoprotein expression *in vitro*, elicit high levels of VNA in skunks upon oral administration, and is highly effective in terms of levels of antibody induced at specified doses (Yarosh *et al*, 1996). In this study, the G gene expression cassette of AdRG1.3 was found to be genetically stable within the adenovirus vector background upon multiple passages of the vaccine in cell culture as well as in a live animal model. Lab-based efficacy trials of this vaccine are currently being carried out in skunks. All findings to date indicate that AdRG1.3 will be a suitable vaccine alternative for animals not protected by the currently licensed vaccines, with the potential to safely and effectively vaccinate all major Canadian terrestrial rabies reservoirs by the oral route with a single vaccine.

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APPENDICES

APPENDIX A: SAMPLE CALCULATIONS

Appendix A-1

Spearman-Karber method for calculation of viral titre

(Lorenz and Bogel, 1973)

$$50\% \text{ endpoint dilution} = \log_{10}(x_0 - d/2 + d\,\Sigma r_i/n_i)$$

x_0 = $\log_{10}$ of reciprocal of the lowest dilution at which all wells are positive + $\log_{10}$ of the reciprocal of the amount inoculated per well

d = $\log_{10}$ of dilution factor (for a 10-fold dilution = 1)

n_i = number of wells used at each dilution

r_i = number of positive wells (out of n_i)(maximum is = n_i)

Example:

Initial titration of AdRG1.3 2001-01B ADRI Lot#301 performed in triplicate (plated 1-4, 5-8, 9-12), 10-fold serially diluting from 10^{-4} to 10^{-11}. Wells scored below, as of 14 days post-infection.

+ = positive for CPE indicative of adenovirus infection
– = negative for CPE indicative of adenovirus infection

			1				2				3	
Dilution	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
10^{-4}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-5}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-6}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-7}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-8}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-9}	+	+	-	-	-	-	-	-	-	-	-	-
10^{-10}	-	-	-	-	-	-	-	-	-	-	-	-
10^{-11}	-	-	-	-	-	-	-	-	-	-	-	-

1	50% endpoint dilution	$= \log_{10}(x_0\text{-}d/2 + d\,\Sigma r_i/n_i)$ $= \log_{10}[(8+1) - (1/2) + 1(4/4 + 2/4)]$ $= \log_{10}[10]$ $= \mathbf{10^{10}\ TCID_{50}/mL}$
2	50% endpoint dilution	$= \log_{10}(x_0\text{-}d/2 + d\,\Sigma r_i/n_i)$ $= \log_{10}[(8+1) - (1/2) + 1(4/4)]$ $= \log_{10}[9.5]$ $= \mathbf{10^{9.5}\ TCID_{50}/mL}$
3	50% endpoint dilution	$= \log_{10}(x_0\text{-}d/2 + d\,\Sigma r_i/n_i)$ $= \log_{10}[(8+1) - (1/2) + 1(4/4)]$ $= \log_{10}[9.5]$ $= \mathbf{10^{9.5}\ TCID_{50}/mL}$

Appendix A-2

Calculation of Geometric Mean Titre (GMT)

Geometric Mean = n^{th} root of $(X_1)(X_2)...(X_n)$

$X_1, X_2 \ldots X_n$ = the individual data points
n = the total number of data points

Example:
The geometric mean titre of AdRG1.3 2001-01B ADRI Lot#301, based on the titrations in Appendix A-1 done in triplicate, is:

$$\begin{aligned} GMT &= \sqrt[3]{(10^{10})(10^{9.5})(10^{9.5})} \\ &= \sqrt[3]{(10^{29})} \\ &= \mathbf{10^{9.66}\ TCID_{50}/mL} \end{aligned}$$

Appendix A-3

Calculation of multiplicity of infection (moi)

Example:
A 100 ml suspension of 293 cells contains $1x10^{6.1}$ cells/ml; a viral stock contains $1 \times 10^{8.5}$ $TCID^{50}$/ml. In order to infect the cells at a moi of 0.1 (where, theoretically, only 1 in 10 cells will become infected), need to infect cells with $1x10^{5.1}$ virus particles/ml.

$$10^{8.5}/10^{5.1} = 10^{3.4} = 2512$$

Therefore, need to dilute the viral stock ~1/2500 in order to obtain a moi of 0.1 during infection.

APPENDIX B: Geometric mean titres of AdRG1.3 achieved upon synchronous infection of 293 cells (raw data)

Time (hours p.i.)	Extracellular[1] GMT ($TCID_{50}$/ml)	Intracellular[2] GMT ($TCID_{50}$/ml)
12	$10^{3.30}$	$10^{2.05}$
14	$10^{3.04}$	$10^{0.97}$
16	$10^{2.85}$	$10^{1.10}$
18	$10^{2.75}$	$10^{2.85}$
20	$10^{2.80}$	$10^{3.75}$
22	$10^{3.14}$	$10^{5.39}$
24	$10^{4.74}$	$10^{5.80}$
26	$10^{3.89}$	$10^{6.60}$
28	$10^{4.50}$	$10^{7.45}$
30	$10^{5.50}$	$10^{7.35}$

[1]Extracellular virus titre determined from infected cell supernatant.

[2]Intracellular virus titres determined from infected cell monolayer following sonication.

$TCID_{50}$/ml = 50% tissue culture infectious dose

APPENDIX C: Geometric mean titres recovered upon 20 serial passages of AdRG1.3 *in vitro* (raw data)

Serial Passage	GMT (FA_{50}/ml)[1]	GMT ($TCID_{50}$/ml)[2]	Discrepancy[3]
1	$10^{9.55}$	$10^{9.10}$	$10^{0.45}$
2	$10^{9.43}$	$10^{9.68}$	$10^{0.25}$
3	$10^{8.80}$	$10^{8.51}$	$10^{0.29}$
4	$10^{8.05}$	$10^{8.60}$	$10^{0.55}$
5	$10^{8.30}$	$10^{8.93}$	$10^{0.63}$
6	$10^{8.10}$	$10^{9.43}$	$10^{1.33}$
7	$10^{8.26}$	$10^{9.39}$	$10^{1.13}$
8	$10^{9.14}$	$10^{9.49}$	$10^{0.35}$
9	$10^{8.99}$	$10^{9.55}$	$10^{0.56}$
10	$10^{9.30}$	$10^{9.51}$	$10^{0.21}$
11	$10^{9.00}$	$10^{9.48}$	$10^{0.48}$
12	$10^{8.85}$	$10^{8.99}$	$10^{0.14}$
13	$10^{8.24}$	$10^{8.55}$	$10^{0.31}$
14	$10^{8.30}$	$10^{8.43}$	$10^{0.13}$
15	$10^{8.60}$	$10^{8.80}$	$10^{0.20}$
16	$10^{8.39}$	$10^{8.49}$	$10^{0.10}$
17	$10^{8.74}$	$10^{8.60}$	$10^{0.14}$
18	$10^{9.10}$	$10^{9.38}$	$10^{0.28}$
19	$10^{9.05}$	$10^{9.05}$	0
20	$10^{9.05}$	$10^{8.80}$	$10^{0.25}$

[1] virus titre determined by indirect immunofluorescence

[2] virus titre determined by endpoint dilution

[3] discrepancy observed between virus titration methods employed

APPENDIX D: **Geometric mean titres recovered upon 5 independent serial passages of AdRG1.3 *in vivo* (raw data)**

Origin of inoculum[1]	Cotton rat inoculated[2]	Virus passage no.	Virus detectable by IF?[3]	log_{10} $TCID_{50}$/ml (GMT)[4]
*	A1	1	yes	4.25
*	A2	1	yes	4.25
*	A3	1	yes	4.48
*	A4	1	yes	3.99
*	A5	1	yes	4.43
A1	B1	2	yes	3.49
A2	B2	2	yes	3.49
A3	B3	2	yes	3.50
A4	B4	2	yes	3.83
A5	B5	2	yes	3.91
B1	C1	3	yes	no titration done
B2	C2	3	yes	no titration done
B3	C3	3	yes	no titration done
B4	C4	3	no	no titration done
B5	C5	3	yes	no titration done
C1	D1	4	no	no titration done
C2	D2	4	no	no titration done
C3	D3	4	no	no titration done
C4	D4	4	no	no titration done
C5	D5	4	no	no titration done

Origin of inoculum[1]	Cotton rat inoculated[2]	Virus passage no.	Virus detectable by IF?[3]	log_{10} $TCID_{50}$/ml (GMT)[4]
A1	Q1	2	yes	4.92
A2	Q2	2	yes	4.08
A3	Q3	2	yes	4.50
A4	Q4	2	yes	4.33
A5	Q5	2	yes	4.99
Q1	R1	3	yes	3.08
Q2	R2	3	no	no virus recovered
Q3	R3	3	yes	2.83
Q4	R4	3	yes	3.66
Q5	R5	3	yes	3.49
R1	S1	4	no	no titration done
R2	S2	4	no	no titration done
R3	S3	4	no	no titration done
R4	S4	4	no	no titration done
R5	S5	4	no	no titration done
not applicable	R1-A	not applicable	yes	7.75
not applicable	Q2-A	not applicable	yes	7.83
not applicable	R3-A	not applicable	yes	7.58
not applicable	R4-A	not applicable	yes	7.33
not applicable	R5-A	not applicable	yes	7.41
R1-A	X1	4	yes	6.00
Q2-A	W2	3	yes	6.17
R3-A	X3	4	yes	6.16
R4-A	X4	4	yes	6.42
R5-A	X5	4	yes	6.16

Origin of inoculum[1]	Cotton rat inoculated[2]	Virus passage no.	Virus detectable by IF?[3]	log_{10} $TCID_{50}$/ml (GMT)[4]
X1	Y1	5	yes	4.67
W2	X2	4	yes	4.58
X3	Y3	5	yes	4.57
X4	Y4	5	yes	4.83
X5	Y5	5	yes	4.50
X2	Y2	5	not applicable	2.99

[1]cotton rat from which the inoculum has been derived

[2]cotton rat to which the inoculum has been administered

[3]indicates whether adenovirus antigen expression was detectable by indirect immunofluorescence

[4]geometric mean titre (logarithmic values) determined by endpoint dilution

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EDUCATION

M.Sc. candidate, Microbiology and Immunology, University of Ottawa, September 2001- present.

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RESEARCH

Response laboratory technician (September 2004 – present)
Emergency and Bioterrorism Response Division, Office of Laboratory Security
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- biological assessment of suspicious packages in Ottawa and surrounding NCR; operate and maintain portable laboratory for emergency response; incorporation of rapid molecular detection of bacterial bioterrorism agents into emergency response; establishment of a respiratory fit-testing program for the EBRD; perform research in CL-2 and CL-3 laboratories; ensure all laboratory maintenance and activities comply with quality assurance objectives; assist in training sessions provided by the OLS; write scientific reports

M.Sc. student (September 2001 – August 2004)
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Research Student (April 2000 – August 2001)
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- *in vitro* pharmacodynamic modelling of fluoroquinolone drugs against ciprofloxacin-resistant strains of *Streptococcus pneumoniae; in vitro* pharmacodynamic modelling of clarithromycin against macrolide-resistant strains of *Haemophilus influenzae*

SKILLS

Microbiology: general bacteriology techniques such as isolation and culture of bacteria; construction of growth/kill curves; media/solution preparation; macro and micro MIC determination
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- Member of the American Society for Microbiology
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HOBBIES

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PUBLICATIONS

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Zhanel, GG., Roberts, D., Waltky, A., Laing, N., Nichol, K., Smith, H., Noreddin, A., Bellyou, T., Hoban, DJ. Pharmacodynamic activity of fluoroquinolones against ciprofloxacin-resistant *Streptococcus pneumoniae. J Antimicrob Chemother.* 2002 May;49(5):807-12.

Abstract contributions

Roberts, D., Nadin-Davis, S., and Wandeler, A. (University of Ottawa and Canadian Food Inspection Agency). Genetic stability of the rabies ERA glycoprotein insert of the AdRG1.3 vaccine upon serial passage in cell culture. 22nd Annual Meeting of the American Society for Virology, UC Davis, California, USA, July 2003.

Zhanel, GG., Roberts, D., Laing, N., Noreddin, A., Nichol, K., and DJ Hoban. Pharmacodynamic (PD) activity simulating free serum concentrations of β-lactams, macrolides and fluoroquinolones against β-lactamase negative and positive *Haemophilus influenzae* using an *in vitro* model. 10th International Congress on Infectious Diseases, Singapore, March 2002.

Zhanel, GG., Noreddin, A., Roberts, D., Nichol, K., Wierzbowski, A., Bellyou, T., Laing, N., and DJ Hoban. *In vitro* pharmacodynamic modelling of clarithromycin simulating serum (S) and ELF concentrations versus *mef*A and *erm*B macrolide-resistant *S.pneumoniae*. International Congress of Chemotherapy (ICC), Amsterdam, 2001.

Zhanel, GG., Roberts, D., Nichol, K., Bellyou, T., Laing, N., and DJ Hoban. Pharmacodynamics of respiratory fluoroquinolones versus ciprofloxacin-resistant *S.pneumoniae*. International Fluoroquinolone Meeting, Edinburgh, Scotland, 2001.